

ANTI-CANCER POTENTIAL OF ETHANOLIC AND WATER
LEAVES EXTRACTS OF *Annona muricata* (GRAVIOLA)

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DECLARATION

This thesis is my original work and has not been submitted to any other University for Examination.

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DEDICATION

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ABSTRACT

Cancer is a major cause of mortality and morbidity globally and overall survival rate is still low despite advances in surgery, radiotherapy and chemotherapy. Plants have played a significant role in the treatment of cancer and infectious diseases for a very long time. *Annona muricata* has been widely used in the treatment of cancer and diseases in many countries. However, there had been no documented scientific study to validate the medicinal importance of *Annona muricata*, despite its wide usage in Uganda. The objective of this study was to determine the phytochemical composition, antioxidant and cytotoxic properties of ethanolic and water leaves extracts of *Annona muricata*. Phytochemical analysis was conducted using standard qualitative methods and a Chi-Square Goodness of fit test was used to assign the relative abundance of the different phytochemicals. Gas Chromatography Mass Spectroscopy Analysis was also conducted. The antioxidant activity was determined using the DPPH and reducing power methods whereas the *in vitro* cytotoxic activity was determined using three different cell lines. Phytochemical screening of the extracts showed them to be rich in secondary class metabolite compounds of alkaloids, saponins, terpenoids, flavonoids, coumarins and lactones, anthraquinones, tannins, Cardiac glycosides, phenols and phytosterols. Total phenolic compounds in the water extract were 683.69 ± 0.09 $\mu\text{g/ml}$ Gallic Acid Equivalent while it was 372.92 ± 0.15 $\mu\text{g/ml}$ Gallic Acid Equivalent in the ethanolic extract. Using Gas Chromatography Mass Spectroscopy Analysis, the ethanolic leaves extract of *Annona muricata* showed 25 constituents of which 12 of the compounds were identified. The major constituents were two unidentified compounds with percentage peak areas of 23.51% and 16.8%. Of the identified compounds, the outstanding in composition were 7-Tetradecenal, (Z) (peak area 9.39%), n-Hexadecanoic acid (peak area 7.12%), Oleryl Alcohol (peak area 6.15%), Phytol (peak area 5.61%), cis, cis, cis-7,10,13-Hexadecatrienal (peak area 4.26%), 2-Pentadecanol (peak area 3.93%), 9,12-Octadecadienoic acid, ethyl ester (peak area 3.21%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (peak area 2.67%), and 1,E-11,Z-13-Octadecatriene (peak area 2.15%), while the rest had less than 2% composition by peak area. The reducing power was 216.41 $\mu\text{g/ml}$ in the ethanolic extract and 470.51 $\mu\text{g/ml}$ Gallic Acid Equivalent in the water extract. *In vitro* antioxidant activity IC_{50} was 2.0456 mg/ml and 0.9077 mg/ml for ethanolic and water leaves extracts of *Annona muricata* respectively. The ethanolic leaves extract was found to be selectively cytotoxic *in vitro* to cancer cell lines (EACC, MDA and SKBR3) with IC_{50} Values of 335.85 $\mu\text{g/ml}$, 248.77 $\mu\text{g/ml}$, 202.33 $\mu\text{g/ml}$ respectively, while it had no cytotoxic effect on normal spleen cells. The data also showed that water leaves extract of *Annona muricata* had no cytotoxic effect at all tested concentrations. TLC fractionation of the ethanolic leaves extracts revealed 11 fractions and tested for the different activities, of which 4 fractions had the best cytotoxic activity. The results showed that *Annona muricata* possesses antioxidant and cytotoxic activities that need to be studied further so as to establish the suitable uses of this plant in cancer therapy.

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ABBREVIATIONS

| | | |
|------------------------|---|---|
| DNA | : | Deoxyribonucleic Acid |
| DPPH | : | 1, 1-diphenyl-2-picrylhydrazyl |
| EACC | : | Ehrlich Ascites Carcinoma Cells |
| EEAM | : | Ethanollic Extract of <i>Annona muricata</i> |
| GAE | : | Gallic Acid Equivalent |
| GC-MS | : | Gas Chromatography Mass Spectroscopy |
| IARC | : | International Agency for Research on Cancer |
| IC₅₀ | : | Half Maximal Inhibitory Concentration |
| L. | : | Linnaeus |
| MTT | : | 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl--tetrazolium bromide |
| OD | : | Optical Density |
| PBS | : | Phosphate Buffer Saline |
| RSA | : | Radical Scavenging Activity |
| SDS | : | Sodium Dodecyl Sulfate |
| TBEA | : | Trypan Blue Exclusion Assay |
| TIC | : | Total Ion Chromatogram |
| TLC | : | Thin Layer Chromatography |
| WHO | : | World Health Organization |

CHAPTER 1: INTRODUCTION

1.1 General Introduction

Cancer is defined as the state characterized by the uncontrolled growth and invasion of normal tissues and spread of cells (Yarbro *et al.*, 2005; Latosińska and Latosińska, 2013). Atawodi, 2011 noted that cancer is a debilitating disease that has afflicted a good proportion of the world population in all generations. According to recent estimates by the WHO (GLOBOCAN, 2008; Atawodi, 2011), annual cancer incidence in sub-Saharan Africa is 551,200 with a mortality of 421,000. Furthermore, WHO reports cancer as a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths) in 2008 (Ferlay *et al.*, 2010). About 70% of all cancer deaths occur in low- and middle-income countries. The deaths from cancer worldwide are projected to continue rising, reaching an estimated 13.1 million in 2030. The number of all cancer cases around the world was estimated to be 12.7 million in 2008 and are expected to increase to 21 million by 2030. Approximately one in five people below the age of 75 will suffer from cancer during their lifetime, while one in ten in this age range is predicted to die due to cancer (Ferlay *et al.*, 2010; Latosińska and Latosińska, 2013). The number of classified carcinogens has increased from 50 in 1987 to 108 in 2012. Although it seems small, this number continuously increases with the evidence of new (probably and possibly) carcinogens (64 and 271 in 2012) to humans (IARC, 2013).

Thirumal *et al.*, 2012 noted that plants are storehouses of “pre-synthesized” molecules that act as lead structures, which can be optimized for new drug development. In practice, a large number of cancer chemotherapeutic agents that are currently available in the market can be traced back to their plant source. Plant derived compounds; in particular have gained importance in anticancer therapy. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development (Thirumal *et al.*, 2012)

Annona muricata L commonly known as Graviola or Soursop belongs to the family of Annonaceae and is the most tropical semi deciduous tree with the largest fruits of the *Annona* genus. It is a typical tropical tree with heart shaped edible fruits and widely distributed and native to Sub-Saharan Africa countries that lie within the tropics including Uganda. The leaves are lanceolate with glossy and dark green in color and had been traditionally used to treat headaches, hypertension, cough, asthma and used as antispasmodic, sedative and nervine for heart condition (Lans, 2006; Hamizah *et al.*, 2012) as well as cancer.

1.2 Problem statement

Molecular targeted agents are currently being studied in all cancer treatment settings including that of chemoprevention, which is defined as the use of natural or synthetic non-essential dietary agents to interrupt the process of carcinogenesis and to prevent or delay tumor growth (Shukla and Pal, 2004; Alagammal *et al.*, 2013). Herbal medicines have been used since the dawn of civilization to maintain health and to treat various diseases. There is an importance to use and scientifically authenticate more medicinally useful herbal products. Many orthodox medicines had been developed from plant and animal materials. These drugs include: chloroquine, artemisinin, nicotine, quinine, among others. Traditional medicinal products no doubts, present the cheapest and easily affordable therapy especially in Uganda and other developing countries of the world. It is hope that traditional medicine will in future provide the cure to many tropical diseases that have defied orthodox prescriptions. *Annona muricata* L is widely used in the traditional treatment of cancer in many countries such as Amazonia, Barbados, Borneo, Brazil, Cook Islands, Curacao, Dominica, Guatemala, Guam, Guyana, Haiti, Jamaica, Madagascar, Malaysia, Peru, Suriname, Togo and West Indies (Schultes and Raffauf; 1990, Morton; 1980, Holdsworth; 1980, and Haddock; 1974) as well as in Uganda. Most parts used are the leaves, bark, roots and fruit seeds. There had been no reported scientific study to validate the anti-cancer potential of *Annona muricata* in the Eastern Africa, despite its wide usage in many countries particularly in Uganda. Therefore, scientific studies of leaves extracts from this plant had to be undertaken in order to establish its potential use as an alternative medicine in the prevention and treatment of cancer in Uganda and other countries.

1.3 Justification

The worldwide use of natural products including medicinal plants has become more and more important in primary health care especially in developing countries. Many pharmacognostical and pharmacological investigations are carried out to identify new drugs or to find new lead structures for the development of novel therapeutic agents for the treatment of human diseases such as cancer and infectious diseases (Newman *et al.*, 2003). In developing countries and particularly in Uganda, a large segment of the population still rely on complementary medicine to treat serious diseases including infections, cancers and different types of inflammations. The current available methods for treatment of cancer are chemotherapy, radiation, and surgery, all of which can induce significant side effects (Mishra *et al.*; 2013). Phytochemicals are relatively safe and abundantly available from dietary sources. Undertaking a scientific evaluation study of the anti-cancer potential of ethanolic and water leaves extracts of *Annona muricata* would help in improving the prevention and treatment of cancer using alternate medicine therapy in the high risk population who at the same time have very little or limited access to modern hospitals and treatments.

1.4 Objectives

1.4.1 General objective

To determine the anti-cancer potential of Ethanolic and water leaves extracts of *Annona muricata*

1.4.2 Specific objectives

- i) To determine the phytochemical composition of ethanolic and water leaves extracts of *Annona muricata*.
- ii) To determine the antioxidant activity of the ethanolic and water leaves extracts of *Annona muricata*.
- iii) To establish the *in vitro* cytotoxic activity of ethanolic and water leaves extracts of *Annona muricata* on selected cancer cell lines.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

2.1.1 The global burden

Cancer is a major public health burden in both developed and developing countries. It is both a national and international health problem. WHO reports show constant rates of mortality from cancer over more than the last six decades (1950–2010) caused by various types of malignancy. Cancer is still the leading cause of death worldwide, regardless of the discovery of several dozens of novel anti-cancer drugs both natural and synthetic. It is a multifactorial, multifaceted and multimechanistic disease requiring a multidimensional approach for its treatment, control and prevention (Jemal *et al.*, 2003).

The major causes of cancer are smoking, dietary imbalances, hormones and chronic infections leading to chronic inflammation (Ames *et al.*, 1995). Age is also a primary risk factor for most cancers, with about 77% of all cancers diagnosed among people aged 55 or older. Breast cancer is the most common form of cancer in women worldwide (Koduru *et al.*, 2007). According to an estimate, 50% of breast cancer and 37% of prostate cancer patients use herbal products (Richardson, 2001). Several chemopreventive agents are used to treat cancer, but they cause toxicity that prevents their usage (Kathiresan *et al.*, 2006). In this context, the natural products derived from medicinal plants have gained significance in the treatment of cancer (Gurib-Fakim, 2006).

2.1.2 Current treatment approaches and use of plants in cancer therapy

Every year 2-3 % of deaths recorded worldwide arise from different types of cancer (Madhuri and Pandey, 2009; Wamidh, 2011). The available treatment methods include surgery, chemotherapy, and radiation (Wamidh, 2011). Mishra *et al.*, 2013 noted that the current available methods of treatment by chemotherapy, radiation, and surgery can all induce significant side effects and therefore the need for alternate adjuvant therapies has arisen.

Plant use in treating diseases is as old as civilization (Fabricant and Farnsworth, 2001; Wamidh, 2011) and complementary medicine is still a major part of habitual treatments of different maladies (Alviano and Alviano, 2009; Wamidh, 2011). Generally, complementary medicine has a long history of serving people all over the world (Cheng, 2000; El-Shemy *et al.*, 2007). In recent times and due to historical, cultural, and other reasons, folk medicine has taken an important place especially in developing

countries where limited health services are available. However, the absence of scientific evaluation of medicinal plants may cause serious adverse effects (Souza *et al.*, 2004; Wamidh, 2011).

Natural products especially plants have been used for the treatment of various diseases for thousands of years. The Ebers Papyrus, the best known Egyptian pharmaceutical record which documented over 700 drugs, represents the history of Egyptian medicine dated from 1500 BC. The Chinese Materia Medica, which describes more than 600 medicinal plants, has been well documented with the first record dating from about 1100 BC (Cragg *et al.*, 1997). Many medicinal plants have anti-cancer activity as *Allium sativum* contains more than 100 biologically useful secondary metabolites, which include alliin, alliinase, allicin, S-allyl-cysteine, Diallyl disulphide, Diallyl trisulphide and Methylallyl trisulphides (Scharfenberg *et al.*, 1990). *Annona* species contain acetogenins, which possess significant cytotoxic activity. *Arctium lappa* contains potent anticancer factors that prevent mutations in the oncogenes. It has been used in the treatment of malignant melanoma, lymphoma and cancers of the pancreas, breast, ovary, oesophagus, bladder, bile duct and the bone. *Gossypium barbadense* contains gossypol and recent studies have revealed that gossypol possesses selective toxicity towards cancerous cells, whereas *Gyrophora esculenta* is a mushroom that inhibits growth of cancer by enhancing activity of the natural killer cells (Ambasta, 2000).

Natural products are extremely an important source of medicinal agents. Although there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the importance of natural products in drug discovery and development (Thirumal *et al.*, 2012). El-Shemy *et al.*, 2007 also noted that many non-natural, synthetic drugs cause severe side effects that were not acceptable except as treatments of last resort for terminal diseases such as cancer and that the metabolites discovered in medicinal plants may avoid the side effect of synthetic drugs, because they must accumulate within living cells.

Molecules derived from plants (e.g., vincristine, taxol and etoposide) have played an important role in cancer therapy and continue to be a promising source of new therapeutic agents (Cragg and Newman, 2003). For the discovery of new anticancer agents, herbal extracts are taken once the plant species are selected (usually based on random, chemosystematic, ecological and/or ethnobotanical criteria) and these are subsequently evaluated using several cancer cell lines. Next, extracts with high *in vitro* cytotoxic activity against tumor cells are prioritized for more in-depth studies such as evaluation of fractions, isolation of possible molecules that may be responsible for the cytotoxic activity, and even *in vivo* studies (Joebe *et al.*, 2010)

2.2 *Annona muricata*

2.2.1 Taxonomy

2.2.1.1 Common Names

It is known by many common names such as; Soursop, Paw-Paw, Graviola, Guanabana, Adunu, Sorasaka, Mullaatha, Thorny custard apple, Shul-ram-fal, Hanuman fal. In Uganda, the Basoga call it *Omusitafeli*, while the Baganda call it *Ekitafeli*.

2.2.1.2 Plant profile

| | |
|----------|--------------------------|
| Kingdom | : Plantae |
| Division | : Angiosperms |
| Class | : Magnoliids |
| Order | : Magnoliales |
| Family | : Annonaceae |
| Genus | : <i>Annona</i> |
| Species | : <i>Annona muricata</i> |

2.2.2 Ecology and physiology

Annona muricata is the most tropical semi deciduous tree with the largest fruits of the *Annona* genus. It is a typical tropical tree with heart shaped edible fruits and widely distributed and native to Sub-Saharan Africa countries that lie within the tropics. Nowadays, it is grown in many areas of Tropical Africa and there are different names for Soursop in different countries.

It is low-branching and bushy but slender because of its upturned limbs, and reaches a height of 25 or 30 feet (7.5-9 m). The malodorous leaves, normally evergreen, are alternate, smooth, glossy, dark green on the upper surface, lighter beneath; oblong, lanceolate, pointed at both ends. The small trees bear their fruit indiscriminately on twigs, branches and trunk. These fruits range in size from four to twelve inches in length, and up to six inches in width. They can be oval or irregularly shaped as one side usually grows faster than the other. The fruit is more or less oval or heart-shaped, sometimes irregular, lopsided or curved, due to improper carper development or insect injury. The fruit is compound and covered with a reticulated, leathery-appearing but tender, inedible, bitter skin from which protrude few or many stubby, or more elongated and curved, soft, pliable "spines". The tips break off easily when the fruit is fully ripe. The thin skin has a leathery appearance, but is surprisingly tender. This skin is a dark green, but later turns yellowish-green, and finally all yellow when over-ripe.



Figure 2.1; *Annona muricata* plant

2.2.3 Ethnobotanical Uses

All parts of the graviola tree have been used medicinally in traditional herbal medicine. Traditional herbal medicine practitioners have attributed graviola with the following properties and actions: anthelmintic, antiparasitic, antipyretic, sedative, antispasmodic, nervine, hypotensive, anticonvulsant and digestive. Traditionally, the leaves are used for headaches, insomnia, cystitis, liver problems, diabetes and as anti-inflammatory, anti-spasmodic and anti-dysenteric. The decoction of the leaves have parasiticide, anti-rheumatic and neuralgic effects when used internally, while the cooked leaves, applied topically, fight rheumatism and abscesses (Orlando *et al.*, 2010)

The traditional use of graviola has been recorded in herbal medicine systems in the following countries: Amazonia, Barbados, Borneo, Brazil, Cook Islands, Curacao, Dominica, Guatemala, Guam, Guyana, Haiti, Jamaica, Madagascar, Malaysia, Peru, Suriname, Togo and West Indies (Schultes and Raffauf, 1990; Morton, 1980; Holdsworth, 1980; Harodock, 1974). In most sub-Saharan countries such as Uganda, where it is equally used, there has been no scientific reporting.

2.2.4 Chemical composition and advances in research

Phytochemically, *Annona muricata* is rich in miscellaneous lactones and isoquinoline alkaloids (Alali *et al.*, 1999; Odeghe *et al.*, 2013). It contains many active compounds and chemicals; these are the natural phytochemicals known as *annonaceous acetogenins* (Alali *et al.*, 1999; Kojima & Tanaka, 2009). Specific acetogenins in extracts of *Annona muricata* have been reported to be selectively toxic *in vitro* to certain types of tumour cells including: lung carcinoma cell lines; human breast solid tumour lines; prostate adenocarcinoma; pancreatic carcinoma cell lines; colon adenocarcinoma cell lines; mammary adenocarcinoma cell lines; liver cancer cell lines; human lymphoma cell lines; and multi-drug resistant human breast adenocarcinoma (Odeghe *et al.*, 2013). Annonaceous acetogenins,

from *Annona muricata* L were found to be a promising new anti-tumor and anticancer agent in numerous *in vitro* studies. These acetogenins demonstrated to be selectively toxic against various types of the cancerous cells without harming healthy cells (Rieser *et al.*, 1993; Wu *et al.*, 1995; Hamizah *et al.*, 2012).

Kumar *et al.*, 2012 concluded that *Annona muricata* extracts might have potential for the development of therapeutically active compounds, which could serve as precursors and/ or chemical templates for the design of an effective, more potent and safe antineoplastic drug which may be more potent than existing drugs of its class.

2.3 Phytochemicals

2.3.1 Introduction to phytochemicals

There have been wide gaps between disease incidence (0.16%), disease burden (5.6%) and mortality caused by cancer (12.5%), (Mathers *et al.*, 2002; Al-Abd *et al.*, 2011), these gaps clearly denoting lack of effective treatment for cancer compared to other causes of mortality. Accordingly, screening of natural products for promising anti-cancer activity is a pertinent field in several countries that have diversity of the gardenia flower. Many plants are widely used medicinally in different countries and are a source of many potent and powerful drugs (Al-Abd *et al.*, 2011).

“Phyto” is the Greek word for plant. Phytochemicals are natural bioactive and non-nutrient compounds that protect plants from bacterial and fungal infections (Doughari *et al.*, 2009; Krishnaiah *et al.*, 2009). Plants produce these chemicals to protect themselves but recent research demonstrates that many phytochemicals can protect humans against diseases. There are many phytochemicals in fruits and herbs and each works differently (Kumar *et al.*, 2009; Afify *et al.*, 2011). Recently, phytochemicals and their effects on human health have been intensively studied. In particular, a search for antioxidants, hypoglycemic agents, and anticancer agents in vegetables, fruits, teas, spices and medicinal herbs has attracted great attention (Afify *et al.*, 2011).

Phytochemicals are categorized as either primary or secondary metabolites. Sugars, amino acids and proteins are example of primary metabolites while secondary constituents include alkaloids, saponins, terpenes, sterols, phenols among others (Krishnaiah *et al.*, 2009).

Alkaloids are basic organic substances containing at least one nitrogen atom in their structure. They are bitter and very toxic and are normally classified according to their pharmacological properties e.g. analgesic, stimulant or anti-malarial alkaloids or according to their sources for example opium, vinca,

and chinchona alkaloids (Kashani *et al.*, 2012). Saponins on the other hand are molecules whose structure consists of a sugar moiety linked to a hydrophobic aglycon. They are classified as either steroidal saponins; commonly found in herbs or triterpenoid glycosides; predominant in cultivated crops (Francis *et al.*, 2007). The phytochemical group terpenoids is the largest and the most structurally diverse group of natural products and they are used as antiallergenic agent, antiseptics agents or as insecticides and in industries they are incorporated in rubber and detergents (Zwebger and Basu, 2008). Sterols (Phytosterols) are triterpenes whose basic structure consists of sterol nucleus and an alkyl side chain (Chumark *et al.*, 2008; Khalafalla *et al.*, 2010) while phenols are compounds possessing one or more aromatic rings with one or more hydroxyl groups (Dai and Mumper, 2010). Phenols are classified as either derivatives of benzoic acids or cinnamic acid and flavonoids, tannins, coumarins and lactones are examples of phenolic compounds (Dai and Mumper, 2010). Flavonoids are responsible for the color of many fruits and vegetables and are categorized as flavones, flavonols, isoflavanones, and anthocyanins (Dai and Mumper, 2010). Tannins on the other hand are classified as either hydrolysable tannins or condensed tannins (Zhang *et al.*, 2010). Hydrolysable tannins have a central core of glucose esterified with gallic acid or with hexahydroxydiphenic acid while condensed tannins are oligomers or polymers of flavan-3-ol linked through interflavan carbon bond (Dai and Mumper, 2010). Coumarins are polyphenolic compound whose structure consists of an aromatic ring linked to a condensed lactone ring while lactones are molecules with an ester group integrated into a carbon ring system, thus coumarins are also a representative of lactones (Lacy and O'Kennedy, 2004)

Ancient herbal medicines may have some advantages over single purified chemicals (Nassr-Allah *et al.*, 2009). Often the different components in a herb have synergistic activities or buffer toxic effects. Mixtures of herbs are even more complex and so might have more therapeutic or preventive activity than single products alone. In fact, several studies have demonstrated that extracts from several herbal medicines or mixtures had an anticancer potential *in vitro* or *in vivo* (El-Shemy *et al.*, 2007; Nassr-Allah *et al.*, 2009). Phenolic and flavonoid contents provide antioxidant activities that may underlie the anticancer potential (Meyers *et al.*, 2003; Nassr-Allah *et al.*, 2009). Aqueous extracts from willow (*Salix* sp.) leaves prevented proliferation of three cancer cells AML, acute ALL and EACC (El-Shemy *et al.*, 2003, El-Shemy *et al.*, 2007; Nassr-Allah *et al.*, 2009). Alcohol extracts of *Ganoderma lucidum* (Hu *et al.*, 2002; Nassr-Allah *et al.*, 2009) induced apoptosis in MCF-7 human breast cancer cells. An aqueous extract of *Paeoniae lactiflora* induced apoptosis in HepG2 and Hep3B hepatoma cells (Lee *et al.*, 2002; Nassr-Allah *et al.*, 2009). An aqueous extract of Bu-Zhong-Yi-Qi-Tang (a mixture of ten herbs) could induce apoptosis in hepatoma cells (Kao *et al.*, 2001; Nassr-Allah *et al.*, 2009). Water-soluble ingredients of Sho-Saiko-To (a mixture of seven herbs) inhibited the proliferation of KIM-1 human hepatoma cells and KMC-1 cholangiocarcinoma cells (Yano *et al.*, 1994; Nassr-Allah *et al.*,

2009). PE-SPES (mixture of eight herbs) had been developed as a clinical treatment of prostate cancer (Bonham *et al.*, 2002). Chemical and pharmacological studies of various extracts or compounds purified from the herbs were found to increase myocardial blood flow, reduce radiation damages and purify blood quality (Wang *et al.*, 2001; Kim *et al.*, 2002; Nassr-Allah *et al.*, 2009). Luffin from the seeds of *Luffa aegyptiaca* is cytotoxic to the human metastatic melanoma, with approximately 10 times greater potency in Ehrlich cells (Nassr-Allah *et al.*, 2009).

2.3.2 Action of extracted phytochemicals

Identifying the mode of action of anticancer agents of plant origin provide helpful information for their future use. Thus it is important to screen the apoptotic potential of plants either in their crude extract form or as pure compounds (Tarapadar *et al.*, 2001; Wamidh, 2011). Due to their multiple intervention strategies, crude plant extracts have been proposed to prevent, arrest, or reverse the cellular and molecular processes of carcinogenesis (Neergheen *et al.*, 2009; Wamidh, 2011).

Since the distribution of bioactive compounds differs according to the plant used, in various studies, different solvents were used to extract these compounds from different plants (Wamidh, 2011). The methanol extract of *Scutellaria orientalis* showed potent anti-leukemic activity against HL-60 cell line (Ozmen *et al.*, 2010). The water extract of *Rheum officinale* exhibited significant anti-proliferative activity by inducing apoptosis in MCF-7 and A549 cell lines (Li *et al.*, 2009). A potent anti-proliferative activity was also reported for the hexane extract of *Casearia sylvestris* stem bark against different cancer cell lines (Mesquita *et al.*, 2009) and the butanol extract of *Pfuffia paniculata* demonstrated high cytotoxic activity against MCF-7 cell line (Nagamine *et al.*, 2009). Additionally, the *Physalis minima* chloroform extract induced apoptosis in human lung adenocarcinoma cell line (Leong *et al.*, 2011). Out of 76 Jordanian plant species, the ethanolic extracts of *Inula graveolens*, *Salvia dominica*, *Conyza canadiensis* and *Achillea santolina* showed potent anti-proliferative activity against MCF-7 cell line (Abu-Dahab and Afifi, 2007). The aqueous methanol of *Ononis hirta* and *Inula viscosa* showed high ability to selectively target MCF-7 cancer cells and induced apoptosis (Talib and Mahasneh, 2010).

Various fractions including different leaf extracts (*Salix safsaf*) by successive solvent extractions were tested for their anti-leukemic activity on acute myeloid leukemia (AML) cells and acute lymphoblastic leukemia (ALL). The fractions of each crude extract were dissolved in a saline solution after removing the solvent and incubated with the leukemia cells. The results showed that a fraction of the willow leaves extracted with nonpolar organic solvents (petroleum ether, ether, and chloroform) had a very low destructive effect on tumor cells as shown by a higher viability 80–90%. Destruction of each extract ranged between 1.5–2.9% of each extract. However, a major destructive effect on AML, ALL

and carcinoma (EACC) cells was obtained by a fraction of the polar organic solvents (water and 70% ethanol), (El-Shemy *et al.*, 2007).

2.4 Antioxidant activity

Antioxidants are a group of substances that are useful for fighting cancer and other processes that potentially lead to diseases such as atherosclerosis, Alzheimer's, Parkinson's, diabetes, and heart disease (Valko *et al.*, 2007; Joabe *et al.*, 2010; Aboul-Enein *et al.*, 2012). Unlike cytotoxic agents that damage tumor cells, antioxidants act by preventing the onset of cancer during carcinogenesis, and they are generally beneficial to cells. Oxidants damage macromolecules, such as proteins, lipids, enzymes, and DNA (Joabe *et al.*, 2010; Aboul-Enein *et al.*, 2012; Sies *et al.*, 1993) and to combat these radicals, living organisms produce enzymes or rely on non-enzymatic molecules such as cysteine, ascorbic acid, flavonoids, and vitamin K for protection (Joabe *et al.*, 2010; Aboul-Enein *et al.*, 2012; Sies *et al.*, 1993).

There has been interest in the contribution of free radicals reaction participating in reactive oxygen species to the overall metabolic perturbation that result in tissue injury and disease. Reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical are generated in specific organelles of cells (Mitochondria and Microsomes) under normal physiological condition. These reactive oxygen species can damage DNA, so as to cause mutation and chromosomal damage, oxidize cellular thiols and abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids (Halliwell and Gutteridge, 1985; Ames *et al.*, 1993).

Antioxidants may offer resistance against the oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation and by other mechanisms and thus prevent disease (Youdim and Joseph, 2001). Recently, various phytochemicals and their effect on health, especially the suppression of active oxygen species by natural antioxidant from tea, spices and herbs, have been intensively studied (Ho *et al.*, 1994; Elmastas *et al.*, 2006). Phenolic compounds play an important role in the oxidative properties of many plant-derived antioxidants (Canadanovic –Brunet *et al.*, 2006). Phenolic substances were also reported to possess a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory and anticancer (Cole *et al.*, 2005; Stevenson and Hurst, 2007).

Several methods have been developed to measure the efficiency of antioxidants as pure compounds or in extract. These methods focus on different mechanisms of the oxidant defense system that is scavenging active oxygen species and hydroxyle radicals, inhibiting of lipid peroxidation, or chelating of metal ions (Dorman *et al.*, 2003)

The DPPH free radical scavenging assay is used for the evaluation of the antioxidant activity. This assay measures the free radical scavenging capacity of the investigated extracts. DPPH is a molecule containing a stable free radical. In the presence of an antioxidant, which can donate an electron to DPPH, the purple colour typical for free DPPH radical decays, and the absorbance change at $\lambda = 517$ nm is measured. This test provides information on the ability of a compound to donate a hydrogen atom, on the number of electrons a given molecule can donate, and on the mechanism of antioxidant action (Mothana *et al.*, 2009).

Although the enzymes are quite efficient in maintaining the cell's REDOX state they must be aided by non-enzymatic molecules. These can be of endogenous (glutathione) and of exogenous origin (ascorbate, tocopherols, carotenes, retinols and polyphenols among others) and their functions are to neutralize free radicals by either acting on their own or in conjunction with the enzymatic systems.

Ascorbate, also known as vitamin C, is one of the exogenous molecules obtained through the diet. It is present in a diverse variety of vegetable foodstuffs such as apples, papaya, mango, guava oranges and others (Oliveira *et al.*, 2010; Suárez-Jacobo *et al.*, 2011). It is known as a free radical scavenger that does not act as a pro-oxidant under normal conditions, and it has been mentioned to function in parallel with glutathione (Cuddihy *et al.*, 2008; Foyer and Noctor, 2011). Because of its ability to neutralize free radicals it has been mentioned as having a possible therapeutic use in disorders such as ischemic stroke, Alzheimer's, Parkinson's and Huntington's diseases (Harrison and May, 2009), and it is sometimes consumed by athletes to neutralize the ROS produced during exercise (Gomez-Cabrera *et al.*, 2008), it can neutralize protein radicals (Domazou *et al.*, 2009) and its role in the improvement of endothelial function (a precursor of atherogenesis) has also been noted (Frikke-Schmidt and Lykkesfeldt, 2010; Sabharwal and May, 2008).

Vitamin E is the collective term for at least eight structurally related molecules: four tocopherols and four tocotrienols (Ravaglia *et al.*, 2008). Since tocopherols are of non-polar nature, their main function lies in the hydrophobic environment of cell and organelle membranes, protecting these structures from free radicals and also by stabilizing them (Atkinson *et al.*, 2008). Tocopherols induce a protective effect against oxidative stress linked to metabolic syndrome as well as other sources (Chung *et al.*, 2010).

Carotenoids are other important molecules with antioxidant activity. They are hydrophobic vegetal pigments derived from isoprenoid units with up to 15 conjugated double bonds (Costantini and Moller,

2008; Tanaka *et al.*, 2008). This large family of molecules contains more than 700 different structures identified so far, and are thought to play an essential role in the life cycle of the plants that produce them (Nishino *et al.*, 2009; Tanaka *et al.*, 2008). Even though some animals like birds, fishes and invertebrates use them in order to generate colorations for structures such as their skin and feathers, none of them have the ability to synthesize them and must therefore obtain them from exogenous plant sources. In animals they have the potential to not only act upon free radicals, but also to modulate critical functions such as immune-stimulation and inhibition of the tumorigenesis process (Nishino *et al.*, 2009).

The last exogenous antioxidant to be mentioned is retinol (Vitamin A), a molecule that is related to carotenoids. β -carotene can in fact be transformed to retinol, and this can in turn be transformed into retinal and retinoic acid, all of which have different biological functions on many tissues (Bremner and McCaffery, 2008). This compound, as well as its other metabolites, affects important processes such as immunity, reproduction, growth, development and, perhaps its best known function, it holds a vital role in the visual cycle (Redmond *et al.*, 2010). β -carotene has been characterized as an antioxidant, nevertheless, it can have toxic effects and has been named as a pro-oxidant when it is administered at higher doses and on certain types of cell cultures (Pasquali *et al.*, 2008; Roehrs *et al.*, 2009). When it functions as an antioxidant it has been positively linked to diverse ailments related to oxidative stress, such as diabetes (Ramakrishna and Jaiikhani, 2008), obesity (Botella-Carretero *et al.*, 2010), low sperm motility (Kao *et al.*, 2008), hearing loss (Michikawa *et al.*, 2009) and others.

Aside from the “antioxidant vitamins” the polyphenol group is another very diverse family of molecules present in fruits and vegetables. They are plant secondary metabolites that can be subdivided into many groups according to their molecular structure. Some of the representative molecules included here are quercetin, coumaric acid, proanthocyanidins resveratrol among others. Although traditionally considered antioxidants, they can also have antimicrobial, antiviral and anti-inflammatory properties (Ignat *et al.*, 2011); they have consequently gained attention because of these *in vivo* health-promoting properties (Massaro *et al.*, 2010). Since fruits and vegetables are rich in all types of exogenous antioxidant molecules, scientists have linked these bioactive molecules to the positive health effects of a fruit and vegetable-rich diet. However, fruit and vegetable intake cannot be replaced by a single antioxidant molecule or supplement; that is, ingesting vitamins or polyphenols in a purified form is not the same as acquiring them from the diet, since other nutrients present in fruits and vegetables may synergize with each other in order to elicit an effect (Liu, 2004).

The most commonly used antioxidant at the present time is butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and *tert*butylhydroxytoluene (TBHQ) (Sherwin, 1990). However, they are suspected of being responsible for liver damage and carcinogenesis in laboratory animals (Wichi, 1988; Grice, 1986). Therefore, the development and utilization of more effective antioxidant of natural origin are desired (Oktay *et al.*, 2003; Abou Elalla and Shalaby, 2009).

2.5 Anti-cancer activity studies

An integrated part of cancer cell development is the resistance to programmed cell death (apoptosis). Re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents (Joshi *et al.*, 1999; Wamidh, 2011). Some plant-derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents (Hirano *et al.*, 1995; Wamidh, 2011).

Mahmoud *et al.*, 2011 concluded that apoptosis has been receiving great attention as a major mechanism of cell death in normal as well as tumor cells. However, the programmed cell death might be interrupted due to defective signaling pathway nonetheless in tumor cells with higher rate of mutation (Tomiyama *et al.*, 2008; Mahmoud *et al.*, 2011). Defective apoptosis has been reflected in the form of cell resistance to apoptotic inducing agents and, consequently, treatment failure.

Annonaceous acetogenins, from *Annona muricata* were found to be promising new anti-tumor and anticancer agent in numerous *in vitro* studies. These acetogenins demonstrated to be selectively toxic against various types of the cancerous cells without harming healthy cells (Rieser *et al.*, 1993; Wu *et al.*, 1995; Hamizah *et al.*, 2012). Specific acetogenins in extracts of *Annona muricata* have been reported to be selectively toxic *in vitro* to certain types of tumour cells including: lung carcinoma cell lines; human breast solid tumour lines; prostate adenocarcinoma; pancreatic carcinoma cell lines; colon adenocarcinoma cell lines; mammary adenocarcinoma cell lines; liver cancer cell lines; human lymphoma cell lines; and multi-drug resistant human breast adenocarcinoma (Odeghe *et al.*, 2013).

An extract is said to have cytotoxic activity if the IC₅₀ value of less than 1000 µg/mL after 24 hours contact time. The smaller the IC₅₀ value of a test compound the more toxic compound (Rachmani *et al.*, 2012).

Cytotoxicity test is a qualitative and quantitative test to determine cell death. The MTT assay is a method used to see cytotoxic effects extracts on cancer cells. The principle of the MTT assay is a spectroscopic method, and is by determining the absorbance value of formazan. MTT will be absorbed

into the cell and entered into the system of cell respiration in mitochondria. The action of the enzyme active mitochondria in cells is to metabolize tetrazolium salts, resulting in termination of tetrazolium ring by dehydrogenase enzymes which lead to tetrazolium formazan being transformed into water-insoluble but soluble in SDS 10%. Formazan formed is colored purple and is proportionate to the number of living cells. Cells that die dissolve in water and remain yellow because the mitochondria of cells that die are not respirational, hence tetrazolium ring is disconnected so it cannot reduce MTT reagent to formazan and the color remains yellow (Rachmani *et al.*, 2012).

A study by Aboul-Enein *et al.*, 2011 on the anticancer activity of *Eichhornia crassipes* showed that the crude extract showed the highest effect compared to all isolated compounds against several tumor types. Some fractions exhibited selective very potent anticancer activity against liver cancer cell line while other fractions exhibited high anticancer activity against hormone dependent tumor types (cervix and breast cancers). The cytotoxicity parameter, IC₅₀ was calculated using E_{max} model. Some isolated compounds showed potency with IC₅₀ as low as 1.6 ± 0.5 µg/ ml. They further noted that it is worth mentioning, that the higher potency of the crude extract against cancer cell lines relative to all fractions from the same extract, might be attributed to auto-synergistic effect of these fractions within the same extract. (Aboul-Enein *et al.*, 2011)

A study conducted by El-Shemy *et al.*, 2007 on willow extracts showed that *in vitro* the extract could kill the majority (75%–80%) of abnormal cells among primary cells harvested from seven patients with acute lymphoblastic leukemia (ALL) and 13 with AML (acute myeloid leukemia). DNA fragmentation patterns within treated cells inferred targeted cell death by apoptosis had occurred. The metabolites within the willow extract may act as tumor inhibitors that promote apoptosis, cause DNA damage, and affect cell membranes and/or denature proteins (El-Shemy *et al.*, 2007).

Another study which was aimed at determining the cytotoxic effects from extracts of leaves of soursop and fraction results in cancer cells T47D was carried out by Rachmani *et al.*, 2012. The research was carried out by extraction using ethanol and fractionation by column chromatography method that used various solvents such as n-hexane, chloroform, ethyl acetate and methanol. The cytotoxic test was performed by the method of MTT assay and apoptosis tests performed by the method of Double Staining. The parameter obtained from the cytotoxic test was IC₅₀ values. Apoptosis assay results were analyzed in a qualitative description. In their conclusion, Ethanol extract of leaves of soursop (*Annona muricata*) had a cytotoxic activity in T47D breast cancer cell lines with IC₅₀ of 17.149 µg/ mL and can induce apoptosis. Ethyl acetate fraction had the best potency of cytotoxic among other fractions against to T47D breast cancer cell lines with value of IC₅₀ was 31.268 µg/ mL.

A study by Ukwubile (2012) showed that seed ethanol extract of *Annona muricata L* had anti-ovarian cancer properties on the experimental animals and can therefore serve as a medication for ovarian cancer problems in females.

Hamizah *et al.*, 2012 evaluated the chemo-preventive effects of ethanolic extracts of leaves of *Annona muricata* (AMLE) in 6-7 week old mouse given a single topical application of 7,12-dimethylbenza(α)anthracene (DMBA 100 μ g/100 μ l acetone) and promotion by repeated application of croton oil (1% in acetone/twice a week) for 10 weeks. And the results suggest that the *Annona muricata* leaves extract was able to suppress tumor initiation as well as tumor promotion even at lower dosage.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

3.1.1 Sample collection and authentication

Fresh leaves of *Annona muricata* L. were collected from the wild in Eastern Uganda in the Districts of Kaliro and Iganga Municipality, during the month of August 2013. The plant was identified and authenticated in the Makerere University Botanical Herbarium (MHU) by Ms. Olivia Wanyana Mangeni. A voucher specimen was deposited in the herbarium under the collection number GY 021-10/13- MB 300-0007/12-001.

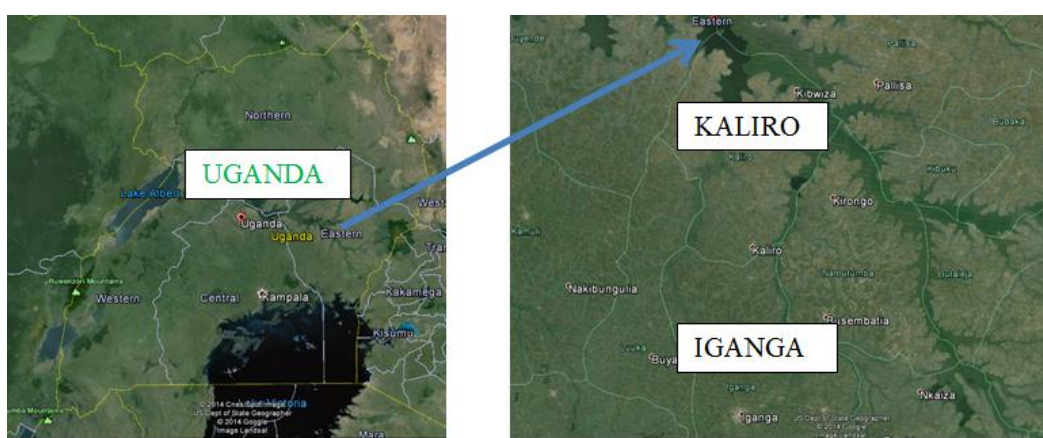


Figure 3.1; Map of Uganda showing the study areas of Kaliro and Iganga

Reference: US Department of State Geographer 2014 (www.google.com/earth)

3.1.2 Samples preparation and Extraction

The leaves of *Annona muricata* were washed with water and cut into small pieces, drying was done at room temperature for three weeks, and the dried leaves were powdered. Equal amounts (350grams) of powdered leaves were extracted using ethanol and distilled water for three days by the plant tissue homogenization method as previously described (Tiwari *et al.*, 2011; Das *et al.*, 2010). The extracts were then concentrated using rotary evaporator and dry block heater respectively and kept at 4°C until used.

3.1.3 Chemicals, reagents and Cell lines

All chemicals and reagents were procured from certified suppliers and were of the highest analytical standard. The Ehrlich Ascites Carcinoma Cells (EACC) had been obtained from the National Cancer Institute (NCI) Cairo, Egypt. The Breast cancer cell lines MDA and SKBR3 were obtained from the

Physiology and Cancer Biology laboratory in the Zoology Department of the Faculty of Science at Cairo University.

3.2 Phytochemical screening of ethanolic and water leaves extracts of *Annona muricata*

3.2.1 Phytochemical Composition of the Extracts

Phytochemical analysis was carried out using standard procedures as previously described (Savithramma *et al.*, 2011; Tiwari *et al.*, 2011; Cai *et al.*, 2011; Sasidharan *et al.*, 2011; Edeoga *et al.*, 2005; Kumar *et al.*, 2007). Samples of both the ethanolic and water leaves extracts of *Annona muricata* were screened for the following phyto constituents; alkaloids, saponins, terpenoids, flavonoids, coumarins and lactones, anthraquinones, tannins, Cardiac glycosides, phenols and phytosterols.

3.2.1.1 Detection of alkaloids

This was conducted using the Wagner's test. To a few ml of filtrate, few drops of Wagner's reagent were added by the side of the test tube, formation of a reddish brown precipitate confirmed the test as positive (Sasidharan *et al.*, 2011; Tiwari *et al.*, 2011).

3.2.1.2 Detection of saponins

The Froth test was used. 0.5 g of the extract were dissolved in 10ml of distilled water in a test tube and shaken vigorously for 30 seconds, and then allowed to stand for 45 Min; the appearance of persistent frothing indicated the presence of saponins (Savithramma *et al.*, 2011; Tiwari *et al.*, 2011)

3.2.1.3 Detection of terpenoids

3.2.1.3.1 Acetic anhydride - Sulphuric acid (H₂SO₄) test

2 ml of the extract were added to 2 ml of acetic anhydride and concentrated H₂SO₄. Formation of green rings indicated the presence of terpenoids (Savithramma *et al.*, 2011).

3.2.1.3.2 Salkowski test

To 5 ml extract were added 2 ml of chloroform and 3 ml of concentrated sulphuric acid H₂SO₄. Reddish brown color of interface indicated presence of Terpenoids (Sasidharan *et al.*, 2011; Edeoga *et al.*, 2005).

3.2.1.4 Detection of flavonoids

The extracts were tested for the presence of flavonoids using the following procedures;

3.2.1.4.1 Ammonia test

The test samples were spotted on a TLC plate and fumigated with ammonia, a yellow spot or a yellow green fluorescence under ultraviolet light (UV) indicated the presence of flavonoids (Cai *et al.*, 2011).

3.2.1.4.2 Hydrochloric acid – Mg reaction

A small amount of magnesium turnings was added into a test tube with test samples, and a few drops of HCl added. Any change in color to red indicated the presence of flavonoids (Cai *et al.*, 2011).

3.2.1.5 Detection of coumarins and lactones

Opened loop- closed loop response method was used. Two drops of 1% sodium hydroxide solution was added to a test tube containing test solution and incubated for 3min in boiling water, 4 drops of 2% HCl were then added to the solution. A cloudy solution implied the presence of coumarins and lactones (Cai *et al.*, 2011).

3.2.1.6 Detection of Anthraquinones

The Borntrager's test was used. About 50mg of extract were heated with 1ml 10% ferric chloride solution and 1ml of concentrated hydrochloric acid. The extract was cooled and filtered. The filtrate was shaken with equal amount of diethyl ether. The ether extract was further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicated presence of Anthraquinones (Sasidharan *et al.*, 2011; Kumar *et al.*, 2007).

3.2.1.7 Detection of Tannins

The Braemer's test was used. 10% alcoholic ferric chloride was added to 2-3ml of the extract. (1:1). Dark blue or greenish grey coloration of the solution indicated presence of Tannins (Sasidharan *et al.*, 2011; Kumar *et al.*, 2007).

3.2.1.8 Detection of Cardiac glycosides

Kellar – Kiliani test was used. 2ml filtrates were added with 1ml of glacial acetic acid, 1ml ferric chloride and 1ml of Concentrated Sulphuric acid. A green-blue coloration of solution indicated presence of Cardiac Glycosides (Sasidharan *et al.*, 2011).

3.2.1.9 Detection of phenols

3.2.1.9.1 Ferric chloride test

The extract (50 mg) was dissolved in 5 ml of distilled water; few drops of neutral 5% ferric chloride solution were added to the extract. A dark green or bluish green color indicated the presence of phenolic compounds (Cai *et al.*, 2011; Tiwari *et al.*, 2011).

3.2.1.9.2 Vanillin- HCl reaction

A drop of the extracts was placed on a filter paper, dried and sprayed with vanillin hydrochloric acid reagent. Appearance of varying degree of red color indicated the presence of phenols (Cai *et al.*, 2011).

3.2.1.10 Detection of phytosterols

The Salkowski test was used. 10 ml of chloroform were added into a test tube containing 1 ml of test samples, equal volume of concentrated H₂SO₄ were then added by the sides of a test tube, appearance of yellowish colour with green fluorescence in the H₂SO₄ layer indicated the presence of phytosterols (Savithramma *et al.*, 2011; Tiwari *et al.*, 2011).

3.2.2 Determination of relative abundance of the phytochemicals present

Following the identification of the different phytochemicals present in both ethanolic and water leaves extracts of *Annona muricata*, the relative abundance of the phytochemicals in each of the extracts was determined (Cai *et al.*, 2011). The results were analyzed using the Chi- Square Goodness of fit test between low and high abundance. For each of the nine runs per phytochemical, we allocated it as either High or low upon which the final allocation of the relative abundance would be based. H₀: The concentration of the phytochemical in the sample is neither high nor low, thus No preference (average); H₁: There is a difference in the concentration of the phytochemical in the sample; $\alpha = 0.1$, Expected value (E) = 4.5, Degrees of freedom = 1 and $\chi^2_{\text{critical}} = 2.7055$; All conditions of the Chi Square test were met, except the standard minimum expected value of 5, for which our expected value was 4.5; as the total data set for each test was 9 values.

3.2.3 Determination of total phenolics

The phenolic content of the *Annona muricata* was determined (Singleton *et al.*, 1999). 20 μ l of the extract was taken from each of the extract and added to 1580 μ l of distilled water. This was followed by adding of 100 μ l of Folin reagent (1%) and left to stand for 2minutes. To each of the samples was then added 300 μ l of Na₂CO₃ (7.5%), mixed thoroughly and left to stand for 2hours at 20°C. All results were expressed as Gallic Acid Equivalent using a standard curve of Gallic acid from which a linear equation was used to calculate the total phenols of the extracts.

3.3 Gas Chromatography Mass Spectroscopy

Gas Chromatography Mass Spectroscopy (GC-MS), a hyphenated system which is a very compatible technique and the most commonly used technique for the identification and quantification purpose was used. The unknown organic compounds in the complex mixture can be determined by interpretation and also by matching the spectra with reference spectra.

3.3.1 Preparation of extracts:

The ethanolic leaves extracts were analyzed using Gas Chromatography Mass Spectroscopy for the identification of the phytochemical compounds present. A solvent blank analysis was first conducted using 1 µl of absolute ethanol. Then 1 µl of the reconstituted ethanolic extract solution was employed for GC/ MS analysis as previously described with modifications (Paranthaman *et al.*, 2012; Komansilan *et al.*, 2012).

3.3.2 Analysis:

GC-MS analysis was carried out on a GC system comprising a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) instrument; Shimadzu GCMS-QP2010, employing the following conditions: Column Elite-1 fused silica capillary column (30×0.25 mm ID×1EM df, composed of 100% Dimethyl poly siloxane), operating in electron impact mode at 70 eV; helium (99.999%) as carrier gas at a constant flow of 1ml/min and a sample injection volume of 1 µl which was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra was taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 550 Da. Total run time was 30 min. The compounds were then identified from the GC-MS peaks, using library data of the corresponding compounds. GC-MS was analyzed using electron impact ionization at 70eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the GC-MS library using NISP Search. The relative % amount of each component was calculated by comparing its average peak area to the total areas. Measurement of peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

3.4 Determination of antioxidant activity of ethanolic and water leaves extracts of *Annona muricata*

3.4.1 Preliminary DPPH radical scavenging assay.

The crude extracts obtained from the leaves were spotted on TLC aluminum plate from Sigma-Aldrich and developed using different solvent system. The TLC plates were air dried and compounds detected under UV lamp at 254 nm. The plates were sprayed with 1, 1-diphenyl-2-picrylhydrazyl (0.04% DPPH) reagent to detect the number of antioxidant compounds present in the extract. The presence of white spots against purple background on the plate was an indication of the crude extract having radical scavenging properties.

3.4.2 Determination of reducing power

The reducing power of the ethanolic and water leaves extracts of *Annona muricata* were determined (Dorman *et al*, 2003). Gallic acid was used as standard. 200µl of each of the samples per extract as well as the standard at different concentrations were taken separately and mixed with 500µl of 0.2M phosphate buffer (pH 6.6) and 500µl of Potassium ferricyanide. The samples were then incubated at 50°C for 20 min. Then 500µl of 10% Trichloroacetic acid were added and centrifuged at 6500 rpm for 16 min at room temperature. 700µl of supernatant were added to 700µl) distilled water, 140µl of freshly prepared ferric chloride, and left to stand for 10 minutes. Finally the absorbance was measured at 700nm. A standard curve for gallic acid was generated and the linear equation was used to calculate the reducing power of the extracts.

3.4.3 Quantification of antioxidant activity using the DPPH method (2,2-diphenyl-2-picrylhydrazyl)

The free radical scavenging activity (RSA) was determined. Different concentrations of the extracts (0, 250, 500, 750, 1000, and 1250 µg/ml) were used. 2.5ml of 0.04% DPPH solution (0.04%) was mixed with 0.5ml of all the concentrations of both extracts separately. After 30 minutes incubation at room temperature in the dark, the absorbance was read at 517 nm, in triplicates for each concentration (Blois, 1958). Butylated Hydroxytoluene (BHT,) and Butylated Hydroxyanisole (BHA) were used as positive control. The percent inhibition of free radical formation was calculated as follows: Radical Scavenging Activity (%) = $[(A_{\text{control}} - A_{\text{sample}}) / (A_{\text{control}})] \times 100$. The Inhibition % was reported as Mean ± standard deviation.

3.5 Determination of *in vitro* cytotoxic activity of ethanolic and water leaves extracts of *Annona muricata*

3.5.1 *In vitro* cytotoxic activity of the extracts on EACC tumor Cell-lines

The culture medium was prepared using RPMI1640 media (Gibco, Grand Island, USA), 10% inactivated fetal bovine serum (Gibco), and 100 units/ ml penicillin and 100 mg/ ml streptomycin were added. A line of Ehrlich Ascites Carcinoma was used. 2 ml of media containing EACC (2×10^4 cells) were transferred into a set of tubes each, then Different concentrations of the extracts both water and ethanolic (0, 250, 500, 750, 1000, and 1250µg/ml) were added. The tubes were incubated at 37°C for 2 h in the presence of 5% CO₂ (Bennett *et al.*, 1976). For each examined materials (and control), a new clean, dry small test tube was used and 10 µl of cell suspension, 80 µl saline and 10 µl trypan blue (0.4%) were added and mixed, then the number of living cells (non-stained) was calculated using a homocytometer slide by microscope (Nikon, TMS). Photomicrographs were captured using an

inverted microscope. The extracts concentration providing 50% inhibition (IC₅₀) was calculated from the graph plotting inhibition percentages against logarithm of concentrations.

3.5.2 MTT Assay for Breast cancer cell lines MDA and SKBR3

The culture medium was prepared using modified Earle's salt with 1.2 g/l sodium carbonate and L-glutamine (Gibco, Grand Island, USA), 10% inactivated fetal bovine serum (Gibco), and 100 units/ml penicillin and 100 mg/ml streptomycin were added. The anticancer effect of the ethanolic leaves extracts on the MDA and SKBR3 cell lines was determined by the MTT assay (Denizot and Lang, 1986). The cell count was adjusted to 1×10^5 cells/0.1 ml and plated in 100 μ l of medium/well in 96-well plates (Costar Corning, Rochester, NY). The cells were then incubated in the presence of various concentrations of the ethanolic extract for 72h at 37°C in triplicates per concentration (750, 500, 250, and 0 μ g/ml). The sample solutions were then removed and washed with PBS (pH 7.4). 20 μ l/well of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT 0.5%) was added. The samples were then incubated for 4 hours. Amount of formazan was determined by measuring the absorbance at 570 nm using an ELISA plate reader (ELx800 universal micro plate reader, Biotech, USA). Concentration required for an inhibition Concentration (IC₅₀) was determined graphically plotting percentage cell death against logarithm of concentration. % Cell death was calculated using the following formula; % Cell death = (Control OD – Sample OD)/Control OD x 100.

3.5.3 Cytotoxicity effect of ethanolic leaves extracts of *Annona muricata* on normal spleen cells

Spleen cells were isolated from normal healthy mice (Lavelle *et al.*, 1972). Spleen cells viability after and before incubation with different concentrations of ethanolic at 37°C in the presence of 5% (v/v) CO₂ for 2hours was calculated using Trypan Blue Technique (Bennett *et al.*, 1976). All concentrations were assayed in triplicates.

3.5.4 TLC Fractionation of the ethanolic leaves extracts of *Annona muricata*

The ethanolic leaves extracts of *Annona muricata* was fractionated using thin layer chromatography (TLC) technique. The extract was applied on silica gel 60 F254 TLC aluminum sheets (20 x20) (Merck, Darmstadt, Germany) at one of extremes to separate the different fractions. Mobile phase was Petroleum ether: Ethyl acetate: Glacial acetic acid (4:1:1). 11 fractions were scratched and named as EEAM1b – EEAM11. All The fractions were tested for anti-oxidant activity, reducing power and anti-cancer activity.

3.6 Data analysis

Quantitative and graphical data was analyzed using Microsoft Excel Package. The results of each series of experiments (performed in triplicates) were expressed as the mean \pm standard deviation. Qualitative data for phytochemical analysis was analyzed using the χ^2 goodness of fit test.

CHAPTER 4: RESULTS

4.1 Phytochemical analysis

4.1.1 Classes of compounds identified from ethanolic and water leaves extracts of *Annona muricata* using the different qualitative methods of analysis

Table 4.1; Classes of compounds identified from ethanolic and water leaves extracts of *Annona muricata* using the different qualitative methods of analysis

| Compound | Test | Ethanolic | Water |
|------------------------|--------------------------------------|-----------|-------|
| Alkaloids | Wagner's test | + | + |
| Saponins | Froth test | + | + |
| Terpenoids | Acetic acid - Sulphuric acid test | + | + |
| | Salkowski test | + | + |
| Flavonoids | Ammonia test | + | + |
| | HCl-Mg reaction test | + | + |
| Coumarins and Lactones | Open loop – close loop response test | + | + |
| Anthraquinones | Borntrager's test | + | + |
| Tannins | Braemer's test | + | + |
| Cardiac glycosides | Keller-Killiani test | + | + |
| Phenols | FeCl ₃ test | + | + |
| | Vanillin- HCl reaction | + | + |
| Phytosterols | Salkowski test | + | + |

Legend: + Present, - Absent

All phytochemicals tested were present in both the ethanolic and water leaves extracts of *Annona muricata* as shown in the table above

4.1.2 Relative abundance of the phytochemicals present in leaves extracts of *Annona muricata*

Table 4.2; Relative abundance of phytochemicals present in Ethanolic leaves extracts of *Annona muricata*

| Phytochemical | High | Low | E | χ^2 Calculated | χ^2 critical | Conclusion |
|------------------------|------|-----|-----|---------------------|-------------------|------------|
| Alkaloid | 7 | 2 | 4.5 | 2.7778 | 2.7055 | High |
| Terpenoids | 8 | 1 | 4.5 | 5.4444 | 2.7055 | High |
| Flavonoids | 7 | 2 | 4.5 | 2.7778 | 2.7055 | High |
| Coumarins and Lactones | 5 | 4 | 4.5 | 0.1111 | 2.7055 | Average |
| Anthraquinones | 8 | 1 | 4.5 | 5.4444 | 2.7055 | High |
| Saponins | 1 | 8 | 4.5 | 5.4444 | 2.7055 | Low |
| Tannins | 7 | 2 | 4.5 | 2.7778 | 2.7055 | High |
| Cardiac glycosides | 6 | 3 | 4.5 | 1 | 2.7055 | Average |
| Phenols | 7 | 2 | 4.5 | 2.7778 | 2.7055 | High |
| Phytosterols | 8 | 1 | 4.5 | 5.4444 | 2.7055 | High |

Table 4.3; Relative abundance of phytochemicals present in water leaves extracts of *Annona muricata*

| Phytochemical | High | Low | E | χ^2 Calculated | χ^2 critical | Conclusion |
|------------------------|------|-----|-----|---------------------|-------------------|------------|
| Alkaloid | 8 | 1 | 4.5 | 5.4444 | 2.7055 | High |
| Terpenoids | 7 | 2 | 4.5 | 2.7778 | 2.7055 | High |
| Flavonoids | 1 | 8 | 4.5 | 5.4444 | 2.7055 | Low |
| Coumarins and Lactones | 6 | 3 | 4.5 | 1 | 2.7055 | Average |
| Anthraquinones | 8 | 1 | 4.5 | 5.4444 | 2.7055 | High |
| Saponins | 7 | 2 | 4.5 | 2.7778 | 2.7055 | High |
| Tannins | 1 | 8 | 4.5 | 5.4444 | 2.7055 | Low |
| Cardiac glycosides | 5 | 4 | 4.5 | 0.1111 | 2.7055 | Average |
| Phenols | 8 | 1 | 4.5 | 5.4444 | 2.7055 | High |
| Phytosterols | 7 | 2 | 4.5 | 2.7778 | 2.7055 | High |

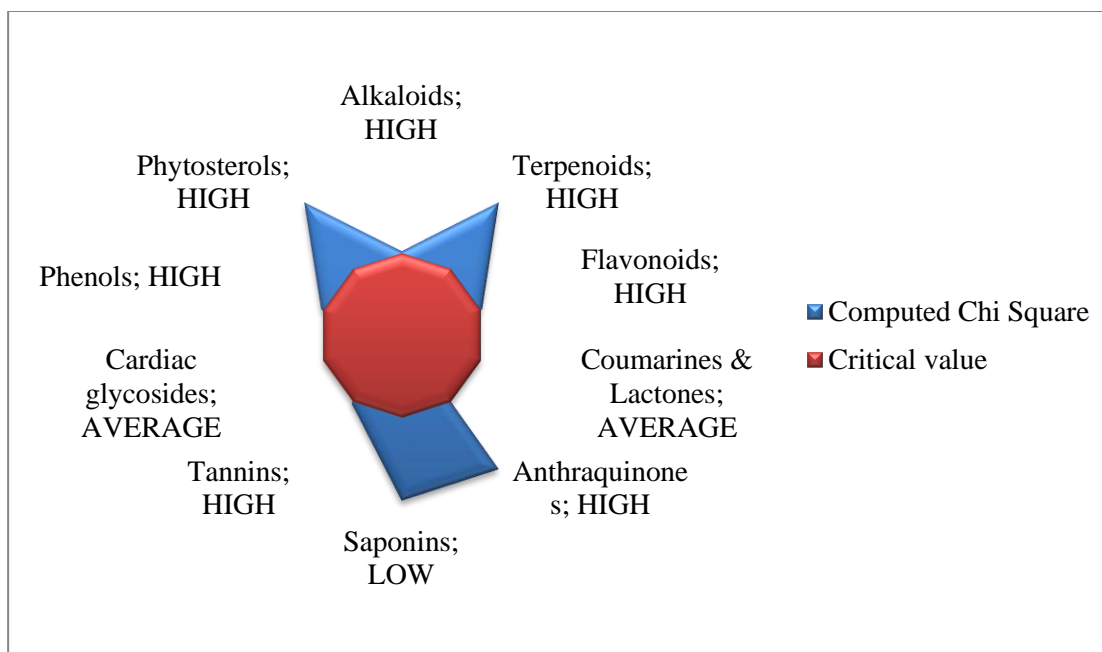


Figure 4.1; Phytochemicals present in Ethanolic leaves extracts of *Annona muricata* with relative abundance computed from the χ^2 Test

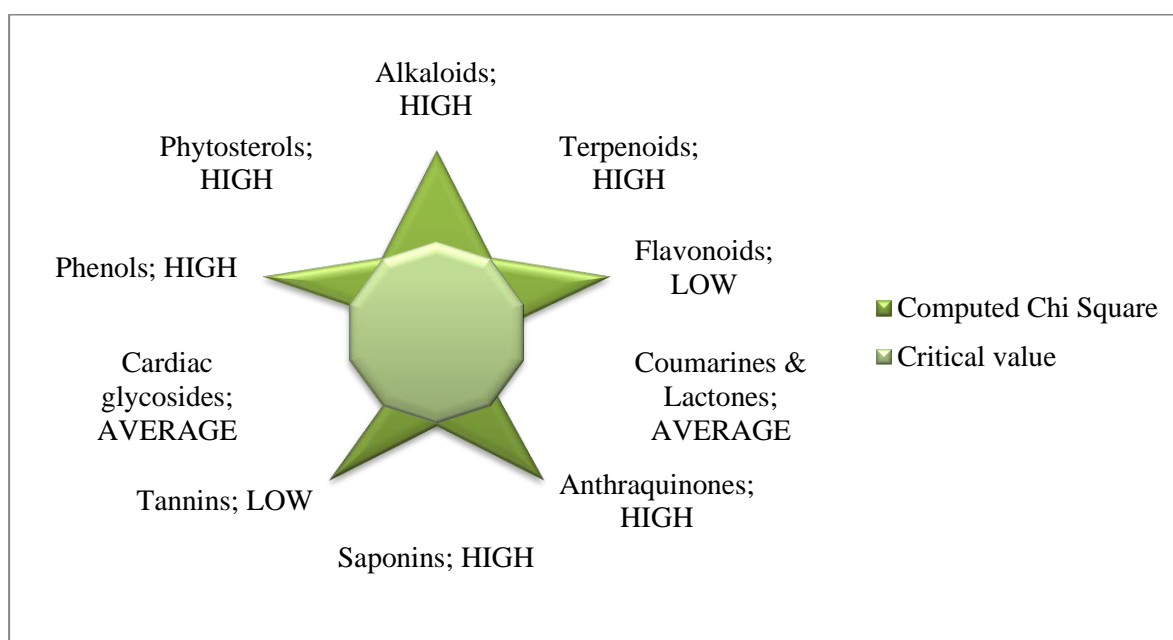


Figure 4.2; Phytochemicals present in water leaf extracts of *Annona muricata* with relative abundance computed from the χ^2 Test

The χ^2 goodness of fit test has been used to allocate the relative abundance of each of the phytochemicals. Phytochemicals with χ^2 values higher than the χ^2_{critical} were designated as high or low, depending on the initial count while those that had χ^2 values less than the χ^2_{critical} were assigned average abundance as shown in figures 4.1 and 4.2

4.1.3 Total Phenolic compounds

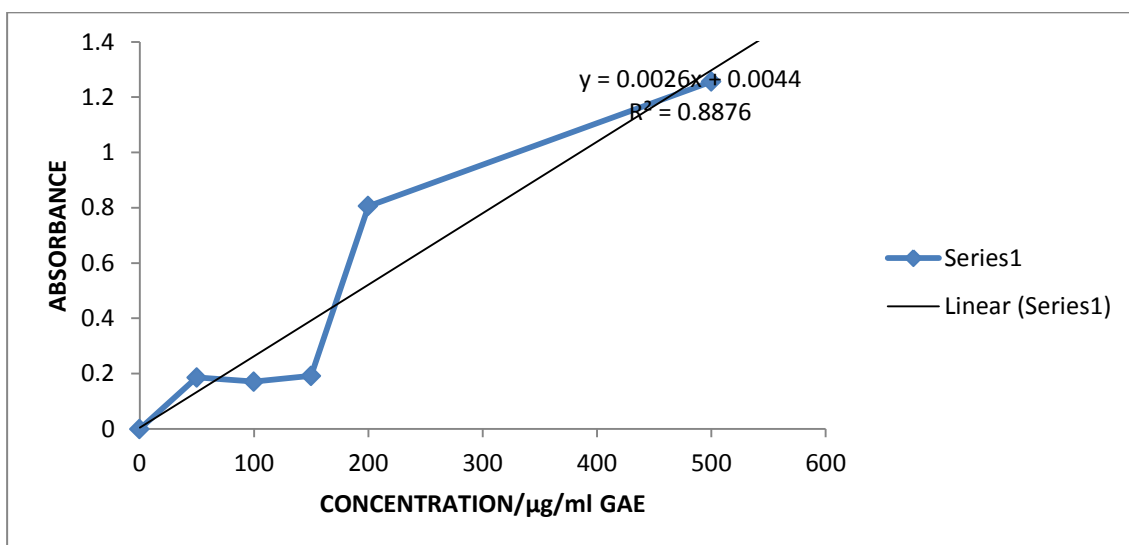


Figure 4.3; Gallic acid standard curve for determination of total phenols

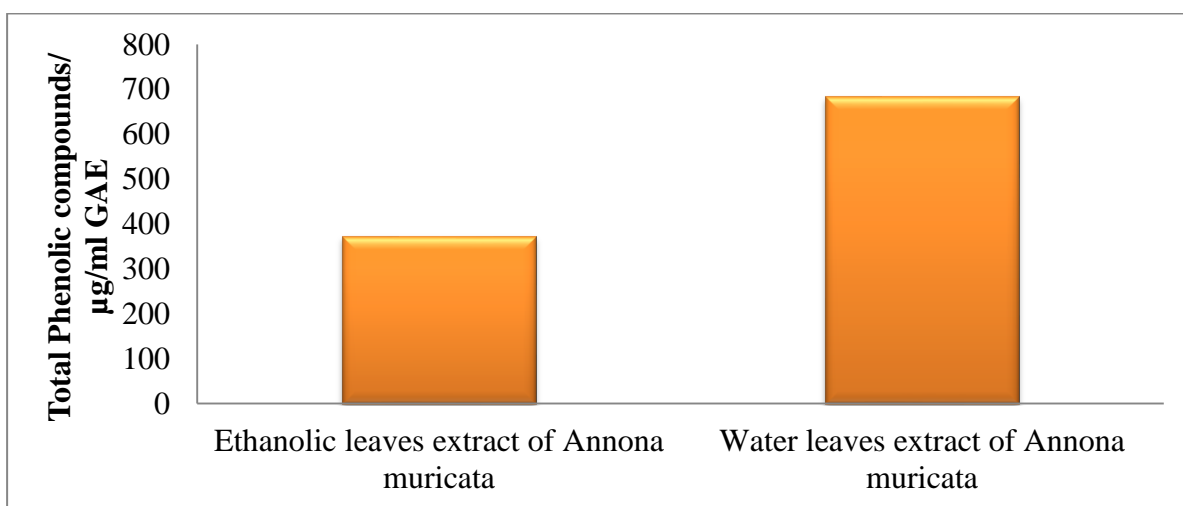


Figure 4.4; Total phenolic compounds in the extracts

Total phenolics ($y=0.0026x + 0.0044$) in the water extract were 683.69 ± 0.09 µg/ml GAE while it was 372.92 ± 0.15 µg/ml GAE in the ethanolic extract

4.2 Gas Chromatography Mass Spectroscopy Analysis

GCMS analysis was carried out on the ethanolic leaves extract of *Annona muricata*. The total ion chromatogram (TIC) of the ethanolic extract, showing the GC-MS profile of the compounds identified is given in figure 4.5 below. The peaks in the chromatogram were integrated and were compared with the database of spectrum of known components stored in the GC-MS NISP library. The detailed tabulations of GC-MS analysis of the extracts are given in Table 4.4. Phytochemical analysis by GC-MS analysis of the ethanolic leaves extract of *Annona muricata* revealed the presence of different fatty acids, heterocyclic compounds, esters etc. 25 peaks were generated, of which 12 compounds matching the peaks were identified, but 13 of the GC-MS peaks remained unidentified, because of lack of library data of corresponding compounds.

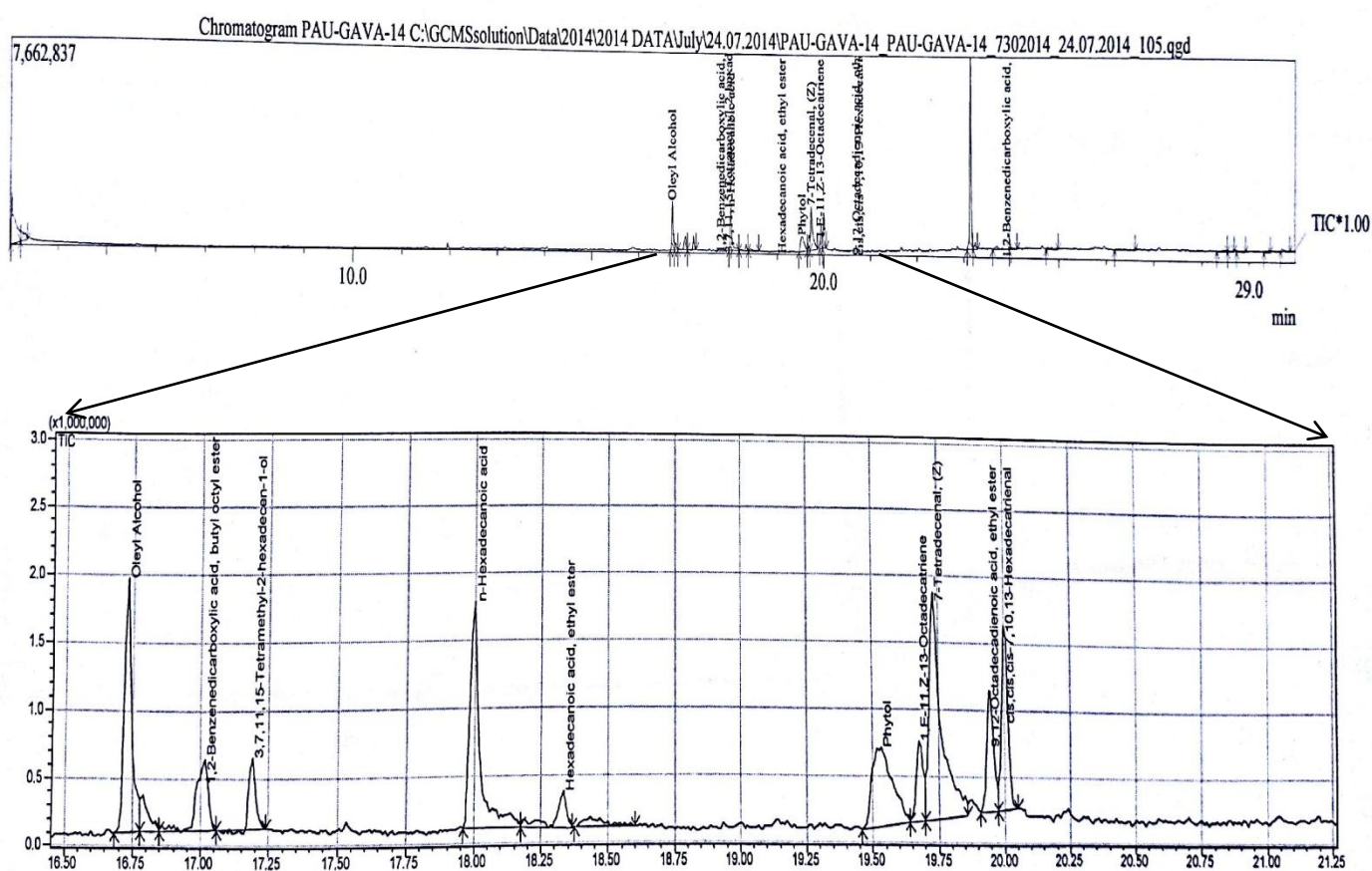


Figure 4.5; Total Ion Chromatogram (TIC) of ethanolic leaves extract of *Annona muricata*

Table 4.4; Phyto-components generated in the ethanolic leaves extract of *Annona muricata* by GC-MS Peak Report TIC

| Peak # | Retention time/ minutes | % Composition by Area | Matched compound IUPAC Name | Chemical Formula | Comment |
|--------|-------------------------|-----------------------|---|--|--------------|
| 1 | 3.034 | 16.8 | - | - | Unidentified |
| 2 | 3.342 | 3.93 | 2-Pentadecanol | C ₁₅ H ₃₂ O | Matched |
| 3 | 16.732 | 6.15 | Oleryl Alcohol | C ₁₈ H ₃₆ O | Matched |
| 4 | 16.792 | 1.04 | - | - | Unidentified |
| 5 | 17.015 | 2.67 | 1,2-Benzenedicarboxylic acid, butyl octyl ester | C ₂₀ H ₃₀ O ₄ | Matched |
| 6 | 17.19 | 1.37 | 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | C ₂₀ H ₄₀ O | Matched |
| 7 | 17.998 | 7.12 | n-Hexadecanoic acid | C ₁₆ H ₃₂ O ₂ | Matched |
| 8 | 18.331 | 1.29 | Hexadecanoic acid, ethyl ester | C ₁₈ H ₃₆ O ₂ | Matched |
| 9 | 18.431 | 0.73 | - | - | Unidentified |
| 10 | 19.531 | 5.61 | Phytol | C ₂₀ H ₄₀ O | Matched |
| 11 | 19.678 | 2.15 | 1,E-11,Z-13-Octadecatriene | C ₁₈ H ₃₂ | Matched |
| 12 | 19.733 | 9.39 | 7-Tetradecenal, (Z) | C ₁₄ H ₂₆ O | Matched |
| 13 | 19.946 | 3.21 | 9,12-Octadecadienoic acid, ethyl ester | C ₂₀ H ₃₆ O ₂ | Matched |
| 14 | 20.003 | 4.26 | cis, cis, cis-7,10,13-Hexadecatrienal | C ₁₆ H ₂₆ O | Matched |
| 15 | 23.143 | 23.51 | - | - | Unidentified |
| 16 | 23.225 | 1.08 | - | - | Unidentified |
| 17 | 23.695 | 1.74 | - | - | Unidentified |
| 18 | 24.025 | 0.75 | 1,2-Benzenedicarboxylic acid, diisooctyl ester | C ₂₄ H ₃₈ O ₄ | Matched |
| 19 | 24.75 | 1.25 | - | - | Unidentified |
| 20 | 26.402 | 1.54 | - | - | Unidentified |
| 21 | 28.35 | 1.12 | - | - | Unidentified |
| 22 | 28.567 | 0.74 | - | - | Unidentified |
| 23 | 28.875 | 0.85 | - | - | Unidentified |
| 24 | 29.331 | 0.74 | - | - | Unidentified |
| 25 | 29.704 | 0.95 | - | - | Unidentified |

4.3 Anti-oxidant analysis

4.3.1 Reducing power of the extracts

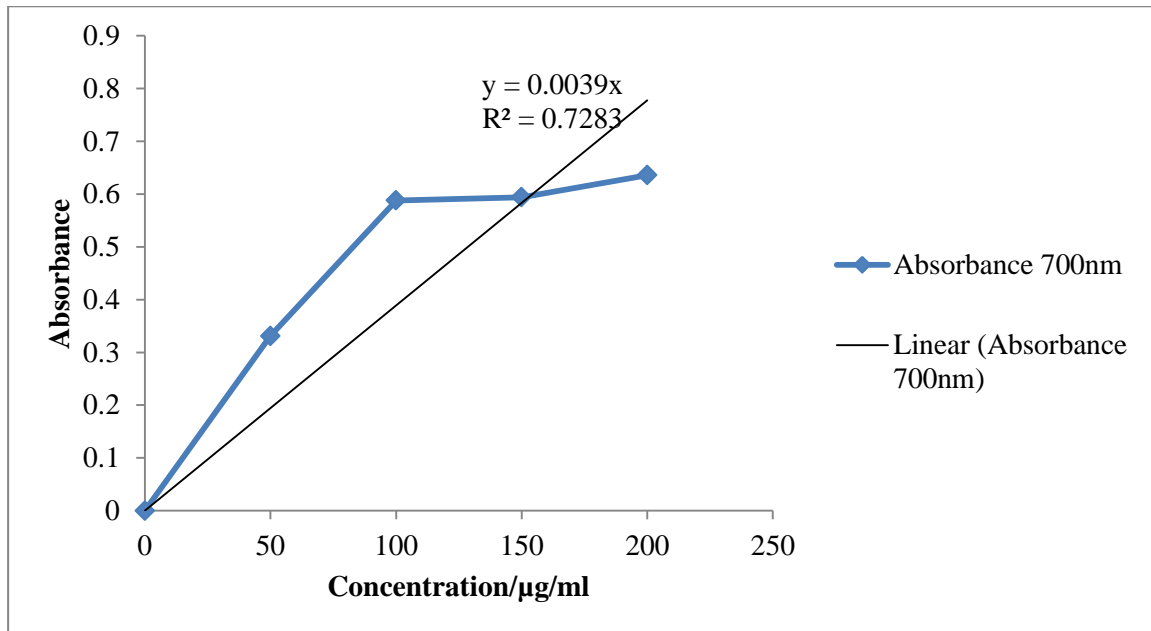


Figure 4.6; Gallic acid standard curve for determination of reducing power

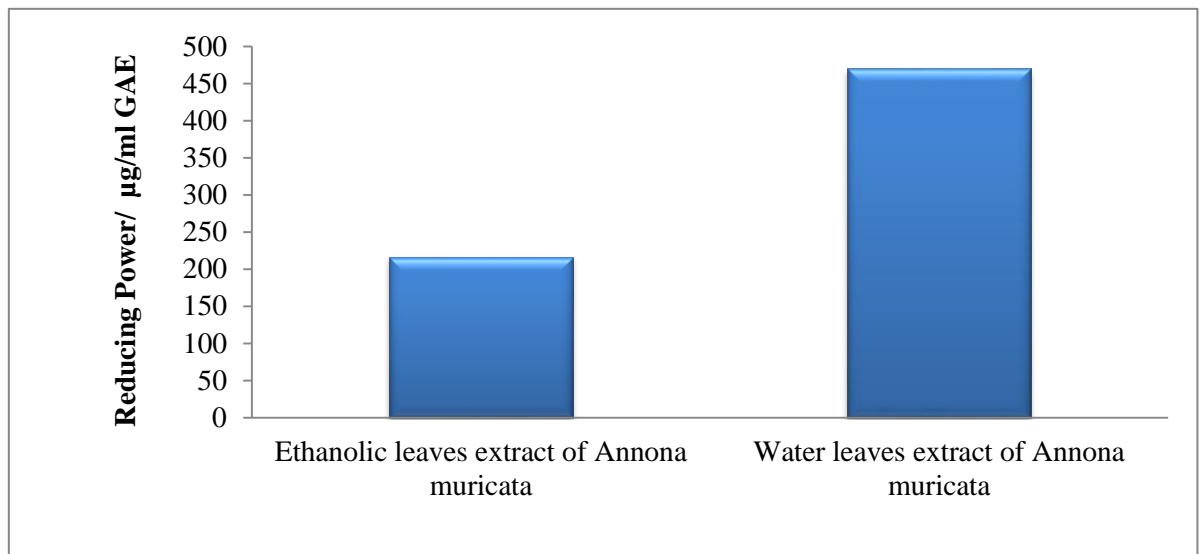


Figure 4.7; Reducing power of both the ethanolic and water leaves extracts of *Annona muricata*

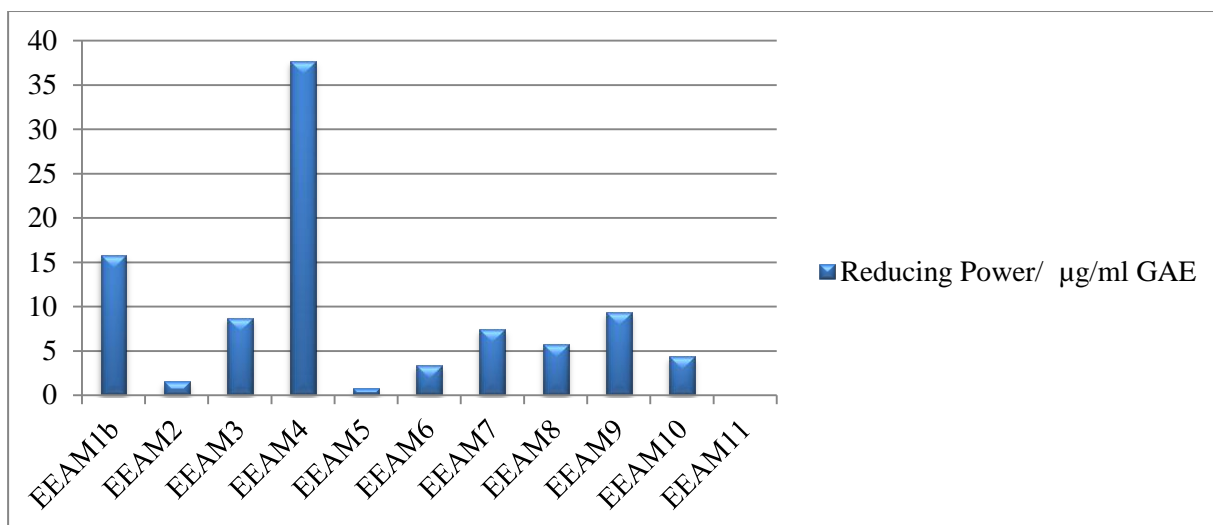


Figure 4.8; Reducing power of the *Annona muricata* Ethanol leaves extract Fractions

The reducing power ($y=0.0039x$) of both the ethanolic and water leaves extracts of *Annona muricata* as well as that of the TLC fractions of the ethanolic leaves extracts are shown by figures 4.7 , 4.8 above. It is evident that the water extract had a higher reducing power than the ethanolic extract. On the other hand, the TLC fraction EEAM4 had the highest reducing power whereas fraction EEAM11 registered no reducing power activity.

4.3.2 Quantification of antioxidant activity using the DPPH method

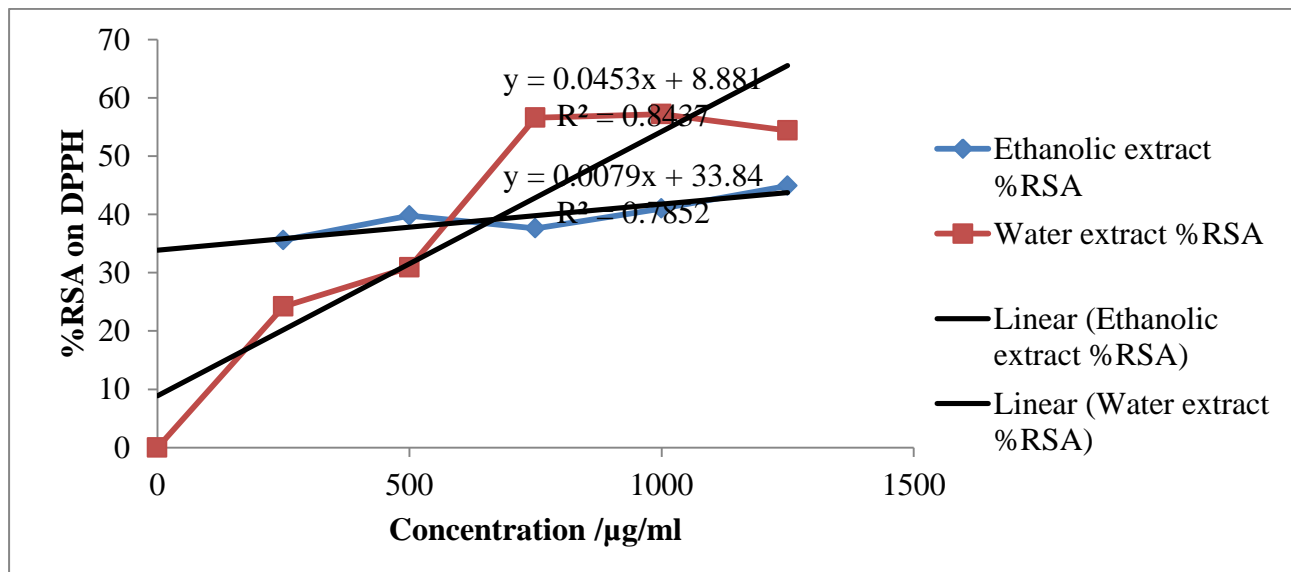


Figure 4.9; Decrease in the concentration of DPPH radical due to the scavenging ability of the soluble constituents in the ethanolic and water leaves extracts of *Annona muricata*

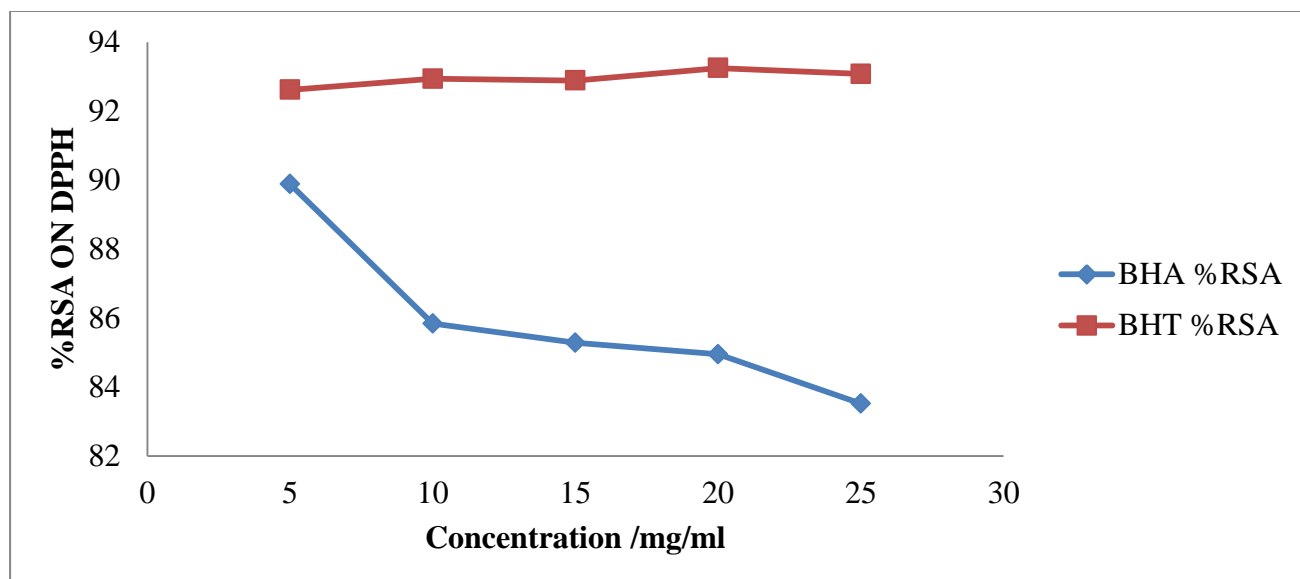


Figure 4.10; Anti-oxidant activity of positive controls; BHA and BHT

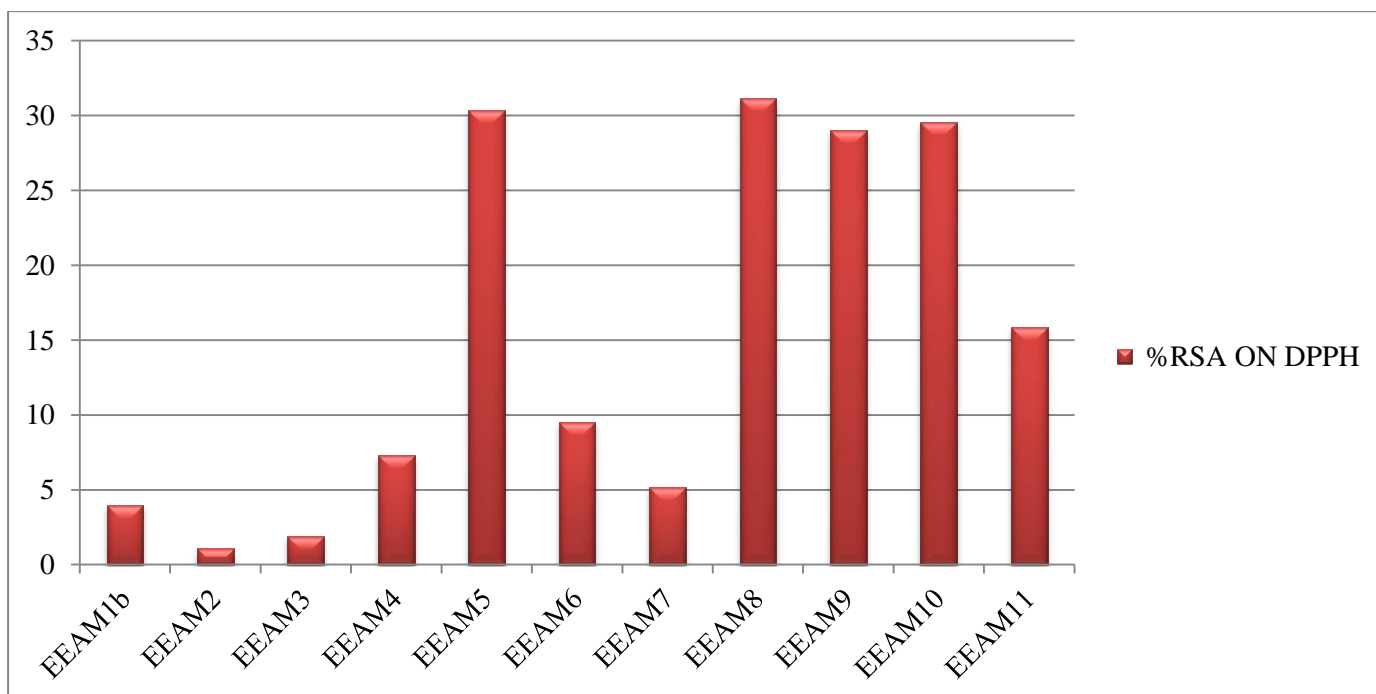


Figure 4.11; Antioxidant activity of the *Annona muricata* Ethanolic leaves extract Fractions on DPPH

The figure above shows the anti-oxidant activities of each of the 11 fractions isolated from the ethanolic leaves extracts of *Annona muricata* by TLC technique. The highest activity was recorded in fraction EEAM8 and the lowest activity was registered by fraction EEAM2.

4.4 *In vitro* cytotoxic activity of leaves extracts of *Annona muricata*

Trypan blue-exclusion assay (TBEA) was used for the evaluation of cytotoxic activity of ethanolic and water leaves extracts of *Annona muricata* against EACC, and for cytotoxicity against normal spleen cells. While the MTT assay was used for the evaluation of cytotoxic activity of ethanolic leaves extracts of *Annona muricata* against two human breast cancer cell lines MDA and SKBR3.

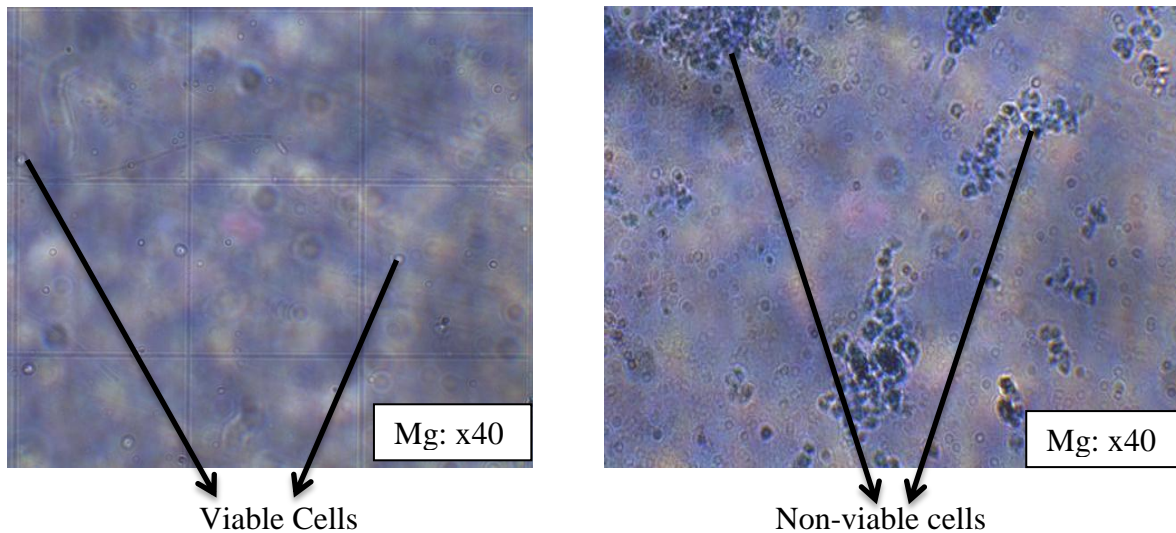


Figure 4.12; Photomicrograph showing viable cells and nonviable cells after treatment with extracts

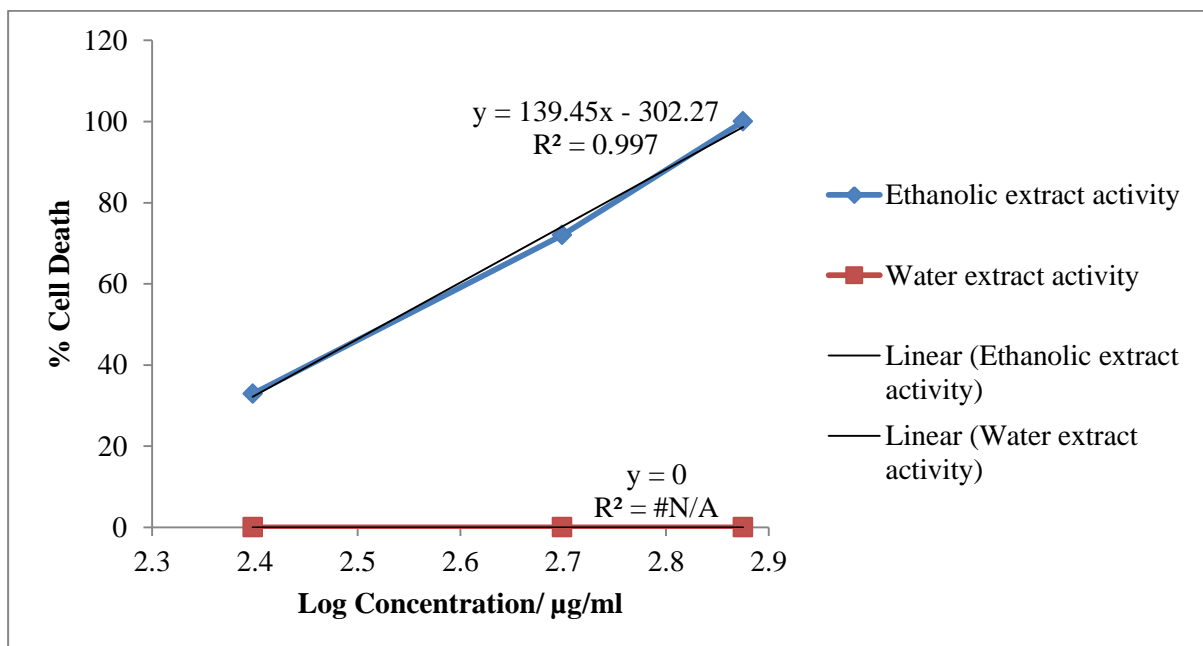


Figure 4.13; Cytotoxic activity of Ethanolic and Water leaves extracts of *Annona muricata* on EACC

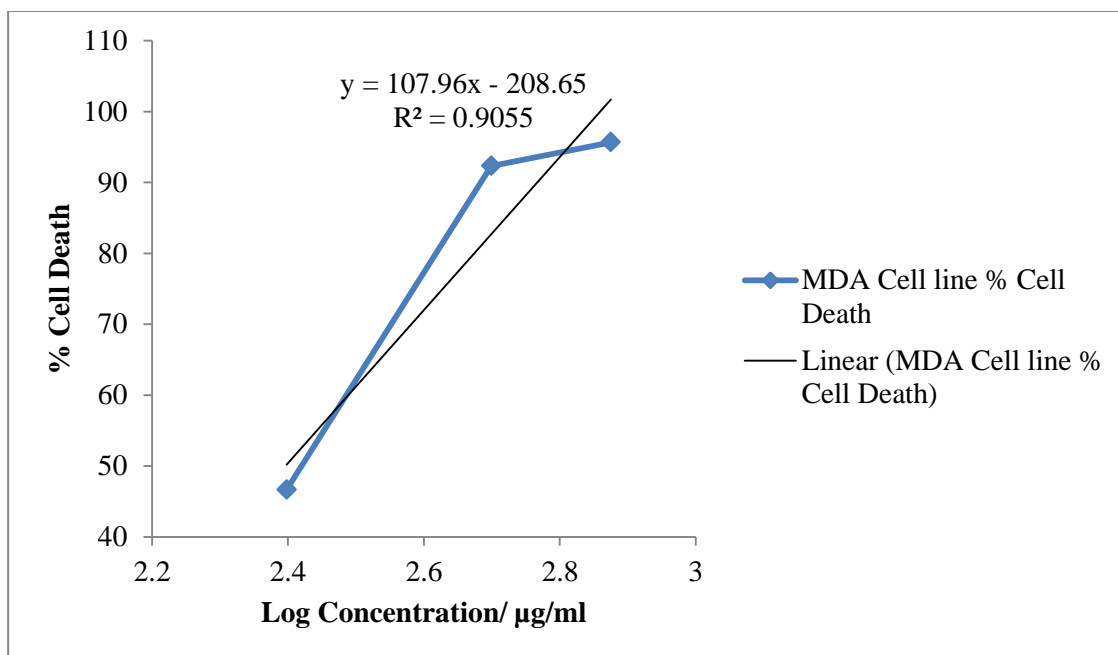


Figure 4.14; Effect of Ethanolic leaves extracts of *Annona muricata* on MDA Cell line using the MTT Assay

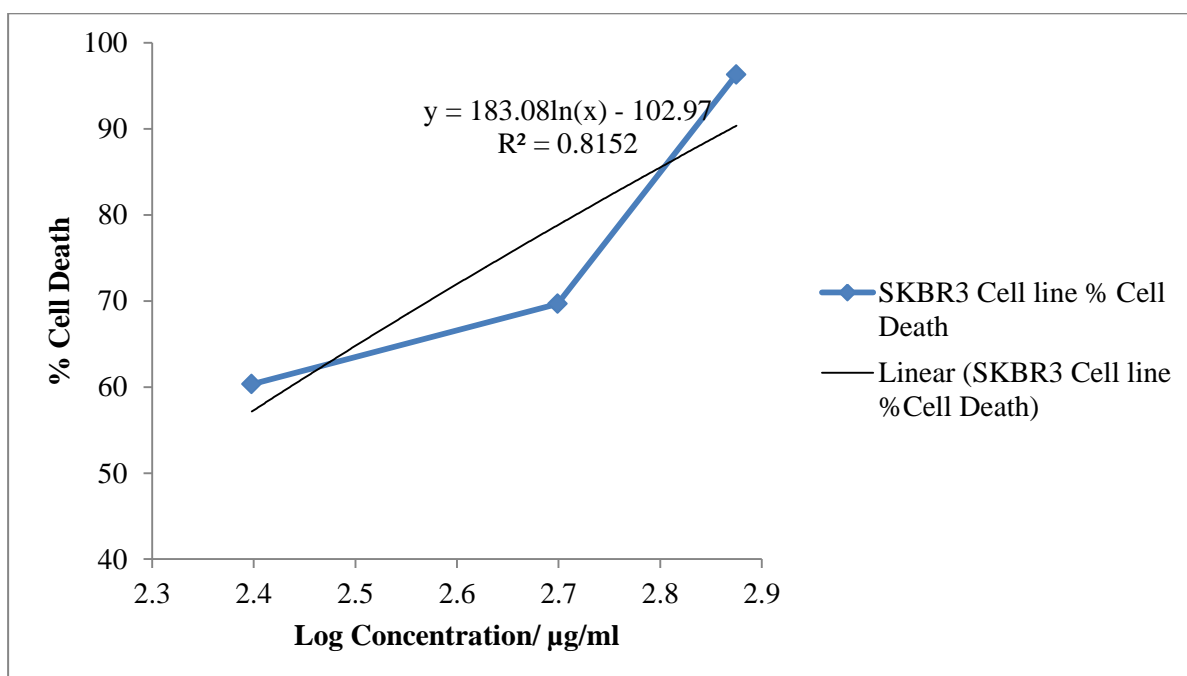


Figure 4.15; Cytotoxic effect of Ethanolic leaves extracts of *Annona muricata* on SKBR3 Cell line using the MTT Assay

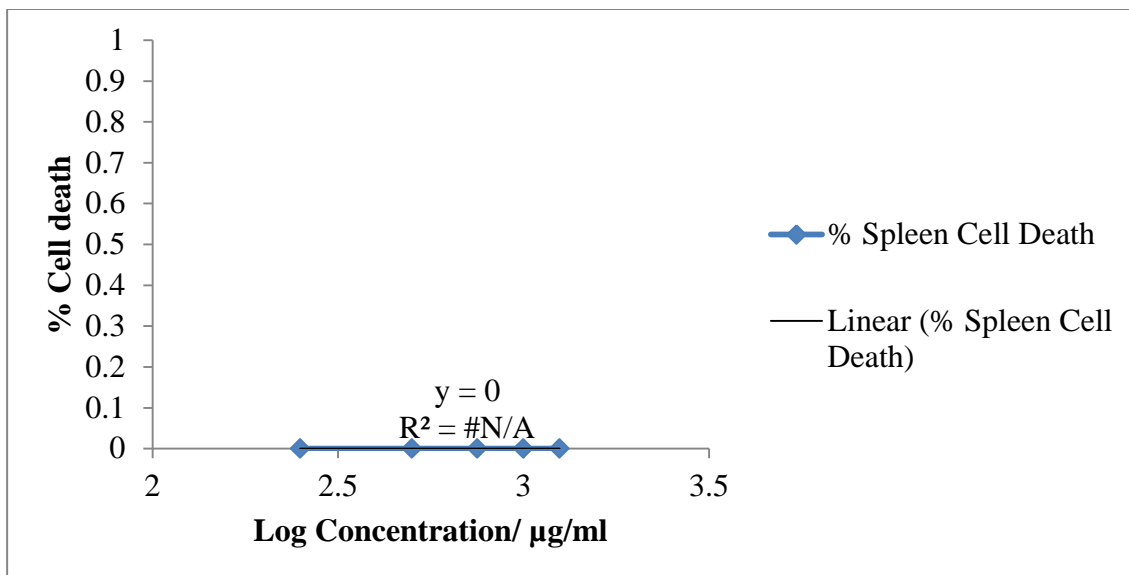


Figure 4.16; Results for the cytotoxicity test for the activity of ethanolic leaves extracts of *Annona muricata* on normal spleen cells using the Trypan Blue Exclusion Assay.

Table 4.5; IC₅₀ Values for the Ethanolic leaves Extracts of *Annona muricata*

| Cell line | Assay | IC ₅₀ / µg/ml |
|-----------------------|-------|--------------------------|
| EACC | TBEA | 335.85 |
| MDA | MTT | 248.77 |
| SKBR3 | MTT | 202.33 |
| Spleen Cells (Normal) | TBEA | #N/A |

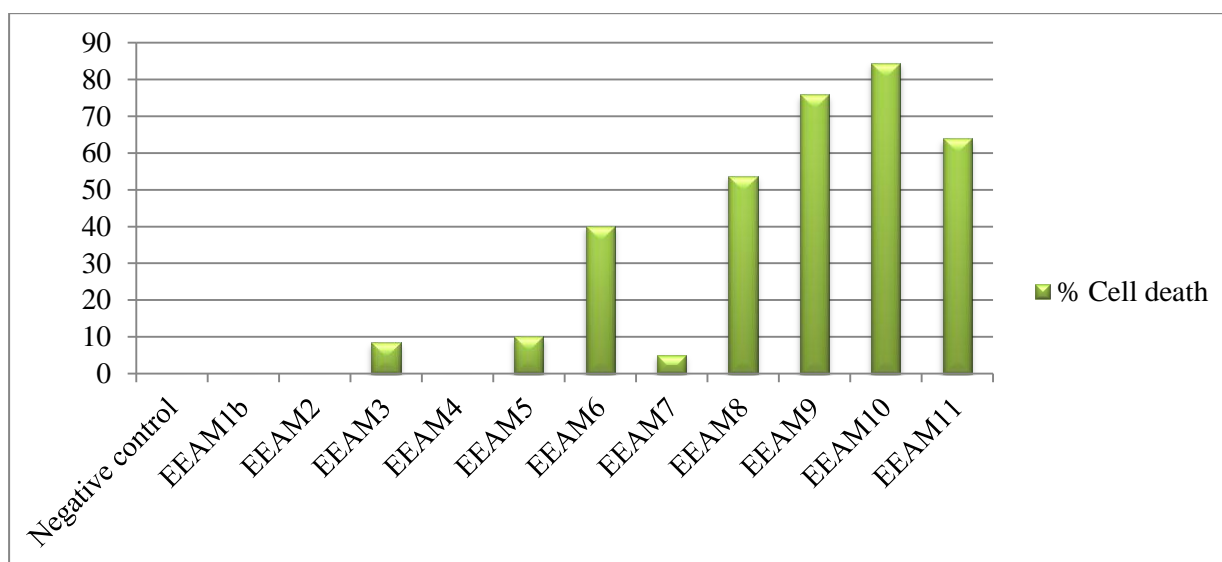


Figure 4.17; Cytotoxic activity of the *Annona muricata* Ethanolic leaves extract TLC Fractions on EACC

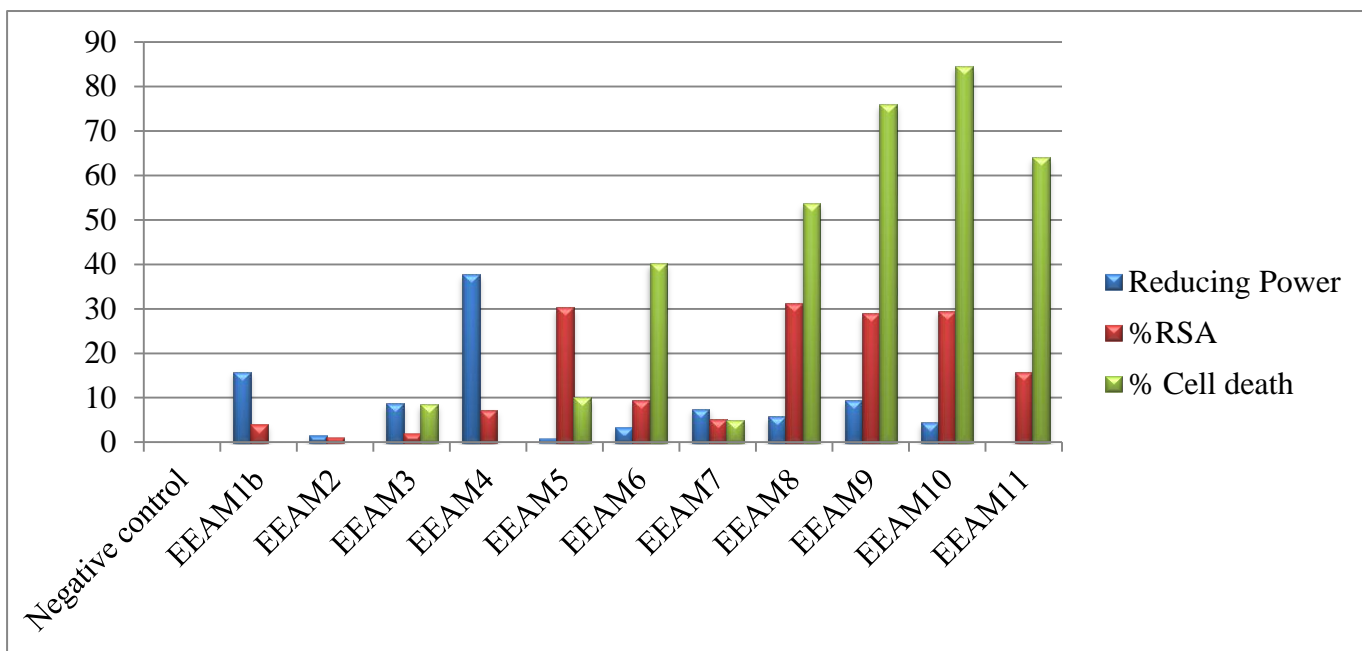


Figure 4.18; Comparison between Anti-oxidant activity (Reducing Power & %RSA) and cytotoxic activity (%Cell death) of Ethanolic leaves extracts TLC Fractions of *Annona muricata*.

CHAPTER 5: DISCUSSION

The objective of this study was to determine the phytochemical composition, anti-oxidant activity and *in vitro* cytotoxic activity of ethanolic and water leaves extracts of *Annona muricata*. Standard procedures were used in assaying for the different components of the study.

Phytochemical screening conducted on leaves extracts of *Annona muricata* revealed the presence of following classes of compounds: alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, Cardiac glycosides, phenols, phytosterols, and saponins. They were present in both the ethanolic and water leaves extracts, but with noticeable differences in relative abundance in both as shown in the figures 4.1 and 4.2. These results are in line with earlier studies that were carried out on the ethanolic seeds extract of *Annona muricata*, and the phytochemical tests showed that ethanol soursop seeds extract contains secondary metabolites compounds group of saponins, alkaloids and triterpenoids, flavonoids, anthraquinones, tannins, and cardiac glycosides, which they noted that they are defense chemical compounds of plants produced in the plant tissue (Ukwubile, 2012; Komansilan *et al.*, 2012).

The extracts were found to be rich in alkaloids which have wide pharmacological effects and thus have been used extensively as drugs in medical field. The detection of high levels of alkaloids in the leaves extracts of *Annona muricata* further reinforces the presence of alkaloid in this species as already outlined by other independent studies (Mishra *et al.*, 2013) that showed that among the chemical constituents found in *Annona muricata*, the alkaloids and essential oils stand out. Cardiac glycosides are molecules used in treatment of heart failure (Kashani *et al.*, 2012), hence the present findings with, finds leaves of extracts suitable for use in treatment of heart diseases.

Generally, presence of alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, Cardiac glycosides, phenols, phytosterols, and saponins confirms that *Annona muricata* leaves extracts contain molecules known for extensive use in the medical field both traditionally and pharmaceutically. This would be an indication for its potential use in anti-inflammatory, anti-allergic, antibacterial, and antiviral, heart failure, antioxidant and anticancer activity among others. These findings emphasize the value of traditional knowledge in the use of plants for medicinal use as well as pharmaceutical development. The use of *Annona muricata* in traditional medicine is validated by presence of these phytochemicals of known health benefits and thus the interest in further studies on this species.

The phenolic content of the *Annona muricata* was determined and all results were expressed as Gallic Acid Equivalents (GAE). Typical phenolics that possess antioxidant activity have been characterized

as phenolic acids and flavonoids (Abou Elalla and Shalaby, 2009; Kahkonen *et al.*, 1999). Phenols are among the non-enzymatic compounds obtained from natural sources, which have received high attention due to their proven antioxidant capabilities. Although phenolic compounds have been related to antioxidant activity, some studies have emphasized specific classes such as flavonoids and tannins (Joabe *et al.*, 2010). Our results revealed the water leaves extract having higher total phenolic content as compared to the ethanolic leaves extract of *Annona muricata* as shown in figure 4.4. The higher phenolic content in the water extract would partly contribute to its higher antioxidant activity.

Using GC-MS Analysis, 25 compounds have been elucidated for the first time in our study on *Annona muricata*, of which 12 compounds were effectively matched and identified as shown in Figure 4.5 and Table 4.4. The ethanolic leaves extract of the plant showed 25 constituents, the major constituents were at peaks 15 (peak area 23.51%), Peak 1 (peak area 16.8%), 7-Tetradecenal, (Z) (peak area 9.39%), n-Hexadecanoic acid (peak area 7.12%), Oleryl Alcohol (peak area 6.15%), Phytol (peak area 5.61%), cis, cis, cis-7,10,13-Hexadecatrienal (peak area 4.26%), 2-Pentadecanol (peak area 3.93%), 9,12-Octadecadienoic acid, ethyl ester (peak area 3.21%), 1,2-Benzenedicarboxylic acid, butyl octyl ester (peak area 2.67%), and 1,E-11,Z-13-Octadecatriene (peak area 2.15%), while the rest had less than 2% composition by peak area.

1,2-Benzenedicarboxylic acid, butyl octyl ester is a Plasticizer compound with Antimicrobial, Antifouling, Antioxidant and Hypo-cholesterolemic activities (Sathish *et al.*, 2012). 3,7,11,15-Tetramethyl-2-hexadecen-1-ol is recorded to have Anti-tuberculosis, insecticidal, anti-inflammatory, antioxidant and antimicrobial activities. n-Hexadecanoic acid on the other hand which is commonly known as Palmitic acid has Nematicide, Pesticide, Lubricant, Anti-androgenic, Flavor, Hemolytic 5-Alpha reductase inhibitor, Antioxidant and Hypo-cholesterolemic properties (Komansilan *et al.*, 2012). Hexadecanoic acid, ethyl ester is a fatty acid ester with nematicide, pesticide, lubricant, anti-androgenic, flavor, and has Hemolytic 5-Alpha reductase inhibitor properties (Venkata-Raman *et al.*, 2012; Aneesh *et al.*, 2013).

Phytol is a Diterpene with Antimicrobial, Anticancer, Anti-inflammatory, anti-diuretic, immune-stimulatory and anti-diabetic properties. 9,12-Octadecadienoic acid, ethyl ester is a Linoleic acid which has Hypo-cholesterolemic, 5-Alpha reductase inhibitor, Antihistaminic, Insectifuge, Anti-eczemic, and Anti-acne properties. Finally, 1,2-Benzenedicarboxylic acid, diisooctyl ester is a Plasticizer Compound with Antimicrobial and Antifouling properties (Paranthaman *et al.*, 2012; Aneesh *et al.*, 2013; Venkata Raman *et al.*, 2012).

It is worth noting that of the major constituents identified in our extract, the compounds with the highest composition at peaks 15 (peak area 23.51%) and Peak 1 (peak area 16.8%) have not been

matched in the library. These two could be very novel compounds that need to be analyzed further in order to elucidate their nature. The same applies to the remaining 11 compounds which have not been identified irrespective of their percentage composition being less than 2%.

GC-MS analysis of the ethanolic leaves extract of *Annona muricata* revealed the presence of different fatty acids, heterocyclic compounds, esters among others. This confirms the results on presence of the various secondary metabolite compounds detected by the qualitative procedures discussed earlier. These mass spectra are fingerprint of the compound which can be identified from the data library. Hence, the identified phytochemicals using GC-MS can be used as a pharmacognostical tool for the identification of adulterants. The current pioneering study suggests that ethanolic extract is a potent therapeutic agent. It paves the way for the development of several treatment regimens based on this extract. In addition, further research is necessary to identify and purify the active compounds responsible for therapeutic activity, as well as the unidentified compounds.

Several methods have been developed to measure the efficiency of antioxidants as pure compounds or in extract. These methods focus on different mechanisms of the oxidant defense system that is scavenging active oxygen species and hydroxyl radicals, inhibiting of lipid peroxidation, or chelating of metal ions (Dorman *et al.*, 2003). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. It was found that in general, the reducing power of the water leaves extracts was higher than that of the ethanolic leaves extracts (Figure 4.7), giving an indication of the potential higher anti-oxidant activity of the extracts.

Figure 4.8 showed the reducing power of 11 fractions of ethanolic extract and it was observed that most of all the fractions had a very low reducing power of less than 50 μ g/ml GAE. This may be translated into the observation that perhaps the final reducing power of the extract is as a result of the combined effect of each of the compounds in the fractions.

Free radical scavenging activity of the leaves extracts was determined. The Data showed that water leaves extracts of *Annona muricata* had a higher free radical inhibition with an IC₅₀ of 0.9077mg/ml as compared to the ethanolic leaves extract with an IC₅₀ of 2.0456mg/ml (Figure 4.9). The standard antioxidants BHA and BHT were used as positive controls and showed ultimately very high antioxidant activities with very little effect with increasing concentrations (Figure 4.10), and an indication that the method used was effective in quantifying the activity. The free radical scavenging activity of the leaves extracts were far lower than the standard Positive controls implying that the extracts, at similar concentrations may not be competitively strong antioxidants. It is however likely that the leaves extract's antioxidant activity of *Annona muricata* may be as strong as standard BHA

and BHT, given that the samples assayed in this study were crude extracts, while the standard controls are usually very purified compounds.

It is not surprising that the water leaves extracts of *Annona muricata* had a stronger antioxidant activity as compared to the ethanolic leaves extract. This is expected as the early tests in this study revealed the total phenolics to be higher in the water leaves extracts two fold to the ethanolic leaves extracts, and phenolics have long been associated with antioxidant activity. Similarly, the water leaves extracts had reducing power almost two times higher than that of the ethanolic leaves extracts. In general however, the relatively strong antioxidant activity makes this plant efficient in managing oxidative stress related diseases; this could be the reason as to why it is used in traditional medicine to manage such diseases where the water extracts are mostly applied.

Earlier studies by Joabe *et al.*, 2010 revealed the antioxidant activity of methanolic bark extract of *Annona muricata* with an IC_{50} of 0.2215 ± 0.01652 mg/ml, which is far higher than our current study. Also in another study, the ethanolic bark extracts of *Annona muricata* registered the IC_{50} values as 0.109mg/ml (Ravishankar *et al.*, 2013). The difference in antioxidant power in results recorded can be partly attributed to the fact that different parts of the plant were used in the current study and the previous studies as well as being attributed to the difference in geographical locations, as both studies were conducted in different areas. The results however agree with Mishra *et al.*, 2013 who noted that *Annona muricata* leaves extracts have antioxidant and molluscicidal properties.

The ethanolic extract fractions showed relatively low reducing power less than 50% inhibition even in the fraction showing the highest activity (Figure 4.11). This suggests that probably the overall antioxidant activity of the extract is as a result of the synergistic combination of the activity of all the compounds in the fractions, especially fractions EEAM5, EEAM8, EEAM9, EEAM10, and EEAM11, which registered activity of higher than 15%. These results provide a possible lead towards the further studies and development of pharmaceutical products with antioxidant properties by targeting the fractions showing the highest activity.

The minimum detectable cytotoxic activity on EACC cell line was observed in the ethanolic leaves extract of *Annona muricata* at a concentration of 250 μ g/ml, with an inhibition of 32.9% Cell death, and reaching a maximum inhibition of 100% cell death at a concentration of 750 μ g/ml (Figure 4.13). IC_{50} of ethanolic extracts was determined to be 335.85 μ g/ml.

The effect of ethanolic extract on two human breast cancer cell lines MDA and SKBR3 was tested at concentrations ranging from 250 to 750 μ g/ml for 72hours, and % of cell death was measured by the MTT assay. The results demonstrated a strong dose-dependent inhibition in treated cell lines. The ethanolic leaves extract were thus found to be highly cytotoxic in vitro against the two human breast

cancer cell lines MDA and SKBR3 (Figures 4.14 and 4.15) with IC_{50} of 248.77 μ g/ml and 202.33 μ g/ml respectively. These results showed that the extract having a very high cytotoxic activity on three cell lines with IC_{50} values which are very close to each other, despite the difference in the method used and source of the cells (Table 4.5). The slightly lower cytotoxic activity against EACC may be attributed to the difference in sensitivity of the Trypan Blue Exclusion assay with respect to the MTT assay used for studying activity against MDA and SKBR3 cell lines.

An integrated part of cancer cell development is the resistance to programmed cell death (apoptosis) and therefore re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents (Joshi *et al.*, 1999). Some plant-derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents (Wamidh, 2011, Joshi *et al.*, 1999). The current study showed the highly effective action of the ethanolic leaves extract of *Annona muricata* and can be used in the management and treatment of cancer. This is in line with a study which showed that any extract has anticancer and cytotoxic activity if it has an IC_{50} value less than 1000 μ g/ml after 24 hours contact time, and that the smaller the IC_{50} value of a test compound the more toxic the compound is (Hirano *et al.*, 1995).

The results of the cytotoxicity test on normal spleen cells of the ethanolic leaves extracts of *Annona muricata* indicate a very high selectivity of the extracts for cancer cells, as they showed no effect on the normal spleen cells throughout the range of concentrations tested. 100% spleen cell viability was observed at all tested concentrations (Figure 4.16). The high selectivity of the extract for cancer cells is a very important aspect for its use in treatment of cancer as normal cells would not be targeted.

The current study confirms earlier studies which showed that extracts of *Annona muricata* have been reported to be selectively toxic *in vitro* to certain types of tumour cells including: lung carcinoma cell lines; human breast solid tumour lines; prostate adenocarcinoma; pancreatic carcinoma cell lines; colon adenocarcinoma cell lines; mammary adenocarcinoma cell lines; liver cancer cell lines; human lymphoma cell lines; and multi-drug resistant human breast adenocarcinoma (Odeghe *et al.*, 2013). Other earlier studies also demonstrated it to be selectively toxic against various types of the cancerous cells without harming healthy cells (Hamizah *et al.*, 2012; Rieser *et al.*, 1993; Wu *et al.*, 1995).

The water extracts however showed no effect throughout the range of tested concentrations. This conspicuous lack of cytotoxic activity of the water leaves extract despite its having a high antioxidant activity and reducing power compared to the ethanolic extract may elicit a number of theories pertaining the mechanism of action of the cytotoxic agents in this plant which may be different from the commonly generalized idea that anticancer activity and cytotoxicity is directly related to antioxidant activity. However, the results from the TLC fractionation of ethanolic leaves extracts can

help in explaining the relationship between antioxidant activity and cytotoxic activity (Figure 4.18). This trend is partly in line with an earlier study by Aboul-Enein *et al.*, 2012 which showed a good relationship between antioxidant efficacy of plant extracts and anticancer potency. All of the extracts which gave high anticancer potency have high antioxidant activity while the opposite trend is not (Aboul-Enein *et al.*, 2012). Our results show that despite its lower antioxidant activity, the ethanolic leaves extract had very high cytotoxic activity.

In this case, we propose that the cytotoxic agents present in the ethanolic leaves extracts may be acting in a very different mechanism from that of the antioxidant mechanism. These compounds related to the cytotoxic activity may also be absent from the water extract and not easily detected by the common phytochemical screening methods. Earlier studies showed that *Annona muricata* contains many active compounds and chemicals which are the natural phytochemicals known as *annonaceous acetogenins* (Alali, *et al.*, 1999; Kojima and Tanaka, 2009) yet there are no readily available methods of identifying them. Some of these may have been present in very high quantities in the ethanolic extract, yet absent in the water extract, leading to the difference in cytotoxic activity. However, more studies need to be conducted to elucidate the root cause of this difference.

The cytotoxic activity of the ethanolic leaves extracts fractions showed the highest single activity to be caused by the EEAM10 fraction at a cytotoxic level of more than 80% cell death. Generally four fractions showed very good promising anticancer activity with cytotoxicity levels of more than 50% Cell death, and these fractions were EEAM8, EEAM9, EEAM10 and EEAM11 (Figure 4.17). These fractions may be responsible for the highest cytotoxic activity of ethanolic extract. These compounds may not be present in water extract, specific that the fractions were in medium in polarity (nature of mobile phase). The encouraging results obtained from this work on cytotoxic activity of ethanolic leaves extracts of *Annona muricata* and isolation of the most active fractions represent an important step towards the effective purification, characterization of the active principles in this extract and to understand the mechanism of cytotoxicity of these extracts. This study showed that *Annona muricata* possesses antioxidant and cytotoxic activities that need to be studied further to establish the use of this plant in cancer therapy.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- i. Phytochemical screening of leaves extracts revealed it to be rich in secondary class metabolite compounds. The use of *Annona muricata* in traditional medicine is validated by presence of these phytochemicals of known health benefits and thus the interest in further studies on this species.
- ii. GC-MS Analysis revealed 25 peaks, of which 12 compounds corresponding to 12 peaks were effectively identified. The two compounds with the highest concentration were not identified, and these would be very novel compounds in the activity of the extracts.
- iii. The *in vitro* antioxidant activity of ethanolic and water leaves extracts of *Annona muricata* revealed a significant antioxidant activity in water extract.
- iv. Our study also showed that ethanolic leaves extracts of *Annona muricata* has a cytotoxic effect on three cell lines EACC MDA and SKBR3. Hence, it is anticipated that *Annona muricata* would be a potentially useful pharmaceutical material to manage breast cancer.

6.2 Recommendations

The current study focused on the ethanolic and water leaves extracts of *Annona muricata* and the results have revealed it to be highly effective in anti-oxidant activity, as well as cytotoxic activity (ethanolic extract). We recommend the following to be done in other studies:

- i. To use higher resolution analyses in order to identify some of the unidentified compounds, due to their absence in the library used.
- ii. Undertake further chemical structure elucidation of the most potent fractions isolated in the current study, so as to act as leads for *in silico* modeling and further drug development.
- iii. Similar studies to be conducted on the other plant parts of *Annona muricata* such as fruits, seeds, backs, and roots, independently and in combinations, as well as using other cell lines.
- iv. Studies need to be undertaken to elucidate the different mechanisms of action of the extracts from this plant at a molecular level as well as *in vivo* studies.

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APPENDICES

Appendix 1: Research time frame

| Activities | Year 2013 | | | | | | | Year 2014 | | | | | | | | |
|-----------------------------------|-----------|------|-----|------|-----|-----|-----|-----------|-----|-----|-----|-----|-----|------|-----|---------|
| | Jun | July | Aug | Sept | Oct | Nov | Dec | Jan | Feb | Mar | Apr | May | Jun | July | Aug | Sep-Oct |
| Literature review | | | | | | | | | | | | | | | | |
| Proposal Building | | | | | | | | | | | | | | | | |
| Samples collection | | | | | | | | | | | | | | | | |
| Proposal presentation and Defense | | | | | | | | | | | | | | | | |
| Phytochemical Analysis | | | | | | | | | | | | | | | | |
| Antioxidant analysis | | | | | | | | | | | | | | | | |
| In vitro studies | | | | | | | | | | | | | | | | |
| Thesis writing | | | | | | | | | | | | | | | | |
| Thesis Submission | | | | | | | | | | | | | | | | |
| Thesis Defense | | | | | | | | | | | | | | | | |
| Final Thesis submission | | | | | | | | | | | | | | | | |

Appendix 2: Budget estimate

The following table presents an estimate of the budget.

| Items | Rate | Subtotals/ US\$ |
|---|------|-----------------|
| Materials and supplies | | |
| Proposal and Report binding | - | 100 |
| Other supplies | - | 150 |
| Subtotal | - | 250 |
| Travel | - | 1500 |
| Subtotal | | 1500 |
| Laboratory tests | | |
| Phytochemical analysis, antioxidant analysis, and <i>In vitro</i> studies | - | 3500 |
| Subtotal | - | 3500 |
| Publications and Conferences | | 600 |
| Subtotal | - | 5,850 |
| Total request US\$ = 5,850 | | |