Investigation of plants used in Gabonese traditional medicine for the treatment of opportunistic infections caused by HIV

by

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DECLARATION OF INDEPENDENT WORK

I, BOUKANDOU MOUNANGA MARLAINE MICHELE (student number: 15015213) hereby declare that this thesis submitted for the award of **PhD** degree in Microbiology at the University of Venda is my original work and has not been previously submitted to this or any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a compiled list of references.

Signature……. Date……

Dieu a une manière particulièrement tordue de mettre des épreuves dans nos vies dont lui seul connaît le dénouement. Au cours de ces épreuves des personnes sortent de nos vies, d'autres y entrent, on fait des choix douloureux, on se découvre une force intérieure insoupçonnée et enfin on finit par découvrir qui on est… …..

DEDICATIONS

This work is dedicated to my farther Michel Mounanga and my mother Marie-Claire Mouguissi without whom I would have never achieved anything, thank you for your commitment towards me, words can never be enough.

I would like to dedicate this work to my two kids Nouria Mouguissi and Anton Mounanga for being so patient, kind and understanding.

I would also like to dedicate this work to Afsatou Ndama Traore, my friend, big sister and mentor who gave me all the strength, all the support and all the encouragements I needed.

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ABSTRACT

Background: Currently, Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome (HIV/AIDS) is one of the deadliest diseases in the world. In Gabon, the prevalence is estimated at 4.1%. Because of the several side effects of highly active antiretroviral therapy on the health of people living with HIV, an emphasis on the utilization of based plant treatments have been noticed highlighted with testimonies of patient health improvement. That led researchers to conduct ethnobotanical surveys aiming at reporting medicinal plants used to treat HIV related opportunistic infections. From the outcomes, 20 plants have been selected based on the frequency of their use but also on the absence of papers in the literature related to HIV research conducted in Gabon. This study aims at evaluating the cytotoxicity, antioxidant, antimicrobial, anti-HIV and immunomodulatory activities of the 20 selected plants and to establish the phytochemical profile of these plants.

Methodology: The plants were extracted using absolute methanol and distilled water. Qualitative (phytochemical screening) and quantitative (TPC and TFC) analysis were performed on the 40 extracts obtained. The extracts were assessed for cytotoxicity on Vero and HeLa cells using MTT and dual staining/fluorescence microscopy. The antimicrobial activity of the extracts was evaluated using well diffusion assay and micro-broth dilution assay. DPPH free radical scavenging assay and ferric reducing power were used to determine the antioxidant potential of the extracts. The anti-HIV effect was evaluated on HIV infected blood using reverse transcriptase inhibitory assay and p24 antigen inhibitory assay. The immunomodulatory effects of the extracts were evaluated on HIV infected blood using IL-2, IL-6 and TNF-α ELISA. *Coula edulis* and *T. iboga* methanol extracts were selected for fractionation using column chromatography. LC-MS was used to establish the phytochemical profile of the two selected extracts.

Results: The results revealed that saponins, alkaloids, terpenoids, phenols, tannins, steroids, flavonoids and cardiac glycosides were detected in almost all the plant extracts. The extracts were found to contain more phenolics than flavonoids especially the methanolic extracts. Both alcoholic and aqueous extracts of *C. edulis, M. cecropioides, S. ochocoa, S. kamerounensis, U. guineensis* and *C. religiosa,* the methanolic extract of *A. hirtella* and *R. vomitoria* and the aqueous extracts of *U. klainei* presented IC_{50} significantly lower ($p<0.05$) than ascorbic acid (38.87±1.54 µg/ml). Both *Coula edulis* methanolic and aqueous extracts, methanolic extracts of *S. kamerounensis, S. gabonensis, M. monandra, U.guineensis* and *A. klaineana* showed antioxidant activity significantly

(p<0.01) higher than Ascorbic acid whose EC_{50} was 152.16±1.09 µg/ml. For the fluorescence microscopy the extracts of interest for Vero cells inhibition were *A. klaineana*, *T. iboga*, *U. klainei* and *C. edulis* while for Hela cells the cytotoxic activity was significant (p<0.05) for the following extracts: *U. klainei*, *U. guineensis*, *P. soyauxii*, *A. klaineana*, *V. conferta*, *M. monandra*, *R. vomitoria*, *R. africanum*, *C. edulis* and *S. ochocoa*. The extracts of *C. edulis*, *M. cecropioides*, *S. ochocoa*, *S. gabonensis, A. hirtella, R. vomitoria, S. kamerunensis, P. soyauxii*, *U. guineensis* and *T. iboga* displayed the highest antimicrobial activity against all the selected bacteria. For the anti-HIV the extracts of interest were *V. conferta* (47%), *C. lucanusianus* (47%), *C. religiosa* (44%), *A. hirtella* (42%), *S. kamerunensis* (41%), *M. puberula* (41%) as well as both aqueous and methanolic extracts of *T. iboga* (48% and 45% respectively), *U. guineensis* (46% and 41% respectively) and *A. klaineana* (41% and 44% respectively). The results for RT inhibition assay revealed that out of 24 selected plant extracts selected only *C. edulis, C. religiosa, C. lucanusianus*, *R. africanum*, *P. soyauxii* and *V. conferta* were able to decrease RT by more than 50%. The modulatory effect of the extracts on the secretion of IL-2, IL-6 and TNF-α by HIV infected cells was found to be insignificant (p>0.05). The fractionation of *T. iboga* and *C. edulis* methanol extracts eluted 4 fractions each. The LC-MS analysis revealed at negative ionization the presence of phenolic compounds (Ellagic acid, Gallic acid, and quercetin) in *C. edulis* samples while alkaloids (ibogaine, ibogaline, voacangine) were mainly found in *T. iboga* samples at positive ionization. The two crude extracts and the fractions showed some levels of antimicrobial, antioxidant, anti-HIV and anti-inflammatory activities.

Conclusion: The present study has validated the use of the selected plants in the management of AIDS in Gabon and provided an explanation of the improvement in HIV individual's life reported. Some of these plants could constitute good candidate for promising anti-HIV molecules.

Key words: Anti-HIV, Anti-inflammatory, Antimicrobial, Antioxidant, Cytotoxicity, Fractions, HIV/AIDS, Immunomodulatory, Medicinal plants, Phytochemical compounds.

LIST OF ABBREVIATIONS

Abbreviations	Definition
%	percentage
°C	Degree Celsius
AIDS	Acquired Immunodeficiency Syndrome
ANOVA	One-Way Analysis of Variance
BEA	Benzene/Ethanol/Ammonia hydroxide
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
CO_2	Carbon Dioxide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
e.g	Example
EC_{50}	Half maximal effective concentration
ELISA	Enzyme-Linked Immunosorbent Assay
$FeCl_3$	Ferric chloride
G	Gram
H_2SO_4	Sulfuric Acid.
HCl	Hydrochloric acids
HIV	Human Immunodeficiency Virus
IC_{50}	Half maximal inhibitory concentration
IFN-γ	Interferon Gamma
IL-2	Interleukin-2
IL-6	Interleukin-6
INT	Iodonitrotetrazolium chloride

µg/ml	Microgram per milliliter
µl	Microliter
M	Mole
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
ml	Milliliter
mm	Millimeter
mg/ml	Milligram per milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MS	Mass spectrometry
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PARP	Poly-ADP Ribose Polymerase
PBMC	Peripheral Blood Mononuclear Cells
Pg/ml	Picograms per milliliter
PHA	Phytohemagglutinin
RPMI	Roswell Park Memorial Institute medium
RSA	Republic of South Africa
St Dev	Standard Deviation
Th	T Helper
TLC	Thin Layer Chromatography
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor Necrosis Factor Alpha
USA	United States of America

UV-light	ultraviolet lights
XTT	2,3-Bis-(2-methoxy-4-nitro5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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CHAPTER ONE

General Introduction

CHAPTER 1

1.1 BACKGROUND

Medicinal plants and their derivatives appear to be an important source of bioactive molecules for the pharmaceutical industry. This is because the skeleton of a structure or a pharmacological response of a known compound can become the base for a new therapeutic drug. In this connection, the World Health Organization (WHO) has reported that from among these plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlated directly with their traditional uses by native cultures (Pathare and Wagh, 2012). It is an accepted fact that the ability of plants to heal is attributable to the impressive number of metabolites that they produce. Moreover, metabolites such as alkaloids, carbohydrates, vitamins, and terpenoids are crucial for plant development, adaptation to stress and defence. In addition, such metabolites determine the nutritional quality of food, colour, taste and smell, and they possess immunomodulatory, antimicrobial, anticancer, antihypertensive, anti-inflammatory, antioxidant and cholesterol-lowering properties which are useful to humans (Aggarwal and Sharma, 2017).

Gabon, which is located in Central Africa is 80% covered by a thick tropical forest. As such, plant-based therapy plays a substantial role in the healthcare of the Gabonese population for economic and cultural reasons. Plant-based therapy is therefore used in the treatment of all kinds of diseases and disorders such as malaria, sterility, cardiovascular disorders, diabetes and AIDS (acquired immune deficiency syndrome) (Borokini and Omotayo, 2012; Hedimbi and Chinsembu, 2012). In 2016, the prevalence of Human immune-deficiency virus (HIV) infected people was reported to be 4.1% and despite the low statistic, the Gabonese authorities implemented measures to fight HIV/AIDS such as making available free highly active antiretroviral therapy (HAART) and HIV testing (Ministère de la santé du Gabon, 2013). As a consequence of the numerous side effects of antiretroviral (ARV) drugs, the long-term medication and because plants are reported to alleviate aids symptoms, the Gabonese population seeks the help of traditional healers who provide plant based alternative medicine (Yaba *et al.,* 2013). Therefore, local researchers have compiled ethnobotanical surveys that are aimed at identifying the medicinal plants used in the treatment of HIV related opportunistic infections (Tchouya *et al.,* 2015). These surveys

have been carried out in selected regions of Gabon and resulted in the selection of 20 plants that have been investigated in this study.

Plants contain many pharmacological compounds which are responsible for a large panel of biological activities (Atanasov *et al.,* 2015). For several decades, many known drugs have been developed from plant compounds including quinine (*Cinchona sp*), codeine (*Papaver somniferum*) or acetylsalicylic acid (*Salix alba*) (Newman *et al.,* 2000). Currently, plants remain the most important source of drugs in the world with many different activities such as antimicrobial, anticancer, antimalarial, antiviral or anti-inflammatory activities. The limitations of the HAART- such as long-term treatment, drug resistance, cost, toxicity, have been largely highlighted as being the principal reasons leading people to combine ARV therapy with plants for their perceived efficacy, safety, non-toxicity, availability and economic backing. Over the last decades, based on affirmation by traditional healers, scientific researches have been directed at investigating plants for their action on HIV or on microorganisms causing opportunistic infections (Gail *et al.,* 2015; Tchouya *et al.,* 2015).

Many traditional healers are managing AIDS patients with some favorable outcomes. The reasons behind this improvement is multi-faceted as it could be due to the capacity of the medicinal plants to fight opportunistic infections, to strengthen the immune system or to control HIV virus replication. Observations on the improvement of patient health are carried out based on weight gain, increase of $CD4^+$ cells in blood or sometimes the viral loads that drop to undetectable levels (Nikiema *et al.,* 2009). Consequently, traditional herbal medicines are being tested in clinical trials in countries like China, India, South Africa and other countries to fight HIV/AIDS, with encouraging results (Nlooto and Naidoo, 2014; Asokan *et al.,* 2013; Tshibangu *et al.,* 2004; Kang *et al.,* 1999). All over the world, the promotion of medicinal plants is considerable. In 1999, the Association for the promotion of Traditional Medicine (PROMETRA) organized the First International Conference on Traditional Medicine and AIDS in Dakar (Senegal). The recommendations have encouraged the use of traditional medicine to manage HIV infected individuals. Based on this, parallel sessions of The Fifth Conference of Parties (COP-5) to the Convention on Biological Diversity and the International Conference on Medicinal Plants. Traditional Medicine and Local Communities in Africa for the first time have placed the role of medicinal plants and HIV/AIDS on the international biodiversity agenda (Sofowora *et al.,* 2013;

Mukthar *et al.,* 2008). The most important recommendation from the sessions was the development and the implementation of secure clinical protocols to evaluate the phytomedicines used to manage AIDS. In this connection, the Canadian AIDS Treatment Information Exchange (CATIE) has implemented a Guide that reports on plants that people living with HIV could utilize to relieve AIDS symptoms and it is called "the CATIE's practical guide to herbal therapies for people living with HIV" (CATIE, 2005).

1.2 THE STUDY RATIONALE

In Gabon, to deal with the HIV epidemic authorities adopted measures aimed at offering ARV treatment, AIDS testing, biological evaluation, management of pregnant seropositive women free of charge and they also increased the generalized resources allocated to fight against HIV/AIDS (Ministère de la santé du Gabon, 2013). It is noteworthy that despite all these efforts, people still continue to seek the help of traditional therapists and this is because of the side effects of ARV treatment (Baudrant *et al.,* 2006). The traditional therapists have pride in themselves that they have found the remedy for AIDS through using traditional practices based on plant recipes. Furthermore, many studies on the anti-HIV activity of medicinal plants find synergy in one another in literature (Gyuris *et al.,* 2009; Klos *et al.,* 2009; Kisangau *et al.,* 2007; Naik *et al.,* 2003). However, even if medicinal plants act to improve the health of people living with AIDS, several cases of death due to consumption of medicinal plants have also been reported (Ghorani-Azam *et al.,* 2018; Ekor, 2014). In Gabon, neither the mechanisms leading to patients' improvement nor the potential toxicity of these medicinal plants used in the treatment of opportunistic diseases caused by HIV, have been systematically elucidated to date. A study conducted at a state hospital in Libreville (Gabon) by Okome-Nkoumou et al. (2006), revealed that opportunistic infections due to HIV/AIDS in terms of prevalence included the following:

- ✓ Bacterial infections (42.35%) with pulmonary tuberculosis, pneumonia, salmonellosis;
- ✓ Fungal infections (24.45%) with candidiasis;
- ✓ Parasitic infections (17.68%) with toxoplasmosis;
- ✓ Viral infections (17.47%) with *Herpes zoster;*
- ✓ Malignancies (1.96%) with Kaposi's sarcoma;
- ✓ Others (38.86%) with prurigo, neuropathy and dermatitis.

An ethnobotanical investigation to collect information on medicinal plants used by traditional healers on opportunistic diseases treated (Unpublished Source) was carried out in several regions of Gabon in consultation with local traditional healers. It was found imperative that the various medicinal plants reported to improve the health of people living with HIV be investigated in order to validate their use and determine their pharmacological properties and toxicity.

1.3 HYPOTHESES OF THE STUDY

It is common practice in Gabon for many people to add plants to their ARV therapy or use plants as alternatives to fight the HIV/AIDS pandemic. This is because some patients have claimed that their health has improved due to the plant alternates. Thus, the hypotheses are:

- Among the selected plants, some might show potential against several AIDS-related opportunistic pathogens;

- Some plants might show potential in inhibiting HIV replication;

- Some plants might show potential in inducing the immune system.

1.4 RESEARCH QUESTIONS

The questions that we tried to answer include:

- ✓ Were the selected plants toxic to cells?
- ✓ Did the plants act on selected opportunistic infections?
- ✓ Did the plants directly act on selected virus enzymes?
- ✓ What were the plants' compositions?
- ✓ What were the plants mode of action?

1.5 OBJECTIVES OF THE STUDY

The general objective of the study was to:

Investigate the effects of Gabonese medicinal plants on specific pathogens responsible for HIV opportunistic infections.

And the specific objectives were to:

- ✓ Evaluate the cytotoxic and antioxidant activities of the selected plants;
- ✓ Evaluate the antimicrobial activities of the plants;

- ✓ Evaluate the effects of the plants on the immune system;
- ✓ Identify the phytochemical composition of the active plants.

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CHAPTER TWO

Literature Review

The following articles were published from information taken from this chapter:

Boukandou Mounanga MM, Traore Afsatou Ndama, Van de Venter Maryna and Potgieter Natasha; Review of Gabonese traditional plants that have reported anti-HIV activity. **Indian Journal of Traditional Knowledge [Appendix I]**

Traoré AN., Sigidi MT., **Boukandou MM.**, Ntlhamu MI., Musoliwa R., Tshisikhawe MP. & Potgieter N. (2017) Anti-microbial and immuno-modulatory properties of indigenous plants found in Central and Southern Africa. **Antimicrobial research: Novel bioknowledge and educational programs (A. Mendes-vilas Ed.) [Appendix II]**

CHAPTER 2

2.1 MEDICINAL PLANTS

The treatment of diseases using plants is one of the oldest forms of practices in medicine used by mankind worldwide. As time went by, different ethnic groups added the medicinal power of the herbs found in their habitat to the indigenous knowledge-base. They collected information on plants and developed well-defined pharmacopoeias. In fact, by the 20th century, much of the pharmacopeia of scientific medicine had been derived from the herbal lore of native peoples. Many drugs generally used today, are of herbal origin (Lawal and Yunusa, 2013). The World Health Organisation (WHO) has estimated that about 80 percent of the world population presently use herbal medicine for some aspect of primary health care in both developed and developing countries. This is due to their attributes of having wide biological and medicinal activities, high safety margins and lesser costs (Pandey *et al.,* 2009). The fact is that Herbal medicine is a major component and a common element in Ayurvedic, homeopathic, naturopathic, traditional oriental in both the African and Native American medicines. The WHO noted that of several plant-derived pharmaceutical medicines, about 74% are used in modern medicine in ways that correlate directly with their traditional uses as plant medicines by native cultures (Pathare and Wagh, 2012). As a result, major pharmaceutical companies are currently conducting extensive research on plant materials for their potential medicinal value (Thalla *et al.,* 2013). Substances derived from plants remain the basis for a large proportion of commercial medications used today in the treatment of heart disease, high blood pressure, pain, asthma and other ailments.

2.1.1 PLANT METABOLITES

2.1.1.1 Primary metabolites

Plants are able to produce chemicals as metabolites (Figure 2.1) as they are widely distributed in nature and can be divided into primary and secondary metabolites. Primary metabolites are generally involved in plant respiration, photosynthesis, growth, and development. The major primary metabolites of plants are:

a) Carbohydrates – These are made of carbon, hydrogen, and oxygen molecules and occur as sugar monosaccharides (glucose, fructose), disaccharides (sucrose, maltose), oligosaccharides (raffinose, fructooligosaccharides), polysaccharides (cellulose, hemicellulose, pectins) and sugar alcohols (sorbitol, mannitol) (Hounsome *et al.,* 2008).

b) Amino acids are composed of an amine (-NH2) and a carboxyl (-COOH) functional groups linked to a side chain (R group). Amino acids include: γ-Aminobutyric acid; arginine; carnitine; citrulline; histidine; proline; threonine; taurine; tyrosine; and valine.

c) Vitamin B complex includes: the water-soluble vitamins such as thiamine (B1); riboflavin (B2); nicotinic acid (B3, niacin); pantothenic acid (B5); pyridoxine (B6); biotin (B7); and folic acid (B9) (Hounsome *et al.,* 2008).

d) Organic acids express carboxylic groups. Organic acids are represented by acid compounds such as ascorbic (known as vitamin C); acetic; citric; oxalic; succinic; fumaric; quinic; tartaric; and malonic acid (Hounsome *et al.,* 2008).

e) Fatty acids are compounds with a hydrophilic carboxylate group attached to a long hydrocarbon chain. These are classified according to the number of double bonds they possess. Thus, saturated fatty acids don't have double bonds but they include palmitic; capric; and stearic acids. Mono-unsaturated acids are acids with only one double bond and they include oleic and palmitoleic acids. Lastly, fatty acids with more than one double bond are called polyunsaturated and include acids like arachidonic and linolenic acids (Hounsome *et al.,* 2008).

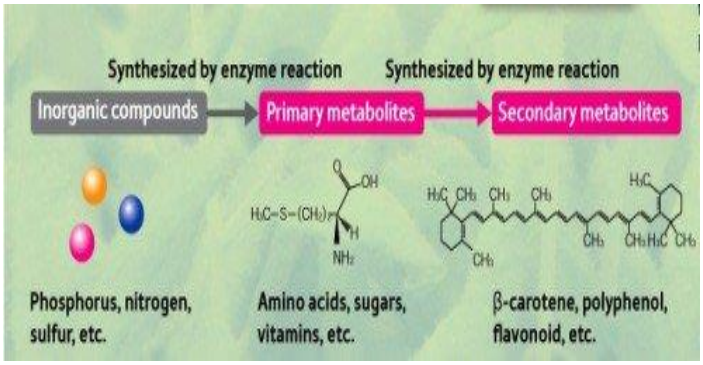

Figure 2.1: Primary and secondary metabolites of plants. (https://www.plantscience4u.com/2013/02/primary-and-secondary-metabolites)

2.1.1.2 Secondary metabolites

The secondary metabolites (Figure 2.2) are derived from primary metabolites and they are the basis for many pharmaceutical drugs. The secondary metabolites determine the color of vegetables, protect plants against herbivores and microorganisms, attract pollinators and seed-dispersing animals, and act as signal molecules under stress conditions (Hounsome *et al.,* 2008). They are produced at different developmental stage into plant specialized cell types. The classification is based on diverse components such as composition, chemical structure, solubility in solvents or the pathway by which they are synthesized. Growth factors such as light, temperature, humidity, type of soil, application of fertilizers, damage caused by microorganisms and insects, stress induced by UV radiation, heavy metals, and pesticides all affect the metabolite composition of plants (Orcutt and Nilsen, 2000). The major secondary metabolites are:

a) Alkaloids which are compounds with nitrogen atom in a heterocyclic ring and are derived from the amino acids. Depending on their biosynthetic precursor and heterocyclic ring system, alkaloids have been classified into different categories including indole, tropane, piperidine, purine, imidazole, pyrrolizidine, pyrrolidine, quinolizidine and isoquinoline alkaloids (Kaur and Arora, 2015). They are responsible for various biological effects such as antimicrobial, antiviral, anticancer, antioxidant, anti-inflammatory, immunomodulatory. Alkaloids are also well known for their toxic properties.

b) Phenolic and polyphenolic compounds are complex compounds composed of one or more aromatic rings attached to at least one hydroxyl group. Phenolics range from simple, low molecular-weight, single aromatic-ringed compounds to large and complex tannins and derived polyphenols. Their classification is based on the number and arrangement of their carbon atoms and they are commonly found conjugated to sugars and organic acids (Crozier *et al.,* 2006). Phenolic compounds include different groups, namely: coumarins; flavonoids (flavonols, flavones); lignans; phenolic acids (stilbenes); and tannins. This group of secondary metabolites is always correlated with antioxidant activity of plants. Phenolic and polyphenol compounds display a wide range of pharmacological activities such as antimicrobial, anti-inflammatory, anti-diabetic, immunomodulatory and anticancer.

c) Terpenes are the principal components of essential oils and are mostly hydrocarbons. Terpenes include compounds such as terpenoids, sterols, carotenes, and steroids. These compounds have shown several biological properties against malaria, cancer, inflammation, and a variety of infectious diseases.

d) Saponins consist of a polycyclic sapogenin (aglycone) linked to one or more carbohydrate chains. The sapogenin unit consists of either a sterol or a triterpene unit. Saponins have the ability to form foam due to the combination of a hydrophobic sapogenin and a hydrophilic sugar part. Some saponins are famous for their toxic properties. Saponins have been reported to have numerous pharmacological actions such as lowering serum cholesterol levels, permeabilizing of the cell membrane, immunomodulatory potential via cytokine interplay, abortifacient properties, cytostatic and cytotoxic effects on malignant tumor cells (Thakur *et al.,* 2011).

e) Cardiac glycosides consisting of an aglycone (cardenolides or bufadienolides) are linked to one or more sugar molecules. They are famous for their cardiovascular activity and their toxicity. Cardiac glycosides also display antiviral and anticancer activity (Morsy, 2017).

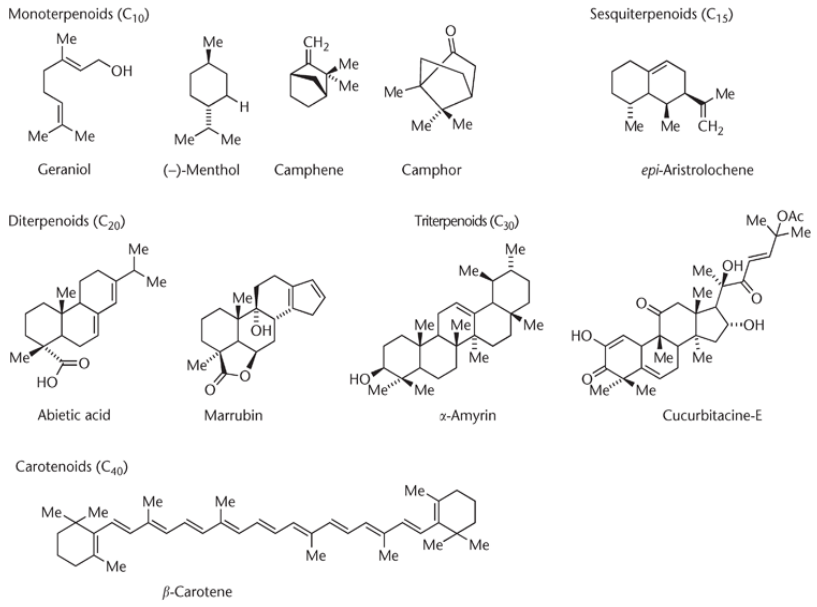

Figure 2.2: Secondary metabolites of plants (www.els.net/WileyCDA)

2.1.2 PHARMACOLOGICAL AND TOXICOLOGICAL PROPERTIES

The consumption of plants and their products (vegetables, seeds, fruits, etc.) and the protection against cancers, bacteria, viruses and other diseases have been correlated and reported (Slavin and Lloyd, 2012). Plants display various pharmacological activities like antimicrobial activity; antioxidant activity; anticancer and anti-inflammatory. However, there are some plants that can have simultaneously more than 10 activities such as the *Jatropha curcas* and *Phyllanthus amarus* (Dahake *et al.,* 2013). Some plants, including *Moringa oleifera*, *Momordica charantia* and *Lawsonia inermis,* have either immunostimulant, antimicrobial, anticancer or antioxidant activities (Laxane *et al.,* 2013; Kadam *et al.,* 2012; Pattel *et al.,* 2011; Kamal *et al.,* 2010; Patel *et al.,* 2009; Amaechina *et al*., 2007). As a result, the plants cited above can be good candidates for anti-HIV therapy, as it is known that the virus weakens the immune system, creates free radicals in the host, and induces different opportunistic infections such as bacterial infections and cancer. The active constituents present in medicinal plants, including essential oils, steroids, phenols and terpenes are responsible for the broad biological activity they exhibit.

The ability of plants to have many activities is widely exploited by traditional healers, who often make mixtures of different plants with the same effects, enhancer or synergistic effects, in order to maximize the chance to heal patients. Yet, the uncertain outcomes of plant combinations could have opposite results to what is expected and may lead to intoxications. Boukandou et al. (2015), demonstrated that plants commonly used in traditional medicine can display deleterious effects in humans. Many plants contain several toxic compounds like alkaloids (Sekhtar *et al.,* 2012) and their level of toxicity might vary from toxic to non-toxic depending on various factors, such as the strength of secondary metabolites, the quantity consumed, the time of exposure, the part of plant used, individual body chemistry, climate and soil (Tülay, 2012). Plants including *Quassia amara*, *Grewia mollis* and *Asparagus racemosus* at certain dosages can cause more or less serious lesions in rodent's organs like liver, kidneys, and even affect the reproductive function (Obidah *et al.,* 2010; Goel *et al.,* 2006; Parveen *et al.,* 2003). Hence, cellular toxicity could be a contributing factor which coupled with a dying cell result in organ lesions then to system failure and this is the basis of diseases that ultimately lead to death.

2.1.3 LIMITATIONS OF IN VITRO ASSAYS

It is not unusual for a plant extract to display excellent bioactivity *in vitro* but have less or no *in vivo* activity. This is often due to poor lipid solubility, or the molecular size of active ingredients, which results in poor absorption and bioavailability (Bruneton, 2002; Chevalier, 2001). Plant extracts contain many secondary metabolites and it is sometimes very laborious to determine or point out one that might be responsible for any potential pharmacological activities. The activity of an extract can also be due to synergistic interactions of several secondary metabolites (Eid *et al.,* 2013; Efferth *et al.,* 2011). Another factor to consider is the effect of *in vivo* drug metabolising pathways on the active compounds which are mostly absent *in vitro*. Following absorption from the intestine to the blood, the secondary metabolites are transported to the liver, where they may undergo enzymatic transformations. A primary product can result from the reduction, oxidation, or hydrolysis of the compounds. Then, this primary product might be eliminated or metabolised by phase II enzymes to more water-soluble conjugates that are easily excreted. These enzymatic transformations might occur by generating an active principle from an initially simple compound or inactivate it. Afterwards, the compound through blood circulation, reaches all the organs and exerts its activity on the targeted cells. The most abundant target in cells are proteins, which function as enzymes, receptors, transcription factors, ion channels, transporters, or cytoskeletal proteins (Wink, 2015).

Secondary metabolites with reactive functional groups that can attack a multitude of proteins in an organism in a non-selective way can result from these transformations. Thus, once in the organism, the plant constituents do not only target specifically the virus or the microorganism, but they also target all the cells that are capable of reacting with them. This leads to the activation of several non-targeted biological processes. Hence with the objective of finding an appropriate HIV infection cure and to provide a good appreciation of the mechanisms of the action of potential drugs, animal models that mimic HIV infection must be used. Unfortunately, there are no appropriate animal models. But several models have been set up to investigate and to find ways and means for the virus eradication. Current research is focusing on the development of mouse, which are non-human primate and feline models that have yielded some good results. But these have so many limitations, which include the absence of certain accessory genes, incomplete colonization of murine tissues with human cells and a

more limited depletion of CD4+ T cells in tissues (Policicchio *et al.,* 2016). The expected outcomes are nevertheless promising and will open the door for medicinal plant testing.

2.1.4 CURRENT RESEARCH

Fear of the side effects from HAART, has driven people living with HIV/AIDS worldwide to drift towards medicinal plants. The evidence of their efficacy and supposedly lesser toxicity has been demonstrated in several studies (Nagaratna *et al.,* 2015; Yang *et al.,* 2014). Several investigations have been undertaken with the aim of discovering new molecules that are able to inhibit viral replication and the suppression of the HIV virus. These studies are mainly focused on the inhibiting activities of plants on various enzymes required for viral development processes like reverse transcriptase, integrase and protease as well as the ability of plants to prevent the entry of the virus into the host cells. From the encouraging outcomes of these studies and WHO supporting the inclusion of traditional healers in the fight against HIV/AIDS, arise the emergence of tests on medicinal plants all over the world.

In Africa, studies investigating plant therapies (Onifade *et al.,* 2013), some with clinical relevance on HIV and related opportunistic infections, have demonstrated an increase in CD4 count, reduction of viral load and/or improvement of quality of life in HIV-infected individuals. Unfortunately, clinical trials that are needed to ensure product development have yet to be conducted (Nlooto and Naidoo, 2014). In Asia, a lot of clinical trials came out with good results including the study on Bevirimat, a derivative of *Syzygium claviflorum* triterpenes (Martin *et al.,* 2008). This promising molecule specifically inhibits the cleavage of the capsid protein from the spacer protein (SP1) at the last step of viral replication. The product has gone through clinical trials until Phase III, but because of the GAG polymorphisms, the study stopped at this phase in 2010 (Wang *et al.,* 2015). American researchers have discovered a promising protein (prostratin), isolated from *Homalanthus nutans* bark commonly used in Samoan traditional medicine (Hezareh, 2005). Research has indicated that prostratin could flush viral reservoirs in latently infected T-cells. Presently, human clinical trials of prostratin are being carried out by the AIDS Research Alliance in the USA (Gulakowski *et al.,* 1997; Gustafson *et al.,* 1992).

Over the past years, more studies are now targeting latent HIV reservoirs in order to extract the virus from the infected cells (Imai *et al.,* 2009; Kulkosky *et al.,* 2001). Once the virus is out of the reservoir it is rendred sensitive to the HAART and the possibility for dealing with it becomes considerable. Recent studies exploring the ability of medicinal plants to induce latent viral reactivation, have identified plants that include *Homolanthus nutans* and *Ocimum labiatum* that have the capacity (Kapewangolo, 2014). Regarding what precedes plants, remain the most promising source of new molecules capable of dealing with this high public health problem safely and efficiently (Figure 2.3).

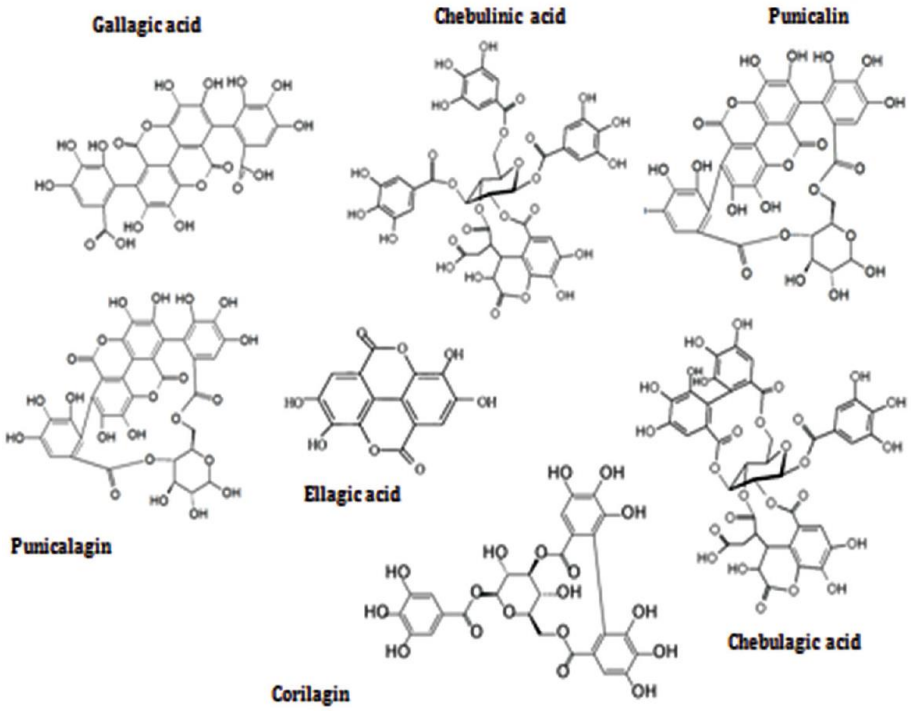

Figure 2.3: Structures of plant-derived compounds with anti-HIV activity (Dwevedi et al., 2016)

2.2 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

2.2.1 HISTORIC

The HIV/AIDS pandemic officially began in 1981 after the discovery of the first cases of unusual immune deficiencies among gay men in America (UNAIDS, 2015). First, it was called gay-related immune deficiency (GRID) because it was detected only among the gay men population. But then, in 1982, AIDS was also reported among people with hemophilia and Haitians (UNAIDS, 2015). The same year, children under the age of 10 with hemophilia including a 20-month-old infant who were transfused with contaminated blood died from AIDS-related infections (UNAIDS, 2015). These last cases provided clear evidence that AIDS was not only transmitted by sexual ways but also via blood. Thus, raising the alarm on blood and blood product supply.

The identification of HIV as the causative agent of AIDS is attributed to both Luc Montagnier (Pasteur Institute, France) and Robert Gallo (National Cancer Institute, USA), who independently isolated virus genomes they suspected caused AIDS (Sharon Leslie, 2016). In 1985, a significant breakthrough came with the United States Food and Drug Administration (FDA) approving the first HIV antibody test (UNAIDS, 2015). Meanwhile, the first mother-to-child transmission of HIV through breastfeeding was reported, and the first international conference on AIDS was held in Atlanta (United States) (UNAIDS, 2015). Alongside those reports, the first cases of infection with an HIV similar (HIV-2) to HIV-1, was reported in West Africa (Clavel, 1987). The same year, clinical trials demonstrated that azidothymidine (AZT) was able to slow down the progression of the virus, which led the FDA to approve the use of this antiretroviral the following year. AZT became the costliest drug ever made to be sold in the world. As research continued, FDA approved the first human testing of a candidate vaccine against HIV in 1987 (UNAIDS, 2015). WHO launched its global AIDS strategy in 1986, and, in 1987, established the Global Program on AIDS (GPA). The role was to raise awareness; formulate evidence-based policies; provide technical and financial support to countries; initiate relevant social, behavioural and biomedical research; promote participation by non-governmental organizations; and champion the rights of people living with HIV (UNAIDS, 2015).

Frustration at the high cost of the medication and discrimination towards HIV infected people led to the formation of several protest units. In 1992, the International

Community of Women Living with HIV/AIDS was created for HIV infected women's rights. Indeed, even though WHO reported that the number of women living with HIV in sub-Saharan Africa exceeded that of men, HIV-related infections experienced specifically by women, such as pelvic inflammatory disease and cervical cancer, were not included in the case definition of AIDS (UNAIDS, 2015). A non-governmental organization dedicated to preventing and eliminating pediatric HIV infection was formed in the 1980s by an HIV-positive mother of HIV-positive children (UNAIDS, 2015). In 2003, International Treatment Preparedness Coalition was formed in Cape Town in order to accelerate access to medication to HIV infected people. Specific groups such as the Global Network of Young People Living with HIV, the Asia Pacific Network of People Living with HIV/AIDS and Interfaith Network of Religious Leaders living with or personally affected by HIV, also emerged.

Interestingly, while the search for an HIV cure was in the spotlight, a community of denialists emerged, including respected scientists with prestigious academic pedigrees. Many people believed that it was a conspiracy implemented by scientists, pharmaceutical industry and governments of the developed countries. A group of people claimed that HIV was deliberately created and infected to people (Sharon Leslie, 2016). This controversy inspired many publications about the topic. For instance, Peter Duesberg, who was an elected member of the prestigious National Academy of Sciences, published an investigation of why he believed AIDS was not caused by a virus, but was the result of using pharmaceutical drugs such as AZT, which are known to be toxic, antibiotic drugs used to treat sexually transmitted diseases which damage the immune system, and also the use of immunity-suppressing recreational drugs (Sharon Leslie, 2016). Another example is the Nobel laureate in chemistry Kary Mullis, who strongly disagreed with evidence showing that HIV causes AIDS.

2.2.2 NOMENCLATURE, STRUCTURE AND LIFE CYCLE OF HIV

The human immunodeficiency virus (HIV) is a member of the Retroviridae which is a family targeting a host cell and regrouping single-stranded positive-sense RNA viruses with an intermediate DNA. The particularity of this family is that it possesses its own reverse transcriptase enzyme which creates a DNA from its RNA genome and *vice versa*. The genus Lentivirus from the subfamily Orthoretrovirinae to which HIV belongs is comprised of many species including HIV-1 and HIV-2. The virus HIV-2 is structurally similar to HIV-1 but the related viral strain is found mainly in West Africa and the infection differs (Clavel *et al.,* 1986). HIV-2 is subdivided into group A to H while HIV-1 which is responsible for the HIV pandemic is subdivided into different groups: M, O, N and P. More than 90% of HIV/AIDS cases are derived from infection with HIV-1 group M, which is further subdivided into about nine subtypes (A-K) (Hemelaar, 2012). HIV-1 group M is more represented both in Gabon and in South-Africa (Lihana *et al.,* 2012).

HIV structure (Figure 2.4) is common to the ones of lentiviruses. All lentiviruses are enveloped by a lipid bilayer that is derived from the membrane of the host cell and contains several cellular membrane proteins (major histocompatibility antigens, actin and ubiquitin) (Arthur *et al.*, 1992). On its surface, HIV presents glycoproteins (gp120) which are bound to the virus *via* interactions with the trans-membrane protein gp41.

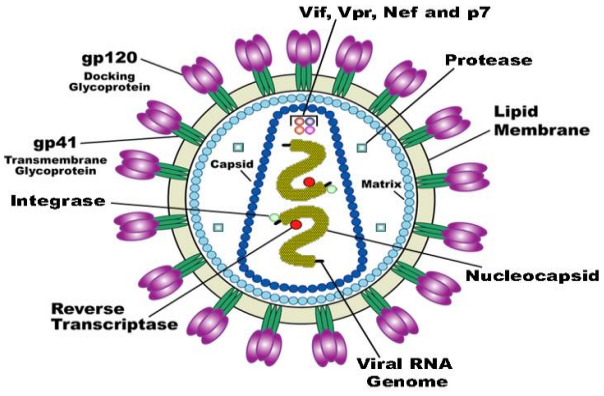

Figure 2.4: Structure of HIV particle (https://commons.wikimedia.org/wiki/File:HI-Virion-en.png#file)

A matrix shell made of many copies of the matrix protein (p17) envelopes the inner surface of the viral membrane, and a conical capsid core particle composed of capsid protein (p24) is located in the centre of the virus. The capsid particle includes two copies of the unspliced viral genome (ribonucleoprotein complex), the nucleocapsid proteins (p7), and the three essential virally encoded enzymes: protease; reverse transcriptase and integrase. The accessory proteins, Nef, Vif, Vpr, Rev, Tat and Vpu can also be found within the virus particle. The HIV-1 life cycle begins with the CD4-dependent invasion of the host cell (Figure 2.5). The viral particle tricks a CD4+ host cell with the gp120 domain of the "Env" protein, which mimics a normal component of the human immune system (the major histocompatibility complex) (Luedtke and Tor, 2003). Binding of CD4 to gp120 initiates endocytosis and fusion of the viral and host membranes. Upon fusion, the viral capsid is partially degraded and reverse transcriptase, transcribes the single-stranded viral RNA genome into double-stranded DNA. The double-stranded DNA copy of the HIV genetic code is then imported into the host's nucleus, where it is permanently integrated into the host's genome (Luedtke and Tor, 2003). Eventually, an RNA copy of the HIV DNA is transcribed.

Early in the replication cycle, viral mRNA becomes highly spliced and is translated into three regulatory proteins: Rev, Tat, and Nef. The Tat protein binds to its cognate RNA site termed TAR and facilitates the transcription of full-length HIV RNA and since Tat itself is an eventual product of this transcription, Tat’s biosynthesis is in a positive feedback loop, leading to a rapid accumulation of viral RNA as well as the Tat, Rev, and Nef proteins. Once the concentration of Rev is sufficient, it polymerizes along the HIV RNA at a site termed the Rev Response Element (RRE), and initiates HIV’s late replication phase. This phase is initiated by the Rev-RRE interaction which prevents the splicing of the HIV RNA. From the un spliced and singly spliced HIV RNA, the proteins needed for viroid construction are translated. Unspliced RNA is then packaged into outgoing viral particles to serve as the primary genome (Frankel and Young, 1998).

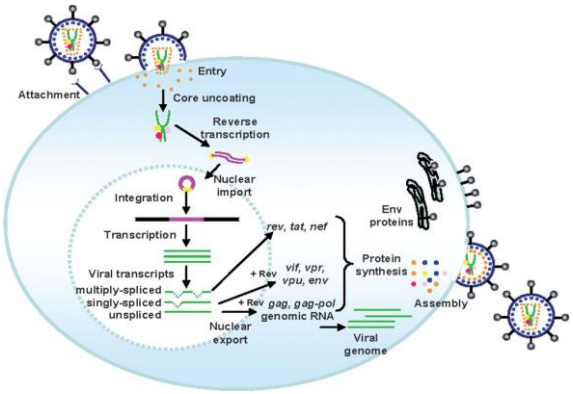

Figure 2.5: Life cycle of HIV (http://www.biochemj.org/bj/443/0603/bj4430603add.htm)

2.2.3 HIV TRANSMISSION AND INFECTION STAGES

As a virus, HIV cannot survive outside a living host. It needs host machinery to reproduce itself and to grow. Therefore, it cannot be transmitted through casual contact. The virus is highly found in body secretions and fluids including blood, semen, breast milk, rectal secretions, vaginal secretions and cervical secretions. Hence, the transmission of the virus occurs via three major ways which are: blood to blood transmission; sexual transmission and mother to child transmission also called vertical transmission. Blood-to-Blood transmission occurs during sharing needles and blood transfusion. Sexual transmission is the predominant mode of transmission. It has been proven that pre-ejaculated semen, vaginal and rectal secretions contain HIV which can penetrate the body through open cuts or sores, the vagina, the penis, and the anus. Regarding the vertical transmission, the blood supplies between the mother and the fetus being well dissociated, do not allow the transmission to occur in the womb but during the parturition. The infant is directly in contact with the mother's blood and vaginal secretion is what increases the risk factor in case of any abrasion on the baby skin. Because the mouth is not a suitable entry point for HIV to infect the body, breast feeding carries a much lower rate of transmission until the baby begins teething. During the teething process, cuts in the gums increase the risk of transmission. When people are infected, the progression of the disease occurs through different stages (Weinberg and Kovarik, 2010; WHO, 2005) namely:

Acute/Primary HIV infection: The first symptoms can appear two to four weeks after exposure to the virus. Even if the symptoms appear early, a diagnosis might not be possible for months. Still, the virus can be transmitted at this stage of the illness.

Asymptomatic HIV infection: In this stage, the infected person doesn't display any symptoms but the infected person is still highly contagious during the stage. The virus lives in white blood cells and it spreads best when the immune system is activated. During this period, the virus is still infecting and killing the T cells.

Early symptomatic HIV infection/AIDS-related complex (ARC): In this stage, symptoms appear, and they include fevers, swollen glands, and thrush. This is when People are diagnosed HIV positive. The HIV positive person has not yet developed opportunistic infections or AIDS.

AIDS: This is the final and most deadly stage of the HIV disease. A person is said to have AIDS when he/she has fewer than 200 CD4+ T cells/mm^3 of blood (as a healthy adult has a CD4+ T-cell count of 1,000 or more). While HIV infects and destroys the cells, the immune system becomes weaker and allows opportunistic infections to develop. From then on, the HIV infected person suffers from many opportunistic infections that can attack the brain, the lungs, and other organs that can be caused by viruses, bacteria, parasites or by cancer cell growth.

2.2.4 OPPORTUNISTIC INFECTIONS ASSOCIATED WITH HIV INFECTION

Generally, people living with HIV start being ill when they develop opportunistic infections which are any form of infections that take advantage of the weakened immune system to spread. Those opportunistic infections are responsible for the high HIV mortality and morbidity rate.

A large spectrum of germs can cause opportunistic infections including those which are normally not pathogenic to immuno-competent people (Table 2.1). Among opportunistic infections (AIDSinfo, 2016; Nissapatorn and Sawangjaroen, 2011; Pinsky and Douglas, 2009), there are:

Cancer: Certain types of cancer such as Kaposi's sarcoma, lymphoma or invasive cervical cancer are more common in people with compromised immune system and are related-HIV condition.

Yeast infections: These infections can spread to the mouth, the throat, genitals, and thighs. In women, this infection can produce severe vaginitis. They are caused by yeast like *Aspergillus sp, Candida sp, Cryptococcus sp,* and *Histoplasma sp*.

Bacterial infections: Infections such as skin infections, tuberculosis, bacterial diarrhoea, and bacterial pneumonia are common in people with AIDS. They are caused by Gram negative and positive bacteria like *Streptococcus pneumoniae, Haemophilus sp, Pseudomonas aeruginosa, Staphylococcus aureus, Mycobacterium tuberculosis, Salmonella enterica, Shigella sp* and *Esherichia coli.*

Viral infections: The co-infection with different viruses including Cytomégalovirus, *Herpes simplex*, *Herpes zoster*, Hepatitis B and C also occurs in HIV patients.

Parasitic infections: Malaria, cryptosporidiosis, leishmaniasis, schistosomiasis, and toxoplasmosis are among parasitic diseases reported in association with HIV.

Table 2.1: Some HIV related opportunistic infections, their causative agents and clinical manifestations (Adapted from Pinsky and Douglas, 2009)

Opportunistic infections	Causative agents	Manifestations
Fungal diseases		
Candidiasis	*Candida sp*	**Oral candidiasis**: Multiple creamy-white, easily removable patches or pseudo-membranous plaques on the tongue, gums, buccal mucosa, and palate, anterior surface of tonsils, posterior wall of throat. **Esophageal candidiasis**: painful swallowing **Vaginal candidiasis**: Itching and burning sensation; creamy white vaginal discharge with cheese-like plaques. Vulvo-vaginal area is erythematous, swollen and painful.
Cryptococcosis	*Cryptococcus sp*	**Septicemia**: skin papule with necrosis, fever, lung infiltration **Meningitis**: headache, photophobia, meningeal syndrome, altered consciousness, focal neurological deficits, fever
Penicilliniosis	*Penicillium Merneffei*	**Isolated skin lesions**: umbilical papules with central darknecrotic crust, without itching or pain, limited to the face or generalized. **Septicemia**: fever, skin lesions, anemia, lymphadenopathy, hepato-splenomegaly, cachexia. **Pneumonia:** dry cough, fever; mild to moderate dyspnea
Pneumonia (PCP)	*Pneumocystis Jiroveci*	Cough, dyspnea, fever, night sweat

Protozoal diseases		
Toxoplasmal Encephalitis	Toxoplasma gondii	Headache, drowsiness, seizures, focal neurological, deficits Fever
Protozoal Diarrhea	Cryptosporidium sp, Microsporidia Isospora	Chronic diarrhea, Vomiting, abdominal pain
Bacterial diseases		
Mycobacterium avium complex (MAC)		Prolonged or recurrent fever, weight loss, fatigue, anemia, hepatosplenomegaly and lymphadenopathy.
Purulent polymyositis	*Staphylococcus aureus, Streptococcus sp*	Pyoderma, folliculitis, cellulitis, abscess of muscle and soft tissues, with or without fever; systemic and other organs involvement.
Pneumonia and pleuritis	*Pneumococcus sp, H. influenzae, P. aeruginosae, S. aureus*	abrupt onset with fever, chills, chest pain, productive cough with thick sputum; dyspnea may present; examination may reveal lung consolidation or pleural effusion, lung crackles;
Bacterial Meningitis	Pneumococcus sp, *R. equi*, Chryseobacterium meningosepticum	Fever, headache, meningeal signs; brain abscess may develop.
Septicemia	*Salmonella sp, E.coli, S. aureus, R.equi Proteus mirabilis, Serratia marcescens, P. aeruginosae*	fever, chills, diarrhea, cellulitis, abscess in the lung and other organs, meningitis and/or brain abscess
Bacterial Diarrhea	*Salmonella sp, Shigella sp, Campylobacter sp* Other enteric bacteria	Fever, frequent bowel movements with watery or bloody mucous stools, severe and prolonged diarrhea frequently accompanied with septicemia; septic metastatic foci may be present in lungs, joints, hepato-biliary tract and bone marrow
Viral diseases		
Varicella	*Herpes Simplex*	Clusters of typical blisters Systemic involvement (HSV encephalitis)
Zona	*Herpes zooster*	Typical painful blisters in clusters within a dermatome. Eye can be involved
Cytomegalovirus (CMV)	*Herpes simplex virus, Epstein-Barr virus*	Retinitis, esophagitis, gastritis encephalitis, skin lesions Polyradiculopathy.
Genital warts (HPV)	Human papilloma virus	Warts present as soft, moist, cauliflower-like papules with peduncle, painless and easily bleeding. - In men warts are found most frequently at the coronal sulcus, prepuce and penis shaft and occasionally at urethral meatus. - In women, warts often occur at clitoris, minor labia, around urethral meatus, perineum. - Genital HPV infection increases the risk of genital cancer.

2.2.5 HIV TREATMENT, DRUG RESISTANCE AND RELATED TOXICITY

In 1986, AZT was the first drug to show promising results in slowing down the progress of HIV. The early 1990s, saw the expansion of the use of AZT for post exposure prophylaxis started by the United States Public Health Service to manage occupational exposure (UNAIDS, 2015). Despite the AZT discovery, the death from HIV continued to rise and new treatments showed limited results. The same year, AZT mono-therapy had proven not to be effective in averting AIDS. In 1996, at the XI International AIDS Conference in Vancouver (Canada), scientists reported a significant treatment breakthrough which was a highly active antiretroviral therapy (HAART) as it was found to reduce the mortality and morbidity rate among patients (UNAIDS, 2015). With the emergence of HAART, deaths related to HIV/AIDS began to dramatically decline. The therapy consists of a combination of at least three drugs taken at the same time, that leads to a viral load below detectable levels with health improvement. Five different classes of antiretroviral (ARV) drugs which target HIV in different ways are in existence and they include: Non-nucleoside reverse transcriptase inhibitors that target the action of reverse transcriptase directly (NNRTIs); Nucleoside reverse transcriptase inhibitors which block the virus genetic material conversion from RNA into DNA (NRTIs); Protease inhibitors prevent the assembly and maturation of infectious viral progeny; Fusion and attachment inhibitors prevent HIV from attaching to a cell; Integrase inhibitors prevent the integration of pro-viral DNA into the host chromosomal DNA (Thakur *et al.,* 2010; Potter *et al.,* 2004).

In addition, a biopharmaceutical company, VaxGen, started the first human trial of the vaccine AIDSVAX in North America and the Netherlands in 1998, and in Thailand the following year (UNAIDS, 2015). The iPrEX (Pre-exposure Prophylaxis Initiative) trial among gay men in Brazil, Ecuador, Peru, South Africa, Thailand and the United States revealed a 44% reduction in HIV acquisition in men taking a single daily tablet of emtricitabine/tenofovir, demonstrating the effectiveness of a new HIV prevention tool, pre-exposure prophylaxis. Atripla (efavirenz/emtricitabine/tenofovir disoproxil fumarate combination), the first multi-class fixed-dose combination pill of antiretroviral drugs, was approved by the United States FDA in 2006. This reduced the pill burden and it also simplified dosing, with the potential to increase adherence. In 2007, the FDA approved the integrase inhibitor, raltegravir which targets a viral enzyme that inserts the viral genome into the DNA of the host cell; as well as the first entry inhibitor

a CCR5 blocker maraviroc. In 2009, a vaccine trial in Thailand showed 31% protection, proving that a preventive HIV vaccine is possible (UNAIDS, 2015). In 2010, a trial in South Africa showed that the vaginal tenofovir gel reduces women's risk of HIV acquisition by 39% over 30 months, thus providing proof of concept that an antiretroviral agent can prevent sexual transmission of HIV in women. The creation of the Medicines Patent Pool by UNITAID in 2010 led to the generic production and affordability improvement of important drugs for pediatric treatment, such as lopinavir, abacavir, and fixed-dose combinations of tenofovir-based regimens (UNAIDS, 2015).

However, despite the significant results of the ARV drugs and because of the high mutation rate of HIV, failure of the treatment started to rise. The most common resistance associated mutations are found within the non-nucleoside reverse transcriptase inhibitors drug class, followed by the nucleoside reverse transcriptase inhibitors and the protease inhibitors (Wheeler *et al.,* 2010). Hence, the drug resistance is firstly due to the lack of regimen adherence of patients under HAART treatment (Klimas *et al.,* 2008). Secondly, the selection of a suitable drug regimen can become troublesome after the patient has developed resistance against some types of ARVs, as this may generate cross resistance towards other antiretroviral drugs. Both the newly infected individuals and treatment naïve patients can be exposed to HIV resistant strains, as the strains can be transferred from infected people under ARVs with developed resistance. Besides, a certain number of factors including the proper behavior of the patient towards the treatment or his/her psychological condition can highly favour drug resistance. WHO released a list of factors increasing drug resistance development that also includes inappropriate selection of drugs, treatment with less than three drugs and interruption of treatment (WHO, 2012). In addition to drug resistance, the long-term medication emerged. Despite the encouraging outcomes of HAART on HIV infection, clinical evidence has demonstrated adverse events compromising quality of life and interference with adherence to ARV. It has been observed that different ARV classes were associated with death from non-AIDS related causes. The impact of HAART exposure on the risk of reduced bone mineral density and osteoporosis in the HIV-infected population was addressed by Todd Brown in a systematic meta-analysis of published literature (Gazola *et al.,* 2010).

Other major complications such as metabolic disorders, neurological disorders, liver disorders and even immune disorders are induced by the long-term use of ARVs

(Bindu and Soumya, 2011). In addition, a concern has now been raised regarding the potential toxic effects of HAART on the infants whose mothers were exposed to this therapy.

2.3 ETHNOBOTANICAL SURVEY AND SELECTION OF PLANTS

2.3.1 PLANT BASED TRADITIONAL MEDICINE IN GABON

Situated in Central Africa, Gabon is bordered to the North by Cameroon and Equatorial Guinea and to the South and East by the Congo Republic. The Atlantic Ocean is to the West coast. Gabon's 9 provinces are 75% covered by forest, and the plant heritage is parted into primary forest (65%), swamps, mangroves, and savannas (FAO, 2010). It is estimated that Gabon's botanical diversity includes more than 8,000 plant species of which 20% are endemic (Ben and Pourtier, 2004) and more than 1600 medicinal plants have been reported (Vliet, 2012).

Despite considerable progress in medicine with conventional synthetic drugs, the majority of the Gabonese population is still reliant on traditional medicine because of cultural reasons (Mvone-Ndong, 2014; Gassita, 1995). As traditional medicine and medicinal plant knowledge has been respected by African traditional practitioners in general, Gabon is no exception to the rule. The Gabonese use mainly plant based traditional medicine to manage all kinds of pain and disease, which extends to those that modern medicine has difficulties in dealing with such as, cancer, kidney failure or HIV (Idyata-Mayombo, 1994). Traditional healers use root barks, stem barks, leaves, or other plant parts for different recipes obtained usually through infusion, maceration or decoction to form the remedy that is given to patients. Medicinal plants are administrated single or as a mixture with other plants that have the same effects, synergistic effects or enhancer effects. Although there are no data available in the literature on the effects of these plant extracts/mixtures on the global health of the population in Gabon, several herbs have been shown to have biological properties. Furthermore, studies on medicinal plants used in the Gabonese traditional medicine have revealed natural compounds that have demonstrated antidiabetic, antitumoral, antimicrobial and antiviral potential such as alkaloids, terpenoids, flavonoids, and phenolics which has resulted in the validation of their use by traditional healers (Verma

et al., 2014; Jung *et al.,* 2006). Plant based traditional medicine remains a valuable way to relieve and treat diseases in Gabon.

2.3.2 ETHNOBOTANICAL SURVEY

The ethnobotanical surveys used in the present work were carried out among traditional healers from various provinces of Gabon (Figure 2.6). The surveys were based on a data sheet which posed questions about the medicinal plants; plant parts used; the modes of preparation and administration of the remedies; and the opportunistic infections (OI) treated. The vernacular names of the plants were also recorded when available. During the surveys, symptoms of HIV/AIDS related OI were described to the traditional healers, to enable them to reveal the appropriate plant species used for the treatment of infections. For this study, the OI that were taken into consideration were: skin rashes; infectious diarrhea and dysentery; pulmonary tract infections; sexually transmitted infections; parasitic infections; cancer; oral infection; and the urogenital or vaginal disorders.

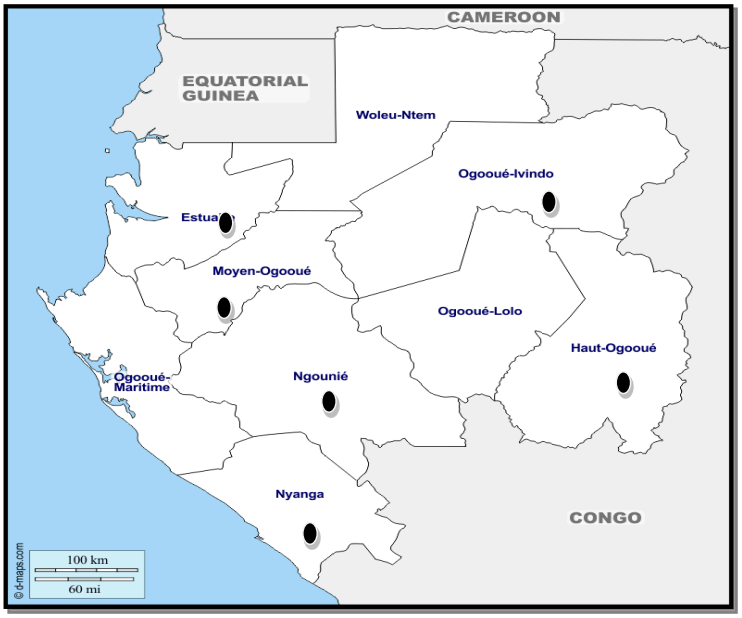

Figure 2.6: Location of areas of the survey indicated on a map of Gabon (https://d-maps.com/carte.php?num_car=4636&lang=fr)

To have an objective view of the collected data, we proceeded to the calculation of a performance index (Betti, 2004) which allowed us to evaluate the effectiveness of the plants listed according to different OI selected, and thus selected the most potentially promising plant for further experimental study. For data analysis, “specific flora” constituted plants used for treating a specific ailment or symptom and the “global flora” were defined as the total plants recorded for all types of ailments cited (Ajibesin *et al.,* 2008). The performance index is the difference between the proportion of the number of citations to the total number of citations and the proportion of specific flora to the global flora (Ajibesin *et al.,* 2008). The proportions used were calculated from the ratios of the number of citations for diseases. The results of the ethnobotanical surveys reveal that a total of 186 plant species belonging to 70 families were used against opportunistic infections. Healers predominantly used stem barks, followed by leaves and roots. Studies related to anti-HIV screening were already undertaken revealing the inhibiting effect on either HIV-1, HIV-2 or both of several plants displaying high IP in the study such as *Alchornea cordifolia, Ocimum gratissimum*, *Euphorbia hirta*, *Annickia chlorantha*, *Picralima nitida* and *Jatropha curcas* (Unpublished Results).

2.3.3 INTRODUCTION TO THE SELECTED PLANTS

The twenty plants described below have been selected as previously stated based on the high index of performance (IP) and the absence of previous studies related to HIV as selected from results of 2.3.2.

ANNONACEAE

***Uvaria klainei* Pierre ex Engl. & Diels.**

U. klainei is a scrambling shrub with descending branches, endemic to Gabon (Figure 2.7). The leaves are used by the population as antiseptic but also to treat venereal diseases and diarrhea. Studies have demonstrated that the polysaccharides that are isolated from this plant possess some immunostimulant, hemostatic and antioxidant capacities (Mengome et al., 2014 a, b, c).

Figure 2.7: *Uvaria klainei* leaves (http://www.tropicos.org/ImagePage.aspx?id=100377606)

APOCYNACEAE

- ***Rauwolfia vomitoria* Afz.**

R. vomitoria is described as a tree that can grow up to 40 m high (Figure 2.8). The fruit is globular to ovoid or ellipsoid orange or red drupe and has one seed. It has white or yellow flowers that are small and robust shaped. This plant can be found in the region stretching from Senegal through Uganda to Tanzania and from Gabon right through the Democratic Republic of Congo to Angola. The roots are used to cure jaundice, diarrhea, rheumatism, venereal diseases, hypertension, snakebites, tumor and can also act as a sedative. Phytochemical studies have revealed the presence of flavonoids, cardiac glycosides, saponins, phenol and alkaloids (Oseni *et al.,* 2014; Sonibare *et al.,* 2011). The pharmacological capacity of *R. vomitoria* when instigated have revealed antitumoral, antimalarial, antioxidant, anti-inflammatory and antipyretic activities (Yu and Chen, 2014; Pesewu *et al.,* 2008; Campbell *et al.,* 2006; Zihiri *et al.,* 2005). *R. vomitoria* extracts have also been found to be active against *Salmonella typhi, Klebsiella pneumonia, Pseudomonas aeruginosa* (Oseni *et al.,* 2014). Teratogenic activity and adverse effects in all the tissues of rodents have been reported (Akanji *et al.,* 2013; Eluwa *et al.,* 2013).

Figure 2.8: *Rauwolfia vomitoria* leaves and fruits
(https://www.naturalmedicinefacts.info/plant/rauvolfia-vomitoria.html)

- ***Tabernanthe iboga* Baill.**

T. iboga is an evergreen shrub of about 4 m in height and native to western Central Africa. The leaves are small and green with white to pink flowers. The orange fruits are oval or round (Figure 2.9). In Gabon, roots are mainly used for initiation ceremonies and can give hallucinations. The roots can be used to relieve hunger, thirst and tiredness but are also taken as aphrodisiacs and febrifuge or to treat coughs, urinary infections, and conjunctivitis. The leaves are anesthetic aphrodisiacs, and are used against toothache (PROTA, 2008). The latex is antiparasitic. About 20 alkaloids including ibogaine, noribogaine have been isolated (Bading *et al.,* 2018).

Figure 2.9: *Tabernanthe iboga* aerial parts and fruits
(https://commons.wikimedia.org/wiki/File:Tabernanthe_iboga_MS_4098.jpg#file)

BURSERACEAE

***Aucoumea klaineana* Pierre.**

Aucoumea klaineana Pierre also known as Okoume (Figure 2.10) in Gabon, is endemic to Gabon, Guinea equatorial and Congo but can also be found in the South of Cameroon. It has been introduced to several countries including Ivory Coast, Indonesia and Malaysia. Okoume is a dioecious, medium-sized to large evergreen tree with a cylindrical bole, often contorted and bent. The bark is lightly fibrous and when cut, exudes a strong-smelling resin which becomes opaque during coagulation (PROTA, 2013). The resin is widely used in Gabon for torches, incense, oil lamps and also for cosmetic applications in skin care products and nail polish. For traditional medicine purposes, *A. klaineana* bark is used to treat abscesses, menstrual disorders, diarrhea and dysentery while the resin is applied to superficial wounds, sores, abscesses and is also used as a water disinfectant (Medzegue *et al.,* 2013). The roots and leaves are used to treat fever, constipation, malaria, diarrhea and jaundice (Obame *et al.,* 2014). Studies on the pharmacological potential of this species is mainly focused on the properties of the volatile components extracted from the resin.

Figure 2.10: *Aucoumea klaineana* leaves and inflorescences (http://tropical.theferns.info/image.php?id=Aucoumea+klaineana)

It has been shown to have an essential oil which contains *p*-cymene, terpinolene, terpineol, α-pinene, β-pinene, limonene, α-phellandrene, β-phellandrene, 3-carene, α-terpineol and eucalyptol (Medzegue *et al.,* 2013; Koudou *et al.,* 2009). This essential oil has displayed some antioxidant activity with a weak DPPH radical scavenging activity and lipid peroxidation inhibition (Koudou *et al.,* 2009). Obame et al. (2014) has

also demonstrated the antimicrobial activity of this essential oil from the resin against several bacterial and fungal strains.

CECROPIACEAE

***Musanga cecropioides* R. Br. ex Tedlie.**

M. cecropioides is a fast-growing and with a short-life spun tree that reaches a height of 45 m with prop roots that are 2-3 m tall (Figure 2.11). The trunk is rough and whitish-yellow while its fruit is yellowish-green and has a succulent flesh and small seeds. *M. cecropioides* is found in many African countries including Equatorial Guinea, Togo, Ethiopia, Gabon and Ghana. This plant is traditionally used as treatment for dysmenorrhea, asthenia, appetite loss, pulmonary diseases, toothache, lumbago, wounds, fever, vaginal disorders and leprosy. The sap from the larger roots is drunk as a galactagogue, blood-purifier, to clean the stomach, and against gonorrhea, coughs and chest infections.

Figure 2.11: *Musanga cecropioides* aerial parts (http://carnetsdevoyages.jeanlou.fr/Autour_du_GABON/Photos_Piste_Kougouleu-Mitzic/files/page351-1020-full.html)

Stem bark and leaves possess saponins, flavonoids, alkaloids, tannins, phlobatannins, glycosides, reducing sugars and anthraquinones (Adeneye *et al.,* 2006). Extracts prepared from the different parts of the plant have shown various biological activities like antibacterial (Fomogne-Fodjo *et al.,* 2014), antidiarrheal (Owolabi *et al.,* 2010), hepatoprotective, antidiabetic, hypotensive (Adeneye, 2009; Adeneye, 2006), oxitocic (Ayinde *et al.,* 2003), antioxidant (Tchouya and Nantia, 2015), diuretic (Ajagbonna *et al.,* 2015) and anti-inflammatory (Sowemimo, 2015). Toxicity studies revealed a LD_{50}>3000mg/kg (Adeneye *et al.,* 2006).

COMPOSITAE

***Vernonia conferta* Benth.**

V. conferta is a shrub or a tree of 9 m in height (Figure 2.12). It can be found in Nigeria, Central Africa, Uganda, Ghana, Gabon and Angola. It is used as an aphrodisiac, diuretic, antiparasitic and tonic (PROTA, 2008). People in many countries including Gabon take *V. conferta* to treat abscesses, bronchitis, stomachache, diarrhea, jaundice, constipation, abdominal pains, coughs and wounds. Mengome et al. (2010) reported antiparasitic activity against Loa-Loa worm and a cytotoxic effect on Vero cells.

Figure 2.12: *Vernonia conferta* leaves (https://www.verticalgardenpatrickblanc.com/inspiration/open-areas?page=3).

COSTACEAE

***Costus lucanusianus* J. Braun.**

C. lucanusianus is a rhizomatous herb up to 3 meters tall (Figure 2.13) found in Tropical western Africa and from East Guinea to western Ethiopia. The traditional uses of this plant include the cure for respiratory tract infections, rheumatism, venereal diseases, parasites, and pain. The inflorescence is used to treat tachycardia and stomach complaints. Its stem sap or fruit is used to treat sore throat, bronchitis and cough whereas its leaf sap is used as eye drops for eye troubles and headache, and in frictions to treat edema and fever (Betti *et al.,* 2013; Termote, 2012).

Figure 2.13: *Costus lucanusianus* leaves and inflorescences (http://www.gingersrus.com/images/ImageFrame.php?ImageID=11153)

Its stem sap is applied to treat urethral discharges, venereal diseases and jaundice, and to prevent miscarriages (Vliet, 2012). *C. lucanusianus* displays many pharmacological activities such as anti-inflammatory, anti-abortive and anti-diarrheal activity (Owolabi *et al.,* 2010). Anti-hyperglycemic, hepatoprotective (Saliu and Fapohunda, 2016), anti-microbial (Baba and Onanuga, 2011), oxytocic (Owolabi *et al.,* 2007) and antinociceptive activities have also been demonstrated (Owolabi and Nworgu, 2009). *Costus lucanusianus* possesses phytochemical compounds such as tannins, saponins, reducing sugars and carbohydrate, myricetin, and flavonols (Kumar *et al.,* 2010). A recent study done on the stem revealed the presence of cardiac glycosides, steroids, alkaloids, flavonoids and terpenoids (Traore *et al.,* 2017).

EUPHORBIACEAE

- ***Alchornea hirtella* Benth.**

A. hirtella is a climbing to straggling shrub or tree that can reach a height of about 15 m (Figure 2.14). This species is native to Central and Southern Africa and can be found in Senegal, Angola, South Africa, Uganda and Kenya. The medicinal uses are multiple and include cure for diarrhea, toothache, stomach ache, worms, headaches and general pain. Previous phytochemical screening revealed the presence of saponins, alkaloids, tannins, flavonoids, sterol and terpenes (Koroma and Ita, 2009). The antimicrobial activity against *Staphylococcus aureus, Streptococcus pyogenes, Proteus vulgaris,* and *Escherichia coli* have been demonstrated (Koroma and Ita, 2009).

Figure 2.14: *Alchornea hirtella* aerial parts (https://www.zambiaflora.com/speciesdata/image-display.php?species_id=134950&image_id=2)

- ***Macaranga monandra* Müll. Arg.**

M. monandra is a shrub or a tree that can reach 25 m tall (Figure 2.15). It is found in Angola, Burundi, Cameroon, Central African Republic, Equatorial Guinea, Gabon and Nigeria. A decoction of the bark of its stem is taken as a galactagogue for the treatment of sterility, dyspnea, in case of threatened abortion. Bioassay-guided fractionation led to the isolation of two clerodane-type diterpenes (Salah *et al.,* 2003). *M. monandra* displayed antifungal activity against *Colletotrichum acutatum*, *C. fragariae, C. gloeosporioides, Fusarium oxysporum, Botrytis cinerea*, *Phomopsis obscurans*, and *P. viticola* (Salah *et al.,* 2003).

Figure 2.15: *Macaranga monandra* leaves and fruits (http://tropical.theferns.info/image.php?id=Macaranga+monandra)

- ***Ricinodendron africanum* Müll.Arg.**

R. africanum synonym *R. heudelotii* is a deciduous tree with a fairly open, spreading, rounded crown reaching 50 m tall (Figure 2.16). It is found in several African countries including Angola, Cameroon, Gabon, Liberia, Senegal, Sudan and Tanzania. All the tree parts are used in traditional medicine. The bark from its stem is taken to prevent abortion and used externally to wash and cicatrize sores. The bark from its root is used as an anti-dysenteric but also to treat constipation. The bark is used in the treatment of gonorrhea, coughs, painful menstruation, it is an antidote to poison, rheumatism, edemas, elephantiasis and fungal infections. The leaves are used to counteract female sterility, dysentery, stomach-pains and edemas and the seeds for treating diarrhea, dysentery and gonorrhea. *R. africanum* contains tannins, steroids, terpenoids, alkaloids, flavonoids, cardiac glycosides, reducing sugars and saponins (Uzoekwe and Hamilton, 2016). Studies on this specie have revealed its antiparasitic, antioxidant and anti-microbial activities (Tekwu *et al.,* 2012; Momeni *et al.,* 2010).

Figure 2.16: *R. africanum* leaves and fruits (https://www.prota4u.org/database/protav8.asp?h=M26&t=Ricinodendron,Ricinodendron_heudelotii&p=Ricinodendron+heudelotii#MajorReferences)

FABACEAE

- ***Copaïfera religiosa* J.Leonard.**

C. religiosa is a tree of 46 m in height (Figure 2.17). This plant is found in several countries including Gabon, Cameroon, Congo, Central African Republic and the Democratic Republic of the Congo. *Copaifera religiosa* is used for the treatment of malaria in Gabon. According to Brink (2012), *C. religiosa* is used to treat cardiovascular disorders, stomach ache, respiratory tract infections, pain and coughs.

Lekana-Douki et al. (2011) showed its anti-plasmodial activity. A study by Traore et al. (2017) revealed the presence of phenolics, cardiac glycosides, tannins, saponins, steroids, alkaloids, flavonoids and terpenoids in the bark.

Figure 2.17: *Copaifera religiosa* aerial part of the tree (http://carnetsdevoyages.jeanlou.fr/Autour_du_GABON/Photos_Piste_Kougouleu-Mitzic/files/page351-1020-full.html).

- ***Pterocarpus soyauxii* Taub.**

P. soyauxii also named Padouk in Gabon is a tree reaching 50 m tall (Figure 2.18). The valuable wood is bright red at first, becoming orange-red when exposed to light and to darkness become purple-brown. It is found in Central Africa (Cameroon, Nigeria, Gabon, Equatorial Guinea, and Congo) and can also be found in Angola. The heartwood is exported mainly from Gabon and Cameroon as a timber but also as a dye (food, clothes and cosmetics). *P. soyauxii* parts are used to treat wounds, skin diseases, yaws, ringworm, dysentery, toothache, gonorrhea, excessive menstruation, uterine hemorrhage, dysentery, hemorrhoids, inflammation, edemas and broncho-pulmonary affections. Osuagwu and Akomas (2013) demonstrated the antimicrobial activity on *Staphylococcus aureus, E. coli, Salmonella typhi, Shigella flexnerii, Klebsiella pneumonia,* and *Candida albicans*. *P. soyauxii* extracts have been found to be safe on rodents with a LD_{50} >10.75 g/kg (Tchamadeu *et al.,* 2011).

Figure 2.18: *Pterocarpus soyauxii* leaves, wood and bark (http://avandje.com/wp-content/uploads/2017/12/padouk-arbre-1.jpg)

HUMIRIACEAE

***Sacoglottis gabonensis* (Baill.) Urb.**

S. gabonensis is a large evergreen tree that can grow up to 40 m or taller (Figure 2.19). The tree is exploited for its timber used locally and also exported. The species stretch from Senegal and the east of the Gambia to the Central African Republic and south of Angola. *S. gabonensis* is generally taken to treat dermatitis, fever, stomach-ache, diarrhea, vaginal infections, gonorrhea and abdominal pains. It can also be used as an antihypertensive, anti-diabetic, emetic and aphrodisiac. Studies on its phytochemical composition revealed the presence of phenol, saponin, alkaloid, oxalate, cyanogenic glycosides, flavonoids and tannins (Tchouya *et al.,* 2016; Ejikeme *et al.,* 2014). The pharmacological properties demonstrated so far are anthelmintic, antioxidant and antimicrobial activity (Tchouya *et al.,* 2015; Maduka and Okoye, 2002). The acute toxicity from its aqueous stem bark extract produced varying degrees of toxicity manifested through depression, drowsiness, unsteady gait, paralysis of the hind limbs, dyspnea, coma and death of the rodents tested (Nwosu *et al.,* 2008).

Figure 2.19: *Sacoglottis gabonensis* leaves and fruits (http://tropical.theferns.info/plantimages/6/1/61363b562f0e05eac108fff3b5ae29fad74e4ff5.jpg)

LOGANIACEAE

Anthocleista vogelii Planch

A. vogelii is an evergreen tree of 20 m in height (Figure 2.20). The tree supplies timber and materials for dyeing and making soap. It is distributed in various African countries such as Sudan, Sierra Leone, Gabon, Kenya, Tanzania and Zambia. *A. vogelii* is used as diuretic and purgative and the other traditional medication qualities that it possesses can treat hepatitis, venereal diseases, leprosy, hernia, elephantiasis, bronchitis, jaundice, fevers, edemas, stomach-ache, ovarian problems as well as the inducement of labor as an abortifacient. It contains carbohydrates, saponins, flavonoids, terpenes, sterols, and phenols (Jegede *et al.,* 2011). Different parts of the plant have demonstrated through several studies (Iroanya *et al.,* 2015; Osadebe *et al.,* 2014; Ateufack *et al.,* 2014; Nguessom *et al.,* 2013; Alaribe *et al.,* 2012) to have properties of anti-ulcer, hematopoietic, hepatoprotective, antioxidant, hypoglycemic, antimicrobial and analgesics.

Figure 2.20: *Anthocleista vogelii* leaves and fruits
(http://phytoimages.siu.edu/imgs/pelserpb/r/Gentianaceae_Anthocleista_vogelii_60945.html0\)

MYRISTICACEAE

- ***Staudtia kamerunensis* Warb.**

S. kamerunensis, also known as *S. gabonensis,* is a tree that can grow up to 35 m tall (Figure 2.21). The bark has a characteristic reddish grey or cinnamon-color while the heartwood is orangey yellow brown to red brown with darker veins and sometimes an oily surface. The species is found to stretch from Nigeria to Central African Republic, DR Congo, Uganda, Angola, and Zambia. It is used to treat anemia, skin disorders, malaria, scabies and hemorrhage. A chemical investigation of the bark and seeds led to the isolation of lignans and glycerol tritetradecanoate (Yankep *et al.,* 1999). Lekana-Douki et al. (2011) revealed it as a potential anti plasmodial.

Figure 2.21: *Staudtia kamerunensis* leaves and fruits
(https://www.flickr.com/photos/juliedewilde/6920905951/in/photostream/)

- ***Scyphocephalium ochocoa* Warb.**

S. ochocoa syn. *S. mannii,* is an evergreen and dioecious tree that reaches to about 45 m tall. It is found in south-eastern Nigeria stretching to Gabon through to Congo (Figure 2.22). It is used to treat abscesses, anemia, general fatigue, women sterility, respiratory tract infections, convulsions and gonorrhea. Phytochemical screening of it revealed the presence of flavonoids, tannins, coumarins, terpenoids, anthocyanins, saponins and sterols. Antioxidant and antimicrobial activities of the extracts were also demonstrated (Tchouya *et al.,* 2015).

Figure 2.22: *Scyphocephalium ochocoa* infloresence
(https://twitter.com/hsauquet_rbgsyd/status/720987673838673922)

OLACACEAE

***Coula edulis* Baill.**

C. edulis is an evergreen tree growing up to about 38 m (Figure 2.23). It is also known as a Gabon nut. It produces an ellipsoidal nut enveloped in a very hard shell and the kernel is oily and tastes like chestnut or hazelnut. *C. edulis* is found in Sierra Leone, Liberia and also in Central Africa which includes Gabon, Cameroon, Nigeria and Ghana. It is widely utilized to treat parasites, diarrhea, dysentery, women problems, skin disorders and stoma-ache. Saponins, alkaloids, tannins, flavonoids, cardiac glycosides, anthraquinone and terpenes have been detected in this species (Bukola *et al.,* 2008) while the antiplasmodial, antidermatophytic and antimicrobial activities have been demonstrated (Tamokou *et al.,* 2011; Zofou *et al.,* 2011; Bukola *et al.,*

2008). Whereas studies by Tamokou et al. (2011) have revealed that *C. edulis* exerted some toxic effects on rodents' liver and kidney.

Figure 2.23: *Coula edulis* shrub and nuts (https://www.sciencenewsforstudents.org/blog/eureka-lab/solving-bad-breath-one-walnut-time)

PANDACEAE

***Microdesmis puberula* Hook.f. ex Planch.**

M. puberula is a shrub or a small tree up to 15 m tall (Figure 2.24 below). The species are found in Nigeria, Central African Republic, Uganda, DR Congo and Gabon. It is generally used against venereal diseases, pain, diarrhea, fever, respiratory tract infections, rheumatism, cough, snakebites, stomach-ache, intestinal worms, malaria, tumors, skin and genital problems. Phytochemical screening indicated the presence of saponins, cardiac glycosides, deoxysugars, alkaloids, terpenes and polyamine (Akpanyung *et al.,* 2013; Roumy *et al.,* 2008). *M. puberula* has been found to exert analgesic and anti-stress effects and general toxicity when used via the intra-peritoneal route (LD_{50}= 1412.50 mg/kg) (Okany *et al.,* 2012).

Figure 2.24: *Microdesmis puberula* aerial parts (http://tropical.theferns.info/image.php?id=Microdesmis+puberula)

PASSIFLORACEAE

Adenia cissampeloides Planch. ex Hook.

Adenia cissampeloides is a robust, climbing plant producing stems up to 30 meters long (Figure 2.25 below). The plant climbs into the surrounding vegetation, attaching itself by means of tendrils. *A. cissampeloides* grows in many African countries such as Liberia, Kenya, Tanzania, Zimbabwe, Botswana, Gabon, DR Congo and Ethiopia. It is traditionally used widely to treat malaria; venereal diseases; fever; respiratory tract infections; cancer; GIT infections; pain; diarrhea and dysentery; edema; inflammation; rheumatism; leprosy; pain; wounds; mental disorders and anemia. Chemical compounds including saponins, alkaloids, tannins, flavonoids and steroids have been detected in the plant (Okunye *et al.,* 2015). This plant has been widely studied and has been proven to exert anti-depressant; anxiolytic; anti-plasmodial; analgesic and anti-microbial activities (Ishola *et al.,* 2015; Okunye *et al.,* 2015; Adebiyi *et al.,* 2013; Annan *et al.,* 2012).

Figure 2.25: *Adenia cissampeloides* leaves
(http://tropical.theferns.info/viewtropical.php?id=Adenia+cissampeloides)

PHYLLANTHACEAE

***Uapaca guineensis* Müll.Arg.**

This species is an evergreen tree with a dense low-branching crown. It grows up to 30 m tall, but its specimens of up to 50 m have been recorded (Figure 2.26 below). It is distributed through the moisten regions of Central Africa from Sierra Leone to south Tanzania through Angola, Zambia and Zimbabwe. Different parts of *U. guineensis* are used in traditional medicine. The leaves serve to mature furuncles and relieve migraine and rheumatism. Roots and bark are taken to treat edemas and gastro-intestinal troubles. Bark decoctions are also used as a treatment for female sterility, nasal cancer, tooth-troubles and rheumatism. Its root bark is used in the treatment of skin disorders such as leprosy, zona and varicella. The roots are considered as anti-abortion but also to treat rhino-pharyngeal and pulmonary infections and fever-pains. It is used as an expectorant and aphrodisiac, and as a good treatment for male impotence. Chemical screening showed the presence of alkaloids, flavonoids, glycoside, phlobatannins, tannins, terpenoids, saponins and steroids (Ezeonu and Ejikeme, 2016). *U. guineensis* has been found to display analgesic, anti-inflammatory and antiulcer activities (Nkeh-Chungag *et al.,* 2009).

Figure 2.26: *Uapaca guineensis* leaves and fruits
(http://www.westafricanplants.senckenberg.de/root/index.php?page_id=14&id=3074)

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CHAPTER THREE

Phytochemical composition, anti-oxidative activities and cytotoxicity of selected Gabonese medicinal plants

Boukandou Mounanga MM, Anokwuru Chinedu, Sigidi Muendi, Traore Afsatou Ndama and Potgieter Natasha; Quantitative phytochemical analysis, cytotoxicity and antioxidant properties of seven medicinal plants used in Gabon for the management of HIV-related opportunistic infections. **BioMed Research International [Appendix III].**

The following article is in preparation.

Boukandou Mounanga MM, Anokwuru Chinedu, Traore Afsatou Ndama and Potgieter Natasha; Phytochemical studies, *in vitro* antioxidant and antiproliferative activities of *Aucoumea klaineana, Macaranga monandra* and *Uvaria klainei* aqueous and methanolic extracts from Gabon

CHAPTER 3

ABSTRACT

HIV infection and antiretroviral therapy lead to pronounced oxidative stress. Researchers believed that antioxidant supplementation may suppress HIV viral loads. Natural products such as plant species are widely used worldwide to alleviate pain, infections, and chronic diseases such as cancer and HIV. The aims of the present chapter are thus to screen for the phytochemical composition; evaluate the antioxidant capacity and assess the cytotoxicity of 20 selected plants. **Methods:** Extraction of the active compounds from the 20 plants collected was performed using water and methanol (100%). Phytochemical investigation was done using standard biochemical testings and determination of TPC/TFC using Folin-Ciocalteu reagent and aluminium chloride colorimetric methods respectively. The biological activities were evaluated using free radical scavenging and reducing power methods for antioxidant activity; MTT assay and quantitative automated fluorescence microscopy with dual staining method on Vero and HeLa cells for cytotoxicity. **Results**: The phytochemical screening of the 40 extracts generated revealed that both aqueous and methanol extracts *of C. edulis; M. cecropioides; S. ochocoa; R. vomitoria; A. hirtella; S. gabonensis; U. klainei; P. soyauxii;* and *U. guineensis* possessed saponins; alkaloids; phenols; tannins; terpenoids; flavonoids; steroids; and cardiac glycosides. The results also indicated that the highest amount of TFC and TPC were detected in methanol extracts of *S. kamerounensis* (4.21±0.31 mg QE/g) and *M. cecropioides* (12.13±0.27 mg GAE/g) respectively. *C. edulis* aqueous extract demonstrated the highest antioxidant activity characterized by the lowest IC_{50} (5.37±1.32 µg/mL) and EC_{50} (58.24±1.31 µg/mL). *A. klaineana* and *T. iboga* were the most toxic on Vero cells with MTT and dual staining/fluorescence microscopy technics. Several plant such as *U. klainei*, *U. guineensis*; *P. soyauxii*; *A. klaineana* and *V. conferta* showed interesting activities against HeLa cells. **Conclusion:** The study has demonstrated that the studied plants possessed interesting antioxidant and anticancer activities. Plants in this study which displayed significant antioxidant activity could be used as supplement for HIV individuals to deal with the oxidative stress induced by HIV and HAART.

Key words: Antioxidant, cytotoxicity, HIV, plant extracts, phytochemical composition.

3.1 INTRODUCTION

The aims of the present chapter were to screen for the phytochemical composition; evaluate the antioxidant capacity and assess the cytotoxicity of the selected plant extracts using respectively biochemical testing, both free radical scavenging and reducing power assays, MTT assay and quantitative automated fluorescence microscopy with dual staining. In general, an ideal antioxidant should be able to scavenge free radicals and chelate redox metals at physiologically relevant levels. The assays conducted aimed at determining the ability of the selected plants to both scavenge free radicals and reduce physiological metal like iron. These two mechanisms could be of interest for people presenting a redox imbalance caused by HIV and ARVs and could be an explanation for the health improvement observed among HIV people who consume those plants.

Cytotoxicity is a global word including both safety and toxic effects of a compound. The safety is about ensuring that the compound under investigation doesn't harm host cells, especially the normal ones. While, the toxic effect is about compounds which can induce the death of normal or cancer cell lines by directly killing them (cytotoxicity) or by stopping their proliferation (antiproliferative effect or cytostasis). In the present study, the cytotoxicity is explored using two different methods; MTT whose principle is to determine the number of viable cells (metabolic activity as a measure of relative cell viability) and fluorescence microscopy with double staining which is more accurate as it will differentiate dead cells from living cells**.** The two methods were used for comparative purpose. Because of the high number of extracts (40), the IC_{50} couldn't be performed thus, only two concentrations of each extract were used in quadruplicate in the experiments.

3.1.1 PHYTOCHEMICAL COMPOSITION OF PLANTS

Interest in plants is constantly increasing because of their importance in pharmaceutical, nutritional and cosmetic application due to the chemicals they contain. Phytochemicals are non-nutrient, bioactive chemical compounds that are produced by plants and within biological systems, they act as prolific dietary antioxidants and render anticarcinogenic; anti-inflammatory; cardioprotective and immunity boosting effects (Aggarwal and Sharma, 2017). These chemicals are present in plant organs such as roots, seeds, leaves, stems, bark, flowers, as well as fruits and include alkaloids,

steroids, tannins, glycosides, volatile oils, fixed oils, resins, phenols and flavonoids (Njila *et al.,* 2017). To date, more than 4000 chemicals from plants have been reported and classified based on their protective function and physicochemical characteristics (Jadaun *et al.,* 2016).

Phytochemicals possess a wide spectrum of biological effects. For instance, flavonoids and phenolic acids have been proven to display numerous properties such as antidiabetic; antimicrobial; antiulcer; anti-inflammatory; antioxidant; cytotoxic and antitumor; antispasmodic; and antidepressant (Ghasemzadeh and Ghasemzadeh, 2011). Tannins are part of the phenolic compounds and possess diverse structures that share abilities to bind and precipitate proteins. Their anti-microbial; antioxidant; anti-parasitic; anti-inflammatory; antiviral and immunomodulation properties are extensively documented (Huang *et al.,* 2018). Alkaloids constitute an important class of structurally diversified compounds and form about 20% of plant based secondary metabolites. This class of chemicals exerts various activities like antimalarial; antioxidant; antimicrobial; anthelmintic; anti-hyperglycemic; antitumor; anti-histaminic; central nervous system stimulant and depressant; cardiotonic; anticonvulsant; anti-inflammatory and analgesic (Kaur and Arora, 2015). Alkaloids are also well known for their toxicity (Street *et al.,* 2017).

Saponins' biological, medical and pharmacological properties such as antiviral; hemolytic; anti-inflammatory; antimicrobial; immunoadjuvants; anticancer; insecticides and molluscicides are due to the wide structural diversity which are in addition to features such as being bitter sweeteners, detergents and emulsifiant (Barbosa, 2014). Steroids have the fundamental structure of four carbon rings called the steroid nucleus to which the addition of different chemical groups at different positions leads to the formation of many different types of steroidal compounds including progesterone and testosterone (sex hormones); corticosteroids (anti-inflammatory); digoxin and digitoxin (cardiac steroids); cholesterol and steroidal glycosides (Patel and Savjani, 2015). Cardiac glycosides are specific steroids with the ability to exert a particular action on the cardiac muscle, but also on tumor and viruses (Morsy, 2017). In addition, many biological activities related to steroids include antimicrobial, antitumor, anti-inflammatory, hepatoprotective, immunosuppressive and antiviral (Patel and Savjani, 2015). All terpenoids are synthesized from two to five-carbon building blocks and based on the number of the building blocks, terpenoids are commonly classified as

monoterpenes, sesquiterpenes, diterpenes, and sesterterpenes. These terpenoids display a wide range of biological activities against cancer, malaria, inflammation, and a variety of infectious diseases (viral and bacterial) (Wang *et al.,* 2005). This panel of phytochemicals are quite essential to the composition of new therapeutic molecules that can be used in treating diseases and infections such as HIV.

3.1.2 ANTI-OXIDATIVE ACTIVITY OF PLANTS

Oxidative stress is defined as an imbalance between the production of reactive oxygen and nitrogen species (ROS and RNS) and antioxidant defence of the organism. Stress; physical damage; viral infection; cytotoxic or carcinogenic compounds may cause peroxidation of cell membrane lipids and liberation of free radicals and oxygen species (Rahal *et al.,* 2014). These toxic substances cause oxidative damage to lipids, proteins and nucleic acids. ROS are crucial in many biological processes including host defence, signal transduction and gene expression (Droge, 2002). But when the levels of ROS increase and cannot be neutralized, they damage biological molecules, alter their functions and act as signalling molecules thus generating a spectrum of pathologies (Ivanov *et al.,* 2016). As a result, they have been implicated in the pathogenesis of many ailments like cardiovascular diseases; cancer; immune/autoimmune diseases; AIDS; inflammation and brain dysfunction (That is: Parkinson's, Alzheimer's and Huntington's diseases) (Khatua *et al.,* 2013).

It is now known that HIV infection and antiretroviral therapy lead to pronounced oxidative stress. HIV-induced oxidative stress plays an important role in the development of a wide spectrum of pathologies including neurotoxicity; dementia; immune imbalance; lung and cardiovascular disorders (Ivanov *et al.,* 2016). For several years, researchers believed that antioxidant supplementation may suppress HIV viral loads, thereby restoring the immune functionality and potentially slowing down the progression of AIDS (Kashou and Agarwal, 2011). Humans have naturally developed highly complex antioxidant systems (enzymatic and non-enzymatic), which work synergistically, and in combination with each other to protect the cells and organ systems of the body against free radical damage (Qusti *et al.,* 2010). The antioxidants can be endogenous or obtained exogenously as part of diet or as supplements. Some dietary compounds that do not neutralize free radicals but instead enhance

endogenous activity may also be classified as antioxidants (Qusti *et al.,* 2010. Endogenous antioxidants play an essential role in maintaining optimal cellular functions and thus ensure systemic health and well-being. However, under some conditions that promote oxidative stress, endogenous antioxidants may not be sufficient and dietary antioxidants may be required to maintain optimal cellular functions (Sandro *et al.,* 2009). The most efficient enzymatic antioxidants involve glutathione peroxidase, catalase and superoxide dismutase. Non-enzymatic antioxidants include Vitamin E and C; thiol antioxidants (glutathione); melatonin; carotenoids; natural flavonoids; and other compounds (Rice-Evans *et al.,* 1996).

A number of methods for the evaluation of antioxidant capacity of foods; beverages; medicinal plants and different natural substances do exist. Based on different principles, these are: peroxyl radical scavenging (Oxygen Radical Absorbance capacity; ORAC); Total radical-trapping antioxidant power (TRAP); metal reducing power (ferric reducing antioxidant power, FRAP); Cupric reducing antioxidant power (CUPRAC); Hydroxyl radical scavenging (deoxyribose assay); organic radical scavenging (2,2-Azino-bis(3-ethylbenz-thiaz-oline-6-sulfonic acid, ABTS); free radical scavenging (2,2-Diphenyl-1-picrylhydrazyl, DPPH); Quantification of the products formed during the lipid peroxidation (Thio- barbituric Acid Reactive Substances, TRAPS); and low-density lipoproteins (LDLs) oxidation (Pérez-Jiménez and Saura-Calixto, 2008). The most widely-used procedures for measuring antioxidant capacity are: DPPH; RP (reducing power); ABTS; FRAP; TEAC (Trolox equivalent antioxidant capacity); and ORAC (Pérez-Jiménez *et al.,* 2008).

The DPPH method is a rapid, simple, accurate and convenient assay for measuring the ability of various compounds to act as free radical scavengers or hydrogen donors as well as to evaluate the global antioxidant activity of different substances such as medicinal plants. The method is unique in carrying out the reaction of the sample with DPPH in methanol or ethanol/water, which facilitates the extraction of antioxidant compounds from the sample. The antioxidant effect is proportional to the disappearance of DPPH radical in test samples. The colour turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant (Figure 3.1). The antioxidant effect can be evaluated by following the decrease of absorption at 517 nm. The IC_{50} (inhibitory concentration) for the interpretation of the results is defined as the concentration of substrate that causes 50% reduction in the

DPPH colour. It is obtained on the basis of the percentages of inhibition of DPPH displayed by a decrease in the absorbance (Kedare and Singh, 2011; Marinova and Batchvarov, 2011).

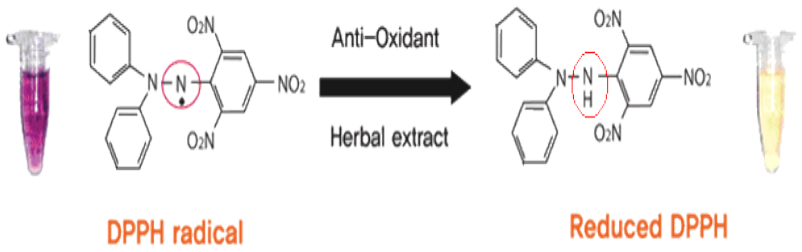

Figure 3.1: Principle of DPPH radical scavenging capacity assay. (http://radio.cuci.udg.mx/bch/EN/Antioxid.html)

On the other hand, the ferric reducing power constitutes as an equal good indicator of the antioxidant capacity of a natural product. The principle of this specific assay is that substances that have reduction potential will react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 595 nm (Figure 3.2). In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. The presence of antioxidant causes the conversion of the Fe^{3+} / ferricyanide complex used in this method to the ferrous form. Therefore, by measuring the formation of pearls Prussian blue, the Fe^{2+} concentration can be monitored; a higher absorbance at 700 nm indicates a higher reducing power (Hemalatha *et al.,* 2010).

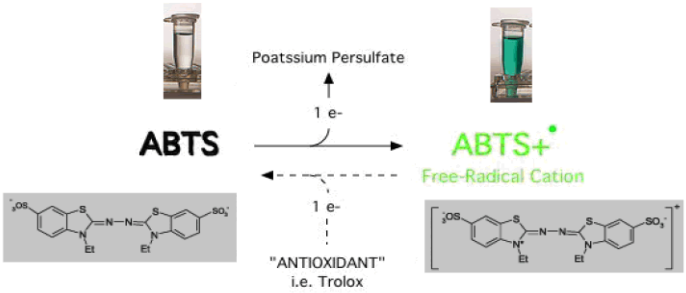

Figure 3.2: Principle of the ferric reducing power assay (Bolignon et al., 2014)

3.1.3 CYTOTOXIC EFFECTS OF PLANT EXTRACTS

It has been established that HIV infects and leads to the death of lymphocyte T-cells (Krasner, 2008). T-cells usually destroy cells infected with cancer-causing viruses. This function of T-cells helps to stop the infections thus inhibiting the development of cancer. People with HIV have low levels of T-cells, significantly lowering the action of T-cells against infections. As a result, viruses like Kaposi's Sarcoma Herpes virus (KSHV); Human Herpes virus-8 (HHV-8); Epstein-Barr virus (EBV) and Human papillomavirus (HPV) have the capacity to make cells grow and divide out of control (NIH, 2018) leading to cancer. The risk of developing certain types of cancer generally called HIV-associated cancers is substantially higher for HIV infected people compared to uninfected people of the same age (Grulich *et al.,* 2007). Kaposi sarcoma; aggressive B-cell non-Hodgkin lymphoma; and cervical cancer are recognized as acquired immunodeficiency syndrome (AIDs)-defining cancers or AIDS-defining malignancies (NIH, 2018). Furthermore, according to a study in the United States of America, HIV infected individuals have a high risk of developing several other types of cancer known as non–AIDS-defining cancers (Hernandez-Ramirez *et al.,* 2017). These other malignancies include Hodgkin lymphoma; anus; liver; pharynx; and lung cancers.

The compromised immune system of HIV infected people is the reason why both immunosuppression and inflammation have direct or indirect roles in the development of some cancers that are elevated in people infected with HIV.

Unfortunately, according to the National Institute of Health (NIH, 2018), the introduction of the chimeric antigen receptor T-cell therapy (CART) has not reduced the incidence of all HIV-related cancers. In fact, there has been an increase in non–AIDS-defining cancers. Experimental data has suggested that certain antiretroviral agents, such as protease inhibitors (PI), may have anti-carcinogenic properties including cell cycle arrest and cell death induction (Chao *et al.,* 2012). Current cancer treatments include chemotherapy, radiotherapy including chemically derived drugs that can put patients under a lot of strain and further damage their health (Greenwell and Rahman, 2015). Therefore, scientists are focusing on the search for alternative treatments and therapies against cancer that can be more effective and less harmful.

Natural products such as plant species are widely used worldwide to alleviate pain, infections, and chronic diseases. This ability is attributed to the secondary metabolites they produce that apparently have no direct role in the plants' growth (Kooti *et al.,* 2017). Such metabolites include alkaloids, phenolic compounds, saponins, and pigments that have been proven to exert a wide spectrum of biological effects in the human system like antibacterial; antiviral; anti-inflammatory; hepatoprotective; cardiotonic; immune-stimulant; anticancer and antioxidant. Nowadays, more than 60% of anticancer compounds are obtained from herbal, marine, and microorganism sources (Kootie *et al.,* 2017).

Medicinal plants have been used in many countries especially in China as treatment for several cancers such as prostate, lung, breast and liver cancers and different improvements have been reported (Zaid *et al.,* 2017; Yin *et al.,* 2013). Studies based on the anticancer activities of medicinal plants have demonstrated various mechanisms that aim at suppressing cancer cell growth stimulating enzymes; inhibiting DNA damage; repairing DNA; stimulating production of antitumor enzymes in cell; increasing body immunity; stopping cell cycle (especially at the G2/M checkpoint); inducing apoptosis; and inhibiting angiogenesis in tumor cells (Kootie *et al.,* 2017; Greenwell and Rahman, 2015). The positive effect of plant compounds in cancer treatment has been studied extensively and has shown positive results.

Compounds such as curcumin; taxol; boswellic acid; quercetin; catechin; cucurbitacin; kaempferol; thymol; and carvacrol have showed remarkable anticancer effects *in vitro (*Kootie *et al.,* 2017). The anticancer therapy derived from plants can be readily consumed as part of a supplementary diet and because they are isolated from plants, they are in general better tolerated and less toxic to human cells (Unnati *et al.,* 2013). Hence if these drugs can demonstrate non-toxic effects to normal cell lines and show cytotoxicity in cancer cell lines, then they could be used in clinical trials for further testing. The elucidation of mechanisms used by plant-derived drugs to counteract cancer led to the classification of these drugs based on activities; mitotic disruptors; methytransferase inhibitors; DNA damage preventive drugs and histone deacetylase inhibitors (Amin *et al.,* 2009). Therefore, looking into medicinal plants as anticancer agents is a positive step towards efficient and less toxic chemotherapy.

Current *in vitro* cytotoxicity assays represent a well-established model system to preliminary assess natural and synthetic substances for anticancer potential and

safety. The objective of these assays is to monitor the response of a cell when exposed to a specific agent. This response is indicated either by a decrease in the number of viable cells and an increase in non-viable cells (cytotoxicity), or by a decrease in the rate at which the cells proliferate (cytostasis). When exposed to a toxic substance, the body's cells initiate physiological processes depending on the type and level of the injury. In the case where the cells have to fatally die, various different pathways can be launched of which necrosis, apoptosis and autophagy are the most well-known. Alternatively, if the injury is not lethal, the cell may just stop dividing and growing.

The assessment of cell viability is usually performed using several vital dyes such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide); INT (Iodonitrotetrazolium chloride); MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-); and XTT ((2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carbox-anilide)). The principle behind these formazan dyes is that once in the cells, the MTT will be reduced by mitochondrial succinate dehydrogenase to an insoluble, coloured (dark purple) formazan product (Figure 3.3). The cells are then solubilised with an organic solvent (DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

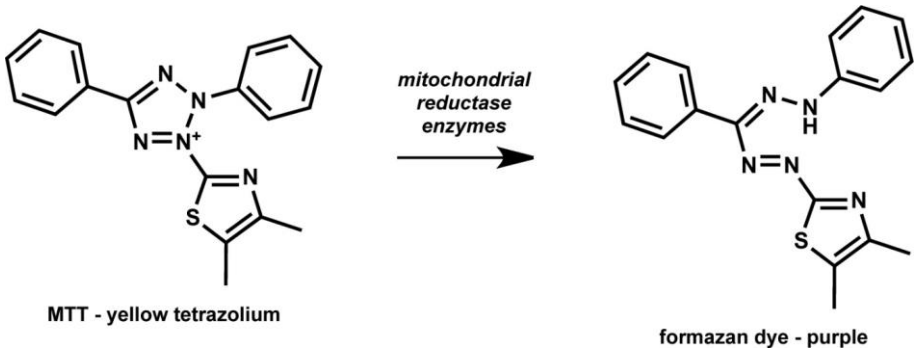

Figure 3.3: Principle of the reduction of Tetrazolium salts to colorful Formazan (http://modernsteroid.blogspot.com/2012/03/potent-antiproliferative-activity-of.html)

The choice of the cells for cytotoxicity assessments is crucial. Different cell culture systems can be used based on their ability to grow attached to a surface (glass, treated plastic substrate) or to float free in the medium. According to what we are focusing on, either the safety or the potential toxic effect of the substance under assessment, healthy or cancerous immortalized cell lines are used and can be either mammalian cells (Vero, HeLa, MeWo, etc.), plant cells (Tobacco BY-2 cells), or insects (C6/36).

It is important to emphasize that while the identification of compounds that induce cytotoxicity in cancer cells is an important initial step in the development of anti-cancer drugs, these compounds need to kill cancer cells at a concentration that does not harm normal cells. In addition, to allow the identification of both cytotoxic and cytostatic compounds, some conditions must be combined such as, the seeding density to ensure that cells are in the log phase of their growth curve during exposure to the samples (e.g. 6000 cells per well), and the drug exposure time (e.g. 48 h) to minimize potential problems such as nutrient deprivation and at the same time allow enough time for at least one population doubling to occur. Therefore, to fully uncover the exact mechanism of cell death and to determine the therapeutic selectivity of promising lead compounds, more studies are required.

3.2 MATERIALS AND METHODS

3.2.1 CHEMICALS AND REAGENTS

Most chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich, Saint 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 (Gauteng, RSA); trichloroacetic acid; di-sodium hydrogen phosphate anhydrous; sodium dihydrogen phosphate and ethanol were obtained from Merck chemical (Kenilworth, NJ; USA). ferric chloride hexahydrate and ascorbic acid were bought from Associated Chemical Enterprise (Gauteng, RSA).

3.2.2 PLANT COLLECTION AND EXTRACTION

3.2.2.1 Selection and collection of plants

Preliminary work entailed the identification of the indigenous plants used in Gabonese traditional medicine for the treatment of common opportunistic infections after an ethnobotanical survey was conducted with local traditional healers. At the end of the survey, 186 species of plants belonging to 70 different families were identified. An index of performance was calculated that allowed the identification of some of the most used plants. Among them, many were already assessed for antimicrobial and anti-HIV activities (Tchouya *et al.,* 2015; Naik *et al.,* 2003). Nevertheless, some plants among those already identified with good performance index were selected along with other plants with unknown anti-HIV activities.

Twenty plants (Table 3.1) were selected and collected all around Libreville in Gabon. These plants were identified and authenticated by Mr Nzabi Thomas, a botanist from the National Herbarium at the Pharmacopeia and Traditional Medicine Institute (IPHAMETRA) in Libreville (Gabon). The collected plant parts were air dried, ground and then stored in a cool dark place till needed at the University of Venda in Thohoyandou (South Africa).

Commented [P1]: After the ethnobotanical survey a literature review was undertaken in order to select plants which were not investigated yet for antimicrobial and antiHIV activities and these authors (Tchouya et al and Naik et al) are among the authors who showed these activities on plants reported in the survey and which were not selected. No voucher numbers were deposited

Table 3.1: Selected plants used in the treatment of OI in Gabon

	Family	Name	Local Name	Localization	Parts collected
1	Annonaceae	*Uvaria klainei* Pierre ex Engl. & Diels.	-	Libreville	Aerial parts
2	Apocynaceae	*Rauwolfia vomitoria* Afz.	Ompepe	Libreville	Leaves
3		*Tabernanthe iboga* Baill.	Dibuga	Libreville	Stem roots
4	Burseraceae	*Aucoumea klaineana* Pierre.	Okumé	Cap	Barks
5	Cecropiaceae.	*Musanga cecropioides* R. Br. ex Tedlie.	Moghómbó	Cap	Barks
6	Compositae	*Vernonia conferta* Benth.	Abingac	Cap	Barks
7	Costaceae	*Costus lucanusianus* J. Braun.	Okosa-kosa	Libreville	Stems
8	Euphorbiaceae	*Alchornea hirtella* Benth.	Mudepa	Kango	Aerial parts

9		*Macaranga monandra* Müll. Arg.	-	Libreville	Barks
10		*Ricinodendron africanum* Müll.Arg.	Mugéla	Cap	Barks
11	Fabaceae	*Copaïfera religiosa* J.Leonard.	Muréï	Kango	Barks
12		*Pterocarpus soyauxii* Taub.	Gisigu	Kango	Barks
13	Humiriaceae	*Sacoglottis gabonensis* (Baill.) Urb.	Ozuka	Cap	Barks
14	Loganiaceae	*Anthocleista vogelii* Planch	Ororo	Cap	Barks
15	Myristicaceae	*Staudtia kamerunensis* Warb.	Mbasisa	Cap	Barks
16		*Scyphocephalium ochocoa* Warb.	Otsoko	Cap	Barks
17	Olacaceae	*Coula edulis* Baill.	Ogula	Kango	Barks
18	Pandaceae	*Microdesmis puberula* Hook.f. ex Planch.	-	Libreville	Aerial parts
19	Passifloraceae	*Adenia cissampeloides* (Planch. ex Hook.) Harms.	Snake climber	Kango	Aerial parts
20	Phyllanthaceae	*Uapaca guineensis* Müll.Arg.	Osambi	Libreville	Barks

3.2.2.2 Extractions

Extraction was performed using water and methanol (100%). Water solvent was selected because water is used by traditional healers for the preparation of the plant-based remedies the objective of selecting water is to be as close as possible to traditional healer preparations. Methanol (100%) was selected as second solvent because methanol is known to extract a high amount of compounds in plants. Briefly, hundred (100) g of the powder of each sample was mixed with 1000 mL of solvent (sterile water or 100% methanol) for 24 hours with constant shaking. Each solution was filtered using Whatman filter paper number 4 (Sigma Aldrich; St Louis, MO; USA).

- The aqueous filtrates were frozen, and water was removed by lyophilization using a FreeZone 2.5 Liter Benchtop Freeze Dryer (LabConco; Kansas City, MO, USA). The obtained powders referred to as crude aqueous extracts were stored until needed.

- The methanolic filtrates were evaporated by rotary evaporation at 50°C using a Buchi Rotavapor R-210 (Büchi Labortechnik AG; Flawil, Switzerland). The crude methanolic extracts obtained were also stored until needed.

Fourty (40) extracts were generated after the extractions, each plant having a methanol and an aqueous crude extract. For the tests, stock solutions of plant extracts were prepared in pure Dimethyl Sulfoxide (DMSO). Stock solutions were diluted to the required test concentrations using assay buffers or culture media appropriate for the relevant assays. The final DMSO concentration never exceeded 0.5% and the vehicle control samples were included in all assays.

3.2.3 DETERMINATION OF PHYTOCHEMICAL COMPOSITION

3.2.3.1 Qualitative phytochemical analysis

The qualitative analysis consisted of colorimetric assays using reagents that show the presence of the desired compounds by colour changes. The screening was performed for terpenoids; steroids; alkaloids; flavonoids; cardiac glycosides; saponins; tannins; and phenolic acids as described by Purena and Bhatt (2018), Harborne (1984) and Farnsworth (1966). The colour intensity or the precipitate formation was used as positive analytical responses to these tests.

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 3.4).

Figure 3.4: 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.

Test for flavonoids

In a test tube, a few drops of a 4 mg/mL sodium hydroxide (NaOH) solution were added to 1 mL 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.

Test for saponins

To 1 mL of crude extracts was added 5 mL of distilled water in test tubes. The test tubes were vigorously shaken for 5 minutes. The formation of a persistent foam was considered an indication for the presence of saponins.

Test for cardiac glycosides (Keller- Kilani test)

In test tubes, crude extracts (1 mL) were mixed with 0.5 mL of glacial acetic (96%) followed by a few drops of a 2% solution of $FeCl_3$ and 1 mL of concentrated Sulphuric acid (H_2SO_4). A brown ring at the interphase or a brown colouration was an indication of the presence of this group of compounds (Figure 3.5).

Figure 3.5: Tubes showing presence of cardiac glycosides. The dark coloration indicates presence of the chemicals, the chemicals (★), the tubes with yellow coloration are the negative (■) controls for each extract.

Test for steroids

One millilitre of crude extracts was mixed with a few drops of concentrated acetic acid and a few drops of concentrated sulphuric acid. A violet or green colouration indicated the presence of steroids.

Test for terpenoids

A volume of 1 mL crude extract was dissolved in about 0.5 mL of chloroform and 1 mL of concentrated Sulphuric acid. A brownish, reddish or dark colouration indicated the presence of the chemical.

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 was added to 1 mL crude extract. The observed turbidity or precipitation was considered as evidence for the presence of alkaloids.

3.2.3.2 Quantitative phytochemical analysis

Quantification of total flavonoid contents (TFC)

The total flavonoids content in the extracts was evaluated by using the aluminium chloride colorimetric method. About 100 µLof plant extracts at 1 mg/mL were mixed with 80 µL of distilled water in individuals wells of 96 well plate and about 100 µL Aluminium chloride solution (stock of 2% in methanol) was poured into the mixture. The plate was incubated for 30 min at room temperature. After incubation, the absorbance of the pink mixture was measured at 420 nm using a spectrophotometer (Multiscan MS, Labsystems; Beverley, MA, USA). Distilled water was used as blank (Figure 3.6). The TFC was expressed in mg of Quercetin (Sigma-Aldrich, Saint Louis, MO, USA) per gram of extract. The experiment was carried out in triplicate. Linearity range of quercetin calibration curve was 10-1000 µg/mL.

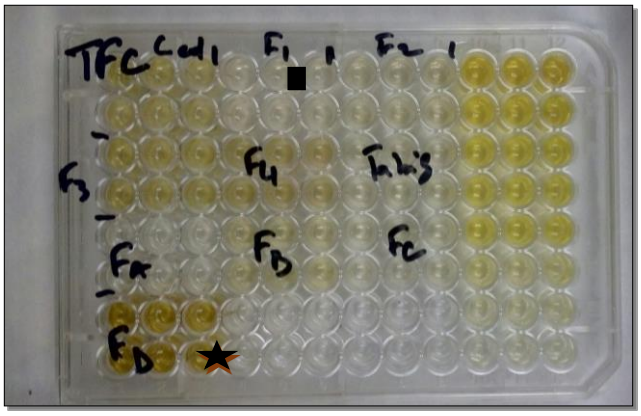

Figure 3.6: Plate showing different concentration of Total flavonoid contents. The star (★) indicates a sample with a high level of flavonoids, while the square (■) indicates a sample with a low quantity of flavonoids.

Quantification of total phenolic contents (TPC)

The Folin-Ciocalteu reagent (FCR) was used for the determination of total phenolic content in the methanol and water plant extracts (1mg/ml). Approximately 80 µL of water was added in each well of the 96 well plate then mixed with 100 µL of extract followed by the addition of 60 µL Sodium carbonate (3.5 g dissolved in 50 mL distilled water) followed by the addition of 60 µL of FCR (10 mL dissolved in 90 mL distilled

water). The absorbance was recorded after 30 minutes at 760 nm spectrometrically (Multiscan MS, Labsystems; Beverley, MA, USA) (Figure 3.7). The total phenolic content of the extracts was calculated as gallic acid (Sigma-Aldrich, Saint Louis, MO, USA) equivalents (mgGAE/g). All the experiments were performed in triplicate.

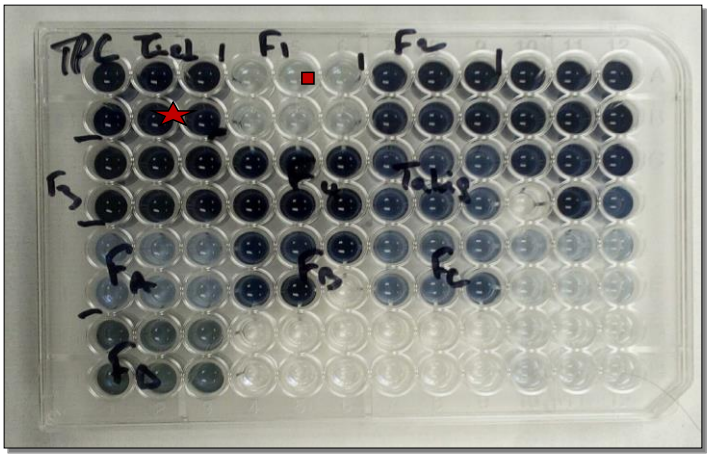

Figure 3.7: Plate showing different concentration of Total phenolic contents. The star (★) indicates a sample with a large quantity of phenolic compounds, while the square (■) indicates a sample with low TPC.

3.2.4 DETERMINATION OF ANTIOXIDANT CAPACITY

3.2.4.1 Determination of antioxidant activity using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method

With this method, the antioxidant activity was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH. The reduction of the radical is followed by a decrease in the absorbance at 517 nm. DPPH assay was run according to the method described by Alkan et al. (2014) with a few modifications. Briefly, in the first row of a 96 well plate, 100 µL distilled water and 100 µL of each extract or controls (Gallic acid and ascorbic acid) were mixed. Then two-fold serial dilutions of the samples were prepared giving the following concentrations in µg/mL: 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.9. A volume of 200 µL of DPPH/ethanol solution was added to each well and the plates were incubated for 30 min in the dark.

After incubation, the absorbance in each well was read on a microplate reader (Multiscan MS, Labsystems; Beverley, MA, USA) at 517 nm (Figure 3.8).

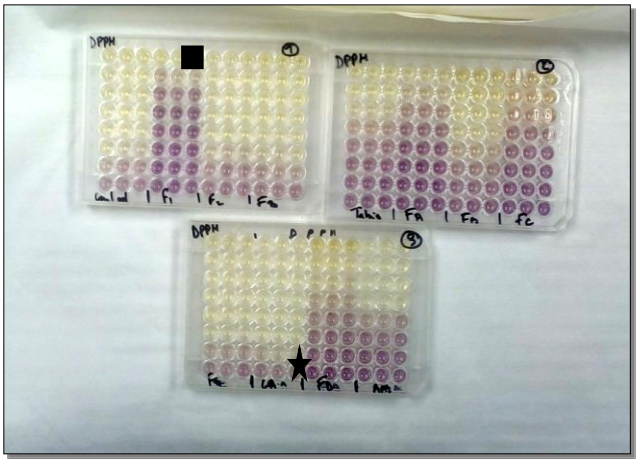

Figure 3.8: Expected representation of DPPH free radical scavenging activity of different samples on a 96 well plate. The star (★) indicates a sample with a strong antioxidant activity, while the square (■) indicates a sample with a weak activity.

The assay was carried out in triplicate. The percentage of inhibition (I%) of DPPH was calculated using the formula that follows:

$$I\% = \frac{(A_{DPPH} - A_{Sample})}{A_{DPPH}} \times 100$$

A = measured absorbance.

This percentage of inhibition allowed us to determine graphically the inhibitory concentration 50 (IC_{50}) which is the concentration of extract/control that causes 50% reduction in the DPPH colour.

3.2.4.2 Determination of Antioxidant Activity Using reducing power

The assay was performed according to Oyaizu (1986) with some slight modifications. Briefly, after adding 50 µL of sodium phosphate buffer (6.6 pH; 0.2M) into each well of a 96 well plate, 50 µL from 1 mg/mL of extracts and control (Gallic acid and Ascorbic acid) were added and two fold serial dilutions made giving the following concentrations in µg/mL: 500, 250, 125, 62.5, 31.25, 15.63, 7.81 and 3.9. Afterward, 50 µL of

potassium hexacyanoferrate (1%) was added to each well which were then incubated for 30 min at 50°C. Next, 50 µL of 10% trichloroacetic acid solution was distributed into each well and 80 µL of the content was transferred into new 96 well plates. A volume of 80 µL of distilled water was added to each well of the new plate, followed by 15 µL of 0.1% ferric chloride solution. The plates were then read (Figure 3.9) at 690 nm on a microplate reader (Multiscan MS, Labsystems; Beverley, MA, USA).

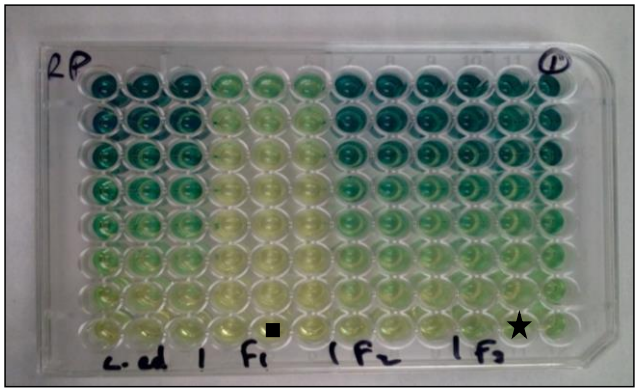

Figure 3.9: Expected representation of ferric reducing activity of a sample on a 96 well plates. The star (★) indicates a sample with a strong antioxidant activity, while the square (■) indicates a sample with a weak activity.

3.2.5 ASSESSING CYTOTOXICITY OF THE PLANTS

3.2.5.1 Reagents

All tissue culture media and fetal bovine serum (FBS) were purchased from Lonza (Basel, Switzerland). Gentamycin sulfate, dimethyl sulphoxide (DMSO), Melphalan, Hoechst 33342 nuclear dye, PI dyes all were also obtained from Sigma-Aldrich.

3.2.5.2 Sample preparation

The plant extracts were reconstituted in dimethyl sulphoxide (DMSO) to give a final concentration of 100 mg/mL and further diluted to working concentrations using culture medium supplemented with 10% FBS. Samples were sonicated if solubility was a problemamples were stored at 4°C until required.

3.2.5.3 Cell cultures

A cervical cancer cell line (HeLa; ATTC # CCL-2, obtained from Cellonex, South Africa) and a control cell line (Vero; African Green monkey kidney epithelial cells; ATTC # CCL-81, obtained from the ATCC) were used in this study. HeLa cells were maintained in Roswell Park Memorial Institute medium 1640 (RPMI) containing 10% foetal bovine serum (FBS) at 37°C in a humidified environment with 5% CO_2 (Thermo Fisher, Waltham, MA USA). Vero cells were maintained using DMEM and 10% FBS at 37°C in a humidified environment with 5% CO_2 (Thermo Fisher, Waltham, MA USA).

3.2.5.4 Screening protocol

Dual staining using Hoechst 33324 and propidium iodide (PI) with fluorescence microscopy

For screening, cells were seeded into 96 well microtiter plates at a density of 6000 cells/well using a volume of 200 µL in each well. The microtiter plates were incubated at 37°C, 5% CO_2 (Thermo-Fisher, Waltham, MA USA), and 100% relative humidity for 24 hours prior to addition of extracts to allow for cell attachment. Two hundred microliter aliquots of diluted extracts in fresh medium was used to treat cells after aspiration of seeding medium. Two concentrations of extract tested in this screening assay were 50 and 100 µg/mL. Melphalan (40 µM) was used as a positive control in all experiments. Treated cells were incubated at 37°C in a humidified 5% CO_2 incubator (Thermo-Fisher, Waltham, MA USA) for 48 hours. Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) and incubated for 10 minutes at 37°C. 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 Image-Xpress Micro XLS Widefield Microscope (Figure 3.10) (Molecular Devices; San Jose, CA; USA).

Figure 3.7: Image-Xpress Micro XLS Widefield Microscope (Molecular Devices)

MTT cytotoxicity assay

After fluorescence imaging, Hoechst and PI dyes were aspirated and replaced with fresh medium containing MTT (Sigma-Aldrich) at a final concentration of 0.5 mg/mL. Cells were further incubated for 3 hours at 37°C after which MTT crystals were solubilized using 100 µL DMSO and absorbance measured (Figure 3.11) at 560 nm using a multi-well scanning spectrophotometer (Multi-scan MS, Labsystems; Waltham, MA; USA).

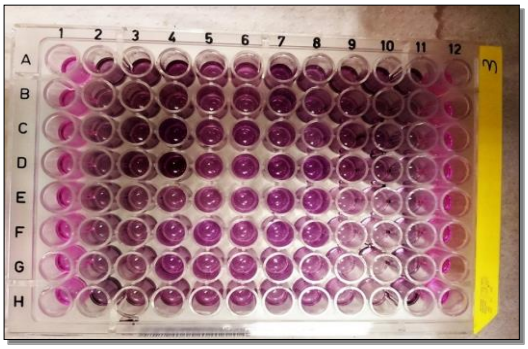

Figure 3.11: Expected MTT plate. The darker the well the higher the number of viable cells.

3.2.5.4 Interpretation of Data

The quantification of live and dead cells for the screening assay was performed using the Image-Xpress Micro XLS Widefield Microscope (Molecular Devices) and acquired data was analysed using the Meta-Xpress software and Multi-Wavelength Cell Scoring Application Module. Acquired data was transferred to an EXCEL spreadsheet and the relative cell viability was determined.

3.2.6 STATISTICAL ANALYSIS

Each sample assessment was performed in quadruplicate. The data were analysed using Graph pad prism 6.0 and Microsoft Excel. The IC_{50} was obtained by nonlinear regression followed by dose response equation. The EC_{50} was determined using linear regression. The results are presented as mean (±SD) of the triplicate measurements. The significance was evaluated using 2-way ANOVA and Tukey's multiple comparisons test. The results were considered as very significant with $p<0.001$ and significant with $p<0.05$.

3.3 RESULTS

3.3.1 PHYTOCHEMICAL COMPOSITION

3.3.1.1 Qualitative analyses

The qualitative phytochemical analysis was used to determine the presence or not of some phytochemicals in both methanol and aqueous extracts of selected plants using biochemical assays. Table 3.2 shows that both aqueous and methanol extracts *of C. edulis; M. cecropioides; S. ochocoa; R. vomitoria; A. hirtella; S. gabonensis; U. klainei; P. soyauxii;* and *U. guineensis* possessed (shown as a +) saponins; alkaloids; phenols; tannins; terpenoids; flavonoids; steroids; and cardiac glycosides.

Table 3.2: Phytochemical composition of aqueous and methanolic extracts of the selected plants

Extracts		Sapo	Alka	Phe	Tan	Ter	Flav	Ster	Glyco
Coula edulis	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Musanga cecropioides	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Scyphocephalium ochocoa	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Alchornea hirtella	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Rauwolfia vomitoria	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Adenia cissampeloides	Methanol	+	+	-	-	+	-	+	-
	Aqueous	+	-	-	-	+	-	+	-
Staudtia kamerounensis	Methanol	+	+	+	+	+	+	+	+
	Aqueous	-	+	+	+	+	+	+	+
Microdesmis puberula	Methanol	+	+	+	+	+	+	+	-
	Aqueous	+	+	+	+	+	+	+	+
Sacoglottis gabonensis	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	-
Macaranga monandra	Methanol	-	+	+	+	+	+	+	-
	Aqueous	-	-	+	+	-	+	+	+
Uvaria klainei	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Pterocarpus soyauxii	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Uapaca guineensis	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Aucoumea klaineana	Methanol	-	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+

Vernonia conferta	Methanol	-	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	+
Anthocleista vogelii	Methanol	-	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	+	+	+	-
Tabernanthe iboga	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	-	+	+	+
Ricinodendron africanum	Methanol	+	+	+	+	+	+	+	+
	Aqueous	+	+	+	+	-	+	+	+

Sapo=saponins; Alka=alkaloids; Phe=phenols; Tan=tannins; Ter=terpenoids; Fla=flavonoids; Ste=steroids; Gly=glycosides

Alkaloids, phenols, tannins, flavonoids and glycosides were not present in both *A. cissampeloides* extracts. Saponins were also absent in both *M. monandra* extracts. Methanol extracts of *A. vogelii, V. conferta, A. klaineana* and the aqueous extract of *S. kamerunensis* did not show the presence of saponins either. Glycosides could not be detected in the *M. puberula* methanol extract or in the aqueous extracts of *S. gabonensis* and *A. vogelii*. Interestingly, both *T. iboga* and *R. africanum* aqueous extracts did not show the presence of terpenoids.

3.3.1.2 Quantitative analyses

The quantitative analyses of the tested plants consisted of determining the total phenolic (TPC) and flavonoid (TFC) contents of each extract (methanol and aqueous) using the calibration curves of Gallic acid and Quercetin respectively (Figures 3.12-a and 3.12-b). These curves allowed the generation of a linear regression equation from which the values of the TPC (mg GAE/g of sample) and TFC (mg QE/g of sample) could be calculated using the obtained absorbance. The calibration curve of Gallic acid showed linearity for Gallic acid in the range of 0.5 – 125 µg/mL, with a correlation coefficient (R^2) of 0.9865 while the one for Quercetin showed linearity in the range 8 – 32 µg/ml with R^2 = 0.9956.

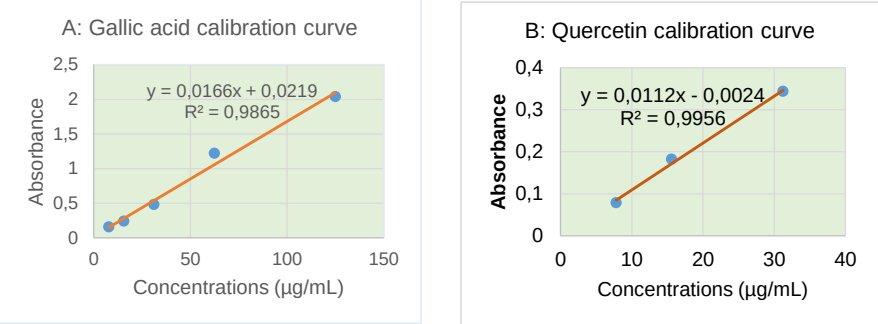

Figure 3.12: Standard calibration curves of Gallic acid (a) and Quercetin (b)

The calculated amount of TPC and TFC in each of the 40 extracts are presented in Table 3.3. The results reveal that in general, methanol extracts were richer in TPC and TFC when compared to the aqueous extracts except for *C. religiosa* (1.63 mg QE/g vs 0.61 mg QE/g), *V. conferta* (1.33 mg QE/g vs 0.68 mg QE/g), *C. lucanusianus* (1.15 mg QE/g vs 0.58 mg QE/g) and *A. vogelii* (1.97 mg QE/g vs 0.68 mg QE/g) where the amount of TFC was significantly ($p<0.05$) higher in the aqueous extracts.

Table 3.3: Total phenolic and flavonoid contents of the aqueous and methanol plant extracts

	TFC (mg QE/g)		TPC (mg GAE/g)	
	Methanol extract	Aqueous extract	Methanol extract	Aqueous extract
Coula edulis	4.15 ± 0.13^{a}	4.05 ± 0.14^{a}	11.88 ± 0.01^{a}	11.87 ± 0.28^{a}
Musanga cecropioides	2.5 ± 0.02^{b}	2.67 ± 0.04^{b}	12.13 ± 0.27^{a}	10.89 ± 0.11^{b}
Scyphocephalium ochocoa	2.51 ± 0.01^{b}	1.59 ± 0.05^{c}	$11.94\pm0,11^{a}$	9.38 ± 0.15^{c}
Alchornea hirtella	$1.25\pm0.01^{c,d}$	1.40 ± 0.24^{c}	7.67 ± 0.58^{d}	2.91 ± 0.07^{e}
Rauwolfia vomitoria	2.38 ± 0.01^{b}	$1.08\pm0^{c,d,e}$	$7.34\pm0.29^{d,f}$	2.04 ± 0.14^{g}
Adenia cissampeloides	$0.83\pm0^{e,f}$	0.54 ± 0.01^{f}	0.82 ± 0.03^{h}	0.31 ± 0.06^{h}
Staudtia kamerounensis	4.21 ± 0.31^{a}	2.43 ± 0.06^{b}	$11.50\pm0.1^{a,b,i}$	$6.64\pm0.18^{f,j}$
Microdesmis puberula	$1.05\pm0.04^{d,e}$	$1.01\pm0.03^{d,e}$	$1.06\pm0.06^{h,k}$	$1.21\pm0.22^{h,k}$
Sacoglottis gabonensis	2.37 ± 0.02^{b}	$0.89\pm0.03^{d,e,f}$	9.97 ± 0.31^{c}	6.07 ± 0.12^{j}
Macaranga monandra	2.26 ± 0.06^{b}	$0.89\pm0^{d,e,f}$	$12.04\pm0.4^{a,i}$	4.53 ± 0.44^{l}
Uvaria klainei	1.93 ± 0.05^{c}	$1.29\pm0.01^{c,d}$	5.07 ± 0.24^{l}	3.06 ± 0.30^{e}

Pterocarpus soyauxii	1.26±0.04^{c,d}	1.76±0.03^{c}	6.42±0.23^{j}	3.42±0.15^{e}
Uapaca guineensis	1.81±0.03^{c}	1.13±0.02^{d,e}	10.91±0.67^{b,i}	8.69±0.5^{c}
Aucoumea klaineana	1.71±0.02^{c}	0.90±0^{d,e,f}	10.95±0.4^{b,i}	2.63±0.23^{e,g}
Vernonia conferta	0.68±0.01^{f}	1.33±0.01^{c,d}	1.09±0.22^{h,k}	1.74±0.09^{g,k}
Anthocleista vogelii	0.68±0.01^{f}	1.97±0.02^{c}	0.80±0.04^{h,k}	2.70±0.09^{e,g}
Tabernanthe iboga	1.03±1.13^{d,e}	1.12±0.12^{d,e}	4.57±0.24^{l}	7.30±0.06^{d,f,j}
Copaifera religiosa	0.61±0.002^{f}	1.63±0.02^{c}	3.14±0.02^{e}	2.61±0.02^{e,g}
Costus lucanusianus	0.58±0.04^{f}	1.15±0.01^{d,e}	0.56±0.01^{h,k}	0.67±0.03^{h,k}
Ricinodendron africanum	0.73±0.01^{e,f}	0.85±0^{e,f}	0.70±0.01^{h,k}	1.30±0.04^{g,h,k}

The numbers with the same index were not significantly different.

In the same manner, *A. vogelii* (2.70±0.09 mg GAE/g) and *T. iboga* (7.30±0.06 mg GAE/g) aqueous extracts presented significantly higher ($p<0.05$) amount of TPC compared to the methanolic extracts (0.80±0.04 mg GAE/g and 4.57±0.24 mg GAE/g respectively). In addition, TPC were significantly ($p<0.05$) higher than TFC for all the extracts tested. The results also indicated that the highest amount of TFC were detected in *S. kamerounensis* methanol extract (4.21±0.31 mg QE/g) followed by *C. edulis*, *M. cecropioides* aqueous (4.05±0.14 and 2.67 mg QE/g respectively) and methanol (4.15±0.13 and 2.5±0.0 mg QE/g respectively) extracts, and *S. ochocoa* methanolic (2.51±0.01 mg QE/g) extracts. The highest levels of TPC were found in the methanolic extracts of *M. cecropioides* (12.13±0.27 mg GAE/g), *M. monandra* (12.04±0.4 mg GAE/g), S. ochocoa (11.94 mg GAE/g), and *S. kamerunens*is (11.50±0.1 mg GAE/g) as well as in both the aqueous and the methanolic extracts of *C. edulis* (11.88±0.01 and 11.87±0.28 mg GAE/g respectively). Plants such as *A. cissampeloides, C. lucanusianus, C. religiosa, R. africanum, A. vogelii, M. monandra, S. gabonensis* and *A. klaineana* displayed the lowest amounts of TPC and TFC.

3.3.2 ANTI-OXIDANT ACTIVITIES

3.3.2.1 Free radical scavenging activity

The free radical scavenging effect of the selected plant extracts was assessed using the DPPH method. The results presented in Table 3.4 below, show the inhibitory concentration 50 (IC_{50}) in µg/mL of methanolic and aqueous extracts determined by

plotting the percentage of inhibition of DPPH with increasing concentrations of the extracts.

The results revealed that the aqueous extract of *C. edulis, U. klainei, A. cissampeloides* and *A. vogelii* were the most active with IC_{50} respectively of 5.37±1.32 µg/mL, 36.46±0.13 µg/mL, 850.30±3.15 µg/mL and 236.77±3.94 µg/mL than their methanolic extracts which showed IC_{50} of 7.66±0.69 µg/mL respectively, 49.23±4.43 µg/mL, 2514.67±2.40 µg/mL and 316.83±0.68 µg/mL. For the other plants, the methanolic extracts were more active when compared to the aqueous extracts. Both alcoholic and aqueous extracts of *C. edulis; M. cecropioides; S. ochocoa; S. kamerounensis; U. guineensis;* and *C. religiosa* showed IC_{50} values lower than ascorbic acid. The methanolic extract of *A. hirtella* and *R. vomitoria* and the aqueous extracts of *U. klainei* presented IC_{50} significantly ($p<0.001$) lower than that of ascorbic acid (38.87±1.54 µg/mL) and higher than that of Gallic acid (2.77±0.25 µg/mL). The IC_{50} of *A. cissampeloides* methanolic extract, *C. lucanusianus* and *M. puberula* aqueous extracts were significantly higher than 1 mg/mL indicating a weak antioxidant activity for these extracts. Both *C. edulis* extracts have demonstrated a very low IC_{50} value compared to the rest of the plants, which is an indication that this plant possesses a very significant antioxidant activity via free radical scavenging mechanism, which can be comparable to that of Gallic acid.

Table 3.4: IC_{50} (µg/mL) of the methanolic and aqueous extracts as determined from the DPPH assay

Plant Name	Methanolic extracts	Aqueous extracts
Coula edulis	7.66±0.69 *	5.37±1.32 *
Musanga cecropioides	30.81±1.35 [a]	32.71±1.19 [a]
Scyphocephalium ochocoa	10.58±0.30 *	19.72±0.51 [a]
Alchornea hirtella	21.21±0.95 [a,*]	52.56±0.81 [a,*]
Rauwolfia vomitoria	19.98±1.81 [a,*]	179.97±0.87 [a,*]
Adenia cissampeloides	2514.67±2.40 [a,*]	850.30±3.15 [a,*]
Staudtia kamerounensis	12.82±0.26 *	32.01±3.44 [a]
Microdesmis puberula	484.97±5.79 [a,*]	3048±3.61 [a,*]
Sacoglottis gabonensis	51.32±0.44 [a]	162.83±0.92 [a,*]
Macaranga monandra	79.97±0.86 [a,*]	184.73±1.57 [a,*]
Uvaria klainei	49.23±4.43 [a]	36.46±0.13 [a]
Pterocarpus soyauxii	71.72±2.04 [a,*]	112.93±3.12 [a,*]
Uapaca guineensis	10.40±0.26 *	24.42±0.88 [a,*]
Aucoumea klaineana	15.21±2.11 *	38.6±2.41 [a]
Vernonia conferta	351.23±1.93 [a,*]	484.67±1.81 [a,*]
Anthocleista vogelii	316.83±0.68 [a,*]	236.77±3.94 [a,*]
Tabernanthe iboga	65.37±0.33 [a,*]	505±3.80 [a,*]
Copaïfera religiosa	14.06±0.09 *	25.36±0.24 [a]
Costus lucanusianus	700.07±0.55 [a,*]	1735±2.89 [a,*]
Ricinodendron africanum	49.29±3.41 [a]	520.47±1.85 [a,*]
Ascorbic acid	**38.87±1.54** [a]	-
Gallic acid	**2.77±0.25***	-

* **(p<0.001)** indicates significant difference between the extracts and Ascorbic acid, while [a] **(p<0.001)** indicates significant difference between the extracts and Gallic acid

3.3.2.2 Reducing power assay

The results of the reducing power of the selected plant extracts are presented in Table 3.5 and show the efficient concentration 50 (EC_{50}) which was obtained by plotting the measured absorbance with the concentrations. A low EC_{50} value indicates a great reducing power/antioxidant effect of the extract. The results show that aqueous extracts of *C. edulis* (58.24±1.31 µg/mL), *M. cecropioides* (221.99±1.47 µg/mL), *A. cissampeloides* (2397.33±1.45 µg/mL), *P. soyauxii* (397.44±2.2 µg/mL) and *A. vogelii* (957±1 µg/mL) were more active than the methanolic extracts which displayed an EC_{50} respectively of 125.29±0.86 µg/mL, 274.07±0.68 µg/mL, 2439.33±2.24 µg/mL, 405.14±2.76 µg/mL and 989.67±1.20 µg/mL.

For the remaining plants, the methanolic extracts demonstrated higher activities when compared to the aqueous ones. Both *Coula edulis* methanolic and aqueous extracts, methanolic extracts of *S. kamerounensis, S. gabonensis, M. monandra, U. guineensis* and *A. klaineana* showed antioxidant activity significantly ($p<0.001$) higher than Ascorbic acid whose EC_{50} was 152.16±1.09 µg/mL but statistically ($p<0.001$) lower than Gallic acid of which the EC_{50} value was 12.45±0.42 µg/mL. Methanolic extract of *Sacoglottis gabonensis* and aqueous extract of *Coula edulis* displayed the best antioxidant activity in this assay while *Adenia cissampeloides, Microdesmis puberula, Costus lucanusianus, Ricinodendron africanum* and *Vernonia conferta* presented the weakest activity with high EC_{50} values.

Table 3.5: EC_{50} in µg/mL of the methanolic and aqueous extracts for the FRAP assay

Plant Name	Methanolic extracts	Aqueous extracts
Coula edulis	125.29±0.86 [a,*]	58.24±1.31 [a,*]
Musanga cecropioides	274.07±0.68 [a,*]	221.99±1.47 [a,*]
Scyphocephalium ochocoa	213.31±2.23 [a,*]	226.37±0.78 [a,*]
Alchornea hirtella	185.77±2.50 [a,*]	314.36±2.54 [a,*]
Rauwolfia vomitoria	246.07±2.22 [a,*]	1221.67±0.83 [a,*]
Adenia cissampeloides	2439.33±2.24 [a,*]	2397.33±1.45 [a,*]
Staudtia kamerounensis	149.90±0.73 [a]	256.97±1.94 [a,*]
Microdesmis puberula	1209.83±1.18 [a,*]	4600±1.16 [a,*]
Sacoglottis gabonensis	89.73±0.68 [a,*]	161.41±2.62 [a]
Macaranga monandra	115.00±0.67 [a,*]	156.08±1.73 [a]
Uvaria klainei	286.74±3.06 [a,*]	339.24±3.15 [a,*]
Pterocarpus soyauxii	405.14±2.76 [a,*]	397.44±2.2 [a,*]
Uapaca guineensis	128.16±0.91 [a,*]	348.33±3.90 [a,*]
Aucoumea klaineana	131.9±1.31 [a,*]	348.46±4.24 [a,*]
Vernonia conferta	1569.44±2.00 [a,*]	1203.75±1.91 [a,*]
Anthocleista vogelii,	989.67±1.20 [a,*]	957±1 [a,*]
Tabernanthe iboga	151.07±0.98 [a]	750±1.72 [a,*]
Copaïfera religiosa	182.72±0.39 [a,*]	325.81±3.49 [a,*]
Costus lucanusianus	1613.89±1.47 [a,*]	2330.83±3.01 [a,*]
Ricinodendron africanum	414.17±0.24 [a,*]	1191.25±5.91 [a,*]
Ascorbic acid	**152.16±1.09 [a]**	-
Gallic acid	**12.45±0.42 ***	-

* **(p<0.001)** indicates significant difference between the samples and Ascorbic acid, while [a] **(p<0.001)** indicates significant difference between the samples and Gallic acid

3.3.3 CORRELATION BETWEEN ANTIOXIDANT ACTIVITY, TPC AND TFC

The correlation between antioxidant activity, total phenolic and total flavonoid contents of the methanolic and the aqueous extracts of the selected plants are presented in Tables 3.6 and 3.7 respectively. The results indicate a very strong correlation between DPPH and reducing power of both methanol and aqueous extracts with r=0.87 and r= 0.95 respectively. The correlation between TPC and TFC of aqueous (r=0.67) and methanol (0.77) extracts was satisfactory. Interestingly, the correlation between TPC and reducing power of methanolic extracts (r=-0.71) was higher than for the aqueous extracts (r=-0.56). Total flavonoid contents were also highly correlated with reducing power for the methanolic extracts (r=-0.57) but weakly correlated with the aqueous extracts (0.37). The results show also that there was a weak correlation between free radical scavenging activity, TPC and TFC of both aqueous (r=-0.45, r=-0.31 respectively) and methanolic extracts (r=-0.39 and r=-0.49 respectively).

Table 3.6: Correlation matrix between antioxidant activity, TPC and TFC of methanolic extracts

	TFC	*TPC*	*DPPH*	*RP*
TFC	1			
TPC	0.771392	1		
DPPH	-0.38588	-0.487	1	
RP	-0.57433	-0.71423	0.867168	1

Table 3.7: Correlation matrix between antioxidant activity, TPC and TFC of aqueous extracts

	TFC	*TPC*	*DPPH*	*RP*
TFC	1			
TPC	0.665661	1		
DPPH	-0.30653	-0.44864	1	
RP	-0.36591	-0.56057	0.953879	1

3.3.4 CYTOTOXICITY RESULTS

Cells were exposed to two concentrations (50 and 100 µg/mL) of aqueous and methanol plant extracts for 48 hours. Melphalan, a well-known chemotherapeutic compound was used as a positive control. Quantification was performed using the Image-Xpress Micro XLS Widefield Microscope and the MTT cytotoxicity assay.

3.3.4.1 Cell viability assessment using MTT assay

The Vero cell viability are pictured in Figure 3.13 and the results showed that viability of cells was significantly ($p<0.0001$) reduced to more than half when compared to the untreated cells after treatment at both concentrations (50 and 100 µg/mL) of *U. guineensis* (23% and 22% viable cells respectively for methanol extracts, 22% and 25% viable cells respectively for aqueous) and *A. klaineana* extracts (20% and 23% viable cells respectively for methanol extract and at 17% and 19% respectively for aqueous) as well as *T. iboga* aqueous (24% viable cells) and methanol (42% viable cells) extracts (100 µg/mL). For methanol extracts of *S. gabunensis* (122% at 50 µg/mL and 141% viable cells at 100 µg/mL) ($p<0.001$), *P. soyauxii* (120% viable cells at 50 µg/mL and 100 µg/mL) ($p<0.0001$) and *A. hirtella* (120% viable cells at 100 µg/mL) ($p<0.0001$) the number of cells was significantly ($p<0.0001$) increased when compared to the untreated cells. For all the samples, the aqueous extracts when compared to the methanol extracts, seem to display a marginally higher cell growth inhibition. This observation did not apply to *A. vogelii* (84% vs 100 at 50 µg/ml, 90% vs 97% at µg/mL) and *R. vomitoria* (82% vs 84% at 50 µg/mL, 76% vs 88% at 100 µg/mL) as well as *R. africanum* (89% vs 101% at 100 µg/mL) where the methanol extracts were slightly more toxic than the aqueous extracts.

The inhibitory activity of the selected plants on HeLa cell line (Figure 3.14) indicated that the extract of interest was *A. klaineana* methanol extract at the higher concentration ($p<0.05$). Also, extracts such as *M. puberula* methanol at 100 µg/mL, *R. vomitoria* methanol at both 50 and 100 µg/mL, *S. kamerounensis* and *A. cissampeloides* aqueous at both concentrations, as well as *M. monandra*, *T. iboga* and *C. religiosa* aqueous extracts at the higher concentration showed cell inhibition ($p>0.05$) ranging between 21% and 31%. Cell proliferation varying from 120% to 149% was observed when the HeLa cells were treated with *A. hirtella* methanol (50 µg/mL) ($p>0.05$), *M. puberula* aqueous (100 µg/mL), Uvaria klainei methanol (50 µg/mL) and

aqueous (100 µg/mL) ($p<0.0001$), both *P. soyauxii* methanol extracts concentrations ($p<0.05$), and aqueous extracts from *U. guineensis* ($p<0.0001$), *V. vonferta* ($p>0.05$) and *T. iboga* at 50 µg/mL ($p>0.05$).

The remaining plant extracts seem to exert a light or insignificant inhibition on cell growth when compared to the untreated cells. Overall the aqueous extracts seemed to exert a toxic effect slightly higher than that of the methanol extracts.

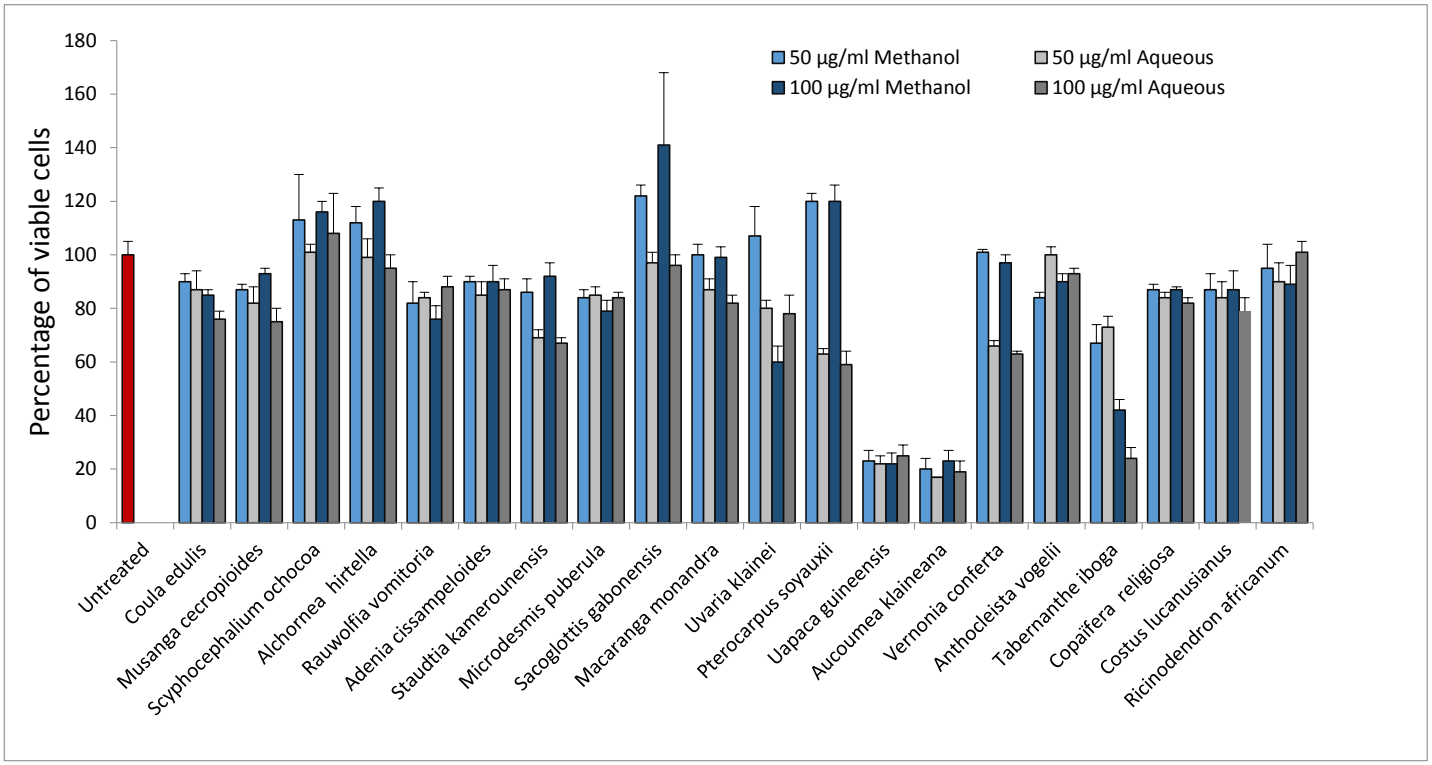

Figure 3.13: Cytotoxicity of 20 plant extracts against Vero cells using the MTT cytotoxicity assay at 50 and 100 µg/mL. Results are indicated as percentage viable cells (i.e. metabolically active) compared to an untreated control. Errors bars denote standard deviation of the experiment performed in quadruplicate.

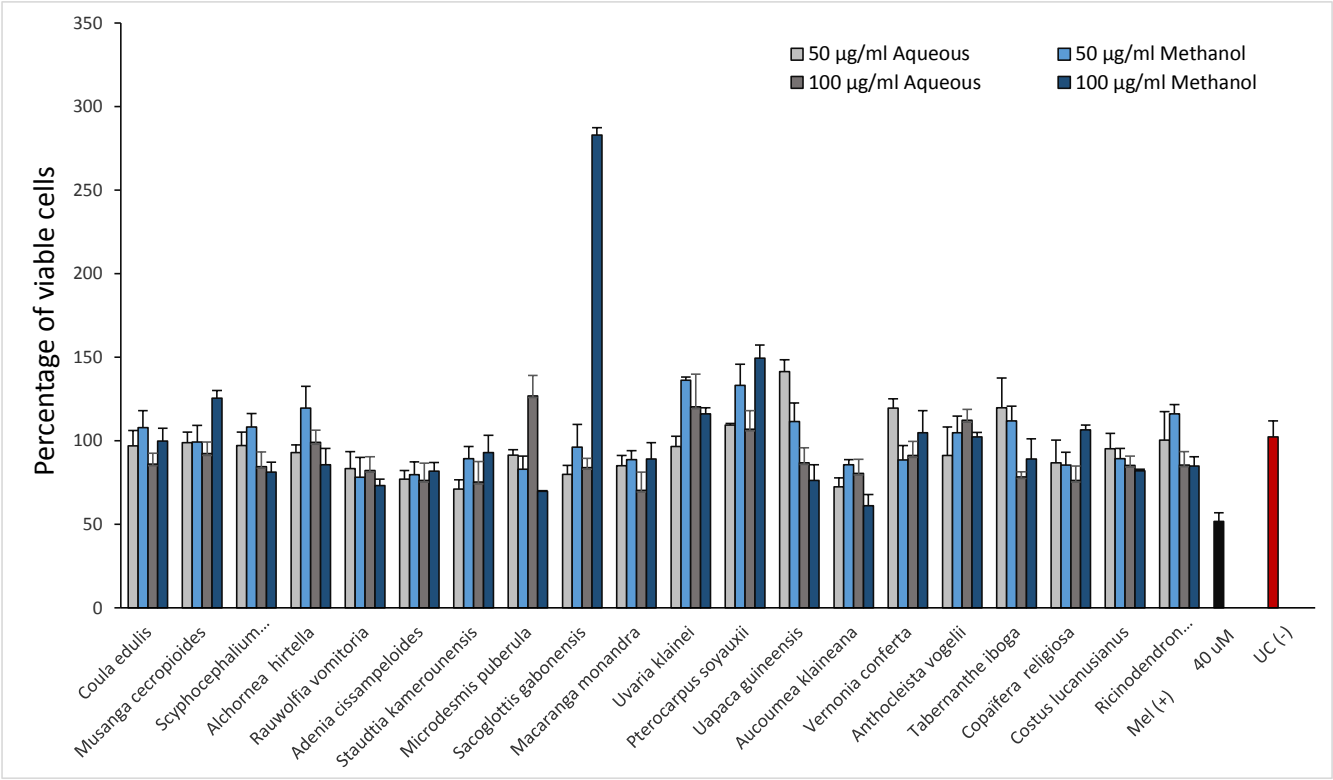

Figure 3.14: Cytotoxicity of 20 plant extracts against HeLa cells using the MTT cytotoxicity assay at 50 µg/mL and 100 µg/mL. Results are indicated as percentage live cells compared to an untreated control. Errors bars denote standard deviation of the experiment performed in quadruplicate

3.3.4.2 Cytotoxicity evaluation using fluorescence microscopy

Cytotoxicity of 40 plant extracts (aqueous and methanol) at two concentrations (50 and 100 µg/mL) against Vero (Figure 3.15 and Figure 3.16) and HeLa (Figure 3.17 and Figure 3.18) cell lines were evaluated using a dual staining technique and fluorescence microscopy. *Sacoglottis gabonensis* methanol extract which was precipitated out in the culture medium interfered with the image analysis. The results are depicted as the number of the live (stained positive with Hoechst only) and dead cells (stained positive with Hoechst and PI) per site. To assis the interpretation of the mechanism by which the cells were killed, the mean nuclei area of the cells was also measured (Figure 3.19 and Figure 3.20). An increase in the nuclei area could be attributed to possible G2/ early M arrest or onset of necrosis when a corresponding increase in propidium iodide staining is observed. A decrease in the nucleus area could suggest the onset of apoptotic cell death.

In Figures 3.15 and 3.16, the number of cells in each well after treatment with the extracts was compared with the number of untreated cells. The results indicated that the number of cells per site were found to be less than 1000 in *A. klaineana* (both extracts and concentrations), *T. iboga* (both extracts and concentrations), *U. klainei* (methanol at both concentrations) and *C. edulis* (aqueous at 100 µg/mL). While for the untreated cells, *C. religiosa* (both extracts and concentrations), *R. africanum* aqueous extract (both 50 and 100 µg/mL), *A. hirtella* and *S. gabonensis* aqueous extracts at 50 µg/mL, the number of cells per site were higher than 1500. The remaining plant extracts showed a number of cells per site ranging between 1000 and 1500 cells. Regarding the number of dead cells, while the untreated cells counted 12 dead cells per site, *A. hirtella* aqueous extract (99 and 196 cells at 50 and 100 µg/mL respectively) and *T. iboga* methanol (112 and 87 cells at 50 and 100 µg/mL respectively) counted the higher rate of dead cells ($p<0.005$). In contrast, both *U. guineensis* and *A. klaineana* aqueous and methanolic extracts at both concentrations showed 0 to 2 dead cells per site. The remaining extracts induced the death of 4 to 64 cells per site.

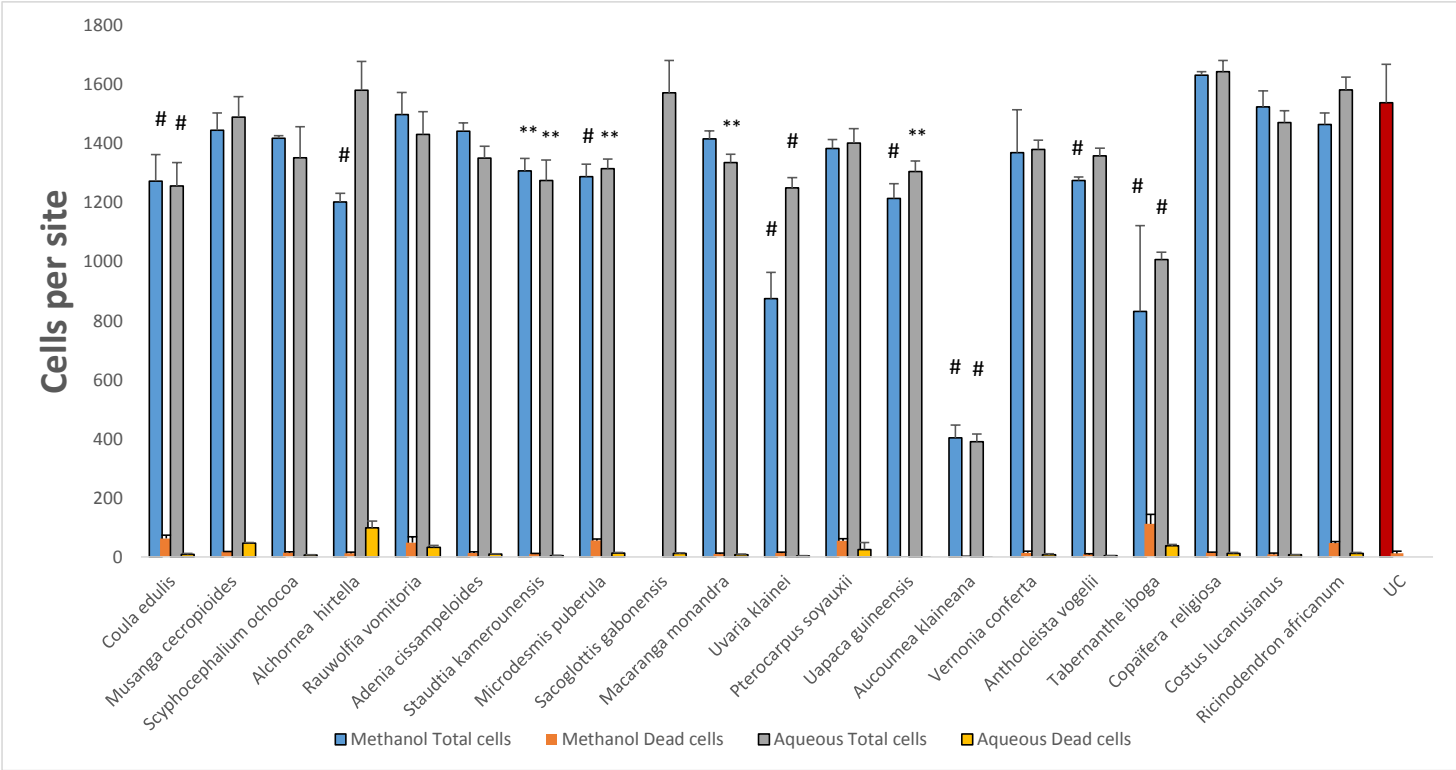

Figure 3.15: Cytotoxicity of 40 extracts (aqueous and methanol) at 50 µg/mL concentrations against Vero cells using dual staining techniques and fluorescence microscopy. Results are indicated as number of total and dead cells per site. Errors bars denote standard deviation. Significant differences are indicated by # (P<0.0001) and ** (P<0.005) compared to the untreated control (UC).

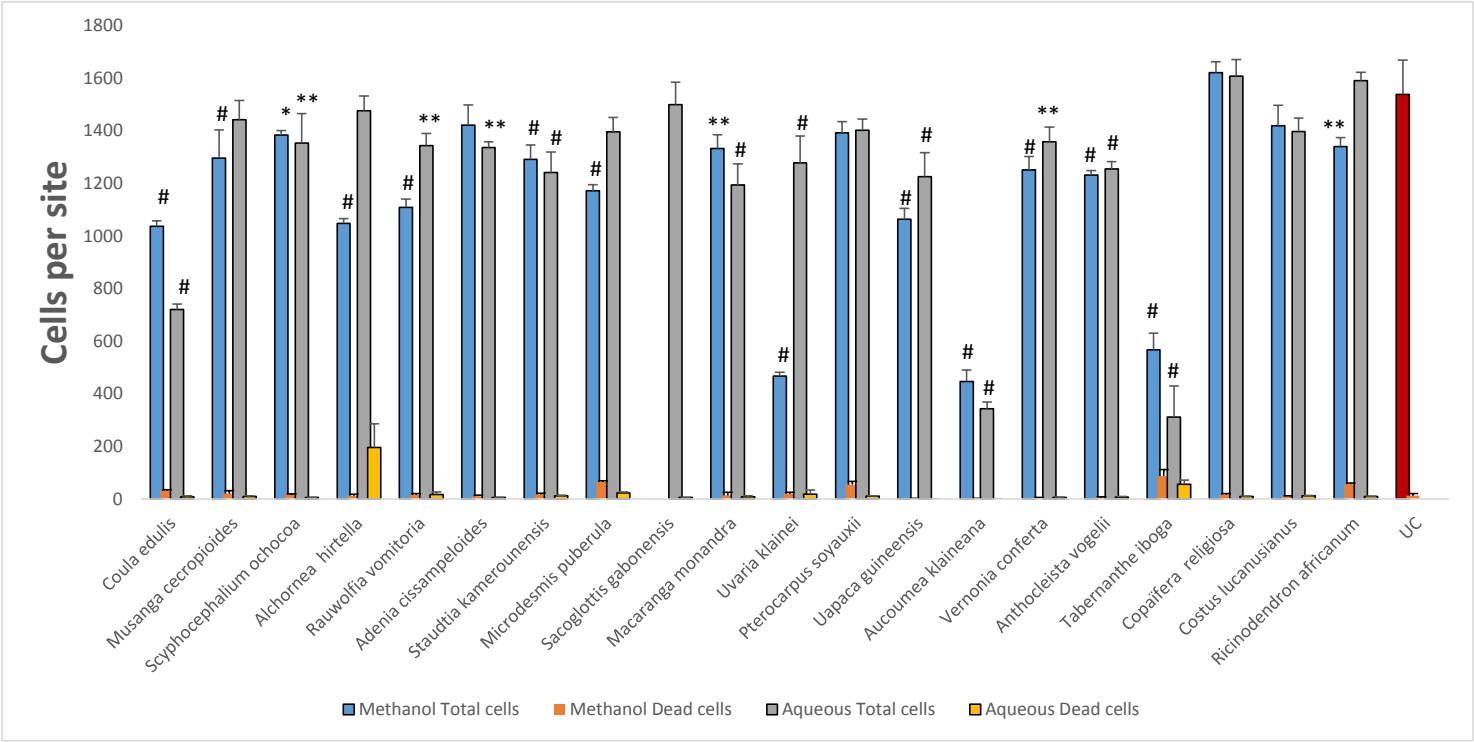

Figure 3.16: Cytotoxicity of 40 extracts (aqueous and methanol) at 100 µg/mL concentrations against Vero cells using dual staining techniques and fluorescent microscopy. Results are indicated as number of total and dead cells per site. Errors bars denote standard deviation. Significant differences are indicated by # (P<0.0001) and ** (P<0.005) compared to the untreated control (UC).

The results of the cytotoxic effects of the 40 plant extracts against HeLa cell line are described in Figure 3.17 and 3.18. Several plant extracts in this study showed interesting activities against HeLa cells. The number of cells per site was significantly ($p<0.0001$) lower than the one of untreated cells for the following extracts; methanol and aqueous extracts of *U. klainei*, *U. guineensis*; *P. soyauxii*; *A. klaineana* and *V. conferta* at all the concentrations tested. *M. monandra* aqueous extracts at both concentrations and methanol extracts at 100 µg/mL; *R. vomitoria;* an *R. africanum* both extracts at 100 µg/mL; *C. edulis* and *S. ochocoa* aqueous extracts at 50 µg/mL.

Regarding the dead cells, Melphalan was able to induce the death of 42.95±7.6 cells per site while for the untreated cells about 30.41±15 cells per site were dead. The number of dead cells in the untreated cells was higher ($p<0.05$) compared to the number of dead cells after treatment with several extracts such as methanol extract of *C. lucanusianus* (50 µg/mL); *R. africanum* (100 µg/mL); *A. Kaineana* (50 µg/mL); *C. religiosa* (100 µg/mL); *A. hirtella* (50 µg/mL); *S. kamerunensis* (100 µg/mL) and aqueous extracts of *A. hirtella* (100 µg/mL); *R. vomitoria* (100 µg/mL); *A. klaineana* (100 µg/mL) and *V. conferta* (100 µg/mL). Some extracts presented very low levels of dead cells per site (less than 10) such as methanol extract of *U. klainei* (50 µg/mL) as well as aqueous extracts of *S. kamerounensis* (100 µg/mL) and *M. puberula* (50 µg/mL). Interestingly, methanol and aqueous extracts of *U. guineensis; A. Klaineana; V. conferta; M. monandra* and *R. vomitoria* were able to both inhibit the HeLa cancer cells growth but also to kill the cells. While *U. klaineana* and *P. soyauxii* showed a promising anti-proliferative potential.

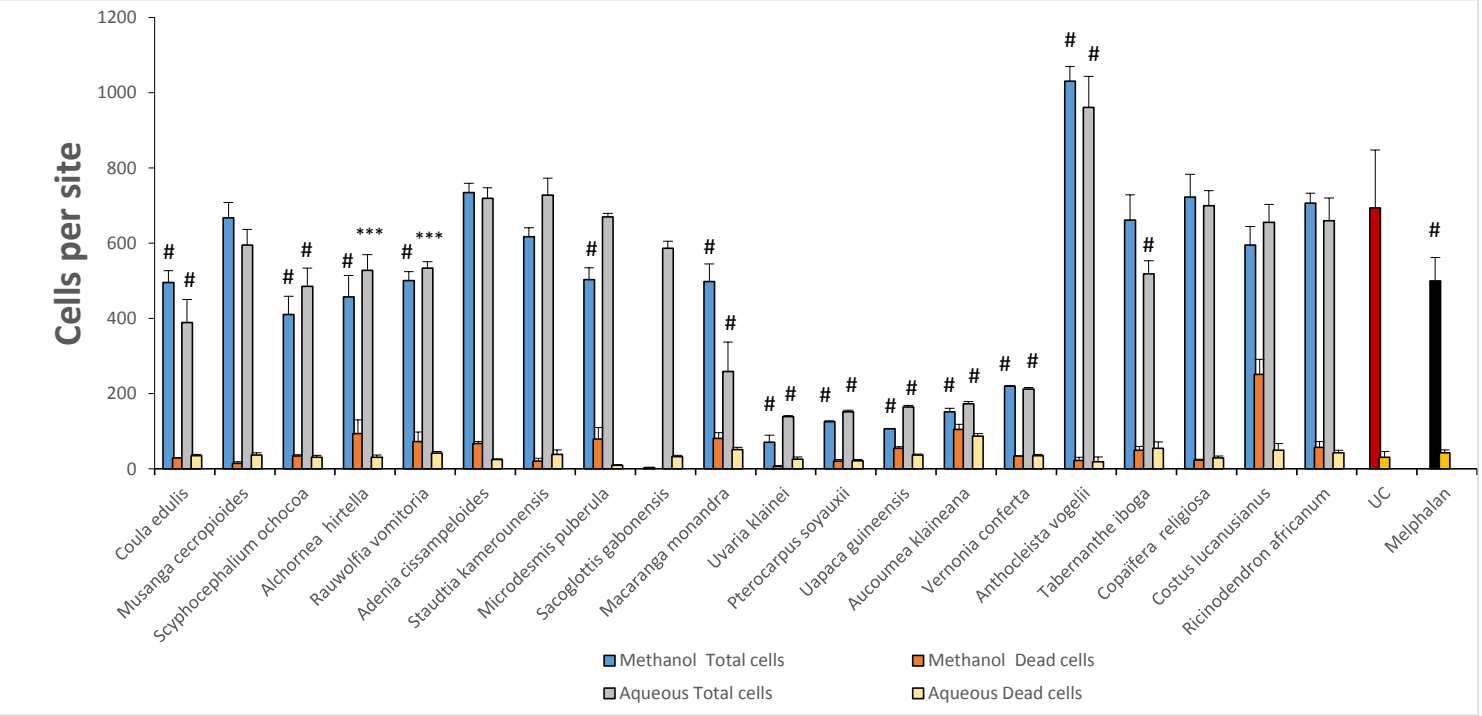

Figure 3.17: Cytotoxicity of 20 plant extracts (aqueous and methanol) at 50 µg/mL against HeLa cells using dual staining techniques and fluorescent microscopy. Results are indicated as number of total and dead cells per site. UC (untreated cells), Errors bars denote standard deviation of one experiment performed as quadruplicate. Significant differences are indicated by # (P<0.0001) and *** (P<0.005) compared to the untreated control (UC).

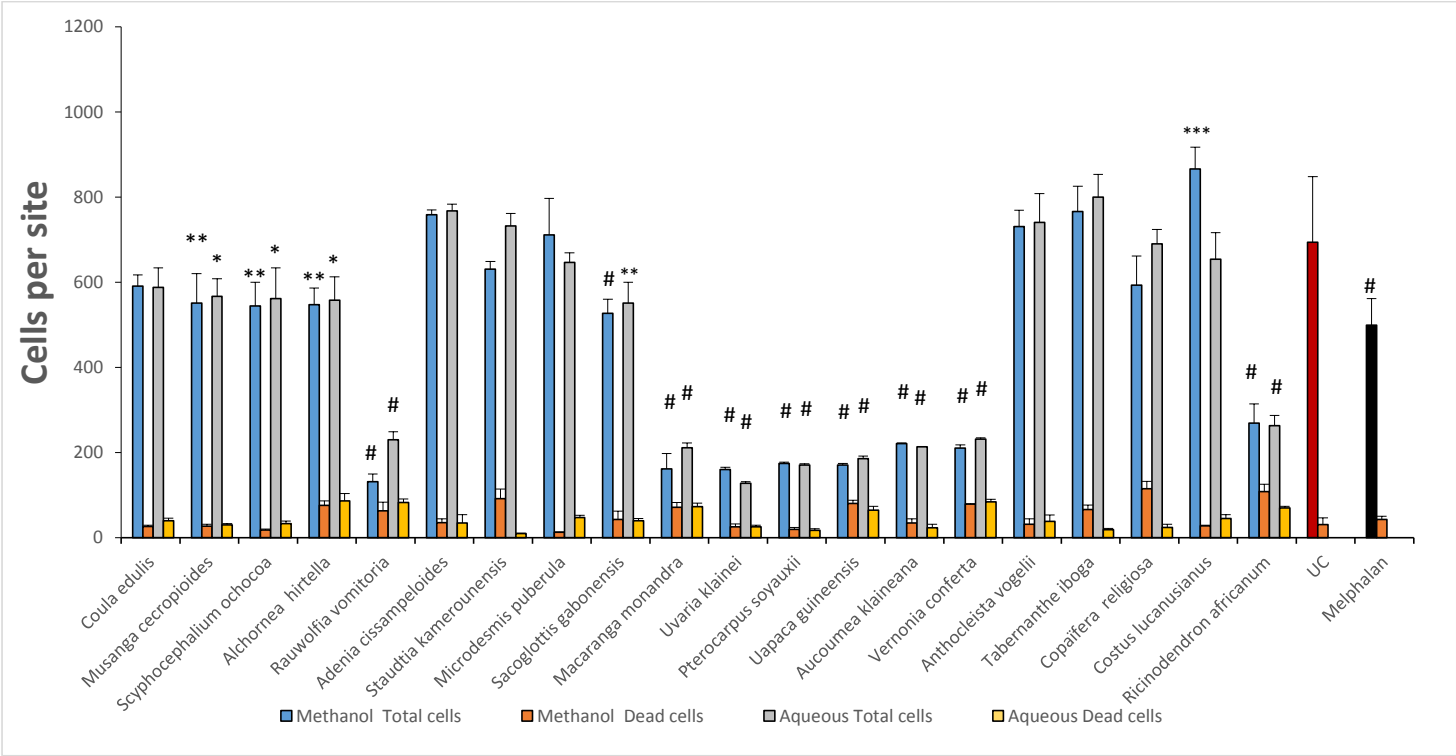

Figure 3.18: Cytotoxicity of 20 plant extracts (aqueous and methanol) at 100 µg/mL concentrations against HeLa cells using dual staining techniques and fluorescent microscopy. Results are indicated as number of total and dead cells per site. UC (untreated cells); Errors bars denote standard deviation of one experiment performed as quadruplicate. Significant differences are indicated by # (P<0.0001) and * (P<0.05) compared to the untreated control (UC)

3.3.4.3 Mechanisms of cytotoxicity of the selected plant extracts

Figure 3.19 shows that when compared to untreated cells, a significant ($p<0.05$) increase in nuclei mean area was observed when cells were treated with both extracts of *T. iboga* (100 µg/mL), *C. lucanusianus* (100 µg/mL), *A. vogelii* (50 µg/mL and 100 µg/mL), methanol extract of *V. confertaI* and *R. vomitoria* (both concentrations); and aqueous extracts of *M. puberula* and *A. klaineana* (both concentrations). An increase in the nuclei area would suggest a possible G2/ early M arrest or onset of necrosis if a corresponding increase in propidium iodide staining was observed. While this trend was not observed for *M. cecropioides* methanol extracts, *S. gabonensis* aqueous extracts, *M. monandra* and *U. guineensis,* both extracts (50 µg/mL and 100 µg/mL) which significantly decreased the mean nuclei area of the cells when compared to untreated cells suggesting cell apoptosis pathway. *C. edulis* methanol extract significantly decreased ($p<0.05$) the mean nuclei area at the lower concentration while the aqueous extract significantly ($p<0.0001$) increased it at the higher concentration.

The results in Figure 3.20 depicted the effect of the extracts on the mean nuclei area of HeLa cancer cell line. A significant increase in the mean nuclei area of the cells was observed after treatment with some extracts. Remarkably, like Melphalan, several extracts were able to increase significantly ($p<0.05$) the mean nuclei area of the cells when compared with untreated cells. Whereas, *S. gabonensis, U. klainei* and *A. vogelii* aqueous extracts (50 µg/mL) on the contrary significantly ($p<0.05$) decreased the mean nuclei area. Yet, a significant lower ($p<0.05$) mean nuclei area of cells treated with plants such as *A. cissampeloides; S. kamerounensis; M. puberula; P. soyauxii; U. klainei; U. guineensis* and *T. iboga* was noticed.

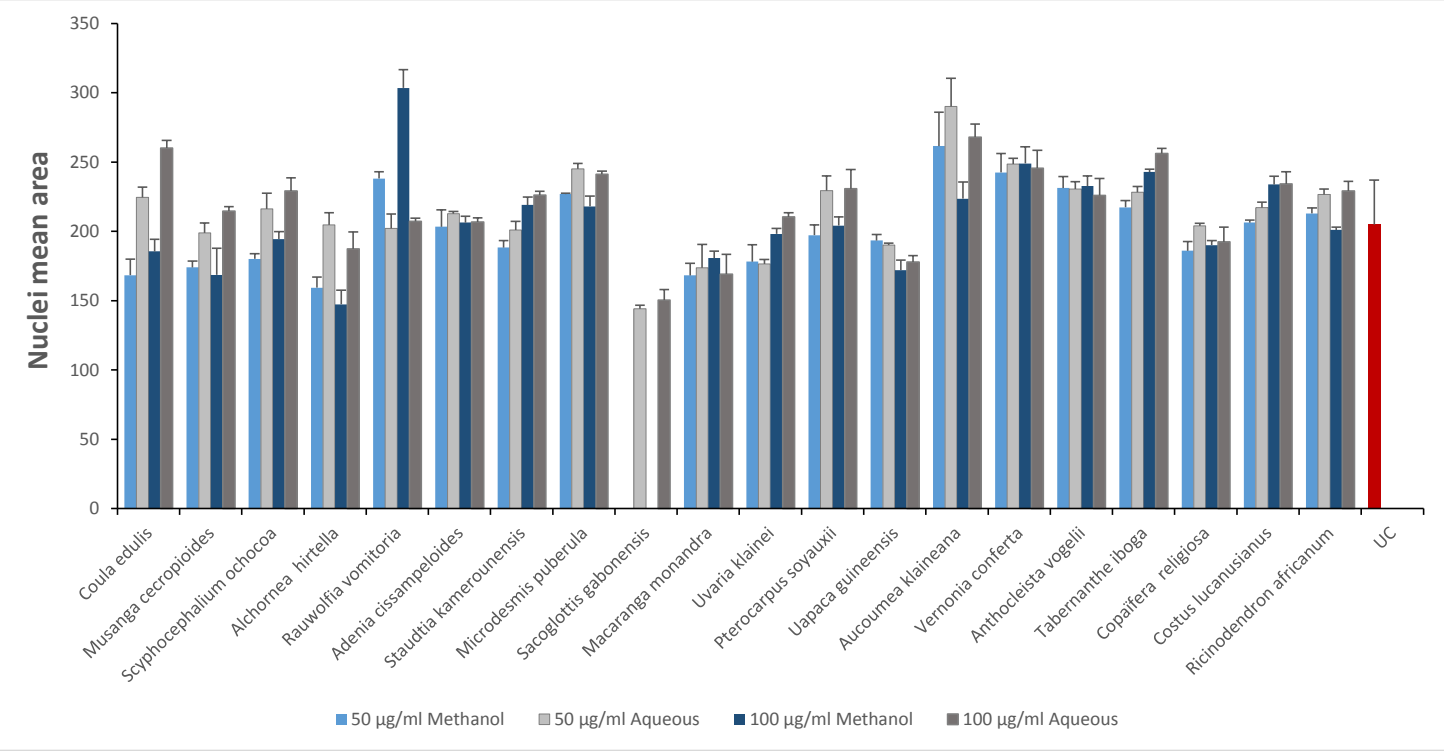

Figure 3.19: Evaluation of the mean nuclei area of Vero cells after treatment with 40 extracts (aqueous and methanol) at two concentrations (50 µg/mL and 100 µg/mL) using dual staining techniques and fluorescent microscopy (UC). Errors bars denote standard deviation of one experiment performed as quadruplicate.

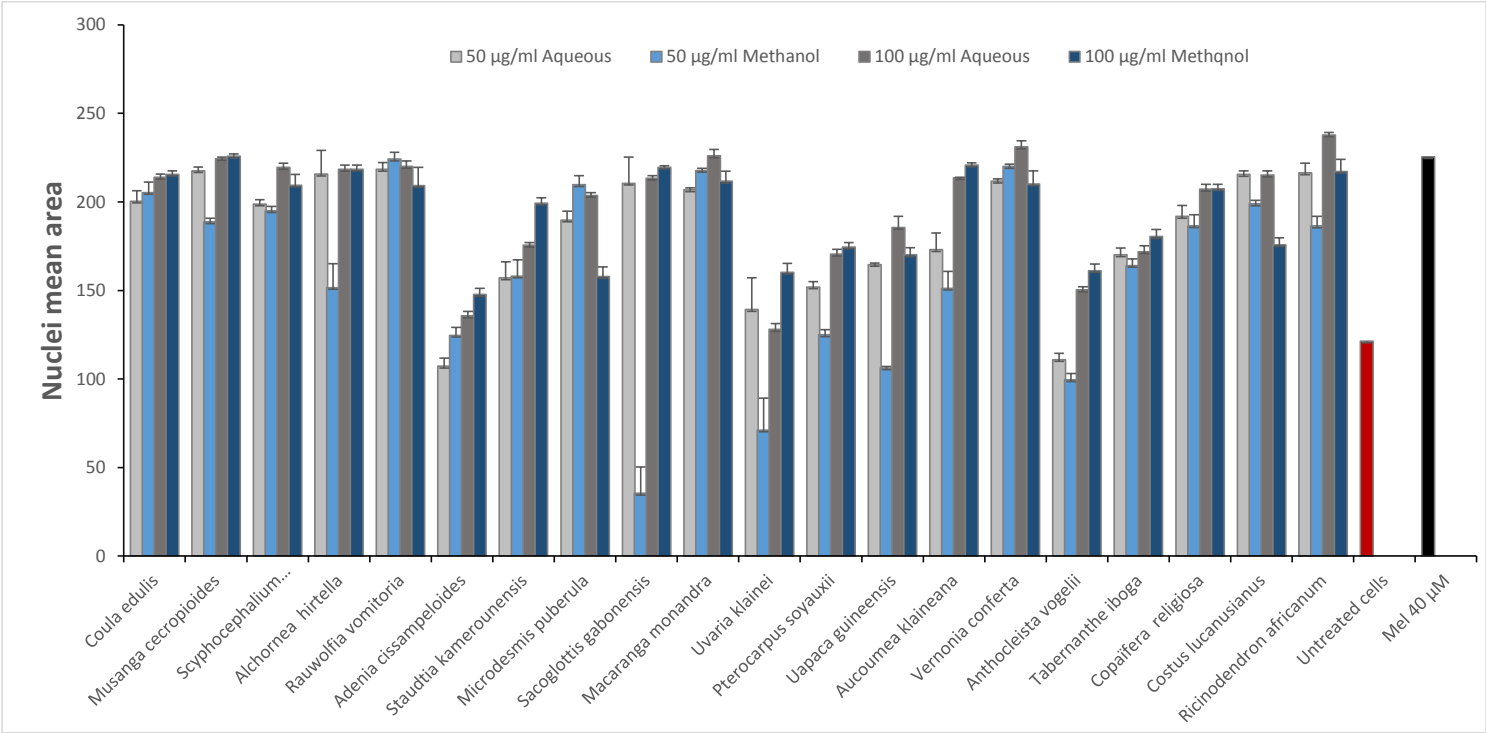

Figure 3.20: Evaluation of the mean nuclei area of HeLa cells after treatment with 40 extracts (aqueous and methanol) at two concentrations (50 µg/mL and 100 µg/mL) using dual staining techniques and fluorescence microscopy. Errors bars denote standard deviation of one experiment performed as quadruplicate.

3.4 DISCUSSION

The objective of this chapter was to determine: the (1) phytochemical profile; (2) anti-oxidative activity; and (3) cytotoxic effects of 40 Gabonese plant extracts (aqueous and methanolic extracts of 20 plants) commonly used to improve the health of HIV patients.

Phytochemical profiling

The qualitative phytochemical screening using colorimetric analysis allowed the detection of several phytochemicals including saponins; tannins; alkaloids; terpenoids; cardiac glycosides; steroids; phenols and flavonoids. The results revealed the presence of steroids in all the plant extracts. Whereas phenols, tannins and flavonoids that were detected in 19 plant extracts, were not found in both aqueous and methanolic extracts of *Adenia cissampeloides*. This peculiar finding is not in agreement with the study by Okunye et al. (2015) who managed to find phenols, tannins and flavonoids in *A. cissampeloides* extracts. The same opposite results were found for *S. gabonensis* aqueous extracts which did not show the presence of cardiac glycosides while Ejikeme et al. (2014) study demonstrated the presence of this compound in *S. gabonensis* extracts. The quality and quantity of phytochemicals extracted from plants vary and depend on the type of solvents and the mode of extraction used as well as the geographical location of the plant, soil composition, temperature and date of collection (Kumar *et al.,* 2017; Dia *et al.,* 2016; Figueiredo *et al.,* 2008). All the above-mentioned parameters have an impact also in the yield of the compounds extracted which explains the different results obtained for the same species in different studies. Nevertheless, according to some authors (Tchouya *et al.,* 2016; Tchouya *et al.,* 2015; Ejikeme *et al.,* 2014) compounds including flavonoids, total phenolics and tannins are present in *Scyphocephalium ochocoa, Musanga cecropioides* and *Sacoglottis gabunensis* extracts which confirms the results found in the present study. Chemicals of plants have proven to display a wide range of biological activities against viruses, bacteria or parasites and some diseases. For instance, steroids possess biological properties such as insecticidal, cardiotonic, and antimicrobial activities whereas tannins are known to exert antiviral, antibacterial, antiparasitic, anti-inflammatory, antiulcer and antioxidant properties (Jayswal *et al.,* 2018). Various studies demonstrated effects on inflammation, microorganisms, cancer, and oxidative stress of saponins and alkaloids (Bribi, 2018; Roy, 2017; Moghimipour and Handali, 2015).

Phenols and flavonoids are part of phenolic compounds which are often correlated to antioxidant activity of plants but also to anticancer, antimicrobial and anti-inflammatory activities (Minatel *et al.,* 2017; Seleem *et al.,* 2017; Panche *et al.,* 2016).

On the other hand, the quantitative phytochemical analysis involved the evaluation of the total phenolic and flavonoid contents of each of the 40 extracts. The results showed that TPC were significantly higher ($P<0.05$) in all the plant extracts compared to TFC. Overall, methanolic extracts were not significantly richer ($p>0.05$) in TPC and TFC as compared to the aqueous extracts except for some extracts such as *C. religiosa*; *C. lucanusianus* and *A. vogelii* where the amounts of TFC were significantly higher ($p<0.05$) in the aqueous extracts. For *A. vogelii* and *T. iboga* aqueous extracts, the amount of TPC was higher compared to the methanolic extracts. The variability in the total phenolic and flavonoid contents in different extracts of a plant might probably be driven by the solvent polarity. According to Yao et al. (2004), methanol compared to water is a suitable solvent to extract phenolic compounds based on its capacity to prevent the oxidation of polyphenols. The results obtained correlate with the findings reported by Anokwuru et al. (2011), where *Hibiscus sabdarifa* methanolic extracts contained significantly higher levels of polyphenols ($p<0.05$) than the aqueous extracts. The results also highlighted the fact that the plants used in our study possess higher content of phenolic compounds as opposed to flavonoid compounds. This also tallies with a previous study reporting on TPC and TFC (Faozia *et al.,* 2017).

Antioxidative activity of plants

The DPPH assay is a common and valuable method to evaluate the antioxidant properties of different substances including plants. In the present study, the plant extracts have displayed very high to weak antioxidant activity, with IC_{50} values ranging from 5.37±1.32 µg/mL to 3048±3.61 µg/mL. According to Blois' classification of antioxidant substances (Blois, 1958), a sample with an $IC_{50} < 50$ µg/mL is a very strong antioxidant, when 50 µg/mL < IC_{50} < 150 µg/mL is a strong to moderate antioxidant, while a weak antioxidant has $IC_{50} > 150$ µg/mL. According to other the classifications for antioxidant activity based on IC_{50} (Qusti *et al.,* 2010; Souri *et al.,* 2008; Leong and Shui, 2002), *C. edulis; M. cecropioides; S. ochoco; A. hirtella; S. kamerounensis; U. guineensis; A. klaineana* and *C. religiosa* demonstrated a very high antioxidant activity

with IC_{50} values lower than 50 µg/mL. The results in the present study support those reported by Tchouya et al. (2015) who found that *S. ochocoa* and *M. cecropioides* have free radical scavenging activity with IC_{50} that are comparable to ascorbic acid (vitamin C) which is one of the greatest natural antioxidants. Our findings also confirmed the antioxidant activity of *S. gabonensis; R. africanum; R. vomitoria; A. vogelii* and *C. lucanusianus* previously demonstrated by several authors (Iroanya *et al.,* 2015; Momeni *et al.,* 2010; Zihiri *et al.,* 2005; Maduka and Okoye, 2002). Interestingly, *C. edulis* seems to have a free radical scavenging activity not significantly different from Gallic acid but greater ($p<0.01$) than ascorbic acid. This indicates that *C. edulis* could be a great antioxidant agent useful in the management of free radicals.

The reducing power assay is used to test the capability of natural products and plant extracts to convert/reduce the potassium ferricyanide (Fe^{3+}) complex to potassium ferrocyanide (Fe^{2+}) which reacts with ferric chloride to form ferrous complex that can absorb maximally at 700 nm. Increased absorbance of the reaction mixture indicates an increase in reduction ability (Subramanian *et al.,* 2013). The EC_{50} values for the 40 extracts ranged from 58.24±1.31 µg/mL to 4600±1.16 µg/mL. The results indicated that the methanolic extracts have the overall lowest EC_{50} values compared to the aqueous extracts, except for *Coula edulis* aqueous extracts that demonstrated EC_{50} value greater than ascorbic acid but lower than gallic acid.

Antioxidant activity of plants is due to compounds such as phenols and flavonoids, which can neutralize unstable and reactive molecules and protect membrane lipids from oxidation. The findings reveal that the plants selected for this study contain various chemicals including phenols; flavonoids; steroids; terpenoids; alkaloids; glycosides; tannins and saponins. The determination of the total phenolic and flavonoid contents in the extracts indicated a high level of these compounds in several plant extracts like *C. edulis*; *M. monandra; S. gabonensis; S. ochocoa* or *S. kamerunensis* which also displayed the highest antioxidant activity. Plant extracts such as *A. cissampeloides*; *C. lucanusianus*; or *M. puberula* that displayed the lowest level of chemicals including TPC and TFC, were also exhibiting weak antioxidant activity. According to various studies, a strong correlation between a high level of TPC and TFC with a strong antioxidant activity is often demonstrated. Mello and Quadros (2014) showed in one study that the antioxidant activity was highly correlated with the TPC.

Yet, in the present study, a weak correlation between free radical scavenging activity, TPC and TFC of both aqueous and methanolic extracts was found. Interestingly, a high correlation between TPC and reducing power of methanolic extracts (r=0.71) and aqueous extracts (r=0.56) were demonstrated (Tables 3.6 and 3.7). This indicates that phenolic compounds contribute to a high level to the reduction power of the plant extracts but not that much to the free radical scavenging activity. Other compounds present in variable quantities in the studied plants could contribute to the free radical scavenging activity displayed by the extracts. In addition, according to Do et al. (2014), aqueous plant extracts may either contain more non-phenolic compounds or possess phenolic compounds that contain a smaller number of active groups than the methanolic extracts. This might be a plausible reason behind almost all the methanolic extracts displaying higher antioxidant activity in contrast to aqueous extracts.

Oxidative stress plays a significant role in the progression of HIV infection. It has been established that people infected with the virus show changes in their antioxidant defence system, which may be the result of the large production of oxygen-derived species during the various phases of the disease (Aquaro *et al.,* 2008). It has further been reported that perturbations in antioxidant defence system, leading to a redox imbalance, are present in many tissues of HIV-infected individuals (Ivanov *et al.,* 2016; Sharma, 2014). Oxidative stress may contribute to several aspects of HIV disease, such as viral replication, inflammatory response and decreased immune cell proliferation. The level of production of free radical species in HIV-1 infected individuals receiving ARVs was reported to be higher than those who harbour HIV-1 infection without receiving any treatment or healthy subjects (Sharma, 2014). In a review by Saeidnia and Abdollahi (2014), it was mentioned that vitamins including vitamin C which is one of the major antioxidants in plasma, was able to suppress HIV replication by inhibiting reverse transcriptase activity. Some studies suggested that antioxidant supplements can significantly reduce the risk of immune decline and morbidity in HIV infected people (Jiménez-Sousa *et al.,* 2018; Baum *et al.,* 2013; Kaiser *et al.,* 2006). Therefore, exogenous supply of antioxidants that scavenge free radicals and have reducing ability such as the plants of the current study, might play an important role in the treatment of HIV or at least in the improvement of HIV infected people's health.

Cytotoxicity of the plants

In vitro bioassays are employed in order to predict the activity of many natural or synthetic substances on different organic systems. The biosafety or cytotoxicity evaluation of compounds is an indispensable means of providing crucial information regarding their potential danger. For the purpose of evaluating the potential cytotoxic effects of 40 extracts from 20 selected plant species on Vero cell lines and HeLa cancer cell lines, two different methods were used for comparison which are MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and dual staining coupled to fluorescence microscopy. Just as many assays using metabolic dyes which measure the metabolic activity of viable cells, the MTT assay presents numerous disadvantages such as interference with plant extracts. The dye (MTT) can interact with the plant compounds and change the metabolism of the cells without being lethal to the cells that lead to false negative or positive results. Whereas high-content imaging-based *in vitro* toxicity assays such as dual staining and fluorescence microscopy show promise for safety and efficacy testing. These assays allow the measure of the number of cells and the differentiation of living cells from dead cells. These assays are more accurate because they can be performed using high throughput systems for simultaneous screening of many compounds (Sirenko *et al.,* 2014). In the present chapter, the biosafety of the plant extracts was assessed on Vero cells while the cytotoxicity or anticancer potential was done on HeLa cancer cells.

The investigation of the biosafety of the selected plants on Vero cells revealed that the outcomes of the two methods were quite similar for several plant extracts. *Coula edulis* (aqueous at 100 µg/mL) showed an anti-proliferative effect and an increase in the mean nuclei area. In addition, *A. hirtella* and *T. iboga* were highly cytotoxic but also quite antiproliferative while *Rauwolfia vomitoria* methanol extracts showed an increase in the mean nuclei area without a remarkable anti-proliferative effect. When cells were treated with *A. klaineana,* an antiproliferative effect was followed by an increase in the mean nuclei area was observed. Interestingly, the results for *U. guineensis* were not matching for MTT and fluorescence microscopy as a cytotoxic effect was observed with MTT assay which was not confirm with the other method. The same observation was elicited with *P. soyauxii* methanol extracts which were causing cell proliferation over the limit of the untreated cells in MTT but not with fluorescence microscopy. The reason could be that the phytochemical compounds present in *U. guineensis* and *P.*

soyauxii may interfere with mitochondrial dehydrogenase activity. The presence of some reducing agents like ascorbic acid and compounds with free thiol groups in the plant extracts is a plausible explanation of the false result observed (Chan *et al.,* 2015). Similar results were found in various studies that were comparing various cell viability methods including MTT and the conclusion was that some plant compounds were interfering with MTT resulting in the activation or the inhibition of mitochondrial dehydrogenases and thus in an over/underestimation of the MTT assay results (Sliwka *et al.,* 2016; Chan *et al.,* 2015).

In the particular case of *Tabernanthe iboga*, which is a plant widely used in Gabon for its numerous properties, our findings support many studies carried out on this plant and demonstrating its toxic effects on various cells and tissues including macrophages and the nervous system. A study done by Mefane et al. (1990) on HIV positive blood showed a decrease of the phagocytic function of macrophages. It has been shown that *T. iboga* toxicity is mainly due to the high amount of indole alkaloids it contains especially ibogain. For many years, ibogain was successfully used as anti-addictive substance. However, studies reporting degeneration of nervous cells and cardiac arrhythmia prompted some countries to prohibit its utilization (Alper *et al.,* 2012; Mazoyer *et al.,* 2012). The toxicity exerted by *T. iboga* in the present study might be due to the presence of alkaloids. Therefore, the findings in this study indicate that the plants which exerted cytotoxicity effects on Vero cells are a potential danger to humans since Vero cells are standard cells used in many laboratories as a model of healthy mammalian cells. And yet, as stated previously, *in vitro* bioassays are employed to predict the activity of many natural or synthetic substances, but *in vivo* testings are necessary to determine the real danger of these substances in humans.

When it came to the anticancer evaluation, the focus was on HeLa cells which are known to derive from cervical cancer (Scherer *et al.,* 1953). Most cases of invasive cervical cancer are caused by the human papilloma virus (HPV) (Melton, 2017). Cervical cancer is a recognized acquired immunodeficiency syndrome defining cancer. Many studies have indicated that HIV-positive women are significantly more likely to develop cervical cancer (Ghebre *et al.,* 2017; Palefsky, 2017; Dryden-peterson *et al.,* 2016). Although studies have associated the introduction of ART with reduced incidence of common AIDS cancers such as Kaposi sarcoma and non-Hodgkin lymphoma, they have not found a similar reduction in the incidence of cervical cancer

(Ghebre *et al.,* 2017; Melton 2017; Palefsky, 2017). In addition, it has been stated that more than half the approved clinical drugs in the USA are of natural sources (Newman and Cragg, 2012). Therefore, plants provide an excellent platform for the isolation and identification of chemotherapeutic agents for the treatment of various cancers including cervical cancer.

From the findings of the current study presented in Figure 3.14, 3.17 and 3.18, several plant extracts exerted a promising anticancer potential. What is interesting here is that as opposed to the Vero cells, the two methods did not show the same results. According to MTT results only *A. klaineana* methanol extract at the higher concentration was able to exert a satisfactory cytotoxic. Whereas in the fluorescence microscopy assay more extracts were potentially inhibiting the HeLa cell growth. The results also demonstrated that *A. hirtella* and *R. vomitoria* extracts (aqueous and methanol) showed both cytotoxic and anti-proliferative potential correlated with a significant increase in the mean nuclei area of HeLa cells. Methanol and aqueous extracts of *U. guineensis; M. monandra* and *R. vomitoria* were able to both inhibit the HeLa cancer cells growth but also to kill the cells via the onset of apoptosis as a decrease in the mean nuclei area was observed for all extracts. Tumor cells have the capacity of resisting apoptosis due to a unique microenvironment and a variety of adaptive responses and mutations that further confer survival advantage (Indran *et al.,* 2011). According to Makin and Hickman (2000), one of the major modes of action of chemotherapeutic drugs is to trigger activation of apoptosis.

Clinical studies as well as experimental approaches have revealed the anticancer properties of a large number of medicinal herbs that are mediated through different mechanisms including altered carcinogen metabolism, induction of DNA repair systems, immune activation, suppression of cell cycle progression and promotion of apoptosis (Parsaee *et al.,* 2013). Therefore, based on the results obtained from this study, several plants among the 20 selected plants provided compounds (flavonoids; alkaloids; saponins; terpenoids; phenols; tannins) with promising cytotoxic and antiproliferative effects for cervical cancer. The proper mechanisms involved in the cytotoxicity activity of the active plant extracts have yet to be elucidated using various other assays such as cell cycle arrest, Histone H3 phosphorylation and caspase activation (Eboji *et al.,* 2017).

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CHAPTER FOUR

Evaluation of selected Gabonese medicinal plants for anti-microbial and anti-HIV activities

The following article is in preparation

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CHAPTER 4

Abstract

In Gabon, some HIV infected people have reported general health improvement after the consumption of plant-based remedies. Therefore, the objectives of the present study are to evaluate the effect of 20 selected plants on some microorganisms causing HIV associated opportunistic infections, and also to evaluate their eventual anti-HIV activity. **Method:** Well diffusion assay followed by micro-broth dilution assay were carried out to determine the antimicrobial activity of 40 aqueous and methanol extracts against several bacterial and fungal strains. The evaluation of antigen p24 levels in PBMCs isolated from HIV infected donors after treatment with the extracts (25 µg/mL) was performed using ELISA kit. The anti-HIV activity was also evaluated using HIV reverse transcriptase kit. **Results:** The plants tested showed great antibacterial and antifungal effects on various strains such as *S. aureus, E. coli, B. cereus, C. albicans* and *C. tropicalis.* Based on the well diffusion assay results *C. edulis*; *M. cecropioides*; *S. ochocoa*; *S. gabonensis; A. hirtella; R. vomitoria; S. kamerunensis; P. soyauxii*; *U. guineensis* and *T. iboga* were selected for micro-broth dilution assay and displayed MIC values ranged between 0.39 and 25 mg/mL. The results of the cell viability assessment of HIV infected PBMCs after treatment with the extracts revealed that globally, aqueous extracts were significantly more toxic than methanol extracts (P=0.0038). In addition, some extracts such as aqueous and methanolic extracts of *C. edulis* (143% and 180% respectively)*;* methanolic extracts of *A. hirtella* (215%) and *P. soyauxii* (222) were able to stimulate the growth of PBMCs at 25 µg/mL. While others such as *R. vomitoria, T. Iboga* and *V. conferta* were toxic to PBMCs. All the plant extracts exerted inhibition (9% to 48%) of antigen p24 levels except *R. vomitoria, U. klainei* methanol and *S. gabonensis* aqueous extracts. Only 8 extracts showed inhibition of RT including *Coula edulis* methanol and aqueous extracts which lowered RT by 34% and 82% respectively at 250 µg/mL and by 84% and 86% respectively at 500 µg/mL. **Conclusion:** This study has demonstrated that plants used in Gabon to deal with OI related to HIV have important antimicrobial activity and also the potential to inhibit the replication of the HIV *in vitro*. These findings validate the reports of HIV patient health improvement and viral load back to undetectable.

Key words: Antimicrobial, anti-HIV, reverse transcriptase, antigen p24, plant extracts.

4.1 INTRODUCTION

The aim of this chapter is to evaluate the effect of 40 aqueous and methanolic plant extracts on selected microorganisms related to HIV associated opportunistic infections (OIs), and also to evaluate their eventual anti-HIV activity. These selected plants are used in Gabon to manage HIV related opportunistic infections. Some HIV infected people have reported general health improvement after the consumption of plant-based remedies. The health improvement is characterised by weight gain; a decrease in the occurrence of OIs; an increase in CD4+ cells and even the registering of an undetectable HIV load. In the light of these positive results, it is therefore necessary to investigate the antimicrobial and antiviral potential of the selected herbs using standardised assays such as well diffusion assay; micro-broth dilution assay; anti-reverse transcriptase assay and p24 assay.

4.1.1 BACKGROUND

Acquired immunodeficiency syndrome (AIDS) is an infection caused by the human immunodeficiency virus (HIV) and is responsible for damaging an individual's ability to fight diseases, which leaves the body susceptible to any normally innocuous pathogens. HIV infects specific cells (lymphocyte T helper and monocytes/macrophages) that play an essential role in the functioning of the immune system. Once infected, the cells are converted into a factory producing copies of the virus, after which they will be destroyed. Thus, a decline in the population of the cells is observed correlating to a weakened immune system. Persistent infections such as yeast infections, shingles, cancers and tuberculosis become part of the infected individual's life. These infections are known as opportunistic infections associated with HIV. In HIV infection, opportunistic infections caused by infectious pathogens are the leading cause of mortality among HIV infected people. Infectious agents take advantage of the immune system weakened by infection with HIV, and provoke numerous infections such as pneumonia; candidiasis; toxoplasmosis and infectious diarrhea that can be lethal to an immunocompromised person. Generally, the prevalence of the OIs varies depending on the climatic environment, the socio-economic level, and the implementation of a suitable prophylaxis (Pang *et al.,* 2018; Katabwa *et al.,* 2017; Okome *et al.,* 2014). In China for example, a study conducted in

a public health center revealed that bacterial pneumonia was the most common OI, while neuromeningeal cryptococcosis was the most reported OI among HIV patients in Congo (Pang *et al.,* 2018; Katabwa *et al.,* 2017). According to a study conducted in Gabon by Okome et al. (2014), pulmonary tuberculosis was among the most common reported OI in HIV patients. Infectious pathogens causing HIV related OIs are numerous and include bacteria (*Staphylococcus sp*, *Enterococcus sp, E. coli; Shigella sp; Salmonella sp*; etc.), parasites (*Plasmodium sp, Toxoplasma sp*, etc.); fungi (*Candida sp, Cryptococcus sp*, etc.) and viruses (*Herpes sp*, Cytomegalovirus, etc.) can be efficiently managed by commercially available drugs (Aidsinfo, 2018).

The emergence of microbial resistance to a high number of available antibiotics is a threat to many people especially HIV infected individuals. The mortality rate for HIV infected people increase dramatically when the infections are caused by drug resistant bacteria in contrast to infections caused by bacteria sensitive to available antibiotics (Aidsinfo, 2018). For instance, unlike immunocompetent people who usually do not require any treatment for Salmonellosis, AIDS patients need to take antibiotics for the treatment but also for the prevention of a recurrent Salmonella infection in the bloodstream (Aidsinfo, 2018). The genetic diversity in infectious organisms can also lead to treatment failure as, like viruses, bacteria can also transfer DNA to other bacteria thus spreading the resistance to antibiotics (Bhattacharjee, 2016).

Unfortunately, only five new antibiotics have been marketed since 2000 and none of these are successful in targeting highly resistant bacteria and currently, only 57 antibiotics are in clinical development phase globally (Patel and Fadaei, 2016). In the light of the increasing cost of drug discovery and development, every new generation of antibiotic is more expensive than its predecessors. The high cost of antibiotics and the bacterial resistance to the available antibiotics necessitate the exploration of novel sources of new antimicrobial drugs. Plant based traditional medicine has been used for centuries to treat infectious diseases. Plants contain various well-known compounds including steroids, flavonoids, saponins and glycosides which have shown extraordinary antimicrobial potential in previous studies (Itelima and Agina, 2014).

4.1.2 ANTIMICROBIAL POTENTIAL OF MEDICINAL PLANTS

The investigation of plant antimicrobial activity starts with biological evaluations to ensure the efficacy but also the safety of those plants. Various *in vitro* standardized methods have been developed in order to evaluate the antimicrobial efficacy of plant extracts that include both well and disk diffusion assays, broth dilution assay, and autobiography (Das *et al.,* 2010). It has been established from previous studies that that plants are able to efficiently inhibit the growth of various microorganisms including multi-resistant bacteria *in vitro* and *in vivo* (Itelima and Agina, 2014). In addition, numerous studies and reviews report on the anti-HIV potential of medicinal plants (Boukandou *et al.,* 2018; Salehi *et al.,* 2018). These studies depict the mechanisms involved in the anti-HIV activity displayed such as inhibiting activities of various enzymes required for viral replication like protease, reverse transcriptase and integrase as well as in preventing virus entry into the host cells.

Furthermore, some reports described the efficiency of plant-based remedies. Thus in a review, Nlooto and Naidoo (2014) summarized studies investigating African traditional medication therapies with clinical relevance which were used for the management of HIV and related opportunistic infections. They depicted results reporting either on the increase of CD4 count or decrease in viral load as well as improvement on the quality of life and HIV related symptoms. Another study reported on a patient who took a plant concoction for 12 weeks and experienced a remarkable rise of CD4 cell count, undetectable virus and negative antibody tests without using anti-retroviral drugs (Mushiwokufa, 2016). Therefore, from the above we can deduce that a possible cure for HIV, using medicinal plant remedies is to be considered.

The main goal of this specific objective of the study was to evaluate the antimicrobial activity and the anti-HIV effect of selected plants against HIV and several selected pathogens responsible for bacterial and fungal HIV related OI.

4.2 MATERIALS AND METHODS

4.2.1 ETHICAL APPROVAL

Ethical approval was obtained from the Health, Safety, and Research Ethics Committee of the University of Venda (SMNS/17/MBY/30/2111). Permission to use health facilities in order to get the HIV infected blood was obtained from the Limpopo Provincial Department of Health. Signed informed consent agreement forms were obtained from all volunteers before the collection of blood specimens. Confidentiality around volunteers' identities was maintained throughout by assigning numerical codes. The blood was used for PBMCs extraction only.

4.2.2 MICROORGANISMS

The selected strains for the current study were *Staphylococcus aureus* (ATCC 25923)*; Salmonella enterica* (ATCC 51741); *Escherichia coli* (ATCC 25922); *Shigella sonnei* (ATCC 25931)*; Klebsiella pneumoniae* (ATCC 27736)*; Pseudomonas aeruginosa* (ATCC 27853)*; Enterobacter cloacae* (ATCC 13047)*; Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (ATCC 10876) and yeast isolates (*Candida tropicalis; Candida albicans* and *Cryptococcus gondii*) (Kolawole et al., 2018; Varghese et al., 2018; Autade et al., 2015).

4.2.3 ANTIMICROBIAL ACTIVITY

Adapted agar-well diffusion susceptibility test for bacteria and fungus as well as broth dilution assay only for bacteria were performed to assess the antimicrobial activity.

4.2.3.1 Well diffusion susceptibility test

The method as described by Balouiri et al. (2016) was used. Briefly, one or two bacterial and fungal colonies from a 24-hour culture plate were emulsified in 5 mL sterile distilled water and standardized to 0.5 MacFarland tube. A sterile cotton swab was soaked with the bacterial inoculum and used to evenly inoculate labelled Muller-Hinton agar plates. With the help of a sterile cork-borer, wells of 6 mm diameter were punched in the inoculated plates. Then, 50 µL of methanol and water extract concentrations of 200, 150, 100, and 50 mg/mL were aseptically added in the labelled corresponding wells. The plates were incubated at 37°C for 24 h for bacteria and 48 h for fungi. The assay was done in triplicate for each strain and each plant extract. The plates were examined for the zone of inhibition (Figure 4.1), which appeared as the

clear area around the wells. Inhibition zone diameter was measured by reading the diameter with a ruler in three different fixed directions in all 3 replicates and the average values were tabulated. Gentamicin was used as control.

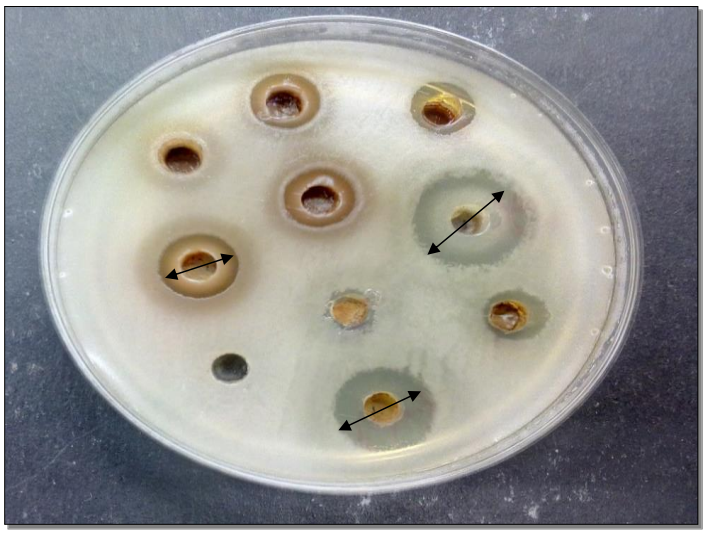

Figure 4.1: Agar plate showing diameter of inhibition

4.2.3.2 Minimal inhibitory concentration (MIC) determination

The minimum inhibitory concentration was evaluated using micro-broth dilution method in sterile 96-well plates (round bottom) as described by Balouiri et al. (2016). The plant extracts were tested against nine Gram positive and Gram-negative bacterial strains. The 96 wells of the plates were filled with 100 µL sterilized brain heart infusion broth. The first row (A1-A12) received an additional 100 µL of plant extracts, positive control (gentamicin) and negative control (sterile distilled water). Two-fold serial dilutions were performed to create a concentration sequence from 50 mg/mL to 0.024 mg/mL. After the serial dilutions of the extracts and gentamicin, 100 µL of 0.5 McFarland bacterial suspensions ($1.5x10^8$ CFU/mL) were added to each well then incubated overnight at 37°C.

The MIC was determined to be where growth was no longer visible by assessment of bacterial viability using 40 µL of 0.4 mg/mL iodo-nitro-tetrazolium (INT) (Sigma-Aldrich). At least three repetitions were performed for each plant extract and strain.

The lowest concentration of the extract which inhibited visible growth of the tested microorganisms was designated as the MIC. Strong activity was defined as MIC < 5 mg/mL.

4.2.3.3 Minimum bactericidal concentration (MBC) determination

From the MIC well and all the wells where no bacterial growth was observed, 25 µL of the culture was streaked on sterile MH agar plates with a glass spreader. The plates were incubated overnight at 37°C. The results were recorded, and the MBC was observed in the plates where there was no bacterial growth and the assays were carried out in triplicate. The ratio MBC/MIC was determined in order to determine if the effect was bactericidal or bacteriostatic.

4.2.4 ASSESSMENT OF ANTI-HIV ACTIVITY

The assessment of the potential antiviral activity of the plant extracts was performed on HIV infected peripheral blood mononuclear cells (PBMCs) by measuring the inhibition of HIV-1 p24 antigen using a commercially available ELISA kit (ZeptoMetrix Corporation, Bufalo, NY). Afterwards, extracts displaying promising activity on HIV were selected to analyze their effect in inhibiting HIV reverse transcriptase.

4.2.4.1 Blood collection

Blood samples were collected from HIV+ patients (3 sets of donors) at the University of Venda clinic by trained nurses of the clinic. Approximately 5 mL of whole blood was collected into a vacutainer tube with EDTA (Ethylenediaminetetraacetic acid) and the tubes were inverted several times to ensure complete mixture of contents. The samples were transported to the School of Mathematical and Natural Sciences' microbiology laboratory for analysis.

4.2.4.2 Culture medium

Cells were maintained and cultured in complete medium RPMI 1640 containing 2mM L-glutamine (Sigma-Aldrich, Missouri, USA), heat-inactivated foetal-calf serum (10% v/v); and gentamycin sulphate (1 ng/mL).

4.2.4.3 Isolation and subculture of PBMCs

Immediately after collection, the 5 mL of blood sample was first diluted with 45 mL of RPMI into 50 mL sterile conical tubes and the tubes were inverted several times to ensure the complete mixing of blood and RPMI. The diluted samples (20 mL) were afterwards transferred to a conical centrifuge tube containing 10 mL of Ficoll (Sigma-Aldrich), then centrifuged for 30 minutes at 2 000 rpm. At the end of the centrifugation, the layer containing the PBMCs was aspirated into a new 15 mL centrifuge conical tube using a pipette (10 mL) and the volume was adjusted to 15 mL with PBS then centrifuged for 10 min at 2000 rpm. The process of washing was repeated twice. Then, the supernatant was removed and the pellet (isolated PBMCs) was resuspended into 10 mL of complete media (RPMI, 10 % FBS, 200 µL, Gentamicin) and PHA was added to stimulate cell growth. The cell suspension was then poured into flasks and incubated at 37°C in a humidified incubator with 5% CO_2 for 24 h.

4.2.4.3 *In vitro* bioassay

Overnight stimulated PBMCs (as described above) were plated in a 24-well plate (Nalgene Nunc, Roskilde, Denmark) at a density of 1×10^6 cells/well and treated with plant extracts at a concentration of 25 µg/mL. Phytohaemagglutinin (PHA) (5 µg/mL) was the positive control and absolute ethanol was the negative control. After 48 h of incubation at 37°C in a humidified incubator with 5% CO_2 (EcoTherm, Hartkirchen, Austria), 1 mL of supernatant were removed for ELISA assays and the remaining cells in media were used for viability assay with XTT. The plant extracts were tested in triplicates.

4.2.4.4 Viability assay using XTT

The viability of infected PBMCs was done by measuring the proliferation of the cultured cells using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) XTT method according to the manufacturer's instructions (Roche, Basel, Switzerland). Briefly, 200 µL of cultured PBMCs from the bio-assay were transferred in 96 well plates and 50 µL of activated XTT solution (0.3 mg/mL) was added to each well of the plates which were then incubated for 4 hours. Absorbance was measured at 570 nm using a microplate reader (Multiscan MS, Labsystems, Vantaa, Finland). A percentage of cell inhibition was calculated from the absorbance of the extract treated cells (Figure 4.2) and the positive control. The experiment was performed in triplicate.

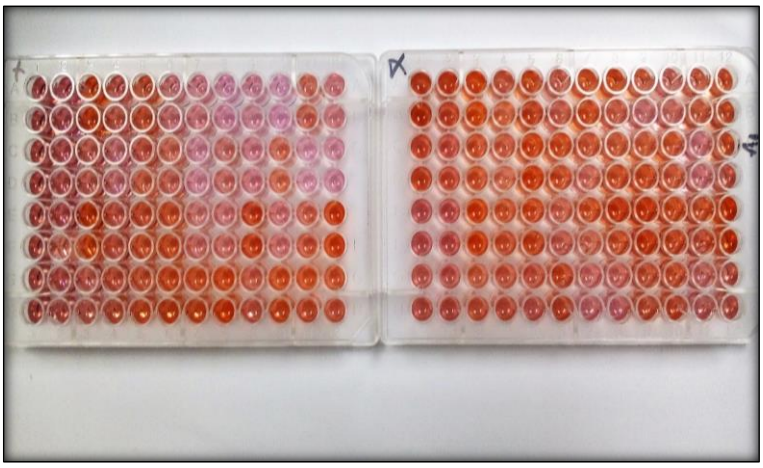

Figure 4.2: Expected XTT plate showing cytotoxic effects. The lighter the coloration, the more toxic the extract

4.2.4.5 P24 ELISA assay

The effect of the selected plant extracts on HIV replication was assessed by measuring viral core protein p24 expression in HIV infected PBMCs using a commercial ELISA kit (ZeptoMetrix Corporation, Bufalo, NY) according to the manufactures' instructions. The supernatants from the bio-assay (as described in section 4.2.4.3) were first subjected to lysis overnight at 4°C and then transferred to wells pre-coated with anti-p24 antibody and assayed by biotinyl- labeled anti-p24 secondary antibody followed by streptavidin-peroxidase conjugate. The p24 expression was evaluated by measuring absorbance of the tetramethylbenzidine substrate at 450 nm. Following the addition of the substrate and incubation, a reactant color develops due to reaction with the HRP enzyme. The measured absorbance is proportional to the amount of HIV-1 p24 antigen present in a given sample.

4.2.4.6 HIV-1 reverse transcriptase inhibition assay

The HIV reverse transcriptase inhibition assay was determined by commercial colorimetric quantitative analysis kit according to the manufacturer's instructions (Roche Diagnostics; Risch-Rotkreuz, Switzerland). The assay is based on the incorporation of dioxigenin and biotin labelled nucleotides into the new DNA synthesis. Lysis buffer with HIV- 1-RT was used as the negative control. Into 96 well plates

containing 10 µL of the lysis buffer, 10 µL of plant extracts (250 and 500 µg/mL) and positive control Efavirenz (75 µM) diluted in lysis buffer were added in triplicate, followed by 10 µL of the RT reaction mixture per well and incubated overnight at an ambient temperature. Thirty (30) µL samples were transferred into the wells. The plates were covered with a cover foil and incubated for 1 hour at 37°C. The solution was then removed completely. After washing them 5 times with the buffer solution, 100 µL of anti-DIG-POD working dilution was added to each well, while the MP modules were covered again and incubated for 1 hour at 37°C. After removing the solution completely and washing, 200 µL of ABTS (2,2'-azino-bis (-ethylbenzthiazoline-6-sulfonic acid)) substrate solution was added per well and incubated at 25°C until colour development (green colour) was sufficient for photometric detection (10-30 minutes) (Figure 4.3). Using a micro plate ELISA reader (Multiscan MS, Labsystems, Vantaa, Finland), the absorbances of the samples were measured at 405 and 490 nm. The percentage RT enzyme inhibition activities of the extract were calculated as follows:

Percentage RT inhibitory= 100− (extract sample $A^{405nm}-A^{492nm}$ / positive control$A^{405nm}-A^{492nm}$) x 100

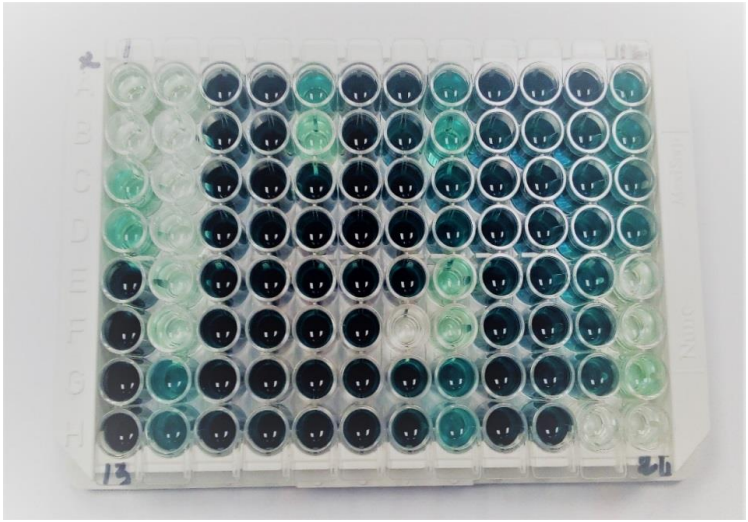

Figure 4.3: Expected plate for RT assay. The lighter the coloration, the more active the extract.

4.2.5 STATISTICAL ANALYSIS

Each sample assessment was performed in triplicate. The data was treated using Graph pad prism 6.0. The results are presented in mean (±SD) of the triplicate. For the anti-HIV data, the non-parametric T-test (Mann Whitney test) was run. A P value <0.05 corresponds to a significant difference between aqueous and methanolic extract means.

4.3 RESULTS

4.3.1 ANTIMICROBIAL ASSESSMENT

4.3.1.1 Well diffusion assay

The antimicrobial screening of 20 selected plants against microorganisms causing opportunistic infections in HIV individuals was done using well diffusion assay. Different concentrations (50, 100, 150 and 200 mg/mL) of 20 aqueous and 20 methanolic extracts were evaluated for their effects on several fungi as well as Gram-positive and Gram-negative bacteria.

The results reported in Figure 4.4 depict the number of extracts that presented diameters of inhibition > 6 mm against the tested microorganisms at the lowest concentration of 50 mg/mL. The results reveal that 28 out of 40 aqueous and methanolic extracts namely, *C. edulis; A. klaineana; M. monandra; U. klainei; S. kamerunensis* and *T. iboga* were active against all the Gram-positive bacteria including *S. pneumonia; S. aureus; E. faecalis* and *B. cereus*. While, 22 plant extracts (such as: *S. ochocoa; M. cecropioides; A. hirtella; R. vomitoria; U. guineensis* and *P. soyauxii*) were able to inhibit the growth of *Shigella sonnei, Salmonella enterica* and *Klebsiella pneumoniae*.

The Gram-negative bacteria *E. coli* and *P. aeruginosa* were susceptible to respectively 20 and 21 plant extracts. *E. cloacae* was less sensitive to the extracts as only 11 plant extracts were able to inhibit its growth at 50 mg/mL. Only 8 aqueous and methanol extracts including *C. edulis; T. iboga; A. hirtella* and *U. guineensis*, exerted inhibition of the fungi tested *(Candida albicans, Candida tropicalis* and *Cryptococcus gondii*). Both aqueous and methanol extracts of *A. cissampeloides; M. puberula; A. vogelii; V. conferta; C. lucanusianus* and *R. africanum* did not show any activity against both

Gram positive and negative bacteria as well as the fungi tested, even at the highest concentration (200 mg/mL).

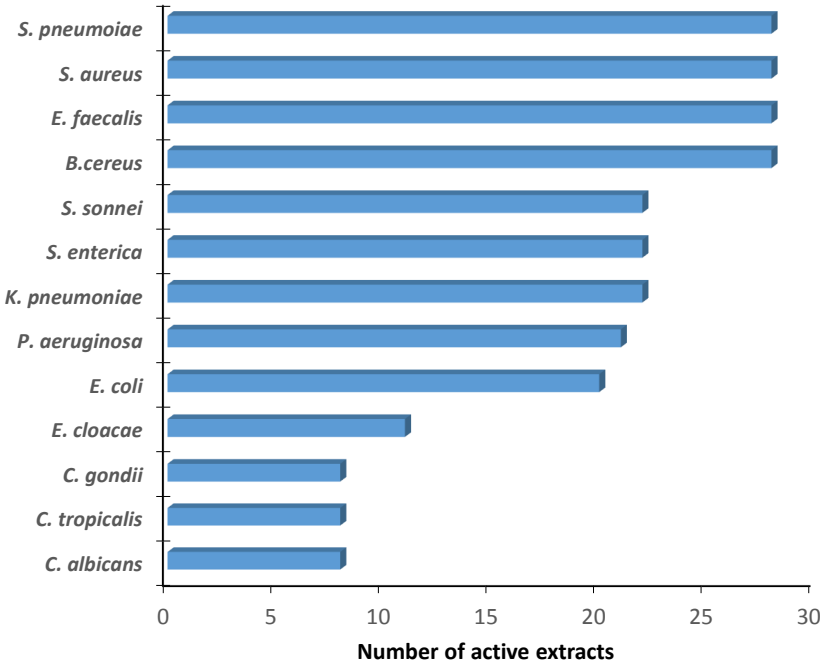

Figure 4.4: Number of active extracts against the selected pathogens

4.3.1.2 Minimum inhibitory concentration

The micro-broth dilution method was used to determine the minimum inhibitory concentration (MIC) of the plant extracts against nine bacterial strains in triplicate.

Based on the well diffusion assay results, only 10 plants (*C. edulis*; *M. cecropioides*; *S. ochocoa*; *S. gabonensis; A. hirtella; R. vomitoria; S. kamerunensis; P. soyauxii*; *U. guineensis* and *T. iboga)* were selected for micro-broth dilution assay. Only plant extracts (aqueous and methanolic) that displayed both antibacterial and antifungal activities against all the microorganisms tested were chosen for MIC determination. The results presented in Table 4.1 indicate the minimal inhibitory concentration (in mg/ml) of the selected plant extracts against gram-positive and gram-negative bacteria. The table shows that, methanol extracts displayed an overall highest activity

compared to the aqueous extracts except for *P. soyauxii* and *U. guineensis*. *P. soyauxii* and *U. guineensis* aqueous extracts which showed the highest activity on all the tested strains compared to the methanol extracts except for *E. cloacae* and *B. cereus* respectively. *E. faecalis* and *S. enterica* were the most sensitive to the different extracts tested with MIC ranging from 0.098 mg/mL to 6.25 mg/mL. *E. cloacae, E. coli, P. aeruginosa* and *K. pneumoniae* were less susceptible to the plant extracts as the MIC values were all higher than 5 mg/mL.

Both methanol and aqueous extracts of *C. edulis* (0.098 mg/mL); *M. cecropioides* (0.39 mg/mL); *S. ochocoa* (0.39 mg/mL); *S. gabonensis* (0.39 mg/mL); *U. guineensis* (0.39 mg/mL for aqueous extract and 0.78 mg/mL for methanol extract) and aqueous extracts of *S. kamerounensis* (0.78 mg/mL) and *P. soyauxii* (0.39 mg/mL) exerted a strong antibacterial activity against *E. faecalis*. *S. ochocoa* (0.39 mg/mL); *A. hirtella* (0.78 mg/mL) both methanol and aqueous extracts and *M. cecropioides* (0.39 mg/mL), *S. gabonensis* (0.78 mg/mL), *P. soyauxii* (0.78 mg/mL) aqueous extracts and *R. vomitoria* (0.39 mg/mL), *S. kamerunensis* (0.39 mg/mL) methanol extracts exerted the strongest antibacterial activity against *S. enterica*. *C. edulis* extracts exerted the strongest antibacterial activity against all the strains tested. Concerning *S. aureus, B. cereus* and *S. sonnei,* the MIC values were ranged between 0.39 and 25 mg/mL.

Table 4.1: Minimum inhibitory concentration (mg/mL) of the plant extracts

Plant extracts		*S. aureus*	*E. faecalis*	*B. cereus*	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*S. enterica*	*E. cloacae*	*S. sonnei*
C. edulis	Methanol	1.56	0.098	1.56	6.25	12.5	25	0.195	6.25	0.39
	Aqueous	1.56	0.098	1.56	6.25	12.5	12.5	0.195	1.56	0.78
M. cecropioides	Methanol	6.25	0.39	6.25	25	25	25	1.56	50	12.5
	Aqueous	12.5	0.39	12.5	25	12.5	25	0.39	50	6.25
S. ochocoa	Methanol	3.125	0.39	3.125	25	12.5	25	0.39	50	3.125
	Aqueous	12.5	0.39	25	25	25	25	0.39	50	3.125
A. hirtella	Methanol	6.25	1.56	6.25	12.5	12.5	12.5	0.78	12.5	3.125
	Aqueous	25	1.56	25	25	12.5	25	0.78	25	3.125
R. vomitoria	Methanol	12.5	3.12	12.5	25	25	25	0.39	25	3.125
	Aqueous	12.5	6.25	6.25	12.5	12.5	25	6.25	50	3.125
S. Kamerounensis	Methanol	6.25	1.56	3.125	12.5	25	25	0.39	50	6.25
	Aqueous	6.25	0.78	6.25	25	12.5	25	1.56	50	3.125
S. gabonensis	Methanol	3.125	0.39	3.125	12.5	12.5	25	1.56	25	3.125
	Aqueous	12.5	0.39	6.25	25	25	25	0.78	50	6.25
P. soyauxii	Methanol	25	3.125	12.5	50	12.5	25	3.125	25	12.5
	Aqueous	6.25	0.39	12.5	12.5	12.5	25	0.78	50	6.25
U. guineensis	Methanol	12.5	0.78	3.125	50	25	25	1.56	50	12.5
	Aqueous	6.25	0.39	12.5	12.5	25	25	1.56	50	6.25
T. iboga	Methanol	3.125	2.06	3.125	6.25	25	6.25	3.125	6.25	4.125
	Aqueous	6.25	3.125	3.125	12.5	50	12.5	3.125	12.5	6.25
Gentamycin		0.024	<0.024	<0.024	1.25	<0.024	1.25	1.25	0.098	0.098

4.3.1.3 Minimum bactericidal concentration and ratio MBC/MIC

The Minimum Bactericidal Concentration (MBC) was confirmed by the absence of bacterial growth of the tested strains streaked from inhibition zone corresponding to their lowest MICs. Table 4.2 reveals MBC values ranging from 0.39 mg/mL to > 25 mg/mL. All the plant extracts displayed MBC values higher than 25 mg/mL for *P. aeruginosa.* For *E. coli* and *K. pneumonia*, only *C. edulis* and *T. iboga* aqueous and methanol extracts showed MBC values ranging from 6.25 mg/mL to 25 mg/mL. Concerning *E. cloacae* only *A. hirtella*, *T. iboga* and *C. edulis* had values that ranged between 1.56 mg/mL and 25 mg/mL. For the Gram-positive bacteria (*S. aureus, E. faecalis* and *B. cereus*), *S. sonnei* and *S. enterica,* the MBC values were 0.39 mg/mL and 25 mg/mL. *C. edulis* presented the lowest MBC values for all the bacteria except *E. faecalis* and *K. pneumoniae*.

The ratio MBC/MIC introduces the bacteriostatic and bactericidal nature of the extracts. Indeed, a ratio MBC/MIC of an antimicrobial agent ≤ 4 implies a bactericidal action of this agent, while a bacteriostatic agent is defined when MBC/MIC > 4 (Spellberg, 2017). The overall mode of action of the extracts presented as a ratio of MBC/MIC (also in Table 4.2) showed a bactericidal action towards the Gram-positive bacteria as well as *S. enterica* and *S. sonnei*. The ratio MBC/MIC was ≤4 for almost all the extracts except for *R. vomitoria* methanol extract towards *S. enterica* (MBC/MIC=8). A bacteriostatic effect was exerted towards *E. faecalis* by *T. iboga* aqueous extracts (8), *C. edulis* aqueous (64) and methanolic (32) extracts, the methanolic extracts of *M. cecropioides* (16) and *S. ochocoa* (8), both aqueous (16) and methanolic (8) extracts of *U. guineensis* and *S. gabonensis.* All the extracts displayed a bacteriostatic effect towards *P. aeruginosa* with a ratio >25. Regarding *E. coli*, *K. pneumonia* and *E. cloacae* the bactericidal effect was demonstrated with *C. edulis* and *T. iboga* extracts.

Table 4.2: MBC in mg/mL and ratio MBC/MIC

		S. aureus		*E. faecalis*		*B. cereus*		*E. coli*		*P. aeruginosa*		*K. pneumoniae*		*S. enterica*		*E. cloacae*		*S. sonnei*	
		Met	**Aqu**	**Met**	**Aqu**	**Met**	**Aqu**	**Met**	**Aqu**	**Met**	**Aqu**	**Met**	**Aqu**	**Met**	**Aqu**	**Met**	**Aqu**	**Met**	**Aqu**
C. edulis	**MBC**	1.56	1.56	3.13	6.25	1.56	1.56	6.25	6.25	>25	>25	25	12.5	0.95	0.95	6.25	1.56	0.39	0.78
	MBC/MIC	**1**	**1**	**32**	**64**	**1**	**1**	**1**	**1**	**>1**	**>1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
M. cecropioides	**MBC**	6.25	12.5	6.25	0.78	6.25	12.5	>25	>25	>25	>25	>25	>25	1.56	1.56	>25	>25	25	6.25
	MBC/MIC	**1**	**1**	**16**	**2**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**1**	**4**	**>1**	**>1**	**2**	**1**
S. ochocoa	**MBC**	3.13	12.5	3.13	0.39	3.13	25	>25	>25	>25	>25	>25	>25	1.56	1.56	>25	>25	12.5	6.25
	MBC/MIC	**1**	**1**	**8**	**1**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**4**	**4**	**>1**	**>1**	**4**	**2**
A. hirtella	**MBC**	6.25	25	3.13	1.56	6.25	25	>25	>25	>25	>25	>25	>25	1.56	3.13	12.5	25	6.25	3.13
	MBC/MIC	**1**	**1**	**2**	**1**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**2**	**4**	**1**	**1**	**3**	**1**
R. vomitoria	**MBC**	12.5	12.5	6.25	6.25	12.5	6.25	>25	>25	>25	>25	>25	>25	3.13	6.25	>25	>25	3.13	6.25
	MBC/MIC	**1**	**1**	**2**	**1**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**8**	**1**	**>1**	**>1**	**1**	**1**
S. kamerunensis	**MBC**	6.25	6.25	1.56	0.78	3.13	6.25	>25	>25	>25	>25	>25	>25	4	1.46	>25	>25	25	3.13
	MBC/MIC	**1**	**1**	**1**	**1**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**4**	**1**	**>1**	**>1**	**4**	**1**
S. gabonensis	**MBC**	3.13	12.5	3.13	6.25	3.13	6.25	>25	>25	>25	>25	>25	>25	1.56	0.78	>25	>25	12.5	6.25
	MBC/MIC	**1**	**1**	**8**	**16**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**1**	**1**	**>1**	**>1**	**4**	**1**
P. soyauxii	**MBC**	25	6.25	3.13	1.56	12.5	12.5	>25	>25	>25	>25	>25	>25	3.13	1.56	>25	>25	12.5	6.25
	MBC/MIC	**1**	**1**	**1**	**4**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**1**	**1**	**>1**	**>1**	**1**	**1**
U. guineensis	**MBC**	12.5	6.25	6.25	6.25	3.13	12.5	>25	>25	>25	>25	>25	>25	1.56	1.56	>25	>25	12.5	6.25
	MBC/MIC	**1**	**1**	**8**	**16**	**1**	**1**	**>1**	**>1**	**>1**	**>1**	**>1**	**>1**	**1**	**1**	**>1**	**>1**	**1**	**11**
T. iboga	**MBC**	3.13	6.25	6.25	25	3.13	6.25	6.25	12.5	>25	>25	6.25	12.5	6.25	6.25	6.25	25	4.125	6.25
	MBC/MIC	**1**	**1**	**2**	**8**	**1**	**2**	**1**	**1**	**>1**	**>1**	**1**	**1**	**2**	**2**	**1**	**2**	**1**	**1**

Met= Methanol extracts; Aqu=Aqueous extracts

4.3.2 ANTI-HIV ASSESSMENT

4.3.2.1 HIV infected PBMCs viability

Before assessing the anti-HIV activity of the selected plant extracts, it was necessary to determine the level of toxicity exerted by the extracts on HIV infected PBMCs. Thus, the cell viability was assessed using XTT assay and the results are presented in Figure 4.5 as percentage of viable cells.

The cell viability of cells treated with extracts was compared to the untreated cells. The data reveals that globally, aqueous extracts were significantly more toxic to HIV infected cells than methanol extracts (P=0.0038). in addition, aqueous and methanolic extracts of *C. edulis* (143% and 180% respectively)*; S. ochocoa* (118% and 206% respectively)*; S. kamerounensis* (182% and 169% respectively)*; S. gabonensis* (116% and 195% respectively) as well as aqueous extract of *M. puberula* (145%); methanolic extracts of *M. cecropioides* (156%)*; A. hirtella* (215%)*; R vomitoria* (165%)*; M. monandra* (15%)*; P. soyauxii* (222%)*; U. klainei* (162%) and *A. klaineana* (141%) were all able to stimulate the growth of PBMCs at the tested concentration (25 µg/mL).

The remaining extracts including aqueous extracts of *R. vomitoria, M. monandra, R. africanum, T. iboga* as well as both aqueous and methanolic extracts of *C. lucanusianus* and *V. conferta* were toxic to the PBMCs as an inhibition (decrease in viability) of more than 20% was observed.

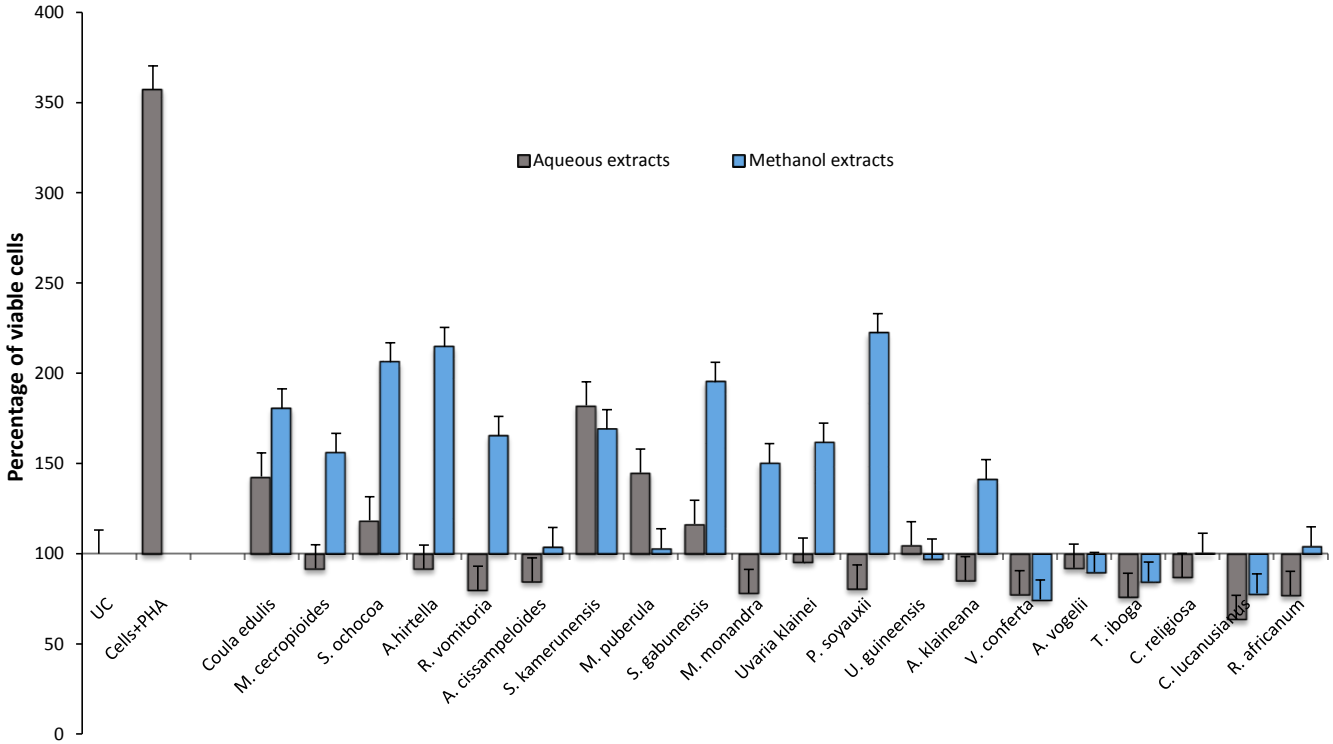

Figure 5.5: HIV-Infected cell viability after treatment with plant extracts at 25 µg/mL. Errors bars denote standard deviation.

4.3.2.2 P24 antigen evaluation

In order to determine the action of the 40 plant extracts on HIV infected PBMCs, the quantification of HIV p24 antigen was performed using a kit (ZeptoMetrix Corporation, Bufalo, NY).

The results described in Figure 4.6 expressed the percentage of inhibition obtained after treatment with the samples at a concentration of 25 µg/mL. No significant difference was detected between aqueous and methanolic extracts (P= 0.32). The effects of plant extracts on cells were compared to untreated cells. Cells treated with PHA showed a decrease of 11% compared to the untreated cells. Moreover, all the plant extracts exerted a high percentage of inhibition of antigen p24 levels except *R. vomitoria* and *U. klainei* methanol extracts as well as *S. gabonensis* aqueous extracts.

Lowest inhibition percentage was displayed by the aqueous extracts of *A. vogelii* (9%), *M. cecropioides* (10%), *R. vomitoria* (11%) and both aqueous and methanol extracts of *M. monandra* (10% and 11% respectively). While highest inhibition was observed for the aqueous extracts of *A. hirtella* (42%); *S. kamerunensis* (41%); *V. conferta* (47%); *C. religiosa* (44%); *C. lucanusianus* (47%); *M. puberula* methanol extracts (41%) and both aqueous and methanolic extracts of *U. guineensis* (46% and 41% respectively); *A. klaineana* (41% and 44% respectively) and *T. iboga* (48% and 45% respectively).

Although the methanol extract of *U. klainei* didn't exhibit an inhibition of the p24 antigen, the aqueous extract showed a promising effect with an inhibition rate reaching 43%.

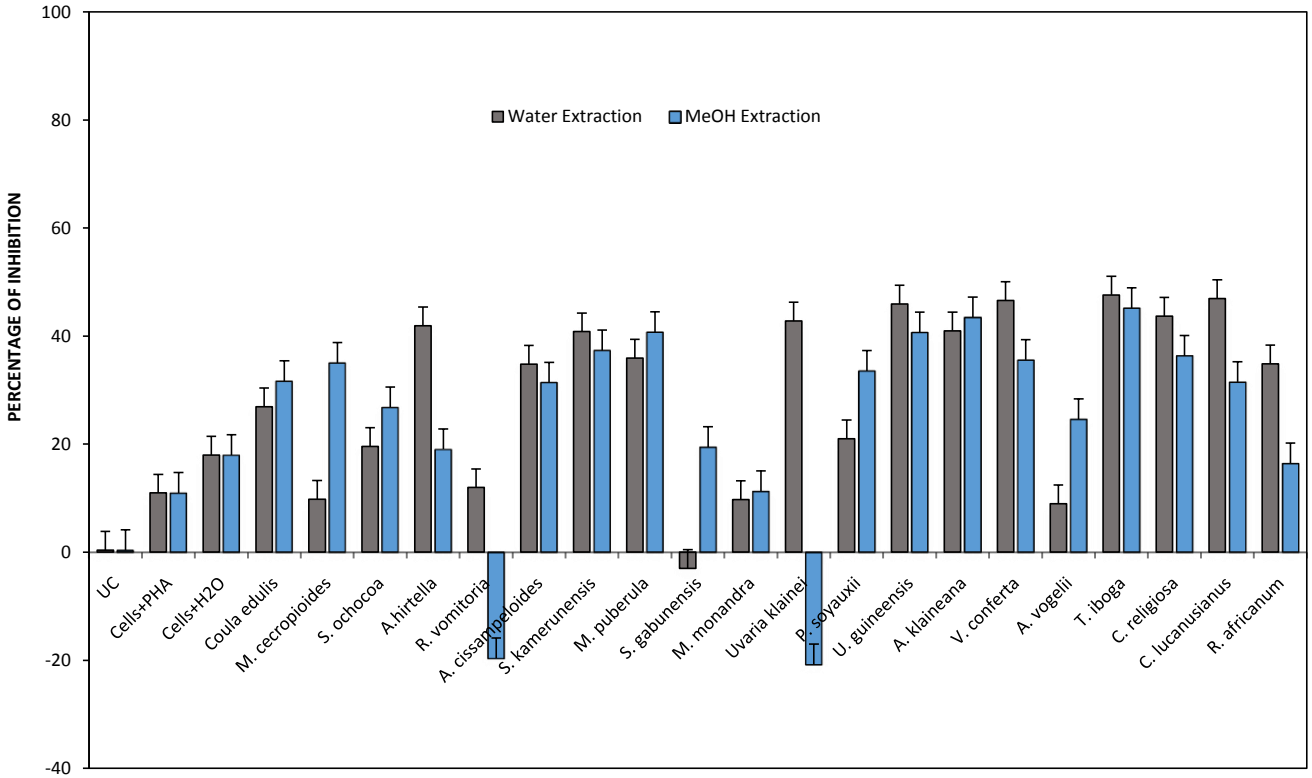

Figure 4.6: P24 antigen expression after treatment with the plant extracts at 25 µg/mL. Errors bars denote standard deviation.

4.3.2.3 HIV-1 reverse transcriptase inhibition assay

The HIV-1 reverse transcriptase inhibition assay was performed with only extracts that were able to substantially inhibit HIV-1 p24 antigen production. Thus, 24 methanol and aqueous extracts were selected.

The results in Figure 4.7 show a report on the percentage of inhibition of HIV-1 reverse transcriptase exhibited by the active extracts. The results revealed that out of 24 selected plant extracts, only 8 extracts showed inhibition of reverse transcriptase activity at either 250 µg/mL or 500 µg/mL or at both concentrations. *Coula edulis* methanol and aqueous extracts were able to lower RT by 34% and 82% respectively at 250 µg/mL and by 84% and 86% respectively at 500 µg/mL. The same trend was observed for *C. religiosa* aqueous extracts at both concentrations with percentage of inhibition of 72% at 250 µg/mL and 81% at 500 µg/mL. Interestingly, *C. lucanusianus* (89%) and *R. africanum* (80%) methanol extracts were inhibiting RT activity only at 250 µg/mL. While the aqueous extracts of *A. hirtella* (23%), *P. soyauxii* (74%) and *V. conferta* (62%) were active only at 500 µg/mL.

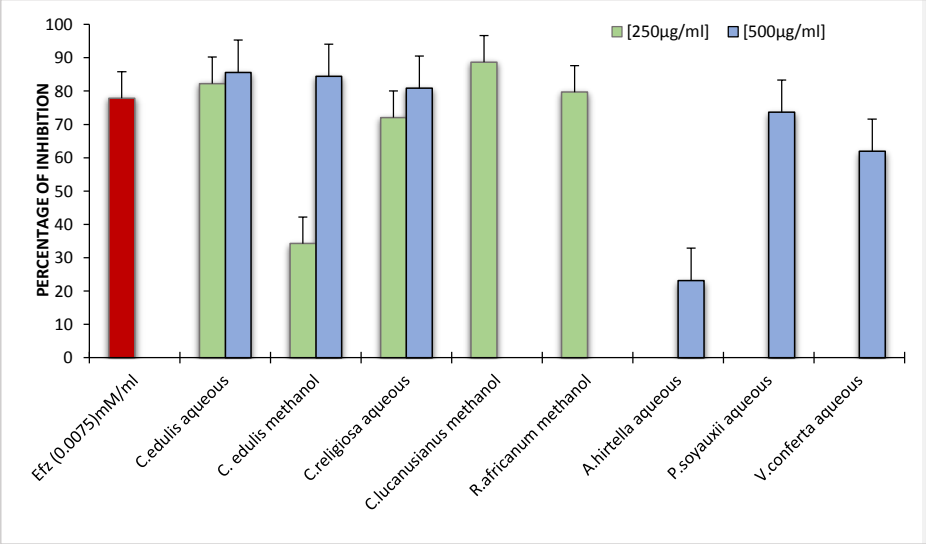

Figure 4.7: HIV reverse transcriptase inhibitory expression after treatment with the plant extracts at 250 and 500 µg/mL. Errors bars denote standard deviation.

4.4 DISCUSSION

Acquired immunodeficiency syndrome (AIDS) is one of the deadliest diseases worldwide and is caused by the human immunodeficiency virus (HIV). This disease constitutes a huge concern for global public health as there is no available cure for HIV/AIDS. Therefore, prevention, antiviral drugs and the treatment of occurring specific OI are the only option in slowing down morbidity and mortality in HIV-infected people. Medicinal plants have the reputation for managing numerous diseases including HIV/AIDS, cancer and various infections with good outcomes. Thus, it is necessary to investigate the plants used in the management of HIV infection and its related opportunistic diseases. For this purpose, the current study aimed at evaluating the antimicrobial activity and the anti-HIV effect of 20 selected plants against HIV and several selected pathogens responsible for bacterial and fungal HIV related OIs.

Anti-microbial activity

The antimicrobial activity of the plant extracts was performed against yeast, Gram-positive and negative bacteria that included: *Candida tropicalis; Candida albicans; Cryptococcus gondii*; *Staphylococcus aureus; Salmonella enterica; Escherichia coli*; *Shigella sonnei; Klebsiella pneumoniae; Pseudomonas aeruginosa; Enterobacter cloacae; Enterococcus faecalis;* and *Bacillus cereus* (Figure 4.4, Table 4.1 and 4.2). Firstly, well diffusion assay was undertaken as a screening in order to select the plant extracts with antimicrobial potential. The results revealed that 28 out of 40 aqueous and methanolic extracts were active against all the Gram-positive bacteria that included *S. pneumonia, S. aureus, E. faecalis* and *B. cereus*. Gram-positive organisms (including bacteria of the genera *Staphylococcus*, *Streptococcus* and *Enterococcus)* are among the most common bacterial responsible for a wide spectrum of pathology, ranging from mild skin and soft tissue infections to life-threatening systemic sepsis and meningitis (Eades *et al.,* 2017). They are susceptible to a wide range of antibiotics in contrast to the Gram-negative bacteria. Several studies reporting on the antimicrobial activity of medicinal plants have shown that Gram-positive bacteria such as the ones tested in this study were sensitive to the inhibitory action of plant extracts. In a study by Masud Rana et al. (2014), most of the plant extracts showed negligible activity against Gram-negative bacteria while the activity against Gram-positive was

promising. The findings in this study are in agreement with those of Tchouya et al. (2015), Fomogne-Fodjo et al. (2014) and Koroma and Ita (2009) who found that *S. ochocoa*, *M. cecropioides* and *A. hirtella* were able to display antimicrobial activity against various Gram-positive bacteria such as *S. aureus*, *Streptococcus spp* or *Bacillus spp*.

The results of the study also indicated that 22 out of 40 extracts were able to inhibit the growth of *Shigella sonnei, Salmonella enterica* and *Klebsiella pneumonia*, while *E. coli* and *P. aeruginosa* were respectively susceptible to 20 and 21 plant extracts. *E. cloacae* was the least susceptible to the extracts as only 11 plant extracts were able to inhibit the bacterial growth at the lowest concentration (50 mg/mL). Gram-negative bacteria are well known for their high resistance to antibiotics as compared to Gram-positive (Mitchell, 2015). This resistance is due to their structural characteristics. Their cell envelope possesses two lipid membranes between which a peptidoglycan cell wall is found. In addition, the outer membrane formed by lipopolysaccharides is an important factor in the pathogenicity of Gram-negative bacteria (Maldonado *et al.,* 2016). Several studies reported on the resistance of Gram-negative bacteria towards antibiotics. According to Miller (2016), out of 2 million illnesses resulting in deaths, many of these infections are caused by gram-negative bacteria in the USA.

Gram-negative bacteria with resistance to commonly used antibiotics, including quinolones, colistins (polymyxins), carbapenems, cephalosporins, and other β-lactam antibiotics, have been isolated from humans with increasing frequency (Miller, 2016). Thus, infections due to Gram-negative bacteria represent a health care concern. Numerous studies related to antimicrobial properties of medicinal plants have shown the efficiency of plants against the mentioned bacteria (Aziz *et al.,* 2018, Oseni *et al.,* 2014). Aziz et al. (2018) showed that methanolic extract of *Microcos Paniculata* exhibited maximum antimicrobial activity against various Gram-negative bacteria especially *Salmonella spp.* with a diameter of inhibition reaching 27 mm. The results for this study support other authors who demonstrated the inhibitory effects of *R. vomitoria, C. edulis* and *P. soyauxii* against various bacteria such as *E. coli, K. pneumonia, S. typhi* and *P. aeruginosa* (Oseni *et al.,* 2014; Osuagwu and Akomas, 2013; Bukola *et al.,* 2008).

Regarding *Candida albicans, Candida tropicalis* and *Cryptococcus gondii*, only aqueous and methanol extracts of *C. edulis, T. iboga, A. hirtella* and *U. guineensis* exhibited an inhibitory effect on the growth of the fungi tested. Worldwide infections resulting from fungi are responsible for hundreds of thousands of deaths each year among individuals with AIDS and HIV (Saloman, 2017). These fungi are causes for numerous infections such as *Pneumocystis pneumonia*, meningitis, histoplasmosis, pulmonary aspergillosis, oropharyngeal and oesophageal candidiasis. Some commensal fungi become harmful and cause infections in immunocompromised individuals. Despite major progress in both translational research and drug development in the field of medical mycology in recent years, both the incidence and mortality of invasive fungal infections in HIV/AIDS remain unacceptably high in developing countries (Armstrong-James *et al.,* 2014). The search for new therapies with better tolerability and efficacy as well as drug resistance and toxicity associated with a prolonged treatment with antifungal drugs, prompted researchers to revert back to medicinal plants which had demonstrated promising results in the management of fungal infections. Ethnobotanical surveys conducted in countries such as South Africa, Togo or Pakistan have reported several medicinal plants used to manage various fungal infections in HIV individuals (Alfa *et al.,* 2018; Sheher *et al*., 2013; Otang *et al.,* 2012). In addition, many other studies have validated the use of plants in fighting fungi associated with HIV such as *Candida spp., Aspergillus spp*. and *Cryptococcus spp* by performing assays including agar well diffusion (Kumari *et al.,* 2017; Murtaza *et al.,* 2015; Ahmad *et al.,* 2010). The findings in this study are in sync with the report by Bukola et al. (2008) who demonstrated the antifungal activity of *C. edulis* against *C. albicans*.

It is interesting to note that, both aqueous and methanol extracts of *A. cissampeloides; M. puberula; A. vogeli; V. conferta; C. lucanusianus* and *R. africanum* did not show any activity against both Gram-positive and negative bacteria as well as the fungi tested, even at the highest concentration (200 mg/mL). Several studies indicated that those plants except *V. conferta* and *M. puberula* possessed some level of antimicrobial activity against various bacteria and yeast strains including *C. albicans* (Okunye *et al.,* 2015; Eze *et al.,* 2014; Tekwu *et al.,* 2012). It is acknowledged that various parameters have an impact in the expression of plant biological activities that include the type of solvent; the mode of extraction used; the geographical location of the plant; the soil

composition; the temperature; the date of collection and also the bacterial strains (isolates or standard ATTC) selected. The variation in any of these parameters may explain the contradictory results observed between the present study and other researchers' results.

The minimum inhibitory concentration (MIC), the minimum bactericidal concentration (MBC) and the ratio MBC/MIC were determined, in order to further investigate the antibacterial activity of the plant extracts (aqueous and methanolic) which displayed both antibacterial and antifungal activities against some of the microorganisms tested. The MIC indicated the lowest amount of an antimicrobial agent that greatly inhibits growth whereas MBC shows the lowest amount of antimicrobial agent that result in microbial death. A ratio MBC/MIC of an antimicrobial agent ≤ 4 implies a bactericidal action of this agent, while a bacteriostatic agent is defined when MBC/MIC > 4 (Spellberg, 2017). The present study demonstrated MIC values ranged between 0.098 mg/mL and 6.25 mg/mL for *E. faecalis* and *S. enterica*, whereas *E. cloacae, E. coli, P. aeruginosa* and *K. pneumoniae* were the least susceptible to the selected extracts with MIC values reaching 50 mg/mL. In Table 4.2 the MBC values were found to be from 0.195 mg/mL to more than 25 mg/mL. The ratios MBC/MIC (Figure 4.2) showed a bactericidal action towards all the Gram-positive bacteria and 2 Gram-negative bacteria (*S. enterica* and *S. sonnei)* where the ratio MBC/MIC was ≤4 for almost all the extracts. On the other side, a bacteriostatic effect was exerted towards *E. faecalis* and *P. aeruginosa.* A common dogma in medicine is the belief that bactericidal agents are more effective than bacteriostatic agents because they are believed to be better at killing bacteria (Aqil *et al.,* 2017; Wald-Dickler *et al.,* 2017). Yet, studies comparing bacteriostatic and bactericidal agents for the treatment of invasive bacterial infections found no differences in clinical outcomes or mortality, thus providing the evidence that bactericidal and bacteriostatic agents are similar in efficacy (Wald-Dickler *et al.,* 2017). Several medicinal plants in the current study including *C. edulis, P. soyauxii, M. monandra, A. klaineana* and *T. iboga* showed great promising antimicrobial effects against bacteria and fungi responsible for a wide range of opportunistic infections associated to HIV/AIDS.

This antimicrobial efficacy of the plant extracts can be attributed to the secondary metabolites they contain such as alkaloids, terpenoids, phenols, tannins and

flavonoids. The literature reports the antimicrobial activity of plant compounds such as alkaloids from which antibacterial drugs like metronidazole and quinolones are derived (Cushnie *et al.,* 2014). A study by Alves et al. (2013) has investigated the antimicrobial effect of some phenolic compounds from various mushrooms and found that phenolic compounds could be used as antimicrobial agents, especially against some micro-organisms resistant to commercial antibiotics. According to Barbieri et al. (2017), several phytochemicals including sulfur-containing phytochemicals, terpenoids and carotenoids, despite having direct antimicrobial activity, exhibited an *in vitro* synergistic effect when tested in combination with conventional antibiotics, thus modifying antibiotic resistance. The present study has demonstrated and confirmed for some plants the extraordinary antibacterial and antifungal potency of medicinal plants such as *C. edulis, T. iboga, A. klaineana* and *M. monandra* used to manage opportunistic infections linked to HIV in Gabon. These results may explain the improvement in HIV peoples' health after taking remedies based on these plants.

Anti-HIV activity

After demonstrating the antimicrobial effects of the selected plants against several pathogens responsible for HIV related OI, it was necessary to explore the potential anti-HIV activity. First the effect of the plant extracts on the viability of isolated HIV infected PBMCs was determined. The results of the cell viability (Figure 4.5) revealed that aqueous extracts of *R. vomitoria, M. monandra, R. africanum, T. iboga* as well as both aqueous and methanolic extracts of *C. lucanusianus* and *V. conferta* were able to inhibit HIV infected PBMC proliferation by more than 20%. Several extracts such as methanolic extracts of *P. soyauxii*, *A. hirtella* or *S. kamerounensis* aqueous extracts, were stimulating the growth of infected cells at the tested concentration (25 µg/ml). These results indicate two things. Firstly, less than 10 extracts exerted highest inhibitory effects on HIV infected cells ranging from 20% to 34%, what is in accordance with viability results of Vero cells treated with the plant extracts as seen in Chapter 3. The results of the cytotoxicity activity of the studied extracts on Vero cells showed that most of the plant extracts except for *A. klaineana* and *T. iboga* exerted a negligible cytotoxic effect against Vero cells. This implies that the plant extracts exhibit an overall weak cytotoxicity towards both healthy cells (Vero) and HIV infected PBMCs.

Secondly, some plants were actively stimulating cell proliferation leading to more than 200% increase when compared to untreated cells. This active proliferation was not observed when Vero cells were treated with the extracts in previous Chapter 3. The highest percentage of Vero cells in the treated wells was less than 110% when compared to untreated wells. These results are in accordance with the study by Klos et al. (2009) who also demonstrated that different plant extracts from *Hypoxis sobolifera, Leonotis leonurus* and *Tulbaghia violacea* were able to stimulate the growth in HIV infected CEM.NKR-CCR5 cells while no significant effect was observed on the viability of uninfected CEM.NKR-CCR5 cells. In the same manner the plant extracts in the present study have the capacity to induce production of new PBMCs. This last observation led us to question whether the newly formed cells are infected by HIV or are healthy.

To have an idea of what is happening, the quantification of the p24 antigen in the treated HIV infected PBMCs was undertaken. It is known that one important HIV component is p24 antigen, a structural protein that constitutes the HIV capsid deriving from the Gag protein of HIV-1 and which is essential in viral core assembly and maturation (Momany *et al.,* 1996). Generally, a viral particle consists approximately 2000 copies of this protein. In early studies of HIV progression, increased levels of p24 antigen was shown to be associated with increased levels of virus in the blood and a reduction of CD4+ which was correlated with more rapid HIV disease progression (Kramer *et al.,* 1992; MacDonell *et al.,* 1990). The p24 antigen has been proposed as a marker for HIV disease progression in patients who, for the most part, have not been treated yet (Sabin *et al.,* 2001). Therefore, many studies related to HIV used this marker to evaluate qualitatively or quantitatively HIV in blood.

In the present study, the evaluation of p24 levels in HIV infected PBMCs exposed to plant extracts, showed that most plant extracts induced a decrease in the levels of p24 antigen except *R. vomitoria* and *U. klainei* methanol extracts and *S. gabonensis* aqueous extracts (Figure 4.6). Many extracts were able to reduce the level of p24 in the infected cells with percentage of inhibition higher than 45% for some extracts such as aqueous extracts of *V. conferta* (47%), *C. lucanusianus* (47%), *U. guineensis* (46%) and both aqueous and methanolic extracts of *T. iboga* (48% and 45% respectively).

For some plants such as *C. edulis, S. ochocoa* and *S. kamerunensis* an increase in cell proliferation was correlated with a decrease in p24 viral antigen. In this case, it can be suggested that the extracts may have the ability to inhibit the production of the virus and destroy it in the cells leading to an active stimulation of the production of new "healthy" cells. That also implies that the plant extracts might interfere somehow with the various steps of virus entry and that the extracts provide a protective effect against cell death. For *R. vomitoria* (methanol), *U. klainei* (methanol) and *S. gabonensis* (aqueous) an increased T-cell proliferation was observed without an expected decrease in p24 viral antigen levels. This may be explained by the inability of the extracts to exert any inhibitory effect on the viral production whereas cells are still proliferating. For plants such as *C. religiosa, C. lucanusianus, R. africanum* and *V. conferta* the inhibition of the cell growth was associated with a decrease in p24 viral antigen levels. In these cases, it can be assumed that the plant extracts exhibited a cytotoxic effect on HIV infected PBMCs that is characterized by a diminution in the number of infected cells and thus in p24 antigen levels. These different results indicate that various mechanisms are involved in the anti-HIV activity displayed by the studied plant extracts including viral entry inhibition, reverse transcription inhibition, viral replication inhibition, integration inhibition, virus maturation inhibition and virion budding (Han *et al.,* 2011). The findings in this study confirmed those obtained by Silva et al. (2004) who showed that an alkaloid from *T. iboga* was inhibiting the replication of HIV-1 in infected PBMCs. The potential anti-HIV effect demonstrated here might be due to the alkaloids present in *T. iboga* extracts. The present work has shown that the studied plants have a promising anti-HIV potential on infected PBMCs'. But further investigations should be undertaken in order to elucidate the mechanisms that really intervene in this antiviral activity.

Reverse transcriptase is one of the most targeted enzymes for anti-HIV drug research (Esposito *et al.,* 2012) as it is among the most important enzymes necessary for HIV dissemination. Its role is to reverse-transcribe virus RNA into DNA, which is then integrated into the host genome and replicated. One cause of the HIV resistance to conventional ARVs is the fact that RT sometimes makes mistakes while reading the RNA sequence, resulting in not all viruses produced in a single infected cell are the same (Clavel, 2004).

In this study, 24 aqueous and methanol extracts that had decreased significantly p24 antigen were selected for the evaluation of their potential in the inhibition of anti-reverse transcriptase. The results reveal that out of 24 selected plant extracts, only 8 showed reverse transcriptase inhibition at either 250 µg/mL or 500 µg/mL or both. *Coula edulis* aqueous and methanol extracts were able to inhibit RT at both concentrations with the percentage of inhibition ranging between 82% and 86%. For *C. religiosa,* only the aqueous extract showed RT inhibition (72% at 250 µg/mL and 81% at 500 µg/mL). *C. lucanusianus* (89%) and *R. africanum* (80%) methanol extracts were inhibiting RT only at 250 µg/mL but not at higher concentration. While the aqueous extracts of *A. hirtella*, *P. soyauxii* and *V. conferta* were active only at 500 µg/mL. The results also indicated that *T. iboga* did not exert a reverse transcriptase inhibition as opposed to Silva et al. (2004) findings. These researchers found that an iboga alkaloid (18-methoxycoronaridine) from *T. iboga* exhibited a moderate HIV-1 RT inhibition with an IC_{50} of 69.4 µM. The reasons behind this difference are multiple. It could be that the concentration of this specific molecule responsible for the activity was low in the crude extracts or it could be that the concentrations used were not suitable for the present study.

In the past years, to find efficient and nontoxic drugs to manage HIV pandemic as well as to overcome drug resistance, scientists reverted to plant based medicine which had many times demonstrated its value in managing many diseases including HIV. Several studies showed that plants constitute an excellent source of anti-HIV agents (Rege *et al.,* 2015; Leteane *et al.,* 2012). This capacity of plants to manage HIV is due to the chemical compounds they contain. Namely: flavonoids; coumarins; alkaloids; terpenoids; phenolics; saponins and tannins have shown anti-HIV properties via multiple mechanisms including reverse transcriptase inhibition; protease inhibition; entry and fusion inhibition or even integrase inhibition (Salehi *et al.,* 2018; Kapewangolo *et al.,* 2017; Silprasit *et al.,* 2011). In chapter 3, it was established that the plants selected for the study contain variable quantities of several phytochemical compounds such as steroids; alkaloids; glycosides; tannins; phenols; flavonoids and saponins. These compounds are most probably responsible for the anti-HIV activities displayed by the different active extracts. Many isolated compounds such as Calanolide; Bevirimat; Prostatin; 3-hydroxymethyl-4-methyl DCK and Conocurvone were isolated from the chemicals mentioned above and were involved in anti-HIV

clinical trials with promising outcomes (Martin *et al.,* 2008; Hezareh, 2005). Therefore, the active plants in the study should be investigated further to facilitate the isolation of promising anti-HIV molecules that could provide efficient and safe drugs to fight HIV/AIDS.

4.5 REFERENCES

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CHAPTER FIVE

Immunomodulatory assessments of selected Gabonese medicinal plants

The article below is in preparation.

Boukandou Mounanga MM, Traore Afsatou Ndama and Potgieter Natasha; Immunomodulatory leads of aqueous and methanolic plant extracts.

CHAPTER 5

Abstract

HIV is responsible for the progressive degeneration of the immune system as the main targets of the virus are the cells such as peripheral blood monocytes and macrophages. This results in an increase or decrease in the production and secretion of specific cytokines responsible for the immune system homeostasis. Thus, the present study aims at evaluating the ability of 40 plant extracts to inhibit or stimulate the production of cytokines in HIV infected cells.

Methods: The concentration of IL-6 and TNF-α in the supernatants obtained from the HIV infected PBMCs treated with the 40 extracts, was tested using ELISA kits specific for human cytokines. The level of IL-2 in HIV infected PBMCs treated with 26 of the less toxic exctracts (amongst the 40 extracts) was determined using ELISA kits.

Results: The results revealed no significant ($p>0.05$) variation in IL-2, IL-6 and TNF-α levels after treatment of HIV infected PBMCs with the extracts although a decrease in IL-6 was observed after treatment with methanolic extracts of *M. monandra, P. soyauxii, U. guineensis, A. klaineana, V. conferta, A. vogelii, T. iboga, C. religiosa, C. lucanusianus* and *R. africanum* and an increase in IL-2 production was observed after treatment with *U. guineensis*, *M. puberula*, *A. hirtella*, *A. cissampeloides*, *S. gabubensis*, *T. iboga* and *A. klaineana.*

Conclusion: The study has demonstrated that some of the plants tested were able down and up regulate the production of some cytokines in HIV infected cells what indicates a promising modulatory effect on the immune system.

Key words: HIV infected PBMCs, IL-2, IL-6, TNF-α, immunomodulatory effect, cytokines.

5.1 INTRODUCTION

The aim of this chapter was to evaluate the ability of the selected plant extracts to inhibit or stimulate the immune system. These specific assays were performed with HIV positive blood in order to determine the action of the plant extracts on targeted cytokines that play an important role in the pathogenesis of HIV infection. Thus, the ability of the selected extracts in the down- or up- regulation of the targeted cytokines would facilitate a better understanding of the mechanisms that the selected plants use in the improvement of the health of the HIV positive individuals.

5.1.1 BACKGROUND

The human immunodeficiency virus (HIV) is responsible for the progressive degeneration of the immune system as the main targets of the virus are the cells that express the CD4 receptor, such as the peripheral blood monocytes (PBMCs) and macrophages. This results in the decrease of the number of CD4+ T cells; a deficiency in the immunological function of both T cells and macrophages as well as the dysfunction of the cytokine production (Kedzierska and Crowe, 2001). This implies an increase or decrease in the production and secretion of specific cytokines responsible for the immune system homeostasis. Previous studies have shown that during HIV infection, there is a shift from a prevalently T-helper type 1 (Th1) response to a Th2 response, leading to a decreased production of interleukin (IL)-2 and interferon (IFN)-γ and increased secretion of IL-4, IL-10 and proinflammatory cytokines (IL-1, IL-6, IL-8 and tumour necrosis factor) (Clerici and Shearer, 1993; Esser *et al.,* 1991). According to Kedzierska and Crowe (2001), cytokines and growth factors that promote activation and differentiation of cells of the immune system can also modulate both HIV-1 infection and replication. This suggests that HIV-1 infection and replication *in vivo* and *in vitro* is under the continuous regulation of a complex cytokine network produced by a variety of cells.

5.1.2 CYTOKINE LEVELS AND HIV INFECTION

Interleukin 6 (IL-6) is a pleiotropic cytokine produced by various cells (macrophages, T and B cells) in response to microbial infection. IL-6 is known to regulate various physiological processes and play an important role in the installation of chronic inflammation (Borges *et al.,* 2015). It has been reported that, when compared to HIV-seronegative subjects, IL-6 levels in HIV-infected individuals is particularly high, especially in the early stages of infection (Breen *et al.,* 1990). *in vitro* stimulation of monocytes and $CD4^+$ T cells from HIV infected subjects with phytohaemagglutinin or lipopolysaccharide, have demonstrated an increase in the secretion of IL-6 in contrast to cells from uninfected individuals (Baqui *et al.,* 2000). Interestingly, production of IL-6 was observed in HIV-infected donor B cells which suggests that HIV is able to induce IL-6 production from a variety of cells of the immune system (Kehrl *et al.,* 1992). A recent study carried out in Zambia that aimed at determining the levels of IL-6 in HIV positive individuals, showed that HIV ART naïve participants had significant higher IL-6 levels than those on ART (Nhhoma *et al.,* 2017).

Interleukin (IL-2) is produced by both $CD4^+$ and $CD8^+$ T cells. The primary role of IL-2 is to stimulate the proliferation of activated T cells, cytotoxic activity of $CD8^+$ T cells and natural killer (NK) cells as well as inducing B cells and monocyte function (Smith, 1988). In HIV infection, low levels of IL-2 are one of the first immunological defects that are detected in patients (Kinter and Fauci, 1996). A study by David et al. (1998) demonstrated that IL-2 receptor expression in HIV patients with high viral load was greatly enhanced on B cells, $CD8^+$ T cells, and monocytes, but not on $CD4^+$ T cells, as opposed to non-infected individuals. IL-2 production by *in vitro* activated PBMCs, isolated from HIV-infected subjects at various stages of disease, is significantly decreased when compared to PBMCs obtained from uninfected subjects (*Meroni et al.,* 1996; Meyaard *et al.,* 1996; Barcellini *et al.,* 1994). IL-2 production is related to $CD4^+$ T cell counts and the clinical status of the patients (Hong *et al.,* 1998). Brezar et al. (2017) highlighted the positive role of IL-2 therapy in HIV infection characterized by an increase in HIV-specific effector CD4+ T cells and a reduction in viral load. The same researcher also emphasized the impact of IL-2 therapy during an autoimmune manifestation, explaining how low-dose IL-2 therapy can increase CD4+ regulatory T

cells, resulting in clinical improvement and how during chronic infection where virus-specific T cells can expand and impact on viral load.

Tumor necrosis factor alpha (TNF-α), is a pro-inflammatory cytokine produced by a wide variety of cells; including monocytes; macrophages; T cells; B cells; NK cells; neutrophils and microglia cells. Just like IL-6, increased levels of TNF-α are detected with HIV infection. HIV-1 infection of PBMCs and $CD4^+$ T cells *in vitro* enhances TNF-α secretion (Vyakarnam *et al.,* 1990). TNF-α actively induce HIV-1 replication in T cells and cells of macrophage lineage through the activation of the cellular transcription factor NF-κB (Naif *et al.,* 1994; Griffin *et al.,* 1991). Several HIV proteins can mimic and regulate TNF-α signaling pathways for use in extending its reservoir (Kumar *et al.,* 2016). The understanding of the TNF signaling pathways by new therapeutic approaches, may contribute in the control of the immune activation and impact both viral replication and viral persistence (Pasquereau *et al.,* 2017). Subsequently, it has been demonstrated that, after treatment with anti-TNF-α neutralizing antibody, several phenomena occur such as a decrease in HIV-1 production, an inhibition of NFκB activation and a prevention in HIV-induced $CD4^+$ T cell depletion *in vitro* (Munoz-Fernandez *et al.,* 1997). Pre-treatment for 3 days before infection withTNF-α, resulted in about 75% inhibition of viral entry in monocyte derived macrophages (Herbein *et al.,* 1996).

The aim of this chapter was to determine the potential immunostimulant or immunosuppressant capacity of selected plant extracts used in the management of HIV individuals in Gabon.

5.2 MATERIAL AND METHODS

5.2.1 ETHICAL APPROVAL

Ethical approval was obtained from the Health, Safety, and Research Ethics Committee of the University of Venda (SMNS/17/MBY/30/2111). Permission to use health facilities was obtained from the Limpopo Provincial Department of Health. Signed informed consent forms were obtained for agreement from all volunteers before blood collection. Confidentiality for volunteers was maintained throughout by assigning numerical codes. The blood was used for PBMCs isolation only.

5.2.2 CELL CULTURE

5.2.2.1 Blood collection

Blood samples were collected from HIV+ patients at the University of Venda clinic by trained nurses of the clinic. Approximately 5 mL of whole blood was collected into vacutainer tubes containing EDTA (Ethylenediaminetetraacetic acid) and the tubes were inverted several times to ensure complete mixture of contents. The samples were transported to the School of mathematical and natural sciences' microbiology laboratory for analysis.

5.2.2.2 Isolation and subculture of PBMCs

Isolation of cells was done as described in detail in Chapter 4 Section 4.2.4.3.

5.2.2.3 Setting up the bio-assay

Overnight PHA-stimulated cells were transferred into 50 mL tubes (Nalgene Nunc) and centrifuged 10 min at 2000 rpm. Afterwards, the supernatants were discarded, and cell pellets were resuspended into 50 mL of complete media (RPMI containing 10% FBS and Gentamicin). Into a 24 well plate, 500 µL of extracts (for a final concentration of 25 µg/mL) and 500 µL of complete media were mixed, then 1 mL of approximately 1×10^6 cells from the cell suspension previously prepared were added to each well. Phytohaemagglutinin (5 µg/mL) was the positive control while water and the absolute methanol were the negative control. After 3 days of culturing at 37°C in a humidified incubator with 5% CO_2 (EcoTherm, Hartkirchen, Austria), the supernatants were collected and stored for more assays.

5.2.3 ASSESSMENT OF THE IMMUNOMODULATORY EFFECTS

5.2.3.1 Assessment of IL-6 and TNF-α production in the supernatants

The concentration of IL-6 and TNF-α in the supernatants obtained from the HIV infected PBMCs as described in the previous section, was tested using ELISA kits specific for human cytokines (BD Biosciences Pharmingen, San Diego, USA) as detailed in the manufacturer's instructions. Briefly, 96-well plates were coated with 100 µL of anti-human TNF-α and IL-6 monoclonal antibodies diluted in a coating buffer and incubated at 4°C overnight. After incubation, the plates were washed 3 times with the

wash buffer (1x) and the blocking diluent (200 µL) was added. Following 1 h incubation, 100 µL of the supernatants were pipetted into appropriate wells in triplicate then incubated for 2 h at room temperature (RT). Afterwards, the plates were washed 5 times with the wash buffer (1x) and 100 µL of the working detector (biotinylated anti-human IL-6/TNF-α monoclonal antibody + enzyme reagent) was added to each well. Following 1 h incubation at RT, the plates were washed with the wash buffer (1x) and 100 µL of substrate solution was added to the wells. The plates were then kept in the dark for 30 min after which the stop solution (50 µL) was added to each well.

The levels of cytokine secreted were detected at 450 nm using a microplate reader (Multiscan MS, Labsystems, Vantaa, Finland). The Absorbance values were translated into concentration of cytokines secreted using the drawn standard curves. The secreted cytokines' levels were compared to the untreated cells (PBMCs only) that was considered as baseline cytokine secretion.

5.2.3.2 Assessment of IL-2 contents in the supernatants

Due to the budget constraints and the large number of plant extracts in the study, the plant extracts (26 extracts) with low toxic effects on PBMCs were selected according to the results obtained in the previous chapter (antimicrobial and anti-HIV) in section 4.3.2.1. The level of IL-2 in supernatants obtained from HIV infected PBMCs as described in section 5.2.2.3, was tested using ELISA kits specific for human cytokines (BD Biosciences Pharmingen, San Diego, USA) as detailed in the manufacturer's instructions. Briefly, 50 µL of ELISA diluent was pipetted into wells coated with antihuman IL-2 monoclonal antibody and 100 µL of extracts were added to each well. After 2 hours incubation at room temperature, the strips were washed 5 times with the wash buffer (1x) followed by the addition of 100 µL of a working detector (biotinylated anti-human IL-2 + Streptavidin-horseradish peroxidase conjugate with BSA). The strips were covered with a plate sealer then left to stand for 1-hour incubation time at room temperature. Afterwards, the wells were washed 7 times with the wash buffer (1x) then TMB one step substrate reagent (100 µL) was added to each well followed by 30 min incubation in the dark. A volume of 50 µL of the stop solution (phosphoric acid) was added to each well. The IL-2 secretion levels were detected at 517 nm using a microplate reader (Multiscan MS, Labsystems, Vantaa, Finland). The Absorbance values were translated into concentration of IL-2 secretion using a drawn standard

curve. IL-2 levels were compared to the untreated cells (PBMCs only) that were considered as baseline cytokine secretions.

5.2.4 STATISTICAL ANALYSIS

Each sample assessment was performed in triplicate. The data were analysed using a Graph pad prism 6.0. The results are presented as means (±SD) of the triplicate. The significance was evaluated using 2-way ANOVA, followed by Dunnet's multiple comparisons test and the results were considered significant when $p<0.05$.

5.3 RESULTS

5.3.1 IL-6 ASSESSMENT

The ability of the 40 plant extracts to enhance or inhibit the pro-inflammatory IL-6 secretion was assessed on HIV-infected PBMCs and the cells were stimulated with PHA, methanol (100%) and water was added as controls.

The results presented in Figure 5.1, indicated that all the water extracts were able to increase the production of IL-6 up to 215% for some plants when compared to the untreated cells (89.28 pg/mL), except for *M. monandra* and *C. lucanusianus* that slightly inhibited the production of the cytokine with values of 83.71 pg/mL and 79.41 pg/mL respectively. The inhibition was not significant ($p>0.05$) when compared to the untreated cells. Its noteworthy, that the methanolic extracts of *M. monandra* (68.80 pg/mL)*; P. soyauxii* (48.13 pg/mL)*; U. guineensis* (50.98 pg/mL)*; A. klaineana* (48.49 pg/mL)*; V. conferta* (44.54 pg/mL)*; A. vogelii* (43.51 pg/mL)*; T. iboga* (60.49 pg/mL)*; C. religiosa* (39.16 pg/mL)*; C. lucanusianus* (36.97 pg/mL); and *R. africanum* (34.23 pg/mL) were able to down regulate IL-6 levels at the tested concentration (25 µg/mL) in contrast to the control (89.28 pg/mL) while the remaining methanol extracts were on the contrary displaying an up-regulation in the cytokine levels up to 155%. However, despite the up regulation and the down regulation noticed, the p value was >0.05 for all the plant extracts showed no significant changes in the expression of Il-6 when compared to the untreated cells.

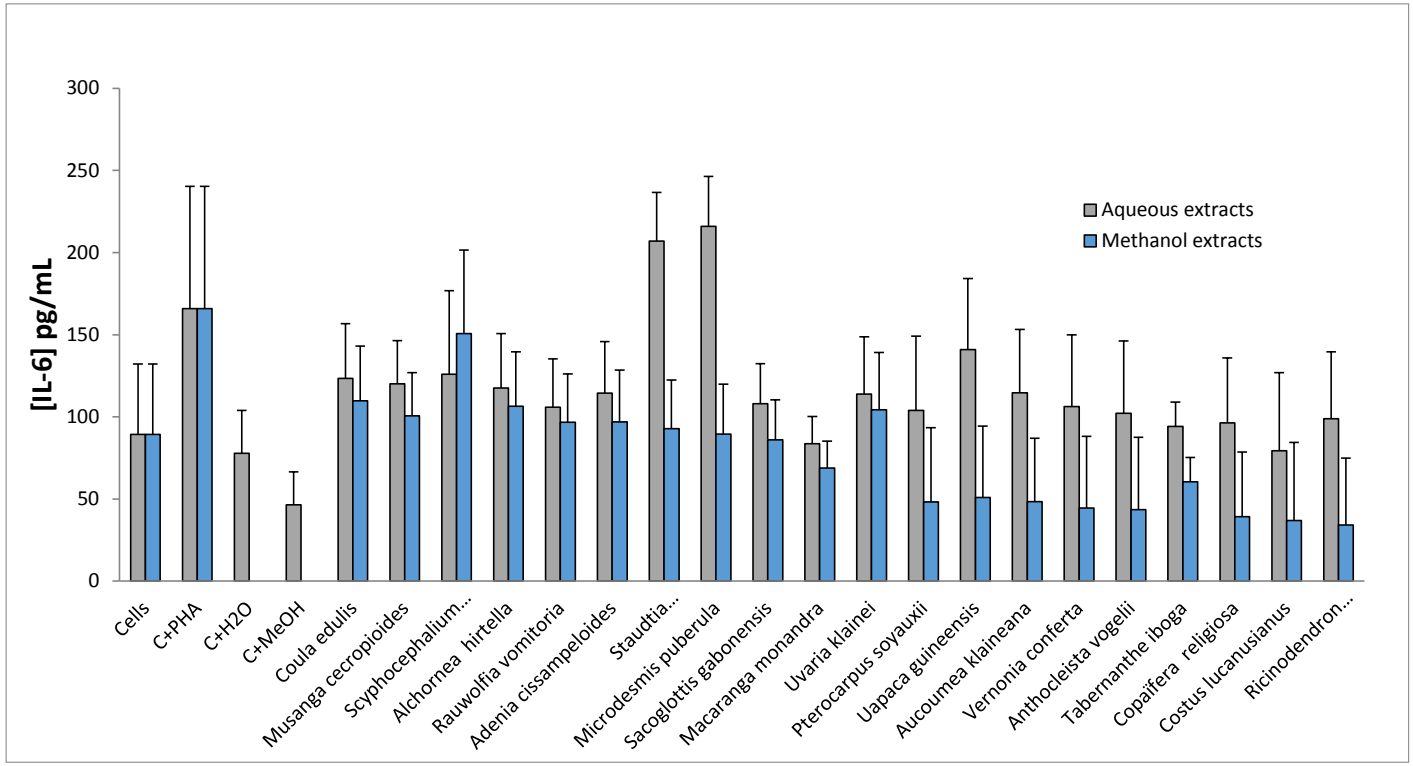

Figure 5.1: Effect of the plant extracts on IL-6 secretion levels by HIV-infected PBMCs. Data represents the mean ± standard deviation for three replicate wells. Errors bars denote standard deviation.

5.3.2 TNF-α ASSESSMENT

The results of the levels of TNF-α from HIV-infected PBMCs after stimulation with 40 plant extracts are shown in Figure 5.2. In this assay, the HIV-infected PBMCs were stimulated with PHA, methanol (100%) and water for control. The results showed inconsistency in secretion after the addition of the plant extracts with small reductions up to 12% or elevations up to 13% that were statistically not significant when compared to the untreated cells (57.63 pg/mL). Indeed, *R. vomitaria* aqueous extract and *C. lucanusianus* methanol extract showed the lowest values of TNF-α at 50.67 pg/mL and 51 pg/mL respectively.

Costus lucanusianus aqueous extract and *S. ochocoa* methanol extract displayed the highest increase in TNF-α levels at 62.96 pg/mL and 65.44 pg/mL respectively. The p value was >0.05 for all the plant extracts showing no significant changes in the expression of TNF-α when compared to the untreated cells.

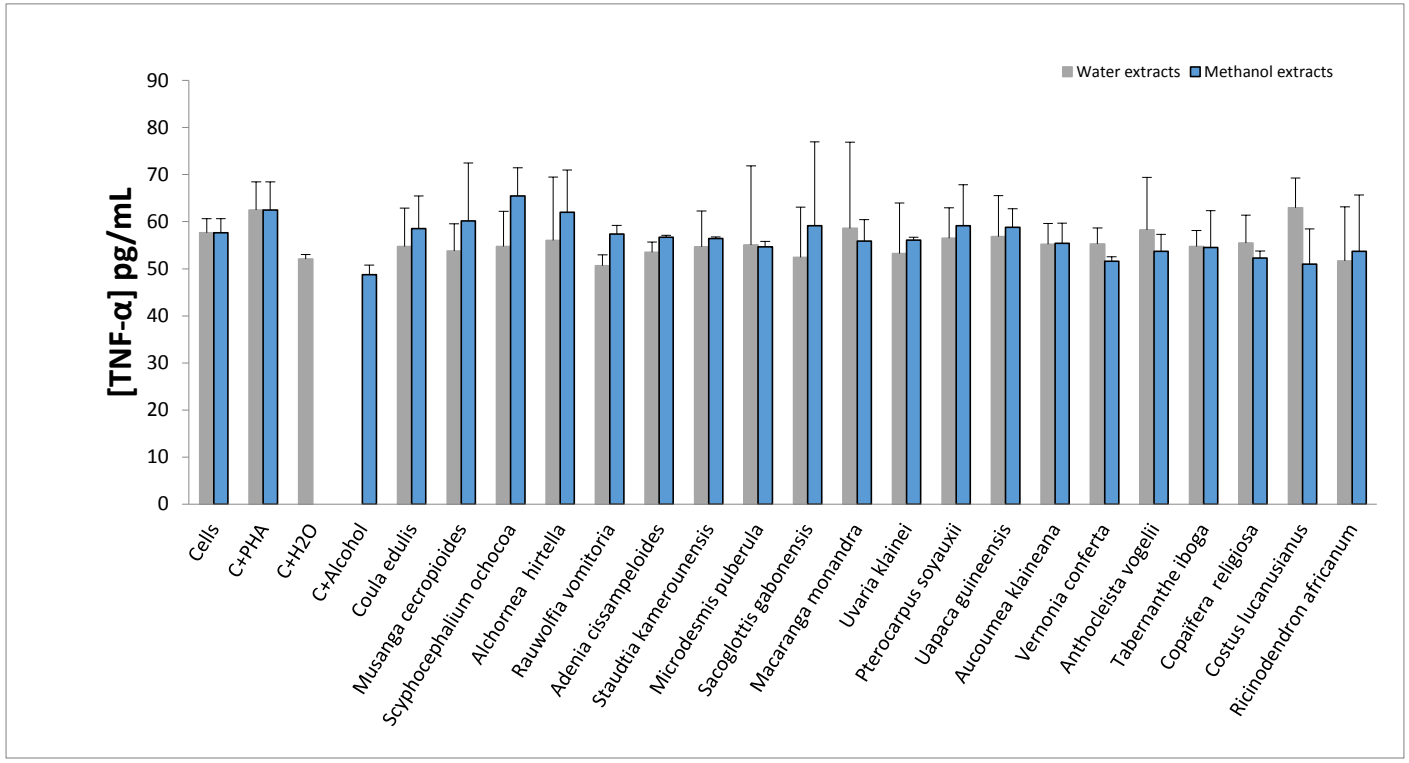

Figure 5.2: Effect of the plant extracts on the secretion of TNF-α by HIV-infected PBMCs. Data represents the mean ± standard deviation for three replicate wells. Errors bars denote standard deviation.

5.3.3 IL-2 ASSESSMENT

After the evaluation of cytotoxic effects of the 40 plant extracts on HIV infected PBMCs, 26 plant extracts were selected from among the less toxic ones.

The results (Figure 5.3) showed no significant difference ($p>0.05$) between aqueous and methanol extracts for all the samples. *Macaranga monandra* aqueous and methanol extracts (191.08 pg/mL and 183.33 pg/mL respectively), the aqueous extracts of *C. edulis* (188.26 pg/mL) and *T. iboga* (199.03 pg/mL) as well as *M. cecropioides* methanol extracts (198.93 pg/mL) were slightly down regulating the expression of IL-2 in the infected PBMCs. Yet the difference was not significant in contrast to the untreated cells (only 2% to 8% of down regulation). The results also showed that some extracts such as *U. guineensis* both extracts, *M. puberula* extracts, the methanol extracts of *A. hirtella*, *A. cissampeloides*, *S. gabubensis* and *T. iboga* as well as the aqueous extract of *A. klaineana* were stimulating the production of IL-2 with values varying between 206.08 pg/mL and 246.56 pg/mL (1% to 21% of stimulation),.

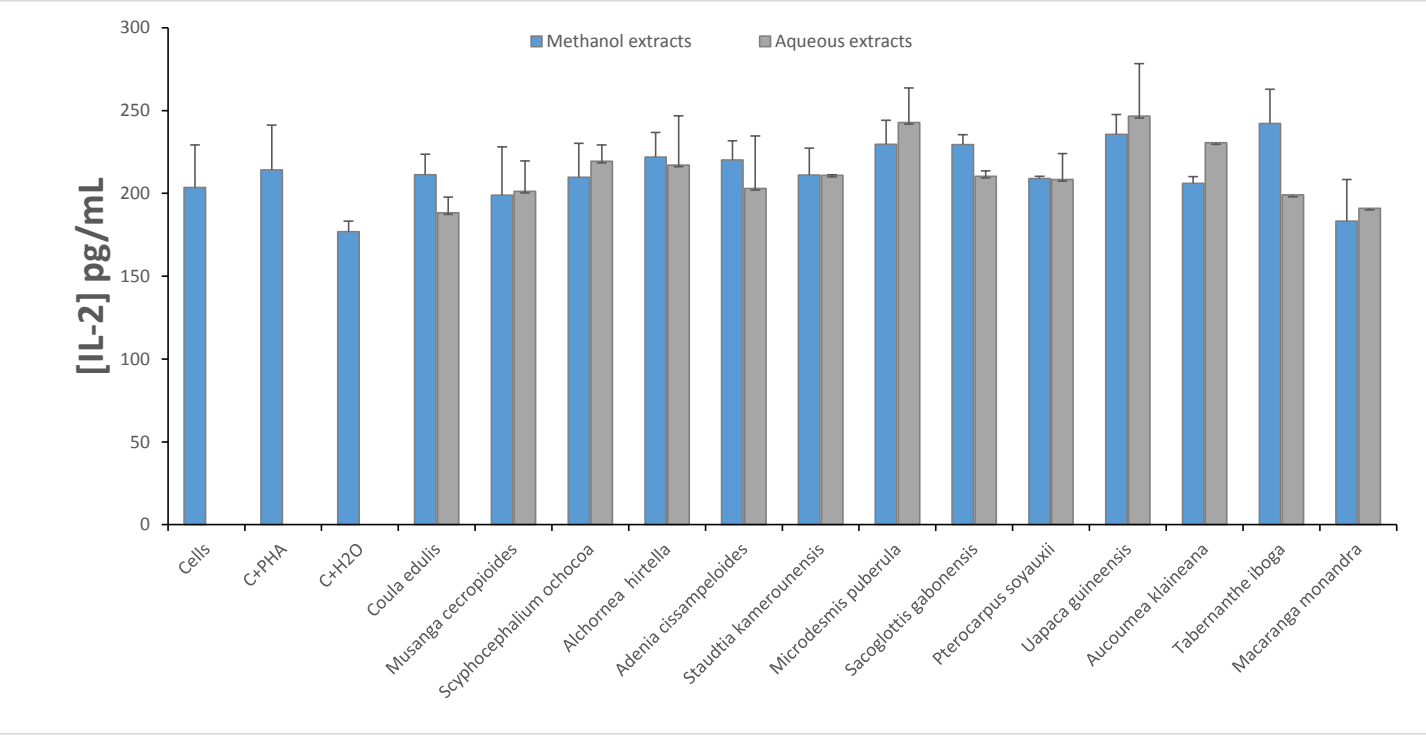

Figure 5.3: Immunomodulatory effects of 26 selected plant extracts on IL-2 secretion by HIV-infected PBMCs. data represents the mean ± standard deviation for three replicate wells. Errors bars denote standard deviation.

5.4 SUMMARY OF RESULTS OBTAINED IN THE STUDY

In summary, a table (Table 5.1) was generated just to get an overall view of how the different plants performed and the rational in choosing only 2 plants for the next chapter.

The activities of the plant extracts were marked as + or -, the total activities were recorded and tabulated. *C. edulis* was found to have anti-HIV activity and both the aqueous and methanolic extracts displayed similar results. Whereas, *T. iboga* displayed high activities with the methanolic extract.

Furthermore, C. edulis and T. iboga showed the best antimicrobial activity against the array of bacteria (Gram positive and Gram negative) tested.

Table 5.1: Summary of the biological activities of the 40 plant extracts assessed

Plant extracts		Safety		Anti-cancer		Antioxidant	XTT	TNF-α	IL-2	IL-6	P24	RT	Antimicrobial			Total (+)
		50 µg/mL	100 µg/mL	50 µg/mL	100 µg/mL								Gram positive	Gram negative	Fungi	
C. edulis	**Aqueous**	-	--	++	+	+++	++	+	-	-	++	+++	+++	+++	+	**21**
	Methanol	-	-	++	+	+++	+++	N.A	N.A	-	++	+++	+++	+++	+	**21**
M. cecropioides	**Aqueous**	N.A	N.A	++	+	+++	-	+	N.A	-	N.A	N.A	++	+	N.A	**10**
	Methanol	N.A	-	+	+	+++	++	N.A	N.A	N.A	++	N.A	++	+	N.A	**12**
S. ochocoa	**Aqueous**	N.A	-	++	+	+++	+	+	N.A	--	+	N.A	++	+	N.A	**12**
	Methanol	N.A	-	++	+	+++	+++	-	N.A	--	++	N.A	++	+	N.A	**14**
A. hirtella	**Aqueous**	+	N.A	++	+	+++	-	+	N.A	-	+++	+	+++	++	N.A	**17**
	Methanol	-	-	++	+	+++	+++	N.A	N.A	N.A	+	N.A	+++	++	+	**16**
R. vomitoria	**Aqueous**	N.A	-	++	+++	+	--	+	N.D	N.A	N.A	N.A	+	+	N.A	**9**
	Methanol	N.A	-	++	+++	+++	++	N.A	N.D	N.A	--	N.A	+	+	N.A	**12**
A. cissampeloides	**Aqueous**	N.A	-	N.A	-	+	+	+	N.A	-	+++	N.A	N.A	N.A	N.A	**6**
	Methanol	N.A	N.A	N.A	-	N.A	+++	N.A	N.A	N.A	+++	N.A	N.A	N.A	N.A	**6**
S. kamerounensis	**Aqueous**	-	-	N.A	N.A	+++	+++	+	N.A	---	+++	N.A	+++	++	+	**16**
	Methanol	-	-	+	N.A	+++	++	N.A	N.A	N.A	+++	N.A	+++	++	+	**15**
M. puberula	**Aqueous**	-	-	+	N.A	N.A	++	+	+	---	+++	N.A	N.A	N.A	N.A	**6**
	Methanol	-	-	++	N.A	+	N.A	+	+	N.A	+++	N.A	N.A	N.A	N.A	**8**
S. gabonensis	**Aqueous**	N.A	N.A	+	+	+	+++	+	N.A	N.A	-	N.A	+	+	N.A	**9**
	Methanol	N.D	ND	N.D	+	+++	+++	N.A	+	+	N.A	N.A	+	+	N.A	**9**
M. monandra	**Aqueous**	-	-	+++	+++	+	--	+	-	+	N.A	N.A	++	+	N.A	**12**

	Methanol	N.A	-	++	+++	++	+++	N.A	-	+	N.A	N.A	++	+	N.A	**14**
U. klainei	**Aqueous**	-	-	+++	+++	+++	N.A	+	N.D	N.A	+++	N.A	+	+	N.A	**15**
	Methanol	--	---	+++	+++	+++	++	N.A	N.D	N.A	--	N.A	+	+	N.A	**13**
P. soyauxii	**Aqueous**	N.A	N.A	+++	+++	++	--	N.A	N.A	N.A	+	++	+	+	N.A	**13**
	Methanol	N.A	N.A	+++	+++	++	+++	N.A	N.A	++	++	N.A	+	+	N.A	**17**
U. guineensis	**Aqueous**	-	-	+++	+++	+++	N.A	N.A	+	--	+++	N.A	+++	++	N.A	**18**
	Methanol	-	-	+++	+++	+++	N.A	N.A	+	+++	+++	N.A	+++	++	N.A	**21**
A. klaineana	**Aqueous**	---	---	+++	+++	+++	--	N.A	+	N.A	+++	N.A	+++	+++	+	**20**
	Methanol	---	---	+++	+++	+++	++	N.A	N.A	+++	+++	N.A	+++	+++	+	**24**
V. conferta	**Aqueous**	N.A	-	+++	+++	+	---	N.A	N.D	N.A	+++	++	N.A	N.A	N.A	**12**
	Methanol	N.A	-	+++	+++	+	---	+	N.D	+++	++	N.A	N.A	N.A	N.A	**13**
A. vogelii	**Aqueous**	N.A	-	---	N.A	+	-	N.A	N.D	N.A	N.A	N.A	N.A	N.A	N.A	**1**
	Methanol	-	-	---	N.A	+	-	N.A	N.D	+++	++	N.A	N.A	N.A	N.A	**5**
T. iboga	**Aqueous**	-	---	++	-	+	--	+	-	+	+++	N.A	+++	+++	+	**13**
	Methanol	--	---	N.A	-	++	--	N.A	+	+++	+++	N.A	+++	+++	+	**16**
C. religiosa	**Aqueous**	+	+	N.A	N.A	+++	--	+	N.D	+	+++	+++	+	N.A	N.A	**14**
	Methanol	+	+	N.A	+	+++	N.A	+	N.D	+++	+++	N.A	+	N.A	N.A	**14**
C. lucanusianus	**Aqueous**	N.A	N.A	N.A	N.A	+	---	N.A	N.D	++	+++	N.A	N.A	N.A	N.A	**6**
	Methanol	N.A	N.A	+	--	N.A	--	+	N.D	+++	+	+++	N.A	N.A	N.A	**9**
R. africanum	**Aqueous**	N.A	N.A	N.A	+++	+	--	+	N.D	N.A	++	N.A	N.A	N.A	N.A	**7**
	Methanol	N.A	-	N.A	+++	+++	N.A	+	N.D	+++	N.A	+++	N.A	N.A	N.A	**13**

+ low activity; **++** moderate activity; **+++** strong activity; **N.A** not active; **N.D** not determined; **-** low unwanted activity; **--** moderate unwanted activity; **---** strong unwanted activity.

5.5 DISCUSSION

In order to shed light on the mechanisms of how the selected plants act to improve HIV-infected individual health, the capacity of the studied plants to inhibit or induce the immune system was assessed. In individuals suffering from AIDS, the decrease in the number of CD4+ T helper cells but also the B-cell activation resulting in a hyperactivity of the immune system are signals of the immune system deficiency. This duality is responsible for both increasing and decreasing cytokine secretion levels that can have direct impact on the progression of HIV infection (Kishimoto, 2010). For this study, 3 cytokines (Interleukin-2, Interleukin-6 and Tumor Necrosis Factor alpha) were selected for their importance in the HIV infection. These cytokines play essential roles in many physiological processes especially the inflammatory responses (Kishimoto, 2010; Hoyer *et al.,* 2008; Popa *et al.,* 2007). It has been proven that persistent inflammation is associated with HIV infection, since the earlier stages of HIV are characterized by an ongoing immune activation and dysregulation (Sokoya *et al.,* 2017; Lederman *et al.,* 2013). Many studies have demonstrated the correlation between high levels of IL-6 and TNF-α with HIV replication while a low expression of IL-2 was predictive for HIV infection clinical progression (Kedzierska and Crowe, 2001).

IL-6 assessment

Under normal conditions, IL-6 displays a wide range of biological events including inflammation, immune regulation, hematopoiesis, and oncogenesis (Kishimoto, 2010). Interleukin-6 is also involved in B-cell stimulation, monocyte differentiation and induction of IL-4 producing cells (Rincon *et al.,* 1997). An overproduction of IL-6 is responsible for the pathogenesis of various inflammatory diseases (Kishimoto, 2010). *In vitro* studies have demonstrated high levels of IL-6 linked with HIV infection in the early stages. Breen et al. (1990) highlighted the elevated levels of plasma IL-6 and IL-6 mRNA in HIV-infected people, but also the elevated production of IL-6 in isolated HIV infected PBMCs. Birx et al. (1990) presented evidence that HIV is able to induce IL-6 production *in vitro*. The same researchers also advanced that HIV may induce a rapid and transient increase in IL-6 gene expression in monocytes without infecting the cells, probably by signal transduction via a cell surface protein.

According to Borges et al. (2015) HIV-infected people under HAART showed a significant high plasma levels of IL-6 compared to uninfected people. Therefore, inhibiting the overproduction of IL-6 by HIV infected cells can be a great strategy in the management of HIV infection.

In the current chapter, the effect of 40 plant extracts on IL-6 secretion by HIV infected PBMCs was determined using an ELISA kit. The results revealed that only the methanolic extracts of *M. monandra; P. soyauxii; U. guineensis*; *A. klaineana; V. conferta; A. vogelii; T. iboga; C. religiosa; C. lucanusianus* and *R. africanum* were able to decrease the levels of IL-6 produced by infected PBMCs when compared to untreated cells but these decreases were statistically insignificant ($p>0.05$). While all the water extracts and the remaining methanol extracts were up-regulating IL-6 levels especially *S. kamerunensis* and *M. puberula* aqueous extracts which induced an increase of 106% and 114% respectively, when compared to the non-treated cells. Again, these changes were statistically insignificant.

Although, several studies have shown the capacity of medicinal plants in modulating cytokine expression *in vitro* and *in vivo*, the present study has demonstrated that the tested plants didn't display a significant immunomodulatory activity in HIV-infected cells. The decrease in the levels of IL-6 induced by *C. lucanusianus*, *T. iboga*, *V. conferta* and *A. vogelii* could be due to the cytotoxic effect demonstrated in the previous chapter (Chapter 4 section anti-HIV activity). A decrease in the number of cells could be responsible for the low levels of IL-6 detected. The decrease observed may also be because of the effect of methanolic plant extracts on mechanisms inducing directly or indirectly the down regulation of IL-6 production by PBMCs. These mechanisms are varied and can involve different pathways such as p38 kinases; Mitogen Activated Protein Kinase (MAPK); Nuclear Factor kappa B (NF-κB); Suppressor of cytokine synthesis (SOCS); but also others cytokines like IL-1 and TNF-α that can modulate IL-6 expression via a cascade of mechanisms (Koch *et al.,* 2014; Tosato and Jones, 1990). *Macaranga monandra*, *P. soyauxii* and *A. klaineana* methanol extracts induced a noticeable proliferation of the peripheral blood monocyte cells (Chapter 4 section anti-HIV activity) and were also able to decrease IL-6 secretion levels. Kapewangolo et al. (2015) also demonstrated an inhibitory effect of *Ocimum labiatum* that leave methanol extract on different pro-inflammatory cytokines

including IL-6. In a study by Assaf et al. (2016) methanolic extracts of various plants were also shown to down-regulate IL-6 expression.

On the other hand, plants such as *C. edulis*, *S. ochocoa, S. kamerunensis, M. puberula* and *U. guineensis* enhanced the production of IL-6 by HIV infected cells. This enhancement could be the result of the cell proliferation induced by these plant extracts as described in the previous chapter (cell viability). An increase in the number of cells might explain the high levels of IL-6 detected. Plant extracts such as *M. cecropioides, A. hirtella, A. cissampeloides* and *P. soyauxii* which displayed non-significant cytotoxicity or cell proliferation showed an up-regulation in IL-6 secretion. Many *in vitro* and *in vivo* studies report similar findings. A study conducted by Wenner et al. (2015) assessing the *in vivo* effects of orally administered *Echinacea purpurea* extracts on humans, showed the potential enhancing effect of these extracts towards IL-6.

The immunomodulatory effects observed in this study are undoubtedly linked to the presence of secondary metabolites present in the plant extracts such as alkaloids; terpenoids; phenols; tannins; steroids and flavonoids (Chapter 3). Many of those compounds have exerted immunostimulant or immunosuppressant effects in different studies. The immune-stimulation exerted by *U. klainei* extracts in IL-6 secretion might be due to the presence of polysaccharides. Mengome et al. (2014) showed that polysaccharides (pectin and hemicellulose) extracts from *Uvaria klainei* were responsible for enhancing IL-6 production levels (370 pg/mL compared to 37 pg/mL for untreated cells) in non-infected PBMCs at a concentration of 1000 µg/ml. The findings are similar to the results in this study which indicate that both methanol and aqueous extracts from *U. klainei* at a concentration of 25 µg/mL were able to up-regulate IL-6 production levels in HIV-infected PBMCs. Further studies should be done in order to determine the real mechanisms involved in the IL-6 immunomodulation of the selected plant extracts. The down regulation of the pleiotropic cytokine IL-6 exerted by plant extracts is of great importance in the search for efficient immunosuppressant drugs as well as molecules that can inhibit chronic inflammation associated with early stages of HIV infection.

TNF-α assessment

Another major pro-inflammatory cytokine that plays a role in HIV infection is the Tumor Necrosis Factor alpha (TNF-α). This T helper type 1 (Th 1) is secreted by various cells such as macrophages, monocytes and T cells. Low levels of TNF-α are reported in healthy individuals while high levels are positively correlated with various physiological disorders including inflammation, cancer and autoimmune diseases (Barrel, 2019). In recent years, the role of TNF-α in HIV infection has been documented extensively. Several studies revealed that elevated levels of TNF-alpha were detected in untreated HIV individuals (Highleymam, 2010). Some studies have reported that in early HIV-1 infection stages, TNF-alpha displays a protective effect against HIV-1 (Kumar *et al.,* 2016). The inhibition of HIV-1 entry into macrophages by TNF-α has also been described *in vitro* by Pasquereau et al. (2017). In a review, Kumar and colleagues have affirmed the contradictory results that surround the changes in TNF levels in HIV patients under HAART (Kumar *et al.,* 2016). Some studies depicted low TNF-alpha levels as marker of HAART efficacy, while others reported an increase in TNF-alpha producing T cells resistant to apoptosis in patients receiving HAART (Brazille *et al.,* 2003; Ledru *et al.,* 2000). Therefore, controlling TNF-α expression in HIV infected patients could constitute a great step in the management of HIV infection.

In this study, the immunomodulatory effects of selected plant extracts on TNF-α expression were assessed. The results indicated no significant variation in the production of specific cytokine by HIV infected PBMCs treated with the extracts at the concentration used in this study (25 µg/mL). *Rauwolfia vomitaria* aqueous extract and *C. lucanusianus* methanol extract showed the lowest values of TNF-α which were 50.67 pg/mL and 51 pg/mL respectively. This corresponds to a decrease of about 12% when compared to the untreated cells. While, *C. lucanusianus* aqueous extract and *S. ochocoa* methanol extract showed the highest increase in TNF-α levels which correspond to an enhancement of 13% when compared to the untreated cells. Medicinal plants have revealed their ability to modulate immune system by various mechanisms including cytokine expression. Plants such as *Echinacea laeviagata,* and *Rhaphidophora korthalsii* were able to enhance *in vitro* the secretion of cytokine like TNF by healthy PBMCs using different signaling pathways including the activation of nuclear factor kappa B (NF-kB), p38 mitogen-activated protein kinase (p38MAPK), JNK (c-Jun N terminal kinase) and apoptotic pathways (Haria *et al.,* 2016; Yeap *et al.,* 2013). In a study by Wenner et al. (2015), oral administration of *Echinacea purpurea*

did not significantly enhance TNF-α responses in mitogen-stimulated PBMC. Furthermore, in another study done in Israel, the researchers also described no significant variation in TNF-α expression by PBMCs after stimulation with *Inula viscosa* derived compounds (Abrham *et al.,* 2010). These findings are quite similar to the current study where the selected plant extracts failed to significantly change TNF levels when compared to untreated cells. Although these specified results could also be due to the unique low concentration (25 µg/mL) used in this study, higher concentrations may be experimented further to really appreciate the immunomodulatory effects of the selected plants on TNF-α secretion by PBMCs.

IL-2 assessment

Lastly, IL-2 production was assessed in the current study. This monomeric glycoprotein is secreted by various activated cells (dendritic cells, CD8 and CD4 cells), and plays a major role in the immune system in contributing to the development of regulatory T cells in the production of cytokines such as TNF-α or IFN-γ cells but also in enhancing cytolytic activity of natural killer cells (Capobianco *et al.,* 2016). In HIV-infection, the destruction and the functional deterioration of CD4+ T cells lead to a deficit in IL-2 levels. Because IL-2 contributes to the proliferation and the activation of lymphocyte T, its induced up-regulation of HIV replication both directly and via increases in TNF-α levels, increases HIV expression by the activation of nuclear factor (NF)-kB (Pett *et al.,* 2010). It has been proven that IL-2 levels are significantly decreased in HIV patients with CD4 cell counts lower than 200 cells/mm3 (Hong *et al.,* 1998). According to Kedzierska and Crowe (2001) IL-2 secretion by *in vitro* activated PBMC isolated from HIV-infected subjects at various stages of the disease, is significantly decreased when compared to PBMCs obtained from uninfected subjects. Stimulating the secretion of this specific cytokine in HIV patients could surely help in improving their health.

Therefore, the effect of 26 selected extracts on IL-2 production by HIV infected PBMCs was evaluated. The results revealed that both aqueous and methanolic extracts *U. guineensis* and *M. puberula*, methanol extracts of *A. hirtella*, *A. cissampeloides*, *S. gabubensis* and *T. iboga* as well as the aqueous extract of *A. klaineana* were stimulating the production of IL-2. When compared to the untreated cells, this up-

regulation was not significant ($p>0.05$) as the increase was only from 1% to 21%. The low concentration (25 µg/mL) used for this assay may explain the weak increase observed in IL-2 levels. Undoubtedly higher concentrations might have provided better results. Nevertheless, these results are promising because if increasing the concentration of the extracts could increase the level of IL-2 produced, then this could provide an explanation of the rise in CD4+ count of HIV patients who take those plants. Because of the role of IL-2 in the modulation of the immune system, scientists started to look at IL-2 as a potent anti-HIV agent alone or in combination with HAART (Joly and Odloak, 2013; Smith, 2001).

Many studies and clinical trials have been led in order to determine the effect of an adjunctive IL-2 therapy in HIV patients (Onwumeh *et al.,* 2017; Heyckendorf *et al.,* 2015; Pett *et al.,* 2010; Mitsuyasu, 2001). It has been demonstrated that, co-administration of HAART and IL-2 showed a rise of CD4 T cell numbers without an increase in viral load (Kedzierska and Crowe, 2001); baseline numbers of circulating CD4+ cells was significantly increased without an expected reduction in the risk for opportunistic diseases or death (Heyckendorf *et al.,* 2015); IL-2 in combination with ART increases the CD4 cell count in HIV-positive adults (Onwumeh *et al.,* 2017). These findings highlight the importance of IL-2 levels in HIV infected individuals as such, enhancing the production of IL-2 in people suffering from AIDS is crucial.

Medicinal plants have the reputation of boosting the immune system by stimulating the production of some cytokines such as IL-2. Yeap et al. (2010) have demonstrated that methanol extract of *Rhaphidophora korthalsii*, a plant used in China to treat cancer, was able to induce the proliferation of immune cells and induce the secretion of IL-2. According to Jantan et al. (2015) a terpenoids (14-deoxyandrographolide) isolated from *Andrographis paniculata* can enhance the production of IL-2 by T-cells. In the same manner, some phenolic compounds such as catechin; epigallocatechin gallate; epicatechin; luteolin; chrysin; quercetin and galangin have also the ability to increase IL-2 secretion (Grigore, 2017). It can therefore be conjectured that phenolic compounds and terpenoids detected in the plant extracts investigated in this study, contribute to the up-regulation of IL-2 in HIV infected PBMCs.

It is noteworthy that our study has highlighted the variability existing among individuals characterized by high standard deviation in the production of cytokines, especially in

Figure 5.1 showing the effect of the plant extracts on IL-6 secretion. The results highly deviated from a donor to another due to several parameters such as the age, ethnicity, genetic heritability but also the fact that the donor was a treatment naïve patient or under ARVs (Burel *et al.,* 2018; Brodin *et al.,* 2015). These parameters might play a crucial role in the responsiveness of HIV infected PBMCs treated with the plant extracts which explains the high standard deviation to the mean generated in our results.

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CHAPTER SIX

Characterization and biological evaluation of fractions isolated from *C. edulis* and *T. iboga*

The article below is in preparation

Boukandou Mounanga MM, Traore Afsatou Ndama and Potgieter Natasha; Evaluation of biological potential of *Coula edulis* fractions.

CHAPTER 6

Abstract

During the previous studies *C. edulis* and *T. iboga* displayed encouraging antimicrobial activity against several strains responsible for O.Is associated to HIV but also promising HIV inhibitory effect, what prompted the selection of methanolic extracts from these plants for fractionation purpose and biological evaluation of the obtained fractions. The aim of the present study is thus to elucidate the active compounds in *C.*

edulis and *T. iboga* crude extracts and isolated fractions and also to evaluate the potential biological activities.

Method: *C. edulis* and *T. iboga* methanol extracts were fractionated using column chromatography and various phytochemical analysis were done using standard phytochemical screening; evaluation of TPC/TFC using Folin-Ciocalteu reagent and aluminium chloride colorimetric methods respectively; and elucidation of structure using UPLC-MS. The biological activities were evaluated using free radical scavenging and reducing power methods for antioxidant activity; ELISA kits specific for human cytokines for immunomodulatory effects (IL-2 and IL-6); Well diffusion assay followed by micro-broth dilution assay for antimicrobial activity; antigen p24 ELISA kit anti-HIV activity.

Results: *C. edulis* methanol extract and the 4 fractions (F1, F2, F3 and F4) eluted after column chromatography contained polyphenol compounds including Ellagic acid and Gallic acid which can be linked to the antibacterial, anti-HIV, anti-inflammatory and antioxidant capacities displayed by *C. edulis* samples. *Tabernanthe iboga* and the 4 fractions eluted (Fa, Fb, Fc and Fd) were found to possess mainly alkaloid compounds including ibogaine, ibogaline and voacangine which have also demonstrated promising antibacterial, anti-HIV and anti-inflammatory effects.

Conclusion: The present findings have showed that the polyphenols and alkaloids present in *C. edulis* and *T. iboga* respectively are the main actors of the biological activities displayed by these plants, what validates the use of the mentioned plants in traditional medicine in Gabon.

Key words: Fractions, anti-inflammatory, anti-HIV, antimicrobial, antioxidant, immunomodulatory effect.

6.1 INTRODUCTION

The aim of the present chapter is to fractionate the two plants namely *T. iboga* Baill. and *C. edulis* Baill. That demonstrated the most promising bioactivity to enable future isolation and identification of the active compounds. These two plants displayed the best antimicrobial activity against several bacteria and fungi causing HIV opportunistic infections. They showed promising anti-HIV potential through the inhibition of p24 antigen and reverse transcriptase. Therefore, performing bio-guided fractionation

using phytochemical tools such as column chromatography followed by biological assays (antioxidant, antibacterial, anti-inflammatory, immunomodulatory and anti-HIV) is a step in the right direction for elucidating the bioactive compounds.

6.1.1 FRACTIONATION AND ISOLATION OF PLANT COMPOUNDS

For centuries plants were used by human beings for various applications like food, cosmetics and medicine. Plants can produce extraordinary complex molecules that are responsible for a tremendous range of pharmacological properties suitable for human health. These molecules also called secondary metabolites consist of organic compounds deriving from primary metabolites. These metabolites can occur in low amounts in specialized cells or tissue and their production may be widespread or restricted to particular families, general or species (Zieger and Taiz, 2006). Secondary metabolites can play numerous roles in plants like helping plants adapt to their environment, protecting them against pathogens and herbivore as well as for defence mechanism. Secondary metabolites also contribute to the specific odours, tastes and colors in plants, serving as attractants for pollinators and seed-dispersing animals (Rungsung *et al.,* 2015). These metabolites can be classified on the basis of biosynthetic pathways, chemical structure or solubility in various composition solvents (Tiwari and Rana, 2015). Since the first isolation of a natural plant compound (morphine) introduced for therapeutic use in 1827, numerous drugs isolated from plants were generated (Rungsung *et al.,* 2015). Because plants usually occur as a combination of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge in the process of identification and characterization of bioactive compounds (Sasidharan *et al.,* 2011). Several extract techniques exist and involve conventional methods of using organic fluid (hexane, acetone, methanol, ethanol etc.) or water carried out generally at atmospheric pressure and new techniques (developed more recently) using pressure and / or elevated temperatures (Ngaha Njila *et al.,* 2017).

The beneficial action of bioactive compounds is due to the merging or synergic work of these secondary metabolites. Yet, only one compound can be responsible for the bioactivity observed. Isolation and elucidation of the bioactive compound is of interest as it can be used as blueprints for the manufacture of synthetic drugs of a similar

structure, serve as building blocks or starting materials for the production of semi-synthetic drugs (Rungsung *et al.,* 2015). This is crucial because many of the medicinal plants are facing extinction or severe genetic loss today due to increasing human population and extensive destruction (Rungsung *et al.,* 2015).

6.1.2 THE TWO PLANTS SHOWING GREAT POTENTIAL

Coula edulis Baill. also known as Gabonese nut, is an evergreen tree growing up to about 38 m. It produces an ellipsoidal nut enveloped in a very hard shell and kernel tasting as chestnut or hazelnut. *Coula edulis* is widely utilized to treat parasites, diarrhea, dysentery, women problems, skin disorders, and stomachache. Saponins, alkaloids, tannins, flavonoids, cardiac glycosides, anthraquinone and terpenes have been detected in this specie (Bukola *et al.,* 2008). The anti-plasmodial, anti-dermatophytic and antimicrobial activities have been demonstrated (Tamokou *et al.,* 2011; Zofou *et al.,* 2011; Bukola *et al.,* 2008). Tamokou et al. (2011) showed that *C. edulis* exerted some toxic effects on rodents' liver and kidney.

Gabonese traditional healers use *C. edulis* to manage HIV and related OIs with somehow good results. The investigation of the various biological activities of barks from this plant is of interest to validate its use in managing AIDS. The results in the present study revealed that the methanolic and aqueous extracts demonstrated important antioxidant and antimicrobial activity. IL-6 levels in isolated HIV infected PBMCs were enhanced after treatment with the *C. edulis* extracts. This study reported the first data on the anti-HIV effects of *C. edulis* extracts. The results demonstrated an inhibition in p24 antigen levels and reverse transcriptase enzyme after treatment with the extracts. The cytotoxicity studies indicated that the extracts showed a mild anti-cancer effect against HeLa cell line and negligible toxic effect on Vero African Green monkey kidney cells.

Tabernanthe iboga Baill. is an evergreen shrub of about 4 m in height and native to western Central Africa. The leaves are small and green with white to pink flowers. In Gabon, roots mainly used in initiation ceremonies can also be used to relieve hunger, thirst and tiredness but is also taken as aphrodisiac and febrifuge or to treat coughs, urinary infections, and conjunctivitis (own observation). The leaves are anesthetic,

aphrodisiac, and used against toothache while the latex is antiparasitic. Bading et al. (2018) demonstrated the antidiabetic effects of the roots. About 20 alkaloids including ibogaine and noribogaine have been isolated so far.

In Gabon, reports on HIV patient health improvement was made after consumption of *T. iboga* (Data not published). The findings in the present study indicated that methanolic and aqueous extracts showed important antimicrobial effect against several selected germs causing OIs. The immunomodulatory assessment revealed a promising effect on pro-inflammatory cytokines characterized by a decrease in the levels of IL-6 in HIV infected PBMCs. The extracts were also able to inhibit p24 levels in the infected cells but did not display an anti-reverse transcriptase effect.

The aim of this last chapter is to fractionate the methanolic extracts of the two selected plants as well as to assess the biological activities of the fractions obtained, and then to elucidate the structure of at least one of the isolated active compounds.

6.2 MATERIALS AND METHODS

6.2.1 ETHICAL APPROVAL

Ethical approval was obtained from the Health, Safety, and Research Ethics Committee of the University of Venda (SMNS/17/MBY/30/2111). Permission to use health facilities in order to get the HIV infected blood was obtained from the Limpopo Provincial Department of Health via collaboration with the HIV and Global Health Research Group at Univen. Signed informed consent forms were obtained for agreement from all volunteers before collection of blood specimens. Confidentiality for volunteers was maintained throughout by assigning numerical code. The blood was used for PBMCs extraction only.

6.2.2 PHYTOCHEMICAL ANALYSIS

Following the biological tests performed with all the plant extracts, two plants (*T. iboga* and *C. edulis*) whose methanolic extracts displayed the best overall biological effects, were selected to be fractionated.

6.2.2.1 Column chromatography

T. iboga (250 g) and *C. edulis* (400 g) dry powders were macerated into 2 L absolute methanol for 24 h. After filtration, the filtrates were collected and kept in the fridge for further utilization while the marcs were soaked for additional 48 hour into 2 L absolute methanol. Following the maceration time, the mixtures were filtered, the marcs discarded, and the filtrates mixed with the previously refrigerated ones. The mixtures were then evaporated with a rotavapor at 45 °C and the crude extracts were collected and weighted. The dried crude extracts, were afterward, resuspend into absolute methanol and silicate gel powder was added in the suspension till all the liquid was absorbed by the silicate gel powder. The glass column was prepared as follows; the stationary phase, 300 mL of hexane were mixed with 300 mL of ethyl acetate and silicate gel powder, was poured into the glass column. Afterwards, the dried powder composed of silicate gel and crude extract, was added to the column and different solvent systems were gradually added to the column to allow the compounds separation. The solvents used for *C. edulis* included hexane/ethyl acetate (50:50) followed by ethyl acetate (100 %), then ethyl acetate/methanol (80:20), and ethyl acetate/methanol (20:80). While for *T. iboga* the solvents were: hexane/ethyl acetate (50:50) followed by ethyl acetate (100 %), then ethyl acetate/methanol (90:10), ethyl acetate/methanol (80:20) and methanol (100 %). The different fractions were collected separately and evaporated at 45°C.

To calculate the yield of each fraction, the followed formula was used;

$$\boldsymbol{FY}\% = \frac{\boldsymbol{Weight\ Fraction}}{\boldsymbol{Weight\ extract}} \boldsymbol{X\ 100}$$

Figure 6.1: Image of the column chromatography technique

6.2.2.2 Comparative phytochemical screening of the obtained fractions and the crude extracts

The screening was done as described in detail in **Chapter 3** Section **3.2.3.1.**

6.2.2.3 TPC/TFC of the crude extracts and the fractions

The determination of the Total phenolic and flavonoid contents was done as described in detail in **Chapter 3** Section **3.2.3.2.**

6.2.2.4 Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry

UPLC analysis

A Waters UPLC (Waters Corporation, Milford, MA; USA) coupled in tandem to a Waters SYNAPT G1 HDMS mass spectrometer (Waters Corporation, Milford, MA; USA) was used to generate accurate mass data. Optimization of the chromatographic separation was done using a Waters HSS T3 C18 column (150 mm x 2.1 mm, 1.8 µm)

and the column temperature controlled at 60°C. A binary solvent mixture was used consisting of water (Eluent A) containing 10 mM formic acid (natural pH of 2.3) and acetonitrile (Eluent B) containing 10 mM formic acid. The initial conditions were 98% A at a flow rate of 0.4 mL/min and were maintained for 1 minute, followed by a linear gradient to 2% A at 25 minutes. The conditions were kept constant for 2 minutes and then changed to the initial conditions. The runtime was 30 minutes and the injection volume was 0.1 - 2 µL depending on the concentration. Samples were kept cool at 6°C in the Sample Manager during the analysis.

TOF Mass Spec analysis

The SYNAPT G1 mass spectrometer (Waters Corporation) was used in V-optics and operated in electrospray mode to enable detection of ESI-compatible compounds. Leucine enkephalin (50 pg/mL) was used as reference calibrant to obtain typical mass accuracies between 1 and 5 mDalton (mDa). The mass spectrometer was operated in both ESI positive and negative modes with a capillary voltage of 2.5 kV, the sampling cone at 30 V and the extraction cone at 5.0 V. The scan time was 0.1 seconds covering the 50 to 1200 Dalton mass range. The source temperature was 120°C and the desolvation temperature was set at 450°C. Nitrogen gas was used as the nebulisation gas at a flow rate of 550 L/h and cone gas was added at 50 L/h. The MassLynx 4.1 (SCN 872) was used to control the hyphenated system and for all data manipulation.

6.2.3 BIOLOGICAL TESTS

6.2.3.1 Antioxidant activity

Determination of the free radical scavenging

The free radical scavenging effect was done as described in detail in **Chapter 3** Section **3.2.4.1.**

Determination of the reducing power assay

The ferric reducing power was done as described in detail in **Chapter 3** Section **3.2.4.2.**

6.2.3.2 Antibacterial activity

Microorganisms

The selected strains for the current study were *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* (ATCC 51741), *Escherichia coli* (ATCC 25922), *Shigella sonnei* (ATCC 25931), *Klebsiella pneumoniae* (ATCC 27736), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter cloacae* (ATCC 13047), *Enterococcus faecalis* (ATCC 29212) and *Bacillus cereus* (ATCC 10876).

Well diffusion susceptibility test

The antimicrobial activity was evaluated as described in detail in **Chapter 4** Sections **4.2.3.1** and **4.2.3.2**

Minimal inhibitory concentration (MIC) determination

The antimicrobial activity was evaluated as described in detail in **Chapter 4** Sections **4.2.3.1** and **4.2.3.2.**

6.2.3.3 Anti-inflammatory activity

Cell lines

The murine macrophage RAW 264.7 cell line was used for this assay. The cells were maintained using DMEM and 10% fetal bovine serum (FBS), 100 µg/L streptomycin and 100 IU/mL penicillin at 37°C in a humidified environment with 5% CO_2.

Cell culture

The mouse macrophage cell line, RAW 264.7, is a well characterized model to investigate the anti-inflammatory potential of test samples. Cells were cultured in multi-well plates and activated by exposure to LPS which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production were determined by measuring the levels of nitrate in the culture medium. Simultaneous evaluation of cell viability (MTT assay) is used to confirm the absence of cytotoxicity of the test sample.

Setting up the bio-assay

In a 96-well plate, RAW 264.7 cells were seeded at a density of $1x10^5$ cells per well and allowed to attach overnight in RPMI medium with 10% FBS. After incubation, the medium was removed and replaced with increased concentrations of test samples (25, 50, 100 and 200 µg/mL) in culture medium at double the desired final concentration. An equal volume of RPMI medium containing 1µg/mL LPS was added then the plates were Incubated at 37°C for 18 hrs. Afterwards, 50 µL conditioned medium was transferred to a new 96-well plate and 50 µL Griess reagent was added. After 10 min incubation at room temperature the plates were read at 540 nm. Silymarin (50 and 100 µM) was used as positive control to illustrate inhibition of LPS induced nitrate levels. To the remaining cells 100 µL culture medium containing 0.5 mg/mL MTT was added and the plate was incubated at 37°C for 1 hr. Next, the medium was removed and 100 µL of DMSO was added in the wells followed by the reading of the plate at 560 nm (Versamax Spectrophotometer) to assess cell viability. The assays were done in triplicate.

6.2.3.4 Immunomodulatory activity

Blood collection and isolation and subculture of PBMCs

Blood samples were collected from HIV positive patients attending local clinics as part of their follow up visits in an ongoing study by the HIV Global Health Research Group at the University of Venda and treated as described in the previous chapters to obtain the isolated PBMCs. Blood was collected from 3 different donors.

In vitro bioassay

The bioassay was performed as described in detail in **Chapter 4** Section **4.2.4.3.**

Cell viability assay

The evaluation of the cell viability was done as described in detail in **Chapter 4** Section **4.2.4.4.**

Assessment of IL-6 production from supernatants

The evaluation of IL-6 expression was performed as described in detail in **Chapter 5** Section **5.2.3.1.**

Assessment of IL-2 production from the supernatants

The evaluation of IL-2 expression was performed as described in detail in **Chapter 5** Section **5.2.3.2.**

6.2.3.5 Assessment of anti-HIV activity

The evaluation p24 antigen expression was done as described in detail in **Chapter 4** Section **4.2.4.5.**

6.2.4 STATISTICAL ANALYSIS

Each assessment was performed in triplicate. The data were treated using Graph pad prism 6.0. The results are presented in mean (±SD) of the triplicate. The difference between the means was considered significant with $p<0.05$.

6.3 RESULTS

6.3.1 PHYTOCHEMICAL PROFILE ANALYSIS

6.3.1.1 Yield result

After extraction of the air-dried plant materials, the final methanolic crude extracts of *C. edulis* weighed 44 g, with a yield of 11%, while *T. iboga* weighed 30.3 g and yielded 12.12%. Each extract produced 4 different fractions; *C. edulis* fractions were noted F1 to F4 while *T. iboga* fractions were named Fa to Fd (Table 6.1).

Fraction F1 and Fa presented the lowest yields (3.79% and 4.29% respectively) of all the fractions, while F3, F4 and Fc gave the highest yields (40.4%, 15.15% and 9.9% respectively). Fractions F2, Fb and Fd presented yield of 7.58%, 8.25% and 8.59% respectively.

Table 6.1: Summary of the fractionation steps

Fraction name		Solvent used	Mass obtained (g)	Percentage yield (%)
Coula edulis	**F1**	Hexane/Ethyl Acetate (50:50)	1.5	3.79
	F2	Ethyl Acetate 100%	3	7.58
	F3	Ethyl Acetate/Methanol (80:20)	16	40.40
	F4	Ethyl Acetate/Methanol (20:80)	6	15.15
Tabernanthe iboga	**Fa**	Hexane/ethyl Acetate (50:50)	1.3	4.29
	Fb	Ethyl Acetate 100%	2.5	8.25
	Fc	Ethyl Acetate/Methanol (80:20)	3	9.90
	Fd	Methanol 100%	2.6	8.59

6.3.1.2 Phytochemical screening

The results of comparative phytochemical screening of the fractions along with the methanolic crude extracts are depicted in Table 6.2. The absence of the chemical is indicated by the sign -, while the presence is represented by the sign +. The amount of the Chemical is represented by + (low), ++ (moderate) and +++ (high).

Coula edulis methanol extract produced a total of 20 (+) indicating the presence of all the compounds screened including saponins, flavonoids, tannins, phenols, alkaloids, steroids, glycosides and terpenoids. The Fraction F4 presented a composition almost similar than the crude extract as 19 (+), describing less alkaloids but more saponins compared to the crude extract. The F3 presented 17 (+) indicating low alkaloids and flavonoids compared to the crude extracts. The F2 with 16 (+) did not show the presence of saponins and alkaloids, while the amount of flavonoids was higher than in *C. edulis* methanol extract. Concerning F1, saponins and flavonoids were not detected while the other chemicals were detected in low amount leading to a total of only 6 (+).

Tabernanthe iboga crude extract produced 13 (+) and was found to possess all the chemicals screened. The Fraction Fd surprisingly yielded more + (16) compared to the crude extracts. More saponins, alkaloids and flavonoids were detected in this fraction compared to the crude. The fraction Fc showed a composition almost similar than the crude extract as 14 (+) were noted, describing more alkaloids and flavonoids while no saponins were detected. The Fb presented 11 (+) indicating high amount of alkaloids compared to the crude, no saponins were detected along to low amount of terpenoids and steroids compared to the crude extracts. Concerning Fa, saponins and flavonoids were not detected while the other chemicals were detected in low amount leading to a total of only 7 (+).

Table 6.2: Comparative phytochemical profile of the crude extracts and the fractions

Extracts	Saponins	Alkaloids	Phenols	Tannins	Flavonoids	Terpenoids	Steroids	Glycosides	Total
C. edulis	++	+++	+++	+++	++	++	++	+++	20
F1	-	+	+	+	-	+	+	+	6
F2	-	-	+++	+++	+++	++	++	+++	16
F3	++	+	+++	+++	+	++	++	+++	17
F4	+++	+	+++	+++	++	++	++	+++	19
T. iboga	+	+	+	+	+	+++	+++	++	13
Fa	-	+	+	+	-	+	+	++	7
Fb	-	+++	+	+	+	++	+	++	11
Fc	-	+++	+	+	+++	++	++	++	14
Fd	++	++	+	+	++	+++	+++	++	16

+ low ; ++ moderate; +++ high; - undetectable

6.3.1.3 TPC/TFC determination

Calibration curves of Gallic acid and Quercetin generated an equation from which the values of the TPC (mg GAE/g of sample) and TFC (mg QE/g of sample) respectively were calculated. The equation used for Quercetin was y = 0.0112x - 0.0024 (R^2 = 0.9956), for Gallic acid: y = 0.0166x + 0.0219 (R^2 = 0.9865).

The comparison of the TPC and TFC between crude extracts and fractions is described in Figure 6.2. The total phenolic and flavonoid contents in F1 (3.67±0.16 mg GAE/g and 1.21±0.04 mg QE/g) was significantly ($p<0.05$) lower than in *C. edulis* crude extracts (23.49±0.34 mg GAE/g and 18.2±0.23 mg QE/g). Total phenolic content on F2 (22.81±0.14 mg GAE/g) was not statistically different than the crude extract while the TFC (5.57±0.31 mg QE/g) was significantly lower. Concerning F3 and F4, the TPC was slightly higher ($P>0.05$) (25.13±0.19 mg GAE/g and 23.86±0.17 mg GAE/g respectively) than the crude extract while for the TFC the difference was statistically higher (19.03±0.42 mg QE/g and 24.6±0.51 mg QE/g respectively).

TPC (0.50±0.05 mg GAE/g) and TFC (0.01±0 mg QE/g) in Fa were very low ($p<0.05$) compared to *T. iboga* (17.12±0.17 mg GAE/g and 2.14±0.06 mg QE/g) crude extract. Fraction b displayed the highest amount of flavonoids (7.67±0.23 mg QE/g) and phenolics (18.42±0.18 mg GAE/g) with a significant difference observed for TFC when compared to the crude. The TFC were not determined in Fd while Fc showed very low amount (0.01±0 mg QE/g). Concerning TPC, Fc (6.2±0.25 mg GAE/g) and Fd (7.21±0.04 mg GAE/g) showed values significantly lower than the crude.

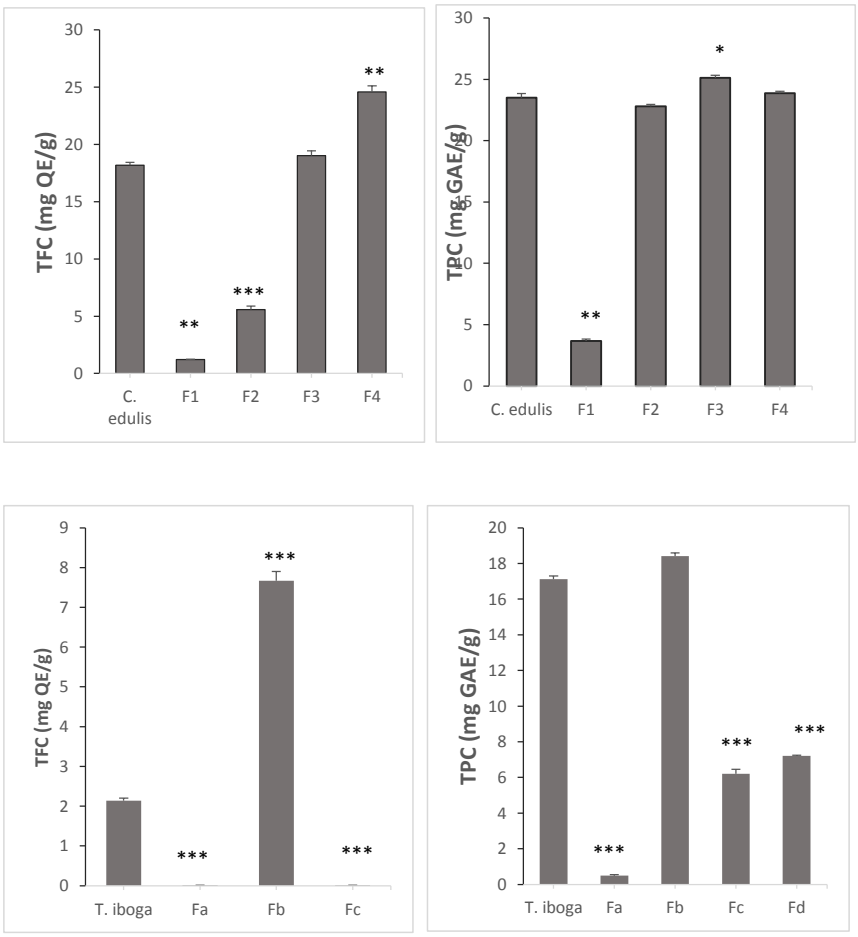


Figure 6.2: TPC and TFC of *C. edulis* and fractions (F1, F2, F3, F4) and *T. iboga* and fractions (Fa, Fb, Fc, Fd). Data represents the mean ± standard deviation for quadruplicate wells. Errors bars denote standard deviation. Significant differences are indicated by * (P<0.05) compared to the respective crude extracts.

6.3.1.4 UPLC-MS analysis

LC–MS/MS allows the simultaneous identification and quantification of compounds found in natural products. These compound structures are derived by comparison to commercially available known substances. The results are read on ESI negative or positive modes.

For the present analysis, F1 and Fa were not evaluated because of the small quantities available. Thus, only the crude extracts (*C. edulis* and *T. iboga*) and the fractions F2, F3, F4, Fb, Fc and Fd were analyzed.

For *C. edulis* samples the results obtained from the UPLC-MS upon ESI negative mode showed that this plant contains structurally diverse metabolites of different physical and chemical properties as seen in Figure 6.3. Upon analysis of the different fractions (Figure 6.4), very minimal differences were observed but all the fractions were seen to contain common metabolites which were further shown to be derivatives of Ellagic acids, appearing as free aglycones (sugar free) at m/z 299/300 or as glycosides (attached to sugar moieties) at m/z 463, 447 and 477 (Figure 6.6). Other forms of Ellagic acid which were methylated were also seen, appearing at m/z 315. The presence of these common metabolites is an indication that these metabolites are the most abundant and inherited features of this plant. It has also been noticed that, from the identified structures, most of the molecules were found to be from the ellagitannins (Appendix Table A1) and they all contain a “fused” Gallic acid (Figure 6.7). These compounds are present in all the fractions assessed from fraction F2 to F4.

Most of the molecules in *T. iboga* crude extract could be seen on the negative ionization chromatogram (Figure 6.8) and showed that alkaloids were mainly detected. Ibogaine and ibogaline (Figure 6.9) are the only molecules which could be detected with positive ionization techniques. The chromatograms of the fractions couldn’t be analysed because of the different settings used which did not lead to conclusive interpretation.

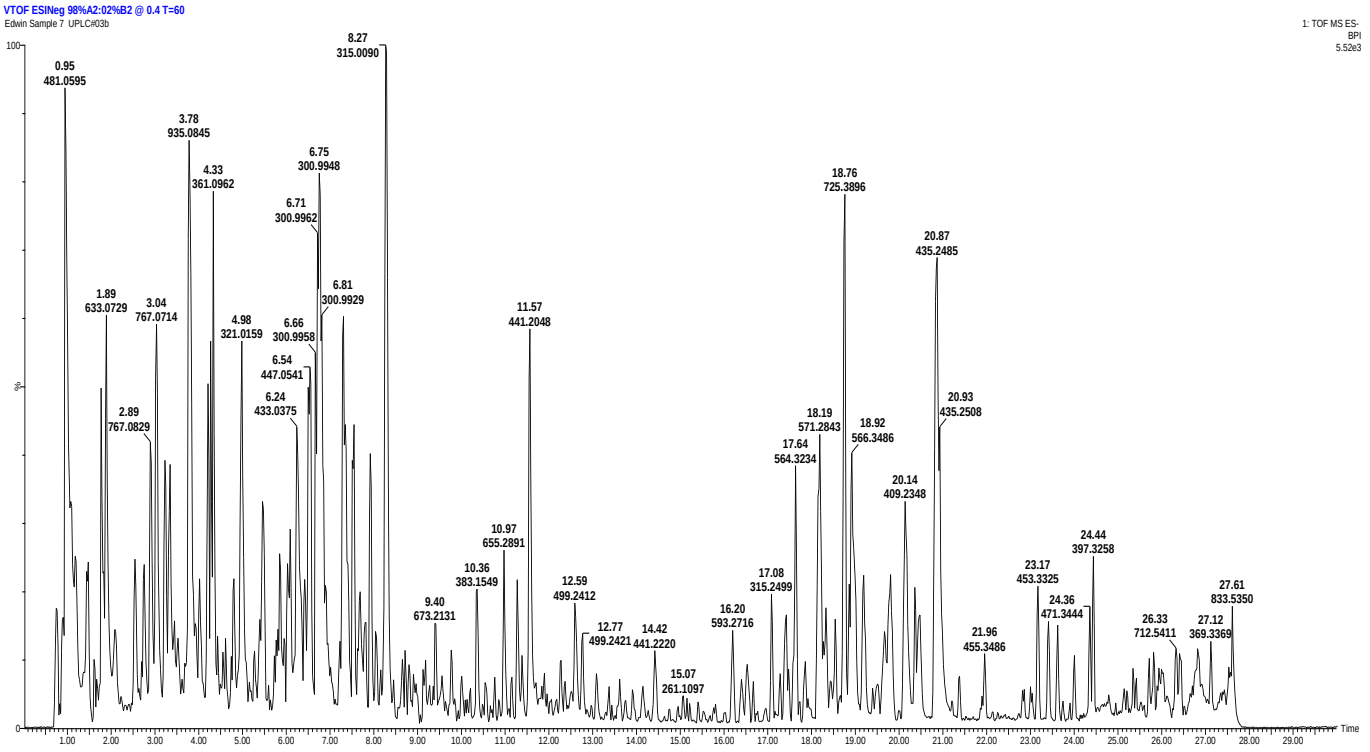


Figure 6.3: Representative LC-MS chromatogram showing different molecules contained in the methanol crude extract of *Coula edulis* in negative iosination

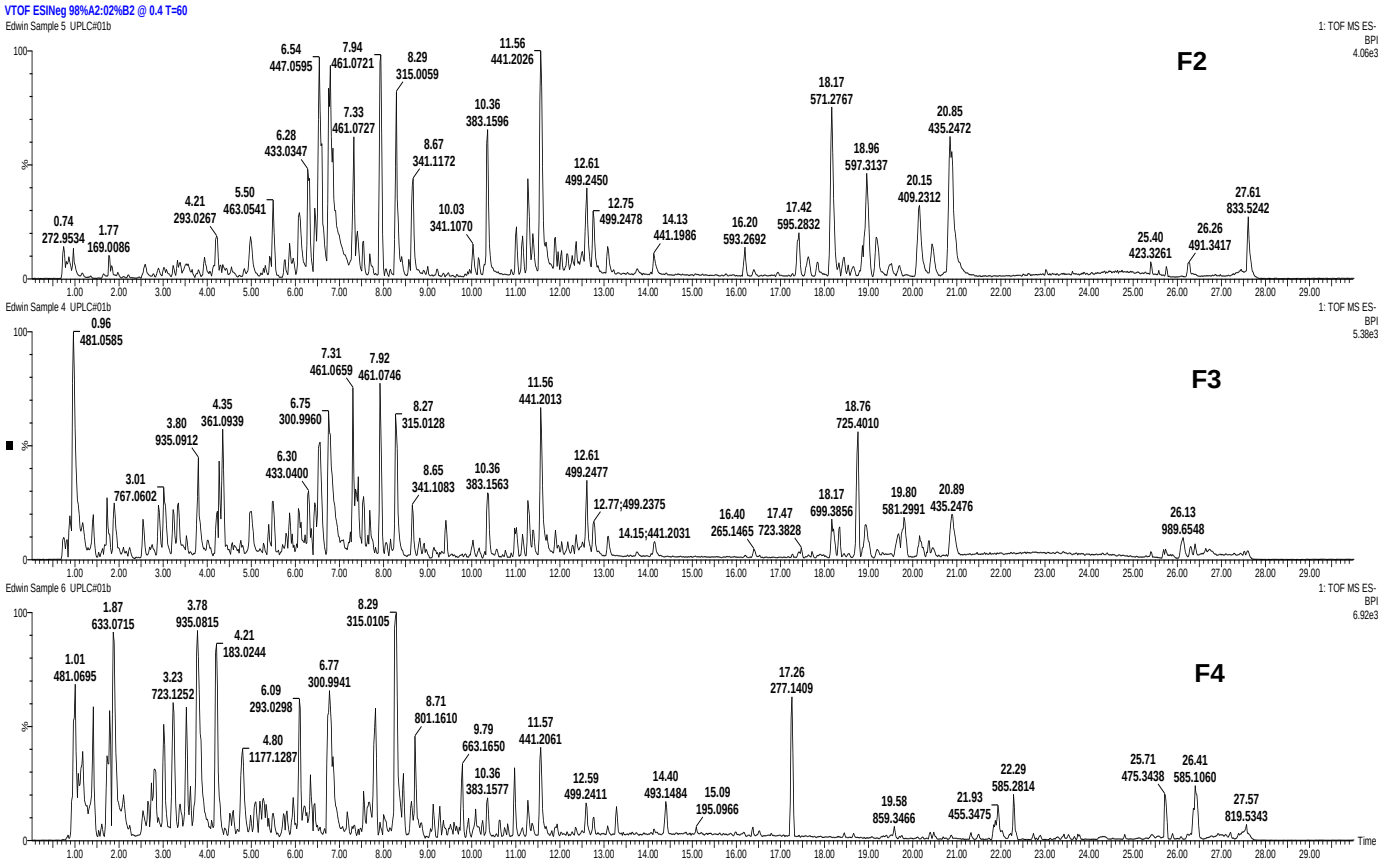


Figure 6.4: Representative LC-MS chromatograms showing elution of different compounds from respective fractions (F2, F3 and F4) of *Coula edulis* methanol extract.

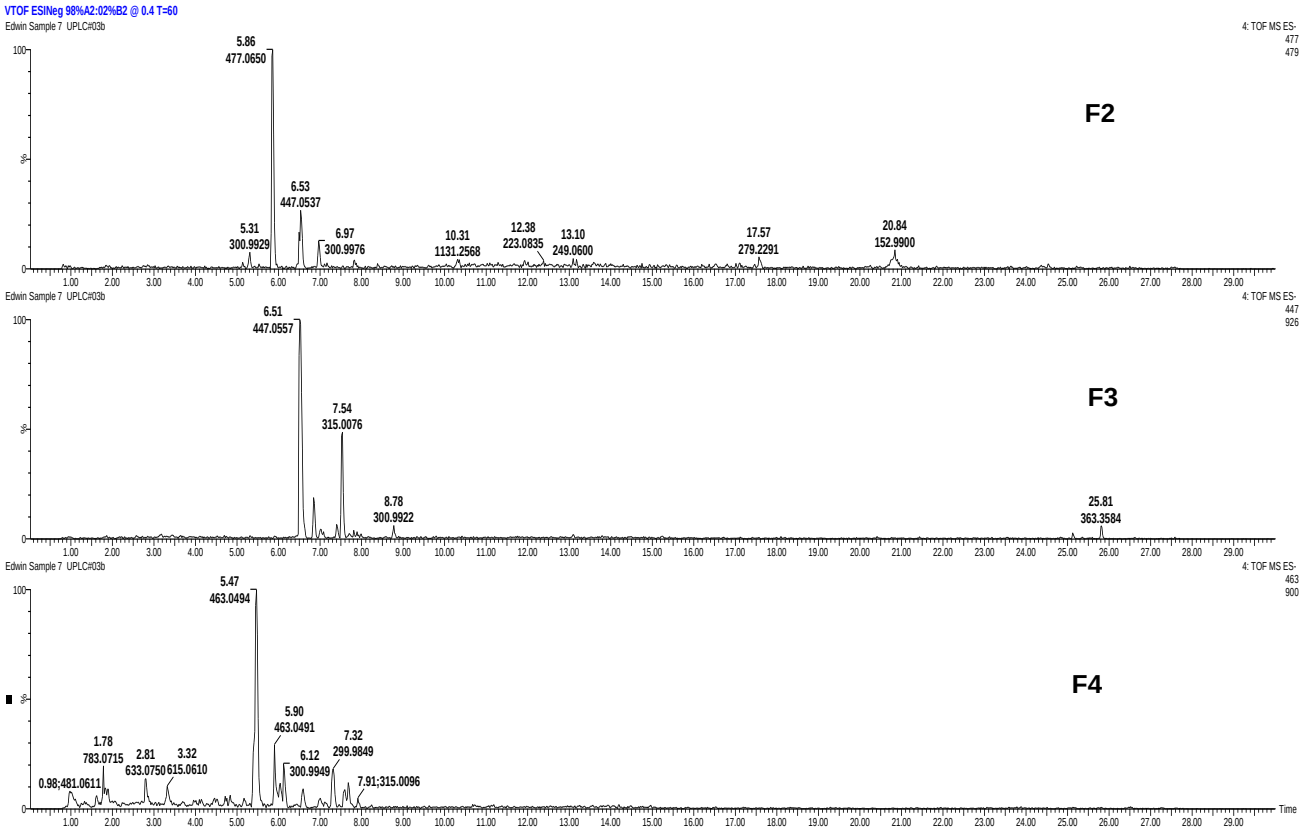
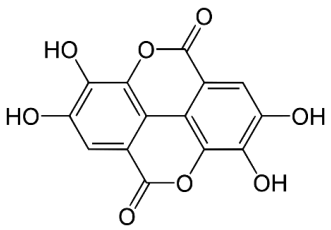
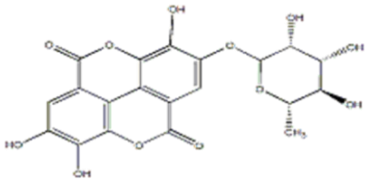


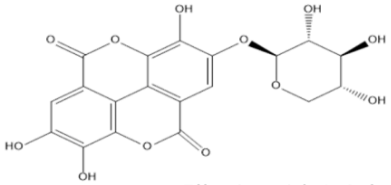
Figure 6.5: Selected ion chromatograms (SIM) of some of the detected Ellagic acid derivatives.



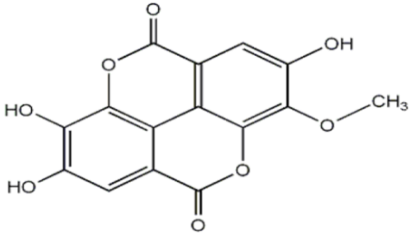
The basic structure of Ellagic acid



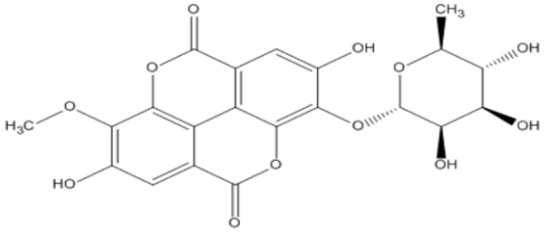
Eschweilenol C



Ellagic acid-4-O-beta-D-xylopyranoside



3-O-methylellagic acid



Eschweilenol C

Figure 6.6: Structures of different Ellagic acid derivatives detected from the methanol extracts of *Coula edulis*.

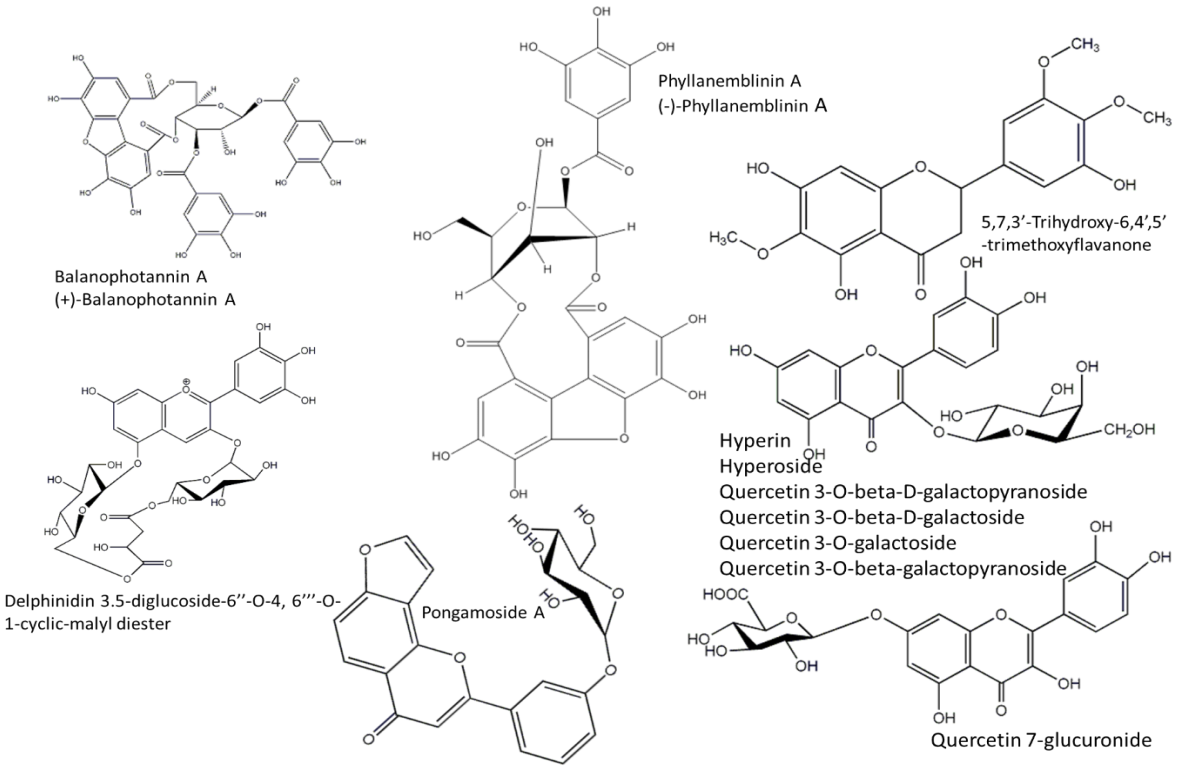


Figure 6.7: Chemical structures of some potential anti-oxidative derivatives detected in *Coula edulis* extracts.

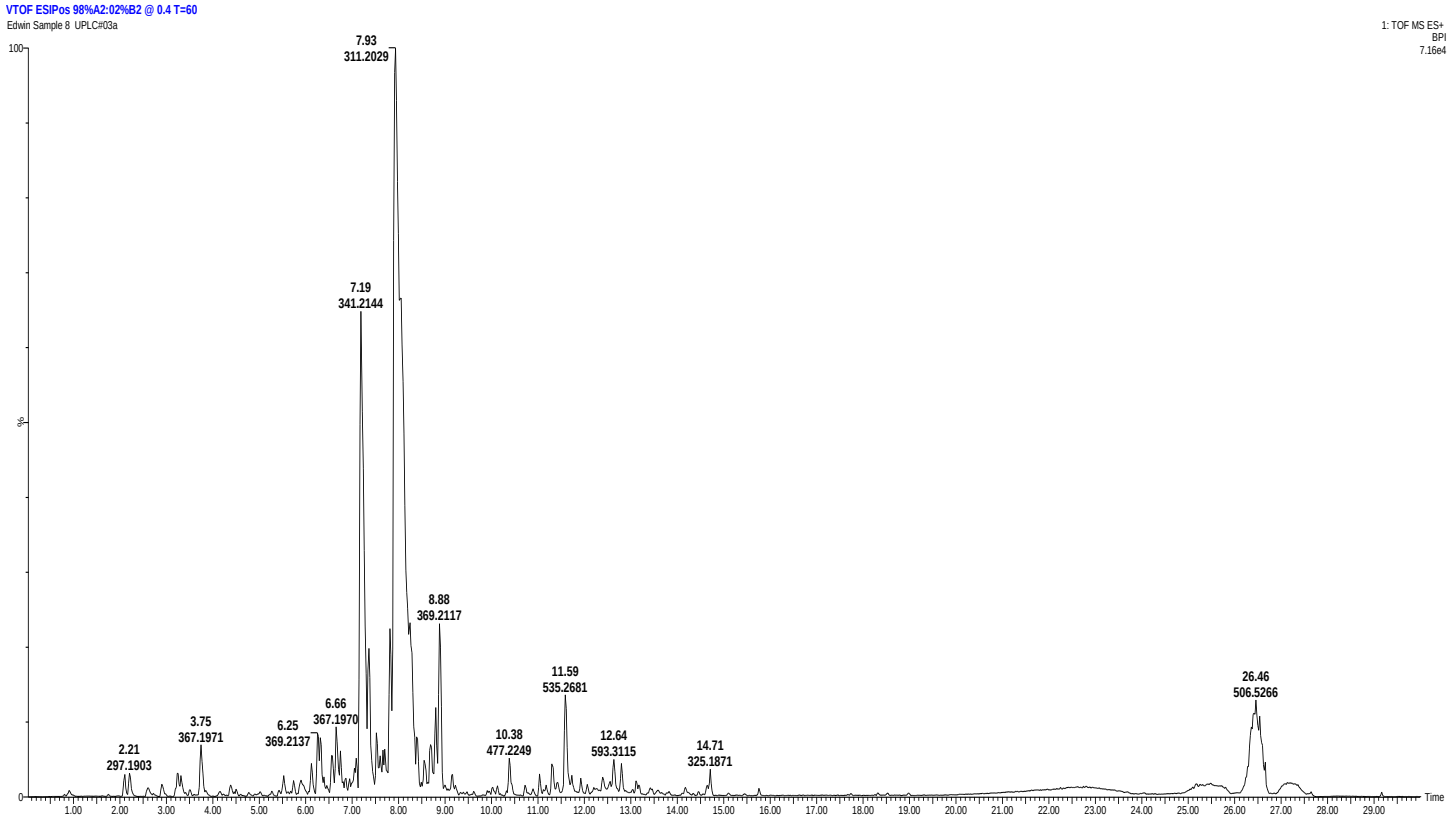


Figure 6.8: Representative LC-MS chromatogram of *T. iboga* collected at positive ionization mode

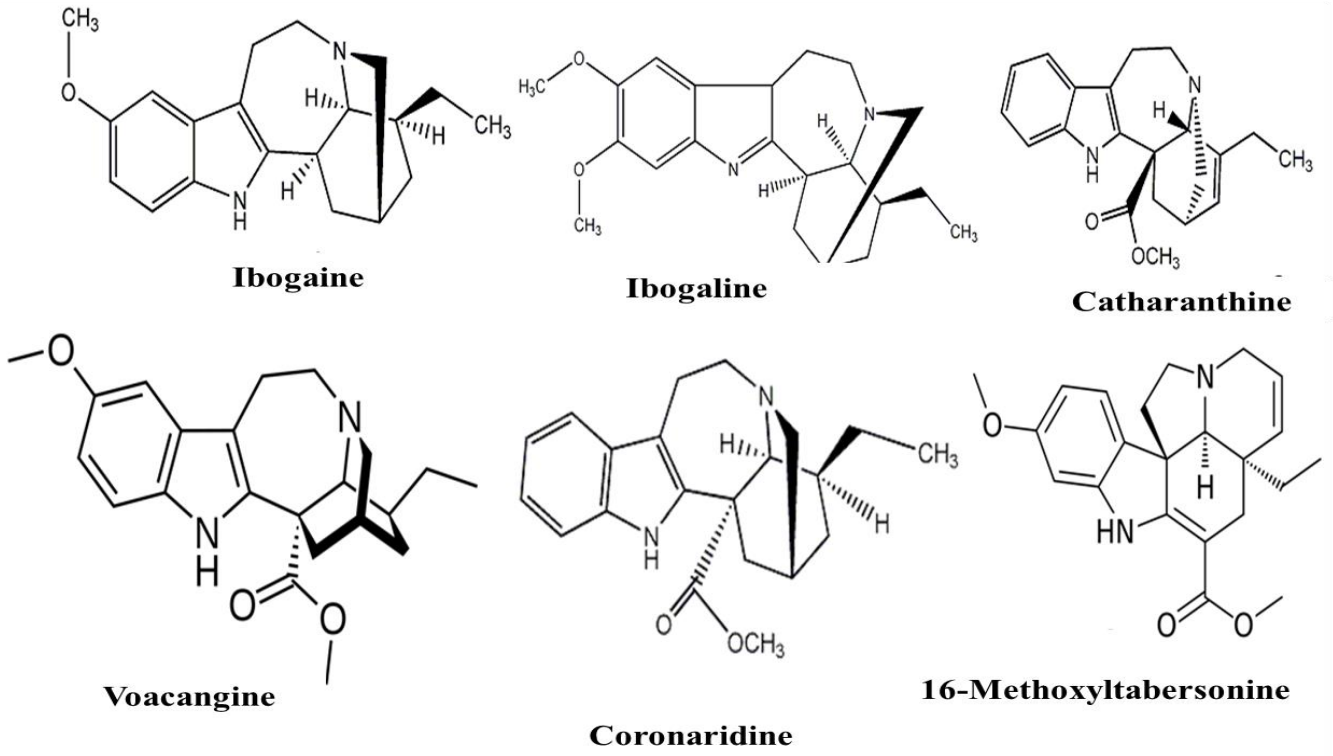


Figure 6.9: Some of the chemical structures detected in *Tabernanthe iboga* extracts

6.3.2 BIOLOGICAL ACTIVITIES

6.3.2.1 Antioxidant activity

The evaluation of the antioxidant activity of the fractions (Figure 6.10) revealed that *C. edulis* crude (8.1±0.21 µg/mL) displayed higher free radical scavenging effect than the Fractions (F3=12.06±0.2 µg/mL and F4=11.73±0.49 µg/mL) especially F2 (21.3±0.4 µg/mL) and F1 (249±0.58 µg/mL) where the difference in IC_{50} values was significant.

Concerning *T. iboga* DPPH activity, Fa (634±0.58 µg/mL), Fc (760±0.58 µg/mL) and Fd (440±0.33 µg/mL) showed lowest activity compared to the crude (430.5±0.29 µg/mL) while Fb IC_{50} was lower meaning that Fb (379±0.58 µg/mL) possess a higher free radical scavenging effect than the *T. iboga* methanol extract.

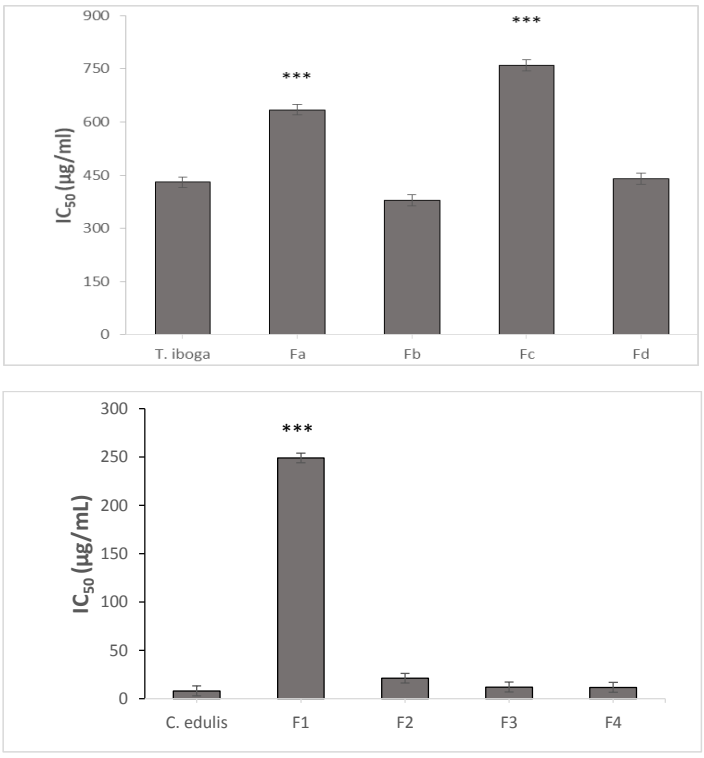


Figure 6.10: Comparative free radical scavenging activity of *T. iboga* and fractions and *C. edulis* and fractions. Data represents the mean ± standard deviation for four replicate wells. Errors bars denote standard deviation. Significant differences are indicated by * (P<0.05) compared to the respective crude extracts.

The reducing power (Figure 6.11) of the crude extracts and the fractions indicated for *C. edulis* that F2 (55.9±0.49 µg/mL), F3 (42.6±0.47 µg/mL) and F4 (67.46±0.44 µg/mL) displayed a higher reducing power than the crude extract (90.53±0.3 µg/mL) with EC_{50} values significantly lower, while F1 (1077.36±1.13 µg/mL) showed the lowest activity.

Regarding *T. iboga*, only Fb (112.49±0.25 µg/mL) showed a greater reducing effect compared to the crude (197.34±0.84 µg/mL) while Fa (2165±0.5 µg/mL), Fc (200±0.58 µg/mL) and Fd (683.6±0.95 µg/mL) displayed a lower activity.

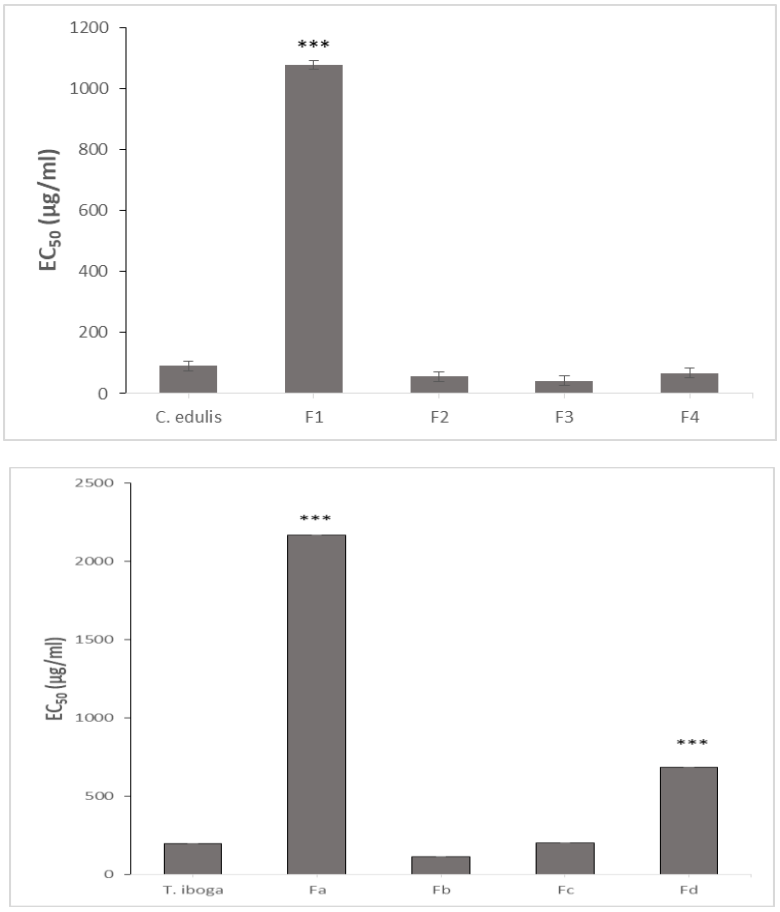


Figure 6.11: Comparative reducing power of *T. iboga* and fractions and *C. edulis* and fractions. Data represents the mean ± standard deviation for four replicate wells. Errors bars denote standard deviation. Significant differences are indicated by * (P<0.05) compared to the respective crude extracts.

6.3.2.2 Antibacterial activity

The antibacterial screening of the fractions against 9 bacterial strains was done using the well diffusion assay with a unique concentration of 50 mg/mL. The results depicted in Table 6.3 showed that the diameters of inhibition of all the active fractions were ranged between 6 mm and 18 mm. Concerning *C. edulis* fractions, F1 was able to inhibit the growth of *S. aureus* and *B. cereus* only with diameters of 14 mm. All the bacteria strains except *E. cloacae* were susceptible to F4 with diameters of inhibition ranged between 6 mm and 11 mm. Fractions F2 and F3 demonstrated an antibacterial activity slightly higher than the crude extract against all the strains tested especially the Gram-positive ones, with diameters of inhibition reaching 17 mm.

Tabernanthe iboga, Fb was found to be exerting an antibacterial activity slightly higher than the crude extracts. Fraction Fb inhibited the growth of all the bacteria with diameters of inhibition ranged between 11 mm and 20 mm, while for the crude the diameters ranged between 10 mm and 16 mm. Fractions Fd (10 mm) was active against *S. aureus* and *B. cereus,* while Fa (6 mm) exerted a very low activity only against *S. aureus*. The fraction c was able to inhibit the growth of 2 Gram positives (*S. aureus* and *B. cereus*) and to Gram negatives (*E. coli* and *K. pneumoniae*) with zones of inhibition measuring 10 mm, 9 mm and 6 mm respectively.

Table 6.3 also edited the Minimum Inhibitory Concentration (MIC) of the fractions against the selected bacteria. The findings indicated that *S. enterica* was the most susceptible to all the fractions except F1 and Fa as the MIC was the lowest (0.01-0.2 mg/mL) for this specific gram-negative bacterium for *C. edulis* crude extracts, F2, F3, Fc and Fd. *Pseudomonas aeruginosa* was the less susceptible to the samples, as the highest MIC values (6.25-25 mg/mL) was generated especially by *T. iboga* crude extract and Fb. The MIC of the remaining bacteria was ranged between 0.02 mg/mL and 12.5 mg/mL. Some fractions did not demonstrate a zone of inhibition during the well diffusion assay, while a MIC was determined. F4 demonstrated a MIC of 1.6 mg/mL against *E. cloacae*; Fa MIC was found to be 6.25 mg/mL against *B. cereus*; Fc showed and activity against *E. faecalis* (1.6 mg/mL), *P. aeruginosa* (12.5 mg/mL), *S. enterica* (0.2 mg/mL) and *E. cloacae* (3.13 mg/mL); and Fd displayed an antibacterial activity against *E. faecalis* (0.79 mg/mL), *E. coli* (3.13 mg/mL), *P. aeruginosa* (12.5

mg/mL), *K. pneumoniae* (1.6 mg/mL), *S. enterica* (0.2 mg/mL) and *E. cloacae* (1.6 mg/mL).

Table 6.3: Antibacterial activity described by zone of inhibition (mm) and MIC (mg/mL)

	S. aureus		***E. faecalis***		***B. cereus***		***E. coli***		***P. aeruginosa***		***K. Pneumoniae***		***S. enterica***		***E. cloacae***	
	ZI (mm)	**MIC (mg/mL)**	**ZI (mm)**	**MIC (mg/mL)**	**ZI (mm)**	**MIC (mg/mL)**	**ZI (mm)**	**MIC (mg/mL)**	**ZI (mm)**	**MIC (mg/mL)**	**ZI (mm)**	**MIC (mg/mL)**	**ZI (mm)**	**MIC (mg/mL)**	**ZI (mm)**	**MIC (mg/mL)**
C. edulis	16	1.6	10	0.39	16	0.2	7	0.39	12	6.25	9	0.79	8	0.01	10	0.79
F1	14	1.6	-	ND	14	0.79	-	ND	-	ND	-	ND	-	ND	-	ND
F2	17	1.6	13	0.7	16	0.39	11	0.79	13	6.25	12	1.6	11	0.01	10	1.6
F3	17	3.13	11	1.6	15	0.39	10	1.6	12	12.5	8	0.79	9	0.2	8	1.6
F4	11	3.13	6	1.6	10	0.39	7	1.6	8	6.25	6	1.6	6	0.2	-	1.6
T. iboga	16	1.6	13	1.6	16	1.6	15	1.6	10	25	10	1.6	10	3.13	11	1.6
Fa	6	12.5	-	ND	-	6.25	-	ND	-	ND	-	ND	-	ND	-	ND
Fb	18	0.79	15	1.6	20	0.79	15	1.6	12	25	14	1.6	11	3.13	14	1.6
Fc	10	3.13	-	1.6	10	1.6	9	3.13	-	12.5	6	1.6	-	0.2	-	3.13
Fd	10	0.79	-	0.79	10	0.79	-	3.13	-	12.5	-	1.6	-	0.2	-	1.6
Gent	28	ND	21	ND	28	ND	22	ND	21	ND	21	ND	22	ND	20	ND

Gent: Gentamicin; ND: Not determine; ZI; Zone of Inhibition; MIC; Minimum Inhibitory Concentration

6.3.2.3 Anti-inflammatory activity

The anti-inflammatory evaluation was performed on mouse macrophage cell line (RAW 264.7). The cells were exposed to LPS which induces the expression of iNOS with concomitant nitric oxide formation. Changes in NO production were determined by measuring the levels of nitrate in the culture medium. Simultaneous evaluation of cell viability using MTT assay was used to confirm for cytotoxicity evaluation of the test samples. Silymarin (50 μM and 100 μM) was used as positive control. The small available quantities of Fractions F1 and Fa did not allow the assessment of these fractions for anti-inflammatory activity. Only the crude extracts (*T. iboga* and *C. edulis*) and fractions F2, F3, F4, Fb, Fc and Fd were assessed. All the tests were done in triplicate.

The results indicated in Figure 6.12 showed that *C. edulis* and F2 did not induce an inhibition in cell viability at all the concentrations tested (25, 50, 100 and 200 μg/mL). The lowest percentage of viable cells was 94% thus an inhibition of 6%. Fractions F3 and F4 seemed to decrease the number of cells in a dose dependent manner especially F4 which induced a decrease of 33% at the highest concentration (200 μg/mL).

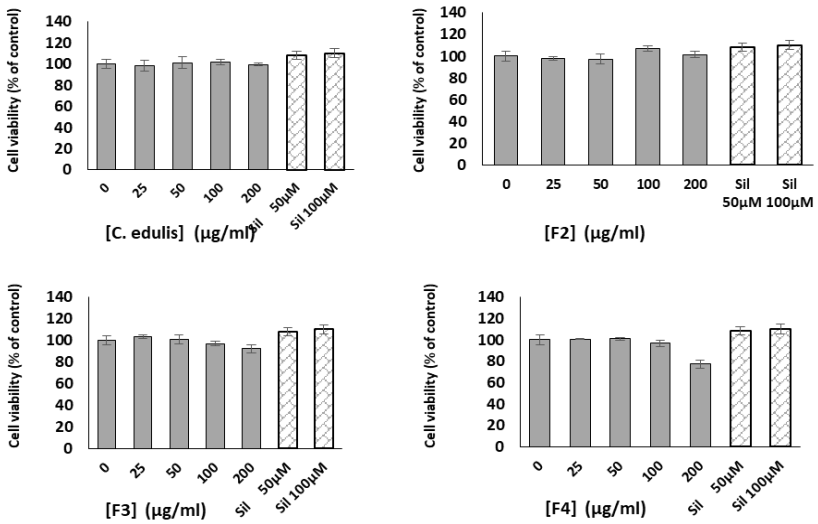


Figure 6.12: Cell viability assessed using MTT assay (*Coula edulis* crude extract, F2, F3, F4). data represents the mean ± standard deviation for three replicate wells. Silymarin was used as positive control. Errors bars denote standard deviation.

Figure 6.13 depicts the evolution of nitrate concentrations (μM) after the RAW cells were treated with the samples at different concentrations. A dose dependent decrease of the nitrate concentration for all the samples was observed. The highest concentrations (200 μg/mL) produced the highest decrease in nitrate concentrations; from 39.54±0.95 for the untreated cells to 10.29±1.8 μM for F4, 11.38±0.62 μM for F3, 13.62±0.45 μM for *C. edulis* and 25±0.75 μM for F2. Thus, as shown in Figure 6.13, F3 and F4 displayed a dose dependent anti-inflammatory activity significantly higher ($p<0.05$) when compared to the nitrate levels in the untreated cells.

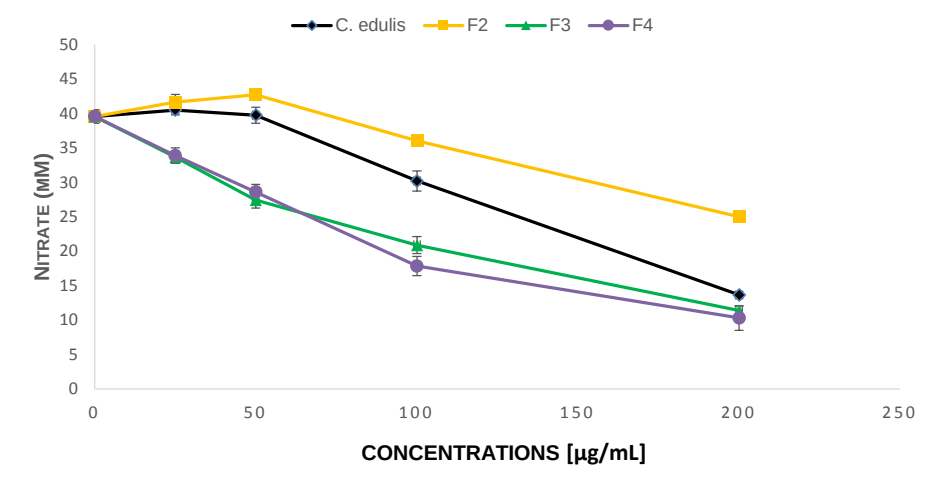


Figure 6.13: Inhibition of LPS induced nitric oxide production in RAW 264.7 cells after treatment with *C. edulis* and fractions (F2, F3, F4). Data points are the mean ± SD of triplicate wells. Errors bars denote standard deviation.

The effects of *T. iboga* and fractions against RAW cell viability showed in Figure 6.14 indicated that *T. iboga* and all the fractions did not affect the cell viability as the lowest percentage of viable cells was 97% at 50 µg/ml while at 200 µg/ml the percentage was back to 100% of viable cells.

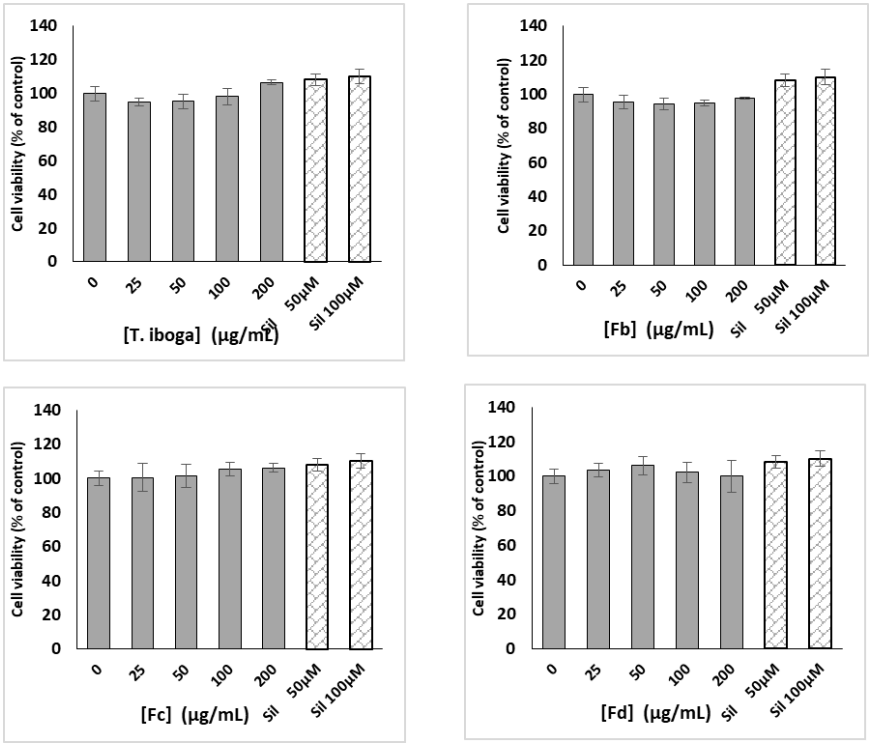


Figure 6.14: Cell viability assessed using MTT assay (*T. iboga*, Fb, Fc, Fd). Data represents the mean ± standard deviation for three replicate wells. Silymarin was used as positive control. Errors bars denote standard deviation.

The anti-inflammatory assessment of *T. iboga* and fractions revealed that the samples showed no significant activity on NO production except for Fb and Fc (Figure 3.15). The highest decrease was induced with the highest concentration and the values of nitrate in the cells after treatment were 8.56±1.96 µM for Fc, 16.44±0.4 µM for Fb, 19.13±0.75 µM for the crude and 32.24±1.74 µM for Fd. The concentration of nitrate in the untreated cells was 39.54±0.95 µM, what implies no significant difference ($p>0.05$) was observed between the activity of the samples and the levels of nitrate in the untreated cells.

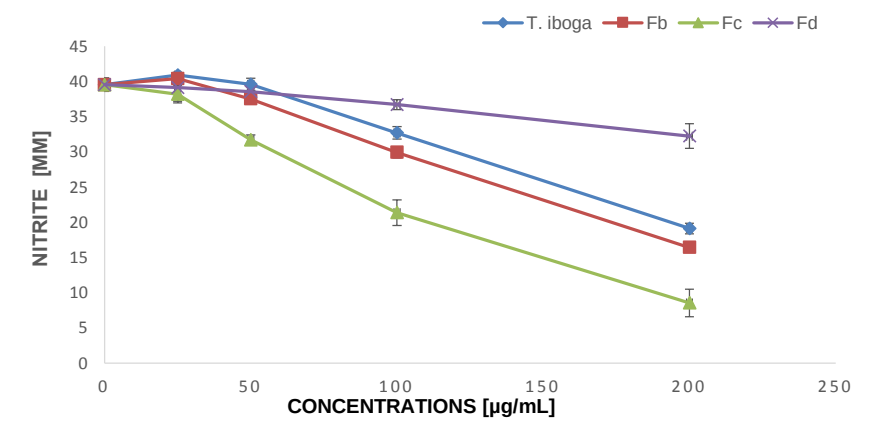


Figure 6.15: Inhibition of LPS induced nitric oxide production in RAW 264.7 cells after treatment with *T. iboga* and fractions (Fb, Fc, Fd). Data points are the mean ± S.D. of triplicate wells. Errors bars denote standard deviation.

6.3.2.4 Immunomodulatory activity

Before assessing the effects of the samples on the expression of selected cytokine in HIV infected PBMCs, we first evaluated the potential of toxicity of the samples on cells. Thus, the cell viability was assessed using XTT assay and the results are depicted in Figure 6.16 as percentage of viable cells. The assay was done in triplicate, and the effect of the samples was compared to untreated cells. A unique concentration of 25 µg/mL was used for the cytotoxicity and the immunomodulatory assays. Here, the levels of TNF-α were not measured because of the low levels obtained in Chapter 5.

The results revealed no significant changes ($p<0.05$) in the percentage of untreated cells vs cells treated with the samples. Despite the cell inhibition induced by *T. iboga* samples the percentage of viable cells was not lower than 70%. On the other hand, F1 showed a toxic effect (70% viable cells) towards the infected cells, while F2 and F3 stimulated the cell growth (128% and 119% of viable cells respectively).

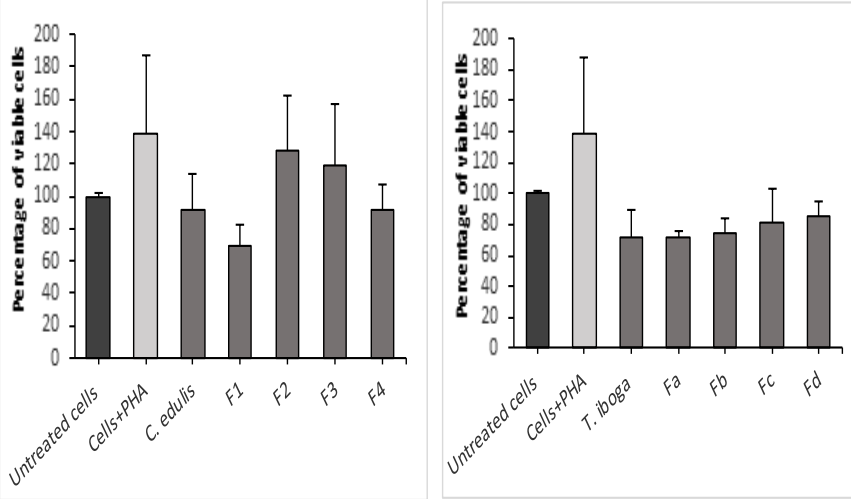


Figure 6.16: HIV infected PBMCs viability assessed using XTT assay after treatment with *C. edulis* and fractions (F1, F2, F3 and F4) and *T. iboga* and fractions (Fa, Fb, Fc and Fd). Data points are the mean ± S.D. of two individuals done in triplicate. Errors bars denote standard deviation. Significant differences are indicated by * ($P<0.05$) compared to the respective crude extracts.

The ability of the tested extracts to modulate the expression of the pro-inflammatory IL-6 HIV-infected PBMCs was assed using an ELISA kit. Cells were stimulated with PHA as control. The results showed in Figure 6.17, demonstrated that the modulation was not significant ($p>0.05$) when compared to the untreated cells. The expression of IL-6 in the cells treated with *Tabernanthe iboga* (102.64±19.26 pg/mL), Fb (125.16±33.87 pg/mL), Fc (102.94±31.38 pg/mL) and Fd (116.02±.8.5 pg/mL) was slightly decreased compared to the untreated cells (140.97±7.8 pg/mL), while Fa (147.22±68.08 pg/mL) was lightly enhancing IL-6 secretion. *Coula edulis* (160.67±96.37 pg/mL), F2 (164.44±64.39 pg/mL), F3 (143.74±63.59 pg/mL) and F4 (142.49±66.31 pg/mL) were able to slightly increase the secretion of IL-6, while IL-6 was lightly decreased by F1 (137.76±47.60 pg/mL).

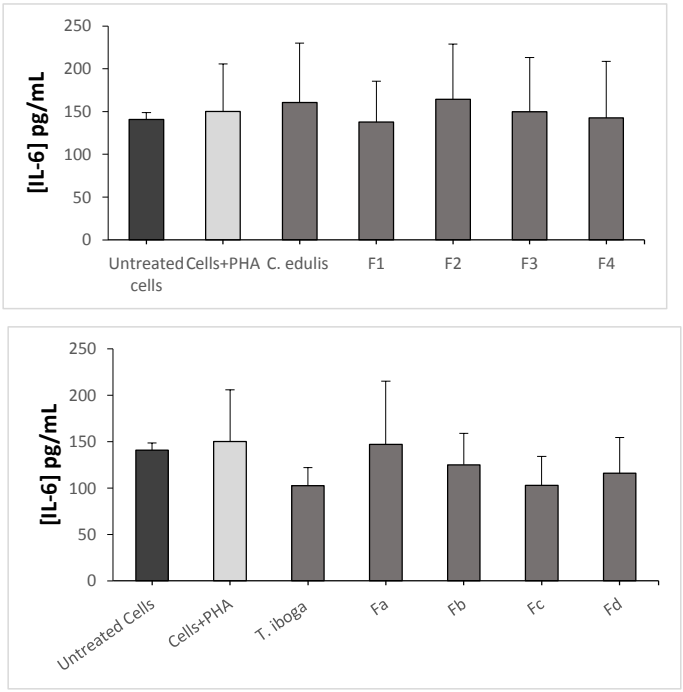


Figure 6.17: IL-6 expression in HIV infected PBMCs after treatment with *C. edulis* and fractions (F1, F2, F3 and F4) and *T. iboga* and fractions (Fa, Fb, Fc and Fd). Data points are the mean ± S.D. of two individuals done in triplicate. Errors bars denote standard deviation.

The samples were also assessed for of IL-2 modulatory activity on HIV-infected PBMCs using ELISA kit. Cells were treated with PHA and water as controls. The results (Figure 6.18) indicated that no significant difference ($p>0.05$) between the treated and the untreated cells was observed. The crude extract *T. iboga* (242.23±20.67 pg/mL), Fa (217.96±18.25 pg/mL), Fb (223.5±11.85 pg/mL), Fc (226.42±21.39 pg/mL), Fd (212.02±4.01 pg/mL), *C. edulis* (211.22±12.36 pg/mL), F2 (224.91±49.25 pg/mL) and F4 (213.93±18.25 pg/mL) were increasing the production of IL-2 in infected PBMCs. The levels of IL-2 in the cells were decreased after treatment with F1 (183.93±36.62 pg/mL) and F3 (151.50±36.77 pg/mL) compared to the levels in the untreated cells (203.56±25.74 pg/mL).

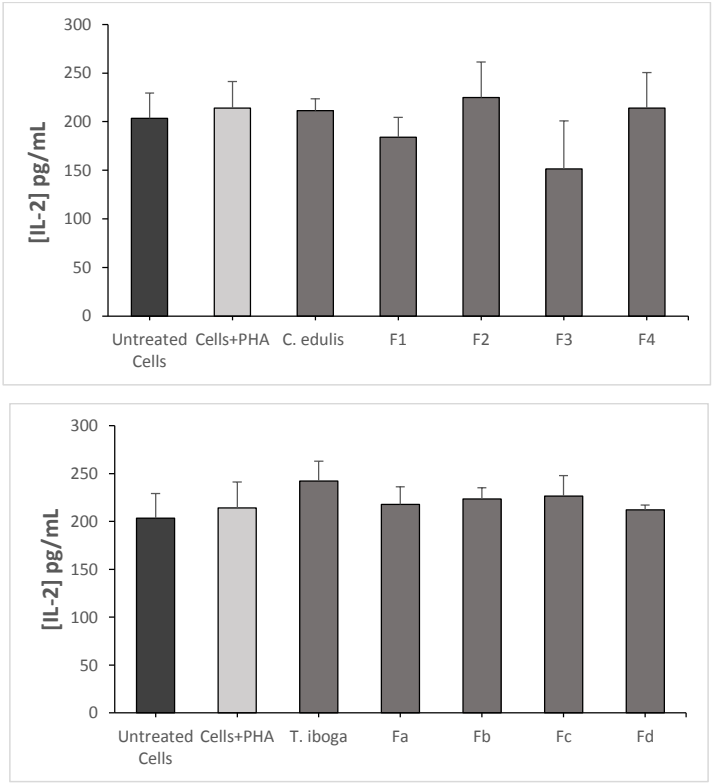


Figure 6.18: IL-2 expression of HIV infected PBMCs after treatment with *C. edulis* and fractions (F1, F2, F3 and F4) and *T. iboga* and fractions (Fa, Fb, Fc and Fd). Data points are the mean ± S.D. of two individuals done in triplicate. Errors bars denote standard deviation.

6.3.2.5 Anti-HIV activity

The anti-HIV activity of the samples was evaluated on HIV infected PBMCs. The percentage of inhibition of the HIV p24 antigen (Figure 6.19) was determined after treatment with the samples at a concentration of 25 µg/mL. The effects of the plant extracts on cells were compared to untreated cells.

The results revealed that all the samples induced a decrease in p24 expression. This activity was not significant except after treatment with Fc (26%) ($p<0.05$). The percentage of p24 expressed after treatment with the other samples was found to be ranged between 30% and 68%. All the fractions were more active than the crude extracts.

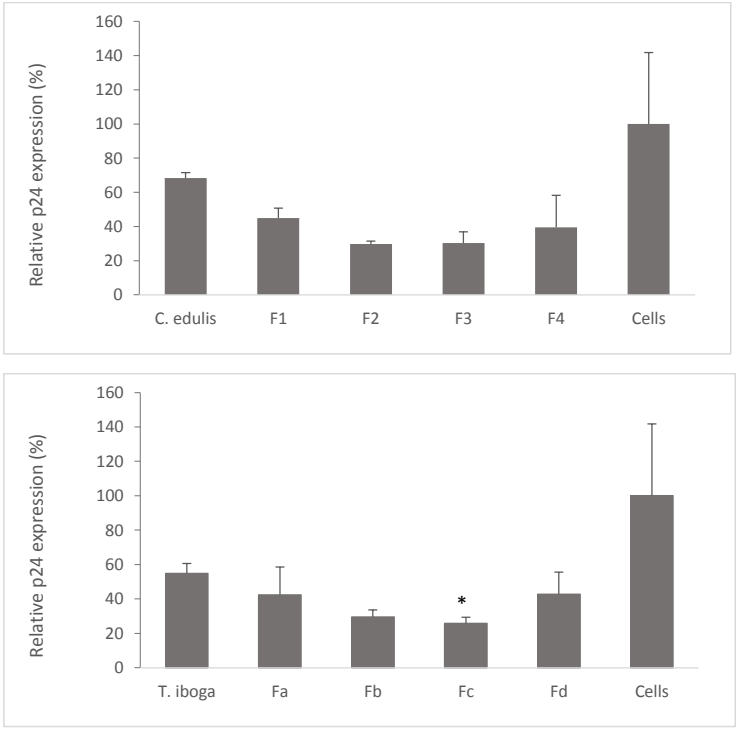


Figure 6.19: Expression of p24 antigen in HIV infected PBMCs after treatment with *T. iboga* and fractions (Fa, Fb, Fc and Fd) and *C. edulis* and fractions (F1, F2, F3 and F4). Data points are the mean ± S.D. of two individuals done in triplicate. . Errors bars denote standard deviation.

6.4 DISCUSSION

The aim of this study was to identify compounds from the methanolic extracts of the two selected plants as well as to assess the biological activities of the fractions obtained, and then to elucidate the structure of at least one of the active compounds. First fractions were obtained after column chromatography performed with the methanolic crude extracts of *C. edulis* and *T. iboga.* The fractions (F1, F2, F3 and F4 for *C. edulis*/ Fa, Fb, Fc and Fd for *T. iboga*) were then subjected to qualitative (phytochemical screening) and quantitative (TPC/TFC) analysis before UPLC-MS could be performed.

Coula edulis

The results of the phytochemical screening and the TPC/TFC revealed that *C. edulis* and fractions (except F1) possessed high amount of polyphenolic compounds (tannins, flavonoids and phenols) (Table 6.2 and Figure 6.2). These findings were later confirmed by UPLC-MS analysis. The crude extract and the fractions were found to share structurally diverse metabolites of different physical and chemical properties such as ellagitannins (Ellagic acid) and gallotannins (Gallic acid) which are polyphenols. *Coula edulis* showed a particular structural modification which could be an evolutionary strategy aiming at a diversification of its metabolites (Masike *et al.,* 2018). Plants are known to produce class of compounds and diversify them through processes such as glycosylation and methylation which ultimately results in complex metabolomes (Masike *et al.,* 2017). These metabolites are used by plants as defensive arsenal against wide spectrum of stressors (Ramabulana *et al.,* 2016). It has been shown that this diversification of metabolites is an evolutionary strategy by plants to store these metabolites and utilize them effectively during times of stress, a phenomenon commonly referred to as “better be ready than sorry” (Ramabulana *et al.,* 2016). The same metabolites, though synthesised by plants, for the sole purpose of defense, have been shown to fight against human ailments (Lin *et al.,* 2016). It is important to mention that, structurally diverse metabolites could be a solution against the ever changing (mutating) pathogens (Makola *et al.,* 2016).

In the current study, different derivatives of Ellagic acids were seen to be the most abundant metabolites in *C. edulis* methanolic extract (Figure 6.6). These metabolites share a common structural moiety (Ellagic acid; Figure 6.7). Ellagic acid is a polyphenolic compound found in the form of Ellagitannins and extensively studied for its numerous biological properties (Bisen *et al.,* 2012). Ellagic acid (EA) is well known for its antioxidants capabilities that include various mechanisms such as free radical scavenging, lipid peroxidation inhibition, metal ion reducing and chelating effects as well as stimulation of glutathione peroxidase, catalase and superoxide dismutase activities (Baek *et al.,* 2016; Sepúlveda *et al.,* 2011). A study by Galano et al. (2014) demonstrated that EA and its metabolites, in aqueous solution at physiological pH, was able of deactivating a wide variety of free radicals. Other properties are attributed to EA and include anticancer, antiviral, antibacterial and anti-inflammatory activities (Garcia-Nino and Zazueta, 2015; Usta *et al.,* 2013; Sepúlveda *et al.,* 2011). Ellagic acid can also prevent damages caused to the nervous system, heart, liver and kydneys (Sepand *et al.,* 2016; Touqeer *et al.,* 2016; Girish *et al.,* 2009; Yuce *et al.,* 2007).

From the identified structures, most of the molecules were found to be from the ellagitannins and they all contain a “fused” Gallic acid (Figure 6.7). What we should highlight here is that this contingent of compounds is present in all the fractions (F2 to F4). Gallic acid (GA) is a naturally occurring low molecule weight phenolic acid largely found in plant tissues. There are several reports on the biological properties of Gallic acid and its derivatives. Like many other phenol acids, GA have demonstrated a great antioxidant power *in vitro, in vivo* and *in silico* (Badhani *et al.,* 2015). Its ability to maintain endogenous defense systems, inhibit lipid peroxidation, chelate metal ion and to scavenge free radical is well documented. Gallic acid also displays anti-inflammatory, antibacterial, antifungal and antiviral activities. From what follows, we can undoubtedly assume that Ellagic acid and Gallic acid contribute to a large scale to the various biological effects uncovered in our study and displayed by *C. edulis* and its fractions.

This chapter has demonstrated that, *C. edulis* methanol extract and its fractions (F1, F2, F3 and F4) possess great antioxidant abilities via free radical scavenging and ferric reducing mechanisms. The IC_{50} values for F2, F3 and F4 were not significantly higher

than that of the crude extract. However, there was a 30.7-fold increase in the IC_{50} of fraction F1, indicating a much lower free radical scavenging activity compared to the extract. For the reducing power F2, F3 and F4 displayed a higher ferric reducing power than the crude extract (EC_{50} values significantly lower). The great antioxidant effect of polyphenol including GA and EA is extensively documented (Badhani *et al.,* 2015; Usta *et al.,* 2013). The fundamental structure of these compounds allows them to transfer an electron (through electron transfer mechanism) or a hydrogen (hydrogen atom transfer mechanism) to free radicals (Velderrain-Rodríguez *et al.,* 2018). Abid et al. (2017) demonstrated the presence of reductones in many polyphenols compounds which can break free radical chains but also prevent the peroxide formation by reacting with its precursors. According to Prihantini et al. (2015) who analyzed the relationship between structure and antioxidant activity of polyphenol compounds including GA and EA, high activity is exerted by compounds having three phenolic hydroxyl groups as well as those having an ortho and para position of hydroxyl phenolic groups. They added that this specific position could be form quinone-like product that could stabilize phenoxyl radicals by trapping two radicals generated from the system. In the current study, some of the identified structures were found to contain the lactone (quinone-like functional groups), an indication that the identified molecules could stabilise free radicals.

The antimicrobial assessment showed that *C. edulis* and fractions except F1 exhibited a promising activity towards the strains tested, with inhibition zones varying from 6 mm to 17 mm. The findings indicated that the overall MICs exhibited by the compounds were not higher than 3.13 mg/mL, with an exception made for *P. aeruginosa* where the MICs were ranged between 6.25 mg/mL and 25 mg/mL. Bussman et al. (2010) indicated that a good antimicrobial agent should display MIC≤5 mg/mL what validate the good antibacterial potential of the test samples. Antimicrobial activities of GA have been reported against various microbial strains such as *Staphylococcus aureus, S. epidermidis, Helicobacter pylori, Escherichia coli, Bacillus cereus, Salmonella typhimurium, S. flexneri* and *Candida albicans* (Fu *et al.,* 2016; Diaz-Gomez *et al.,* 2013; Martini *et al.,* 2009). Some studies have indicated that GA was able to inhibit the growth of many methicillin-sensitive *S. aureus* and multi-resistant *S. aureus* (MRSA) (Borges et al., 2013; Chanwitheesuk et al., 2007). The capacity of EA to inhibit microbial growth has also been reported on various Gram-positive and Gram-negative

bacteria including methicillin-resistant *Staphylococcus aureus* strains and fungi. The mechanisms involved in the antimicrobial activity displayed by these two compounds are multiple. Gallic acid and EA can inhibit bacteria motility, adherence and biofilm formation as well as DNA gyrase activity during the replication process (Fu *et al.,* 2016; Shao *et al.,* 2015). These chemicals can also interfere with the membrane permeability of certain bacteria leading to an increase in antibiotic accumulation in these bacteria (Oh and Jeon, 2015). Gallic acid and EA are able to destroy the outer membrane of gram-negative bacteria via chelation of divalent cations explaining the good antimicrobial activity observed against Gram negative bacteria such as *Pseudomonas aeruginosa*, *Salmonella typhimurium* or *E. coli* (Kahkeshani *et al.,* 2019). It has been reported that these compounds can inflict irreversible changes in bacterial membrane properties through hydrophobicity changes, changes of the surface charge and occurrence of pore formation in the cell membranes (Borges *et al.,* 2013).

The immunomodulatory and the anti-inflammatory potential of *C. edulis'* fractions was also investigated. The anti-inflammatory effect was evaluated on RAW 264.7 cells (mouse macrophage cell line) and the immunomodulatory effects on HIV infected PBMCs (human cells). The results revealed that *C. edulis* and fractions were not inhibiting the growth of the Raw cells at all the concentrations tested, while the toxicity was significant in the infected PBMCs when treated with F1 (31%). The fractions F2 and F3 seemed to induce the production of the infected cells whereas a slight cell inhibition was noticed for the crude extract and F4. The compound assessed didn't show a significant immunomodulatory effect on IL-2 and IL-6 production. When compared to the untreated cells no significant changes in the expression of the cytokines were observed at the tested concentration (25 µg/mL). While, the anti-inflammatory investigation indicated a decrease in nitrate levels in a dose dependent manner. A significant diminution of nitrate in RAW cells was observed at the higher concentrations (100-200 µg/mL) of the crude extract and the fractions suggesting a promising anti-inflammatory effect. Plants are known to induce anti-inflammatory responses in human via cytokine expression modulation, NO inhibition, and molecular signalling pathways regulation. This activity is attributed to compounds contained in the plant such as GA and EA. These compounds are reported to have anti-inflammatory activity via inhibition of proinflammatory cytokines (il-6, TNF-α) (Seo *et al.,* 2016). The test samples did not display satisfactorily immunomodulatory effect

towards the selected cytokines, other cytokines must be targeted to have a larger view of the effects of these samples on the immune system. The concentration used in the present study may be too low to induce immunomodulatory activity, higher concentrations should be assessed for the extracts which did not show a toxic effect towards the cells.

The anti-HIV potential of *C. edulis* and fractions was evaluated on HIV infected PBMCs. The results indicated that although the fractions were able to induce an inhibition reaching 60% especially F2, the inhibition of p24 antigen in the cells after treatment with all the compounds was not significant ($p>0.05$). These results indicated that the compounds responsible for the anti-HIV activity depicted is present in all the fractions. These compounds include GA and EA (Figure 6.6 and 6.7) which have been found in various form in all the fractions. The presence of these particular compounds may explain the anti-HIV activity exhibited by the fractions and the crude extract. Bessong et al. (2005) demonstrated that a similar tannin molecule containing hydroxyl-benzoic acid was shown to possess anti-HIV activity via inhibition of the RDDP and RNase H functions of RT and abolition of the 3 -end processing activity of integrase. Ellagic acid and GA have demonstrated in several studies the inhibition of reverse transcriptase, protease and syncytia formation (Nutan *et al.,* 2013; Rasheed *et al.,* 2012). Lu and collaborators (2004) showed that tannins and its metabolites constitute non-uniform polyphenolic compounds that can significantly inhibit p24 production, the fusion of HIV-1IIIB-infected of H9 cells with uninfected MT-2 cells and also target the viral proteins that mediate the late steps of HIV replication (Lu *et al.,* 2004). A More recent study has confirmed the activity of GA and EA against HIV and added that they can also prevent HIV-1 from entering into the cells through binding with proteins such as gp120 (Zang *et al.,* 2017).

Tabernanthe iboga

Tabernanthe iboga methanol extract and the 4 fractions (Fa, Fb, Fc and Fd) obtained after fractionation were suggested to different phytochemical analysis including phytochemical screening, total phenolic contents, total flavonoids contents and LC-MS. The results of the phytochemical screening reported that *Tabernanthe iboga* methanol possessed in a low amount all the chemicals screened. The determination

of TPC and TFC indicated that low amounts of these compounds were detected in the samples. These results were later not confirmed by LC-MS analysis at the positive ionization where the alkaloids were the compounds the most represented. The phenols and flavonoids detected with the screening and the TPC/TFC determination couldn't be identified with LC-MS at the positive ionization. In a study by Bading et al. (2018) five phenolic compounds, including 3-O-caffeoylquinic acid, and 30 alkaloids, were identified from the water extract of *T. iboga* roots at the positive ionization. Additional phytochemical analysis must be performed with *T. iboga* samples to detect the group of compounds detected in this study by the qualitative phytochemical analysis. *Tabernanthe iboga* is well known for its numerous alkaloids including ibogaine, ibogamine and noribogaine which display many biological activities such as antidiabetic, anti-addictive, anti-HIV and antiparasitic (Bading *et al.,* 2018; Maciulaitis *et al.,* 2008; Silva *et al.,* 2004; Delorenzi *et al.,* 2002). These findings confirmed the results in the present study in terms of alkaloids detected. Ibogaine which was the major alkaloid represented in this study is a naturally occurring alkaloid which has been found to provide metabotropic and psychotropic (House *et al.,* 1995). Ibogaine's anti-addictive capacity has been extensively reported (Dos Santos *et al.,* 2017; Maciulaitis *et al.,* 2008; Alper and Lotsof, 2007). Another study by House et al. (1995) assessing T-cell regulatory and effector function, B-cell function, macrophage function, and natural killer-cell function after ibogaine treatment indicated that this alkaloid was able to suppress all the immune functions in a dose-related way except macrophage function. In a study aiming at determining ibogaine mechanism of action via the measurement of its effects in human erythrocytes *in vitro* and antioxidant enzymes activity, it was conclude that ibogaine was acting as a pro-antioxidant by increasing activity of antioxidative enzymes and as an adaptagene in oxidative distress (Nikolic-Kokic *et al.,* 2015). Other alkaloids (Coronaridine, 18-Methoxycoronaridine) detected in this study were shown to have antiparasitic and anti-HIV activities (Silva *et al.,* 2004; Delorenzi *et al.,* 2002). There is no doubt that the alkaloids detected in this study contribute to the biological activities displayed by *T. iboga* methanol extract and fractions.

The evaluation of the antioxidant potential of *T. iboga* samples revealed a weak free radical scavenging effect with IC_{50} ranged between 379±0.58 µg/mL and 760±0.58 µg/mL. Blois (1958) indicated that a weak antioxidant activity is characterized by IC_{50}

> 150 µg/mL. The reducing power assay showed EC_{50} ranged from 112.49±0.25 µg/mL to 2165±0.5 µg/mL) which are high values compared to *C. edulis* and fractions EC_{50} which were ranged between 42.6±0.47 µg/mL and 90.53±0.3 µg/mL except for the F1. Strong antioxidant activity has been several times correlated with high level of polyphenols (tannins, flavonoids, phenols). In the present study, the phenolics compounds were not found in high amount after TPC/TFC determination and the LC-MS analysis did not detect these compounds. That can explain the weak antioxidant potential displayed by *T. iboga* and fractions. But some studies have demonstrated the antioxidant capacity of alkaloids via free radical scavenging effects (Gutiérrez *et al.,* 2014; Oloyede *et al.,* 2010; Maiza *et al.,* 2007). Although studies reviewed the antioxidant potential of natural alkaloids, these compounds do not have the reputation to be great antioxidant agents (Rehman and Khan 2017; Sudheer *et al.,* 2005). Yet, because alkaloids are the most represented in this plant compared to phenolic compounds which are known to be great antioxidant agents, one can suggest that the antioxidant activity displayed by *T. iboga* samples are due to the alkaloids.

The antibacterial activity of *T. iboga* and fractions was evaluated towards various Gram negative and Gram-positive bacteria. The results indicated a promising antibiotic potential of the methanol extract, Fb, Fc and Fd. These samples were able to produce a diameter of inhibition reaching 20 mm especially with the Gram-positive bacteria. The MICs were not higher than 3.13 mg/mL except for Fa and on *P. aeruginosa*. Antibacterial activity displayed by alkaloids compounds has been intensively reviewed (Compean and Ynalvez, 2014; Cushnie *et al.,* 2014). Various mechanisms such as: the inhibition of the nucleic acid synthesis; the inhibition of biofilm formation; the inhibition of exotoxin-mediated effects; the impairment of the membrane and cytoplasmic membrane integrity have been suggested. The alkaloids possess a proton-accepting nitrogen atom and one or more proton-donating amine hydrogen atoms that allows hydrogen bonding with enzymes receptors and proteins. This specificity of alkaloids confers to these compounds exceptional antimicrobial power (Kittakoop *et al.,* 2014). In chapter 4 of this study, it was demonstrated that *T. iboga* possess bactericidal properties. Many studies reported that alkaloids display bactericidal effects against various Gram negative and Gram-positive bacteria which can reach 99% in a few hours. The antibacterial effect shown by *T. iboga* samples might be linked to the numerous alkaloids that this plant is composed of. Yet, more

studies aiming at isolating the compounds that confer antimicrobial activity to this plant should be undertaken as this plant constitutes an interesting source of antibiotic agents that can efficiently fight infectious diseases and drug resistance.

Tabernanthe iboga and fractions were also assessed for immunomodulatory and anti-inflammatory abilities. The results indicated that the changes observed in IL-2 and IL-6 expression by HIV infected PBMCs after treatment with *T. iboga* samples were not significant ($p>0.05$) compared to the levels of cytokines in the untreated cells. The samples *Tabernanthe iboga,* Fb, Fc and Fd were down regulating (10-20%) IL-6 expression in HIV infected cells, while IL-2 was slightly increased (4% to 19%). The anti-inflammatory evaluation indicated a decrease in nitrate levels in RAW cells after treatment with high concentrations (100-200 µg/mL) of the samples indicating an anti-inflammatory potential. Many plants are used to treat various diseases due to anti-inflammatory and immunomodulatory activities conferred by plant secondary metabolites. Alkaloids have been reported to have an effect on the regulation of the immune response (cytokine regulation, inflammation) *in vitro* and *in vivo* (Bachhav and Sambathkumar, 2016; Venkatalakshm *et al.,* 2016). Inflammation is a normal process by which the immune system recognizes and suppresses harmful situations (damaged tissues, microorganisms, toxic compounds) and starts the healing mechanism. This normal process becomes noxious when it turns chronic and can lead to several diseases such as cancer, heart diseases and rheumatoid arthritis, or participate to the severity of some others such as AIDS (Ahmed, 2011). In Chapter 4 of this study, the role of inflammation in HIV infection has been depicted, and the importance of fighting inflammation to attenuate HIV infection has been highlighted. Plant compounds such as alkaloids present in *T. iboga* and fractions are known to have an influence on immune responses but also on inflammation. Souto et al. (2011) reviewed the naturally sourced alkaloids with *in vitro* and *in vivo* anti-inflammatory effects reported from 2000 to 2010. The researchers found that many of the alkaloids reported in their review offer considerable promise as anti-inflammatory compounds or drug candidates. Venkatalakshm et al. (2016) also reported the remarkable immunomodulatory power of alkaloids and added that a large number of alkaloids were being investigated for their immunostimulant properties. In the present study the unique dose assessed for the immunomodulatory evaluation did not show the real potential of the samples.

Higher concentrations and other cytokines should be investigated to determine the proper effects of these samples on the immune response.

The interest for less toxic and more efficient new molecules from the plant kingdom is growing continuously. In the last decades, many plants have been investigated for anti-HIV activity based on several mechanisms such as enzyme inhibition (reverse transcriptase, protease and integrase) and entry inhibition or p24 antigen inhibition. Many of these plants have demonstrated promising antiviral activity, some of them are even under trial. These encouraging outcomes prompted the evaluation of the anti-HIV potential of *T. iboga* which is consumed in Gabon to manage AIDS. The anti-HIV activity of *T. iboga* samples were evaluated using p24 antigen inhibition assay in HIV infected PBMCs. The results revealed that although no significant difference ($p>0.05$) between untreated cells and treated cells was observed except for Fc ($p<0.05$), an inhibition reaching 68% was recorded. In chapter 4 of the current study, it was shown that both *T. iboga* aqueous and methanol extracts were displaying an inhibitory effect of p24 antigen in HIV infected cells. In a study by Silva et al. (2004), it was reported that an iboga alkaloid congener 18-methoxycoronaridine showed activity on HIV-1. The virus was used to infect isolated macrophages and PBMCs from healthy donors and the inhibition of HIV-1 replication after treatment with the compound was observed. This same alkaloid also displayed a reverse transcriptase inhibitory effect *in vitro*. The alkaloids which have been mainly found in *T. iboga* samples might be the main responsible for the anti-HIV effect reported in this study. These results validate the use of *T. iboga* in the management of AIDS in Gabon. Yet, the consumption of this plant should be monitored because toxic effects have been reported (Mazoyer *et al.,* 2012; Mefane *et al.,* 1990). In this study, the evaluation of the cell viability after treatment with the samples showed that the highest percentage of inhibition was 30% which is quite high considering the low concentration used (25 µg/mL). It has been positively acknowledged that plant alkaloids can display toxic effects on the nervous system, liver, respiratory tract and the kidney (Diaz, 2015; Matsura and Fett-Neto, 2015). The toxicity of ibogaine from *T. iboga* has also been previously reported (Steinberg and Deyell, 2018; Hanneman *et al.,* 2016; Alper *et al.*, 2012). Despite *Tabernanthe iboga* relevant biological properties, this plant should be consumed in low doses.

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CHAPTER SEVEN

Conclusions and Recommendations

CHAPTER 7

7.1 CONCLUSIONS

Worldwide like in Gabon, people use plant-based remedies or add plant products to their ARV therapy to fight HIV/AIDS and some of the HIV infected people claim their health has improved due to certain plants. The main goal of the present study was to investigate 20 selected medicinal plants reported to improve the health of people living with HIV in Gabon in order to validate their use, their pharmacological properties and their toxicity. The hypothesis was that, among the selected plants, some would show potential against several OI causing pathogens, immune system or directly inhibit HIV replication. Thus, the main objective of this study was achieved through four derived specific objectives.

The first objective was to evaluate the cytotoxic and antioxidant activities of 20 selected plants (20 aqueous and 20 methanolic extracts in total). The results of the investigation revealed that the plant extracts studied except *A. klaineana* and *T. iboga* exerted a weak either cytotoxic or antiproliferative effect against Vero cells. The investigation of anticancer effects on HeLa cell line indicated that most of the plant extracts especially *U. klainei, U. guineensis, P. soyauxii, A. klaineana* and *V. conferta* exhibited either an antiproliferative or cytotoxic effect or both effects. It is noteworthy that the active plant extracts seemed to activate apoptosis pathway in dying cells, which is a great characteristic for a good chemotherapeutic agent.

The study has also demonstrated that among the two methods used MTT was the less reliable compared to fluorescence microscopy because many false results were reported in the study mostly resulting in phytochemical compounds interfering with MTT salts. The antioxidant studies demonstrated that the studied plants possessed significant free radical scavenging activity as well as reducing capacity, especially *C. edulis* which showed an activity close to the positive control Gallic acid. These results may explain the ability of the tested plants to improve HIV individual's health and to treat HIV related opportunistic infections such as respiratory tract infections, diarrhea and dysentery.This validates the use of the selected plants in the management of HIV patient's health by traditional healers as well as the many reports of life quality and health improvement. Therefore, plants in this study which displayed significant

antioxidant activity could be used as supplements for HIV individuals to deal with the oxidative stress induced by HIV and HAART.

The second objective was to evaluate the anti-HIV effect but also the antimicrobial activities of the 40 extracts against several bacterial and fungal strains associated with HIV OIs. The plants tested showed great antibacterial and antifungal effects on various strains such as *S. aureus, E. coli, B. cereus, C. albicans* and *C. tropicalis*. The active plant extracts were found to display bactericidal or bacteriostatic effects that are of great value for the research on new antibiotic molecules. Some plants demonstrated promising anti-HIV activity on HIV infected PBMCs, characterized by an inhibition of reverse transcriptase enzyme and p24 antigen expression. Some extracts were also able to both inhibit the production of the virus and to destroy it in the cells. This study has demonstrated that plants used in Gabon to deal with OI related to HIV have important antimicrobial activity and also the potential to inhibit the replication of HIV *in vitro*. These findings validate the reports of HIV patients' health improvement and viral load back to undetectable levels.

The third objective was to evaluate the effects on the immune system. This effect was evaluated on the production of cytokines IL-6, IL-2 and TNF-α by HIV infected cells. The results showed that only the methanolic extracts of *M. monandra; P. soyauxii; U. guineensis; A. klaineana; V. conferta; A. vogelii; T. iboga; C. religiosa; C. lucanusianus;* and *R. africanum* were able to decrease not significantly ($p>0.05$) the levels of IL-6 produced by infected PBMCs when compared to untreated cells. All the water extracts and the remaining methanol extracts were up-regulating IL-6 levels especially *S. kamerunensis* and *M. puberula* aqueous extracts. The evaluation of TNF-α in infected cells after treatment with the extracts indicated no significant variation in the production of this cytokine at the concentration used. Plants such as *U. guineensis*; *M. puberula*; *A. hirtella*; *A. cissampeloides*; *S. gabubensis T. iboga* and *A. klaineana* were stimulating the production of IL-2 in HIV infected cells. The study has demonstrated that some of the plants tested were able to down and up regulate cytokine production in HIV infected cells which is an indication of a promising modulatory effect of the active plants on the immune system. But more studies need to be conducted with higher concentrations and targeting other cytokines or mechanisms in order to characterize the real effects of the active plant extracts on the

immune system with a large number of blood donors in order to eliminate inter-variation in responses.

The final aim of this study was to determine the phytochemical composition of the 40 plant extracts and to characterize the active compounds of 2 plants. The phytochemical profiling of the 40 extracts revealed the presence of alkaloids; saponins; tannins; flavonoids; steroids; cardiac glycosides; terpenoids and phenols in almost all the extracts. The total phenolic and flavonoid contents were found to be higher in almost all the methanolic extracts compared to the aqueous extracts. *C. edulis* and *T. iboga* were selected for characterization of active compounds on the basis of good antimicrobial activity displayed. The findings in this study were that *C. edulis* methanol extract and the 4 fractions eluted after column chromatography contained polyphenol compounds including Ellagic acid and Gallic acid which can be linked to the antibacterial, anti-HIV, anti-inflammatory and antioxidant capacities displayed by *C. edulis* samples. *Tabernanthe iboga* and fractions were found to possess mainly alkaloid compounds including ibogaine, ibogaline and voacangine which have also demonstrated promising antibacterial, anti-HIV and anti-inflammatory effects. Further investigations need to be carried out to isolate these molecules in order to conduct animal testing for a better comprehension of the mechanisms involved in the biological activities exhibited.

This study has answered several questions related to the effects of 20 plants used in Gabon against HIV associated OIs. To the question ‘are the selected plants toxic to cells’, the study has demonstrated that the plant extracts were relatively safe to normal cells but toxic to cervical cancer cells.

To the question ‘do the plants act on selected opportunistic infections’, the study showed that several plants have antimicrobial activity against fungi, Gram positive and Gram-negative bacteria.

To the question ‘do the plants act directly on selected HIV enzymes’, the findings indicated that several extracts were able to inhibit HIV reverse transcriptase but also p24 antigen in HIV infected cells.

To the question 'what are the plant composition', the results showed the presence of many compounds including alkaloids, polyphenols and saponins.

To the question 'what are the modes of action of the plants', the analysis of the different results suggest that the plants used in Gabon can either control the oxidative stress generated by HIV infection and HAART, modulate the immune response via stimulation or suppression of various cytokine expression, inhibit HIV related OIs causing pathogens and inhibit HIV replication via inhibition of some essential enzymes and proteins or both mechanisms.

In conclusion, this study has validated the use of the selected plants in the management of AIDS in Gabon and provided an explanation to the improvement of HIV individual's life quality reported. Some of these plants could constitute good candidate molecules for promising anti-HIV drug.

7.2 RECOMMENDATIONS

Nowadays, plant-based medicine constitutes an essential alternative to conventional medicine because plants are better tolerated by human organism and also less toxic than synthetized drugs. Besides, their use to manage numerous diseases has been intensively validated by scientific studies. In addition, our study provided the evidence that several plants used to manage HIV related opportunistic infections showed promising anti-HIV, antibacterial and immunomodulatory potential. We thus recommend:

- To investigate plant extracts that displayed great antimicrobial, anti-HIV and immunostimulant effects in order to study their mechanisms and to elucidate the bioactive compounds;
- To elaborate improved traditional medicines from the above-mentioned plants in order to provide affordable and efficient anti-HIV alternative drugs to patients;
- To investigate traditional healer's remedies and to monitor their effects on HIV individuals in order to validate or invalidate these remedies.

APPENDICES

Table A1: Some molecules identified from *Coula edulis* using ULC-MS

Molecule number	Retention time	m/z	Molecule name
1-2	3.39	767.0730	Balanophotannin A (+)-Balanophotannin A
3	3.85	723.1204	Delphinidin 3.5-diglucoside-6''-O-4, 6'''-O-1-cyclic-malyl diester
4	4.26	615.0580	Phyllanemblinin A (-)-Phyllanemblinin A
6	5.65	361.0945	5,7,3'-Trihydroxy-6,4',5'-trimethoxyflavanone
7	7.39	463.0459	Hyperin Hyperoside Quercetin 3-O-beta-D-galactopyranoside Quercetin 3-O-beta-D-galactoside Quercetin 3-O-galactoside Quercetin 3-O-beta-galactopyranoside
8	7.57	439.1029	Pongamoside A
9	7.70	477.0630	Quercetin 7-glucuronide
10	8.80	461.0793	3'-mono-O-methylellagic acid 4-O-alpha-L-rhamnopyranoside
11	9.03	447.0585	Eschweilenol C
12	9.58	299.9904	3-O-methylellagic acid
13	8.08	433.0385	Ellagic acid-4-O-beta-D-xylopyranoside
14	8.29	447.0538	Eschweilenol C

Anti-microbial and immunomodulatory properties of indigenous plants found in Central and Southern Africa

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Plant-derived medicinal products are currently in high demand both by traditional healers and the herbal drug industries, and their popularity is growing. In developing countries, traditional medicine is used to meet the primary health care needs where infectious diseases are endemic and modern health facilities and services are inadequate. In both Gabon and the Venda Region of South Africa, indigenous plants have been used for the treatment of several ailments like malaria, jaundice, diarrhoea and HIV/AIDS. Some medicinal plants are reported to have immunomodulatory properties; but a large number of indigenous plants such as *Cassia abbreviata, Copaifera relogiosa, Costus lucanusianus, Ziziphus mucronata* and *Lannea edulis*; have yet to be thoroughly investigated. Studies done in some countries have reported on their biological activities in order to justify their utilization in traditional medicine and few investigations have been carried out on their anti-microbial and immunomodulatory potentials. This review aimed to highlight studies done on these indigenous plants based on information compiled from scientific databases (Science Direct and PubMed) and the most recent results obtained in house.

Keywords: Anti-microbial, immunomodulation, indigenous plants, primary health, traditional medicine.

1. Introduction

1.1 Background

Medicinal plants have been used throughout the history of humans [1]. In Africa, the population uses traditional medicine for the treatment of various diseases and ailments like malaria, typhoid, ulcers, skin diseases, diabetes, reproductive problems, aches and pains for various socio-cultural and economic reasons [2,3]. In addition, it is estimated that 80% of the Asian and African populations use herbal medicine as aspect of primary health care (World Health Organization [4].

Plants are able to produce different kinds of chemical compounds for performing different biological activities and defence against insects, fungi, animals, and microbes. Nowadays, natural products of plant sources have been the centre of focus as the main source of new, safer and effective bioactive compounds with medicinal properties. Medicinal plants possess ingredients which can be used to develop drugs and synthesize them [5]. Most of the prescribed drugs used today are made from plants [2].

Indeed, several natural products and their derivatives constitute over 50% of all drugs with various chemical structures and disease preventive properties. These compounds also called phytochemicals or secondary metabolites which include the alkaloids, steroids, flavonoids, terpenoids, tannins, and many others [6]; are known to be responsible for several pharmacological activities of plants such as anti-malarial, anti-asthma, anti-cancer, cholinomimetic, vasodilatory, anti-arrhythmic, analgesic, anti-bacterial, and anti-hyperglycemic activities [7-10]. The selection of plants based on their ethno-medicinal usage increases the likelihood of finding an effective therapeutic agent as opposed to random selection of plants [11,12].

New chemotherapeutic compounds which are effective and of low cost are urgently needed [13]. In recent days herbal products represent safety compared to synthetics which are considered to be harmful to people and their surroundings [14]. Moreover, the dependence on synthetics is over and people are returning to natural remedies with hope of safety and security [14].

South Africa like the rest of Africa has a rich diversity of more than 24 000 indigenous plant species, representing approximately 10% of all higher plants on Earth [15]. The people of South Africa have a long tradition of medicinal plant use with an estimated 70% of the population harvesting one if not more from the approximated 3 000 species of plants used as traditional medicines [15-17]. The country's vast variety of indigenous floral species together with their diverse medicinal uses indicates a great potential for uncovering new bioactive chemicals [3,18,17].

Over the past few years, various research groups from different fields have investigated the ethnobotanical, chemical and health characteristics of medicinal plants in the Vhembe district from the Venda region of South Africa [19-21]. The traditional knowledge of plants and animals in relation to their use within the district ought to be documented for future

generations [22]; however, it has not been well explored scientifically. The shortage of scientific evidence allows the native medicinal plants of Africa to be considered as relatively unexploited potential sources of novel anti-retroviral compounds [23].

A number of surveys have highlighted the fact that rural people heavily rely on traditional medicine for treatment of many ailments [24,20,25-27]. The use of such plants could be attributed to their attainability, affordability [28] and cultural beliefs [29]. For some villagers, the dependency on medicinal plants is due to lack of access to modern medical facilities or lack of effective services within the facilities if ever present.

It is also of great importance to understand the mechanisms of the immune system during infection to help improve its capacity to defend itself against subsequent infections. Few studies have been conducted to assess the effects of medicinal plants on the immune system of those diagnosed with various life threatening infections. A study conducted by Bessong et al [21], evaluated the biological activities of *Terminalia sericea, Bridelia micrantha* and *Combretum molle* against HIV-1 (reverse transcriptase (RT) and RnaseH). Another study was conducted to evaluate *Bridelia micrantha* activity against viral enzymes [30]. Additional efforts have also been made to investigate the immunomodulatory effects of *Combretum hereroense* and *Canthium mundianum* [20].

The current paper aimed to highlight studies done on these indigenous plants based on claims by traditional healers on their healing properties against life threatening infections and the most recent results obtained in house.

2. Methods used

2.1 Literature search on plant materials

The focus of this review was on *Cassia abbreviata*, *Copaifera relogiosa*, *Costus lucanusianus*, *Ziziphus mucronata* and *Lannea edulis* used as therapeutic plants. The search term used was "*name of plant*", on several online databases such as PubMed, Science Direct, and Google. The information found from this search was then matched with information gathered from interviews with traditional healers. Then, a compilation of each plant on their description usages and pharmacological activities such as anti-microbial, anti-cancer, anti-oxidant, and toxicity was done.

2.2 Reagents and equipment

RPMI-1640, foetal calf serum (FCS), streptomycin and L-glutamine were purchased from *Thermo Fisher* (SA). Blood was obtained from volunteer donors from the University of Venda community. Histopaque 1077 and the MTT salts were purchased from *Sigma Aldrich* (SA). All chemicals were bought from *Merck Chemicals* (SA). The culture plates and plastic ware were obtained from *AEC Amersham* (SA). The ELISA kits were purchased from *BD Biosciences* (San Jose, CA, USA). The 96-well plate reader was acquired from *Separations* (SA). Most laboratory equipment was purchased from NETZSCH (Germany) and BÜCHI Labortechnik AG (Switzerland).

2.3 Collection of plant materials

Medicinal plants were selected on the basis of their intense use for the treatment of different ailments and also a literature search was conducted to corroborate the scientific findings with traditional findings. Numerous traditional healers were interviewed to understand how they treat and "prescribe" medications for their patients.

Stem barks of *Copaifera religiosa* and stems of *Costus lucanusianus* were collected in Libreville (Gabon) after being identified by a botanist of the National Herbarium of Gabon.

Cassia abbreviata, Ziziphus mucronata and *Lannea edulis* were collected from within the geographic locations of the Vhembe District Municipality of the Limpopo Province (South Africa) with assistance from a traditional leader. These plants were identified by their vernacular names then later using taxonomic keys by an ethnobotanical specialist at the University of Venda (Limpopo province; South Africa). Voucher specimens were deposited in the herbarium of the Department of Botany (University of Venda).

The plants were dried under the sun light, grounded and then stored in a cool dark place until further use.

2.4 Plant preparation and extraction

Extraction was performed using water and methanol or Ethyl Acetate. After 24 h maceration and filtration, the aqueous filtrate was frozen and water was removed by freeze drying.

The methanolic or ethylic filtrates were evaporated by rotary evaporation at 50°C. The crude extracts obtained were then stored.

2.4 Phytochemical analysis

Both aqueous and methanol or ethyl acetate extracts were completely dissolved in their respective solvents (water and methanol or ethyl acetate). The obtained stock solutions were used for phytochemical screening as reported by Harborne [31] and Hossain et al [32] using chemical standard methods. The methods used are colorimetric assays that depend on color change of plant extracts after reaction.

2.5 Antimicrobial assays

2.5.1 Microorganisms tested

The selected strains for the current study were *Staphylococcus aureus* (ATCC 25923), *Salmonella enterica* (ATCC 51741), *Escherichia coli* (ATCC 25922), *Shigella sonnei* (ATCC 25931), *Klebsiella pneumoniae* (ATCC 27736), *Pseudomonas aeruginosa* (ATCC 27853), *Enterobacter cloacae* (ATCC 13047), *Streptococcus agalactiae* (ATCC 12386) *Enterococcus faecalis* (ATCC 29212), and *Bacillus cereus* (ATCC 10876) and were obtained from ANATECH (SA). The drugs used as positive controls were Gentamicin sulphate, Vancomycin hydrochloride, Imipenem dehydrate, Amicillin, Penicillin G and Chloramphenicol.

2.5.2 The well diffusion assay

The different plant extracts were studied for anti-microbial activity against pathogenic microorganisms using agar well diffusion assay as described by Perez et al [33] with some modifications. Antimicrobial activity of extracts was tested against Gram-positive and Gram-negative strains.

The test strains were cultured by inoculation into sterile nutrient agar plate (Mueller Hinton media) and incubated overnight at 37◦C. The cultured strains were transferred into test tubes with 5 ml of sterile water and the turbidity of the inoculumn was measured using the McFarland standard of 0.5.

The test bacteria were aseptically swabbed on nutrient agar plates using sterile cotton swabs. With the help of a sterile cork borer, wells of 6 mm diameter were punched in the inoculated plate. 50 µl of extracts at concentrations of 200, 150, 100 and 50 mg/ml were tested against the strains, and then incubated overnight at 37◦C. The plates were examined for the zone of inhibition, which appeared as clear areas around the wells.

Inhibition zone diameters were recorded.

2.6 Testing for immunomodulatory effects of the plants

Whole blood from healthy volunteers (collected in EDTA coated tubes, ethics approval number SMNS/14/MBY/30/1210) was used for the isolation of peripheral Blood mononuclear cells (PBMCs) as described by Ngcobo et al [34].

PBMCs (5 × 105) in culture medium (RPMI 1640 containing FBS 10% and 100 µg/ml of streptomycin) were added to a 24 multi-well culture plates and treated with 50 and 100 µg/ml concentrations of *Cassia abbreviate, L. edulis* and *Z. mucronata* extracts for 72 h in 5% CO_2 atmosphere at 37 °C. Three control groups consisted of PBMCs in medium as baseline control, PBMCs in media containing DMSO as negative control and PBMCs in medium plus PHA as positive control were used.

After incubation, the supernatant of cell cultures were used to assessed the production of cytokines (IL-2, IL-6 and TNF-α) according to the manufacturer's instructions and the cells were used for viability using the MTT assay (as described by Mosmann [35]. All experiments were carried out with three different donors and each test was performed in triplicate.

3. Finding and Discussion

3.1 Description of the indigenous plants

This section gives the ethno-botanical and pharmacological description of *Cassia abbreviata, Copaifera relogiosa, Costus lucanusianus, Ziziphus mucronata* and *Lannea edulis* as summarized in Table 1.

3.1.1 *Copaifera religiosa* J.Leonard

Description: *Copaifera religiosa* is a species belonging to the family of Caesalpineaceae (Leguminosae) family. It is a tree with a height of 46 m. The bole is straight, cylindrical, and up to 30 m in length, with swollen base in older trees. Buttresses are absent. The trunk diameter reaches up to 200 cm [36]. It mostly occurs in dense, primary forests and more rarely in the sedimentary basin in mixed stands

Uses: According to Brink [37], traditional uses of this plant include treatments against malaria symptoms, cardiovascular diseases, stomach ache, and cough. *Copaifera religiosa* is utilized in Gabon to treat malaria, whereas the bark is used in fumigations against headache and kidney pain and it is added to a bath together with other ingredients to treat leprosy [37,38].

Table 1 Properties of the selected plants

Family	Botanical name	Plant parts	Traditional used	Pharmacological properties	Toxicity	Phytochemical composition
Caesalpiniaceae	*Copaifera religiosa* J.Leonard	Roots, Leaves, Stem bark, Fruits	Abdominal pains, dysentery, fever, malaria, hernia, wounds, impotency, snake bite, diarrhoea, aphrodisiac, arbotifacient, venereal diseases, stomach ache, skin rashes AIDS, sterility, cough, emetic, epilepsy, bilharzia, jaundice, hernia	Antibacterial, antifungal, Antimalarial, Antiviral Anthelmintic, Antioxidant, Antidiabetic, anti-inflammatory, antiHIV	Not cytotoxic on PBMCs but toxic in shrimps.	Alkaloids, saponins, phenols, flavonoids, anthraquinones, glycosides, coumarins, sterols and alcohols derivatives
Caesalpineaceae (Leguminosae)	*Cassia abbreviata* Oliv	Barks	Malaria, cardiovascular diseases, stomach ache, cough, sterility, head ache, leprosy, kidney pains	In vitro antiplasmodial	Cytotoxic	Not found in the literature
Costaceae	*Costus lucanusianus* J.Braun & K.Schum	Inflorescences, Leaves, Stems, Juice	Filariasis, tachycardia, stomach ache, cough bronchitis, eye troubles, headache, oedema, fever, urethral discharges, jaundice	Anti-inflammatory, antioxidant, cytotoxic, antihyperglycemic, hepatoprotective, renoprotective, antidiarrheal, antiabortive, antimicrobial	Hepatotoxic	Glycosides, tanins, saponins, reducing sugars, flavonols carbohydrates, myricetin
Anacardiaceae	*Lannea edulis* Engl.	Leaves, Roots	Stomachaches, Wounds, diarrhoea, eye troubles	Antioxidant, antimicrobial	Moderately toxic	dihydroalkyl-hexenones Flavonoids, tanins alkylphenols, cardonols,
Rhamnaceae	*Ziziphus mucronata* Willd	Roots, Leaves, Barks, Fruits	Emetic, cough, skin disorders, pain, diarrhoea, venereal diseases, stomach ulcers rheumatism, respiratory infections, gastrointestinal complaints, snake bites, swellings, wounds, diabetes	Mutagenic effects, antioxidant, antimicrobial, α-glucosidase and α-amylase inhibitor, antidiabetic	Not toxic	cardiac glycosides, saponins, flavonoids, proanthocyanidin, tannins

Known Research Findings: It has some interesting anti-plasmodial activity and its dichloromethane bark extract has shown considerable in-vitro cytotoxicity [38]. A phytochemical screening has demonstrated the presence of tannin [37].

3. 1. 2 *Costus lucanusianus* J.Braun & K.Schum

Description: *Costus lucanusianus* J.Braun & K.Schum is also called Spiral Ginger. The plant belongs to the family of Costaceae [39]. Spiral ginger is a perennial, rhizomatous herb, which attains up to 3 meters in height. This plant is mainly found in Africa specifically in DR Congo and Tropical western Africa – Guinea east to western Ethiopia.
Uses: The plant is mostly used for religious and ceremonial purposes [40-42]. Inflorescence infusion treats tachycardia and stomach aches [43]. A decoction of a stem or fruit is used for the treatment of cough, bronchitis and a sore throat. Leaf sap treats eye troubles, headache, oedema and fever [43,44] and can be used as nose drop. Leaf pulp can slow down insanity when it is rubbed. Stem sap is also able to cure/treat urethral discharges, jaundice, and also to prevent miscarriage [45]. In Gabon, stem sap controls filariasis when applied as eye drops [46].
Known Research Findings: Numerous studies have been done on *C. lucanusianus* based on their ethnobotanical uses. *C. lucanusianus* display many pharmacological activities such as anti-inflammatory activity, anti-nociceptive activity, anti-abortive activities, dysmenorrhea, pyrexia, anti-diarrhoeal activity [47]. *Costus lucanusianus* possesses phytochemical compounds such as tannins, saponins, reducing sugars, carbohydrates, myricetin, and flavonols [48].

3. 1.3 *Cassia abbreviata* Oliv.

Description: *Cassia abbreviata* (Caesalpiniaceae) is a shrub which grows up to 10 m in height. It has a light brown bark, rounded crown and yellowish leaves. It has compound leaves, with 5 to 12 pairs, and brown black pods which are cylindrical in shape. Flowers are yellow, sweet-scented, large, loose, becoming brown-veinted with age and fruits are long cylindrical dark brown and hanging pod [49]. It is widespread in Africa, from Somalia to South Africa and occurs mostly at low to medium altitudes (between 220 and 1520 m above sea level), in open bushveld, woodland or wooded grasslands, along rivers, on hillsides and frequently on termite mounds [50].
Uses: In South Africa and Botswana the root is ground into powder, mixed with water and used to wash dirty blood, referring to a woman who has miscarried and need to be cleansed [51]. In Tanzania, the decoction of the root is made to treat abdominal pains, dysentery, fever, malaria, hernia, wounds, syphilis, impotency and snake bite [52,53]. In Mozambique, the decoction of root bark may be taken orally to treat diarrhea [54]. It is an aphrodisiac and is an arbotifacient [17]. Decoction of stem bark is taken orally to treat stomach ache and malaria, while infusion of roots, leaves and stem bark mixed together is taken to treat stomach ache [54]. Bark and roots may also be used to treat stomach ache of a mother during pregnancy, close fontanelle of newborn babies, dysentery, blood vomits, venereal diseases, bilharzia, hernia, post-partum pains and menstrual cycle problems [55]. It may also be used to treat skin rashes associated with human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) infections [56].
Known Research Findings: Many studies have demonstrated its various pharmacological properties such as anti-malarial, anti-helmintic, anti-viral, anti-oxidant, anti-diabetic, anti-bacterial and anti-fungal activities [57]. Methanol extracts of the root exhibited high toxicity in a brine shrimp lethality test [58]. Ethanol root extracts did not show cytotoxicity on PBMCs [59]. Its phytochemical composition includes alkaloids, saponins, phenols, flavonoids, anthraquinones, glycosides, coumarins, sterols and alcohols derivatives [59].

3.1.4 *Ziziphus mucronata* Willd.

Description: *Ziziphus mucronata* is a small to medium-sized tree species with a spreading canopy. It is commonly known as the Buffalo thorn, a species in the Rhamnaceae family. It is distributed throughout the summer rainfall areas of sub-Saharan Africa, extending from South Africa northwards to Ethiopia and Arabia. It is up to 8 m tall with a rounded crown, which grows on loamy sands. The leaves are three veined from the base and often; there is one straight and one hooked spine at the leaf axil. The flowers are said to be small and yellowish forming clusters. The fruit is almost circular, about 1.5 cm in diameter and bright red when ripe. The fleshy drupes are rich in sugars and vitamins [60]. The plant is common at medium altitudes mainly in mixed woodland, in areas with medium to lower rainfall, also on termite mounds.
Uses: The Zulu nation takes the powdered leaf and bark in water as an emetic in chest troubles. They also use hot infusions of the bark for cough. In general a poultice of the leaf is applied to boils, carbuncles and other septic swellings of the skin. Decoction of the root is taken internally and a paste of the leaf is applied to tubercular glandular swellings. For Pain of any sort, a poultice of meal made with a decoction or of powdered baked root is applied. People do inhale the vapor and gargles with a decoction of the leaf and shoot for measles and scarlet fever [61]. The roots can be used to treat bloody diarrhoea or stomach ulcers. *Ziziphus* is used to treat gonorrhea [60]. The root decoction is taken internally and a paste of the leaf is applied to tubercular glandular swellings. Hot infusion of the bark is used for coughs and the powdered leaf and bark in water is used as an emetic in chest troubles.

Known Research Findings: *Ziziphus mucronata* in the presence of metabolic activation showed mutagenic effects [62]. The phytochemistry of the plant has not yet been exhaustively studied; current literature survey showed one other article indicating that the leaf extract contains low amounts of tannins [63].

3.1.5 *Lannea edulis* Engl.

Description: *Lannea edulis* is a species that belongs in the Anacardiaceae family. It is a small perennial shrub, which grows from, branched underground stems 30-300mm high from large woody root-crown, or from a creeping rhizome. It occurs on deep sand and grows up to 20cm tall. The alternate pinnate leaves have 5 to 7 pairs of shiny leaflets which are variable in shape and size. The flowers are small, about 2mm long and yellow borne in a congested false spike or panicle and can be seen in September. The fruits are about 1cm long, green but turning red when ripe. They occur from September onwards and can be seen on the plant at same time as flowers [60]. The shrub is common in most Zimbabwean areas and is found in open woodland. The fruit which is purplish-black in color when ripe has a juicy pulp and is pleasantly sour.
Uses: Traditionally the leaf infusion is used to treat stomach aches. The powdered leaves are applied on wounds for healing. A cold infusion of the roots of *Lannea edulis* is used for treating diarrhoea. Africans take a decoction of the roots particularly of its bark in frequent large doses for blackwater fever. The Lobedu use a cold infusion of the leaf as a local application to the eyelashes to loosen the dried pus in sore eyes [64].
Known Research Findings: It has been found that the dichloromethane root extracts of *Lannea edulis* do contain some important phytochemical groups like alkylphenols, cardonols and dihydroalkylhexenones [65]. Lannea has also been shown to be able to induce frameshift mutations in Salmonella [66].

3.2 Phytochemical screening

In this study, the phytochemical analysis of the methanol and aqueous extract of *Copaifera religiosa* and *Costus lucanusianus* showed the presence of different groups of secondary metabolites such as alkaloids, cardiac glycosides, flavonoids, saponins, terpenoids, steroids and phenolics (Table 2). They were present in both aqueous and methanolic extracts of *Copaifera religiosa* and *Costus lucanusianus.* The presence of glycosides and Saponins in the methanolic extract of *C. lucanusianus* correlates with the phytochemical results obtained respectively by Baba and Onanuga [41] and Owolabi et al [42].

Phenolics were only present in the methanolic extract of *Copaifera religiosa.* Alkaloids in *Copaifera religiosa* were found only in the aqueous extracts while in *Costus lucanusianus* it was the methanolic extracts which contained them. Saponins were present in both aqueous and methanolic extracts of *Copaifera religiosa.* But in *Costus lucanusianus,* saponins were present only in the aqueous extract.

Table 2 Phytochemical screening on the methanol and aqueous extracts of the bark parts of *Copaifera religiosa* and *Costus lucanusianus*

Class of compounds	***Copaifera religiosa***		***Costus lucanusianus***	
	Aqueous	Methanolic	Aqueous	Methanolic
Flavonoids	+++	+	++	+++
Steroids	+	++	+	++
Cardiac glycosides	++	+++	+	++
Phenolics	-	+++	-	-
Saponins	++	+	+	-
Terpenoids	+	+++	+	++
Alkaloids	+	-	-	+

+ = Presence of constituents (+++= High, ++= Moderate, += Low); – = Absence of constituents

Furthermore, the phytochemical analysis of the aqueous and ethylic extracts of *Cossia abbreviata* and *Lannea edulis* revealed the presence of cardiac glycosides, flavonoids, saponins, steroids and phenolics as mentioned in Table 3.

Both the ethyl acetate and water extracts yielded the same compounds except for saponins which were positively tested in the water extracts only. The tested plant extracts exhibited 100% positive presence for phenols, flavonoids, steroids, cardiac glycosides and saponins was present in all the water extracts. *L. edulis* possessed the most phenol concentration whereas *C. abbreviate* possessed more flavonoid.

The extracts of *C. abbreviatta* had higher concentrations of saponins. Both plants showed higher concentration of cardiac glycosides.

Table 3 Phytochemical screening on the Ethyl Acetate (EtAc) and aqueous extracts of *Cossia abbreviata* and *Lannea edulis*

Class of components	*Cassia abbreviata*		*Lannea edulis*	
	Aqueous	EtAc	Aqueous	EtAc
Phenolics	++	++	+++	+++
Flavonoids	+++	++	+	+
Saponins	+++	-	+	-
Steroids	+++	+++	+	+
Cardiac glycosides	+++	+++	+++	+++

+ = Presence of constituents (+++= High, ++= Moderate, += Low); – = Absence of constituents

The phytochemical constituents of the plants tested demonstrated compounds including steroids, flavonoids, cardiac glycosides, phenols and saponins. These compounds are known to display medicinal activity as well as physiological activity [67]. The presence of different phytochemicals in the extracts accounts for varying effects they exert either directly on the pathogens or by neutralizing the body's defense by-products through anti-oxidation. Researches have shown that saponins are well known to possess detergent properties and to exhibit anti-inflammatory properties [68].

Furthermore, cardiac glycosides have been reported to increase the concentration of Ca^{2+} ions by inhibiting the Na^{+}/K^{+} pump. The increase of such ions controls the contraction of the heart muscles thus has been employed in the treatment of congestive heart failure [69].

Moreover, flavonoids have been reported to be highly potent antioxidant compound that aids in the reduction of stroke incidences, cancer and heart failure [67].

3.3 Anti-microbial activity of selected plants

The antimicrobial activity of the crude extracts (methanol and aqueous) of *Copaifera religiosa* and *Costus lucanusianus* were studied at different concentrations against 9 pathogenic gram (+) and gram (-) bacteria (*E. faecalis, B. cereus, S. aureus*, *K. pneumoniae, S. sonnei, S. enterica, E. cloacae, P. aeruginosa, E. coli*).

C. lucanusianus aqueous extract did not show any inhibition against all strains, while methanol and aqueous extract of *C. religiosa* exhibited potent antimicrobial activity towards *B. cereus, S. aureus, E. faecalis*, and *P. aeruginosa*. The zones of inhibition values compared with gentamicin as positive control are presented in (Table 4).

S. aureus was found to be more susceptible towards the aqueous extract of *C. religiosa* with a maximum inhibitory zone (33.33±1.52 mm), followed by methanol extract (23.33±1.52mm). *B. cereus* was found to be more sensitive to the methanol extract with a maximum inhibitory zone (15.66±0.57mm),

E. faecalis was found to be more sensitive to the *C. religiosa* methanol extract, with a maximum inhibitory zone (10.66±0.57mm), followed by aqueous (9.66±0.57mm). *P. aeruginosa* was found to be more sensitive to the methanol extract of *C. religiosa* with a maximum inhibitory zone (7.33±0.57mm), followed by the least sensitive exhibited to the aqueous extract (6.66±0.57mm).

The anti-microbial activity of aqueous extracts of *C. abbreviate, L. edulis and Z. mucronata* are shown in Table 5. From observation, it is apparent that the plant extracts did not possess any form of anti-bacterial activity against the panel of microorganisms or the bacterial species were resistant to the extracts.

The increasing trend of drug resistant mutations is raising greater health concerns. The majority of people residing in rural based settings are interested in herbal remedies that they perceive as being safe and effective [70]. Such advancements have prompted scientists to investigate the efficacy of indigenous medicinal plants with the hope of discovering potential drug candidates that might eradicate different diseases and overcome the issue of resistance. The type and level of biological activities which might be exhibited by any plant extracts depends on many factors such as the plants' part used, geographical location of the plant, plant drying methods and storage conditions. The efficacy of the extracts can be affected by elevated temperature during extraction steps. Such adversity might affect the level and composition of the secondary metabolites within the extract [71].

Table 4 Antimicrobial activity of *Copaifera religiosa* and *C. Lucanusianus* indicating inhibition zones (mm) ± SD

Microorganisms	*C. religiosa*								*C. Lucanusianus*				Positive control
	Methanol extracts				Aqueous extracts				Aqueous extracts				Gentamicin
	Inhibition zones (mm)												
	200 mg/ml	150 mg/ml	100 mg/ml	50 mg/ml	200 mg/ml	150 mg/ml	100 mg/ml	50 mg/ml	200 mg/ml	150 mg/ml	100 mg/ml	50 mg/ml	
B. cereus	15.66±0.57	14.33±0.57	12.66±0.57	12±0	12.33±2.31	12±1.73	9.33±1.15	6.33±0.57	-	-	-	-	25±1.73
S. aureus	22.66±0.57	25±1.73	23.33±1.52	16±2.0	30±0	33.33±1.52	29±1.73	26±2.64	-	-	-	-	25.66±1.15
E. faecalis	10.66±0.57	9.66±0.57	8.33±1.15	6.33±0.57	9.33±1.52	9.66±0.57	7±0	-	-	-	-	-	15.33±0.57
E. coli	-	-	-	-	-	-	-	-	-	-	-	-	21.33±0.57
P. aeruginosa	7.33±0.57	-	-	-	6.66±0.57	-	-	-	-	-	-	-	21±1.00
S. enterica	-	-	-	-	-	-	-	-	-	-	-	-	19±0
E. cloacae	-	-	-	-	-	-	-	-	-	-	-	-	20±0
S. sonnei	-	-	-	-	-	-	-	-	-	-	-	-	18.33±2.08

(- = No activity)

Concerning the antimicrobial activity of *Copaifera religiosa* and *Costus lucanusianus*, only *Copaifera religiosa* extracts displayed activity against some strains tested. Indeed, *C. religiosa* extracts showed inhibition of *P. aeruginosa, E. faecalis, B. cereus* and *S. aureus*. Methanolic *Copaifera regiosa* extract exhibited the highest inhibition on *S. aureus*. Yet, regarding *C. lucanusianus* extracts which did not possess any antimicrobial in this study Baba and Onanuga [41] demonstrated the inhibitory activity of *C. lucanusianus* leaves on *S aureus*, *P. aeruginosa* and *E. coli* at a concentration of 20 mg/ml. This difference on the results may be due to the plant parts used as in this study we worked with stem instead of leaves, also the mode of extract preparation or the geographical location.

The results of the anti-bacterial activity of *C. abbreviate, L. edulis* and *Z. mucronata* demonstrated no activity against the clinical bacterial strains. These results are in contradiction to findings by Olajuyigbe and Afolayan [72]. The bacterial isolates *E. faecalis, E. coli, K. pneumoniae* were shown to exhibit varied degree of susceptibility with zones of inhibition ranging between 17 and 27 ± 1.0 mm; though the difference in results can be attributed to the extraction solvent used.

In addition, a study published by Samie et al [73] showed that ethanolic extracted compounds from *P. angolensis* inhibited bacterial growth of *Staphylococcus aureus* with median inhibitory concentration of 25 µg/ml.

In this study, methanolic extracts displayed higher anti-microbial activity than the aqueous extracts. The results obtained in this study are not surprising because previous reports have shown that certain plant water extracts do not possess any biological activity [74].

Table 5 Antimicrobial activity of *C. abbreviate, L. edulis and Z. mucronata* indicating inhibition zones (mm) ± SD

Organism	Positive control	MIC (µg/ml)	***C. abbreviata***	***L. edulis***	***Z. mucronata***
Enterococcus faecalis	Vancomycin hydrochloride	2	-	-	-
Escherichia coli	Gentamicin sulfate	8	-	-	-
Klebsiella pneumonia	Gentamicin sulfate	2	-	-	-
Salmonella	Gentamicin sulfate/ imipenem dehydrate/ amicillin/ penicillin G/ chloramphenicol	Resistant	-	-	-
Streptococcus agalactiae	Vancomycin hydrochloride	2	-	-	-

(- = No activity)

3.4 Immunomodulatory effects of plants

Several medicinal plants and their compounds have been recorded to possess immunomodulatory potentials [75].

However, no reports were found that have documented on the potential of *C. abbreviata* as an immunomodulator. In this review, the aqueous extracts of *C. abbreviate, L. edulis* and *Z. mucronata* were investigated for their effects on the production of IL-2, IL-6 and TNF-α in PBMCs (Table 6). Firstly, the viability of cells was assessed at two concentrations (50 and 150 µg/ml) using the MTT assay. Proliferation of cells was induced with high concentrations of *C. abbraviata* and *L.edulis* extracts. IL-2 production was increased by low concentrations of *C. abbraviata* and *Z. Mucronata* while *L.edulis* extract induced secretion of IL-2 at both concentrations. An increase in IL-6 production was observed for all 3 plants at both concentrations tested. The secretion of TNF-α was induced by *C. abbraviata* only.

Brendler and Van Wyk [76] showed that phenolic compounds have strong immunomodulatory activities. A study done by Gayathri et al [77] have indicated that the ability of plants to suppress immune system response was associated with the presence of some compounds such as glycosides, saponins, flavonoids, and steroids. Most of these compounds were found to be present in both *C. abbreviata* and *L. edulis*. Indeed, plants have been used as immunomodulators in the treatment of various diseases [78] even in the treatment of autoimmune diseases [79,80]. Therefore, medicinal plants able to inhibit excessive immune responses might have useful applications in the treatment of immunological disorders [81].

Table 6 Immunomodulatory effects of aqueous extracts *C. abbreviate, L. edulis* and *Z. mucronata* on PBMCs

Plants tested		Viability (Absorbance)	IL-2 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
Control	Cells only	0.16	52	133	17
	Positive control (PHA)	0.327	95	488	41
	Negative control (DMSO)	0.02	0	109	16
C. abbraviata	100 µg/ml	0.318	39	179	26
	50 µg/ml	0.13	96	498	21
Z. mucronata	100 µg/ml	0.145	69	350	16
	50 µg/ml	0.135	85	471	15
L. edulis	100 µg/ml	0.244	64	142	13
	50 µg/ml	0.155	107	211	12

4. Conclusion

The present review aimed at highlighting studies done on 5 indigenous plants from Gabon and South Africa and giving the most recent results obtained in house. The literature review indicated the plants to possess a wide range of compounds with known pharmacological properties. These findings were emphasized by the results obtained in house demonstrating their phytochemical composition, immunomodulatory and antimicrobial activities. The compounds found could be used as therapeutic agents in preventing or treating diseases caused by oxidative stress and different pathogenic strains such as *S. aureus, B. cereus, E. faecalis,* and *P. aeruginosa*. In addition, the results obtained, validate traditional healers' claims in treating various infections. However, it should be reported that the remedies made by traditional healers are a composition of various parts of different plant species, in order to improve the efficacy and the quality of the remedies given to people. This study may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control.

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PROJECT NO: SMNS/17/MBY/30/2111

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Date Considered: November 2017
Decision by Ethical Clearance Committee Granted
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