EVALUATION OF ANTIOXIDANT AND CYTOTOXIC PROPERTIES OF *CITRULLUS LANATUS* EXTRACTS

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DECLARATION

I, OFOSU-AIKINS PEARL AFRAM, of the Department of Chemical Pathology, School of Biomedical and Allied Health Sciences (SBAHS) of the University of Ghana (UG), do hereby declare that this research study is my own work, with the exception of references that have been acknowledged. I carried out this work at the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research (NMIMR) under the supervision of Prof. Henry Asare-Anane of the Department of Chemical Pathology (UG) and Prof. (Mrs.) Regina Appiah-Opong of the Department of Clinical Pathology, NMIMR, UG.

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DEDICATION

This work is firstly dedicated to God Almighty for His grace and guidance throughout my MPhil studies. Additionally, I dedicate this work to my mother, Doris Afram, and my late dad, Emman Kwesi Ofosu-Aikins whose prayers and support sustained me throughout my course of study in this noble University.

ACKNOWLEDGEMENT

I give thanks to God Almighty for His mercies and protection that sustained me throughout my studies. My profound gratitude goes to my supervisors, Prof. Henry Asare-Anane and Prof. (Mrs.) Regina Appiah-Opong for their guidance and mentorship throughout my MPhil. studies. Special thanks to my coach, Ms. Eunice Dotse for her assistance. I also thank the staff of Department of Chemical Pathology, NMIMR for the support they offered during my research work. I am sincerely grateful.

ABBREVIATIONS

A

ALL: Acute lymphocytic leukaemia

AML: Acute myeloid leukaemia

Aq MES: Aqueous mesocarp extract

Aq SD: Aqueous seed extract

Aq SE MES: Aqueous mesocarp extract (from sequential extraction)

Aq SE SD: Aqueous seed extract (from sequential extraction)

ATP: Adenosine triphosphate

B

BHT: Butylated hydroxytoluene

C

CHLO MES: Chloroform mesocarp extract

CHLO SD: Chloroform seed extract

CLL: Chronic lymphoid leukaemia

CML: Chronic myeloid leukaemia

D

dH_2O: Distilled water

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPPH: 2, 2- diphenyl-1-picryl hydrazyl

E

EC_{50}: Effective concentration at 50%

SD: Standard deviation

EtAc MES: Ethylacetate mesocarp extract

EtAc SD: Ethylacetate seed extract

EtOH MES: Ethanolic mesocarp extract

EtOH SD: Ethanolic seed extract

F

FBS: Fetal Bovine Serum

G

GAE: Gallic acid equivalent

GSH: Glutathione

GSSG: Glutathione disulfide

GST: Glutathione S-Transferase

H

HEX MES: Hexane mesocarp extract

HEX SD: Hexane seed extract

I

IC_{50}: Inhibitory concentration at 50%

M

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

N

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor – kappa B

NMIMR: Noguchi Memorial Institute for Medical Research

NSAIDs: Non-Steroidal Anti-Inflammatory Drugs

O

OD: Optical density

P

PBS: Phosphate buffered saline

pH: Power of hydrogen

PSG: Penicillin Streptomycin L-glutamine

R

RBC: Red blood cell

RNA: Ribonucleic acid

ROS: Reactive oxygen species

RPMI: Rose Park Memorial Institute

S

SI: Selectivity Index

SOD: Superoxide dismutase

STAT3: Signal transducer and activator of transcription 3

T

TFC: Total flavonoid content

TPC: Total phenolic content

U

UV: Ultraviolet

W

WBC: White blood cell

W.H.O: World Health Organization

ABSTRACT

INTRODUCTION

Citrullus lanatus (watermelon) is well noted for its nutritional and therapeutic uses. The seeds and mesocarp of the fruit, which were of interest in this study are rich sources of phytochemicals including flavonoids and phenols. These bestow upon the fruit its medicinal properties such as antioxidant, anti-inflammatory, analgesic and anti-hypertensive activities. Cancer has been a public health menace for long and it currently stands as the world's second killer disease. This owes to the fact that generally, lifestyle modifications keep occurring in the developing world, and the modes of treatment; chemotherapy, radiation and biological therapy are costly. Patients also tend to be resistant to therapy, and exhibit antagonistic side effects. Alternative sources of cancer therapy, particularly from plant sources, which transcend the accompanying problems of these current treatment methods thus are being researched into rigorously.

This project therefore was aimed at evaluating antioxidant and cytotoxic activities of aqueous and organic *Citrullus lanatus* extracts.

METHODS

The study was comparative and employed the use of purposive sampling in the selection of the *C. lanatus* fruits. Aqueous extracts of *C. lanatus* mesocarp and seeds were prepared from distilled water by heating, filtration and centrifugation. The organic extracts (hexane, ethanol, ethyl acetate and chloroform) however were prepared by cold maceration and rotary evaporation. Antioxidant capacity of the extracts was assessed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteau and total flavonoids assays. Cytotoxic activity of extracts on HL-60, Jurkat and Chang liver cell lines was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)- 2, 5-diphenyltetrazolium bromide (MTT) assay.

RESULTS

The outcome of the project showed that the ethanolic seed and aqueous seed extracts have good total antioxidant activity with EC_{50} values of 0.57 mg/ml and 0.75 mg/ml respectively. Phenols were established to be present in all the extracts except the ethanolic mesocarp, with the ethanolic seed having the highest total phenolic content of 3943.23 mg gallic acid equivalent /100g of *C.*

lanatus extract. Only five of the extracts had significant total flavonoid content, with the hexane seed having the highest of 1013.82 mg quercetin equivalent /100g of *C. lanatus* extract. For the cytotoxic assay, ethanolic seed extract exhibited the highest cytotoxic activity against HL-60 cells, whilst hexane mesocarp extract showed the highest anticancer activity against Jurkat cells with IC_{50} values of 65.39 µg/ml and 165.33 µg/ml respectively. Also, the ethanolic seed extract proved to be the most promising anticancer agent with a selectivity index of 12.6 for HL-60 cells.

CONCLUSION

The study demonstrated that five of the *C. lanatus* extracts possess significant antioxidant activity and most of the extracts exhibited significant anticancer activity. The organic and seed extracts generally exhibited greater activities than their aqueous and mesocarp counterparts, respectively.

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CHAPTER ONE

1.0 INTRODUCTION

Citrullus lanatus is popularly grown in the tropics and some parts of Asia. The fruit is highly nutritious and is regularly consumed as a snack, smoothie for breakfast, or as part of diet plans. According to the United States Food and Drug Administration (2014), a 280-gram sliced piece of *C. lanatus* fruit contains the following nutrients: sugars (20 g), fibre (1 g), amino acids (1 g), potassium (270 mg), fat and cholesterol (0 g).

In addition, *C. lanatus* fruit contains up to 92 % water, and small amounts of minerals such as magnesium, copper, zinc and selenium. Therefore, consumption of the fruit on sunny days especially may help in the prevention of dehydration. The American National Watermelon Promotion Board in 2013 stated that the fruit contains more lycopene than most fruits or vegetables. This is significant as lycopene has been reported to have a key role in managing diseases such as cardiovascular pathologies and cancer (Senthil *et al*., 2004; Charoensiri *et al*., 2009). Gloria *et al*. (2014) reported that lycopene and other carotenoids inhibited human breast cancer cell line growth by halting the cell cycle in different phases, and increasing apoptosis.

Free radicals such as Reactive Oxygen Species (ROS) are generated in the body due to metabolism, and cause damage to biomolecules such as DNA and lipids by binding to them and interfering with normal cellular processes. Reactive Oxygen Species are detoxified in the body by free radical scavengers or antioxidants such as glutathione and Vitamins C and E, to prevent unwanted occurrences facilitated by the ROS. These undesirable effects of ROS include mutations, which eventually lead to cancer and other degenerative diseases. However, cells and their constituents are protected from free radicals by antioxidants to prevent oxidative stress (Riso *et al.*, 2005). Some of these antioxidants are present in foods and they usually come from plants in the form of phytochemicals such as carotenoids and flavonoids.

Cancer is a group of diseases characterized by unregulated cell growth. According to Cancer Research UK, in the year 2012, 14.1 million new cases of cancer occurred whilst an estimated 8.2 million people died of cancer worldwide (Ferlay *et al*., 2015). Narrowing the demographic region down to Ghana, it was reported in 2012 that the estimated cancer incidence Age Adjusted Standardised Rate for males was 10.9/100,000 and 22.4/100,000 for females (Laryea *et al.*, 2014). There are over 100 cancers known to affect humans, one of which is leukemia. Between 70% and

90% of reported cancer cases tend to be as a result of environmental risk factors such as exposure to radiation, prior chemotherapy experience and tobacco (Wu *et al*., 2016).

A majority of these factors are lifestyle choices that can be regulated, hence cancer is generally a preventable disease (Danaei *et al*., 2005).

Leukemia is a form of cancer that affects the blood. Like any other cancer, it is a public health menace. In 2000, approximately 256,000 children and adults around the world developed some form of leukemia, and 209,000 were killed by it (Mathers *et al*., 2001). In 2012, 352,000 men and women were diagnosed of leukemia, and this number is expected to rise to approximately 600,000 by 2035 (Ferlay *et al*., 2015).

Also, hematopoietic cancer types, which includes leukemia caused 15.57% and 14.69% of male and female cancer deaths out of 2,008 and 1,651 cases respectively from 1991-2000, in Ghana (Wiredu and Armah, 2006). Hence, it has become prudent that prevention and treatment methods are enhanced to curb the rate of incidence.

1.1 PROBLEM STATEMENT

Reactive Oxygen Species (ROS) are known to cause oxidative stress in cells. The generation of oxidative stress has been observed in the progression of cancer (Punnonen *et al*., 1993). In this process, ROS damage lipids, proteins and DNA, therefore inducing unwanted genetic events in some cells leading to unregulated and abnormal growth of cells, and finally cancer (Atawodi, 2005).

Current cancer treatment methods include surgery, chemotherapy, radiation and biological therapy. However, cancer cases are still burdensome to manage because of high cost of these treatment methods, resistance to therapy, and adverse side effects (Mukherjee *et al*., 2001). According to the World Cancer Report by the World Health Organization (W.H.O) in 2014, the financial expenses of cancer were valued at $1.16 trillion per annum as of 2010.

Mortality rate is also high due to late reporting, diagnostic and treatment problems (Segbefia *et al*., 2013). In addition, the occurrence rate of cancer is significantly on the increase as major lifestyle changes arise in the developing world (Jemal *et al*., 2011).

It is thus important to research into more efficacious therapeutic agents which are not associated with problems of current treatment methods.

Discovering novel anticancer agents, and therapy using antioxidants or free-radical scavengers that have the likelihood to prevent, improve or cure malignancy, offer interesting challenges to scientists (Acheampong *et al*., 2015). Antioxidants are known to reduce the risk of cancer development by mopping up ROS hence preventing oxidative stress, and these antioxidants can be found abundantly in some natural plant extracts (Appiah-Opong *et al*., 2016). Thus, more efficacious and naturally occurring antioxidant sources need to be researched into, as these are less expensive and can be consumed on a regular basis to serve as prevention and management of oxidative stress and its related diseases such as cancer.

These research endeavours create room for publications to be done as there is scarce availability of supporting literature on the effect of *C. lanatus* extracts on human leukemia cell lines.

1.2 JUSTIFICATION

Citrullus lanatus extracts are a rich source of natural chemicals that have disease preventive and curative properties (Melo *et al*., 2006; Yuan *et al*., 2006; Oseni and Okoye, 2013). The lycopene content of *C. lanatus* suggests possible cytotoxic (antiproliferative) activity as lycopene has been associated with decreased risk of cancer (Rhodes and Zhang, 1999; Venket Rao and Agarwal, 2000). Also, the significant cytotoxicity of compounds isolated from *C. lanatus* chloroform extract on brine shrimps suggests the presence of antitumor compounds (Hassan *et al*., 2015). Thus, *C. lanatus* could be a source of interesting anticancer drug candidate(s). Evaluation of antioxidant activity of anticancer agents is also necessary in determining the mechanism of action of these agents due to the important role of mopping up of free radicals (which could cause cancer) by antioxidants. Therefore, finding antioxidants that can serve as anticancer agents is of interest in modern science (Acheampong *et al*., 2015).

In addition, *C. lanatus* aqueous extract unlike current anticancer agents are affordable and have been reported to have little or no toxicity even at extremely high doses: acute toxicity studies did not show any toxic symptoms or death in any of the mice used up to the doses of 3000 mg/kg body weight (Kumari *et al*., 2013).

1.3 HYPOTHESIS

Citrullus lanatus extracts do not possess significant antioxidant and anti-leukemic activities.

1.4 AIM

The goal of this study was to evaluate the antioxidant and anti-leukemic activities of aqueous and organic *Citrullus lanatus* extracts.

1.5 OBJECTIVES OF STUDY

- To determine antioxidant activity of the extracts using the free radical scavenging (DPPH) assay
- To quantify the total phenolic and flavonoid contents of *C. lanatus* extracts using the Folin-Ciocalteau and Total flavonoids content assays
- To investigate the cytotoxic property of aqueous and organic fractions of *C. lanatus* using the leukemia cell lines HL-60 and Jurkat, and Chang liver cells via the MTT assay

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BOTANY OF *CITRULLUS LANATUS*

2.1.1 Origin and distribution of *Citrullus lanatus*

The *C. lanatus* plant is an annual, dicotyledonous and flowering plant and belongs to the family *Cucurbitaceae* (Fig. 1a). The fruit, which is a pepo, is usually consumed as a snack, smoothie for breakfast, or as part of diet plans (Fig. 1b). Also, they are typically round, oblong or elongated with a size normally ranging from 1.5 to 15 kg (Kyriacou *et al*., 2018).

Citrullus lanatus fruits were originally found in North Eastern Africa as a wild plant approximately fifty centuries ago (Renner, 2017). They are thought to be first cultivated in Egypt as there are illustrations of *C. lanatus* in Egyptian hieroglyphics on walls, and Pharoahs were buried with theses melons to serve as nourishment in the afterlife (Zohary and Hopf, 2000). The spread of *C. lanatus* to other parts of the world by missionaries and African slaves however occurred predominantly from the 7th to 17th century (Maynard and Maynard, 2012). According to the Food and Agriculture Organization of the United Nations, China is presently the world's largest *C. lanatus* producer. Other large scale producers of the fruit include Russia, United States, Egypt, Turkey and Brazil. Some common names of *C. lanatus* are *Xigua* (Mandarin), *Mtango* (Swahili), *Arbuz* (Russian) and *Pastèque* (French).

(a)  (b) 

Figure 2.1.1: (a) *Citrullus lanatus* plant and (b) *Citrullus lanatus* fruit.
Source: Google Images, 2008

2.1.2 Varieties of *C. lanatus*

Citrullus lanatus has a number of cultivar groups, but the three main ones are the *Vulgaris*, *Citroides* and *Lanatus*. According to the Ministry of Food and Agriculture, the most suitable varieties for cultivation in Ghana are Florida Giant, Black Diamond, Sugar Baby and Charleston Gray. Worldwide, varieties cultivated include the Carolina Cross, Densuke, Moon and Stars, and Orangeglo.

With the advancement of science, some *C. lanatus* varieties such as the Crimson sweet are no longer cultivated on a large scale. However, their heredity has been adopted to produce hybrids with better quality, yield and appearance (Maynard and Maynard, 2012). Seedless triploid *C. lanatus* fruits which are costly and produced genetically by crossing a diploid parent with a tetraploid parent have been introduced to the market in advanced countries (Maynard and Maynard, 2012).

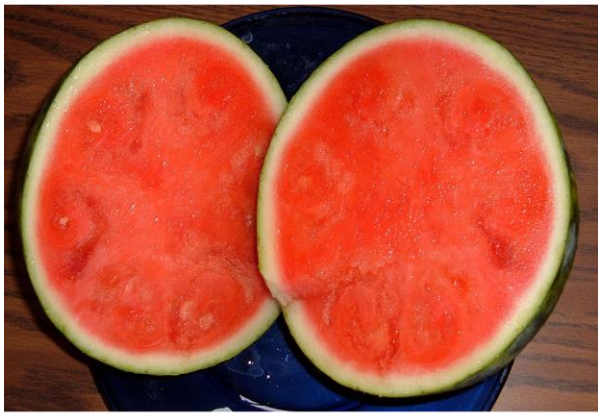

Figure 2.1.2a: Seedless variety of *C. lanatus* fruit (extazy) split into two

This variety is spherical and has a cycle of 80 - 85 days. It also has an ex cellent post harvest storage life due to the lack of seeds.

(Google Images, 2005)

Figure 2.1.2b: Orangeglo variety of *C. lanatus* fruit

This variety is noted for its extremely sweet orange pulp and oblong shape. In its cultivation, it takes about 90 -100 days after planting to harvest fruits.

(Google Images, 2010)

Figure 2.1.2c: Diana variety of *C. lanatus* fruit

This variety is noted for its beautiful yellow rind. It is also oblong in shape and has red and juicy flesh.

(Google Images, 2007)

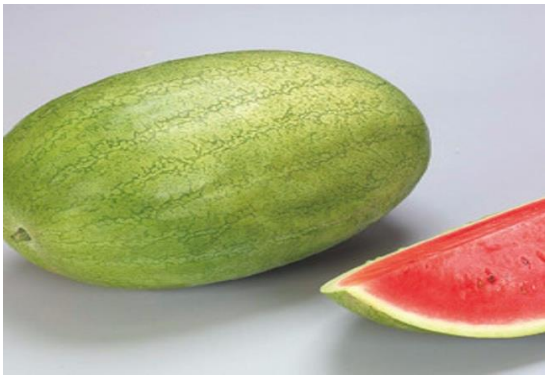

Figure 2.1.2d: Charleston Gray variety of *C. lanatus* fruit

This is one of the common varieties grown in Ghana. It has a greyish green rind with red mesocarp.

(Google images, 2018)

2.1.3 Ecology and climate

Citrullus lanatus is tailored to grow in tropical, and tropical-like areas. Usually, plantations of *C. lanatus* are situated in regions with temperature of 25 ^{o}C or greater. Heavy rains and frost do not favour cultivation as these make the leaves susceptible to diseases and affect fruit quality respectively. Ideally, *C. lanatus* seeds should be planted in areas with soils that have good water retention ability, such as sandy loam, and a slightly acidic pH. The presence of insects for example bees, is also necessary as these serve as pollination agents.

2.1.4 Nutritional uses of *C. lanatus*

Citrullus lanatus fruits are usually consumed in slices, or mixed with other fruits to make fruit salad and juice. It can also undergo fermentation with other fruits using the bacterium *Saccharomyces cerevisiae* to produce wine (Ogodo *et al*., 2015).

Due to the massive water and significant electrolyte content of the fruit, it is largely consumed during hot weather conditions such as summer and heat waves to quench thirst and provide relief for dehydration. The seeds taste like nuts and can be roasted and eaten raw as a snack, or grounded into flour. The rind, which is the hard exterior of the fruit is used in some countries as a vegetable for making stews, and pickles (Todd, 2008; Bryant, 2009).

2.1.5 Cosmetic uses of *C. lanatus*

Oil extracted from the seeds is known to be of good cosmetic value owing mainly to its composition. The oil has a high unsaturated fatty acid content, with linoleic (63%) and oleic (15%) as the main acids, and stearic (10%) and palmitic (11%) as the remaining acids (El-Adawy and Taha, 2001). *Citrullus lanatus* seed oil is used for its anti-aging, skin exfoliating, anti-hyperpigmentation, moisturizing and hair growth properties.

2.1.6 Health benefits of *C. lanatus*

The medicinal properties of the fruit have largely been attributed to the presence of phytochemicals or antioxidant compounds such as flavonoids and carotenoids including lycopene (Melo *et al*., 2006; Oseni and Okoye, 2013).

According to Kumari *et al*. (2013), aqueous extract of *C. lanatus* exocarp has exhibited good analgesic tendency in a time- and concentration-dependent manner, and may be adopted as an

alternative to conventional Non-Steroidal Anti-inflammatory Drugs (NSAIDs). Similarly, *C. lanatus* pomace which is a rich source of L-citrulline, attenuated metabolic syndrome in diabetic, overweight rats (Rimando and Perkins-Veazie, 2005; Wu *et al*., 2007). Figueroa *et al*. (2012) found that middle-aged overweight subjects with stage 1 hypertension who were administered with a *C. lanatus* fruit extract containing 6 g of L-citrulline and L-arginine daily, for 6 weeks, experienced a decrease in ankle blood pressure and improved arterial function. A study by Gunasekera *et al*. (2007) determined that the proliferation of malignant AT3 breast cancer cells treated with the carotenoid lycopene, which is found richly in *C. lanatus* fruit was inhibited in a time- and concentration-dependent mode.

2.2 ANTIOXIDANT ACTIVITY

2.2.1 Reactive oxygen species

Free radicals are species; atoms or molecules with unpaired electron(s). They include reactive oxygen species (ROS) such as peroxides, hydroxyl radical and superoxides, and which are highly reactive and have their levels increased exponentially during environmental stress (Devasagayam *et al.*, 2004; Herrling *et al*., 2008). According to Aruoma (1998), free radicals are constitutively generated in a cell and these can be overproduced resulting in oxidative stress which leads to damage to biomolecules such as proteins and nucleic acids. In addition, ROS when amassed in cells and the body at large have the tendency to cause aging and degenerative diseases including myocardial infarction, atherosclerosis, cancer and rheumatoid arthritis (Harman, 2009; Hekimi *et al*., 2011).

Radicals usually are unstable hence require that they accept electron(s) to become stable. When there are inadequate antioxidant levels, these radicals take electrons from other molecules. These molecules are then rendered unstable and in turn can cause oxidative stress and tissue damage. This phenomenon has been adapted in certain cancer therapy such as chemotherapy and radiotherapy as these are implicated in the production and accumulation of free radicals that subsequently cause DNA damage and cell death in the malignant cells (Chawda *et al.*, 2011).

It is imperative therefore that there exists a balance between the production and mopping up of these radicals to ensure a prolonged lifespan in normal cells.

2.2.2 Antioxidants

These are essential chemical compounds that work to avert cellular damage by reacting with ROS and consequently mopping them up. Also known as free radical scavengers, they could be endogenous, or from exogenous sources such as diet. Typical examples of endogenous antioxidants include enzymes such as superoxide dismutase, glutathione peroxidase and catalase. Examples of exogenous antioxidants are glutathione, Vitamins A, C and E, and phytochemicals such as carotenoids and flavonoids. These antioxidants when used individually, or in combination have the tendency to provide protection against chromosomal damage caused by UV radiation and xenobiotic oxidative stress (Chawda *et al.*, 2011).

The human body has systems responsible for maintaining this balance and these involve the use of antioxidants including ascorbic acid, and enzymes such as superoxide dismutase, glutathione peroxidase and catalase. Figures 2.2.2a and 2.2.2b show chemical reactions involving the scavenging of free radicals by ascorbate and antioxidant enzymes, respectively.

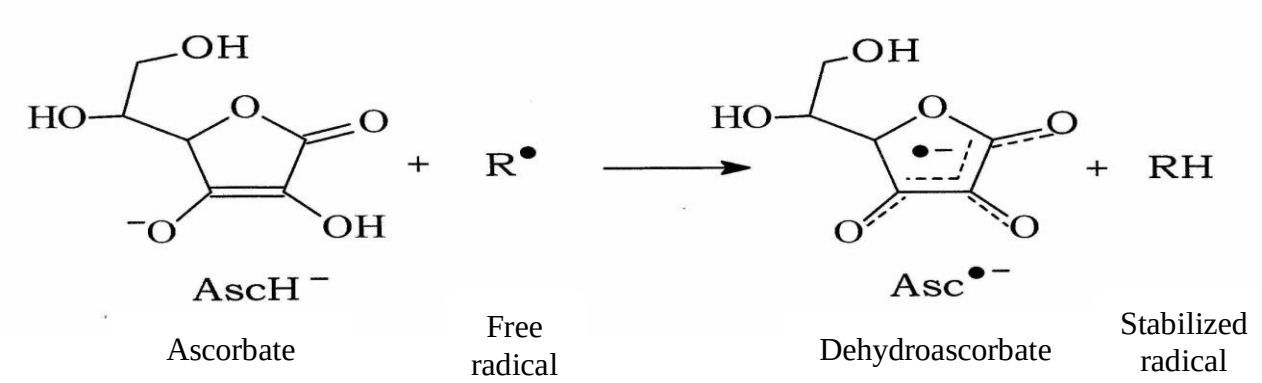

Figure 2.2.2a: Reaction showing free radical scavenging activity of ascorbic acid

(Google images, 2013).

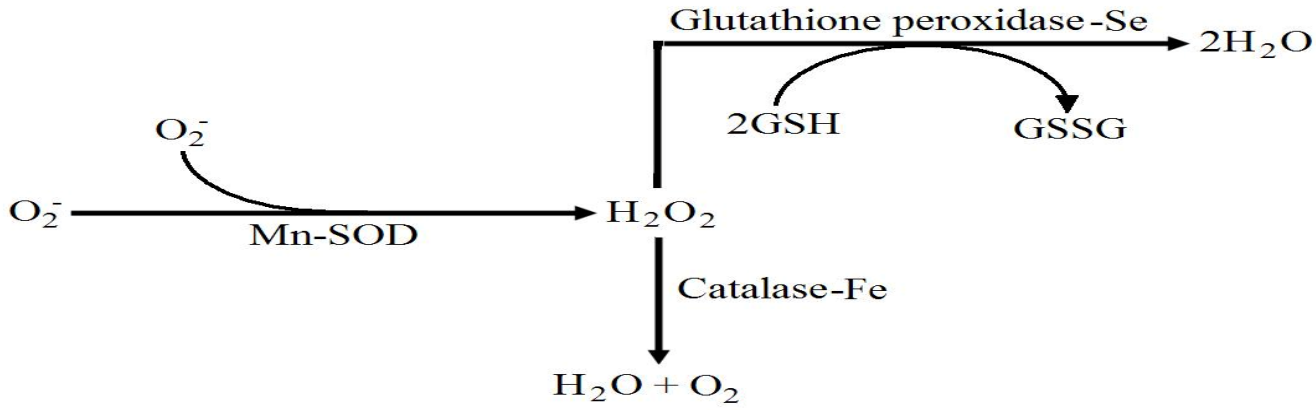

Figure 2.2.2b: Reaction showing free radical scavenging activity of antioxidant enzymes
(Google images, 2013).

These enzymes require specific cofactors, without which the enzyme activity is halted. Superoxide dismutase (SOD) requires manganese (Mn), catalase requires iron (Fe) and glutathione peroxidase requires selenium (Se) (Pham-Huy *et al*., 2008; Sen *et al*., 2010).

2.3 CANCER AND CYTOTOXIC ACTIVITY

2.3.1 Cancer

According to W.H.O, cancer refers to a vast group of diseases characterized by excessive cellular proliferation. It is a multi-step disease that originates from cells that are mutated (Alison, 2001). For normal cells to turn tumorous, there must be an alteration in multiple genes that code for proteins that partake in cellular growth and differentiation, resulting in these mutated cells (Knudson, 2001; Croce, 2008). Cancerous cells can form malignant tumours and invade neighbouring as well as distal parts of the body by a process called metastasis (Alison, 2001). These malignant tumours can be formed as a result of internal factors; metabolic mutations, hormonal and immune conditions, or external factors such as viruses, smoke, alcohol, sedentary lifestyle and radiation (Anand *et al*., 2008; Hejmadi, 2010; Jayasekara *et al*., 2016). Cancerous cells can also form benign tumours, which are relatively not harmful and are mild. According to Luo *et al*. (2009), these mild tumours are a result of consecutive mutations following the uncontrolled growth of abnormal cells. Irrespective of the ability of cancerous cells to evade other parts of the body, and their genetic predisposition being able to be passed on from parent to

progeny, cancers generally are not considered as contagious diseases (Tolar and Neglia, 2003). There are more than 100 human cancers as stated by the United States National Cancer Institute, and these include lung, brain, cervical, prostate, and breast cancer.

2.3.2 Diagnosing cancer

A majority of cancers are detected through physical signs and symptoms, or screening. These are evaluated via blood tests, scans and X-rays among others. However, these detection modes do not lead to a conclusive diagnosis. The only definitive way of diagnosing cancer is by examining a biopsy of the affected area or body organ. Diagnosis by biopsy is advantageous as this provides information on the exact cell type that has been transformed, the stage of cancer, prognosis and most appropriate treatment mode. It is prudent to note though that when diagnosis is done on time, prognosis is better and management is less burdensome (Segbefia *et al*., 2013).

2.3.3 Managing cancer

There are a number of treatment modes for cancer. Commonly used ones include chemotherapy, surgery and radiation therapy. The choice of treatment method depends largely on the type of cancer, stage, and the preference and health status of the patient.

2.3.3.1 Chemotherapy

As the core means of managing the disease, chemotherapy involves the use of cytotoxic drugs, that are designed to inhibit growth of cancerous cells by various mechanisms; the drugs can act as antimetabolites, microtubule stabilizers or DNA alkylating agents (Lind, 2008; Palumbo *et al*., 2013). Typical drugs administered for chemotherapy include avastin, gleevec and revlimid. The efficacy of these cytotoxic drugs is dependent mainly on the cancer type and stage. Some leukemias tend to be susceptible to chemotherapy, whereas some brain tumours are not (Rampling *et al*., 2004; Freedman, 2012; Nastoupil *et al*., 2012). Additionally, chemotherapy has proven needless in most non-melanoma skin cancers (Madan *et al*., 2010). It is noteworthy however that

chemotherapy nevertheless serves as an adjunct in management cases involving radiation therapy or surgery, as they are useful in relieving symptoms such as pain and occasionally, tumour size (Lind, 2008).

2.3.3.2 Radiation therapy

This mode of treatment, also known as radiotherapy, employs the use of radiation capable of ionizing atoms. Examples of such radiation include gamma rays, X-rays and charged particles. In cancer therapy, the ionizing radiation is targeted to cancerous cells to cause their deaths by DNA damage. Radiotherapy tends to be more efficacious in benign cancers such as those affecting the lung, cervix and prostate (Sabesan *et al*., 2018). It is also used as an adjunct to surgical therapy in some cases of brain, breast, and head and neck cancers (Sabesan *et al*., 2018). Cancers such as melanoma and renal cell cancer however are markedly resistant to radiation therapy as they require more radiation doses than usual for management. A combination of immunotherapy with radiotherapy thus has emerged as a promising active investigation area for the management of these so-called radio-resistant cancers (Maverakis *et al*., 2015). Radiotherapy is painless, but can cause side effects such as diarrhoea, hair loss and cardiovascular complications, which depend on the administered dose and affected area (Cox and Ang, 2009; Mahmood, 2016).

2.3.3.3 Surgery

Surgery involves the medical removal of the tumour, or the entire affected organ (Subotic *et al*., 2012). It is usually done in the case of non-haematological and benign cancers such as breast cancer. This is because metastasis renders complete management by surgery impossible as various parts of the body have been affected. In such cases neoadjuvant therapy, which involves the use of other treatment modes prior to surgery is often considered when surgery must be done. However, it is not always relevant as survival rate of patients undergoing only surgical treatment is no different than those undergoing surgery with adjuncts, e.g. in some breast cancer cases (Mieog *et al*., 2007).

2.3.4 The human blood

The blood is a reddish fluid present in humans that is responsible for the transport of vital substances such as nutrients, oxygen and hormones. Other roles of blood include homeostasis, removal of waste such as carbon dioxide, coagulation and immune functions. It makes up about 7% of the body weight, and the average adult has nearly 5 litres of blood, made up of plasma and various cells (Elert, 2012). These cells are primarily erythrocytes, thrombocytes and leukocytes. According to Ganong (2003), a volume of 1 µL of blood comprises 4.2 - 6.1 million erythrocytes, 200,000 – 500,000 thrombocytes and 4,000 – 11,000 leukocytes. Plasma on the other hand is mainly water with substances such as nutrients, blood proteins and electrolytes dissolved in it. Normally, blood is slightly basic and is carefully adjusted to stay in the pH range of 7.35 - 7.45 via acid-base regulation (Waugh and Grant, 2007). Blood cells are made principally in the bone marrow by a process called hematopoiesis. Other glands are responsible for the production of specialized blood cells. The thymus for example is a key source of T-lymphocytes during childhood (Williams and Gray, 1989). The liver chiefly makes blood proteins such as albumin, endocrine glands make hormones, and the hypothalamus and kidney regulate the water component of blood.

2.3.5 Leukemia

Leukemia is a cancer of bone marrow or blood-forming tissues that results from spontaneous mutations in the DNA, leading to unregulated proliferation of hematopoietic stem cells (Wang and Dick, 2005; Davis *et al*., 2014; Radivoyevitch *et al*., 2016). This usually results in reduced thrombocyte levels, and the formation of immature leukocytes known as leukoblasts, in large quantities. The cause of this disease is not precisely known, but a blend of factors (internal and external) tend to make one susceptible to developing leukemia (Hutter, 2010). These factors include familial history of leukemia, genetic disorders such as Down syndrome, and lifestyle influences; exposure to radiation, previous chemotherapy, smoking etc. (Stass *et al*., 2000; Robinette *et al*., 2001). A study by Rudant *et al*. (2013) determined that mothers who induced ovulation via fertility drugs had children who are greater than twice prone to leukemia during their childhoods than other children.

The disease can be developed abruptly or chronicly, can be of myeloid or lymphoid origin, and is characterized by excessive bleeding, fever, and the compromising of the immune system, leading

to increased risk of illnesses (Davis *et al*., 2014). Leukemia can be diagnosed using bone marrow examination and complete blood counts (Döhner *et al*., 2015). Treatment of the disease is via radiation therapy, chemotherapy using drugs such as imatinib and dexamethasone, and sometimes, bone marrow transplant from a compatible donor. According to the World Cancer Report (2014), leukemia occurs more commonly in the developed world and in children, but treatment outcomes in the developed world are being enhanced with the advancement of science.

Clinicians categorise leukemia into various groups depending mainly on the origin of cancerous cells (myelogenic or lymphoblastic), and the time period for development (acute or chronic). As a result, there are four main classifications of the disease. These are acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute mylogenic leukemia (AML) and chronic myelogenic leukemia (CML). There are other rare leukemic conditions that affect adults mostly. These include hairy cell leukemia, T-cell prolymphocytic leukemia and adult T-cell leukemia.

2.3.5.1 Acute lymphoblastic leukemia

This is the most prevalent leukemia type in young children and has a peak frequency among 2 – 5 year olds (Inaba *et al*., 2013). It can also affect adults, particularly those older than 65 years. Symptoms of ALL include anaemia, fever, fatigue and increased risk of infection. The prognosis of this disease is better for these young children than adults as there is an 85% survival rate for children, and 50% for adults (Jameson *et al*., 2005). Typical treatment for ALL is chemotherapy using drugs such as dexamethasone and vincristine, and radiotherapy for painful bony parts. Surgery is not an ideal treatment mode for this type of leukemia due to the fact that the cancerous cells are easily spread across the body. However, patients who undergo relapse may appropriately be managed by bone marrow transplantation (Hoffbrand *et al*., 2006).

2.3.5.2 Chronic lymphocytic leukemia

This is a slowly progressive leukemia type that occurs predominantly in old people (> 55 years), occasionally in young adults and very rarely in children (Inaba *et al*., 2013). Symptoms of CLL include fatigue, hepatomegaly and abnormal bruising (Davis *et al*., 2014). A familial history of CLL and being male, tend to predispose one to the disease. There is currently no cure for CLL, but it can be effectively managed by combination drug therapy using chlorambucil or

cyclophosphamide, and a corticosteroid. Younger patients however may find single drug therapy with agents such as fludarabine useful, or can undergo bone marrow transplantation (Eichhorst *et al*., 2006; Gribben, 2008). According to Inaba *et al*. (2013), a significant difference between ALL and CLL is that malignant B lymphocytes in CLL seem more mature but are not functional. These cells are additionally able to evade apoptotic signals.

2.3.5.3 Acute myelogenic leukemia

Acute myelogenic leukemia is characterised by unregulated growth of myeloblasts; precursors in the production of myelocytes. According to Wiernik, 2001, some genetic diseases such as Down syndrome and Fanconi anaemia markedly increase the risk of AML development. In adults the use of tobacco causes a slight increase in the susceptibility to AML development (Wiernik, 2001). Symptoms develop suddenly and may include thrombocytopenia, neutropenia, fever and lethargy. Acute myelogenic leukemia patients are additionally extremely prone to infections as the myeloblasts have no ability to fight infections (Hoffman, 2005). Clinicians diagnose AML by suitable bone marrow examination; Vitamin B_{12}, folic acid and copper deficiencies are ruled out to make the diagnosis conclusive (Aitelli *et al*., 2004; Barzi *et al*., 2010; Zuo *et al*., 2010). Treatment is done mainly via chemotherapy. However, when chemotherapy is ineffective or relapse occurs, stem cell transplantation is considered. Research is underway to ascertain the use of tyrosine kinase inhibitors for chemotherapy (Kayser and Levis, 2014).

2.3.5.4 Chronic myelogenic leukemia

This is also a slowly progressive leukemia type that affects adults mainly. It is characterised by the development of differentiated granulocytes that are not functional (Wang & Dick, 2005). CML is the first cancer that was associated with a well-defined genetic aberration, the Philadelphia chromosome that arises from a translocation. Thus definitive CML diagnosis requires the detection of the Philadelphia chromosome (Calabretta and Perrotti, 2004). Symptoms of the disease include splenomegaly, shortness of breath and inexplicable weight loss. Standard curative treatment involves bone marrow or stem cell transplantation. Chemotherapy is also employed using cytotoxic agents such as imatinib (mainly), dasatinib and radotinib. Since the introduction of imatinib in chemotherapy, CML has emerged the first cancer with a regular management mode that may give a patient a normal lifespan (Gambacorti-Passerini *et al*., 2011).

2.3.6 REVIEW OF METHODS

2.3.6.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

Molecules that have hydrogen atoms to donate can serve as antioxidants. The radical species, DPPH, possesses a lone electron pair situated on the central nitrogen. Thus in the presence of an antioxidant compound, DPPH can accept protons to form stabilized reduced DPPH. This electron pairing hence reduction triggers a colour change from purple to yellow, and the intensity of yellow colour is quantitative as it depends on the number of electrons paired (Sannigrahi *et al*., 2009). The assay quantifies the colour change by measuring the decrease in absorbance at a wavelength of 517nm. Figure 2.3.6.1 shows the reduction of DPPH radical by an antioxidant.

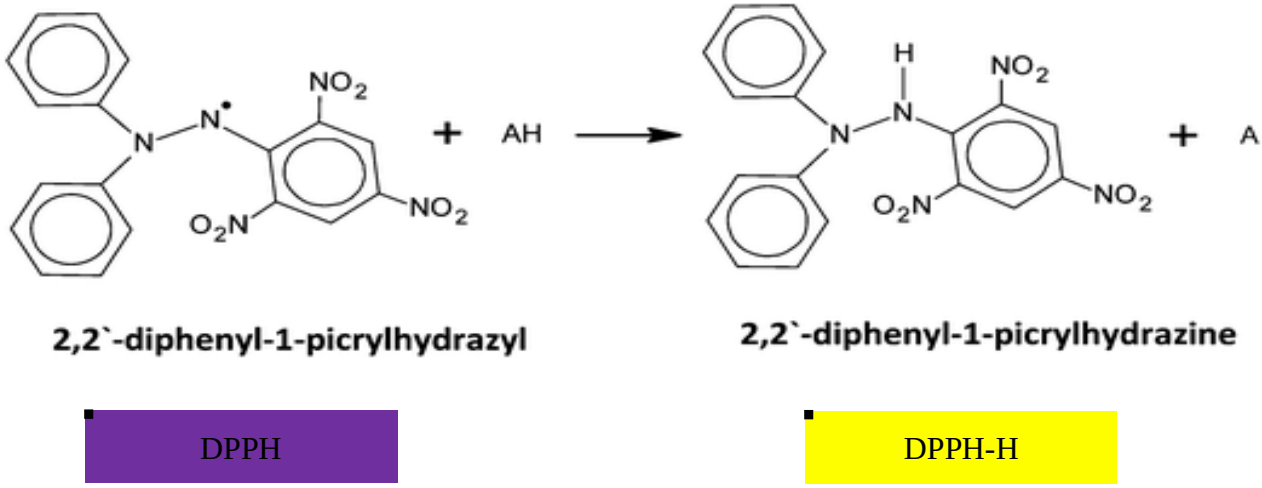

Figure 2.3.6.1: Reaction showing the reduction of DPPH radical by an antioxidant

(Google images, 2013)

2.3.6.2 Folin-Ciocalteau assay

This assay employs the use of Folin-Ciocalteau reagent (developed by Folin Dennis) to cause the oxidization of phenols (Folin and Ciocalteau, 1927). The reagent was originally developed for tyrosine level determination in proteins. The Folin-Ciocalteau reagent comprises phosphotungstic and phosphomolybdic acids. After the phenolic oxidization, the metal ions in the reagent gets reduced resulting in the formation of blue oxides of molybdenum and tungsten (Agbor *et al*., 2014). The total phenol composition of an extract is thus quantified by the measurement of the blue intensity of the solution at a wavelength of 760nm.

2.3.6.3 Total Flavonoids Content assay

Flavonoids are a group of phytochemicals with a 15-carbon skeleton structure. The total flavonoid content assay involves the use of aluminium chloride which reacts with specific functional groups (ketone and hydroxyl) on the fourth and either the third or fifth carbon atoms of flavonoids to form stable complexes of acids (Kalita *et al*., 2013). This causes a colour change to yellow that is quantifiable at a wavelength of 415 nm.

2.3.6.4 Cytotoxicity and estimation of cell viability

Medicinal plants have been used largely in research to identify drug candidates for various diseases of public health concern, including cancer (Cheuka *et al*., 2017). Immortalized cancer cells have been helpful in cancer research thus, in this study, the leukemia cell lines HL-60 and Jurkat will be used. The HL-60 cell line was derived from a thirty-six year old woman with acute promyelocytic leukemia (Gallagher *et al*., 1979). The Jurkat cells on the other hand are immortalized human T-lymphocyte leukemia cells obtained from a fourteen-year old boy's peripheral blood (Schneider *et al*., 1977; Abraham and Weiss, 2004).

The anti-leukemic or cytotoxic activity of an extract can be evaluated using MTT assay. The MTT assay analyses cell viability by employing the use of the yellow-coloured tetrazolium dye compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Reduction of this dye is dependent on NAD(P)H-dependent oxidoreductase enzymes which are found mainly in the cytosol of viable cells (Berridge and Tan, 1993; Berridge, Herst and Tan, 2005). This enzyme acts on MTT to change the dye from its characteristic yellow colour to an insoluble, purple coloured compound known as formazan as follows:

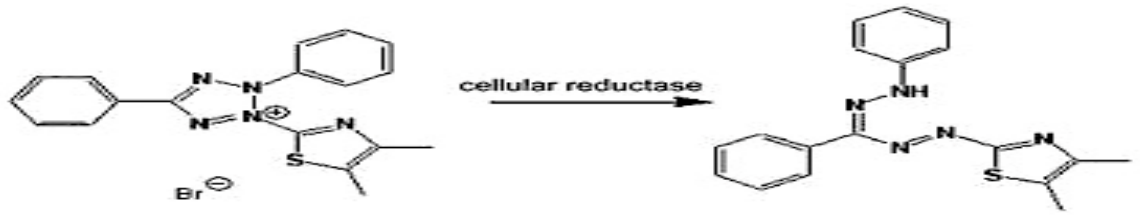

Figure 2.3.6.4: Reaction showing the conversion of MTT to formazan by cellular reductases

(Google images)

Since the reduction of the MTT compound occurs in metabolically active cells only, a decrease in MTT levels during the assay is an indication of cell viability (Stoddart, 2011).

The use of trypan blue dye is commonly employed for staining cells in viability assays. This dye uses the principle that viable cells with intact cell membranes are impenetrable to the dye; inviable cells are thus stained blue as they are able to pick up the dye. There is a shortcoming however to this as the dye is unable to differentiate between healthy cells and non-functional cells, although alive (Riss, 2005).

CHAPTER THREE

METHODOLOGY

3.0 MATERIALS AND METHOD

3.1 CELL LINES AND REAGENTS

Analytical grade solvents including hexane, chloroform, ethylacetate and ethanol (obtained from Sigma-Aldrich Company, Missouri - USA, as well as distilled water from NMIMR were used in this study. Other reagents obtained from Sigma-Aldrich Company, Missouri – USA include Folin-Ciocalteau reagent, sodium carbonate solution and phosphate buffered saline (PBS).

Penicillin streptomycin L-glutamine (PSG), trypsin, trypan blue dye and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were acquired from Wako Pure Chem. Ind., Tokyo, Japan. Other reagents such as curcumin, dimethyl sulphoxide (DMSO), butylated hydroxyl toluene (BHT), gallic acid and 2, 2- diphenyl-1-picryl hydrazyl free radical reagent (DPPH) were obtained from Hamburg, Germany.

Culture medium, which is Rosewell Park Memorial Institute (RPMI)- 1640 was acquired from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. Fetal Bovine Serum (FBS) for supplementing the culture medium was obtained from Hyclone Lab. Inc., South Logan, Utah, USA. Human leukaemia-immortalized T lymphocytes (Jurkat), human acute promyelocytic leukemia (HL-60) and Chang liver cell lines which had been passaged for a maximum of fourteen times were acquired from the Clinical Pathology Department, NMIMR, where the study was undertaken.

3.2 STUDY DESIGN

The study employed the use of experimental and control groups. The experimental groups were used for *in vitro* testing. For the chemical analysis of antioxidants and cytotoxicity assays, tests were ran in triplicates as this produced verifiable results.

The control groups however aided in validating results obtained for experimental groups. Among the controls, there were standards (positive controls), colour controls and blanks for the chemical analysis of antioxidants. For the cytotoxicity assays, the use of negative controls were employed in addition to the standard, colour controls and blanks.

Six sets of extraction were done on the mesocarp and seeds of watermelon to obtain six extracts of each. For each extract, the total antioxidant activity, total phenolics content, total flavonoids content and ferric reducing power activity were evaluated. The cytotoxic activity of each extract was also determined on each of the three cell lines used.

3.3 ETHICAL CONSIDERATIONS

Approval was obtained from the Ethics and Protocol Review Committee, and the Scientific and Technical Committee of the Noguchi Memorial Institute for Medical Research (NMIMR) of the College of Health Sciences, University of Ghana.

3.4 SAMPLE COLLECTION

Seventeen ripe *C. lanatus* fruits of average mass, 6.0 kg, were procured from Ada in the Greater Accra region of Ghana for the study. These selected fruits were green in colour, heavy to lift, and made a hollow sound when shaken or tapped on the outside. Authentication of the fruits was done at the Ghana Herbarium, Department of Plant and Environmental Science, University of Ghana.

3.5 PREPARATION OF EXTRACTS

The fleshy mesocarp of the fruits were homogenized and subsequently freeze dried using a Lyotrap freeze dryer (ultra LF/LYO/04/1, England). The seeds on the other hand were air dried and grinded using a dry blender. Both powdered samples were stored at -20 ^{o}C in a freezer. Subsequently, 100 g of each sample was weighed using a balance, and added to 1000 ml of each solvent to attain a 10% (w/v) concentration of each organic solvent, extracted sequentially in the order:

hexane → chloroform → ethylacetate → ethanol, by cold maceration at room temperature (26 ^{o}C). This sequential extraction was undertaken by shaking a mixture of two 50 g samples of powdered mesocarp or seed and 200 ml each of solvent for the first 3 days using a Yamato shaker model SA-31 at 3.5 rpm speed. The resulting mixtures were filtered with Whatman No.1 filter papers and the residues were subjected to shaking and filtration again using 200 ml each of solvent for next 3 days and 100 ml each of solvent for the last 3 days. Hence for each solvent used, the shaking was performed for 9 continuous days. The solvents were then taken out of the filtrates by rotary evaporation at 45^{o}C and 150 mBar, using Rotavapor R-205 (Buchi, Switzerland).

Aqueous extracts of the fleshy mesocarp and seeds however were prepared by extraction of powdered samples with distilled water. A 10% (w/v) concentration of each sample was prepared by heating 100 g of each sample in 1000 ml distilled water at 80^{o}C for 20 min, followed by cooling and filtration using a clean net as a sieve. The filtrates were then centrifuged at 3500 rpm for 15 min via an Eppendorf 5810R centrifuge and the resulting supernatants were frozen and subsequently freeze-dried by use of the Lyotrap freeze dryer.

The percentage yield of each aqueous and organic extract was subsequently determined from the formula:

$$\frac{\text{Mass of extract (g)}}{100\text{g}} \text{ X } 100$$

where 100 g is the mass of powdered sample used for extraction.

3.6 DETERMINATION OF ANTIOXIDANT CAPACITY

3.6.1 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay

The total free radical scavenging activity of each extract was determined using the DPPH assay (Blois, 1958). A mass of 20 mg of each extract was weighed into Eppendorf tubes (using a Mettler Toledo electronic balance) and dissolved in 1 ml of appropriate solvent [distilled water (dH_2O), absolute ethanol or DMSO]. The resulting mixtures were vortexed to attain complete dissolution and subsequently underwent 2-fold serial dilution to attain seven different concentrations in the range 0.03125 – 20 mg/ml. A mass of 1 mg of BHT was weighed also, dissolved in 1 ml of absolute methanol and serially diluted in 2-folds to attain seven different concentrations (0.015625 - 1 mg/ml). Aliquots of 100 µl of each extract was transferred in triplicate into the wells of a well-labelled 96-well microtitre plate. A volume of 100 µl of 0.5 mM DPPH was pipetted into each of the wells and mixed gently. The plate was subsequently covered with aluminium foil and incubated in the dark for 20 min. Finally, the absorbance readings were taken at a wavelength of 517 nm by use of a Tecan Infinite M200 Pro, microplate reader (Austria) to quantify the amount of reduced radical (DPPH) in solution. The procedure was repeated using BHT as positive control and methanol as negative control. Percentage DPPH scavenging activity was determined using the formula:

$$\%\ \text{Antioxidant activity} = \left(\frac{\text{OD control} - \text{OD sample}}{\text{OD control}}\right) \times 100$$

where OD control = Optical density of control

and OD sample = Optical density of extract or BHT

The mean percentage scavenging (antioxidant) activity for each triplicate experiment was plotted against extract or BHT concentrations to obtain the EC_{50} value via nonlinear regression analysis. The EC_{50} value is the concentration of extract that decreases the initial DPPH concentration by 50%.

3.6.2 Total phenolic content assay

The total phenolic content of each extract was determined using the Folin-Ciocalteau method as described by Ghasemi *et al*. (2009). A mass of 20 mg of each extract was weighed into Eppendorf tubes using a Mettler Toledo electronic balance, and dissolved in 1 ml of appropriate solvent (dH_2O, absolute ethanol or DMSO). Two additional concentrations (5 and 10 mg/ml) of each extract were prepared by 2-fold serial dilution. To obtain a standard curve using gallic acid, 5 mg of gallic acid was weighed and completely dissolved in 100 µl of absolute ethanol. This was further made up to 1 ml by the addition of distilled water. Seven different concentrations of the resulting gallic acid solution were prepared by 2-fold serial dilution (0.015625 - 1 mg/ml). For blank, 50% ethanolic solution was used. A volume of 10 µl of each sample or gallic acid standard was aliquoted into the wells of a 24-well plate, in triplicates. Subsequently, 790 µl of dH_2O and 50 µl of Folin-Ciocalteau reagent were added to each of these wells, mixed and incubated at room temperature for 8 min. Then, 150 µl of Na_2CO_3 (20% w/v) was added and the mixture was further incubated at room temperature for 2 h. After the incubation, absorbance was read at a wavelength of 750 nm using the microplate reader (Austria). From the absorbance readings, the total phenolic content of each extract was calculated from the regression equation of the gallic acid curve ($y = 0.3394x – 0.0031$, $R^2 = 0.99577$) and expressed as gallic acid equivalents (GAE).

3.6.3 Total flavonoid content assay

To assess the entire flavonoid composition of each extract, the aluminum chloride ($AlCl_3$) colorimetric method (Ordónez *et al*., 2006) was used. In this evaluation, 20 mg of each extract was weighed using a Mettler Toledo electronic balance, and dissolved in 1 ml of appropriate solvent (dH_2O, absolute ethanol or DMSO). Two additional concentrations (5 and 10 mg/ml) of each extract were prepared by 2-fold serial dilution. To obtain a standard curve using quercetin, 1 mg of quercetin was weighed and completely dissolved in 1 ml of absolute methanol. Seven different concentrations of the resulting quercetin solution were prepared serially in 2-folds (0.015625 - 1 mg/ml). Then, 100 µl of each extract or quercetin concentration was aliquoted into wells of a well-labelled 96-well plate. A volume of 100 µl of 2% aluminium chloride ($AlCl_3$) was subsequently added to each well. The plate was well shaken and incubated at room temperature for 20 min. After the incubation, absorbance was read at a wavelength of 415 nm using the microplate reader

(Austria). From the absorbance readings, the total flavonoid content of each extract was calculated from the regression equation of the quercetin standard curve ($y = 2.2762x + 0.0012$, $R^2 = 0.9982$) and expressed as quercetin equivalents.

3.7 CYTOTOXIC ANALYSIS

3.7.1 Cell culture

The HL-60 and Jurkat cells as well as Chang liver cells were cultured in Rosewell Park Memorial Institute (RPMI)-1640 medium. This was supplemented with fetal bovine serum and 1% penicillin-streptomycin. Subsequently, the cells were maintained in a Panasonic MCO-18AC-PE humidified incubator (Japan) at 37 ^{o}C and in the presence of 5% CO_2.

The HL-60 and Jurkat are suspension cells, hence at the time of culturing, the spent medium was poured off. Then, the cells were washed with Phosphate Buffered Saline (PBS). Following this, incomplete medium (RPMI–1640, 1% streptomycin-penicillin, glutamate and sodium bicarbonate) was added and spun using a centrifuge (Tomy LC200 centrifuge, Japan) at 1000 rpm for 5 min. The resulting supernatant was poured off and the cells were re-suspended in complete medium (RPMI–1640, 1% streptomycin-penicillin, glutamate, sodium bicarbonate and fetal bovine serum). Finally, the cells were incubated at 37 ^{o}C in a humidified CO_2 incubator.

Normal human liver cells on the other hand are adhesion cells hence were cultured and passaged as follows:

The spent medium which was obtained by centrifuging the cells at 1000 rpm for 5 min was pipetted off into a 25 cc flask. A volume of 5 ml of PBS was added and swirled gently over attached cells. Then, the added PBS was aspirated off. Following that, a volume of 1 ml of trypsin was added and the flask was incubated at 37 ^{o}C for 2 min in a CO_2 incubator to detach cells. Undetached cells underwent removal by firmly hitting the sides of the flask and observation under a (Zeiss Primo Vert, Germany) microscope to confirm detachment. A volume of 5 ml of fresh RPMI culture medium was added to the flask, cells were re-suspended and transferred into a 15 ml centrifuge tube for centrifugation for 5 min at 1000 rpm using a Tomy LC200 centrifuge, Japan. The cells were then incubated under conditions stated above.

3.7.2 MTT assay

The cytotoxic effect of each extract on the human leukemia cell lines and normal liver cells were determined using the MTT assay (Appiah-Opong *et al*., 2016). Starting from a stock concentration of 50 mg/ml, five different concentrations (0.625 - 10 mg/ml) of each extract were prepared by serial dilution. Then, 100 µl of RPMI culture medium was added to the wells in rows 1 and 8 of a well-labelled 96-well plate to prevent evaporation in test wells, and 100 µl of cell suspension (1 x 10^5 cells/ml) was transferred into the remaining wells. Subsequently, 10 µl of extract (0.625 - 10 mg/ml) was added in triplicate (vertically) to wells containing cells. A volume of 10 µl of RPMI culture medium was added to wells for negative control. Curcumin was used as a positive control in the concentration range of 23.00 – 368.04 µg/ml. The plate was incubated as stated above for 72 h. After incubation, 20 µl of 2.5 mg/ml MTT solution in PBS was added and the incubation was continued for 4 h. One hundred and fifty microliters of acidified isopropanol (1.7% (*v/v*) aqueous HCl in isopropanol) containing 1% Triton-X was added to each well to terminate the reaction and the plate was incubated in the dark room at room temperature (26 ^{o}C) overnight. Finally, the absorbance readings were taken at a wavelength of 570 nm using a Tecan Infinite M200 Pro microplate reader and recorded. The percentage cell viability was determined as follows:

$$\% \text{ cell viability} = \left(\frac{(\text{ABCDEBAFGH DI IEHAIHK GHLLC M ABCDEBAFGH DI GDIDNE GDFIEDL})}{(\text{ABCDEBAFGH DI NFJEHAJHK GHLLC M ABCDEBAFGH DI BLAFO})} \right) \times 100$$

The calculated values were plotted against concentrations of extracts, and the IC_{50} value which is the concentration of the extract with 50% inhibitory activity was determined for each extract.

The calculated IC_{50} values were used to determine the selectivity indices (SI) of the extracts using the formula:

$$SI = \frac{IC50 \text{ of extract in normal cell}}{IC50 \text{ of extract in cancer cell line}}$$

SI values ≥ 2 were considered as significant (Badisa *et al*, 2009).

3.8 CALCULATIONS

The percent cell viability was calculated using the formula of Ayisi *et al.*, 1991, which is a ratio of the absorbance of treated cells to the absorbance of untreated cells with consideration to absorbance of the blank and colour control, expressed as a percentage. These values were plotted against concentrations of extracts, and the concentrations at which 50% inhibitory activities was attained (IC_{50}) were determined. The selectivity indices of the extracts were also determined as the ratio of the IC_{50} values in normal cells to that in cancer cells. The mean percentage scavenging (antioxidant) activity was plotted against extract or BHT concentrations to obtain the EC_{50} value via nonlinear regression analysis. The EC_{50} value is the concentration of extract that decreases the initial DPPH concentration by 50%.

3.9 STATISTICAL ANALYSIS

All data were expressed as means of three experiments ± SD. Significant differences between the control and test experiments were determined using Student's t-test from Microsoft Excel 2016 version. P-values less than 0.05 were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 Yield of extracts

The seeds were obtained from 17 fruits. After extraction, the yield was calculated as described in the method section. The aqueous extract recorded the highest yield whilst the ethanolic extract gave the least.

Table 4.1a: Yield of *C. lanatus* seed extracts

SEED EXTRACT	PERCENTAGE YIELD
Hexane	8.68
Chloroform	1.05
Ethylacetate	0.97
Ethanol	0.52
Aqueous (Sequentially extracted)	13.82
Aqueous	14.57

The mesocarp used was obtained from 4 fruits. The samples were blended, freeze-dried and subsequently extracted with various solvents. Following extraction, the yield was calculated as described in the method section. The aqueous extract recorded the highest yield whilst the ethyl acetate extract gave the least.

Table 4.1b: Yield of *C. lanatus* mesocarp extracts

MESOCARP EXTRACT	PERCENTAGE YIELD
Hexane	0.19
Chloroform	0.27
Ethylacetate	0.16
Ethanol	50.46
Aqueous (Sequentially extracted)	24.31
Aqueous	66.17

4.2 Chemical analysis of antioxidants

4.2.1 Total antioxidant activity

The total antioxidant activity of the various extracts and positive control, BHT, are shown in Figures 4.2.1a – 4.2.1g. The antioxidant activity is a measure of DPPH free radical scavenging ability of each extract. Absorbance readings were taken at a wavelength of 517 nm. The extracts generally demonstrated a concentration-dependent antioxidant potency with seed extracts generally exhibiting higher antioxidant activity than the mesocarp extracts. Ethanolic seed extract showed the strongest antioxidant activity with a calculated EC_{50} value of 0.58 ± 0.06 mg/ml. The hexane and ethanolic mesocarp extracts however exhibited the weakest antioxidant activity with EC_{50} value of >10 mg/ml.

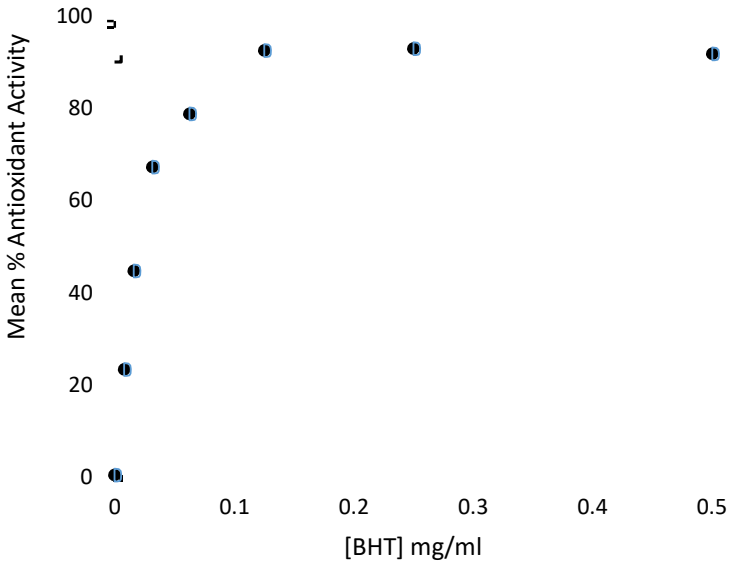

Figure 4.2.1a: Total antioxidant activity of the standard, butylated hydroxytoluene (BHT)

Each point is the mean of triplicate experiments (n=3)

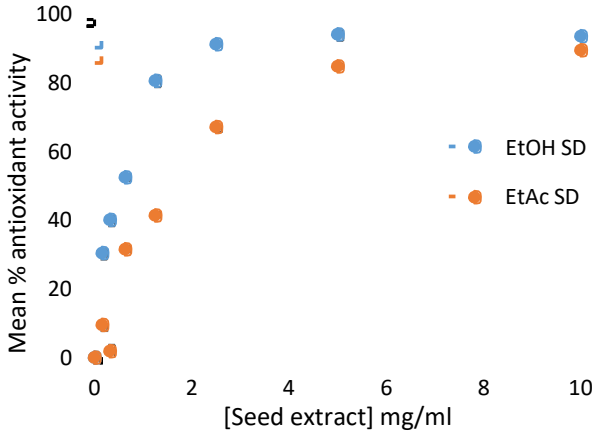

Figure 4.2.1b: Total antioxidant activity of ethanolic and ethylacetate seed extracts

EtOH SD represents the ethanolic seed extract and EtAc SD represents the ethyl acetate seed extract. The ethanolic seed extract possesses stronger antioxidant activity than the ethyl acetate seed extract.

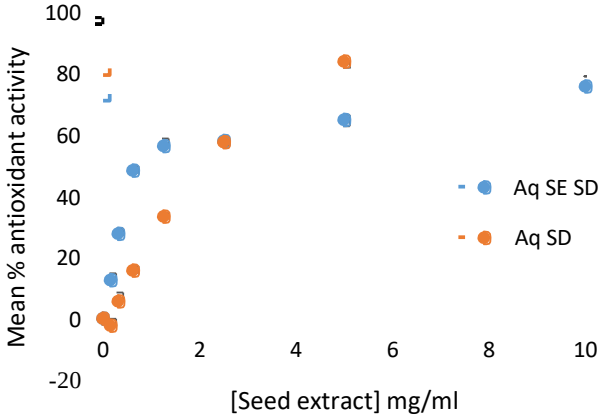

Figure 4.2.1c: Total antioxidant activity of aqueous and aqueous (sequentially extracted) seed extracts

Aq SE SD represents the sequentially extracted aqueous seed extract and Aq SD represents the aqueous seed extract. The (SE) aqueous seed extract possesses stronger antioxidant activity than the aqueous seed extract although both were extracted using distilled water.

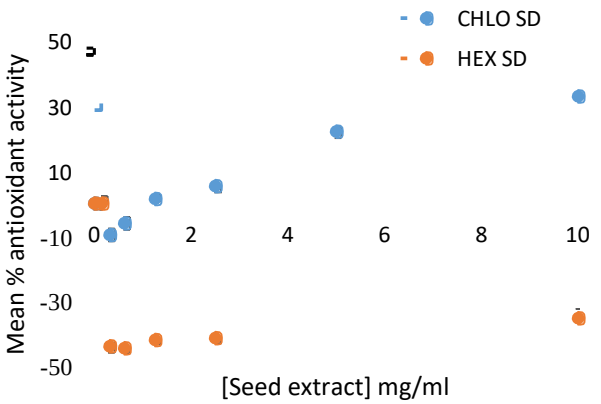

Figure 4.2.1d: Total antioxidant activity of chloroform and hexane seed extracts

CHLO SD represents the chloroform seed extract and HEX SD represents the hexane seed extract. The chloroform seed extract exhibited moderate antioxidant activity whilst the hexane seed extract showed no antioxidant activity.

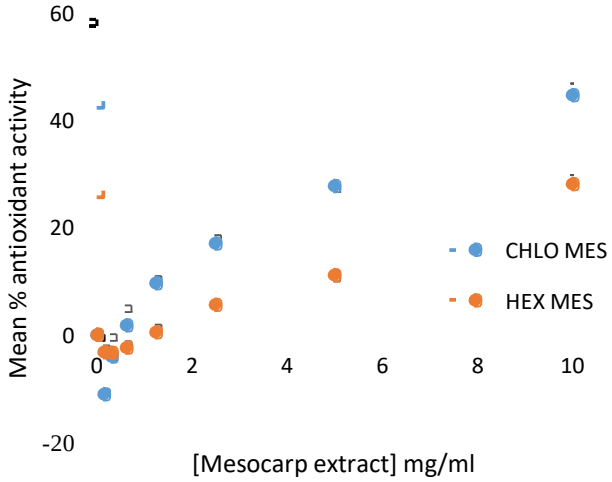

Figure 4.2.1e: Total antioxidant activity of chloroform and hexane mesocarp extracts

CHLO MES represents the chloroform mesocarp extract and HEX MES represents the hexane mesocarp extract. The chloroform mesocarp extract exhibited stronger antioxidant activity than the hexane mesocarp extract.

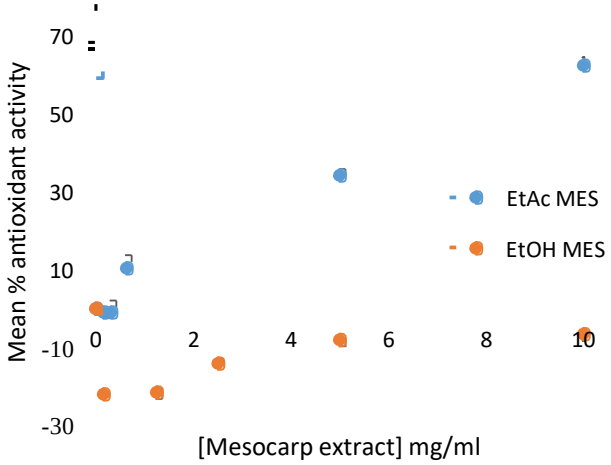

Figure 4.2.1f: Total antioxidant activity of ethanolic and ethylacetate mesocarp extracts

EtAc MES represents the ethyl acetate mesocarp extract and EtOH MES represents the ethanolic mesocarp extract. The ethyl acetate mesocarp extract exhibited significant antioxidant activity whilst the ethanolic mesocarp extract showed no antioxidant activity.

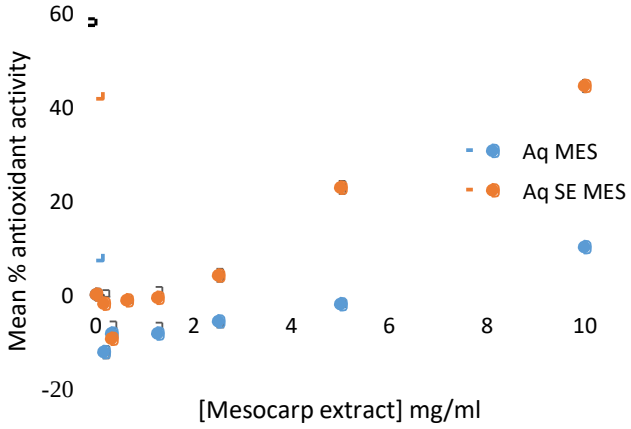

Figure 4.2.1g: Total antioxidant activity of aqueous and aqueous (sequentially extracted) mesocarp extracts

Aq SE MES represents the sequentially extracted mesocarp extract and Aq MES represents the aqueous mesocarp extract. The SE mesocarp extract exhibited moderate antioxidant activity whilst the aqueous mesocarp extract showed weak antioxidant activity.

Table 4.2.1 shows the comparison of total antioxidant activities of various extracts and the standard, BHT, is done by the use of EC_{50} values. Each calculated EC_{50} value is the concentration of extract at which 50% DPPH scavenging ability is exhibited.

Table 4.2.1: Antioxidant activity of *C. lanatus* extracts and BHT

	EC_{50} VALUES (mg/ml)		
SAMPLE	**SEED**	**MESOCARP**	**BHT**
Hexane	> 10	> 10	
Chloroform	> 10	> 10	
Ethylacetate	1.69 ± 0.06*	7.82 ± 0.36*	
Ethanol	0.58 ± 0.06*	> 10	0.02 ± 0.00
Aqueous (SE)	0.85 ± 0.26*	> 10	
Aqueous	2.01 ± 0.12*	> 10	

Each value is the mean ± SD of three experiments. Values with * connote significant difference ($p < 0.05$) in antioxidant activity in comparison to BHT, the positive control.

4.2.2 Total phenolic content

The gallic acid standard curve (Figure 4.2.2a) was plotted using the absorbance readings taken at a wavelength of 750 nm for the concentrations tested. The total phenolic content of 20 mg/ml of each extract was extrapolated from the gallic acid curve (Figures 4.2.2a and 4.2.2b). The seed extracts were found to generally have a higher phenolic content than the mesocarp extracts. Ethanolic seed extract recorded the highest phenolic content among all the extracts (3851.90 ± 202.22 mg gallic acid equivalent/ 100 g of seed extract). The chloroform mesocarp recorded the highest phenolic content among the mesocarp extracts (1678.45 ± 35.21 mg gallic acid equivalent/ 100 g of mesocarp extract), and the ethanolic mesocarp recorded no phenolic content.

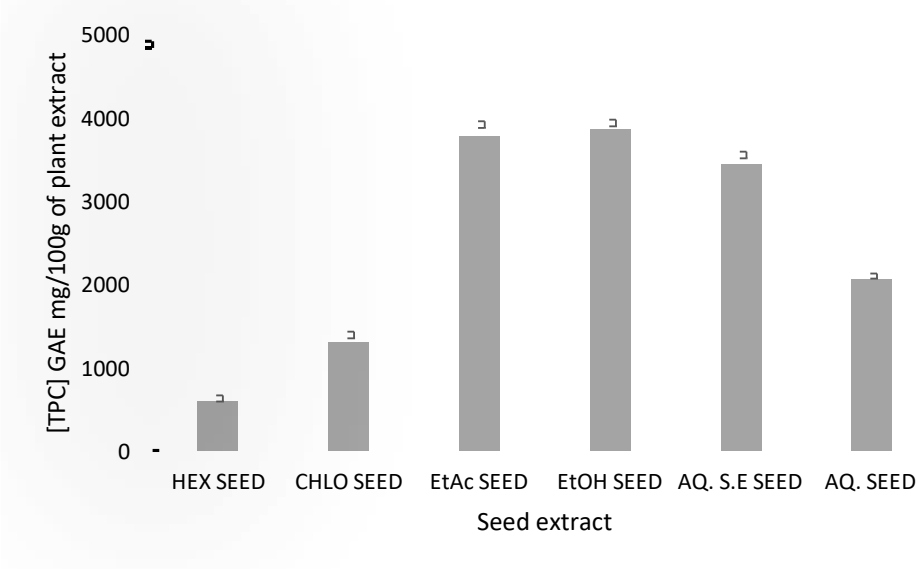

Figure 4.2.2a: Total phenolic content of seed extracts

The seed extracts possess significant phenolic content except for the hexane extract which exhibited low phenolic content.

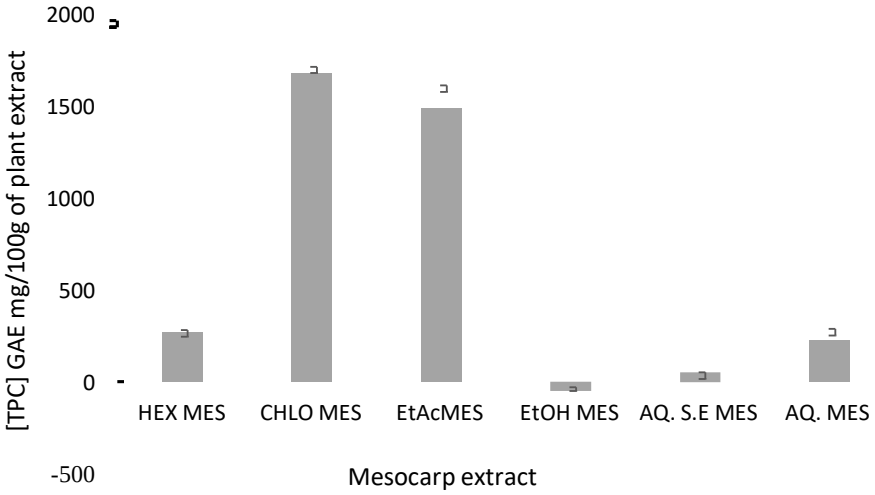

Figure 4.2.2b: Total phenolic content of mesocarp extracts

The mesocarp extracts generally possess low total phenolic content except for the chloroform and ethylacetate extracts that possess significant total phenolic content.

4.2.3 Total flavonoid content

The quercetin standard curve (Figure 4.2.3a) was plotted using the absorbance readings taken at a wavelength of 415 nm for the concentrations tested. The total flavonoid content of 20 mg/ml of each extract was extrapolated from the quercetin standard curve (Figures 4.2.3a and 4.2.3b). The seed extracts were found to generally have a higher flavonoid content than the mesocarp extracts. Hexane seed extract recorded the highest flavonoid content among all the extracts (1013.82 ± 110.56 mg quercetin equivalent/ 100 g of seed extract). The chloroform mesocarp recorded the highest flavonoid content among the mesocarp extracts (807.05 ± 119.46 mg quercetin equivalent/ 100 g of mesocarp extract), and the hexane mesocarp and aqueous (SE) seed recorded no flavonoid content.

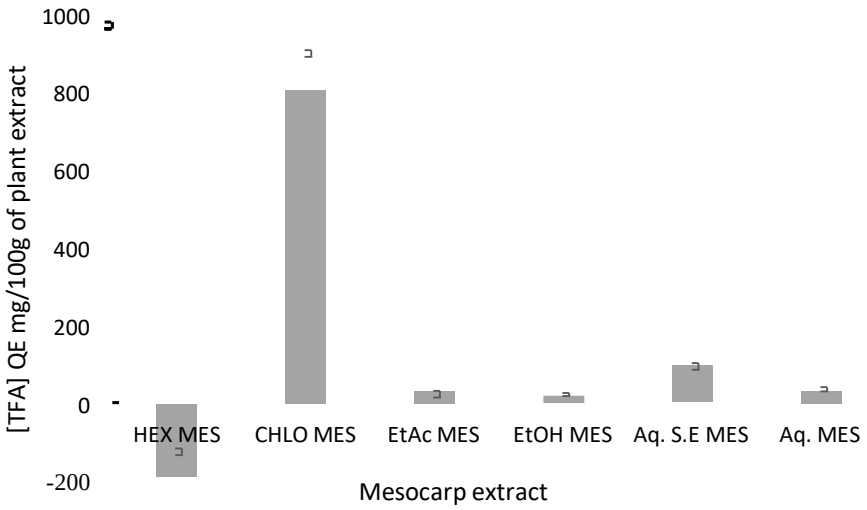

Figure 4.2.3a: Total flavonoid content of mesocarp extracts

The mesocarp extracts possess little or no flavonoids content except for the chloroform extract which exhibited significant phenolic content.

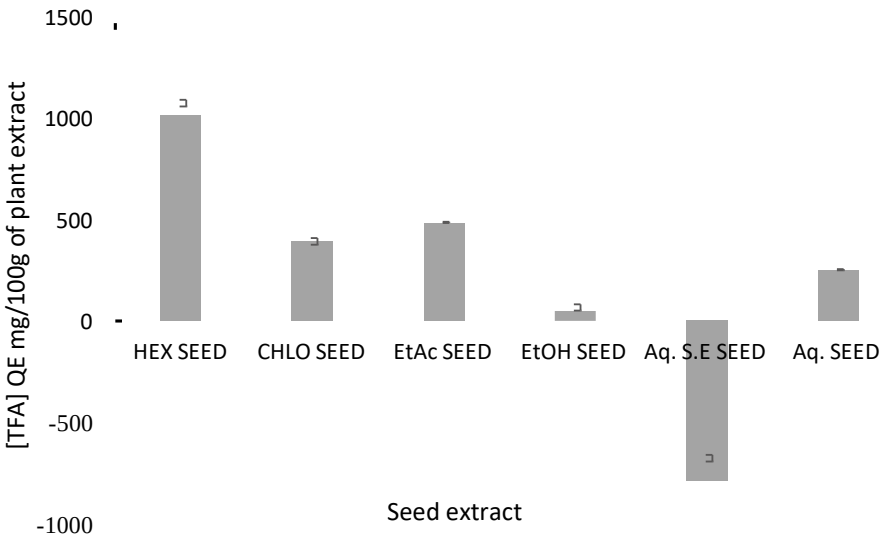

Figure 4.2.3b: Total flavonoid content of seed extracts

The seed extracts possess little or no flavonoid content except for the hexane extract which exhibited significant flavonoid content.

4.3 *In vitro* cell viability assay

4.3.1 Cytotoxic effect of extracts on Jurkat cell line

The anti-proliferative effect of the extracts on the leukemic cell line, Jurkat, was evaluated as shown in Figures 4.3.1a – 4.3.1g. Six of the extracts showed significant cytotoxicity differences comparative to the positive control, curcumin. The hexane mesocarp extract exhibited the highest anti-proliferative effect on this cell line (IC_{50} = 154.36 ± 0.92 µg/ml, $p < 0.05$) followed by the chloroform seed extract (IC_{50} = 194.38 ± 0.67 µg/ml, $p < 0.05$), and the chloroform mesocarp extract (IC_{50} = 198.25 ± 2.12 µg/ml, $p < 0.05$). The ethanolic, hexane and ethylacetate seed extracts also exhibited significant cytotoxic activity on the Jurkat cell line. However, the remaining six extracts (aqueous and aqueous (SE) seed, ethylacetate, ethanolic, aqueous and aqueous (SE) mesocarp) did not show significant cytotoxic activity on the cells over the concentration range used (0 – 1000 µg/ml).

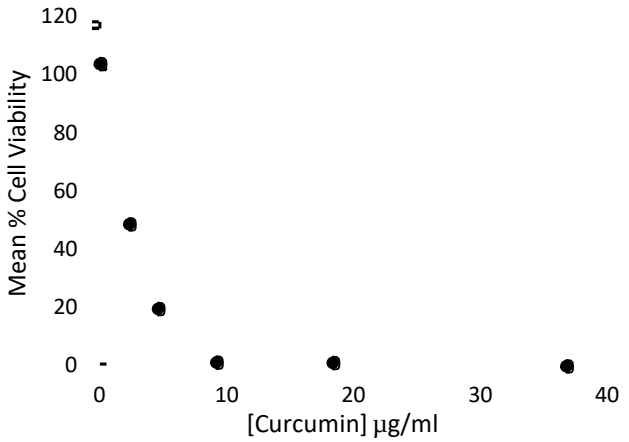

Figure 4.3.1a: Cytotoxic effect of curcumin on Jurkat cell line

Each test point is a mean ± SD of three experiments. There exists an inverse relationship between curcumin concentration and percent viability of cells.

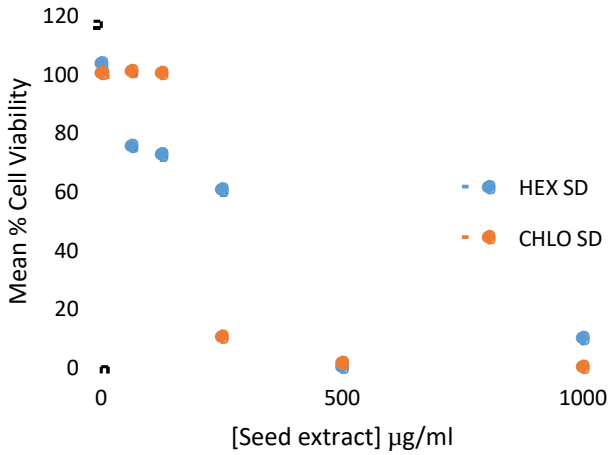

Figure 4.3.1b: Cytotoxic effect of hexane and chloroform seed extracts on Jurkat cell line

Each point on the chart is the mean ± SD of three experiments. The curves are generally shown to be concentration-dependent; percent cell viability decreases as extract concentration increases. The chloroform seed extract is more cytotoxic to the cells than the hexane seed extract.

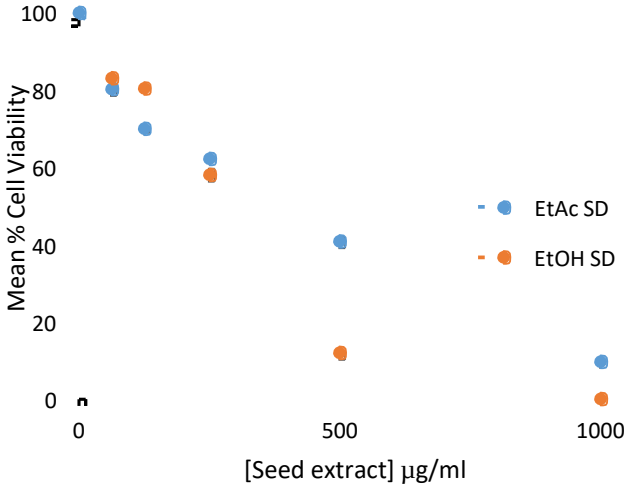

Figure 4.3.1c: Cytotoxic effect of ethyl acetate and ethanolic seed extracts on Jurkat cell line

Each point on the chart is the mean ± SD of three experiments. The curves are generally shown to be concentration-dependent; percent cell viability decreases as extract concentration increases. The ethanolic seed extract is more cytotoxic to the cells than the ethylacetate seed extract.

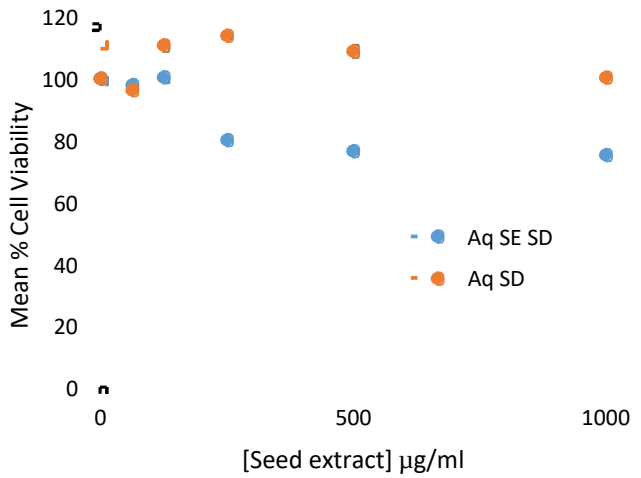

Figure 4.3.1d: Cytotoxic effect of aqueous and sequentially extracted aqueous seed extracts on Jurkat cell line

Each point on the chart is the mean ± SD of three experiments. The AQ SD extract did not inhibit the growth of Jurkat cells. However, AQ SE SD slightly inhibited the growth of Jurkat cells.

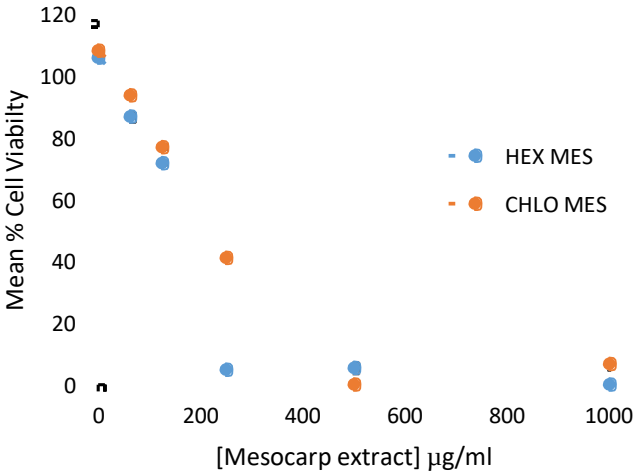

Figure 4.3.1e: Cytotoxic effect of hexane and chloroform mesocarp extracts on Jurkat cell line

Each point on the chart is the mean ± SD of triplicate experiments. The curves are generally shown to have a concentration-dependent pattern; percent cell viability decreases as extract concentration increases. The hexane mesocarp extract is more cytotoxic to the cells than the chloroform mesocarp extract.

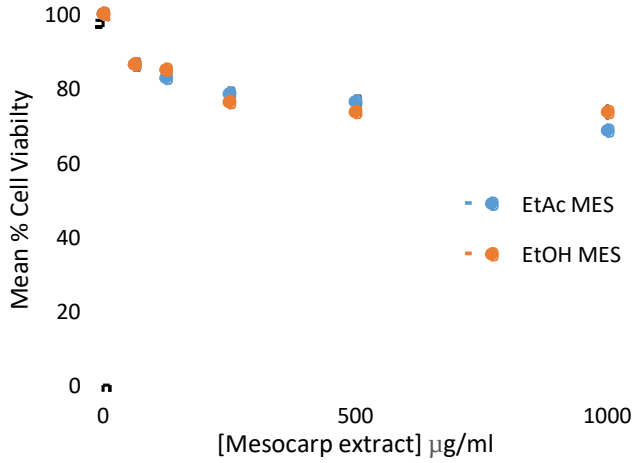

Figure 4.3.1f: Cytotoxic effect of ethyl acetate and ethanolic mesocarp extracts on Jurkat cell line

Each point on the chart is the mean ± SD of triplicate experiments. Both extracts did not significantly inhibit the growth of Jurkat cells.

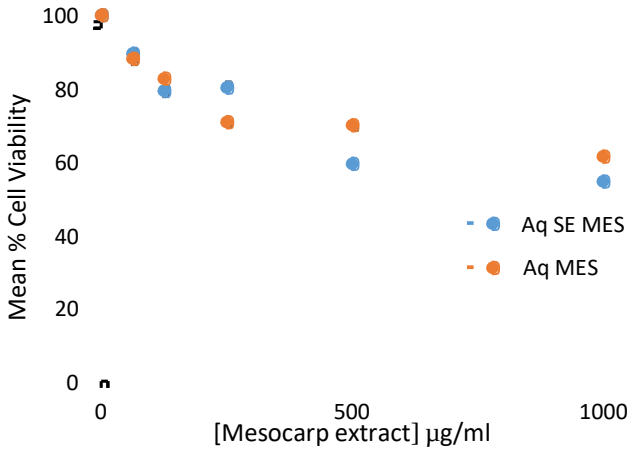

Figure 4.3.1g: Cytotoxic effect of aqueous and sequentially extracted aqueous mesocarp extracts on Jurkat cell line

Each point on the chart is the mean ± SD of triplicate experiments. Both extracts did not significantly inhibit the growth of Jurkat cells.

4.3.2 Cytotoxic effect of extracts on HL-60 cell line

The anti-proliferative effect of the extracts on the leukemic cell line, HL-60 was evaluated as shown in Figures 4.3.2a – 4.3.2g. Seven of the extracts showed significant cytotoxicity differences comparative to the positive control, curcumin. The ethanolic seed extract showed the highest cytotoxicity (IC_{50} = 65.22 ± 2.99 µg/ml, $p < 0.05$) followed by the chloroform seed extract (IC_{50} = 92.74 ± 1.65 µg/ml, $p < 0.05$), and the hexane mesocarp extract (IC_{50} = 98.02 ± 2.29 µg/ml, $p < 0.05$). The ethylacetate and hexane seed extracts, as well as the chloroform and aqueous mesocarp extracts also exhibited significant cytotoxic activity on the HL-60 cell line. All the other extracts (aqueous and aqueous sequentially extracted seed, ethylacetate, ethanolic and aqueous sequentially extracted mesocarp) however did not show significant cytotoxic activity on the cells.

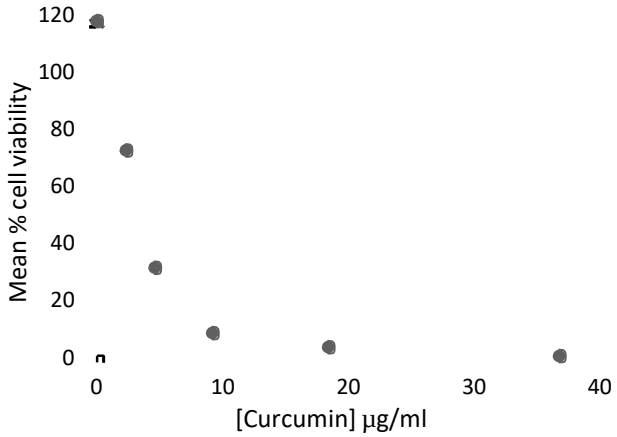

Figure 4.3.2a: Cytotoxic effect of curcumin on HL-60 cell line

Each point on the chart is the mean ± SD of three experiments. There exists an inverse relationship between curcumin concentration and percent viability of cells.

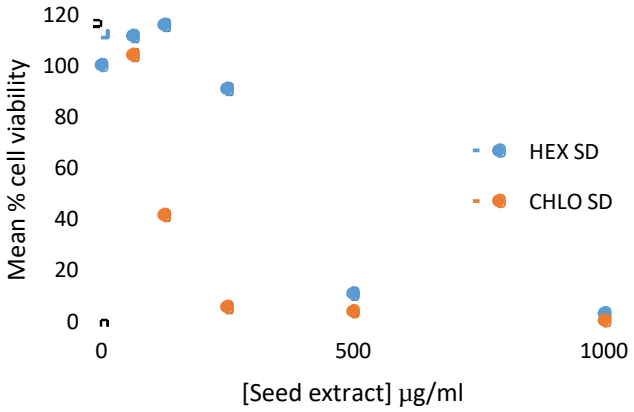

Figure 4.3.2b: Cytotoxic effect of hexane and chloroform seed extracts on HL-60 cell line

Each point on the chart is the mean ± SD of triplicate experiments. The curves are generally shown to be concentration-dependent; percent cell viability decreases as extract concentration increases. The chloroform seed extract is more cytotoxic to the cells than the hexane seed extract.

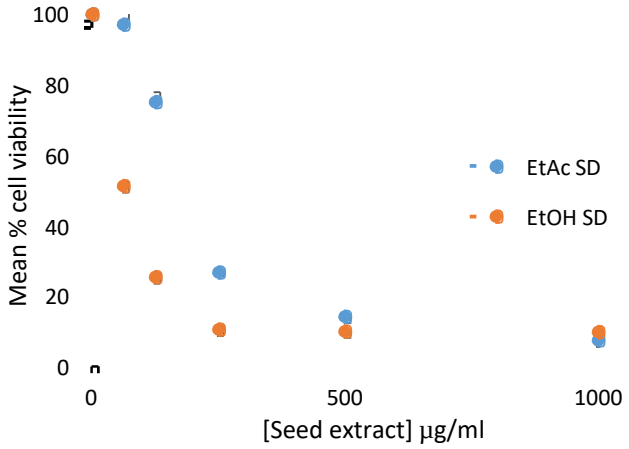

Figure 4.3.2c: Cytotoxic effect of ethyl acetate and ethanolic seed extracts on HL-60 cell line

Each point on the chart is the mean ± SD of triplicate experiments. The curves are generally shown to be concentration-dependent; percent cell viability decreases as extract concentration increases. The ethanolic seed extract is more cytotoxic to the cells than the ethylacetate seed extract.

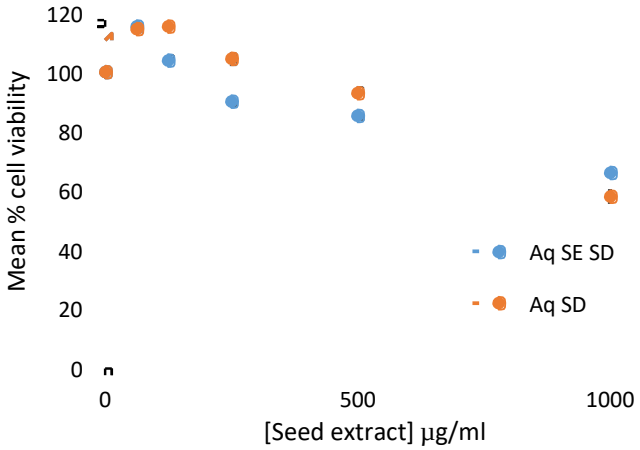

Figure 4.3.2d: Cytotoxic effect of aqueous and sequentially extracted aqueous seed extracts on HL-60 cell line

Each point on the chart is the mean ± SD of three experiments. Both extracts did not significantly inhibit the growth of HL-60 cells.

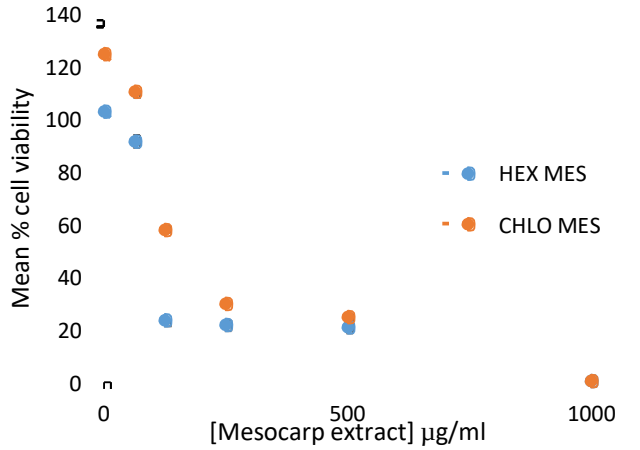

Figure 4.3.2e: Cytotoxic effect of hexane and chloroform mesocarp extracts on HL-60 cell line

Each point on the chart is the mean ± SD of triplicate experiments. The curves are generally shown to have a concentration-dependent pattern; percent cell viability decreases as extract concentration increases. The hexane mesocarp extract is more cytotoxic to the cells than the chloroform mesocarp extract.

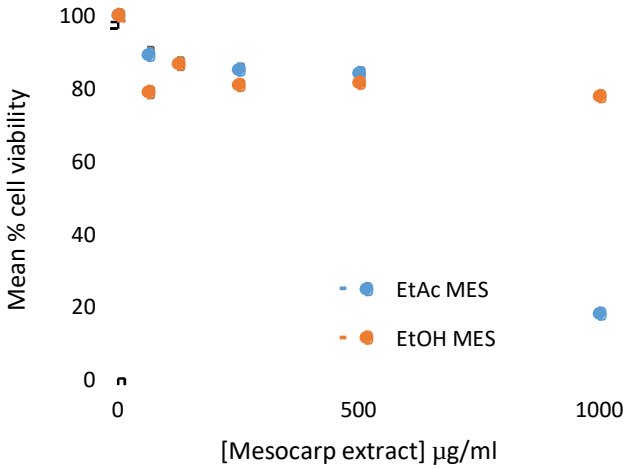

Figure 4.3.2f: Cytotoxic effect of ethylacetate and ethanolic mesocarp extracts on HL-60 cell line

Each point on the chart is the mean ± SD of triplicate experiments. The ethyl acetate mesocarp extract is shown to have greater cytotoxicity than the ethanolic mesocarp extract, which did not significantly inhibit the proliferation of HL-60 cells.

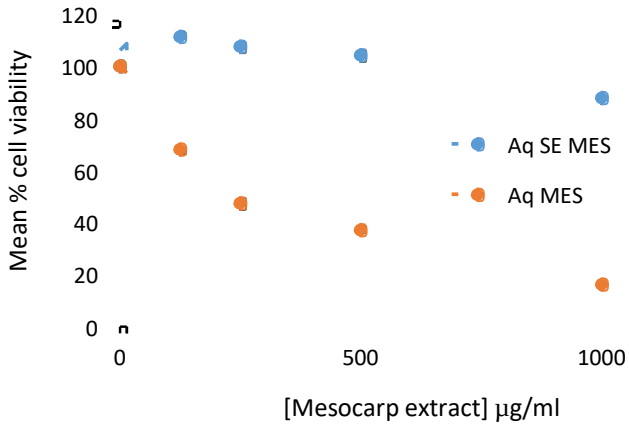

Figure 4.3.2g: Cytotoxic effect of aqueous and sequentially extracted aqueous mesocarp extracts on HL-60 cell line

Each point on the chart is the mean ± SD of triplicate experiments. The aqueous mesocarp extract is shown to have greater cytotoxicity than the aqueous (S.E) mesocarp extract, which did not significantly inhibit the proliferation of HL-60 cells.

4.3.3 Cytotoxic effect of extracts on Chang cell line

The anti-proliferative effect of the extracts on normal human Chang liver cells was evaluated as shown in Figures 4.3.3a – 4.3.3g. Six of the extracts showed significant cytotoxicity differences comparative to the positive control, curcumin. Chloroform mesocarp extract was the most toxic to these normal Chang liver cells with an IC_{50} value of 422.82 ± 8.16 µg/ml, $p < 0.05$. This was followed by the chloroform seed extract (447.43 ± 24.98 µg/ml, $p < 0.05$) and the ethyl acetate seed extract (617.10 ± 14.72 µg/ml, $p < 0.05$). The hexane and ethanolic seed extracts, as well as the hexane mesocarp extract also exhibited slightly significant cytotoxic activity on the normal Chang liver cell line. The other extracts (aqueous and aqueous SE seed, ethylacetate, ethanolic, aqueous and aqueous sequentially extracted mesocarp) however did not show significant cytotoxic activity on the normal cells.

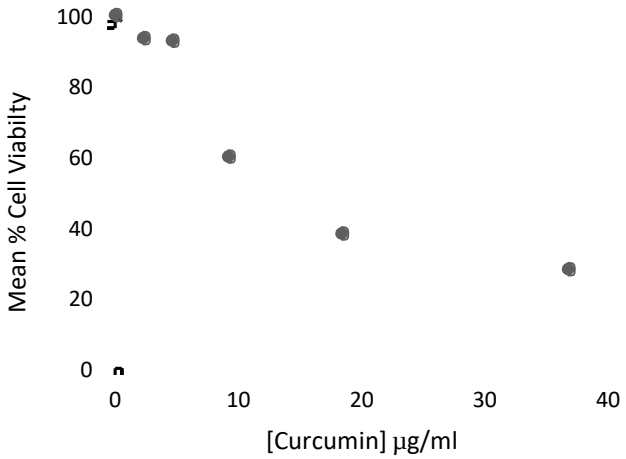

Figure 4.3.3a: Cytotoxic effect of curcumin on Chang cell line

Each point on the chart is the mean ± SD of three experiments. There exists an inverse relationship between curcumin concentration and percent viability of cells.

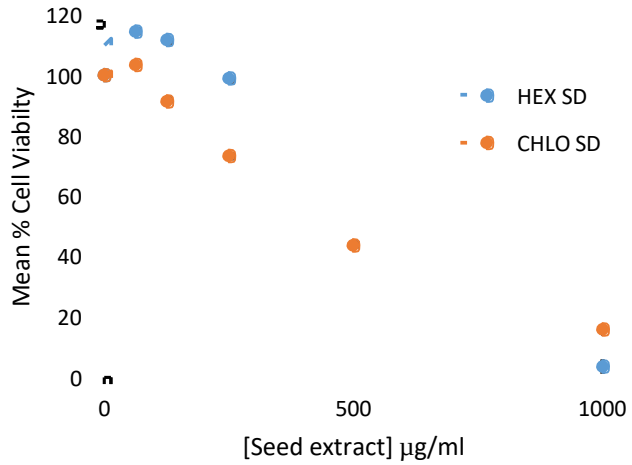

Figure 4.3.3b: Cytotoxic effect of hexane and chloroform seed extracts on Chang cell line

Each point on the chart is the mean ± SD of triplicate experiments. The curves are generally shown to be concentration-dependent; percent cell viability decreases as extract concentration increases. The chloroform seed extract is more cytotoxic to the cells than the hexane seed extract.

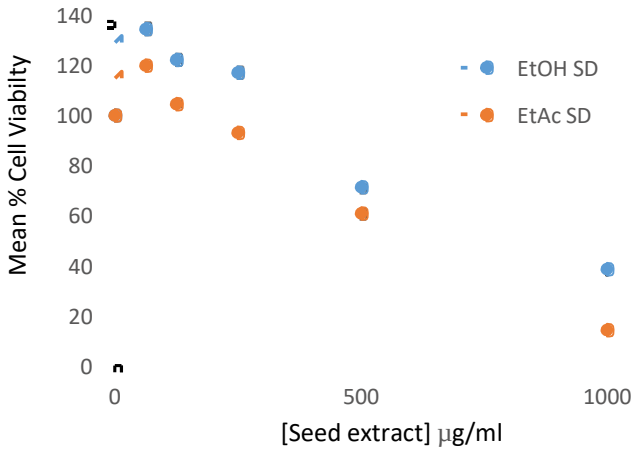

Figure 4.3.3c: Cytotoxic effect of ethyl acetate and ethanolic seed extracts on Chang cell line

Each point on the chart is the mean ± SD of triplicate experiments. The curves are generally shown to be concentration-dependent; percent cell viability decreases as extract concentration increases. The ethyl acetate seed extract is more cytotoxic to the cells than the ethanolic seed extract.

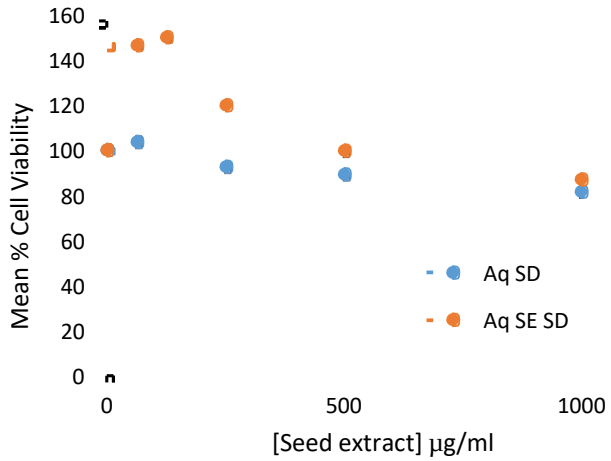

Figure 4.3.3d: Cytotoxic effect of aqueous and sequentially extracted aqueous seed extracts on Chang cell line

Each point on the chart is the mean ± SD of triplicate experiments. Both extracts did not significantly inhibit the growth of Chang cells.

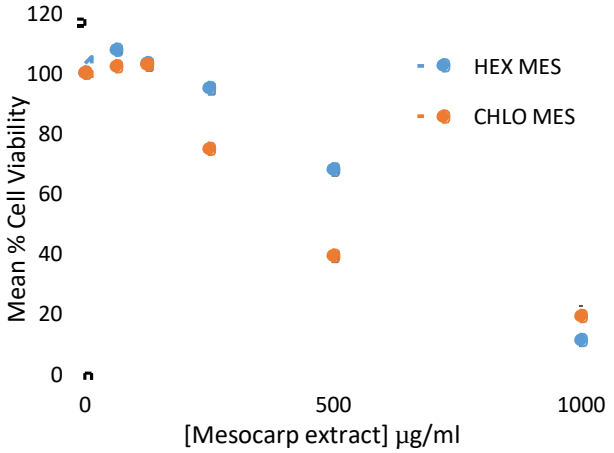

Figure 4.3.3e: Cytotoxic effect of hexane and chloroform mesocarp extracts on Chang cell line

Each point on the chart is the mean ± SD of triplicate experiments. The curves are generally shown to have a concentration-dependent pattern; percent cell viability decreases as extract concentration increases. The chloroform mesocarp extract is more cytotoxic to the cells than the hexane mesocarp extract.

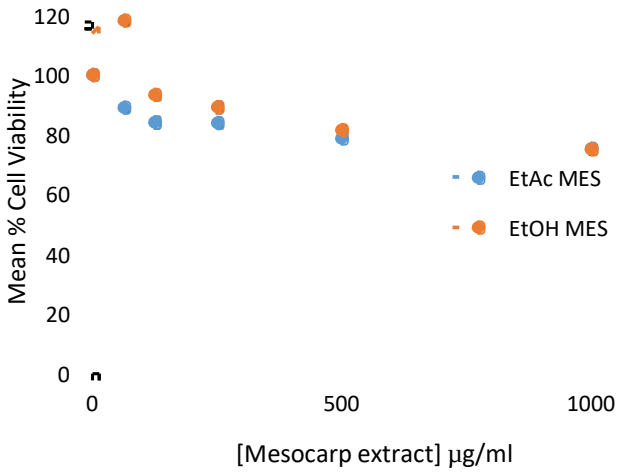

Figure 4.3.3f: Cytotoxic effect of ethylacetate and ethanolic mesocarp extracts on Chang cell line

Each point on the chart is the mean ± SD of three experiments. Both extracts did not significantly inhibit the growth of Chang cells.

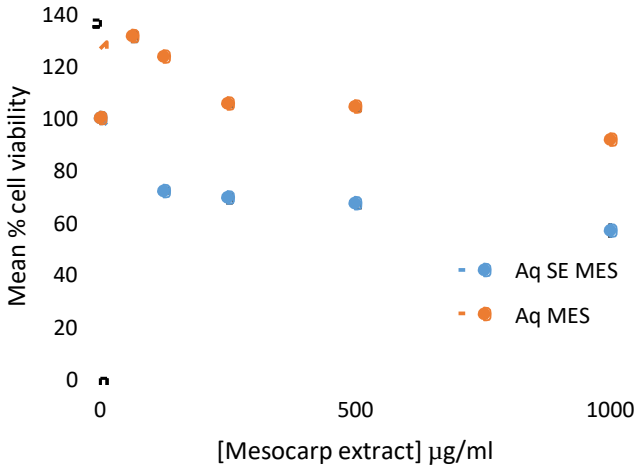

Figure 4.3.3g: Cytotoxic effect of aqueous and sequentially extracted aqueous mesocarp extracts on Chang cell line

Each point on the chart is the mean ± SD of three experiments. Aq SE MES showed stronger inhibitory activity towards the growth of Chang cells than Aq MES.

4.3.4 IC_{50} values of extracts

The IC_{50} value is the concentration of extract at which 50% of treated cells are no longer viable. The IC_{50} value of each extract on each cell line was determined using their corresponding cell viability curve. Lower IC_{50} value connotes greater cytotoxicity. For the range of concentrations used (0 – 1000 µg/ml), hexane seed extract was the most cytotoxic to Jurkat cells (154.36 ± 0.92 µg/ml), ethanolic seed extract was the most cytotoxic to HL-60 cells (65.22 ± 2.99 µg/ml) and the chloroform mesocarp extract had the lowest IC_{50} value of 422.82 ± 8.16 µg/ml for Chang cells. Extracts such as aqueous and aqueous SE seed, ethanolic and aqueous SE mesocarp did not inhibit cell growth significantly for the concentration range used, hence their IC_{50} values were concluded to be higher than the highest concentration used (>1000 µg/ml).

Table 4.3.4: Cytotoxic activity of *C. lanatus* extracts and standard, curcumin

	IC_{50} VALUES (μg/ml)		
EXTRACT	**JURKAT**	**HL-60**	**CHANG**
Curcumin	2.09 ± 0.02	3.08 ± 0.24	13.41 ± 0.60
Hexane seed	351.87 ± 15.14*	376.90 ± 0.70*	634.67 ± 22.11*
Chloroform seed	194.38 ± 0.67*	92.74 ± 1.65*	447.43 ± 24.98*
Ethylacetate seed	392.82 ± 6.60*	189.22 ± 8.16*	617.10 ± 14.72*
Ethanolic seed	284.02 ± 7.93*	65.22 ± 2.99*	824.64 ± 19.11*
Aqueous (SE) seed	> 1000	> 1000	> 1000
Aqueous seed	> 1000	> 1000	> 1000
Hexane mesocarp	154.36 ± 0.92*	98.02 ± 2.29*	656.08 ± 14.99*
Chloroform mesocarp	198.25 ± 2.12*	129.14 ± 3.03*	422.82 ± 8.16*
Ethylacetate mesocarp	> 1000	757.91 ± 1.99*	> 1000
Ethanolic mesocarp	> 1000	> 1000	> 1000
Aqueous (SE) mesocarp	> 1000	> 1000	> 1000
Aqueous mesocarp	> 1000	236.73 ± 24.79*	> 1000

Each determination is the mean ± SD of triplicate experiments. Values with * connote significant difference ($p < 0.05$) in cytotoxicity in comparison to curcumin, the positive control.

4.3.5 Selectivity indices of extracts

The selectivity index (SI) of each extract was calculated as a ratio of normal cell IC_{50} value to cancer cell IC_{50} value. The hexane mesocarp extract exhibited the greatest SI for Jurkat cells (4.51) followed by the ethanolic seed extract (2.90). For HL-60 cells, the ethanolic seed extract possessed the the greatest SI (12.64) and this is about 3-fold higher than SI for curcumin, the positive control (4.35). Additionally, the hexane mesocarp and chloroform seed extracts superseded the SI of curcumin with values of 6.69 and 4.82, respectively. Extracts with no calculable IC_{50} value over the concentration range tested (0 – 1000 µg/ml) for any of the cancer cells did not have an SI determined for that cancer cell line.

Table 4.3.5: Selectivity indices of *C. lanatus* extracts and standard, curcumin

	Selectivity indices	
EXTRACT	**JURKAT**	**HL-60**
Curcumin	6.42	4.35
Hexane seed	1.80	1.68
Chloroform seed	2.30	4.82
Ethylacetate seed	1.57	3.26
Ethanolic seed	2.90	12.64
Aqueous (SE) seed	N/A	N/A
Aqueous seed	N/A	N/A
Hexane mesocarp	4.51	6.69
Chloroform mesocarp	2.13	3.27
Ethylacetate mesocarp	N/A	1.32
Ethanolic mesocarp	N/A	N/A
Aqueous (SE) mesocarp	N/A	N/A
Aqueous mesocarp	N/A	4.22

NB: N/A means Not Applicable

CHAPTER FIVE

5.0 DISCUSSION

As a prevalent disease, cancer remains a public health menace globally. Irrespective of strides made in the management of the disease, there are still challenges faced by clinicians. Discovering drugs has risen to become a significant endeavour to overcome many life-claiming ailments. In lieu of this, substantial awareness has been raised and this has sparked the interest of scientists and pharmaceutical enterprises in unearthing novel anticancer agents, particularly from natural or medicinal plants. These medicinal plants may comprise antioxidants which scavenge ROS that can cause cancer.

The study was aimed at evaluating the antioxidant and anti-leukemic activities of *C. lanatus*. Hexane, chloroform, ethylacetate, ethanolic and aqueous extracts of the seed and mesocarp were used. *Citrullus lanatus* extracts have been reported to comprise a variety of phytochemicals including phenolics and flavonoids (Melo *et al*., 2006; Oseni and Okoye, 2013; El Zawawy, 2015). The findings of this study align with these conclusions earlier made as the ethyl acetate, ethanolic and aqueous seed extracts as well as ethyl acetate mesocarp extract exhibited significant phenolic content shown in Figures 4.2.2a and 4.2.2b. These phenol-containing extracts aforementioned were also observed to possess significant total antioxidant activity (Figures 4.2.1b, 4.2.1c, 4.2.1f and Table 4.2.1). This can be explained by the reasoning of Hassan *et al*. (1998) that phenolic compounds can undergo redox action hence can be reducing agents, neutralizers of singlet oxygen and hydrogen donors which are of relevance in free radical scavenging. The total phenolic content of an extract is thus a good indicator of its antioxidant potency (Iqbal *et al*., 2005).

As determined by Rolim *et al*. (2018), aqueous extracts of cantaloupe seeds possessed a total phenolic content of 75.2 ± 7.39 mg gallic acid equivalent/ 100g of extract. From this study, it was determined that the aqueous extract of *C. lanatus* seeds possessed a total phenolic content of 2058.04 ± 67.04 mg gallic acid equivalent/ 100g of extract. Also, the ethanolic extract of *Ficus religiosa* fruit (sacred fig) was shown to possess a total phenol content of 2670 ± 0.16 mg gallic acid equivalent/ 100g of extract dry weight (Sultana, Anwar and Ashraf, 2009). The ethanolic mesocarp extract of the *C. lanatus* fruit according to this study did not possess any significant phenolic content (-51.07 ± 20.10 mg gallic acid equivalent/ 100g of extract). The cantaloupe and *C. lanatus* both belong to the *Cucurbitaceae* family. The sacred fig on the other hand belongs to

the *Moraceae* family. These findings suggest that plant extracts from different families may contain differential amounts of phenolic compounds.

As one of the most common phytochemicals in plants, flavonoids function in biological systems to aid in blood clotting, and also possess antioxidant, antiallergenic, anti-inflammatory and antitumor activity (Bouallagui *et al*., 2011; Alirezaei *et al*., 2014). They have also been proven to prevent oxidative stress, possess strong anticancer property and offer protection against carcinogenesis (Sodipo and Akiniyi, 2000). This study determined that only the chloroform mesocarp extract in addition to the hexane, chloroform, ethyl acetate and aqueous seed extracts possessed significant total flavonoid content as shown in Figures 4.2.3a and 4.2.3b. The total flavonoid content of chloroform and hexane fractions of *Ficus sycomorus* fruit were determined to be 0.477 ± 0.52 and 0.100 ± 0.09 mg quercetin equivalent/ 100 g of extract, respectively (Al-matani *et al*., 2015). This study showed the total flavonoid content of chloroform mesocarp, chloroform seed and hexane seed extracts of *C. lanatus* fruit to be 807.046 ± 119.46, 395.029 ± 47.23 and 1013.824 ± 110.56, respectively. This shows the relative enhanced flavonoid composition of the chloroform and hexane extracts of *C. lanatus*.

Generally, the organic extracts exhibited stronger activity than aqueous extracts in this study. This could be due to the fact that phytochemicals are more soluble in organic solvents (Djeridane *et al.*, 2006). This solubility phenomenon has been observed in other studies too. Chatha *et al*. (2006) reported the presence of phytochemicals were more in organic extracts than aqueous extracts of rice bran. Singh *et al*. (2016) also reported that phytochemicals including tannins and carotenoids from watermelon and ash gourd were soluble in organic solvents and insoluble in water.
Additionally, the study showed the seed extracts generally possessed stronger total antioxidant activity than the mesocarp extracts. This is because the phenolic and flavonoid contents were generally more pronounced in seed extracts, and aligns with the assertion that these phenolic and flavonoid compounds have been known to infer antioxidant activity (Alirezaei *et al*., 2014).

The total antioxidant ability of the extracts in this study was generally observed to be in a concentration-dependent manner for most extracts as compared to positive control, BHT. This is in agreement with observations from other studies such as Acheampong *et al*. (2015) and El Zawawy (2015).

There are various studies that have validated the correlation between antioxidant and cell growth inhibitory activities of medicinal plant compounds (Lee *et al*., 2012). According to Acheampong *et al*. (2015), antioxidants serve as anticancer agents. Chon *et al*., (2009) also determined there exists a strong association between the total phenolic content of extracts and their antitumor activity. This is not surprising as the ethanolic seed extract possessed the highest total content of phenols and demonstrated the strongest antiproliferative activity against the HL-60 cells, as shown in Figures 4.2.2a and 4.3.2c. Contrary to this, the chloroform mesocarp, chloroform seed and hexane mesocarp extracts of *C. lanatus* were shown in this study to be cytotoxic although they did not exhibit significant total antioxidant activity (Tables 4.2.1 and 4.3.4). This means the extracts exerted their growth inhibitory effect via other mechanism(s). They may have played a role in inducing cell death by triggering the release of apoptotic signals, or inhibiting the release of growth signals. The cytotoxic effect was observed to be in a concentration-dependent manner for most extracts as compared to positive control, curcumin.

The overgrowth of HL-60 cells for the negative control (concentration = 0 µg/ml) of Figure 4.3.2a may have been as a result of the absence of curcumin in those test wells. HL-60 cells thus were not inhibited over the 72 h test period. For the aqueous mesocarp extract (sequentially extracted), the lower concentrations yielded an overgrowth of HL-60 cells as shown in Figure 4.3.2g. This may have been as a due to the inability of these concentrations to inhibit cell growth over the 72 h test period. Although the aqueous seed, ethanolic mesocarp and aqueous mesocarp extracts did not show significant cytotoxicity in any of the three cell lines used as shown in Table 4.3.4, their corresponding % cell viabilities were shown in Figures 4.3.1-d, -f and -g, 4.3.2-d, -f and –g, and 4.3.3-d, -f and -g to be slightly reduced after the 72h incubation period. This reduction of cell viability may have been due to competition between the cells for survival, resulting in cell death. Curcumin inhibits glutathione s-transferase enzymes (GSTs) which function to catalyze detoxification of cells and mopping up of ROS using glutathione as a cofactor Also, curcumin is known to inhibit the signal transducer and activator of transcription 3 (STAT3) and nuclear factor kappa B (NF-κB) signaling pathways which function to promote cell growth and survival (Vallianou *et al*., 2015). High levels of GSTs and uncontrollable activation of the STAT3 and NF-κB pathways have been implicated in cancer cell growth. It can therefore be said that curcumin inhibits the growth of the cancer cells via its inhibition of GSTs leading to the accumulation of

ROS, which cause oxidative stress and overwhelms the cells. The pattern of cytotoxic activity exhibited by ethanolic seed and ethyl acetate seed extracts were observed to be very similar to that of standard, curcumin. This suggests the ethanolic seed and ethyl acetate seed extracts may function via either or both mechanisms by which curcumin inhibits the growth of cancer cells.

Extracts with significant cytotoxicity to cancer cells but reduced toxicity to normal cells are tagged as promising anticancer agents (Al-Rashidi *et al*., 2011). A selectivity index ≥ 2 connotes a good anticancer agent (Badisa *et al.*, 2009). As shown in Table 4.3.4, the chloroform and ethanolic seed extracts, as well as the hexane and chloroform mesocarp extracts had SI >2 for both Jurkat and HL-60 cells.

According to the findings of this study, the ethanolic seed extract is the most promising cancer therapeutic agent for acute myelogenic leukemia as it had the greatest SI of 12.64 for HL-60 cells. The hexane mesocarp extract on the other hand is the most promising cancer therapeutic agent for T-lymphocyte leukemia as it had the highest SI of 4.51 for Jurkat cells.

Approaches for prevention of cancer by natural foods, especially of plant sources, are deemed as one of the essential modes for cancer control (Li *et al*., 2012). Cucurbits, especially those with palatable fruits are an economically significant family of plants (Singh *et al*., 2016). These include the *C. lanatus* fruit which is of a high nutritional value. Food plants which exhibit good and diverse medicinal properties may be considerable sources of nutraceuticals.

CHAPTER SIX

6.0 CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.1 CONCLUSION

The study demonstrated that five of the *C. lanatus* extracts possess significant antioxidant activity. The seeds extracts generally had a higher capacity as they contain more phenols and flavonoids than the mesocarp. Cytotoxic activity consequently was significant for most *C. lanatus* extracts and was more pronounced in seeds than mesocarp. However, only five of the extracts exhibited good selective toxicity to the cancer cells, hence may be exploited as a source of novel anticancer agent or nutraceutical.

6.2 LIMITATIONS

Finances served as a constraint as the reagents and disposables were costly. It also served as a barrier to performing apoptotic inductive studies and antioxidant animal work using the extracts.

6.2 RECOMMENDATIONS

The use of *C. lanatus* rind and whole fruit for antioxidant and cytotoxic analysis should be evaluated to complement the findings of this study. The antioxidant activity of potent extracts should be experimented in animal models too. Additionally, isolation and characterization of bioactive compounds from potent *C. lanatus* extracts, as well as the investigation into the apoptotic inductive mechanism is highly recommended.

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APPENDICES

APPENDIX 1

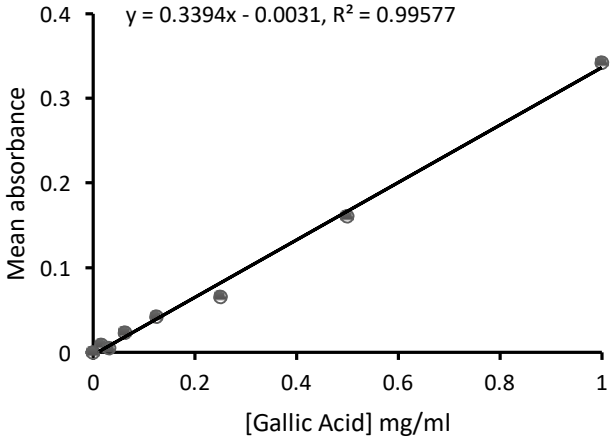

Figure A1: Gallic acid standard curve

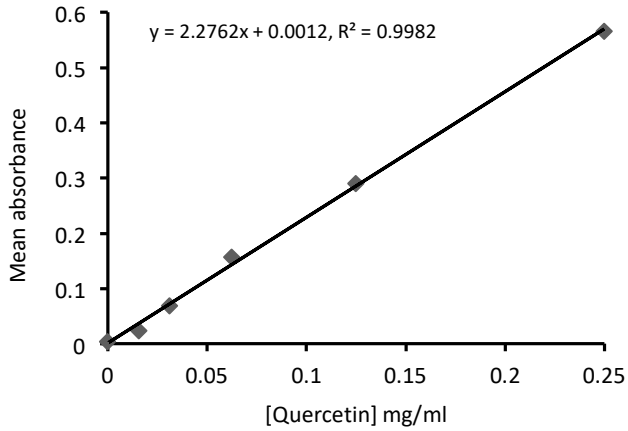

Figure A2: Quercetin standard curve

APPENDIX 2

UNIVERSITY OF GHANA
COLLEGE OF HEALTH SCIENCES
ETHICAL AND PROTOCOL REVIEW COMMITTEE

Ref. No.:.....

23rd November, 2017

Pearl Afram Ofosu-Aikins
Dept. of Chemical Pathology
SBAHS
Korle –Bu.

ETHICAL CLEARANCE

Protocol Identification Number: **CHS-Et/M.2 – P1.5/2017-2018**

The Ethical and Protocol Review Committee of the College of Health Sciences on the 2nd of November, 2017 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Evaluation of antioxidant and cytotoxic activity of *Citrullus lanatus* extracts and compounds"**

PRINCIPAL INVESTIGATOR: **Pearl Afram Ofosu-Aikins**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

This ethical clearance is valid till 25nd November, 2018.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed: .....
PROFESSOR ANDREW A. ADJEI
CHAIRPERSON, ETHICAL AND PROTOCOL REVIEW COMMITTEE

cc: Provost, CHS
Dean, SBAHS
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Figure A3: Ethical clearance obtained from the Ethical and Protocol Review Committee, College of Health Sciences, University of Ghana