



Faculty of Sciences

Department of Biological Sciences and Biotechnology

**AN EVALUATION OF ANTIOXIDANT PROPERTIES AND DEVELOPMENT OF ANTICANCER INGREDIENTS
FROM *SCLEROCARYA BIRREA* FRUITS EXOCARP AND *TERMINALIA PRUNOIDES* PODS**

By

Phazha Bushe BAETI

Student ID number: 19100075

BSc (Agricultural education) (University of Botswana, Gaborone)

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Supervisor: Dr. KWAPE Tebogo E.

Department of Biological Sciences and Biotechnology

Faculty of Sciences, BIUST

kwapet@biust.ac.bw

Supervisor: Dr. MASISI Kabo

Department of Biological Sciences and biotechnology

Faculty of Sciences, BIUST

masisik@biust.ac.bw

Supervisor: Dr. GAOBOTSE Goabaone

Department of Biological Sciences and biotechnology

Faculty of Sciences, BIUST

gaobotseg@biust.ac.bw

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LIST OF ABBREVIATIONS

CAT; Catalase

CD95; Cluster differentiation 95

CYFRA 21-1; Cytokeratin fragment 21-1

EGFR; Epidermal growth factor receptor

GAPDH; Glyceraldehyde 3-phosphate dehydrogenase

GSH; Glutathione

M1E1M2E2; *Sclerocarya birrea* fruit exocarp methanol extracts + ethyl acetate extracts + *Terminalia prunoides* pods methanol extracts + ethyl acetate (1:1:1:1)

M1M2; *Sclerocarya birrea* fruit exocarp methanol extracts + *Terminalia prunoides* pods methanol extracts (1:1)

M2E2; *Terminalia prunoides* pods methanol extracts + ethyl acetate extracts (1:1)

MACC1; Metastasis associated with colon cancer 1

mRNA; messenger ribose nucleic acid

ROS; Reactive oxygen species

RTqPCR; Real time quantitative polymerase chain reaction

SOD; Superoxide dismutase

VEGF; Vascular endothelial growth factor

DECLARATION REGARDING THE WORK AND COPYRIGHT

Candidate: PHAZHA BUSHE BAETI

Student ID: 19100075

Thesis Title: AN EVALUATION OF ANTIOXIDANT PROPERTIES AND DEVELOPMENT OF ANTICANCER INGREDIENTS FROM *SCLEROCARYA BIRREA* FRUITS EXOCARP AND *TERMINALIA PRUNOIDES* PODS

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Primary Supervisor: DR. TEBOGO E. KWAPE

I, **DR. TEBOGO E. KWAPE**, hereby confirm that I have inspected the above titled thesis and, to the best of my knowledge, it is based on the original work of the candidate.

Signed:



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ABSTRACT

Oxidative stress has been implicated as one of the causes of cancer. Cancer ranks top amongst diseases with a high mortality rate. Conventional drugs are expensive and have been reported to have severe side effects. This creates a need to come up with a solution that is inexpensive with less or no side effects. Therefore, this study aimed to evaluate antioxidant properties and formulate anticancer ingredients from *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods. Hexane, chloroform, ethyl acetate and methanol extracts from *Sclerocarya birrea* fruit exocarp, and *Terminalia prunoides* pods underwent phytochemical screening, qualitative phytochemical analyses, potential antioxidant activity. Furthermore, three anticancer ingredients were formulated from the 2 plants, and then evaluated for their antiproliferative effects on HeLa cells. Two individual extracts; *Sclerocarya birrea* fruit exocarp methanol extracts (M1) and *Terminalia prunoides* pods methanol extracts (M2), and two ingredients; *Sclerocarya birrea* fruit exocarp methanol extracts + *Terminalia prunoides* pods methanol extracts (M1M2), and methanol + ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp + methanol + ethyl acetate from *Terminalia prunoides* pods (M1E1M2E2) with the lowest IC₅₀ values on HeLa cells were evaluated for their effects on the expression of cervical cancer markers; Epidermal Growth Factor Receptor (EGFR), Metastasis-Associated in Colon Cancer 1 (MACC1), Vascular Endothelial Growth Factor (VEGF), Cytokeratin Fragment (CYFRA 21-1), and Cluster differentiation 95 (CD95). Methanol and ethyl acetate extracts from both plants tested positive for saponins, tannins, flavonoids, phenols, terpenoids, cardiac glucoside and steroids. Methanol extracts from both plants recorded total flavonoid content (TFC) of 55.2± 3.8mg QE/g and 67.8 ±10.4 mg QE/g for *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods respectively, and total phenolic contents (TPC) of 105.4 ± 1.4 mg GAE/g and 113.2 ± 7.6 mg GAE/g for *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods respectively. Methanol extracts from *Sclerocarya birrea* fruit exocarp recorded IC₅₀ values of 0.12 mg/mL, 0.09 mg/mL, and 0.2 mg/mL of 2, 2-diphenyl-1-picryl hydrazyl (DPPH), Ferric reducing antioxidant power (FRAP), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assays respectively. Methanol

extracts from *Terminalia prunoides* pods recorded IC₅₀ values of 0.06 mg/mL, 0.07 mg/mL, and 0.24 mg/mL after DPPH, FRAP and ABTS assays respectively. Ethyl acetate and methanol extracts of both plants significantly increased Catalase (CAT), reduced glutathione (GSH), and Superoxide Dismutase (SOD) activities. For antiproliferative effects, the IC₅₀ values of methanol extracts of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods were 75 µg/mL and 190 µg/mL respectively. The M1E1M2E2 and M1M2 ingredients had IC₅₀ values of 77 µg/mL and 83 µg/mL respectively. All treatments: M1E1M2E2, M1M2, M1 and M2 inhibited the growth of HeLa cells by significantly downregulating the expression of EGFR, MACC1, VEGF, CYFRA 21-1 genes, and upregulating the Cluster differentiation 95 (CD95) gene. Ingredients were more effective through effectively regulating gene expression. However, extracts need to be isolated and studied more.

CHAPTER 1: INTRODUCTION

1.1 Background

Dysregulation of reactive oxygen species (ROS) has frequently been linked to development of numerous diseases, including heart diseases, liver diseases, pancreatic diseases and several types of cancer (Yang & Lian., 2020; Liu *et al.*, 2018). Reactive oxygen species are a family of highly reactive and highly unstable oxygen-based radicals, generated from normal physiological pathways such as oxidative phosphorylation and other cell signalling pathways (Sinenko *et al.*, 2021). Excessive levels of ROS create a toxic cellular environment known as oxidative stress (Sies, 2020). Oxidative stress is toxic to the anatomy and physiology of cells, as it has been reported to damage macromolecules (Wang *et al.*, 2016; Lee & Hüttemann, 2014). The action of endogenous antioxidants has been shown to be inhibited by oxidative stress, weakening the cell's antioxidant defences and increasing the cell's vulnerability to oxidative damage (Gottfredsen *et al.*, 2013). Furthermore, oxidative stress may damage DNA, resulting in the changes of gene expression may lead to diseases such as cancer (Rezatabar *et al.*, 2019; Yung & Lian., 2020). Oxidative stress is normally prevented and alleviated by endogenous antioxidants (Ighodaron & Akinloy, 2018). In many cases, endogenous antioxidants alone do not match the physiological required levels to effectively protect cells (Poljsak *et al.*, 2013). Hence the need to supplement with exogenous antioxidants. Antioxidants derived from plants have been found to be effective, and some have been reported to have anticancer properties (Amorati & Valgimigli *et al.*, 2018; Xu *et al.*, 2016).

Through binding and electron/hydrogen reduction, ROS induce oxidative damage on lipids, proteins, nucleic acids (Adwas *et al.*, 2019). Oxidative damage on DNA can alter the chemical and physical form of the DNA strands (Srinivas *et al.*, 2019; Turgeon *et al.*, 2018). Deoxyribose

sugar oxidation by ROS may result in the breaking of the DNA single and/or double strands (Szebeni *et al.*, 2017). Another common DNA oxidation is when free radicals oxidize the nucleobases, causing nucleobase lesions, strand breaks, and proteins DNA cross links (Abdul Salam *et al.*, 2016). Every cell in the human body has a DNA repair mechanism (Iyama & Wilson, 2013). In some cases, due to irregularities in the biomechanisms of DNA repair, genes constituting damaged DNA may be expressed. Expression of altered genes (damaged DNA segments) consequently upsets downstream pathways. Depending on the location of the deoxyribose sugar(s) and/or the nucleotide base being oxidized, some genes may be activated, deactivated, over-expressed or under-expressed (Barnes *et al.*, 2018; Kay *et al.*, 2019 ; Cadet & Douki, 2018).

The cellular oxidative stress defense mechanism uses homeostatic redox by the action of antioxidants to slow down and/or prevent DNA oxidation (Rajendran *et al.*, 2014). Antioxidants are molecules that lower the oxidation potential of or neutralize ROS by donating electrons or hydrogen atoms (Barzegar, 2012). A successful homeostatic redox reaction of antioxidants and ROS give cells antioxidant protection against oxidative stress (Zeb, 2020; Jamshidi-Kia *et al.*, 2020). The significance of antioxidants resulted in the development of artificial antioxidants by pharmaceutical companies as an attempt to supplement the body's naturally produced antioxidants. Some of the artificial antioxidants include butylhydroxyanisol and butylhydroxytoluene which are fortified in processed foods such as sweets, raisins, processed cheese, and peanut butter (Garg *et al.*, 2022). Although synthetic antioxidants are said to be effective, their safety for both humans and animals remains questionable (Islam, 2017).

Cancer is a non-communicable disease characterized by abnormal cells with a distinct advantage of cell proliferation and delayed programmed cell death (Poetsch, 2020). Cancer ranks top among diseases with extremely high mortality rate worldwide (WHO, 2022). The constantly

accelerating prevalence of cancer is attributed to physiological disorders that may be a result of poor diet, dormant physical exercise, environmental factors such as exposure to UV radiation, and pollution (Shankar *et al.*, 2019). These elements contribute to cancer through upregulating the concentrations of ROS (oxidative damage), thus upregulating the detrimental effects of oxidative damage such as DNA damage, and changes in gene expression (Sharifi-Rad *et al.*, 2020; Rezatabar *et al.*, 2019). There are multiple variants of cancer, such as cervical cancer, lung cancer, bone cancer and skin cancer (Hoadley *et al.*, 2018). Cancer is a result of changes in the expression of genes responsible for cell cycle progression, cell growth and cell death (Dhar *et al.*, 2018; Campbell & Tait, 2018). While some mutations are random, some are due to epigenetic modifications (DNA methylation and acetylation), and some are induced by oxidative stress (Pilié *et al.*, 2019 & Barnes *et al.*, 2019).

Existing evidence suggests that DNA oxidation in regions that constitute proto-oncogenes results in the transformation of proto-oncogenes to oncogenes. Oncogenes are genes that are responsible for cell growth and proliferation (Campbell & Tait, 2018). This oxidative stress-based transformation results in the overexpression of oncogenes (Jopkiewicz, 2019; Rezatabar *et al.*, 2019). Multiple researches have shown that, some cancer cells have upregulated growth genes, such as Epidermal Growth Factor Receptor (EGFR), Metastasis-Associated in Colon Cancer 1 (MACC1), Vascular Endothelial Growth Factor (VEGF), and Cytokeratin Fragment (CYFRA 21-1) (Lin *et al.*, 2017; Pastukh *et al.*, 2015; Zheng *et al.*, 2020; Dohmoto *et al.*, 2000). The EGFR gene codes for a tyrosine transmembrane receptor. This receptor is activated by an epidermal growth factor, forming a complex which dictates a signaling pathway that results in the activation of a CREB transcription factor (TF). The CREB TF translocates to transcription initiation sites of cell growth and cell division genes (Li *et al.*, 2017). VEGF is a key signalling protein that, through its binding to the VEGF receptor, initiates a cascade of phosphorylations that lead to the activation of TFs for cell survival, proliferation, cell migration and cohesion (Sawada *et al.*, 2019 & Melincovici *et al.*, 2018). CYFRA 21-1 codes for a cytoplasmic

intermediate fragment, which is part of the cytoskeleton (Caviglia *et al.*, 2020). MACC1 codes for the MACC protein 1, which regulates the c-met/hepatocyte growth factor (HGF) pathways for cell proliferation, cell survival and metastasis (Tan *et al.*, 2016).

Tumour suppressor genes are reportedly downregulated or knocked out as a result of carcinogenesis (Zhao *et al.*, 2016). Tumour suppressor genes code for proteins that prevent the initiation and progression of cancer. These genes monitor precursors of cancer such as DNA damage and prevent cancer by suspending the proliferation of damaged cells (Vurusaner & Basaga, 2012; Rayess *et al.*, 2012). Tumour suppressor genes also code for proteins that dictates and regulate cancer-inhibiting pathways, by inducing apoptosis (Campbell & Tait, 2018; Gossage *et al.*, 2015). Studies show that Fas (CD95) gene is downregulated in several of cancer types including cervical and colorectal cancers (Wilson *et al.*, 2013; Putzbach *et al.*, 2018). The Fas receptor, also known as the cluster differentiation 95(CD95), is a membrane death receptor coded for by the CD95 gene (Martin-Villalba *et al.*, 2013). A death-inducing signalling complex is generated when CD95 ligand (CD95L) binds to the CD95 receptor, leading to apoptosis (Lavrik & Krammer *et al.*, 2012).

Conventional clinical drugs have been given a opportunity to guard against and combat cancer. In 2015, Botswana had 80.5 % of the World Health Organization (WHO) essential medicine list for cancer, including Calcium folinate, Docetaxel and Carboplatin, and then the country ran out of at least 40 % of necessary cancer medication for roughly 30 days (Chiyapo *et al.*, 2018). Since Botswana is still not manufacturing anti-cancer drugs, the situation may repeat itself, and affect the nation in a far more devastating away. This situation exposes the need for a local solution. One approach to curbing oxidative stress-induced diseases like cancer is the neutralization of free radicals (ROS) and the reduction of their adverse effects by using plant-based approaches. Botswana is home to a wide variety of both edible and non-edible flora. For many years, indigenous people have been using indigenous plants for medicinal purposes (Balde

et al., 2020; Maroyi, 2016; Mocheki *et al.*, 2018). Only a few indigenous plants have been studied for their medicinal properties. Possibly, with no or fewer side effects, the competency of alternative medicine from natural products may be based on the concept of reverse pharmacognosy.

Amongst the many native plants of Botswana are *Sclerocarya birrea* and *Terminalia prunoides*. Existing evidence from phytochemical analysis of different parts of the *Sclerocarya birrea* suggests that this plant has high phenolic content (Virginie *et al.*, 2016). Phytochemical screening and antioxidant potential of *Sclerocarya birrea* fruit exocarp have not been studied in Botswana. Phytochemical extracts from the exocarp of the *Sclerocarya birrea* fruit may help in curbing cancer. Amongst the many phytochemicals that constitute *Sclerocarya birrea* are flavonoids, indoles and isoflavones amongst others (Louis *et al.*, 2018). *Terminalia prunoides* is one of the most understudied local plant species, nonetheless, analysis of other species of the genus *Terminalia* have shown the presence of tannins, triterpenes, flavonoids, and phenols in *Terminalia spinosa*, *Terminalia stenotrachya* and *Terminalia parvilora* amongst others (Zhang *et al.*, 2019). *Terminalia* species have been part of the African culture as diet or remedy. They have also been used as traditional medicine for diabetes, bilharzia, cancer, coughs and colds, fever, diarrhea, venereal illnesses, heart problems (Das *et al.*, 2020). The solution of ROS based diseases maybe through plant-based approaches, as plants extracts have been reported to have bioactive compounds. The plant-based approach maybe inexpensive compared to the current conventional medication. The general study aim was (i) to extract phytochemicals, from *T. prunoides* pods and *S. birrea* fruit exocarp, and analyze their antioxidants properties (ii) analyse the protective effect of extracts on HeLa cell lines against free-radical induced cell death and (iii) to formulate potential anticancer ingredients from the crude extracts of these 2 plant species, and (iv) to analyse the effect of the formulated ingredients on HeLa cell lines.

1.2 Statement of the problem

Cancer is a disease that continues to devastate human civilization. Every year, millions of people are diagnosed with different kinds of cancer all over the world, and a reasonable portion of these patients will eventually die (WHO, 2022). In Botswana, an estimated 5 253 people are said to have cancer, 2 116 males and 3 137 females. In Botswana the total death toll was 2 116, in a population of about 2 351 625 for the year 2020 (WHO, 2020). In the year 2020, there was 10 million cancer-related fatalities worldwide, and an estimated 9.3 new cases (Sung *et al.*, 2021; WHO, 2022). In most countries, cancer ranks high amongst the causes of deaths alongside cardiovascular diseases (WHO, 2022).

Some of the present-day interventions on treating cancer require the use of medical equipment such as radiation machines that are not easily accessible in Botswana (Ralefala *et al.*, 2021). Some clinical anti-cancer drugs are only available at significantly higher financial cost to patients, and the cost is expected to rise (Prasad *et al.*, 2017). There have been reports on the side effects with conventional cancer medications, including cardiovascular toxicity, electrolyte imbalances, diarrhea, vomiting, and nausea (Coppola *et al.*, 2018; Nurgali *et al.*, 2018). Recently, the thrust of cancer research has shifted to developing novel treatments for the disease utilizing natural resources. There are numerous edible plants in Botswana that have been consumed by local population. Although most of the plant species in Botswana have not been scientifically explored for their full medicinal capacity, they may contribute to the disruption or prevention of the biomechanisms of cancer. Amongst those plant species is a common wild fruit that exist in all regions of Botswana called *Sclerocarya birrea* or Morula in the local language. The *Sclerocarya birrea* plant has been a part of the African diet for ages. It is distributed throughout Botswana and has been used as medicine by both the native and present-day Africans (Mocheki *et al.*, 2018). *Terminalia prunoides* is a largely under-studied plant that is found in the Southeast, Central, and Northern parts of Botswana. *Terminalia prunoides* has been used as tea and as traditional medicine for coughs, cold, constipation, abdominal pain and sore throat (Lawal &

Tshikalange, 2020). The use of dietary antioxidants against free radical induced diseases like cancer is simple and inexpensive.

1.3 Hypothesis

The exocarp of *Sclerocarya birrea* fruits and pods of *Terminalia prunoides* are good sources of secondary metabolites that may act as antioxidants and anticancer agents.

1.4 Aim

To determine the antioxidant and anticancer potential of the *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods as well as the synergistic anticancer potential of the formulated ingredients.

1.5 Objectives

Objective 1: To extract secondary metabolites from *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods.

Objective 2: To perform qualitative and quantitative phytochemical analysis of the acquired extracts.

Objective 3: To evaluate antioxidant properties of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods extracts.

Objective 4: To evaluate the cytoprotective effects of *Sclerocarya birrea* fruit exocarp, and *Terminalia prunoides* pods extracts, from hydrogen peroxide induced cell death.

Objective 5: To evaluate the effects of the extracts on oxidative stress parameters.

Objective 6: To assess the antiproliferative effects of the extracts, and the formulated anticancer ingredients from the extracts as well as to assess their synergistic anticancer potential.

Objective 7: To perform gene expression analysis of specific cancer biomarkers.

1.6 Justification of the study

In the year 2020, a record number of 10 million cancer related fatalities, and 9.3 million new cancer diagnoses were reported (Sung *et al.*, 2021; WHO, 2022). As of now, there is no broad-spectrum cure for cancer. Existing synthetic cancer drugs cause harm as they have been alleged to have negative consequences such as cardiovascular toxicity, electrolyte abnormalities, nausea, vomiting and diarrhea. Bioactive compounds extracted from plants have been used in many studies of many diseases and have given significantly positive results with fewer or no side effects. There is an opportunity in cancer drug development to exploit plants for their ethnopharmacology. This study aimed to explore and evaluate antioxidant and anticancer properties of the exocarp of *Sclerocarya birrea* fruit, and pods of *Terminalia prunoides*, and formulate potential anticancer ingredients. This study will contribute to the already existing knowledge on medicinal plants and their potential in cancer management.

CHAPTER 2: LITERATURE REVIEW

2.1 Oxygen and Reactive oxygen species (ROS)

In nature, oxygen (O_2) is a diatomic molecule. Oxygen has 2 unpaired electrons in each electron orbital in the same spin. It is this configuration that makes O_2 relatively stable and unreactive because of a very low energy state that is also referred to as a ground state. Oxygen reacts by reduction (accepting electrons) (Afanas'ev, 2017). Molecular oxygen reduction happens in a sequential manner where one electron is accepted at a time. The partial reduction of O_2 excites its energy state, giving rise to unstable and highly reactive oxygen-based molecules (reactive oxygen species) (Bergdoll *et al.*, 2016). Table 1 demonstrates the reduction of O_2 by single electron, forming superoxide anion, when superoxide is reduced by one electron, it forms a peroxide anion. Peroxide is reduced by one electron to form hydroxyl radical (Collin, 2019; Hoare, 2017). Accumulation of ROS brings about oxidative stress. This might very well lead to a number of disorders, such as anaemia, cancer, degenerative diseases (Yang & Lian, 2020).

Table 1. Names of reactive oxygen species, chemical formulars and chemical reactions resulting in the generation of ROS

Sequential reduction of oxygen	Chemical formular of ROS	Names of ROS
$O_2 + 1e \rightarrow O_2^{\cdot -}$	$O_2^{\cdot -}$	Superoxide anion
$O_2^{\cdot -} + 1e \rightarrow H_2O_2$	O_2^{2-} / H_2O_2	Peroxide/Hydrogen peroxide
$H_2O_2 + 1e \rightarrow OH^{\cdot}$	$OH^{\cdot}$	Hydroxyl radical

2.2 Sources of Reactive Oxygen Species (ROS)

Throughout routine cell physiology, successive molecular oxygen reduction results in reactive oxygen species (ROS) generation (Bergdoll *et al.*, 2016). In the course of oxidative phosphorylation, some ROS are generated in the membrane of mitochondrion. Figure 1 shows molecular oxygen being partially reduced by 1 electron leaked from protein complex 1, CoQ, and complex 3 forming superoxide anion in the matrix of mitochondrion, p66SHC binds to Cyt C and shuttles electrons to oxygen, forming hydrogen peroxide in the intermembrane space of mitochondrion (Bazil *et al.*, 2016; Li & Qin 2018). Some ROS are generated in endoplasmic reticulum, by protein disulphide isomerase (PDI) enzymes (Konno *et al.*, 2021). The PDI generates ROS during the breaking and forming of disulphide bonds between cysteine residues, during the formation of tertiary structures of proteins (Lee, 2017; Khan *et al.*, 2016; Shergalis *et al.*, 2020). In the peroxisome, other free radicals are produced during inflammation (through respiratory burst), when a pathogen invades a cell, phagocytes engulf the pathogen, forming phagolysosome (Ganguli *et al.*, 2019). Phagolysosome has NADPH oxidase, which reduces O₂ molecule by 1electron, forming superoxide anion (Boonla, 2018). When the flavin coenzymes Flavin Mono Nucleotide and Flavin Dinucleotide undergo one electron transfer reduction in the peroxisome, they create a radical ion (Ganguli *et al.*, 2019; Wander *et al.*, 2016; Boonla, 2018). When L-amino and D-amino acids are oxidatively deaminated to form-keto acids by L-amino and D-amino acids oxidase, some ROS are produced in liver and kidney.(Nagano *et al.*, 2019; Yu *et al.*, 2017). Reactive oxygen species are also generated when xenobiotic material build up in cells, in conditions such as hemochromatosis where unusually high amounts of iron are absorbed. Iron (2) is involved in the Fenton process, iron is oxidized by hydrogen peroxide into iron (3), hydroxyl radical and hydroxide ion (Ahmad *et al.*, 2017).

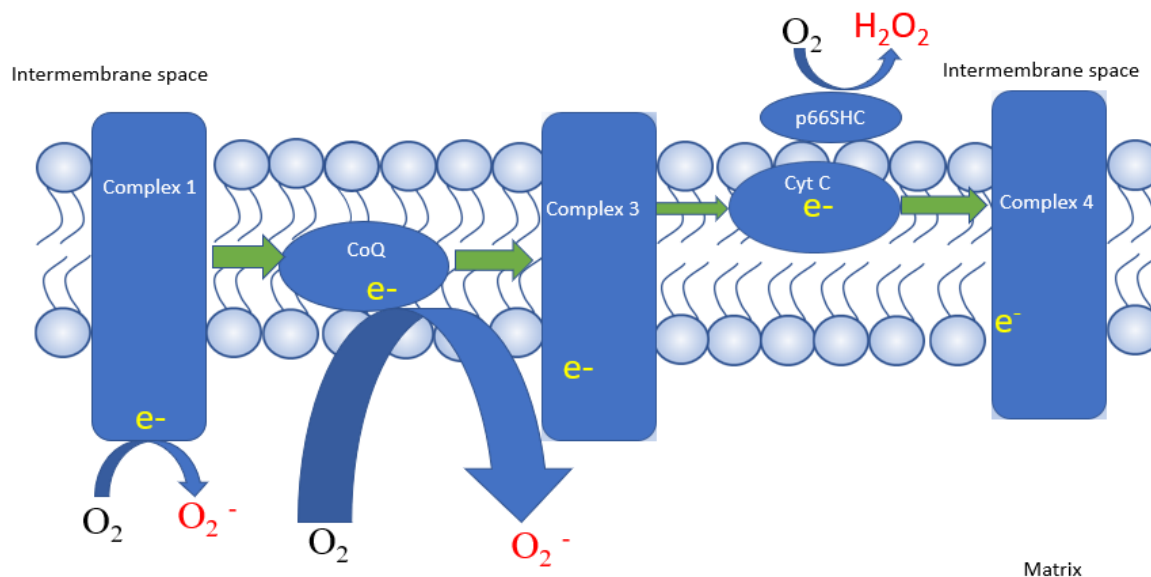


Figure 1. Partial reduction of oxygen during electron transport chain in the mitochondrion. Protein complex 1,3 and CoQ reduce oxygen by 1 electron to form super oxide radical. Cytochrome C binds and leak electrons to p66SHC, p66SHC reduces oxygen by 2 electrons, forming hydrogen peroxide which is discharged into the intermembrane space.

2.3 Oxidative stress and DNA damage

Oxidative stress arises as a result of disequilibrium between antioxidants and reactive oxygen species (Liguori *et al.*, 2018). Oxidative stress build-up has been tied to the pathogenesis of cancer (Klaunig, 2018). Oxidative stress can damage DNA by single electron oxidation. The most prevalent site of oxidation in DNA nucleobases (T, G, A, C), is guanine(G), which is converted to 8-oxo-7,8-dihydroguanine (8-OxoG) or 8-oxo-7,8-dihydro-2' -deoxyguanosine (8-oxodG) (Murata *et al.*, 2012). The oxidized guanine (8-oxoG) has an oxidation potential much lower than the normal G, making it readily susceptible to further oxidation which depends on the concentration of oxidants (Suzuki & Kamiya ,2017). The rate of nucleobase oxidation increases with increasing oxidant concentration. Another most frequent reaction of DNA oxidation occurs at the thymine base by a sequential process (Cadet & Wagner, 2013). With this type of DNA T

oxidation, the nucleotides develop an electron hole. Then, by hopping from one nucleobase to its neighbouring nucleobases, it moves through the DNA strand in a reversible manner until it is trapped in an irreversible state (Zhang *et al.*, 2020; Borrás *et al.*, 2019; Serrano *et al.*, 2016). Figure 2 shows some of the results of oxidative damage on DNA, oxidative stress can cause base pair mismatches, single strand and double strand breaks, inter and intra strand crosslinks. The damaged DNA may result in the rearrangement of DNA sequence, covalent attachment of chemical adducts to DNA, development of oxidative stress induced lesions the DNA strand, DNA-protein cross-links, modification of bases, and gene amplification (Yu *et al.*, 2016; Yu *et al.*, 2018; Barnes *et al.*, 2018) These outcomes are implicated in the initiation phase and progression of several carcinogenesis (Fagin & Petrini, 2020; Srinivas *et al.*, 2019).

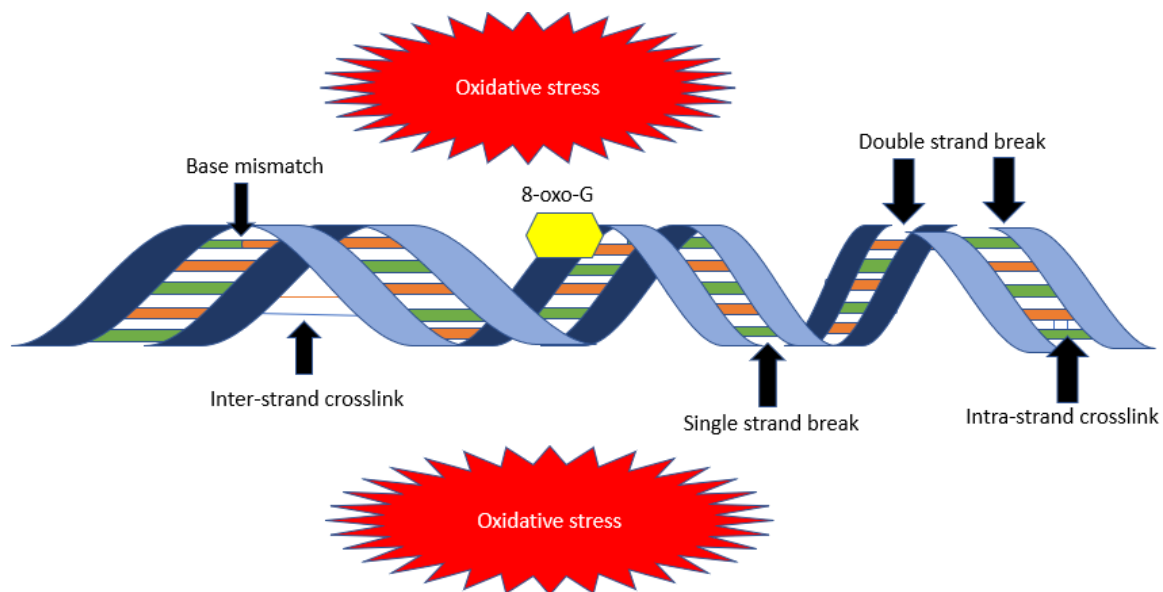


Figure 2. DNA helix being damaged by oxidative stress, resulting in DNA single or double strand breaks, inter and/or intra strand crosslinks, base pair mismatch, and transformation of guanine into 8-oxo-G.

2.4 Antioxidant defenses

The body has antioxidants as an adaptation response to the prevention, and to lessen the adverse consequences of oxidative stress. Antioxidants, which are chemical substances and proteins that considerably slow down / stop oxidative damage, when they are present in low quantities against oxidants (Pisoschi *et al.*, 2021; Barzegar, 2012). The 1st line of defence is by endogenous antioxidants including a non enzymatic reduced Glutathione (GSH), enzymatic Superoxide Dismutase (SOD), and enzymatic Catalase (CAT) (Zhao *et al.*, 2021; Ighodaro & Akinloye, 2018; Lee *et al.*, 2019). An enzymatic antioxidant called Super Oxide Dismutase (SOD) converts super oxide anion into diatomic oxygen and hydrogen peroxide (Tiwari & Mishra 2016; Hao & Liu 2019). Catalase (CAT) is an enzymatic antioxidant that converts hydrogen peroxide into water, and sometimes through the Fenton's reaction, into hydroxyl anion (Davood *et al.*, 2018; van Lith & Amaeer, 2016). Reduced glutathione (GSH) is a non-enzymatic antioxidant, GSH reduces hydroxyl radical to water (Misak *et al.*, 2018). The reaction between GSH and hydroxyl radical results in the reduction of GSH to GSSG (oxidized GSH) (Jelinek *et al.*, 2018; Mirończuk-Chodakowska *et al.*, 2018). Oxidized GSH requires electrons and/ or hydrogen atom donors to be reduced to GSH. Studies have shown that exogenous antioxidants from drugs and plants reduce GSSH to GSH (Yin, 2019; Jeong *et al.*, 2019). It has been noted that exogenous reducing substances like vitamins (A and C), tannins, flavonoids, and phenolics provide electrons and hydrogen to GSH (Dias *et al.*, 2020; Neha *et al.*, 2019; Amarowicz & Pegg 2019). Exogenous antioxidants of plant origin scavenge ROS through several mechanisms, such as single electron transfer (SET), chelating transition metals, and hydrogen atom transfer (HAT) (Santos-Sánchez *et al.*, 2019; Muraro *et al.*, 2020). Additionally, endogenous antioxidants like CAT, SOD, and GSH have been shown to express and function favourably when antioxidants from plants are present (Li *et al.*, 2007; Chiang *et al.*, 2006; Yeh *et al.*, 2009).

2.5 Cancer

2.5.1 Cancer progression

Mutations cause alterations in gene expression that cause normal cells to become cancer cells (Srinivas *et al.*, 2019; Botezatu *et al.*, 2019). Mutations can be caused by many things such as exposure to radiation, epigenetic modification, and DNA oxidation (by ROS) (Poetsch, 2020). In some cases, oncogenesis leads to the overexpression of oncogenes and upregulation of signalling pathways for growth of cells, cell activity and survival of cells (Chen *et al.*, 2018). The overexpression of oncogenes give cells the innate ability to bypass cell cycle check points and overrule cell cycle arrest. This allow cells to progress rapidly through the stages of the cycle. Some of the onco-proteins inhibit tumour suppressor proteins (Senft & Ze'ev, 2018). Cancer cells also downregulate tumour suppresser genes in terms of level of expression, protein activity and altering the 3D conformational structure of tumour suppresser proteins (Newton & Brognard, 2017). These are proteins that inhibit damaged cells from proliferating and instead cause cell cycle arrest or apoptosis. (Campbell & Tait, 2018). The downregulation of tumour supressor proteins reduces the concentration of these proteins, reducing the progression of tumour suppressor proteins mediated apoptosis pathways. The change in conformational structure prevents the binding of these proteins to the intended sites, defying cell cycle arrest and/or apoptosis (Dhar *et al.*, 2018; Newton & Brognard, 2017). Figure 3 shows cancer cells growing bigger and faster than normal cells. Cancer cells postpone and bypass programmed cell death. This gives rise to a colony of damaged cells that rapidly divides to form a tumour (Rajani *et al.*, 2020).

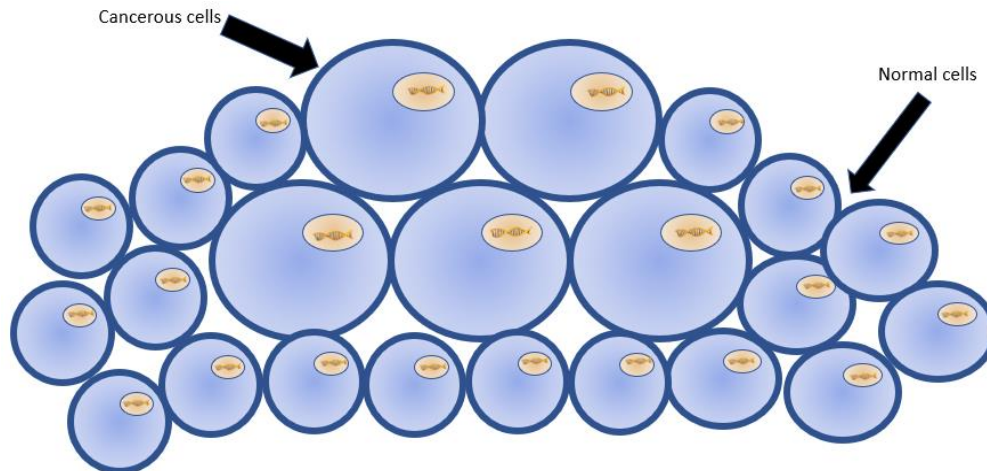


Figure 3. Cancer cells growing faster and larger than normal cells as a result of upregulated oncogenes expression and downregulated tumour genes express.

2.5.2 Types of cancer

There are many types of cancer that are named based on the organs they affect such as breast cancer, lung cancer, skin cancer, brain cancer, bladder cancer, bone cancer ,colorectal cancer, esophageal cancer, fallopian tube cancer, gastric cancer, bile duct cancer, liver cancer, mouth cancer, pancreatic cancer, prostate cancer, thyroid cancer, vulva cancer and cervical cancer amongst others (Hoadley *et al.*, 2018; Bonneville *et al.*, 2017). According to (WHO, 2020), breast and lung cancers are the top two most prevalent with 2.26 million cases. They are followed by colorectal cancer with 2.21 million for the year 2020 (WHO, 2022). According to WHO, (2020) Botswana recorded a total of 2 010 new cancer cases for the year 2020. Figure 4 shows that Cervix uteri was highest type of cancer recorded in Botswana for the year 2020, with 18.6 % of all cases. Followed by breast cancer (10.4 %), Kaposi sarcoma (10%), prostate cancer (9%) and Oesophagus cancer with 5.4%. (WHO, 2020).

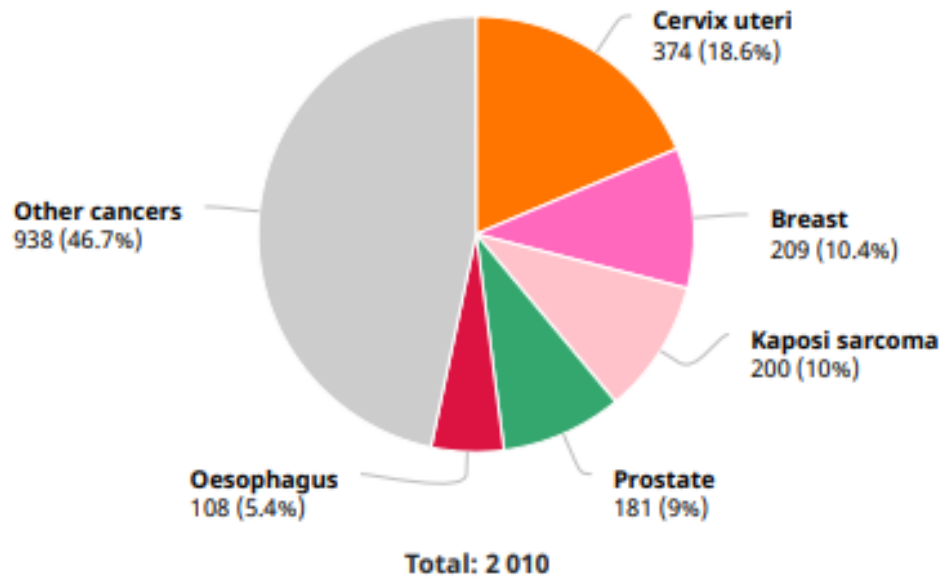


Figure 4. Cancer statistics for Botswana. Cervix uteri is the highest constituent of all 2 010 cases recorded in the year 2020, with 18.6 %. Followed by Breast cancer (10.4%), Kaposi sarcoma (10%), Prostate cancer (9%) and Oesophagus cancer (5.4%) (WHO, 2020).

2.6 Cervical cancer biomarkers and their modes of action

2.6.1. Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor gene codes for a tyrosine transmembrane receptor that mediates extra cellular signaling (Cheng *et al.*, 2021). As shown in Figure 5, EGF receptor is a complex that has two separated monomers. The two monomers dimerize and cross phosphorylate upon binding to an external epidermal growth factor (Toyam *et al.*, 2018). This results in the intercellular part of the receptor having a phosphorylated tyrosine residue. This residue is an active site for binding with Growth factor receptor-bound 2 (Grb2) protein. The Grb2 protein is an adaptor and activator for the Sone of Senvenless (SOS) protein (Surve *et al.*, 2021). The SOS protein has an active site for binding with Ras-GTP complex. The Ras-GTP complex then moves to activate a Rapid accelerated fibrosarcoma (Raf) protein which then activates Mitogen-

Activate Protein Kinase (MEK) kinase protein (Lin *et al.*, 2021). MEK activates Mitogen-Activate Protein Kinase (Map) kinase, transcription factor cAMP response element-binding protein (CREB) is signalled by activated kinase to migrate into the nucleus to the location of other genes that code for cell growth, proliferation, division, and survival. The overexpression of EGFR has been observed in several cancer cells such as cervical cancer and colorectal cancer (Chaihian *et al.*, 2020; Li *et al.*, 2017; Moritsugu *et al.*, 2020).

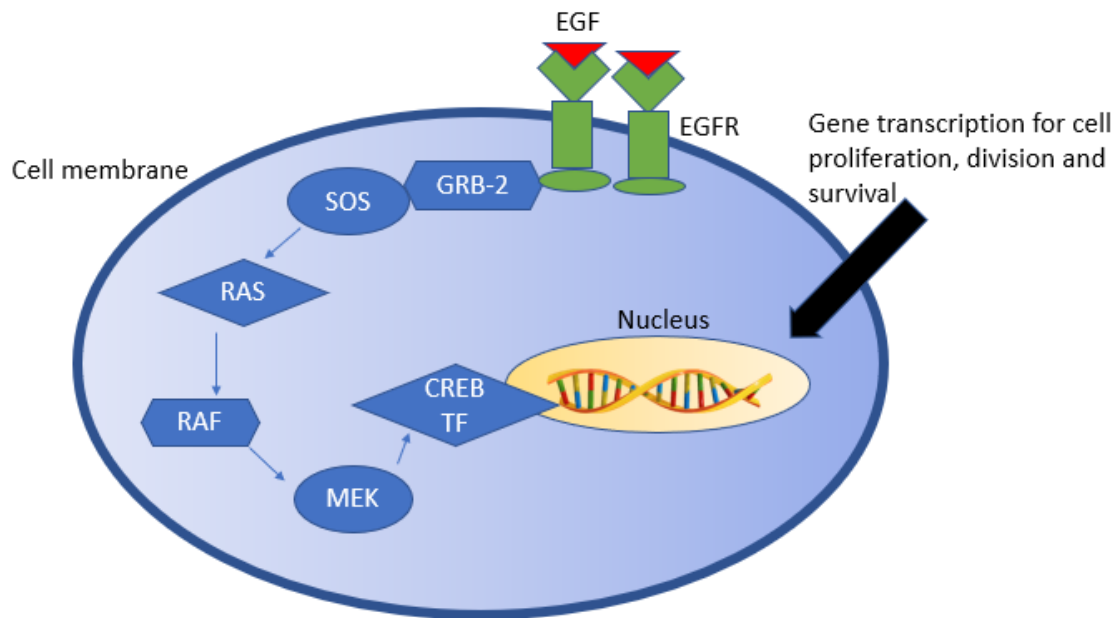


Figure 5. The EGFR mediated pathway for cell proliferation, division and survival. The EGF binds to EGF receptor to activate the GRB-2 protein. The sos protein is bound to and activated by activated GRB-2 protein. The sos protein moves to activate the RAS protein, which then activates the MEK protein. Activated MEK activates the CREB transcription factor, which moves to the nucleus to transcribe genes for cell proliferation, survival and division.

2.6.2 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor gene is part of the Platelet-Derived Growth Factor (PDGF) family of proteins (Kieran *et al.*, 2012). This gene (VEGF) has been found to be expressed in several cell types such as platelets, renal mesangial cells, and tumour cells (Peterson *et al.*, 2012; Laengsri *et al.*, 2018). The VEGF gene codes for the VEGF signalling protein, which, through its binding to the VEGF receptor, initiates a cascade of phosphorylation reactions that regulate transcription factor (TF) for genes coding for the blood vessels development, and the restoration of oxygen supply (Abhinand *et al.*, 2016). Vascular endothelial growth factor is upregulated in cancer cells to match oxygen and blood demand by the rapidly growing cancer cells. VEGF is not only limited to vascular pathways, but also take part in the physiological development of a cell and cell survival (Karali *et al.*, 2014; Xu *et al.*, 2019). Figure 6 illustrates VEGF activating the VEGF receptor. This activates the Shc adopter protein (Abhinand *et al.*, 2016). The Shc attracts, binds and activates the Rat sarcoma (RAS) protein (Carter *et al.*, 2016). The RAS protein then moves to activate Mitogen-Activated Protein Kinase (MAPK), which will then activate the Protein-1 (AP-1) transcription factor (Guo *et al.*, 2020; Sawada *et al.*, 2015). The AP-1 protein moves into the nucleus, targets and bind sequences constituting promoter region for the coding of cell proliferation and cell survival genes (Jia *et al.*, 2016; Atsaves *et al.*, 2019). The survival of a tumour also depends in the VEGF paracrine and endocrine signalling pathways (Shi *et al.*, 2021). The overexpression of VEGF has been recorded in several known cancer cells. The level for expression is correlated to the tumour stage and progression (Sawada *et al.*, 2019; Laengsri *et al.*, 2018; Samouellian *et al.*, 2018).

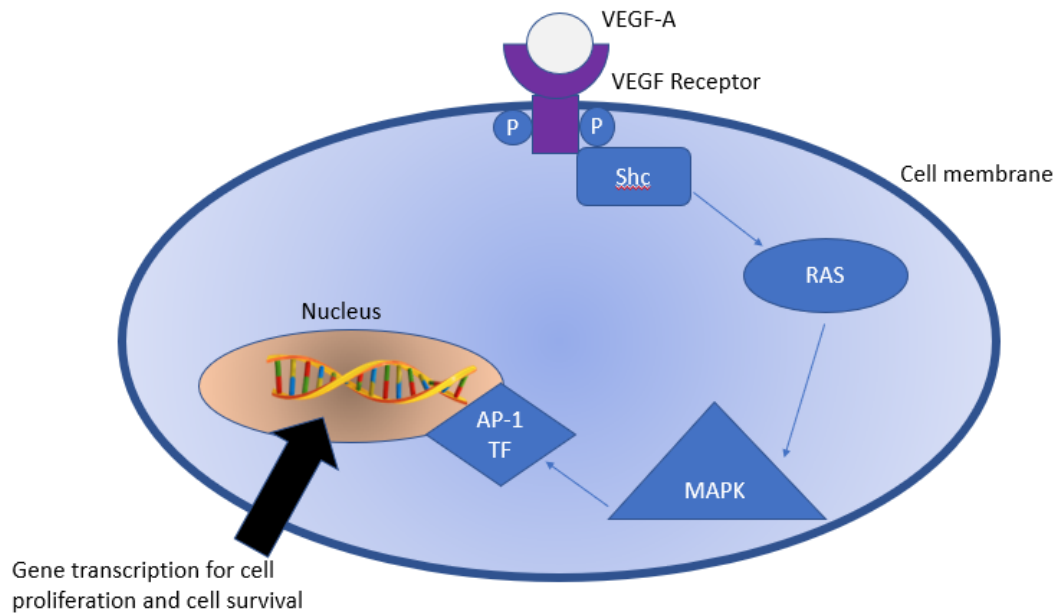


Figure 6. VEGF- A binds to and activates the VEGF receptor, which activates the Shc adaptor. The Shc adaptor activates the RAS, which then activates MAPK. Activated MAPK activates the AP-1 TF for cell proliferation and survival genes.

2.6.3 Metastasis -Associated in Colon Cancer 1(MACC1)

Metastasis – Associated in Colon Cancer 1, which is also expressed in cervical cancer cells, is a regulatory gene that codes for the MACC protein 1 (Arlt & Stein, 2009). The MACC1 is transcription factor for hepatocyte growth factor (HGF). Figure 7 shows MACC 1 gene being transcribed and translated into a MACC 1 protein, which then connects with the promoter sequence of the Met gene for the enhanced expression of Met receptor. The HGF/Met is involved in cell growth (Zheng *et al.*, 2020; Zhu *et al.*, 2018). The MACC1 mRNA is a biomarker for colorectal, breast and cervical cancer studies. It is also a target in diagnostic and prognostic studies of many cancer studies (Chandrasinghe *et al.*, 2017; Tan *et al.*, 2016; Zincke, 2020).

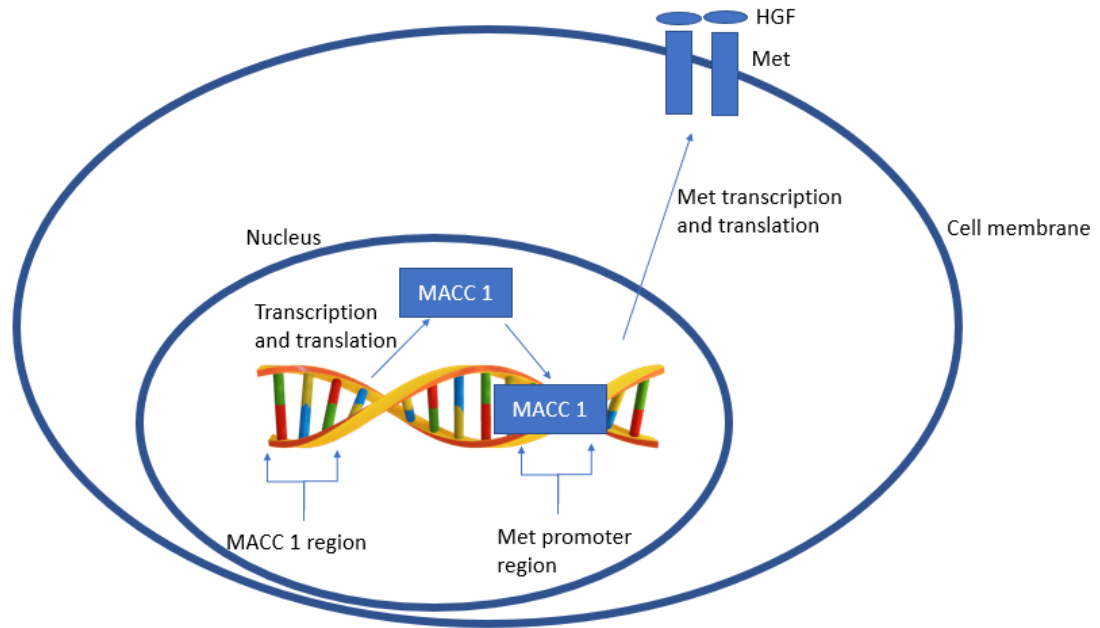


Figure 7. Metastasis -Associated in Colon Cancer 1 is transcribed, translated, then it targets and binds the Met promoter region for enhanced expression of Met receptor protein.

2.7.4 Cytokeratin Fragment (CYFRA 21-1)

Cytokeratin Fragment 21-1 gene codes for a cytoplasmic fibrous protein fragment of a soluble cytokeratin 19 family (Holdenrieder *et al.*, 2017). The CYFRA 21-1 protein, through polymerization, makes up part of the cytoplasmic intermediate filament (IF) network (Dohmoto *et al.*, 2000; Suzuki *et al.*, 2000). The IF holds the cell organelles in place, maintains the 3D structure of cells, and protects the cells from both mechanical and non-mechanical stress (Suzuki *et al.*, 2000). CYFRA 21-1 has been widely used as a prognosis and surveillance tool. It has also been used as a treatment and monitoring tool in numerous cancer studies (Caviglia *et al.*, 2020; Holdenrieder *et al.*, 2017; Gu & Long 2016).

2.8.5 Cluster differentiation 95 (CD95)/FAS gene

Cluster differentiation 95 is a subfamily of the death receptor (DR) family, under tumour necrosis factor (TNF) super family of proteins (Fu *et al.*, 2016; Lavrik, 2014). As shown in figure 8, CD95 is a receptor to CD95L, forming the death-inducing signalling complex (DISC) upon binding (Sessler *et al.*, 2013). The DISC activates the death domain (DD), which is the intracellular part of the receptor. This triggers the clustering of other components to come to and bind to the receptor for activation. Fas-associated protein with death domain (FADD), is an adapter molecule. It has a death effector domain (DED) which binds to the DD of the CD95 receptor leading to the binding of pro-caspase 8 to FADD (Seyrek & Lavrik, 2019; Siegmund *et al.*, 2017). Upon binding to the FADD adaptor, pro-caspase 8 is activated into caspase 8. Caspase 8 then detaches from the DISC into the cytosol, where it will activate pro-caspase 3 (Tummers & Green, 2017; Fox *et al.*, 2021). Activated caspase 8 has 2 down stream pathways, intrinsic and extrinsic apoptosis. For the intrinsic pathway, caspase 3 activates apoptotic effectors such as caspase dependent DNase (CAD) and proteolytic enzymes (Seyrek & Lavrik, 2019). These apoptotic effectors cleave multiples points of the DNA, inducing cell death (Koyama *et al.*, 2016; Al-Ghorbani *et al.*, 2016). For the extracellular apoptosis signalling, caspase 8 activates the bid protein into tbid, consequently activating Bax / Bak proteins. The Bax / Bak proteins induce apoptosis on the neighbouring cells (Kim *et al.*, 2017; Svandova *et al.*, 2017). The level of expression of CD95 gene is used marker in many cancer studies (He *et al.*, 2014; Laytragoon-Lewin *et al.*, 2014; Martin-Villalba *et al.*, 2013).

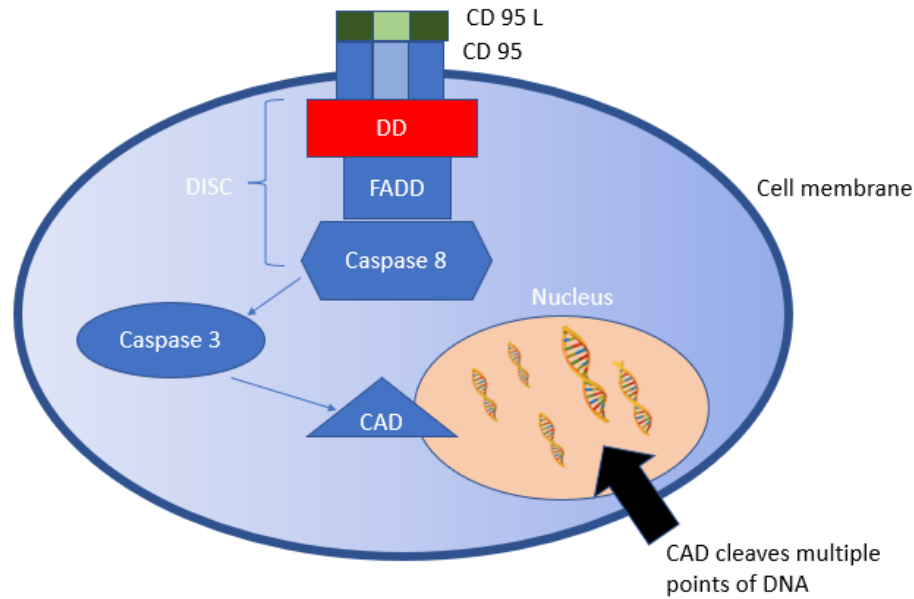


Figure 8. The CD 95L binds and activates the CD 95. Activated CD 95 activates the DISC by activating the DD, FADD and caspase 8. Caspase 8 detaches from DISC and move to activate caspase 3. Activated caspase 3 activates CAD, which gets into the nucleus and induce cell death by cleaving multiple points of the DNA.

2.9 Plants extracts in the treatment of cancer

A study published by Borek. (2004) eluded that, phytochemicals protected against oncogenic transformation and induced apoptosis in cancer cells. Experimental data by Yang *et al.* (2017), essential oils from Gannan Navel Orange peel, showed positive effects in inhibiting proliferation of A549 cells and 22RV-1 cells. In a study of Dietary Antioxidant trans-Cinnamaldehyed (CA) against the progression of Visfatin-Induced Breast Cancer, conducted by Chiang *et al.* (2019), a combination of FK866(visfatin inhibitor) and CA significantly inhibited Visfatin-Induced carcinogenic effects in *Vivo* studies. On another study by Kang *et al.* (2017), luteolin induced mitochondrial membrane action potential loss, activated mitochondrial mediated caspase pathway, caused the release of cytochrome C into the cytosol (from the mitochondria),

upregulated Ca^{2+} and Bax, downregulated Bcl-2 thereby inducing apoptosis on HT-19 cells. The same study (Kang *et al.*, 2017) also revealed that luteolin activated intracellular and mitochondrial ROS neutralization, by activating endogenous antioxidants. In Xu *et al.* (2016)'s study, natural antioxidants from *Thelephora gangbajun* mushroom exhibited antiproliferative activities in several cancers like A549, HT-29, HepG2, and MCF-7 cells. Findings published by Ahmed *et al.*, (2016), of anticancer, antioxidants and antimicrobial of pharmacologically active flavonoids extracts of *Cassia angustifolia*, results showed a significant anticancer activity against MCF-7 cell lines with IC_{50} values of 4.0 $\mu\text{g}/\mu\text{L}$, and 5.45 $\mu\text{g}/\mu\text{L}$ in Hela cell lines.

2.10 *Sclerocarya birrea* plant taxonomy and plant description

Distributed throughout Africa, *Sclerocarya birrea* is a savanna tree that belongs to a family of ANARCARDIACEOUS. The *Sclerocarya birrea* is single stemmed with a wide spreading crown. Characterized by a grey mottled bark, it can grow up to 18meters. The exocarp is initially green and turn yellow when ripe, with a light grey jelly mesocarp (fruit flesh). The tastes of mesocarp ranges from sweet, sour and a blend of sweet sour. The endocarp may encase between 1 and 4 seeds.

Phytochemical screening, isolation, and analysis of *Sclerocarya birrea* stem barks conducted by Virginie *et al.* (2016), Daniela Russo *et al.* (2018), and Manzo *et al.* (2017), revealed the presence of polyphenols, flavonoids, saponites, triterpenes and tannins. Another study of phytochemical screening of stem bark, leafs, and roots extracts from *Sclerocarya birrea* by Manzo *et al.* (2017) revealed the availability of flavonoid, saponin and tannin.

Sclerocarya birrea has been a fundamental part of the African community for many years. Besides its fruits being used for diet, the *Sclerocarya birrea* has been used as traditional medicine. A study conducted in south central Zimbabwe by Maroyi, (2016) and another study by

Mocheki *et al.*, (2018) in Limpopo province, South Africa, revealed that the *Sclerocarya birrea* has been used medicinally for cold and flu, diarrhea, malaria, dysentery, schistomiasis, sore eyes and toothache. On findings published by Masoko *et al.* (2008), pathogenic yeasts (*Candida parapsilosis*, *Cryptococcus albidus* and *Rhodoturula mucilaginosus*) were inhibited by extracts from the bark of the *Sclerocarya birrea*. A study by Ojewole, (2003), studied the hypoglycaemic activity of *Sclerocarya birrea* stem-bark aqueous extract in rats. The results of this in vivo study indicate that *Sclerocarya birrea* possesses hypoglycaemic activity. A study conducted by Prinsloo & Street, (2013) revealed that the bark and leaves have hypoglycaemic activity, anti-diarrheal, antioxidants, antimicrobial, anti-plasmodial, anti-inflammatory, anti-diabetic, anti-hypertension, and anti-septic properties. Existing literature does not show any studies on specific secondary metabolites of *Sclerocarya birrea* fruit exocarp.



Figure 9. *Sclerocarya birrea* tree



Figure 10. *Sclerocarya birrea* fruits exocarp

2.11 *Terminalia prunoides* plant taxonomy and Description

Terminalia prunoides is a multi-stemmed shrub or small tree that belongs to COMBRETACEAE family. *Terminalia prunoides* can grow up to heights of 2.5meters to 15meters, with a bark that is pale grey to grey black, spur shoots at the end of lateral shoots, creamy or white flowers. *Terminalia prunoides* bears purple pods.

Genus *Terminalia* has over 43 recorded species all over the world. *Terminalia subspathulata* located in Malaysia and Indonesia, *Terminalia parvilora* located in Sri lanka, *Terminalia mulleri* located in Indonesia and Southern Africa. *Terminalia mollis*, *terminalia schimperiana*, *Terminalia spinosa*, *Terminalia stenotrachya* and *Terminalia prunoides* have been reported to have tannins, triterpenes, flavonoids. *Terminalia piniculata* in India has been reported to have phenols, lignans, monoterpenes, carotenoids, and glycosides. *Terminalia trifloral* in Argentina has has been reported to have ellagitannins and phenolic compounds. *Terminalia sericea* in Southern Africa has been reported to have stilbenes, glycosides, and phenolic compounds (Cock, 2015; Fahmy *et al.*,2015; Beigi *et al.*,2018).

In southern Africa, many species of *Terminalia* have been used as medicine by native people for many years. *Terminalia mollis* has been used as an anti fungal, antiseptic, antimalaria and has been reported to have some anti-HIV activities (Cock, 2015). *Terminalia prunoides* has been used to relief abdominal pains. *Terminalia schimperiana* and *Terminalia stenotrachya* has have been used for oral hygiene, and as an antibiotic and antifungal agent (Balde *et al.*, 2020). *Terminalia spinosa* has been used as medicine for abdominal disorders, bilharzia, anticancer, influenza, diarrhoea, heart problems, hypertension, and diabetes (Cock, 2015; Beigi *et al.*,2018; Balde *et al.*, 2020).



Figure 11. *Terminalia prunoides* tree



Figure 12. *Terminalia prunoides* Pods

CHAPTER 3: MATERIAL AND METHODS

3.1 Reagent and Chemical list

For extraction, qualitative and quantitative phytochemical analysis, and antioxidants assays, Hexane, Chloroform, Ethyl acetate, Methanol, Sodium hydroxide, Hydrochloric acid, Acetic anhydride, Sulphuric acid, Distilled water, Ferric chloride, Acetic acid, Aluminium chloride, Ammonium molybdate, Potassium acetate, Sodium phosphate, Gallic acid, Folin-ciocalteu reagent, Quercetin, Potassium ferrocyanide, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Sodium dodecylsulphate, Potassium persulfate, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and Phosphomolybdenum were used. In cell culture, enzyme activity and gene expression studies, the following reagents and materials were used; Dulbecco's minimal essential medium (DMEM) (Capricorn-Scientific), Dimethyl-sulfoxide (DMSO) (Capricorn-Scientific), Penicillin-streptomycin (10 000 U/mL: 10 000 µg/mL) (Capricorn-Scientific), Fetal bovine serum (FBS), Trypsin-EDTA (0.25%) (ThermoFisher Scientific, USA), 4-[-3(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate (WST-1) assay kit (Roche Diagnostics, Germany), Hydrogen peroxide, Phosphate buffer saline (PBS), Superoxide dismutase (SOD), Catalase (CAT), and reduced Glutathione (GSH) ELISA kits (Elabscience, USA), RNeasy minprep kit (ZYMO RESEARCH), ProtoScript[®] II First strand cDNA synthesis kit (NEW ENGLAND Biolabs[®] inc), Luna[®] Universal qPCR Master Mix and primers.

3.2 Sample collection and preparation of extracts

Pods of *Terminalia prunoides* were collected at Majwanaadipitse (22°00'27.4''S 26 ° 47'48.9'' E), while the fruits of *Sclerocarya birrea* were collected in Palapye (22 °35'35.7'' S 27° 07' 28.5'' E) both in the central region. Both the pods and the fruits were washed with distilled water at the Department of Biological Sciences and Biotechnology of Botswana International University of Science and Technology (BIUST). The pods were dried in a Lasec MMMERT UF

110 laboratory oven at 40°C for a period of 48 hours. The fruits were peeled to separate the exocarp from the mesocarp. The peeled exocarps were washed again with distilled water, and dried using a laboratory oven (Lasec MMMERT UF 110) at 40°C for 24 hours. The exocarps and pods were separately ground to powder using a Salton SB400E blender. All 4 solvents; hexane (nonpolar), chloroform (low polar), ethyl acetate (moderately high polar) and methanol (high polar), were purified by distillation, using a Heidolph Hei-VAP Platinum 2 rotary vapour before use. Following, sequential extraction as stated by (Mustafa *et al.*, 2020), with some modifications, 3 kilograms (kg) of both samples were soaked separately in 7 L of hexane for 76 hours in round bottom flasks. After 76 hours, hexane was decanted to another container. The samples were air dried for a period of 6 hours and soaked again on chloroform (7L) for 76 hours. After soaking, chloroform was decanted, and the sample was air dried for 6 hours. After drying, the sample was soaked again on ethyl acetate (7L) for 76 hours. After soaking, ethyl acetate was decanted, and the sample was air dried for 6 hours. After drying, the sample was soaked again on methanol (7L) for 76 hours, after soaking, methanol was decanted. The decanted solvents were evaporated using Heidolph Hei-VAP Platinum 2 rotary vapour and a fume hood. The dried extracts were measured to estimate the efficiency of solvents by calculating the percentage extractive yield. After calculating percentage extractive yield, extracts were stored at room temperature in the dark. Percentage extractive yield was calculated as;

$$\% \text{ Extractive yield} = (\text{mass of dry extracts} / \text{mass of powder sample}) \times 100$$

3.3 Qualitative phytochemical screening

Extracts from *Sclerocarya birrea* fruit exocarps and *Terminalia prunoides* pods were screened for the presence of some known metabolites. The screenings were executed as described below.

3.3.1 Test for flavonoids

About 0.2 g of both extracts was dissolved in 5 mL of distilled water in a test tube. Ten (10) % aqueous sodium hydroxide (NaOH) was added, turning the mixture into a yellow solution. Dilute hydrochloric acid (HCL) was then added causing mixture decolorization. This was considered as an indication of the presence of flavonoids.

3.3.2 Test for terpenoids

About 0.2 g of sample was dissolved in 2 mL of ethanol in a test tube. A mL of acetic anhydride (CH_3CO)₂O, and 1 mL of concentrated sulphuric acid (H_2SO_4) was added. The color changed from pink to purple, an indication of the presence of terpenoids.

3.3.3 Test for saponins

Five millilitre of distilled water was used to boil 1 gram of sample. Three millilitres of distilled water was then added, and vigorously agitated for 5 minutes. The presence of saponins was indicated by foaming of a light mass after warming.

3.3.4 Test for tannins

About 0.5g of sample was dissolved in 10mL of distilled water in a test tube. To 2mL of the filtrate, a few drops of 1% ferric chloride were added. The development of a green or blue-black precipitate was an indication of the presence of tannins.

3.3.5 Test for phenols

About 0.2 g of sample was dissolved in 2 mL of distilled water in a test tube. Then, a few drops of neutral ferric chloride (FeCl_3) were added, and the mixture turned dark green. This was an indication of the presence of phenols.

3.3.6 Test of Cardiac glycosides

In a test tube, 5 mL of the extract was mixed with 2 mL of glacial acetic acid and 1 drop of FeCl_3 in a test tube. One millilitre (1mL) of concentrated H_2SO_4 was gradually added to the mixture. The emergence of a brown ring was an indication of the presence of cardiac glycosides.

3.3.7 Test steroids

Extracts (0.2g) were dissolved in acetic acid (2mL) in test tubes with screw caps, the placed in a hot water bath and the mixture was then cooled before the careful addition of concentrated H_2SO_4 (2mL). The presence of a steroidal ring was indicated by a shift in colour from violet to blue.

3.4 Quantitative phytochemical analysis

For phytochemical analysis, 10 mg of extracts, quercetin (positive control for total flavonoid content), gallic acid (positive control for total phenolic content) were resuspended in 10 mL of methanol to make a concentration of 1mg/mL. This concentration (1mg/mL) was adopted from (Chatatikun & Chiabchalar, 2013).

3.4.1 Determination of total flavonoid content (TFC)

The TFC of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods were determined using a method described by (Sembiring *et al.*, 2018). About 2 mL of methanol, 200 μL of 10% aluminium chloride, 200 μL of 1 M potassium acetate, 600 μL of distilled water, and 1 mL of quercetin at concentrations of 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL were mixed in test tubes. After mixing, the solution was incubated in the dark for 30 minutes at room temperature, to allow the ingredients to react completely. Absorbance was measured at 420 nm using a

Thermo Scientific™ SPECTRONIC™ 200 Spectrophotometer. The results of quercetin were used to plot a standard curve which was used for calculating TFC of extracts. About 2 mL of methanol, 200 µL of 10% aluminium chloride, 200 µL of 1 M potassium acetate, 600 µL of distilled water, and 1 mL of extracts at a concentration of 1mg/mL, were mixed in test tubes and incubated for 30 mins in the dark at room temperature. Absorbance was measured at 420 nm using a Spec200E UV-V (Thermofisher Scientific, United States of America). TFC was calculated as,

$$TFC = \frac{cV}{m}$$

Where c = concentration of extract equivalence to that of quercetin (quercetin standard curve),
 V = volume of extracts in mL and m = mass of extracts in grams.

3.4.3 Determination of total phenolic content (TPC)

The TPC of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods were determined using a method by (Singleton *et al.*, 1999). About 200µL of gallic acid at concentrations, 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL, 2mL distilled water and 200 µL of 10 % Folin-ciocalteu reagent, were mixed and incubated for 8 minutes in the dark. About 600 µL of 20% sodium carbonate was added and left to incubate in the dark for 2 hours at room temperature. Absorbance was read at 760 nm using a Spec200E UV-V, and the results for gallic acid were used to plot a standard curve that was used in calculating TPC of extracts About 200µL of 1 mg/mL extracts, 2mL distilled water and 200 µL of 10 % Folin-ciocalteu reagent, were mixed and incubated for 8 minutes in the dark. About 600 µL of 20 % sodium carbonate was added and left to incubate in the dark for 2 hours at room at temperature. Absorbance was read at 760 nm using a Spec200E UV-V (Thermofisher Scientific, United States of America). The TPC was calculated as,

$$TPC = \frac{cV}{m}$$

Where c = concentration of extract equivalence to that of gallic acid (from gallic acid standard curve), V = volume of extracts in mL and m = mass of extracts in grams.

3.5 Antioxidants assays

For antioxidants assays, 10mg of extracts were resuspended in 10mL absolute methanol to make a concentration of 1mg/mL, this concentration was adopted from (Uddin *et al.*, 2018). 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay measures hydrogen atom transfer (HAT) (Lv *et al.*, 2019), Total Antioxidants Capacity (TAC) assay measures electron transfer (ET) (Phatak & Hendre, 2014), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay measures HAT/ET (Kooy *et al.*, 2016), Ferric reducing antioxidants powder (FRAP) measures ET (Berker *et al.*, 2007).

3.5.1 Determination of antioxidant activity using 2, 2-diphenyl-1-picrylhydrazyl (DPPH)

As described by (Lv *et al.*, 2019), this method was used with some modifications to determine the radical scavenging activity of extracts on 2, 2-diphenyl-1-picrylhydrazyl (DPPH). About 0.0039 grams(g) of DPPH powder was dissolved in 100mL of methanol, to make 0.1M DPPH. The dissolved DPPH was incubated for 2 hours in the dark. About 1mL of extracts (1mg/mL) and gallic acid (1 mg/mL) were serial diluted to make concentrations of 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL. After DPPH incubation, 150µL of 0.1M DPPH was transferred to wells of a 96 well plate. For wells designated as treatment, DPPH was treated with 50 µL of plant extracts. For wells designated as positive control, DPPH was treated with 50 µL of gallic acid. For wells designated as negative control, DPPH was not treated. The reactions were incubated for 30 mins in the dark at room temperature. Optical density (OD) was measured after 30

minutes at 518 nm using a MULTISKAN FC microplate reader (Thermo Scientific, USA). The percentage inhibition (decolorization) was determined as follows,

$$\text{DPPH inhibition (\%)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{control}}}{\text{OD}_{\text{control}}} \times 100$$

3.5.2 Determination of antioxidant activity using ferric reducing antioxidant powder (FRAP)

This assay was used as described by (Berker *et al.*, 2007) with some modifications. About 1.0 N hydrochloric acid (HCL) was prepared by adding 8.3 mL of HCL into 100mL of distilled water. Plant extracts and gallic acid were serial diluted into concentrations of 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL. About 40µL of each concentration of plant extracts and gallic acid, were added to the wells of a 96 well plate. About 100 µL of 1.0 N HCL was added to the 96 well plates. Followed by 20 µL of 1% sodium dodecylsulphate and 30 µL 1% of potassium ferrocyanide. The reaction was left to incubate for 20minutes at 50°C. Absorbance was read at 750nm using a MULTISKAN FC microplate reader (Thermo Scientific, USA).

$$\text{FRAP reduction (\%)} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100$$

3.5.3 Determination of antioxidant activity using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method

ABTS method was executed as described by (Kooy *et al.*, 2016). This method is based on ABTS radical cation decolorization. About 100mL of 7mM ABTS and 100 mL of 2.4mM of potassium persulfate (1:1), were mixed to make ABTS working solution, and stored in the dark for 16 hours at room temperature. About 1mL of ABTS solution was diluted gradually with methanol until an absorbance of 0.706± 0.01 was obtained at 734 nm. Then 1 mL of the ABTS solution was added to 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL concentrations of extracts and gallic acid. After mixing, the reaction was left to incubate for 7 minutes in the dark at room temperature.

Absorbance was measured at 734 nm using a MULTISKAN FC microplate reader (Thermo Scientific, USA). Gallic acid was used as standard. Percentage inhibition was determined as follows:

$$\text{ABTS inhibition (\%)} = \frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \times 100$$

3.5.4 Total antioxidants capacity (TAC) using phosphomolybdenum

As described by (Phatak & Hendre, 2014), TAC is an assay that is based on the formation of a stable green phosphate-Mo(V) complex, as a result of electron reduction of Mo (VI) to Mo(V). About 0.1 mL of extracts and gallic acid was mixed with 1mL of phosphomolybdenum (prepared by mixing 31.4 mL of 0.6M H₂SO₄, 397.49 mg of 28mM sodium phosphate and 494.36mg of 4mM of ammonium molybdate). The mixture was incubated at 95°C in a IncoCool LABOTEC (LAMWORLD TECHNOLOGIES PTY LTD) for 90 minutes, then cooled to room temperature. Absorbance was measured at 950 nm using a Spec200E UV-V (Thermofisher Scientific, United States of America). Gallic acid was used to make a standard curve, from which TAC of extracts was interpolated from.

3.6 Cell culture studies

3.6.1 Preparation of plant extracts and cisplatin concentrations

About 100mg of plant extracts was dissolved in 10mL of 10 % dimethyl-sulfoxide (DMSO) to make a stock solution of 10mg/mL. About 10mL of stock solution was added to 25mL DMEM to make a concentration of 400 µg/mL. The 400 µg/mL was adopted from (Damodaran *et al.*, 2019). The 400 µg/mL was serially diluted to make concentrations of 12.5, 25, 50, 100, 200, and 400 µg/mL. Cisplatin (standard) is one of the most effective chemotherapeutic drugs (Raudneska *et al.*, 2019; Gosh, 2019). About 10mg of cisplatin was dissolved in 1mL of 10 % DMSO to make a stock solution of 10mg/mL, 1mL of cisplatin stock solution was added to 10mL of

DMEM to make a concentration of 100 µg/mL. This concentration was adopted from (Mamat *et al.*, 2020), in which the highest concentration of cisplatin used as positive control was 100 µg/mL. Then 100 µg/mL of cisplatin was diluted into 50, 25, 12.5, 6.25, 3.125, and 1.5625 µg/mL.

3.6.2 Cell culture

HeLa cells were cultured in preparation for performing several experiments on the cytoprotective and anticancer potential of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods extracts. HeLa cells were grown in high glucose Dulbecco's minimal essential medium (DMEM), supplemented with penicillin-streptomycin (10 000 U/mL: 10 000 µg/mL) and 10% of fetal bovine serum (FBS). Cells were incubated in a Thermo Scientific Forma SERIES 11 Water Jacket CO₂ Incubator at 37°C and 5% CO₂. A Countess™ 3 Automated Cell Counter was used to count cells before being seeded into flat bottom 96-well plates, and the concentrations of cells were prepared as per the output of the cell counter to attain 1×10^5 /mL.

3.6.2.1 4-[-3(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate (WST-1) assay

The effect of *Sclerocarya birrea* exocarp and *Terminalia prunoides* pods extracts were assessed on the cancer cell line HeLa. The determination of the effect of extracts on HeLa cells was done using WST-1 assay (Roche Diagnostics, Germany). WST-1 is a quantitative colorimetric assay used to measure cell proliferation, growth, and survival. This assay is based on concept that live cells reduce tetrazolium salt WST-1 (4-[-3(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate) to a dark red water-soluble formazan, using a mitochondrial dehydrogenase enzyme. The intensity of the formazan dye is proportional to the concentration of live cells. This cell viability assay was used for cytoprotective effects and antiproliferative effects of extracts.

3.7 Determination of the cytoprotective effect of extracts against hydrogen peroxide induced cell death using WST-1

Ninety-eight (98) % confluent HeLa cells at day 6 of passage number 5 were trypsinized, seeded in flat bottom 96-well plates at a concentration of $1 \times 10^5/\text{mL}$, and left to attach to the bottom of the plate for 72 hours. After the incubation, media was aspirated and replaced with 100 μL of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods extracts (400 $\mu\text{g}/\text{mL}$), for wells designated as treatment. For positive control wells, cells were treated with PBS only. After treatment cells were incubated for 2 hours. After 2 hours, cells were exposed to hydrogen peroxide induced oxidative stress by adding 10 μL of 1mM hydrogen peroxide (H_2O_2) to the wells containing extracts and PBS (positive control) and incubated for 4 hours. The 1mM H_2O_2 was adopted from (Mezghani *et al.*, 2015). For negative control wells, cells were not treated. After incubation, 10 μL of WST-1 was added to the wells and incubated for 3 hours. Absorbance was taken at 480 nm using a MULTISKAN FC microplate reader (Thermo Scientific, USA). Absorbance of wells containing media and 10 μL of WST-1 was regarded as background and was subtracted from that of treatment and control. The protective effect was determined as percentage of viable cells which was calculated as absorbance comparison with that of control.

$$\text{Viable cells (\%)} = \frac{\text{OD negative control} - \text{OD treated cells}}{\text{OD negative control}} \times 100$$

3.8 Effects of extracts on oxidative stress biomarkers

About 90% confluent HeLa cells (day 6 of passage of number 5) at a concentration of $1 \times 10^5/\text{mL}$ were treated with 100 μL of extracts (400 $\mu\text{g}/\text{mL}$) for 2 hours, and untreated cells were used as a control for the experiment. The 400 $\mu\text{g}/\text{mL}$ of extract concentration was adopted from cytoprotective assay above (section 3.7). Cells were detached using trypsin and centrifuged at 1000 g for 10 minutes. The pellet was resuspended in cold PBS and centrifuged at 1000 g for 10

minutes. The pellet was suspended on cold PBS, then frozen at – 20 °C. After freezing, the pellet was thawed, frozen again, and thawed again, this was repeated 3 times to break the cells. The cell homogenate was centrifuged at 1500 g for 10 minutes and preserved in ice for further studies. The effects of extracts on the activity of superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were evaluated using ELISA kits (Elabscience, USA) following the manufacturer's instructions.

3.9 Antiproliferative effects of extracts

About 93% confluent cells HeLa cells (day 6 of passage number 5) were trypsinised and seeded in flat bottom 96-well plates at a concentration of 1×10^5 /mL. They were then left to attach to the bottom of the plate for 72 hours. After incubation, media was aspirated and replaced with 100 μ L of cisplatin (positive control), *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods extracts and incubated for 24 hours. Phosphate buffer saline (PBS) was added to wells containing untreated HeLa cells (negative control). After incubation, 10 μ L of WST-1 was added to the wells and incubated for 3 hours. Absorbance was taken at 480 nm using a MULTISKAN FC microplate reader (Thermo Scientific, USA). Absorbance of wells containing media and 10 μ L of WST-1 was regarded as background and was subtracted from that of treatment and control. The cytotoxicity was determined as,

$$\text{Cytotoxicity (\%)} = \frac{OD \text{ control} - OD \text{ treated cells}}{OD \text{ control}} \times 100$$

3.10 Potential synergistic antiproliferative effect of ingredients on HeLa cells

Results from antiproliferative effects of individual extracts were used in choosing which extracts were selected to formulate 3 potential anticancer ingredients. The first ingredient was formulated by combining 1 extract with the lowest IC₅₀ value from *Sclerocarya birrea* fruit exocarp, and 1 extract with the lowest IC₅₀ value from *Terminalia prunoides* pods, at a ratio of 1:1. The second ingredient was formulated by combining 1 extract with the 2nd lowest IC₅₀ values

from *Sclerocarya birrea* fruit exocarp, and 1 extract with the 2nd lowest IC₅₀ value from *Terminalia prunoides* pods, at a ratio of 1: 1. The third ingredient was formulated by combining 2 best anticancer extracts (lowest IC₅₀ values) of *Sclerocarya birrea* fruit exocarp, and 2 best anticancer from *Terminalia prunoides* pods, at a ratio of 1:1:1:1. The antiproliferative effects of ingredients was executed as mention in section 3.6.2.1.

3.11 Gene expression analysis using qPCR

About 25 x 10⁴ HeLa cells were cultured and left to attach to the bottom of the plates for 24 hours. Following cell attachment, 93% confluent HeLa cells at day 6 of passage number 5 were treated with 100 µL of extracts and formulated ingredients (400 µg/mL) and incubated for 12 hours. Total RNA was extracted using RNAeasy minprep kit (ZYMO RESEARCH) following the manufacturer's instructions. The NanoDrop One spectrophotometer (ThermoFischer Scientific, USA) at 260 nm, was used to determine the amount and purity of RNA. Complementary DNA was synthesized from 290ng of RNA suing a ProtoScript[®] II First strand cDNA synthesis kit (NEW ENGLAND Biolabs[®] inc). A Luna[®] Universal qPCR Master Mix and primers (Table 2) were used for gene expression studies. The primers used was from published literature. Changes in mRNA was quantified using Applied Biosystems 7500 Fast cyclers (Life Technologies, Singapore) RTqPCR. The Real time PCR was set to initial denaturing for 2 minutes at 95⁰C, denaturing at 95⁰C for 20 seconds, and extension was set at 60⁰C for 30 seconds. The melt curve was run from 60⁰C - 95⁰C. The 2^{-ΔΔCT} Livak method (Livak & Schmittegen, 2001) was used to analyse fold changes in gene expression, normalising expression of target genes to the expression of a house keeping gene (GAPDH).

Table 2. Primer sequences for genes of interest and a house keeping gene.

Gene	Forward primer	Reverse primer
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EGFR (Qi <i>et al.</i> , 2016)	5'-GACGGGCAACGGCGTATTCTCAG-3'	5'- CCCACCTTG CCTTTGTCT CCTGTC - 3'
VEGF (Psoma <i>et al.</i> , 2020)	5'- AGGATGGGGCTGACTAGGTAA- 3'	5'- CCCCCTTTT CCTCCAAC CTC - 3'
MACC1 (Li <i>et al.</i> , 2019)	5'-TTCTTTTGATTCCCTCCGGTGA - 3'	5'- ACTCTGATG GGCATGTGC TG-3'
CYFRA 21-1 (Marrakchi <i>et al.</i> ,2008)	5' AAGCTAACCATGCAGAACCTCAACGACCGC- 3'	5'- TTATTGGAG GTCAGGAGA AGAGCC - 3'
CD95 (Wang <i>et al.</i> ,2015)	5'-CACTCGAGCTTTCACTTCGGAGGATTGC - 3'	5'- GTGAATTCT GACCAAGCT TTGGATTTC ATTTC - 3'
GAPDH (Nazet <i>et al.</i> , 2019)	5'-AGCCACATCGCTCAGACAC - 3'	5'- AATACGACC AAATCCGTT GACT - 3'

3.12 Data Analysis

All results were analysed using Origin Pro 2018 software. Data was presented as mean $\pm$ standard error (n=3). Means were compared using One way- analysis of variance (ANOVA) with Tukey-Kramer's range test. Means were considered significant at 95% confidence level ($p < 0.05$).

CHAPTER 4: RESULTS

4.1. Percentage extractive yield from plant material

This study began with collection of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods, followed by preparation of extracts. Three (3) kg of dried powder of each sample was used in a 4 solvent sequential extraction protocol. The 4 solvents were of ascending polarity. The dried extracts were weighed, and the mass was used to deduce percentage of extractive yield. Figures 3 and 4 show that all 4 extracts did not have the same yield. The texture, colour and viscosity of the extracts were also different. The results revealed extract polarity dependent yield. Hexane had the lowest yield with 3.6% and 2.7% yields for *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods, followed by chloroform as the second lowest yielding solvent. Methanol had the highest yield followed with 18.1 % and 19 % yields for *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods respectively, followed by ethyl acetate in both plants, as depicted in tables 3 and 4.

Table 3. Percentage extractive yield from 3 kg of sample powder and description of extracts of *Sclerocarya birrea* fruit exocarp.

Extract	Raw mass(g)	% Yield	Physical description
Hexane	107.938	3.6	Oily dark brown paste
Chloroform	236.267	7.9	Sticky and oily dark brown paste
Ethyl acetate	464.322	15.5	Brown dry paste
Methanol	541.851	18.1	Dark brown oily gel

Table 4. Percentage extractive yield from 3 kg of sample powder and description of extracts of *Terminalia prunoides* pods.

Extract	Raw mass(g)	% Yield	Physical description
Hexane	87.216	2.9	Green dry paste
Chloroform	196.492	6.5	Black dry paste
Ethyl acetate	501.132	16.7	Dark green sticky thick oily paste
Methanol	569.723	19.0	Dark brown oily paste

4.2 Phytochemical screening

4.2.1. Qualitative phytochemical screening

Tests were performed on extracts to determine whether they contained tannins, flavonoids, phenols, cardiac glycosides, steroids, alkaloids and saponins.

4.2.1.1 Qualitative phytochemical screening of *Sclerocarya birrea* fruit exocarp extracts

Different extracts of *Sclerocarya birrea* fruit exocarp tested positive for different metabolites at different concentrations. As shown in Table 5, hexane extracts only tested positive for saponins and steroids. Chloroform extracts tested negative for saponins, positive for tannins at mild concentrations, flavonoids at low concentrations, phenols at low concentrations, terpenoids at low concentrations, cardiac glucoside at mild concentrations and steroids at low concentrations. Ethyl acetate extracts tested positive for saponins at mild concentrations, tannins at low concentrations, flavonoids at mild concentrations, phenols at high concentrations, terpenoids at mild concentrations, cardiac glucoside at low concentrations and steroids at low concentrations. Methanol extracts test positive for saponins at high concentrations, tannins at low concentrations, flavonoids at mild concentrations, phenols at high concentrations, terpenoids at high concentrations, cardiac glucoside at mild concentrations, and steroids at low concentrations.

Table 5. Qualitative phytochemical screening of *Sclerocarya birrea* fruit exocarp extracts.

Test	Hexane	Chloroform	Ethyl acetate	Methanol
Tannins	-	++	+	+
Saponins	+	-	++	+++
Flavonoids	-	+	++	++
Phenols	-	+	+++	+++
Terpenoids	-	+	++	+++
Cardiac glucoside	-	++	+	++

Steroids + + + +

(-) = absent, (+) = present at very low concentrations, (++) = present at mild concentrations, (+++) = present at high concentrations.

4.2.1.2 Qualitative phytochemical screening of *Terminalia prunoides* pods extracts

As depicted in table 6, hexane extracts tested negative for all the phytochemicals. Chloroform extracts tested positive for tannins at low concentrations, flavonoids at low concentrations, phenols at low concentrations, cardiac glucoside at low concentrations, steroids at low concentrations, and tested negative for saponins and terpenoids. Ethyl acetate extracts tested positive for saponins at low concentrations, tannins at mild concentrations, flavonoids to mild concentrations, phenols to mild concentrations, terpenoids to mild concentrations, cardiac glucoside to low concentrations and steroids at low concentrations. Methanol extracts tested positive for saponins at high concentrations, tannins at mild concentrations, flavonoids at high concentrations, phenols at high concentrations, terpenoids at mild concentrations, cardiac glucoside at low concentrations and steroids at mild concentrations.

Table 6. Qualitative phytochemical screening of *Terminalia prunoides* pods extracts.

Test	Hexane	Chloroform	Ethyl acetate	Methanol
Tannins	-	+	++	++
Saponins	-	-	+	+++
Flavonoids	-	+	++	+++
Phenols	-	+	++	+++

Terpenoids	-	-	++	++
Cardiac glucoside	-	+	+	+
Steroids	-	+	+	++

(-) = absent, (+) = present at very low concentrations, (++) = present at mild concentrations, (+++) = present at high concentrations.

4.3 Quantitative analysis of phytochemicals

Extracts were subjected to tests that estimated the sum quantities of phenolic content and flavonoid content. The estimations were based on interpolating standard curves of isolated and known flavonoids and phenolic compounds.

4.3.1. Determination of total flavonoid content (TFC)

Quercetin has been isolated and found to be a flavanol, which belongs to a group of flavonoids (Lesjak *et al.*, 2018). Quercetin was used to establish a standard curve in Figure 13, from which TFC of extracts was interpolated from.

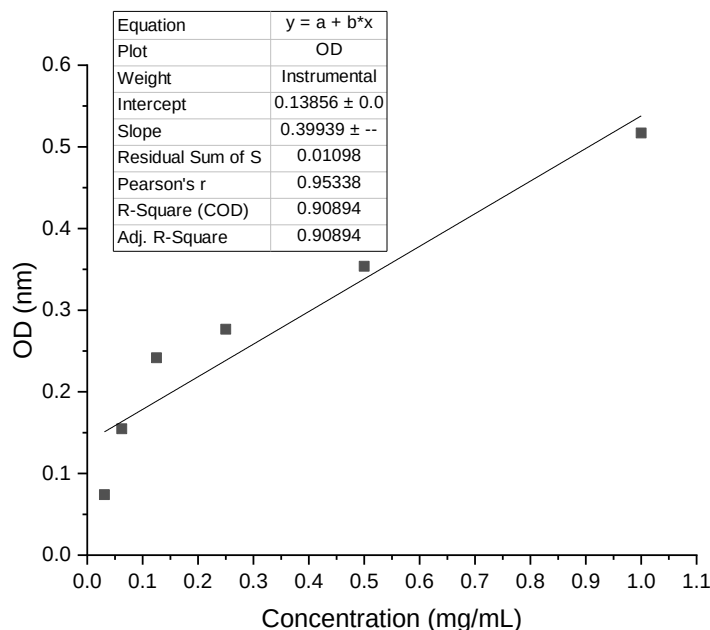


Figure 13. Optical densities (OD) of quercetin for different concentrations of 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL at 750nm.

4.3.2. Total flavonoid content of extracts from *Sclerocarya birrea* fruit exocarp

As shown in figure 14, extracts of *Sclerocarya birrea* fruit exocarp showed different contents of flavonoids. Results show that TFC values were dependent on the polarity of the extraction solvents. This is because extracts of methanol, which is the most polar of the solvents, showed the highest TFC values while extracts of hexane, which is the least polar of the solvents, showed the lowest TFC values. Methanol extracts showed the highest TFC of 55.2 ± 3.8 mg QE/g followed by ethyl acetate (47.7 ± 2.9 mg QE/g) and chloroform (27.7 ± 5.0 mg QE/g). Hexane had the lowest TFC value of 20.2 ± 5.3 mg QE/g. The TFC values of hexane and chloroform extracts were not significantly different from each other ($p < 0.05$). The TFC values of ethyl acetate and methanol extracts were not significantly different from each other.

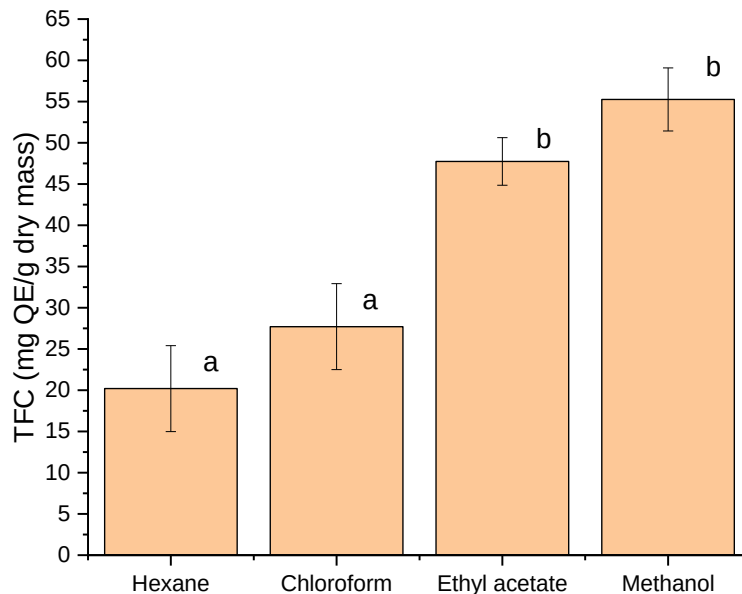


Figure 14. Total flavonoid content of one gram of *Sclerocarya birrea* fruit exocarp, expressed in mg of quercetin equivalence per one gram of dry mass of an extract (mg QE/g). Data presented as mean $\pm$ standard error (n=3). Letters above bars indicate significant differences between average means ($p < 0.05$), where different letters depict significant difference between means. Same letters indicate that means are not significantly different from each other.

4.3.3. Total flavonoid content of extracts from *Terminalia prunoides* pods

Figure 15 shows that *Terminalia prunoides* pods extracts showed different values of total flavonoid content. There was a proportional relationship between polarity of extraction solvents and TFC values. Hexane extracts had the lowest TFC values of 4.3 ± 2.9 mg QE/g. Methanol extracts had the highest TFC value of 67.8 ± 10.4 mg QE/g, followed by ethyl acetate extracts (59.4 ± 3.8 mg QE/g) and chloroform extracts (19.4 ± 5.2 mg QE/g). The TFC value of hexane and chloroform extracts were not significantly different from each other ($p < 0.05$).

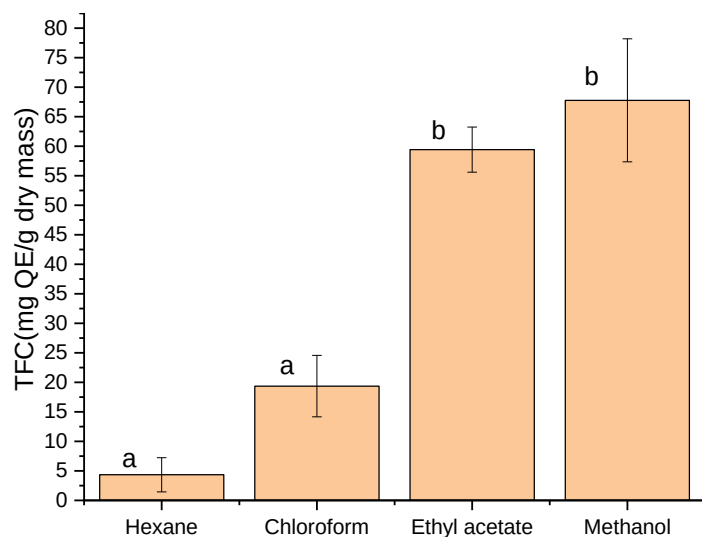


Figure 15. Total flavonoid content of one gram of extracts from *Terminalia prunoides* pods, expressed in mg of quercetin equivalence per one gram of dry mass of an extract (mg QE/g). Data presented as mean $\pm$ standard error (n=3). Letters above bars indicate significant difference ($p < 0.05$), where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

4.4 Determination of total phenolic content (TPC)

Gallic acid (GA) is a known phenolic compound (Phuyal *et al.*, 2020). Gallic acid was used to establish a standard curve (figure 16), from which the TPC of extracts were interpolated.

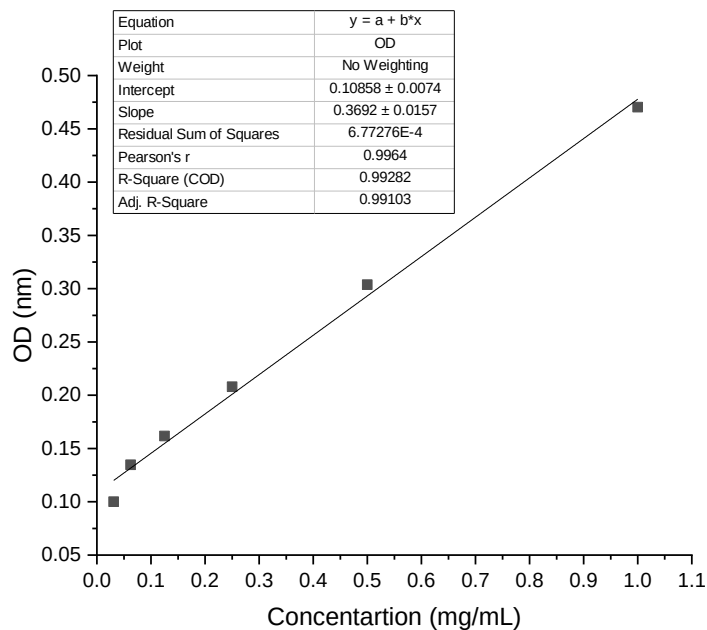


Figure 16. Optical densities (OD) of Gallic acid for different concentrations 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL at 750 nm. This curve was used as a standard curve for calculating total phenolic content (TPC) of extracts.

4.4.1 Total phenolic content of extracts of *Sclerocarya birrea* fruit exocarp

Extracts from *Sclerocarya birrea* fruit exocarp showed significant TPC values. Figure 17 shows that there was a positive relationship between extraction solvent polarity and TPC values. Hexane extracts had the lowest TPC value of 42.3 ± 5.4 mg GAE/g. Chloroform extracts had the 2nd lowest TPC value of 67.8 ± 1.12 mg GAE/g. Methanol extracts recorded the highest TPC value of 105.4 ± 1.4 mg GAE/g, followed by ethyl acetate with 84.3 ± 2.6 mg GAE/g. The TPC values of all extracts were significantly ($p < 0.05$) different from each other.

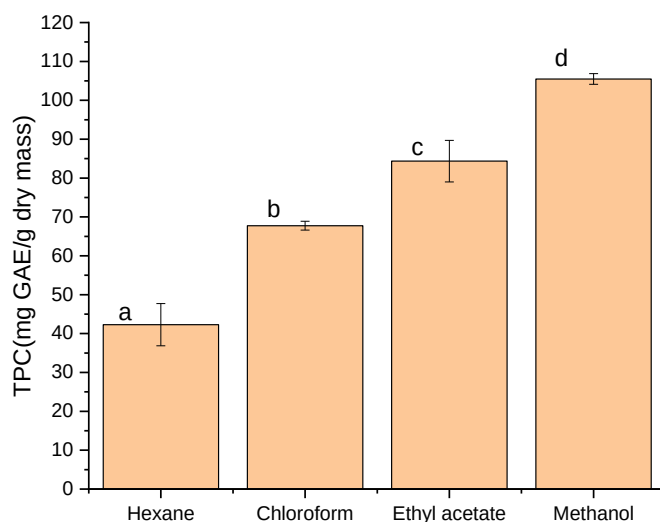


Figure 17. Total phenolic content of one gram of extracts of *Sclerocarya birrea* fruit exocarp, expressed in mg of gallic acid equivalence per 1 gram of dry mass of an extract (mg GAE/g). Data presented as mean $\pm$ standard error (n=3). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

4.4.2 Total phenolic compound of extracts of *Terminalia prunoides* pods

All *Terminalia prunoides* extracts showed different TPC levels (Figure 18). TPC values increased with increasing extraction solvent polarity. Hexane extracts recorded the lowest TPC value of 3.6 ± 5.0 mg GAE/g. Methanol extracts showed the highest TPC value of 113.2 ± 7.6 mg GAE/g, followed by ethyl acetate extracts (89.6 ± 2.4 mg GAE/g), and chloroform extracts (36.9 ± 14.5 mg GAE/g). The TPC values of hexane and chloroform extracts were significantly different from all the other extracts. However, TPC values of ethyl acetate and methanol extracts were not significantly different from each.

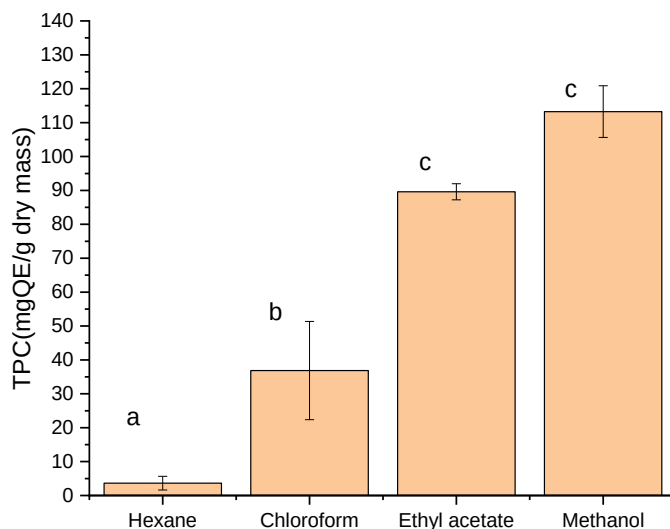


Figure 18. Total phenolic content of one gram of extracts of *Terminalia prunoides* pods, expressed in mg of gallic acid equivalence per 1 gram of dry mass of an extract (mg GAE/g). Data presented as mean $\pm$ standard error (n=3). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

4.5 Screening of extracts for antioxidant properties

Plants have many electron-rich metabolites, that act as antioxidants by neutralising oxidants such as reactive oxygen species (ROS). These metabolites help in reducing or preventing oxidative damage, and oxidative stress-based diseases. Extracts from *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods were screened for their antioxidant properties using 4 assays.

4.5.1 Determination of potential antioxidant activity of extracts using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

DPPH assay is based on the concept of electron transfer as part of redox system, DPPH is an oxidant/radical that changes colour when reduced (Lv *et al.*, 2019). Extracts from *Sclerocarya*

birrea fruit exocarp and *Terminalia prunoides* pods were assessed for their ability to reduce DPPH. Gallic acid was used as a standard for the experiments.

4.5.1.1 Determination of DPPH inhibition by extracts of *Sclerocarya birrea* fruit exocarp

Figure 19 demonstrates that extracts of *Sclerocarya birrea* fruit exocarp showed an effective DPPH inhibition capacity. DPPH inhibition increased with an increase in both extraction solvent polarity and concentration. At the highest concentration used in the experiment (1 mg/mL), gallic acid showed the highest inhibition of $86.93 \pm 0.17\%$, followed by methanol extracts with $71.44 \pm 0.44\%$, followed by ethyl acetate ($55.73 \pm 0.25\%$), then chloroform and hexane with $27.21 \pm 0.31\%$ and $16.42 \pm 0.37\%$ respectively. At the lowest concentration used in the experiment (0.0312 mg/mL), the inhibitions were in this order, gallic acid ($39.26 \pm 0.92\%$) > methanol ($36.53 \pm 3.53\%$) > ethyl acetate ($19.51 \pm 1.67\%$) > chloroform ($4.65 \pm 0.36\%$) > hexane ($1.61 \pm 0.21\%$).

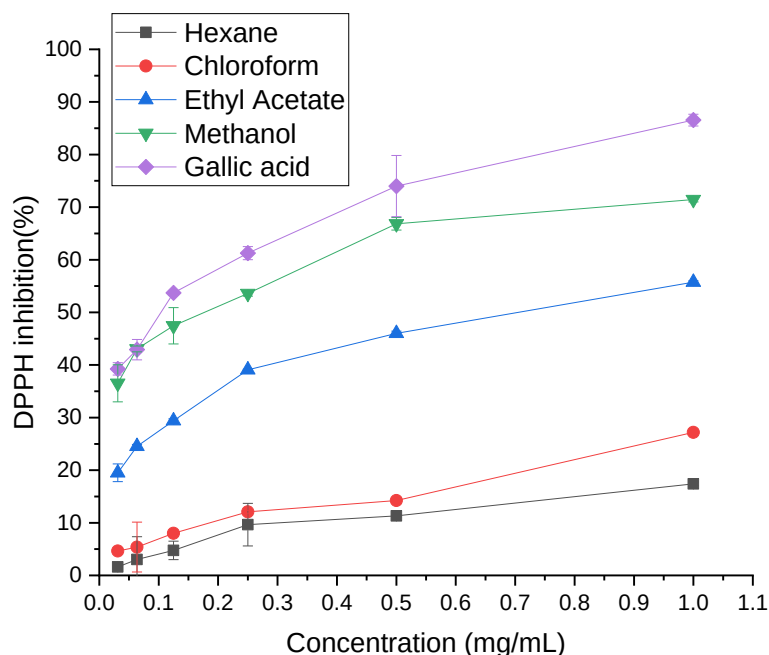


Figure 19. DPPH inhibition by *Sclerocarya birrea* fruit exocarp extracts at different concentrations, expressed as % inhibition. Data presented as mean $\pm$ standard error (n=3).

4.5.1.2 Determination of DPPH inhibition by extracts of *Terminalia prunoides* pods

Figure 20 shows that DPPH inhibition was dependent on extraction solvent polarity and concentration. At the highest concentration (1mg/mL), methanol extracts from *Terminalia prunoides* pods had the highest inhibition of $91.13 \pm 2.24\%$ amongst extracts, followed by ethyl acetate, chloroform, and hexane with $89.46 \pm 2.6\%$, $34.99 \pm 1.36\%$ and $19.22 \pm 3.12\%$ respectively. All extracts had an inhibition lower than that caused by gallic acid (control), which was $94.73 \pm 21\%$. At 0.0312 mg/mL (lowest concentration), the inhibitions were in this order, gallic acid ($47.26 \pm 0.21\%$) > methanol ($50.28 \pm 1.96\%$) > ethyl acetate ($41.71 \pm 2.18\%$) > chloroform ($5.36 \pm 0.29\%$) > hexane ($4.81 \pm 2.96\%$).

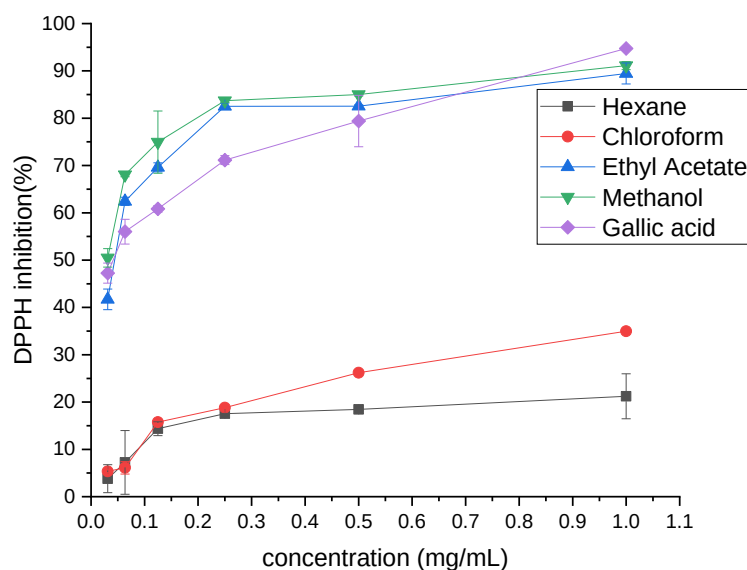


Figure 20. DPPH inhibition by *Terminalia prunoides* pods extracts at different concentrations, expressed as % inhibition. Data presented as mean $\pm$ standard error (n=3).

4.5.2 Antioxidant activity using Ferric Reducing Antioxidant Powder assay (FRAP)

This assay assesses the samples' ability to donate an electron and reduce potassium ferricyanide ($K_3Fe(CN)_6$) to potassium ferrocyanide ($K_4Fe(CN)_6$) (Henderson *et al.*, 2015). Gallic acid was used as a standard.

4.5.2.1 Determination of reducing power of extracts of *Sclerocarya birrea* fruit exocarp using FRAP

Figure 21 shows that generally, extracts showed a positive relationship between FRAP reduction and extraction solvent polarity. At the highest concentration used in the experiment (1mg/mL), methanol, and ethyl acetate extracts of *Sclerocarya birrea* fruit exocarp had the highest reducing power amongst extracts, with 94.89 ± 0.03 % and 91.19 ± 0.10 % respectively. Followed by chloroform (29.53 ± 0.12 %), and hexane (8.67 ± 0.01 %). The reducing power of gallic acid was greater than that of any extract, with 95.1 ± 0.04 %. At the lowest concentration (0.0312 mg/mL), the reducing powers were in this order, methanol (18.86 ± 0.08 %) > gallic acid (15.41 ± 0.10 %) > ethyl acetate (3.82 ± 0.12 %) chloroform (2.76 ± 0.19 %) > hexane (0.57 ± 0.01 %).

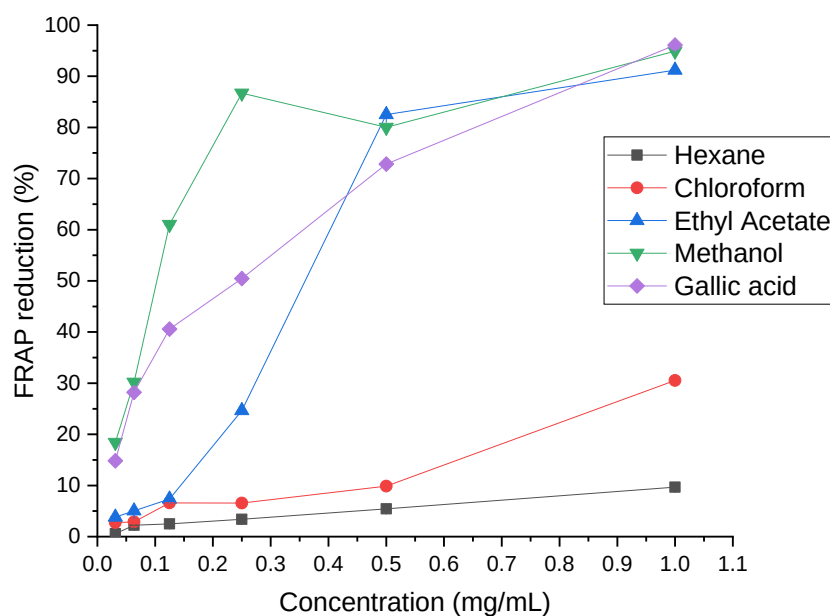


Figure 21. Frap reduction (%) by *Sclerocarya birrea* fruit exocarp extracts, at different concentrations. Data presented as mean $\pm$ standard error (n=3).

4.5.2.2 Determination of reducing power of extracts of *Terminalia prunoides* pods using FRAP assay

Figure 22 shows that FRAP reduction was dependent on both extract polarity and concentration. At the highest concentration (1 mg/mL), Gallic acid (standard) recorded the highest reducing power with $95.1 \pm 0.04\%$. Followed by methanol ($94.93 \pm 0.01\%$), ethyl acetate ($90.09 \pm 0.03\%$), chloroform ($13.39 \pm 0.01\%$) and hexane extracts ($12.66 \pm 0.13\%$), at 1mg/mL (highest concentration). At the lowest concentration (0.0312 mg/mL), the reducing powers were in the following order, methanol ($26.86 \pm 0.32\%$) > gallic acid ($14.83 \pm 0.63\%$) > ethyl acetate ($12.96 \pm 0.06\%$) > hexane ($3.83 \pm 0.08\%$) > chloroform ($0.29 \pm 0.12\%$).

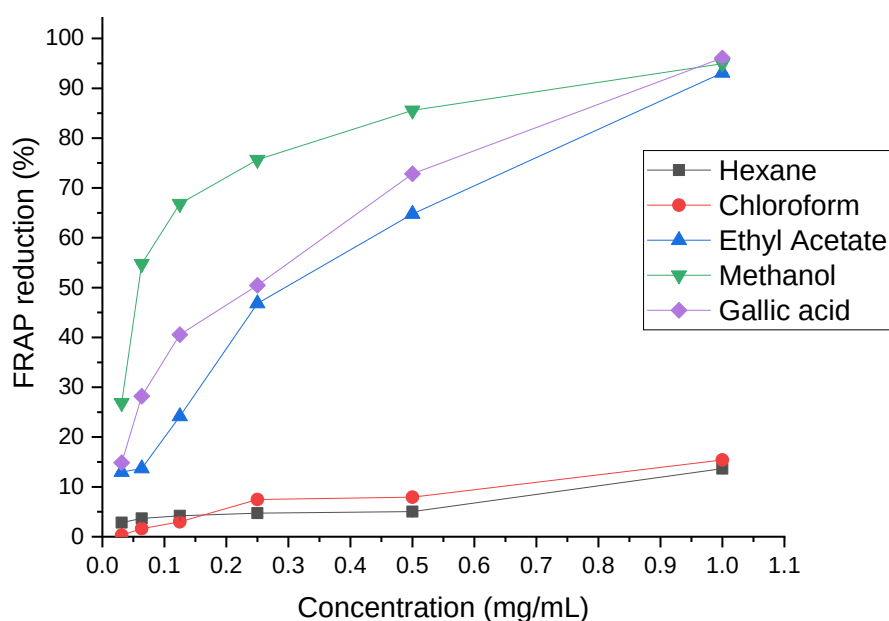


Figure 22. FRAP reduction (%) by *Terminalia prunoides* pods extracts at different concentrations. Data presented as mean $\pm$ standard error (n=3).

4.5.3 Antioxidant activity using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method

ABTS assay is an antioxidant assay that is based on the concept of reduction in redox, it measures the ability of a sample to reduce ABTS radical by single electron transfer (Kooy *et al.*, 2016).

4.5.3.1 Determination of the antioxidant activity *Sclerocarya birrea* fruit exocarp extracts using ABTS assay

Figure 23 shows that all extracts of *Sclerocarya birrea* exocarp displayed a positive relationship between extract solvent polarity and ABTS inhibition at 1mg/mL, gallic acid (standard) showed the highest ABTS scavenging with 86.72 ± 2.19 %. Amongst the extracts, methanol extracts had the highest radical scavenging activity ($82.75 \pm 3.48\%$) followed by ethyl acetate ($59.01 \pm 0.84\%$), hexane ($15.43 \pm 0.94\%$), and. chloroform ($13.27 \pm 1.69\%$). At 0.0312 mg/mL (lowest concentration), the radical scavenging of ABTS were in this order gallic acid ($24.99 \pm 1.17\%$) > gallic acid methanol ($17.08 \pm 1.61\%$) > ethyl acetate ($15.23 \pm 1.56\%$) > chloroform ($7.75 \pm 1.26\%$) > Hexane ($6.61 \pm 1.05\%$).

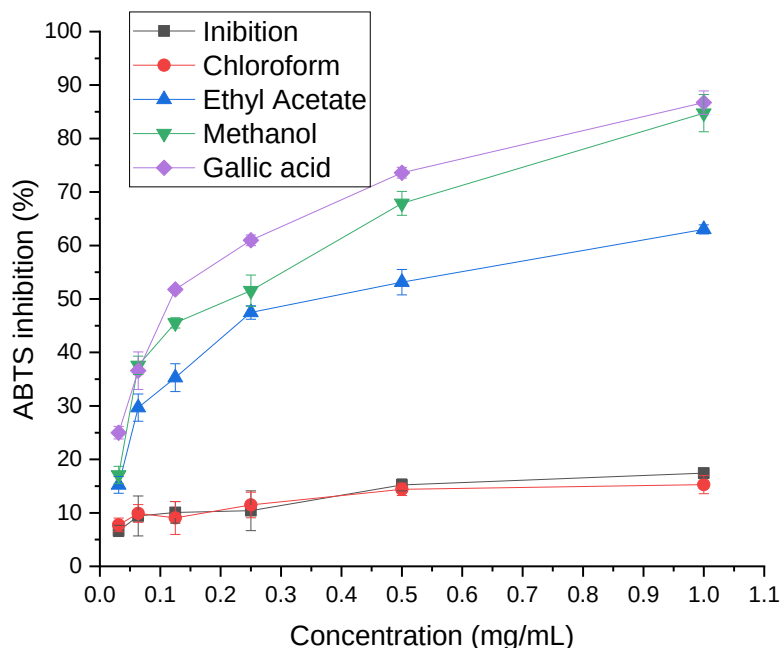


Figure 23. ABTS inhibition by *Sclerocarya birrea* fruit exocarp extracts at different concentrations, expressed as % inhibition. Data presented as mean $\pm$ standard error (n=3).

4.5.3.2 Determination of the antioxidant activity of *Terminalia prunoides* pods extracts using ABTS assay

Terminalia prunoides pods extracts all showed extraction solvent polarity dependent ABTS radical scavenging activity (Figure 24). Gallic acid (standard) showed the highest radical scavenging activity with (86.54 $\pm$ 1.16%). It was followed by methanol (76.4 $\pm$ 3.44%), ethyl acetate (72.73 $\pm$ 3.25%), chloroform (24.21 $\pm$ 0.31%), and hexane (21.42 $\pm$ 0.36%). At 0.0312 mg/mL (lowest concentration), the radical scavenging was in this order gallic acid (39.26 $\pm$ 1.18%)> methanol (36.57 $\pm$ 1.67%)> ethyl acetate (19.51 $\pm$ 3.52%)> hexane (4.65 $\pm$ 0.36%)> chloroform (1.61 $\pm$ 0.21%).

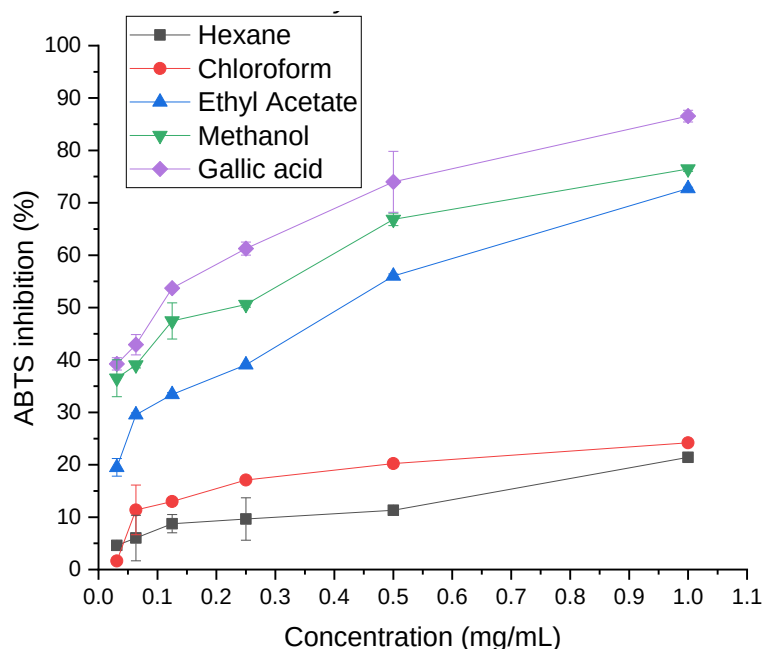


Figure 24. ABTS inhibition by *Terminalia prunoides* pods extracts at different concentrations, expressed as % inhibition. Data presented as mean $\pm$ standard error (n=3).

Summary of antioxidants assays (IC₅₀)

Based on antioxidant assays results, IC₅₀ values were interpolated from graphical presentations to make a summary. Table 7 show that methanol extracts of *Sclerocarya birrea* fruit exocarp are more efficient antioxidants compared to other *Sclerocarya birrea* fruit exocarp extracts. Methanol extracts of *Sclerocarya birrea* fruit exocarp recorded IC₅₀ values of 0.12, 0.09, 0.25 mg/mL for DPPH, FRAP and ABTS assays respectively. The same observation was recorded for methanol extracts of *Terminalia prunoides* pods, more effective antioxidants activity compared to ethyl acetate, chloroform, and hexane extracts from *Terminalia prunoides*. The IC₅₀ values of *Terminalia prunoides* methanol extracts on DPPH, FRAP, and ABTS were 0.06 mg/mL, 0.07 mg/mL, and 0.24 mg/mL respectively.

Table 7. IC₅₀ values of extracts after DPPH, FRAP and ABTS antioxidant assays.

	<i>Sclerocarya birrea</i> fruit exocarp				<i>Terminalia prunoides</i> pods			
	Hexane	Chloroform	Ethyl acetate	Methanol	Hexane	Chloroform	Ethyl acetate	Methanol
DPPH(IC ₅₀) (mg/mL)	>1	>1	0.89	0.12	>1	>1	0.09	0.06
FRAP(IC ₅₀) (mg/mL)	>1	>1	0.43	0.09	>1	>1	0.21	0.07
ABTS(IC ₅₀) (mg/mL)	>1	>1	0.29	0.25	>1	>1	0.42	0.24

4.6 Total antioxidant capacity (TAC) of extracts using phosphomolybdenum

TAC is an assay that is based on the formation of a stable green phosphate-Mo(V) complex, as a result of electron reduction of Mo (VI) to Mo(V) (Phatak & Hendre, 2014). Gallic acid was used as a standard to create a standard curve (Figure 25).

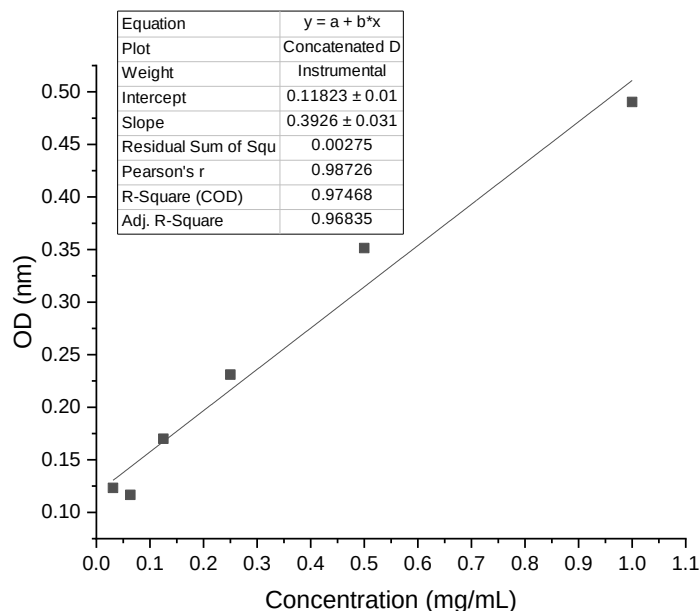


Figure 25. Optical densities (OD) of Gallic acid for different concentrations 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL at 750nm. This curve was used as a standard curve for total antioxidant capacity (TAC) determination.

4.6.1 Determination of total antioxidant capacity (TAC) of *Sclerocarya birrea* fruit exocarp extracts using phosphomolybdenum

Figure 26 depicts that all extracts showed a TAC that was proportional to the extract polarity. Methanol extracts showed the highest TAC value of 41.43 ± 1.65 mg GAE/g. It was followed by ethyl acetate (38.3 ± 1.13 mg GAE/g), chloroform (19.86 ± 1.02 mg GAE/g) and hexane with the lowest TAC value of 7.1 ± 0.2 mg GAE/g. The TAC of ethyl acetate and methanol extracts were not significantly ($p < 0.05$) different from each other but were significantly different from hexane and chloroform.

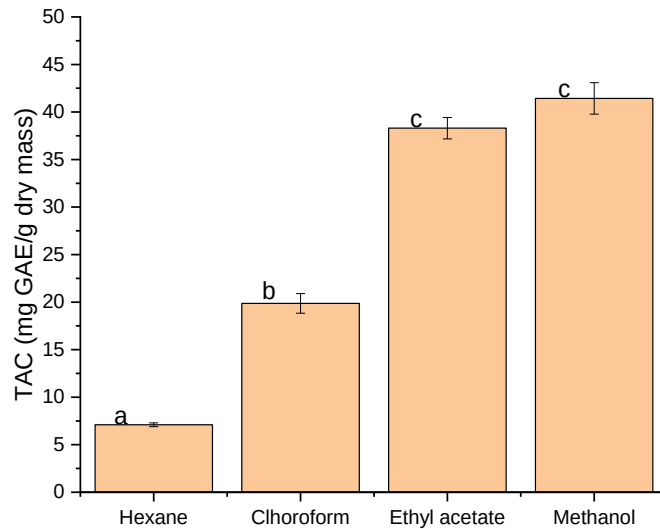


Figure 26. Total antioxidant capacity (TAC) of one gram of *Sclerocarya birrea* fruit exocarp, expressed in mg of gallic acid equivalence per one gram of dry mass of an extract (mg GAE/g). Data presented as mean $\pm$ standard error (n=3). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different

4.6.2 Determination of total antioxidant capacity (TAC) of *Terminalia prunoides* pods extracts using phosphomolybdenum

Figure 27 shows that there was a positive relationship between extraction solvent polarity and TAC. Methanol extracts had the highest TAC value of 43.9 ± 0.7 mg GAE/g, followed by ethyl acetate (41.73 ± 0.36 mg GAE/g), however TAC values of methanol extracts were not significantly different ($p < 0.05$) from TAC values of ethyl acetate extracts. They were followed chloroform (15.63 ± 0.64 mg GAE/g) and hexane (4.03 ± 0.31 mg GAE/g). TAC values of all extracts were significantly different from each other ($p < 0.05$).

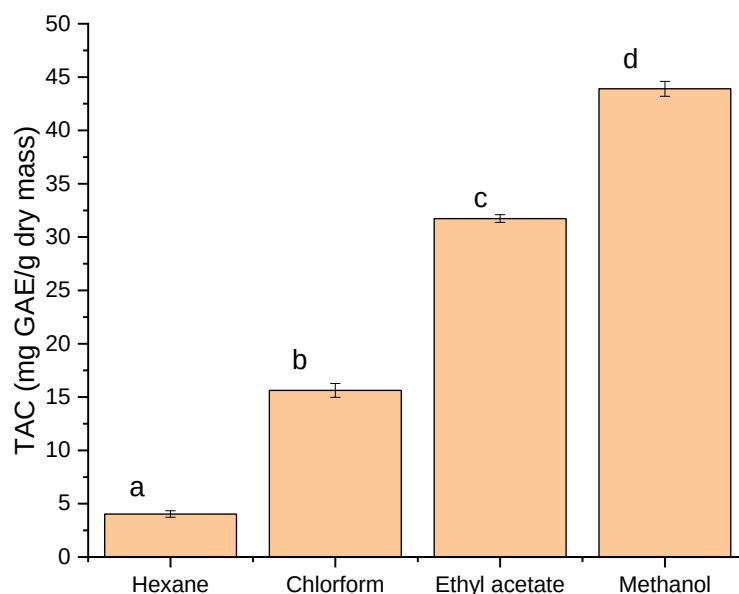


Figure 27. Total antioxidant capacity (TAC) of one gram of *Terminalia prunoides* pods extracts, expressed in mg of gallic acid equivalence per one gram of dry mass of an extract (mg GAE/g). Data presented as mean $\pm$ standard error (n=3). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

4.7 Cytoprotective effects of plant extracts

Extracts from *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods were evaluated for their protective effect, in protecting HeLa cells from hydrogen peroxide induced cell death.

4.7.1 Determination of the cytoprotective effects of *Sclerocarya birrea* fruit exocarp extracts against H₂O₂ induced cell death

The ability of *Sclerocarya birrea* fruit exocarp extracts to protect 98% confluent HeLa cells at passage number 5 against H₂O₂ induced cell death was evaluated using WST-1 cell viability assay as described in subsection 3.6.2.1. Figure 28 details that the most efficient dose was 400 μ g/mL (highest concentration used in the experiment). HeLa cells that were exposed to 1mM of

H₂O₂ without any protective agent (positive control), had only 27.3 ± 0.23 % of the initial 98 % confluent HeLa cells viable. HeLa cells that were only treated with PBS only and not exposed to 1mM H₂O₂ (negative control) had 98.08 ± 0.6 % viable cells. All extracts significantly ($p < 0.05$) protected HeLa cells from H₂O₂ induced cell death at 400 µg/mL. Hexane extracts had the least high protection of 78 ± 2.96 % cells, Ethyl acetate had the highest protective effect at 400 µg/mL, by protecting 94 ± 2.3 % cells, however the protective effects of ethyl acetate extracts and methanol extracts (92.2 ± 0.2 %) were not significantly different ($p < 0.05$) from each other. The degree of cytoprotection decreased with decreasing concentrations of extraction solvents. At the lowest extract concentration (12.5 µg/mL), hexane had the lowest protective effect of only 32 ± 3.12 % cells remaining viable, which was not significantly different from that of positive control. Methanol extracts had the highest protection at 12.5 µg/mL, protecting 74 ± 2.61 % of HeLa cells.

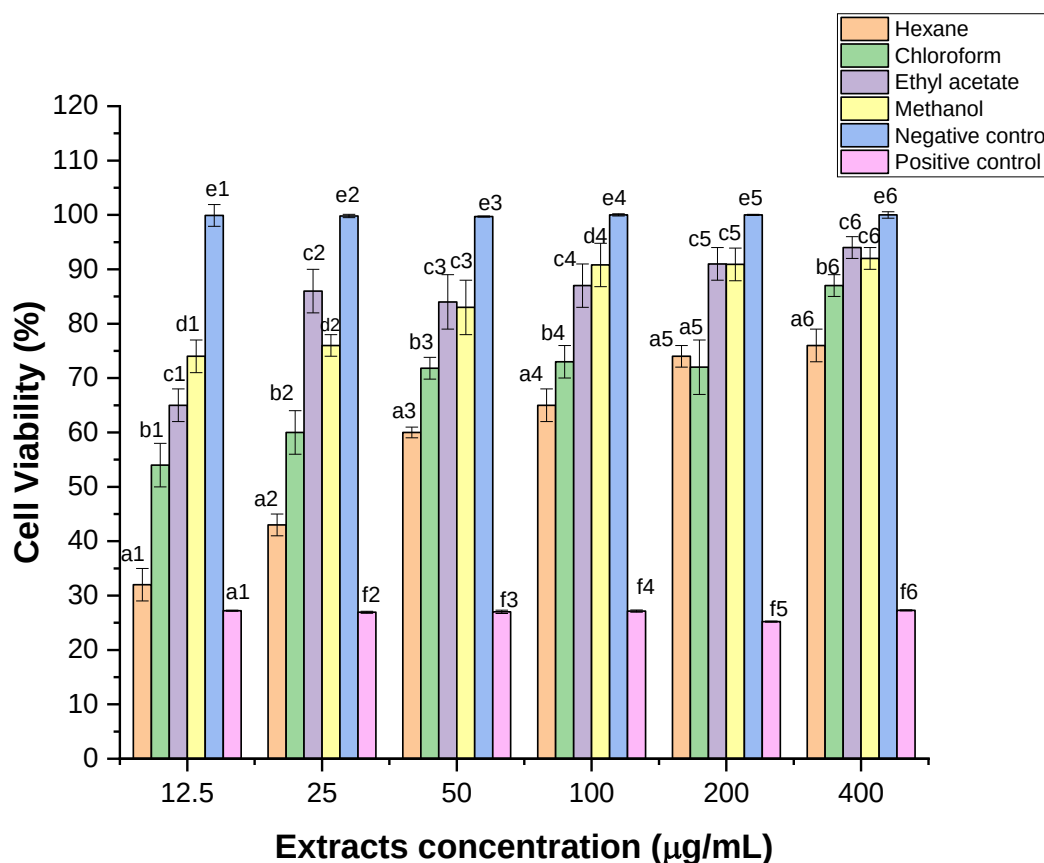


Figure 28. Cytoprotective effect of *Sclerocarya birrea* fruit exocarp extracts at different concentrations on 98 % confluent HeLa cells (passage number 5) against H₂O₂ induced cell death. Negative control= untreated HeLa cells + PBS only. Positive control= HeLa cells exposed to 1mM H₂O₂ only. Data is presented in mean± standard error (n=3). Letters above bars indicates significant differences (p< 0.05) between means. The numbers attached to the letters indicates comparison between means in the same concentration.

4.7.2 Determination of the cytoprotective effects of *Terminalia prunoides* pods extracts against H₂O₂ induced cell death

The ability of *Terminalia prunoides* pods extracts to protect 98 % confluent HeLa cells at passage number 5 against oxidative stress induced cell death was evaluated using WST-1 cell viability assay. Figure 29 shows HeLa cells that were exposed to 1mM of H₂O₂ without any protective agent (positive control), had only 25.3 ± 0.01 % of the initial 98 % confluent HeLa cells viable. HeLa cells that were only treated with PBS only (negative control) had 96. ± 0.6% of viable cells. At a concentration of 400 µg/mL (the highest concentration), methanol extracts had the highest protective activity resulting in 95.9±2.3% of cells remaining viable, which was not significantly different (p< 0.05) from negative control. Hexane extracts had the least protective activity with 83±3.1 % viable cells. The protective activity of the extracts decreased with their decreasing concentrations. At a concentration of 12.5 µg/mL (the lowest concentration), methanol extracts had the highest protective activity of only 54± 2.76 %, which was not significantly different (p< 0.05) from the protective activity of ethyl acetate extracts. Hexane extracts had the least cytoprotective activity which was numerically (29 ±3.1%) higher, but not significantly different (p< 0.05) from that of positive control.

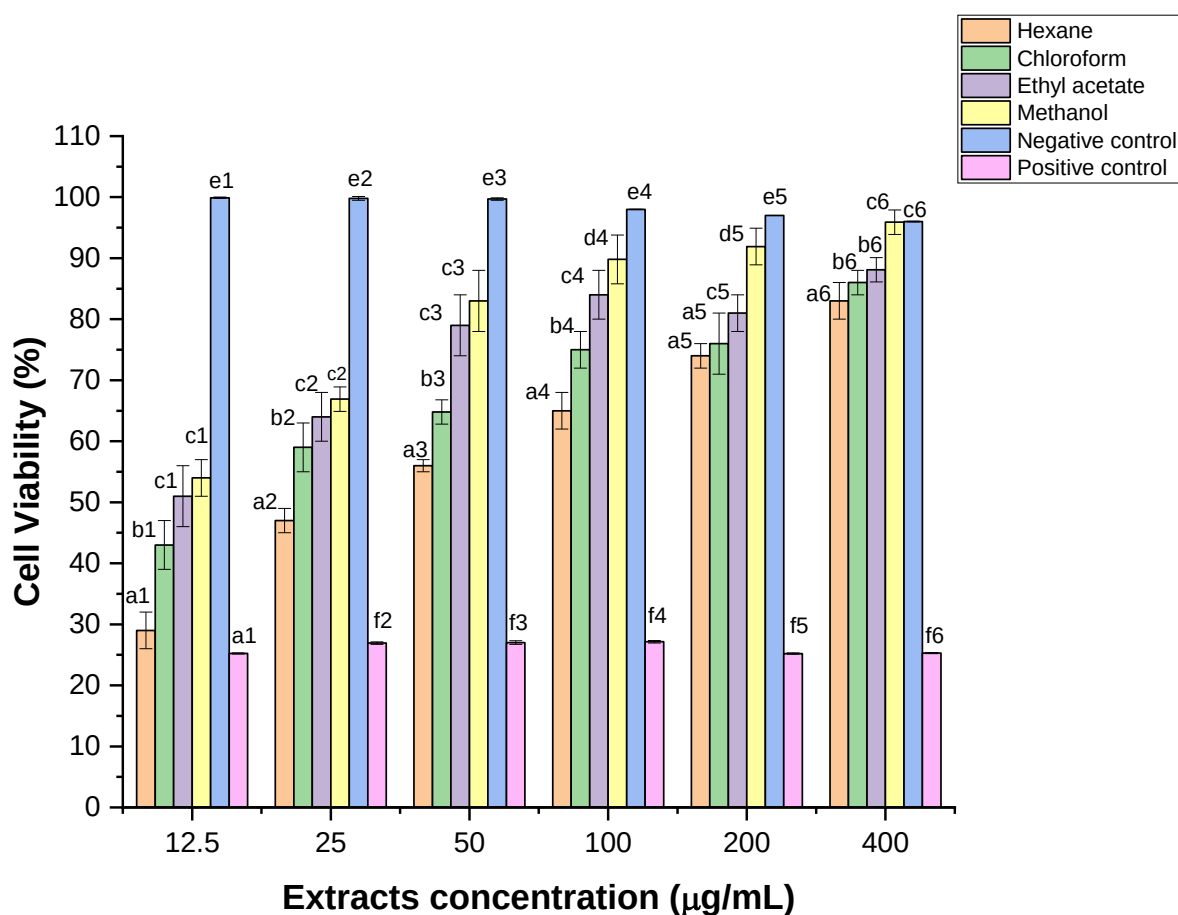


Figure 29. Cytoprotective effect of *Terminalia prunoides* pods extracts at different concentrations on 98 % confluent HeLa cells passage number 5 against H₂O₂. Negative control= untreated HeLa cells + PBS only. Positive control= HeLa cells exposed to 1mM H₂O₂ only. Data is presented in mean± standard error (n=3). Letters above bars indicates significant differences (p< 0.05) between means. The numbers attached to the letters indicates comparison between means in the same concentration.

4.8 Effects of extracts on the oxidative stress markers

4.8.1 Effects of extracts on super oxidise dismutase (SOD) activity

The effects of *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods extracts on the activity of superoxide dismutase (SOD) of 90 % confluent HeLa cells at passage number 5 was evaluated, compared to that of control (untreated HeLa cells from the same stating colony at the same passage number and confluency). Figure 30 show that except for hexane, all extracts from *Sclerocarya birrea* fruit exocarp significantly ($p < 0.05$) increased the activity of SOD. The results revealed an extraction solvent polarity dependent activity, where low polar extracts had low effects on SOD activity, and high polar extracts showed high effects on SOD activity. Though methanol and ethyl acetate are not significantly different ($p < 0.05$) from each other, methanol extracts recorded the most increased SOD activity with 9 ± 2.41 U/mL. Methanol extracts were followed by ethyl acetate (9.22 ± 0.44 U/mL), chloroform extracts (7.6 ± 0.6 U/mL), and hexane extracts (4.8 ± 0.4 U/mL). Figure 31 show that all extracts of *Terminalia prunoides* pods significantly increased ($p < 0.05$) the activity of SOD. There was no significant difference ($p < 0.05$) between chloroform (8.44 ± 0.54 U/mL), ethyl acetate (9.06 ± 0.7 U/mL), and methanol extracts. Methanol extracts recorded the highest SOD activity with 9.19 ± 0.29 U/mL.

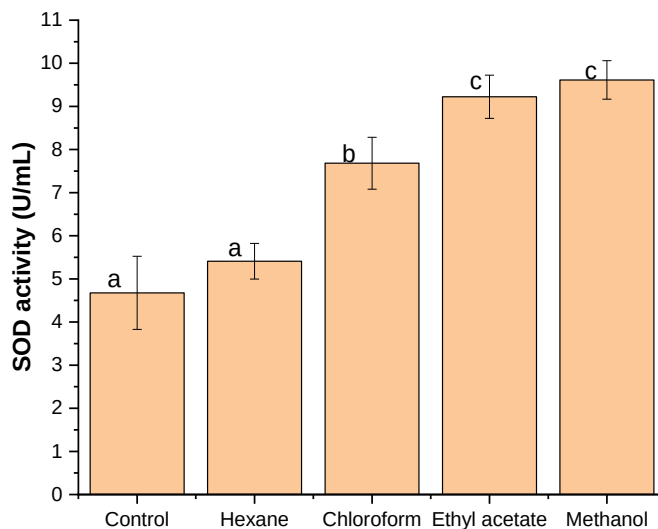


Figure 30. Effects of extracts of *Sclerocarya birrea* fruit exocarp on superoxide dismutase (SOD) activity on 90% confluent Hela cells of passage number 5. Control = SOD activity in untreated HeLa cells. Data presented as mean $\pm$ standard error (n=3) Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

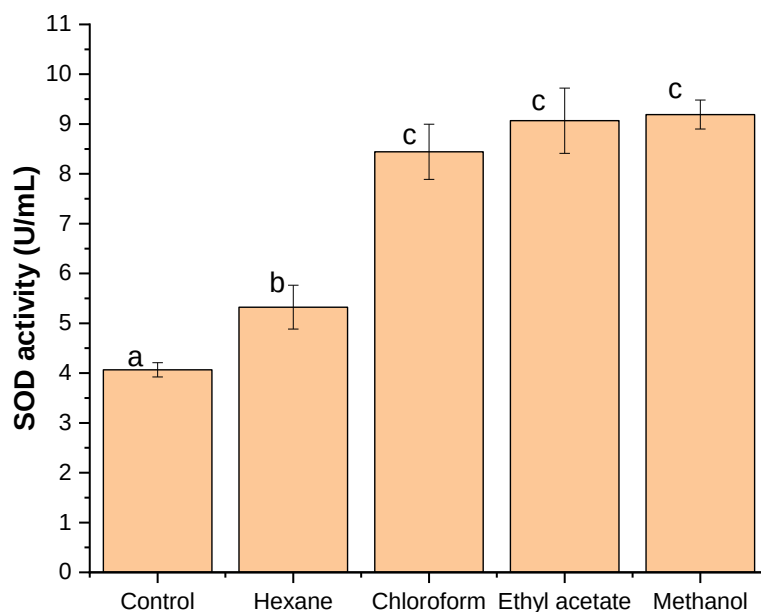


Figure 31. Effects of extracts of *Terminalia prunoides* pods on superoxide dismutase (SOD) activity on 90% confluent HeLa cells of passage number 5. Control = SOD activity in untreated HeLa cells. Data presented as mean $\pm$ standard error (n=3) Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

4.8.2 Effects of extracts on catalase (CAT) activity

The effects of extracts on the activity of catalase (CAT) were evaluated on 90% confluent HeLa cells at passage number 5 and compared to that of the control (untreated HeLa cells from the same starting colony at the same passage number and confluency). Figure of 32 shows that there

was an extraction solvent polarity dependent effect on the activity of CAT. Amongst *Sclerocarya birrea* fruit exocarp extracts, hexane extracts had the least effect on CAT activity (33.70 ± 5.95 U/mL). Chloroform extracts had the 2nd least effect on CAT activity. There was no significant difference between CAT activity of untreated cells (control cells) and the CAT activity caused by hexane and chloroform extracts. Methanol extracts had the most effect (49.18 ± 7.39 U/mL) on CAT activity, followed by ethyl acetate extracts. However, there was no significant difference between the effects caused by methanol and ethyl acetate extracts of *Sclerocarya birrea* fruit exocarp on CAT activity. For *Terminalia prunoides* pods (Figure 33), all extracts significantly ($p < 0.05$) elevated the activity of CAT. Extracts did not exhibit extraction solvent polarity dependent activity; chloroform recorded the least effect on the activity of CAT (47.98 ± 2.68 U/mL). Hexane extracts showed the 3rd highest increase in CAT activity behind ethyl acetate and methanol extracts (54.14 ± 4.11 U/mL) which had the most effect on CAT activity. There was no significant difference between effects of hexane, chloroform, ethyl acetate and methanol extracts.

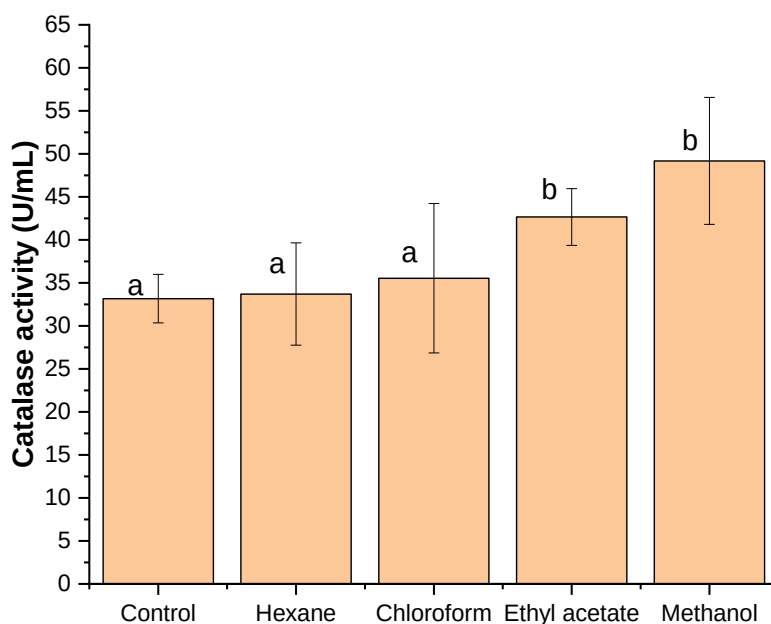


Figure 32. Effects of extracts of *Sclerocarya birrea* fruit exocarp on catalase (CAT) activity on 90% confluent HeLa cells at passage number 5. Control = CAT activity in untreated HeLa cells. Data presented as mean $\pm$ standard error ($n=3$). Significant differences ($p < 0.05$) are indicated by

letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

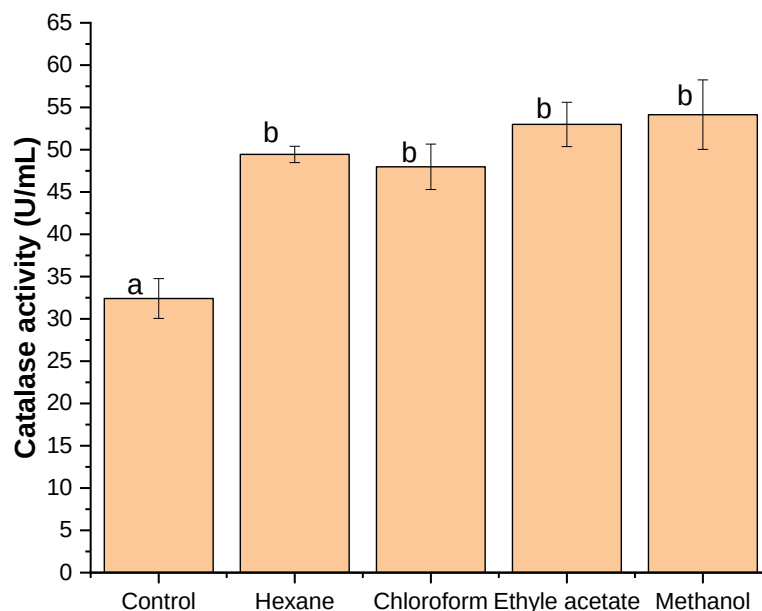


Figure 33. Effects of extracts of *Terminalia prunoides* pods on catalase (CAT) activity 90% confluent HeLa cells of passage number 5. Control = CAT activity in untreated HeLa cells. Data presented as mean $\pm$ standard error (n=3). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

4.8.3 Effects of extracts on the activity of reduced glutathione (GSH)

The effects of extracts from both plants on the activity of reduced glutathione (GSH) on 90% confluent HeLa cells at passage number 5 were compared to that in control (untreated HeLa cells cells from the same stating colony at the same passage number and confluency). Figure 34 shows that all extracts from *Sclerocarya birrea* fruit exocarp increased the activity of GSH. The effects of hexane extracts on GSH activity were not significantly different from that in control cells. Ethyl acetate extracts had the most effect (34.86 ± 5.32 mmol/L), followed by methanol extracts.

The effects of ethyl acetate and methanol extracts on GSH activity were not significantly different from each other ($p < 0.05$). Hexane extracts had the least effect on GSH activity (22.03 ± 5.96 mmol/L). For effects of extracts of *Terminalia prunoides* pods on GSH activity (Figure 35), all extracts significantly increased GSH activity. However, the effects of all 4 extracts on GSH activity were not significantly different ($p < 0.05$) from each other. Methanol extracts showed the most effect (47.97 ± 2.30), followed by; ethyl acetate extracts (46.27 ± 5.03 mmol/L), hexane extracts (44.66 ± 1.15 mmol/L) and chloroform extracts (45 ± 4.46 mmol/L).

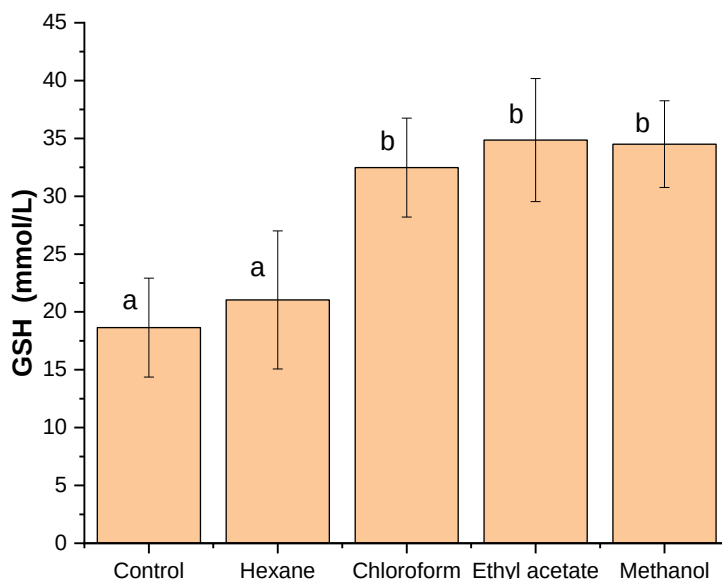


Figure 34. Effects of extracts of *Sclerocarya birrea* fruit exocarp on reduced Glutathione (GSH) activity on 90% confluent HeLa cells at passage number 5. Control = GSH activity of untreated HeLa cells. Data presented as mean $\pm$ standard error ($n=3$). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

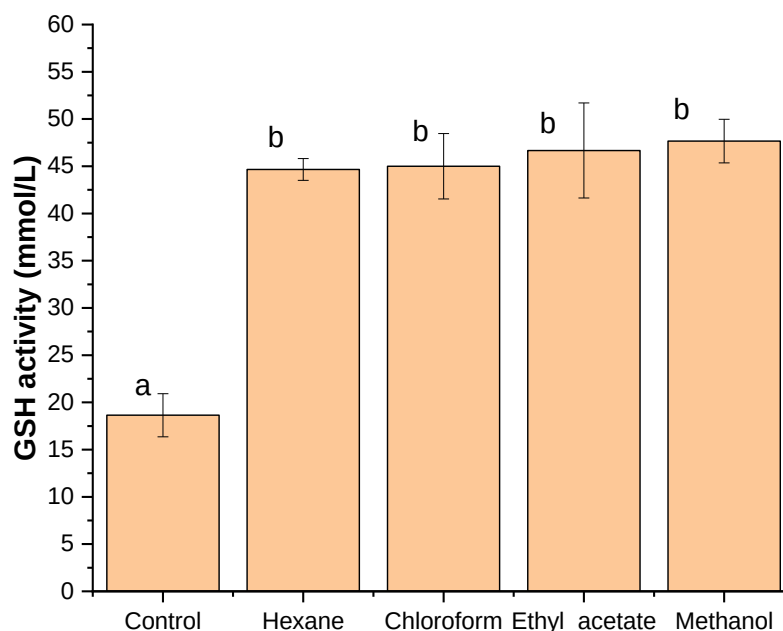


Figure 35. Effects from *Terminalia prunoides* pods on reduced Glutathione (GSH) activity on 90% confluent HeLa cells at passage number 5. Control = GSH activity of untreated HeLa cells. Data presented as mean $\pm$ standard error (n=3). Significant differences ($p < 0.05$) are indicated by letters above the bars, where different letters depict significant difference between average means. Same letters indicate that means are not significantly different.

4.9 Antiproliferative effects of extracts on HeLa cells

Extracts from *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods were evaluated in the inhibition of the growth of 93 confluent HeLa cancer cells at passage number 5. This inhibition was compared to the inhibition induced by the anticancer drug cisplatin.

4.9.1 Antiproliferative effects of Cisplatin (standard) on HeLa cells

WST-1 assay was used to establish the degree of inhibition of cisplatin as a verified positive control on 93% confluent HeLa cells at passage number 5. A graph (Figure 36) was then used to

interpolate the concentration that inhibits 50% of the cells. At a concentration of 100 $\mu\text{g/mL}$ (highest concentration), cisplatin inhibited 78 ± 2.13 % of HeLa cells. The inhibition decreased with decreased concentrations. At 1.56 $\mu\text{g/mL}$, 33 ± 4.10 % of cells were inhibited. At 12.5 $\mu\text{g/mL}$, 50 % of HeLa cells were inhibited (IC_{50}).

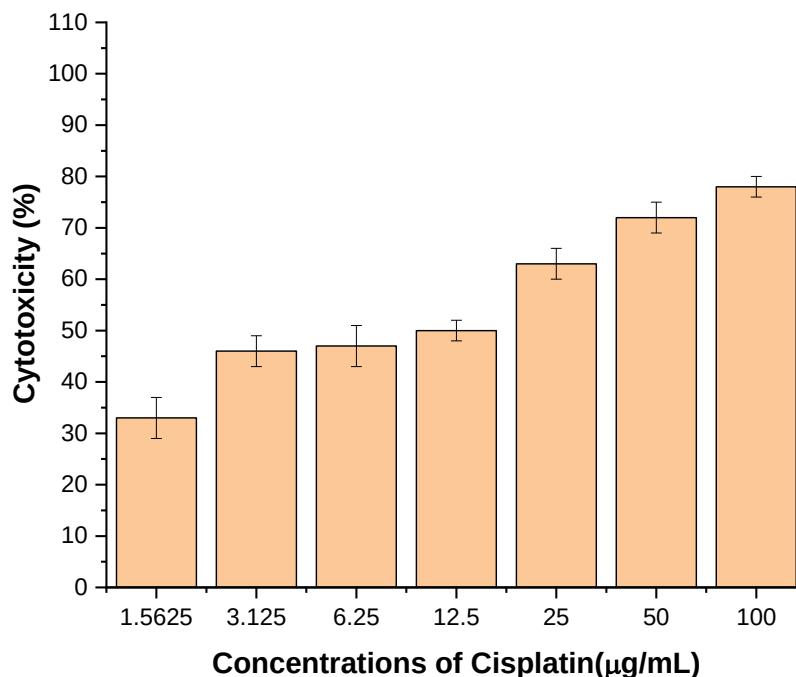


Figure 36. Cytotoxicity of cisplatin on 93% confluent HeLa cells at passage number 5, at different concentrations. Results are presented as mean $\pm$ standard error ($n=3$).

4.9.2 Antiproliferative effects of extracts of *Sclerocarya birrea* fruit exocarp on HeLa cells

WST-1 assay was used to evaluate antiproliferative properties of *Sclerocarya birrea* fruit exocarp extracts on 93% confluent HeLa cells at passage number 5. Figure 37 shows that at a concentration of 400 $\mu\text{g/mL}$ (the highest concentration), methanol extracts exhibited the most antiproliferative effect of 77.6 ± 1.05 %. Hexane exhibited the least antiproliferative effect at 400 $\mu\text{g/mL}$, with 55.2 ± 2.02 % of HeLa cells inhibited. At concentrations of 12.5 $\mu\text{g/mL}$ (lowest concentration), methanol extracts exhibited the least anticancer activity with cytotoxicity levels

of 3.17 ± 2.00 % but was not significantly different ($p < 0.05$) from anticancer activity of chloroform and hexane extracts. Ethyl acetate extracts exhibited the most anticancer activity at $12.5 \mu\text{g/mL}$, with 5.97 ± 1.03 % of HeLa cells inhibited. Figure 38 shows that for concentrations that inhibit 50% (IC_{50}) of HeLa cells, extraction solvent polarity was inversely proportional to the IC_{50} values. Hexane extracts had the highest IC_{50} value of $350 \mu\text{g/mL}$, and methanol had the lowest IC_{50} value of $75 \mu\text{g/mL}$ amongst extracts.

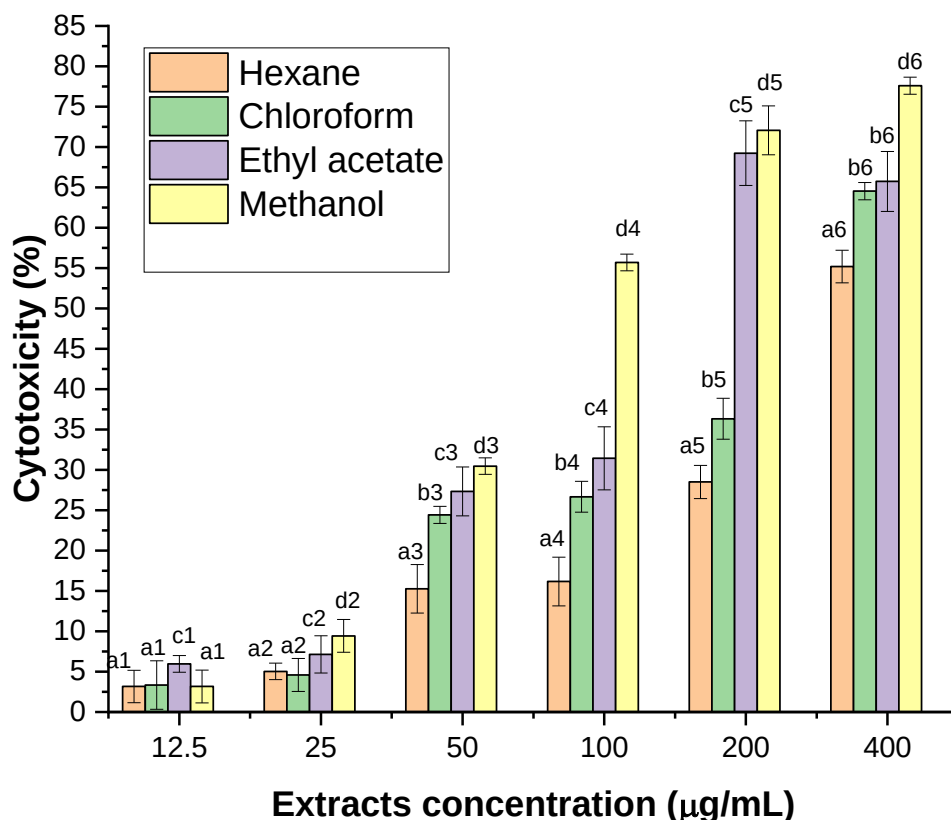


Figure 37. Antiproliferative effects of *Sclerocarya birrea* fruit exocarp extracts at different concentrations on 93% confluent HeLa cells at passage number 5. Data presented as mean $\pm$ standard error ($n=3$). Letters above bars indicate significant differences ($p < 0.05$) between means. The numbers attached to the letters indicates comparison between means in the same concentration.

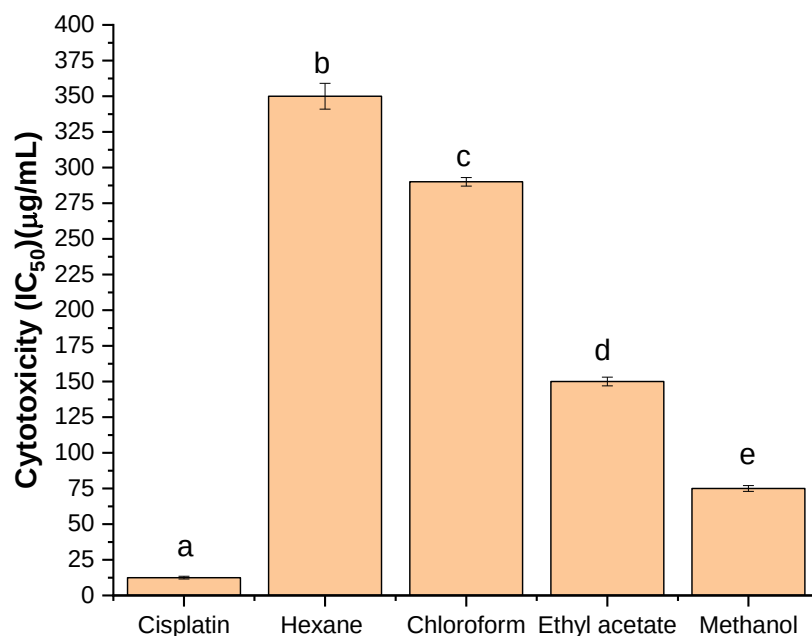


Figure 38. Concentrations of *Sclerocarya birrea* fruit exocarp extracts and cisplatin that inhibits 50 % (IC₅₀) of 93% confluent HeLa cells at passage number 5. Data presented as mean± standard error (n=3). Letters above bars indicate significant differences (p< 0.05) between means.

4.9.3 Antiproliferative effects of extracts of *Terminalia prunoides* pods on HeLa cells

WST-1 assay was used to evaluate the antiproliferative effects of extracts of *Terminalia prunoides* pods on 93% confluent HeLa cells at passage number 5. From the relationship between extracts concentrations and the level of toxicity, IC₅₀ values were interpolated. Figure 39 shows that at a concentration of 400 µg/mL (highest concentrations), methanol extracts showed the most antiproliferative effect by inhibiting 70.47 ± 3.06 % of HeLa cells. Hexane extracts showed the least antiproliferative effect by inhibiting 33.78 ± 2.40 % cells. Cytotoxicity decreased with decreasing concentrations of extracts. At a concentration of 12.5 µg/mL (lowest concentration), methanol extracts had the most antiproliferative effect by inhibiting 7.0 ± 1.91 % of cells but not significantly different (p< 0.05) from the inhibition caused by ethyl acetate extracts. Hexane extracts had the least antiproliferative effect by inhibiting 3.1±2.3 % of cells but

not significantly different ($p < 0.05$) from the inhibition caused by chloroform extracts. The IC_{50} values of extracts were inversely proportional to the polarity of extraction solvents as shown in Figure 40, IC_{50} values decreased with increasing extraction solvent polarity. For hexane extracts, it would require a concentration greater than the maximum concentration used in the experiment ($IC_{50} > 400 \mu\text{g/mL}$). Methanol extracts had the lowest IC_{50} value of $190 \mu\text{g/mL}$.

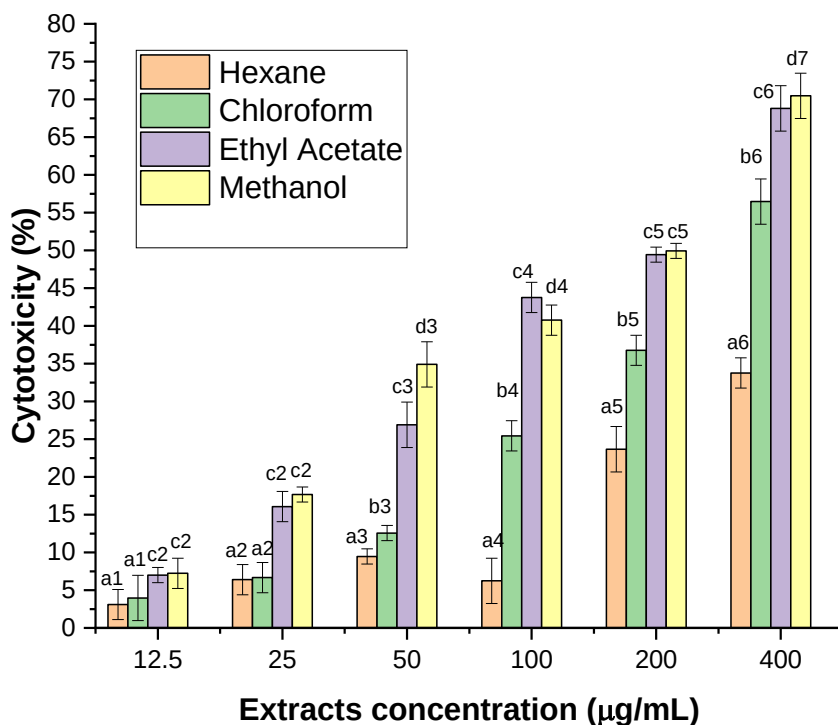


Figure 39. Antiproliferative effects of *Terminalia prunoides* pods extracts at different concentrations on 93% confluent HeLa cells at passage number 5. Data presented as mean $\pm$ standard error ($n=3$). Letters above bars indicate significant different difference ($p < 0.05$) between means. The numbers attached to the letters indicates comparison between means in the same concentration.

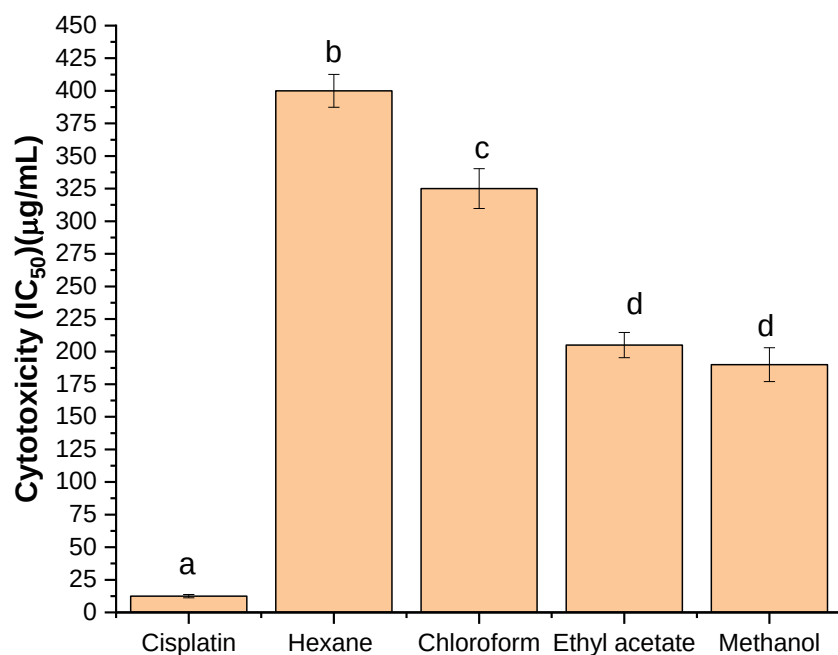


Figure 40. Concentrations of *Terminalia prunoides* pods extracts and cisplatin that inhibits 50 % (IC₅₀) of HeLa cells. Data presented as mean± standard error (n=3). Letters above bars indicate significant difference ($p < 0.05$) between means.

4.10 Synergistic antiproliferative effects of formulated anticancer ingredients on HeLa cells

The results from antiproliferative effects of individual extracts were used to formulate 3 anticancer ingredients. The first ingredient was formulated by combining methanol extracts of *Sclerocarya birrea* fruit exocarp (M1) and methanol extracts of *Terminalia prunoides* pods (M2) at a ratio of 1:1. This ingredient was named M1M2. The second ingredient was formulated by combining ethyl acetate extracts of *Sclerocarya birrea* exocarp (E1) and ethyl acetate extracts of *Terminalia prunoides* pods (E2) at a ratio of 1:1 and this ingredient was named E1E2. The third ingredient was formulated by combining M1, E1, M2 and E2 at 1:1:1:1 ratio and this ingredient was named M1E1M2E2. The 3 formulations were evaluated for their synergistic antiproliferative effects on 93% confluent HeLa cells at passage number 5, using WST-1 as described above

(subsection 3.6.2.1). At a concentration of 400 $\mu\text{g/mL}$ (the highest concentration), the M1E1M2E2 formulation showed the most antiproliferative effect with $86\pm4.3\%$. Cytotoxicity of M1E1M2E2 was not significantly different ($p < 0.05$) from the cytotoxicity of M1M2 ($83\pm3.1\%$) and E1E2 ($77\pm4.6\%$) as shown in figure 41. At a concentration of 12.53 $\mu\text{g/mL}$, the antiproliferative effects were as follows M1E1M2E2($16\pm3.6\%$)>M1M2($13\pm2.4\%$)>E1E2($9\pm2.0\%$). The data from antiproliferative effects of formulations was used to interpolate IC_{50} values as shown in figure 42. M1E1M2E2 had the lowest IC_{50} value of 77 $\mu\text{g/mL}$, followed by M1M2 (83 $\mu\text{g/mL}$) and E1E2 (130 $\mu\text{g/mL}$).

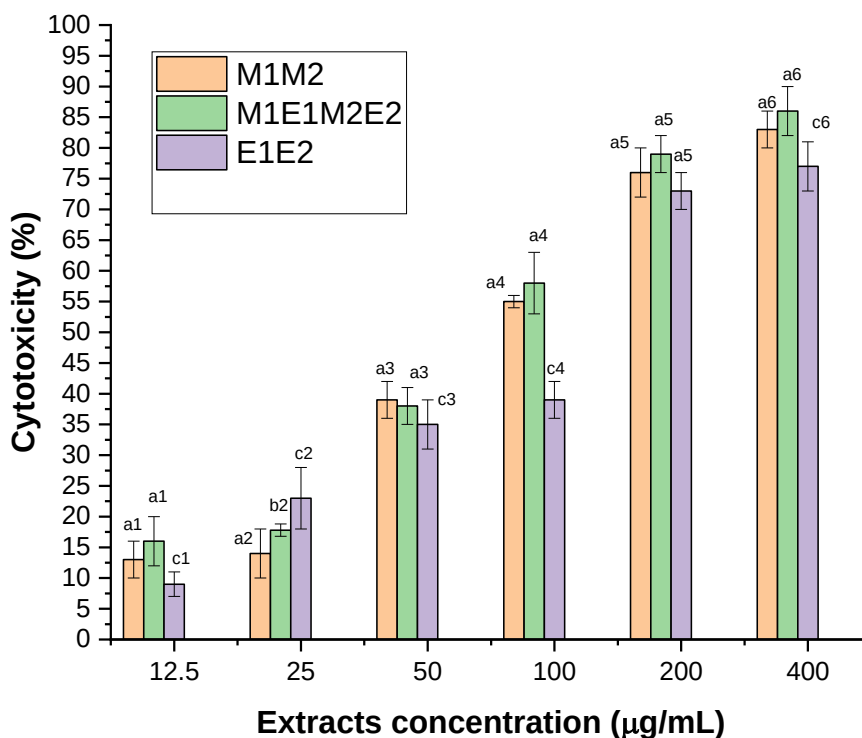


Figure 41. Antiproliferative effects of ingredients at different concentrations on 93% confluent HeLa cells at passage number 5. Data presented as mean $\pm$ standard error ($n=3$). Letters above bars indicate significant difference ($p < 0.05$) between means. The numbers attached to the letters indicates comparison between means in the same concentration.

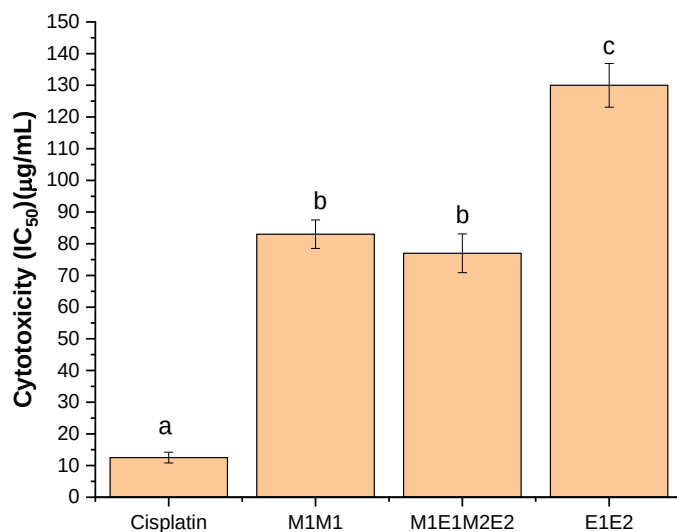


Figure 42. Concentrations of formulations and cisplatin that inhibit 50% (IC₅₀) of 93% confluence HeLa cells at passage number 5. Data presented as mean± standard deviation (n=3). Letters above bars indicate significant difference ($p < 0.05$) between means.

4.11 Gene expression analysis by RT-qPCR

HeLa cells treated with methanol extracts of *Sclerocarya birrea* fruit exocarp (M1), methanol extracts of *Terminalia prunoides* (M2), M1E1 ingredient, and M1E2M2E2 ingredient were subjected to gene expression analysis. These are extracts and ingredients with the most antiproliferative activity on HeLa cells (93 % confluency) at passage number 5. They were subjected to gene expression analysis of Epidermal growth factor receptor (EGFR), Vascular endothelial growth factor (VEGF), Metastasis associated with colon cancer 1 (MACC1), Cytokeratin fragment 21-1 (CYFRA 21-1), and Cluster of differentiation 95 (CD95) using RT-qPCR. The expression of EGFR, VEGF, MACC1, CYFRA 21-1, and CD 95 genes have been used in the diagnosis and prognosis of cervical cancer.

4.11.1 Gene expression profiles of cervical cancer marker

4.11.1.1 Relative mRNA expression of Epidermal growth factor receptor (EGFR)

Figure 43 shows that there was a significant ($p < 0.05$) downregulation of EGFR expression on 93% confluent HeLa cells at passage number 5, treated with extracts and ingredients, compared to that of untreated HeLa cells (control). The formulated ingredients (M1E1M2E2 and M1M2) downregulated EGFR expression more than individual extracts (M1 and M2). The M1E1M2E2 ingredient led to the most downregulation of EGFR with a 0.51-fold decrease while M1 (*Sclerocarya birrea* fruit exocarp methanol extracts) resulted in the least downregulation with a 0.28-fold decrease of EGFR expression.

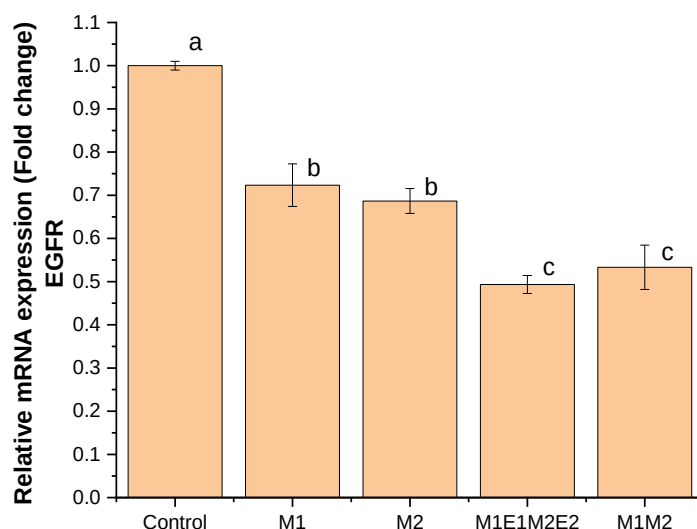


Figure 43. Relative EGFR mRNA expression in HeLa cells passage number 5 treated with extracts and formulated ingredients. Data presented as mean $\pm$ standard deviation ($n=3$). Letters above bars indicate significant difference ($p < 0.05$), where different letters depict significant difference between average means. Same letters indicate that means are not significantly different. Control: untreated HeLa cells. M1: methanol extracts from *Sclerocarya birrea* fruit exocarp. M2: methanol extracts from *Terminalia prunoides* pods. M1M2: methanol extracts from *Sclerocarya birrea* fruit exocarp + methanol extracts from *Terminalia prunoides* pods

(1:1). M1E1M2E2: ((methanol + ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp) + (methanol + ethyl acetate *Terminalia prunoides* pods)).

4.11.1.2 Relative mRNA expression of Vascular endothelial growth factor (VEGF)

Figure 44 depicts a significant ($p < 0.05$) downregulation of VEGF expression in HeLa cells (passage number 5) treated with extracts and formulated ingredients, compared to that of untreated HeLa cells (control). The formulated ingredients (M1E1M2E2 and M1M2) downregulated VEGF expression more than individual extracts (M1 and M2). The M2 (*Terminalia prunoides* pods methanol extracts) led to the least downregulation with a 0.22-fold decrease in VEGF expression. The M1E1M2E2 ingredients led to the most downregulation of 0.5-fold decrease.

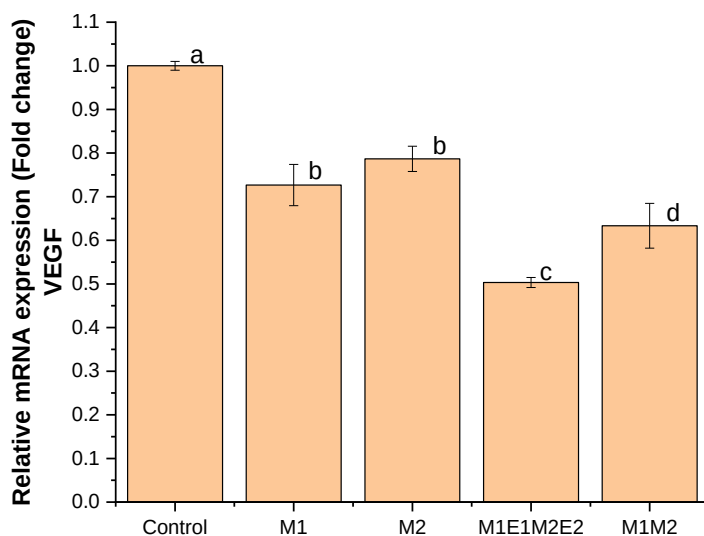


Figure 44. Relative VEGF mRNA expression in HeLa cells passage number 5 treated with extracts and formulated ingredients. Data presented as mean $\pm$ standard deviation ($n=3$). Letters above bars indicate significant difference ($p < 0.05$), where different letters depict significant difference between average means. Same letters indicate that means are not significantly different. Control: untreated HeLa cells. M1: methanol extracts from *Sclerocarya birrea* fruit exocarp. M2: methanol extracts from *Terminalia prunoides* pods. M1M2: methanol extracts

from *Sclerocarya birrea* fruit exocarp + methanol extracts from *Terminalia prunoides* pods (1:1). M1E1M2E2: ((methanol + ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp) + (methanol + ethyl acetate from *Terminalia prunoides* pods)).

4.11.1.3 Relative mRNA expression of Metastasis associated with colon cancer 1 (MACC1)

Figure 45 shows a significant ($p < 0.05$) downregulation of MACC1 expression on HeLa cells passage number 5 treated with extracts and formulated ingredients, compared to that of untreated HeLa cells (control). Although there was no significant difference ($p < 0.05$) amongst all treatments, M1E1M2E2 led to the most downregulation (0.51-fold decrease) while M2 resulted in the least downregulation of 0.45-fold decrease of MACC1 expression.

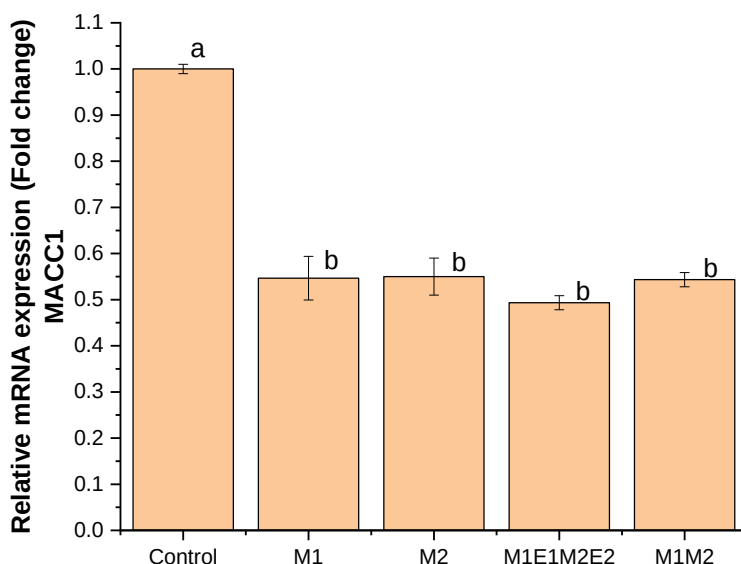


Figure 45. Relative MACC1 mRNA expression in HeLa cells (passage number 5) treated with extracts and formulated ingredients. Data presented as mean $\pm$ standard deviation ($n=3$). Letters above bars indicate significant difference ($p < 0.05$), where different letters depict significant difference between average means. Same letters indicate that means are not significantly different. Control: untreated HeLa cells. M1: methanol extracts from *Sclerocarya birrea* fruit exocarp. M2: methanol extracts from *Terminalia prunoides* pods. M1M2: methanol extracts

from *Sclerocarya birrea* fruit exocarp + methanol extracts from *Terminalia prunoides* pods (1:1). M1E1M2E2: ((methanol + ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp) + (methanol + ethyl acetate from *Terminalia prunoides* pods)).

4.11.1.4 Relative mRNA expression of Cytokeratin fragment 21-1 (CYFRA 21-1)

Figure 46 shows that there was a significant ($p < 0.05$) downregulation of CYFRA 21-1 expression on HeLa cells passage number 5 treated with extracts and formulated ingredients, compared to that of untreated HeLa cells (control). The formulated ingredients (M1E1M2E2 and M1M2) downregulated CYFRA 21-1 expression more than individual extracts (M1 and M2). The M1E1M2E2 treatment led to a 0.46-fold decrease, and M1 was led to a 0.34-decrease in the expression of CYFRA 21-1.

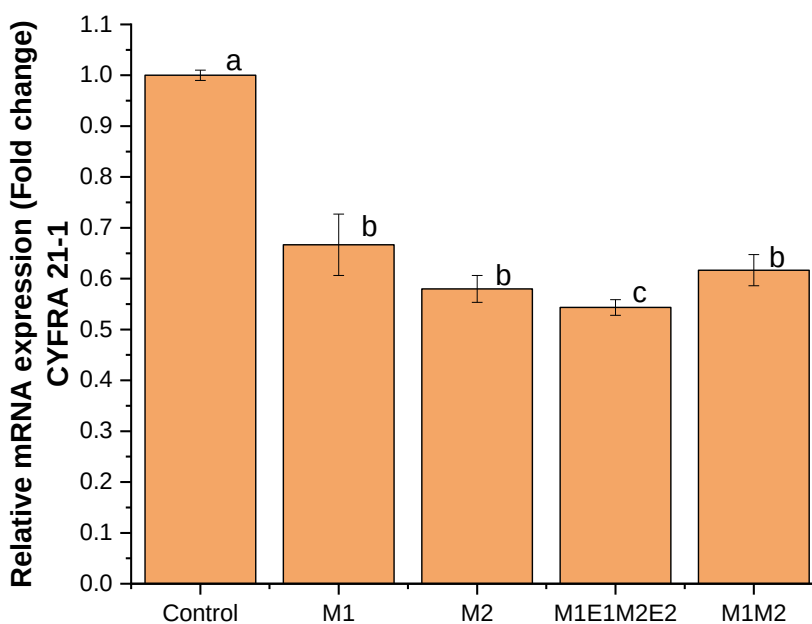


Figure 46. Relative CYFRA 21-1 mRNA expression in HeLa cells (passage number 5) treated with extracts and formulated ingredients. Data presented as mean $\pm$ standard deviation ($n=3$). Letters above bars indicate significant difference ($p < 0.05$), where different letters depict significant difference between average means. Same letters indicate that means are not

significantly different. Control: untreated HeLa cells. M1: methanol extracts from *Sclerocarya birrea* fruit exocarp. M2: methanol extracts from *Terminalia prunoides* pods. M1M2: methanol extracts from *Sclerocarya birrea* fruit exocarp + methanol extracts from *Terminalia prunoides* pods (1:1). M1E1M2E2: ((methanol + ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp) + (methanol + ethyl acetate from *Terminalia prunoides* pods)).

4.11.2 Gene expression profile of the tumour suppressor gene Cluster of differentiation 95 (CD95)

4.11.2.1 Relative mRNA expression of CD95

Figure 47 depicts that all treatments significantly ($p < 0.05$) upregulated the expression of CD95, compared to that of untreated HeLa cells (control). Formulated ingredients (M1E1M2E2 and M1M2) led to the most upregulation of CD95 expression compared to individual extracts (M1 and M2). The most upregulation was observed for the M1E1M2E2 ingredient with a 1.9-fold increase, and the least upregulation was observed for the M1 treatment with a 0.6fold increase in the expression of CD95.

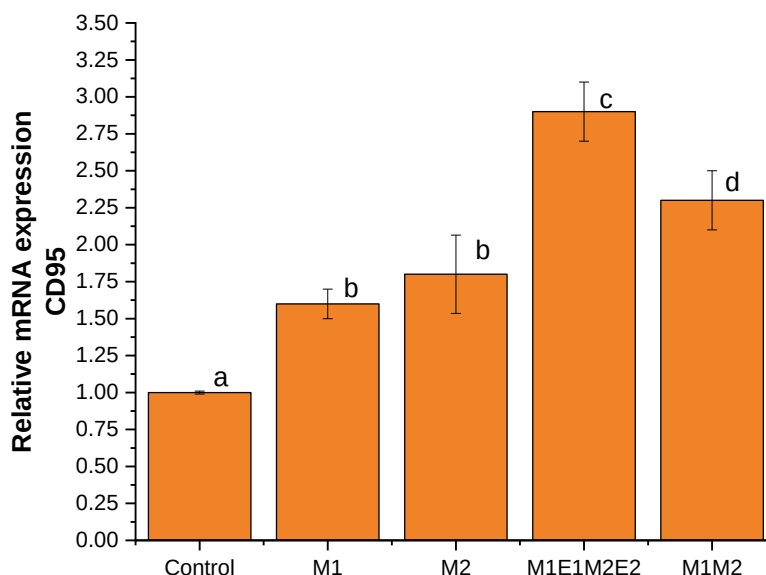


Figure 47. Relative CD95 mRNA expression in HeLa cells (passage number 5) treated with extracts and formulated ingredients. Data presented as mean $\pm$ standard deviation (n=3). Letters above bars indicate significant difference ($p < 0.05$), where different letters depict significant difference between average means. Same letters indicate that means are not significantly different. Control: untreated HeLa cells. M1: methanol extracts from *Sclerocarya birrea* fruit exocarp. M2: methanol extracts from *Terminalia prunoides* pods. M1M2: methanol extracts from *Sclerocarya birrea* fruit exocarp + methanol extracts from *Terminalia prunoides* pods (1:1). M1E1M2E2: ((methanol + ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp) + (methanol + ethyl acetate from *Terminalia prunoides* pods)).

CHAPTER 5: DISCUSSION

Ethnopharmacology has always been a part of human survival for a long time either as traditional medicine or through diet. Many of indigenous plants have therapeutic grade metabolites that are used to manage diseases. To understand the vast potential of medicinal plants, there is a need to study their phytochemistry and explore their metabolites in halting, disrupting, or stopping the pathophysiology of diseases progression. In this study, the antioxidant and anticancer properties of *Sclerocarya birrea* fruit exocarp, and *Terminalia prunoides* pods were studied.

Samples were collected, washed, dried, pound to powder, subjected to sequential extraction and computed for extractive yield. The selected solvents; hexane, chloroform, ethyl acetate and methanol, were of ascending polarity. Hexane is relatively nonpolar, chloroform has a low polar index, ethyl acetate has a moderately higher polarity and methanol is a high polar solvent (Al-Saeed *et al.*, 2016). Different solvents extracted different quantities of extracts, with low polar solvents having lowest yields and high polar solvents having much higher extractive yields. The results obtained from this study (Table 3 and Table 4) show that hexane had a very low percentage extractive yield of 3.6 % for *Sclerocarya birrea* fruit exocarp, and 2.9 % for *Terminalia prunoides* pods. Chloroform displayed the second lowest extractive yield, and ethyl acetate had the second highest extractive yield in both plant samples. Methanol had the highest extractive yields of 18.1% and 19.0%, for *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods respectively. Extractive yield increased with increasing polar index of solvents. This proportional relationship between solvent polarity and extractive yield has been observed by (Zullaikah *et al.*, 2019; Aruwa *et al.*, 2019; Simorangkir *et al.*, 2018). The efficiency of higher polar solvents may be due to their higher separation capacity. This may also be due to a higher levels, and solubility of high polar phytochemicals (Xu *et al.*, 2016; Xu *et al.*, 2017).

The consecutive extraction products were examined for the presence and intensity of saponins, tannins, flavonoids, phenols, terpenoids, cardiac glucosides and steroids. After the phytochemical screening of extracts from *Sclerocarya birrea* fruit exocarp (Table 5), hexane extracts only tested positive for saponins and steroids. *Sclerocarya birrea* fruit exocarp chloroform extracts tested negative for saponins, positive for tannins (in mild concentrations), flavonoids (low concentrations), phenols (low concentrations), terpenoids (low concentrations), cardiac glucoside (mild concentrations) and steroids (low concentrations). Ethyl acetate extracts showed availability of saponins (mild concentrations), tannins (low concentrations), flavonoids (mild concentrations), phenols (high concentrations), terpenoids (mild concentrations), cardiac glucoside (low concentrations) and steroids (low concentrations). *Sclerocarya birrea* fruit exocarp methanol extracts test positive for saponins (high concentrations), tannins (low concentrations), flavonoids (mild concentrations), phenols (high concentrations), terpenoids (high concentrations), cardiac glucoside (mild concentrations) and steroids (low concentrations). Saponin , tannins, flavonoids, phenols, terpenoids, cardiac glucoside and steroids have been reported in other parts of the *Sclerocarya birrea* plant such as the stem bark, roots and leaves (Virginie *et al.*, 2016; Russo *et al.*, 2018; Manzo *et al.*, 2018). The phytochemical screening of extracts from *Terminalia prunoides* pods (Table 6) revealed that hexane extracts tested negative for all the tested compounds. Chloroform extracts tested positive for tannins (low concentrations), flavonoids (low concentrations), phenols (low concentrations), cardiac glucoside (low concentrations), steroids (low concentrations), and tested negative for saponins and terpenoids. Ethyl acetate extracts tested positive for saponins (low concentrations), tannins (mild concentrations), flavonoids (mild concentrations), phenols (mild concentrations), terpenoids (mild concentrations), cardiac glucoside (low concentrations) and steroids (low concentrations). *Terminalia prunoides* pods methanol extracts tested positive for saponins (high concentrations), tannins (mild concentrations), flavonoids (high concentrations), phenols (high concentrations), terpenoids (mild concentrations), cardiac glucoside (low concentrations) and steroids (mild concentrations). Saponins, tannins, flavonoids, phenols, terpenoids, cardiac glucoside and steroids have been reported by (El-Rafie & Hammed, 2014; Cock 2015; Fahmy *et al.*,2015:

Beigi *et al.*, 2018) in other species of genus *Terminalia*. The presence phenols and flavonoids at mild and high concentrations, may be the reason behind the reported folk pharmacological properties of different parts of *Sclerocarya birrea* and *Terminalia prunoides* (Fahmy *et al.*, 2015; Russo *et al.*, 2018).

Total flavonoid content (TFC) and total phenolic content (TPC) of extracts from both plant species were quantified. Quercetin has been isolated and studied as a natural flavonoid (Lesjak *et al.*, 2018). Quercetin was used as a standard in estimating TFC of extracts from both plant species. All extracts from the 2 plant species (Figures 14 and 15), displayed different content of flavonoid. Hexane extracts from both plant species recorded the lowest TFC. Total flavonoid content of *Sclerocarya birrea* fruit exocarp (Figure 14) hexane extracts was 20.2 ± 5.3 mg QE/g. Results (Figure 15) showed hexane extracts TFC value of 4.34 ± 2.90 mg QE/g, suggesting that there were other non-polar flavonoids present in the pods of *Terminalia prunoides*. This has been observed in other studies (Hegazy & Ibrahim, 2012; Formisano *et al.*, 2014; Yakubu *et al.*, 2014), where low polar solvents recorded the lowest TFC values compared to high polar solvents. In comparison, methanol extracts possessed the highest TFC values in both plants with 55.25 ± 3.8 mg QE/g observed for *Sclerocarya birrea* fruit exocarp methanol extracts, and 67.78 ± 10.4 mg QE/g observed for *Terminalia prunoides* pods methanol extracts. Ethyl acetate extracts had the second highest TFC for both *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods followed by chloroform extracts.

Gallic acid is a well studied and known phenol that has been used in many antioxidants and phytochemistry studies (Gopalasatheeskumar *et al.*, 2019; Phuyal *et al.*, 2020). For this study, gallic acid was used as a standard for TPC and subsequent antioxidant assays. Figures 17 and 18 show that all extracts recorded a significant content of phenols except hexane extracts from *Terminalia prunoides* pods. Methanol extracts recorded the highest TPC in extracts of both plants, with 105 ± 1.4 mg GAE/g for *Sclerocarya birrea* fruit exocarp extracts, and 113.25 ± 7.6

mg GAE/g for *Terminalia prunoides* pods extracts. These results are consistent with other findings (Russo *et al.*, 2018; Manzo *et al.*, 2018; Formisano *et al.*, 2014; Yakubu *et al.*, 2014; Akonto *et al.*, 2020), in which higher polar solvents recorded the highest TPC in *Sclerocarya birrea* and other species of genus *Terminalia*. Methanol extracts were followed by ethyl acetate extracts, chloroform extracts, and hexane extracts in TPC in descending order.

Sclerocarya birrea has been used traditionally as medicine for influenza, malaria, dysentery, sore eyes, and toothache (Maroyi, 2016; Mocheki *et al.*, 2018). *Terminalia prunoides* has been used traditionally in treating abdominal pains (Cock, 2015). The folk medicinal uses of these two plants may be due to presence of saponins, tannins, flavonoids, phenols, terpenoids, cardiac glucoside and steroids, with a high content of phenols and flavonoids. Many studies have alluded that phenols and flavonoids are amongst many of plant chemical entities with electron and hydrogen rich chemical structures (Kumar & Goel, 2016; Minatel *et al.*, 2017). It is because of these configurations that they are commonly referred to as antioxidants because they can reduce oxidants by electron and/or hydrogen transfer (Manna & Maiti, 2016; Wang & Bi, 2018; Shen *et al.*, 2019).

A lot of research points out that transition of healthy cells to cancerous cells is a result of oxidative damage by amongst others reactive oxygen species (ROS) (Moloney & Cotter, 2018). ROS are neutralised by antioxidants which are normally produced by the body. Oxidative stress is a condition where antioxidants are at a concentration that does not effectively neutralise ROS (Sies, 2020). The complete action and efficiency of endogenous antioxidants in radical scavenging pathways is co-dependent on exogenous antioxidants (Aguilar *et al.*, 2016). This is one of many reasons why findings in medical research suggest that people supplement with natural antioxidants from vegetables and fruits (Liu, 2013). In this study, extracts were analysed for their antioxidant properties compared to gallic acid. Antioxidant assays determine the ability

and the degree to which samples can reduce laboratory prepared oxidants such as ABTS, DPPH, phosphomolybdate and potassium ferricyanide.

Table 7 indicates methanol extracts from *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods, showing high radical scavenging activity. Methanol extracts from *Sclerocarya birrea* fruit exocarp recorded IC₅₀ values of 0.12 mg/mL, 0.09 mg/mL, and 0.25 mg/mL for DPPH, FRAP and ABTS assays respectively. These observations are in accordance with those of Da costa Mousinoh *et al.* (2019), where methanol extracts of *Sclerocarya birrea* roots recorded IC₅₀ values of 9.8 µg/mL and 2.2 µg/mL for DPPH and ABTS assays respectively. Results are also parallel with Niang *et al.* (2021)'s findings, where *Sclerocarya birrea* bark methanol extracts showed antioxidants activity with recorded IC₅₀ values of 0.156 mg/mL, 0.248 mg/mL, and 0.213 mg/mL for DPPH, ABTS, and FRAP respectively. Methanol extracts of *Terminalia prunoides* pods recorded IC₅₀ values of 0.06 mg/mL, 0.07 mg/mL, and 0.24 mg/mL for DPPH, FRAP and ABTS assays respectively. These have also been observed by Das *et al.* (2020), Sobeh *et al.* (2019), and Arya *et al.*, (2012) where methanol extracts of *Terminalia chebula*, *Terminalia bellerica*, and *Terminalia arjuna* recorded IC₅₀ values of 11.6 µg/mL, 16.4 µg/mL, 21.8 µg/mL for DPPH, and 241 µg/mL, 256 µg/mL, and 232 µg/mL for FRAP respectively. Hexane and chloroform extracts of both plant species required concentrations greater than 1 (>1) mg/mL to inhibit 50 % of DPPH, FRAP and ABTS. The high radical scavenging activity of methanol extracts may be due to high total flavonoid and phenolic contents of these extracts. Phytochemicals identified in this study such as phenols and flavonoids have been studied and reported to have hydrogen and electron rich chemical structures that give them high radical scavenging capacity (Santo-Sánchez *et al.*, 2019; Flieger *et al.*, 2021).

Additionally, extracts were investigated for their capacity to prevent cell death induced by H₂O₂. In general, extracts demonstrated a dose dependent activity. Methanol and ethyl acetate extracts displayed more protection when compared to chloroform and hexane extracts. At a concentration

of 400 µg/mL, methanol extracts showed the highest protective activity of 92.2 ± 0.2 %, and 95.9 ± 2.3 % viable cells for *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods respectively (Figures 28 and 29). Effective cytoprotective effects by *Sclerocarya birrea* extracts has also been observed in other members of the family ANACARDIACEAE as reported by Noguera-Artiaga & García-Romo (2019) and, Xanthis *et al.* (2021), where *Hydrosos pistachio* and *Pistacia lentiscus* effectively protected retinal pigment epithelia (RPE) cells, and human keratinocyte cells (HaCaT) cells from H₂O₂ induced cell death. Additionally, it was also found in investigations by Jamarkattel-Pandit *et al.* (2012) and Gaire & Kim (2014), that *Terminalia chebula* and *Fructus chebulae* (COMBRETACEAE family) significantly protected PC12 rat cell lines from H₂O₂ induced cell death.

Sclerocarya birrea fruit exocarp and *Terminalia prunoides* pods extracts display a possible ability to shield HeLa cells from H₂O₂ induced cell death through the upregulation of the activity of both the endogenous enzymatic antioxidants (Superoxide dismutase and Catalase) and nonenzymatic antioxidant (Glutathione). Catalase (CAT) detoxifies H₂O₂ into diatomic oxygen and water (Kaushal *et al.*, 2018). Other H₂O₂ reactions proceed backward, producing superoxide radical, in which SOD is required to neutralise (Hayyan *et al.*, 2016). Other H₂O₂ reactions results in the production of a hydroxyl radical which is neutralised to water by GSH (Kabel, 2014). The activities of SOD were significantly upregulated in cells treated with chloroform, ethyl acetate and methanol extracts from *Sclerocarya birrea* fruit exocarp, as well as in cells treated with all extracts from *Terminalia prunoides* pods as shown in figures 30 and 31. Activities of CAT were significantly upregulated in cells treated with methanol and ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp (Figure 32). All extracts of *Terminalia prunoides* pods significantly increased the activities of CAT (Figure 33). All extracts except hexane extracts from *Sclerocarya birrea* fruit exocarp significantly upregulated GSH activity as shown in figures 34 and 35. The increase in SOD, CAT and GSH activity after treatment by plant extracts, was mentioned by Nguegium *et al.* (2015) and Abubakar *et al.* (2021), where stem bark

extracts of *Sclerocarya birrea* elevated activities of SOD, CAT and GSH. This is also in keeping with results obtained by Kanthe *et al.* (2021), Pandya *et al.* (2013) and Gupta *et al.* (2021), where *Terminalia arjuna*, *Terminalia catappa*, and *Terminalia bellirica* elevated activities of SOD, CAT and GSH. In absence of hydrogen/electron donors, peroxidise radicals can act as oxidising or reducing agents, they have been reported to inhibit SOD, CAT, and GSH activities by oxidizing and binding to amino acids residues in the spatial proximity to the active sites in reactions termed peroxide-peroxide inhibition (García-Molina *et al.*, 2005; Gottfredsen *et al.*, 2013; Armogida *et al.*, 2012; Hajizadeh *et al.*, 2021). The presence of saponins, tannins, flavonoids, phenols, terpenoids, cardiac glucoside, steroids may have upregulated the activities of SOD, CAT and GSH by reducing hydrogen peroxide, by so doing, setting the SOD, CAT and GSH free from hydrogen peroxide.

Sclerocarya birrea fruit exocarp and *Terminalia prunoides* pods extracts were also evaluated for their antiproliferative activity on HeLa cells. Their antiproliferative effects were compared to that of the anticancer drug cisplatin (control). The comparison was based on the minimum concentration that inhibits 50% (IC₅₀) of a HeLa cell population. Figures 38 and 40 show that methanol extracts were the most effective with IC₅₀ values of 75 µg/mL and 190 µg/mL for *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods respectively. These results suggest that methanol and ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp and *Terminalia prunoides* pods have high antiproliferative effects compared to chloroform and hexane extracts in HeLa cells. The antiproliferative effects of *Sclerocarya birrea* fruit exocarp extracts are supported by published works of Tanih & Ndip (2013), where *Sclerocarya birrea* stem bark extracts recorded IC₅₀ values of 87.6 µg/mL, 686.65 µg/mL and 326.01 µg/mL on MCF-7 cells, HT-29, and HeLa cells respectively. Gospodinova *et al.* (2017) likewise stated that leaf extracts from *Cotinus coggryrian scop* (member of ANACARDIACEAE family) recorded IC₅₀ values of 30.8 µg/mL, 55 µg/mL, 61.1 µg/mL, 122.7 µg/mL for A2780 cell, MCF7 cells, T47D cells, and HeLa cells respectively. The antiproliferative effects of *Terminalia prunoides*

Pods extracts are similar to findings by Gu *et al.* (2018), where *Terminalia sericea* recorded IC₅₀ values of 120 µg/mL and 528 µg/mL for HeLa cells and Caco2 cells respectively. Shalom *et al.* (2018) indicated that, leaf extracts of *Terminalia ferdinandiana kino* recorded IC₅₀ values 259 µg/mL and 275 µg/mL for HeLa cells and Caco-2 cells respectively. The antiproliferative effects of these two plants may be linked to the availability of saponins, tannins, flavonoids, phenols, terpenoids, cardiac glucoside, and steroids.

Three (3) anticancer ingredients were formulated from the most effective and second most effective antiproliferative extracts of the 2 plant species (based on IC₅₀ values). The anticancer ingredients were evaluated for their synergistic antiproliferative effects on HeLa cells. Figure 42 shows that the methanol + ethyl acetate extracts from *Sclerocarya birrea* fruit exocarp) + (methanol + ethyl acetate from *Terminalia prunoides* pods (M1E1M2E2) ingredient was the most potent HeLa cell proliferation inhibitor with an IC₅₀ value of 77 µg/mL, followed by M1M2 (83 µg/mL) and E1E2 (130 µg/mL). These results show that the antiproliferative effects of the extracts are more effective when they are combined together than when they are applied individually. This is in consistence with studies by Kumar *et al.* (2016), where Curcumin (compound from *Curcuma longa*) and Ellagic acid (a polyphenol commonly found in fruits) recorded IC₅₀ values of 16.52 µM, 19.47 µM individually, and IC₅₀ values of 10 µM synergistically (Curcumin + Ellagic acid) on HeLa cells. Saeedifar *et al.* (2021) reported that Ginger ethanolic extracts (GEE) and Licorice methanol extracts (LME) recorded IC₅₀ values of 170 µg/mL, and 300 µg/mL respectively, and recorded 155 µg/mL for the combined 2 (GEE+LME) on CT26 mouse colon carcinoma cells. This may be due to the synergistic effects of saponins, tannins, flavonoids, phenols, terpenoids, cardiac glucoside, and steroids, as they have been reported to downregulate transcriptional and translational expression oncogenes such as HPV E6, and upregulated tumour suppressor genes such as p21 and Bax/ Bcl 2 expressions (Kumar *et al.*, 2016; Saeedifar *et al.*, 2021).

The underlying bio-mechanism of antiproliferative effects on HeLa cells by extracts and formulated ingredients may be through changes in the regulation of oncogenes and tumour suppressor gene expression. Cancer cells thrive on the overexpression of oncogenes. All treatments may have inhibited the growth and survival of HeLa cells by significantly reducing the expression of EGFR gene (Figure 43). These outcomes concur with studies published by Istiqomah *et al.* (2020) and Qurrohman *et al.* (2020) where 5000 µg/mL of polyisoprene extracts from *Nypa fructicans* leaves, and 10 µM of polyisoprene from *Avicennia marina* leaves extracts exhibited anticancer activities through downregulating the expression of EGFR genes on WiDr cells. The significant downregulation of EGFR suggests that the transcription and translation of the EGF receptor may have been significantly reduced and therefore significantly reducing the concentration of the EGF receptor on the surface of the cell as indicated by Dai *et al.* (2011) and Chadalapaka *et al.* (2010). This in turn may have significantly downregulated the EGF receptor mediated pathway that results in the formation of the CREB transcription factor (Linder *et al.*, 2018; Tao *et al.*, 2020). The downregulation of the CREB TF pathway may have consequently downregulated the expression of other cell proliferation genes, resulting in the growth inhibition of HeLa cells (Steven & Seliger, 2016; Yang *et al.*, 2012).

All treatments may have also inhibited HeLa cells proliferation through significantly downregulating ($p < 0.05$) the transcription of VEGF (Figure 44). This is in accordance with Jahanban- Esfahlan *et al.* (2017), where 100 µg/mL of *Nelissa offinalis* leaves extracts downregulated VEGF expression on A549, MCF-7 and PC3 cell. Suboj *et al.* (2012) reported that 10, 20 and 40 µM of Aloe emodin inhibited the proliferation of cancer cells through the downregulation of the VEGF gene in WiDr cells. These findings imply that the transcription and translation of an extracellular VEGF signalling molecules may have been decreased (Cascio *et al.*, 2010; Printer *et al.*, 2013), possibly reducing the binding to, and the activation of the VEGF receptor, and subsequently inhibiting the VEGF dependant cell proliferation and survival pathways. The antiproliferative properties of treatments may also have been through the

downregulation of the MACC1 gene expression (Figure 45). Güllü *et al.* (2020) revealed that saffron crudes: 5mM safranal, 5mM crocin and 5 μ M crocetin downregulated MACC1 expression in CRC cells. Juneja *et al.* (2017) reported that 2.5 μ M rottlerin (a polyphenol) inhibited CRC cells by downregulating MACC1 expression. The significant transcriptional downregulation of MACC1 in this study may have downregulated the expression of the MACC protein 1 (Che *et al.*, 2015; Tong *et al.*, 2019). The MACC protein 1 is a regulator (TF) of the hepatocyte growth factor (HGF) pathway, which drives cell proliferation, motility, morphogenesis and angiogenesis (Ma *et al.*, 2013). Downregulation of MACC protein 1 may have downregulated the HGF/MET mediated pathways for cell proliferation and survival. Growth inhibition of HeLa cells may also have been due to the downregulation of the CYFRA 21-1 gene (Figure 46). Suzuki *et al.* (2000) reported that radiation therapy resulted in the downregulation of CYFRA 21-1 levels in cervical cancer patients. Yuan *et al.* (1997) reported that monoclonal antibody Mab Cx-99 induced apoptosis on HeLa cells by downregulating CYFRA 21-1 expression. The downregulation of CYFRA 21-1 gene expression may have reduced the translation of the CYFRA 21-1 protein fragment (Dohmoto *et al.*, 2000; Suzuki *et al.*, 2000; Wu *et al.*, 2014), leading to the downregulation of the CYFRA 21-1 mediated proliferation.

The treatments may have induced cell death by upregulating the expression of CD95 gene (Figure 47). These results are parallel to findings by Li *et al.* (2015), where 40 μ g/mL and 60 μ g/mL of β - elemene extracted from *Curcuma wenyujin* induced apoptosis in U87 Glioma cells by upregulating CD95 mRNA expression. Lin *et al.* (2012) reported that 10 μ M (-)- Epigallocatechin Gallate extracted from *Camellia sinensis* induced apoptosis on HNSCC cell lines through the upregulation of CD95 gene expression. The upregulation of transcriptional CD95 expression may have also upregulated the protein expression of the CD95 receptor (Wu *et al.*, 2011; Wu *et al.*, 2009; Ma *et al.*, 2020), increasing the concentration of the receptors in the cell membrane. This may have upregulated the CD95 receptor mediated apoptosis (El-Missiry *et al.*, 2014; Stolpman *et al.*, 2012).

CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In sum up, findings of this study have illustrated that extracts of *Sclerocarya birrea* fruits exocarp and *Terminalia prunoides* pods have antioxidant and anticancer activities. Extracts of these two plants were found to contain phenolic and flavonoid compounds. Extracts demonstrated antioxidant activity through scavenging and reducing radicals. Extracts also demonstrated protective effects on HeLa cells against hydrogen peroxide induced cell death. Extracts upregulated the activity of endogenous antioxidants. Individual extracts and formulated ingredients demonstrated antiproliferative effects by inducing the death on HeLa cells. Methanol extracts of both plants and formulated anticancer ingredients (M1M2 and M1E1M2E2) demonstrated anticancer activity by reducing the expressions of EGFR, VEGF, MACC1 and CYFRA 21-1 genes in HeLa cells. Additionally, methanol extracts of both plants and formulated anticancer ingredients (M1M2 and M1E1M2E2) induced cell death on HeLa cells by upregulating the expression of the tumour suppressor gene CD95. Formulated ingredients proved to be more effective than individual extracts.

6.2 Study limitations and recommendations

This study used crude extracts. Therefore, anticancer ingredients were formulated from crude extracts. It is recommended that extracts be isolated and studied further for their antioxidants, protective effects, and anticancer activities.

This study was focussed on the transcriptional expression of genes only, it is recommended that the effects of extracts be studied on transcriptional and translational expression of receptors and their ligands, and also the activities of other proteins involved in proliferation and apoptosis pathways be studied.

Other future work recommendations

- Try extracts on other cancer cell lines.
- Determine gene expression of cancer markers at the protein level (Western Blot)
- Try cytotoxicity studies at concentrations above 400 µg/mL.
- Try other parts of the same plant species.
- Use of LC-MS to study the metabolome of the 2 plants, predict antioxidant, cytoprotective, and anticancer effects of the plants.

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APPENDIX



APPENDIX. Three kilograms of *Sclerocarya birrea* fruit exocarp (A1) and *Terminalia prunoides* pods (A2) sample after blending. Maceration in round bottom flasks, and filtration of *Sclerocarya birrea* fruit exocarp (C) and *Terminalia prunoides* pods (B) extracts. Dried extracts.

