IN VITRO ASSESSMENT OF PHYTOCONSTITUENTS, EFFICACY AND CYTOTOXICITY OF EXTRACTS FROM MEDICINAL PLANTS ON PROSTATE CANCER C4-2 CELLS

by

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A THESIS

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DEDICATION

I am dedicating this thesis to my family. You have inspired me to be curious, put my best into my work and never give up. Without your prayers and support, I would not have made it this far. My sincere heartfelt thanks go to you all.

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***In vitro* Assessment of Phytoconstituents, Efficacy and Cytotoxicity of Extracts from Medicinal Plants on Prostate Cancer C4-2 Cells**

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Faculty Advisor: Dr. Alberta N.A. Aryee

ABSTRACT

Phenolic compounds are products of secondary plant metabolism known for their biological activity including their antimicrobial, antioxidant, analgesic, stimulant, anti-carcinogenic, and aphrodisiac properties. The main objective of this study was to assess the content and properties of bioactive phytochemicals in the extracts of *Prunus africana*, *Pausinystalia yohimbe, Moringa oleifera, Momordica charantia and Orthero spp* and determine their potency/cytotoxic effects. Total phenolics (TPC), carotenoids, anthocyanins, and flavonoids and their antioxidant properties in water, ethanol, methanol, acetone, and dichloromethane extracts of the different plant parts of these five plants were measured using the Folin-Ciocalteu, 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging (ABTS) assays. For most of the plant samples, extraction yields were highest in ethanol or methanol extraction solvents. The highest total phenolic content (1397.33 mg GAE/g) was seen in the methanol extract of *P. africana* bark from Cameroon, while the acetone extract of *M. charantia* leaves yielded the highest total flavonoid content (217.33 mg RU/g). The FRAP values in this study ranged from 7.09 in the DCM extract of *P. africana* bark (Kenya) to 131.57 mM Fe^{2+}/g in ACE extract of *M. charantia* leaf. The EC_{50} values for the acetone and methanol extracts of *P. africana* bark (Cameroon), methanol and ethanol extracts of *P. yohimbe* leaf and the methanol extract of *P. yohimbe* root were comparable to ascorbic acid (0.18 mg/mL). TPC showed a strong positive

correlation with TFC of acetone extracts of *P. yohimbe* and *Orthero* roots, FRAP of ethanol and methanol extracts of *P. africana* (Cameroon) root, acetone and methanol extracts of *P. africana* (Cameroon) leaf, methanol extracts of *P. yohimbe* leaf, *M. charantia* leaf and the TEAC of *P. africana* (Cameroon) bark water extract, ethanol extracts of *P. yohimbe* leaf and *Orthero* root. Using high performance liquid chromatography (HPLC), seven phenolic acids, namely methyl 4-hydroxybenzoate, protocatechuic acid ethyl ester, trans-sinapic acid, vanillic acid, trans-ferulic acid, p-coumaric acid and caffeic acid were isolated from nine extracts of *P. africana* and *P. yohimbe*. The most abundant phenolic acids were vanillic acid (116.41 mg/g dry extract in methanol extract of *P. yohimbe* leaf) and trans-sinapic acid (102.22 mg/g dry extract in water extract of *P. africana* bark). On gas chromatographic phytosterol analysis, stigmasterol, β-sitosterol and campesterol were present in all plant parts of *P. africana* and *P. yohimbe* except for *P. africana* root and bark, where campesterol was not detected. β-sitosterol showed the highest concentration and variation between plant parts, ranging from 0.55-2.26 in the bark and leaf and 0.35-0.46 mg/g in the root and leaf of *P. africana* and *P. yohimbe*, respectively. Using different concentrations of *P. africana* extracts, prostate cancer C4-2 cells, a hormonally insensitive subline of LNCaP cells, were treated in a proliferation assay. A concentration dependent inhibition of cell growth in cells treated with *P. africana* bark and root extracts was present from days 1 through 3 of incubation, with the methanol extract of the bark showing the strongest effect. Compared to other plant parts, leaf extracts were significantly less cytotoxic at the same concentrations. All plant part extracts contained significant amounts of phenolic compounds and pigments with potent antioxidant activity comparable to that of ascorbic acid, in the case of *P. africana*, demonstrated *in vitro* cytotoxicity.

Keywords: Phenolic compounds, antioxidants, medicinal plants, C4-2 cells, cytotoxicity

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABTS	2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging assay
ACE	Acetone
ASE	Accelerated solvent extraction
ATP	Adenosine triphosphate
BPH	Benign prostatic hypertrophy
c-AMP	Cyclic adenosine monophosphate
CCC	Countercurrent chromatography
CPC	Centrifugal partition chromatography
CO_2	Carbon dioxide
DCM	Dichloromethane
DMCA	Dimethylaminocinnamaldehyde assay
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity
DW	Dry weight
EAD	Electro-array detection
ECD	Electrochemical detection
EC_{50}	50% effective concentration
ELISA	Enzyme-linked immunosorbent assay
EMEM	Earl's minimum essential media
ETH	Ethanol
FBS	Fetal bovine serum
FCS	Fetal calf serum
FMAE	Focused microwave assisted extraction
FRAP	Ferric reducing antioxidant power
FTIR	Fourier-transform infrared spectroscopy

GAE	Gallic acid equivalent
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GPCR	G-protein coupled receptor
HCl	Hydrochloric acid
HEPES	4 (2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
H_20	Water
IR	Infrared spectroscopy
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LDL	Low density lipoprotein
MAbs	Monoclonal antibodies
MAE	Microwave assisted extraction
MEM	Minimum essential medium
MET	Methanol
MPa	Megapascal
MS	Mass spectrometry
MTS tetrazolium	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBBS	N-butylbenzene-sulfonamide
NMR	Nuclear magnetic resonance spectroscopy
PDA	Photodiode array

PFE	Pressurized fluid extraction
PKA	Protein kinase A
PKM2	Pyruvate kinase isoenzyme M2
PLE	Pressurized liquid extraction
PMAE	Pressurized microwave assisted extraction
RB	Retinoblastoma
R_f	Retardation factor
RNA	Ribonucleic acid
RU	Rutin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFE	Supercritical fluid extraction
siRNA	Small interfering ribonucleic acid
SRB	Sulforhodamine B assay
SWE	Subcritical water extraction
TEAC	Trolox equivalent antioxidant capacity
TFC	Total flavonoid content
TLC	Thin layer chromatography
TPC	Total phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
UAE	Ultrasound assisted extraction
UV	Ultraviolet
UV/VIS	Ultraviolet/visible spectrum
WHO	World health organization

CHAPTER 1: INTRODUCTION

1.0 Background and significance

When cells use oxygen to generate energy, free radicals are created as a consequence of endogenous metabolic processes in the human body (Hajhashemi, Vaseghi, Pourfarzam, & Abdollahi, 2010; Pham-Huy, He, & Pham-Huy, 2008). Free radicals and oxidants generate a phenomenon called oxidative stress, a process that can change the cell membranes and other structures such as proteins, lipids, lipoproteins, and deoxyribonucleic acids (DNA) (Chandra et al., 2012; Hajhashemi et al., 2010; Z. Liu et al., 2018; Pham-Huy et al., 2008). In humans, oxidative damage and free radicals are associated with a number of chronic diseases including atherosclerosis, Alzheimer's disease, cancer, ocular disease, diabetes mellitus, rheumatoid arthritis and motor neuron disease (Hajhashemi et al., 2010; Z. Liu et al., 2018; Pham-Huy et al., 2008). According to a previous study, approximately 125 million Americans (45% of the population) had one chronic condition, and 61 million (21% of the population) had multiple chronic conditions in 2000 (Anderson & Horvath, 2004). Cancer is a major cause of mortality worldwide, contributing to 7.6 million deaths in 2008 (Soerjomataram et al., 2012). It is also the second leading cause of death globally, and was responsible for an estimated 9.6 million deaths in 2018 (Bray et al., 2018; Riboux, 2018). Globally, about 1 in 6 deaths is due to cancer; and cancer incidence and mortality are rapidly growing world-wide (Bray et al., 2018).

The most common cancers are lung, breast, colorectal, prostate, non-melanoma skin, and stomach cancer, while the most common causes of cancer death include cancers of the lung, colon/rectum, prostate, stomach, liver, and breast (Riboux, 2018). Tobacco use, alcohol use, an unhealthy diet, and physical inactivity are major cancer risk factors worldwide; some chronic infections are also risk factors for cancer and have major relevance in low- and middle-income

countries (Danaei et al., 2005; Riboux, 2018). The rising prominence of cancer as a leading cause of death partly reflects marked declines in mortality rates of stroke and coronary heart disease, relative to cancer, in many countries (Bray et al., 2018; Danaei, Vander Hoorn, Lopez, Murray, & Ezzati, 2005; Vineis & Wild, 2014). Although a combination of screening and treatment is increasingly effective in reducing mortality from some cancers, limitations in availability of clinical interventions for other cancers, and in access to and use of existing technologies, clearly constrain the effects of treatment on population trends in cancer mortality, even in developed countries (Danaei et al., 2005).

1.1 Statement of problem and hypotheses

Prostate cancer is a significant cause of morbidity and mortality worldwide and with an estimated 232,000 new cases and 33,000 deaths in 2016, it is the most frequently diagnosed cancer and second most frequent cause of cancer deaths in US males (Scher, Solo, Valant, Todd, & Mehra, 2015; Shenouda et al., 2007). Approximately, 9 - 11% of men are at risk of clinically suffering from prostate cancer in their life-time globally (Komakech, Kang, Lee, & Omujal, 2017). Prostate cancer is a dynamic disease that changes over time as a function of the intrinsic properties of the tumor, patient factors, and the specific therapies to which the tumor has been exposed (Scher et al., 2015). For years, prostate cancer has been managed through conventional treatment modalities such as surgery, radiation therapy, cryosurgery, and hormone therapies, but there is still no effective treatment for advanced stages of prostate cancer, which is hormonally resistant. Chemoprevention and chemotherapy have been identified as effective approaches by which the prevalence of prostate cancer can be reduced, suppressed, or reversed (Komakech et al., 2017).

In the last decades, several plants have been confirmed to contain chemo-preventive and therapeutic agents for various cancers including prostate cancer (Komakech et al., 2017; Shenouda et al., 2007). Among plants with anti-prostate cancer potential, researchers identified *Prunus africana* (African cherry), with its unique combination of phytochemicals and *Momordica charantia* (Komakech et al., 2017; Shenouda et al., 2007). *P. africana* has been used in African traditional medicine to treat prostate cancer and related conditions across various communities for many years (Komakech et al., 2017) while the different parts of *M. charantia* have been used as an anti-viral, anti-microbial, hypoglycemic and anti-cancer agents in traditional medicine(Barbieri et al., 2015; J.-C. Chen et al., 2009; Lee-Huang et al., 1990; Lo, Ho, Lin, Li, & Hsiang, 2013; Patel Subhashchandra , Patel Tushar, Parmar Kaushal, Bhatt Yagnesh, Patel Yogesh, 2009; Xiong et al., 2009). Several studies on other plants such as *Moringa oleifera* (used in traditional medicine in several parts of the world) have highlighted its hypotensive, vibriocidal, enzyme-inhibiting and anti-cancer properties (Al-Asmari et al., 2015; Albuquerque Costa et al., 2017; Bijina et al., 2011; Gilani et al., 1994; Jansakul, C; Wun-noi, A; Croft, K; Byrne, 1997; Murakami, Kitazono, Jiwajinda, Koshimizu, & Ohigashi, 1998).

The most commonly known use of *Pausinystalia yohimbe* in traditional medicine has been in the treatment for erectile dysfunction. Nevertheless, it has other benefits, including lipid metabolism regulation and antioxidant, anti-atherosclerotic, anti-fungal and anti-carcinogenic effects (Eweka, Om'Iniabohs, & Momodu, 2010; Igwe, Madubuike, Ikenga, Otuokere, & Amaku, 2016; Morales, 2000; Sala et al., 1988). Previous studies have confirmed the effectiveness of the bark extract of *P. africana* in prostate disorders and attributed this to the synergistic effects of pentacyclic triterpenoids, ferulic esters of long-chain fatty alcohols, and phytosterols (Karan, Kumar Jena, Sharma, Vasisht, & Efferth, 2017; Komakech et al., 2017; Nyamai et al., 2015).

Researchers have discovered that polyphenols are very good antioxidants capable of neutralizing the destructive reactivity of undesired reactive oxygen/nitrogen species produced as byproducts during metabolic processes in the body (Pandey & Rizvi, 2009). Also, epidemiological studies have revealed that polyphenols provide a significant protection against development of several chronic conditions such as cardiovascular diseases (CVDs), cancer, diabetes, infections, aging and asthma (Pandey & Rizvi, 2009). We hypothesize, therefore, that phenolic compounds in selected medicinal plants play a significant role in chronic disease prevention and chemotherapy and set out to test this hypothesis *in vitro*, with the goal of finding new drugs for prostate cancer.

1.2 Specific objectives

The main aim of this study was to identify the phytochemical composition of different plant parts from five commonly used African medicinal plant species, namely *Moringa oleifera*, *Prunus africana*, *Pausinystalia yohimbe*, *Momordica charantia* and *Orthero spp*, and to determine the biological activity of the extracts on cancer cells. The specific research objectives were:

i. Identify and quantify phytochemicals from *Moringa oleifera*, *Prunus africana*, *Pausinystalia yohimbe*, *Momordica charantia* and *Orthero spp*
ii. Determine the antioxidant activity of plant extracts from the different plant parts
iii. Evaluate the cytotoxicity of *Prunus africana* extracts on prostate cancer cells

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

From time immemorial, plants have been used for medicinal purposes in most parts of the world for their unique properties and administered in various preparations which have been passed down from preceding generations (B. Joshi, Prasad Sah, et al., 2011). In Hindu culture, the "Rigveda", said to have been written between 4500 - 1600 B.C and thought to be one of the oldest repository of human knowledge, has records of medicinal use of plants (B. Joshi, Prasad Sah, et al., 2011). The World Health Organization (WHO) estimates that nearly 20,000 medicinal plants exist in over 91 countries of the world and over 80% of the world's population relies to some extent on traditional medicine for their primary health care needs (Sasidharan, Chen, Saravanan, Sundram, & Yoga Latha, 2011). It is important, however, to note that not all plants are medicinal. The distinguishing characteristic of medicinal plants is the presence of chemical substances, phytochemicals, that can produce a definite physiological action in the human body (Jeruto, Mutai, Catherine, & Ouma, 2011; Yadav & Agarwala, 2011).

Phytochemicals are classified as primary or secondary metabolites based on their role in plant metabolism (Krishnaiah, Sarbatly, & Bono, 2007). Primary metabolites include simple sugars, amino acids, proteins, and lipids, all of which are involved in cellular processes (Kumari, Kumari, & Singh, 2017). On the other hand, secondary metabolites are chemically active compounds including alkaloids, anthocyanins, flavonoids, terpenoids, tannins, steroids, saponins, coumarins, phenolics and antioxidants. These are often produced in response to stress, are more complex in structure, and are less widely distributed than the primary metabolites (Krishnaiah et al., 2007; Kumari et al., 2017). They are pharmacologically active as anti-oxidative, anti-allergic, anti-bacterial, anti-fungal, anti-diabetic, anti-inflammatory and anti-carcinogenic compounds (A.

Gupta, Naraniwal, & Kothari, 2012; Kumari et al., 2017; Ncube, Afolayan, & Okoh, 2008). It is common for a single plant to produce many secondary metabolites with a wide range of chemical and biological properties, providing a range for bioactive substances (Ncube et al., 2008).

2.2 Secondary metabolites

2.2.1 Alkaloids

Naturally occurring alkaloids are nitrogenous compounds that represent pharmacologically active compounds in flowering plants and many of the earliest isolated pure compounds with biological activity in plant extracts (Ncube et al., 2008). Based on the precursors and final structure, alkaloids can be grouped into three major classes: monoterpenoid indole alkaloids (e.g. quinine, with anti-malarial and camptothecin, vincristine and vinblastine with anti-neoplastic activity), tropane and nicotine alkaloids (e.g. cocaine with analgesic and scopolamine with anticholinergic activity) and benzylisoquilonone alkaloids (e.g. colchicine with anti-inflammatory and emetic with anti-amoebic activity) (Kutchan, 1995).

Alkaloids are a large group of alkaline secondary metabolites, with considerably varying degrees of alkalinity, which result from the structure of the molecule, and the presence and location of the functional groups (Doughari, 2012). They can react with acids to form crystalline salts without the production of water. Most alkaloids dissolve readily in alcohol and, although sparingly soluble in water, their salts are usually water-soluble. The solutions of alkaloids are intensely bitter. These nitrogenous compounds defend plants against herbivores and pathogens and have been widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities (Doughari, 2012). Alkaloids' many pharmacological properties include anti-hypertensive effects (many indole alkaloids), anti-arrhythmic effect (quinidine, spareien), anti-

malarial activity (quinine), stimulant properties (caffeine and nicotine), analgesic activity (morphine), and anti-cancer activity (dimeric indoles, vincristine, vinblastine) (Saxena, Saxena, Nema, Singh, & Gupta, 2013).

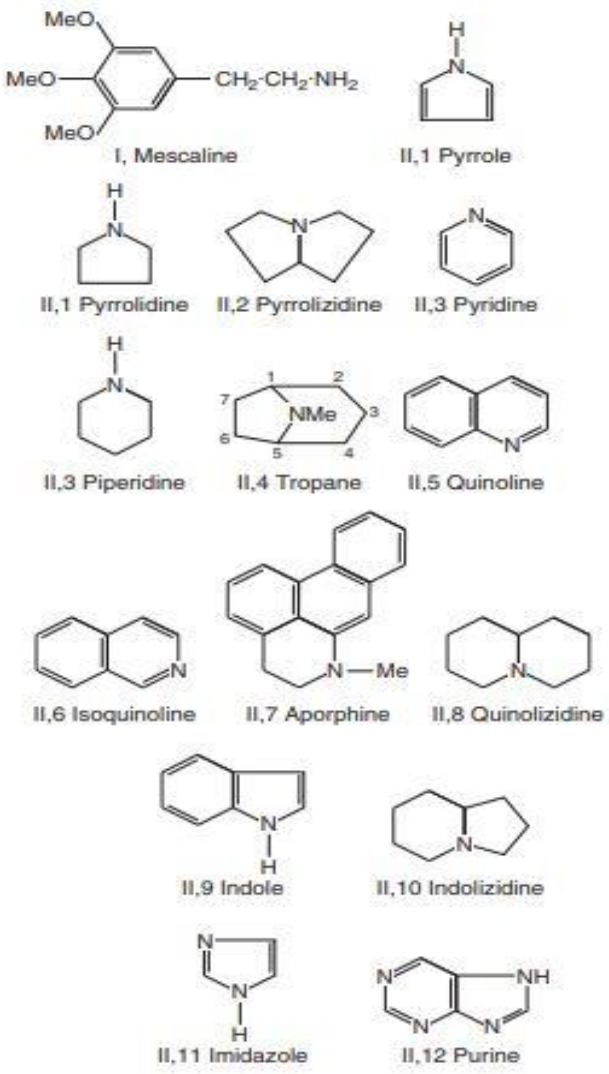


Figure 1: Skeletal structures of alkaloids found in medicinal plants

Source: Evans, 2009

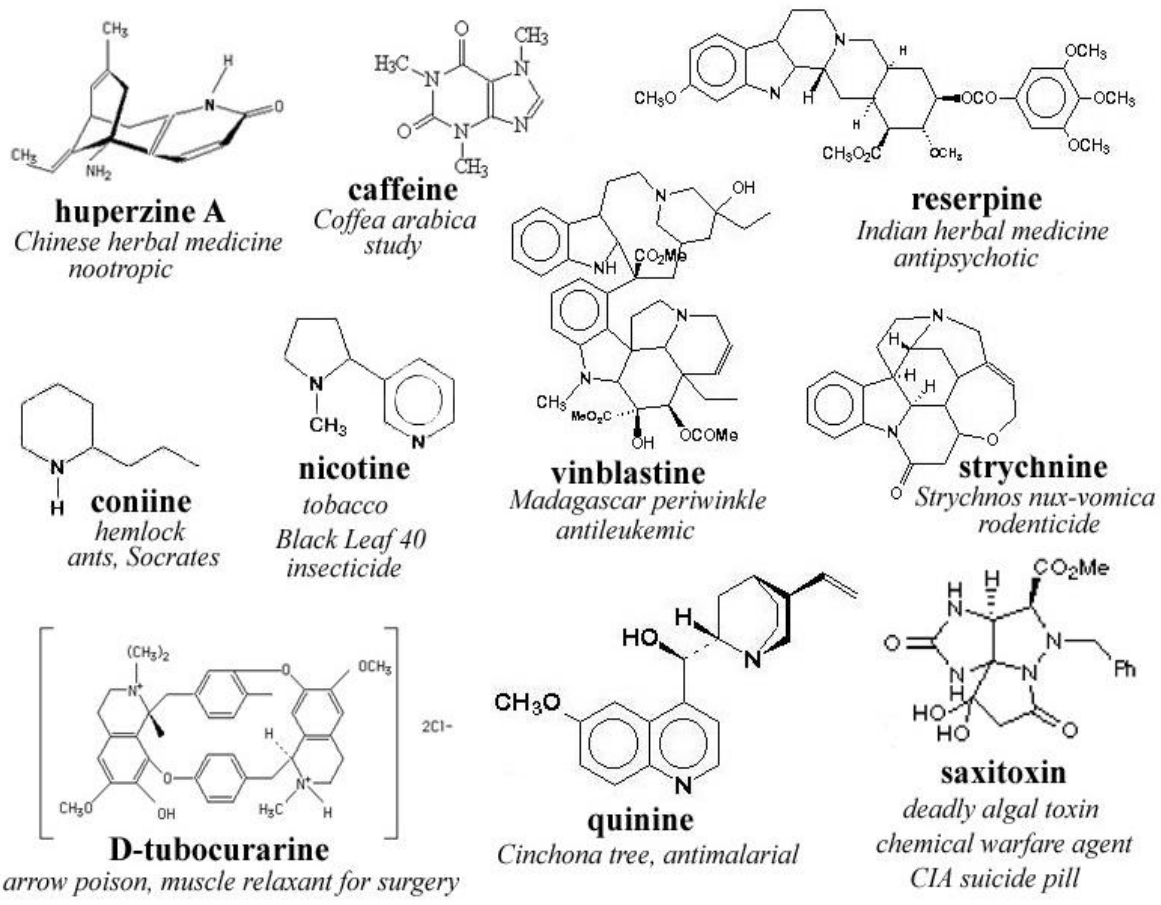


Figure 2: Basic structures of some pharmacologically important plant-derived alkaloids

Source: Chikezie, Ibegbulam, & Mbagwu, 2015

2.2.2 Phenols

Compounds possessing one or more aromatic rings with one or more hydroxyl groups in their structures are called phenolics (Chandra et al., 2012; R. Liu, 2004, 2013b, 2013a). Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom (Saxena et al., 2013). They are generally classified into the following subgroups: phenolic acids, flavonoids, stilbenes, coumarins, and tannins. Phenolics, resulting from the secondary metabolism in plants, play vital roles in the reproduction and growth of plants, provide defense mechanisms against pathogens, parasites, predators, and UV irradiation, and contribute to

the color of plants (R. Liu, 2013a). Furthermore, phenolic compounds in the human diet provide additional health benefits associated with reduced risk of developing chronic diseases (Chandra et al., 2012; R. Liu, 2004, 2013a, 2013b).

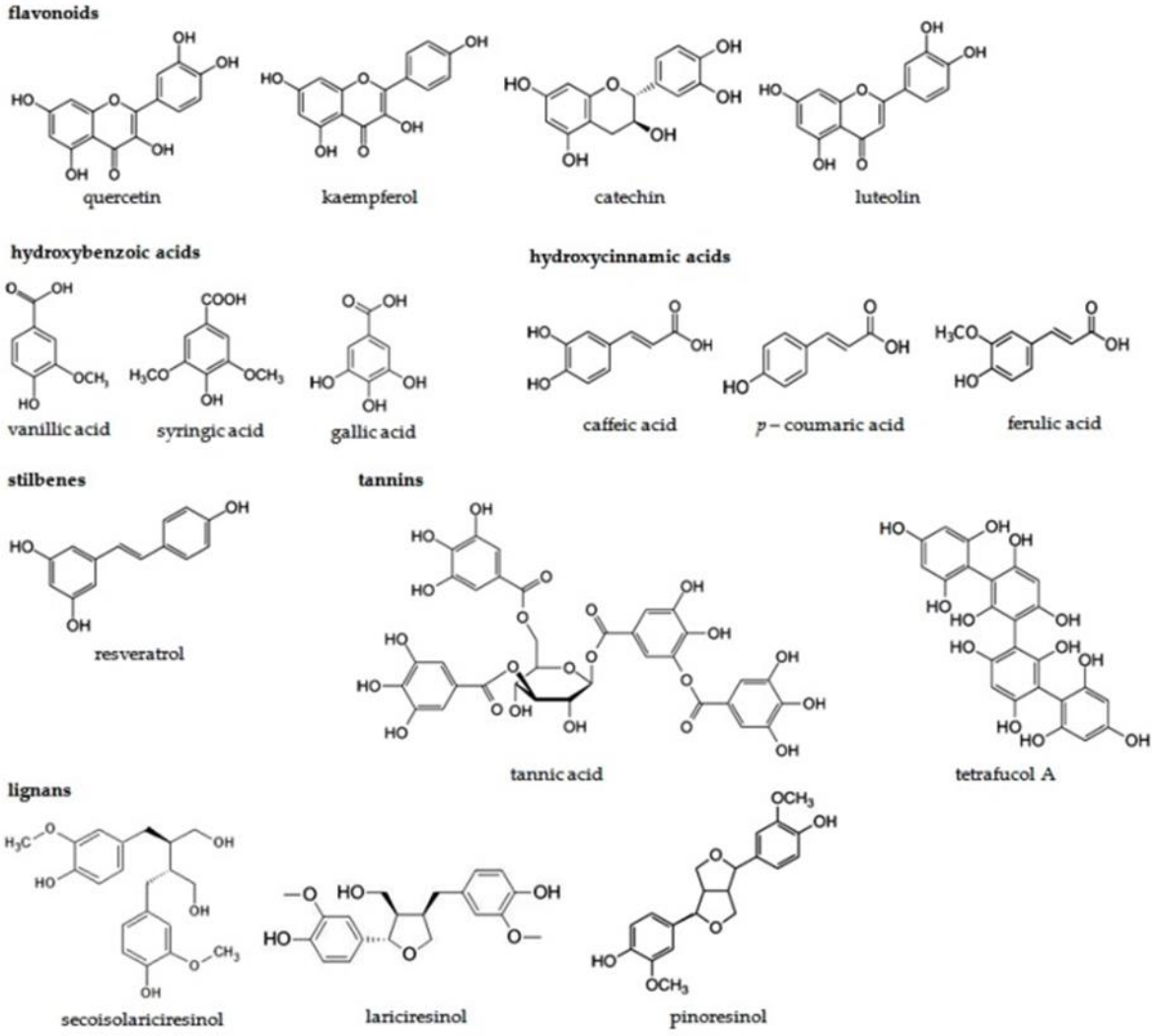


Figure 3: Basic structures of some pharmacologically important plant-derived phenolics

Source: Dzialo et al., 2016

Phenolic acids are phenols that possess at least one functional carboxylic acid group and can be further subdivided into two subgroups: hydroxybenzoic acid and hydroxycinnamic acid derivatives (Doughari, 2012; Saxena et al., 2013). Both are found in bound form in plants;

hydroxybenzoic acid derivatives usually as components of complex structures like lignin and hydrolysable tannins, and hydroxycinnamic acid derivatives typically connected through ester bonds to cell wall structural components like cellulose, lignin and proteins (Chandra et al., 2012; R. Liu, 2004, 2013a, 2013b). Phenolics acids possess diverse biological activities, including anti-ulcer, anti-inflammatory, antioxidant, cytotoxic and anti-tumor, anti-spasmodic, and anti-depressant activities (Saxena et al., 2013).

Flavonoids, a major group of phenolic compounds have a generic structure composed of a couple of aromatic A and B rings, which are linked by three carbons that are usually in an oxygenated heterocycle ring, or C ring. They are further classified as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids based on differences in the generic structure of the heterocycle C ring (Chandra et al., 2012; R. Liu, 2004, 2013a, 2013b). Flavonoids possess many useful properties, such as anti-inflammatory activity, enzyme inhibition, anti-microbial activity, estrogenic activity, anti-allergic activity, vascular activity and cytotoxic/anti-tumor activity but the best-described property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species (Saxena et al., 2013).

Flavonoid consumption has been linked to a reduced risk of major chronic diseases (Chandra et al., 2012; R. Liu, 2013a, 2013b). They also extend the activity of vitamin C, protect low density lipoprotein cholesterol (LDL-C) from oxidation to the unsafe cholesterol oxides and inhibit platelet aggregation (Chandra et al., 2012). The capacity of flavonoids to act as antioxidants depends upon their molecular structure as the position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities (Saxena et al., 2013).

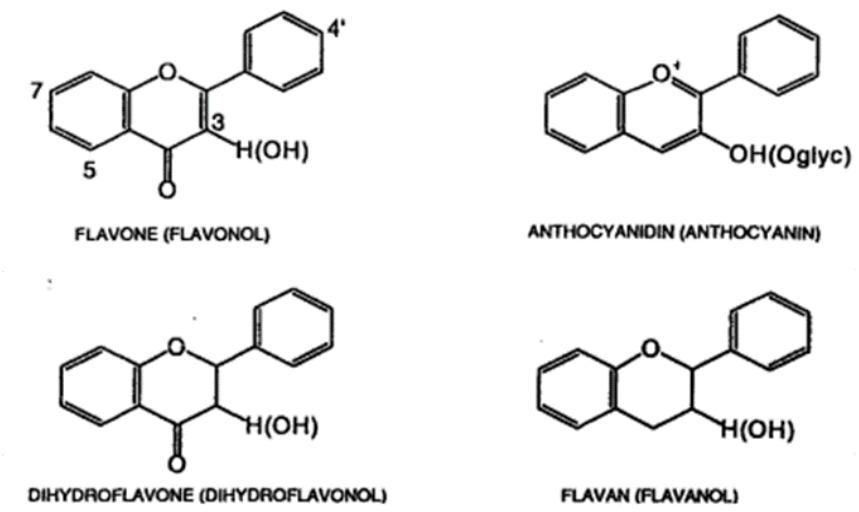


Figure 4: Basic structures of some pharmacologically important plant-derived flavonoids

Source: Doughari, 2012

Coumarins owe their class name to 'Coumarou', the vernacular name of the tonka bean (*Dipteryx odorata*), from which coumarin was isolated in 1820 (Jain & Joshi, 2012). There are four main coumarin sub-types: the simple coumarins, furanocoumarins, pyranocoumarins, and pyrone-substituted coumarins (Jain & Joshi, 2012). Coumarins are able to subsidize swelling in high protein edema states, have a stimulatory effect on macrophages, activate other cells of the immune system, and are competitive inhibitors of vitamin-K in the biosynthesis of prothrombin making them good anti-coagulants (Jain & Joshi, 2012; Mortimer, 1997; Rohini & Srikumar, 2014). Coumarins and their derivatives are also highly effective against the inflammatory response and have anti-microbial, antioxidant, neuroprotective and anti-human immunodeficiency virus (HIV) activities (Bubols et al., 2013; Jain & Joshi, 2012). They are potential antioxidants with the ability to chelate metal ions and scavenge free radicals (Kumari et al., 2017).

Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with mainly proteins, but also

polysaccharides (cellulose, hemicellulose, pectin), alkaloids, nucleic acids and minerals (Saxena et al., 2013). They can be divided, based on their structural characteristics, into four major groups: gallotannins, ellagitannins, complex tannins, and condensed tannins (Saxena et al., 2013). Tannin-containing plant extracts are used as astringents, anti-diarrheals, diuretics, anti-stomach and duodenal tumors and as anti-inflammatory, antiseptic, antioxidant and hemostatic pharmaceuticals (Kumari et al., 2017; Saxena et al., 2013). The food industry uses tannins to clarify wine, beer, and fruit juices and as antioxidants in the fruit juice, beer, and wine industries (Jaiswal, Singh, Chauhan, Sahu, & Dv, 2018; Saxena et al., 2013).

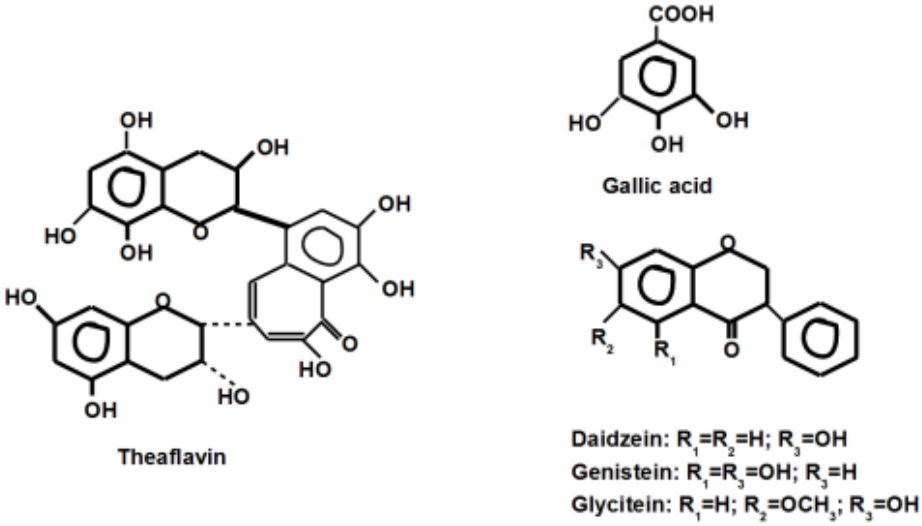


Figure 5: Basic structures of some pharmacologically important plant-derived tannins

Source: Doughari, 2012

2.2.3 Terpenes

Terpenes are one of the most diverse groups of secondary metabolites (Ncube et al., 2008; Saxena et al., 2013). They include sterols and triterpenes, which are complex compounds formed by the cyclization of 2,3-oxidosqualene and can accumulate as glycoside conjugates in plants. These glycosides, commonly called saponins, have *in vitro* inhibitory effects on protozoa

according to a previous study (e.g. saponins from *Quillaja saponaria*) (Ncube et al., 2008). Essential oils, another sub-class of compounds under terpenes, possess anti-bacterial, anti-viral, anti-fungal and anti-inflammatory properties (e.g. oils from *Cinnamomum osmophloeum* with anti-bacterial activity against *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* (including methicillin resistant *S. aureus*) and *Vibrio parahaemolyticus* (Ncube et al., 2008; Saxena et al., 2013). Monoterpenes, diterpenes and sesquiterpenes form most of this subclass (Ncube et al., 2008). Terpenes have medicinal properties such as anti- carcinogenic (e.g. perilla alcohol, taxol), anti-malarial (e.g. artemisinin), anti-ulcer, anti-microbial and diuretic properties (e.g. glycyrrhizin) (Saxena et al., 2013).

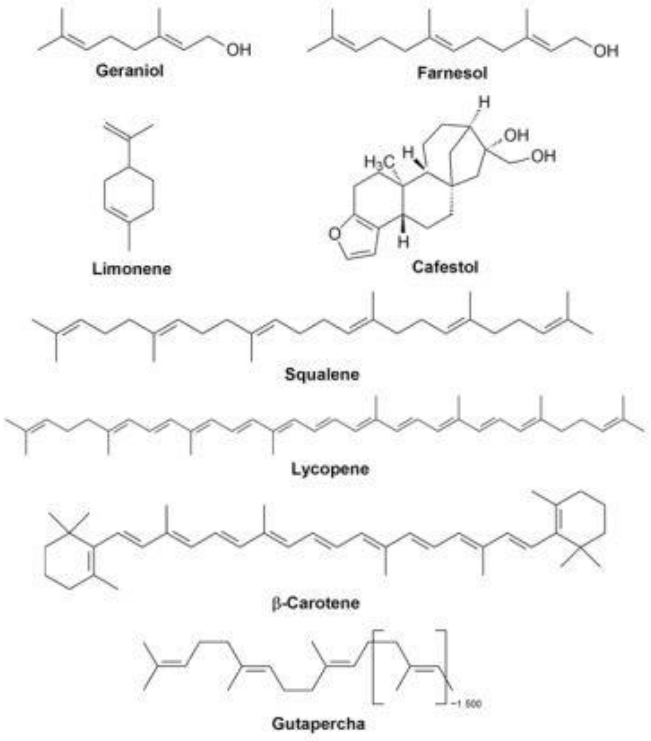


Figure 6: Basic structures of some pharmacologically important plant-derived terpenes

Source: Todorova et al., 2012

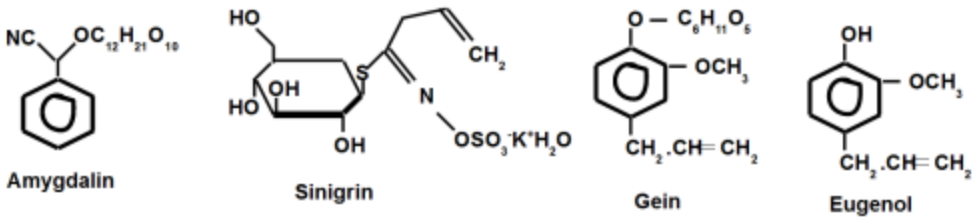


Figure 7: Basic structures of some pharmacologically important plant-derived essential oils

Source: Doughari, 2012

2.2.4 Saponins

Saponins are a group of secondary metabolites that form a stable foam in aqueous solutions such as soap, hence the name "saponin" (Moghimipour & Handali, 2015; Saxena et al., 2013). They consist of a polycyclic aglycone attached to one or more sugar side chains. The aglycone part, which is also called sapogenin, is either a steroid (27 carbon atoms with a 6-ring spirostane or a 5-ring furostane skeleton) or a triterpene (30 carbon atoms) (Guclu-Ustundag & Mazza, 2007; Moghimipour & Handali, 2015; Saxena et al., 2013). Saponins that have one sugar molecule attached at the C-3 position are called monodesmoside saponins, and those that have a minimum of two sugars, one attached to C-3 and one to C-22, are called bidesmoside saponins (Saxena et al., 2013). Plant saponin mixtures possess diverse biological effects including membrane-permeabilising, immunostimulatory, hypocholesterolemic, anti-microbial, anti-cancer, antioxidant, anti-pyretic, hepato-protective, hemolytic, and anti-inflammatory activities, in addition to having the ability to kill protozoans and mollusks, to cause hypoglycemia, be used as adjuvant in vaccines and to act as anti-fungal and anti-viral agents (Guclu-Ustundag & Mazza, 2007; Moghimipour & Handali, 2015; Saxena et al., 2013).

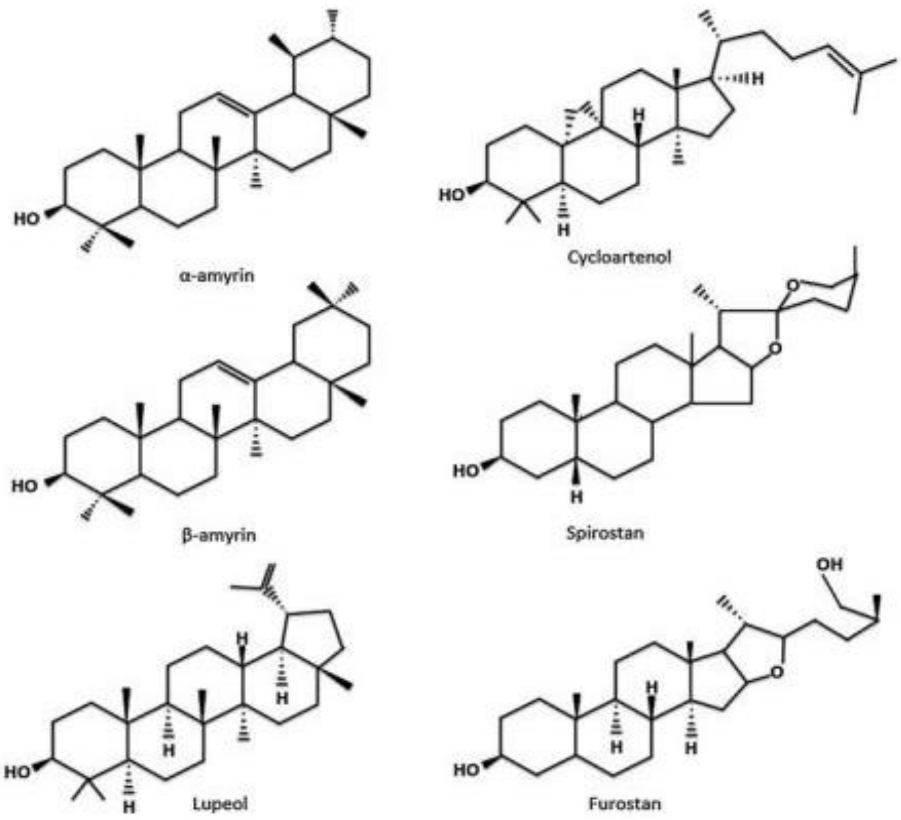


Figure 8: Basic (a) Triterpene (b) Steroid ponins

Source: Faizal and Geelen, 2013

2.2.5 Pigments

Pigments are chemical compounds that absorb light in the wavelength range of the visible region, producing color due to the presence of chromophores (Delgado-Vargas, Jiménez, Paredes-López, & Francis, 2000). In plants, pigments have the primary function of photosynthesis, capturing the radiant energy of the sun and converting it into organic food (Upadhyay, 2018). In addition, they also perform important functions in maintaining vitality, growth, and development of plants. Pigments have evolved in nature to serve a variety of purposes, including protection from oxidative stress, bioactivity against skin diseases, and act as antioxidants, anti-inflammatory, anti-analgesic, and chemo preventive agents against cancer (Saviola, 2014; Upadhyay, 2018). The

most important pigments in higher plants are chlorophylls, carotenoids, betalains and anthocyanins when considering their capacity to impart colors (Delgado-Vargas et al., 2000).

Chlorophylls are fat soluble green pigments that occur in the plastids of most plants, algae, and certain bacteria (B. Simpson, Benjakul, & Klomlao, 2012; Upadhyay, 2018). They are the most widely distributed natural plant pigments and exist in nature in different forms (e.g., chlorophylls a, b, c1, c2, and d) as a result of subtle structural changes (B. Simpson et al., 2012). Commercial sources of chlorophylls include the green algae chlorella, the blue-green algae Spirulina, string lettuce (*Enteromorpha*) and sea lettuce (*Ulva*), all of which are used as human food (B. Simpson et al., 2012). Chlorophyllin, a chlorophyll derivative, is used as a food-coloring agent, known as natural green 3 with E number E141 (İnanç, 2011). The health benefits of chlorophylls for humans include their ability to manage anemia by supplying nutrients for red cell synthesis (İnanç, 2011; B. Simpson et al., 2012). The high magnesium content in chlorophyll promotes fertility by increasing the levels and activities of the enzymes that regulate sex hormones (B. Simpson et al., 2012). Chlorophylls have antibacterial properties and positive effects on inflammation, oxidation and wound healing (İnanç, 2011). They are also used in deodorizers, to inhibit oral bacterial infections, promote healing of rectal sores, and reduce typhoid fevers (B. Simpson et al., 2012). In concentrations found in green vegetables, it can provide substantial cancer chemo-protection, possibly by reducing carcinogen bioavailability through the formation of complexes with carcinogens (İnanç, 2011; McQuistan et al., 2012). In addition, chlorophyll from *Carica papaya* leaves shows cytotoxicity toward human squamous cell carcinoma (Upadhyay, 2018).

Carotenoids are isoprenoid compounds (mostly C40) with polyene chains that may contain several conjugated double bonds (Delgado-Vargas et al., 2000; Tanaka, Sasaki, & Ohmiya, 2008).

Figure 9: Basic structures of some pharmacologically important plant-derived chlorophylls

Source: Pareek et al., 2018

To date, over 700 naturally occurring carotenoids have been identified (Fiedor & Burda, 2014; Tanaka et al., 2008). Most carotenoids are fat soluble and heat stable and are found ubiquitously in both plants and animals (Fiedor & Burda, 2014; B. Simpson et al., 2012; Upadhyay, 2018). Carotenoids like α-carotene, β-carotene, zeaxanthin, and β-cryptoxanthin are important sources of dietary vitamin A and are thus, classified as having provitamin A activity (B. Simpson et al., 2012). They are important constituents of photosynthetic organelles of all higher plants, mosses, ferns and algae and are known to be very efficient physical and chemical quenchers of singlet oxygen, as well as potent scavengers of other reactive oxygen species (ROS) (Fiedor & Burda, 2014; Perera & Yen, 2007; B. Simpson et al., 2012). Carotenoids and some of their metabolites are suggested to play a protective role in a number of ROS-mediated disorders, such as cardiovascular diseases, several types of cancer, and neurological and photosensitive or eye-related disorders (Fiedor & Burda, 2014; Perera & Yen, 2007; B. Simpson et al., 2012; Tanaka et al., 2008; Upadhyay, 2018). Carotenoids have also been suggested to participate in the stimulation of the immune system, the modulation of intracellular signaling pathways (gap junction communication), the regulation of the cell cycle and cell apoptosis, the modulation of growth factors, cell differentiation and the modulation of receptors or adhesion molecules (Fiedor & Burda, 2014; Perera & Yen, 2007).

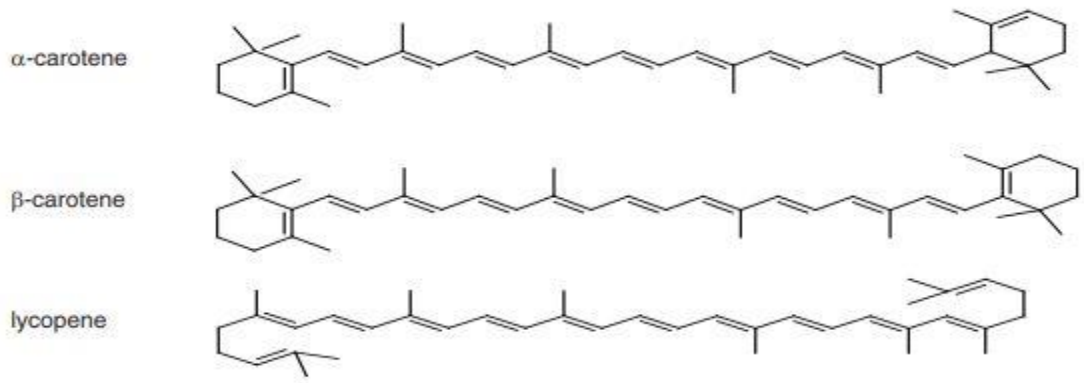


Figure 10: Basic structures of some pharmacologically important plant-derived carotenoids

Source: Kiokias, Proestos, & Varzakas, 2016

Betalains are red/violet and yellow/orange water-soluble aromatic indole pigments found in plants and fungi (Gandía-Herrero et al, 2016; Rahimi et al, 2018; Simpson et al., 2012; Upadhyay, 2018). The occurrence of betalains and anthocyanins is mutually exclusive in plants (Rahimi et al., 2018; Tanaka et al., 2008; Upadhyay, 2018). They are subdivided into betacyanins (reddish to violet) and betaxanthins (yellowish to orange), based on their colors (B. Simpson et al., 2012; Upadhyay, 2018). Betalains are the red pigments found in beets and are established food colorants (B. Simpson et al., 2012; Upadhyay, 2018). Betalains belong to the class of dietary cationized antioxidants; and betanin and its aglycone counterpart have lipoperoxyl radical scavenging and antioxidative effects (Rahimi et al., 2018). Results from studies suggest that in human liver cells, betanin may induce the expression of phase II detoxifying enzymes through nuclear factor erythroid 2-related factor 2 (Nrf2) activation as a result of mitogen-activated protein (MAP) kinases stimulation, preventing liver injury and cancer (Khan, 2016; Krajka-Kuźniak et al, 2013; Rahimi et al., 2018). Betalains block the proliferation of tumor cells and inhibit their pro-survival pathways (B. Simpson et al., 2012; Upadhyay, 2018), thought to be due to their

antioxidant and free radical scavenging behavior (B. Simpson et al., 2012). Betanin/isobetanin (both found in beetroot) significantly decreased cancer cell proliferation and viability (Upadhyay, 2018). Several betalain-rich formulations have been patented with claims of a wide range of health benefits including increased high density lipoprotein/low density lipoprotein (HDL/LDL) cholesterol ratios, reduced oxidized LDL concentrations, normalized blood glucose, and inhibition of oxidative stress responsive transcription factor nuclear factor (NF)-κB (Khan, 2016).

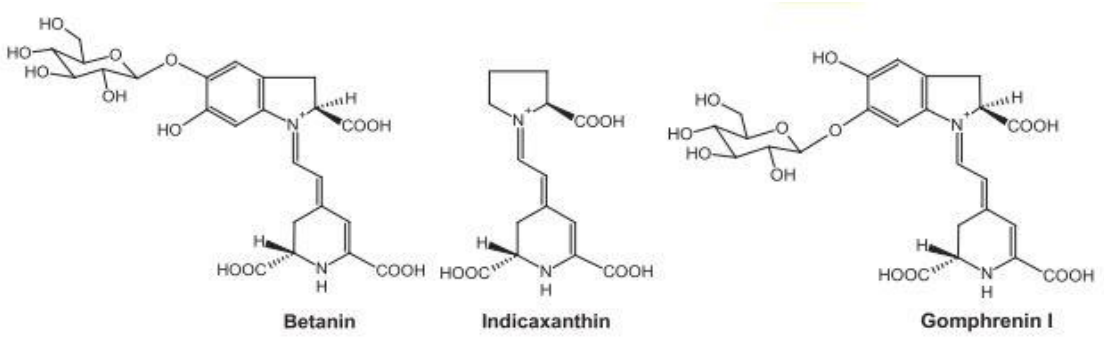


Figure 11: Basic structures of some pharmacologically important plant-derived betalains

Source: Khan, 2016

Anthocyanins are water-soluble, pH-sensitive, red, purple, and blue pigments found in higher plants (Peña-Sanhueza et al, 2017; Simpson et al., 2012). Considered members of the flavonoid group of compounds they have a positive charge at the oxygen atom of the C-ring of the basic flavonoid structure (Khoo, Azlan, Tang, & Lim, 2017; Lila, 2004; B. Simpson et al., 2012). Anthocyanins are red under acidic conditions and turn blue at neutral to alkaline pHs (Khoo et al., 2017; B. Simpson et al., 2012; Tanaka et al., 2008). Based on their chemical structures, two types of anthocyanin are distinguished: the anthocyanidin aglycones (or anthocyanidins) that are devoid of sugar moieties, and the true anthocyanins, glycosides or sugar esters of the anthocyanidins (B. Simpson et al., 2012). Anthocyanins are potent antioxidants (Khoo et al., 2017; Luna-Vital & De

Mejia, 2018; B. Simpson et al., 2012; Upadhyay, 2018). In addition, they possess antidiabetic, anticancer, anti-inflammatory, antimicrobial, and anti-obesogenic effects, as well as prevention of cardiovascular diseases (Khoo et al., 2017; B. Simpson et al., 2012). Its anti-diabetic effect is achieved through several mechanisms including enhanced glucose utilization, suppressed hepatic gluconeogenesis, modulated glucose metabolism in liver cells, improved glucose tolerance and insulin sensitivity and prevention of diabetic complications through inhibition of the aldoreductase enzyme (Luna-Vital & De Mejia, 2018; B. Simpson et al., 2012).

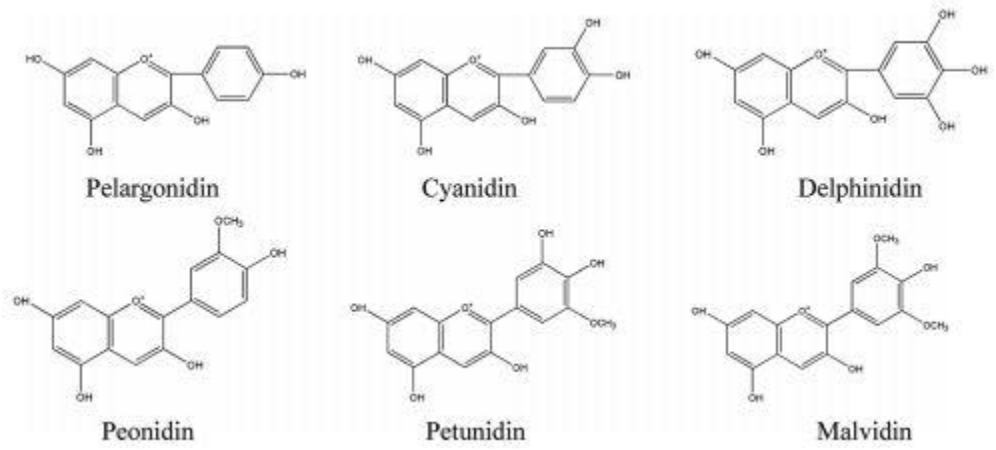


Figure 12: Basic structures of some pharmacologically important plant-derived anthocyanins

Source: Martin, Navas, Jiménez-Moreno, & Asuero, 2017

2.2.6 Sources of secondary metabolites

Secondary metabolites may be extracted from various parts of medicinal plants; they may be derived from barks, leaves, flowers, roots, fruits or seeds (Azwanida, 2015; A. Gupta et al., 2012; Saxena et al., 2013; Yadav & Agarwala, 2011). The medicinal properties of plant extracts are unique to a particular plant species, which is in keeping with the concept that the combination of secondary products in a particular plant is taxonomically distinct. These secondary metabolites

vary in concentration by tissue (higher concentrations occur in bark, heartwood, roots, branch bases and wound tissues), among species, from tree to tree and from season to season (Ncube et al., 2008). Mitscher et al. (1972) found that extracts are generally richest in anti-bacterial agents, after the flowering or sexual stage of their growth is complete, and that plants taken from stressful environments had a higher content. However, the decision of what plant material to use in extract preparation is usually guided by the traditional use of the plant and the ease of handling of the different plant parts (Ncube et al., 2008).

2.3 Methods used in the extraction of secondary metabolites

The first step in the analysis of medicinal plants is extraction, because the desired compounds have to be extracted from the plant materials before they can be isolated and characterized (Sasidharan et al., 2011). The physical and chemical properties of secondary metabolites are highly variable, making it imperative that appropriate extraction protocols be chosen, as the optimum extraction conditions vary widely for the different metabolites (Krishnaiah et al., 2007). Usually before extraction is done, plant samples are treated by milling, grinding and homogenization, often proceeded by air-drying or freeze-drying (Dai & Mumper, 2010; Ncube et al., 2008). It is important to note, however, that drying processes can cause undesirable effects on the constituent profiles of plant samples; therefore, caution must be taken when planning and analyzing experimental studies on the medicinal properties of plants (Dai & Mumper, 2010). For several reasons, though, researchers identifying and analyzing secondary plant metabolites tend to use dried plant materials. Traditionally, many plants are used in the dried form or as an aqueous extract. The differences in water content of the plant extracts may affect solubility of subsequent separations by liquid extraction (Ncube et al., 2008).

2.3.1 Solvent extraction

Solvent extraction is the most commonly used method of extraction because of the ease of use, efficiency, and wide applicability (Dai & Mumper, 2010; Krishnaiah et al., 2007). The type of solvent used in extraction determines to a large extent how successful the separation and identification of bioactive compounds from plant extracts is. Therefore, a good solvent for plant extractions should have the following properties: low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, and inability to cause the extract to complex or dissociate (Ncube et al., 2008). The product of the extraction process will contain trace amounts of the solvent used in extraction, making it necessary that the solvent be non-toxic and cause no interference in bioassays.

The compounds targeted for extraction also influence the choice of solvent. For example, aqueous acetone was found to be better at extracting total phenolics than aqueous methanol, while chloroform was found to be the best solvent among 20 others evaluated for extraction of non-polar, biologically active compounds from the roots of *Angelica archangelica*. Plant extracts from organic solvents were reported to provide more consistent anti-microbial activity compared to water extracts, although traditional healers have primarily used water (Ncube et al., 2008).

Other factors influencing the yield of metabolites in solvent extraction include temperature, pressure, pH, sample-to-solvent ratio, extraction time, and the chemical composition and physical characteristics of the samples (Dai & Mumper, 2010; Ncube et al., 2008).

2.3.1.1 Effect of temperature on extraction

The use of elevated temperature in solvent extraction increases the capacity of solvents to solubilize analytes. This means that the solubility of any solute in solvent increases with the

increase of solvent temperature. An increase in temperature leads to faster diffusion rates so that analytes move more quickly from the boundary layer to near the surface of the matrix from which they are extracted to the bulk solvent at higher temperatures. In addition, higher temperature reduces viscosity and surface tension, such that solvent can penetrate the pores of the matrix more easily (Dai & Mumper, 2010; Mottaleb & Sarker, 2012). Increased temperatures have the ability to easily disrupt the strong solute-matrix interactions caused by van der Waals forces, hydrogen bonding, and dipole attractions and remove the solute from matrix with ease (Mottaleb & Sarker, 2012). However, many phenolic compounds are easily hydrolyzed and oxidized. The use of high temperatures increases the chance of oxidizing phenolics which decreases their yield in the extracts (Dai & Mumper, 2010). For example, conventional extraction and concentration of anthocyanins is typically conducted at temperatures ranging from 20 to 50°C because temperatures >70°C have been shown to cause rapid anthocyanin degradation (Dai & Mumper, 2010). It is therefore preferable to start at 25°C below the thermal degradation point if the decomposition temperature of the target analyte is known (Mottaleb & Sarker, 2012).

2.3.1.2 Effect of pressure on extraction

A number of organic solvents used in extraction boil at relatively low temperatures, making the use of high temperatures impractical (Mottaleb & Sarker, 2012). One of the ways of circumventing this limitation is to exert sufficient pressure on the solvent during extraction. Doing this will realize all advantages of working at elevated temperature even with solvents of relatively low boiling point (Mottaleb & Sarker, 2012). Implementing extraction at elevated pressure also speeds up the overall extraction process since pumping the solvent through a matrix-packed bed cell is easier at elevated pressure (Mottaleb & Sarker, 2012). Pressurized solvent is forced into the

pores of the sample matrix, resulting in more close contact with the analytes in those areas. Hence, the combination of elevated temperature and pressure allows effective, rapid, complete, and efficient extraction (Mottaleb & Sarker, 2012).

2.3.1.3 Effect of pH on extraction

Nostro et al. (2000) compared sequential extractions with various solvents at room temperature to extraction in a water bath at 37^{o}C for 30 min with distilled water adjusted to pH 2.0 using hydrochloric acid (HCl), neutralized with sodium hydroxide (NaOH) prior to extraction with diethyl ether. The superior activity of this method was ascribed to the acidified aqueous extraction media (Ncube et al., 2008). In another study, Eddine et al. (2016) suggested that the total phenolic content, flavonoids and flavonols from leaves of *Matricaria pubescens* were the highest in extracts at pH 5, but gradually decreased at a higher pH. The ferric reducing antioxidant power (FRAP) assay and diammonium salt (ABTS) scavenging capacity was found to be the highest for extract at pH between 4 and 5 (Eddine, Djamila, & Redha, 2016).

2.3.1.4 Effect of sample-to-solvent ratio on extraction

The sample-to-solvent ratio influences the quantity and quality of extracted constituents (Ncube et al., 2008). In a study to identify the optimal conditions for extracting sugars from non-defatted soybean, a solvent to sample ratio of 5:1 at 25^{o}C or 50^{o}C for 15 min yielded more sugar. Others reported that solvent to sample ratios of 10 mL:1 g solvent (dry weight ratio) is ideal (Ncube et al., 2008). According to Tan & Ho (2011), a high solid-to-solvent ratio was favorable in extracting phenolic compounds from pegaga (*Centella asiatica*). A solid to solvent ratio of 1:15 (w/v) showed high yield of total phenolic content (TPC) and total flavonoid content (TFC) with a

value of 967.2 mg gallic acid equivalent (GAE)/100 g dry weight (DW) and 908.3 mg catechin equivalent (CE)/100 g dry weight (DW), respectively. A high solid-to-solvent ratio could promote an increasing concentration gradient, resulting in an increase of diffusion rate that allows greater extraction of solids by solvent, and an increased likelihood of bioactive components coming into contact with extracting solvent when the quantity of extraction solvent is higher, leading in higher leaching-out rates. However, active component yields will not increase further once an equilibrium is reached. A further increase in solid to solvent ratio to 1:20 (w/v) did neither increase TPC nor TFC yield significantly (P. W. Tan, Tan, & Ho, 2011).

2.3.1.5 Effect of extraction time on extraction

Extraction of molecules from biological materials by conventional techniques, such as simple maceration, is time consuming because bioactive components within pores and other structures within the cell membranes and sample matrices have limited accessibility (Falleh et al., 2012; Thermo Scientific, 2013). Thus, the longer the contact between solvent and materials results in higher phytochemical extraction until all possible metabolites have been extracted (Ncube et al., 2008). Increasing the extraction time, and combining this with elevated temperatures allows these metabolites to diffuse into the extraction solvent (Thermo Scientific, 2013). Falleh et al., (2012) reported that longer extraction duration yielded higher polyphenol content for the solvents used in the extraction of *Mesembryanthemum edule* shoots.

2.3.1.6 Effect of chemical composition and physical characteristics of samples on extraction

The chemical composition and physical characteristics of the samples also influence extraction of secondary metabolites. The solubility of phenolics is governed, to a large extent, by

the properties of the plant sample and the polarity of the solvents used (Dai & Mumper, 2010). Methanol tends to be more efficient in extracting lower molecular weight polyphenols and aqueous acetone with higher molecular weight flavanols (Dai & Mumper, 2010). The nature of the plant material, its origin, the degree of processing, the moisture content of the sample and the particle size all influence extraction yield (Ncube et al., 2008). Differences in water content may affect solubility of subsequent separation by liquid-liquid extraction. The secondary metabolic plant components should be relatively stable, especially if it is to be used as an anti-microbial agent, because most antimicrobial active components that have been found are not water soluble. In addition, plants may have different constituents and concentrations of constituents, depending on the climatic conditions in which they are growing (Ncube et al., 2008). Reducing the particle size through grinding or milling improves extraction of plant metabolites because of the larger surface area available for contact with the solvent. Efficient extraction requires a minimum particle size of less than 0.5 mm (Thermo Scientific, 2013). Homogenization in solvent and serial exhaustive extraction are some of the routinely utilized in solvent extraction techniques (Ncube et al., 2008).

2.3.2 Other extraction methods

Other conventional extraction methods include heating under reflux, maceration, and Soxhlet extraction which are inefficient and can pollute the environment because of the large volumes of organic solvents (sometimes hazardous compounds) and long extraction time (Dai & Mumper, 2010). In addition, degradation of thermolabile compounds, limited solvent choice, and need for further clean-up and concentration steps are other disadvantages of the Soxhlet method (Dai & Mumper, 2010; Krishnaiah et al., 2007). Other more recently developed methods include microwave-assisted extraction (MAE), ultrasound-assisted extractions, and techniques based on

use of compressed fluids as extracting agents. The latter are comprised of subcritical water extraction (SWE), supercritical fluid extraction (SFE), pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE) for extraction from plant materials (Dai & Mumper, 2010; Krishnaiah et al., 2007; Ncube et al., 2008; Sasidharan et al., 2011).

2.3.2.1 Microwave assisted extraction (MAE)

MAE presents an alternative laboratory-scale extraction method. Considerably faster, it requires less solvent and provides higher recoveries than the Soxhlet extraction (Dai & Mumper, 2010; Krishnaiah et al., 2007). Microwave waves interact with dipoles of polar and polarizable materials (e.g. solvents and sample) causing heating near the surface of the materials and heat transfer by conduction. Dipole rotation of the molecules induced by microwave electromagnetic fields disrupts hydrogen bonding, enhancing the migration of dissolved ions and promotes solvent penetration into the matrix (Azwanida, 2015; A. Gupta et al., 2012). Since sample components absorb microwave energy in accordance to their dielectric constants, MAE is a selective method that favors polar molecules and solvents with high dielectric constants such as acetone, methanol, and ethanol (Azwanida, 2015).

When plant material is immersed inside a microwave transparent solvent, the heat of microwave radiation directly reaches to the solid without being absorbed by the solvent, resulting in instantaneous heating of the residual moisture in the solid (A. Gupta et al., 2012). Heating causes the moisture to evaporate and creates a high vapor pressure that breaks the cell walls hold on the substrate, releasing the content into the solvent. Microwave transparent solvents like acetone proved to be best for extraction of phenolic compounds (A. Gupta et al., 2012). In a previous study, MAE was reported to have superior extraction efficiency, particularly for extraction of phenolics

and tannins, along with a significantly higher antioxidant activity when compared to hot aqueous and sonication extracts of the *Terminalia chebula* fruit (A. Gupta et al., 2012; Thomas, Tripathi, Kamat, & Kamat, 2012). MAE can be a closed vessel operation, performed under controlled/elevated pressure and temperature, or an open vessel operation, performed at atmospheric pressure. These technologies are called pressurized microwave-assisted extraction (PMAE) and focused microwave-assisted extraction (FMAE), respectively (A. Gupta et al., 2012). Solvents may be heated to much higher temperatures than their atmospheric boiling points in PMAE, which enhances both extraction speed and efficiency and is most suitable for volatile compounds, while FMAE offers increased safety in sample handling and can be used in the extraction of larger sample sizes (A. Gupta et al., 2012). The major disadvantage MAE is that it is usually performed at higher temperatures (110 - 150^{o}C), which may lead to the denaturation of thermolabile compounds (Krishnaiah et al., 2007).

2.3.2.2 Ultrasound-assisted extraction (UAE)

Ultrasound-assisted extraction (UAE), using ultrasound ranging from 20 to 2000 kHz (Azwanida, 2015), does not require complex instruments and is relatively low-cost. It can also be adapted to both small and large scale extractions in the phytopharmaceutical extraction industry (Dai & Mumper, 2010). Cell disruption, improved penetration and enhanced swelling, a capillary effect, and the hydration process have been proposed as mechanisms for the effectiveness of UAE (A. Gupta et al., 2012). The physical and chemical properties of the materials subjected to ultrasound are altered, disrupting the plant cell wall, facilitating the release of compounds and enhancing mass transport of the solvents into the plant cells (Azwanida, 2015). UAE has the advantage of reduced extraction time and solvent consumption. Application of UAE significantly

accelerated nicotine plant extraction, as each extraction step which requires 24 h for the conventional cold extraction technique, took less than 20 min to achieve the same extraction efficiency. UAE also yielded more flavonoids such as tectoridin, iristectorin B, iristectorin A, tectorigenin, iris-tectorigenin A, and total isoflavonesin less time when compared to maceration and Soxhlet extraction (A. Gupta et al., 2012). In addition, higher efficacy on phenolics was observed in *Cratoxylum formosum* extraction by ultrasound at 45 kHz, 50.33% ethanol, at 65°C for 15 min. However, the use of ultrasound energy greater than 20 kHz may result in the formation of free radicals from the phytochemicals (Azwanida, 2015).

2.3.2.3 Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), is a relatively new technology for extraction of phytochemicals under high temperature and pressure. In PLE, pressure is applied to allow the use of liquids as extraction solvents at temperatures greater than their normal boiling point. The combined use of high pressure (3.3 - 20.3 MPa) and temperature (40 - 200°C) provides faster extraction that requires only small amounts of solvents (e.g., 20 min using 10 - 50 mL of solvent in PLE compared to traditional extraction for 10-48 h and up to 200 mL). High temperature and pressure also improve analyte solubility and the desorption kinetics from the matrices, so that extraction solvents, including water, which show low efficiency in extracting phytochemicals at lower temperatures may be much more efficient at elevated PLE temperatures (Dai & Mumper, 2010). The use of water as the extraction solvent in PLE is called subcritical water extraction (SWE), in which water is heated up to 200°C and the change in the dielectric constant of water with temperature causes water to behave as an organic solvent (Dai & Mumper, 2010). Supercritical fluid extraction (SFE) with carbon dioxide as

supercritical fluid is a very attractive method for extraction because CO_2 is an inert, non-flammable, non-explosive, inexpensive, odorless, colorless, clean solvent that leaves no solvent residue in the product (Krishnaiah et al., 2007). The critical temperature of CO_2 at 30^{o}C is conducive for extracting thermolabile compounds. However, the usefulness of CO_2 is restricted by its inadequate solvating power for highly polar analytes; using an appropriate modifier such as ethanol can offset this limitation to some extent (Dai & Mumper, 2010; Kumari et al., 2017). Since SFE is performed in the absence of both light and air, degradation and oxidation processes are significantly reduced compared with other extraction techniques and are more environmentally friendly (Dai & Mumper, 2010).

In general, all the compressed fluid-base extraction techniques are more environmentally friendly and reduce the use of organic solvents (e.g., PLE), allowing extraction to be performed with non-polluting, non-toxic solvents, such as water (e.g., SWE) and supercritical CO_2 fluid (e.g., SFE) (Dai & Mumper, 2010). However, the need for high pressure in these techniques necessitates equipment that makes it so costly on an industrial scale that this often outweighs the technical benefits (Dai & Mumper, 2010). Other modern extraction techniques include solid-phase micro-extraction and surfactant-mediated techniques, which require less organic solvent, degrade samples efficiently degradation, eliminate additional sample clean-up and concentration steps before chromatographic analysis, and improve extraction efficiency, selectivity, and speed of extraction (Sasidharan et al., 2011). The ease of automation for these techniques also favors their usage for the extraction of plants materials (Sasidharan et al., 2011).

2.4 Isolation and characterization

Most plant extracts naturally contain a combination of bioactive compounds so that identification of the different metabolites after extraction is necessary (Sasidharan et al., 2011).

Several separation techniques achieve this, including thin layer chromatography (TLC), column chromatography, flash chromatography, Sephadex chromatography and high-performance liquid chromatography (HPLC). Other non-chromatographic techniques employ include immunoassays (with the use of monoclonal antibodies), phytochemical screening assays and Fourier-transform infrared spectroscopy (Sasidharan et al., 2011). For polyphenolic-rich extracts in particular, sequential extraction, liquid-liquid extraction, and solid phase extraction have been used for metabolite separation and isolation (Dai & Mumper, 2010). Due to high solvent costs and low recoveries, the classical liquid-liquid extraction is less attractive, especially since it is also tedious and time-consuming, (Dai & Mumper, 2010).

2.4.1 Countercurrent chromatography (CCC)

CCC is an all-liquid chromatographic technique, based on partitioning compounds between two immiscible liquid phases, a liquid stationary phase and a liquid mobile phase. Solutes are separated according to their partition coefficients based on their hydrophobicity between the two solvent phases (Sasidharan et al., 2011). Both CCC and centrifugal partition chromatography (CPC) are support-free and have the advantage of eliminating the irreversible adsorption of the sample unto the solid support, unlike conventional chromatography (Wei, Zhang, Xu, & Ito, 2001; Zhang, Liu, Qi, Li, & Li, 2015). However, CCC and CPC differ in terms of the hydrodynamic and hydrostatic mechanisms and, thus, operate based on different principles, so that the compounds separated by CCC and CPC using the same set of two-phase solvent systems are usually different. Using both CCC and CPC may result in enhanced separation efficiency (Zhang et al., 2015). Wei et al., (2001) performed a preparative high-speed CCC separation of a crude extract of tomato paste with a non-aqueous solvent system composed of n-hexane: dichloromethane: acetonitrile at

an optimum volume ratio of 10:3.5:6.5, which yielded 8.6 mg of lycopene at over 98.5% purity as determined by HPLC analysis. They reported that using CCC had the advantage of a greater capacity, with excellent sample recovery and the ability to introduce crude extracts directly into the column without need for purification, compared to high performance liquid chromatography (Wei et al., 2001).

2.4.2 Thin layer chromatography (TLC)

TLC is a simple, quick and inexpensive procedure useful for rapidly determining the component profile of a given mixture (Sasidharan et al., 2011). It is also used to support the identity of a compound in a mixture when the retardation factor (R_f) of the compound is compared with the R_f of a known compound. TLC is employed extensively for a number of reasons: it enables rapid analysis of herbal extracts, requiring minimal sample clean-up, provides qualitative and semi-quantitative information on the resolved compounds and also enables the quantification of chemical constituents (Doughari, 2012). Separation using TLC depends on several factors: solubility (metabolites more soluble in the solvent move up the plate faster), attractions between the compound and the silica (the more the compound interacts with silica, the less distance it moves on the plate) and the size of the compound (larger compounds move more slowly up the plate) (Genene & Hazare, 2017). Sonam et al., (2017) explored the scientific basis of the ethno-medicinal potential of different solvent extracts of *Reinwardtia indica* using TLC. They reported the presence of flavonoids in all extracts, alkaloids and tannins in acetone and methanol extracts and phenol in the methanol extract only by comparing the R_f of the crude extracts to that of phytochemicals suspected to be present in the plant (Sonam, Singh, & Saklani, 2017).

2.4.3 Column chromatography

Column chromatography is often labor-intensive and consumes solvent, but it provides more fractions for subsequent isolation and identification of pure substances in the fractionation of crude extracts (Dai & Mumper, 2010). Some of the more commonly utilized column sorbents are reverse phase C18 (RP-C18), Toyopearl, Sephadex LH-20 and less commonly, polyamide resin, commonly utilizing ethanol, methanol, acetone, and water and their combinations as eluents. The isolation of proanthocyanidins (which are condensed tannins) is routinely carried out by employing Sephadex LH-20 column. Methanol is more commonly used than ethanol to elute non-tannin compounds when using LH-20 column chromatography and acetone-water is a much better solvent than ethanol-water to elute procyanidins from the column, especially polymeric procyanidins (Dai & Mumper, 2010). Using seven solvent/solvent mixtures as eluates, Natarajan and Dhas (2015) reported between 24 and 38 fractions from each of the eluates in *Leptadenia reticulata* ethanolic extracts. Fractions from chloroform: methanol eluate had significant antidiabetic effect after 3 weeks of administration to diabetic rats, which prompted further purification and analysis using TLC and FTIR (Natarajan & Dhas, 2015).

2.4.4 High performance liquid chromatography (HPLC)

HPLC is a versatile and widely used technique for the isolation of natural compounds since the resolving power of HPLC is ideally suited for rapid processing of multicomponent samples even when the biologically active entity is present only as a minor component in the extract. Separation is based on the difference in migration rates of different compounds on a given particular column/stationary phase and mobile phase, with the extent of separation mostly being determined by the choice of stationary and mobile phases (Sasidharan et al., 2011). In general, the

identification and separation of phytochemicals can be accomplished using an isocratic system (a single unchanging mobile phase system) (Genene & Hazare, 2017). Altering the proportion of organic solvent to water with time may be desirable if more than one sample component is being studied and the components differ significantly in retention under the conditions employed from each other (Sasidharan et al., 2011).

Identification of compounds by HPLC is an essential part of the HPLC technique (Genene & Hazare, 2017). To identify any compound by HPLC, a detector must first be selected and set to optimal settings, before developing a separation assay. The parameters of this assay should be such that a clean peak of the known sample is observed from the chromatograph and the identifying peak should have a reasonable retention time and be distinct from extraneous peaks at the detection levels at which the assay will be performed. Ultraviolet (UV) detectors are popular among the detectors because they offer high sensitivity; and the majority of naturally occurring compounds have some absorbance at low wavelengths (190 - 210 nm). The high sensitivity of UV detection is a bonus when the quantity of the compound of interest is small (Genene & Hazare, 2017). Other HPLC detectors in use include fluorescence, electrochemical separation, radioactivity, conductivity, chemiluminescent nitrogen, chiral, refractive index and light scattering detectors. The choice of detector for separation may depend on the chemical nature of the analytes and on potential interferences, availability/cost of the detector, compatibility with the HPLC system and required limit of detection (Swartz, 2010). Sovrlić et al. (2014) reported that the HPLC analysis of *Daphne blagayana*, which has antimicrobial properties, resulted in the identification of phenols and flavonoids. They identified daphnetin, a dihydroxycoumarin, which is biologically active and may be responsible, possibly in synergy with other metabolites, for the antimicrobial properties of the plant (Sovrlić et al, 2014).

2.4.5 Immunoassay

Immunoassays use monoclonal antibodies (MAbs) against low molecular weight natural bioactive compounds and show high specificity and sensitivity for receptor binding analyses, enzyme assays, qualitative and quantitative analytical techniques. Enzyme-linked immunosorbent assays (ELISA) based on MAbs (which can be produced in specialized cells using hybridoma technology) are more sensitive in many cases than conventional HPLC methods (Sasidharan et al., 2011). Shoyama et al. (1999) utilized ELISA in the assay of forskolin, a labdane diterpenoid, isolated from *Coleus forskohlii*, which used in traditional medicine in India and a typical activator of adenylate cyclase. Forskolin was detected with a range of 0.003 - 5.97 µg/mg dry weight in the different plant parts, establishing that ELISA can be utilized for the assay of a wide range of forskolin contents and suggesting that it is possible to study many cultured plantlets containing a small amount of forskolin. In the same study, ELISA was utilized to measure the contents of Ginsenoside Rb 1 (GRb1), a major ginseng component with sedative effect and a unique immunostaining of ginseng organs in various ginsengs. They compared their findings to reports of HPLC analysis of ginsengosides and concluded that the ELISA and HPLC methods closely correlate over the range of concentrations found in the plant materials. In addition, ELISA was more sensitive than the TLC or HPLC methods and did not require pretreatment of crude extracts (Shoyama, Tanaka, & Fukuda, 1999).

2.4.6 Phytochemical screening

Phytochemical screening assay provides a simple, quick, and inexpensive way to determine the various types of phytochemicals in a mixture (Kumar et al., 2007; Parekh, Karathia, & Chanda, 2006; Sasidharan et al., 2011). There are numerous methods for phytochemical screening, with

sometimes more than one test for a particular group of compounds. For example, both the Wagner and Dragendorff's tests are used for alkaloids (Sasidharan et al., 2011). Other tests include the Borntrager's test for anthraquinones; the Shinoda test for flavonoids; the phenol test for phenols; the frothing or foam test for saponins; the Braemer's test for tannins; and the Salkowski test for terpenoids (Edeoga, Okwu, & Mbaebie, 2005; Kumar et al., 2007; Parekh et al., 2006; Sasidharan et al., 2011). After obtaining the crude extract from plant material, researchers employ a quick qualitative test to determine the type of phytochemicals in the extract mixture or fraction. Edeoga et al., (2005) utilized phytochemical screening for the assessment and comparison of secondary metabolites in ten indigenous medicinal plants from different families. The Salkowski test, phenol test, steroid test, and test for phlobotannins demonstrated that the leaves and stems were rich in alkaloids, flavonoids, tannins, and saponins (Edeoga et al., 2005).

2.4.7 Fourier-transform infrared spectroscopy (FTIR)

Researchers can identify the spectra of most unknown plant compounds by comparing them to a library of known compound spectra using FTIR. FTIR is a valuable tool for the identification and characterization of compounds or functional groups present in an unknown mixture of plants extract. Plant samples for FTIR can be prepared in different ways: for liquid samples it is easiest to place one drop of sample between two plates of sodium chloride, which then forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) and compressed into a thin pellet for analysis or may be dissolved in a solvent such as methylene chloride, placing the solution onto a single salt plate and evaporating the solvent, to leave behind a thin film of the original material on the plate (Sasidharan et al., 2011). FTIR was successfully used in the isolation and identification of antibacterial pentahydroxy flavones from

the seeds of *Mimusops elengi*, 2,3-dihyro-3,3'4'5,7-pentahydroxyflavone and 3,3',4',5,7-pentahydroxyflavone, which have strong inhibitory activity against gram positive and gram negative bacteria (Hazra, Roy, Sen, & Laskar, 2007)

2.5 Methods of extraction, isolation and characterization of secondary metabolites

2.5.1 Phenolics

Phenolics can be extracted from fresh, frozen or dried plant samples. Plant materials contain phenolics varying from simple (e.g. phenolic acids, anthocyanins) to highly polymerized substances (e.g. tannins) in different quantities and may be associated with other plant components like carbohydrates and proteins. Hence, there is no universal extraction procedure suitable for extraction of all plant phenolics (Dai & Mumper, 2010). Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials, often with different proportions of water (Bisignano et al., 2000; Lourens et al, 2004; Ncube et al., 2008; Nostro et al, 2000; Parekh et al, 2005; Rojas et al, 2006; Salie et al, 1996). Research suggests that methanol is more efficient in extracting lower molecular weight polyphenols while aqueous acetone works better with higher molecular weight flavanols. Ethanol, also a good solvent for polyphenol extraction, has the benefit of being safe for human consumption (Dai & Mumper, 2010).

In general, solvent extraction, microwave and ultrasound-assisted extractions, subcritical water extraction (SWE), supercritical fluid extraction (SFE), pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE) have all been successfully applied in the extraction of phenolic compounds from plant materials (Dai & Mumper, 2010). Plant crude extracts usually contain large amounts of carbohydrates and/or lipids while the concentration of the phenolics in

the crude extract may be low. To concentrate and obtain polyphenol-rich fractions before analysis, strategies including sequential extraction or liquid-liquid partitioning and/or solid phase extraction (SPE) based on polarity and acidity have been commonly used (Dai & Mumper, 2010; Lemberkovics et al, 2004; Vilegas et al, 1997).

Lipids are removed by washing the crude extract with non-polar solvents (e.g., hexane, dichloromethane, chloroform) while polar non-phenolic compounds such as sugars, organic acids, and other water-soluble compounds are removed through the SPE process (Dai & Mumper, 2010). This involves passing the aqueous sample through preconditioned C18 cartridges, washing the cartridges with acidified water to remove sugar, organic acids and other water-soluble constituents, and then eluting the polyphenols with absolute methanol or aqueous acetone. Further separation of phenolic compounds can be achieved by adjusting the pH of the sample and the pH and polarity of eluents (Dai & Mumper, 2010; Pinelo, Laurie, & Waterhouse, 2006). Column chromatography has been employed for fractionation of phenolic extracts, but the classical liquid-liquid extraction procedure is not as commonly used because it is tedious, very time-consuming, costly due to the amount of solvents required, and inefficient in its recovery (Dai & Mumper, 2010; Klejdus & Kubáň, 2000). An alternative, countercurrent chromatography (CCC), effectively fractionates the various classes of phenolic compounds (Dai & Mumper, 2010).

The chemical nature of the analyte, assay method, selection of standards, and presence of interfering substances influences the quantification of phenolic compounds in plant extracts. None of the methods used to determine total phenolics are perfect (Dai & Mumper, 2010; Singleton, Orthofer, & Lamuela-Raventos, 1999). They include the Folin-Denis method, Folin-Ciocalteu method, permanganate titration, colorimetry with iron salts, and ultraviolet absorbance. The Folin-Ciocalteu method is preferable to the other methods because it is simple, reproducible, and relies

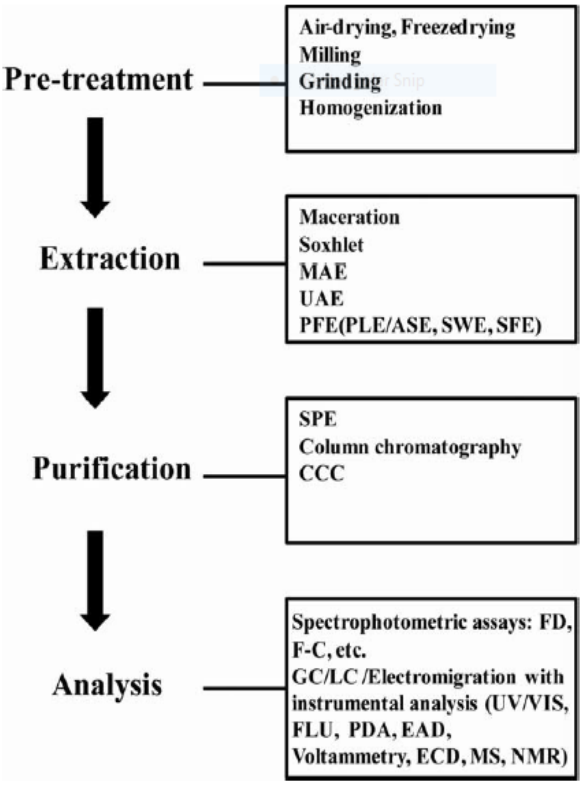
on the transfer of electrons from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes in alkaline medium to form blue complexes that are determined spectroscopically at approximately 760 nm with the antioxidant gallic acid being widely used as the comparison standard. Results are expressed in milligram of gallic acid equivalents per kilogram or liter of extract (Dai & Mumper, 2010).

The simplest assay for the quantification of anthocyanins is based on the measurement of absorption at a wavelength between 490 and 550 nm, where all anthocyanins show a maximum. This band is far from the absorption bands of other phenolics, which have spectral maxima in the UV range (Dai & Mumper, 2010). Different colorimetric methods measure total proanthocyanidin (condensed tannin) content in plant samples: the proanthocyanidin assay, the vanillin assay, and the dimethylaminocinnamaldehyde (DMCA) assay. However, these colorimetric methods for quantification of total proanthocyanidins are limited due to low yield due to the formation of side reaction products such as phlobatannins (Dai & Mumper, 2010; Schofield, Mbugua, & Pell, 2001). Recently validated, the methyl cellulose precipitable tannin assay precipitates condensed tannins from the sample by forming insoluble polymer-tannin complexes with methyl cellulose. Its concentration is determined by subtracting the phenolics contents in the sample, dtermined by measuring the absorbance at 280 nm before and after the methyl cellulose treatment (Dai & Mumper, 2010; Mercurio et al., , 2007; Sarneckis et al., 2006). This method quantified condensed tannins in grape extracts and red wine and was reported to be simple and robust, selective for condensed tannins, and correlating well with tannins measured by reverse phase HPLC (Sarneckis et al., 2006).

Modern high-performance chromatographic techniques combined with instrumental analysis are the “state of the art” for the profiling and quantification of phenolic compounds (Dai

& Mumper, 2010). Gas chromatographic (GC) techniques have been widely used especially for separation and quantification of phenolic acids and flavonoids (Stalikas, 2007). The major concern with this technique is the low volatility of phenolic compounds. However, HPLC currently represents the most popular and reliable technique for analysis of phenolic compounds (Lin & Harnly, 2007; Ruiz, Egea, Gil, & Tomas-Barberan, 2005). This is because various supports and mobile phases are available for the analysis of phenolics, and HPLC techniques offer a unique opportunity to simultaneously analyze all components of interest together with their possible derivatives or degradation products (Anttonen & Karjalainen, 2006; Dai & Mumper, 2010; Vrhovsek, Rigo, Diego Tonon, & Mattivi, 2004).

All phenols exhibit varying absorptions in either ultraviolet (UV) or ultraviolet/visible (UV/VIS) regions, making these the most common means of detection, in addition to liquid chromatography (LC), UV/VIS, photodiode array (PDA), and UV fluorescence detectors (Dai & Mumper, 2010). Other methods employed to detect phenolic compounds include the electrochemical detection (ECD) (Novak, Janeiro, Seruga, & Oliveira-Brett, 2008), voltammetry technique, on-line connected PDA and electro-array detection (EAD) (Harris et al., 2007; Mattila, Astola, & Kumpulainen, 2000), chemical reaction detection techniques, mass spectrometry(MS) and nuclear magnetic resonance (NMR) detection (Cavaliere et al., 2008; Christophoridou & Dais, 2009; Oh et al., 2008; Pawlowska et al., 2008).



Abbreviations: MAE, microwave-assisted extraction; UAE, ultrasound-assisted extraction; PFE, pressurized fluid extraction; PLE, pressurized liquid extraction; ASE, accelerated solvent extraction; SWE, subcritical water extraction; SFE, supercritical fluid extraction; SPE, solid phase extraction; CCC, countercurrent chromatography; FD, Folin-Denis method (FD), F-C, Folin-Ciocalteu method; GC, gas chromatography; LC, Liquid chromatography; FLU, fluorescence; PDA, photodiode array; EAD, electro-array detection; ECD, electrochemical detection; MS, mass spectrometric; NMR, nuclear magnetic resonance (Source: Dai & Mumper, 2010).

Figure 13: Strategies for preparation and characterization of phenolic samples from plant materials.

2.5.2 Terpenes

The optimal extraction of a particular terpene depends on its properties, but in general, the methods include the following steps: 1) breaking the plant cells to release their chemical constituents; 2) extracting the sample using a suitable solvent (or through distillation or the trapping of compounds); 3) separating the desired terpene from other undesired contents of the extracts that confound analysis and quantification; and 4) using an appropriate method of analysis (e.g. thin layer chromatography (TLC), gas chromatography (GC), or liquid chromatography (LC)) (Ding et al., 2004; Jiang et al., 2016). Depending on the polarity and size of the target terpenoid, the final three steps usually vary with several protocols for terpenes with specific characteristics.

However, these protocols can also be combined, with mixing and matching of specific steps to optimize extraction of the desired terpene.

Non-volatile, non-polar terpenes (like squalene) can be extracted using very non-polar organic solvents like hexane (Çitoğlu & Acıkara, 2012; Jiang et al., 2016; Mawa, Jantan, & Husain, 2016). Using silica as a stationary phase in chromatography is another good method to separate these terpenes from other compounds in the extract. Cyclized terpenes tend to elute faster than the corresponding non-cyclized terpenes with the same carbon number because they are more compact., although lower molecular weight compound terpenes generally elute more quickly (Jiang et al., 2016). Many monoterpenes and sesquiterpenes are volatile and require special methods for their analysis. Volatile terpenes in specific plant tissues can be studied directly using traditional extraction techniques (e.g., hydro-distillation, extraction by organic solvent) and newer techniques, such as solid-phase micro extraction or microwave-assisted extraction (Chemat, et al., 2013; Jiang et al., 2016). However, terpenes that are emitted to the atmosphere accumulate only temporarily in small concentrations in leaf aqueous and lipid phases and therefore require molecular trapping techniques for their analysis (Jiang et al., 2016; S. Wu et al., 2006).

Some terpenes can be modified by the addition of substituent groups (like the addition of hydroxyl groups or oxidation of a methyl group to the corresponding carboxylic acidic function) to increase their polarity (Çitoğlu & Acıkara, 2012; Jiang et al., 2016). Although this addition may allow for analysis by GC, care must be exercised because addition of many such modifications or a very polar group (e.g. glycosylation) will make it necessary to use LC instead of GC. Such moderately polar terpenes (like capsidiol) can be extracted using a chloroform partitioning method. To analyze terpenes that form esters with fatty acids or other acyl derivatives (like phytosterols), saponification can break those bonds, exposing the hydroxyl group on the terpenoid backbone

which can be used in derivatization, to allow for better volatility and analysis using GC (Jiang et al., 2016).

Naturally polar terpenes may contain multiple cyclized structures and various types of groups (hydroxyl groups, fatty acids, sugars, benzyl rings), which significantly increase the polarity of the molecule. They cannot be extracted using non-polar solvents or GC because they generally are unable to partition into the organic phase and be efficiently volatilized for GC analysis (Jiang et al., 2016). More polar solvents should be used for extraction (e.g. methanol) and a liquid chromatography-mass spectrometry (LC-MS) system would be more suitable for analysis. Steam distillation has also been successfully used for extraction of terpenes (Çitoğlu & Acıkara, 2012; Jiang et al., 2016). Whichever extraction method is chosen, the sample should be prepared by the aforementioned methods prior to extraction (Jiang et al., 2016).

2.5.3 Alkaloids

The major scheme for the isolation of alkaloids is based on the following steps: extraction of the plant raw material, separation of the total alkaloids from other extracted substances, separation of the alkaloids, and purification of the individual compounds (Al-Shahwany, Al-Hemiri, & Abed, 2013; Maldoni, 1991; Tolkachev, Shemeryankin, & Pronina, 1983). Alkaloids are not distributed uniformly throughout plant organs. At the end of the vegetation period, they accumulate in the overwintering parts, such as the root system, seeds, and bark, while, at the beginning of the vegetation period, they pass from the roots, seeds, and bark into the shoots and then also into the leaves (Tolkachev et al., 1983). The vegetation material can be selected after precipitation reactions using Mayer, Bouchardat and Dragendorff reagents to detect levels of

alkaloids present in the material (Maldoni, 1991). The material is then dried and ground to increase the surface area for extraction.

Extraction of alkaloids is usually achieved using the Soxhlet extraction method (Gonzales & Tolentino, 2014; Maldoni, 1991). Petroleum ether first extracts lipids and carotenoids, which are discarded (Maldoni, 1991; Meshram, Kumar, & Srivastava, 2015). The material is then subjected to an alcohol extraction (e.g., ethanol or methanol), and that extract is evaporated to obtain a crude alkaloids mixture which is treated with hydrochloric acid to remove the bases as soluble salts. The resulting acid solution is made basic with 15% NaOH, and the resulting free bases are extracted in chloroform (secondary and tertiary alkaloids). The residual aqueous solution may have water-soluble alkaloids (quaternary alkaloids), which can be determined by precipitation reagents (Reinecke or Mayer) in acid solution. The precipitate is dissolved in acetone: methanol (1:1 v/v) solution, and the solution is passed through a column of anion exchange resin in the chloride form, leaving a mixture of crude chlorides after evaporation. This method is used specifically for alkaloids with quaternary nitrogen (Maldoni, 1991).

To separate the individual alkaloid compounds from the mixture of bases, different chromatographic techniques may be employed (Maldoni, 1991; Tolkachev et al., 1983). Column chromatography can be performed using either a silica gel or neutral alumina; chloroform and chloroform with increasing amounts of methanol are the most common eluents, and the progress of the columns is monitored by TLC (Gonzales & Tolentino, 2014; Maldoni, 1991). Preparative-layer chromatography can also be used for isolation and purification of alkaloids, with detection using UV examination and chromogenic reactions, i.e., Munier reagent or potassium iodoplatinate (Maldoni, 1991). The use of GC has extended and increased the types of isolated alkaloids because of improvements in the equipment (more efficient columns and packing, operations at higher

temperatures) and improved working techniques that increase stability and volatility of the alkaloids. This is because the main requirement for an analyte in GC is that it should be volatile enough to be present in detectable amounts in the mobile phase (Stashenko & Martínez, 2014). However, many alkaloids have relatively high molecular weights, making their GC analysis almost impossible and very polar, thermolabile, ionic and high-molecular weight compounds are not compatible with regular GC analysis (Stashenko & Martínez, 2014). This can be overcome using HPLC, which separates and quantifies alkaloids effectively. HPLC is also used to examine the purity of alkaloids isolated by TLC and column chromatography (e.g., potato glycoalkaloids) (Maldoni, 1991). Finally, GC-MS has revolutionized the study of some alkaloids groups, i.e., arylalkylamines and quinolones (Maldoni, 1991; Meshram et al., 2015).

2.5.4 Saponins

As with any extraction process, the extraction solvent, extraction conditions and the properties of the plant material (such as composition and particle size) are the main factors determining process efficiency and the properties of the end product (Guclu-Ustundag & Mazza, 2007). To increase the efficiency of the extraction, sample drying, particle size reduction, and defatting (using a lipophilic solvent such as ethyl acetate or hexane) can pretreat the samples (Guclu-Ustundag & Mazza, 2007). The conventional extraction techniques for saponin extraction are maceration, Soxhlet, and reflux extraction (Guclu-Ustundag & Mazza, 2007; Moghimipour & Handali, 2015). Some green technologies that improve the extraction efficiency by reducing extraction time and solvent consumption/waste without compromising sample quality include microwave-assisted, ultrasound-assisted and accelerated solvent extraction, and pressurized liquid

extraction (Guclu-Ustundag & Mazza, 2007; Moghimipour & Handali, 2015; Vongsangnak, Gua, Chauvatcharin, & Zhong, 2004; J. Wu, Lin, & Chau, 2001).

Water, lower alcohols (methanol and ethanol), or aqueous alcohol mixtures have been widely used for extraction of saponins from plant matrices (Guclu-Ustundag & Mazza, 2007; Majinda, 2012). Other solvents for extraction of saponins include aqueous and alcoholic surfactant solutions, glycerin, and the addition of ammonia to solvents for glycyrrhizic acid extraction, based on chemical complexation of glycyrrhizic acid with ammonia, which results in an increased extraction yield (Choi, Chan, Leung, & Huie, 2003; Fang, Yeung, Leung, & Huie, 2000; Guclu-Ustundag & Mazza, 2007). The solvent choice for a particular application is based on the effect of the solvent on saponin yield and purity, and the composition of the saponin mixture. Differences in yield and composition of extracts arise from the varying solvent selectivities for individual saponins and other plant sample components (Guclu-Ustundag & Mazza, 2007; J. Wu et al., 2001).

2.5.5 Pigments

Considering the chemical and physical properties of secondary metabolites, most extractions are based on solubility of the chemicals of interest (Butnariu, 2016). Pigment solutions containing chlorophyll react easily with oxygen when exposed to light, resulting in the formation of activated oxygen species (Benoıˆt, 2002). For this reason, care should be taken during extraction and analysis. Independent of solvent and extraction method, successful quantitative extraction requires thorough tissue maceration and disintegration,, and repeated extraction under continual grinding (Petering, Wolman, & Hibbard, 1940; Rajalakshmi & Banu, 2013). Acetone and alcohol are often used in the extraction of chlorophylls (Petering et al., 1940; Rajalakshmi & Banu, 2013), while the lipophilic carotenoids are usually extracted using non-polar solvents (Butnariu, 2016;

Delgado-Vargas et al., 2000). On the other hand, anthocyanins are highly soluble in water, requiring polar solvents (Martín et al., 2017; Oancea & Oprean, 2011), and betalains can also be water-extracted, although, in most cases, the use of methanol or ethanol solutions (20 - 50%) is required to complete extraction (Azeredo, 2009; Delgado-Vargas et al., 2000).

A variety of modern techniques have been developed as alternatives to solvent extraction, including SPE, CCC, MAE, SFE and high hydrostatic pressure among others. They have the advantage of increasing selectivity or specificity of extraction, improving recovery of pigments and greater automation (Martín et al., 2017). Absorbance spectroscopy, however, appears to be the simplest way to identify major pigments present in a mixture (Azeredo, 2009; Benoıˆt, 2002; B. Simpson et al., 2012). The absorbance spectrum reflects the organization of the conjugated double bond system and constitutes the fingerprint of pigments. Once the absorbance spectra of the respective pigments are identified by comparing them to that of pure pigments, it is possible to estimate their respective concentrations using a set of equations. However, this process becomes much less efficient if the number of pigments in the extract is higher than three (Benoıˆt, 2002). To this end, chromatographic separation using column or TLC can be used to analyze a pigment mixture further (Benoıˆt, 2002; Butnariu, 2016; Delgado-Vargas et al., 2000). Non-chromatographic methods, such as phase partition using petroleum ether and aqueous methanol (90%) have also been used in the separation of carotenoids (Delgado-Vargas et al., 2000).

The HPLC technique has become the method of choice for chromatographic separation, rapid quantification, and identification of pigments (Azeredo, 2009; Benoıˆt, 2002; Delgado-Vargas et al., 2000; Martín et al., 2017; Rivera & Canela-Garayoa, 2012; B. Simpson et al., 2012). In general, HPLC shows greater sensitivity, resolution, reproducibility, and ability to use inert conditions and speed of analysis than traditional methods (Delgado-Vargas et al., 2000). Diode

array detection (DAD) and MS or tandem mass spectrometry (MS/MS) are among the most widely used detection techniques (Azeredo, 2009; Delgado-Vargas et al., 2000; Martín et al., 2017). DAD permits detection at several wavelengths and simultaneous identification by UV- spectral analyses, while also providing information about the purity of compounds (Delgado-Vargas et al., 2000). MS/MS is particularly suited for structure elucidation and compound identification (Martín et al., 2017). Other techniques used in pigment analysis include infrared spectroscopy, Raman spectroscopy, NMR spectroscopy (Azeredo, 2009; Butnariu, 2016; Delgado-Vargas et al., 2000; Rivera & Canela-Garayoa, 2012), FTIR (B. Simpson et al., 2012) and LC-MS (Rivera & Canela-Garayoa, 2012). Additional investigations can be conducted on crude or purified plant extracts to further elucidate the functional properties of the extracts and the mechanisms of action by which they exert their physiological effects. One very important procedure for analysis of plants, suspected to have anti-carcinogenic properties, is cell culture analysis.

Table 1: Specific examples of medicinal plants, their methods of extraction and isolation and relevant remarks

Plant	Method of extraction	Method of identification	Compounds isolated	General remarks	References
Moringa oleifera leaves	Extraction with methanol	HPLC	Niaziminin	Inhibitor of tumor promoter induced Ebstein Barr Virus (EBV) activation	Murakami, Kitazono, Jiwajinda, Koshimizu, & Ohigashi, 1998
Moringa oleifera leaves	Ethanol extraction of fresh leaves	Bioassay directed fractionation using HPLC	Niazinin A, niazinin B, niazimicin and niazinin A + B	Blood pressure lowering effect in rats mediated possibly through a calcium antagonist effect	Anwar, Latif, Ashraf, & Gilani, 2007; Faizi et al., 1994; Gilani et al., 1994
Moringa oleifera seeds	Homogenization with 300 mL hexane x 3 every 24 h	Fractionation and HPLC	Niazirine and niazimicine	Vibriocidal	Albuquerque Costa et al., 2017
Moringa oleifera pods and seeds	Extraction with warm (60ºC) 70% methanol (in water) for 3 h twice	HPLC	Niazimicin and niaziminin A and B	Hypotensive and negative chronotropic effects in anesthetized rats as well as vasodilation	Jansakul, C; Wun-noi, A; Croft, K; Byrne, 1997
Moringa oleifera leaves and bark	Homogenization in 60 mL of ethanol in soxhlet apparatus for 6-8 h	GC-MS	Eugenol, D-allose, isopropyl isothiocynate, hexadecanoic acid	Anti-cancer properties	Al-Asmari et al., 2015
Moringa oleifera leaves and seeds	Homogenization in 100 mL of solvent at room temperature for 30 min. Filtration followed by centrifuging of filtrate at 10,000 rpm for 15 min at 4ºC	Ion-exchange chromatography and gel filtration chromatography	Small protein with a molecular mass of 23.6 kDa belonging to the Kunitz type of serine protease inhibitor family	Serine protease inhibitor	Bijina et al., 2011
Ocimum gratissimum flowering aerial parts	Steam distillation, with hexane and dichloromethane extraction of the distillate	GC-MS	Eugenol	Anti-microbial and antioxidant properties	R. K. Joshi, 2013
Ocimum gratissimum leaves	100 g powdered sample extracted with acetone, with filtration of the extract and evaporation after concentration in a vacuum at 40ºC	GC-MS	Volatile oils (especially thymol)	Anti-microbial activity	Yuvarajan, Mekala, Srinivasan, & Natarajan, 2014
Ocimum gratissimum aerial parts	Steam distillation followed by extraction of distillate with dichloromethane	NMR, IR and GC-MS	Eugenol	Anti-fungal activity	Faria et al., 2006; Prabhu, Lobo, Shirwaikar, & Shirwaikar, 2009
Ocimum gratissimum leaves	Steam distillation with petroleum ether extraction of the distillate	NMR supported by GC/MS	Eugenol	Anti-bacterial activity	Nakamura et al., 1999
Ocimum gratissimum leaves	Steam distillation using a steam generator	GC-MS	Eugenol	Hypotensive effect through calcium channel blocking mechanism	Interaminense et al., 2007
Ocimum gratissimum plant materials	Hydro-distillation of semi-dried samples in a Clevenger like apparatus	GC	Essential oil components-eugenol, Z-β ocimene, p-cymene and thymol	Anti-fungal and free radical scavenging activity	Dambolena et al., 2010
Ocimum gratissimum whole plant samples	Hydro-distillation of whole plant samples using modified Clevenger apparatus	GC	Eugenol, Z-β ocimene	Fumigant and repellant effects against food insect pests	Ogendo et al., 2008

Momordica charantia seeds	Extraction with 60 mL of phosphate-buffered saline PBS overnight, followed by centrifugation at 15000 g for 15 min	LC-MS/MS	TI, a 68-amino acid polypeptide of 7 kDa	Anti-glycemic effect	Lo, Ho, Lin, Li, & Hsiang, 2013
Momordica charantia fruit	Percolation with 80% ethanol followed by concentration in vacuum, suspension in 95% ethanol and alkalization with KOH to around pH 10	FTIR, MS and 1H-NMR	Charantin	Anti-microbial activity	Patel Subhashchandra , Patel Tushar, Parmar Kaushal, Bhatt Yagnesh, Patel Yogesh, 2009
Momordica charantia vines and leaves	Extraction with ethanol under conditions of reflux and concentrated under vacuum	Silica gel and Sephadex LH-20 GS MS	Kuguacins	Anti-HIV 1 activity *in vitro*	Chen et al., 2009
Momordica charantia seeds	Homogenization of pulverized seeds with 10 mM sodium phosphate and treatment of supernatant with acetone	GC, SDS-PAGE and MS	MCP 30 containing 2 highly related ribosome-inactivating proteins, α-momorcharin and β-momorcharin	Induces apoptosis in prostatic intraepithelial neoplasia and prostate cancer cell lines in vitro and suppresses PC-3 cell line growth in vivo with no effect on normal prostate cells	Xiong et al., 2009
Momordica charantia seeds	Homogenization with ice-cold 0.15 M followed by centrifugation at 12 000 x g for 30 min	SDS-PAGE and GC	MAP 30, a basic protein of about 30 kDa	Inhibition of HIV-1 infection and replication	Lee-Huang et al., 1990
Momordica charantia seeds	Extraction with 0.2 M NaCl containing 0.005 M sodium phosphate buffer, overnight, followed by centrifugation at 11000 g for 20 min.	GC	2 proteins, an inhibitor with MW 23000 and a haemagglutinating lectin with MW 115000 consisting of 4 subunits of MW 30500, 29000, 28500 and 27 000.	Potent inhibition of protein synthesis and hem agglutination	Barbieri, Zamboni, Lorenzoni, & Montanaro, 1980
Prunus africana bark and leaves	Sequential extraction with hexane, dichloromethane and methanol	GC and GC-MS	β-sitosterol-3-O-glucoside and β-amyrin	*In vitro* cytotoxic and apoptotic activity on cancer cells	Maiyo, Moodley, & Singh, 2016
Prunus africana bark	Homogenization in n-hexane, followed by percolation with n-hexane, dichloromethane, methanol and water	Silica gel GC and HPLC	N-butylbenzene sulfonamide (NBBS)	Antiandrogenic activity	Schleich, Papaioannou, Baniahmad, & Matusch, 2006b
Prunus africana stem bark	Homogenization in n-hexane and percolation with n-hexane, dichloromethane, methanol, methanol-water 1:1 and water at room temperature and evaporated to dryness under vacuum at 30^0C.	Silica gel GC and HPLC	Methyl 2,4-dihydroxy-3,6-dimethylbenzoate (artraric acid), ethyl 2,4-dihydroxy-3,6-dimethylbenzoate, n-propyl 2,4-dihydroxy-3,6-dimethylbenzoate and n-butyl 2,4-dihydroxy-3,6-dimethylbenzoate	Antiandrogenic activity	Papaioannou et al., 2009; Schleich, Papaioannou, Baniahmad, & Matusch, 2006a
Prunus africana bark	Ethanolic extraction of dried plant samples (30% w/v)	-	β-sitosterol and campesterol	Regulation of prostate cancer *in vitro* and *in vivo*	Shenouda et al., 2007

2.6 Prostate cancer

Prostate cancer is a significant cause of morbidity and mortality worldwide and with an estimated 232,000 new cases and 33,000 deaths in 2016, it is the most frequently diagnosed cancer and second most frequent cause of cancer deaths in U.S. males (Q. Chen et al., 2006; Scher et al., 2015; Shenouda et al., 2007). It is caused by unregulated prostate cell division, which leads to abnormal growth and potential metastases (Komakech et al., 2017). Approximately 9 - 11% of men will be diagnosed with prostate cancer in their lifetime. Since prostate cancer progresses slowly, is a target candidate for chemo-prevention (Komakech et al., 2017; Shenouda et al., 2007). Chemoprevention and chemotherapy, including the administration of one or more naturally occurring anti-prostate cancer agents have been identified as approaches by which the prevalence of prostate cancer can be reduced, suppressed, or reversed (Komakech et al., 2017).

The etiology of prostate cancer is still under research, but a strong correlation exists with age, with increased relative risk for individuals with a family history (Cunningham & You, 2015). African-American men are at significantly higher risk, and environmental risk factors, which have been quantified in adoption studies at 4.8%, include the consumption of long chain polyunsaturated fatty acids found in smoked or over-cooked fish, vitamin D deficiency in people with reduced tanning potential, smoking and dietary factors such as intake of red meat (Castillejos-Molina & Gabilondo-Navarro, 2016; Cunningham & You, 2015). The most consistent correlation for prostate cancer prevention is a significant consumption of fruits, vegetables, and whole grains, which are potential sources of phytoestrogens (Shenouda et al., 2007). Phytoestrogens are also found in many plants, which are commonly used in traditional medicine (Azadbakht, 2007; B. Joshi, Sah, et al., 2011; Shenouda et al., 2007).

Observations at autopsy indicate that early prostate cancer evolves in a multifocal pattern within the gland. Although the treatment of localized disease has significantly improved, once prostate cancer progresses to the periprostatic space by penetration and perforation of the prostate capsule and/or by invasion of the perineural spaces to the lymph nodes, few therapeutic options with limited durability are available (Thalmann et al., 2000). Androgen ablation therapy, either by chemical or by surgical castration, is the last line of defense and has been gold standard for the treatment of advanced prostate cancer since Charles Huggins first pioneered this approach in 1941 (Q. Chen et al., 2006). Although the initial response is a dramatic reduction and palliation of symptoms, prostate cancer eventually progresses to a lethal, hormone-refractory stage, for which no curative therapies currently exist (Q. Chen et al., 2006; Thalmann et al., 2000). It has become clear that the progression from the AS stage to the androgen-independent (AI) or hormone-refractory stage is the critical step that determines whether an individual's disease can be cured (Q. Chen et al., 2006).

2.6.1 Role of medicinal plants in cancer treatment and management

In the last decades, several plants have been confirmed to contain chemo-preventive and therapeutic agents for various cancers including prostate cancer (Komakech et al., 2017). In addition, over 60% of currently used anti-cancer agents are estimated to be derived from natural sources (Komakech et al., 2017). Among plants identified to possess significant anti-prostate cancer potential is *Prunus africana* (Komakech et al., 2017; Shenouda et al., 2007).

2.6.1.1 *Prunus africana*

African cherry (*Prunus africana* (Hook.f.) Kalkman) is a long-lived monoecious evergreen tree found in Afromontane forests growing from Ethiopia in the north to South Africa in the south, from Nigeria in the west to Madagascar in the east (Kadu et al., 2012; Nyamai et al., 2015). It is also called *Pygeum africanum* (Hook. f.), bitter almond, African prune, and red stinkwood. The African cherry belongs to Rosaceae family and is a medium to large tree with a spreading crown of 10 to 20 m when mature, becoming quite huge in forest regions, but is usually medium-sized in gardens (Das, 2017; Jeruto et al., 2011). Besides its use for timber, it is employed as a medicinal plant, whose leaves, roots and bark are used in traditional medicine in Africa. This is not surprising since various bioactive substances with anti-inflammatory, anti-cancer, and anti-viral properties have been identified in different members of the genus *Prunus* (Jeruto et al., 2011; Kadu et al., 2012; Ngule, Ndiku, & Ramesh, 2014; Schleich et al., 2006b).

P. africana has great medicinal value, as it is used traditionally in the treatment of a wide range of clinical conditions such as chest pain, fever, malaria, stomach ache, diarrhea, allergies, kidney disease, epilepsy, arthritis, hemorrhage, hypertension, sexually transmitted diseases, and benign prostatic hyperplasia (BPH) (Das, 2017; Jeruto et al., 2011; Komakech et al., 2017). *P. africana* extracts have also been reported to have anti-bacterial and anti-fungal activity, and the bark is used for liver problems and constipation (Jeruto et al., 2011; Madivoli et al., 2018; Ngule et al., 2014). However, the tree is predominantly used by brewing bark extracts to treat BPH, a disorder of the prostate, common in older men. The extracts significantly improve urologic symptoms having anti-proliferate and apoptotic effects on the prostate (Kadu et al., 2012; Komakech et al., 2017). *P. africana* is one of the many medicinal plants containing large quantities of bioactive compounds that can be used for prostate cancer management. Its use in cancer

chemotherapy and chemoprevention has been discussed in a few peer reviewed journal articles (Boulbès et al., 2006; Komakech et al., 2017; Roell & Baniahmad, 2011; Schleich et al., 2006a; Shenouda et al., 2007).

2.6.1.2 Phytochemical constituents of *Prunus africana*

The pharmacological efficacy of *P. africana* is believed to be due to various known and unknown compounds (Kadu et al., 2012; Komakech et al., 2017; Nyamai et al., 2015; Shenouda et al., 2007). Among the known compounds, three groups are of great importance: (1) phytosterols, especially β-sitosterol, with its anti-inflammatory properties that inhibit the swelling of the prostate gland (Kadu et al., 2012; Komakech et al., 2017; Nyamai et al., 2015); (2) pentacyclic triterpenoids that are anti-edematous by inhibiting glucosyl transferase activity (Kadu et al., 2012; Nyamai et al., 2015); and (3) ferulic acid esters, or their chemical derivatives, which inhibit angiogenic pathways, thus preventing the growth of new blood vessels from preexisting vessels, as well as the growth and spread of prostate cancer (Komakech et al., 2017; Nyamai et al., 2015).

Other secondary metabolites from this species include β-sitostenone, campesterol, n-tetracosanol and n-docosanol, myristic acid, linoleic acid, lauric acid, methyl myristate, methyl laurate, methyl linoleate, lup-20(29)-en-3-one, palmitic acid, (3.β.,5.α)- stigmast-7-en-3-ol, stigmastan-3,5-diene, α-tocopherol, cyanidin-O-galactoside, cyanidin-3-O-rutinoside, procyanidin B5, and robinetinidol-(4-α-8) catechin-(6,4-α) robinetinol (Mugaka et al., 2013; Nyamai et al., 2015). Phytochemicals from *P. africana*, suggested for the treatment of prostate cancer, include ursolic acid, oleanolic acid, β-amyrin, atraric acid (AA), N-butylbenzene-sulfonamide (NBBS), β-sitosterol, β-sitosterol-3-O-glucoside, ferulic acid, and lauric acid.

Previous studies suggested that *P. africana* extracts target fast dividing prostate cancer cells by impairing mitosis or causing target cells apoptosis (Komakech et al., 2017).

P. africana's anti-prostate cancer phytochemicals can be divided into three major categories based on their targets and pharmacological effects: (i) phytochemicals that kill the tumor cells through apoptotic pathways, a common mode of action of chemotherapeutic agents against a wide variety of cancer cells, (ii) phytochemicals that alter the signaling pathways required for the maintenance of prostate cancer cells, and (iii) phytochemicals that exhibit strong antiandrogenic and antiangiogenic activities (Komakech et al., 2017).

2.6.2 Other selected medicinal plants

In addition to *P. africana*, other medicinal plants were included, of which most have been used in traditional medicine and some were known to have anti-cancer properties.

Pausinystalia yohimbe bark is a traditional treatment for sexual disorders (i.e., erectile dysfunction) in addition to treatments of dementia, diabetic complications (e.g., neuropathy), exhaustion, fevers, insomnia, leprosy, low blood pressure, obesity and syncope and is currently used as a dietary supplement to enhance athletic performance and weight loss. Yohimbine, an alkaloid in *P. yohimbe* has also been reported to inhibit the proliferation of PC-2 and PC-3 cell lines via apoptosis induction (Shen et al., 2008). Most of its effects are attributed to the presence of alkaloids (yohimbine in particular) in the bark (Anadón, Martínez-Larrañaga, Ares, & Martínez, 2016).

Moringa oleifera is an important food commodity, often referred to as the "natural nutrition of the tropics". In many countries, particularly in India, Pakistan, the Philippines, Hawaii, and many parts of Africa, the population consumes the leaves, fruit, flowers and immature pods of this

tree as a vegetable. Indigenous south Asian medicine treats inflammation and infectious diseases, cardiovascular, gastrointestinal, hematological, and hepatorenal disorders with its root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil. Leaf and bark extracts have been reported to markedly reduce (70-90%) colony formation as well as cell motility, and increase apoptosis in MDA-MB-231(breast) and HCT-8 (colorectal) cancer cell lines (Al-Asmari et al., 2015). The leaf extracts have also been reported to be effective against human B-lymphocyte plasmacytoma (U266B1) cell line, cervical cancer cells (HeLa cell line), hepatocarcinoma (HepG2), colorectal adenocarcinoma (Caco-2), and breast adenocarcinoma (MCF-7) cell lines among others (Khor, Lim, Moses, & Abdul Samad, 2018). The seeds of *Moringa* are considered to be anti-pyretic, and reported to show anti-microbial activity due to the synergistic effects of several bioactive compounds, including sterols and phenols (Anwar et al., 2007).

Momordica charantia, also known as bitter melon, karela, balsam pear, or bitter gourd, is a popular plant used for the treatment of diabetes-related comorbidities amongst the indigenous populations of Asia including India, South America, the Caribbean and East Africa (Joseph & Jini, 2013). It is also valued for its anti-viral, anti-bacterial and anthelmintic properties in treating a number of infections and diseases; its potential as antifertility agent in male animals has also been extensively evaluated (Odusoga et al., 2014). The leaves in particular have been found to possess hypoglycemic, anti-tumor and immunity enhancing properties attributed to the synergistic activity of several compounds, including triterpenoids and saponins (Li, Lin, Yang, Wang, & Qiao, 2015). *M. charantia* extracts and its monomer components have shown strong anticancer activity against various tumors such as lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin cancer and prostate cancer (Jia, Shen, Zhang, & Xie, 2017).

2.7 Cell culture

Cells provide a useful model for studying physiological processes and assessing the toxicity and therapeutic potential of compounds (Invitrogen, 2010). Researchers use cell culture systems for basic research and a wide range of clinical in vitro studies (Goodspeed et al., 2016), testing the potency and cellular toxicity of plant extracts with potential medicinal benefits. This includes their use in cancer biology research, where cell cultures have provided insight in the workings of cancer cells and the therapies for prevention and treatment (Ferreira et al., 2013). One of the initial steps in the development of chemotherapeutic agents involves testing these agents in vitro using human cancer cell lines as experimental models (Lovitt et al., 2014; Wright Muelas et al., 2018).

Cell culture techniques involve the removal of cells from a plant or animal and their subsequent growth in an artificially favorable environment (Invitrogen, 2010). The cells may be removed from the tissue directly and separated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established. Researchers throughout the world study cell cultures extensively. The techniques required to allow cells to grow and be maintained outside the body have been developed throughout the 20th century (Langdon et al., 2004). The *in vitro* cultivation of organs, tissues, and cells is collectively known as tissue culture, and is used in many areas of science (Langdon & Macleod, 2011; Unchern, 1999).

2.7.1 Brief history of cell culture analysis

Cell cultures can be traced back to Sir William Harvey in the 16th century, who observed that a piece of myocardium kept in the palm of his hand covered in his own saliva could remain contractile for extended periods of time (Allen et al., 2005). In 1911, Warren Lewis began studies to identify factors required for cell growth in culture (Langdon & Macleod, 2011). Further investigation revealed that cultured cells can be propagated in a defined mixture of small molecules

supplemented with serum proteins. Soon, researchers recognized that different cell lines require differing mixtures of hormones and growth factors to grow in a serum-free medium (Allen et al., 2005). The 1950s and 1960s were marked by detailed studies by a host of investigators, defining the nutritional requirements of cells in culture leading to the development of the media in current use (Langdon & Macleod, 2011). By the middle of the 20th century, Earle and colleagues isolated single cells of the L cell line and demonstrated that they form clones of cells in tissue culture. Gey and colleagues established probably the best-known continuous cell line, derived from a human cervical carcinoma, which later became the HeLa cell line (Allen et al., 2005; Langdon & Macleod, 2011).

A primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate or reach confluence. These then must be sub-cultured by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. After the first sub-culture, the primary culture becomes known as a cell line or sub-clone (Invitrogen, 2010; Langdon & Macleod, 2011). Cell lines derived from primary cultures have a limited life span. As they are sub-cultured, the cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population (Invitrogen, 2010). Either cell lines exist as adherent cultures or they grow in "suspension." Most cell types will adhere to a "substrate" such as plastic or glass and proliferate as a monolayer, in contrast to suspension cultures, which do not attach to a substrate and will grow floating in a medium (Langdon & Macleod, 2011).

Usually, normal usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence; their cell lines are known as finite. However, some cell lines become immortal through a process called

transformation, which can occur spontaneously or can be chemically or virally induced by reactivating telomerase and inactivating the p53 and retinoblastoma (RB) tumor suppressor pathways (Invitrogen, 2010; Vela & Chen, 2015). When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line (Hull et al., 2018; Invitrogen, 2010).

2.7.2 Cancer cell lines

Cancer cell lines are useful tools in genetic research because they provide a versatile model of the biological mechanisms involved in cancer. The use of cancer cell lines improved the knowledge of deregulated genes and signaling pathways in disease, which is crucial to study of genetic, epigenetic and cellular pathways, of proliferation deregulation, apoptosis and cancer progression, to define potential molecular markers, and to screen and characterize cancer therapeutic agents (Ferreira et al., 2013). Numerous cell lines with their unique properties and characteristics are currently available for *in vitro* study of different diseases, especially cancer, (Cunningham & You, 2015).

For several reasons, the use of cell lines is particularly advantageous in cancer research. Cell lines are easy to handle and manipulate genetically/epigenetically by using demethylation agents, small interfering ribonucleic acid (siRNA), expression vectors, and can be pharmacologically manipulated through cytostatics (cell growth inhibitors). Cell lines are very homogenous providing a homogeneous population of tumor cells for easier analysis rather than the heterogeneous solid tumors. However, to mimic *in vivo* tumor characteristics (including heterogeneity) as closely as possible, a panel of cancer cell lines representative of the heterogeneity observed in the primary tumors can be used. Cancer cell lines are pure populations of tumor cells

and have a high degree of similarity with the initial tumor, while being readily accessible to the scientific community. Because of this, results of experiments using correct conditions are easily reproducible (Ferreira et al., 2013). In addition, there is a substantial number and variety of cancer cell lines available.

Despite these many advantages, there are disadvantages in using cancer cell lines. Some cell lines may be cross contaminated with HeLa cells. In addition, genomic instability may cause differences between the original tumor and the respective cell line. Culture conditions required to maintain the cell lines could change the morphology, the gene expression, and several cellular pathways. Infections with mycoplasma could also alter culture properties (Ferreira et al., 2013). Furthermore, it is difficult to establish long-term cancer cell lines of certain types of tumors including prostate cancer tumors (Ferreira et al., 2013; Vela & Chen, 2015). The limited number of cell line models for prostate cancer research stems from the difficulty in propagating prostate cancer cells in vitro for extended periods. Many investigators have been able to generate only seven cell lines that were previously available through public cell line repositories, but these do not represent the spectrum of clinical disease. New cell lines, which demonstrate the commonly observed clinical phenotypes, are clearly needed (Vela & Chen, 2015).

A cancer cell line is more valuable in an *in vitro* model if it is properly molecularly characterized. Molecular characterization allows for a more detailed study of the genetic/epigenetic events and cellular pathways associated with oncogenesis, where the micro-evolutionary progression of the tumor is understood due to the molecular profiling in different subcultures and the molecular patterns associated with resistance/sensitivity to anti-cancer drugs are unveiled. The molecular characterization of cell lines can be done using different and complementary platforms: cytogenomic, genomic, epigenomic, transcriptomic and proteomic

platforms. The anchorage independency (soft agarose assay) of cancer cell lines should also be characterized, which is important when studying the interaction of drugs with the cells. In addition, researchers should molecularly profile cell lines to disclose alterations in the cell cycle regulators and other signaling molecules because this is useful for targeting anti-cancer drugs for cell cycle defects. The molecular profiling of cancer cell lines also enables easier assessment of cancer types and subtypes, defining which cell lines are more suitable for different investigations, and thereby enhancing the screening and study of anti-cancer drugs (Ferreira et al., 2013).

There are many prostate cancer cell lines in use today, most of which have been established from metastatic deposits (Pamela J. Russell & Kingsley, 2003). The LNCaP cell line was isolated from a subclavian lymph node metastasis of prostate cancer. The cell line retains several key markers including: prostate specific antigen (PSA), prostate specific membrane antigen (PSMA) and the androgen receptor (AR) (Q. Chen et al., 2006). Through passage and hormonal manipulation in vivo, the lineage-related LNCaP sublines have resulted in a series of cells that mimic the progression of prostate cancer from the original androgen sensitive (AS) LNCaP cell line to the androgen-independent (AI) C4-2 and C4-2B cell lines (Q. Chen et al., 2006; Thalmann et al., 2000).

An AI cell line, C4-2, reproducibly and consistently follows the metastatic patterns of hormone-refractory prostate cancer by producing lymph node and bone metastases when injected either subcutaneously or orthotopically in either hormonally intact or castrated hosts. This model permits the study of factors that determine the tropism of prostate cancer cells for the skeletal microenvironment (Thalmann et al., 2000). These C4-2 cells have a doubling time of about 48 h, are androgen independent, express an androgen receptor, metastasize to lymph nodes, and produce PSA (Cunningham & You, 2015; Pamela J. Russell & Kingsley, 2003). The AI C4-2 cell line

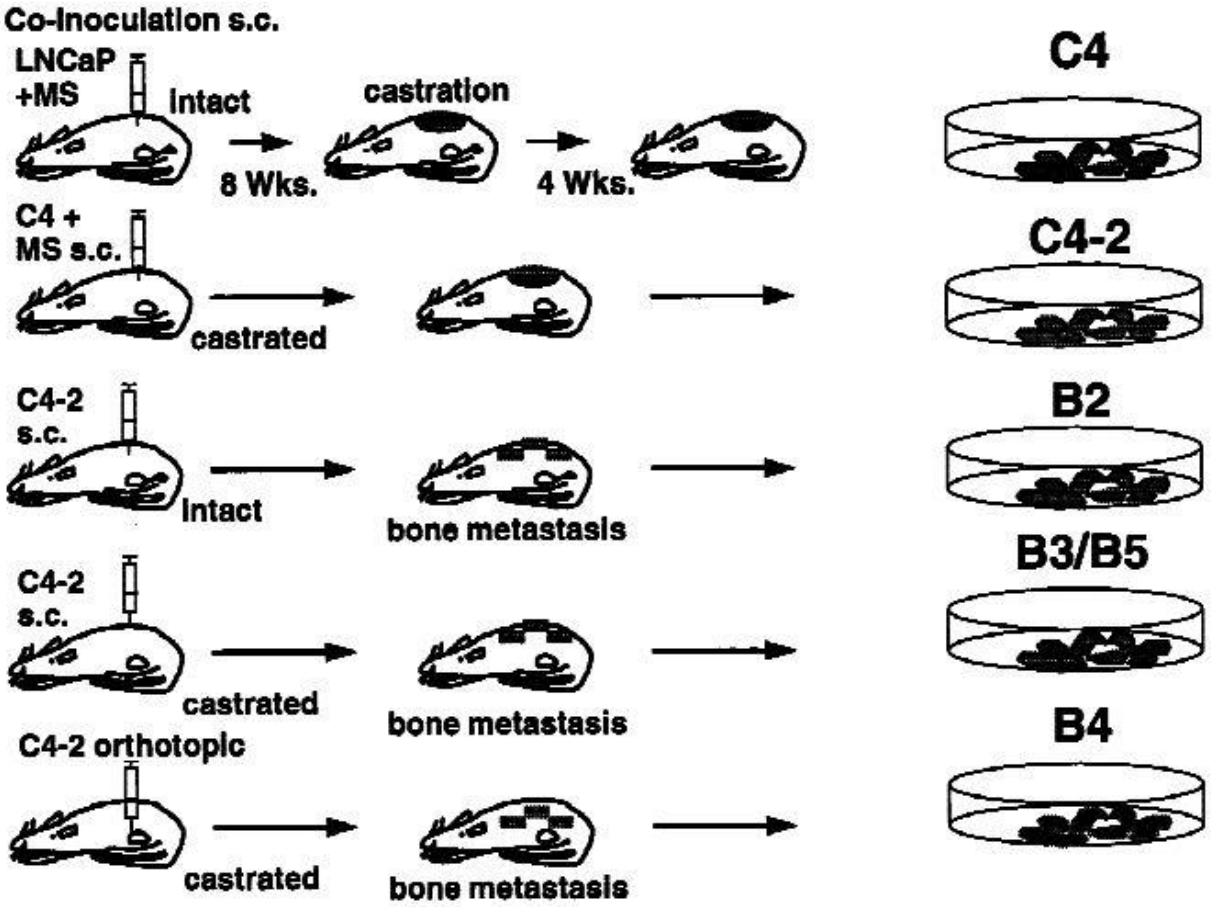


Figure 14: Schematic derivation of LNCaP sublines from tumors maintained in intact and castrated athymic male mice MS, fibroblasts derived from a human osteosarcoma.

Source: Thalmann et al., 2000

differs from its parent AS LNCaP, with differential expression of 38 genes between the two cell lines (≥ 2-fold change, 95% CI), 14 of which expressed at higher levels in LNCaP than in C4-2 cells, while the remaining 24 were expressed at lower levels in LNCaP than in C4-2 cells. In addition, the AI C4-2 cell line is highly tumorigenic and metastatic, including spontaneous metastasis to bone, whereas the AS LNCaP cell line is only weakly tumorigenic and is non-metastatic (Q. Chen et al., 2006). Some of the cancer cell lines that used in anti-cancer bioassays of medicinal plants include the following (Table 2).

Table 2: Some cell lines used in cell culture analysis of medicinal plants

Cell line	Culture medium	Supplementation	Culture conditions	Medicinal plant	Reference
Androgen-dependent growing human prostate cancer cell line, LNCaP (lymph node prostate cancer)	Roswell Park Memorial Institute (RPMI) 1640 medium	10% fetal calf serum (FCS), penicillin (100 U/mL), streptomycin (100 U/mL), and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	5% CO_2-95% air, humidified atmosphere at 37°C	*Prunus africana*	Hessenkemper et al., 2014
Human hepatoma Hep3B cells stably expressing green fluorescent protein (GFP)-AR or yellow fluorescent protein (YFP)-AR-cyan fluorescent protein (CFP)	Minimum Essential Medium Eagle, alpha modification (α-MEM)	5% FCS, 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 U/mL), and Genticin (G418)	5% CO_2-95% air, humidified atmosphere at 37°C	*Prunus africana*	Hessenkemper et al., 2014
Monkey kidney CV1 cells	Dulbecco's modified Eagle's medium (DMEM)	10% (v/v) FCS, penicillin (100 IU/ml) and streptomycin (100 IU/ml)	37ºC and 5% CO_2	*Prunus africana*	Schleich et al., 2006a
Mouse mammary breast cancer cell line, mouse colon cancer cell line and Vero cells (monkey kidney cells)	Earl's Minimum Essential Media (EMEM)	Penicillin, streptomycin and 10% fetal bovine serum (FBS)	37°C in a humidified atmosphere of 5% CO_2	*Prunus africana*	Nabende, Karanja, Mwatha, & Wachira, 2015
Human embryonic kidney cells, HEK293	EMEM	Glutamine, 10% FBS and antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin)	37°C in 5% CO_2	*Moringa oleifera*, *Prunus africana*	Chepkoech, 2014
Colorectal adenocarcinoma cell line, Caco-2	EMEM	Glutamine, 10% FBS and antibiotics	37°C in 5% CO_2	*Moringa oleifera*, *Prunus africana*	Chepkoech, 2014
Hepatocellular carcinoma cell line, HepG2	EMEM	Glutamine, 10% FBS and antibiotics	37°C in 5% CO_2	*Moringa oleifera*, *Prunus africana*	Chepkoech, 2014
HepG-2, Caco-2 and the non-cancer cell line HEK293	EMEM + glutamine	10% FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin	37°C and 5% CO_2	*Prunus africana*	Maiyo et al., 2016
Human prostate carcinoma LNCaP cells	RPMI-1640 medium	10% (v/v) FCS, 1% (v/v) penicillin and streptomycin, 1% (v/v) L-glutamin and 1% (v/v) sodium pyruvate	Humidified 5% CO_2 incubator	*Prunus africana*	Papaioannou et al., 2009
Human prostate carcinoma cell lines PC3, PC3-ARwt	DMEM	10% (v/v) FCS, 1% (v/v) penicillin and streptomycin, 1% (v/v) L-glutamin (and 600 µg/ml geneticin for PC3- ARwt)	Humidified 5% CO_2 incubator	*Prunus africana*	Papaioannou et al., 2009
Human prostate cancer C4-2 cells	DMEM	10% (v/v) FCS, 20% F12, 5 µg/mL Insulin, 13.6 pg/mL T3 (3,3′,5-triiodo-L-thyronine sodium salt), 5 µg/ml apotransferrin, 0.25 µg/ml Biotin, 1% (v/v) penicillin and streptomycin	Humidified 5% CO_2 incubator	*Prunus africana*	Papaioannou et al., 2009
Cells removed directly from human prostate cancer tissue	Endothelial basal medium MCDB 131	1 × L-glutamine, 5% FCS, 1 × MEM vitamins solution, 1× insulin-tranferrin-selenium liquid media supplement, and 1% (v/v) antimycotic/ antibiotic solution	-	*Prunus africana*	Boulbès et al., 2006
Madin-Darby canine kidney epithelial cell line (MDCK cells)	DMEM	1 × L-glutamine, 5% FCS and 1% (v/v) antibiotic solution	-	*Prunus africana*	Boulbès et al., 2006
Vero E6, CT 26-CL 25 colon cancer cells and Hep2 throat cancer cells	MEM medium	10% FBS, 1% L-glutamine and 1% antibiotic solution	High humidity environment at 37ºC and 5% CO_2	*Prunus africana*	Yiaile, 2017
Human ileoceacal adenocarcinoma, HCT-8 cell line	RPMI-1640	10% heat inactivated FBS, 2 mM L-glutamine, 50 µg/mL of penicillin-G, and 50 µg/mL of streptomycin sulfate	37ºC and 5% CO_2	*Moringa oleifera*	Al-Asmari et al., 2015
Human breast cancer, MDA-MB-231 cell line	DMEM	10% heat inactivated FBS, 2 mM L-glutamine, 50 µg/mL of penicillin-G, and 50 µg/mL of streptomycin sulfate	37ºC and 5% CO_2	*Moringa oleifera*	Al-Asmari et al., 2015
Human B-lymphoblastoid cells, Raji	RPMI-1640	10% fetal calf serum (FCS) containing n-butyric acid (3 mM) and teleocidin B-4 (50 nM)	37ºC and 5% CO_2	*Moringa oleifera*	Murakami et al., 1998

2.7.3 Anti-cancer bioassays

In vitro anti-cancer screening has long been used by researchers as a rapid tool in screening natural and synthetic compounds for drug development (Chepkoech, 2014). To assess preliminary anti-cancer activity in terms of cell viability, the 3(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) *in vitro* cytotoxicity assays are considered two of the most economic, reliable, and convenient methods (Chepkoech, 2014; McCauley, Zivanovic, & Skropeta, 2013). This is based on their ease of use, accuracy, rapid indication of toxicity, and sensitivity and specificity (McCauley et al., 2013). Both assays are *in vitro* whole cell toxicity assays that employ colorimetric methods for determining the number of viable cells based on mitochondrial dehydrogenase activity measurement and differ only in the reagent employed (McCauley et al., 2013).

In the MTT assay, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is bio-reduced by dehydrogenase inside living cells using the succinate-tetrazolium reductase system, to form a colored formazan dye, while in the MTS assay, a similar bioconversion takes places utilizing 3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and an electron coupling reagent, phenazine ethosulfate (PES) (Chepkoech, 2014; McCauley et al., 2013). In addition, the MTT assay requires the addition of solubilizing agents to dissolve the insoluble formazan product, while the MTS assay generates a water-soluble formazan product that simplifies the assay. The number of viable cells is measured calorimetrically, based on the principle that the mitochondrial dehydrogenase enzymes which produce NADH or NADPH, reduce the colorless tetrazolium salt to a colored aqueous soluble formazan product through the mitochondrial activity of viable cells at 37°C (McCauley et al.,

2013). The quantity of the colored product is directly proportional to the number of live cells in the culture since only metabolically active cells can reduce the MTT/MTS reagent to formazan (Chepkoech, 2014; McCauley et al., 2013).

The MTT and MTS assays assess the toxicity of a compound to a particular, but not anti-cancer activity in particular. When these assays are used, most researchers also screen for cytotoxicity to either murine (mouse) or human cancer cell lines and to normal cell lines such as peripheral blood lymphocytesto determine a selectivity index of the compound for cancer cells over normal cells. Although whole cell assays are essential, further assays are required if the researcher desires to determine activity against a specific molecular or cellular target to confirm the mechanism of action, to assess the selectivity for different targets, and identify off-target effects. This can be achieved using *in vitro* inhibition assays with either purified enzymes or cell-free extracts enriched with the enzyme target of interest or apoptosis assays (McCauley et al., 2013).

The sulforhodamine B (SRB) assay is a rapid, sensitive, and inexpensive method for determining cell growth, utilizing a bright pink anionic dye that binds electrostatically to basic amino acids of trichloroacetic acid fixed cells. The protein-bound dye is extracted with Tris (tris (hydroxymethyl) aminomethane) base to quantify the protein content indirectly with spectrophotometry (S. Kumar et al., 2016). The endpoint of the SRB assay is non-destructive, stable, and comparable with other fluorescence assays. However, it is labor intensive, requiring several washing steps (S. Kumar, Bajaj, & Bodla, 2016; Vichai & Kirtikara, 2006).

A known characteristic of cancer cell growth and metastasis is the ability of the cells to escape apoptosis because of a gene mutation that causes production of proteins that inhibit apoptosis. Induction of apoptosis is thus used as an important indicator of the ability of

chemotherapeutic agents to inhibit tumor growth and progression. The acridine orange/ethidium bromide (AO/EB) apoptosis assay, which is both qualitative and quantitative, is used to study changes in cellular and nuclear morphology and characteristics of apoptosis under a fluorescent microscope (Chepkoech, 2014). Although acridine orange and ethidium bromide bind to DNA and RNA by intercalation between adjacent base pairs causing the helical structure to stretch, acridine orange stains both live and dead cells while ethidium bromide stains dead cells only. Therefore, live cells appear green under the microscope and early apoptotic cells have a bright green nucleus due to chromatin condensation and nuclear fragmentation, while late apoptotic cells appear orange because they take up ethidium bromide and necrotic cells will stain orange but have a normal nuclear morphology (Chepkoech, 2014; K. Liu et al., 2015). After cells are counted under the microscope an apoptotic index is calculated.

The living status of a cell can be determined by measuring the amount of ATP in the cell, since ATP is necessary for life and function of all cells, and levels of cytoplasmic ATP decrease in cases of injury and hypoxia. After a cell is lysed, ATP is free to react with luciferin and luciferase, resulting in the generation of high-quantum chemiluminescence, the intensity of which is linearly proportional to the ATP concentration under optimum conditions. When compared to the MTT assay, the luciferase assay had higher sensitivity and reproducibility over several days and was able to detect the viability of cells with cell counts as low as 2000 cells/well compared to a minimum of 25,000 cells/well required for the MTT assay for above background readings (Kumar et al., 2016). Mueller et al (2014) reported that the ATP assay was the most sensitive of the ATP, MTT and calcein assays, used to determine the potency of cytotoxic agents. This high sensitivity of the ATP assay allowed for detection of cytotoxic agent-induced ATP breakdown after incubation periods as short as 1 h, which provides and additional advantage over the MTT

assay that requires approximately 72 h of incubation. A further advantage of the ATP assay was the short measurement time of 15 s per well, compared to the MTT assay which required a 1-2 h solubilization step of the formazan prior to a subsequent absorption measurement (Mueller, Kassack, & Wiese, 2004).

Enzymes are popular drug targets as they play a significant role in several disease processes and are susceptible to inhibition by small drug-like molecules. Most drugs used today demonstrate their bioactivity by acting as either receptor antagonists or as enzyme inhibitors (McCauley et al., 2013). Protein kinases are the second most important anti-cancer drug targets being pursued today after G protein-coupled receptors (GPCRs), accounting for a quarter of all current drug discovery research and development efforts (Bhullar et al., 2018; McCauley et al., 2013). With approximately 518 different protein kinases, they are abundant in the human body. Encoded in the human genome, they catalyze the chemical transfer of a phosphate group from a high energy molecule such as adenine triphosphate (ATP) to a hydroxyl-containing amino acid such as serine, threonine and tyrosine. Based on their selectivity for these amino acids, they are divided into different families (McCauley et al., 2013).

Pyruvate kinase isoenzyme M2 (PKM2) is the most significant regulatory enzyme functioning in the intracellular control of glucose consumption specific to cells with overgrowth tendency. Since inhibition and activation of this enzyme controls intracellular consumption of glucose, it is an excellent target for cancer treatment. In addition, PKM2 enzyme inhibition reduces metabolites, such as pyruvate and lactate, which are involved in drug resistance (Aslan et al., 2016). Another enzyme, cyclic adenosine monophosphate (c-AMP) dependent protein kinase A, regulates several physiological processes. The c-AMP-protein kinase A (PKA) pathway has been linked to the promotion of malignant phenotypes of head and neck squamous cell carcinoma and

was activated by a range of different tumors (Iqbal et al., 2017; McCauley et al., 2013). PKA inhibitors display both in vitro and in vivo anti-tumor activity against various human cancer cell lines, making the ability to selectively inhibit PKA a new way of potentially modulating cancer (McCauley et al., 2013).

Since all these assays have been used successfully as anti-cancer bioassays, the choice depends to a large extent on the target cancer cells and the phytochemical composition of the medicinal plant. Prostate cancer is an example of a target that has attracted much attention in recent times.

CHAPTER 3: Connecting Statement

Medicinal plants contain secondary metabolites with varying biological activity. To further understand the potential of medicinal plants in managing and treating oxidative stress-related diseases among other health conditions, including cancer, investigations to determine the phytochemical profile and antioxidant potential of these plants was carried out. Total phenolics, pigment and total flavonoid contents, antioxidant properties and phenolic acids concentration of different extracts of *Prunus africana, Pausinystalia yohimbe, Moringa oleifera, Momordica charantia* and *Orthero spp* were analyzed spectrophotometrically and chromatographically.

3.0 Materials and Methods

3.1 Materials

Prunus africana, *Pausinystalia yohimbe*, *Moringa oleifera*, *Momordica charantia* and *Orthero spp* were obtained from Cameroon, in addition to another sample of *Prunus africana* bark from Kenya. The phenolic acid standards used in this study: methyl 4-hydroxybenzoate, protocatechuic acid ethyl ester, trans-sinapic acid, vanillic acid, trans-ferulic acid, p-coumaric acid, caffeic acid and syringic acid and all other solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Waltham, MA) except for ethanol which was purchased from Decon Labs (King of Prussia, PA).

3.2 Methods

3.2.1 Sample preparation and extraction

After the plant parts from the selected medicinal plants were pulverized in a coffee grinder the particles that could pass through a screen with apertures of 1.3 mm were labelled and stored at

4°C until needed. A 10 g sample of each plant part was homogenized at a ratio of 1:10 (w/v) with one of five solvents: acetone (ACE), dichloromethane (DCM), methanol (MET), ethanol (ETH) or water (H_2O). The slurry, stirred at room temperature on an Orbi Shaker (Benchmark, Edmonton, AB) for 24 h at 140 rpm, was subsequently filtered using a cheesecloth. The filtrate was then centrifuged at 1200 rpm for 20 min before the supernatant was decanted carefully into pre-weighed aluminum drying pans and evaporated to dryness under a fume hood. The dried extracts were weighed and stored at 4°C until needed.

3.2.2 Qualitative screening for yohimbine

The presence of yohimbine in *P. yohimbe* extracts was determined qualitatively using sodium carbonate (Na_2CO_3). In brief, a drop of a 5% solution of Na_2CO_3 was placed on a microscope slide, prior to adding a drop of 10 mg/mL *P. yohimbe* extract. This was dried in the oven at 50°C, and then viewed under a microscope. Fine needles in sheaf-like bundles and rosettes indicated the presence of yohimbine.

3.2.3 Determination of total phenolic content (TPC)

The total phenolic content was determined following the procedure described by Taga et al. (1984). In brief, 5 mg of each extract was dispersed in 1 mL of 60:40 (v/v) acidified methanol: water buffer solution, from which 100 µL were drawn, mixed thoroughly with 2 mL of 2% sodium carbonate solution (Na_2CO_3), and incubated at room temperature for 2 min. To this mixture (and a blank containing 100 µL of buffer solution mixed with 2 mL of Na_2CO_3) 100 µL of 50% Folin-Ciocalteu reagent was then added before an additional incubation at room temperature for 30 min. The absorbance of the samples and different concentrations of gallic acid standard in the range of

0.05 - 0.11 mg/mL were measured at 750 nm using a DU 720 General-purpose Spectrophotometer (Beckman Coulter Inc., Brea, CA), the concentration of phenolic compounds in the extracts calculated from the gallic acid standard curve and expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g).

3.2.4 Determination of total flavonoid content (TFC)

Total flavonoid content was determined using the Stankovic method (2011). In brief, 1 mL of 2% aluminum chloride solution was added to 4 mg of each extract dispersed in 1 mL of methanol before incubation at room temperature for 1 h. The absorbance of the samples and the rutin standard of concentration range 0.005 - 0.03 mg/mL were measured at 415 nm using a DU 720 General-purpose Spectrophotometer (Beckman Coulter Inc., Brea, CA). The flavonoid content was calculated from the rutin standard curve and expressed as mg rutin per gram of extract.

3.2.5 Determination of pigment content

The chlorophyll, xanthophyll, carotenoid, anthocyanin and lycopene contents of the extracts were determined following the procedure described by Nagata and Yamashita (1992). In brief, 20 mg of each extract was dissolved in 10 mL of 80% ethanol before the absorbance of the filtrate was measured in triplicate at 453, 470, 480, 485, 505, 537, 645, 647 and 663 nm using a DU 720 General-purpose Spectrophotometer (Beckman Coulter Inc., Brea, CA). The following calculations were then used to obtain the pigment content in mg/g of sample:

- Lycopene = $-0.0458 \times Abs_{663} + 0.372 \times Abs_{505} - 0.0806 \times Abs_{453}$
- β-carotene = $0.216 \times Abs_{663} - 0.304 \times Abs_{505} + 0.452 \times Abs_{453}$
- Carotenoids = $Abs_{480} + (0.114 \times Abs_{663} - 0.638 \times Abs_{645})$

- Chlorophyll A = 12.7 x Abs_{663} – 2.69 x Abs_{645}
- Chlorophyll B= 22.4 x Abs_{645} – 4.68 x Abs_{663}
- Total chlorophyll = 8.02 x Abs_{663} + 20.2 x Abs_{645}
- Xanthophyll = 2026.1 x Abs_{470} – 2288.6 x Abs_{485} + 0.0036 (A) -0.06518 (B)
- Anthocyanin = 0.08173 x Abs_{537} – 0.00697 x Abs_{647} – 0.002228 x Abs_{663}

3.2.6 Antioxidant assays

Three antioxidant assays; ferric ion reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity were chosen because of their wide application in previous studies and rapidity of the tests. The FRAP assay measures the reducing potential of antioxidant-containing compounds which exert their action by breaking the free radical chain while donating a hydrogen atom through the single electron transfer method. The DPPH and TEAC assays combine both hydrogen atom transfer and electron transfer reactions, measuring the ability of an antioxidant to scavenge free radicals via hydrogen donation to form stable compounds and the ability of an antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and free radicals. Their methods are as described below:

3.2.6.1 Ferric ion reducing antioxidant power (FRAP)

The FRAP was determined following the procedure described by Benzie and Strain (1999). In brief, 4 mg of each extract was dissolved in 1 mL of methanol, from which 0.1 mL was added to 3 mL of working FRAP reagent (300 mM acetate buffer at pH 3.6, 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM iron chloride solution ($FeCl_3.6H_2O$) in a ratio of 10:1:1 (v/v/v) just

before use). The absorbance of the samples and the 1 mM iron sulfate solution ($FeSO_4.7H_2O$) standard were measured at 593 nm after incubating for 4 min in a water bath (BÜCHI, New Castle, DE) at 37ºC using a DU 720 General-purpose Spectrophotometer (Beckman Coulter Inc., Brea, CA). The FRAP value was calculated from the equation of the $FeSO_4.7H_2O$ standard (concentration range of 5 - 100 µM) curve and expressed as µM Fe^{2+} per gram of extract.

3.2.6.2 Trolox equivalent antioxidant capacity (TEAC)

The TEAC was determined following the procedure described by Benzie and Strain (1999). A 2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) working solution was prepared by combining 7 mM ABTS solution and 2.45 mM potassium persulfate ($K_2S_2O_8$) at a ratio of 1:1 (v/v). This mixture was incubated for 12-16 h at room temperature in the dark, before diluting it with ethanol to an absorbance reading of 0.7±0.05 at 734 nm. After blanking the spectrophotometer with ethanol, the absorbance of 3 mL of the working ABTS solution was read at 0 min using a DU 720 General-purpose Spectrophotometer (Beckman Coulter Inc., Brea, CA). A 150 µL aliquot from 3 mg of each extract dispersed in 1 mL of ethanol was then added to the cuvette, mixed immediately prior to incubation at room temperature for 5 min. A second absorbance reading was taken at 5 min and the TEAC was calculated from the Trolox standard curve with a concentration range of 0.045 - 0.330 mM.

3.2.6.3 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was determined using the method described by Garcia et al. (2012). In brief, 1 mL of a 5 mg/mL methanol solution of each extract was added to 3 mL of methanol in a test tube and mixed thoroughly. Afterwards, 1 mL of 0.12 mg/mL of DPPH

in methanol solution was added and the mixture incubated at room temperature for 30 min in the dark. After blanking with a solution made up of 3 mL of methanol and 1 mL of test solution, the absorbance of the samples and the control (3 mL methanol and 1 mL DPPH) were measured under dim lighting at 515 nm using a DU 720 General-purpose Spectrophotometer (Beckman Coulter Inc., Brea, CA). The DPPH radical scavenging activity was calculated using the following formula: %DPPH scavenging = [(Absorbance of control - Absorbance of sample) * 100]/Absorbance of control.

3.2.7 Qualitative and quantitative analysis of selected extracts using HPLC analysis

Based on the EC_{50} results, nine *P. africana* and *P. yohimbe* extracts with EC_{50} values comparable to ascorbic acid (±0.08) were selected for HPLC analysis of phenolic content. A Shimadzu LC-20AB Prominence chromatograph equipped with a Shimadzu SPD-M20A PDA detector (Kyoto, Kyoto prefecture, Japan) was used for HPLC analysis. Chromatographic separation was managed, chromatograms were recorded, and data was processed with the LabSolutions software (Kyoto, Kyoto Prefecture, Japan). Chromatographic separations were implemented using a Phenomenex LC column (5 µm, C18, 250 x 4.6 mm i.d.) (Phenomenex, Torrance, CA), operated at a constant temperature of 25^{o}C. Then, 10 µL of the selected extracts were injected and eluted with a mobile phase of 2.5% (v/v) acetic acid in water (solvent A) and 100% (v/v) acetonitrile (solvent B). The flow rate was 1 mL/min, and gradient elution was used. The following conditions of elution were applied: 0 - 10 min, 0 - 3% B; 10 - 12 min, 3 - 10% B; 12 - 64 min, 10 - 15% B; 64 - 69 min, 15 - 24% B; 69 - 74 min, 24 - 50% B; and 74 - 75 min, 50 - 95% B. The phenolic acid standards were prepared as stock solutions at 10 mg/mL in methanol. Chromatographic peaks were identified by comparing the retention times and spectral

characteristics of the eluting peaks with those of standard compounds at λ = 250 - 350 nm. For quantitative analysis, relative response factors were calculated by injecting known concentrations of the different standard compounds.

3.2.8 Phytosterol content analysis using gas chromatography

Analysis of phytosterols in *P. africana* and *P. yohimbe* leaf, bark and root was performed as described by Hwang et al. (2003). In brief, 10 mg/mL stock solutions of the sterol standards (stigmasterol, campesterol, β-sitosterol and cholesterol) were prepared in ethanol. Each 0.3 g portion of pulverized sample for each plant part was spiked with 50 µL of cholesterol (internal standard), before adding 6 mL of saponification reagent (33% potassium hydroxide (KOH) with ethanol in the ratio 1: 15.67 (v/v)) prior to vortexing the solution vigorously and incubating it in a water bath at 50^{o}C for 1 h. Afterwards, 2 mL each of potassium chloride and hexane were added, vortexed, and after phase separation, the hexane layer was passed through an anhydrous sodium sulfate column to dry it before placing it in GC vials. Chromatographic analysis was carried out using a Shimadzu GC 2010 Plus, equipped with an online auto-injector and a FID detector (Kyoto, Kyoto prefecture, Japan). A Zebron ZB-5MS capillary column, 0.25 mm (i.d) x 30 m with 0.25 µm film thickness (Phenomenex, Torrance CA) was used for chromatographic separations at an initial temperature of 278°C, held for 5 min. Then the temperature was increased to 286°C for 6 min, and then to 305^{o}C for 8 min. The inlet temperature was 295°C and the detector temperature 305°C. Helium was the carrier gas with a constant flow of 30 mL/min. The area of each peak was integrated using the LabSolutions software (Kyoto, Kyoto Prefecture, Japan) and the amount of phytosterols were calculated using relative response factors achieved by injecting known concentrations of the different standard compounds.

3.3 Statistical Analysis

Data were analyzed using Statistical Package for Social Sciences (SPSS), version 25.0 (SPSS Inc., Chicago, IL). Following one-way analysis of variance (ANOVA), $p \leq 0.05$ was considered significant, and treatment means were compared using Tukey's post hoc comparisons tests. Each assay was repeated in triplicates unless otherwise stated. Correlation between the TPC, TFC, DPPH, TEAC values and among the antioxidant tests were conducted using the Pearson's method.

3.5 Results and Discussion

Extraction yield varied from 0.24% (*Yohimbe* bark dichloromethane extract) to 20.07% (*M. oleifera* seed ethanol extract) (Table 3). For most of the plant samples, extraction yields were highest when ethanol or methanol were used as extraction solvents, except for *P. africana* root (Cameroon), *P. africana* bark (Kenya) and *M. charantia* leaves where dichloromethane for both *P. africana* (Cameroon and Kenya) and acetone for *M. charantia* provided higher yields than ethanol. Sultana et al. (2009) reported comparable results, indicating that maximum extract yield from seven medicinal plants parts, including *M. oleifera* leaves were 17.9 and 21.1 g/100g DW, by shaker and reflux respectively. This involved extraction with aqueous methanol, followed by aqueous ethanol (Sultana et al., 2009). The differences in the extraction yields in their study may be due to the varied chemical composition of the plants, the vigor of the extraction procedure (shaker vs reflux) and the efficiency of the extracting solvent to dissolve endogenous compounds (Sasidharan et al., 2011; Sultana et al., 2009). Results from the Sultana et al. (2009) study suggested that yields from an extract were better when compounds were extracted under reflux, regardless of the plant material and solvent used indicating that hot solvent systems under reflux state are more efficient for the recovery of antioxidant components.

Qualitative analysis for presence of yohimbine revealed fine needles in sheaf-like bundles and rosettes, indicating its presence. This is supported by previous studies which found yohimbine was the major alkaloid in *P. yohimbe* extracts (Evans, 2009; Igwe et al., 2016; Morales, 2000).

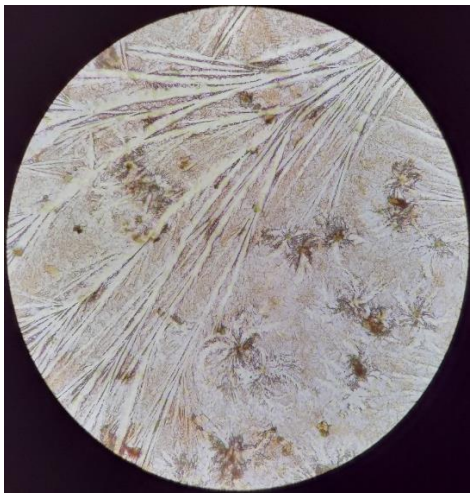


Figure 15: Needles and rosettes of yohimbine crystals.

The methanol extract of *P. africana* bark (Cameroon) had the highest TPC (1397.33 mg GAE/g) (Table 3) while the dichloromethane extract of *P. africana* bark had the lowest TPC value (0.24% of methanol extract), potentially because phenolics are often extracted in higher amounts in more polar solvents as several studies have suggested. The most suitable solvents for phenolic extraction in those studies were (hot or cold) aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (Peschel et al., 2006; Qasim et al., 2016; Sultana et al., 2009). Interestingly, the TPC values in this study (3.30 - 1397.33 mg GAE/g) were much higher than those reported in previous studies (12.20 - 69.2 mg GAE/g) (Johnson et al., 2008; Karan et al., 2017; Sultana et al., 2009). This may be due to differences in the concentration of sample used or the methodology used for determining TPC, as previous studies utilized different modifications

Table 3: Sample yield, total phenolic content, total flavonoid content and antioxidant properties of selected plants

Plant	Part	Extraction solvent	Yield (%)	TPC (mg GAE/g)	TFC (mg RU/g)	FRAP (mM Fe^{2+}/g)	DPPH (%)	TEAC (mM TE/g)
Prunus africana (C)	Bark	Acetone	9.81±2.87^{b}	1348.00±35.55^{d}	25.13±0.81^{d}	26.26±2.60^{c}	89.07±0.65^{d}	185.64±0.56^{c}
		Dichloromethane	0.38±0.12^{a}	3.30±0.38^{a}	1.45±0.43^{a}	10.22±0.63^{a}	76.18±0.70^{a}	162.59±0.09^{b}
		Methanol	12.03±1.70^{b}	1397.33±36.90^{d}	17.84±0.58^{c}	32.41±2.19^{d}	83.63±0.60^{b}	175.15±2.56^{bc}
		Ethanol	31.4±0.07^{c}	982.66±11.55^{b}	38.63±1.23^{e}	24.70±3.13^{c}	85.95±0.28^{c}	318.55±1.41^{d}
		Water	6.75±1.26^{ab}	1054.67±13.32^{c}	7.85±0.37^{b}	16.57±1.57^{b}	93.29±0.33^{e}	133.19±10.30^{a}
	Leaf	Acetone	4.83±0.16^{a}	82.67±2.31^{b}	118.06±1.34^{d}	83.03±1.36^{c}	43.71±0.63^{a}	255.12±4.66^{b}
		Dichloromethane	4.82±1.41^{a}	66.67±4.62^{a}	61.71±1.34^{b}	22.51±1.72^{b}	70.89±0.48^{c}	101.41±2.14^{a}
		Methanol	11.62±0.59^{b}	349.33±2.30^{d}	55.00±1.34^{a}	16.36±0.36^{a}	78.32±0.62^{d}	332.38±3.68^{c}
		Ethanol	6.09±0.69^{a}	178.66±2.30^{c}	76.02±4.31^{c}	25.32±1.78^{b}	62.83±1.23^{b}	325.67±5.33^{c}
	Root	Acetone	5.00±0.00^{a}	316.00±8.00^{b}	6.44±0.52^{b}	19.18±0.48^{a}	89.30±0.17^{b}	332.99±2.88^{c}
		Dichloromethane	7.50±3.54^{a}	16.00±3.46^{a}	4.36±0.35^{a}	56.78±1.90^{c}	87.72±0.89^{ab}	82.32±2.08^{a}
		Methanol	8.15±1.54^{a}	430.66±6.11^{c}	8.67±0.16^{c}	20.11±0.48^{ab}	87.65±0.82^{ab}	324.45±2.47^{b}
		Ethanol	5.72±0.12^{a}	584.00±10.58^{d}	6.79±0.27^{b}	24.70±3.13^{b}	86.77±1.02^{a}	322.62±0.70^{b}
Prunus africana (K)	Bark	Acetone	2.03±0.42^{a}	214.67±4.62^{c}	17.78±0.42^{b}	19.91±0.31^{b}	91.96±0.93^{a}	159.56±3.40^{c}
		Dichloromethane	3.57±2.76^{a}	194.67±2.31^{b}	9.44±0.10^{a}	7.09±1.74^{a}	94.52±0.38^{b}	32.90±2.54^{a}
		Methanol	2.82±0.01^{a}	209.33±2.30^{c}	21.53±0.27^{c}	21.16±1.65^{b}	91.93±0.60^{a}	229.30±0.70^{d}
		Ethanol	1.60±0.37^{a}	122.67±2.30^{a}	28.97±0.30^{d}	40.95±1.10^{c}	97.58±0.53^{c}	128.45±3.71^{b}
Pausinystalia yohimbe	Bark	Acetone	2.71±3.20^{a}	378.67±2.31^{c}	31.79±0.26^{ab}	26.36±0.65^{ab}	77.98±1.48^{c}	311.03±3.01^{b}
		Dichloromethane	0.24±0.02^{a}	42.67±1.15^{a}	9.48±0.25^{a}	96.36±2.84^{d}	90.64±0.15^{d}	264.47±3.23^{a}
		Methanol	13.01±1.63^{c}	913.33±30.55^{d}	58.40±1.05^{b}	32.09±1.25^{b}	61.43±1.24^{b}	312.25±1.53^{b}
		Ethanol	10.99±0.72^{bc}	897.33±88.48^{d}	111.61±28.53^{c}	53.14±4.02^{c}	56.38±0.53^{a}	313.27±0.00^{b}
		Water	4.95±0.15^{ab}	182.67±2.31^{b}	8.69±0.15^{a}	19.39±2.97^{a}	92.775±0.15^{d}	265.89±1.27^{a}
	Leaf	Acetone	9.99±0.04^{b}	994.67±9.24^{d}	175.65±11.00^{d}	92.41±3.26^{c}	71.12±0.13^{a}	328.51±0.00^{a}
		Dichloromethane	4.41±0.44^{a}	362.67±15.14^{a}	126.37±12.29^{c}	65.22±2.72^{b}	83.65±0.55^{d}	327.50±0.93^{a}
		Methanol	12.84±1.36^{b}	550.67±8.32^{b}	66.76±0.07^{a}	23.55±1.48^{a}	75.58±0.26^{c}	340.51±1.96^{c}
		Ethanol	8.57±1.82^{ab}	650.67±6.11^{c}	101.06±2.79^{b}	20.11±0.48^{a}	80.22±1.13^{b}	336.24±1.53^{b}
	Root	Acetone	5.42±0.82^{a}	316.00±8.00^{b}	13.10±1.10^{a}	28.66±1.08^{a}	85.95±0.47^{c}	354.74±1.53^{b}
		Dichloromethane	1.96±0.63^{a}	16.00±3.46^{a}	13.48±1.87^{a}	32.93±2.22^{a}	85.28±0.92^{c}	345.19±4.15^{a}
		Methanol	10.63±1.77^{b}	430.67±6.11^{c}	10.01±0.14^{a}	31.05±3.21^{a}	71.71±1.28^{a}	346.81±2.80^{ab}
		Ethanol	15.40±1.10^{c}	584.00±10.58^{d}	27.88±1.89^{b}	29.18±0.95^{a}	77.31±1.08^{b}	347.42±1.06^{ab}
Orthero	Root	Acetone	5.42±2.28^{a}	250.00±0.00^{b}	6.73±0.22^{a}	13.14±0.79^{b}	93.83±0.19^{b}	346.00±0.93^{b}
		Dichloromethane	4.39±0.25^{a}	272.67±2.31^{d}	11.13±0.23^{b}	14.59±0.83^{b}	92.74±0.07^{a}	343.97±1.27^{ab}
		Methanol	8.23±0.06^{a}	254.00±0.00^{c}	15.31±0.20^{c}	10.74±1.10^{a}	92.91±0.13^{a}	343.36±0.35^{ab}
		Ethanol	6.57±0.90^{a}	244.00±2.00^{a}	6.52±0.03^{a}	13.76±0.72^{b}	94.40±0.07^{c}	340.92±2.47^{a}
Momordica charantia	Leaf	Acetone	4.97±0.66^{a}	264.00±16.00^{b}	217.33±26.19^{c}	131.57±6.13^{d}	74.95±1.15^{a}	149.80±3.71^{a}
		Dichloromethane	2.88±0.82^{a}	50.67±4.62^{a}	152.49±2.79^{b}	88.14±0.95^{c}	77.48±0.33^{b}	146.75±14.29^{a}
		Methanol	16.5±2.12^{b}	318.67±2.31^{c}	118.14±14.37^{b}	34.49±1.80^{b}	89.49±0.16^{c}	146.35±0.70^{a}
		Ethanol	3.97±0.31^{a}	62.67±6.11^{a}	61.64±1.29^{a}	8.03±0.31^{a}	87.40±0.41^{d}	156.51±12.00^{a}
Moringa oleifera	Seed	Ethanol	20.07±1.27	6.07±0.61	2.01±0.06	33.45±0.48	38.69±0.44	-

C - Cameron; K - Kenya. Values are mean of three determinations (n = 3) ± standard deviation (SD). Values in the same column (in the same block) followed by the same letter are not significant at $p \leq 0.05$.

of the Folin-Ciocalteu method (Johnson et al., 2008; Karan et al., 2017; Nagarani et al., 2014; Sultana et al., 2009).

All tested plant parts contained significant amounts of flavonoids. The acetone extract of *M. charantia* leaves and dichloromethane extract of *P. africana* (Cameroon) contained the highest and lowest TFC with 217.33 and 1.45 mgRU/g, respectively. Tan et al. (2014) also reported that acetone was the best solvent by far for extracting flavonoids compared with other solvents. They suggested that the solubility of flavonoids highly correlates with their chemical structures and the nature of the extraction solvent and cited similar studies where TFC was higher in acetone extracts than in extracts of other solvents (S. P. Tan, Parks, Stathopoulos, & Roach, 2014). Other factors proposed to affect TFC of plants include extraction methods, plant to solvent ratios, and granular size of plant materials (Nobre et al., 2005; Sultana et al., 2009). TFC values obtained in this study were generally higher than reported in previous studies, which is probably due to differences in the methodologies (Nobre et al., 2005; Subhashchandra et al., 2011; S. P. Tan et al., 2014).

The FRAP values in this study ranged from 7.09 in *P. africana* (Kenya) bark dichloromethane extract to 131.57 mM Fe^{2+}/g in *M. charantia* leaf acetone extract (Table 3). There were variations between plant parts. For *P. africana* (Cameroon), the FRAP values were highest in leaf extracts followed by root extracts and finally bark extracts while for *P. yohimbe* the FRAP values were highest in leaf extracts followed by bark extracts and finally root extracts. These values were lower than results reported in previous studies (Al-Laith et al., 2015; Nagarani et al., 2014), but similar in range to values reported for some Chinese and Japanese medicinal plants (0.3 - 120 mmol Fe^{2+}/g) (Dragland et al., 2003).

The DPPH and TEAC assays, both hydrogen atom transfer and electron transfer reaction-based methods, measure the ability of an antioxidant to scavenge free radicals via hydrogen

donation to form stable compounds and the ability of an antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and free radicals (Al-Laith et al., 2015; Kasote, Katyare et al., 2015). The extracts of all the tested medicinal plant materials possessed free radical scavenging properties to varying degrees. The concentrations required to obtain a 50% antioxidant effect or 50% effective concentration (EC_{50}) were calculated using the %DPPH remaining. Lower EC_{50} values indicate a stronger antioxidant effect than higher values. The EC_{50} values for acetone

Table 4: EC_{50} and antiradical power (ARP) of medicinal plant sample extracts

Plant	Part	Extraction solvent	EC_{50} (mg/mL)	ARP
Prunus africana (Cameroon)	Bark	Acetone	0.10	9.65
		Dichloromethane	-	-
		Methanol	0.10	10.50
		Ethanol	0.37	2.69
		Water	0.22	4.64
	Leaf	Acetone	1.68	0.59
		Dichloromethane	-	-
		Methanol	0.49	2.05
		Ethanol	0.47	2.13
	Root	Acetone	0.51	1.95
		Dichloromethane	4.72	0.21
		Methanol	0.33	3.04
		Ethanol	0.24	4.14
Prunus africana (Kenya)	Bark	Acetone	1.94	0.52
		Dichloromethane	9.41	0.11
		Methanol	1.90	0.53
		Ethanol	2.70	0.37
Pausinystalia yohimbe	Bark	Acetone	2.72	0.37
		Dichloromethane	-	-
		Methanol	2.86	0.35
		Ethanol	2.15	0.47
		Water	11.09	0.09
	Leaf	Acetone	0.23	4.31
		Dichloromethane	0.78	1.29
		Methanol	0.18	5.48
		Ethanol	0.15	6.64
	Root	Acetone	0.24	4.22
		Dichloromethane	0.39	2.55
		Methanol	0.18	5.57
		Ethanol	0.34	2.91
Orthero	Root	Acetone	3.24	0.31
		Dichloromethane	4.63	0.22
		Methanol	1.89	0.53
		Ethanol	3.26	0.31
Momordica charantia	Leaf	Acetone	9.54	0.10
		Dichloromethane	10.52	0.10
		Methanol	7.86	0.13
		Ethanol	8.97	0.11
Moringa oleifera	Seed	Ethanol	163.85	0.01
Ascorbic acid			0.18	5.58

Values are mean of two determinations (n = 2) ± standard deviation (SD).

and methanol extracts of *P. africana* bark (Cameroon), methanol and ethanol extracts of *P. yohimbe* leaf, and methanol extract of *P. yohimbe* root were comparable to those of ascorbic acid (0.18 mg/mL) (Table 4), demonstrating that *P. africana* bark (Cameroon) and *P. yohimbe* leaf and root extracts contain potent antioxidants. The EC_{50} values for *P. africana* in this study (0.10 - 9.41 mg/mL) were higher than values reported by Madivoli et al. (2018) with 35.87 µg/mL. This may be due to differences in the methodology as the EC_{50} value reported for ascorbic acid in the same study was 10.14 µg/mL.

M. charantia had an EC_{50} value of 7.86 mg/mL for the methanol extract of the leaves, which was higher than the EC_{50} of 2.22 mg/mL for the hot water extract of unripe *M. charantia* fruits reported by Jia et al. (2017). and EC_{50} values of 0.15 mg/mL in aerial parts of wild *M. charantia* reported by Svobodova et al. (2017). The ethanol extract of *P. yohimbe* root had the highest TEAC value with 347.42 mM TE/g while the dichloromethane extract of *P. africana* (Kenya) bark had the lowest with 32.90 mM TE/g. This may be due to differences in the stability of DPPH versus ABTS radicals. DPPH is a stable free radical while ABTS was stabilized with potassium persulfate in this study. Other factors that may have influenced the results include the time of incubation to reach a steady state (the reaction with $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ with many phenolic extracts occurs rather slowly, requiring 1 - 6 h) and the ratio of sample quantity to radical concentrations (Cerretani & Bendini, 2010; Ratnavathi et al., 2016).

Most plant parts in this study contained significant amounts of pigments (Table 5). The highest values obtained for lycopene, β-carotene, carotenoids, chlorophyll a, chlorophyll b, total chlorophyll and anthocyanins in mg/g of dry sample were 0.32 (*P. yohimbe* bark methanol extract), 0.50 (*P. yohimbe* bark methanol extract and *M. charantia* ethanol extract), 1.45 (*P. yohimbe* bark methanol extract), 8.09 (*M. charantia* ethanol extract), 8.89 (*P. africana* bark (Kenya) methanol

Table 5: Pigments content in sample extracts

Plant	Part	Extraction solvent	Pigments (mg/g)						
			Lycopene	β-carotene	Carotenoids	Chlorophyll a	Chlorophyll b	Total chlorophyll	Anthocyanin
Prunus africana (C)	Bark	Acetone	0.01±0.00^{bc}	0.08±0.00^{a}	0.30±0.00c	0.14±0.02^{a}	0.04±0.03^{a}	0.53±0.04^{a}	0.01±0.00^{c}
		Dichloromethane	0.01±0.00^{ab}	0.01±0.00^{a}	0.02±0.00^{a}	0.02±0.01^{ab}	0.40±0.01^{a}	0.64±0.01^{ab}	0.00±0.00^{a}
		Methanol	0.11±0.00^{c}	0.18±0.00^{a}	0.60±0.00^{e}	0.58±0.02^{c}	1.28±0.04^{c}	1.89±0.06^{d}	0.02±0.00^{d}
		Ethanol	-	0.14±0.17^{a}	0.04±0.00^{b}	0.15±0.02^{a}	0.31±0.05^{a}	0.47±0.07^{b}	0.00±0.00^{a}
		Water	0.08±0.00^{c}	0.14±0.00^{a}	0.04±0.01^{d}	0.03±0.06^{b}	0.81±0.10^{b}	1.17±0.16^{c}	0.02±0.00^{d}
	Leaf	Acetone	0.03±0.03^{a}	0.03±0.07^{b}	0.26±0.11^{a}	4.15±2.96^{a}	3.22±3.63^{a}	7.48±0.73^{b}	0.01±0.01^{a}
		Dichloromethane	0.01±0.00^{a}	0.17±0.00^{a}	0.16±0.00^{a}	4.20±0.35^{a}	0.72±0.17^{a}	4.97±0.53^{a}	0.00±0.00^{a}
		Methanol	0.00±0.01^{a}	0.24±0.03^{b}	0.22±0.02^{a}	5.73±0.74^{a}	1.43±0.43^{a}	7.24±1.19^{b}	0.01±0.00^{a}
		Ethanol	0.01±0.00^{a}	0.06±0.01^{a}	0.05±0.01^{a}	1.69±0.19^{a}	0.37±0.14^{a}	2.09±0.04^{a}	0.00±0.00^{a}
	Root	Acetone	0.01±0.00^{b}	0.02±0.00^{a}	0.05±0.01^{a}	0.16±0.04^{a}	0.25±0.03^{a}	0.42±0.07^{a}	0.00±0.00^{a}
		Dichloromethane	-	0.02±0.00^{a}	0.03±0.01^{a}	0.31±0.10^{a}	0.42±0.30^{a}	0.75±0.40^{a}	0.00±0.00^{a}
		Methanol	0.00±0.00^{ab}	0.01±0.00^{a}	0.01±0.00^{a}	0.09±0.06^{a}	0.15±0.08^{a}	0.24±0.15^{a}	0.00±0.00^{a}
		Ethanol	0.01±0.00^{ab}	0.01±0.01^{a}	0.02±0.02^{a}	0.14±0.03^{a}	0.24±0.09^{a}	0.38±0.13^{a}	0.00±0.00^{a}
Prunus africana (K)	Bark	Acetone	0.16±0.00^{a}	-	0.43±0.01^{a}	3.75±0.02^{a}	7.51±0.12^{a}	11.46±0.15^{a}	0.03±0.00^{a}
		Dichloromethane	0.09±0.00^{a}	0.18±0.01^{ab}	0.28±0.00^{a}	3.90±0.03^{a}	6.17±0.02^{a}	10.24±0.00^{a}	0.02±0.00^{a}
		Methanol	-	0.30±0.11^{b}	0.46±0.16^{a}	5.29±2.28^{a}	8.89±3.29^{a}	14.43±5.66^{a}	0.03±0.01^{a}
		Ethanol	0.08±0.00^{a}	0.29±0.02^{b}	0.42±0.02^{a}	4.86±0.22^{a}	4.94±0.26^{a}	9.95±0.49^{a}	0.02±0.00^{a}
Pausinystalia yohimbe	Bark	Acetone	0.12±0.00^{c}	0.40±0.00^{d}	1.23±0.00^{d}	0.18±0.00^{b}	0.44±0.00^{b}	0.64±0.00^{b}	0.02±0.00^{c}
		Dichloromethane	0.10±0.00^{b}	0.26±0.00^{c}	0.47±0.00^{b}	2.60±0.00^{e}	4.10±0.01^{d}	6.82±0.01^{d}	0.02±0.00^{d}
		Methanol	0.32±0.00^{d}	0.50±0.00^{e}	1.45±0.00^{e}	0.36±0.00^{d}	1.04±0.03^{c}	1.41±0.00^{c}	0.03±0.00^{e}
		Ethanol	0.03±0.00^{a}	0.06±0.01^{b}	0.12±0.01^{b}	0.31±0.02^{c}	0.43±0.06^{b}	0.75±0.09^{b}	0.00±0.00^{a}
		Water	-	0.03±0.00^{a}	0.07±0.00^{a}	0.06±0.00^{a}	0.10±0.00^{a}	0.16±0.00^{a}	0.00±0.00
	Leaf	Acetone	0.01±0.00^{a}	0.24±0.00^{b}	0.36±0.00^{a}	2.32±0.04^{a}	2.44±0.07^{c}	4.83±0.12^{b}	0.00±0.00^{ab}
		Dichloromethane	0.00±0.01^{a}	0.12±0.01^{a}	0.17±0.01^{a}	1.84±0.22^{a}	0.60±0.19^{a}	2.47±0.41^{a}	0.00±0.00^{a}
		Methanol	0.01±0.00^{a}	0.30±0.01^{b}	0.48±0.02^{a}	3.49±0.14^{b}	1.46±0.08^{b}	5.01±0.23^{b}	0.00±0.00^{ab}
		Ethanol	0.03±0.03^{a}	0.41±0.03^{c}	0.71±0.10^{a}	5.55±0.03^{c}	2.30±0.01^{c}	7.95±0.05^{c}	0.01±0.00^{b}
	Root	Acetone	0.04±0.01^{a}	0.14±0.00^{a}	0.38±0.02^{a}	0.26±0.07^{a}	0.54±0.15^{a}	0.81±0.23^{a}	0.00±0.00^{a}
		Dichloromethane	0.15±0.01^{b}	0.30±0.00c	0.56±0.021^{a}	4.36±0.14^{c}	7.85±0.33^{c}	12.43±0.49^{c}	0.04±0.00^{c}
		Methanol	0.05±0.01^{a}	0.14±0.00^{a}	0.38±0.021^{a}	0.25±0.07^{a}	0.55±0.15^{a}	0.81±0.23^{a}	0.00±0.00^{a}
		Ethanol	0.01±0.02^{a}	0.24±0.03b	0.58±0.10^{a}	0.87±0.16^{b}	1.73±0.33^{b}	2.65±0.50^{b}	0.01±0.00^{b}
Orthero	Root	Acetone	0.05±0.00^{a}	0.10±0.00^{a}	0.14±0.01^{a}	1.54±0.12^{ab}	2.86±0.24^{a}	4.49±0.37^{a}	0.01±0.00^{a}
		Dichloromethane	0.05±0.01^{a}	0.13±0.01^{a}	0.18±0.01^{b}	1.85±0.31^{b}	3.24±0.53^{a}	5.19±0.86^{a}	0.01±0.00a
		Methanol	0.04±0.00^{a}	0.12±0.00^{a}	0.17±0.00^{ab}	1.10±0.09^{a}	2.01±0.14^{a}	3.16±24^{a}	0.01±0.00^{a}
		Ethanol	0.08±0.00^{b}	0.22±0.01^{b}	0.25±0.00^{c}	2.98±0.02^{c}	5.44±0.41^{b}	8.57±40^{b}	0.02±0.00^{b}
Momordica charantia	Leaf	Acetone	0.15±0.22^{a}	0.08±0.18^{a}	0.21±0.01^{b}	3.11±0.04^{b}	2.03±0.05	5.22±0.10^{b}	0.00±0.00^{b}
		Dichloromethane	-	0.19±0.00^{a}	0.23±0.00^{b}	3.34±0.00^{ab}	1.56±0.01	4.97±0.01^{b}	0.00±0.00^{b}
		Methanol	-	0.11±0.00^{a}	0.10±0.00^{a}	1.68±0.08^{a}	0.79±0.15	2.51±0.23^{a}	0.00±0.00^{a}
		Ethanol	-	0.50±0.05^{a}	0.43±0.05^{c}	8.09±0.71^{c}	4.32±0.42	12.58±0.28^{c}	0.00±0.00^{c}
Moringa oleifera	Seed	Ethanol	0.08±0.01	0.12±0.02	0.18±0.01	2.60±0.23	5.25±0.47	8.01±0.72	0.02±0.00

Values in the same column (in the same block) followed by the same letter are not significant at $p \leq 0.05$.

extract), 14.43 (*P. africana* bark (K) methanol extract), and 0.04 (*P. yohimbe* root dichloromethane extract) respectively. Since *P. yohimbe* extracts contained more pigments in all plant parts it would be a potentially valuable resource for the food and pharmaceutical industry. The antioxidant role of carotenoids is well known, serving to protect membranes and tissues from oxidative damage, preventing or minimizing serious diseases such as cancer, heart disease, and nutritional blindness (B. Simpson et al., 2012). The pro-vitamin A activity of β-carotene, the suppression of oxidation of low-density lipoprotein (LDL) and lowering of blood cholesterol levels by lycopene and the increased activities of lymphocytes, and protection of the immune system from damage by ultraviolet (UV) light and x-rays are some of the benefits of carotenoids (A. Shamina, 2007; B. Simpson et al., 2012).

The anthocyanin content of the plants tested was low, compared to the other pigments. In *P. yohimbe* dichloromethane extract, the anthocyanin content was 0.04 mg/g, which amounts to 7.1% of the carotenoid content in the same extract. The low anthocyanin content was not unexpected as anthocyanins are commonly found in flowers and fruits of many plants (Khoo et al., 2017; Sadikoğlu & Cevahir, 2004). They are nevertheless important because they possess antioxidative and anti-microbial properties, improving visual and neurological health, and protecting against various non-communicable diseases (Khoo et al., 2017; B. Simpson et al., 2012). They are also used as food colorant in drinks, food spreads (jams and jellies), gelatins, pastries, and confectioneries because of their intense blue or red colorations (B. Simpson et al., 2012). Among the solvents used, methanol was superior in extracting pigments from *P. yohimbe* and *P. africana* while ethanol was superior for *M. charantia* and *Othero spp* (Table 5). The β-carotene content of *M. charantia* leaf ethanol extract in this study (0.50 mg/g) was comparable to that by Zhang et al., (2009), who reported 0.42 mg/g in the dried vegetable, but higher than that by Cuong

et al. with 287.03 µg/g. This may be due to differences in methodologies as HPLC was used in β-carotene quantification rather that spectrophotometric analysis (Cuong et al., 2017). The contents of chlorophyll A and B in *M. oleifera* ethanol extract (2.60 and 5.25 mg/g respectively) in this study were higher than that reported in Asuit region, Egypt by Barakat and Ghazal (2016) (1.78 and 4.89 mg/100g DW, respectively). Potentially, this is due to the seeds being defatted prior to testing, which could have resulted in some pigment loss. To our knowledge, there have not been any studies on the pigment content of *P. africana* and *P. yohimbe* and none comparing pigment contents between different plant parts of the same plants.

For the total carotenoid content, *P. africana* (Kenya) bark ethanol extract is comparable to the value reported by Wijesundara et al. (2017) for the ethanol extract of ginger rhizome (455.0 µg/g). The ethanol extract of *P. yohimbe* bark is slightly lower than the ethanol extract of liquorice root (157.4 µg/g), and the ethanol extract of *P. yohimbe* root is about a fourth of the value reported for the ethanol extract of thyme flowering shoots (2373.2 µg/g) reported by Wijesundara et al., 2017. The carotenoid content for *P. africana* (Cameroon) in descending order in the different plant parts was bark > leaf > root and for *P. yohimbe* bark > root > leaf while the total chlorophyll content for *Prunus* (Cameroon) and *P. yohimbe* plants, respectively, was in descending order leaf > bark > root and leaf > root > bark for *Prunus* (Cameroon) and *P. yohimbe* plants, respectively.

The Pearson's correlation coefficients between TPC, TFC and antioxidant tests for the different plant part extracts as presented in Table 6 were obtained to investigate the relationship or strength of association between the different tests. The TPC correlated strongly with TFC of acetone extracts of *P. yohimbe* and *Orthero* roots, FRAP of ethanol and methanol extracts of *P. africana* (Cameroon) root, acetone and methanol extracts of *P. africana* (Cameroon) leaf, methanol extracts of *P. yohimbe* leaf, *M. charantia* leaf and the TEAC of *P. africana* (Cameroon)

bark water extract, ethanol extracts of *P. yohimbe* leaf and *Orthero* root. This indicates that the phenolic compounds in these plant extracts are largely responsible for their antioxidant effect. There was little consistency in results when comparing different solvent extracts of plant parts or the various parts of the same plant used. This may be due to the method used to obtain a total

Table 6: Pearson's correlations between total phenolic content and total flavonoid content (TFC), ferric ion reducing antioxidant power (FRAP) and Trolox equivalent antioxidant capacity (TEAC)

Plant	Part	Extraction solvent	Total phenolic content		
			TFC	FRAP	TEAC
Prunus africana (C)	Bark	Acetone	0.32	-0.89	0.59
		Dichloromethane	0.30	0.00	-0.50
		Methanol	0.65	-0.63	-0.68
		Ethanol	-0.76	-0.89	0.50
		Water	-0.97	0.19	0.95
Prunus africana (C)	Root	Acetone	-0.90	-0.33	0.74
		Dichloromethane	0.33	-0.57	-0.96
		Methanol	-1.00	1.00	-0.54
		Ethanol	-0.65	0.96	-0.33
Prunus africana (C)	Leaf	Acetone	0.00	0.99	0.76
		Dichloromethane	-0.87	0.89	-0.90
		Methanol	0.00	1.00	-0.91
		Ethanol	-0.63	-0.71	0.23
Prunus africana (K)	Bark	Acetone	0.28	0.86	-0.78
		Dichloromethane	-0.76	-0.78	0.69
		Methanol	0.87	-0.33	-1.00
		Ethanol	0.76	-0.91	0.43
Pausinystalia yohimbe	Bark	Acetone	-0.87	-0.97	-0.99
		Dichloromethane	0.61	-0.92	0.33
		Methanol	-0.55	-0.33	-0.22
		Ethanol	0.48	-0.84	-0.91
		Water	0.46	-0.88	-0.28
Pausinystalia yohimbe	Root	Acetone	0.98	0.50	-0.92
		Dichloromethane	0.82	0.77	0.59
		Methanol	-0.50	-0.86	0.89
		Ethanol	0.78	0.14	-0.76
Pausinystalia yohimbe	Leaf	Acetone	0.86	0.58	-0.50
		Dichloromethane	0.32	-0.70	-0.32
		Methanol	0.28	1.00	0.42
		Ethanol	-0.89	-0.93	1.00
Orthero	Root	Acetone	0.99	-0.12	0.76
		Dichloromethane	0.50	-0.65	-0.69
		Methanol	0.19	0.08	-0.50
		Ethanol	-0.87	-0.87	0.99
Momordica charantia	Leaf	Acetone	0.62	-0.41	-0.90
		Dichloromethane	-0.69	0.19	0.44
		Methanol	-0.61	1.00	-0.50
		Ethanol	-0.98	0.33	-0.20
Moringa oleifera	Seed	Ethanol	-0.33	-1.00	_

C - Cameroon; K - Kenya

	Top 10% positive correlations
	Top 10% negative correlations
	Between 0.65 and top 10% positive correlations
	Between -0.65 and top 10% negative correlations
	Between -0.65 and 0.65

estimate of the amounts of phenolic compounds present in an extract. The Folin-Ciocalteu assay has been shown to be non-specific not only for polyphenols but also any other compound that

Table 7: Pearson's correlations between the antioxidant assays: ferric ion reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Plant	Part	Extraction solvent	FRAP	
			DPPH	TEAC
Prunus africana (C)	Bark	Acetone	0.53	-0.89
		Dichloromethane	0.58	0.87
		Methanol	0.00	-0.14
		Ethanol	0.27	-0.06
		Water	-0.83	0.49
Prunus africana (C)	Root	Acetone	0.94	-0.88
		Dichloromethane	0.56	0.33
		Methanol	-0.87	-0.54
		Ethanol	-0.81	-0.06
Prunus africana (C)	Leaf	Acetone	0.92	0.83
		Dichloromethane	-0.68	-0.61
		Methanol	-0.63	-0.91
		Ethanol	1.00	0.52
Prunus africana (K)	Bark	Acetone	-0.91	-0.99
		Dichloromethane	-0.99	-0.99
		Methanol	-0.43	0.33
		Ethanol	-0.74	-0.77
Pausinystalia yohimbe	Bark	Acetone	-0.55	0.99
		Dichloromethane	-0.39	0.06
		Methanol	-0.17	0.99
		Ethanol	-0.93	0.99
		Water	-1.00	0.70
Pausinystalia yohimbe	Root	Acetone	-0.50	-0.11
		Dichloromethane	-0.87	-0.06
		Methanol	0.86	-0.53
		Ethanol	-0.55	-0.76
Pausinystalia yohimbe	Leaf	Acetone	1.00	0.42
		Dichloromethane	0.96	0.90
		Methanol	0.61	0.45
		Ethanol	-0.50	-0.95
Orthero	Root	Acetone	-0.44	-0.74
		Dichloromethane	0.65	-0.09
		Methanol	-0.43	-0.90
		Ethanol	-0.50	-0.93
Momordica charantia	Leaf	Acetone	-0.18	0.76
		Dichloromethane	-0.65	-0.80
		Methanol	0.65	-0.50
		Ethanol	0.00	-0.99
Moringa oleifera	Seed	Ethanol	-0.54	-

C - Cameroon; K - Kenya.

	Top 10% positive correlations
	Top 10% negative correlations
	Between 0.65 and top 10% positive correlations
	Between -0.65 and top 10% negative correlations
	Between -0.65 and 0.65

could be oxidized by the Folin reagent, leading to a poor specificity of the assay (Fernandes de Oliveira et al., 2012). This suggests that there may be other non-phenolic compounds present in the plant extracts that influenced the antioxidant activity of some of the solvent extracts as also indicated by Anagnostopoulou et al. (2006), who reported an R-value of 0.42 between TPC and DPPH for extract obtained from sweet orange peel.

The Pearson's correlation coefficients between the antioxidant tests for the different plant part extracts are presented in Table 7. The FRAP showed a strong positive correlation with the DPPH of the acetone extract of *P. africana root*, ethanol extract of *P. africana* leaf, acetone and dichloromethane extracts of *P. yohimbe* leaf and a strong positive correlation between the FRAP and TEAC of the acetone, methanol and ethanol extracts of *P. yohimbe* bark. There was also a strong negative correlation between the FRAP and DPPH of dichloromethane and water extracts of *P. africana* (Kenya) bark and *P. yohimbe* bark respectively, and a strong negative correlation between the FRAP and TEAC of the acetone, dichloromethane extracts of *P. africana* (Kenya) bark, ethanol extracts of *P. yohimbe* and *M. charantia* leaves. This differs from results from a previous study where the R value was 0.808 and above for the antioxidant tests (Utakod et al., 2017) but is similar to the varied correlation results in a study by Thaipong et al. (2006), where they reported no consistent correlations between assays to measure antioxidant activity between methanol and dichloromethane extracts of guava fruit, as well as between the different assays for the dichloromethane extract. As previously reported the antioxidant capacity determined by *in vitro* assays differs as *in vitro* models do not assess all of the antioxidant activities in plant materials, may be influenced by highly pigmented samples and depend on the solvent used for extraction (Floegel et al., 2011).

Based on the EC_{50} results, nine extracts from *P. africana* and *P. yohimbe* were selected for HPLC analysis of phenolic content. Methanol and water extract of *P. africana* (C) bark had higher amounts of phenolic acids compared to acetone bark and ethanol root extracts of the same plant and all the extracts from *P. yohimbe*. Both contained all the phenolic acids except trans-ferulic acid for the methanol extract and caffeic acid for the water extract. Vanillic acid was the only phenolic acid found in all tested samples, while trans-sinapic acid was found only in methanol and water extracts of *P. africana* bark. The acetone extract of *P. yohimbe* leaf contained the highest amount of caffeic acid (13.54 mg/g). This acid is one of the major hydroxycinnamic acid components in wine and it is a well-known antioxidant, boosting immunity controlling lipid levels in blood, and serving as anti-mutagenic agents. The caffeic acid content is higher than in cauliflower (0.058 mg/g), carrots (0.09 mg/g), lettuce (1.57 mg/g), potatoes (2.80 mg/g) and *Z. acanthopodium* (0.136 mg/g). The ferulic acid content in the same extract, however, is comparable to results reported for ferulic acid in methanol extract of *Z. acanthopodium* (0.23 mg/g) (Seal, 2016).

Vanillic acid, with a range of 38.91 - 116.41 mg/g dry extract, was the major phenolic acid in most of the tested extracts and was highest in the methanol extract of *P. yohimbe* leaf, even higher than the methanol extract of *H. strigosum* (38.58 mg/g) and >100 fold higher than the water extract of mountain germander extract (0.165 mg/g) (Qayyum et al., 2016; Tumbas et al., 2004). In a previous study, Qayyum et al. (2016) reported that the amount of phenolic components is diversified at the sub-cellular level in plants, which may explain the differences in concentration of the phenolic acids in the different parts of the same plant and between. To the best of our knowledge, the phenolic composition of different extracts of *P. africana* and *P. yohimbe* plant parts is new, adding to previous studies on antioxidant activity and other screening assays.

Table 8: Phenolic acid content of selected extracts of *P. africana* and *P. yohimbe*

Plant	Part	Extraction solvent	Phenolic acids (mg/g of extract)						
			Methyl 4-hydroxy	Protocatechuic acid ethyl	Trans sinapic acid	Vanillic acid	Trans ferulic acid	p-coumaric	Caffeic acid
Prunus africana (C)	Bark	Acetone	0.24±0.01^{ab}	0.25±0.01^{e}	ND	45.55±0.15^{ab}	ND	0.25±0.02^{a}	1.83±0.04^{ab}
		Methanol	0.15±0.12^{ab}	0.22±0.00^{d}	81.11±2.68^{a}	51.94±0.74^{bc}	ND	0.34±0.02^{a}	1.45±1.20^{ab}
		Water	0.20±0.01^{ab}	0.38±0.01^{f}	102.22±2.12^{b}	71.87±4.60^{d}	0.10±0.01^{a}	0.57±0.03^{b}	ND
	Root	Ethanol	ND	0.15±0.01^{c}	ND	42.21±3.73^{a}	0.38±0.05^{c}	ND	3.05±0.16^{b}
Pausinystalia yohimbe	Leaf	Acetone	2.43±0.14^{c}	0.22±0.01^{d}	ND	56.29±1.00^{c}	0.22±0.06^{b}	2.13±0.09^{d}	13.54±0.68^{e}
		Methanol	5.21±0.27^{d}	ND	ND	116.41±0.57^{f}	ND	2.72±0.08^{e}	5.22±0.31^{c}
		Ethanol	0.31±0.00^{b}	ND	ND	82.15±3.13^{e}	ND	1.54±0.13^{c}	8.30±0.51^{d}
	Root	Acetone	0.03±0.00^{a}	0.06±0.00^{b}	ND	38.91±3.00^{a}	ND	4.41±0.14^{f}	2.97±1.44^{b}
		Methanol	0.03±0.00^{a}	0.04±0.00^{a}	ND	54.17±1.06^{c}	ND	ND	5.79±0.39^{c}

C - Cameroon; ND - not detected. Values are mean of three determinations (n = 3) ± standard deviation (SD). Values in the same column (in the same block) followed by the same letter are not significant at $p \leq 0.05$.

Table 9: Phytosterol content of different plant parts of *P. africana* and *P. yohimbe*

Plant	Part	Sterols (mg/g of dry sample)		
		Stigmasterol	β-sitosterol	Campesterol
Prunus africana (C)	Root	0.09±0.02^{a}	0.73±0.04^{c}	ND
	Leaf	0.09±0.01^{a}	2.26±0.05^{d}	0.15±0.02^{c}
	Bark	0.08±0.01^{a}	0.55±0.09^{b}	ND
Pausinystalia yohimbe	Root	0.08±0.01^{a}	0.35±0.07^{a}	0.02±0.00^{a}
	Leaf	0.10±0.00^{a}	0.46±0.08^{ab}	0.11±0.01^{b}
	Bark	0.08±0.02^{a}	0.38±0.03^{a}	0.03±0.01^{a}

C - Cameroon; ND - not detected. Values are mean of three determinations (n = 3) ± standard deviation (SD). Values in the same column (in the same block) followed by the same letter are not significant at $p \leq 0.05$.

Analysis of phytosterols in *P. africana* and *P. yohimbe* revealed that stigmasterol, β-sitosterol, and campesterol were present in all plant parts tested except for *P. africana* root and bark (Table 9). Beta-sitosterol varied most significantly between plant parts, ranging from 0.55 - 2.26 mg/g of sample and 0.35 - 0.46 mg/g of sample, compared to stigmasterol with a range of 0.08 - 0.09 mg/g of sample and 0.08 - 0.10 mg/g of sample in *P. africana* and *P. yohimbe*, respectively. The concentration of β-sitosterol in the leaf and bark of *P. africana* in this study were higher than those reported by Mugaka et al. (2013); this may be because of differences in methodology, as HPLC was used in that study. However, in agreement with the same study, the concentration of β-sitosterol in the stem barks of *P. africana* was lower than in the leaves (Mugaka et al., 2013). *P. africana* bark's β-sitosterol values in this study were comparable with values reported by Kadu et al. (2012) who conducted a bioactive component study of *P. africana* bark extracts from different populations using GC-MS analysis (349 - 583 mg/kg). The β-sitosterol content of *P. africana* and *P. yohimbe* in this study were higher than values reported for many fruits and vegetables; and content in the roots of *P. africana* are comparable with those found in avocado, which is considered a very rich source of β-sitosterol (76.4 mg/100g). The campesterol and stigmasterol concentrations were more comparable to values reported for common fruits and vegetables (Duester, 2001; Kadu et al., 2012; Piironen et al., 2003).

3.6 Conclusion

The extraction yield of the medicinal plants increased with the polarity of the extracting solvent, supporting findings from previous studies that polar solvents are preferable for extraction of phenolic compounds. All plant parts tested contained significant amounts of phenolic compounds and pigments, making them a potentially valuable natural source of antioxidants with activities comparable to ascorbic acid. There were strong positive correlations between the TPC

and TFC, FRAP and TEAC of several extracts, indicating that a significant part of the antioxidative effects of these plant extracts was due to the presence of phenolic compounds. To our knowledge, there have been no previous studies on the pigment content or phenolic composition of *P. africana* and *P. yohimbe*. HPLC analysis of phenolic acids showed the presence of seven phenolic acids, with vanillic acid being the major phenolic acid in most of the plant extracts. GC analysis of phytosterols showed a higher β-sitosterol content in *P. africana* and *P. yohimbe* than found in many fruits and vegetables evaluated in previous studies. The qualitative analysis of *P. yohimbe* revealed the presence of yohimbine. Our findings also show significant carotenoid and chlorophyll contents, which may explain some of the mechanisms of action of these plants as used in traditional medicines. Further studies need to investigate this possibility and to identify other possible uses for these medicinal plants.

CHAPTER 4: Connecting Statement

The toxicity of chemotherapeutic drugs frequently interferes with the treatment of cancer using current medications. Natural compounds from flowering plants could provide an alternative or addition to cancer chemotherapy. Testing the effects of compounds on the viability of cells grown in culture is widely used as a predictor of potential toxic effects on live animals. Cell lines have a long history as models to study molecular mechanisms of disease and cancer biology. Connecting genomic alterations to drug responses can aid in understanding cancer patient's response to therapy. Extracts from different parts of *Prunus africana* were tested on their ability to inhibit the growth of cancer cells on the human prostate cancer C4-2 cells for the first time.

4.0 Materials and Methods

4.1 Materials

Bark, root and leaf plant parts of *Prunus africana* were obtained from Cameroon. Dr. Robert A. Sikes of the University of Delaware donated the C4-2 prostate cancer cell lines. Roswell Park Memorial Institute (RPMI) media, trypsin/EDTA, phosphate buffered saline (PBS), dimethyl sulfoxide and ethanol were purchased from Sigma-Aldrich (St. Louis, MO) and Thermo Fisher Scientific (Waltham, MA) and ethanol was purchased from Decon Labs (King of Prussia, PA).

4.2 Methods

4.2.1 Sample preparation and extraction

Dried *P. africana* plant parts (bark, root and leaves) were pulverized in a coffee grinder, and 10 g sample of each plant part was homogenized with acetone, dichloromethane, methanol, or ethanol, at a ratio of 1:10 (w/v) as previously described in section 3.2.1.

4.2.2 Cell culture

The cells were grown to confluency in a T-75 tissue culture flask in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with glutamine, 10% fetal bovine serum and antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin). Cells at a seeding cell density of 8 x 10^5 cells per well were plated into six well plates containing 2 mL of medium, with sufficient plates for a 4-day proliferation study. The cells were then incubated overnight at 37°C in 5% CO_2. The *Prunus* extracts were first dissolved in 100% ethanol or 1% dimethyl sulfoxide and further diluted with RPMI 1640 medium for the cytotoxicity assay.

Different concentrations of the extracts were tested for effect in a preliminary assay with concentrations ranging from 1 mg/mL to 0.001 mg/mL. Based on the results of the preliminary testing, extracts at concentrations of 0.025 mg/mL, 0.01 mg/mL, 0.005 mg/mL, 0.0025 mg/mL and 0.001 mg/mL were then added in triplicate to the cells and incubated for 24 h at 37°C with an ethanol/ DMSO vehicle control. After 24 h, media from one set of plates was removed, and the cells were stripped and counted using a NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA), while the media from the remaining set of cells were removed and replaced with the same concentration of extracts in 2 mL of media. This was repeated every 24 h up to the 4^{th} day and a proliferation profile of the treated cells was plotted and compared to the profile of the control cells.

4.3 Results and Discussion

A concentration-dependent cytotoxicity was observed in the preliminary testing of bark and root extracts on C4-2 cells, with the methanol extract of the bark showing a stronger effect than the others. There was complete lysis of all cells above 0.05 mg/mL for all bark and root samples. Differences between the different extracts was most obvious at 0.05 mg/mL (Fig. 16 A-

G). A proliferation effect was also observed, especially in concentrations below 0.01 mg/mL when incubated for more than 24 h. This may be due to some previously undescribed compounds in the extracts or a different composition of the same compounds in this sample of *P. africana*, resulting from its genetic composition and epigenetic factors. Compared to other plant parts, leaf extracts showed much less cytotoxicity at the same concentrations, although there was marked clustering of cells and proliferation compared to the control. This may indicate a higher percentage of the compound or combination of compounds responsible for the proliferation seen with treatment of cells using bark and root extracts. In support of this hypothesis, Mugaka et al. (2013) reported differences in secondary metabolite (β-sitosterol and n-docosanol) content between the different plant parts of *P. africana* (bark and leaves) and differences between plants from different geographical regions in Tanzania (Mugaka et al., 2013).

Mostly dead cells are visible with some granulation-like material in areas of cell clusters in Fig. 16B. Most cells in Fig. 16c are round but still appear attached to the bottom of the well. Many dead or round cells are present in Fig. 16d, with a few healthy-looking cells as well. Most cells in Fig. 16e are dead or round with significant debris but a few healthy-looking cells are present. Most cells in Fig. 16f are also dead or round with a few healthy-looking cells present, but healthy cells are fewer than those seen in Fig. 16e. Very little cell death is seen in Figs. 16f and h compared to cells treated with extracts from the bark and root. However, there is marked cell clustering, and rapid proliferation appears to be occurring in both treatments. The phenomenon hormesis, which is a dose-response phenomenon characterized by a low-dose stimulation and a high-dose inhibition may account for the proliferation seen at lower doses (V. Calabrese et al., 2010). Southam and Ehrlich first presented the term hormesis in published literature in 1943 by, reporting that low doses of extracts from the red cider tree enhanced the proliferation of fungi with

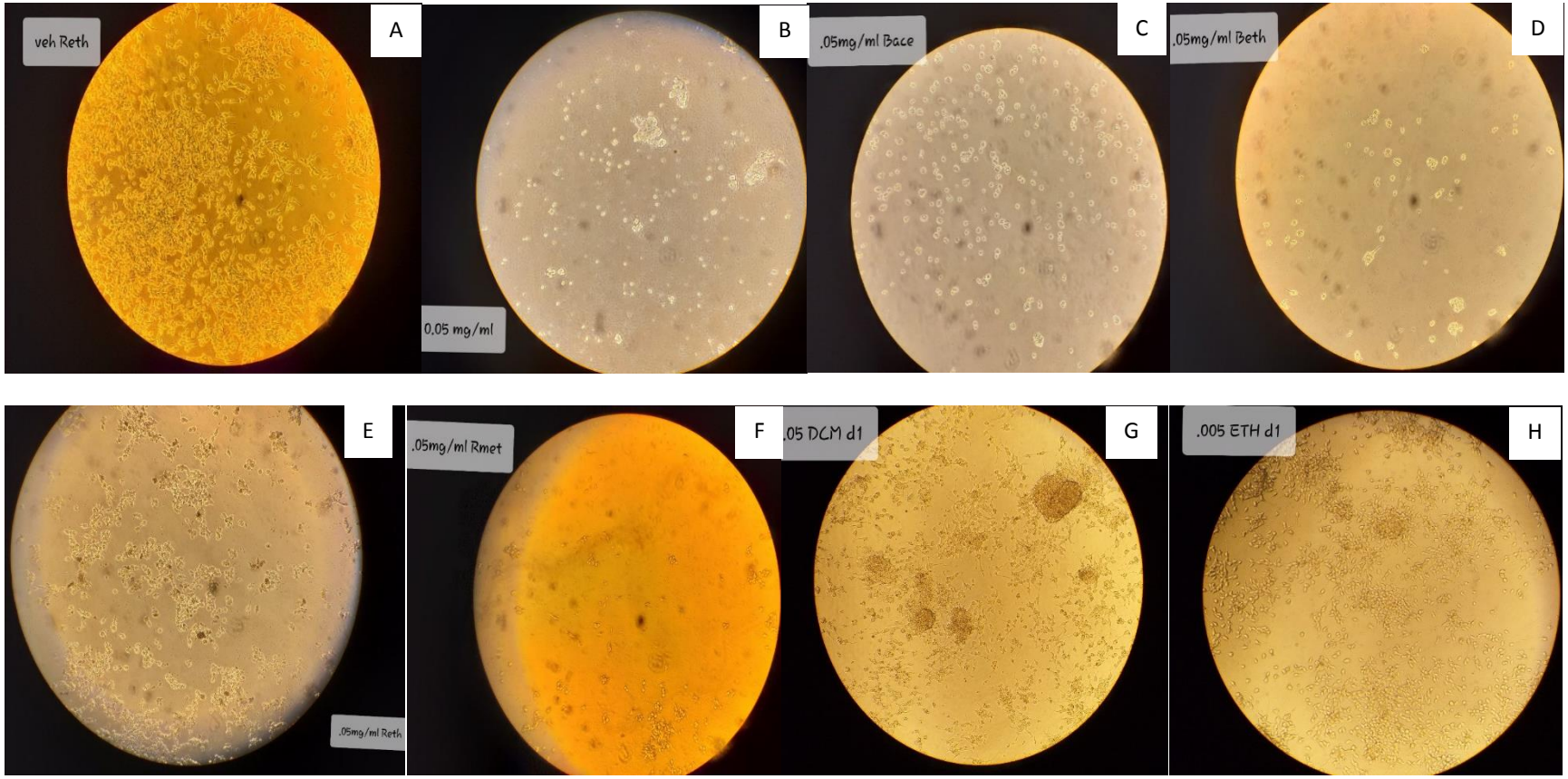


Figure 16: C4-2 cells after 24 h exposure to treatment with different *P. africana* plant part extracts. Cells treated with ethanol in media (control) (A), 0.05 mg/mL *Prunus* bark methanol extract (B), *Prunus* bark acetone extract (C), *Prunus* bark ethanol extract (D), *Prunus* root ethanol extract (E), *Prunus* root methanol extract (F), *Prunus* leaf dichloromethane extract (G) and *Prunus* leaf ethanol extract (H).

the overall shape of the dose response being biphasic. Similar types of dose-response observations have been subsequently reported by other researchers (E. J. Calabrese, 1999, 2005; V. Calabrese et al., 2010). This low-dose stimulation that manifests immediately below the pharmacological and toxicological thresholds is modest in magnitude, being at most about 30 - 60% greater than the control group response and may result from either a direct stimulation or via an overcompensation stimulatory response after disruption in homeostasis (E. J. Calabrese, 1999; V. Calabrese et al., 2010). Hormetic-like biphasic dose responses have been reported and demonstrated in 136 tumor cell lines from over 30 tissue types for over 120 different agents (E. J. Calabrese, 2005; V. Calabrese et al., 2010).

In vitro cytotoxicity screening is a valuable tool in drug discovery and is used widely by researchers, especially when bio-prospecting for potentially active cancer drugs (Maiyo et al., 2016). Results from the proliferation assay showed growth inhibition of C4-2 cells by *P. africana* bark and root extracts in a dose dependent manner. Shenouda et al. (2007) reported similar results in a tissue culture study, where they suggested that ethanolic extracts of *P. africana* bark inhibited the growth in human prostate cancer cell lines (PC-3 and LNCaP) by 50% at 2.5 µL/mL and induced significant apoptosis in both cell lines (PC-3 and LNCaP) at 2.5 µL/mL compared to untreated cells (Shenouda et al., 2007). This shows that crude extracts of *P. africana* inhibit growth of both hormonally sensitive (LNCaP) and hormonally insensitive (C4-2) cells modelling advanced prostate cancer at similar concentrations. Thus, the crude extract has the potential for treatment of advanced prostate cancer which is hormonally insensitive. Although there was appreciable growth inhibition in this study, there was a significant difference only between the control cells and cells treated with the highest concentration (0.025 mg/mL) of bark methanol

extract ($p < 0.05$) (Figs. 17 and 18), indicating the need of a significant dose to control advanced stage prostate cancer cells.

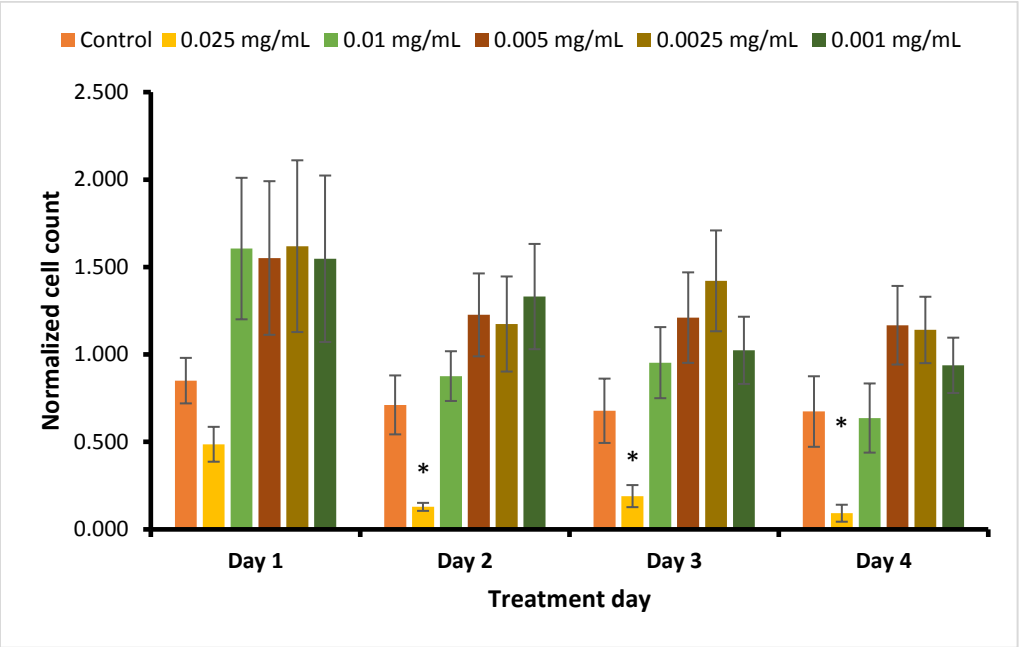


Figure 17: Proliferation assay for *P. africana* bark methanol extract.

Values are mean of three determinations (n = 3) ± SE. Asterisk (*) denotes the presence of statistical significance.

The inhibition of the growth of C4-2 cells was present from day 1 through day 4 of incubation with the *P. africana* extract as seen in the study by Shenouda et al. (2007) but there were variations in the percentages of inhibition. The maximum inhibition by treatment with 0.025 mg/mL methanol bark extract was seen on day 2 (82% reduction), and for methanol root extract on day 3 (37% reduction) unlike in the previous study where a more consistent inhibition was seen (50%) compared to the control. This may be due to differences in metabolite composition between bark and root extracts and methodology as the previous study assayed for the total DNA concentration using the thymidine incorporation assay, while simple cell counting was used in this

study (Shenouda et al., 2007). In addition, C4-2 cells differ from the cell lines used in the previous study by being hormone insensitive. However, it is clear that the bark extract showed more potent inhibition of cell growth than did the root extracts Figs. 17 and 18.

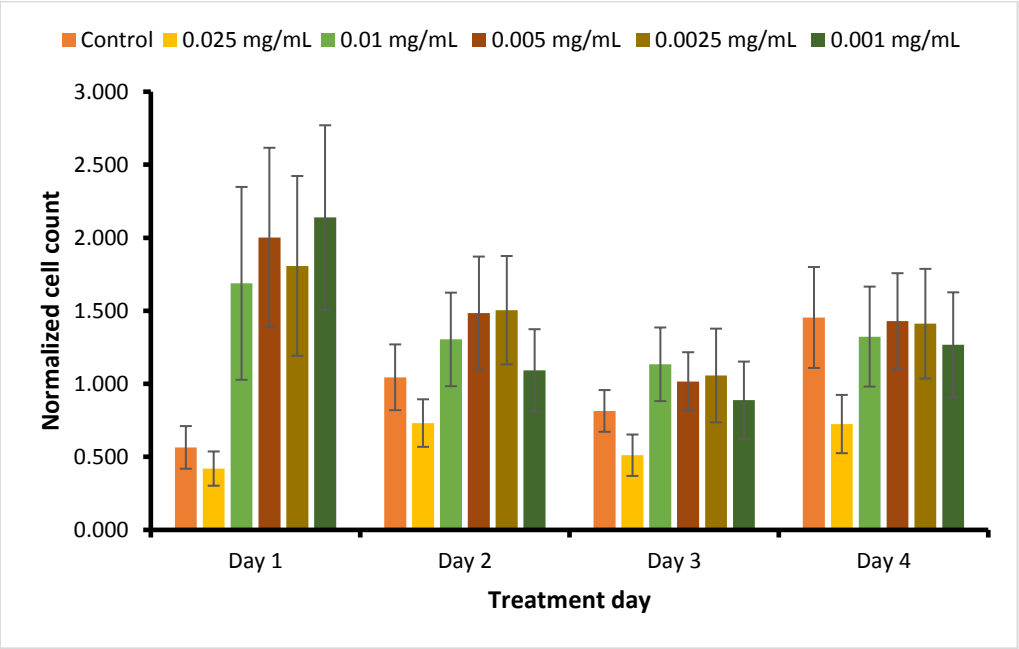


Figure 18: Proliferation assay for *P. africana* root methanol extract.
Values are mean of three determinations (n = 3) ± SE.

The growth inhibition effect of *P. africana* root extract is notable because although there have been a number of studies on the cytotoxic effects of *P. africana* bark extract, or components of bark extract, there have been few on the effects of other parts of the *P. africana* tree (Boulbès et al., 2006; Kadu et al., 2012; Karan et al., 2017; Komakech et al., 2017; Nabende et al., 2015; Shenouda et al., 2007). Comparing extracts from the bark and root obtained by using the same solvent excludes any possible effect of extraction solvent on the extract composition and suggests that any differences seen are due to the natural composition of the plant parts. Although bark

extracts had a higher inhibitory effect, the inhibitory effect of the root extracts was also evident, signifying that plant parts other than the bark can be utilized.

This is particularly important because *P. africana* bark has been exploited for use in the management of benign prostatic hypertrophy (BPH). Consequently, it is now an endangered species in Africa, needing to be conserved. This also has been recommended at international level by various organizations including UNESCO (Mugaka et al., 2013; Nabende et al., 2015). Utilization of other plant parts will hopefully lead to a less destructive exploitation of the *P. africana* tree, allowing for its use for medicinal purposes without the danger of extinction of the species. In addition, further analysis of other plant parts may lead to the discovery of additional secondary metabolites that may be useful for many other purposes. As more evidence emerges of the role secondary metabolites play in human health and nutrition, there is a need for more research into the composition of such medicinal plants and their potential uses. In light of the challenges facing chemotherapy and modern medicine such as drug toxicity and resistance, it has become imperative to find alternative drugs with improved specificity and efficiency.

4.4 Conclusion

The first experimental study testing use of bark and root extracts of *P. africana* on C4-2 cells showed a concentration-dependent cytotoxicity, with the bark methanol extract showing the strongest effect. There was complete lysis of all cells above 0.05 mg/mL for all cells treated with bark and root extracts, while cells treated with leaf extracts showed a proliferation effect and clustering of cells compared to the control. The proliferation effect may be explained by the phenomenon hormesis, which is a dose-response phenomenon characterized by a low-dose stimulation and a high-dose inhibition or by a different composition of active secondary

metabolites in the leaves than seen in the bark and root extracts. A four-day proliferation assay, using *P. africana* bark and root extracts at concentrations between 0.025 mg/mL and 0.001 mg/mL, showed growth inhibition of C4-2 cells in a dose dependent manner on all four days of the assay. There was a significant difference between control cells and cells treated with 0.025 mg/mL of the bark methanol extracts but not the root methanol extracts. As C4-2 cells are hormonally insensitive and designed to mimic advanced prostate cancer, crude extracts of *P. africana* are a possible treatment option, not only for hormone sensitive prostate cancer, but also advanced, hormonally insensitive prostate cancer. Further research is however needed, to understand the mechanisms through which the extract causes inhibition of hormonally insensitive cancer.

5. General Conclusion and Recommendations

5.1 General conclusions

The medicinal plants investigated in this study (*Prunus africana*, *Pausinystalia yohimbe, Moringa oleifera, Momordica charantia* and *Orthero spp*) contain significant amounts of secondary metabolites, with phenolic acids being a major constituent of their extracts. They also possess potent antioxidant activities, with some being comparable with the antioxidant activity of ascorbic acid, a well-known antioxidant. In addition, *P. africana* extracts demonstrated a concentration-dependent growth inhibition of C4-2 prostate cancer cells in *in vitro* tests. As C4-2 cells are hormonally insensitive and designed to mimic advanced prostate cancer, crude extracts of *P. africana* are a possible treatment option, not only for hormone sensitive prostate cancer, but also advanced, hormonally insensitive prostate cancer. To our knowledge this is the first study examining use of *P. africana* extracts on C4-2 prostate cancer cells. Further research is however needed, to understand the mechanisms through which the extract causes inhibition of hormonally insensitive cancer. This study validates the use of these plants in traditional medicine and highlights the potential of these plants to serve as new prophylactic and treatment options in the management of chronic diseases in general, and cancer in particular

5.1 Recommendations for future studies

Having established the cytotoxic effects of *P. africana* extracts on C4-2 cells, it will be necessary to determine the selectivity of its cytotoxic activity to cancer cells by comparing its effect on cancer cells and control non-cancer cell lines grown under the same conditions. Further investigation on possible mechanisms of action of the extracts is needed, including an apoptosis assay. Our preliminary study also shows that certain concentrations of the unpurified extracts may

be upregulation mediators, attenuating proliferation, increasing the survival of some of the prostate cancer cells. RNA sequencing (RNA-seq) can be used to compare the transcriptomes of up-and down regulated genes to evaluate this observation. In addition, a comparison of the effects on C4-2 prostate cancer cells of crude and isolated phenolic acids found in *P. africana*, alone or in combination to determine potential synergistic effects of those compounds or a combination of different classes of secondary metabolites will add to existing knowledge on the phytochemical efficacy of *P. africana.*

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