A PRELIMINARY STUDY OF THE ANTIPROLIFERATIVE PROPERTIES OF CRUDE *BLIGHIA SAPIDA* SEEDS EXTRACTS ON LUNG, PROSTATE, SKIN, LEUKEMIC CANCERS AND NORMAL HUMAN LIVER CELLS

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DECLARATION

I hereby declare that this submission is my own work towards the MSc Biotechnology and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

To my wonderful and supportive family and lovely my wife, Anita Appiah-Kyere.

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ABSTRACT

The plant kingdom and its rich diversity still remains a potential source of varied bioactive phytochemicals with extensive applications especially in the mitigation of human diseases. In this study, the medicinal capabilities of *Blighia sapida* seeds are investigated by evaluating the antioxidant and antiproliferative activities on, lung, prostate, skin, leukemic cancers and normal liver cells using methods such as GC-MS, MTT and Resazurin based cell viability assays and DPPH/FRAP assays. In the MTT assay, the methanolic and hexane extracts all demonstrated a dose-dependent decrease in both Chang and Jurkat cell viabilities. The effective inhibitory concentrations (IC_{50}'s) of the methanolic extract in Jurkat and Chang cells were 0.59 ± 0.15 mg/ml and 0.82 ± 0.05 mg/ml respectively and that for hexane extract were 0.96 ± 0.44 mg/ml and 0.79 ± 0.13 mg/ml respectively. However, the sub-fractions of the hexane extract namely: chloroform, petroleum ether and hexane base exhibited growth inhibitory effects only on Normal livers cells at effective concentrations of 0.54 ± 0.31, 0.45 ± 0.16, 0.36 ± 0.24 respectively in a dose-dependent manner. In the Resazurin assay, the methanolic extract reduced the viabilities of DU145, PC3, H460, A549, A431 cancer cells in a dose-dependent manner with calculated IC_{50} values of 14.22 ± 1.51 mg/ml, 3.17 ± 0.88 mg/ml, 1.95 ± 0.15 mg/ml, 1.56 ± 0.09 mg/ml, 1.3 ± 0.2 mg/ml respectively. Also, the hexane extract likewise inhibited the growth of DU145, PC3, H460, A549, A431 cancer cells with calculated IC_{50} values of 1.51 ± 0.59 mg/ml, 1.06 ± 0.27 mg/ml, 24.7 ± 2.2 mg/ml, 1.12 ± 0.42 mg/ml, 0.35 ± 0.04 mg/ml respectively. Among all the cell lines tested, both methanolic and hexane extracts demonstrated the most significant growth inhibitory effect on human epidermoid carcinoma cells (A431) at I.C_{50}'s of 1.3 ± 0.2 mg/ml and 0.35 ± 0.04 mg/ml respectively. Overall, the highest inhibitory effects was observed in the non-polar hexane extract on A431 at 0.35 ± 0.04 mg/ml. GC-MS analyses of both extracts of the seeds revealed the presence of both polar and nonpolar phytochemicals with the identification of a known Glycoside; 3-n-hexylthiane-S,S-dioxide possessing anticancer properties. Sub-fractions of hexane extract (chloroform, petroleum ether and hexane base) demonstrated effective antioxidant potentials (EC50's) at 5.08±0.33 mg/ml; 4.98 ± 0.3 mg/ml; and 19.02 ± 0.35 mg/ml respectively in the DPPH assay and 15.77 ± 0.78 mg/ml; 24.20 ± 0.27 mg/ml; and 87.03 ± 2.28 mg/ml respectively in the FRAP assay. Overall, the chloroform sub-fraction exhibited the highest DPPH scavenging activity with the methanolic extract showing the highest Ferric reducing potential. These findings suggest that crude extracts of *B. sapida* seeds may hold great potential as a natural product against squamous cell carcinoma and other inflammatory cutaneous conditions such as contact dermatitis, psoriasis etc., however further studies are required to improve its bioactivities.

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LIST OF ABBREVIATIONS

A431	Human Epermoid Carcinoma cell line
A549	Human Lung cancer cell line
AKT	Protein Kinase B
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BHT	Butyl Hydroxy Toluene
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhdrazyl
DU145	Prostate cancer cell line
EC50	50% Effective Concentration
ER-MDA-MB-453	Breast Cancer cell line
FBS	Fetal Bovine Serum
FRAP	Ferric reducing antioxidant power
GC-MS	Gas-Chromatography Mass Spectrometry
H460	Human Lung cancer cell line
HCT	Colorectal carcinoma cell line
IC50	50% Inhibitory Concentration
LDH	Lactate dehydrogenase
M.W	Molecular weight
MCF	Human breast adenocarcinoma
MCPA	Methylene-cyclopropyl acetyl
MeOH	Methanol
mRNA	Messenger Ribonucleic acid
MTT	Dimethylthiazoyl-2-yl-diphenyltetrazolium
NAD	Nicotinamide adenine dinucleotide
NADH	Reduced Nicotinamide adenine dinucleotide
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NMIMR	Noguichi Memorial Institute of Medical Research
O_2-	Oxygen radical
OH-	Hydroxyl radical
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol 3-kinase
PKCδ	Phosphokinase C
PSG	Phosphate Streptomycin Glutamine
PUFA	Polyunsaturated Fatty Acid
R.T.	Retention time
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
TCA	Tricarboxylic acid cycle
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

Owing to the increase in the incidence of cancer the world over, the quest to search for anti-cancer agents from the natural kingdom has increased. The plant kingdom upon subjection to centuries of extensive scrutiny has yielded very modest results culminating in some effective drugs ranked among the most common chemotherapeutic agents employed today. Nevertheless, due to the wide diversity and complexity of phytochemicals produced by plants, they still serve as indispensible source of naturally derived anti-cancer agents (Cragg et al., 2005).

The major cause of cancer is the uncontrolled proliferation of cells in response to damage to DNA from over-production of free radicals arising from oxidation of biomolecules (Klaunig and Kamendulis, 2007). According to a report published by the American Cancer Society and the Livestrong organization, cancer is identified as having the greatest impact on the economy worldwide compared to that of premature death and disability of all causes of death with an estimated total annual economic cost of US$1.16 trillion (World Cancer Report, 2014). Furthermore, the worldwide cancer statistics provided by Ferley *et al.,* 2012, estimated the incidence of cancer in 2012 to be 14 million; with lung, female breast, bowel and prostate being the four most common. According to the World Health Organisation, 2017, in 2015, 8.8 million deaths were recorded globally and projects deaths arising from cancer to be 11.8 million by 2030.

Several types of treatment options are available for the management of cancers. Medically, surgical interventions alongside radiotherapy and chemotherapy are employed, however, a more holistic approach has come to include the involvement of complementary and alternative medical practices such as massage, acupuncture, tai chi, etc. (National Cancer

Institute, 2015) owing to development of resistance, high cost, unbearable side effects, and some limitations in their effectiveness.

A comprehensive survey of the literature describing plants used in cancer management dates back to several centuries and lists over 1,400 plant genera indicating that, plant material use in cancer mitigation is an age-old endeavor and still a viable and unexhausted source for better chemotherapeutic alternatives. More recently, systematic scientific inquisition into the plant kingdom for naturally derived therapeutic agents has come to be guided by a folkloric history of use against the disease condition. For instance, podophyllin an antitumour agent marketed under the trade names warticon, wartec, condylox, etc., was used over 2000 years ago diverse cultures as an anti-tumour drug (Cragg *et al.*, 2005; Guerram *et al.*, 2012).

The Sapindaceae family in the order Sapindales, has been extensively studied for its pharmacological effects (Diaz *et al.*, 2012). The family made up of about 2000 species, distributed from tropical to temperate regions, produce fruits and wood of economically high value (Rodriguez, 1958).

As a particularly rich source of the phytochemical saponins, members of this family also contain a range of other phytochemicals including flavonoids, linear triterpenes, caffeine, xanthanes and cathequines (Getie *et al.*, 2003; Mahmoud *et al.*, 2001; Benlekehal *et al.*, 2001; Sousa *et al.*, 2009; Diaz *et al.*, 2012).

Blighia sapida, popularly known in Jamaica as Ackee, is a distinguished species that grows in West Africa and the Caribbean (Gledhill, 1972). *B. sapida* has proved very useful in African folkloric medicine. In Cote D'Ivoire for instance, it is utilized as a liniment for oedema and intercostal pains (Odesamni *et al.*,2013). Irvin (1965) also reported the use of the ash of the dry husks and seeds in the formulation of local soap whereas the pulp as well

as its leafy juice are instilled as opthalmic drops, however little is known about the medicinal use of its root. However, a recent investigation of the root extract in combination with *Xylopia aethiopica* in Nigeria revealed its abortifacient activity (Odesamni *et al.*, 2013).

B. sapida is also employed in folk medical practice for treatment of psoriasis (Psoriasis Foundation of Ghana, 2012), for its anti-inflammatory, anticancer, fertility enhancer (Folorunsho and Oluwatoyin, 2014), epilepsy, dysentery, diabetes and yellow fever (Gbolade, 2009). Furthermore, another publication has reported the discovery of three new triterpene saponins, namely: blighoside A, B and C as well as two known steroids, stigmasta-5,22-dien-3-ol and stigmasta-5,22-dien-3-O-glucopyranoside in the ethylacetate fraction of B. sapida pods. The three new monodesmosidic triterpene saponins isolated, exhibited significant antiproliferative effect on human breast cancer cells at concentrations below 11 μM. More so, in a free radical scavenging study, the ethylacetate fractions of B. sapida pod and seed showed strong DPPH antioxidant activity as well as high total phenolic and flavonoid content. Via an activity-guided fractionation of selected fractions, six known polyphenolic antioxidants namely: methyl gallate, ellagic acid, isoquercitrin, protocatechuic acid, gallic acid, and quercetin were recovered and identified from the ethylacetate fractions of the ackee pod for the first time (Parkinson, 2007).

Ubulom *et al.* (2013) detected several phytochemicals such as saponins, tannins, phlobatannins, flavonoids, terpenes, cardiac glycosides, alkaloids and combined anthraquinones in the leaf and stem bark of *B. sapida*. Its arils have also been recently reported to contain polyphenols that have been shown to prevented an N-nitrosodiethylamine-mediated oxidative onslaught on microsomal protein, lipid and DNA (Hamzah *et al.*, 2013). Omosuli (2003), also documented the seeds and arils of *B. sapida* to contain 15.26 % fat whose composition of fatty acids included palmitic acid, margaric acid,

myristic acid, stearic acid, arachidic acid, behenic acid, linoleic acid, lignoceric acid, palmitoleic acid, oleic acid, linolenic acid and erucic acid.

The phytoconstituents as well as the *in vitro* antioxidant properties of the methanolic extracts of the fruits of *B. sapida* has also been recently evaluated by Hamzah *et al.* (2013). The high proportion of flavonoids and other phenols in the methanolic extract of fruits of *B. sapida, Vitellaria paradoxa and Vitex doniana* make them a valuable source of antioxidants which are beneficial in the mitigation of various ailments arising from oxidative stress such as cancer. Also, the excessive oxidative attack on intracellular components such as microsomal proteins, lipids and DNA pose a major risk in cancer initiation and development, however, Hussein *et al.,* (2013) have also demonstrated the polyphenolic extracts of *B. sapida* to arrest these events in carcinogenesis.

Fatty acids on the other hand, have also been demonstrated to influence interactions between cells of the immune system, thus exhibit immunomudulatory effects. Molecular studies conducted have revealed some fatty acids to inhibit inflammatory responses through inhibition of NFκB (Breton *et al.*,2015, Fiona et al, 2001).

The Jamaican Ackee is greatly exploited for its nutritional benefits. In Jamaica for instance, it is regarded as a national fruit and in 2009 alone generated export revenue of 1.2 billion Jamaican dollars (The Gleaner, 2010). However, in Ghana, it is commonly referred to as "Akye fufuo" by the Akans and utilised mainly as an ornamental plant, as timber, and in folk remedy for treatment of psoriasis (Antwi *et al.*, 2009; Psoriasis Foundation of Ghana). The anti-proliferative effect of ackee in successful management of psoriasis forms the basis of this research in which the cytotoxic effects of extracts of ackee seeds are tested on some cancer cell lines. The seeds of the fruits, though rich in oils and other interesting bioactive chemicals, have not been given much attention as a potential source for therapeutic

principles especially towards cancer which is poorly controlled in developing countries such as Ghana (World Cancer Report, 2014).

1.1 Aims and Objectives

With the aforementioned recent scientific revelations in terms of its potential as a medicinal agent, investigating extractive preparations of its seeds for immunomodulatory effects and activity against a panel of cancer cells becomes a laudable scientific inquiry. The main purpose of this research is to evaluate the antiproliferative and antioxidant capabilities of crude methanolic and hexane extracts of *B. sapida* seed extract on normal and cancer cell lines.

The specific objectives include:

i. To undertake a qualitative characterization of the crude seed extracts using Gas chromatography-mass spectrometry (GC-MS)
ii. To investigate the anti-oxidant potentials of the extracts.
iii. To study the antiproliferative effect of the extracts on Jurkat (T lymphocyte), Chang (Normal Human liver), H460 (Human lung cancer), A549(Human lung cancer) PC3(Prostate cancer), A431(Human epidermal carcinoma).

1.2 Limitation of the study

Blighia sapida are known to contain the toxic phytochemical: hypoglycin A and B which are known to be the causative agent of the Jamaican Vomiting Sickness (Katibi *et al.*, 2015). However, since the in vitro cytotoxic effects of these chemicals have not been established, they may pose as a confounding effect on the interpretation of our findings.

CHAPTER TWO

LITERATURE REVIEW

2.1 Jamaican Ackee (Blighia sapida)

2.1.1 History and distribution of Ackee

Ackee (Blighia sapida) of the family Sapindaceae is native to tropical West Africa (USDA, 2002) and the botanical name *Blighia sapida* was chosen to honour Captain William Bligh who took some of its fruits from Jamaica to England in 1793 exposing this plant to scientific scrutiny. The more familiar name Ackee derives from the Akan language Ankye fufo native to West Africa. Following its introduction to Jamaica from West Africa in the 1700's, it gained massive popularity and has now become the nation`s national fruit just as cocoa is to Ghana. Though ackee originates from West Africa, it has little cultural and historical significance in this terrain. Figure 2.4 shows the parts of ackee fruit.

Figure 2.1 Ackee Fruit and its various Parts

2.1.2 Bioactive Natural Products from Sapindaceae

Fruits from *Blighia sapida, Litchi chinensis, Nephelium lappaceum, Dimocarpus longan,* represents some of the edible species of the Sapindaceae family widely patronized as a delicacy in Australia and Asia (Vichitrananda and Somsri 2008; Diczbalis et al., 2010). They derive their name from Sapindus saponaria tree (Emmanuel and Benkeblia, 2011), which is rich in saponins. Locally they are used as pest control agent and also as soap. Extensive pharmacological investigations into this plant family have revealed the anti-inflammatory, antioxidant, and anti-diabetic properties of some species (Sofidiya et al., 2008; Muthukumran et al., 2011).

The sapindaceae species have been extensively scrutinized as potential source of biologically active compounds. This endeavor resulted in the discovery of a special group of secondary metabolites called the cyanolipids in some of the species (Avato et al., 2005).

These toxic metabolites that occur in the seeds appear to serve a protective physiological role and are restricted to this family and are produced in characteristic amounts ranging between 3-58% (Mikolajczak and Smith, 1971; Dinesh and Hasan; Selmar et al., 1990; Hasan et al., 1994; Ucciani et al., 1994; Hasan and Roomi 1996; Sarita et al., 2002; Avato et al., 2006

The cyanolipids have been classified into four types and differentiated by their fatty acid moieties. Although there exist conflicting reports in literature as regards this classification, it suffices that only one of two types is present at a time in each specie with of several studies reporting eicosanoic acid (20:0) and eicosanoic acid (20:1 n-9) as the two main two fatty acids esterified to them (Aichholz et al., 1997).

2.1.3 Medicinal uses of Ackee

Aside the nutritionally valuable properties of Ackee, it is also known for its medicinal use in the West African country, Benin, where its roots, bark, leaves, capsules and seeds are used for management of some common ailments such as malaria, internal haemorrhage, dysentery, constipation, yellow fever, fever and epilepsy (Adeyeye and Oyarekua, 2008; Kean and Hare, 1980), the leaves and pulp in treatment of conjunctivitis and the roots for diabetes (Atolani, 2009). In Jamaica the nutritional benefits of Ackee is recognized more than its therapeutic benefit (Rashford, 2001).

The hypoglycaemic effect of ackee root bark was demonstrated by Saidu et al. (2012) substantiating its use in the management of diabetes in Nigeria.

2.1.4 Toxicity of Ackee Fruits

The Jamaicans being the chief consumers of ackee have conducted series of toxicity studies on Blighia sapida and these have revealed the existence and possible dietary exposure to its natural toxins: hypoglycin A (L-α-amino-β-methylenecyclopropyl propanoic acid) and hypoglycin B (γ-glutamylhypoglycin). The successful isolation and characterisation of hypoglycins from the seeds of the ackee fruit enabled the scientific community to obtain analytical standards with which to quantify levels of the toxin in typical diets.

Of the two hypoglycins, the A type has been found to be more toxic and known to cause the medical condition which has come to be known as the Jamaican Vomiting Sickness. The clinical features include: hypoglycaemia, vomiting, comatose and subsequent death. Hypoglycin A is metabolised in the same manner as the branched-chain amino acids, valine, leucine and isoleucine which involves an initial deamination followed by decarboxylation step. This metabolic pathway was discovered to generate an acetyl-CoA derivative of methylene cyclopropyl acetic acid (MCPA) which has been identified to be the toxic

metabolite of hypoglycin A (Kean, 1976). MCPA-CoA is also an inhibitor of gluconeogenesis (hence the reason for hypoglycemia); it allosterically inhibits pyruvate carboxylase, a key enzyme in gluconeogenesis (Tanaka et al., 1976).

2.2 Proliferative Diseases

Proliferative diseases are characterized by excessive production of cells and their extracellular matrix and includes conditions such as atherosclerosis, tumours, rheumatoid arthritis, scleroderma, psoriasis, idiopathic pulmonary fibrosis, and liver cirrhosis (Sporn and Harris, 1981).The early developmental stages of this group of diseases are believed to be driven by proliferative responses elicited by some cellular products and which culminates in the proliferation of cells and/or extracellular matrix. Sporn and Harris have also further identified five other biochemical concepts that governs the pathogenesis of diseases described as being proliferative; these include the involvement of hormones and some hormone-like mediators; alterations in collagen metabolism; direct involvement of post-translational processing of polypeptide hormones; and inhibition of proteinases. Alterations in these metabolic activities leading to excessive proliferation of cells may therefore contribute to understanding of the pathogenesis of the disease as well as their prevention.

Reich *et al.* (1975) highlighted the crucial role proteinases play in the malignant transformation of cells. In this same vein, Rossman *et al.*, 1980 also reports *in vitro* and animal experimental findings that show that inhibitors of proteinases can prevent carcinogenesis. Much investigation over the years suggest that one laudable approach toward studying the molecular basis and management of proliferative diseases was in the active search for polypeptide inhibitors and other proliferative factors.

It can be hypothesized that the plant kingdom can be a potential source for the discovery of these agent as already, retinoids, naturally present in plants play a role in inhibiting the effects of proliferative polypeptide mediators including sarcoma growth factor (Todaro *et al.,* 1978), prolactin (Moon *et al.,* 1979), and mononuclear cell factor (Brinckerhoff *et al.,*1980). Retinoids have successfully suppressed the malignant transformation of cells *in vitro* (Merriman and Bertram, 1979), prevent malignant cellular transformations in experimental animals (Sporn and Newton, 1979), halted *in vitro* collagenase synthesis by rheumatoid synovial cells (Brinckerhoff *et al.,*1980) and to treat patients with psoriasis (Goerz and Orfanos, 1978).

2.2.1 Cell Proliferation and Oncogenesis

A critical factor in embryogenesis, tumourigenesis, growth and the proper functioning of several tissues in living organisms is cell proliferation. In view of this, much effort has been dedicated to understanding this indispensable element in cell survival. The mechanistic regulation of cell proliferation has been the focus of investigation for scientists in the field of cell biology for decades.

As a key composite element of cell metabolism, several growth-factors involved in signal transduction pathways, transcriptional networks and cell cycling have been identified (DeBerardinis *et al.,* 2007). Following the interception of cell proliferating signals, the metabolic activity of the cell is reorganized to prepare the quiescent type to undergo proliferation through the uptake and metabolism of extracellular nutrients (Figure 2.1).

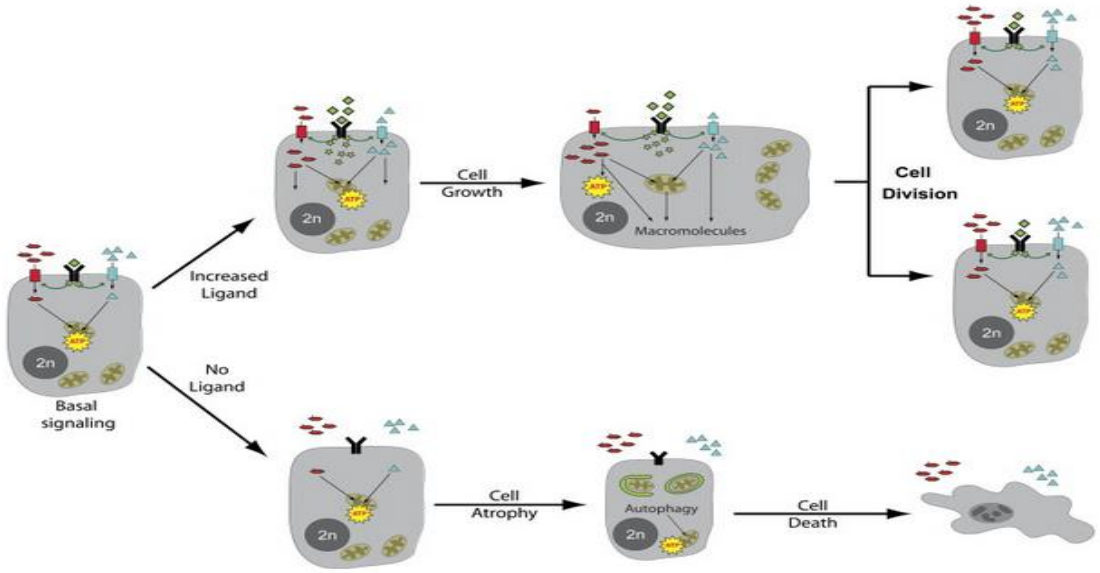


Figure 2.2: Regulation of Absorption and Metabolism of Extracellular Nutrients via growth-factor Signaling

(Source: DeBerardinis *et al.*, 2007).

According to DeBerardinis *et al.,* (2007), at rest, cells are stimulated to absorb nutrients, such as glucose (red), and amino acids (blue) by residual levels of lineage-specific growth factors to increase ATP production as well as lipids, proteins, and nucleic acid synthesis needed to sustain homeostasis.

However, the absence of extrinsic signals (no ligand) results in the loss nutrient transporters on the surface of cells. Autophagic breakdown of macromolecules and organelles is initiated in growth-factor-deprived cells to survive the absence of extracellular-nutrient uptake. Increases in ligand signaling on the other hand direct cells to initiate a high rate absorption of nutrients and to channel them into metabolic generation of ATP and macromolecules culminating a net increase in biomass (growth) and, subsequently, the production of daughter cells.

Quiescent lymphocytic and other hematopoietic cells are stimulated under in vitro conditions to undergo proliferation and the associated signaling mechanisms are characterized in metabolic regulatory studies.

One notable metabolic characteristic that is critical in a proliferating cell is the change in carbon flux. Figure 2.2 shows a schematic of change in carbon flux in quiescent and proliferating cells.

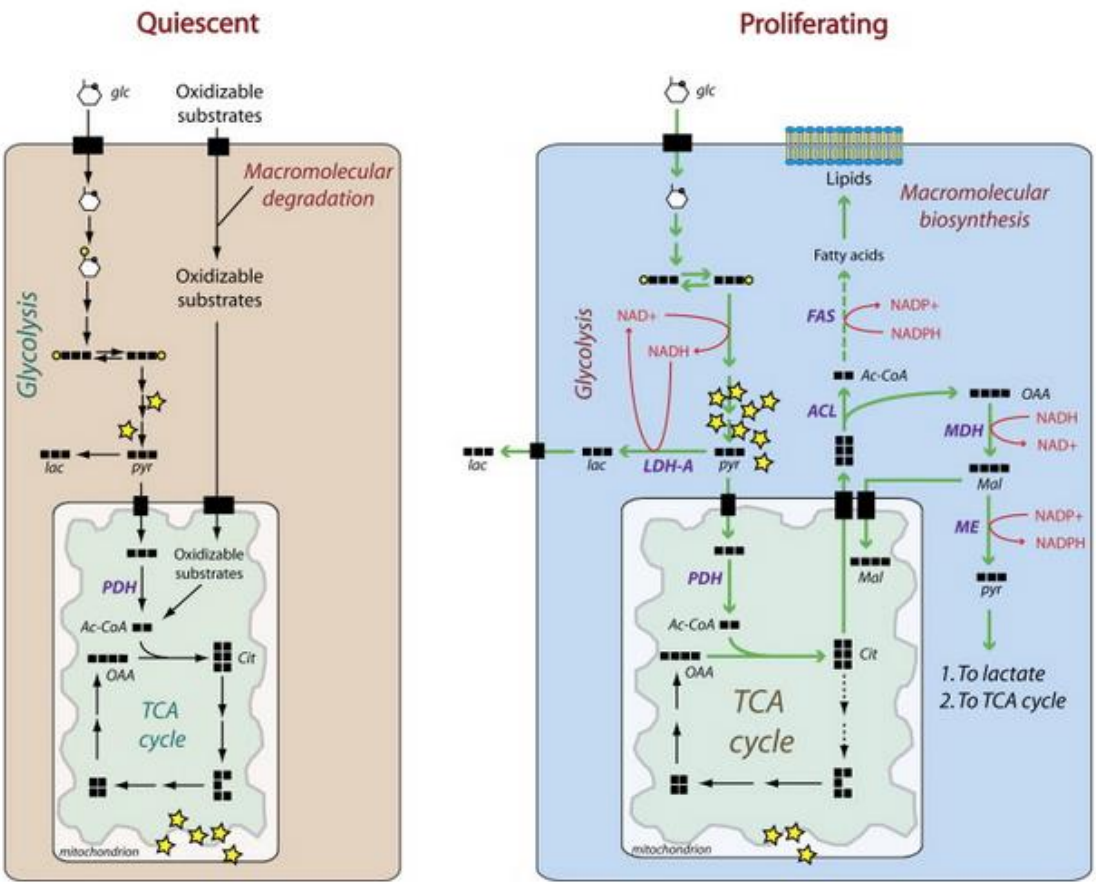


Figure 2.3: Differences between Carbon Flux in Quiescent and Proliferating Cells

The schematic on the left depicts glycolytic transformation of glucose (gly) to pyruvate (pyr) which is subsequently oxidized in the TCA cycle in non-dividing cells. Excessive growth (depicted in right schematic) is characterized by an increase in ATP production in the cytoplasm via the glycolytic pathway. This increase results in a subsequent decrease in the cytoplasmic NAD^+/NADH ratio. Most of the resulting pyruvate is converted to lactate

(lac) by lactate dehydrogenase A (LDH-A) transforms most of the pyruvated generated into lactate (lac) with the rest being converted to acetyl-CoA (Ac-CoA) through the action of pyruvate dehydrogenase (PDH). Resulting acetyl-CoA moves and enters the Tricarboxylic Acid pathway, where intermediates such as citrate (cit) is generated for use in macromolecular biosynthesis. Source: DeBerardinis *et al.* (2007).

Aberrations in cellular metabolism that promote cells to proliferate in a disregulated fashion triggers cell death, however, for normal cells to transform into cancerous cell, alterations in genes that regulate cell growth and differentiation must take place.

Oncogenesis is the process initiating and promoting the development of a neoplasm through the action of biological, chemical, or physical agents. Cancer is basically a disease of tissue-growth regulation. In simple terms, it is a disease of a highly complex nature in which cells in a specific tissue are no longer fully responsive to the signals within the tissue that controls cellular differentiation, survival, proliferation and death. Subsequent to this, cells accumulate within the tissue, causing local damage and inflammation (Safarzadeh *et al.*, 2014).

Extracellular signals contribute signicantly in the homeostatic regulation of cell growth and proliferation. Via a linear series of signaling molecules, the intracellular machinery that effects cell growth and division is linked to the cell surface hence enabling extracellular signals to influence intracellular cell regulatory mechanisms. Mutations in genes that code for any of the signaling molecules could establish the oncogenic conditions that prevail in cancers. Cancer cells also exhibit ability to sustain their proliferation even in the absence of a controlled signaling input in five ways (Hanahan and Weinberg, 2011):

- Increasing growth factor production
- Stimulating production of growth factors by normal cells in their microenvironment
- Increasing the population of growth factor receptors on the cell surface

- Structurally changing receptor to facilitate cancer cell signaling
- Activating proteins in the downstream signaling pathway

cancer cells' propensity to disengage the negative feedback loops that constitute a safety mechanism in the event of mitogenic hyperactivity has also been established. By disrupting the negative feedback loops in the oncogenic Ras signaling pathway for example, tumour cells gain ability to sustain their proliferative signals (Bardeesy and Sharpless, 2006).

There are over 200 different types of cancers identified (www.CancerResearchuk.org). At the cellular level, cancer can be described as a genetic disease; as genetic mutations play a critical role in its pathogenesis. The initiating event in carcinogenesis is usually a non-lethal genetic damage from a carcinogen that may affect four principal group of genes namely, tumour suppressor genes, proto-oncogenes, genes involved in DNA repair, and apoptosis.

2.2.2 Cancer Prevalence in Ghana

Cancer demographics in Ghana has seen significant change over the last few decades (Laryea, *et al.*, 2014). While this may not be apparent in disease pattern, there has been drastic decline in the prevalence of skin cancers and this is attributed to change in the demography of Caucasians to blacks (Wright, 1961; Wiredu and Armah, (2006). Most developing countries especially in sub-saharan Africa lack quality data on cancer (Silva, 1999). However, in recent times, improvement in cancer data collection is becoming more apparent as efforts are being put in place by governments and non-governmental agencies in the establishment of national cancer registries for systematic collection of data on cancers. These establishments become useful when planning cancer prevention and control activities. Retrospective studies conducted by Wiredu and Armah (2006), which involved the reviewing a total of 3,659 cancer deaths over a ten-year period (1991-2000) at the Korle Bu Teaching Hospital, represents one of the relatively few cancer epidemiology studies in Ghana. Among females, breast, haematopoietic organs, liver and cervix malignancies were

reported to be the commonest cases in cancer mortality in females while that in males was the malignancies of the liver, prostate, haematopoietic organs and stomach. Annually, about 1200 cases of paediatric cancers which affects children between the ages of 0-14 years were reported. According to recent findings by the Paediatric Society of Ghana, about 100 new children beginning from 1998 were diagnosed with cancer on yearly basis at the Paediatric cancer unit of the Komfo Anokye Teaching Hospital (KATH) with more than 2,000 cases seen at the unit in the year 2013 (Paediatric Society of Ghana, 2014).

With the establishment of the Kumasi Cancer Registry, it is envisaged that, population-based cancer registries would soon take over from the institutionally-based cancer registries which have traditionally been the source of most cancer data. Data collated so far by this establishment indicates breast, cervix uteri, ovary, liver and prostate cancers were the most common among both sexes while among males, cancers of the liver, prostate, lung and stomach were more prevalent. Breast, cervix, ovary and endometrium cancers were exclusively recorded in females (Laryea *et al.*, 2014).

2.3 Cancer Treatment Options

Surgery together with chemotherapy and radiotherapy presents as the most common options in cancer management (Safarzadeh *et al.*, 2014) with other less common ones such as acupuncture, tai chi, yoga also available (www.cancer.gov.). Even though these common orthodox treatment strategies have served as mainstream treatment for cancers especially in the developed world for many decades, some are not effective and the clinical outcomes not acceptable. Consequently, other unorthodox or traditional methods have sufficed.

In developing countries where traditional medicines form a major part in their healthcare delivery, herbal medicines are accepted in cancer management (De Araujo *et al.*, 2012) The main aims of utilising herbal medicines in cancer management include: the primary

prevention and recurrence of cancer, strengthening of the body's immune system, and reduction of chemotherapy and radiotherapy induced side effects (Tavakoli *et al.*, 2012; Wheat and Currie, 2008).

2.3.1 Cancer Chemotherapy

Chemotherapy is a treatment strategy that involves the administration of drugs directed toward interference of the DNA of fast-growing cells. Traditionally categorized into specific groups; alkylating agents, antimetabolites, anthracyclines and topoisomerase inhibitors, their success hinge mainly on their selective toxicity to tumor cells which typically is defined by abnormally increased mitotic rate and increased reliance on the constant availability of macromolecules for growth.

Basically, there are two types of chemotherapy, that is, adjuvant and primary (neoadjuvant) chemotherapy and a variety of factors usually determine the type of chemotherapy to adopt. The administration of anti-cancer drugs, following the complete removal of a primary tumor is referred to as adjuvant chemotherapy. Primary chemotherapy (neoadjuvant chemotherapy) or induction chemotherapy, is when conventional cytotoxic agents are employed in mainstay treatment.

2.3.1.1 Alkylating Chemotherapy Drugs

The use of alkylating chemotherapy drugs dates back to the 1940's. These cytotoxic agents work by covalently attaching to preferentially the nitrogen atom of both guanine and adenine and also to two different sites on DNA generating both intra or inter-strand crosslinks which may be referred to as DNA adducts (Jackson *et al.*, 1960). This modification results in the inhibition of DNA synthesis and transcription and subsequently in cell death. Typical examples include nitrogen mustards, alkyl sulfonates, ethylene imines, nitroureas and the triazenes.

2.3.1.2 Antimetabolite Drugs

The antimetabolite class are low weight molecular compounds that have similar structures as naturally occurring molecules utilized in nucleic acid synthesis. These include folate antagonists and pyrimidine and purine analogues. By interfering with the S-phase of cellular growth where they get incorporated into either DNA or RNA they exhibit their cytotoxic effect. Additionally, they inhibit enzymes required for nucleic acid production. Methotrexate, a dihydrofolate reductase inhibitor is one of the earliest antimetabolites to be discovered. For over thirty years, methotrexate was employed chiefly as an anticancer agent, however, it has also been put into different uses such as, in the management of both psoriasis and rheumatic arthritis. Mucositis, myelosuppression, and thrombocytopenia are the commonest side effects identified with this group (Espinoza *et al.*, 1992).

2.3.1.3 Anthracyclines

The anthracyclines are *Streptomyces*-derived anticancer agents developed in the 1960's (Brockmann, 1963). These aromatic polyketide compounds are considered one of the most effective anticancer treatments ever developed and are effective against many types of cancers than any other class of chemotherapeutic agents (Krohn, 2008).

A species of actinobacteria, *Streptomyces peucetius* produced the first structurally and stereochemically-characterised anthracycline compound which came to be known as daunorubicin. Other related compounds of this class have since been discovered mainly via chemical derivation. The search for better and improved versions of daunorubicin over the years has resulted in the generation of about 2,000 different analogues, however, Doxorubicin, Epirubicin, Idarubicin, and Valrubicin are the major examples in this class approved for clinical use (Minotti, 2004). As a result of its broad spectrum of activity, the hydroxyl derivative of daunorubicin, doxorubicin, has gained the reputation as one of the most patronised anticancer drugs in clinical use. In combination with other classes of

anticancer agents, doxorubicin may be employed in the management of different types of cancers, including stomach, bladder, hepatic and thyroid tumours as well as Wilms tumuor and others. In addition, its potency in several types of leukaemia and cutaneous T-cell lymphoma as well as solid tumuors is recognised and hence considered the prudent choice.

The mechanism of action of anthracyclines is still poorly elucidated with multiple mechanisms proposed, all attempting to explain the cytostatic and cytotoxic effects. Covalent bond formation and base modification, as well as intercalation with DNA and Topoisomerases are popularly proposed mechanisms that are believed to interfere with DNA replication and transcription and subsequently triggering apoptotic cell death (Szulawska and Cyzyz, 2006).

2.3.1.4 Topoisomerase Inhibitors

Cardinal to the maintenance of the integrity of transcription, replication and recombination processes are the DNA topoisomerases. These enzymes regulate DNA topology and occur in two forms designated as I and II.

While Topoisomerase I initiate the breakdown of a single strand of deoxyribonucleic acid molecule, Topoisomerase II cleaves both strands. These molecular interventions ensure the continual replication of DNA which when compromised results in the demise of the cell.

Wall and Wani (1966), in a systematic screening of natural products for anticancer agents discovered a quinoline alkaloid that had the ability to inhibit the DNA enzyme Topoisomerase 1 from the bark and stem of *Camptotheca acuminate*, a tree native to China.

Following its discovery, synthetic and medicinal chemists modified its chemistry to improve on its low solubility and its pronounced side effects of sepsis, fatigue, stomatitis, constipation, neutropenia, dyspnea, alopecia, leukopenia, thrombocytopenia, anemia,

nausea, vomiting, abdominal pain, headache, fever, weakness, granulocytopenia, diarrhea, and anorexia.

This endeavor has resulted in a plethora of camptothecan derivatives, a few of which have entered clinical use. Notable examples include Irinotecan and Topotecan which are employed in the management of metastatic ovarian cancers, small cell lung cancers, and advanced cervical cancers.

2.4 Targeted Therapy and Immunotherapy

The application of cytotoxic chemical agents in cancer management has over the years enjoyed some notable successes, however, a considerable number of cancer patients, either are irresponsive or relapse after treatment. Targeted therapy and immunotherapy which in some time past was an intellectual concept has now become a reality driven by advancements in molecular and genetic studies(Alfarouk, 2015).

The capacity to identify specific gene mutations in tumours, and in-depth comprehension of cancer molecular biology, has led to the advancement of targeted agents and the blossoming of the field of cancer immunotherapeutics (Palumbo *et al.*, 2013).

This new class of cancer therapeutic agents appears to be more efficient at delivering growth inhibitory or cytotoxic effects in a much more cell-specific manner. More so, these agents have better toxicological profiles than most of the traditional chemotherapy drugs.

These novel therapies presently, form the main nexus of many anticancer drug development agenda since they are envisaged to be the pivot of precision medicine. The initial identification of key molecular targets involved in cancer cell growth and survival sets the stage for the development of these agents (Li *et al.*, 2012).

A comparison of the individual amounts of proteins of normal cell with those of cancer cells serves as one strategy for identifying these targets. The highly expressed epidermal growth factor receptor 2 protein on the surface of some cancer cells, form a typical example of a differentially expressed protein that has been utilized in the development of several targeted therapies against certain breast and stomach cancers (Li *et al.*, 2012).

Targeted therapies mostly comprise of small molecules or monoclonal antibodies. Small-molecule compounds are typically designed to interact with intracellular targets because they are able to penetrate cells relatively easily. Monoclonal antibodies, on the other hand, are relatively large and generally cannot cross the cell surface membrane and thus, are used only for extracellular targets. High-throughput screening exercises, involving large number of test compounds on a specific target protein are employed in the identification of these small molecule compounds. Once identified, close relatives of these lead molecules are synthesized and then tested to identify hit molecules.

As pharmaceutical biotechnological agents, monoclonal antibodies are prepared using animals, usually mice. These animals are used to synthesize several types of antibodies against the target which is injected as purified proteins. Ligand-protein binding studies are then conducted to identify ones that preferentially bind to the target while sparing the nontargeted ones (Lui *et al.*, 2016). However, before use in humans, portions of the mouse antibody are replaced with corresponding portions of human antibodies to prevent their destruction by the human immune system (www.cancer.gov).

Drug Regulatory Authorities over the world have approved many different targeted therapies for clinical application. These therapies include hormone therapies, angiogenesis inhibitors, gene expression modulators, signal transduction inhibitors, apoptosis inducers, immunotherapies, and toxin delivery molecules.

Regulatory measures governing use of targeted therapies include lack of response to other treatments, spread or inoperable cancer. The limitations of targeted cancer therapies include drug resistance as with antibiotics and chemotherapeutic agents. Two main ways through which this phenomenon of resistance occurs have been described; one is via genetic mutation of the gene that codes for that specific protein molecular target, and the other, through the identification of new pathways for tumour growth not dependent on the targeted molecule.

To mitigate this threat, it is proposed that multiple targeted therapies be employed. Flaherty *et al.,* (2012) validated this strategy by employing a dual therapy approach in which different entities of the biochemical signaling pathway that has been remodeled in melanoma by the BRAF V600E mutation was targeted. Resistance and disease progression was significantly reduced with this new strategy compared to the single targeted therapy.

Other commonly described side-effects of targeted therapies include diarrhea and liver function discrepancies, acneiform rash, dry skin, nail changes, hair depigmentation, poor blood clotting, high blood pressure and gastrointestinal perforations (Petrelli *et al.,* 2012; Cai *et al.*, 2013; Gore *et al.*, 2013).

A limitation of targeted therapy worth mentioning is the difficulty encountered in the development of some identified targets owing to the target's structure and/or the way its function is regulated in the cell. A signaling protein that is mutated in many cancers known as Ras, presents such a challenge (www.cancer.gov). It is hoped that advancements in more cutting-edge drug development technologies would surmount this challenge in the near future. Table 2.1 lists some selected cancers and their available targeted therapies.

Table 2.1: Selected Cancers and their available targeted Therapies

Cancer Type	Targeted therapy Available
Lymphoma	Denileukindiftitox (Ontak), Ibritumomabtiuxetan, (Zevalin),
T-Cell Lymphoma	Brentuximab vedotin (Adcetris), Ibritumomab tiuxetanden Ileukindiftitox (Ontak),
Chronic lymphocytic leukemia	Alemtuzumab (Campath), Ofatumumab (Arzerra)
Melanoma	Trametinib (Mekinist), Ipilimumab (Yervoy), Vemurafenib (Zelboraf),
Multiple myeloma	Carfilzomib (Kyprolis Bortezomib (Velcade),), panobinostat (Farydak)
Glioblastoma	Bevacizumab (Avastin)
Colon cancer	Panitumumab (Vectibix), Cetuximab (Erbitux)
Pancreatic cancer	Erlotinib (Tarceva), Everolimus (Afinitor)
Breast cancer	Everolimus (Afinitor), Tamoxifen (Nolvadex), Toremifene (Fareston)
Prostate cancer	Cabazitaxel (Jevtana), Enzalutamide (Xtandi)
Lung cancer	Bevacizumab (Avastin), Crizotinib (Xalkori,
Cervical cancer:	Bevacizumab (Avastin)

Source: (www.cancer.gov).

2.45 Antitumour activities of some medicinal plants in Ghana

In Ghana, several plants and herbal products are used in the management and treatment of cancers, however, most of them lack proper documentation as they are usually passed on verbally from generation to generation. Recently, some crop of researcher's in various academic and research institutes in Ghana have begun investigations that seek to properly define the activity, efficacy, and potency of antitumour medicinal plants used by traditional healers. For instance, Bayor *et al*., in 2007 demonstrated the antitumour activities of various parts of ten (10) medicinal plants provided by the Centre for Scientific Research into Plant Medicine (CSRPM), Mampong-Akuapem and the Forestry commission, Kumasi as commonly used antitumour medicinal agents utilizing colorectal carcinoma (DLD-1), breast cancer (MCF-7), and melanoma (M14) cancer cell models in *in vitro* experiments. These

plants included *Ficus asperifolia* (leaves), *Paullinia pinnata* (root), *Thoninga sanguinea* (roots), *Adenia Lobata* (roots), *Clerodendrum capitatum* (leaves), *Garcinia Kola* (stem bark), *Plumbago zeylanica* (leaves), *Vernonia conferta* (roots), *Croton membranaceus* (roots) and *Zanthoxylum xanthoxyloides* (stem bark). Below is a table showing their cytotoxic activities expressed as $I.C._{50}$'s

Table 2.2 Cytotoxic Activities of Crude Methanolic Plant Extrcacts and Standard Cytotoxic Drugs against DLD-1, MCF-7 and M14 cells

Plant species/drug	**($I.C._{50}$ µg/ml ± SD)**		
	DLD-1	**MCF-7**	**M14**
Adenia Lobata	170 ± 3.50	230 ± 1.2	>300
Croton membranaceus	16.0 ± 1.0	17.40±1.6	33.5 ± 0.5
Clerodendrum capitatum	86.5 ± 5.2	80 ± 4.0	128 ± 2.0
Ficus asperifolia	45.0 ± 1.0	49 ± 3.2	83.7 ± 1.6
Garcinia Kola	112 ± 4.5	212 ± 1.8	210 ± 3.7
Paullinia pinnata	52.0 ± 2.5	55.0 ± 0.5	>100
Plumbago zeylanica	68.0 ± 3.4	84.5 ± 3.8	>100
Thoninga sanguinea	40.0 ± 1.0	55.0 ± 1.2	43.2 ± 2.0
Vernonia conferta	92.50 ± 2.6	235.0 ± 3.5	283 ± 1.5
Zanthoxylum xanthoxyloides	16.0 ± 1.8	43.3 ± 3.1	44.3 ± 1.5
5-Fluorouracil	1.5 ± 1.0	2.5 ± 0.2	27.5 ± 1.2
Doxorubicin	0.6 ± 0.1[a]	0.4 ± 0.2[a]	1.0 ± 1.5[a]
Etoposide	2.0 ± 1.0	1.8 ± 1.4	5.0 ± 1.2

[a]Value in ng/ml S.D - Standard Deviation

Source: Bayor et al., 2007

Similarly, plant species native to Ghana such as *Ocimum canum* Sim (Lamiaceae), *Solanum nigrum* (Solanaceae).

Heliotropium indicum (Boraginaceae), *Alchonea cordifolia* (Euphorbiaceae), *Ficus exasperata P. Beauv* (Moraceace), *Momordica charantia* (Cucurbitaceae), *Ocimum gratissimum* (Lamiaceae), *Albizia zygia, Petersianthus macrocarpus* (Lecythidaceae), *Aframomum melegueta* (Zingiberaceae), *Secamone afzelii* Rhoem. (Asclepiadaceae), *Ageratum conyzoides* L. (Asteraceae), *Mallotus oppositifolius* (Euphorbiaceae) have been evaluated to possess anticancer activity against breast cancer (Narh, 2015).

2.6 Reactive Oxygen Species and Cancer

In almost all types of cancers, elevated levels of ROS are implicated (Liou and Storz, 2010; Schumacker, 2015). Chemical carcinogenesis proceeds in multiple stages of which the initiation and promotional stages have been widely studied (Klaunig *et al.*, 2010). Initiation stages begin a misrepair of damaged DNA caused by a physical or chemical carcinogen with

the promotional step being marked by a selective clonal growth of an initiated cell population. In the promotional stage, modulation of gene expression appears to influence cell division and apoptosis (Klaunig and Kamendulis, 2007*).*

Oxidative stress in tissues may be induced following the metabolic transformation of an environment carcinogen into potent primary radical intermediates (Rice-Evans and Burdon, 1993; Klaunig *et al.*, 1997). Also, exposure to xenobiotics of varied structures and activities such as the chlorinated compounds, barbiturates, radiation, phorbol esters etc. have been observed to be responsible for the induction of oxidative stress and damage. Several groups of researchers over the years have demonstrated the ability of acetonitrile a chemical agent

to induce events of oxidative stress in rat brain tissue and cultured rat astrocytes (Bigner *et al.,* 1986; Johannsen and Levinskas, 2002).

Notable endogenous sources of ROS such as hydroxyl (OH·), superoxide (O_2^-), and hydrogen peroxide (H_2O_2) generated from mitochondrial, peroxisomal as well as inflammatory activities also play crucial role in tumour initiation and propagation (Kumar *et al.,* 2008; Ishikawa *et al.,* 2008: Liou and Storz, 2010). By converting both superoxide and hydrogen peroxide into water, superoxide dismutase and catalase reduce their damaging effects.

$2\ H^+ + {}^{\bullet}O^-_2 + {}^{\bullet}O^-_2 \rightarrow H_2O_2 + O_2$

$H_2O_2 + e^- \rightarrow HO^- + {}^{\bullet}OH$

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ (catalase)

$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$ (glutathione peroxidase)

Generation of reactive oxygen species from the Ubiquinone cycle of Complex III tends to also regulate the activities of a group of transcription factors that controls cell proliferation and angiogenesis (Bell *et al.,* 2007). Although peroxisomes appear to be rich in catalases and endogenous antioxidants such as glutathione, copper and zinc, superoxide dismutase and peroxiredoxin I (Dhaunsi *et al.*, 1992; Asayama *et al.*,1994; Immenschuh *et al*, 2003) they still are implicated in the generation of free radicals utilizing their acyl-COA oxidase and xanthine oxidase components (Schrader and Fahini, 2006).

Eosinophils, neutrophils, and macrophages also contribute significantly to cellular ROS generation via their oxygen-dependent antimicrobial system (Robinson and Badwey, 1995). In their defensive role, phagocytes for instance, consume oxygen and undergo a respiratory burst that engages NADPH oxidase complex to produce superoxides. The superoxide then

undergoes dismutation to yield hydrogen peroxide (Robinson *et al.*, 2004; Griending *et al.*, 2000)

$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^- + H^+$

$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

The H_2O_2 and O_2- become cytotoxic when they react chemically with products of other microbicidal systems of leukocytes to increase further the levels of ROS. For instance, hypochlorous acid together with nitric oxide is generated through the activities of myeloperoxidase and nitric oxide synthase, respectively (Harrison and Schultz 1976; Xie *et al.*, 1992) as illustrated in the equations below:

$O^-_2 + HOCl \xrightarrow{\text{myeloperoxidase}} OH\cdot + O_2 + Cl^-$ (hypochlorous acid)

$O^-_2 + NO\cdot + H^+ \xrightarrow{\text{nitric oxide synthase}} OH\cdot + NO2$ (nitric oxide)

In many types of cancers, ROS-sensitive signaling pathways are persistently elevated; these include: the PI 3K/Akt pathway, the IKK/NF-κB pathway, MAPK/Erk1/2 pathway. Also, there is the upregulation of mRNA levels of cyclins by reactive oxygen species expediting transition from G1 to S phase (Felty, 2005). The environmental carcinogen sodium arsenite has also be identified to potentiates S phase progression and subsequent cell proliferation in breast cancer cells. (Ruiz-Ramos, 2009). Figure 2.4 summarises the role of ROS in the development of cancers.

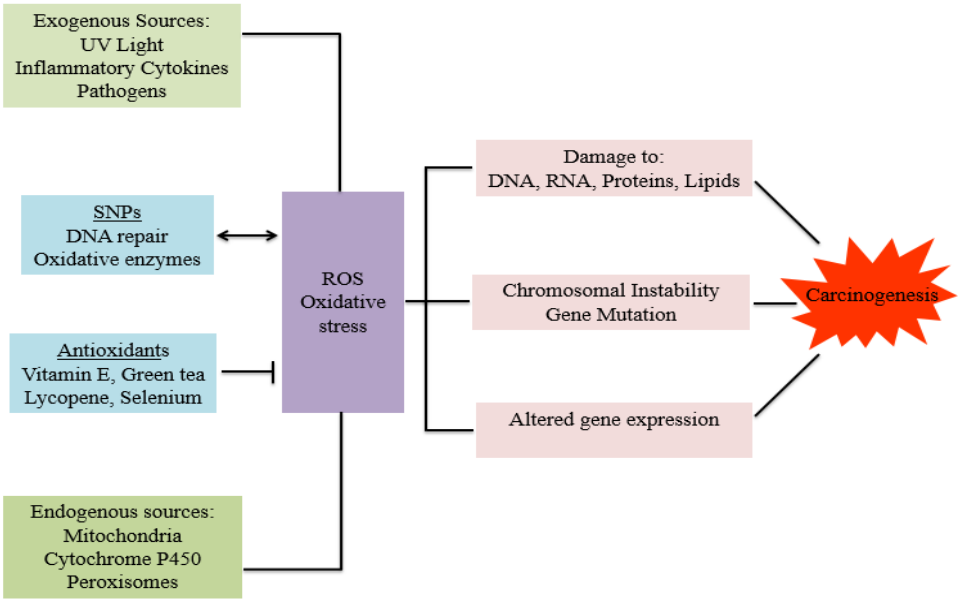


Figure 2.4 Impact of ROS in Human Cancer Development

(Adapted from: Klaunig *et al.*, 2010).

2.6.1 Effects of Anti-Oxidation on Carcinogenesis

For centuries, depressing the levels of oxidative stress in organisms has been one of the main strategies adopted in the fight against carcinogenic tendencies in humans. This has been achieved naturally through the consumption of fresh fruits and vegetables. Antioxidant phytochemicals which occur naturally in vegetables, fruits, cereal grains, and medicinal plants, have been the success and principle behind this endeavor (Deng *et al.*, 2012; Guo *et al.*, 2012; Wu *et al.*, 2012).

Phytochemicals with anti-oxidative property may regulate the initiation of carcinogenic processes by protecting against DNA damage. According to a recent study, about two-thirds of human cancer mortalities could have been prevented through dietary lifestyle modification (Sak, 2014). For instance, antioxidant phytochemicals in ethanolic extract of *Amaranthus paniculatus* (Sreelatha *et al.*, 2012), has been demonstrated, alleviate oxidative stress rationally substantiating its anti-tumour effects. Also, the protective activity of green

tea polyphenols, milk thistle silymarins, and grape seed proanthocyanidins against the adverse effects of UV radiation on skin and risk of skin cancers,have been reported (Nichols and Katiyar, 2010). Further evidence is provided by Li and Zhang, 2014 to the antiproliferative and apoptosis-inducing effects of the plant-derived antioxidant flavonol, catechin and cucurmin in human colon adenocarcinoma HCT 15, HCT 116 and larynx carcinoma cell lines.

The free radical scavenging assays as well as the ferric reducing power assays have become popular in the determination of the antioxidant potentials of natural products. Free radical scavenging ability is governed by the proton-donating chemical species in plants. When the stable diphenylpicrylhydral (DPPH) radical accepts an electron from inthe antioxidant compound, the purple color of the DPPH radical is reduced to yellow colored DPPH radical which is measured colorimetrically. The ferric reducing assay on the other hand is governed by the ability of antioxidant bio-constituents to reduce Fe3+-ferricyanide complexes to the ferrous (Fe2+) form in the presence of FeCl3. The formation of the Prussian blue colored complex is aided by the FeCl3 and then ferric form (Fe2+) converted to the ferrous form (Fe2+). The amount of reduction was subsequently determined by measuring the formation of Perl's Prussian blue at 700 nm (Chung et al., 2002; Gülçin, 2012). In this assay, the yellow color of the test solution changes to green or blue depending on the reducing power of the antioxidant. A higher absorbance indicates higher ferric reducing power. This assay has been widely used in the evaluation of antioxidant component in dietary polyphenols (Zahin et al., 2013).

2.7 Constituents of Ackee Seeds and Arils

The seeds and arils of ackee have been demonstrated to a wide variety of constituents. It's fatty content is rich in fatty acids such as palmitic acid, myristic acid, margaric acid, stearic

acid, arachidic acid, behenic acid, lignoceric acid, palmitoleic acid, oleic acid, linoleic acid, linolenic acid and erucic acid (Omosuli, 2003).

Three new triterpenes (blighoside A, B, C) together with stigmasta-5,22-dien-3-ol and stigmasta-5,22-dien-3-O-glucopyranoside have also been discovered in the pods (Parkinson, 2007).

2.7.1 Cytotoxic Principles of Ackee

The diverse phytochemistry (saponins, alkaloids, lipids, flavonoids, tannins, cardiac glycosides and proteins) of *B. sapida* makes it a potential source for cytotoxic compounds.

2.7.1.1 Saponins

These are a class of bioactive compounds derived mainly from plants (Thakur *et al.,* 2011) and contain a sugar moiety that is glycosidically linked to a hydrophobic aglycone (Harlev *et al.,* 2012). They derive the name saponin from Latin origin based on the word "sapo", which means soap-like (Melzig *et al.,* 2001).

Friess *et al.* (1960) were the first to apply saponins to tumours, demonstrating the anti-tumour potentials of these natural compounds from the sea cucumber, subsequently several similar reports have been published (Thakur *et al.,* 2011). A major limitation to the application of saponins as anti-tumour agents in clinical setting is their high singular toxicity. (Thakur *et al.,* 2011). Nevertheless, more studies continue to validate the tumour suppressive properties of saponins. One interesting study worth mentioning is that undertaken by Gutterman and his colleagues, two saponin derivatives termed avicin A and D which elicited marked inhibitory effect on selected cancer cell lines: human foreskin fibroblast, mouse fibroblast, and immortalized breast epithelial cells, were isolated in their study. Also, these compounds induced a cell cycle (G1) arrest in human MDA-MB-453 breast cancer cell and apoptosis in Jurkat cells (T cell Leukemia).

Some synthetically modified saponins have however have been successfully applied to treat many experimental tumuors (Liby *et al.,* 2008). Championing this course was the Sporn Research group who utilized tumour models such as the estrogen receptor negative breast cancer cell lines and the androgen responsive and non-responsive prostate cancer cells, PC3 and DU145 respectively in their tests. Quill saponins, steroid saponins isolated from *Quillajia saponaria* and the biangeloyl saponins from *Xanthocera sorbifolia* both been patented as novel anticancer agents.

2.7.1.2 Polyphenols

The cytotoxic as well as antioxidant properties of polyphenols on a panel of cancer cells have been substantiated (Azmi *et al.,* 2006; Siriwantanmetanon *et al.,* 2010). Typical examples of this class of compounds include flavonoids, silibinins, quercetin curcumin, resveratrol and Apigenin (Abdal *et al.*, 2016).

These phytochemicals are hypthesized to exhibit their cyototoxic effects by regulating various signaling pathways, such as the PI3K/AKT, RAS/RAF/ERK, PKCδ, and AMPK which tend to affect autophagic processes (Rodrigo and Gil-Becerra, 2014). This ability makes them very desirable in anticancer therapy. Resveratrol a good example of this group abounds in fruits such as grapes and peanuts whereas curcumin is present in turmeric, curry powder, and mango ginger. Green tea, onions, parsley are also rich in flavonoids (Dhillon *et al.*, 2008).

2.7.1.3 Lipids

Some groups of saturated and PUFAs fatty acids, have been discovered to be capable of controlling the viability of tumor cells. Cytochrome P450 enzymes together with lipooxygenases and cyclooxygenases have been identified be the molecular targets that mediate this cytotoxic event. These regulate the conversion of ω-6 polyunsaturated fatty

acids (PUFAs) into the potent eicosanoid regulators of tumor cell proliferation and cell death in many tumors.

However, the biotransformation of ω-3 PUFAs into analogous products disrupts particular tumorigenic pathways. For instance, the ω-3 17, 18-epoxide of eicosapentaenoic acid activates cytotoxic and proapoptotic signaling cascades in tumor cells and the lipoxygenase-derived resolvins are effective inhibitors of inflammatory pathways that may drive tumor expansion (Murray *et al.,* 2015; Devi *et al.,* 2015). None the less, the development of potential anti-cancer drugs based on these molecules is complex, with in vivo stability being a major issue.

2.8 In Vitro Assessment of Cellular Response to Bioactive Constituents

2.8.1 Cytotoxicity Assays

Basically, cytotoxicity can be described as the propensity of a chemical compound to induce cell death. Basal cytotoxicity data has for some time now gained considerable recognition as a tool for predicting the acute toxic effects of compounds *in vivo* (Walum *et al.,*1992) If a compound is acutely toxic, it is envisaged that, in most cases, it will reflect as an insult to the biochemical integrity of cells (Eisenbrand *et al.,* 2002a).

Most *in vitro* cytotoxicity assays measures necrosis; one of the two known pattern of cell death, however, the other form also referred to as apoptosis can also be assessed using in vitro assays. The use of cytotoxicity data as a prediction of acute systemic toxicity has successfully being applied in validated *in vitro* methods to assess for instance; the phototoxic effects of some compounds based on ATP-dependent neutral red uptake into lysosomes (Borenfreund *et al.,* 1985; Spielman *et al.,* 1998).

Studies conducted decades ago highlighted positive correlations between *in vitro* cytotoxicity and acute toxicity in animals and humans using a wide range of chemical compounds (Clemedson *et al.,* 2000).

The ability of cytotoxicity assays to help establish the concentration range of xenobiotics enable more comprehensive *in vitro* tests to be conducted and from which meaningful information on some parameters such as genotoxicity, induction of mutations or programmed cell death can be gathered (Eisenbrand et al, 2002b). Colorimetric and Fluorometric based in vitro cell viability assays such as the MTT and Resazurin have been extensively applied in this endeavor owing to their reproducibility and commercial availability. These methods are based on the reduction of specially synthesized salts by actively growing cells to a colored absorbing product that can be quantified with a spectrophotometer/fluorometer.

2.8.2 Cell lines for Anticancer Research

In vitro methods are widely patronised for the screening and ranking of therapeutic agents. This was made possible with the advent of cell lines. The unlimited lifespan of continuous or immortalized cell lines creates the convenience of subculturing cells many times. This reduces the cost of establishing fresh primary cultures for in vitro cell and molecular biology studies.

Cancer cell lines have enabled the biology of cancers to be studied and also allow the testing of cancer treatments. There are about 974 different human cancer cell lines in existence; however only a handful have become routine in anticancer drug sensitivity studies (Barretina *et al.*, 2012). Typical example includes: HeLa, PC3, H460, A549, A431, DU145, HL-60, ER- MDA-MB-453. These cell lines are usually given simplified code names based on their characteristics (phenotypic and genotypic).

2.8.2.1 Leukemic cell line (Jurkat)

The Jurkat cell line established in the late 1970s from the peripheral blood of a 14-year-old boy with T cell leukemia (Schneider *et al.*, 1977) is used primarily to elucidate the mechanism of differential susceptibility of cancers to drugs and radiation. However, different varieties obtained through the mutation of some genes are also now available for specific research endeavors (American Type Culture Collection). Examples include: J-Lat, J-CaM1.6, D1.1.

2.8.2.2 Human lung cancer cell line (A549 andH460)

A549 cells are adenocarcinomic human alveolar basal epithelial cells that aid in the diffusion of water and electrolytes, across the alveoli of lungs. It was first established by D. J. Giard, *et al.* in 1972 through the removal and culturing of cancerous lung tissue in the explanted tumor of a 58-year-old Caucasian male (Giard *et al.*, 1973). One characteristic feature of this cell is its ability to produce lecithin and high levels of polyunsaturated fatty acids. It has gained much popularity as an *in vitro* model for type II pulmonary epithelial cell in drug metabolism and host transfection experiments (ATCC, 2012).

The NCI-H460 cell linear hypotriploid human cell line was established by A.F. Gazdar and his colleagues from the pleural fluid of a patient with large cell cancer of the lung back in the 1980's (Banks-Schlegel *et al.,* 1985).

2.8.2.3 Prostate Cancer Cell Line (DU145 and PC3)

The classical cell lines of prostatic cancers include, D145 and PC3 (Alimirah *et al.*, 2006). Sourced from a CNS-invasive form of a metastatic prostate adenocarcinoma DU145 has lost its responsiveness to hormones and hence devoid of the prostate-specific antigen (PSA) (Stone *et al.,* 1978). PC3 on the other hand was developed in 1979 from bone metastasis of grade IV prostate cancer in a 62-year-old Caucasian male (Kaighn *et al,* 1979) and is characterised by a low testosterone-5-alpha reductase and acidic phosphatase activity

(ATCC, 2012). PC3 cells intrinsically demonstrate higher metastatic potential than DU145 and LNCaP cells (Pulukuri, *et al.*, 2005).

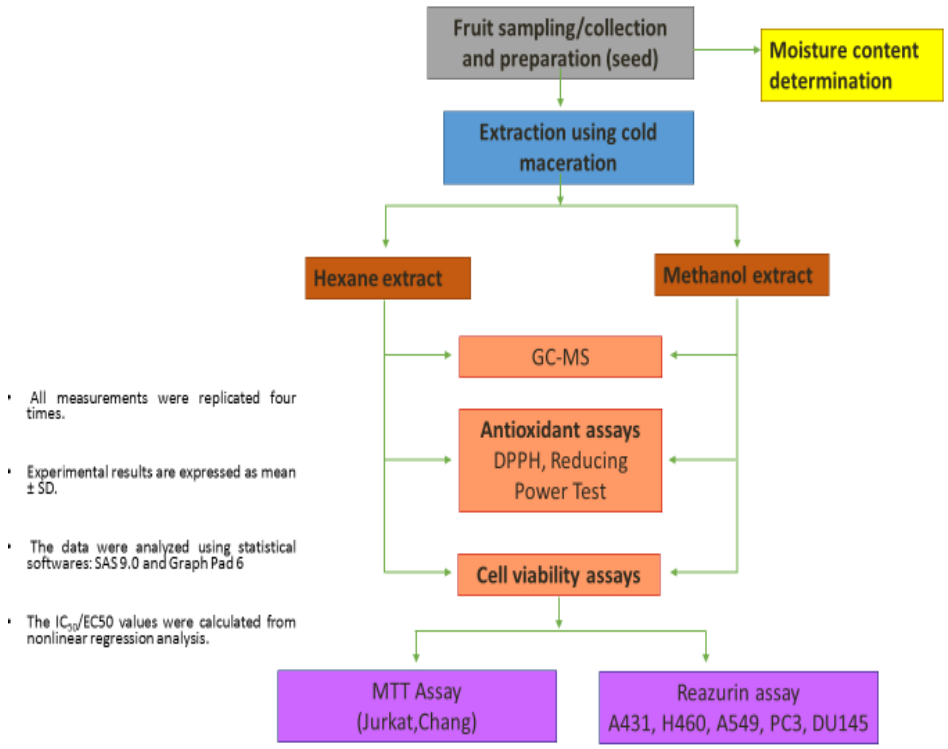
2.8.2.4 Human Epidermal Carcinoma Cell Line (A431)

Established from the skin/epidermis of a 85 year old caucasian female, it expresses increased levels of EGFR, and lacks the functional tumor suppressor gene P53 which makes it highly sensitive to mitogenic stimuli, and hence used often in cell cycle and tumour cell signalling studies.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental design



Schematic diagram of the experimental design

3.2 Seed sampling and preparation

Seed samples of *B. sapida* were collected from two geographical areas in Ghana, namely, Kwame Nkrumah University of Science and Technology, Kumasi, and Daamango in the Northern region. Only seeds from split-opened fruits were picked for the investigation. Samples were prepared by removing the fleshly arils, washed in water to clean all aril debris, after which they were air-dried. The seeds were bulked and then divided into three replicates each for preparation of methanolic and hexane extracts.

3.2 Determination of Moisture Content of *B. sapida* Seeds

The standard method of Trease and Evans (2007) was used to determine the moisture content. Briefly, a cleaned porcelain crucible was dried in the oven at 105 °C to constant weight (M_0). A 20-gram seed sample was weighed into the crucible (M_1) and seeds dried in a thermostatically regulated electric oven at 105 °C for 5 h. The crucibles were transferred into a desiccator for cooling after which they were weighed (M_2). Moisture content was determined by difference in weights and expressed as a percentage using equation 3.1

Moisture content = $(M_1-M_2)/(M_1-M_0)$ ……. (3.1)

3.3 Preparation of Plant Extracts

Dried seeds were pulverized using a Hammer mill (Christy and Norris 8" Lab Mill, England) and screened through a 127.5 µm mesh sieve. One-kilogram (1kg) each of the pulverized seeds was weighed and transferred into two (2) 5L conical flasks. Using the cold maceration method, 1 litre of each of two (2) solvents, methanol and hexane were added and the flasks shaken intermittently for 72 h. The resulting supernatants were decanted, filtered with Whatman No. 1 paper, and concentrated in a rotary evaporator (Buchi Rotavapor R-205, Switzerland) to obtain a hexane extract and a methanolic extract. These were stored in a desiccator at room temperature in an amber-coloured glass bottle and transparent glass container respectively. Each extract was bulked across the three replications for subsequent studies.

3.4 Qualitative characterisation of Crude Methanolic and Hexane Extracts

3.4.1 Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Gas Chromatography/Mass Spectroscopy analyses were carried out on both methanol and hexane extracts. Analyses of the samples were performed with a Perkin Elmer Clares SQ8S equipped with a Clares 580 MS fused silica capillary column (30m × 0.25mm internal

diameter, film thickness 0.25μm). Helium was employed as carrier gas at a flow rate of 1ml/min. Injector and MS transfer line temperatures were set at 220 ^{o}C and 290 ^{o}C respectively. Programmed from 50^{o}C to 150 ^{o}C at 3 ^{o}C/min, the oven temperature was held isothermal for 10min, and raised to 250^{o}C at 10 ^{o}C/min**.** Diluted samples (1/100, v/v, in methylene chloride) of 1.0μL were injected manually in the splitless mode.

Gas Chromatography/Mass Spectroscopy was evaluated by electron impact ionization at 70 eV and data was analysed using total ion count (TIC) for compound identification and quantification. The spectrums of the components were matched with the database of known spectra of compounds contained in the GC-MS library. Spectrums were visualized and data processed using the Turbo-Mass- OCPTVS-Demo SPL software. The retention time, molecular weight, name and structure of compounds were then established using standard protocols.

3.5 Reagents

All reagents used for extraction such as methanol, petroleum ether, chloroform and hexane were of analytical grade (Sigma-Aldrich, Illnois, U.S.A.). Rose Park Memorial Institute (RPMI)-1640 and Dulbecco modified Eagle's culture media, fetal bovine serum (FBS), penicillin streptomycin L-glutamine (PSG), curcumin, 2,2-diphenyl-1-picrylhydrazyl free radical reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, Dimethylsulphoxide (DMSO), butylated hydroxyl toluene (BHT),ferric chloride (FeCl3), potassium ferricyanide (K3Fe(CN)6, trichloroacetic acid, phosphate buffered saline (PBS) and trypan blue were all procured from Sigma-Aldrich (Illinois, U.S.A.) and used as specified by the manufacturer.

3.6 Cancer Cell Lines

Human leukaemia-immortalized T lymphocyte (Jurkat) and Normal human liver cell (Chang liver) were purchased from RIKEN Bio Resource Center Cell Bank (Japan) and provided by the Clinical Pathology Department, Noguchi Memorial Institute for Medical Research (NMIMR) Cell Bank where the *in vitro* work was carried out. Human lung cancer (A549), Human lung cancer (H460) cells, Prostate cancer (PC3) cells, Human prostate cancer (DU145) and Human epidermal carcinoma A431 cells were purchased from Invitrogen, Carlsbad, CA., U.S.A. and used as specified by the manufacturer.

3.7 Total Antioxidant activity (2, 2- diphenyl-1-picryl hydrazyl-DPPH) Assay

Stock solutions of the extracts were prepared by dissolving 10 mg of each of crude extracts in 1 ml of Dimethylsulfoxide (DMSO). Stock solutions of 10 mM of standard (Butylated hydroxyl toluene, BHT) and 0.5 mM of DPPH were also prepared (Appendix A).

The antioxidant potentials of the extracts were ascertained according to the procedures described by Brand-Williams *et al.*, (1995) but with slight modification. Briefly, in an 8 x 12 well plate, the extracts were serially diluted in DMSO to produce a range of 0.3125–10 mg/ml to obtain seven concentrations. The resulting mixture constituted of 100 μL of 0.5 mM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), and 100 μL of each concentration of the extract served as test samples.. Butylated hydroxy toluene (BHT) at a concentration range of 0.315–10 mM in methanol served as the standard. The solvents, methanol, DMSO, were used as blanks. Experiments were repeated thrice. The plates which were covered with aluminum foil, were gently shaken and kept in the dark for 20 min after which the absorbance was read on a Tecan-PC infinite M200 Pro Plate reader (Austria), at a wavelength of 517 nm.

The percentage scavenging activity was determined by equation (2)

$$\% \text{ scavenging} = \frac{\text{Absorbance of blank-Absorbance of test}}{\text{Absorbance of blank}} \times 100 \quad (2)$$

Graphs of mean percentage antioxidant activity against concentration were plotted for the standard and samples and their 50% effective concentration (EC_{50}) established from nonlinear regression analysis (Appendix C).

3.8 Reducing Power Assay

In this method, a 200 µL of the extracts at each of the seven concentrations in the range 0.15625 -10 mg/ml as before were transferred into 1.5-ml Eppendorf tubes. These mixed with 200 µL of $K_3Fe(CN)_6$ and incubated at 50 $^oC$for 20min. Following this, 200 µL tetrachloroacetic acid was added and then centrifuged at 300×g for 10min. A 200 µL of the upper layer was then transferred into a new tube and 200 µL of distilled water and 40 µL of $FeCl_3$ added. This mixture was then incubated for 30min and the absorbance read at 700 nm. From a plot of Absorbances against the different concentrations, effective concentrations (EC_{50}'s) were calculated and used as indices for comparing antioxidant activity of the extracts and the Butyl hydroxyl Toluene (BHT) standard.

3.9 Cell culture and Cell Viability Assays

Cytotoxicity studies were performed on seven (7) cell lines made up of Chang (Normal Human liver) and six (6) cancerous cell lines, namely, Jurkat, H460, A549, PC3, DU145 and A431. All cultures were prepared following the method of Ham *et al.,* (2012) with slight modifications. Chang and Jurkat (T lymphocyte) employed in the MTT assay, as well as Human lung cancer (H460) cells were cultured in RPMI 1640 medium.A549 (Human lung cancer) and PC3 (Prostate cancer) () cells were also cultured in F12K media. lastly, DU145 (Human prostate cancer) and A431 (Human epidermal carcinoma) were cultured in Dulbecco's modified eagle's medium (DMEM). . All culture media were supplemented with 1 % Penicillin-Streptomycin-Glutamine (PSG) and 10 % foetal bovine serum (FBS)

and cultures were grown in T75 culture vessels. The cells were maintained in an incubator with 5 % CO_2 concentration at 37 °C, and relative humidity of 95 % and passaged on reaching about 80 % confluence by means of an inverted microscope (Primovert, Germany).

3.9.1 MTT Assay

A 50 mg/ml stock solution of each of the extracts and fractions in DMSO was prepared. The solutions were vortexed and filter sterilized through 0.45µm membrane filters into cryo tubes in a bio-safety cabinet and stored at -20 ^{o}C until use. The procedure of MTT assay of Ayisi *et al.,* (2011) was followed with slight modifications. In this method, serial dilutions of the DMSO stock solutions of each plant extract were prepared to six concentrations of 0.0625, 0.125, 0.25, 0.5, and 1 mg/mL.

Suspension cells (Jurkat) in culture flask were then transferred into 50 mL centrifuge tubes, spun down at 3000 × g for 60 sec. Pellets were resuspended in 5 % FBS and mixed with 10 µL Trypan blue dye at 1:1 (v/v) and immediately counted. Cells were counted under 10× magnification using Zeiss compound microscope (Primovert, Germany) equipped with a hemocytometer. Viable cells appeared transparent while dead cells were blue. An average of 2 x 10^6 cells/ml was obtained which was further diluted to a final density of 1 ×10^5 cells/ml in 5% FBS. A volume of 100 µL of the diluted cell suspension was seeded into wells of a 96-well plate.

The cells were immediately subjected to treatment with 10 µL of each extract dilutions in triplicates and incubated with 5 % CO_2 concentration at 37 °C, relative humidity of 95 % for 72 hours. The final concentrations of the sub-fractions of the hexane extract were in the range of 62.5-1000 µg/ml. Since the oxidoreductases of metabolically active cells reduce MTT to form a formazan dye, only viable cells would develop the characteristic purple

colour. The intensity of the colour was measured at 570 nm using a spectrophotometer (Tecan Infinite M200 Pro Plate Reader, Austria).

A 50 µM curcumin standard was serially diluted to obtain a range of concentrations: 3.125, 6.25, 12.5, 25, 50 µM in 1 % DMSO. In order to appreciate the cytotoxic effect of ackee extracts, a standard cytotoxic reagent, curcumin (Vallianou *et al.*, 2015) was tested along with the extract. Contents of wells in this plate were made up of 10 µL of sample or control in 100µL of DMEM. Control wells were prepared with all reagents and cells without extracts. All spectrophotometer readings were corrected against a blank made up of all reagents without cells. Calculations of cell viability for the extract and for curcumin were carried out using equations (4) and (5), respectively.

$$\% \text{ Cell Viability} = [(A_{control} - A_{test}) / (A_{control})] \times 100 \quad \ldots\ldots\ldots (4)$$

$$\% \text{ Cell Viability} = [(A_{control} - A_{test}) / (A_{control})] \times 100 \quad \ldots\ldots\ldots (5)$$

Where: $A_{control}$ is Absorbance for control

A_{test} is Absorbance for test

Mean percentage cell viability for triplicate experiments was plotted against concentration of ackee extract. Same was done for the curcumin standard for comparison. From the plots, the effective concentration that i reduced the population of viable cells by 50% (EC_{50}) were calculated for both the standard and test samples in equation (6).

$$y = d + a - d/1 + (x/c)^b \ldots\ldots\ldots\ldots (6)$$

Where:

a = the minimum value that can be obtained (i.e. what happens at 0 dose)

d = the maximum value that can be obtained (i.e. what happens at infinite dose)

c = the point of inflection (i.e. the point on the S shaped curve halfway between a and d)

b = Hill's slope of the curve (i.e. this is related to the steepness of the curve at point c).

3.9.2 Resazurin Assay

Cells were seeded onto 96-well plates at a density of 2.5 x $10^4$and incubated for 24 h at 37°C in 5% CO_2/95% humidified air. The cells were then exposed to varying concentrations of the various fractions (0-10 mg/ml) either dissolved in acetone (for hexane extract or water (for methanolic extract) in 5% FBS-containing media daily for 48 h. The final concentration of the acetone in each well was 1%. No standard drug was included in this screening.

Resazurin (20 µL) was added to each well and the contents gently mixed and incubated in the dark for 2 h at room temperature before measurement of the fluorescence with excitation at 560 nm and detection at 590 nm using FLx 800 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, VM). Cell Titer-Blue Cell Viability Assay kit (CTB) with fluorescence readout was used to measure resazurin reduction as a marker of metabolic activity according to the vendor's instructions. Cell viability was expressed as the percentage of the measured fluorescence in the treated cells (Test samples) relative to that of the controls in equation (6).

% Cell viability = $\text{fluorescence}_{\text{control}}$-$\text{fluorescence}_{\text{test}}$/$\text{fluorescence}_{\text{control}}$……. (6)

3.10 Statistical Analysis

Descriptive analyses, including minimum, maximum, means, standard deviations and coefficient of variation were carried out on triplicate determinations for all tests. In the extractability tests, analysis of variance was performed and differences in means were located by the least significant difference (LSD) test (Steel *et al.*, 1998). The resazurin assay was conducted in four s replicates while the antioxidant and MTT assay were replicated thrice (due to limited resources). Differences in means were based on the student's t-test and ANOVA. Significance of results were tested at the $P<0.05$ significance

level. Nonlinear regression analysis was carried out to determine dependence of cytotoxicity on concentration of ackee extract. All statistical computations were carried out using SAS 9.3 (SAS Institute, Cary, 2011) and GraphPad Prism 6 (GraphPad Software, Inc, USA) was employed for construction of graphs.

CHAPTER FOUR

RESULTS

4.1 Moisture Content of *B. sapida* Seeds

Moisture content of *B. sapida* seeds from two sampling sites in Ghana, were determined. The values ranged from 18.86 to 46.55 % with a mean of 30.70 ± 10.17 %. Moisture content was lowest (22.70 ± 4.39 %) in seeds sampled fromr the northern region seeds and not significantly different from the relatively higher moisture contents of seeds from Kumasi , 38.71 ± 6.84%. (Appendix C).

4.2 Physical Appearance and Yield of Extracts

Following extraction of the bulked and milled seeds, the extracts were examined for their physical appearance and yield of extractions calculated. All three replicates of methanolic extracts were solid whereas the hexane extracts were liquid and oily. The yield of hexane and methanolic extracts across the three replicates are presented in Table 4.1. Yield from hexane extraction was significantly higher (11.53 ± 5.49 %) than that from the methanol (1.60 ± 0.24 %).

Table 4.1 Percentage yield of Hexane and Methanol Extracts of *B. sapida* seeds

Solvent	Mean	SD[1]	Min	Max	CV[2]
Hexane	11.53	5.49	5.20	15.00	47.63
Methanol	1.60	0.24	1.32	1.76	15.09

[1]Standard Deviation; [2]Coefficient of Variation

A preliminary study on the hexane and methanolic extract failed to demonstrate inhibitory action on DPPH hence the hexane extract was fractionated with chloroform and petroleum

ether producing chloroform, petroleum ether and hexane base fractions. The fractions generated were however not quantified due to paucity. The methanolic extract was not fractionated for further analysis due to logistical constraints.

4.3 GC-MS analysis of phytochemical constituents from *B. sapida* Extracts

The methanolic and hexane extracts of *B. sapida* seeds were subjected to GC-MS analysis to qualitatively characterize the phytochemical constituents that may be present. Of The many peaks produced by the mass spectra 23 distinct peaks were clearly identified, of which 21 were well resolved (Figure 4.1A and B).

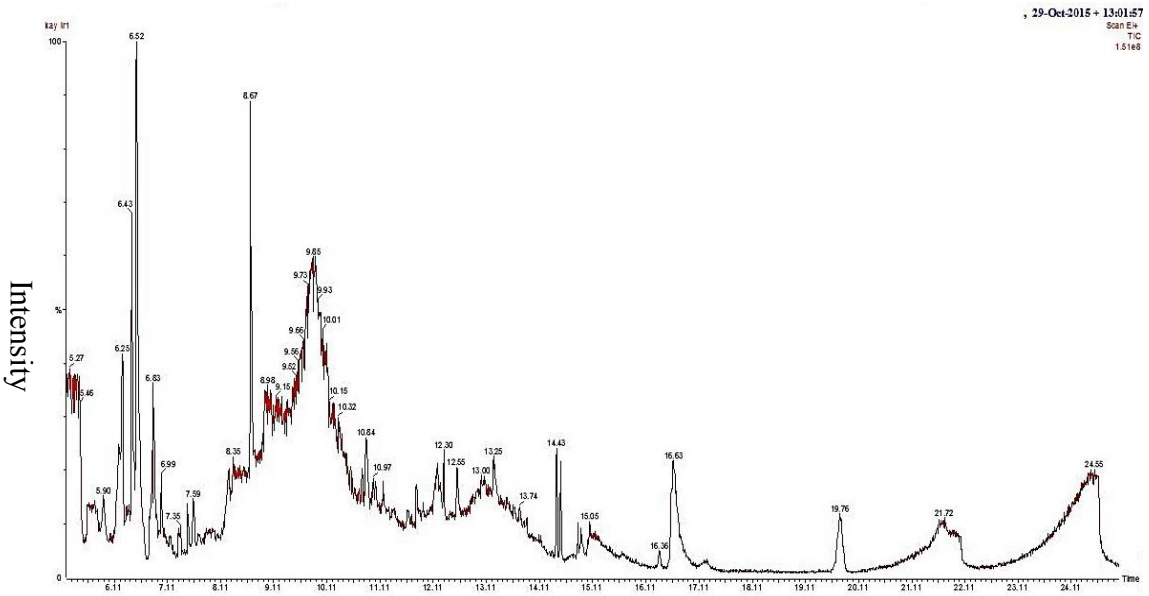


Figure 4.1A Gas chromatogram of methanolic extract.

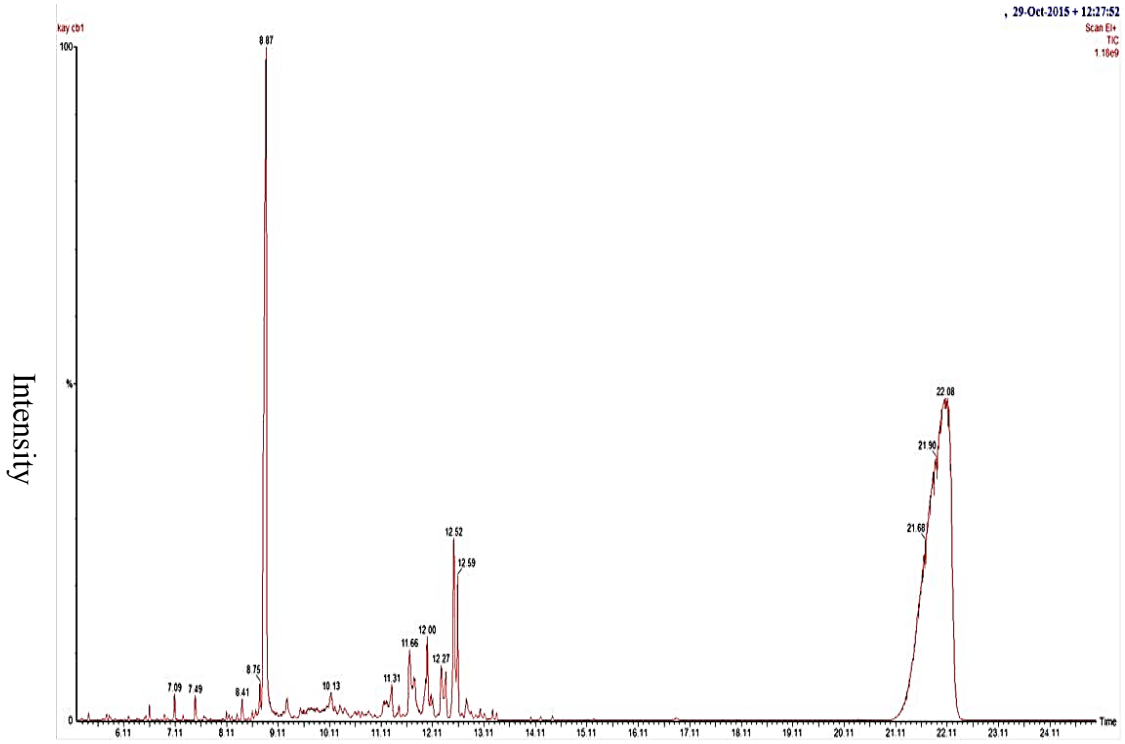


Figure 4.1B Gas Chromatogram of Hexane Extract

The probable compounds identified from the National Institute of Standards and Technology GC-MS library in each extract are presented in Table 4.2. The mass spectra obtained from the distinct peaks of the gas chromatograms are presented in Appendix B.

Figures 4.2 A and B show the structures of the resolved compounds in hexane and methanolic extracts. Compounds of the hexane extract were predominantly non-polar consisting of one ester, cyclohexane ethanol, and three long chain aliphatic compounds whereas the methanolic extract consisted of polar compounds including 1-aminocyclopentane hydroxamic acid and 3-n-hexylthiane,S,S, dioxide.

Table 4.2 List of hit Compounds from hexane and extracts

HEXANE EXTRACT					
SN	**Retention Time (min)**	**Compound**	**Molecular Formula**	**Molecular Weight (Daltons)**	**Peak Area %**
1	7.093	Unknown		128	0.38
2	7.493	Unknown		323	0.38
3	8.840	Unknown		116	40.87
4	10.134	Cyclohexaneethanol		128	0.76
5	11.314	Dodecane	$C_{12}H_{26}$	170	0.48
6	11.681	1-undecyne	$C_{11}H_{20}$	152	2.85
7	12.015	Unknown	$C_{13}H_{24}$	136	3.14
8	12.295	1-tridecyne		180	0.38
9	12.508	Unknown		152	6.84
10	22.091	3-methylpentadecanoate	$C_{16}H_{34}O_2$	270	43.92
METHANOLIC EXTRACT					
1	6.240	Unknown		83	12.35
2	6.853	Unknown		96	10.40
3	9.881	1-aminocyclopentane hydroxamic acid	$C_6H_{12}O_2N_2$	144	
		But-2-enylethylcarbonate	$C_7H_{12}O_3$		26.32
		Hexanoic acid-5-methyl-methyl ester	$C_8H_{16}O_2$		
4	12.61	Unknown		342	1.46
5	13.242	3-n-Hexylthiane,S,S,dioxide	$C_{11}H_{22}O_2S$	218	1.62
6	14.515	Unknown		239	1.79
7	15.129	Unknown		179	2.03
8	16.623	Unknown		273	8.12
9	19.784	Unknown		270	3.25
10	21.711	Unknown		256	4.87
11	24.458	10-(tetrahydro-pyran-2-yloxy)-tricyclo [4,2,1,1]^{2,5}decan-9-ol		256	27.78

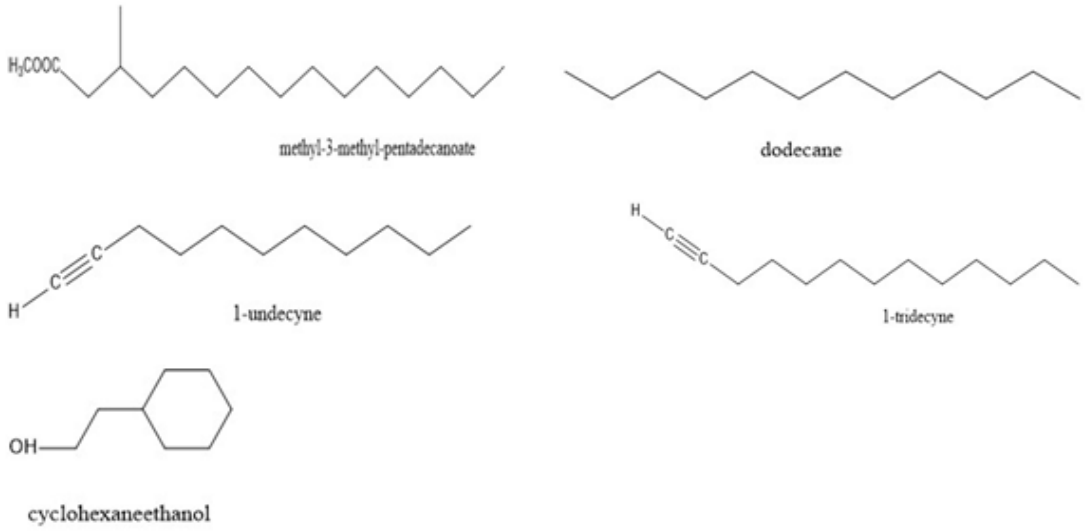


Figure 4.2A Chemical structures of hit compounds of hexane extract

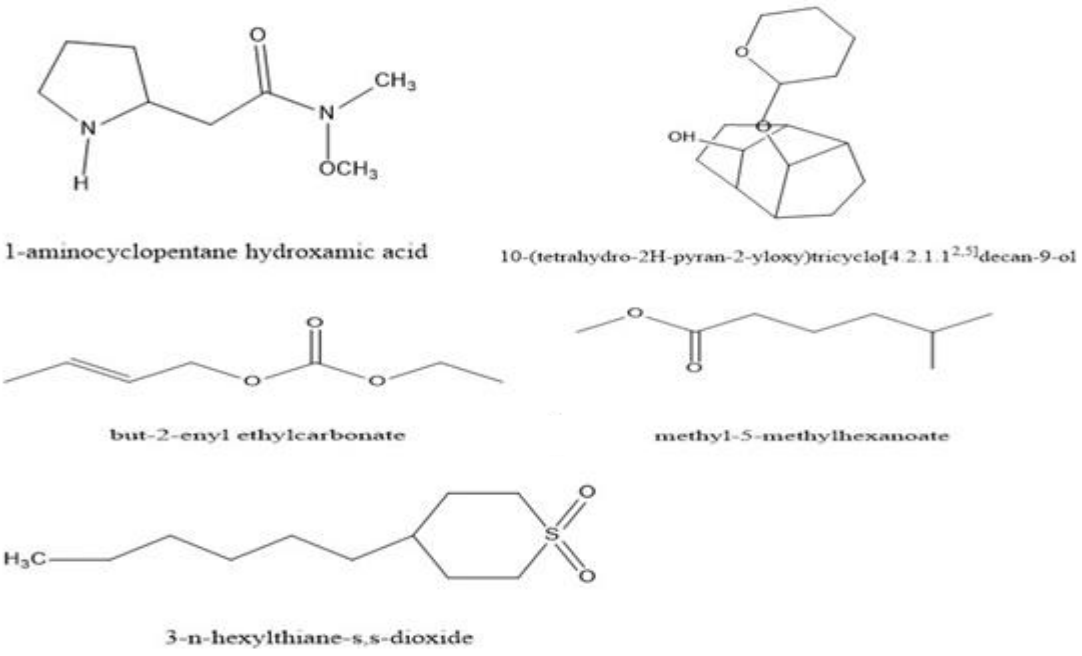


Figure 4.2B Chemical structures of hit compounds of methanolic extract

4.4 Anti-oxidant Assay

The anti-oxidant capacity of methanolic and hexane extracts, chloroform and petroleum ether fractions, and hexane base of *B. sapida* seeds were evaluated by means of the 1,1-

diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging assay and the reducing power assay. Fig. 4.3A below, shows the DPPH scavenging ability as well as the ferric reducing abilities of the extracts with BHT as standard. At all the concentrations tested, the antioxidant standard (BHT), demonstrated higher scavenging potential than the *B. sapida* extracts, showing a consistent increase in inhibition of 23.03 ± 0.26 % to 96.69 ± 0.11 % at concentrations of 0.016 to 10 mg/ml (Fig. 4.3). Surprisingly, the methanolic and hexane extracts failed to exhibit inhibition at all the concentrations tested but all the hexane sub-fractions exhibited the ability to reduce DPPH concentration. The sub-fractions exhibited a dose-dependent scavenging ability with increasing concentration over the range of 0.016 - 10 mg/ml at 20.96 ± 2.36 % to 75.18 ± 1.46 % (Figure 4.3A). Significant difference ($P < 0.05$) in scavenging ability was identified among the fractions at each level of concentration tested. Gradual increases in concentrations of hexane base and chloroform sub-fractions produced no appreciable change in scavenging activity, this trend changed at 1.25 mg/ml, increasing from 27.00 to 75 % at 10 mg/ml. (Appendix C). Compared to the scavenging activity of petroleum ether sub-fraction i.e. 15.35 ± 0.71 % to 31.16 ± 0.1 % at 0.16 to 10 mg/ml, they were higher.

Furthermore, Figure 4.3B shows the results of reducing power of extracts, fractions, and the standard BHT. All extracts and fractions exhibited a lower reducing power than the standard at each concentration. The petroleum ether and chloroform fractions as well as the hexane extract showed consistent low reducing activities at all concentrations tested. Reducing activity expressed in absorbance at 700 nm ranged from 0.01 to 0.72. The extracts and fractions showed large differences in reducing activity. Reducing activity increased from 0.01 ± 0.001 to 0.03 ± 0.01in hexane extract, 0.001±0.001 to 0.10 ± 0.003 in petroleum ether fraction, 0.02 ± 0.001 to 0.25±0.0001 in chloroform fraction, 0.01 ± 0.0001 to 0.04 ±

0.01 in hexane base, to a highest value of 0.04 ± 0.001 to 0.43 ± 0.03 in the methanolic extract.

Fifty percent inhibitory concentration (IC_{50}) of the extracts and fractions for both methods are presented in Table 4.3 below.

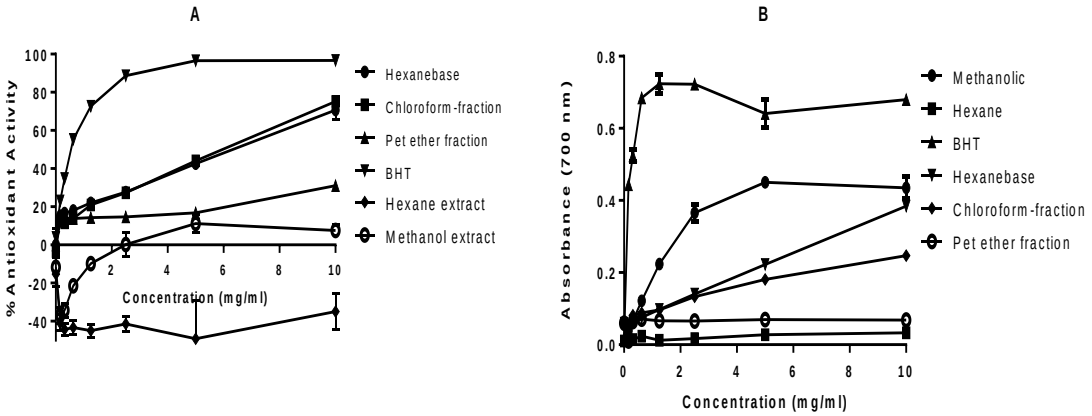


Figure 4.3

A) DPPH free radical scavenging activity of both extracts and hexane sub-fractions and BHT

B) Ferric reducing power of extracts and sub-fractions.

Table 4.3 IC_{50} values of DPPH scavenging and reducing power activities of extracts and fractions of *B. sapida* seeds.

Extract/ Fraction	50% Effective Concentration(mg/ml)	
	DPPH	**Reducing Power**
BHT	0.49 ± 0.01a*	0.36 ± 0.04a
Methanolic	NA	6.8 ± 0.25b
Hexane	NA	232.63 ± 17.18c
Chloroform fraction	4.98 ± 0.3b	24.20 ± 0.27d
Petroleum ether fraction	19.02 ± 0.35c	87.03 ± 2.28e
Hexane base	5.08 ± 0.33b	15.77 ± 0.78f

Values are means of three replicates

NA -Not Accessible

*Means with different letters are significantly different.

4.5 Antiproliferative assays.

Effect of methanolic and hexane extracts on five cancer cell lines, namely, A431 (human epidermal carcinoma), A549 (human lung cancer), PC3 (prostate cancer 1), H460 (human lung cancer) and DU145 (prostate cancer 2) were studied in a Resazurin based cell viability assay. The antiproliferative effects of the hexane sub-fractions were not tested, however these were tested in a separate MTT cell viability assay using Jurkat (T lymphocytic cell) and Chang (Normal human liver cell).

Figure 4.4A-E shows results of the resazurin assay. Both extracts exhibited growth inhibitory effects against all cancer cell lines, decreasing cell viability from 100 % to 0 %

over a 0-10 mg/ml concentration range, though in varying fashion across the cell lines. For the methanolic extract, reduction in cell viabilities were 89.50 ± 3.26 % to 55.87 ± 1.70 % in DU145; 109.87 ± 9.00 % to 3.16 ± 0.66 % in PC3; 77.11 ± 4.80 % to 1.56 ± 1.06 % in A549; 79.72 ± 0.50 % to 4.38 ± 0.82 % in H460; 84.02 ± 1.99 % to 2.61 ± 0.71 % in A431 over a concentration range of 0.16 to 10 mg/ml. However, in both types of prostate cancer cell lines (DU145 and PC3) an unusual trend was observed. In DU145, a decrease in viability was observed from concentration 0.00 to 0.50 mg/ml after which viability increased from 1.00 to 2.00 mg/ml and then decreased again from 2.00 to 10.00mg/ml (Figure 4.4 & Table 4.4).

For the hexane extract, reduction in cell viabilities were 105.00 ± 1.38 % to 7.41 ± 2.00 % in DU145; 98.91 ± 6.15 % to 0.32 ± 0.40 % in PC3; 83.21 ± 7.32 % to 4.96 ± 1.46 % in A549; 96.06 ± 1.61% to 49.86 ± 1.00 % in H460; 67.78 ± 5.05 % to 1.67 ± 1.43 % in A431 over a concentration range of 0.16 to 10 mg/ml as indicated in Figure 4.4. Fifty percent inhibitory concentrations ($I.C_{50}$) of the extracts for all five (5) cell line are presented in Table 4.4 below.

Table 4.4 Inhibitory concentrations (IC_{50}) of methanolic and hexane extracts of *B. sapida* seeds in five cell cancer lines

	50% Inhibitory Concentration(mg/ml)				
EXTRACT	**DU145**	**PC3**	**H460**	**A549**	**A431**
Methanolic	14.22 ±1.51a*	3.17± 0.88c	1.95 ± 0.15b	1.56 ± 0.09b	1.3 ± 0.2b
Hexane	1.51± 0.59b	1.06 ± 0.27b	24.7 ±9.22a	1.12 ± 0.42b	0.35 ± 0.04d

Values are means ± standard error of means of four replicates

*Means with different letters are significantly different.

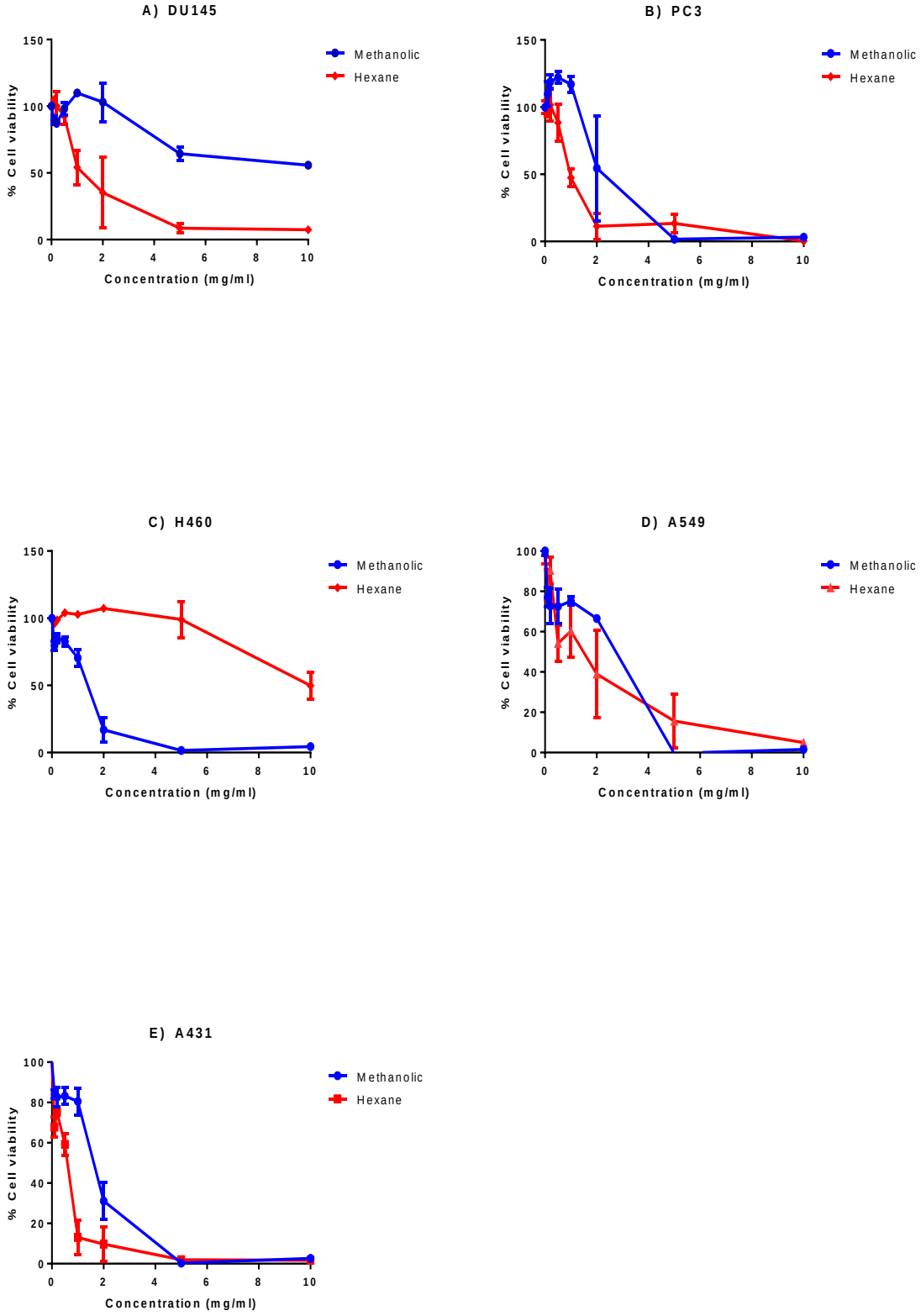


Figure 4.4: Dose-response curves of methanolic and hexane extracts on cancer cell lines. A) DU145 B) PC3 C) H460 D) A549 E) A431

The MTT assay was used to investigate the antiproliferative effect of the methanolic and hexane crude extracts, as well as the fractions of *B. sapida* on Jurkat (leukemia) cell line and Chang (normal human liver) cell. Curcumin was used as a positive control and exhibited significant growth inhibitory effects on both Jurkat and Chang cells over concentration range: 0.0 to 18.00 µg/ml. Methanolic and hexane extracts exhibited no cytotoxicity to Jurkat cell lines at concentrations up to 250 µg/ml. Above 250 µg/ml, the extracts showed cytotoxicity, reducing cell viability from 92.54 ± 18.29 to 0.13 ± 0.00 and 104.73 ± 2.62 to 4.44 ± 1.93 for methanolic and hexane respectively (Fig. 4.5E). All the fractions demonstrated no cytotoxicity on Jurkat cells over the entire concentration range of 0 to 1000 µg/ml (Fig. 4.5F). In contrast, effect of extracts and fractions on Chang cells were different. Increasing concentrations of both methanolic and hexane extracts that is between 62.50 and 500 µg/ml did not produce any significant decrease in Chang cell viability (Fig. 4.5B; Appendix C). However, at 1000 µg/ml concentration, a significant ($P< 0.05$) decrease was observed, that is from 90.16 ± 1.76% to 8.81 ± 7.89% and 92.86 ± 5.21% to 8.64 ± 8.10% for methanolic and hexane extracts respectively (Appendix C). The fractions were moderately cytotoxic to Chang cells significantly ($P<0.05$) reducing cell viability from 100% to 59.60 ± 3.77 %; 63.90 ± 5.71 % and 68.16 ± 1.98 % at 500µg/ml for chloroform, hexane base and petroleum ether respectively (Figure 4.5C; Appendix C). Calculated effective inhibitory concentration (IC_{50}) of the extracts and hexane sub-fractions as well the standard drug (curcumin) in both cell lines are presented in Table 4.5.

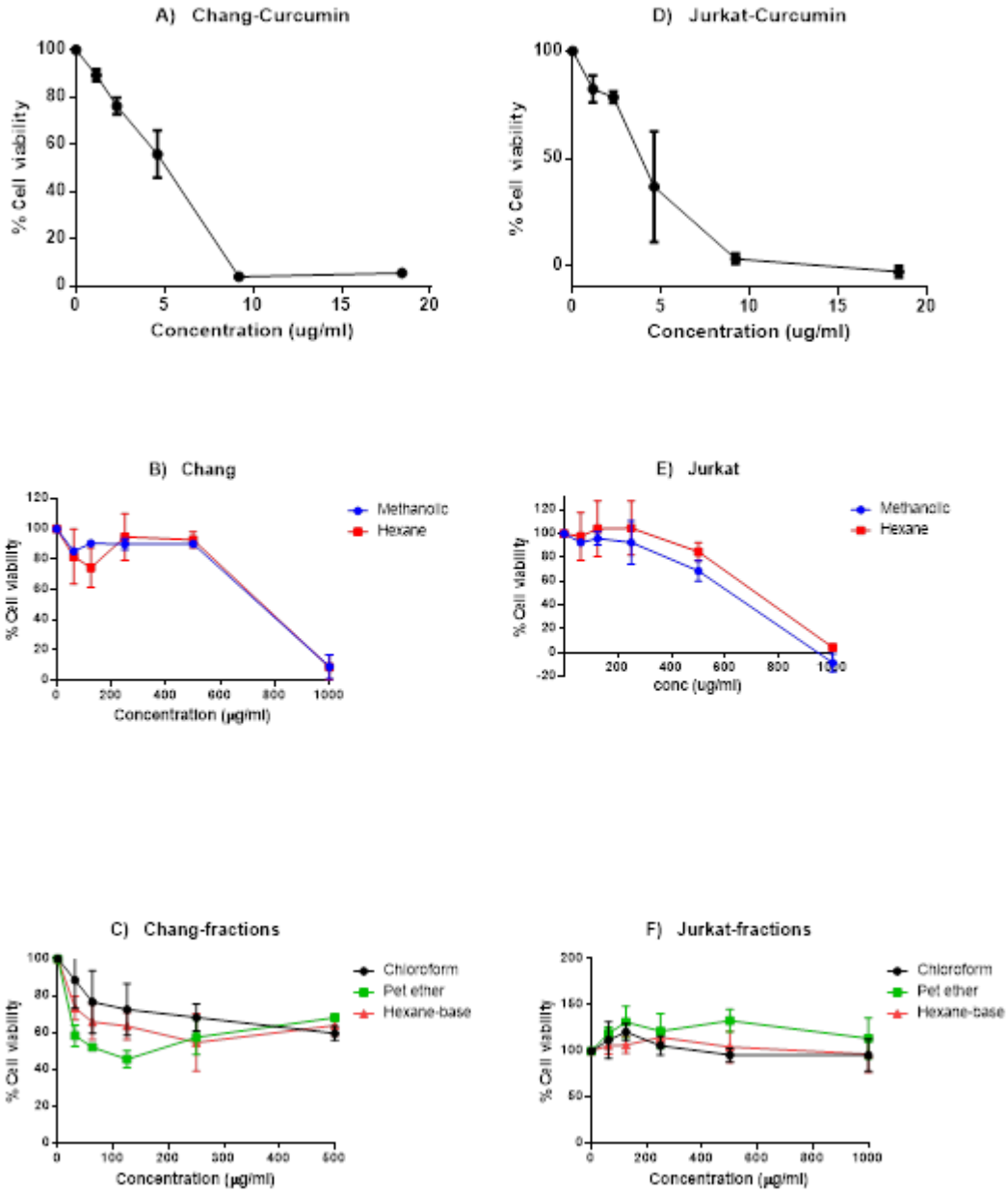


Figure 4.5: Antiproliferative effect of A) Curcumin on Chang, B) Extracts on Chang, C) Fractions on Chang, D) Curcumin on Jurkat, E) Extracts on Jurkat, F) Fractions on Jurkat.

Table 4.5 Effective inhibitory concentrations (IC_{50}) of methanolic and hexane extracts and fractions of hexane extracts of *B. sapida* seeds.

EXTRACTS/FRACTIONS	**50% Inhibitory Concentration(mg/ml)**	
	JURKAT	**CHANG**
Methanolic	0.59± 0.15a*	0.82 ± 0.05a
Hexane	0.96 ± 0.44a	0.79 ± 0.13a
Chloroform	>1.0	0.54 ± 0.31a
Petroleum ether	>1.0	0.45 ± 0.16a
Hexane base	>1.0	0.36 ± 0.24a
Curcumin	0.0032 ± 0.0008b	0.004 ± 0.0004b

Values are means ± standard deviation of three replicates

*Means with different letters are significantly different.

*Means with same letters are not significantly different

CHAPTER FIVE

DISCUSSION, CONLUSION AND RECOMMENDATIONS

Cancer as a proliferative disease kills over 8 million people annually and has been ranked as the second deadliest disease to cardiovascular diseases (Institute of Health Metrics and Evaluation, 2016). In Ghana alone, it is estimated that about 16,000 cases of cancers are diagnosed each year making cancers a major public health concern (International Agency for Research on Cancer, 2015). Nonetheless, even with recent advances in drug discovery, the therapeutic arsenal against cancers is gradually becoming limited. Factors such as their expensive cost, resistance by cancers, serious side-effects may account for this deficiency hence necessitating the need for expansion of cancer therapy arsenal.

Natural products especially from the plant kingdom has served as indispensable sources for classical anticancer drugs such as Vincristine, Vinblastine, Taxol, Topotecan etc (Cragg et al., 2005; Guerram et al., 2012). Despite centuries of extensive scrutiny, the plant kingdom still holds great potential for the discovery of more anticancer agent to combat this disease. Findings from the study of the antiproliferative properties of *Blighia sapida* seed extracts are a presented and discussion hereafter.

5.1 Moisture Content

The mean percentage moisture contents of *B. sapida* seeds from two different geographical zones was determined to be 30.70 ±10.17 %. Earlier reports on the moisture content of whole seeds were, however lacking in literature, nonetheless, this is lower compared to 66% in freshly sampled arils as reported by Falloon *et al.*, (2014). Moisture is not only a significant factor in preservation, food quality, and resistance to deterioration (Nielsen, 2010) but also in the preparation of plant materials for modern therapeutic use. As an inevitable component of crude drugs, measures to eliminate practical amounts is deemed

essential since it protects bioactive constituents from undergoing enzymatic or hydrolytic reactions which may lead to loss of bioactivity (Mukherjee, 2002). High moisture content has been identified to also cause caking to occur on the grinding wheels of milling machines affecting the particle size distribution of pulverized materials and subsequently the efficiency of solvent extraction (ISTA, 2008). This observation however also informed the choice of an absolute methanol as suitable polar solvent in this study.

5.2 Yield of Extract

Following successive extraction with hexane and methanol, the mean percentage yield of *B. sapida* seeds was determined to be 11.53 ± 5.49 % and 1.60 ± 0.24 % respectively. The hexane extract yield is consistent with a 5.62 ± 1.03% crude fat seed harvest using petroleum ether extraction observed of *B. sapida* seeds. (Peart et al). An oily extract with yield of 14.7% is consistent with yields of 15.61 ± 0.01% and 14.05 ± 0.01% for both ripe and unripe seeds respectively as reported by Onuekwusi *et al.*, (2014). In comparison to other well-known oil seeds such as sunflower (35%), safflower (30%), olive (30%) and soya bean (20%) the yield of hexane extract was lower, but, it was comparable to that of cotton seed (15%) but higher than that of corn (3.0 to 6.5%) (Alexander, 2009).

The significant difference ($p<0.05$) in the yield of extract from these two solvents can be ascribed to the different composition of polar and non-polar phytoconstituents in the seeds (Hsu *et al.*, 2006) The observed higher yield from the hexane solvent than methanol is expected as *B. sapida* seeds as well as its arils are known to be a rich source of natural oil (Goldson *et al.*, 2014; Onuekwusi *et al.*, 2014; Oladiji *et al.*, 2009). Seed oils in general have extensive demands both for human consumption and for industrial applications (Kyari, 2008) and also have been ranked as the second most valuable commodity in the world trade today (Ige *et al.*, 1984).

5.3 Characterisation of Phytoconstituents

Utilising pet ether oily extract of both ripe and unripe ackee seeds in a gas chromatography analyses, Onuekwusi *et al.*, (2014), demonstrated *B.sapida* seeds to contain predominantly straight chain hydrocarbons, aromatic compounds and monounsaturated fatty acids. Findings from this study and others informed the choice of the gas chromatography-mass spectrometry technique for the qualitative analysis of the extracts. Results from this analysis revealed the presence of ten (10) putatively identified phytochemical compounds; five (5) in the methanolic extract namely 1-aminocyclopentane hydroxamic acid, But-2-enylethylcarbonate, hexanoic acid-5-methyl-methyl ester, 3-n-Hexylthiane,S,S,dioxide and 10-(tetrahydro-pyran-2-yloxy)-tricyclo [4,2,1,1]2,5decan-9-ol and five (5) in the hexane extract: Cyclohexane ethanol, dodecane, 1-undecyne, 1-tridecyne and 3-methylpentadecanoate. The presence of both straight chain and cyclic hydrocarbons well as compounds of a relatively higher molecular weight in the methanolic extract are consistent with the findings of Onuekwusi *et al.*, (2014).

The non-polar straight chain hydrocarbon, dodecane present in the hexane extract has also been identified in the fixed oils of seeds of *salvia* species however, its biological activity has not been established (Taghreed, 2012; Pourhosseini and Asgarpanah, 2015). Also 1-undecyne has been reported in the seed oils of *Azadirachta indica* (Shivashankar *et al.*, 2012). Derivatives of this hydrocarbon such as the undecylenic acid are incorporated food supplement formulations due to its antibiotic and antifungal properties (www.naturedoc.shop). Again, its 1-tridecyne phytoconstiuent has also been identified in the leaf extracts of the plant *Vitex doniana*, likewise no biological activity has been ascribed to this element (Nweke *et al.*, 2015). The 15-carbon fatty ester, 3-methypentadecanoate present in most seed extracts of plants such as sheanut, sesame (Denloye and Adegboye,), Nigella sativa (Singh et al., 2012) was also identified.

The methanolic extract derived glycoside 3-n-Hexylthiane, S,S dioxide which has also been identified in ethanolic extracts of *Hibiscus rosa sinensis* has been reported by to possesses anticancer properties(Anusha et.al., 2011). Also, 1-amino cyclopentane hydroxamic acid of Jatropha curcus seed oil has been reported to have antimicrobial properties (Rafiee-Moghaddam et., 2014).

5.4 Antioxidant Properties of Extracts

Polyphenols, as well as nitrogen containing compounds (alkaloids, chlorophyll derivatives, amino acids and amines), ascorbic acid and carotenoids are classical examples of compounds with antioxidant propeties (Hall and Cuppett, 1997; Larson, 1988; Anagnostopoulou *et al*., 2006). The presence of unsaturation in these compounds also makes them preferentially oxidized thus protecting intracellular biomolecules against oxidation (Dehpour *et al.*, 2009).

Findings from the antioxidant assays revealed that the BHT positive control demonstrated a dose dependent increase in both scavenging activity and ferric reducing power with EC_{50} indices of 0.49 ± 0.01 mg/ml and 0.36 ± 0.04 mg/ml respectively.

The reducing potential of all fractions and extracts was concentration-dependent. The synthetic standard, BHT exhibited maximum reducing activity among the fractions. The activity at 10.00 mg/mL concentration was in the order of BHT> methanol extract> hexane base > chloroform > hexane and least in petroleum ether fraction.

The hexane base, chloroform and pet ether sub-fractions of the hexane extract which were prepared following its inability to demonstrated antioxidant scavenging potentials demonstrated activity at effective concentrations of 5.08±0.33 mg/ml; 4.98 ± 0.3 mg/ml; and 19.02 ± 0.35 mg/ml respectively in the DPPH assay and 15.77 ± 0.78 mg/ml; 24.20 ± 0.27 mg/ml; and 87.03 ± 2.28 mg/ml respectively in the ferric reducing assay.

Failure of hexane and methanolic extracts of *B. sapida* to inhibit DPPH free radical was unexpected; however, these exhibited ferric reducing activities at effective concentrations of 232.63 ± 63 mg/ml and 6.80 ± 17.18 mg/ml respectively. This may be attributed to the presence of some constituents in the extracts that may have antagonized the reactivity of DPPH in vitro (Sonibare *et al.*, 2011). This observation also highlights the importance of utilizing several methods in the antioxidant activity evaluation as some methods may be less sensitive than others given the nature of the plant extract.

In this work, fractionation of the hexane extract produced appreciable increase in scavenging activity possibly due to a purification effect. Scavenging ability of *B. sapida* fractions varied with solvents. The values were low compared to previous work on other plant extracts. For instance, ethanolic extract of leaf and bark of *Monodora myristica*, a medicinal plant in West Africa, gave scavenging activity of 25.00 ± 0.25 % to 95.25 ± 0.23 % at much lower concentration of 0.025 - 0.300 mg/ml (Moukette *et al.*, 2015). Similarly, Norshazila *et al.* (2010) reported stronger free radical scavenging abilities of ethanolic extracts of 95.12 ± 1.55 % for mango seed, 93.59 ± 2.44 % for guava seed and 93.19 ± 0.25 % for papaya seed at 1.0 mg/ml.

According to Sultana *et al.* (2009), the type of solvent and extraction technique employed in preparation of extract could influence the bioactivity of its constituents. Failure of the methanolic extract in our study to demonstrate scavenging ability may have been caused by some polar phytoconstituents that could antagonize the antioxidant effect. In addition, Alzeer *et al.* (2014) confirmed the influence of type of solvent on bioactivities of phytoconstituents in medicinal plants. The relatively low antioxidant activity observed in this study could be attributed to the method of preparation of the extract used. The cold maceration method is less efficient compared to other methods such as the infusions, decoction, soxhlation, and supercritical fluid extraction. This method was however chosen

to simulate its ethnomedical preparation of *B. sapida* plant materials in Ghana. In addition, auto-oxidation of some constituents of the oil extract; such as the oleic acids which are present in high levels in ackee oils could be responsible for the reduced antioxidant activity (Anderson-Foster *et al.,* 2012). This may imply that the hexane oily extract may have undergone rancidification during storage prior to the *in vitro* studies (Wang *et al.*, 2011).

5.5 Antiproliferative Effects on Cancerous Cells

Cytotoxicity testing, which generally depends on the quantification of cell number and viability, has become one of the most critical steps in early-phase drug discovery programs (Zang *et al.*, 2012).

In this study, both methanolic and hexane extracts relatively reduced the viabilities of DU145, PC3, H460, A549, A431 cancer cells in a dose-dependent manner *in vitro* (Fig. 4.4) with calculated 50% inhibitory concentrations ranging between 24.7 and 0.35 mg/ml (Table 4.4).

A significant ($P<0.001$) difference was observed in the inhibitory effects of the methanolic extract on the prostate cancer cell lines (DU145 and PC3), whereas no significant ($P<0.05$) difference was observed among these cells for the hexane extract. However, the inhibitory effect of the two extracts on DU145 were signicantly different ($P<0.05$) with the hexane extract exhibiting a 9-fold stronger inhibitory effect. A similar observation was made in PC3 cells, again with the hexane extract exhibiting a 3-fold stronger inhibitory effect than the methanolic. The results further indicate that hexane extracts of *B. sapida* was selectively cytotoxic to DU145 than the highly metastastic, androgen-independent and aggressive PC3 cells. Findings from a study conducted by Bali et al however demonstrated no significant growth inhibitory effects of methanolic extracts of *Achillea teretifolia* on both DU145 and PC3. This could be attributed to difference in the biochemical pathways (particularly in

signal transduction events) and hence the different targets employed by the growth inhibiory principles of the hexane extract. (Knudsen and Edlund, 2004). *A. teretifolia* whole plant in a different study ($I.C_{50}$:0.14 ± 0.04mg/ml) appear to demonstrate a relatively stronger growth inhibitory effect on DU145 prostate cancer compared to methanolic extract of *B. sapida* seeds (Bali *et al.*, 2015).

In the two-human lung cancer cell lines (H460 and A549) no significant ($P<0.05$) difference in inhibitory effect was observed after the methanolic extract treatment whereas a significant ($P<0.05$) difference was observed in the hexane extract treatment. The inhibitory effect of the two extracts on H460 were statistically different ($P<0.05$) with the hexane extract exhibiting a 12-fold stronger inhibitory effect than its methanolic counterpart. However, the inhibitory effect on A549 was not significant ($P<0.05$). These findings further suggest that hexane extracts of *B. sapida* was selectively cytotoxic to H460 than A549. This observation compares contrary to finding of a study by Chang and Chen, where no significant effect in growth inhibition in both H460 and A549 following exposure to curcuminoid extract (Chang and Chen, 2015).The antiproliferative effects of hydroethanolic seed extracts of *Litchi chinensis*, another member of the Sapindaceae family on A549 (22.49 ± 1.02µg/ml) appear to be stronger than both methanolic and hexane extracts of *B. sapida* seeds. (Chung, 2017).

Comparing the antiproliferative effects of *B. sapida* hexane extracts in this study (IC_{50}: 0.35 ± 0.04 mg/ml) to an ethyl acetate extract of grape seeds (IC_{50}: 0.48 ± 0.01 mg/ml) in a recent work by Mohansrinivasan *et al.,* 2015, *B. sapida* demonstrated a stronger effect on A431, however, in a similar study it was comparable to peel extracts of *Vitis vinifera* (IC_{50}:319.14 µg/mL) but weaker than its seed extracts (IC_{50}:111.11 µg/ml) (Nirmala *et al.*, 2017). Over all, hexane extract of *B. sapida* appear to be most cytotoxic to human epidermal carcinoma among all the cancerous cells tested in the resazurin assay.

In the MTT assay, both methanolic and hexane extracts exhibited a dose-dependent decrease in both leukemic (Jurkat) and Normal human liver (Chang) cell viabilities with estimated IC_{50}'s ranging between 0.59- 0.96 mg/ml (Table 4.5). This observation may suggest the non-selectivity in growth inhibitory effects of the extracts on both normal and cancerous cells. On the contrary the hexane sub-fractions (chloroform, petroleum ether, and hexane base) exhibited a cytostatic rather than a dose-dependent cytotoxic effect in Jurkat but not Chang cells implying that the phytoconstituents of *B. sapida* hexane extract act synergistically in its cytotoxic effects on Jurkat cells. In Chang cells, the estimated effective concentrations of the hexane sub-fractions that is Chloroform, petroleum ether, and hexane base were 0.54 ± 0.31mg/ml; 0.45 ± 0.16 mg/ml and 0.36 ± 0.24 mg/ml respectively. The growth inhibitory effects of ethanolic leaf extract of *Ageratum conzyoides* (15.08 ± 0.28 µg/ml) (Acheampong *et al.*, 2015) and methanolic leaf extract of *Pachystachya cecropis* (51.00 ± 14.13 µg/ml) (Almeida *et al.*, 2016) were higher compared to all extracts and fractions tested and hence relatively safer.

A plant extract is generally considered to have substantial cytotoxicity if its IC_{50} value, following incubation between 48 to 72 h, is 30 µg/mL or less (Suffness, 1990; Lee and Houghton, 2005; Malek *et al.*, 2011) and considered as potential hits for further development into a drug. Finding from this study shows that, all the crude extracts tested in both bioassays had IC_{50}'s greater than 30µg/ml and therefore not potent enough, requiring further refinements to improve its bioactivity since already, Parkinson in 2007 has demonstrated the anticancer activities of three triterpinoid compounds from *B. sapida* pods making the seeds a good source for prospecting. Several studies also highlight the fact that in vitro exposure concentrations of drug preparation used in most experiments does not exhibit any direct correlation to the maximum plasma concentrations achieved *in vivo* (Mckim and James, 2010), hence necessitating further *in vivo* studies for activity validation.

In light of this, the topical application of the crude extract tends to be a more desirable approach to harnessing its therapeutic potentials as the effective *in vitro* concentrations does not limit the application of *B. sapida* seed extract as a natural prospective anticancer agent since in topical therapy, the drug concentration in the skin can significantly exceed free concentrations in plasma as the topical formulations deliver the drug directly to or near the intended site of action (Nair *et al.*, 2013). More so, studies have revealed no correlation between the availability of drug in the skin and the resulting blood levels (Borsadia *et al.*, 1992; Behl *et al.*, 1993). Typically, topical drug doses are relatively smaller ranging between 2–5 mg of product/cm^2) such that serum and/or urine concentrations are usually undetectable when analyzed using conventional assay techniques (Herkenne, *et al.*, 2008).

At 1 mg/ml and 5 mg/ml concentrations the viabilities of both leukemic T cells and epidermal squamous carcinoma cells (A431) were significantly reduced from 100 to 1% respectively. A431 is regarded as a keratinocyte cell line and has been used extensively as a dermatological *in vitro* model in several previous experimentations (Groves, *et al.*, 1993; Banerjee, *et al.*, 2004).

Banerjee et al, in 2004 demonstrated the key role A431 play in antigen presentation and polarization of human T lymphocytes in the human skin. This suggests that crude extracts of *B. sapida* seeds could target keratinocytes and dermal T lymphocyte and control their participation in the immunological cascade that underlies most dermatologic diseases

5.6 Conclusion

By employing relatively simple methods for the preparation of crude extracts of *B. sapida* seeds, the antioxidant and antiproliferative potentials of the extracts have been confirmed to follow concentration-dependent manner providing some evidence to the efficacy of traditional preparations. However, the cold maceration method used in the ethnomedical

preparation *of B. sapida* seeds may limit its therapeutic potential. Considering the results of this study and other previous works, it can be asserted that the activity of *B. sapida* extracts may be largely influenced by the type of solvent and extraction method used.

To the best of our knowledge, the antiproliferative activity of the crude extract of *B. sapida* seeds on the selected cancer cells, is the first to be reported and the effect on normal liver cells appear to support some toxicological concerns raised against *B. sapida* seeds. This goes to re-emphasize the importance of dosage as a key element in its ethnomedical applications. However, its selective antiproliferative activity on human keratinocytes gives some indication of its great potential as a dermatological therapeutic agent.

5.7 Recommendations for Further Work

To improve on the bioactivity of *B. sapida* extracts, more efficient and modernized methods of extraction such as supercritical fluid extraction should be employed in future works. Bioassay-guided fractionation of the extracts is also recommended for future investigations. In addition, molecular investigations should be conduct to shed more light on the molecular mechanisms underlying some of its bioactivities especially its antiproliferative effects. More so its anti-inflammatory potentials such be exploited in the bid to establish the extent of its bioactivity. Lastly, in vivo experimentations are also recommended in future investigations.

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APPENDICES

Appendix A

PREPARATION OF SOLUTIONS

1. **10 mM of standard butylated hydroxyl toluene (BHT)**

2.2 mg of BHT (220.35 g/mol) dissolved in 1mL absolute methanol. The solutions were then vortexed until complete dissolution was achieved.

2. **0.5 mM of 2,2-diphenyl-1-picrylhydrazyl (DPPH)**

3 mg of DPPH (394.32 g/mol) was weighed and dissolved in 15 mL absolute methanol. The solution was then vortexed until complete dissolution was achieved. To avoid photobleaching of the DPPH solution, it was immediately kept in the dark by wrapping in aluminium foil until ready for use.

3. **1% Penicillin,Streptomycin, Glutamine and 10% Fetal Bovine Serum**

Heat activated FBS and PSG stocks were thawed at room temperature. 50mL of FBS and 5mL of PSG stocks were added to the growth medium and the additions recorded and dated on the medium bottle after which it was refrigerated at 4ºC.

Appendix B

GAS-CHROMATOGRAPHY MASS SPECTRA

Methanolic extract

TC1

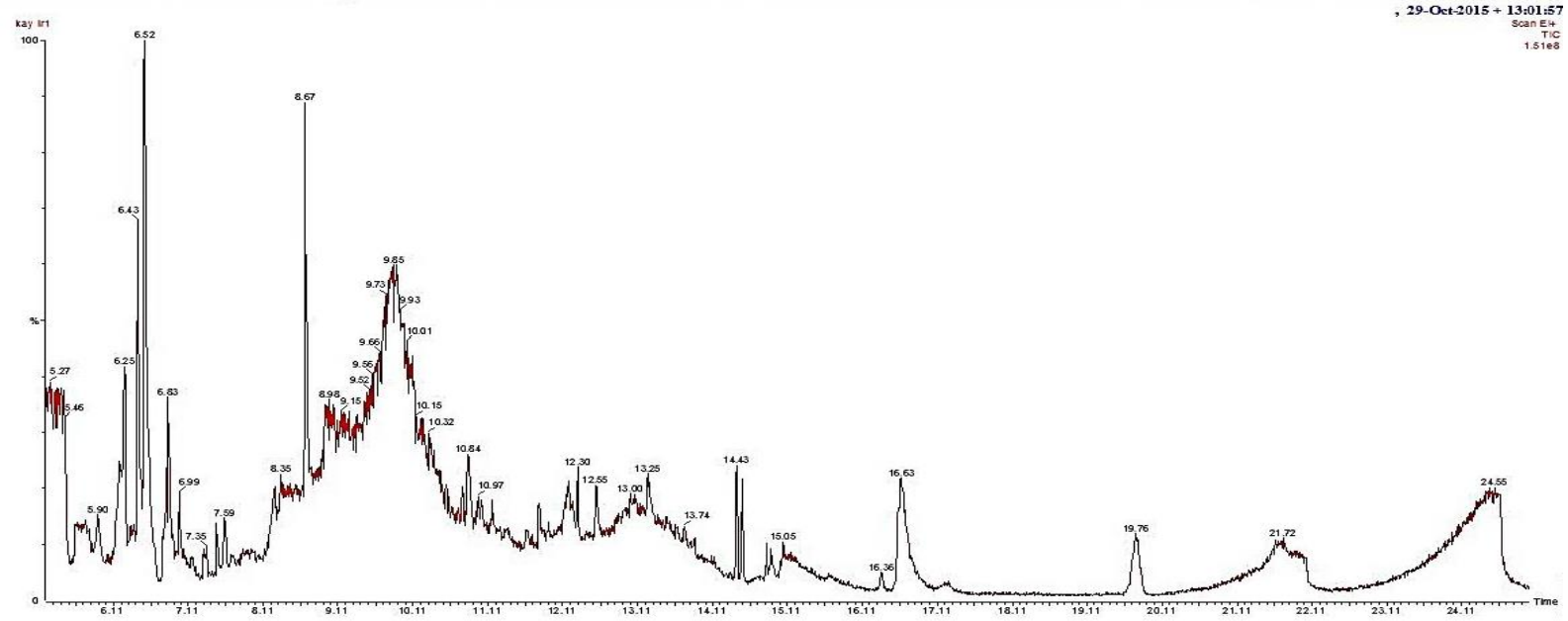

Figure B-1: Gas Chromatogram of Methanolic extracts

MS1

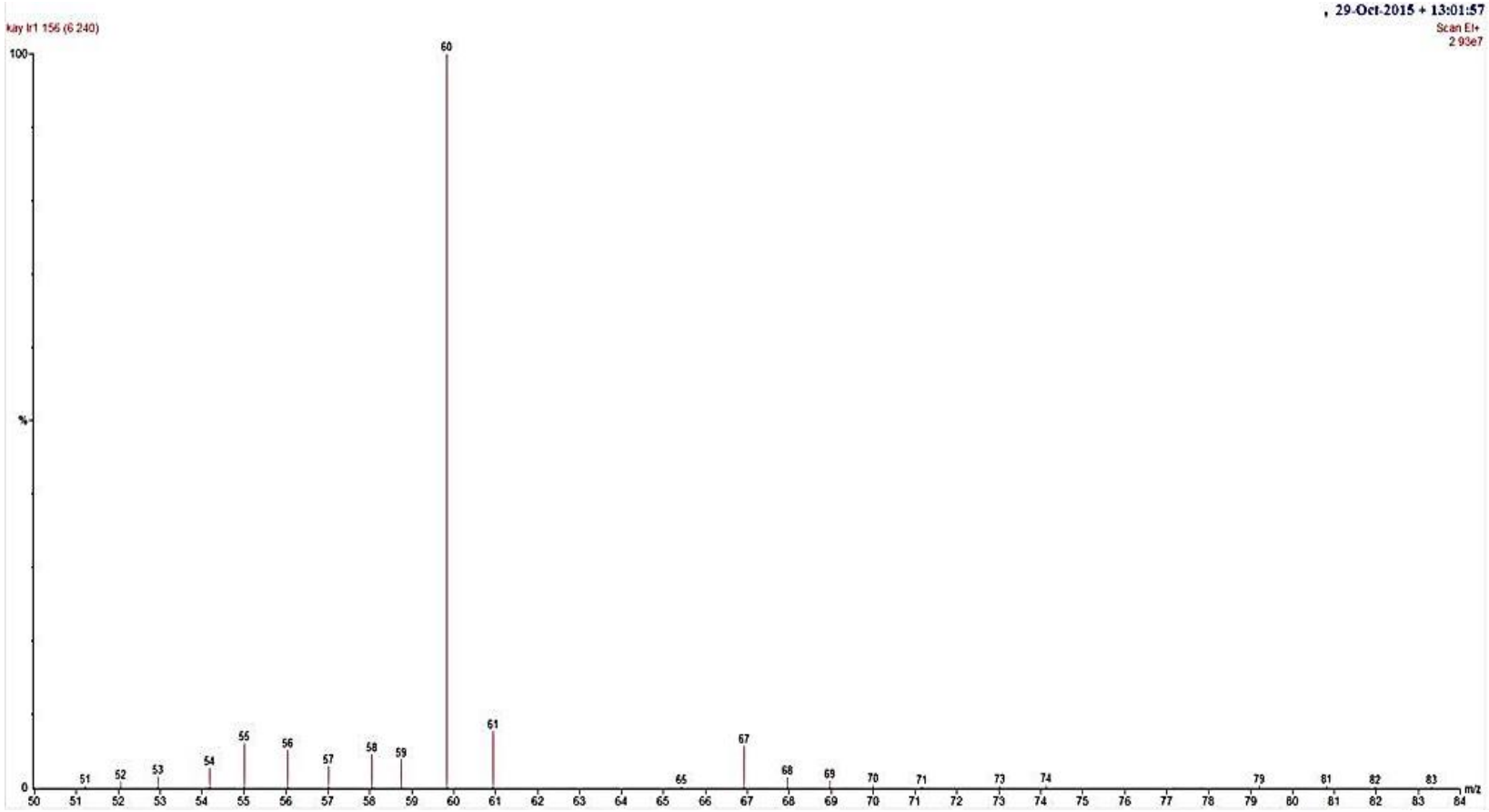

Figure B-2: Mass spectrum of chromatogram peak at 6.240 min Retention time.

MS2

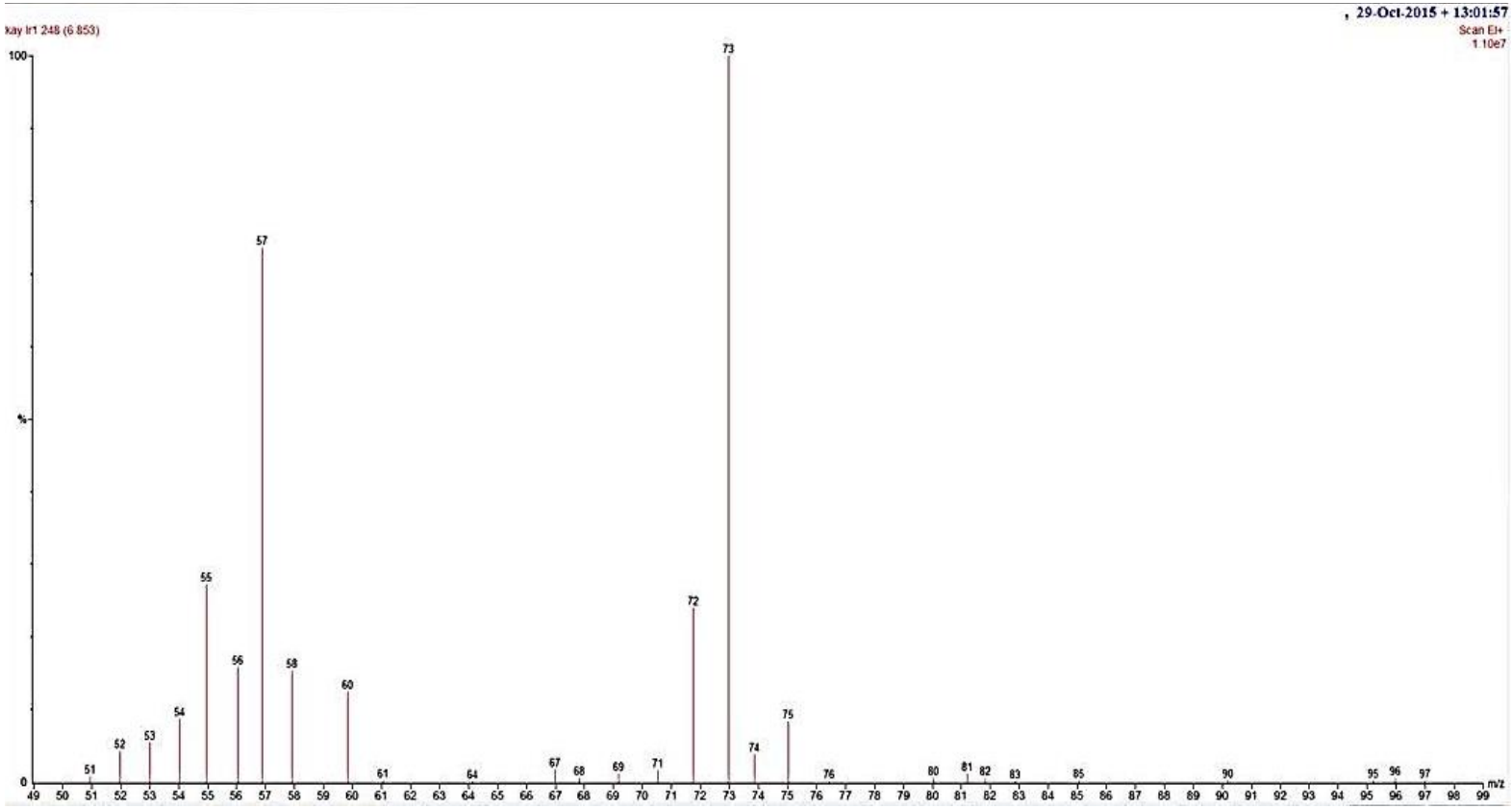

Figure B-3: Mass spectrum of chromatogram peak at 6.853 min Retention time.

MS3

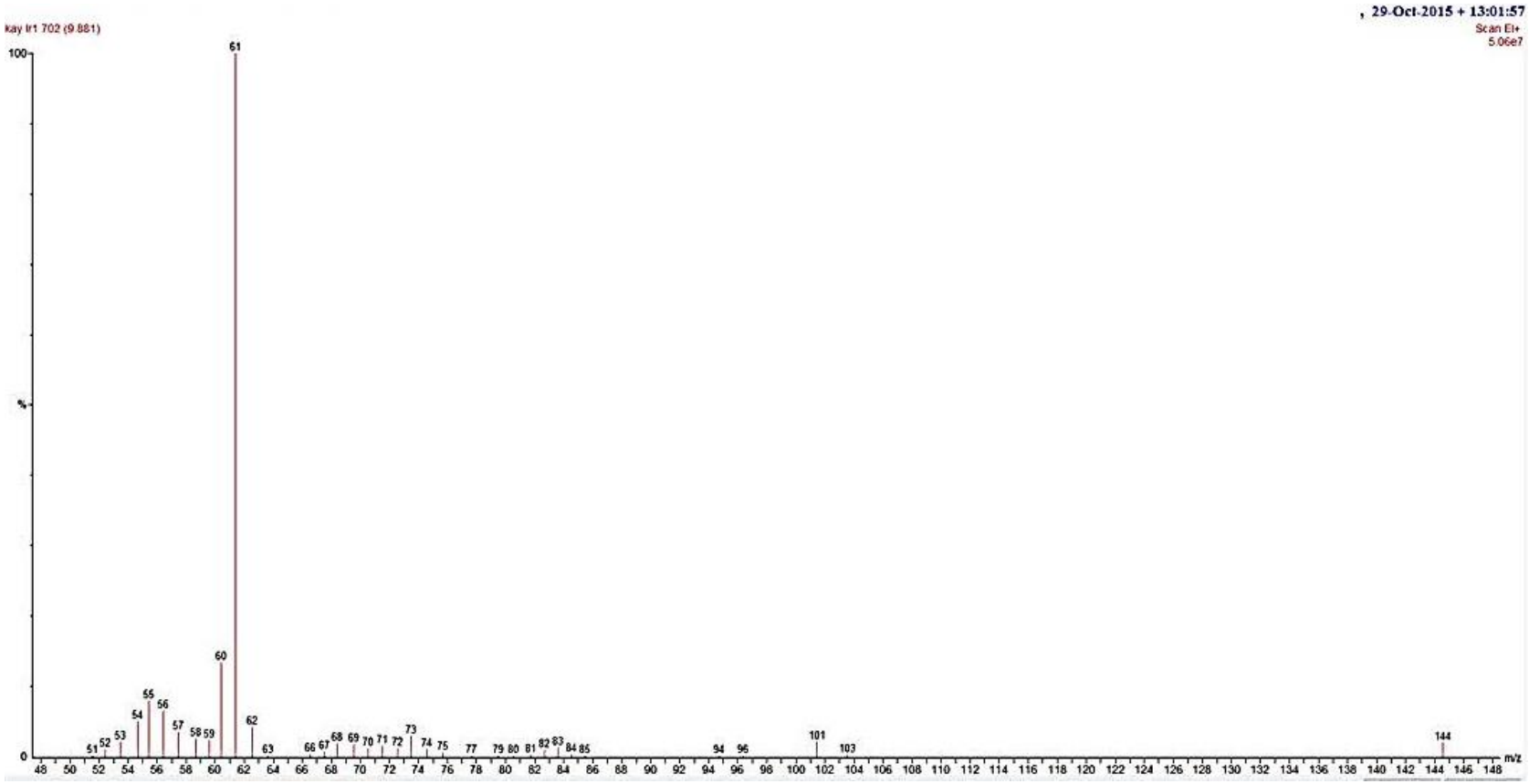

Figure B-4: Mass spectrum of chromatogram peak at 9.881 min Retention time.

MS4

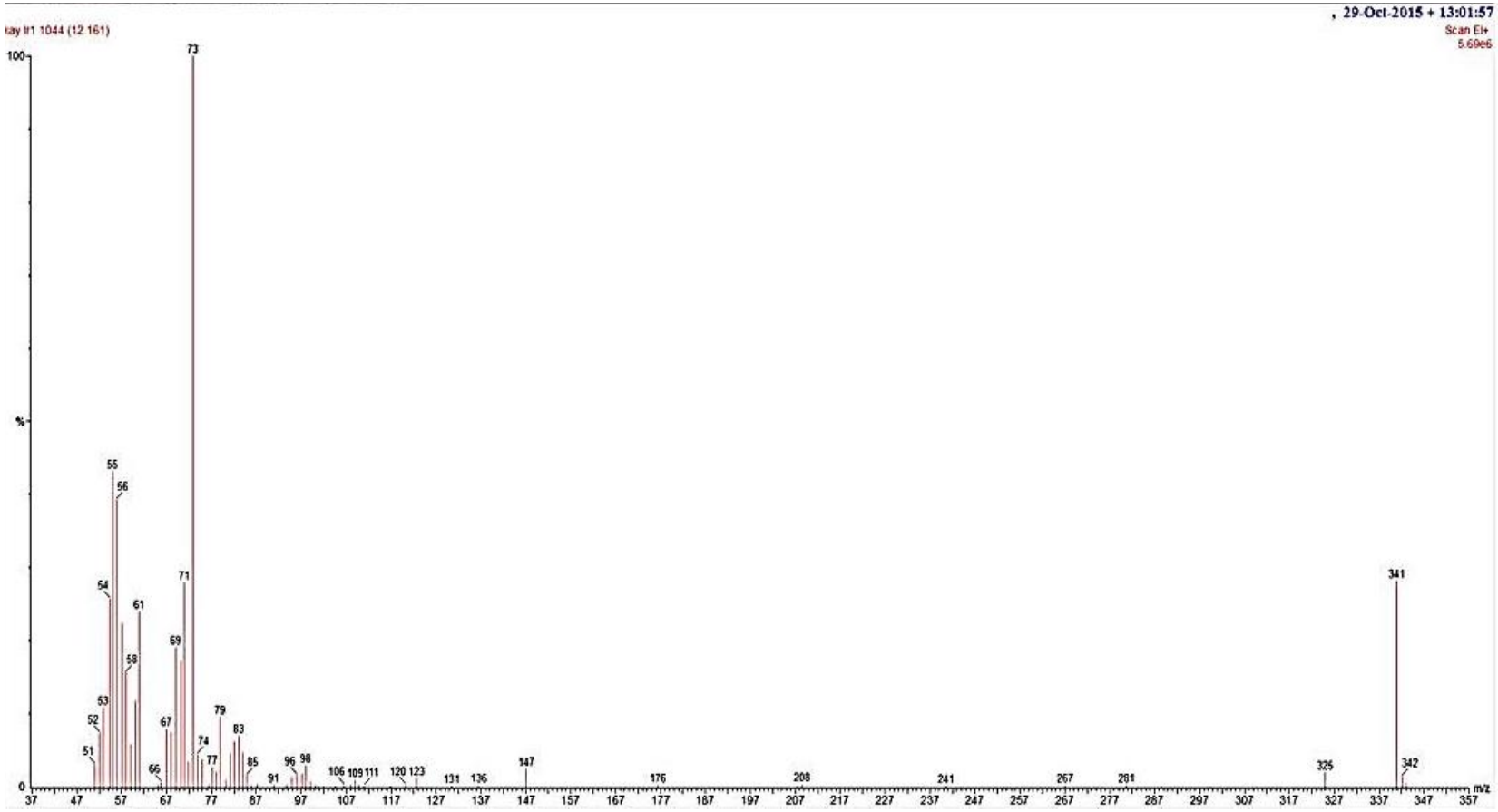

Figure B-5: Mass spectrum of chromatogram peak at 12.161 min Retention time.

MS5

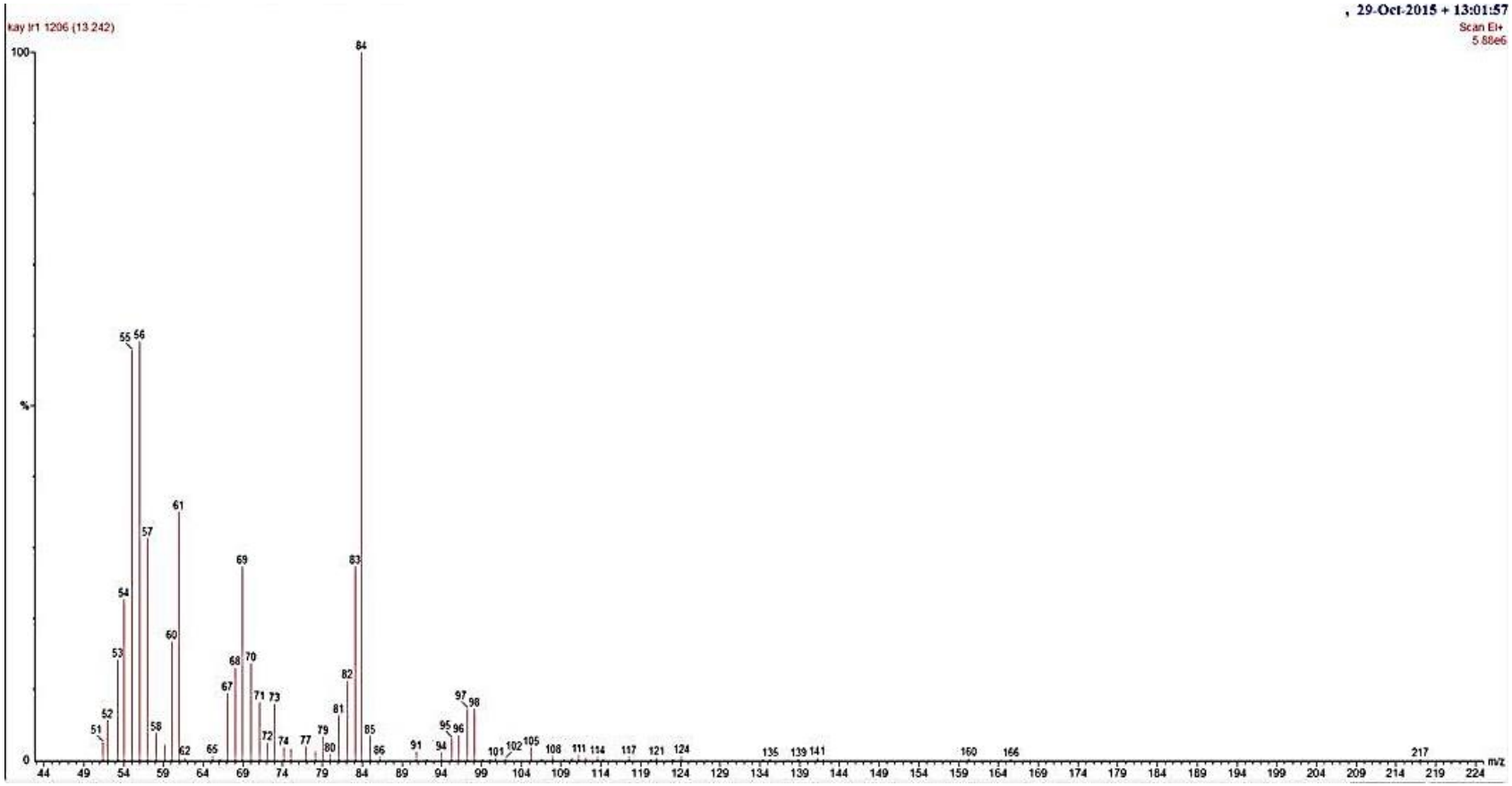

Figure B-6: Mass spectrum of chromatogram peak at 13.242 min Retention time.

MS6

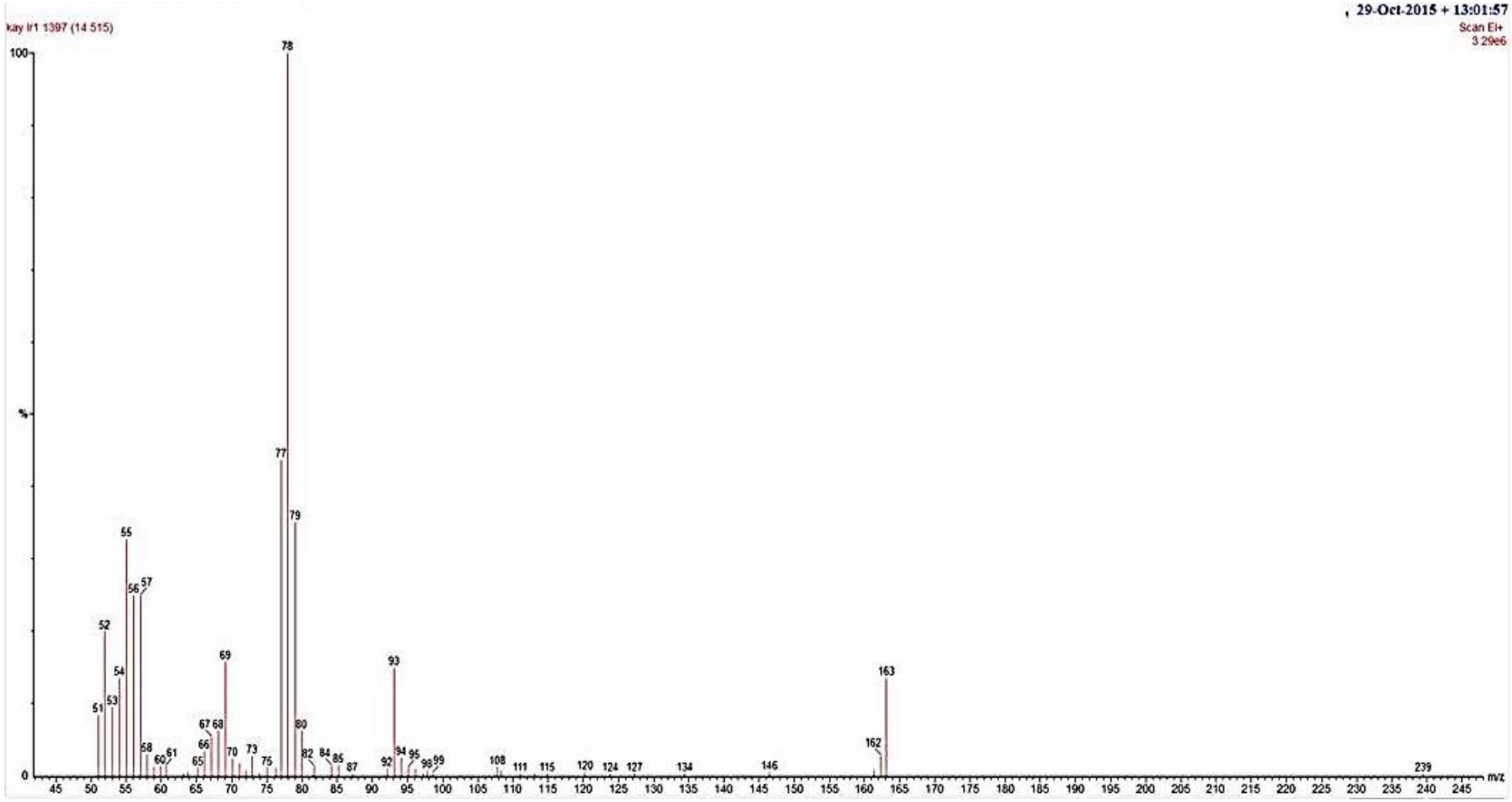

Figure B-7: Mass spectrum of chromatogram peak at 14.515 min Retention time.

MS7

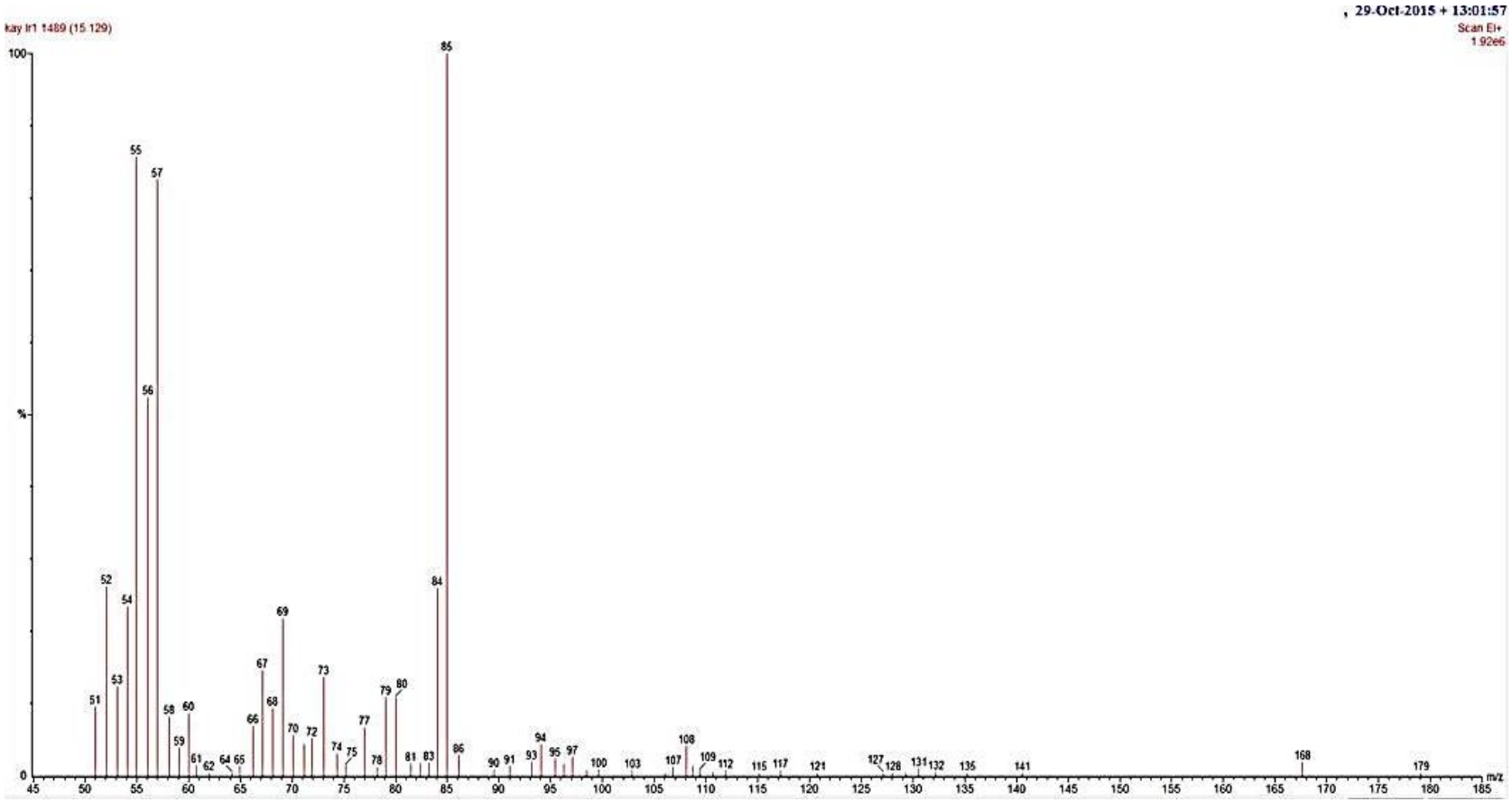

Figure B-8: Mass spectrum of chromatogram peak at 15.129 min Retention time.

MS8

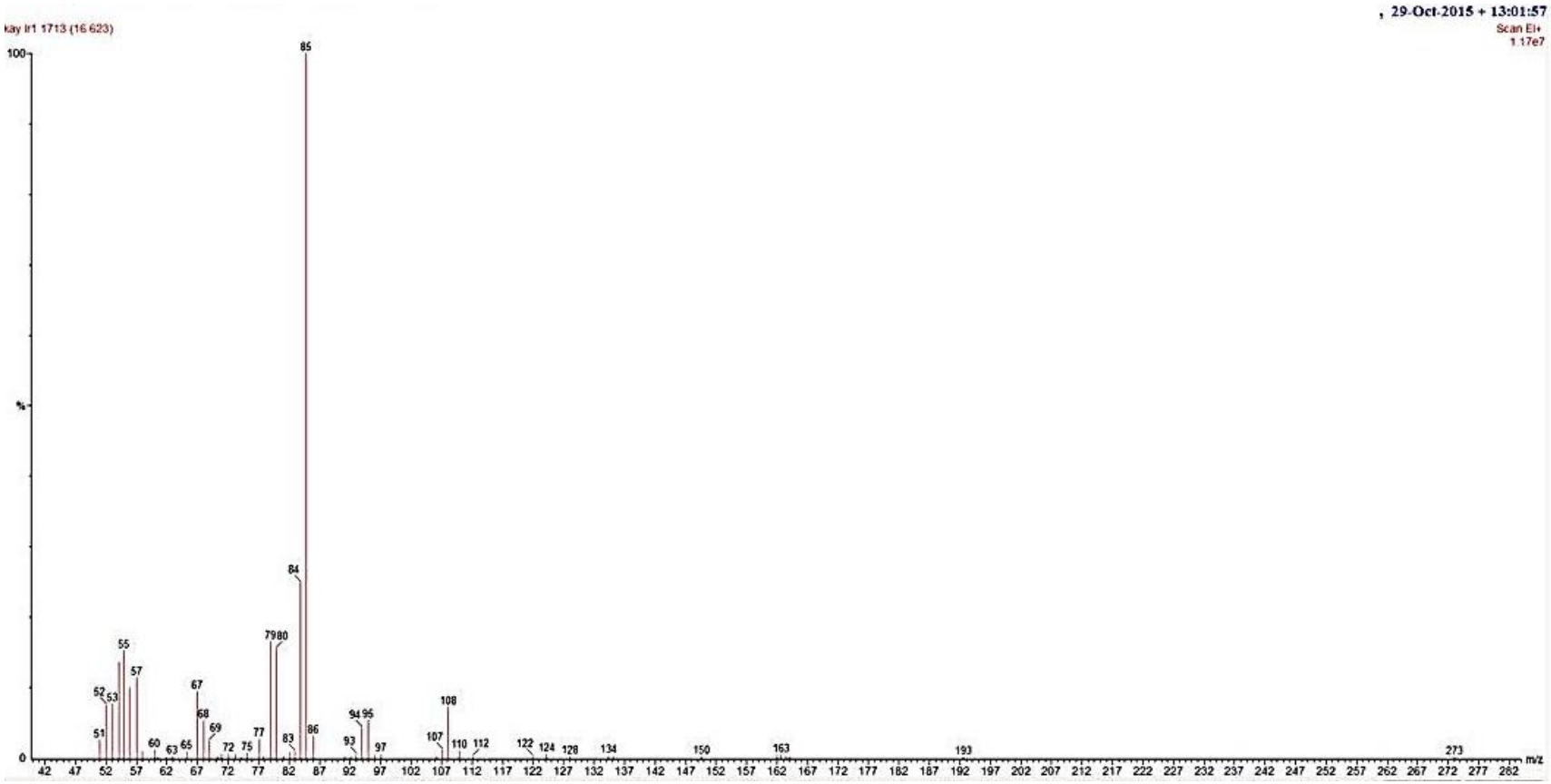

Figure B-9: Mass spectrum of chromatogram peak at 16.623 min Retention time.

MS9

Figure B-10: Mass spectrum of chromatogram peak at 19.784 min Retention time.

MS10

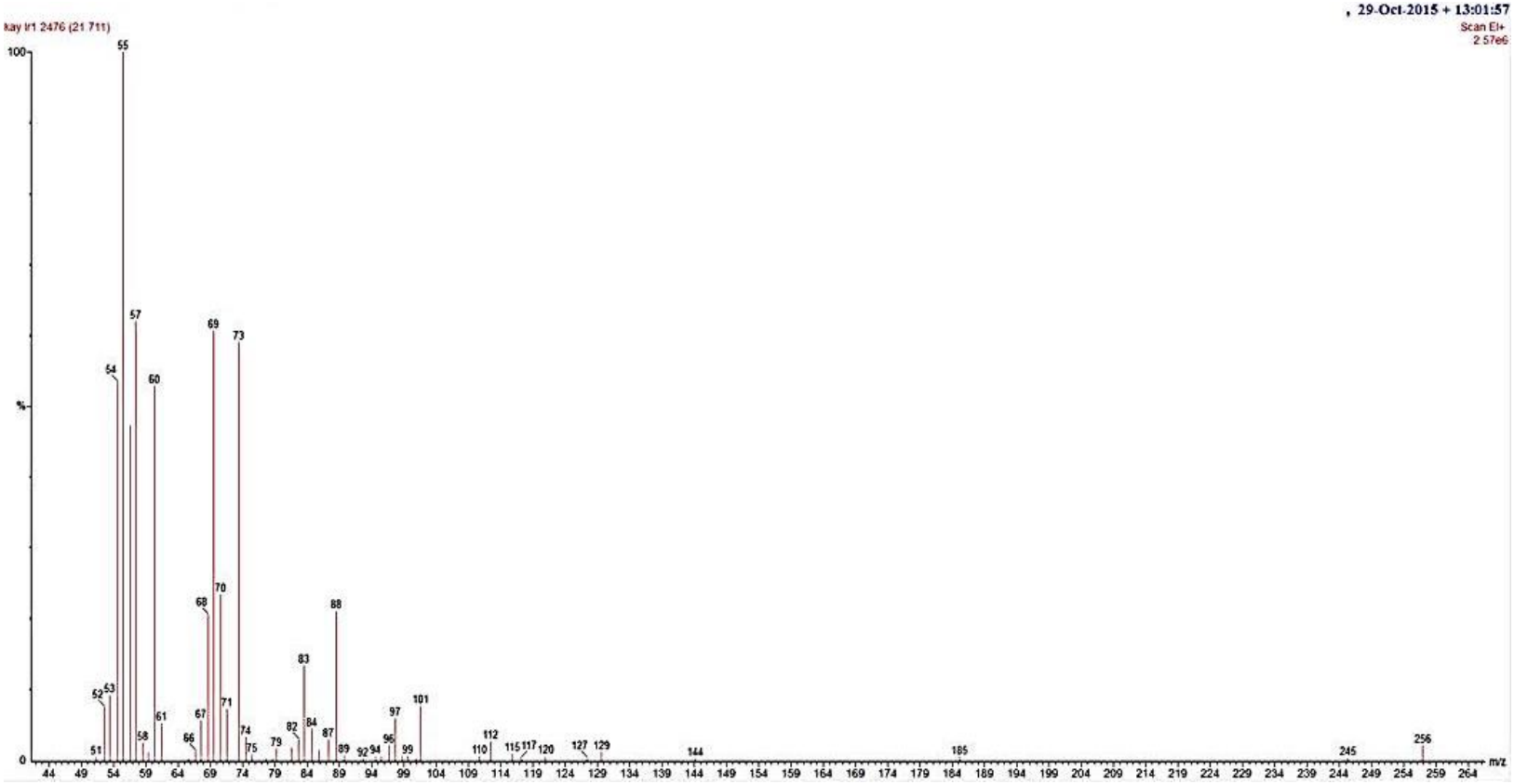

Figure B-11: Mass spectrum of chromatogram peak at 27.711 min Retention time.

MS11

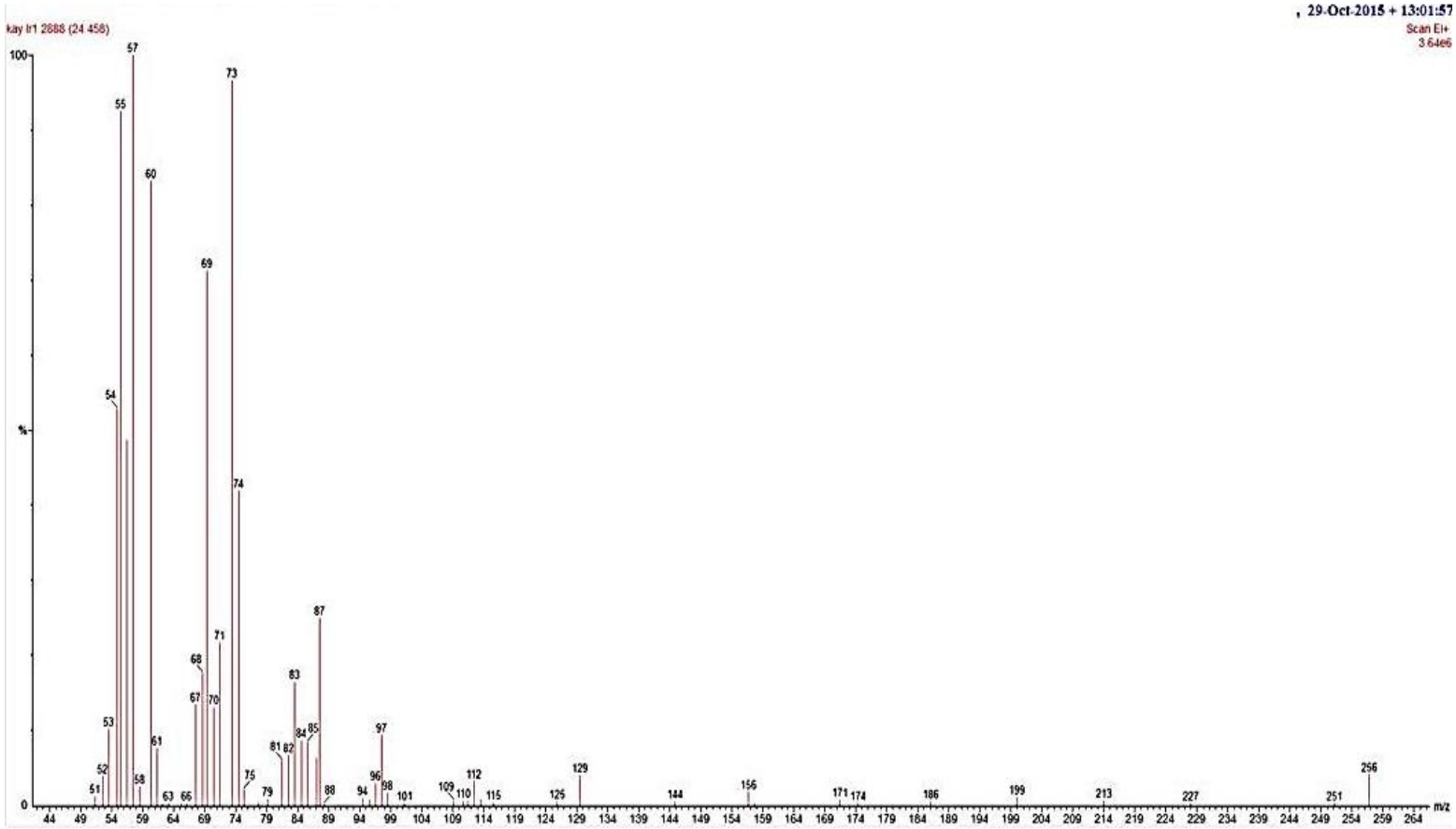

Figure B-12: Mass spectrum of chromatogram peak at 24.458 min Retention time.

Hexane Extract

TC1

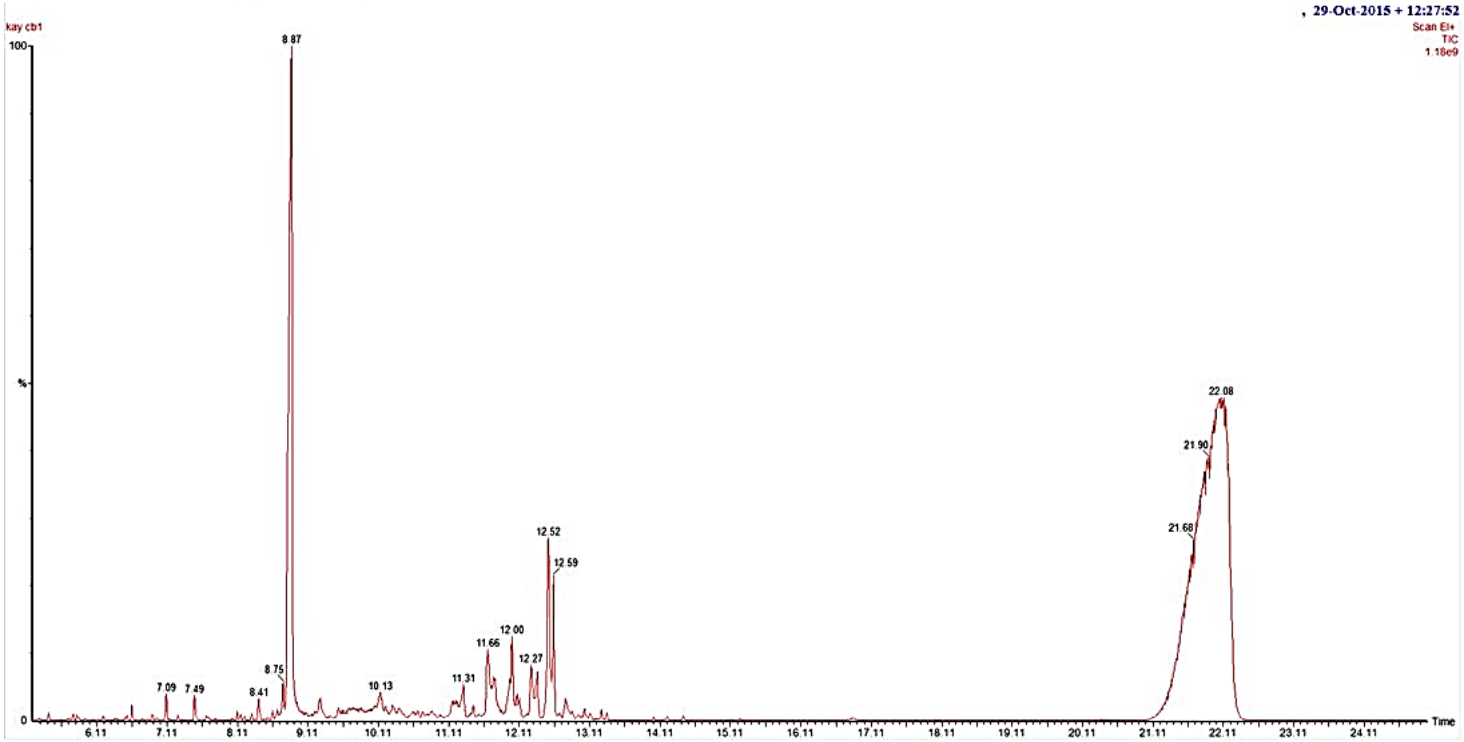

Figure B-13: Gas Chromatogram of hexane extract.

MS1

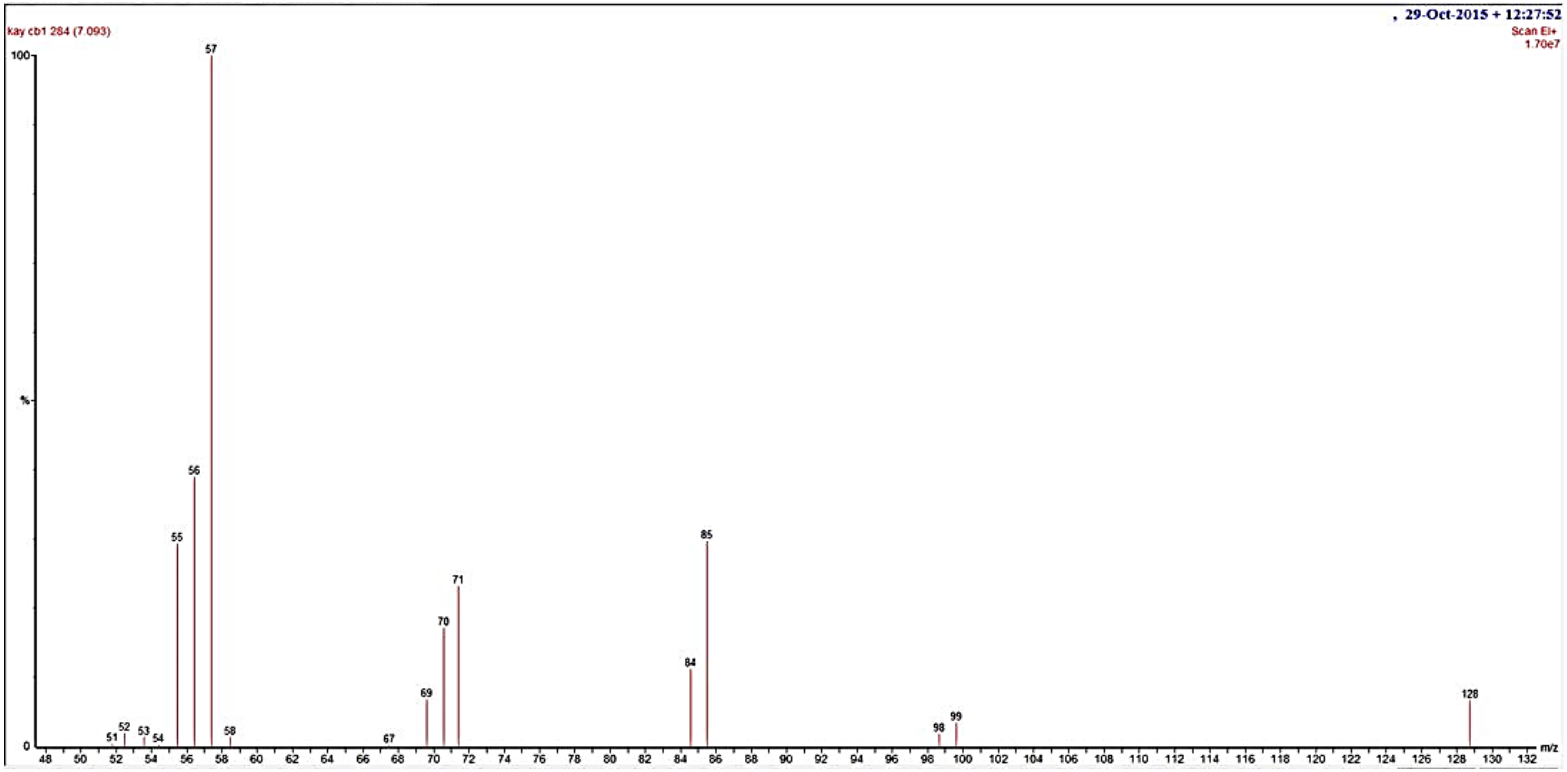

Figure B-14: Mass spectrum of chromatogram peak at 7.093 min Retention time

MS2

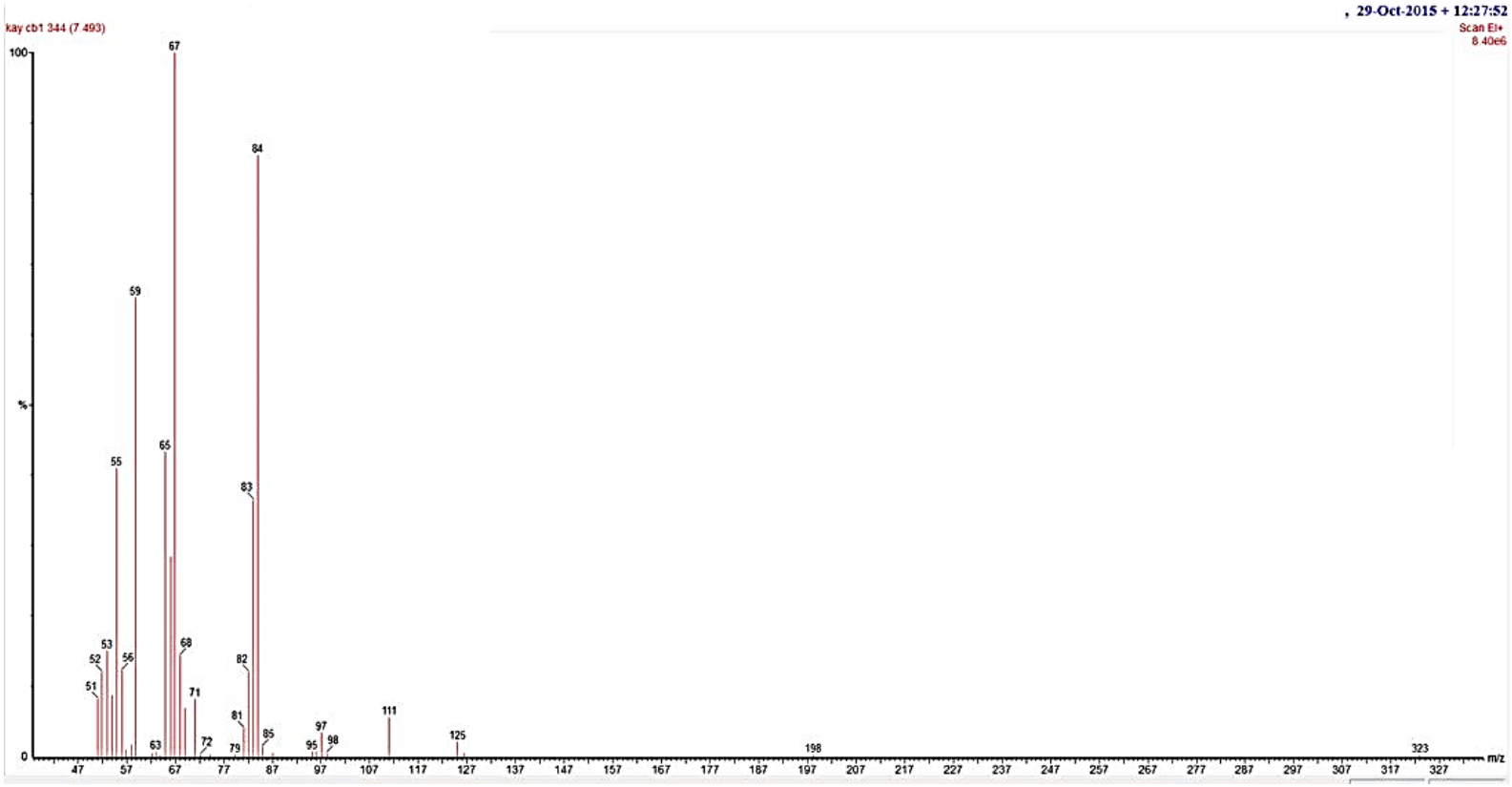

Figure B-15: Mass spectrum of chromatogram peak at 7.493 min Retention time

MS3

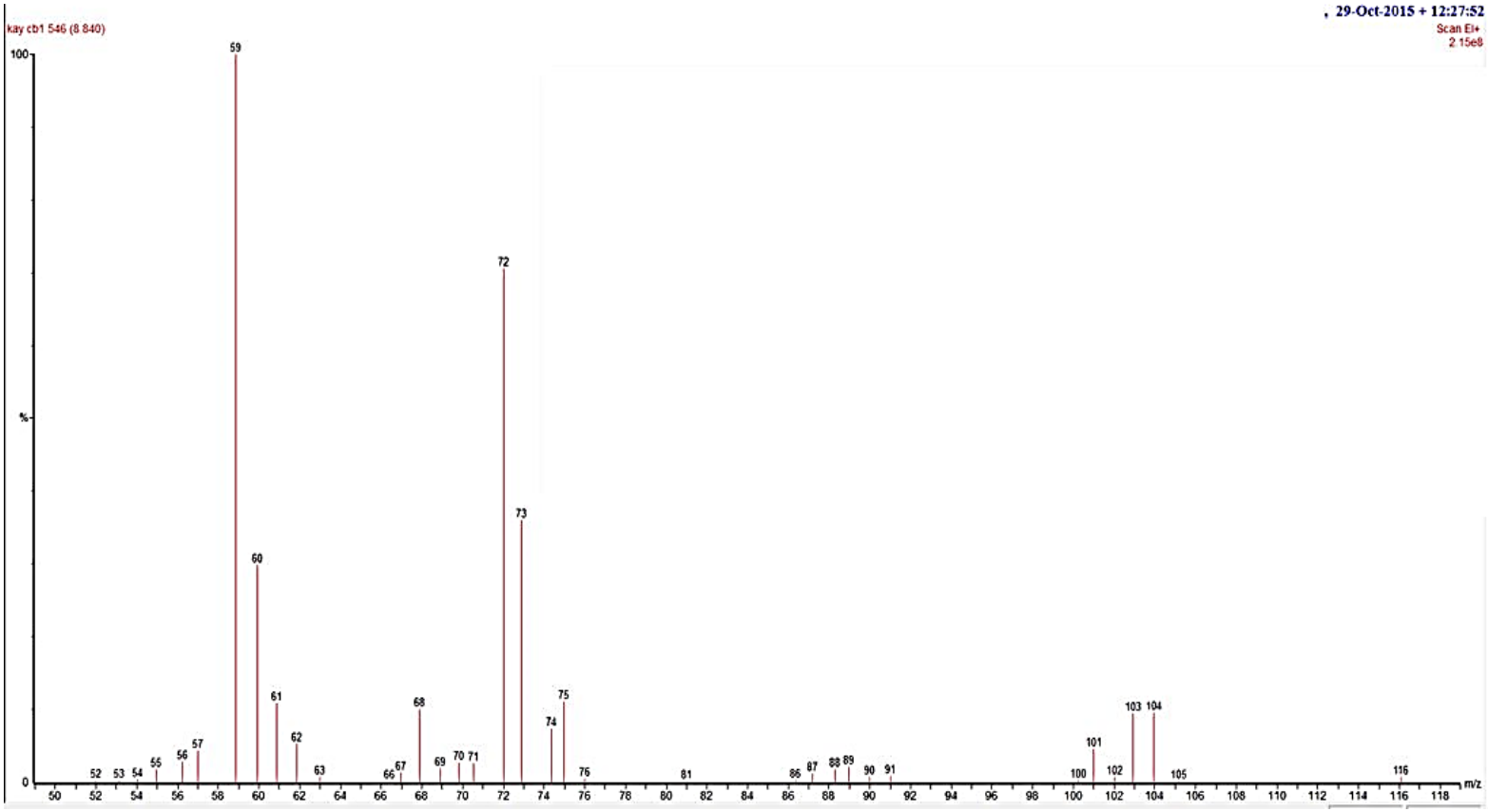

Figure B-16: Mass spectrum of chromatogram peak at 8.840 min Retention time

MS4

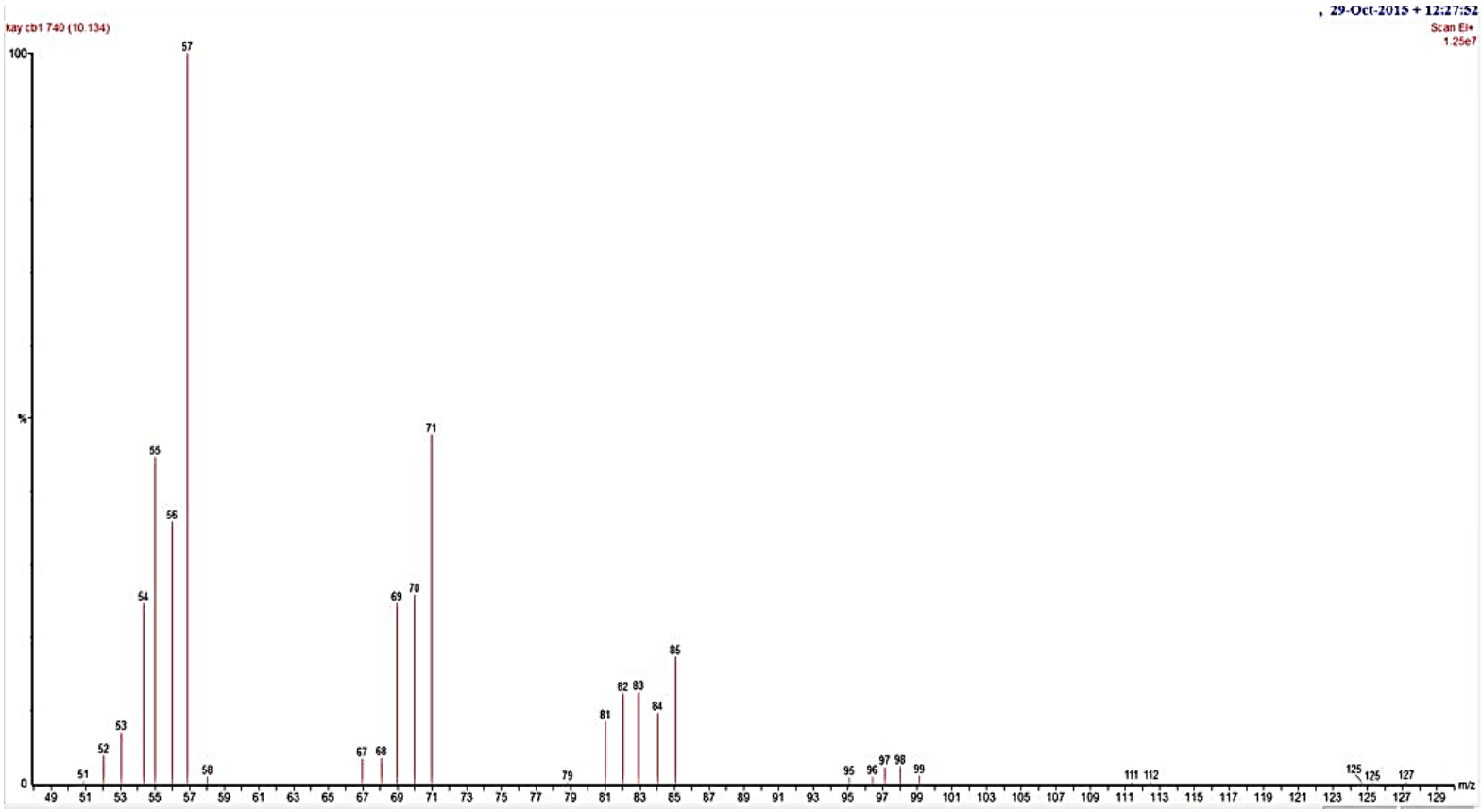

Figure B-17: Mass spectrum of chromatogram peak at 10.134 min Retention time

MS5

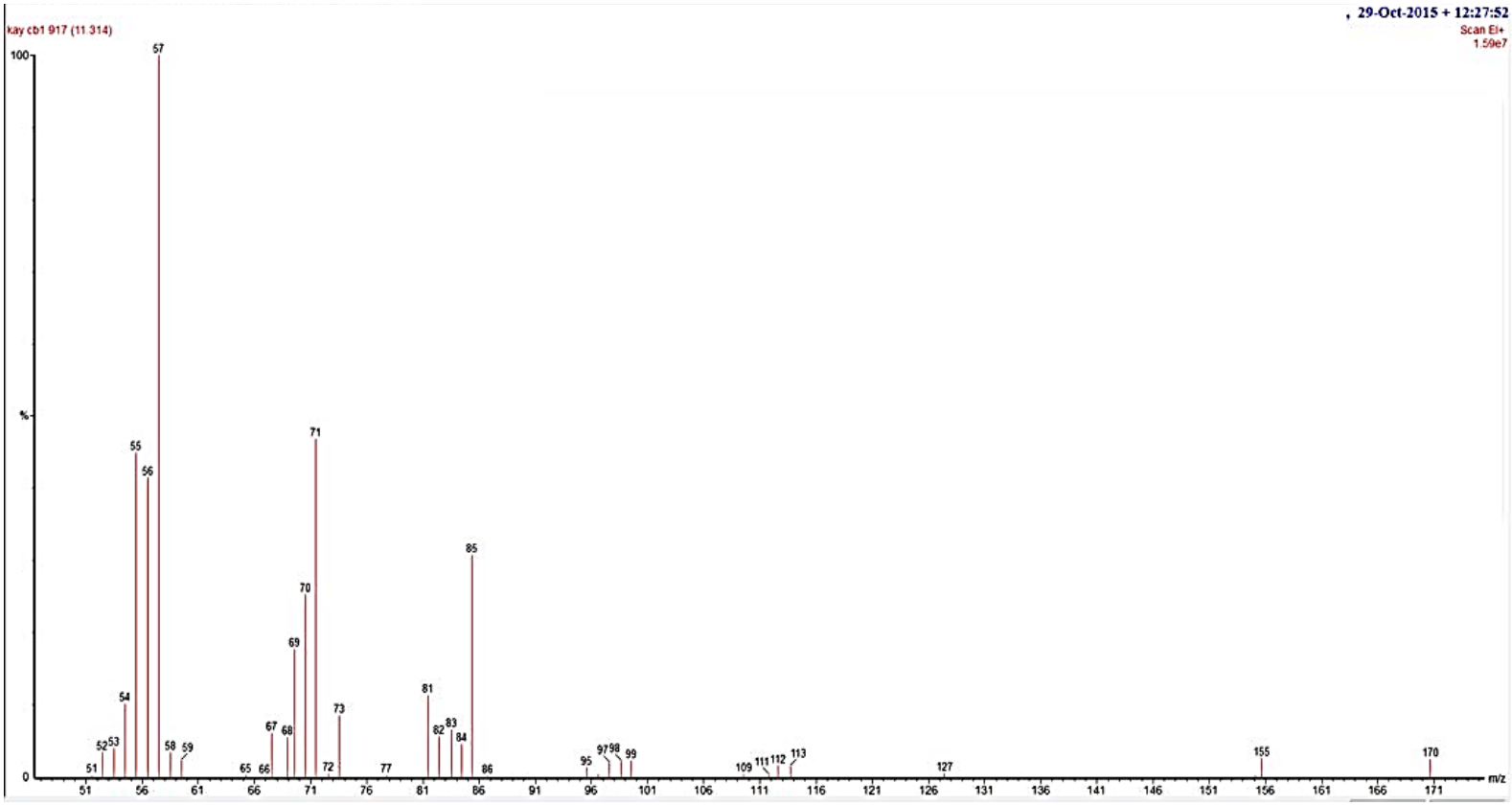

Figure B-18: Mass spectrum of chromatogram peak at 11.314 min Retention time

MS6

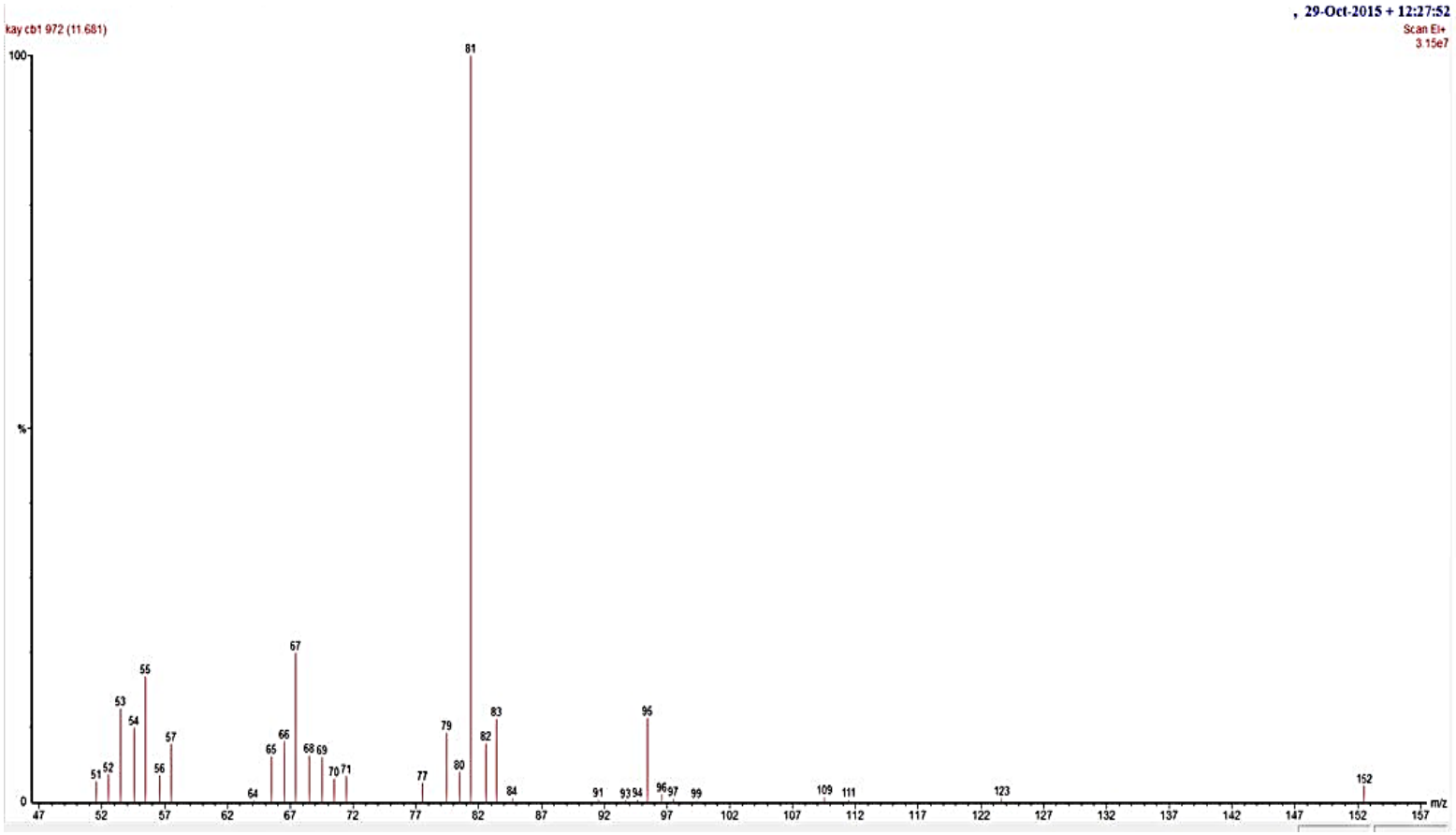

Figure B-19: Mass spectrum of chromatogram peak at 11.681 min Retention time

MS7

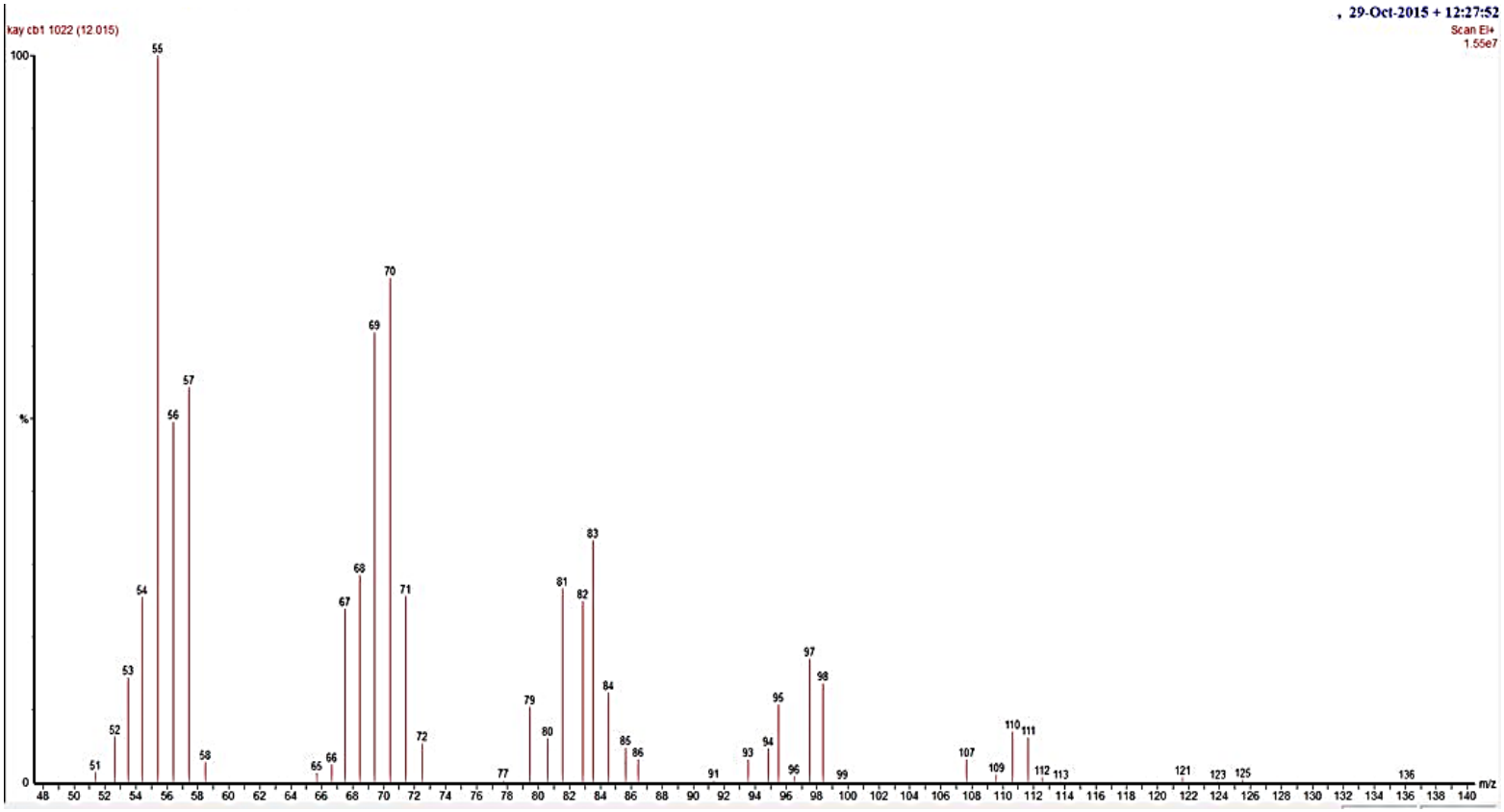

Figure B-20: Mass spectrum of chromatogram peak at 12.015 min Retention time

MS8

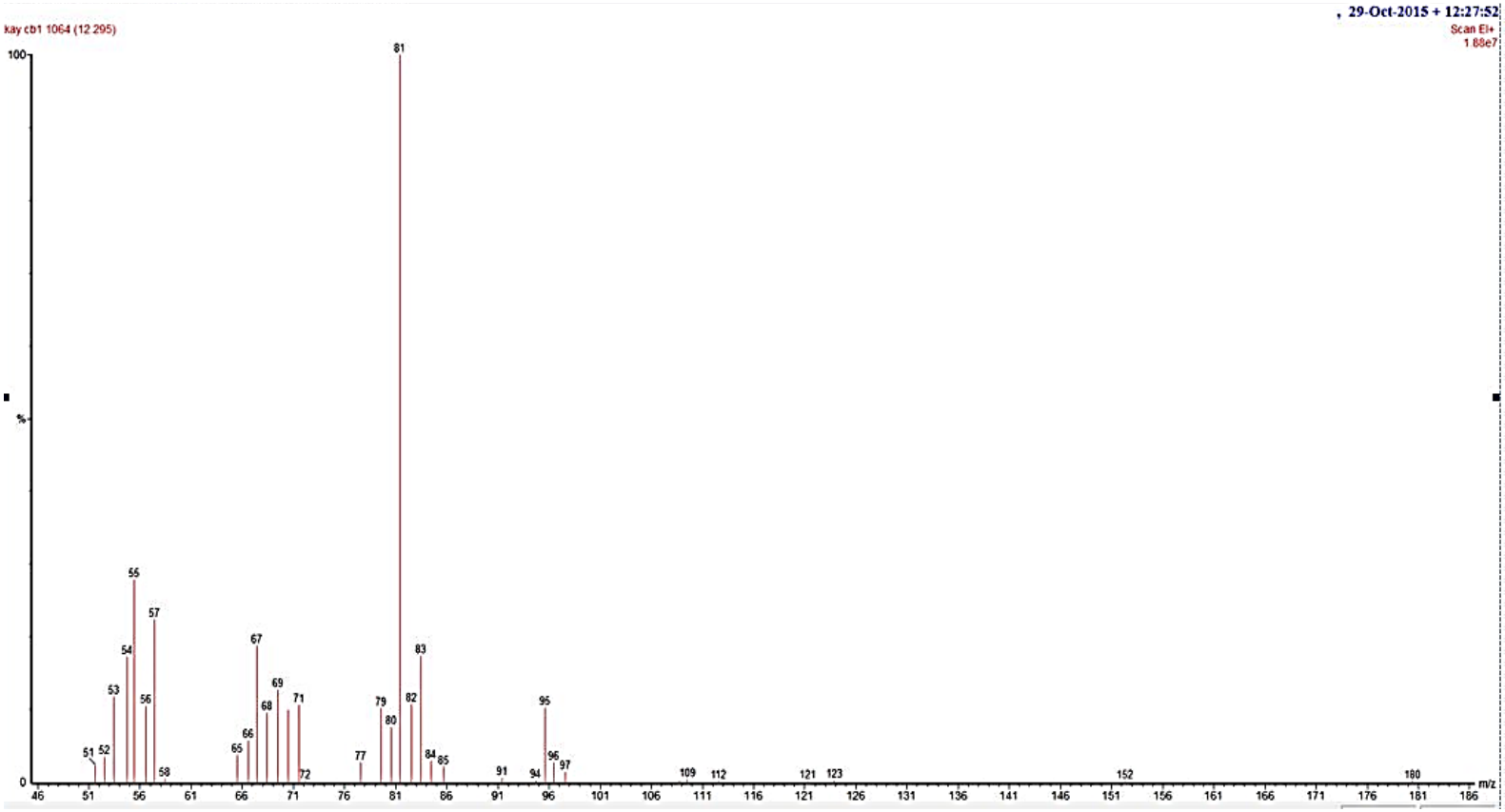

Figure B-21: Mass spectrum of chromatogram peak at 12.295 min Retention time

MS9

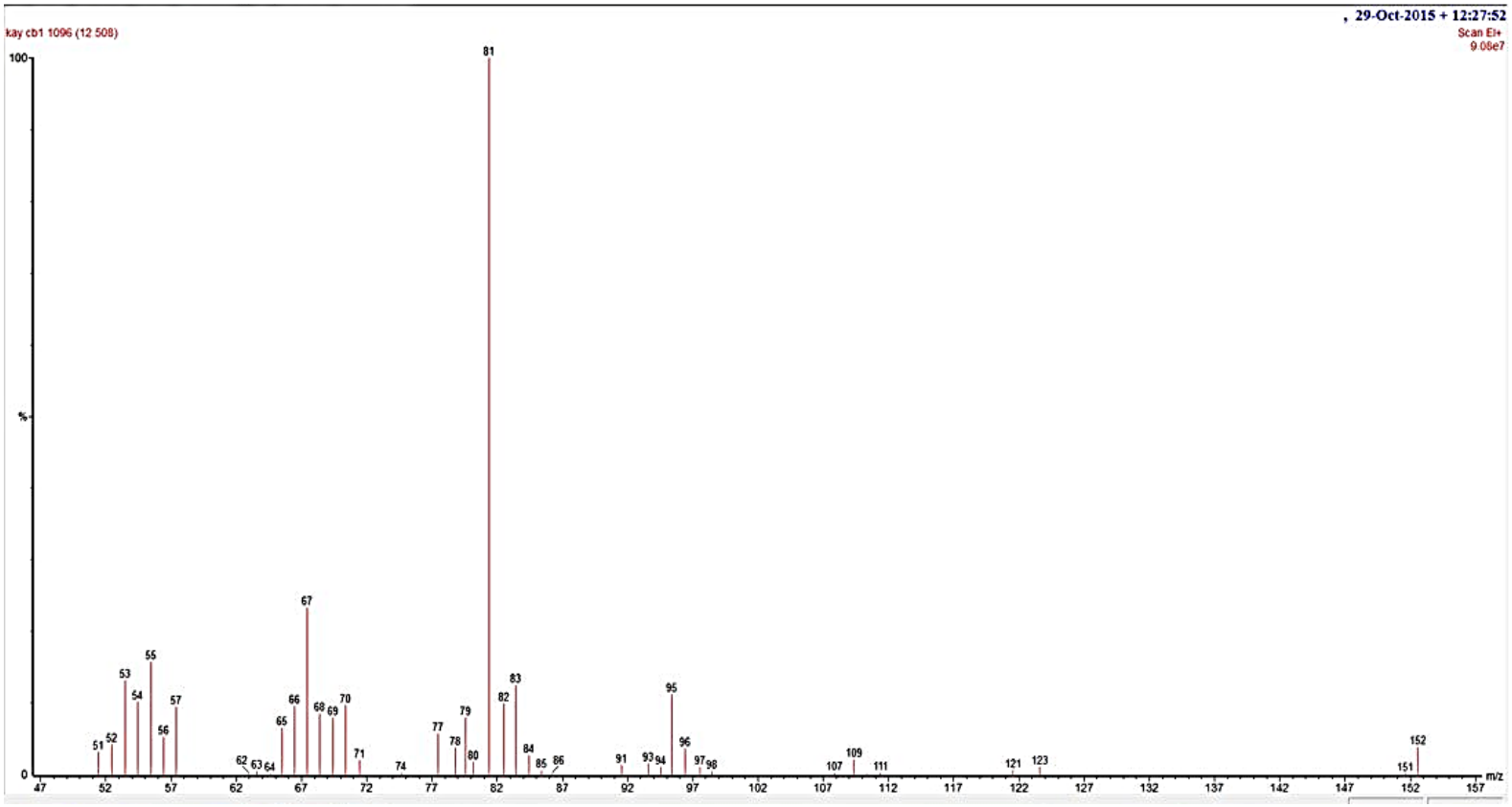

Figure B-22: Mass spectrum of chromatogram peak at 12.508 min Retention time

MS10

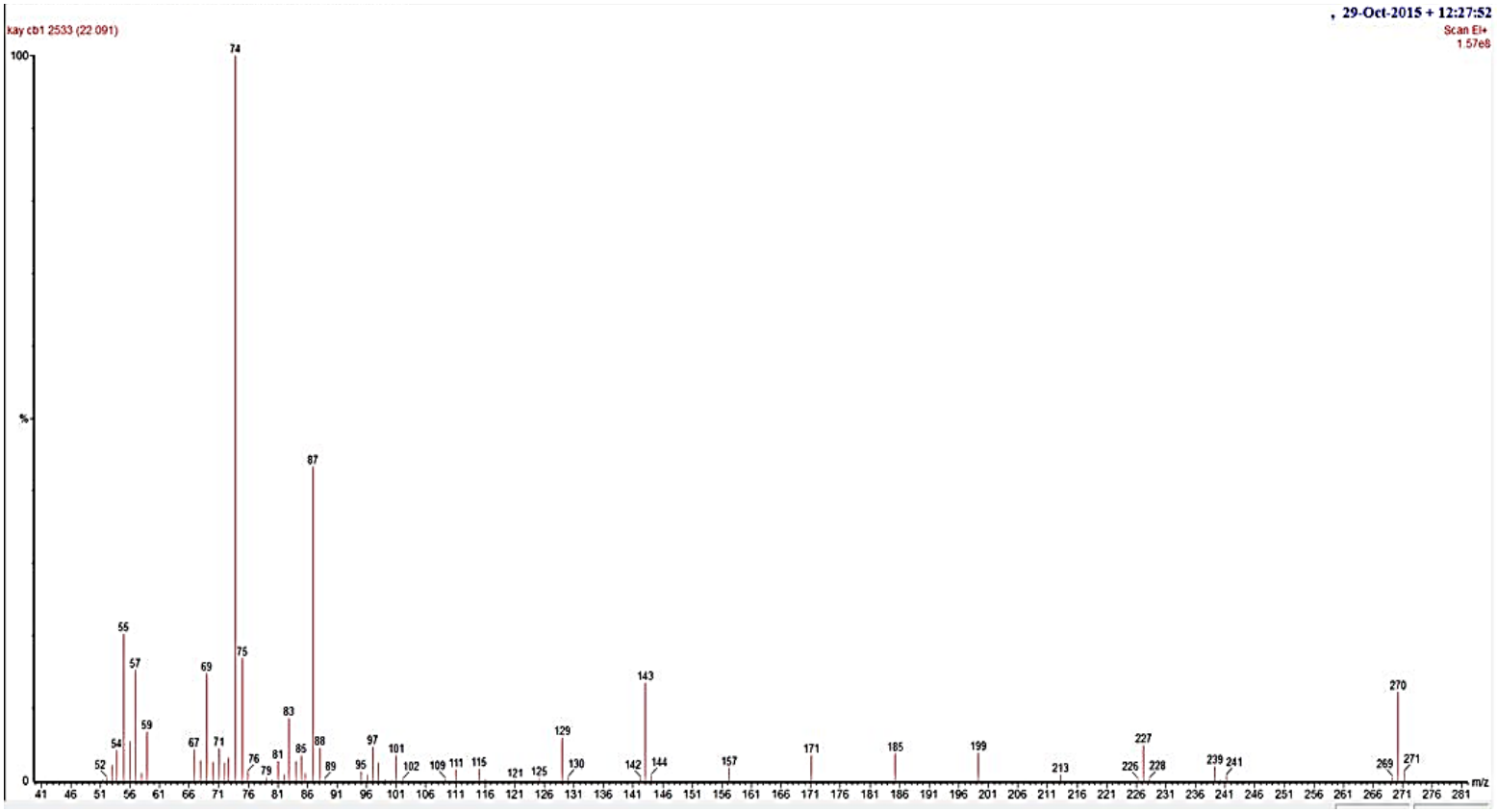

Figure B-23: Mass spectrum of chromatogram peak at 7.493 min Retention time

Appendix C

STATISTICAL TABLES

Table 1Moisture Content Mean and Standard Deviation

Analysis Variable: Moisture Content					
Mean	**N**	**Std Dev**	**Minimum**	**Maximum**	**Coeff of Variation**
34.01	9	9.71	18.86	46.55	28.54

Table 2 ANOVA table for DPPH Antioxidant activity of Extract/fractions

Source	**DF**	**Type I SS**	**Mean square**	**F Value**	**Pr > F**
Solvent	3	29044.10133	9681.36711	4932.17	< .0001
concentration	6	24608.68255	4101.44709	2089.48	< .0001
Solv & Conc	18	9159.07467	508.83748	259.23	< .0001

Table 3 ANOVA table for means of DPPH antioxidant activity of fractions

Solvent	**BHT**	**Hexane base**	**chloroform**	**Pet ether**
Mean of activity	66.8358[a]	30.4115[b]	29.3218[c]	17.1214[d]
N	21	21	21	21

***Means with the same letter are not significantly different**

Table 4 ANOVA table for the different concentrations of fractions used in DPPH assay

Concentration(mg/ml)	**0.16**	**0.31**	**0.63**	**1.25**	**2.50**	**5.00**	**10.00**
Mean	16.46[a]	19.22[b]	25.25[c]	32.50[d]	39.60[e]	50.02[f]	68.41[g]
N	12	12	12	12	12	12	12

*Means with the same letter are not significantly different

Table 5 ANOVA table comparing scavenging activities of different concentrations among extracts (DPPH)

	Concentration(mg/ml)						
Extracts/fractions	**0.16**	**0.31**	**0.63**	**1.25**	**2.50**	**5.00**	**10.00**
Methanol	--	--	--	--	--	--	--
Hexane	--	--	--	--	--	--	--
Chloroform	12.29±1.31c	11.62±0.85d	13.77±1.48c	20.97±2.40b	27.35±1.14b	44.08±1.51b	75.18±1.50b
Hexane base	15.16±1.18b	16.23±0.91b	18.03±0.98b	22.07±0.55b	27.68±0.70b	42.62±1.50b	70.69±5.11b
Petroleum ether	15.35±0.71b	13.77±0.80c	13.86±0.91c	14.29±0.64c	14.68±0.24c	16.81±0.23c	31.08±0.10c
BHT	23.04±0.26a	34.87±1.02a	55.34±0.90a	72.67±1.49a	88.69±0.38a	96.56±0.10a	96.68±0.11a

Table 6 ANOVA table for the different concentrations of fractions used in Reducing power assay

	Concentration(mg/ml)						
Extracts/fractions	**0.16**	**0.31**	**0.63**	**1.25**	**2.50**	**5.00**	**10.00**
Methanol	0.04±0.001b	0.10±0.002c	0.12±0.001b	0.22±0.01b	0.40±0.02b	0.50±0.01	0.43±0.03b
hexane	0.01±0.001c	0.01±0.001d	0.02±0.002e	0.01±0.001e	0.02±0.001e	0.03±0.01	0.03±0.01f
Chloroform	0.02±0.001c	0.08±0.001b	0.10±0.002c	0.10±0.01c	0.13±0.002c	0.20±0.01	0.25±0.001d
Hexane base	0.01±0.001c	0.10±0.001c	0.10±0.001d	0.10±0.001c	0.14±0.004c	0.22±0.01	0.40±0.01c
Petroleum ether	0.001±0.001c	0.10±0.001c	0.10±0.003d	0.10±0.003d	0.10±0.002d	0.10±0.002	0.10±0.003e
BHT	0.44±0.001a	0.52±0.02a	0.70±0.01a	0.72±0.03a	0.72±0.01a	0.64±0.04	0.70±0.00a

Table 7 ANOVA table comparing Antiproliferative activities of different concentrations among extracts/fractions on Chang and Jurkat cells

		Concentration(µg/ml)						
Extracts/Fractions	**Cell line**	**0.00**	**31.25**	**62.50**	**125.00**	**250.00**	**500.00**	**1000.00**
Methanol	Chang	100.00±0.00a		85.34±0.77	90.54±1.16	89.95±3.56	90.16±1.76	8.81±7.89
Hexane		100.00±0.00a		81.78±18.27	74.40±13.17	94.72±15.40	92.86±5.21	8.64±8.10
Chloroform		100.00±0.00a	88.48±15.37a	76.66±17.04	72.52±14.05	68.19±7.30	59.60±3.77	
Hexane base		100.00±0.00a	73.32±6.50ab	65.75±9.48	63.51±7.89	54.66±15.61	63.90±5.71	
Petroleum ether		100.00±0.00a	58.37±5.73b	52.01±1.51	45.56±4.68	90.35±9.35	68.16±1.98	
Methanol	Jurkat	100.00±0.00a	±	92.57±0.12	95.19±5.60	92.54±18.29	68.75±8.39	0.13±0.00
Hexane		100.00±0.00a	±	97.64±19.92	104.12±23.43	104.73±22.62	84.84±7.58	4.44±1.93
Chloroform		100.00±0.00a	111.73±19.92c	120.82±9.00	105.82±10.02	95.83±7.32	95.38±17.34	
Hexane base		100.00±0.00a	105.82±9.44ac	106.60±9.00	114.88±5.48	104.73±17.03	96.55±19.96	
Petroleum ether		100.00±0.00a	119.04±6.66c	131.49±17.17	121.58±19.13	132.98±12.12	113.67±22.10	

Table 8 ANOVA table comparing Antiproliferative activities of different concentrations among extracts/fractions on different cancer cells

Source	Solvent	Cell line	0.00	0.10	0.20	0.50	1.00	2.00	5.00	10.00
Skin	Hexane	A431	100.00±3.63a	67.78±5.05a	75.00±0.64ac	59.17±5.32a	13.06±8.43a	9.72±8.50a	1.94±0.56a	1.67±1.43ab
	Meth	A431	100.00±1.89a	84.02±1.99bdf	82.64±4.84ab	83.22±4.05b	80.51±6.65b	31.13±9.30a	0.69±1.38a	2.61±0.71a
Lung	Hex	A549	100.00±1.05a	83.21±7.32b	90.46±6.64bd	54.19±9.03a	60.31±13.04c	38.93±21.79ab	15.65±13.56b	4.96±1.46a
Lung	Meth	A549	100.00±2.36a	77.11±4.80be	72.68±8.75c	72.49±5.97c	75.19±2.10b	66.55±1.25c	0.35±0.70a	1.56±1.06a
Prostate	Hex	DU145	100.00±1.22a	105.00±1.38e	100.17±10.70df	92.42±5.97bd	54.21±12.88c	35.20±26.32a	8.51±3.42a	7.41±2.00b
	Meth	DU145	100.00±0.41a	89.50±3.26f	87.19±1.69b	98.22±4.69de	109.96±1.88e	103.02±14.53de	64.41±5.21c	55.87±1.70c
Lung	Hex	H460	100.00±4.79a	96.06±1.61cbfh	98.74±2.21df	104.02±1.63ef	102.86±2.76e	107.34±2.11e	99.06±13.50d	49.86±1.00d
	Meth	H460	100.00±0.84a	79.72±0.50dg	85.28±3.57b	82.58±3.24b	70.48±6.24bd	16.94±9.14a	1.60±0.17a	4.38±0.82ab
Prostate	Hex	PC3	100.00±4.79a	98.91±6.15ce	101.63±12.24ef	88.49±13.58b	47.38±6.39cf	11.27±9.72a	13.47±6.80b	0.32±0.40a
	Meth	PC3	100.00±1.08a	109.87±9.06g	118.86±5.50g	122.11±4.59g	116.99±5.76eh	54.55±39.05bc	1.63±0.48ac	3.16±0.66ab