Studies on the antimicrobial, antioxidant and antiproliferative potential of the ethyl acetate extract and compounds of *Peltophorum africanum*

By

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DECLARATION

I, Benjamin Ifeoluwa Okeleye declare that the results of experimental work of this thesis for the award of a Doctor of Philosophy degree in Microbiology at the University of Fort Hare under the supervision of Prof. Roland N. Ndip and Prof. Noxolo T. Mkwetshana, hereby submitted by me, is original and has not been previously submitted for a degree at this or any other university. All the reference materials contained in this thesis have been properly acknowledged.

Signature……...

Supervisor's signature……...

Co-supervisor's signature……..

Date……...

DEDICATION

To my father, Mr. Gabriel O. Okeleye, my mother, Mrs. Rebecca O. Okeleye, my wife, Ndidi Amaka Okeleye (Nee Joseph), my lovely daughter, Olivia O. Okeleye and to all those who cherish education and strive hard to attain it

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GENERAL ABSTRACT

Cells are constantly exposed to a variety of oxidizing agents, some of which are necessary for life. Oxidants produced in excess can cause an imbalance, leading to oxidative stress, especially in chronic bacterial, viral, and parasitic infections. This can result to damage of biomolecules such as lipids, proteins, and DNA, hence, an increased risk for cancer. Plants have a long history of use in the treatment of cancer. Plant secondary metabolites have proved to be an excellent reservoir of new medical compounds. Fruits, vegetables, and whole grains contain a wide variety of antioxidant phytochemicals, such as phenolics and carotenoids, and may help protect cellular systems from oxidative damage and also may lower the risk of chronic diseases. *Peltophorum africanum*, a member of the family Fabaceae (Sond) is also known as the African weeping wattle and is used in traditional medicine in South Africa. This study investigated the antimicrobial, antioxidant and antiproliferative potential of the ethyl acetate extract and compounds of *Peltophorum africanum* in order to validate its pharmacological use.

The study assessed the *in vitro* antimicrobial activity of ethyl acetate extract (EAE) of *Peltophorum africanum* stem bark and its fractions by the agar well and macrodilution methods. The toxicity on a normal human liver cell (Chang liver cell) and antiproliferation of human breast (MCF-7), colon (HT-29) and cervical (HeLa) cancer cell lines were determined using the CellTiter-Blue cell viability assay and the mechanism of action delineated using the Nucleic Acid and Protein Purification Nucleospin® Tissue Kit, Scanning Electron Microscopy (SEM), Propidium iodide (PI) and Acridine orange (AO) double-staining techniques, the Cleaved Caspase 3 (Asp 175) Alexa Fluor® 488 Antibody and the Coulter® DNA Prep™ Reagents Kit. Purification and identification of the compounds from EAE and fractions as well as the morphological alteration of bacteria, yeast and cancer cells were determined using thin layer chromatography, infrared spectra fingerprint and GC-MS

analysis, micro-dilution and scanning electron microscopy with energy-dispersive X-ray analysis.

In vitro antioxidant activity of EAE was determined by means of radical scavenging and ferric reducing power analysis using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2`-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) kit, hydrogen peroxide (H_2O_2), iron (iii) chloride (Fe^{3+}) and nitric oxide (NO). To assess the likely effects of secondary metabolites on the activities observed; total proanthocyanidins, phenolics, flavonols, and flavonoids were determined using standard phytochemical methods. Data were analyzed by one way analysis of variance (ANOVA; SPSS Version 17.0, 2011), regression analysis (MINITAB, version 12 for windows), probit analysis test (software NCSS, 2007) and GraphPad Prism4 software package. The *p*-values < 0.05 were considered significant.

Marked activity of the extract was observed against *Plesiomonas shigelloides* ATCC 51903, with MIC and MLC values of 0.15625 and 0.3125mg/mL, respectively. The extract was both bactericidal (MICindex ≤ 2) and bacteriostatic/fungistatic (MICindex > 2) in activity. Lethal dose at 50 (LD50) showed 82.64 ± 1.40 degree of toxicity at 24 hrs, and 95 percentile of cell death dose activity ranged from log 3.12 ± 0.01 to 4.59 ± 0.03. The activity of the eight fractions tested ranged from 1.0 ± 0.5 to 3.7 ± 1.6 mg/mL (IC_{50}) and from 2.1 ± 0.8 to 6.25 ± 0 mg/mL (IC_{90}) (Chapter 3). Due to the effect of compounds present in the crude extract and fractions, the *P. aeruginosa* treated with EAE had a reduction of sodium from 5.55 % (untreated) - 1.50 %. For *C. albicans*, pottasium was reduced from 4.16 % (untreated) - 0.76 % (T1). Remarkable morphological alterations were observed including deformation of the germ tubes and perforation of the cell wall (Chapter 4). Extract scavenging activity of 88.73± 6.69 % (25 µg mL^{-1}), 53.93±1.09 % (25 µg mL^{-1}) were recorded for H_2O_2 and NO respectively with proanthocyanidins (92.18±4.68 mg/g) occurring more ($p < 0.05$) in the

extract compared to all other phenolics compounds (Chapter 5). Significant reduction in cell viability of the cells was noted as the MCF-7 cells were reduced from 100 - 54.33±1.84 % after 72 hrs of treatment with 5 µg/mL of EAE (*P. value* < 0.05). TEt10 was cytotoxic against human normal cells (chang liver cell) at EC_{50} of 37 µg/mL and 74 µg/mL after 24 and 48 h of treatment respectively. Marked antiproliferative activity of 13.2 µg/mL (EC_{50}) was observed when HeLa cells were treated for 48 h. Internucleosomal DNA of MCF-7, HT-29 and HeLa cells randomly fragmented into an uninterrupted spectrum of sizes, complemented by the intercalation of nucleic acid-specific fluorochromes by PI and AO spotting two phases of apoptosis; early (EA) and late (LA) apoptosis. Distinctive ultramorphological changes observed include; cell shrinkage, membrane blebbing, and typical cell induced death. The study also recorded 705.102 ± 28.56 % TEt10 caspase-3 activity compared to curcumin 592.857 ± 165.76 % (positive control) and untreated (negative control; 100 ± 15.81 %) cells. Percentage HeLa cell with Sub-G1 DNA phase increased from 0.13 ± 0.06 % (negative control) to 13.8 ± 3.04 % compared to curcumin (8.17 ± 2.20 %) after treatment with TEt10. The compounds identified in the fractions including Colchicine, N-(trifluoroacetyl)methyl-N-deacetyl-, Lupeol and .gamma.-Sitosterol may be responsible for the induction of apoptosis observed and could be further studied *in vivo* as a potential template for new anticancer treatment (Chapter 6 & 7).

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AO	Acridine Orange
ATCC	American Type Culture Collection
cagA	Cytotoxin associated gene
CCA	Cholangiocarcinoma
CDKs	Cyclin Dependent Kinases
CPD	Critical Point Dried
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethyl Sulfoxide
EA	Early Apoptosis
EAE	Ethyl Acetate Extract
EBV	Epstein - Barr virus
EC	Effective Concentration
EDXS	Energy Dispersive X-ray
FOBT	Fecal Occult Blood Test
GC-MS	Gas Chromatography Mass Spectrophotometer
HIV/AIDS	Human Immunodeficiency Virus/ Acquired Immune Deficiency Syndrome
HPV	Human Papilloma Virus
LA	Late Apoptosis
LD	Lethal Dose
MBC	Minimum Bactericidal Concentration
MFC	Minimum Fungicidal Concentration
MIC	Minimum Inhibitory Concentration
MiRNAs	MicroRNAs
MLC	Minimum Lethal Concentration
NCSS	Number Cruncher Statistical System

NCTC	National Collection of Type Cultures
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
QM	Quality Match
R_f	Retention Factor
ROS	Reactive Oxygen Species
RT	Retention Time
SEM	Scanning Electron Microscopy
SN	Secondary Necrosis
SPSS	Statistical Package for the Social Sciences
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TP	Total Percentage
UV	Ultraviolet
VC	Viable Cell

CHAPTER ONE

GENERAL INTRODUCTION

1.1. INTRODUCTION

Radicals are chemical atoms that cause oxidative damage to various biomolecules and they have been directly linked to the oxidative stress which mostly results in various diseases such as cancer and aging (Mandal *et al*., 2009). Cancer is the uncontrolled growth of abnormal cells in the human body with invasion that intrudes upon and destroys adjacent tissues, and sometimes metastasis, or spreading to other locations in the body via lymph or blood. These malignant properties of cancers differentiate them from benign tumors, which do not invade or metastasize (Anand *et al.,* 2008).

There are over hundred different types of cancer, and each is classified by the type of cell that is initially affected and can develop in almost any organ or tissue, such as the lung, colon, breast, skin, bones, or nerve tissue (Stadtlander and Waterbor, 1999). Some cancers are more common in certain parts of the world. For example, in Japan, there are many cases of stomach cancer, but in the United States, this type of cancer is pretty rare. Gastric cancer causes nearly one million deaths worldwide per year and is one of the most common cancers worldwide, ranking fourth in overall frequency and more common in men than in women (Carl-McGrath *et al*., 2007).

Telomerase is critical to unrestricted cell growth. The enzyme maintains the telomeres that cap the ends of each chromosome, keeping them long enough so that DNA replication and cell division go without a hitch (Gonzalez, 2004). Most tumors and cancer cells are covered by a sticky resistant mucous/protein coating, which makes them safe from immune cells and even protects them to a degree, from chemotherapy. The removal of the protein covering leaves the cancer cell exposed and the body's natural defense leukocytes (white blood cells)

comes into effect killing the cancer cells (Campus News, 2002; Hartkamp *et al*., 2010). The inflammatory environment dramatically impacts the formation of cancer at many levels, acting on the stem cell to foster the initiation of cancer all the way through its contribution to metastatic disease. Marrow-derived stromal cells orchestrate growth and remodelling through secreted factors and cell–cell communication. This understanding of the inflammatory environment and its many effects on cancer throughout its natural history provides intervention targets directed at the unique aspects of cancer behavior (Stoicov *et al*., 2009).

Merrouche *et al*. (2006) reported the use of irinotecan and oxaliplatin to be significant in the treatment of advanced colorectal cancer, but with possible treatment-related acute promyelocytic leukemia and sometimes liver metastases after sequential chemotherapy with these cytotoxic agents. Irinotecan belongs to the class of topoisomerase I inhibitors, the mutagenic properties of which *in vitro* are similar to those induced by topoisomerase II inhibitors, and which are therefore expected to cause similar clinical manifestations, such as acute leukemia. Single-agent, such as, fluorouracil has demonstrated no carcinogenic potential, either in animals or in humans. Although platinum derivatives like cisplatin and carboplatin have been shown to be leukemogenic (Merrouche *et al*., 2006; Shapiro *et al*., 2007).

As chemotherapy affects cell division, tumors with high growth fractions (such as acute myelogenous leukemia and the aggressive lymphomas, including Hodgkin's disease) are more sensitive to chemotherapy, as a larger proportion of the targeted cells are undergoing cell division at any time. Malignancies with slower growth rates, such as indolent lymphomas, tend to respond to chemotherapy much more modestly (Hirsch, 2006; Joensuu, 2008).

Various factors play a role in cancer, including the effects of diet, chemicals, genetic factors (MicroRNAs are frequently altered in abnormal cell, revealing their functions as oncogenes or tumor suppressors), infectious agents and pathological conditions. When the human body is exposed to carcinogens, free radicals are formed that try to steal electrons from other molecules in the body. These free radicals damage cells and affect their ability to function normally. Carcinogens are a class of substances that are directly responsible for damaging DNA, promoting or aiding cancer. Tobacco, asbestos, arsenic, radiation, such as gamma and X-rays, the sun, and compounds in car exhaust fumes are all examples of carcinogens (Anand *et al.*, 2008; Volkov *et al*., 2009). Antioxidants (AO) play an important role protecting against damage by reactive oxygen species. Plants containing flavonoids have been reported to possess strong antioxidant properties. According to several researchers, the quantitative content of compounds in plant extracts that react with 2,2-diphenyl-1-picrylhydrazyl (DPPH) correlates well with the concentration of phenolic compounds (Adedapo *et al*., 2008).

Several viruses (Oncovirus) are usual infectious agents associated with certain types of cancer; however some bacteria and parasites also increase cancer risk. Besides *Helicobacter pylori* and some parasites (*Schistosoma haematobium*, liver flukes), most of the associations remain controversial or contested (Mager, 2006). About 18% of cancers are related to infectious diseases and complications are the recurrent causes of morbidity and mortality in cancer patients, repeatedly replacing the main disease as the foremost cause of death (Mager, 2006; Tanih *et al*., 2010). Ordinarily; infectious bacteria can easily be identified in disease because they can be observed microscopically in tissue sections. At times careful special staining of tissue sections is required to make microbes more observable and identifiable. Foremost research on cancer microbe, first revealed tuberculosis type acid fast staining bacteria in scleroderma, a fatal autoimmune connective tissue disease that causes hardening of the skin (Cantwell *et al*., 1981).

Other bacteria such as, *Mycoplasma neurolyticum*, *Porphyromonas gingivalis*, *Propionibacterium acnes*, *Eubacterium saburreum*, *Leptotrichia buccalis*, *Exiguobacterium oxidotolerans* and *Veillonella parvula* have been identified and associated with different forms of cancer (Ebbesen and Lind, 1969; Chocolatewala *et al*., 2010). Viruses including Epstein-Barr virus, Human T lymphotropic virus type 1, simian virus 40 as well as parasites namely; *Schistosoma helminths*, *Opistorchis felineus*, *Blastocystes hominis* and *Toxoplasma gondii* have been implicated in cancer (Cheever *et al*., 1976; Giuseppe *et al*., 2006). Cancer is considered to be one of the manifestation of microbial infections including fungi (e.g., *Candida tropicalis*) and therefore suggesting that the eradication of fungal mycotoxins, bacteria, virus and parasite from the body is the best form of cure or prevention against cancers (Jha *et al*., 2006).

The anti-inflammatory properties of several phytomedicines that contain substances like phytoestrogens, flavonoids and its derivatives, phytosterol, tocopherol, ascorbic acid, curcumin, genistein, and others can inhibit the molecular targets of pro-inflammatory mediators in inflammatory responses. There are other plants that contain alkaloids, tannin, saponins, anthraquinones, triterpenoids and other constituents which have been reported to possess a diverse range of bioactivities including anticancer, immunostimulatory, antibacterial, antimalarial and antituberculosis activities bearing in mind that some of the causative organisms and factors responsible for initiating and promoting inflammation could be removed or neutralised to suppress the expression of pro-inflammatory agents (Iwalewa *et al*., 2007). Based on the molecular events that lead to inflammation, it has been suggested that the process of sustained inflammation that accompanies chronic diseases like gastritis, ulcers and cancer can be ameliorated and possibly even prevented by phytomedicines (Iwalewa *et al*., 2007).

Phytomedicines remain promising in the treatment of infectious and metabolic diseases in Africa (Iwu *et al.,* 1999; Ndip *et al*., 2007). Plant materials have been reported to be present in or have provided the basis for about 50% of Western drugs (Harbone, 1998); herbal combinations are still being applied in the cure of diseases (Tabuti *et al.,* 2003; Ndip *et al.,* 2007). *Peltophorum africanum* (Fabaceae), also known as Weeping wattle; African wattle; huilboom (Afrikaans); isiKhaba-mkhombe (Xhoza); mosethla (Tswana); umThobo (Zulu); Musese (Venda), is widespread in South Africa. Leaves and bark have been traditionally employed to clear intestinal parasites and relieve stomach problems (Iwalewa *et al.,* 2007; Theo *et al.,* 2009; Okeleye *et al*., 2013). It has also been reported to be active against some pathogenic bacterial including *E. coli, S. aureus, A. hydrophila, S. sonei*, *C. jejuni* and *H. pylori* (Samie *et al.,* 2005; Okeleye *et al*., 2010; Okeleye *et al*., 2013). Extraction solvent may have major impact on the antioxidant activity and cytotoxicity of plant extracts (Khonkarn *et al*., 2010). Antioxidant activity has been linked with the inhibition of proliferation, an indicator of anticancer potential (Wang and Lewers, 2007). Human cancer cell lines are commonly used for most initial anticancer screening because of ethical considerations and the considerable time and expense needed when using animal models or human subjects.

1.2. Rationale of the study

Acute myeloid leukaemia and metastases secondary to treatment with commonly used drugs such as capecitabine, irinotecan and oxaliplatin for metastatic colorectal cancer and others have frequently been reported, hence, the need for alternative approaches to treatment (Merrouche *et al*., 2006; Shapiro *et al*., 2007). Body cells use oxygen, and naturally produce free radicals (by-products) which can cause damage or death to the cells. Antioxidants; example of which are polyphenols act as "free radical scavengers" and hence prevent and repair damage done by these free radicals. Health problems such as heart disease, macular

degeneration, diabetes, cancer are all contributed by oxidative damage. Antioxidants may also enhance immune defense and therefore lower the risk of cancer and infection (Nagulendran *et al.,* 2007). There are currently many phytochemicals possibly having medicinal properties in clinical trials for a variety of diseases. Lycopene, for example, from tomatoes has been tested in clinical trials for cardiovascular diseases and prostate cancer (Krishnadev *et al.,* 2010).

Cancer is the uncontrolled growth of cells coupled with malignant behavior: invasion and metastasis. Cancer is thought to be caused by the interaction between genetic susceptibility and environmental toxins. In the broad sense, most chemotherapeutic drugs work by impairing mitosis (cell division), effectively targeting fast-dividing cells therefore causing damage to the cells. Some drugs cause cells to undergo apoptosis (so-called "self programmed cell death"), however, some have a better side effect profile than others (Hirsch, 2006). Over time, cancer cells become more resistant to chemotherapy treatments. Recently, scientists have identified small pumps on the surface of cancer cells that actively move drugs from inside to outside of the cell (Hirsch, 2006; Joensuu, 2008).

Scientists have designated the cancer microbe as progenitor cryptocides; meaning, hidden killer and asserted that the microbe is present in every cell (Diller and Diller, 1965; Wuerthele-Caspe and Livingston, 1972; Cantwell et al., 1981). Scientific reports have shown that strikingly larger numbers of microbes can be observed in the areas of the tumour than that of the normal tissue, although these microbes can be identified in pre-cancerous conditions, suggesting their presence before the induction of the cancer (Mager, 2006; Broniscer *et al*., 2010). After cancer radiation and chemotherapy, the microbes can still be found in the previously cancerous areas and most people who die from cancer actually die from the cancer treatments (Broniscer *et al*., 2010). Cancer is believed to be caused by

infected injuries with most fungi, viruses, bacteria and parasites which are relatively easy to treat to reduce the risk of cancer. Many treatments are claimed to cure cancer by killing bacteria or parasites, often by supposedly emitting some sort of electromagnetic fields. Interestingly, some bacteria and bacterial toxins may also be used as a treatment for cancer, such as Coley's toxins, a type of immunotherapy invented back in the 1800s (Mager, 2006).

This study is therefore designed to establish the antibacterial, antifungal, antioxidant and anticancer potential of the crude ethyl acetate extract and the compounds of *Peltophorum africanum* in order to seek cheap alternative and readily available tools of eradicating or preventing debilitating diseases; therefore addressing the limiting factors of commercial drugs, including side effect or metastases secondary to treatment, poor patient compliance and the significant cost of therapy.

1.3. Hypotheses

The following hypotheses were investigated:

(a). *P. africanum* has potent antimicrobial potential.

(b). It can provide potent and safe antioxidant and anticancer compounds.

1.4. Overall Objective

The overall objective of this study was to establish the antimicrobial, antioxidant and anticancer activity of *Peltophorum africanum*.

1.5. Specific Objectives

The specific objectives of the study were to:

1. Determine the antibacterial and antifungal activity of the crude extract and it's compounds

2. Ascertain the total polyphenolic content of the extract
3. Determine the antioxidant activity of the extract and compounds
4. Isolate and identify the bioactive compounds
5. Evaluate the cytotoxicity of the extract and compounds
6. Determine the *in vitro* anticancer activity of the extract and compounds

1.6. Overview of the thesis

This thesis contains eight chapters and each tie with the objectives this study investigated. Chapter one introduces the general concept of the thesis with the rationale, objectives and scope of the study. Chapter two is a logical review on medicinal plant, microorganisms and cancer. It details the literature review on the effect of phytochemicals, antioxidant, bacterial metabolites, mycotoxins, parasitic infection, food substances, genetics and immunological basis of cancer and drug development. Antibacterial and antifungal potential of the extract and its toxicological effect on human Chang liver cell is covered in chapter three (Published). The mechanism of action of the compounds and heavy metals in the ethyl acetate extract and their morphological effects on the pathogenic bacteria and yeast cell is reported in chapter four.

The presence and effects of phytochemical, polyphenolic content and antioxidant compounds in many plants tend to make them of medicinal importance in the society; this was also investigated in chapter five. Chapter six elaborates on the *in-vitro* assessment of the antiproliferative and apoptotic potential of the ethyl acetate extract on different cancer cell lines together with the ultrastructural characterization of the treated cells. *In-vitro* antiproliferation of cervical cancer cell (HeLa) by the fraction TEt10 of *P. africanum* is contained in chapter seven. It expresses the functionality of caspases' enzymatic activity, cell

cycle (G1, G2 and Sub G1) analysis and compounds identification. Chapter eight discuses the general findings, concludes and offers recommendations.

REFERENCES

Adedapo, A.A., Jimoh, F.O., Koduru, S., Afolayan, A.J. and Masika, P.J. (2008). Antibacterial and antioxidant properties of the methanol extracts of the leaves and stems of Calpurnia aurea. *BMC Complementary and Alternative Medicine*. **8:** 53.

Adewusi, E.A. and Afolayan, A.J. (2009). Antibacterial, antifungal and antioxidant activity of the roots and leaves of *Pelargonium reniforme* Curtis (Geraniaceae). *African Journal of Biotechnology*. **8** (22): 6425-6433.

Alexander-Jackson, E. (1954). A specific type of microorganism isolated from animal and human cancer: bacteriology of the organism. *Growth*. **18**: 37-51.

Anand, P., Kunnumakkara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B. and Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Journal of Pharmaceutical Research*. **25** (9): 2097-2116.

Broniscer, A., Baker, J.N., Tagen, M., Onar, A., Chin, T.K., Gilbertson, R.J., Onar-Thomas, A., Gilbertson, R.J., Davidoff, A.M., Pai Panandiker., A.S., Leung, W., Chin., T.K., Stewart, C.F., Kocak, M., Rowland, C., Merchant, T.E, Kaste, S.C. and Gajjar, A. (2010). Phase I study of vandetanib during and after radiation therapy in children with diffuse intrinsic pontine glioma. *Journal of Clinical Oncology*. **28**: 4762-4768.

Cantwell, A.R Jr., (1981). Histologic observations of variably acid-fast coccoid forms suggestive of cell wall deficient bacteria in Hodgkin's disease. A report of four cases. *Growth*. **45**: 168-187.

Carl-McGrath, S., Ebert, M. and Rocken, C. (2007). Gastric adenocarcinoma: epidemiology, pathology and pathogenesis. *Cancer Therapy*. **5:** 877-894.

Cheever, A.W., Kuntz, R.E., Moore, J.A, Bryan, G.T. and Brown, R.R. (1976). Animal model of human disease: carcinoma of the urinary bladder in *Schistosoma haematobium* infection. *American Journal of Pathology*. **84**: 673-676.

Chocolatewala, N., Chaturvedi, P. and Desale R. (2010). The role of bacteria in oral cancer. *Indian Journal of Medical and Paediatric Oncology*. **31**: 126-131.

Diller, I.C. and Diller, W.F. (1965). Intracellular acid-fast organisms isolated from malignant tissues. *Transactions of the American Microscopical Society*. **84**: 138-148.

Ebbesen, P. and Lind, K. (1969). Lack of evidence for oncogenic or amyloid inducing qualities of *Mycoplasma neurolyticum* inoculated into Balb/C Mice. *Acta Pathologica Microbiologica Scandinavica*. **76**: 594-600.

Eloff, J.N., Famakin, J.O. and Katerere, D.R.P. (2005). Isolation of an antibacterial stilbene from *Combretum woodii* (Combretaceae) leaves. *African Journal of Biotechnology*. **4** (10): 1167-1171.

Eloff, J.N., Masoko, P. and Picard, J. (2007). Resistance of animal fungal pathogens to solvents used in bioassays. *South African Journal of Botany*. **73** (4): 667-669.

Giuseppe, B.B., Silvia, S., Fernanda, M., Massimo, N., Alfredo, C. and Mauro, T. (2006). BK Virus, JC Virus and Simian Virus 40 infection in humans, and association with human tumors. *Advances in Experimental Medicine and Biology*. **577**: 319-341.

Gonzalez, T.N. (2004). Pancreatic enzyme extract improves survival in murine pancreatic cancer. *Pancrease*. **28** (4): 401-412.

Harbone, J.B. (I998). Phytochemical methods: a guide to modern techniques of plant analysis. Chapman and Hall, London. 23-30.

Hartkamp, J., Carpenter, B. and Roberts, S.G.E. (2010). The Wilms' tumor suppressor protein WT 1 is processed by the serine protease Htr A2/0mi. *Journal of Molecular Cell*. **37** (2): 159-171.

Hirsch, J. (2006). An anniversary for cancer chemotherapy. *Journal of the American Medical Association*. **296** (12): 1518-1520.

Iwalewa, E.O., McGaw, L.J., Naidoo, V. and Eloff, J.N. (2007). Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. *African Journal of Biotechnology*. **25:** 2868-2885.

Jha, B.J., Dey, S., Tamang, M.D., Joshy, M.E., Shivananda, P.G. and Brahmadatan, K.N. (2006). Characterization of *Candida* species isolated from cases of lower respiratory tract infection. *Kathmandu University Medical Journal*. **4**: 290-294.

Joensuu, H. (2008). Systemic chemotherapy of cancer: from weapon to treatment. *Lancet Oncology*. **9** (3): 304.

Kotze, M. and Eloff, J.N. (2002). Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). *South African Journal of Botany*. **68:** 62-67.

Krishnadev, N., Meleth, A.D. and Chew, E.Y. (2010). Nutritional supplements for age-related macular degeneration. *Current Opinion in Ophthalmology*. **21** (3): 184-189.

Kumaran, A. and Karunakaran, R.J. (2007). *In vitro* antioxidant activity of methanol extracts of *Phyllanthus* species from India. *Lebens-Wiss Technologie*. **40**: 344-352.

Liyana-Pathiranan, C.M. and Shahidi, F. (2005). Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L) as affected by gastric pH conditions. *Journal of Agricultural Food Chemicals*. **53:** 2433-2440.

Mager, D.L. (2006). Bacteria and cancer: cause, coincidence or cure. *Journal of Translational Medicine*. **28**: 9-14.

Mandal M, Misra T.K. and Ghosal M. (2009). Free-radical scavenging activity and phytochemical analysis in the leaf and stem of *Drymaria diandra* Blume. *International Journal of Integrative Biology*. **7**: 80-84.

Merrouche, Y., Mugneret, F. and Cahn, JF. (2006). Secondary acute promyelocytic leukemia following irinotecan and oxaliplatin for advanced colon cancer. *Annals of Oncology*. **17** (6): 1025-1026.

Mosman, T. (1983). Rapid, colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. **65:** 55-63.

Nagulendran, K.R., Velavan, S., Mahesh, R. and Hazeena, B.V. (2007). *In vitro* antioxidant activity and total polyphenolic content of *Cyperus rotundus rhizomes*. *E-Journal of Chemistry*. **4** (3): 440-449.

Ndip, R.N., Ajonglefac, A.N., Mbullah, S.M., Tanih, N.F., Akoachere J-F, T.K., Ndip, L.M., Luma, H.N., Wirmum, C., Ngwa, F. and Efange, S.M.N. (2008). *In vitro* anti-*Helicobacter pylori* activity of *Lycopodium cernuum* (Linn) Pic. Serm. *African Journal of Biotechnology*. **7** (22): 3989-3994.

Ndip, R.N., Malange, A.E., Tarkang, A.E., Mbullah, S.M., Luma, H.N., Malongue, A., Ndip, L.M., Nyongbela, K., Wirmum, C. and Efange, S.M. (2007). *In vitro* anti-*Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. *Journal of Ethnopharmacology*. **114:** 452-457.

Okeleye, B.I., Samie, A., Bessong, P.O., Mkwetshana, N.F., Green, E., Clarke, A.M. and Ndip, R.N. (2010). Crude ethyl acetate extract of the stem bark of *Peltophorum africanum* (Sond, Fabaceae) possessing *in vitro* inhibitory and bactericidal activity against clinical isolates of *Helicobacter pylori*. *Journal of Medicinal Plants Research*. **4** (14): 1432-1440.

Okeleye, B.I., Mkwetshana, N.T. and Ndip, R.N. (2013). Evaluation of the Antibacterial and Antifungal Potential of Peltophorum africanum: Toxicological Effect on Human Chang Liver Cell Line. Scientific World Journal. 2013:878735.doi:10.1155/2013/878735.

Samie, A., Obi, C.L., Lall, N. and Meyer, J.J. (2009). *In-vitro* cytotoxicity and antimicrobial activities, against clinical isolates of *Campylobacter* species and *Entamoeba histolytica*, of local medicinal plants from the Venda region, in South Africa**.** *Annals of Tropical Medicine & Parasitology*. **103:** 159-70.

Samie, A., Obi, CL., Bessong, P.O. and Namrita, L. (2005). Activity profiles of fourteen selected medicinal plants from rural Venda communities in South Africa against fifteen clinical bacterial species. *African Journal of Biotechnology*. **4** (12): 1443-1451.

Shapiro, S., Hughes, G., Al-Obaidi, M.J., O'Reilly, E., Ramesh, S., Smith, J., Ahmad, R., Dawson, C., Riddle, P. and Sekhar, M. (2007). Acute myeloid leukaemia secondary to treatment with capecitabine for metastatic colorectal cancer. *European Journal of Haematology*. **78** (6): 543-544.

Stadtlander, C.T.K.H. and Waterbor, JW. (1999). Molecular epidemiology, pathogenesis and prevention of gastric cancer. *Carcinogenesis*. **20** (12): 2195-2207.

Stoicov, C., Li, H., Cerny, J. and Houghton, J.M. (2009). How the study of *Helicobacter* infection can contribute to the understanding of carcinoma development. *Clinical Microbiological Infection*. **15:** 813-822.

Tabuti, J.R.S., Dhillion, S.S. and Lye, K.A. (2003). Traditional medicines in Bulamogi County, Uganda: its practitioners, users and viability. *Journal of Ethnopharmacology*. **85:** 119-129.

Tanih, N.F., Okeleye, B.I., Ndip, L.M., Clarke, A.M., Naidoo, N., Mkwetshana, N.T., Green, E. and Ndip, R.N. (2010). *Helicobacter pylori* prevalence in dyspeptic patients in the

Eastern Cape Province – race and disease status. *South African Medical Journal.* **100**: 734-737.

Theo, A., Masebe, T., Suzuki, Y., Kikuchi, H., Wada, S., Obi, L.C., Bessong, P.O., Usuzawa, M., Oshima, Y. and Hattori, T. (2009). *Peltophorum Africanum*, a Traditional South African Medicinal Plant, Contains an Anti HIV-1 Constituent, Betulinic Acid. *Tohoku Journal of Experimental Medicine*. **217** (2): 93-99.

Volkov, V.A., Dorofeeva, N.A. and Pakhomov, P.M. (2009). Kinetic method for studying the antiradical activity of medicinal plant extracts. *Pharmaceutical Chemistry Journal*. **43** (6): 27-31.

Wei, D., Zhengyuo, L., Guomin, W., Yingwu, Y., Yingguo, L. and Yuxian, X. (2006). Seperation and purification of natural pyrethrins by reversed phase high performance Liquid chromatography. *China Journal of Analytical Chemistry,* **34** (12): 1776-1778.

Wolfe, K., Wu, X. and Liu, R.H. (2003). Antioxidant activity of apple peels. *Journal of Agricultural Food Chemicals*. **51:** 609-614.

Wuerthele-Caspe, L.V. and Livingston, A.M. (1972). Demonstration of Progenitor cryptocides in the blood of patients with collagen and neoplastic diseases. *Transactions of the New York Academy of Sciences*. **174**: 636-654.

Xingming, M., Yanping, L., Hongjuan, Y. and Yan, C. (2006). Ethanolic extracts of *Sophora moorcroftiana* seeds induce apoptosis of human stomach cancer cell line SGC-7901 *in vitro*. *African Journal of Biotechnology*. **5** (18): 1669-1674.

CHAPTER TWO

LITERATURE REVIEW

2.1. Phytochemical, antioxidant and antimicrobial potentials of medicinal plants

Modern medicine now tends to use the active ingredients of plants rather than the whole plants. Phamarcognosy studies the physical, chemical, biochemical and biological properties of medicines derived from natural sources, including plants. Plants synthesize a wide variety of chemical compounds which perform important biological functions (Lai and Roy, 2004). These phytochemicals comprise primary metabolites such as sugars and fats; and secondary metabolites compounds, such as toxins, inulin, quinine, morphine, codeine and digoxin with a more specific function. The phytochemicals may be synthesized, compounded or otherwise transformed to make pharmaceuticals (Lai and Roy, 2004; Cai *et al*., 2007).

Medicinal plants mostly contain a wide variety of scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes and tannins), nitrogen compounds (alkaloids, amines and betalains), vitamins, terpenoids, carotenoids and some other rich endogenous antioxidant metabolites (Larson, 1988; Cotelle *et al*., 1996; Zheng and Wang, 2001; Cai *et al*., 2003). For the fact that there are no exceptionally effective drugs to treat most cancers, natural compounds isolated from medicinal plants, as rich sources of novel anticancer drugs, have been of increasing interest since 1960 (Monks *et al*., 2002). Generally, free radical scavenging and antioxidant activity of phenolics (e.g. flavonoids, phenolic acids) mainly depend on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, glycosylation of aglycones, H-donating groups (-NH, -SH) and other factors (Nakatani, 2000; Zheng and Wang, 2001).

More than 4,000 kinds of flavonoids and hundreds of coumarins and lignans have been reported as naturally occurring compounds (Iwashina, 2000; Xiao *et al*., 2000). Phenolics

prevent cancer through antioxidant action and/ or the modulation of several protein functions. It can also inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages (Yang *et al*., 2001). Phenolics have been reported to demonstrate antagonism and/or agonism of carcinogenesis-related receptors such as arylhydrocarbon receptor, epidermal growth factor and estrogen receptor. They transformed the secretion of protein kinases in tumor cell proliferation, and stimulated the expression of anticarcinogenic enzymes or repressed induction of cancer-promoting enzymes (Owen *et al*., 2000; Sakakibara *et al*., 2003).

Phytochemicals are plant bioactive chemicals linked to reducing the risk of major chronic diseases. More than five thousand phytochemicals have been identified with several still unknown and the mechanisms by which dietary phytochemicals may prevent cancer has been proposed to include; antioxidant activity, scavenge free radicals and reduce oxidative stress, inhibition of cell proliferation, induction of cell differentiation, inhibition of oncogene expression, induction of tumor suppress gene expression, induction of cell-cycle arrest and induction of apoptosis (Liu, 2003; Liu, 2004; Dai & Mumper, 2010). Others include inhibition of signal transduction pathways, enzyme induction and enhancing detoxification, phase II enzyme, glutathione peroxidase, catalase, superoxide dismutase, enzyme inhibition, phase I enzyme (block activation of carcinogens), cyclooxygenase-2, inducible nitric oxide synthase, xanthine oxide, enhancement of immune functions and surveillance, antiangiogenesis, as well as inhibition of cell adhesion and invasion, inhibition of nitrosation and nitration, prevention of DNA binding, regulation of steroid hormone metabolism, regulation of estrogen metabolism and antibacterial and antiviral effects (Liu, 2004; Ramos, 2008).

It has been envisaged that between 2000 and 2020, the total number of cases of cancer would have been increased by 29% and 73% in the developed and developing world respectively (Parkin, 2001). The objective of cancer chemotherapy and discovery of anticancer agents must be related to novel molecular targets, a unique mechanism of action for specific types of cancer with as little damage as possible to normal cells (Pezzuto, 1997). Nature for decades has long been an important source of medicinal agents and notable number of modern drugs have been isolated or derived from it (Cragg and Newman, 2001). Of the plant derived anticancer drugs in clinical use, the best known is the so-called vinca alkaloids, which include vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthus roseus* (Linn.). Vinorelbine (VRLB) and Vindesine (VDS) have been reported to show potential activity against leukemia's, lymphomas, advanced testicular cancer, breast cancer, lung cancer and Kaposi's sarcoma (Cragg and Newman, 2005). Other biologic compounds are aloe-emodin, lapachol, colchicines, indicine-N-oxide, bruceantin and elephantopin including etoposide, teniposide, which are semi-synthetic derivatives of the epipodophyllotoxin, an isomer of podophyllotoxin isolated as the active anti-tumor agent from the genus *Podophyllum* (Berberidaceae) (Itharat and Ooraikul, 2007). The benzophenanthridine derivatives are present in *Chelidonium majus* (Papaveraceae), a plant with substantial folklore history of use in the treatment of cancers (Dewick, 2002). More recent additions to the armamentarium of the naturally derived chemotherapeutic agents are the taxanes and camptothecins. Paclitaxel, docetaxel and baccatins were initially isolated from the bark of the Pacific or American yew tree, *Taxus brevifolia* Nutt. (Taxaceae) (Cragg *et al*., 1993; Cortes and Pazdur, 1995). Topotecan (hycamptamine), irinotecan, 9- amino and 9-nitrocamptothecin are semi-synthetically derived from camptothecins, isolated from the Chinese ornamental tree, *Camptotheca acuminate* Decne (Family Cornacea) (Potmeisel and Pinedo, 1995).

Foods are generally recognized as harmless; therefore its use in chemotherapeutic purposes has drawn the interest of scientists (Steinmetz and Potter, 1991). Oxidative stress causes toxic effects including altering DNA structure, decreasing DNA repair and activating signal transduction pathways. As a consequence, oxidative stress has been considered a contributing factor in the development of many human chronic diseases including cancer (Franco *et al*., 2008; Christian & Kunal, 2009). The increasing prevalence of antimicrobial resistance and negative impact on the eradication treatment regimens has prompted the search for novel therapeutic approaches (Ernst and Gold, 2000). Prenylflavanones from *Sophora tomentosa* and *Sophora moorcroftiana* has been reported to show tumor-specific cytotoxic activity and antimicrobial activity (Shirataki *et al*., 2001). Allium vegetables, including garlic (Allium sativum L.) exhibit a broad antibiotic spectrum against Gram-positive and Gram negative bacteria (Mahady and Pendland, 2000; Sivam, 2001). The extracts of *Garcinia kola* Heckel seeds have been reported to show potentials of synergy in combination with some antibiotics against pathogenic organisms often presenting with problems of drug resistance (Sibanda and Okoh, 2008). Capsaicin (hot pepper) consumed as a flavoring spice, has been reported to possess pharmacological, physiological and antimicrobial effects (Molina-Torres, 1999). Antifungal activity of the hexane and acetone extracts of *Arctotis arctotoides* has been observed to be potent against some opportunistic fungi associated with HIV/AIDS, including *Candida glabrata*, *C. krusei* and *Microsporum canis* (Otang *et al*., 2011).

2.2. Cancer development and prognosis

Cancer is known medically as a malignant neoplasm of a broad group of diseases involving unregulated cell growth which can spread to more distant parts of the body through the lymphatic system or bloodstream (Anand *et al*., 2008). Cancer pathogenesis is traceable back to DNA mutations that impact cell growth and metastasis. There are frequent epigenetic

alterations of the DNA sequences coding for small RNAs called microRNAs (or miRNAs), hence, revealing their functions as oncogenes or tumour suppressors. MiRNAs do not code for proteins, but can target protein-coding genes and reduce their expression (Croce, 2008). Decades of research has demonstrated that tobacco smoke contains over fifty known carcinogens, including nitrosamines and has been associated with the lung, larynx, stomach, bladder, kidney, esophagus and pancreas cancer. Approximately, 2-20% of all cancer cases are related to one's occupation (e.g., leukemia from exposure to benzene). Diet, physical inactivity, and obesity are related to approximately 30–35% of cancer deaths based on the negative effects on the immune and endocrine systems. Worldwide approximately 18 % of cancer deaths are related to infectious diseases, from a high of 25% in Africa to less than 10% in the developed world (Kushi *et al*., 2012). Cancer can be detected by the signs and symptoms depending on the type of cancer, screening tests, or medical imaging and then diagnosed by microscopic examination of a tissue sample. The tissue diagnosis determines the type of cell that is proliferating, its histological grade and genetic abnormalities. Cytogenetics and immunohistochemistry are other types of test perform on the tissue specimen that provides information about the cell molecular changes (Wilson and Jungner, 1968). Cancer is usually treated with chemotherapy, radiation therapy and surgery. COX-2 inhibitor and NSAIDs reduce the risk of colorectal cancer and others, but with known cardiovascular and gastrointestinal side effects. Aspirin has been found to reduce the risk of death from cancer by about 7%. Tamoxifen or raloxifene has been demonstrated to reduce the risk of developing breast cancer (Rostom *et al*., 2007; Rothwell *et al*., 2011). Vaccines have been developed that prevent some infection by some viruses. For example, human papillomavirus vaccine (Gardasil and Cervarix) decreases the risk of developing cervical cancer, while, hepatitis B vaccine prevents infection with hepatitis B virus and thus decreases the risk of liver cancer (NCI, 2008).

2.3. Breast Cancer

Breast cancer is the most frequently diagnosed cancer in women worldwide with an estimated 1.4 million new cases and leading cause of cancer death among women worldwide. An estimated 458,400 breast cancer deaths occurred in women in 2008. Between 1980 and the late 1990s, breast cancer incidence rates rose approximately 30% in westernized countries. Breast cancer incidence rates have been rising in many African and Asian countries including Japan (Cronin *et al*., 2009). This has been associated with the changes in reproductive patterns, obesity, physical inactivity and some breast cancer screening activity (Parkin, 2009; Sankaranarayanan *et al*., 2010).

2.3.1. Signs and symptoms

A painless mass is the most common physical sign of breast cancer (Hulka and Moorman, 2001). Less common signs and symptoms include breast pain and persistent changes to the breast, such as thickening, swelling, skin irritation or distortion, and nipple abnormalities such as spontaneous discharge, erosion, inversion, or tenderness (Seradour *et al*., 2009).

2.3.2. Risk factors

Age, inherited mutations in breast cancer susceptibility genes *BRCA1* and *BRCA2*, family history, reproductive and hormonal changes are the main risk factor of breast cancer (Chen and Parmigiani, 2007). Overweight or obese after menopause, use of menopausal hormone therapy (MHT) can increase the risk (Seradour *et al*., 2009; Worsham *et al*., 2009).

2.3.3. Prevention and early detection

Avoidance of the risk factors and by maintaining a healthy body weight, increasing physical activity, and lessen alcohol intake are the best approach to reduce the risk of developing

breast cancer. Breast cancer can be detected by screening using mammography and clinical breast examination methods (Hulka and Moorman, 2001; Seradour *et al*., 2009).

2.3.4. Treatment

Lumpectomy (surgical removal of the tumor with clear margins) or mastectomy (surgical removal of the breast) with removal of some of the axillary (underarm) lymph nodes, radiation therapy, chemotherapy (before or after surgery), hormone therapy or targeted biologic therapy (Anderson *et al*., 2006; Anderson *et al*., 2007) are the best known treatment for breast cancer.

2.4. Colorectal Cancer

Colorectal cancer is the third most common cancer in men and the second in women. Worldwide, an estimated 1.2 million new cases of colorectal cancer occurred (Edwards *et al*., 2010). About 608,700 deaths from colorectal cancer occured in 2008 worldwide, accounting for 8% of all cancer deaths. The decrease in colorectal cancer incidence in developed countries reflects the increase in detection and removal of precancerous lesions, although increases in mortality rates are still persist in developing countries (Center *et al*., 2009; Sankaranarayanan *et al*., 2010).

2.4.1. Signs and symptoms

Rectal bleeding, blood in the stool, a change in bowel habits and cramping pain in the lower abdomen and in some cases, anemia resulting from blood loss (Sankaranarayanan *et al*., 2010; Edwards *et al*., 2010).

2.4.2. Risk factors

Age, inherited genetic mutations, family history of colorectal cancer and chronic inflammatory bowel disease are the common risk factors (Edwards *et al*., 2010). However, lifestyle factors such as physical inactivity, obesity, a diet high in red or processed meat, heavy alcohol consumption, and smoking are also important determinants of colorectal cancer risk (Martin *et al*., 2006).

2.4.3. Prevention and early detection

Screening and removal of precancerous lesions can prevent colorectal cancer (Winawer, 2007). Fecal occult blood test (FOBT), flexible sigmoidoscopy, double-contrast enema, and colonoscopy are accepted colorectal cancer screening methods. Maintaining a healthy body weight, being physically active, minimizing consumption of red meat and alcohol, and not smoking are the other preventive measures (Hawk *et al*., 2005; Winawer, 2007).

2.4.4. Treatment

Surgery is the most common treatment for colorectal cancer (Edwards *et al*., 2010). A permanent colostomy (creation of an abdominal opening for elimination of body wastes) is very rarely needed for colon cancer and is frequently required for rectal cancer. For rectal cancer, chemotherapy alone, or in combination with radiation, is often given before or after surgery (Hawk *et al*., 2005).

2.5. Cervical Cancer

Cervical cancer is the third most commonly diagnosed cancer in women, with an estimated 529,800 new cases worldwide and more than 85% of which were in developing countries (Parkin *et al*., 2008). The disease is the fourth leading cause of cancer death in women

worldwide with an estimated 275,100 deaths; 90% of which occured in developing countries (Vizcaino *et al*., 2000; Maucort-Boulch *et al*., 2008).

2.5.1. Signs and symptoms

The most common symptom is abnormal vaginal bleeding. Bleeding may start and stop between regular menstrual periods, or it may occur after sexual intercourse or douching. Menstrual bleeding may last longer and be heavier than usual. Bleeding after menopause or increased vaginal discharge may also be symptoms of cervical cancer (Gustafsson *et al*., 1997; Chen *et al*., 2009).

2.5.2. Risk factors

The primary cause of cervical cancer is infection with certain types of human papilloma virus (HPV). About 15 HPV types are associated with cervical cancer. Among these, HPV 16 and 18 are most common among cervical cancer patients (associated with 50.5% and 13.1% of cervical cancers, respectively) (Parkin *et al*., 2008). Sexually active women are at high risk of a cervical infection with at least one type of HPV. Progression from the infection to cancer may be influenced by many factors, such as immunosuppression, high number of lifetime sexual partners and continuing use of oral contraceptives (Castellsague *et al*., 2006).

2.5.3. Prevention and early detection

The Pap test is a simple procedure in which a small sample of cells is collected from the cervix and examined under a microscope (Sherris *et al*., 2009). The most cost effective screening techniques include visual inspection using either acetic acid or Lugol's iodine and DNA testing for HPV in cervical cell samples. Vaccines which protect against 70% of viruses

that cause cervical cancer are the new promise for preventing cervical cancer (Sankaranarayanan *et al*., 2009; Sankaranarayanan, 2009).

2.5.4. Treatment

The best known treatments are electrocoagulation (the destruction of tissue through intense heat by electric current), cryotherapy (the destruction of cells by extreme cold), laser ablation, or local surgery. Invasive cervical cancers generally are treated by surgery, radiation and chemotherapy in some cases (ACS, 2006).

2.6. Prostate Cancer

Prostate cancer is the second most frequently diagnosed and the sixth leading cause of cancer death in men, with 903,500 new cases and virtually three quarters of these cases diagnosed in developed countries (Baade *et al*., 2009). Differences in genetic susceptibility may be the reason for the high prostate cancer risk among some populations of Africans. However, over the years, a remarkable progress in survival has been observed, which is linked to early diagnosis of asymptomatic cases and improvements in treatment (ACS, 2006; Altekruse *et al*., 2010).

2.6.1. Signs and symptoms

Weak or interrupted urine flow, blood in the urine and continual pain in the lower back, pelvis, or upper thighs may be an indication of spread of the disease to the bones (Wolf *et al*., 2010).

2.6.2. Risk factors

Older age, race (black), and family history of the disease are the established risk factors for prostate cancer. Some studies suggest that a diet high in processed meat may also be a risk factor and the risk of dying from prostate cancer is increased by obesity (Oliver *et al*., 2001; Schroder *et al*., 2009; Wolf *et al*., 2010).

2.6.3. Prevention and early detection

Factors that may reduce risk include maintaining a healthy body weight, regular physical activity, and consuming a diet low in animal fat and high in fruits and vegetables (Oliver *et al*., 2001; Smith *et al*., 2009; Schroder *et al*., 2009).

2.6.4. Treatment

Surgery (open, laparoscopic, or robotic-assisted), external beam radiation, or radioactive seed implants (brachytherapy) may be used to treat early stage disease. Hormonal therapy, chemotherapy, and radiation (or combinations of these treatments) are used for metastatic disease and as a supplemental or additional therapy for early stage disease. Hormone treatment may control prostate cancer for long periods by shrinking the size or limiting the growth of the cancer, thus relieving pain and other symptoms (ACS, 2006).

2.7. Lung Cancer

Despite some improvements in surgical techniques and combined therapies over the past several decades, lung cancer is one of the most lethal cancers (Altekruse *et al*., 2010). In men, the highest lung cancer incidence rates are in North America and the lowest rates are in Africa. Among women, the highest lung cancer rates are in North America and Northern Europe (Youlden *et al*., 2008; Bray and Weiderpass, 2010). Lung cancer is the leading cause

of cancer death in men and the second leading cause of cancer death in women, with an estimated 951,000 deaths in men and 427,400 deaths in women in 2008 worldwide (Global Cancer, 2008).

2.7.1. Signs and symptoms

Symptoms may include persistent cough, sputum streaked with blood, chest pain, voice change, and recurrent pneumonia or bronchitis (Ezzati *et al*., 2005).

2.7.2. Risk factors

Cigarette smoking is the most important risk factor for lung cancer, accounting for about 80% of lung cancer cases in men and 50% in women worldwide (Ezzati *et al*., 2005). Other risk factors include secondhand smoke, occupational or environmental exposures to radon and asbestos (particularly among smokers), certain metals (chromium, cadmium, and arsenic), some organic chemicals, radiation, air pollution, coal smoke, and indoor emissions from burning other fuels. Genetic susceptibility contributes to risk, especially in those who develop the disease at a younger age (Li and Hemminki, 2004; Matakidou *et al*., 2005).

2.7.3. Prevention and early detection

Lung cancer is one of the most preventable cancers. Most lung cancers could be prevented by reducing smoking initiation among adolescents. Newer tests, such as low-dose spiral computed tomography (CT) scans and molecular markers in sputum have produced promising results in detecting lung cancers at early stages (Shafey *et al*., 2009; NCI, 2010).

2.7.4. Treatment

Treatments include surgery, radiation therapy, chemotherapy, and targeted therapies. For localized cancers, surgery is usually the treatment of choice (Global Cancer, 2008).

2.8. Gastric Cancer

Gastric cancer was the fourth most common malignancy in the world; approximately 72% of new cases occured in developing countries (Bertuccio *et al*., 2009). Generally, stomach cancer rates are about twice as high in men as in women. In general, the highest incidence rates are in Asia and many parts of South America and the lowest rates are in North America and most parts of Africa (Lee *et al*., 2006; Bertuccio *et al*., 2009; Yeh *et al*., 2009).

2.8.1. Signs and symptoms

Common signs of gastric cancer are indigestion, a bloated sensation after eating, and heartburn. Symptoms may include nausea, abdominal pain or discomfort in the upper abdomen, diarrhea or constipation, bloody stools, vomiting blood, loss of appetite, weight loss, anemia, and feelings of fullness or pressure in the stomach (Global Cancer, 2008).

2.8.2. Risk factors

H. pylori infection, a bacterium that colonizes in the stomach is the main risk factor for gastric cancer. Eating diets rich in smoked foods, salted meat or fish, and pickled vegetables can contribute, together with smoking in particularly for cancers of the upper portion of the stomach (Yang *et al*., 2005; Parkin, 2006).

2.8.3. Prevention and early detection

By reducing known dietary risks and increasing consumption of fresh fruits and vegetables, one can prevent the development of gastric cancer. Avoiding *H. pylori* infection by improvement of hygienic conditions may also reduce risk. Antibiotics are sometimes utilized to treat persons with known *H. pylori* infection; however, their effectiveness is suboptimal, and extensive use of antibiotics leads to concerns for the development of antibiotic-resistant strains of *H. pylori* (Lee *et al*., 2006; Tanih *et al*., 2010a; Tanih *et al*., 2010b).

2.8.4. Treatment

Cancer of the stomach is difficult to cure unless it is found in an early stage and the main treatments are surgery, chemotherapy, and radiation therapy (Global Cancer, 2008).

2.9. Cancer induced by bacterial metabolites

In 1931, Cornell University pathologist, Elise L'Esperance reported the presence of acid-fast, tuberculosis-like organisms in Hodgkin's disease (L'Esperance *et al*., 1931). Beginning in 1963; the National Cancer Institute (NCI) launched preliminary investigation on the etiology of cancer bacteria, including *Mycoplasma orale*, *Mycoplasma fermentans*, *Mycoplasma neurolyticum* and *Mycoplasma pneumonia* mostly in leukemic bloods (Ebbesen and Lind, 1969).

Recently, bacteria have been linked to cancer by two mechanisms: induction of chronic inflammation and production of carcinogenic bacterial metabolites. *Mycoplasma hyorhinis* encodes a protein known as p37, which could infect humans and facilitate tumor invasiveness (Ketcham *et al*., 2005). A considerably high presence of *Mycoplasma sp.* DNA has been reported in the tissues of patients with predictable renal cell carcinoma as contrasting to normal controls (Pehlivan *et al*., 2005).

Streptococcus mutans and *Streptococcus sanguis* are the predominant bacterial species in dental plaque that prevent oxygen from reaching areas under the gum line and eventually leads to oral cancer. Pancreatic cancer, is one of the most difficult cancers to treat with no clear symptoms in its early stages; it usually spreads very quickly, and only around one in 20 patients live five years after diagnosis and responsible for 40,000 deaths a year in the United States (Cantwell and Kelso, 1981; Parsonnet, 1995). *Porphyromonas gingivalis* (two fold causative), *Granulicatella adjacens* (associated with systemic inflammation) and antibodies for multiple oral bacteria have been commonly identified in patients with pancreatic cancer. A considerable reduction of *Streptococcus mitis* plays a protective role against inflammation and this helps in the early detection of the disease. Reports have indicated that an unusual microbe can be observed microscopically in cancer tissue, tumors and blood (Cantwell and Kelso, 1981; Michaud *et al*., 2012).

The cancer microbes are considered to be monomorphic and pleomorphic, competent of generating tiny sub-microscopic virus-like and mycoplasma-like forms, as well as large fungal-like forms (large bodies) (Cantwell, 2012). By the use of the acid-fast stain, due to the substandard or lack of cell walls (amorphous), the cancer microbe appears primarily as purple-stained variably-sized, round coccoid forms similar to the size and shape of staphylococci (Hooper *et al*., 2009; Cantwell, 2012). *Helicobacter pylori* is a pleomorphic microbe, exhibiting spiral, coccoid, and degenerative forms which in recent times has been accepted as the cause of stomach ulcers and secondary gastric cancer (Cantwell, 1981; Tanih *et al*., 2010c; Cantwell, 2012). *H. pylori's* ability to cause disease is closely associated with a virulence protein called *CagA*. It was revealed that *CagA* and RUNX3, a protein that guards against tumor formation physically interact with each other in human epithelial cells. *CagA* targets RUNX3 for degradation and loss of expression of RUNX3 has been reported to be causally associated with the development of gastric cancer (Tsutsumi *et al*., 2003). The

organism plays a role in adenocarcinoma of the distal stomach, mucosa-associated lymphoid tissue lymphoma (MALT), primary gastric non-Hodgkin's lymphoma and pancreatic cancer in many societies (Tanih *et al*., 2010).

Salmonella typhi appears to be a strong risk factor for gallbladder cancer and other biliary tract cancers while *Chlamydia pneumoniae* and *Mycobacterium tuberculosis* may increase the risk of lung cancer. *Borrelia burgdorferi* (Lyme disease), *Chlamydia psittaci* (psittacosis) and *Campylobacter jejuni* (which causes food poisoning) have been associated with lymphomas while *Streptococcus anginosus* has been connected to esophageal cancer and oral cancer (McGarr *et al*., 2005, Mager, 2006). Some cases of colorectal cancer have been linked to *Firmicutes*, *Bacteroides*, *Proteobacteria*, *Fusobacterium*, *Streptococcus bovis*, *Escherichia coli*, *Clostridium septicum* which are also associated with inflammatory bowel diseases, such as ulcerative colitis, bowel cancer and gas gangrene (Larson *et al*., 1995; Mager, 2006; Keku, 2010). *E.coli*, which carry *pks* genes that encode a toxin which damage DNA in the cells of the gut lining, are more commonly found in the colon of patients with inflammatory bowel disease and colon cancer. *Fuscobacterium* is a known player in disorders characterized by inflammation, such as gum disease and appendicitis which has now been detected in colon tumors, suggesting that it may set a stage for colorectal cancer, the second-deadliest malignancy (Larson *et al*., 1995; Hu, 2011).

Prostate tissues from patients with prostate cancer and benign prostatic hyperplasia (BPH) often contain histological inflammation, and a proportion of these patients show indication of *Propionibacterium acnes* infection in the prostate gland. A chronological analysis of prostate tissue from individual patients suggested that *P. acnes* can create an importunate infection for up to 6 years in the prostate gland (Krieger *et al*., 2000; Alexeyev *et al*., 2007). Adaptations of *Salmonella enterica* to harsh evironments, including low pH conditions of the stomach,

have given this strain its infamy in human infections such as gastroenteritis. Such adaptations may also be the basis of *Salmonella enterica*'s ability to preferentially populate tumors and cancerous lesions (Flentie *et al*., 2008; Leschner and Weiss, 2010). *Agrobacterium tumefasciens* is a bacterium that produces tumors (crown gall) in plants by inserting a bit of its DNA into the plant cell. The *Agrobacterium* host range is not limited to the plant kingdom; the microbe has been shown to also transform many species of fungi. *Agrobacterium* can genetically transform human-derived cancerous HeLa cells and therefore implicated in cancer in humans (Tzfira *et al*., 2006).

Hidradenitis suppurativa (HS) is a recurrent chronic inflammatory disease of the apocrine glands. Association between chronic HS and squamous cell carcinoma exists and the predominant bacteria commonly isolated include *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa*, *Peptostreptococcus spp*., *Prevotella spp*. and *Streptococci* (Brook and Frazier 1999; Rosenzweig *et al*., 2005). Osteomyelitis (Marjolin ulcer) has been linked with cancer (cell carcinoma) in humans associated pathogens like *Staphylococcus aureus, Streptococcus pyogenes, Haemophilus influenzae* (Mousa, 2003). *Prevotella melaninogenica, Capnocytophaga gingivalis, Capnocytophaga ochracea, Eubacterium saburreum, Leptotrichia buccalis and Streptococcus mitis, Exiguobacterium oxidotolerans, Staphylococcus aureus, Veillonella parvula* have also been implicated in oral cancer (Mager *et al*., 2005; Chocolatewala *et al*., 2010).

Lung cancer is one of the most common cancers worldwide and the etiologic role of chronic pulmonary infection in lung carcinogenesis increases the risk. Studies have identified *Mycoplasma* strains in patients which suggest association of infection with tumorigenesis and a higher ability to metastasize *in vivo* than non-mycoplasma-infected cells (Ushio *et al*., 1995). Other bacteria reported to have been isolated on the cancerous lung tissue include,

Staphylococcus epidermidis, *Streptococcus mitis*, *Legionella pneumophila*, *Bacillus* strains, *Chlamydia*, *Listeria*, and *Haemophilus influenzae* (Apostolou *et al*., 2011).

2.10. Oncoviruses

Several viruses have been linked to certain types of cancer in humans with different ways of reproduction representing several diverse virus families. Cancer viruses are grouped into DNA tumor viruses (permissive and non-permissive cell) and RNA tumor viruses (retroviruses) (Lam *et al*., 2012). It has been estimated that 90% of the world's adult population, including people in the most distant corners of the world, are infected by the Epstein-Barr virus (EBV) and commonly associated with multiple histologic types of breast cancer (Bonnet *et al*., 1999). The variable expression of EBER (small EBV RNA molecules) in neoplastic epithelial cells also leaves open the possibility that EBV may be associated with a broader range of tumors than previously thought; such as, lymphomas and nasopharyngeal cancer (Magrath and Bhatia, 1999). A gene known as the *p53* gene, when mutated has been linked to many cancers including inherited cases, accounting for 60% of all human cancers (e.g., cervix, liver, breast, lung, bladder, skin, prostate and colon cancer) and 80% of colon cancers (Lam *et al*., 2012).

Kaposi sarcoma associate herpes virus (KSHV) the most common cancer in AIDS patients, belongs to the human γ-herpesvirus family, and can establish lifelong persistence in the host after primary infection similar to EBV in which lytic and latent antigens have been shown to block cell cycle regulatory checkpoints, apoptosis control machinery and importantly, the immune response regulatory mechanisms. Human T lymphotropic virus type 1 (HTLV-1) and type 2 (HTLV-2), have been implicated in adult T-cell leukemia and hairy-cell leukemia, respectively (Soffritti *et al*., 2005; Saha *et al*., 2010). Adrenal tumor, Lymphomas, chest

cancer, anaplastic thyroid carcinomas (ATC), bone cancer and brain (Glioblastoma multiforme) tumor have been linked with simian virus 40 (Giuseppe *et al*., 2006).

Infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is the major contributor to hepatocellular carcinoma (HCC), the fifth most common cancer and the third leading cause of cancer death worldwide. However, the oncogenic mechanisms of these two viruses are significantly different at the molecular level (Verma and Robertson, 2003). Marked complexity of cellular deregulation has been reported induced by the expression of viral-oncoproteins (O'Nions and Allday, 2004). In human cancers, mutations have been observed in genes encoding cyclins, cyclin dependent kinases (CDKs), CDK-activating enzymes, CDK inhibitors (CKI), CDK substrates and checkpoint proteins (McDonald and El-Deiry, 2000).

There are scientific evidences linking human papillomaviruses (HPVs) with cervical and anal cancer. HPVs are a group of more than 150 related viruses (Robertson, 2011). Virtually all cervical cancers are caused by HPV infections, with just two HPV types, 16 and 18, responsible for about 70 percent of all cases. HPV also causes anal cancer, with about 85 percent of all cases caused by HPV-16. HPV types 16 and 18 have also been found to cause close to half of vaginal, vulvar, and penile cancers (Schiffman *et al*., 2007; Watson *et al*., 2008). HPV types 16, 18, 34, or 35 are detected in more than 60% of cases of subungual squamous cell carcinoma (SCC) of the nails. HIV-positive patients are especially susceptible to developing HPV-associated squamous cell carcinoma. It has been approximated that, by 2020, HPV will cause more oropharyngeal cancers than cervical cancers in the United States (Phatak and Kolwadkar, 2005; Chaturvedi *et al*., 2011). BK virus (BKV), John Cunningham virus (JCV) and human mammary tumor virus have been linked with prostate cancer, brain cancer and breast cancer respectively (McLaughlin-Drubin and Munger, 2009; Lam *et al*., 2012). Human endogenous retroviruses are associated with germ cell tumors, breast cancer,

ovarian cancer, and melanoma, while torque teno virus has been suggested to play a role in myeloma, gastrointestinal, lung and breast cancer (McLaughlin-Drubin and Munger, 2009).

2.11. Mycotoxin-induced malignancies

Fungi or mycotoxins have been for the most part ignored as the cause of many malignancies and auto-immune diseases (Center for Cancer, 2006). Diseases associated with inhalation of fungal spores can include toxic pneumonitis, hypersensitivity pneumonitis, tremors, chronic fatigue syndrome, kidney failure and cancer (Robey *et al*., 2009). Some of the mycotoxin-induced malignancies are: hepato-cellular carcinoma, esophageal cancer, lung cancer, colon cancer, kidney cancer, breast cancer, endometrial cancer, leukemia, lymphoma, astrocytoma and Kaposi's sarcoma (Takeuchi, 1983; Center for Cancer, 2006; Robey *et al*., 2009). It has been reported that some fungi secrete substances that can cause cancer and a disease called Sick Building Syndrome (SBS) with symptoms including irritation of the nose and eyes, headaches, dematitis, listlessness and other symptoms, caused by inhabiting a building that has bad air quality (Mays *et al*., 2006; Lillis *et al*., 2010); *Candida tropicalis* has been implicated in lung cancer (Apostolou *et al*., 2011).

Candida excretes toxins that weaken and harm the body; the major waste product of *Candida* is acetaldehyde that results in excessive fatigue, destroys enzymes needed for cell energy and causes the release of free radicals that can damage DNA. Ethanol formed by acetaldehyde can reduce the absorption of iron and as a result low oxygen levels (Jha *et al*., 2006). Grains such as corn, wheat, barley, sorghum, and other foods such as peanuts, are commonly contaminated with cancer-causing fungal poisons called mycotoxins. One of them, aflatoxin (*Aspergillus* species), is one of the most carcinogenic substances on earth; others include ochratoxins (*Aspergillus* and *Penicillium* species), sterigmatocystin (*Aspergillus* species especially *Aspergillus versicolour*) and trichothecenes (*Stachybotrys* and *Fusarium*).

Antibiotic plays a negative role in this as it destroys the normal, protective gut bacteria, permitting the intestinal yeast and fungi to grow unchecked, resulting in *Candida* overgrowth. This can lead to immune suppression, symptoms of autoimmune diseases, or even cancer (Weig *et al*., 1999).

Fungi plays a role in the etiology of cancer e.g., leukemia (Seibert *et al*., 1967; Gdanski, 2001; Gdanski, 2003). Fungal tissue (ergosterol) and enzyme (fumonisin) are mostly targeted in the chemotherapy of cancer tumors. Smoking, for example, causes cancer by causing fungal infections. Tobacco contains fungal residue, spores and fumonisin. Antifungal agents are classified according to their chemical structure as macrolides, azoles, allylamines and pyrimidine analogs (Gdanski, 2001; Gdanski, 2003).

Dermatophyte fungi (*T. rubrum, T. mentagrophytes, T. tonsurans*, and *E. floccosum*), yeasts (especially *Candida albicans*) and nondermatophyte molds are implicated in the cause of onychomycosis which has been linked with subungual squamous cell carcinoma (SCC) and subungual melanoma of the nails (Scher, 1990; Cohen *et al*., 1992; Zaias *et al*., 1996; Phatak *et al*., 2005). Several researches have reported the presence of yeast and fungus in cancer patients but mostly after cancer treatment has begun. It has been revealed that cancer therapies, aimed at destroying cancer, also destroy the immune system of the patient; hence, proliferation of disease-causing germs and secondary fungal infections. Attention is needed to be fixed on methods of controlling fungal proliferation while administering chemotherapy. Yeasts used to make bread and many alcoholic beverages, are reported to cause breast cancer (van den Brandt *et al*., 1990).

2.12. Parasitic infection and human cancer development

Parasites such as round worm, pin worm, seat worm, hook worm, and tape worm and many more can be found all through the human system. They are in the lungs, liver, blood and over 80% of human population harbour them (Mager, 2006). Mucin-type O-glycosylated antigen is the only existing expression of tumor-associated antigens by helminth parasites. One of the best-characterized cross-reactive antigens between cancer and helminth parasites is the *Sm23* integral membrane protein of *Schistosoma mansoni*. This antigen, identified in infected humans, is strikingly similar with respect to both amino acid sequence and putative domain structure to ME491 (Osinaga, 2007).

Besides bladder cancer linked to a trematode *Schistosoma helminths*, several other *Schistosoma* (e.g., *Schistosoma haematobium*) species have been implicated in colorectal, liver (*Schistosoma japonicum*) and other gastrointestinal cancers (Cheever *et al*., 1976; Mager, 2006). Parasitic trematode; *Opistorchis felineus, Opistorchis viverrini* (liver fluke) and *Clonorchis sinensis* are strongly associated with cholangiocarcinoma (CCA; cancer of the bile ducts). *Blastocystes hominis* a protozoan intestinal parasite appears to enhance the growth of colorectal cancer (Mager, 2006).

Cholangiocarcinoma (CCA) is responsible for about 24% of liver cancers in the U.S. compared with 87% in Khon Kaen, Thailand. CCA is extremely prevalent in Northeast Thailand, areas where uncooked cyprinoid fish are a staple of the diet. In Northeast Thailand and Laos, an estimated 6 million people are infected with *Opisthorchis viverrini* and despite widespread drug treatment that has been implemented in the past, the prevalence of *Opisthorchis viverrini* in some endemic areas approaches 70 percent (Pairojkul *et al*., 1991). *Toxoplasma gondii*, a parasitic protozoa has been reported to infect about a third of human population and is one of the mind altering parasites already associated with neurosis and

schizophrenia which has been suggested to be linked to brain cancer. Infection can be as a result of contact with soil contaminated by cat faeces, or by eating infected meat (Thomas *et al*., 2011).

2.13. Food substances and cancer proliferation

According to the National Cancer Institute (NCI, 2009), artificial sweeteners (sugar substitutes) including saccharin, aspartame, acesulfame potassium, sucralose, neotame, and cyclamate have been linked with one kind of disease (e.g., cancer) or the other. Studies showed that saccharin caused urinary bladder cancer in laboratory animals, though with no clear evidence of an association with cancer in humans. Lymphomas and leukemias in rats fed with very high doses of aspartame have been reported to be likely linked to brain tumor (Soffritti *et al*., 2005; Lim *et al*., 2006).

It is estimated by NCI (2012) that at least 35% of all cancers are related to diet; in women this rises to at least 50% with a similar trend to the link between tobacco and lung cancer. It has been noted that lung cancer patients eat fewer fruits and vegetables and hence have lower serum levels of beta carotene and other botanical factors (Bertone *et al*., 2002; NCI, 2012). Other essential nutrients that are lower in the diet of people eating less than five servings of fruits and vegetables each day include folic acid and ascorbic acid (Vit C). Consumption of more than 50 % of energy food from fat could result in ovarian cancer in women (Bertone *et al*., 2002).

Other cancers associated with diet include breast, colorectal, pancreatic, stomach, esophageal and uterine cancers. It has been reported that association between diet and cancer has risen to as high as 80% (Katzin, 2012). The typical American diet of fast food hamburgers with fries, pizza, turkey and sandwiches has very little dietary fiber and is associated with a relatively

high incidence of breast cancer; furthermore, epidemiologic studies have identified obesity as an important negative prognostic factor for several cancers (e.g., oesophagus, pancreas, colon and rectum, breast cancer after menopause, endometrium lining of the uterus, kidney, thyroid, gallbladder) type (Rock and Demark-Wahnefried, 2002). Studies have revealed the benefit of healthy lifestyle on specific breast cancer subtypes. For example reports have shown that there are considerable advantages for estrogen receptor positive, progesterone receptor negative (ER+/PR-) and ER+/PR+ groups. Meanwhile there was no survival advantage for healthy lifestyle in ER-, PR- group and a limited advantage for ER-/PR+ group (Pierce *et al*., 2007).

Synthetic food dyes have been linked to a wide variety of health concerns including behavioural problems, hyperactivity, allergic reactions, and even cancers. For example, Red 3 has been identified to be carcinogenic resulting in thyroid tumors and DNA damage while Citrus Red 2 and Yellow 6 added to the diet results in bladder tumors (Schab and Trinh, 2004; McCann *et al*., 2007). Red meat and corn are heavy sources of fungal mycotoxins, over consumption of which can result in inflammatory bowel disease, cancer of the breast, prostate, kidney, and pancreas (van den Brandt *et al*., 1990). Meat is devoid of fiber and other nutrients that have a protective effect and also contains animal protein, saturated fat, and, in some cases, carcinogenic compounds such as heterocyclic amines (HCA) and polycyclic aromatic hydrocarbons (PAH) formed during the processing or cooking (Skog *et al*., 1998).

Food additives such as sodium nitrate, sodium nitrite, acrylamide, aspartame, potassium bromate, butylated hydroxyanisole (BHA) and the related compound, butylated hydroxytoluene (BHT) have been linked or associated with cancer risk (Food democracy, 2008). Acrylamide, one of the many cancer causing agents is formed not only by potatoes but also by certain foods when fried, baked or roasted. Endometrial and ovarian cancers are

associated with exposure to high levels of acrylamide. This chemical is used in the production of paper, plastics and dyes and is found to cause damage to brain (van den Brandt *et al*., 1990; Weig *et al*., 1999).

2.14. Genetics and Immunological Basis of Cancer

Cancer development is a multi-step process and several mutations are necessary for a gene to induce cancer (Lynch *et al*., 2008). Oncogene such as *SIS*, the gene for platelet-derived growth factor (PDGF) and *EGFR* (HER1), the gene encoding the receptor for epidermal growth factor (EGF) act as dominants; the over expressed products of these genes drive the malignant process stimulating mitosis even though normal growth signals are absent (Kadouri *et al*., 2007). *BRCA1* and *BRCA2* genes in normal cell prevent uncontrolled cell growth and ensure the stability of the genetic material (Thompson and Easton, 2002). Mutation of these genes and other genes such as *TP53*, *PTEN*, *STK11*/*LKB1*, *CDH1*, *CHEK2*, *ATM*, *MLH1*, and *MSH2* has been correlated to the development of hereditary breast and ovarian cancer. *BRCA1* mutations may increase the risk of developing cervical, uterine, pancreatic, and colon cancer in woman (Walsh *et al*., 2006; Kadouri *et al*., 2007). Men with harmful *BRCA1* mutations also have an increased risk of breast cancer and, possibly, of pancreatic cancer, testicular cancer, and early onset of prostate cancer. However, male breast cancer, pancreatic cancer, and prostate cancer appear to be more strongly associated with *BRCA2* gene mutations (Kadouri *et al*., 2007; Lynch *et al*., 2008; Campeau *et al*., 2008).

Studies have shown that cancer cells polyps contain one or two mutations associated with cancer and frequently are the deletion of a healthy adenomatous polyposis coli (APC) gene on chromosome 5, a gene product that destroys the transcription factor β-catenin, therefore; preventing it from turning on gene that causes cell division (Rubinfeld *et al*., 1996; Laken *et*

al., 1997). Another important regulatory protein, *p53*, prevents replication of damaged DNA in normal cells and promotes cell death (apoptosis) in cells with abnormal DNA. Inactive or altered *p53* allows cells with abnormal DNA to survive and divide, subsequently; conferring a high probability of neoplastic transformation (Evans *et al*., 1998). There are several other oncogenes that may contribute to human neoplastic transformation. The *ras* gene encodes the Ras protein, which regulates cell division. Mutations may result in the inappropriate activation of the Ras protein, leading to uncontrolled cell growth and division (Kalikaki *et al*., 2008). Other oncogenes have been implicated in specific cancers and these include; *Her2/neu* (breast cancer), *BCR-ABL* (chronic myelocytic leukemia, B-cell acute lymphocytic leukemia), *C-myc* (Burkett's lymphoma), *N-myc* (small cell lung cancer, neuroblastoma) (Makower *et al*., 1998). Among the several genes participating in DNA mismatch repair (MMR), germ-line mutations in *hMLH1* (on chromosome 3p21.3) and *hMSH2* (on 2p22-p21) account for more than 90% of mutations detected in hereditary nonpolyposis colorectal cancer (HNPCC) (Peltomaki and Vasen, 1997). In malignant melanoma, three putative melanoma-susceptibility genes have been proposed, namely; *CMM1* (chromosome 1p36), *CDKN2* (chromosome 9p21) and *CDK4* gene (chromosome 12q14) (Ranade *et al*., 1995).

The human body elaborates more than one million different immune responses and there is considerable evidence that tumor cells are being attacked by the body T-cells, linked with the breakdown of immune surveillance; hence, cancer cell proliferation. Lymphocyte development can go off beam due to more than 50 known genetic defects in the immune system (Sullivan, 1999). The lifelong DNA rearrangements in lymphocytes are error prone and can lead to consequent formation of lymphoid cancers (lymphomas). Immune system dysfunction as a result of inherited genetic mutation, acquired disorders, aging, or immunosuppression interferes with normal immune surveillance of early tumors and results in higher rates of cancer (Makower *et al*., 1998). Known cancer-associated immune disorders

include; Ataxia-telangiectasia (acute lymphocytic leukemia [ALL], brain tumors, gastric cancer), Wiskott-Aldrich syndrome (lymphoma, ALL), X-linked agammaglobulinemia (lymphoma, ALL), Immune deficiency secondary to immunosuppressants or HIV infection (large cell lymphoma, Kaposi's sarcoma), Rheumatologic conditions, such as Systemic lupus erythematosus, Rheumatoid arthritis, and Sjogren's syndrome (B-type lymphoma) and general immune disorders (lymphoreticular neoplasia) (Hartley *et al*., 1994; Savitsky *et al*., 1995; Makower *et al*., 1998).

2.15. Cancer common risk factors

Cancer is a collection of more than hundred types and each with different set of risk factors. Developing cancer can be greatly reduced by avoiding risk factors, although not all cancers are preventable. The risk of developing cancer increases as with age, gender, race, personal and family medical history, lifestyle choices, occupational exposures, genetic, environmental factors (Harras, 1996; ACS, 2002; Thompson and Easton, 2002; Kadouri *et al*., 2007; NCI, 2012) and some certain bacterial (Cohen, 1991; Hawkins *et al*., 1992; Banas and Vickerman, 2003; Banas, 2004; Guiney, 2005; Biswas and Biswas, 2011; Spano and Galan, 2012), fungal (Silva *et al*., 2001; Yang, 2003), parasitic (Hitanant *et al*., 1987; Sripa and Kaewkes, 2000; Rim, 2005) and viral (Seiki *et al*., 1983; Walboomers *et al*., 1999; Wells *et al*., 2000; Coskun and Sutton, 2005) infections.

It has been reported that red meat increased the risk of bowel cancer to about 17-30% in relation to 100-120g/day and a 9-50% in relation to 25-50g/day of processed meat and risk reduction by 10% for every 10g/day total dietary fibre and cereal fibre (Sandhu *et al*., 2001; Aune *et al*., 2011). In comparison to healthy-weight men (BMI less than 25 kg/m2), overweight men (BMI 25-29.9kg/m2) have a 23% higher risk of colon cancer with substantial evidence that the most active men can reduce their risk of colon cancer by 19-28%, and the

most active women can reduce their risk by 11-32% (Moghaddam *et al*., 2007; Wolin *et al*., 2009). About 21% of colon and rectal cancers risk have been reported with an alcohol intake of around 1.6-6.2 units per day, similar to 20-21% of cigarette smokers and 42% risk reduction with regular low-dose of aspirin for bowel cancer (Tsoi *et al*., 2009; Fedirko *et al*., 2011; Algra and Rothwell, 2012)

Table 2.1.: Cancers and their common risk factors (American Cancer Society. Cancer Facts & Figures, 2002. Harras A, editor. Cancer Rates and Risks, USDHHS, NIH 1996).

	Liver	Pancreas	Leukemia	Gallbladder	Mouth	Breast	Ovary	Colorectal	Brain	Stomach	Endometrium	Bladder	Hodgkin Lymphoma	Cervix	Esophagus	Prostate	Lung	Melanoma	Larynx	Kidney	Multiple myeloma
Smoking	*	**			**			*	*	*		**		**	**	*	***		**	**	*
Alcoholism	**	*			**	**		*	*						**	*			**		
Diet low in fruits and vegetables					*			**		**					*		*				
Physical inactivity						*										*					
Chemical/ compound	**		*						**			**					**		**	**	*
Anemia					*					**					*						
Race																*		**			
Obesity				**		**		*			*					*				**	
Coffee, or tea consumption		*										*									
Diabetes		*									*										
Vitamins deficiencies														*	*						
Use of estrogen				**		**					**										
Ionizing radiation			**			**			**	*							**				**
Use of tamoxifen											*										
Infection	**	*	**	*	*			**		**		*	**	**				*			*
Diet high in meat or fat		*		**		*	*	**			*					*				*	
Occupational exposure	**		**						**			**					**		**	*	*
Use of steroids	*																				
Air pollution																	*				
Early age sexual intercourse/ many sexual partner														**							
Cirrhosis	**	*																			

Sexually transmitted agent																*				
Multiple births														**						
Dietary nitrites or pickled, salted, and smoked foods										**										
Increasing age				**		*														
Alkylating drugs			**																	
Allergic conditions		*																		*
Genetic factors	*		**			**	**	**	**	*		*	*					*			*
Aflatoxin ingestion	**																			
Reduced immune function																		**			*
Family history			**			**	**	**					*			**		**		
Gallstones				**																
Infertility							**				**									
Ultraviolet radiation																		**		
Socioeconomic status						**														
Hypertension											*									
Hormonal factors														*		*				

*, Low risk factor; **, High risk factor; ***, Very high risk factor

2.16. Cancer and drug development

Cancer is considered to be the ultimate healing mechanism the human system adopted to get rid of waste and toxic materials in the body. Cancers are treated by mainstream medicine usually bombarded with radiation and subjected to toxic chemotherapy that destroys healthy cells and weakens the body (Baker *et a*l., 2008; Broniscer *et al*., 2010). For example, pioglitazone, a diabetes medication if used for more than one year has been scientifically linked with an increased risk of bladder cancer (Lewis *et al*., 2011). Chemically, antibiotics are much more complex than anti-cancer or anti-viral drugs and of all the antibiotics that have been produced over the last 60 years, 99% are derived from microorganisms, primarily bacteria and fungi in the soil (Barton, 2012). Bacteria found in caves (isolated environments) could provide the clues to help produce antibiotics needed in the fight against drug-resistant superbugs and cancer cells. There have been assertions that anti-sera derived from cancer or associated bacteria could be used therapeutically (June, 2007; Restifo *et al*., 2012). This could be explained with Coley's Toxins, where patients are deliberately injected with bacteria and the fever developed combat the infection and in some cases, fight the cancer (Decker and Safdar, 2009; McCarthy, 2006).

Scientists have raised the prospect of vaccine against bowel cancer, revealed to be caused by *E. coli*. A therapy using *Clostridium sporogenes*, a bacterium common in dirt has been developed with the anticipation that the strain will be used in a clinical trial. The spores of this bacterium when injected can only grow in solid tumors and a specific enzyme produced, activates a cancer drug and therefore causes the destruction of only the cancer cells without any negative effect on healthy cells (Patyar *et al*., 2010).

Genome of the parasite *Leishmania* has been discovered to possess three kinds of TOR kinases, proteins that are linked to cell growth and cancer with a longstanding target for drug

development (Sripa and Kaewkes, 2000; da Silva and Beverley, 2010). Mammals have only one TOR kinase protein, and drug developers have targeted it to block immune system rejection of transplanted organs and to treat certain forms of cancer (Rim, 2005; da Silva and Beverley, 2010). The properties of the parasite with mutated TOR kinase 3 confirmed prior research that suggested the acidocalcisome ability to help cells regulate the flow of fluids across the cell membrane or cope with stress and loss of access to the sugar glucose (da Silva and Beverley, 2010). Much research is ongoing to verify if it were possible to introduce healthy *p53* genes into cells to shut down tumor growth. The gene *p53* is a tumor suppressor which has been referred to as The Guardian of the Genome since it regulates multiple components of the DNA damage control system. It is a transcription factor and when builds up can stop DNA replication and set the cell on course to apoptosis (Lam *et al*., 2012).

Sodium bicarbonate salts therapy, which is harmless, fast and effective, used in the treatment of proliferation of fungal (Candidas) colonies has been reported to be effective in the treatment of leukaemia and other extremely advanced cancers (e.g., prostate and breast cancer), suggesting that cancer is the manifestation of fungi infections (Seibert *et al*., 1967; Robey *et al*., 2009). The use of olive oil and its leaf have also been recommended as a natural killer of fungi. Anti-fungal drug may help treat cancer; therefore fungal mycotoxins and their eradication from the body are the best form of prevention against cancers; e.g., breast cancer (van den Brandt *et al*., 1990). Scientists have taken a step further with plans to use special bugs of *Salmonella enterica* for cancer treatment. For example, genes that produced bacterial toxins like the Shiga toxin, could be engineered into tumor-hunting bioluminescent *Salmonella* bacteria under the control of bacterial genes expressed (Flentie *et al*., 2008; Leschner and Weiss 2010).

Mushrooms (fungi) including *Lentinula edodes* (Shiitake), *Pleurotus djamor* (Pink oyster), *Grifola frondosa* (Maitake), *Flammulina velutipes* (Enokitake), *Agaricus bisporus* (Portobello, champignon, white button), *Coriolus versicolor* (Turkey Tail), *Ganoderma lucidum*, Dong chong xia *cao*, *Yun zhi*, *Agaricus Blazei Murrill* and *Reshi* have gained prominence as therapeutic agents (Kodoma *et al*., 2002; Fang *et al*., 2006). Researchers have been studying the anti-cancer, anti-viral, anti-fungal, anti-bacterial and immunity enhancing effects of many different species of Mushrooms. Polysaccharides (e.g., Beta-(1-3)-D-glucan, Beta-(1-4)-a-D-glucan & Beta-(1-6)-D-glucan) contained within *Agaricus Blazei Murrill*, *Yun zhi* and *Reshi* enhance the body's immune response to diseases like cancer (Borchers *et al*., 1999; Cao and Lin, 2004). They contain enzymes and anti-tumor substances that stimulate natural killer cells and activate macrophage activity, which engulf and destroy foreign materials. Dong chong xia cao, another mushroom that grows on the bodies of dead ants, has been shown to reduce fatigue associated with radiation and chemotherapies (Borchers *et al*., 1999; Fang *et al*., 2006; Martin and Brophy, 2010). Compounds in shiitake mushrooms have been shown to trigger programmed cell death in breast cancer cells; frequent consumption of mushrooms decreased the risk of breast, stomach and colorectal cancers by up to 60 - 70% (Kodoma *et al*., 2002; Fang *et al*., 2006). Button mushrooms have been found to inhibit enzymes that contribute to breast and prostate cancer (deVere White *et al*., 2002; Zhang *et al*., 2009; Martin and Brophy, 2010).

Gene damage is an important event in cancer and certain bacterial species in the colon (*Streptococcus bovis* and *Escherichia coli*) produce harmful substances that are linked to cancer. Beneficial bacteria may prevent the growth of these organisms, and some beneficial species even produce anti-carcinogenic substances (Boyle and Langman, 2000). Since probiotics modify the balance of gut bacteria in favour of beneficial bacteria, it seems more than a possibility that

probiotics might help reduce colorectal cancer (CRC or bowel cancer) (Boyle and Langman, 2000; Gill and Rowland, 2002). Several studies have established the prospective use of recombinant soluble tumor necrosis ligand (TRAIL) which functions as a cytokine (cancer therapeutic agent) to selectively induce apoptosis of various cancer cells without toxicity to most normal cells (Zheng *et al*., 2012).

Relatively few studies have reported inverse associations between prostate cancer risk and consumption of plant-derived foods (Steinmetz and Potter, 1996). There has been particular interest in the potential anticarcinogenic properties of the major carotenoids, including β-carotene and lycopene, the most effective oxygen radical quenching agents among the carotenoids (Blumberg, 1995). *Fagonia cretica*, also known as Virgon's Mantlem or Mantle of the Virgin, belongs to the Caltrop family and grows in arid desert areas. The extracts of this plant has been reported to cause apoptosis on two human breast cancer cell lines without any damage to normal breast cells. The antimicrobial activity of this plant against such common bacteria as *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* have also been documented (Razi *et al*., 2011; Lam *et al*., 2012). Notable anticancer potential of the extracts from *Ballochia atro-virgata*, *Eureiandra balfourii* and *Hypoestes pubescens*, with EC_{50} values ranging between 0.8 and 8.2 μg/mL were reported by Mothana *et al*. (2009). Costa-Lotufo *et al*. (2005) also in his study evaluated the anticancer potential of 11 plants used in Bangladeshi folk medicine including *Oroxylum indicum* (EC_{50} of 14.2 μg/ml for HL-60), *Moringa oleifera* and *Aegles marmelos* as potential sources of anticancer compounds.

There are several other species of plants that have been reported to posess anticancer potential including; Castor oil (*Ricinus communis*) Zedoary (*Curcuma zedoaria*), Rodent Tuber (*Typhonium flagelliforme*), Happy Tree (*Camptotheca acuminata*), Madagaskar Periwinkle

(*Catharanthus rosens*), Artocarpus Integer (*Selaginella corymbosa*), Wild lemon (*Podophyllum peltatum*), Bamboo Grass (*Loathatreum Gracies*), fruit makasar (*Brucca javanica*), Garlic (*Allium sativum*), Sunflower (*Helianthus annus*), God's Crown (*Phaleria macrocarpa*), Leunca (*Solanum nigrum*), Job's Tears (*Coix Lachryma-Jobi*), Bamboo Rope (*Asparagus cochinchinensis*), Yew (*Taxus brevifolia*) and others (Gruenwald, 2004; Kushi *et al*., 2012; Umadevi *et al*., 2013).

REFERENCES

Alexeyev, O.A., Marklund, I., Shannon, B., Golovleva, I., Olsson, J., Andersson, C., Eriksson, I., Cohen, R. and Elgh, F. (2007). Direct visualization of *Propionibacterium acnes* in prostate tissue by multicolor fluorescent in situ hybridization assay. *Journal of Clinical Microbiology*. **45**: 3721-3728.

Algra, A.M. and Rothwell, P.M. (2012). Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol*ogy. **13**: 518-527.

Altekruse, S. F., Kosary, C. L., Krapcho, M., Neyman, N., Aminou, R., Waldron, W., Ruhl, J., Howlader, N., Tatalovich, Z., Cho, H., Mariotto, A., Eisner, M. P., Lewis, D. R., Cronin, K., Chen, H. S., Feuer, E. J., Stinchcomb, D. G. and Edwards, B. K (eds). (2010). *SEER Cancer Statistics Review*, 1975-2007, National Cancer Institute. Bethesda, MD, 2009 SEER data submission.

American Cancer Society. (2002). *Cancer Facts & Figures*.

American Cancer Society. (2006). Cancer Facts & Figures. Atlanta.

American Cancer Society. (2006). The Worldwide Cancer Burden. Atlanta.

Anand, P., Kunnumakkara, A.B., Kunnumakara, A.B., Sundaram, C., Harikumar, K.B., Tharakan, S.T., Lai, O.S., Sung, B. and Aggarwal, B.B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical Research*. **25** (9): 2097-116.

Anderson, B.O., Yip, C.H., Ramsey, S.D., *et al*. (2006). Breast cancer in limited-resource countries: health care systems and public policy. *Breast Journal*. **12** (1): 54-69.

Anderson, B.O., Yip, C.H., Smith, R.A., *et al*. (2008). Guideline implementation for breast healthcare in low-income and middle-income countries: overview of the Breast Health Global Initiative Global Summit 2007. *Cancer*. **113** (8): 2221-2243.

Anghelescu, D.L., Faughnan, L.G, Baker, J.N, Yang, J. and Kane, J.R. Use of epidural and peripheral nerve blocks at the end of life in children and young adults with cancer: the collaboration between a pain service and a palliative care service. *Pediatric Anesthesia*. 2010, **20**: 1070-1077.

Apostolou, P., Tsantsaridou, A., Papasotiriou, I., Toloudi, M., Chatziioannou, M., Giamouzis, G. (2011). Bacterial and fungal microflora in surgically removed lung cancer samples. *Journal of Cardiothoracic Surgery*. **6**: 137.

Aune, D., Chan, D.S.M., Lau, R., Vieira, R., Greenwood, D.C. and Kampman, E., *et al*. (2011). Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies. *British Medical Journal* . **343**: 6617.

Baade, P.D., Youlden, D.R. and Krnjacki, L.J. (2009). International epidemiology of prostate cancer: geographical distribution and secular trends. *Molecular Nutrition & Food Research*. **53** (2): 171-184.

Baker, J.N., Hinds, P.S., Spunt, S.L., Barfield, R.C., Allen, C., Powell, B.C., *et al*. (2008). Integration of Palliative Care Principles into the Ongoing Care of Children with Cancer: Individualized Care Planning and Coordination. *Pediatric Clinics of North America*. **55**: 223-250.

Banas, J.A. (2004). Virulence properties of *Streptococcus mutans*. *Frontiers in Bioscience*. **9**: 1267-1277.

Banas, J.A. and Vickerman, M.M. (2003). Glucan-binding proteins of the oral *Streptococci*. *Critical Reviews in Oral Biology & Medicine*. **14**: 89-99.

Barton, H. (2012). Cave Bacteria Could Help Develop Future Antibiotics. Ed. Hibaq. *Tutor Hunt Discover Learning Possibility*. **1**:1-2.

Bertone, E.R., Rosner, B.A., Hunter, D.J., Stampfer, M.J., Speizer, F.E., Colditz, G.A., *et al*. (2002). Dietary fat intake and ovarian cancer in a cohort of US women. *American Journal of Epidemiology*. **156**: 22-31.

Bertuccio, P., Chatenoud, L., Levi, F., et al. (2009). Recent patterns in gastric cancer: a global overview. *International Journal of Cancer*. **125** (3): 666-673.

Biswas, S. and Biswas, I. (2011). Role of *VltAB*, an ABC transporter complex, in viologen tolerance in *Streptococcus mutans*. *Antimicrobial Agents and Chemotherapy*. **55** (4): 1460-9.

Blumberg, J.B. (1995). Considerations of the scientific substantiation for antioxidant vitamins and beta-carotene in disease prevention. *Journal of American Clinical Nutrition*. **62**: 1521-1526.

Bonnet, M., Guinebretiere, J.M., Kremmer, E., Grunewald, V., Behhamou, E. and Contesso, G. (1999). Detection of Epstein-Barr virus in invasive breast cancers. *Journal of the National Cancer Institute*. **91**: 1376-1381.

Borchers, A.T., Stern, J.S., Hackman, R.M., Keen, C.L. and Gershwin, M.E. (1999). Mushrooms, tumors, and immunity. *Proceedings of the Society for Experimental Biology and Medicine*. **221**: 281-293.

Boyle, P. and Langman, J.S. (2000). ABS of colorectal cancer – epidemiology. *British Medical Journal*. **321**: 805-808.

Bray, F.I. and Weiderpass, E. (2010). Lung cancer mortality trends in 36 European countries: secular trends and birth cohort patterns by sex and region 1970-2007. *International Journal of Cancer*. **126** (6): 1454-1466.

Broniscer, A., Baker, J.N., Tagen, M., Onar, A., Chin, T.K., Gilbertson, R.J., Onar-Thomas, A., Gilbertson, R.J., Davidoff, A.M., Pai Panandiker., A.S., Leung, W., Chin., T.K., Stewart, C.F., Kocak, M., Rowland, C., Merchant, T.E, Kaste, S.C. and Gajjar, A. (2010). Phase I study of Vandetanib during and after radiation therapy in children with diffuse intrinsic Pontine Glioma. *Journal of Clinical Oncology*. **28**: 4762-4768.

Brook, I. and Frazier, E.H. (1999). Aerobic and anaerobic microbiology of axillary hidradenitis suppurativa. *Journal of Medical Microbiology*. **48**: 103-105.

Cai, Y.Z., Sun, M. and Corke, H. (2003). Antioxidant activity of betalains from plants of the Amaranthaceae. *Journal of Agricultural and Food Chemistry*. **51** (8): 2288-2294.

Cai, Yun., Wang, Rui., Pei, Fei., Liang, B.B. (2007). Antibacterial Activity of Allicin Alone and in Combination with β-Lactams against Staphylococcus spp. and *Pseudomonas aeruginosa*. *The Journal of Antibiotics*. **60** (5): 335-8.

Campeau, P.M., Foulkes, W.D. and Tischkowitz, M.D. (2008). Hereditary breast cancer: New genetic developments, new therapeutic avenues. *Human Genetics*. **124** (1): 31-42.

Cantwell, A. (2012). Bacteria cause cancer - the microscopic evidence. *Aries rising press*. **2**: 1-5.

Cantwell, A.R Jr., (1981). Histologic observations of variably acid-fast coccoid forms suggestive of cell wall deficient bacteria in Hodgkin's disease. A report of four cases. *Growth*. **45**: 168-187.

Cantwell, A.R. Jr. and Kelso, D.W. (1981). Microbial findings in cancer of the breast and in their metastases to the skin. *Journal of Dermatologic Surgery and Oncology*. **7**: 483-491.

Cao, Q.Z. and Lin, Z.B. (2004). Antitumor and anti-angiogenic activity of Ganoderma lucidum polysaccharides peptide. *Acta Pharmacologica Sinica*. **25**: 833-838.

Castellsague, X., Diaz, M., de Sanjose, S., et al. (2006). Worldwide human papillomavirus etiology of cervical adenocarcinoma and its cofactors: implications for screening and prevention. *Journal of the National Cancer Institute*. **5**: 303-315.

Center for Cancer. (2006). Alternative Cancer Treatment.

Center, M.M., Jemal, A. and Ward, E. (2009). International trends in colorectal cancer incidence rates. *Cancer Epidemiology, Biomarkers & Prevention*. **18** (6): 1688-1694.

Chaturvedi, A.K., Engels, E.A. and Pfeiffer, R.M. (2011). Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of Clinical Oncology*. **29**: 4294-4301.

Cheever, A.W., Kuntz, R.E., Moore, J.A., Bryan, G.T. and Brown, R.R. (1976). Animal model of human disease: carcinoma of the urinary bladder in *Schistosoma haematobium* infection. *American Journal of Pathology*. **84**: 673-676.

Chen, S. and Parmigiani, G. (2007). Meta-analysis of BRCA1 and BRCA2 penetrance. *Journal of Clinical Oncology*. **25** (11): 1329-1333.

Chen, Y.Y., You, S.L., Chen, C.A., *et al*. (2009). Effectiveness of national cervical cancer screening programme in Taiwan: 12-year experiences. *British Journal of Cancer*. **101** (1): 174-177.

Chocolatewala, N., Chaturvedi, P. and Desale, R. (2010). The role of bacteria in oral cancer. *The Indian Journal of Medical and Paediatric Oncology*. **31**: 126-131.

Christian, K. R., and Kunal, K. S. (2009). Oxidative stress and metabolic syndrome. *Life Sciences*. **84**: 705-712.

Cohen, J.I. (1991). Epstein-Barr virus lymphoproliferative disease associated with acquired immunodeficiency. *Johns Hopkins Medical Journal, Baltimore*. **70**: 137-160.

Cohen, J.L., Scher, R.K. and Pappert, A.S. (1992). The nail and fungus infections. In: Cutaneous fungal infections. New York, N.Y: Igaku-Shoin Inc. 106-122.

Cortes, J.E. and Pazdur, R. (1995). *Docetaxel Journal of Clinical Oncology*. **13**: 2643-2655.

Coskun, A.K. and Sutton, R.E. (2005). Expression of glucose transporter 1 confers susceptibility to human T-cell leukemia virus envelope-mediated fusion. *Journal of Virology*. **79**: 4150.

Costa-Lotufoa, L. V., Khanb, M. T. H., Atherc, A., Wilkea, D. V., Jimeneza, P. C., Pessoaa, C., Amaral de Moraesa, M. E., Odorico de Moraesa, M. (2005). Studies of the anticancer potential of plants used in Bangladeshi folk medicine. *Journal of Ethnopharmacology*. **99** (1): 21-30.

Cotelle, N., Bernier, J.L., Catteau, J.P., Pommery, J., Wallet, J.C. and Gaydou, E.M. (1996). Antioxidant properties of hydroxyflavones. *Free Radical Biology and Medicine*. **20** (1): 35-43.

Cragg, G.M. and Newman, D.J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology*. **100**: 72-79.

Cragg, G.M., Schepartz, S.A., Suffness, M. and Grever, M.R. (1993). The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. *Journal of Natatural Products*. **56**: 1657-1668.

Cragg, M.G. and Newman, D.J. (2001). Natural Product Drugs Discovery in Next Millennium, National Cancer Institute, Frederick, USA.

Croce, C.M. (2008). Oncogenes and cancer. N. Engl. J. Med. **358** (5): 502-11. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nature Reviews Cancer*. **2**: 107-116.

Cronin, K.A., Ravdin, P.M. and Edwards, B.K. (2009). Sustained lower rates of breast cancer in the United States. *Breast Cancer Research and Treatment*. **117** (1): 223-224.

da Silva, L.M. and Beverley, S.M. (2010). Expansion of the target of rapamycin (TOR) kinase family and function in Leishmania shows that TOR3 is required for acidocalcisome biogenesis and animal infectivity**.** *Proceedings of the National Academy of Sciences*. **107**: 1073-11965.

Dai, J., and Mumper, R. J. (2010). Plant Phenolics: Extraction, Analysis and Their Antioxidant and Anticancer Properties. *Molecules*. **15**: 7313-7352.

Decker, W. K. and Safdar, S. (2009). Bioimmunoadjuvants for the treatment of neoplastic and infectious diseases: Coley's legacy revisited. *Cytokine & Growth Factor Reviews*. 20(4):271-81.

deVere White, R.W., Hackman, R.M., Soares, S.E., Beckett, L.A. and Sun, B. (2002). Effects of a mushroom mycelium extract on the treatment of prostate cancer. *Urology*. **60**: 640-644.

Dewick, P.M. (2002). Tumor inhibitors from plants. In Evans, W.C. (ed.) Trease and Evans Pharmacognosy, London, W.B. Saunders. pp. 394-407.

Ebbesen, P. and Lind, K. (1969). Lack of evidence for oncogenic or amyloid inducing qualities of *Mycoplasma neurolyticum* inoculated into Balb/C Mice. *Acta Pathologica Microbiologica Scandinavica*. **76**: 594-600.

Edwards, B.K., Ward, E., Kohler, B.A., et al. (2010). Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates. *Cancer*. **116**: 544-573.

Ernst, P. B. and Gold, B. D. (2000). The disease spectrum of *Helicobacter pylori*: the immunopathogenesis of gastro duodenal ulcer and gastric cancer. *Annual Review of Microbiology*. **54**: 615-640.

Evans, S.C., Mims, B., McMasters, K.M., *et al*. (1998). Exclusion of a *p53* germline mutation in a classic Li-Fraumeni syndrome family. *Human Genetics*. **102**: 681-686.

Ezzati, M., Henley, S.J., Lopez, A.D. and Thun, M.J. (2005). Role of smoking in global and regional cancer epidemiology: current patterns and data needs. *International Journal of Cancer*. **116** (6): 963-971.

Fang, N., Li, Q., Yu, S., Zhang, J., He, L., Ronis, M.J., *et al*. (2006). Inhibition of growth and induction of apoptosis in human cancer cell lines by an ethyl acetate fraction from shiitake mushrooms. *Journal of Alternative Complementary Medicine*. **12**: 125-132.

Fedirko, V., Tramacere, I., Bagnardi, V., Rota, M., Scotti, L. and Islami, F., *et al*. (2011). Alcohol drinking and colorectal cancer risk: an overall and dose-response meta-analysis of published studies. *Annals of Oncology*. **22**: 1958-72.

Flentie, K.N., Qi, M., Gammon, S.T., Razia, Y., Lui, F., Marpegan, L., Manglik, A., Piwnica-Worms, D. and McKinney, J.S. (2008). Stably integrated luxCDABE for assessment of Salmonella invasion kinetics. *Molecular Imaging*. **7**: 222-233.

Franco, R., Schoneveld, O., Georgakilas, A. G. and Panayiotidis, M. I. (2008). Oxidative stress, DNA methylation and carcinogenesis. *Cancer Letters*. **266**: 6-11.

Gdanski, R. (2001). Cancer is caused by infected injuries. *Cancer, Cause, Cure and Cover-up*. ISBN 0-9685665-0-2.

Gdanski, R. (2003). The Fungal Nature of Cancer. *The Planetary Association for Clean Energy Newsletter*. **11**:26-28.

Gill, C.I.R. and Rowland, I.R (2002) Diet and cancer: assessing the risk. *British Journal of Nutrition*. **88**: 73-87.

Giuseppe, B.B., Silvia, S., Fernanda, M., Massimo, N., Alfredo, C. and Mauro, T. (2006). BK Virus, JC Virus and Simian Virus 40 infection in humans, and association with human tumors. *Advances in Experimental Medicine and Biology*. **577**: 319-341.

Gruenwald, J. (2004). *PDR for Herbal Medicines*. 3rd ed. Montvale, N. J.: Thomson PDR.

Guiney, D.G. (2005). The role of host cell death in Salmonella infections. *Current Topics in Microbiology and Immunology*. **289**: 131-50.

Gustafsson, L., Ponten, J., Bergstrom, R. and Adami, H.O. (1997). International incidence rates of invasive cervical cancer before cytological screening. *International Journal of Cancer*. **71** (2): 159-165.

Harras, A., Editor. (1996). Cancer Rates and Risks, *USDHHS, NIH* 1996.

Hartley, A.L., Birch, J.M., Harris, M., *et al*. (1994). Leukemia, lymphoma, and related disorders in families of children diagnosed with Wilms' tumor. *Cancer Genetics and Cytogenetics*. **77**: 129-133.

Hawk, E.T., Umar, A., Richmond, E. and JL, V. (2005). Prevention and therapy of colorectal cancer. *Medical Clinics of North America*. **89**: 85-110.

Hawkins, R.E., Rickman, L.S., Vermund, S.H. and Carl, M. (1992). Association of mycoplasma and human immunodeficiency virus infection: detection of amplified *Mycoplasma fermentans* DNA in blood. *Journal of Infectious Diseases*. **165**: 581-585.

Hitanant, S., Trong, D.T., Damrongsak, C., Chinapak, O., Boonyapisit, S., Plengvanit, U., *et al*. (1987). Peritoneoscopic findings in 203 patients with *Opisthorchis viverrini* infection. *Gastrointestinal Endoscopy*. **33**: 18-20.

Hooper, S., Wilson, M., Crean, S. and Myers, J.N. (2009). Exploring the link between microorganisms and oral cancer: a systematic review of the literature. *Head neck*. **31**: 1228-1239.

Hu, B., Elinav, E. and Flavell, R.A. (2011). Inflammasome-mediated suppression of inflammation- induced colorectal cancer progression is mediated by direct regulation of epithelial cell proliferation. *Cell cycle*. **10**: 1936-1939.

Hulka, B.S. and Moorman, P.G. (2001). Breast cancer: hormones and other risk factors. *Maturitas*. **38** (1): 103-113.

Itharat, A. and Ooraikul, B. (2007). Research on Thai medicinal plants for cancer treatment. *Advances in Medicinal Plant Research*. **13**: 287-317.

Iwashina, T., 2000. The structure and distribution of the flavonoids in plants. *Journal of Plant Research*. **113** (1111): 287-299.

Jha, B.J., Dey, S., Tamang, M.D., Joshy, M.E., Shivananda, P.G. and Brahmadatan, K.N. (2006). Characterization of Candida species isolated from cases of lower respiratory tract infection. *Kathmandu University Medical Journal*. **4**: 290-294.

June, C. H. (2007). Adoptive T cell therapy for cancer in the clinic. *The Journal of clinical investigation*. **117** (6): 1466-76.

Kadouri, L., Hubert, A., Rotenberg, Y., Hamburger, T., Sagi, M., Nechushtan, C., Abeliovich, D. and Peretz, T. (2007). Cancer risks in carriers of the BRCA1/2 Ashkenazi founder mutations. *Journal of Medical Genetics*. **44**: 467-471.

Kalikaki, A., Koutsopoulos, A., Trypaki, M., Souglakos, J., Stathopoulos, E., Georgoulias, V., Mavroudis, D., and Voutsina, A. (2008). Comparison of *EGFR* and *K-RAS* gene status between primary tumours and corresponding metastases in NSCLC. *British Journal of Cancer*. **99**: 923-929.

Katzin, C. (2012). Healthy nutrition choices for breast cancer and other women's cancers (endometrial, ovarian and cervical). *Cancer Nutrition Center*. **1**:1

Keku, T.O. (2010). Study identifies bacteria associated with colorectal cancer. *Oncology Times*. **32**: 33.

Ketcham, C.M., Anai, S., Reutzel, R., Sheng, S., Schuster, S.M., Brenes, R.B., Agbandje-McKenna, M., McKenna, R., Rosser, C.J. and Boehlein, S.K. (2005). *p-37* Induces tumor invasiveness. *Molecular Cancer Therapeutics*. **4**: 1031-1038.

Khonkarn, R., Okonogi, S., Ampasavate, C. and Anuchapreeda, S. (2010). Investigation of fruit peel extracts as sources for compounds with antioxidant and antiproliferative activities against human cell lines. *Food and Chemical Toxicology*. **48**: 2122-2129.

Kodoma, N., Komuta, K. and Nanba, H. (2002). Can maitake MD-fraction aid cancer patients? *Alternative Medicine Review*. **7**: 236-239.

Krieger, J.N., Riley, D.E., Vesella, R.L., Miner, D.C., Ross, S.O. and Lange, P.H. (2000). Bacterial DNA sequences in prostate tissue from patients with prostate cancer and chronic prostatitis. *Journal of Urology*. **164**: 1221-1228.

Kushi, L. H., Doyle, C., McCullough, M. *et al*. (2012). American Cancer Society guidelines on nutrition and physical activity for cancer prevention: Reducing the risk of cancer with healthy food choices and physical activity. *CA Cancer Journal for Clinicians*. **62** (1): 30-67.

L'Esperance, E. (1931). Studies in Hodgkin's disease. *Annals of Surgery*. **93**: 162-168.

Lai, P.K. and Roy, J. (2004). Antimicrobial and chemopreventive properties of herbs and spices. *Current Medicinal Chemistry*. **11** (11): 1451-60.

Laken, S.J., Petersen, G.M., Gruber, S.B., *et al*. (1997). Familial colorectal cancer in Ashkenazim due to a hypermutable tract in *APC*. *Nature Genetics*. **17**: 79-83.

Lam, M., Carmichael, A.R. and Griffiths, H.R. (2012). An aqueous extract of *Fagonia cretica* induces DNA damage, cell cycle arrest and apoptosis in breast cancer cells via FOXO3a and p53 expression. *PLoS ONE*. **7**: 401-452.

Larson, C.M., Bubrick, M.P., Jacobs, D.M. and West, M.A. (1995). Malignancy, mortality, and medicosurgical management of *Clostridium septicum* infection. *Surgery*. **118**: 592-598.

Larson, R.A. (1988). The antioxidants of higher plants. *Phytochemistry*. **27** (4): 969-978.

Lee, J.K., Inoue, M., Otani, T., Iwasaki, M., Sasazuki, S. and Tsugane, S. (2006). Gastric cancer screening and subsequent risk of gastric cancer: A large-scale population-based cohort study, with a 13-year follow-up in Japan. *International Journal of Cancer*. **118**: 2315-2321.

Leschner, S. and Weiss, S. (2010). Salmonella-allies in the fight against cancer. *Journal of Molecular Medicine*. **88**:763-773.

Lewis, J.D., Ferrara, A., Peng, T., Hedderson, M., Bilker, W.B., Quesenberry, C.P. Jr., *et al*. (2011). Risk of bladder cancer among diabetic patients treated with pioglitazone: interim report of a longitudinal cohort study. *Diabetes Care*. **34**: 916-922.

Li, X. and Hemminki, K. (2004). Inherited predisposition to early onset lung cancer according to histological type. *International Journal of Cancer*. **112** (3): 451-457.

Lillis, J.V., Dawson, E.S., Chang, R. and White Jr, C.R. (2010). Disseminated dermal *Trichophyton rubrum* infection - an expression of dermatophyte dimorphism? *Journal of Clinical Pathology*. **37**: 1168-1169.

Lim, U., Subar, A.F. and Mouw, T. (2006). Consumption of aspartame-containing beverages and incidence of hematopoietic and brain malignancies. *Cancer Epidemiology, Biomarkers & Prevention*. **15**: 1654-1659.

Liu, R. H. (2003). Health benefits of fruits and vegetables are from additive and synergistic combination of phytochemicals. *American Journal of Clinical Nutrition*. **78**: 517-520.

Liu, R. H. (2004). Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action. *American Society for Nutritional Sciences*. **134**: 3479-3485.

Lynch, H.T., Silva, E., Snyder, C. and Lynch, J.F. (2008). Hereditary breast cancer: Part I. Diagnosing hereditary breast cancer syndromes. *The Breast Journal*. **14** (1): 3-13.

Mager, D.L. (2006). Bacteria and cancer: cause, coincidence or cure. *Journal of Translational Medicine*. **28**: 9-14.

Mager, D.L., Haffajee, A.D., Devlin, P.M., Norris, C.M., Posner, M.R. and Goodson, J.M. (2005). The salivary microbiota as a diagnostic indicator of oral cancer: A descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. *J Journal of Translational Medicine*. **3**: 27.

Magrath, I. and Bhatia, K. (1999). Breast cancer: a new Epstein-Barr virus-associated disease. *Journal of National Cancer Institute*. **91**: 1349-1350.

Mahady, G., B. and Pendland, S. (2000). Garlic and *Helicobacter pylori*. *American Journal of Gastroenterology*. **95**(1): 209.

Makower, D., Marino, P., Frank, M. and Wiernik, P.H. (1998). Familial hairy cell leukemia. *Leukemia Lymphoma*. **29**: 193-197.

Martin, J.J., Hernandez, L.S., Gonzalez, M.G., Mendez, C.P., Rey Galan, C. and Guerrero, S.M. Trends in childhood and adolescent obesity prevalence in Oviedo (Asturias, Spain) 1992-2006. *Acta Paediatrica*. **97** (7): 955-958.

Martin, K.R. and Brophy, S.K. (2010). Commonly consumed and specialty dietary mushrooms reduce cellular proliferation in MCF-7 human breast cancer cells. *Experimental Biology and Medicine*. **235**: 1306-1314.

Matakidou, A., Eisen, T. and Houlston, R.S. (2005). Systematic review of the relationship between family history and lung cancer risk. *British Journal of Cancer*. **93** (7): 825-833.

Maucort-Boulch, D., Franceschi, S. and Plummer, M. (2008). International correlation between human papillomavirus prevalence and cervical cancer incidence. *Cancer Epidemiology, Biomarkers & Prevention*. **17** (3): 717-720.

Mays, S.R., Bogle, M.A. and Bodey, G.P. (2006). Cutaneous fungal infections in the oncology patient: recognition and management. *American Journal of Clinical Dermatology*. **7**: 31-43.

McCann, D., Barrett, A. and Cooper, A. (2007). Food additives and hyperactive behaviour in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial. *Lancet*. **370** (3): 1560-1567.

McCarthy, E. F. (2006). The Toxins of William B. Coley and the Treatment of Bone and Soft-Tissue Sarcomas. *Iowa Orthopaedic Journal*. **26**: 154-158.

McDonald, E.R. and El-Deiry, W.S. (2000). Cell cycle control as a basis for cancer drug development (Review). *International Journal of Oncology*. **16**: 871-886.

McGarr, S., Ridlon, J. and Hylemon, P. (2005). Diet, anaerobic bacteria metabolism, and colon cancer: a review of the literature. *Journal of Clinical Gastroenterology*. **39**: 98-109.

McLaughlin-Drubin, M.E. and Munger, K. (2009). Viruses associated with human cancer. *Biochimica et Biophysica Acta*. **1782**: 127-150.

Michaud, D.S., Izard, J., Wilhelm-Benartzi, C.S., You, D.H., Grote, V.A., Tjonneland, A., Dahm, C.C., Overvad, K., Jenab, M., Fedirko, V. *et al*. (2012). Plasma antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study. *Gut*. doi:/2012/10.1136/gutjnl-2012-303006.

Moghaddam, A.A., Woodward, M. and Huxley, R. (2007). Obesity and risk of colorectal cancer: a meta-analysis of 31 studies with 70,000 events. *Cancer Epidemiology, Biomarkers & Prevention*. **16**: 2533-47.

Molina-Torres, J., Garcia-chavez, A. and Ramirez-chavez, E. (1999). Antimicrobial properties of alkamides present in flavouring plants traditionally used in Masoamerica: affinin and capsaicin. *Journal of Ethnopharmacology*. **64**: 241-248.

Monks, N.R., Bordignon, S.A.L., Ferraz, A., Machado, K.R., Faria, D.H., Lopes, R.M., Mondin, C.A., de Souza, I.C.C., Lima, M.F.S., da Rocha, A.B. and Schwartsmann, G. (2002). Anti-tumor screening of Brazilian plants. *Pharmaceutical Biology*. **40** (8): 603-616.

Mothana, R. A., Lindequist, U., Gruenert, R. and Bednarski, P. J. (2009). Studies of the *in vitro* anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. *BMC Complementary and Alternative Medicine*. **9** (7): 1-11.

Mousa, H.A. (2003). Bone infection. *East Mediterranean Health Journal*. **9**: 208-214.

Nakatani, N. (2000). Phenolic antioxidants from herbs and spices. *Biofactors*. **13** (1-4): 141-146.

National Cancer Institute. (2008). Cancer Vaccine Fact Sheet. Retrieved 15 November 2008.

National Cancer Institute. (2010). Lung cancer trial results show mortality benefit with low dose CT. NLST results Release.

National Cancer Institute. (2012). Cancer Trends Progress Report Update. *NIH, DHHS, Bethesda, MD*.

O'Nions, J. and Allday, M.J. (2004). Deregulation of the cell cycle by the Epstein-Barr virus. *Advance Cancer Research*. **92**: 119-186.

Oliver, S.E., May, M.T. and Gunnel, D. (2001). International trends in prostate-cancer mortality in the 'PSA ERA'. *International Journal of Cancer*. **92**: 893-898.

Osinaga, E. (2007). Expression of cancer-associated simple mucin-type O-glycosylated antigens in parasites. *Life*. **59**: 269-273.

Otang, W. M., Grierson, D. S. and Ndip, R. N. (2011). The effect of the acetone extract of *Arctotis arctotoides* (Asteraceae) on the growth and ultrastructure of some opportunistic fungi associated with HIV/AIDS. *International Journal of Molecular Sciences*. **12**: 9226-9235.

Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Spiegelhalder, B. and Bartsch, H. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer*. **36** (10): 1235-1247.

Pairojkul, C., Shirai, T., Hirohashi, S., Thamavit, W., Bhudhisawat, W., Uttaravicien, T., Itoh, M., Ito, N. (1991). Multistage carcinogenesis of liver-fluke-associated cholangiocarcinoma in Thailand. *Princess Takamatsu Symposia*. **22**: 77-86.

Parkin, D.M. (2001). Global cancer statistics in the year 2000. *Lancet Oncology*. **2**: 533-543.

Parkin, D.M. (2006). The global health burden of infection-associated cancers in the year 2002. *International Journal of Cancer*. **118**: 3030-3044.

Parkin, D.M. (2009). Is the recent fall in incidence of post-menopausal breast cancer in UK related to changes in use of hormone replacement therapy? *European Journal of Cancer*. **45** (9): 1649-1653.

Parkin, D.M., Almonte, M., Bruni, L., Clifford, G., Curado, M.P. and Pineros, M. (2008). Burden and trends of type-specific human papillomavirus infections and related diseases in the Latin America and Caribbean region. *Vaccine*. **26** (11): 1-15.

Parsonnet, J. (1995). Bacterial infection as a cause of cancer. *Environmental Health Perspectives*. **8**: 263-268.

Patyar, S., Joshi, R., Byrav, D.S.P., Prakash, A., Medhi, B. and Das, B.K. (2010). Bacteria in cancer therapy: a novel experimental strategy. *Journal of Biomedical Sciences*. **17**: 21.

Pehlivan, M., Pehlivan, S., Onay, H., Koyuncuoglu, M. and Kirkali, Z. (2005). Can mycoplasma-mediated oncogenesis be responsible for formation of conventional renal cell carcinoma? *Urology*. **65**: 411-414.

Peltomaki, P. and Vasen, H.F.A. (1997). The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. *Gastroenterology*. **113**: 1146-1158.

Pezzuto, J.M. (1997). Plant-derived anticancer agents. *Biochemical Pharmacology*. **53**: 121-133.

Phatak, S.V. and Kolwadkar, P.K. (2005). Images: Squamous cell carcinoma of the hand and wrist developing in burn scar. *Indian Journal of Radiological Imaging*. **15**: 467-8.

Pierce, J.P., Stefanick, M.L., Flatt, S.W., Natarajan, L., Sternfeld, B., Madlensky, L., *et al*. (2007). Greater survival after breast cancer in physically active women with high vegetable-fruit intake regardless of obesity. *Journal of Clinical Oncology*. **25**: 2345-51.

Potmeisel, M. and Pinedo, H. (1995). Camptothecins. New Anticancer Agents. CRC Press, Boca Raton, Florida.

Ramos, S. (2008). Cancer chemoprevention and chemotherapy: Dietary polyphenols and signalling pathways. *Molecular Nutrition & Food Research*. **52**: 507-526.

Ranade, K., Hussussian, C.J., Sikorski, R.S., *et al*. (1995). Mutations associated with familial melanoma impair $p16^{INK4}$ function. *Nature Genetics*. **10**: 114-116.

Razi, M.T., Asad, M.H., Khan, T., Chaudhary, M.Z., Ansari, M.T., Arshad, M.A., *et al*. (2011). Antihaemorrhagic potentials of Fagonia cretica against Naja naja karachiensis (black Pakistan cobra) venom. *Natural Products Research*. **25**: 1902-1907.

Restifo, N. P., Dudley, M. E. and Rosenberg, S. A. (2012). Adoptive immunotherapy for cancer: harnessing the T cell response. *Nature Reviews Immunology*. **12** (4): 269-81.

Rim, H.J. (2005). Clonorchiasis: An update. *Journal of Helminthology*. **79**: 269-281.

Robertson, E.S. (2011). Cancer Associated Viruses. *Springer*. 1-866.

Robey, I.F., Baggett, B.K., Kirkpatrick, N.D., Roe, D.J., Dosescu, J., Sloane, B.F. *et al*. (2009). Bicarbonate Increases Tumor pH and Inhibits Spontaneous Metastases. *Cancer Research*. **69**: 2260-2268.

Rock, C.L. and Demark-Wahnefried, W. (2002). Can lifestyle modification increase survival in women diagnosed with breast cancer? *Journal of Nutrition*. **132**: 3504-3507.

Rosenzweig, L.B., Brett, A.S., Lefaivre, J.F., Vandersteenhoven, J.J. (2005). Hidradenitis suppurativa complicated by squamous cell carcinoma and paraneoplastic neuropathy. *American Journal of Medical Sciences*. **329**: 150-152.

Rostom, A., Dube, C., Lewin, G., Tsertsvadze, A., Barrowman, N., Code, C., Sampson, M. and Moher, D. (2007). Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: a systematic review prepared for the U.S. Preventive Services Task Force. *Annals of International Medicine*. **146** (5): 376-89.

Rothwell, P.M., Fowkes, F.G., Belch, J.F., Ogawa, H., Warlow, C.P. and Meade, T.W. (2011). Effect of daily aspirin on long-term risk of death due to cancer: analysis of individual patient data from randomised trials. *Lancet*. **377** (9759): 31-41.

Rubinfeld, B., Albert, I., Profiri, E., *et al*. (1996). Binding of GSK-β with APC/β-catenin complex and regulation of complex assembly. *Science*. **272**: 1023-1026.

Saha, A., Kaul, R., Murakami, M. and Robertson, E.S. (2010). Tumor viruses and cancer biology modulating signalling pathways for therapeutic intervention. *Cancer biology & therapy*. **10**: 1-18.

Sakakibara, H., Honda, Y., Nakagawa, S., Ashida, H. and Kanazawa, K. (2003). Simultaneous determination of all polyphenols in vegetables, fruits, and teas. *Journal of Agricultural and Food Chemistry*. **51** (3): 571-581.

Sandhu, M.S., White, I.R. and McPherson, K. (2001). Systematic review of the prospective cohort studies on meat consumption and colorectal cancer risk: a meta-analytical approach. *Cancer Epidemiology, Biomarkers & Prevention*. **10**: 439-446.

Sankaranarayanan R, Swaminathan R, Brenner H, et al. (2010). Cancer survival in Africa, Asia, and Central America: a population-based study. *Lancet Oncology*. **11**: 165-173.

Sankaranarayanan, R. (2009). HPV vaccination: the promise & problems. *Indian Journal of Medical Research*. **130** (3): 322-326.

Sankaranarayanan, R., Black, R.J. and Parkin, D.M. (1999). Cancer survival in developing countries. Lyon: IARC Scientific Publications. 145.

Sankaranarayanan, R., Nene, B.M., Shastri, S.S., *et al*. (2009). HPV screening for cervical cancer in rural India. *New England Journal of Medicine*. **360** (14): 1385-1394.

Savitsky, K., Bar-Shira, A., Gilad, S., *et al*. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*. **268**: 1749-1753.

Schab, D.W. and Trinh, N.H. (2004). Do artificial food colors promote hyperactivity in children with hyperactive syndromes? A meta-analysis of double-blind placebo-controlled trials. *Journal of Developmental & Behavioral Pediatrics*. **25**: 423-434.

Scher, R.K. (1990). Diseases of the nails. In: Current therapy. Philadelphia, Pa: The W. B. Saunders Co. 736-742.

Schiffman, M., Castle, P.E., Jeronimo, J., Rodriguez, A.C and Wacholder, S. (2007). Human papillomavirus and cervical cancer. *Lancet*. **370**: 890-907.

Schroder, F.H., Hugosson, J., Roobol, M.J., *et al*. (2009). Screening and prostate-cancer mortality in a randomized European study. *New England Journal of Medicine*. **360** (13): 1320-1328.

Seibert, F.B., Farrelly, F.K. and Shepherd, C.C. (1967). DMSO and other combatants against bacteria isolated from leukemia and cancer patients. *Annals of the New York Academy of Sciences*. **141**: 175-201.

Seiki, M., Hattori, S., Hirayama, Y. and Yoshida, M. (1983). Human adult T-cell leukemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. *Proceedings of the National Academy of Sciences USA*. **80**: 3618.

Seradour, B., Allemand, H., Weill, A. and Ricordeau, P. Changes by age in breast cancer incidence, mammography screening and hormone therapy use in France from 2000 to 2006. *Bull Cancer*. **96** (4): 1-6.

Shafey, O., Eriksen, M., Ross, H. and Mackay, J. (2009). The Tobacco Atlas Third Edition. Atlanta, GA: American Cancer Society.

Sherris, J., Wittet, S., Kleine, A., *et al*. (2009). Evidence-based, alternative cervical cancer screening approaches in low-resource settings. *International Perspective on Sex and Reproductive Health*. **35** (3): 147-154.

Shirataki, Y., Motohashi, N., Tani, S., Sakagami, H., Satoh, K., Nakashima, H. and Mahapatra, S. K. (2001). *In* vitro biological activity of prenylflavanones. *Anticancer Research*. **21**:275-280.

Sibanda, T., and Okoh, A. I. (2008) *In vitro* evaluation of the interactions between acetone extracts of *Garcinia kola* seeds and some antibiotics. *African Journal of Biotechnology*. **7**: 1672-1678.

Silva, S., Hooper, S.J., Henriques, M., Oliveira, R., Azeredo, J. and Williams, D.W. (2011). The role of secreted aspartyl proteinases in *Candida tropicalis* invasion and damage of oral mucosa. *Clinical Microbiology and Infection*. **17**: 264-272.

Sivam, G. P. (2001). Protection against *Helicobacter pylori* and other bacterial infection by garlic. *Journal of Nutrition*. **131**(3s): 1106S-1108S.

Skog, K.I., Johansson, M.A.E. and Jagerstad, M.I. (1998). Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurrence, and intake. *Food and Chemical Toxicology*. **36**: 879-896.

Smith, R.A., Cokkinides, V. and Brawley, O.W. (2009). Cancer screening in the United States, 2009: a review of current American Cancer Society guidelines and issues in cancer screening. *CA Cancer Journal for Clinicians*. **59** (1): 27-41.

Soffritti, M., Belpoggi, F., Esposti, D.D. and Lambertini, L. (2005). Aspartame induces lymphomas and leukaemias in rats. *European Journal of Oncolology*. **10**: 107-116.

Spano, S. and Galan, J. (2012). A Rab32-Dependent Pathway Contributes to *Salmonella* Typhi Host Restriction. *Sciences***. 338**: 960-963.

Sripa, B. and Kaewkes, S. (2000). Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. *International Journal of Parasitology*. **30**: 735-740.

Steinmetz, K. A. and Potter, J. D. (1991). Vegetables, fruits and cancer. I. Epidemiology. *Cancer Causes & Control*. **2**: 325-357.

Steinmetz, K. and Potter, J.D. (1996). Vegetables, fruit and cancer prevention: a review. *Journal of American Diet Association*. **96**: 1027-39.

Sullivan, J.L. (1999). The abnormal gene in X-linked lymphoproliferative syndrome. *Current Opinion in Immunology*. **11**: 431-434.

Takeuchi, H., Arai, Y., Konami, T., Ikeda, T., Tomoyoshi, T. and Tatewaki, K. (1983). A study on urinary fungal infection. *Hinyokika Kiyo*. **29**: 1273-1277.

Tanih, N. F., McMillan, M., Naidoo, N., Ndip, L. M., Weaver, L. T. and Ndip, R. N. (2010b). Prevalence of *Helicobacter pylori vacA, cagA* and *iceA* genotypes in South African patients with upper gastrointestinal diseases. *Acta Tropica*. **116** (1): 68-73.

Tanih, N. F., Okeleye, B. I., Green, E., Mkwetshana, N., Clarke, A. M., Ndip, L. M. and Ndip, R. N. (2010a). Marked susceptibility of South African *Helicobacter pylori* strains to ciprofloxacin and amoxicillin: clinical implications. *South African Medical Journal.* **100**(1):49-52.

Tanih, N.F., Okeleye, B.I., Ndip, L.M., Clarke, A.M., Naidoo, N., Mkwetshana, N.T., Green, E. and Ndip, R.N. (2010c). *Helicobacter pylori* prevalence in dyspeptic patients in the Eastern Cape Province – race and disease status. *South African Medical Journal.* **100**: 734-737.

Thomas, F., Lafferty, J., Brodeur, E. and Elguero, M. (2011). Incidence of adult brain cancers is higher in countries where the protozoan parasite Toxoplasma gondii is common. *Biology Letters*. doi: 10.1098/rsbl.2011.0588.

Thompson, D. and Easton, D.F. (2002). The Breast Cancer Linkage Consortium. Cancer incidence in *BRCA1* mutation carriers. *Journal of the National Cancer Institute*. **94** (18): 1358-1365.

Thompson, D. and Easton, D.F. (2002). The Breast Cancer Linkage Consortium. Cancer incidence in *BRCA1* mutation carriers. *Journal of the National Cancer Institute* **94**: 1358-1365.

Tsoi, K.K., Pau, C.Y., Wu, W.K., Chan, F.K., Griffiths, S. and Sung, J.J. (2009). Cigarette smoking and the risk of colorectal cancer: a meta-analysis of prospective cohort studies. *Clinical Gastroenterology and Hepatology*. **7**: 682-688.

Tsutsumi, R., Higashi, H., Higuchi, M., Okada, M. and Hatakeyama, M. (2003). Attenuation of *Helicobacter pylori CagA x SHP-2* signaling by interaction between *CagA* and C-terminal *Src* kinase. *Journal of Biological Chemistry*. **278**: 3664-3670.

Tzfira, T., Kunik, T., Gafni, Y., Citovsky, V. (2006). Mammalian cells. *Methods in Molecular Biology*. **344**: 435-51.

Umadevi, M., Kumar, K. P. S., Bhowmik, D., Duraivel, S. (2013). Traditionally used anticancer herbs in India. *Journal of Medicinal Plants Studies*. **1** (3): 56-74.

Ushio, S., Iwaki, K., Taniai, M., Otha, T., Fukuda, S. and Sugimura, K. (1995). Metastasis-promoting activity of a novel molecule, Ag 243-5, derived from mycoplasma, and the complete nucleotide sequence. *Microbiology and Immunology*. **39**: 393-400.

van den Brandt, P.A., Goldbohm, R.A., van't Veer, P., Volovics, A., Hermus, R.J., Sturmans, F. (1990). A large-scale prospective cohort study on diet and cancer in the Netherlands. *Journal of Clinical Epidemiology*. **43**: 285-295.

Verma, S.C. and Robertson, E.S. (2003). Molecular biology and pathogenesis of Kaposi sarcoma-associated herpesvirus. *Federation of European Microbiological Societies Microbiology Letters*. **222**: 155-63.

Vizcaino, A.P., Moreno, V., Bosch, F.X., *et al*. (2000). International trends in incidence of cervical cancer: II. Squamous-cell carcinoma. *International Journal of Cancer*. **86** (3): 429-435.

Walboomers, J.M., Jacobs, M.V., Manos, M.M., Bosch, F.X., Kummer, J.A., Shah, K.V., *et al*. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology*. **189**: 12-19.

Walsh, T., Casadei, S., Coats, K.H., *et al*. (2006). Spectrum of mutations in *BRCA1*, *BRCA2*, *CHEK2*, and *TP53* in families at high risk of breast cancer. *Journal of the American Medical Association*. **295** (12): 1379-1388.

Wang, S.Y. and Lewers, K.S. (2007). Antioxidant activities and anticancer cell proliferation properties of wild strawberries. *Journal of the American Society for Horticultural Science*. **132**: 647-658.

Watson, M., Saraiya, M. and Ahmed, F. (2008). Using population-based cancer registry data to assess the burden of human papillomavirus-associated cancers in the United States: overview of methods. *Cancer*. **113**: 2841-2854.

Weig, M., Werner, E., Frosh, M. and Kasper, H. (1999). Limited effect of refined carbohydrate dietary supplementation on colonization of the gastrointestinal tract of healthy subjects by *Candida albicans*. *American Journal of Clinical Nutrition*. **69**: 1170-1173.

Wells, S.I., Francis, D.A., Karpova, A.Y., Dowhanick, J.J., Benson, J.D. and Howley, P.M. (2000). Papillomavirus *E2* induces senescence in HPV-positive cells via *pRB*- and *p21* (CIP)-dependent pathways. *EMBO J*. **19**: 5762-5771.

Wilson, J.M.G. and Jungner, G. (1968). Principles and practice of screening for disease. Geneva: World Health Organization. *Public Health Papers*. **34**.

Winawer, S.J. (2007). The multidisciplinary management of gastrointestinal cancer. Colorectal cancer screening. *Best Practice & Research Clinical Gastroenterology*. **21** (6): 1031-1048.

Wolf, A.M., Wender, R.C., Etzioni, R.B., *et al*. (2010). American Cancer Society guideline for the early detection of prostate cancer: update 2010. *CA Cancer Journal for Clinicians*. **60** (2): 70-98.

Wolin, K.Y., Yan, Y., Colditz, G.A. and Lee, I.M. (2009). Physical activity and colon cancer prevention: a meta-analysis. *British Journal of Cancer*. **100**: 611-616.

Worsham, M.J., Raju, U., Lu, M., *et al*. (2009). Risk factors for breast cancer from benign breast disease in a diverse population. *Breast Cancer Research and Treatment*. **118** (1): 1-7.

Xiao, C.H., Yang, S.S. and Hong, X.K. (2000). The Chemistry of Traditional Chinese Medicines. Shanghai Science and Technology Publishing House, Shanghai.

Yang, C.S., Landau, J.M., Huang, M.T. and Newmark, H.L. (2001). Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annual Review Nutrition*. **21**: 381-406.

Yang, G., Ma, J., Liu, N. and Zhou, L. (2005). Smoking and passive smoking in Chinese, 2002. *Chinese Journal of Epidemiology*. **26** (2): 77-83.

Yang, Y. (2003). Virulence factors of Candida species. *Journal of Microbiology, Immunology and Infection*. **36**: 223-228.

Yeh, J.M., Goldie, S.J., Kuntz, K.M. and Ezzati, M. (2009). Effects of Helicobacter pylori infection and smoking on gastric cancer incidence in China: a population-level analysis of trends and projections. *Cancer Causes Control*. **1**: 3-9.

Youlden, D.R., Cramb, S.M. and Baade, P.D. (2008). The International Epidemiology of Lung Cancer: geographical distribution and secular trends. *Journal of Thoracic Oncology*. **3** (8): 819-831.

Zaias, N., Glick, B. and Rebell, G. (1996). Diagnosing and treating onychomycosis. *Journal of Family Practice*. **42**: 513-518.

Zhang, M., Huang, J. and Xie, X. (2009). Dietary intakes of mushrooms and green tea combine to reduce the risk of breast cancer in Chinese women. *International Journal of Cancer*. **124**: 1404-1408.

Zheng, L., Weilun, Z., Minghong, J., Yaxi, Z., Shilian, L., Yanxin, L., *et al*. (2012). Adeno-associated virus-mediated doxycycline-regulatable TRAIL expression suppresses growth of human breast carcinoma in nude mice. *BMC Cancer*. **12**: 153.

Zheng, W. and Wang, S.Y. (2001). Antioxidant activity and phenolic compounds in selected herbs. *Journal of Agricultural and Food Chemistry*. **49** (11): 5165-5170.

CHAPTER THREE

Evaluation of the Antibacterial and Antifungal Potential of *Peltophorum africanum*: Toxicological Effect on Human Chang Liver Cell Line

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ABSTRACT

We assessed the *in vitro* antimicrobial activity of *Peltophorum africanum* by means of the agar well and macrodilution methods. The toxicity on a normal human liver cell (Chang liver cell) was determined using the CellTiter-Blue cell viability assay, and the compounds contained in the fractions were identified using GC-MS. Zone diameter of inhibition of the extract ranged from 12.5 ± 0.7 to 32 ± 2.8 mm for bacteria and from 7.5 ± 0.7 to 26.4 ± 3.4 mm for yeast. Marked activity of the extract was observed against *Plesiomonas shigelloides* ATCC 51903, with MIC and MLC values of 0.15625 and 0.3125mg/mL, respectively. The extract was both bactericidal (MICindex $\leq$ 2) and bacteriostatic/fungistatic (MICindex > 2) in activity. Lethal dose at 50 (LD_{50}) showed 82.64 ± 1.40 degree of toxicity at 24 hrs, and 95 percentile of cell death dose activity ranged from log 3.12 ± 0.01 to 4.59 ± 0.03. The activity of the eight fractions tested ranged from 1.0 ± 0.5 to 3.7 ± 1.6 mg/mL (IC_{50}) and from 2.1 ± 0.8 to 6.25 ± 0 mg/mL (IC_{90}). The extract was toxic to human Chang liver cell lines.

3.1. INTRODUCTION

Following the invention of modern medicine, herbal medicine suffered a setback, but recent advances in photochemistry and identification of plant compounds that are effective against life-threatening diseases have enhanced interest in it. In many developing countries, traditional medicine is one of the primary healthcare systems (Farnsworth, 1993; Houghton, 1995). Extracts and bioactive compounds with known antimicrobial activities isolated from plants can be of great impact in the formulation of new drugs, and the potential of higher plants as source for new drugs is still largely unexplored (Satish *et al*., 2008). The increasing prevalence of multidrug-resistant microbial strains due to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agent, and continuous epidemics of HIV infection adds urgency to the search for alternative treatment. Most horrible human diseases are caused by bacteria, viruses, fungi, and parasitic worms (Mahesh and Satish, 2008). *Staphylococcus aureus*, a Gram positive bacterium and an opportunistic pathogen of the human skin, has been incriminated in wound infection, toxic shock syndrome, and food poisoning (Halcon and Milkus, 2004), while *Pseudomonas aeruginosa* is a pathogen associated with pyogenic and urinary tract infections (Kumar *et al*., 2012). *Plesiomonas shigelloides* infections occur in the summer months and correlate with environmental contamination of freshwater and have been implicated in gastroenteritis (Krovacek *et al*., 2000). Fungi especially *Candida* sp. and *Cryptococcus* sp. are increasingly being recognized as major pathogens in critically ill patients (Mishra *et al*., 2010).

Peltophorum africanum (Sond Fabaceae), an African wattle (isiKhaba-mkhombe in Xhoza and Musese in Venda) is a semideciduous to deciduous trees, traditionally used in South Africa to alleviate gastric problems, human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), and infertility (Okeleye *et al*., 2010). Though the ethyl acetate extract of

the stem bark of *P. africanum* has been previously investigated for its antimicrobial activity against clinical strains of *Helicobacter pylori* and other pathogens (Okeleye *et al*., 2010); there is a dearth of information on its toxicity. This study was therefore aimed at elucidating the probable compounds responsible for the antimicrobial activity of the plant extract as well as evaluating its safety in an effort to validate its folkloric use in the treatment of microbial infections.

3.2. MATERIALS AND METHODS

3.2.1. Plant extract

The ethyl acetate extract of the stem bark of *P. africanum* was selected based on the remarkable activity reported in our previous study (Okeleye *et al*., 2010). The extract was prepared as described in our previous study with modifications. Briefly, dried powder of the stem bark (200 g) was extracted with 96% ethyl acetate (800 mL) and filtered after 48 hrs. The plant residue was reextracted exhaustively (three filtration processes), and the filtrate was concentrated on a rotary evaporator (Strike 202 Steroglass, Italy) at 70 ^{o}C to remove the ethyl acetate. Fresh working stock of the extract was prepared by sterilizing in 100% DMSO for each bioassay analysis. The extract was aseptically bottled using Acrodisc 25mm PF Syringe (Pall, USA) and then tested for sterility by putting 0.5mL of the extract into 2.5mL of nutrient broth. A sterile extract was indicated by a clear broth (absence of turbidity) after incubation at 37 ^{o}C for 24 hrs. The extracts were kept at 4 ^{o}C until use.

3.2.2. Test organisms

The microorganisms used were obtained from our microbial stock collection in the Department of Biochemistry and Microbiology University of Fort Hare, South Africa. The bacteria included *Staphylococcus aureus* NCTC 6571, *Pseudomonas aeruginosa* ATCC 15442, *Plesiomonas shigelloides* ATCC 51903, *Helicobacter pylori* ATCC 43526, *Streptococcus pyogenes* ATCC 49399, *Aeromonas hydrophila* ATCC 35654, *Shigella sonnei* ATCC 29930, and *Salmonella Typhimurium* ATCC 13311. The fungi included *Aspergillus flavus* ATCC 204304, *Aspergillus niger* ATCC 16888, *Candida albicans* ATCC 2091, and *Cryptococcus neoformans* ATCC 66031. All bacteria and fungi cultures were subcultured thrice for purity. The fungi were

inoculated in Sabouraud dextrose broth and bacteria into nutrient broth (*H. pylori* was inoculated in brain heart infusion broth with Skirrow's supplement and 10% horse serum) and incubated for 24 hrs at 37 ^{o}C (*H. pylori* was incubated microaerophilically in anaerobic jar with gas pack). The turbidity of the culture was adjusted with sterile saline solution to match 0.5McFarland standards.

3.2.3. Cell line Growth and Maintenance

The human Chang liver cell line used in this study was a kind donation from Professor Maryna van de Venter of Nelson Mandela Metropolitan University, South Africa. Briefly, vials containing cells were taken from liquid nitrogen stocks. The cells were thawed in a water bath (37 ^{o}C) and transferred to a 25mm3 culture flask (TPP, Switzerland). A 1mL thawed cell stock was diluted with 9mL prewarmed Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were incubated in a 37 ^{o}C humidified incubator (Shel Lab, USA), 5% CO2 for multiplication and adherence. Maintenance of cells was achieved by splitting the cells until the desired cell number and confluence was reached.

3.2.4. Antimicrobial Assay

The agar well diffusion method was used as previously described (Ndip *et al*., 2007; Okeleye *et al*., 2011). Agar plates were prepared using sterile brain heart infusion agar (Oxoid, England) with Skirrow's supplement and 10% horse serum for *H. pylori*, Mueller-Hinton (MH) agar (Merck, Gauteng, South Africa) for bacteria and potato dextrose agar (PDA) (Lab M, UK) for fungi. Strains of standardized cultures

were evenly spread onto the surface of the agar plates using sterile swab sticks. Wells were punched in the plates using a sterile stainless 6mm cork borer. The wells were filled with 100 μL of 25mg/mL, 50mg/mL, and 100mg/mL of the extract. Ten percent DMSO was used as a negative control and 30 μg/ mL of amoxicillin and tetracycline as positive controls. Diffusion of the extracts, antibiotics, and DMSO was allowed at room temperature for 30mins in a laminar flow cabinet and then incubated at 37 ^{o}C (fungi, 27 ^{o}C) for 24–72 hrs. Each experiment was conducted in duplicate, and the zone diameters of inhibition (mean ± SD) produced by each of the concentrations of the solutions were measured in millimeters and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guideline (Wikler, 2008).

3.2.5. Minimum inhibitory concentration (MIC) determination

The MIC was determined according to the method of Delahaye *et al*. (2009) with modification. Different concentrations (0.005–10.0mg/mL) of the extract were prepared by two fold serial dilutions in brain heart infusion broth (Oxoid, England) with Skirrow's supplement and 10% horse serum, Mueller-Hinton broth (Merck, Gauteng, South Africa) and sabouraud dextrose broth (Lab M, UK) for *H. pylori*, other bacterial strains, and fungi, respectively. Each tube was inoculated with 100 μL of each of the adjusted microbial strain and incubated at 37 ^{o}C (fungi, 27 ^{o}C) for 24–72 hrs. Tetracycline and amoxicillin (30 μg/mL) were used as positive controls, and two tubes each containing medium with/without a microbial strain and treatment were used as negative control. After the incubation period, the first tube in the series of concentrations that showed no visible trace of growth was taken as the MIC.

3.2.6. Minimum lethal concentration (MLC) assay

Fresh nutrient agar (Merck, Gauteng, South Africa), PDA (Lab M, UK), and Columbia blood agar (Oxoid, England) plates prepared for bacteria, fungi, and *H. pylori*, respectively, were inoculated with one loopful of culture taken from each of the MIC tubes for the determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC). After the incubation periods, the lowest concentration of the extract that did not produce any or >90% bacterial or fungal growth on the solid medium was regarded as the minimum lethal concentration (MLC) of the extract. The mechanism of antibiosis of the extracts was calculated using the ratio of MLC/MIC. When the ratio of MLC/MICindex was ≤2, the extract was considered as bactericidal/ fungicidal otherwise as bacteriostatic/fungistatic. If the ratio was ≥16 the extract was considered as ineffective (Delahaye *et al*., 2009; Shanmughapriya *et al*., 2008).

3.2.7. Assay for cytotoxicity

Toxicity of the extract was evaluated on human Chang liver cell lines using microculture CellTiter-Blue viability (Promega, USA) assay (Merghoub *et al*., 2009). For the assay, 96-well microplates were seeded with 100 μL DMEM + high glucose, L-glutamine and sodium pyruvate (Thermo Sceintific, South Logan, Utah, USA) containing 3.0×10^3 cells in suspension and incubated in a CO_2 incubator regulated at 37 ^oC and 5% CO2. After 24 hrs incubation and attachment, the cells were treated with 1000, 500, 250, 125, 75, 25, and 5 μg/mL concentration of the extract. Exactly 60 μM of curcumin (Sigma-Aldrich, South Africa) was used as positive control and 0.1 % DMSO as negative control. After 24, 48, and 72 hrs of incubation, cell viability was determined by adding CellTiter-Blue as an indicator and further incubated for 4hrs.

Fluorescence was read at 570/620 nm using Analytical & Diagnostic Product Gen spectrophotometer (BioTek, USA).

3.2.8. Fractionation of the extract and antimicrobial activity of the fractions

The ethyl acetate extract of *P. africanum* was fractionated using two solvent systems; toluene/ethanol (TEt; 90 : 5) and benzene/ethanol/ammonium hydroxide (BEtA; 90 : 10 : 1) using thin layer chromatography (TLC) and column chromatography methods (Yrjönen *et al*., 2003; Masoko and Eloff, 2006). The inhibitory effect of the fractions was carried out in accordance with our previously reported method (Okeleye *et al*., 2011). Briefly, twofold dilutions of the fractions (0.049–6.25mg/mL) were prepared in 96-well plates containing brain heart infusion broth (Oxoid, England) with Skirrow's supplement and 10% horse serum, Mueller-Hinton broth (Merck, Gauteng, South Africa), sabouraud dextrose broth (Lab M, UK) for *H. pylori*, other bacteria, and yeast cell, respectively. Exactly 25 μL of each strain (0.5 McFarland standards) was added into the wells and the assay performed in duplicate. After incubation at 37 ^{o}C for 24 hrs (bacteria) and 27 ^{o}C for 3 days (yeast), the absorbance was read at 620nm with Analytical & Diagnostic Product Gen spectrophotometer (BioTek, USA). Concentration at which 50% (IC_{50}) and 90% (IC_{90}) of microbial growth were inhibited was determined.

3.2.9. GC-MS analysis for the identification of compounds in the fractions

The fractions were dissolved and injected onto a GC column and helium was used as the carrier gas with injection volume of 1 μL. Injection temperature and MS transfer were set at 280 ^{o}C with acquisition mode scanning mass range of 40 to 550 m/z (electron ionization at 70 Ev). Agilent

6890N GC with CTC Combi-PAL Auto sampler and Agilent 5975BMS with Rtx-5MS (30 m, 0.25mm ID, 0.5 μm film thickness) Restek 12723-127 were used for the analysis (Adams, 1989).

3.2.10. Ethical Consideration

This study which is a continuation of our line of studies on microbial pathogens and anti-infective from medicinal plants had been approved by the institutional review board of the University of Fort Hare.

3.3. RESULTS

3.3.1. Susceptibility and resistant phenotype

The ethyl acetate extract of *P. africanum* was tested against pathogenic bacteria, yeast, and mold in this study. All the eight bacteria and two yeast tested were susceptible at the three concentrations of 25, 50, and 100mg/mL, with the zone diameter of inhibition ranging from 12.5 ± 0.7 to 32 ± 2.8 mm. For yeast, it ranged from 7.5 ± 0.7 to 26.4 ± 3.4 mm, while, for mold, no activity was observed. *Helicobacter pylori* ATCC 43526, *Streptococcus pyogenes* ATCC 49399, *Shigella sonnei* ATCC 29930, *Salmonella Typhimurium* ATCC 13311, *Aspergillus flavus* ATCC 204304, *Aspergillus niger* ATCC 16888, and *Cryptococcus neoformans* ATCC 66031 were resistant against tetracycline (positive control antibiotic) at 30 μg/mL (Table 3.1).

Table 3.1: Antimicrobial susceptibility test of the ethyl acetate extract compared with standard antibiotics

Test bacteria and fungi	**EAE.* 25mg/mL**	**EAE. 50mg/mL**	**EAE. 100mg/mL**	**Tetra.* 30µg/mL**	**Amox.* 30µg/mL**
Helicobacter pylori (ATCC 43526)	12.5±0.7	14.25±0.4	17.11±0.2	0	0
Pseudomonas aeruginosa (ATCC 15442)	16±1.4	16.5±0.7	18±1.4	7.5±0.7	0
Plesiomonas shigelloides (ATCC 51903)	24.5±3.5	26.5±2.1	32±2.8	26.7±2.3	0
Streptococcus pyogenes (ATCC 49399)	17.6±0.5	19.9±1.2	24.1±2.4	0	0
Aeromonas hydrophila (ATCC 35654)	17.5±0.7	21.5±2.1	24.9±2.8	28.5±2.1	0
Staphylococcus aureus (NCTC 6571)	12.8±0.4	20.7±0.4	25.6±0.6	27.1±1.3	0
Shigella sonnei (ATCC 29930)	18.6±0.9	19.1±0.2	21.9±1.2	0	0
Salmonella Typhimurium (ATCC 13311)	19.1±0.2	21.5±2.1	24.1±2.4	0	0
Aspergillus flavus (16870)	0	0	0	0	0
Aspergillus niger (ATCC 16888)	0	0	0	0	0
Candida albicans (ATCC 2091)	16.9±0.2	21.5±2.1	26.4±3.4	38.1±0.2	36.9±1.7
Cryptococcus neoformans (ATCC 66031)	7.5±0.7	12.5±0.7	14.25±0.4	0	0

*EAE., Ethyl acetate extract; Tetra., Tetracycline; Amox., Amoxicillin.

3.3.2. Mechanism of antibiosis

The extract had bactericidal effect (MLC/MICindex ≤ 2) on *P. aeruginosa, P. shigelloides, A. hydrophila*, *S. aureus*, and *S. Typhimurium*. Bacteriostatic/ fungistatic effect (MLC/MICindex > 2) was noted against *H. pylori*, *S. pyogenes*, *S. sonnei*, and *C. albicans*. Marked activity was observed against *P. shigelloides* with MIC and MLC values of 0.15625 and 0.3125mg/mL, respectively (Table 3.2).

Table 3.2: Mechanism of antibiosis using MLC/MIC_{index} of the extract against test organisms

	Ethyl acetate extract (mg/mL)			Tetracycline (µg/mL)	
Test bacteria and yeast	**MIC***	**MLC***	**MLC/MIC_{index}**	**MIC**	**MBC***
Helicobacter pylori (ATCC 43526)	2.5	10	4	ND*	ND
Pseudomonas aeruginosa (ATCC 15442)	0.625	1.25	2	ND	ND
Plesiomonas shigelloides (ATCC 51903)	0.15625	0.3125	2	10	> 10
Streptococcus pyogenes (ATCC 49399)	0.625	2.5	4	ND	ND
Aeromonas hydrophila (ATCC 35654)	2.5	5	2	10	> 10
Staphylococcus aureus (NCTC 6571)	2.5	5	2	> 10	> 10
Shigella sonnei (ATCC 29930)	1.25	5	4	ND	ND
Salmonella Typhimurium (ATCC 13311)	1.25	2.5	2	ND	ND
Candida albicans (ATCC 2091)	5	> 10	> 2	10	> 10
Cryptococcus neoformans (ATCC 66031)	10	ND	ND	ND	ND

*MIC, Minimum inhibitory concentration; MLC, Minimum lethal concentration; MBC, Minimum bactericidal concentration; ND, Not determined.

3.3.3. Cytotoxicity effect

We observed that at 5, 25, 50, 75, and 95 percentile of cell death after 24, 48, and 72 hrs of incubation, the extract dose activity ranged from log to 0.75 ± 0.03–1.05 ± 0.01, 0.82 ± 0.02 to 1.66 ± 0.01, 1.92 ± 0.01 to 2.15 ± 0.00, 2.51 ± 0.00 to 3.01 ± 0.01, and 3.12 ± 0.01 to 4.59 ± 0.03, respectively (Table 3.3). Lethal dose at 50 (LD_{50}) showed 82.64 ± 1.40, 140.09 ± 1.33, and 121.07 ± 0.92 μg/mL degree of toxicity at 24, 48 and 72 hrs, respectively (Table 3.4).

Table 3.3: Effective concentration and time interval on the percentage cell death

Percentile	**Probit**			**Log (Dose)***		
	24 hrs	**48 hrs**	**72 hrs**	**24 hrs**	**48 hrs**	**72 hrs**
5	3.36	3.36	3.36	-0.75±0.03	0.76±0.01	1.05±0.01
25	4.33	4.33	4.33	0.82±0.02	1.58±0.01	1.66±0.01
50	5.00	5.00	5.00	1.92±0.01	2.15±0.00	2.08±0.00
75	5.68	5.68	5.68	3.01±0.01	2.72±0.01	2.51±0.00
95	6.65	6.65	6.65	4.59±0.03	3.54±0.01	3.12±0.01

*Antilog which gives lethal dose in µg/mL. Probit analysis NCSS 2007 used to determine log (Dose), percentile and probit values.

Table 3.4: Lethal dose at 50 percent reduction of the cell population

Dose (µg/mL)	Probit percent			Actual percent*			LD_{50} (µg/mL)*		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
5	25.24	4.34	1.37	19.23	3.87	5.21	-	-	-
25	31.51	18.80	13.75	29.48	10.68	5.34	-	-	-
75	38.36	37.41	37.02	34.96	24.65	16.96	82.64±1.40	-	-
125	45.59	47.66	50.88	55.96	65.03	70.68	-	140.09±1.33	121.07±0.92
250	51.03	61.70	69.21	60.12	71.17	79.41	-	-	-
500	62.54	74.33	83.68	61.85	72.16	82.88	-	-	-
1000	77.35	84.37	92.80	70.98	80.86	88.43	-	-	-

*LD_{50} (50 % of the cells have been killed); actual and probit percent were calculated using probit statistical analysis software "NCSS 2007"; *Actual % = Actual formulas (n is the number of cells in a group); -, not applicable.

3.3.4. *In vitro* antimicrobial activity of the fractions from the ethyl acetate extract of *P. africanum*

Activity of the fractions on the bacteria and yeast cells are shown in Table 3.5. Fractions BEtA2, BEtA4, T1, and BEtA3 inhibited 50 % of test organisms (IC_{50}) at 1.0 ± 0.5, 1.0 ± 0.6, 1.6 ± 1.7, and 1.7 ± 0.9 (mg/mL), meanwhile 90 % were inhibited (IC_{90}) at 2.5 ± 0.8, 3.1 ± 2.3, 2.1 ± 0.8, and 3.8 ± 1.9 (mg/mL), respectively. Of the four fractions, the best activity against yeast (*C. albicans*) was demonstrated by BEtA4 with IC_{50} and IC_{90} values of 0.391 and 1.563 mg/mL, respectively, while for the bacteria, the same fraction exhibited marked activity against *P. aeruginosa* with IC_{50} and IC_{90} of 0.024 and 0.097 mg/mL, respectively.

Table 3.5: Inhibitory concentration (IC_{50} & $_{90}$) of the eight isolated bioactive fractions (mg/mL)

BACTERIA/YEAST	T1	T2	TEt1	TEt9	TEt10	BEtA2	BEtA3	BEtA4
A. hydrophila IC_{50}	0.391	3.125	3.125	1.563	1.563	1.563	1.563	1.563
IC_{90}	1.563	ND*	ND	3.125	3.125	3.125	3.125	3.125
H. pylori IC_{50}	6.25	3.125	6.25	3.125	6.25	1.563	ND	1.563
IC_{90}	ND	ND	ND	ND	ND	3.125	ND	3.125
P. aeruginosa IC_{50}	1.563	3.125	3.125	3.125	1.563	0.781	1.563	0.024
IC_{90}	ND	ND	ND	ND	6.25	1.563	3.125	0.097
P. shigelloides IC_{50}	1.563	3.125	3.125	3.125	3.125	1.563	1.563	0.781
IC_{90}	3.125	ND	ND	ND	ND	3.125	6.25	1.563
S. aureus IC_{50}	0.781	3.125	3.125	1.563	1.563	0.781	0.781	0.781
IC_{90}	1.563	6.25	6.25	3.125	3.125	3.125	1.563	6.25
S. pyogenes IC_{50}	0.781	3.125	1.563	3.125	3.125	0.781	1.563	0.391
IC_{90}	3.125	ND	6.25	ND	ND	1.563	3.125	1.563
S. sonnei IC_{50}	0.781	3.125	3.125	1.563	1.563	0.391	1.563	1.563
IC_{90}	1.563	ND	ND	3.125	ND	1.563	3.125	6.25
S. Typhimurium IC_{50}	0.391	3.125	3.125	1.563	3.125	0.781	3.125	1.563
IC_{90}	1.563	ND	ND	3.125	ND	3.125	6.25	1.563
C. albicans IC_{50}	1.563	3.125	ND	0.781	1.563	0.781	0.781	0.391
IC_{90}	ND	6.25	ND	6.25	ND	1.563	1.563	1.563
C. neoformans IC_{50}	1.563	3.125	6.25	1.563	ND	1.563	3.125	1.563
IC_{90}	ND	ND	ND	3.125	ND	3.125	6.25	6.25
IC_{50}	1.6±1.7	3.125±0	3.7±1.6	2.1±0.9	2.6±1.6	1.0±0.5	1.7±0.9	1.0±0.6
IC_{90}	2.1±0.8	6.25±0	6.25±0	3.7±1.3	4.2±1.8	2.5±0.8	3.8±1.9	3.1±2.3

*ND., Not determined.

3.3.5. Identified compounds in the fractions of ethyl acetate extract of *P. africanum*

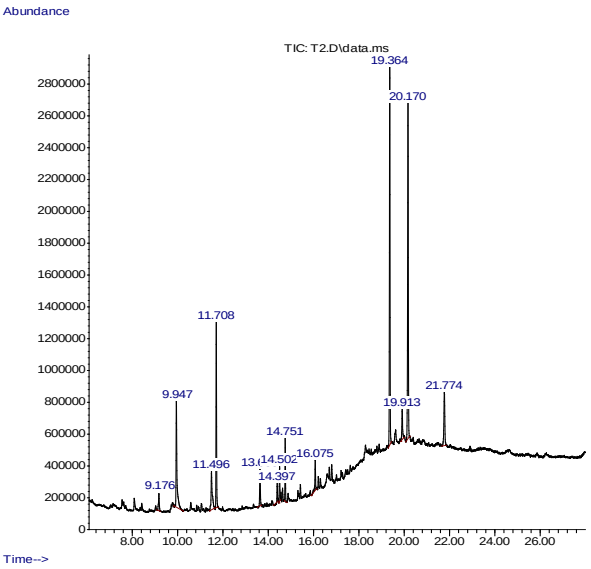
Though we proceeded to determine the possible compounds responsible for the observed activities in these fractions, Table 3.6 gives only the compounds identified in BEtA4, which had the best activity. However, volatile compounds identified in the fractions are those mostly found in food and medicine which are grouped into family of compounds such as hydrocarbon (cholest-2- eno[3,2-a]naphthalene, 4'-dimethylamino; 0.2% TP; BEtA3), alcohol (9-O-pivaloyl-N acetylcolchinol; 17% TP; TEt1), carbonyls (6-phenylamino-1H-pyrimidine-2,4-dione; 2.7% TP; BEtA3), acids (isopimaric acid TMS; 0.1% TP; BEtA2), esters (stig-masta-5,22-dien-3-ol, acetate, (3.beta., 22Z); 0.8% TP; T1), ethers (1-monolinoleoylglycerol trimethylsilyl ether, 2.1% TP, BEtA3; beta.-Sitosterol trimethylsilyl ether, 8.8% TP, BEtA2), acetals (cyclic 3,23-acetal with acetone, 0.2% TP, BEtA2; 3,4-dimethyl-1-dimethyl (allyl)-silyloxycyclohexane, 0.9% TP, BEtA3), halogens (3- bromomethyl-3,6,6-trimethyl-cyclo-hexene; 2.6% TP; T2), amides (N-methyl-1-adamantaneac- etamide; 3.4% TP; TEt10), phenols (phenol, 4-chloro-6-iodo-2-(4-iodophenyliminomethyl) and silane, trimethyl[5-methyl-2-(1-methylethyl) phenoxy]; 0.9% TP; BEtA3), bases (19-Norpregna-1, 3,5(10)-trien-20-yn-2-amine, 3,17-bis[(trimethylsilyl)oxy], (17.alpha.); 1.8% TP; BEtA4), ketones (1-(2-naphthyloxy)- 4-nitroanthraquinone; 0.2% TP; BEtA2), furans (fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl), 3.4% TP, T2; Furan, 2-(diphenylamino)-4-(morpholinocar- bonyl)-5-(pnitrophenyl), 1%TP, BEtA3 and BEtA2 (1.3% TP)), coumarins (2H-1,3-dithiolo[4,5-c]coumarine, 2-dicyanomethylene-8- nitro; 0.4% TP; BEtA3), and sulfur compounds (2-(4-methoxy- phenyl)-7-oxo-7H-[1,2,4]triazolo[5,1-b][1,3]thiazine- 5-carboxylic acid, methyl ester; 0.3 % TP; BEtA3) (Table 3.6 and Figure 4.1).

Table 3.6: Identified compounds (twenty) from the BEtA4 fraction and their total percentage present

BEtA4				
PK	**RT**	**NIST05 Library Identified Compounds**	**QM**	**TP (%)**
1	7.2866	Benzoic acid, 3-methoxy-4-[(trimethylsilyl)oxy]-, trimethylsilyl ester	96	6.9
2	7.5432	Azelaic acid, bis(trimethylsilyl) ester	74	0.8
3	8.5248	Trimethylsilyl 3,5-dimethoxy-4-(trimethylsilyloxy)benzoate	99	2.0
4	8.7557	Cinnamic acid, 4-methoxy-3-(trimethylsiloxy)-, trimethylsilyl ester	99	1.5
5	9.9169	Hexadecanoic acid, trimethylsilyl ester	96	1.7
6	10.3852	Ferulic acid, trimethylsiloxy, trimethylsilyl ester	99	2.9
7	11.6811	Octadecanoic acid, trimethylsilyl ester	99	1.2
8	13.6057	Unknown	46	2.8
10	14.8823	Unknown	50	9.8
11	15.5816	Lanost-8-en-3-one	15	0.4
12	16.2937	Tetracosanoic acid, trimethylsilyl ester	98	10.7
13	16.6978	3-Oxo-9b-lanosta-7-en-26,23-olide	35	0.9
14	16.9609	Hexacosanoic acid, 2-[(trimethylsilyl)oxy]-, trimethylsilyl ester	22	2.6
15	17.6345	Lanosta-9(11),24-dien-3-ol, acetate, (3.beta.)-	27	15.4
17	17.8911	19-Norpregna-1,3,5(10)-trien-20-yn-2-amine,3,17-	43	1.8

		bis[(trimethylsilyl)oxy]-, (17.alpha.)-		
18	18.2696	Unknown	38	1.9
21	18.9047	Unknown	3	9.8
28	22.0354	Hop-22(29)-en-3.beta.-ol	70	11.0
29	22.6577	Unknown	35	2.8
30	25.1853	Unknown	38	4.1

* PK, Peak; RT, Retention Time; QM, Quality March; TP, Total Percentage.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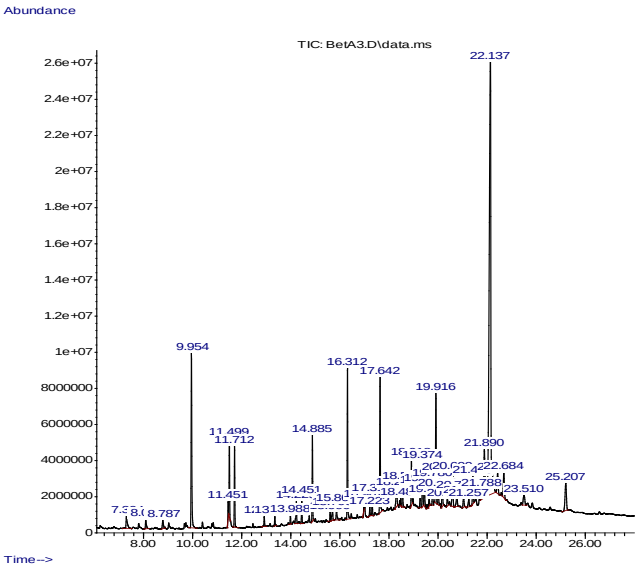
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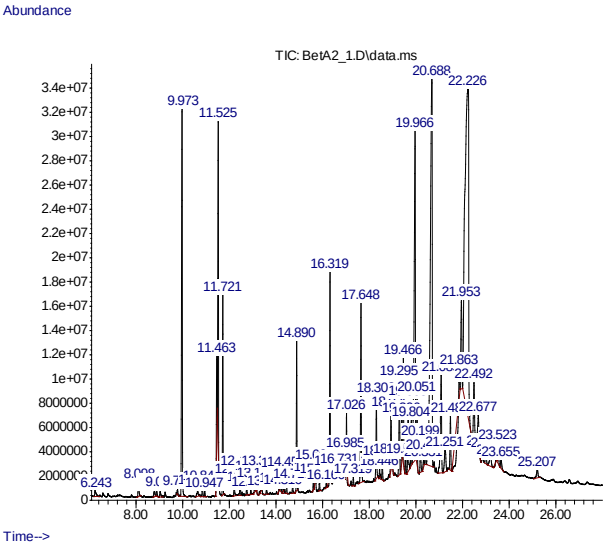
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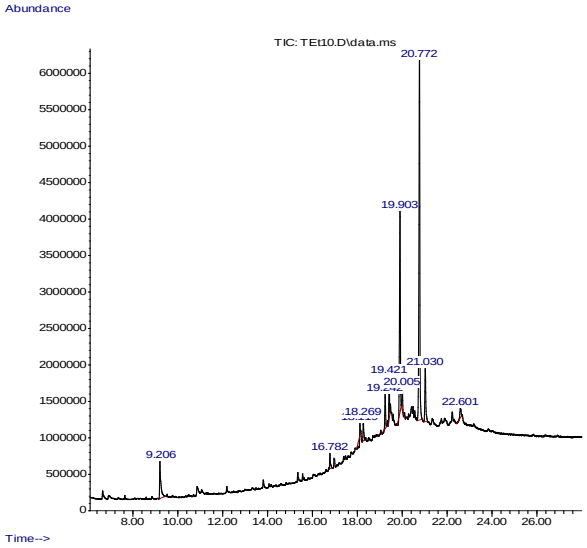
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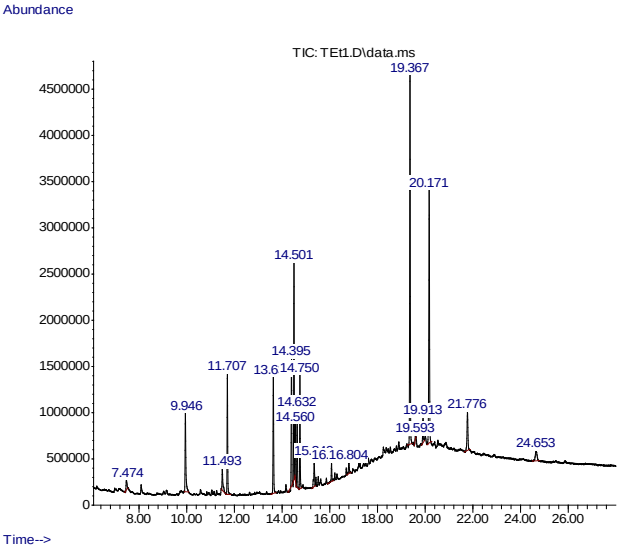
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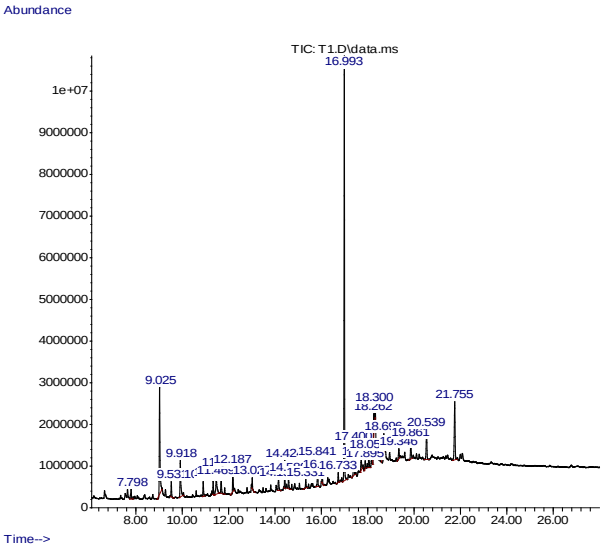
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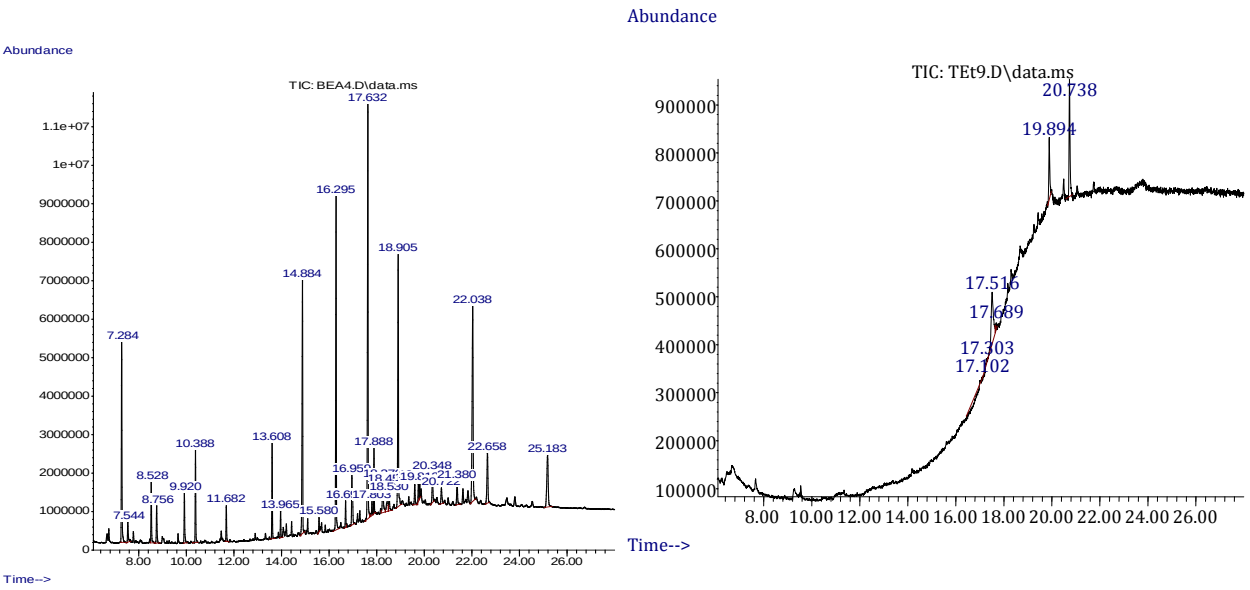


Figure 3.1: GC-MS total ion chromatogram (TIC) showing the retention time of the compounds identified from the fractions

3.4. DISCUSSION

Resistance of microorganisms to currently available antimicrobial agents used in the treatment of infectious diseases requires new assessment. The antimicrobial susceptibility observed in the current study was concentration dependent. For example, 24.5 ± 3.5, 26.5 ± 2.1, and 32 ± 2.8 mm zones of inhibition were observed for *P. shigelloides* and 16.9 ± 0.2, 21.5 ± 2.1, and 26.4 ± 3.4 mm for *C. albicans* at 25, 50, and 100 mg/mL concentration of the extract, respectively. This is similar to the observation reported in our previous study of this extract against clinical strains of *H. pylori* (Okeleye *et al*., 2010). Some of the bacteria and fungi species on the other hand were resistant to tetracycline. The incessant spread of multidrug-resistant pathogens has become a serious threat and a major public health concern worldwide, leading to the re-emergence of previously controlled diseases, which contributes extensively to the high incidence of opportunistic and chronic infection cases all over the world (Satish *et al*., 2008; Mishra *et al*., 2010; Ndip *et al*., 2007; Okeleye *et al*., 2011).

The extract had MIC and MLC values of 0.15625 and 0.3125 mg/mL, respectively, against *P. shigelloides*, a Gram negative bacterium that causes gastroenteritis. *P. shigelloides* has mostly been isolated from freshwater fish, shell fish, cattles, goats, swine, cats, dogs, monkeys, and snakes; and shares similar antigens with *S. sonnei* which has been implicated in dysentery (Niedziela *et al*., 2002). *Salmonella Typhimurium* mostly found in the intestinal lumen has been etiologically linked to diarrhea and typhoid fever, while *H. pylori* is a major risk factor in peptic ulcer, gastritis, and gastric cancer in later life (Miao and Miller, 2000; Tanih *et al*., 2010; Okeleye *et al*., 2010). There was no discrepancy of activity observed with the extract in both Gram-negative and -positive (*S. pyogenes* and *S. aureus*) bacteria in the current study, an

interesting finding which points to the fact that this plant could be used in the treatment of gastrointestinal related and other morbidities.

The mechanism of antibiosis revealed that the extract was bacteriostatic/fungistatic against 3 organisms, bactericidal against 5, and was not considered ineffective against any of the organisms tested in our study (MLC/MICindex ≥ 16). Fractions BEtA2, BEtA4, T1, and BEtA3 inhibited 90% of the test organisms (IC_{90}) at 2.5 ± 0.8, 3.1 ± 2.3, 2.1 ± 0.8, and 3.8 ± 1.9 (mg/mL), respectively. Although it might seem reasonable that bactericidal drugs would have a preference to bacteriostatic drugs, the nature of infection is important in deciding which kind of drug to apply. For example, high concentrations of some bacteriostatic agents are also bactericidal, while low concentrations of some bactericidal agents are bacteriostatic (Pankey and Sabath, 2004). Furthermore, in central nervous system infections, a bactericidal drug can discharge bacterial products that stimulate inflammation. Certain bacteriostatic drugs may be ideal in cases of streptococcal and clostridial gangrene, since they inhibit the production of the toxins that cause a great deal of the morbidity (Delahaye *et al*., 2009; Shanmughapriya *et al*., 2008; Pankey and Sabath, 2004).

Although there is a dearth of information in the literature on the microbial and cytotoxic effect of the ethyl acetate extract of the stem bark of *P. africanum*, other solvents extract (methanol, acetone, dichloromethane hexane etc.) and plant parts have been reported to be active against some bacteria including *Campylobacter* spp., *Enterococcus fecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, and *Helicobacter pylori* (Okeleye *et al*., 2010; Samie *et al*., 2005; Bizimenyera *et al*., 2005). However, the effect of a drug on an organism is complex and involves interactions at multiple levels that cannot be predicted using biochemical assays. To understand this complexity, increase use of cell-based screening assays as more biologically

relevant surrogates are being employed to predict the response of the organism. Moreover, in the drug discovery process, predicting cellular toxicity is essential and eukaryotic cell culture has been recognized as the model system of choice to get an approximation of toxicity (Riss and Moravec, 2004).

The LD_{50} (lethal dose, 50 %) indicates the quantity of extracts/compounds/drugs that, if administered to a population of organisms, will cause 50% of the organisms to perish. A high LD_{50} implies it would take a large quantity of the extract to cause a toxic response, while small LD_{50} values are highly toxic and could be dangerous. It was observed that the dose of the extract appeared to be more toxic after 24 hrs (log 1.92 ± 0.01; LD_{50} = 82.64 ± 1.40) of treatment than at 48 (log 2.15 ± 0.00; LD_{50} = 140.09 ± 1.33) and 72 hrs (2.08 ± 0.00; LD_{50} = 121.07 ± 0.92). Cell-based lethality assay is an indication of cytotoxicity, bactericidal and fungicidal activities, pesticidal effects, and various pharmacologic actions. The LD_{50} value obtained in the current study indicates that the extract has high pharmacological actions (MacLaughin *et al*., 1991; Gupta *et al*., 1996). The activity observed against bacteria, fungi, and human Chang liver cell may be ascribed to the compounds identified in the fractions of the extract. For example, phytosterols (e.g., silane,[[(3.beta.,24R)- ergost-5-en-3-yl]oxy]trimethyl) are recognized as save ingredients that lower blood cholesterol (Jones, 1999). Gallic acid, [(benzoic acid, 3,4,5-tris(trimethylsiloxy), trimethylsilyl)] is a phenolic compound that helps to protect human cells against oxidative damage and cancer (Chanwitheesuk *et al*., 2007). Lupeol, ferulic acid, and Hop-22(29)-en-3.beta.-ol acts as an antioxidant, anti inflammatory, and anti ulcerogenic agent (Lewis and Hanson, 1991).

3.5. CONCLUSIONS

The ethyl acetate extract of *P. africanum* exhibited *in vitro* antibacterial (both Gram-negative and positive species) and antifungal activity. The major chemical compounds revealed by GC-MS analysis are believed to be responsible for the antimicrobial activity. However, since the extracts were toxic to the human Chang liver cells, we recommend that this plant extract should be used with caution, and further studies using *in vivo* (animal model) approach should be conducted to confirm this finding.

REFERENCES

Farnsworth, N.R. (1993). Ethno pharmacology and future drug development: The North American experience. *Journal of Ethnopharmacology*. **38**: 145-152.

Houghton, P.J. (1995). The role of plants in traditional medicine and current therapy. *Journal of Alternative and Complementary Medicine*. **1** (2): 131-143.

Satish, S., Raghavendra, M.P. and Raveesha, K.A. (2008). Evaluation of antibacterial potential of some plants against human pathogenic Bacteria. *Advances in Biological Research*. **2** (3-4): 44-48.

Mahesh, B. and Satish, S. (2008). Antimicrobial activity of some important medicinal plant against plant and human pathogens. *World Journal of Agricultural Sciences*. **4**: 839-843.

Halcon, L. and Milkus, K. (2004). Staphylococcus aureus and wounds: A review of tea tree oil as a promising antimicrobial. *American Journal of infection Control*. **32** (7): 402-408.

Kumar, A., Kumar, A., Thakur, P., Patil, S., Payal, C., Kumar, A. and Sharma, P. (2012). Antibacterial activity of green tea (*Camellia sinensis*) extracts against various bacteria isolated from environmental sources," *Recent Research in Science and Technology*. **4** (1): 19-23.

Krovacek, K., Eriksson, M.L., Gonzalez- Rey, C., Rosinsky, J. and Ciznar, I. (2000). Isolation, biochemical and serological characterisation of *Plesiomonas shigelloides* from freshwater in Northern Europe. *Comparative Immunology, Microbiology and Infectious Diseases*. **23** (1): 45-51.

Mishra, A., Shrivastava, A. and Jain, S.K. (2010). Screening of some plant extracts against *Alternaria sp*. isolated from foot infections in cancer patients. *International Journal of PharmTech Research*. **2** (2): 1165-1170.

Okeleye, B.I., Samie, A., Bessong, P.O., Mkwetshana, N.T., Green, E., Clarke, A.M. and Ndip, R.N. (2010). Crude ethyl acetate extract of the stem bark of *Peltophorum africanum* (Sond, Fabaceae) possessing *in vitro* inhibitory and bactericidal activity against clinical isolates of *Helicobacter pylori*. *Journal of Medicinal Plants Research*. **4** (14): 1432-1440.

Ndip, R.N., Malange, A.E., Tarkang, A.E., Mbullah, S.M., Luma, H.N., Malongue, A., Ndip, L.M., Nyongbela, K., Wirmum, C. and Efange, S.M. (2007). *In vitro* anti-*Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. *Journal of Ethnopharmacology*. **114** (3): 452-457.

Okeleye, B.I., Bessong, P.O. and Ndip, R.N. (2011). Preliminary phytochemical screening and *in vitro* anti-*Helicobacter pylori* activity of extracts of the stem bark of *Bridelia micrantha* (Hochst., Baill., Euphorbiaceae). *Molecules*. **16** (8): 6193-6205.

Wikler, M.A. (2008). Performance Standards for Antimicrobial Susceptibility Testing; Eighteenth Informational Supplement M100-S18. *Clinical and Laboratory Standards Institute*. **28** (1): 46-52.

Delahaye, C., Rainford, L., Nicholson, A., Mitchell, S., Lindo, J. and Ahmad, M. (2009). Antibacterial and antifungal analysis of crude extracts from the leaves of *Callistemon viminalis*. *Journal of Medical and Biological Sciences*. **3** (1): 1-7.

Shanmughapriya, S., Manilal, A., Sujith, S., Selvin, J., Kiran, G.S. and Natarajaseenivasan, K. (2008). Antimicrobial activity of seaweeds extracts against multiresistant pathogens. *Annals of Microbiology*. **58** (3): 535-541.

Merghoub, N., Benbacer, L., Amzazi, S., Morjani, H. and El mzibri, M. (2009). Cytotoxic effect of some Moroccan medicinal plant extracts on human cervical cell lines. *Journal of Medicinal Plants Research*. **3** (12): 1045-1050.

Yrjönen, T., Peiwu, L., Summanen, J., Hopia, A. and Vuorela, H. (2003). Free radical-scavenging activity of phenolics by reversed-phase TLC. *Journal of the American Oil Chemists' Society*. **80** (1): 9-14.

Masoko, P.J. and Eloff, J.N. (2006). Bioautography indicates the multiplicity of antifungal Compounds from twenty-four southern African *Combretum* species. *African Journal of Biotechnology*. **5** (18): 1625-1647.

Adams, P.R. (1989). Identification of Essential Oil Components by Ion Trap Mass Spectroscopy. *Academic Press*, New York, NY, USA.

Niedziela, T., Lukasiewicz, J., Jachymek, W., Dzieciatkowska, M., Lugowski, C. and Kenne, L. (2002). Core oligosaccharides of *Plesiomonas shigelloides* O54:H2 (strain CNCTC 113/92): structural and serological analysis of the lipopolysaccharide core region, the O-antigen biological repeating unit, and the linkage between them. *Journal of Biological Chemistry*. **277** (14): 11653-11663.

Miao, E.A. and Miller, S.I. (2000). A conserved amino acid sequence directing intracellular type III secretion by *Salmonella typhimurium*. *Proceedings of National Academy of Sciences of the United States of America*. **97** (13): 7539-7544.

Tanih, N.F., Okeleye, B.I., Ndip, L.M., Clarke, A.M., Naidoo, N., Mkwetshana, N.T., Green, E. and Ndip, R.N. (2010). *Helicobacter pylori* prevalence in dyspeptic patients in the Eastern Cape Province – race and disease status. *South African Medical Journal*. **100** (11): 734-737.

Pankey, G.A. and Sabath, L.D. (2004). Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. *Clinical Infectious Diseases*. **38** (6): 864-870.

Samie, A., Obi, C.L., Bessong, P.O. and Namrita, L. (2005). Activity profiles of fourteen selected medicinal plants from rural Venda communities in South Africa against fifteen clinical bacterial species. *African Journal of Biotechnology*. **4** (12): 1443-1451.

Bizimenyera, S.E., Swan, G.E., Chikoto, H. and Eloff, J.N. (2005). Rationale for using *Peltophorum africanum* (Fabaceae) extracts in veterinary medicine. *Journal of the South African Veterinary Association*. **76** (2): 54-58.

Riss, T. and Moravec, R. (2004). Use of multiple assay endpoints to investigate the effects of incubation time, dose of toxin, and plating density in cell-based cytotoxicity assays. *Assay and Drug Development Technologies*. **2** (1): 51-62.

MacLaughin, J.L., Chnag, C.J. and D.L. Smith, (1991). Bench-top bioassays for the discovery of bioactive natural product: An update," *Studies in Natural Product Chemistry*. **9**: 388-409.

Gupta, M.P., Monge, A., Karitas, G., Lopez, C.A., Solis, P.N., Leon, E., de Trujillo, M., Surez, O., Wilson, F., Montenegro, G., Noriega, Y., Santana, A.I., Correa, M. and Sanchez, C. Screening of Panamanian medicinal Plants for brine shrimp toxicity, crown gall tumor inhibition, cytotoxicity and DNA interaction. *Pharmaceutical Biology*. **34** (1): 19-27.

Jones, P.J.H. (1999). Cholesterol-lowering action of plant sterols. *Current Atherosclerosis Reports*. **1** (3): 230-235.

Chanwitheesuk, A., Teerawutgulrag, A., Kilburn, J.D. and Rakariyatham, N. (2007). Antimicrobial gallic acid from *Caesalpinia mimosoides* Lamk. *Food Chemistry*. **100**: 1044-1048.

Lewis, D.A. and Hanson, P.J. (1991). Anti-ulcer drugs of plant origin. *Progress in Medicinal Chemistry*. **28**: 201-231.

CHAPTER FOUR

Evaluation of compounds and heavy metals in the ethyl acetate extract of *Peltophorum africanum*: Morphological effects on pathogenic bacteria and yeast cell

ABSTRACT

The study purified the crude ethyl acetate extract (EAE) and fractions (T1 and BEtA2) of *Peltophorum africanum*, identified the compounds and determined their role in the morphological alteration of bacteria and yeast cell in order to determine the antimicrobial activity and the probable mechanism of action; using thin layer chromatography, infrared spectra fingerprint and GC-MS analysis, micro-dilution and scanning electron microscopy with energy-dispersive X-ray analysis. The common characteristic of all the four pathogenic bacteria and yeast was the presence of sodium, pottasium, thallium and magnesium. Activity of sulphur, calcium, nitrogen and chlorine in combination with compounds identified such as Benzoic acid, 3,4,5-tris(trimethylsiloxy)-,trimethylsilyl ester were noted as *P. aeruginosa* treated with EAE had a reduction of sodium from 5.55 % - 1.50 %. For *C. albicans*, pottasium was reduced from 4.16 % - 0.76 % (T1). Remarkable morphological alterations were observed including deformation of the germ tubes and perforation of the cell wall. At the highest concentration of T1 (3.125 mg/mL), 57.8, 89.0 and 96.2 % growth inhibition were observed, while for BEtA2, 96.9, 96.5 and 91.4 % were recorded for *C. albicans*, *S. pyogenes* and *S. aureus* respectively. These findings indicate the potential of *P. africanum* in preventing bacterial and fungal invasion.

4.1. INTRODUCTION

Herbal medicines known to have many essential and nutritional elements are being used worldwide since ancient time as readily available source for curing various diseases and to reinforce the body immune system (Khan *et al*., 2006; Lokhande *et al*., 2010). Most known proteins contain metal (Na^{2+}, K^{+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{+}, and Mn^{+}) cofactor(s), which carry out an array of tasks ranging from protein structure stabilization to enzyme catalysis, activating many fundamental life processes such as photosynthesis and respiration (Dudev and Lim, 2007).

Oxidative stress is the attack caused by free radicals (charged molecules), the natural by-products of human metabolism, against the cells, breaking cellular membranes and reacting with the proteins, nucleic acids and enzymes which can result in loss of structure and cell dysfunction (Carreno *et al*., 2006). Zinc as trace element emerges to shield against oxidative stress by preventing or reducing reactive oxygen species development and by lessening the primary contact of such stress on the cell (Hfaiedh *et al*., 2012). Natural products show evidence of a remarkable collection of structural motifs. Some plant metabolites have notable biological activities whose probable benefit serves as a model or template in the development of new antifungal, anticancer and antiviral agents. Among the many plant compounds of biological and structural interest are quinone and /or hydroquinone, phenolics compounds, acetylsalicylic acid, etc (Sorensen *et al*., 2000; Ling *et al*., 2002; Carreno *et al*., 2006).

Metabolism of many aromatic types of ether involves cleavage to produce phenolics metabolites. For example, p-nitrophenyl ethers, phenacetin, p-phenetidine and a diversity of nuclear-substituted anisoles are converted into their corresponding phenols in the body (Axelrod, 1956). Azoles, the five-membered nitrogen heterocyclic ring compounds containing at least one other

non-carbon atom is very important building blocks of many important compounds widely used in medicine (Zhang *et al*., 2012). Carbonyl compound (C=O), one of the major components in drug development of pain reliever, antitumor (e.g., naphthoquinones; vitamins K_1 and K_2), occurs in all amino acids and is central to the metabolism of carbohydrates; fats, and acts as an electron carrier and transporter of compounds and proteins (Janausch *et al*., 2002; Ling *et al*., 2002; Nic *et al*., 2006; Zhang *et al*., 2012).

Candida albicans infection is one of the opportunistic fungal infections (OFIs) and appears mostly when CD4 count is between 200–500/mm^3 which may be the initial sign of immunodeficiency (Miceli *et al*., 2011). *Streptococcus pyogenes* and *Staphylococcus aureus* are Gram-positive and facultative anaerobic bacteria that are able to invade via the mucous membrane while *P. shigelloides* (enterobacteriaceae) is a Gram-negative rod commonly isolated in fresh water fish, shell fish, cattles e.t.c., and a major cause of gastroenteritis. Infections caused by *S. pyogenes* and *S. aureus* include pharyngitis, rheumatic fever, pneumonia, and urinary tract infections. These organisms have been reported to have the aptitude to build up resistance gene to antibiotics (Krovacek *et al*., 2003 Kumar *et al*., 2012).

In view of the fact that the systematic screening of medicinal plant extracts for anticancer, antimicrobial and antioxidant activities signifies a constant attempt to finding new potent compounds, this study was aimed at isolating and characterizing compounds contained in the ethyl acetate extract of *P. africanum* as well as evaluating their antimicrobial effect on selected bacteria and yeast of medical importance in an effort to justify its folkloric use and likely potential as lead in the development of new antimicrobials.

4.2. MATERIALS AND METHODS

4.2.1. Preparation of the extract

Stem bark of *P. africanum* was collected and identified in collaboration with a botanist at the University of Venda, South Africa (voucher specimens number, BP01). The samples were dried in an oven at 30 – 40 °C for 2 – 5 days. Milled samples were percolated with ethyl acetate in the ratio 1:3 (w/v) and the mixture was put in a shaker incubator (Edison, N.J. U.S.A.) regulated at room temperature (RT) for 48 hours. The extract was filtered with Whatman No. 1 filter paper, after which it was concentrated in a rotavapor (Buchi R461, Switzerland) and kept in a storage vial at 4 °C (Okeleye *et al*., 2010).

4.2.2. Antimicrobial screening

Following preliminary screening of the extracts by the agar dilution method; the minimum inhibitory concentration (MIC_{90}) determination was carried out in accordance with the method of Okeleye *et al*. (2011) with modifications. Two-fold dilutions of the extract (EAE) was prepared in 96-well test plates containing Mueller Hinton broth (Merck, Gauteng, South Africa) and sabouraud dextrose broth (LAB M, UK) for bacteria and yeast cell respectively; the final extract concentrations ranged from 0.0048 – 10 mg/mL and the tests were carried out in duplicate. Each strain applied into the wells was serially diluted to correspond to 0.5 McFarland standards and incubated at 37 °C for 24 hours (bacteria) and 27 °C for 3 days (yeast). After incubation, resazurin solution was added per well and further incubated at 37 °C for 1 hour. The absorbance was read with Analytical & Diagnostic Product Gen™ spectrophotometer (BioTek, USA) adjusted to 620 nm and the MIC_{90} was taken as lowest concentration of the test extract resulting in inhibition of 90 % of microbial growth.

4.2.3. Thin layer chromatography (TLC) analysis

TLC was used to isolate compounds from the extract using Silica gel TLC plates (Merck, Kieselgel 60 F_{254}) according to the method of Masoko and Ellof (2006) with modification. Exactly 20 µL of ethyl acetate extract dissolved in dichloromethane and DMSO, at a concentration of 100 mg/mL was spotted on TLC plates, and developed using fifteen different mobile solvent systems at diverse ratio. Solvent system used were optimized to separate components of polar, intermediate and non polar compounds respectively. The plates were sprayed with vanillin sulphuric acid spray reagent (2mg of vanillin in 28 mL of methanol plus 1mL of concentrated sulphuric acid) and carefully heated for 5-10 minutes at 105 ^{o}C to allow colour development. The plates were visuallized under ultraviolet light at 365 nm (BioDoc-It™; 230~50Hz, 0.8 Amps, USA). The retention factor value (R_f) of the visible bands were marked and calculated using the formular: R_f = distance moved by analyte (compound)/distance moved by solvent.

4.2.4. Determination of antioxidant compounds

Adopting the method of Yrjönen *et al*. (2003) and Rashid *et al*. (2010), the antioxidant compounds were determined using toluene/ethanol (90:5) and benzene/ethanol/ammonium hydroxide (90:10:1) solvent system based on the number of compounds (higher) separated in the above experiment. After development, the plates were air-dried for 15 minutes and then dipped into a 0.05 % (wt/vol) solution of DPPH prepared in methanol for 5 seconds using a chromatogram immersion tank. Instant purple colour of DPPH reagent bleaching to yellow spots and confirmed as white under UVP-Transilluminator (BioDoc-It™; 230~50Hz, 0.8 Amps, USA) was an indication of positive antioxidant activity.The plates were visuallized under ultraviolet

light at 365 nm. The R_f of the visible bands were calculated using the formular: R_f = distance moved by analyte (compound)/distance moved by solvent.

4.2.5. Fingerprint for the identification of functional group of compounds

The functional groups of compounds of the crude extract were characterised using infrared spectra fingerprint according to the method of ZiAmons *et al*. (2004) and Muruganantham *et al*. (2009). Dried ethyl acetate extract, pulverized into powder with potassium bromide (KBr) in ratio a 1:100, was subjected to FT-IR analysis (Perkin Elmer System, 2000, England). The Frequency wavelengths (cm-1) were interpreted using "Interpretation of infrared spectra, a practical approach" in encyclopedia of analytical chemistry (Coates, 2000).

4.2.6. Purification and separation process of ethyl acetate extract by column chromatography

Two 40 cm long × 2.5 cm diameter glass columns were packed to a height of 35 cm with a slurry of silica gel 60 (Merck, Germany; particle size 0.063 to 0.2 mm/ 70 to 230 mesh). The columns were equilibrated with 100 % toluene and benzene respectively for 30 min. The mixture of seven grams of ethyl acetate extract and 14 g of silica gel powder were ground to very fine particles and then loaded onto a silica gel column. The solvent system which gave good separation, toluene/ethanol (90:5) and benzene/ethanol/ammonium hydroxide (90:10:1) were used to elute the column; fractions (200 ml twice; 50 ml) were collected and concentrated on a rotary evaporator (Strike 202 Steroglass, Italy). Fractions were weighed and stored in air tight containers for further bioassay (Ndip *et al*., 2009).

4.2.7. Assay for growth inhibition of T1 and BEtA2

Based on the preliminary screening of the fractions; the inhibitory effect of T1 and BEtA2 were carried out in accordance with the method of Okeleye *et al*. (2011) and Das *et al*. (2010) with modifications. Two-fold dilutions of the T1 and BEtA2 were prepared in test 96-well plates of complete Mueller Hinton broth (Merck, Gauteng, South Africa) sabouraud dextrose broth (LAB M, UK) for bacteria and yeast cell respectively; the final extract concentrations ranged from 0.024 – 3.125 mg/mL. The tests were carried out in duplicate and each strain applied into the wells was serially diluted to correspond to 0.5 McFarland standards. After the incubation at 37 °C for 24 hrs (bacteria) and 27 °C for 3 days (yeast), the absorbance was read at 620 nm with Analytical & Diagnostic Product Gen™ spectrophotometer (BioTek, USA). Percentage inhibition of microbial growth was calculated as: % Microbial inhibition = [(Microbial growth (control) − Microbial growth (treatment) / (Microbial growth (control))] × 100.

4.2.8. Morphological analysis of bacteria and yeast cells

The morphological characteristics of the bacteria and yeast cells were determined using scanning electron microscope (SEM; JSM-6390LV, Jeol, Japan) and Energy Dispersive X-ray Spectroscopy (SEM-EDXS; thermo super dry II Xray detector, Jeol, Japan). The treated and untreated cells (controls) were washed in phosphate buffered saline (PBS), centrifuged (1000 rpm for 5 mins; 2 times), then fixed in 2.5 % gluteraldehyde prepared in 0.1 M PBS. The cells were washed twice and deposited on poly-L-lysine-coated glass coverslip, post fixed with 1 % osmium tetroxide (OsO_4) in 0.2 M PBS. Cells were washed with PBS, dehydrated through graded ethanol (30, 50, 70, 85 and 95 %), mounted onto stubs and coated using IB3 Ion Coater (EIKO, Japan). Different sections of the cells were micro-analyzed and the representative spectra

presented. A focused beam of electrons was used to scan the germ tubes (yeast) and bacteria cell wall at the point where examination of its chemical composition was desired. The detection and determination of elements with the EDXS was based on the emission of characteristic X-rays under bombardment with electrons. The quantitative data of the elements produced were analyzed and represented as previously described (Otang *et al*., 2011).

4.2.9. Gas chromatography-mass spectrometry (GC-MS) analysis of the fractions

Agilent 6890N GC with CTC Combi-PAL Auto-sampler and Agilent 5975B MS with Rtx®-5MS (30 m, 0.25 mm ID, 0.5 µm film thickness) Restek 12723-127 were used for the analysis. The T1 and BEtA2 samples were dissolved in pyridine and dichloromethane respectively, derivatized with pyridine and MSTFA before injection onto a GC column. Oven temperature were programmed at 150 °C, 180 °C and 325 °C with hold minutes of 1, 4 and 20 minutes for Initial, Ramp1 (50 °C/min) and Ramp2 (10 °C/min) respectively. Helium was used as the carrier gas with injection volume of 1 µL (Split mode ratio: 15:1) and flow rate of 1 mL/min. Injection temperature and MS transfer were set at 280 °C with acquisition mode scanning mass range of 40 to 550 m/z (EI+; Electron energy 70 eV) (Adams, 1989; Wei *et al*., 2006).

4.3. RESULTS

4.3.1. Antimicrobial susceptibility

Preliminary susceptibility screening revealed that the ethyl acetate extract was active against bacteria (14 – 32 mm) than fungi (0 – 26 mm). The activity observed ranged from 0.3125 – 2.5 mg/mL for bacteria and 10 mg/mL for *Candida albicans* at MIC_{90} as shown in Table 4.1.

Table 4.1: Minimum inhibitory concentration (MIC_{90}) of the extract against microorganisms

Test organisms	**MIC_{90}[a] (mg/mL)**
Candida albicans ATCC 2091	10
Streptococcus pyogenes ATCC 49399	1.25
Staphylococcus aureus NCTC 6571	2.5
Plesiomonas shigelloides ATCC 51903	0.3125
Pseudomonas aeruginosa ATCC 15442	1.25

[a] Concentration at which 90% of the test organisms were inhibited

4.3.2. Retention factor of fractionated compounds

Of the 15 solvent systems used, 76 compounds were isolated. The solvent systems that separated the highest number of compounds were TEt (toluene/ethanol), BEtA (benzene/ethanol/ammonium hydroxide) and TE (toluene/ethyl acetate) with 12, 10 and 6 compounds respectively (Table 4.2). This study revealed that toluene; benzene, ethanol and ethyl acetate if used in appropriate ratio are potential solvents that could separate significant number of compounds from the ethyl acetate extract of *P. africanum*. The separated compounds appeared on the plate as pink, purple, yellow and lemon after staining with vanillin (Figure 4.1).

Table 4.2: Effect of solvent system and different ratio on isolated compounds

Solvent system/ratio	No. of compounds separated	R_f (cm)
CEF/5:4:1	3	0.581, 0.205, 0.170
HE/5:1	5	0.976, 0.536, 0.301, 0.142, 0.090
CM/9.5:0.5	4	0.823, 0.750, 0.269, 0.094
TEF/50:40:10	5	0.955, 0.903, 0.5, 0.349, 0.181
TE/95:5	6	0.909, 0.267, 0.172, 0.089, 0.048, 0.030
EH/5:1	4	0.445, 0.219, 0.136, 0.084,
TEt/90:5	12	0.970, 0.658, 0.545, 0.478, 0.379, 0.296, 0.258, 0.217, 0.187, 0.144, 0.113, 0.048
EH/9:1	3	0.801, 0.209, 0.070
TEH/12.5:10:7.5	5	0.936, 0.752, 0.613, 0.563, 0.059
EH/1:1	5	0.988, 0.897, 0.848, 0.188, 0.044
HE/9:1	4	0.579, 0.334, 0.110, 0.062
BEH/12.5:20:10	5	0.970, 0.924, 0.884, 0.244, 0.299
BEtA/90:10:1	10	0.996, 0.968, 0.944, 0.890, 0.652, 0.258, 0.189, 0.146, 0.052, 0.032
EMH/20:7.5:12.5	2	0.941, 0.868
CMH/19:5:10	3	0.862, 0.150, 0.050

C, Chloroform; E, Ethyl acetate; Et, Ethanol; F, Formic acid; M, Methanol; B, Benzene; A, Ammonium hydroxide; T, Toluene; H, Hexane.

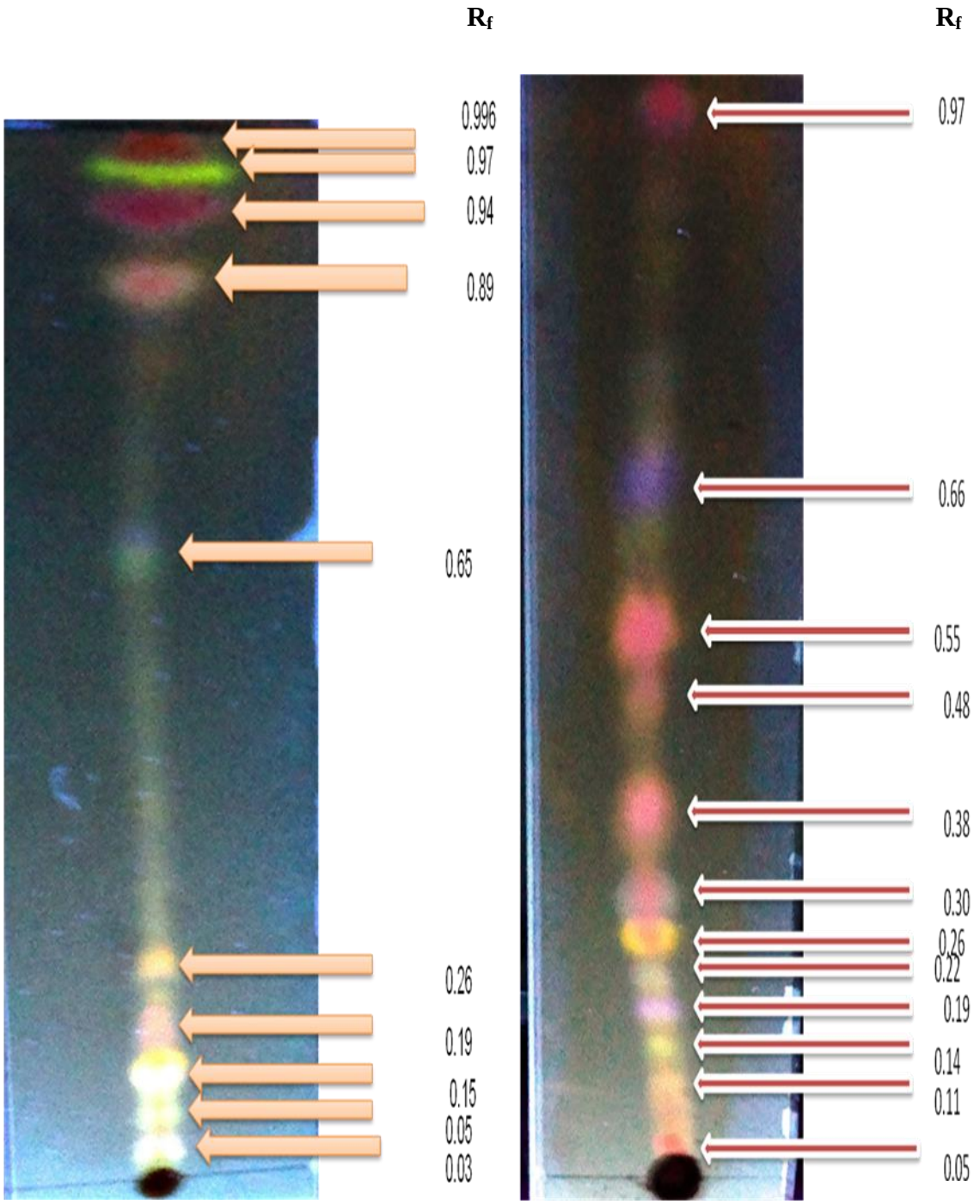


Figure 4.1: Separated compounds on TLC plate with two solvent systems **(a)** BEtA and **(b)** TEt visuallized under ultraviolet light at 365 nm (BioDoc-It™; 230~50Hz, 0.8 Amps, USA).

4.3.3. Fractionated antioxidant compounds

Eight (8) antioxidant compounds were isolated from the two solvent systems (TEt10 and BEtA). This study recorded retention factors of 0.96, 0.64, 0.21, 0.16, 0.11, and 0.07 which have been previously identified as acetylsalicylic acid (aspirin), phenol, caffeine, resorcinol, hydroquinine and theophylline respectively, with considerable medicinal uses. The antioxidant compounds appeared white on the TLC plate under the uv-light (Figure 4.2).

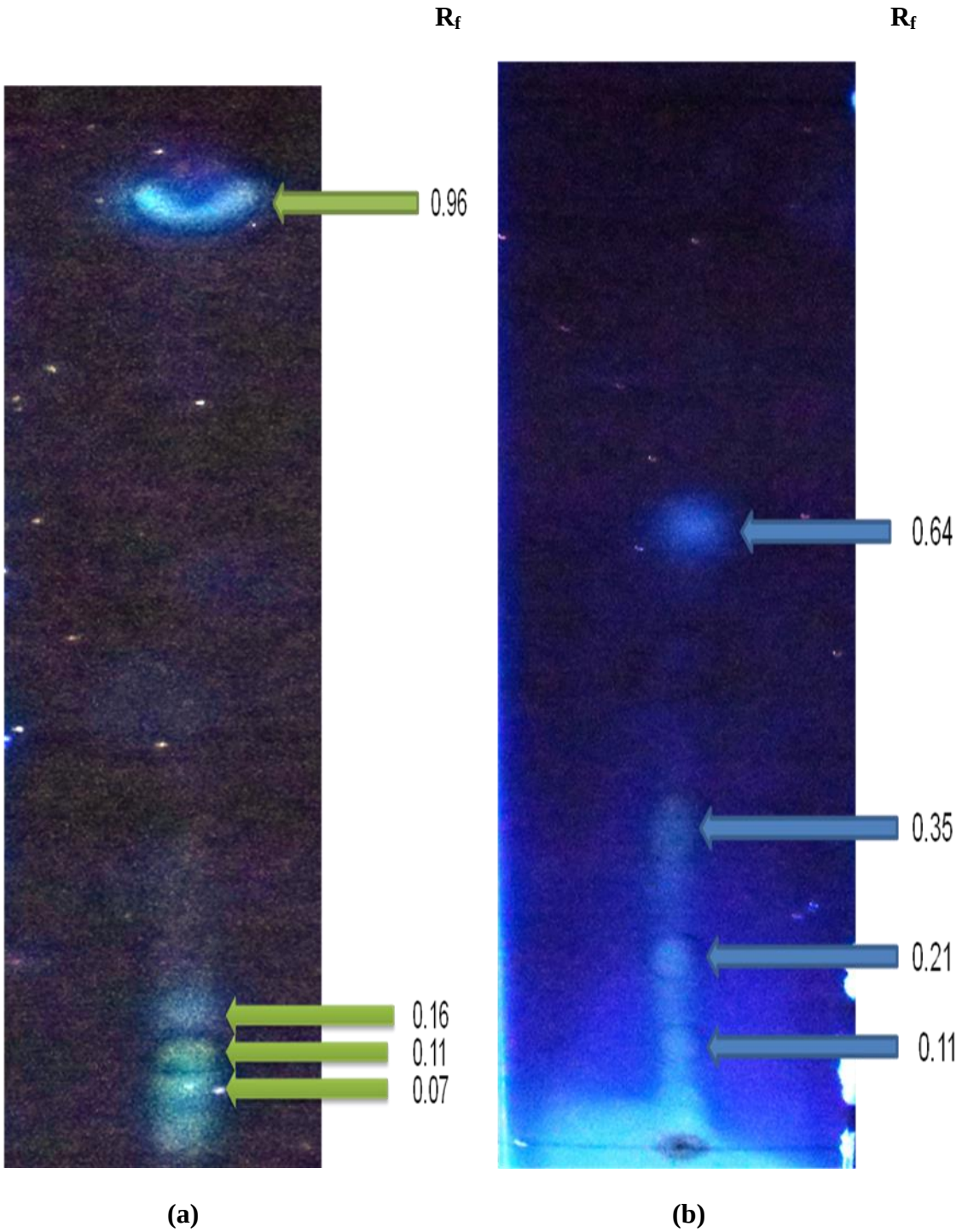


Figure 4.2: Antioxidant compounds on TLC plate using two solvent system **(a)** BEtA and **(b)** TEt visuallized under UV-light at 365 nm (BioDoc-It™; 230~50Hz, 0.8 Amps, USA).

4.3.4. Characteristic of the chemical compounds

The infrared frequency wavelength (cm-1) observed ranged from 420.23 - 3430.02 cm-1 (Figure 4.3); seventeen (17) functional groups that characterised the compounds in the extract were delineated. Due to the extract's relevance in conventional medicine, the compounds structural potency in bio-system and medicine were searched and the salient ones reported. Functional groups noted were of a wide collection: >N-H, $>CH_2$, C=O, P=O, Si-O-C, C-O-, C-Cl and S-S with varying group of compounds including; saturated aliphatic, aromatic, secondary amino, carbonyl, halogenated, unsaturated aliphatic, silicon-oxy and phosphorus-oxy compounds (Table 4.3).

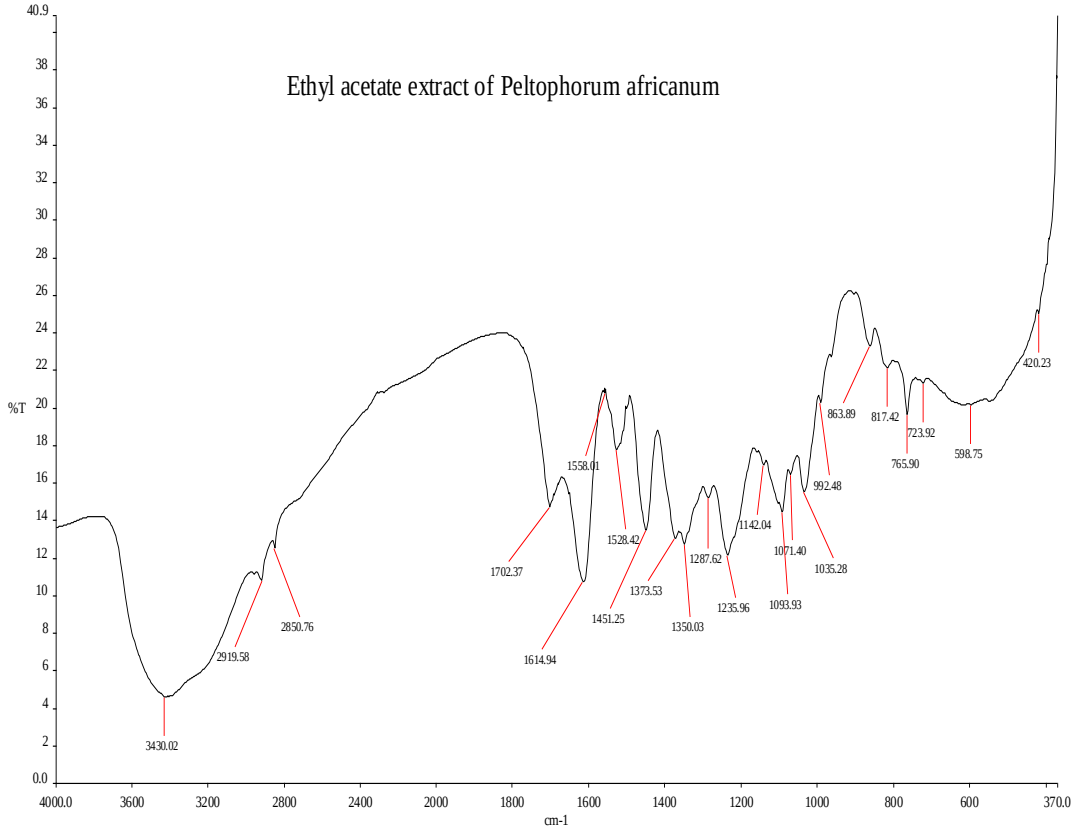


Figure 4.3: FT-IR fingerprint analysis of percentage transmition (%T) against frequency wavelenght (cm-1) of the compounds in the extract.

Table 4.3: Chemical compounds in the ethyl acetate extract based on their FT-IR wavelength (cm-1)

Origin/ functional group	Frequency wavelength (cm^{-1})	Assignment	Compound
>N-H	3430.02	Heterocyclic amine, NH stretch	Amino compound (secondary amino)
Methylene (>CH_2)	2919.58	Methylene C-H asym./sym. stretch	Saturated aliphatic compound
C=O	1702.37	Carboxylic acid	Carbonyl compound
C=O/-N=N-	1614.94	Quinone or conjugated ketone/open-chain azo	Carbonyl compound/thiols and thio-substituted compound
C=O	1558.01	Carboxylate (carboxylic acid salt)	Carbonyl compound
C=C-C	1451.25	Aromatic ring stretch	Aromatic compound
P=O	1287.62	phosphate	Phosphorus-oxy compound
Φ-O-H	1235.96	Aromatic ethers	Ether and oxy compound
C-F	1142.04	Aliphatic fluoro compound C-F stretch	Halogenated compound
Si-O-C	1093.93	Organic siloxane or silicone	Silicon-oxy compound
C-H	992.48	Vinyl C-H out-of-plane bend (Olefinic alkene group)	Unsaturated aliphatic compound
C-O-O-C	863.89	Peroxides, C-O-O-stretch	Ether and oxy compound
C-O-	817.42	Epoxy and oxirane rings	Ether and oxy compound
C-H	765.90	1,3-Disubstitution (meta) aromatic ring (aryl) group	Aromatic compound
C-Cl	723.92	Aliphatic chloro compound (organohalogen)	Halogenated compound
C-S/C-I	598.75	Disulfides (C-S stretch)/Aliphatic iodo compound C-I stretch	Thiols and thio-substituted compound/Halogenated compound
S-S/ClCN	420.23	Aryl disulfides (S-S stretch)/ triatomic inorganic molecule	Thiols and thio-substituted compound/ linear molecule

4.3.5. Inhibitory effect of fractionated compounds on test organisms

The response of the test organism to T1 and BEtA2 fractions are presented in Table 4.4. The least concentration at which activity was observed in T1 against *C. albicans* and *S. aureus* were 0.391 mg/mL with 13.2 % and 30.1 % growth inhibition respectively. At highest concentration of 3.125 mg/mL, 57.8 and 96.2 % inhibition were observed. A wide spectrum of activity was observed against *S. pyogenes* from 8.3 – 89.0 % rate of inhibition across the 8 concentrations tested (0.024 – 3.125 mg/mL). A much wider range of activity was observed in the BEtA2 compound with 1.8 – 96.9 % cell growth inhibited at all concentrations except for *C. albicans* (0.024 mg/mL).

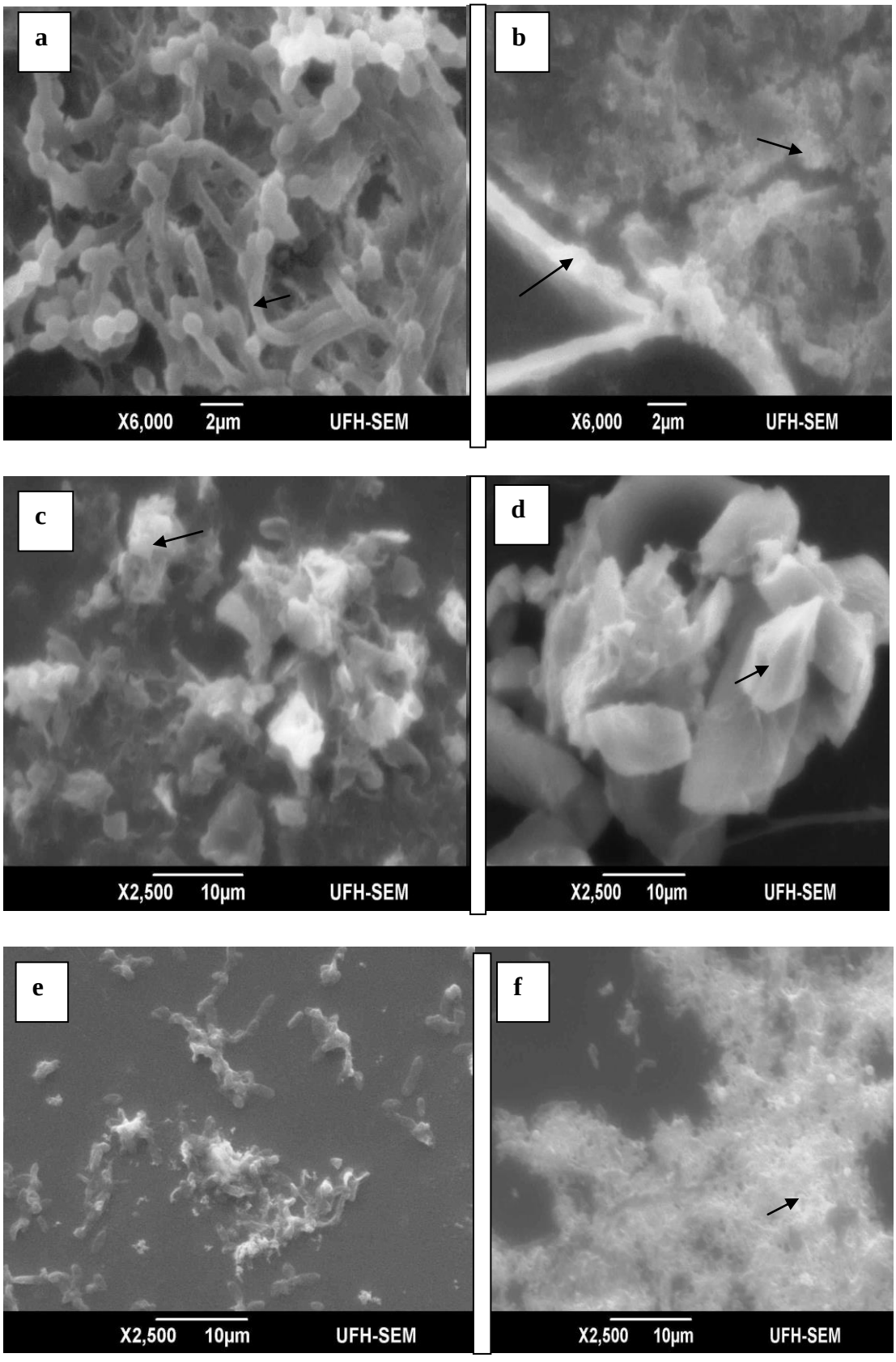
Table 4.4: Percentage growth inhibition of the yeast and bacteria cells by the fractionated compounds

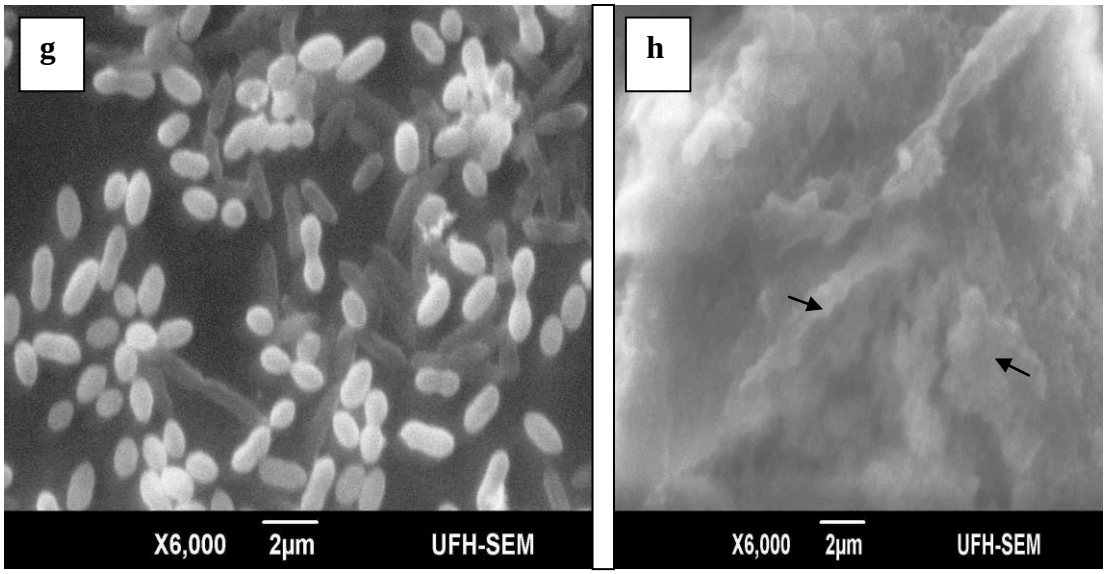
Fractions	Concentrations (mg/mL)	Growth inhibition (%)*		
		Ca	Sp	Sa
T1	3.125	57.8	89.0	96.2
	1.563	48.6	57.9	75.3
	0.781	30.7	46.8	32.6
	0.391	13.2	31.2	30.1
	0.195	0	30.2	0
	0.098	0	12.1	0
	0.049	0	8.6	0
	0.024	0	8.3	0
BEtA2	3.125	96.9	96.5	91.4
	1.563	60.0	80.4	66.9
	0.781	51.3	22.9	43.6
	0.391	49.8	15.8	41.1
	0.195	40.5	14.6	19.0
	0.098	27.0	9.2	11.0
	0.049	2.4	8.9	8.0
	0.024	0	7.8	1.8

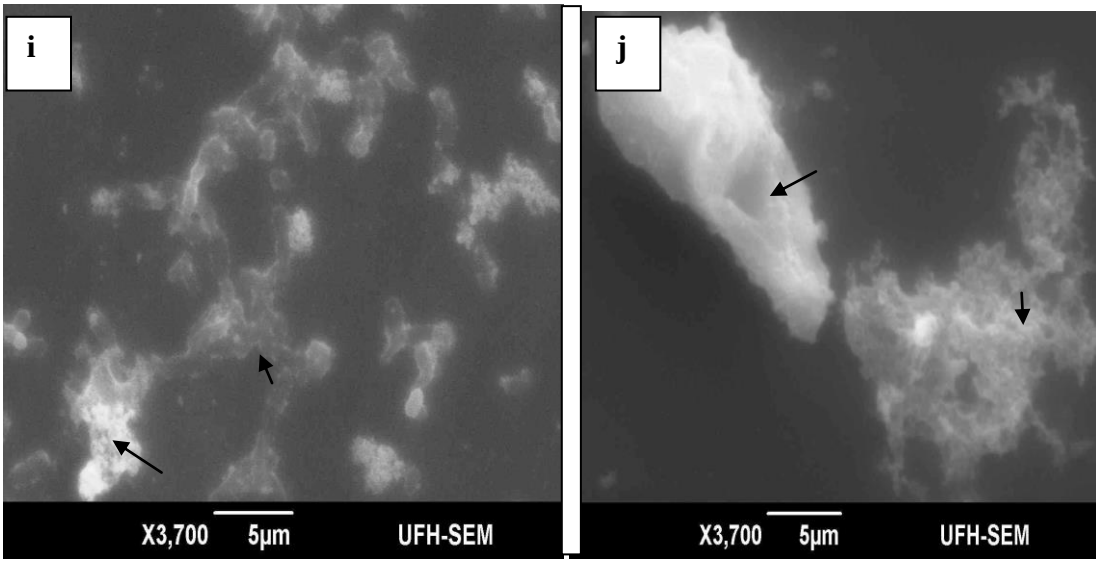
* Values are percentage means of cell growth inhibition. The control showed 0% inhibition for all bacteria and yeast species. Ca, *Candida albicans* (ATCC 2091); Sp, *Streptococcus pyogenes* (ATCC 49399); Sa, *Staphylococcus aureus* (NCTC 6571).

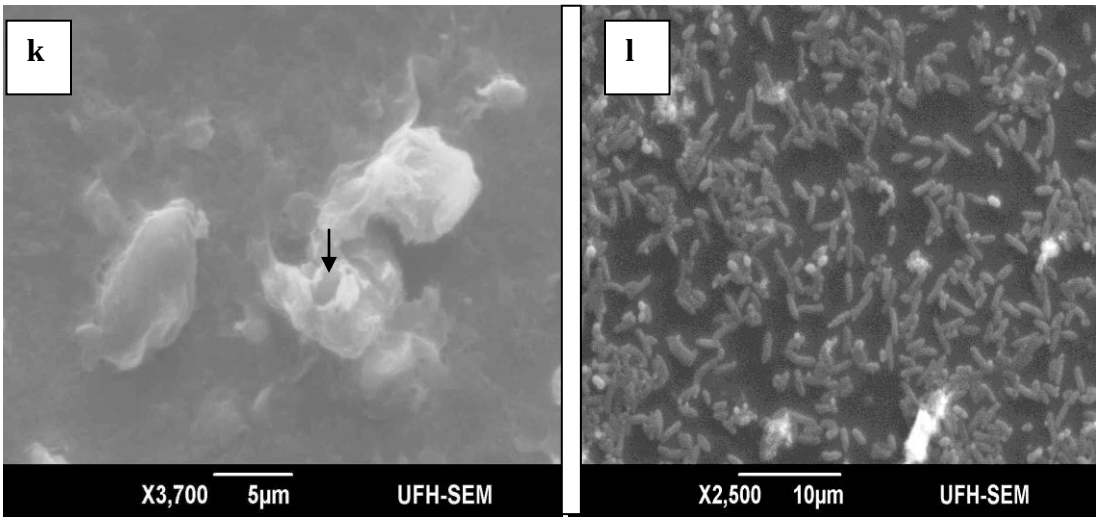
4.3.6. Morphological alterations of bacteria and yeast cells using SEM-EDSX

Notable morphological alterations were observed in treated *C. albicans*; including deformation of the germ tubes, pseudohyphae appeared rough, flattened and distorted in EAE (Figure 4.4b) and completely disintegrated with fraction BEtA2 and T1 as shown in figures 4.4c & d respectively, while the pseudohyphae of the control appeared smooth, clear and turgid (Figure 4.4a). The cells of *P. aeruginosa* and *P. shigelloides* were shrunk, warped, and became stifling (Figure 4.4f and h) as compared to control (Figure 4.4e and i). *S. pyogenes* treated with BEtA2 and T1 showed visible perforation of the cell wall while *S. aureus* treated appeared emaciated and muggy (Figure 4.4n and m).


a
X6,000 2µm UFH-SEM
b
X6,000 2µm UFH-SEM
c
X2,500 10µm UFH-SEM
d
X2,500 10µm UFH-SEM
e
X2,500 10µm UFH-SEM
f
X2,500 10µm UFH-SEM


g
X6,000 2µm UFH-SEM
h
X6,000 2µm UFH-SEM


i
X3,700 5µm UFH-SEM
j
X3,700 5µm UFH-SEM


k
X3,700 5µm UFH-SEM
l
X2,500 10µm UFH-SEM

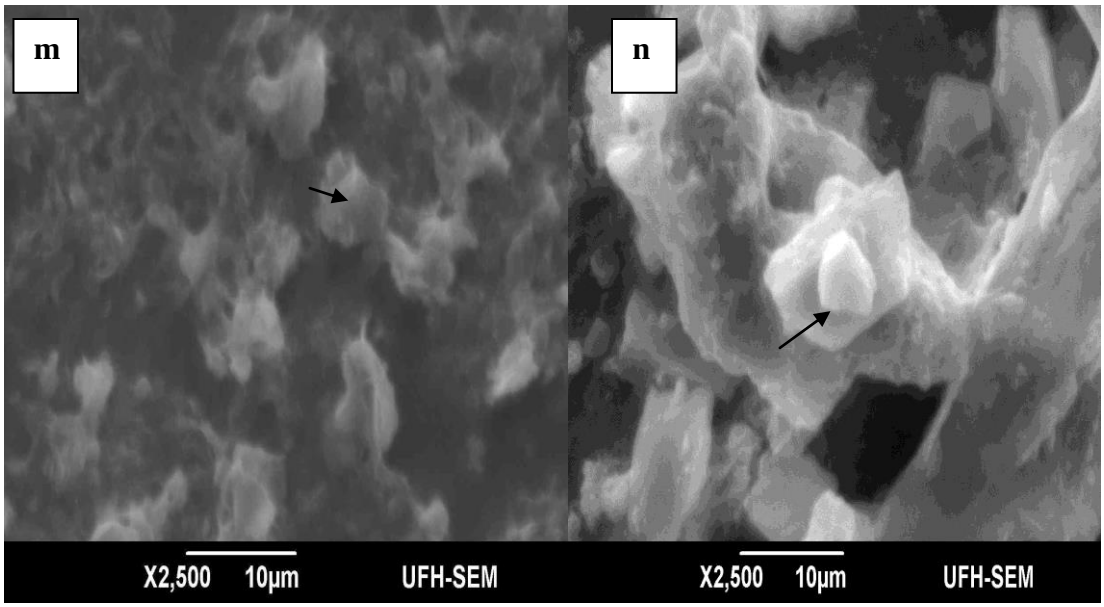
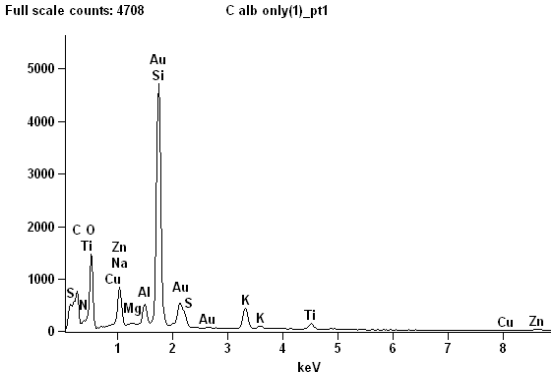


Figure 4.4: Morphological changes in tested bacteria and yeast treated with crude extract and fractions using scanning electron microscope (SEM): **(a)** Germ tubes of *C. albicans* untreated **(b)** *C. albicans* treated with 10 mg/mL EAE appeared rough and trodden. **(c)** Treated with 1.5625 mg/mL BEtA2 showing withered and disintegration of *C. albicans* cells. **(d)** 3.125 mg/mL T1 treated *C. albicans* trodden and disintegration. **(e)** *P. shigelloides* untreated. **(f)** Collapsed *P. shigelloides* 0.3125 mg/mL EAE treated. **(g)** *P. aeruginosa* untreated. **(h)** shrunken and sticky *P. aeruginosa* 1.25 mg/mL EAE treated. **(i)** *S. pyogenes* untreated **(j)** visible perforation of the cell wall of *S. pyogenes* 1.5625 mg/mL T1 treated. **(k)** *S. pyogenes* 1.5625 mg/mL BEtA2 treated with perforated cell wall. **(l)** *S. aureus* untreated **(m)** Muggy *S. aureus* 1.5625 mg/mL BEtA2 treated **(n)** Emaciated *S. aureus* 1.5625 mg/mL T1 treated cells.

4.3.7. X-ray microanalysis of the treated and untreated cells

The common characteristic of all the four pathogenic bacteria and yeast was the presence of sodium (Na), potassium (K), thallium (Ti) and magnessium (Mg) [only in *C. albicans* which is necessary for the formation of germ tubes]. The activity and potency of the extract (EAE) and fractions (T1 & BEtA2) against the organisms were noted by the reduction in the quantity of these elements (treated) when compared to untreated. For example, *P. aeruginosa* teated with EAE had a reduction of sodium from 5.55 % - 1.50 %. For *S. aureus*, thallium was reduced from 2.25 % (untreated) - 0.36 & 0.77 % for T1 and BEtA2 treated respectively while for *C. albicans* pottasium was reduced from 4.16 % - 0.76 % (T1; Table 4.5). The natural mineral element in living tissues, such as carbon and oxygen were expected, while gold (AU) and high silicon (Si) content were assumed to be derived from the spur coater and poly-L-lysine coated glass used respectively (Figure 4.5). Although small quantity of silicon and aluminium was present in the extract and fractions (Table 4.3 and Figure 4.5), the presence or increase of calcium (Ca), sulphur (S), nitrogen (N) and chlorine (Cl) observed in *C. albicans*, *S. pyogenes*, *S. aureus* and *P.aeruginosa* might indicate the lethal effect caused by EAE, T1 and BEtA2 (Table 4.5).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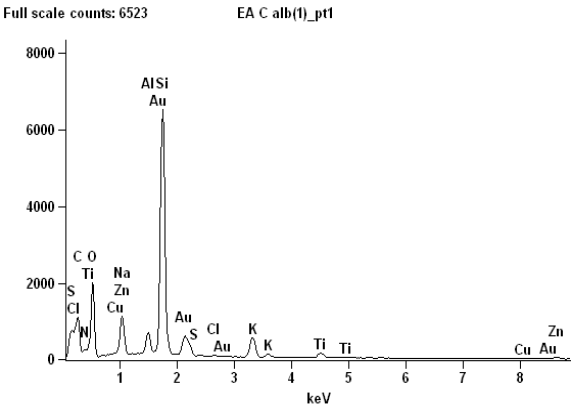
C alb only(1)_pt1
keV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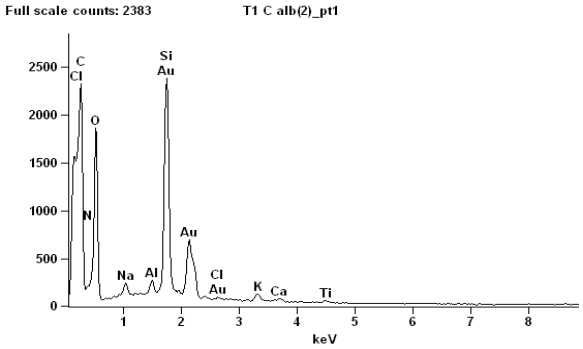
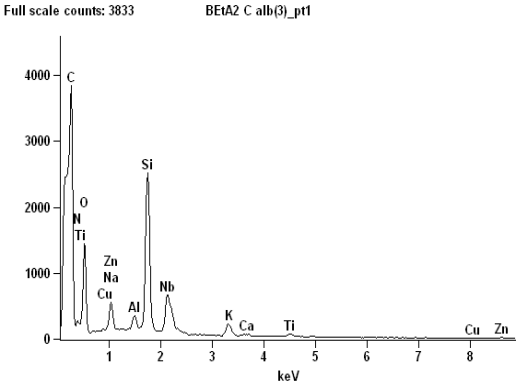
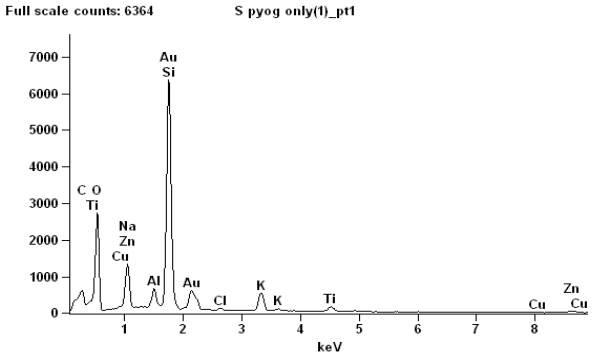
EA C alb(1)_pt1
keV


Full scale counts: 2383
T1 C alb(2)_pt1
keV


Full scale counts: 3833
BEtA2 C alb(3)_pt1
keV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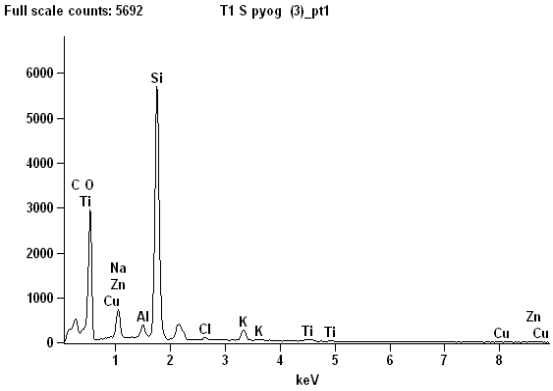
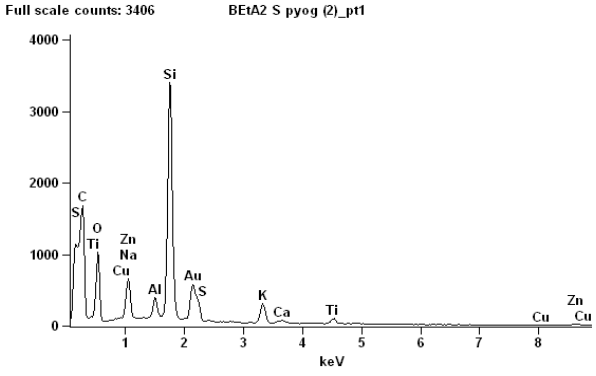
S pyog only(1)_pt1
keV


Full scale counts: 5692
T1 S pyog (3)_pt1
keV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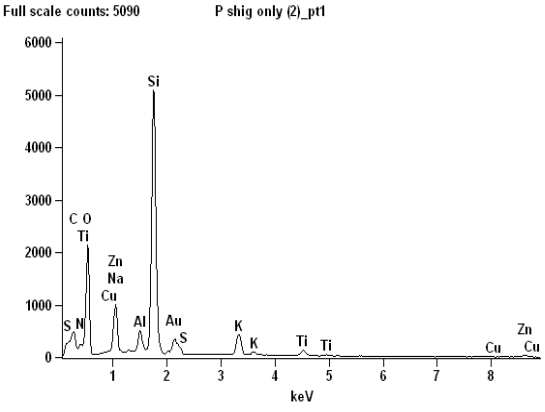
BEtA2 S pyog (2)_pt1
keV


Full scale counts: 5090
P shig only (2)_pt1
keV

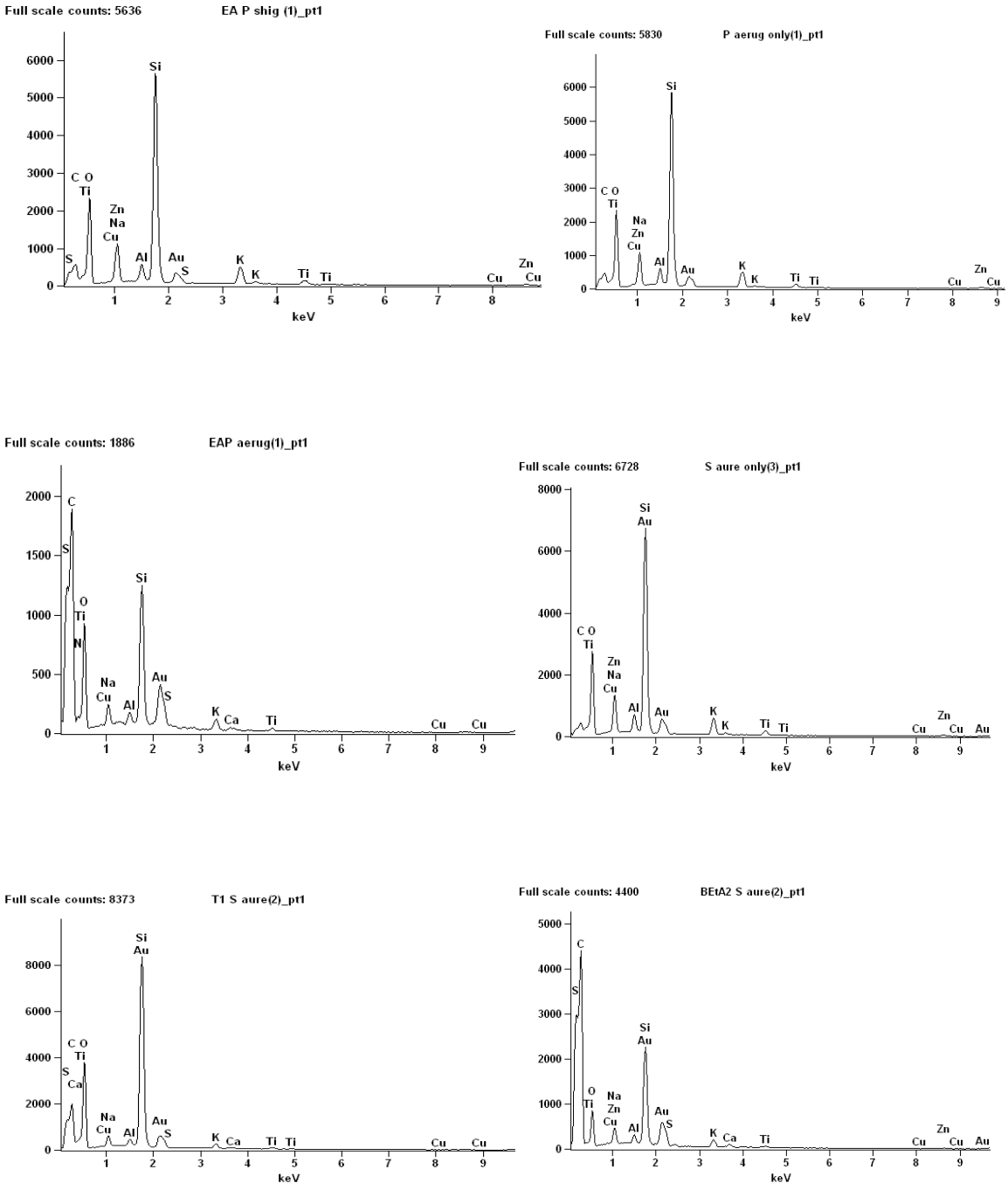


Figure 4.5: EDXS chromatogram of the elements detected in the treated and untreated pathogenic microorganisms

Table 4.5: Quantitative analysis of the elements detected*

Treated & untreated cells	**Mg**	**S**	**N**	**Na**	**Zn**	**Cu**	**K**	**Ca**	**Cl**	**Ti**
C. albicans	0.26	0	0.65	4.27	3.64	0.40	4.16	0	0	2.19
C. albicans + T1	0	0	0	1.0	0	0	0.76	0.38	0.23	0.69
C. albicans + BEtA2	0	0	0.21	3.52	3.14	0	3.49	0	0	0.94
C. albicans + EAE	0	0	0	4.23	3.94	0.12	4.32	0	0	2.09
S. pyogenes	0	0	0	5.56	4.21	0.08	4.37	0	0.36	2.13
S. pyogenes + T1	0	0	0	4.21	3.28	0.16	2.87	0	0.54	1.31
S. pyogenes + BEtA2	0	0	0	3.35	2.56	0.16	2.80	0.41	0	1.37
S. aureus	0	0	0	5.65	3.94	0	4.81	0	0	2.25
S. aureus + T1	0	0.02	0	1.65	0	0	1.32	0.15	0	0.36
S. aureus + BEtA2	0	0	0	1.85	1.88	0	1.60	0.68	0	0.77
P. shigelloides	0	0	1.69	5.14	4.69	0	4.16	0	0	2.22
P. shigelloides +EAE	0	0	0	4.93	4.39	0	4.65	0	0	2.23
P. aeruginosa	0	0	0	5.55	5.44	0.16	5.19	0	0	2.43
P. aeruginosa + EAE	0	0.12	2.04	1.50	0	0	1.58	0.31	0	0.61

*, Values represent % reduction of elements. Mg, Magnesium; S, Sulphur; N, Nitrogen; Na, Sodium; Zn, Zinc; Cu, Copper; K, Potassium; Ca, Calcium; Cl, chlorine; Ti, Thallium.

4.3.8. GC-MS analysis of the T1 and BEtA2

The compounds present in fraction T1 and BEtA2 were analysed using GC-MS. Each peak of the chromatograms represents a distinct chemical compound (Figure 4.6 & 4.7). Among the compounds revealed were benzoic acid, 3,4,5-tris(trimethylsiloxy)-,trimethylsilyl ester (gallic acid) of 99 % quality match to library identification with 11.8 total percentage (RT-9.025; Figure 4.6 & Table 4.6), followed by .beta.-Sitosterol trimethylsilyl ether (silicon hydride) with 8.8174 % (RT-19.963; Figure 4.7 & Table 4.7) and 7.0031 % for hexadecanoic acid, trimethylsilyl ester (palmitic acid; RT-9.9744; Figure 4.7 & Table 4.7). T1 analysis showed 4 unknown compounds one of which had the highest total percentage of 35.6 % (Table 4.6), while 7 were observed in BEtA2 (Table 4.7).

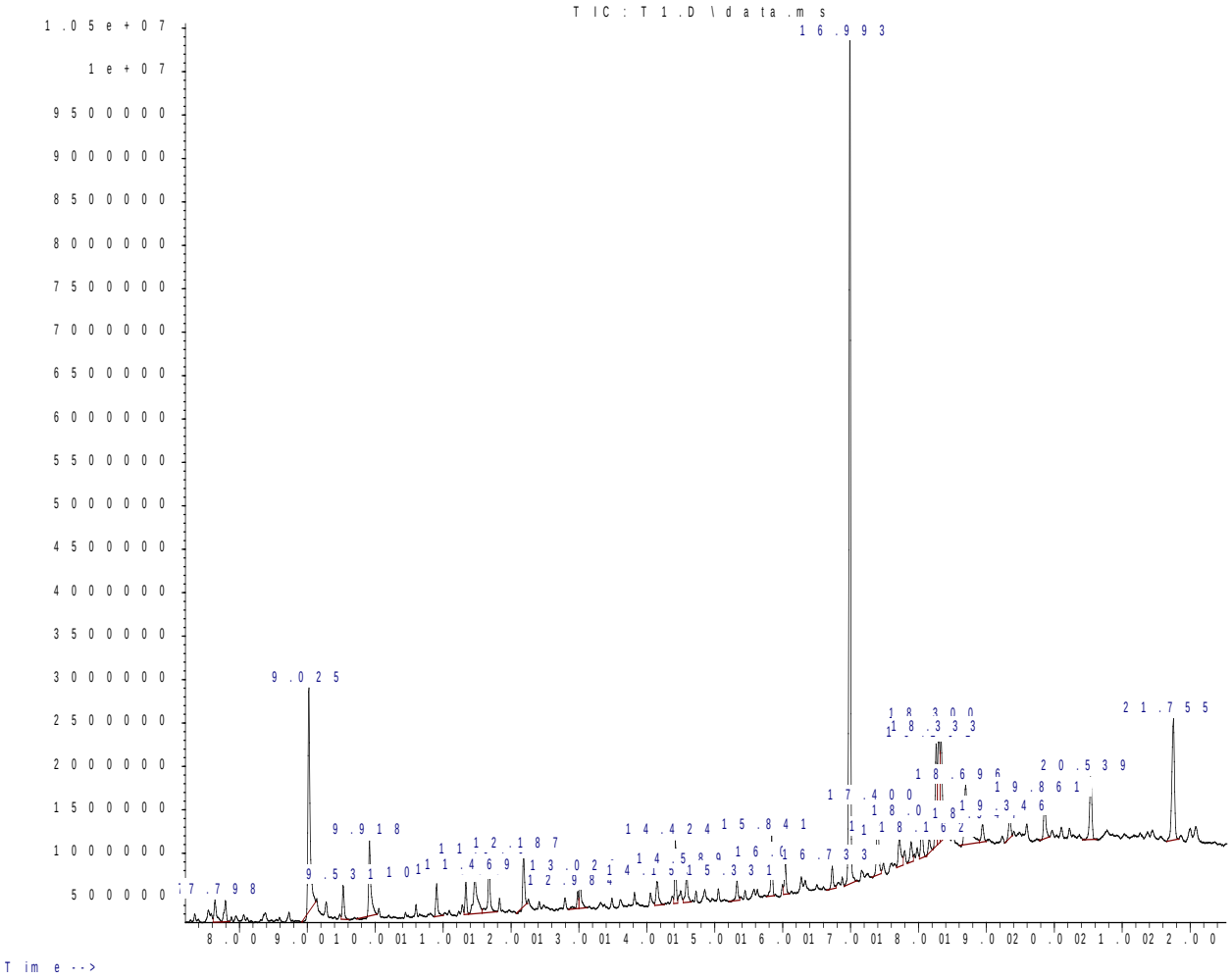


Figure 4.6: Chromatogram of the T1 fraction compounds with their retention time.

Table 4.6: Analysis of compounds identified from T1 fraction

		T1		
PK	**RT (mins)**	**Compounds Identified**	**QM**	**TP (%)**
2	9.0251	Benzoic acid, 3,4,5-tris(trimethylsiloxy)-,trimethylsilyl ester	99	11.8
4	9.9169	Hexadecanoic acid, trimethylsilyl ester	98	4.5
5	10.9048	1-Cyclohexyldimethylsilyloxyoctadecane	96	1.5
7	11.4694	trans-9-Octadecenoic acid, trimethylsilyl ester	95	3.2
8	11.6811	Octadecanoic acid, trimethylsilyl ester	99	2.3
12	14.4268	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	87	2.1
15	15.8382	Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	46	2.4
18	16.993	Unknown	35	35.6
19	17.4035	Cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-, pivalate	55	3.2
20	17.7179	Estra-1,3,5(10),7-tetraen-17-one, 3-[(trimethylsilyl)oxy]-, O-methyloxime	47	1.3
21	17.8975	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.,22Z)-	46	0.8
23	18.2632	Olean-18-en-28-oic acid, 3-oxo-, methyl ester	30	1.0
24	18.3017	Clionasterol acetate	95	1.1
26	19.3474	Estra-1,3,5(10),6-tetraen-17-one, 3-[(trimethylsilyl)oxy]-, O methyloxime	38	1.2
27	19.8606	Unknown	38	2.2
28	20.5406	Unknown	64	3.7
29	21.7531	Unknown	35	7.7

* PK, Peak; RT, Retention Time; TP, Total Percentage

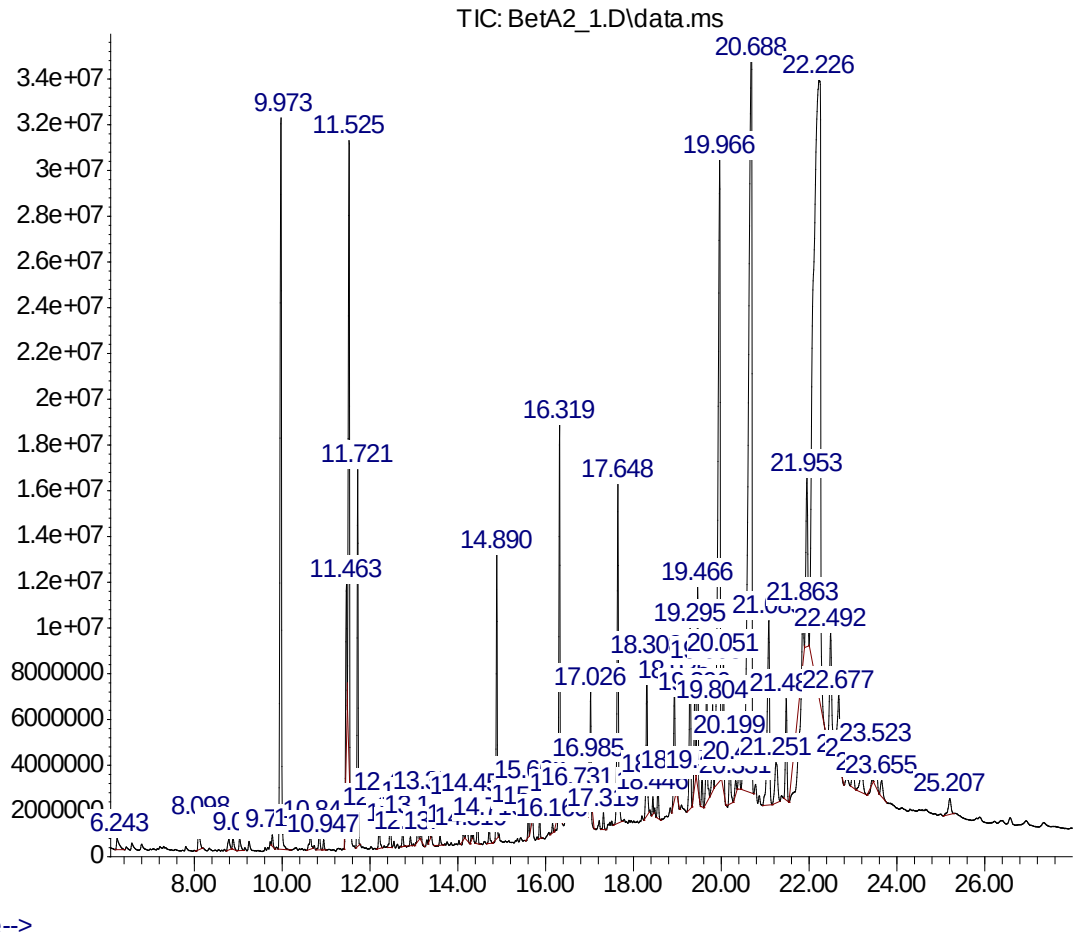


Figure 4.7: Chromatogram of the BEtA2 fraction compounds with their retention time.

Table 4.7: Analysis of compounds identified from BEtA2 fraction

		BEtA2		
PK	**RT (mins)**	**Compounds Identified**	**QM**	**TP (%)**
1	6.2407	Dodecanoic acid, trimethylsilyl ester	99	0.1935
2	8.1012	Tetradecanoic acid, trimethylsilyl ester	98	0.2854
5	9.0314	n-Pentadecanoic acid, trimethylsilyl ester	90	0.1429
6	9.7755	Palmitelaidic acid, trimethylsilyl ester	91	0.0886
7	9.9744	Hexadecanoic acid, trimethylsilyl ester	98	7.0031
8	10.648	6-Methyl-2-phenyl-7-(3,4-dimethylphenylmethyl)indolizine	60	0.1912
9	10.8469	Heptadecanoic acid, trimethylsilyl ester	99	0.2008
11	11.4628	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	95	0.9016
12	11.5269	Oleic acid, trimethylsilyl ester	94	4.8036
13	11.7194	Octadecanoic acid, trimethylsilyl ester	99	2.404
15	12.4636	Nonadecanoic acid, trimethylsilyl ester	68	0.3782
16	12.7458	Isopimaric acid TMS	97	0.1361
18	13.0923	2H-1,3-Dithiolo[4,5-c]coumarine, 2-dicyanomethylene-8-nitro-	95	0.2438
19	13.1628	11-Eicosenoic acid, trimethylsilyl ester	95	0.1093
20	13.3553	Eicosanoic acid, trimethylsilyl ester	99	0.2511
25	14.4523	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	70	0.298
26	14.7153	1-(2-Naphthyloxy)-4-nitroanthraquinone	46	0.166
27	14.8885	Docosanoic acid, trimethylsilyl ester	86	1.7986
28	15.607	Lanostol	64	0.146
29	15.6648	Tricosanoic acid, tert-butyldimethylsilyl ester	50	0.2488
30	15.8701	Octadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	58	0.1648
32	16.3192	Tetracosanoic acid, trimethylsilyl ester	60	2.4069
36	17.0248	Colchiceine, N-desacetyl-N-TFA-	50	0.4058
38	17.6471	Lanosta-8,24-dien-3-ol, acetate, (3.beta.)-	43	2.8456
39	18.3079	Furan, 2-(diphenylamino)-4-(morpholinocarbonyl)-5-(p-nitrophenyl)-	2	1.2651

40	18.4426	19-Norpregna-1,3,5(10)-trien-20-yn-4-amine, 3,17-bis[(trimethylsilyl)oxy]-, (17.alpha.)-	27	0.1029
41	18.5645	3.beta.-(Trimethylsiloxy)cholest-4-ene	38	0.2982
42	18.9366	Cholestano[2,3-d]cinnoline-3',6'-dicarboxylic acid, 4',5'-dihydro-4'-(1-pyrrolidinyl)-, dimethyl ester	12	0.9593
43	19.0008	Ferrocene, [benzoyl(phenylmethyl)amino]-	32	0.1162
44	19.2959	Silane, [[(3.beta.,24R)-ergost-5-en-3-yl]oxy]trimethyl-	99	1.6754
45	19.3985	3,6-Dioxa-2,4,5,7-tetrasilaoctane, 2,2,4,4,5,5,7,7-octamethyl-	43	0.6284
46	19.4691	Stigmasterol trimethylsilyl ether	95	1.5827
47	19.5653	24-Ethyl-.delta.(22)-coprostenol, TMS	38	0.2418
48	19.6679	Unknown	1	1.2063
49	19.8027	Unknown	2	0.694
50	19.963	.beta.-Sitosterol trimethylsilyl ether	94	8.8174
51	20.0528	Unknown	38	1.0601
52	20.2004	9-O-Pivaloyl-N-acetylcolchinol????	40	0.5812
53	20.3287	A-Norcholestan-3-one, 5-ethenyl-, (5.beta.)-	70	0.1255

* PK, Peak; RT, Retention Time; TP, Total Percentage

4.4. DISCUSSION

Thin layer chromatography (TLC) is used to separate and identify intricate mixtures of compounds by comparing R_f values (Skibinski, 2003). The two solvent systems that gave higher separation of compounds, TEt (90:5) and BEtA (90:10:1) have relatively low polarity (Table 2; Figure 1). Toluene and benzene in the combination slightly increased the eluting power of the solvent. Compounds have a high affinity for solvents with a similar polarity; and separation occurs due to different affinities of the compounds within a mixture to the stationary (higher polarity) and the mobile (lower polarity) phases (Ndip *et al*., 2009; Skibinski, 2003). Other factors such as Lewis acid-Lewis base interactions, hydrogen bonding interactions, and Van der Waals interactions have been reported to affect a compound's affinity for the stationary or liquid phase (Skibinski, 2003).

Compounds with the same R_f are likely to be the same. The retention factors of the antioxidant compounds observed; 0.96, 0.64, 0.21, 0.16, 0.11 & 0.35, and 0.07 (Figure 2) have been identified by other investigators to include acetylsalicylic acid, phenol, caffaine, hydroquinone, resorcinol and theophylline compounds (Muijselaar *et al*., 1995; Skibinski, 2003; Silver *et al*., 2010). Phenol is a plant hormone used as a short-acting intravenous anesthetic agent with analgesic, anti-inflammatory and antipyretic properties (Carreno *et al*., 2006). Quinone, a class of aromatic compounds (anti-inflammatory) found widely in plants with the functional group C=O (carbonyl compounds; Table 3) was also identified in the extract. This can also be formed from reactive aromatic compounds with phenol. Naphthoquinones are quinones derived from naphthalene (vitamins K_1 and K_2); they are commonly found in the blood and responsible for proper blood clotting, electron carrier and antitumor activity (Ling *et al*., 2002; Nic *et al*., 2006).

Resorcinol, a white crystalline compound is mainly used in the treatment of psoriasis or eczema, exploited for its antioxidant, analgesic and haemostatic properties and in the management of nausea, asthma, whooping cough, gastric ulcers and diarrhea (Hahn *et al*., 2006). Aryl disulfides (S-S stretch) is a potential antibiotic for the treatment of infections caused by methicillin-resistant *S. aureus* e.g., infection of the bloodstream, bones or joints (Turos *et al*., 2008; Boucher *et al*., 2010). The aliphatic chloro compound (e.g., β-chloro substituted haloalkanoates, organohalogen) C-Cl in the extract can be carcinogenic and genotoxic (Table 3) in both man and animal (Jing and Huyop, 2007), while, aspirin also known as acetylsalicylic acid can enhance excessive gastrointestinal bleeding (Sorensen *et al*., 2000; Meds-cape, 2011). Current evidence suggests a likely correlation between zinc deficiency and leptin levels in the pathogenesis of anorexia in chronic kidney disease (Exley *et al*., 2007). Zinc shield cells against oxidative stress by averting or reducing reactive oxygen species (ROS) formation and by dwindling the early brunt of such stress on the cell (Hfaiedh *et al*., 2012). Copper-zinc superoxide dismutase presence in the nucleus, peroxisomes, and mitochondria of cells acts as an antioxidant enzyme, therefore easing the steady-state concentration of superoxide (Valentine *et al*., 2005).

Benzoic acid, 3,4,5-tris(trimethylsiloxy)-,trimethylsilyl ester identified in T1 fraction (Table 6; Figure 6) is also known as Gallic acid, a phenolics acid and antioxidant that helps to protect human cells against oxidative damage. It has been reported to have anti-fungal, anti-viral properties and cytotoxicity effect against cancer cells, without harming healthy cells (Chanwitheesuk *et al*., 2007). Clionasterol acetate an inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt which has a critical role in cell survival was also identified in the T1 fraction and has a better activity when compared to .beta.-Sitosterol trimethylsilyl ether [also known as silane (silicon hydride)], a compound formed by the action of hydrochloric acid on aluminum silicide

identified in BEtA2 fraction (Table 7; Figure 7). It has been used in traditional medicine for the management of inflammation, dementia, amnesia, neurasthenia and cancer (Cerqueira *et al*., 2003; Le *et al*., 2012). Most other compounds identified are fatty acids e.g., hexadecanoic acid, trimethylsilyl ester, a palmitic acid. Other compounds identified (e.g., Lanostol) have dearth of information in the literature as to their structure and medicinal uses.

Several studies have reported that heavy metals control microorganisms by adversly affecting their growth, morphology, and biochemical activities. It is observed that both Cu and Zn are essential trace elements for bacterial growth which if accumulated in various subcellular compartments of cells, can result in cell death (Gad El-Rab *et al*., 2006). Thallium ionic radius is similar to that of potassium and the toxicity of these elements from most pathogenic organisms mimic each other in many metabolic processes. Thallium may also bind with sulfhydryl groups of protein to inactivate many enzymatic reactions (Smith and Carson, 1977). Thallium along with potassium was considerably reduced in *C. albicans*, *S. aureus* and *P. aeruginosa* by EAE, T1 and BEtA2 in the current study. It has been documented that during the development of some pathogenic organisms, they secrete extracellular material that includes some elements that give them their characteristic virulence (Figueras and Guarro, 1997). These ions may enhance their pathogenicity by affecting the permeability and subsequent invasion of hosts' membranes (Das *et al*., 2010). A change in cell permeability might result in an imbalance in intracellular osmotic pressure, subsequent disruption of intracellular organelles, leakage of cytoplasmic contents and finally cell death (Tolouee *et al*., 2010).

C. albicans requires Magnesium (Mg) for the formation of germ-tube (Walker *et al*., 1984). The germ-tube formation in this study corresponded with the increase in Mg (0.26 %) concentration within the cells. Treated *C. albicans*, which did not form germ-tubes or withered in the process,

had no Mg (0 %) content after incubation under the same conditions for germ-tube formation (Table 5; Figure 5). There was no expression of calcium (0 %) with the presence of Mg in untreated *C. albicans*, whereas calcium uptake of 0.38 % and absence of Mg were observed in the cells treated with T1 fraction which contributed to the morphological changes noted (Figure 4). This is in accordance with the findings of Holmes *et al*. (1991) that inhibition of germ tube formation by calcium does not occur in the presence of magnesium and chelation of Ca(2+) ions increased *C. albicans* cell death to almost 50 % (Alby *et al*., 2010). Therefore unrestricted uptake of magnesium and calcium by the cells resulted in the rapid growth and inhibition of *C. albicans* respectively, demonstrating the essential regulatory function for magnesium and calcium in the morphogenesis of *C. albicans* (Walker *et al*., 1984; Holmes *et al*., 1991).

The stimulation and secretion of chlorine, sulphur, nitrogen and calcium by the compounds describes further mechanisms by which the lethal effect was caused by EAE, T1 and BEtA2 against the yeast and bacteria tested. For example, calcium hypochlorite is mostly used for the disinfection of drinking or swimming pool water (Grohmann and Carlson, 1977; Grohmann, 1991).

4.5. CONCLUSION

A variety of bioactive compounds and elemental nutrients were identified in the extracts and fractions of *P. africanum* which exerted remarkable morphological alterations of the microorganisms. X-ray microanalysis showed the specific spectra of sodium, potassium, thallium and magnessium as the principal intersection of the microorganisms. Since these ions have the potential of fostering microbial invasion by altering the permeability of hosts' membranes, their presence could be important makers and targets for antimicrobial chemotherapy.

REFERENCES

Adams, P.R. (1989). Identification of Essential Oil Components by Ion Trap Mass Spectroscopy. *Academic Press New York*. 302.

Alby, K., Schaefer, D., Sherwood, R.K., Jones, S.K. Jr., and Bennett, R.J. (2010). Identification of a cell death pathway in *Candida albicans* during the response to pheromone. *Eukaryotic Cell*. **9** (11): 1690-1701.

Axelrod, J. (1956). The Enzymic Cleavage of Aromatic Ethers. *Biochemical Journal*. **63**: 634-639.

Boucher, H., Miller, L.G. and Razonable, R.R. (2010). Serious infections caused by methicillin-resistant *Staphylococcus aureus*. *Clinical Infectious Diseases*. **51** (2): 183-197.

Carreno, M.C., Lopez, M.G. and Urbano, A. (2006). Oxidative de-aromatization of para-alkyl phenols into para-peroxyquinols and para-quinols mediated by oxone as a source of singlet oxygen. *Angewandte Chemie International Edition*. **45** (17): 2737-2741.

Cerqueira, F., Watanadilok, R., Sonchaeng, P., Kijjoa, A., Pinto, M., Quarles, U.H., Kroes, B., Beukelman, C. and Nascimento, M.S. (2003). Clionasterol: a potent inhibitor of complement component C1. *Planta Medica*. **69** (2): 174-176.

Chanwitheesuk, A., Teerawutgulrag, A., Kilburn, J.D. and Rakariyatham, N. (2007). Antimicrobial gallic acid from *Caesalpinia mimosoides* Lamk. *Food Chemistry*. **100** (3): 1044-1048.

Coates, J. (2000). Interpretation of Infrared Spectra, A Practical Approach. *Encyclopedia of Analytical Chemistry*. 10815-10837.

Das, K., Tiwari, R.K.S. and Shrivastava, D.K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*. **4** (2): 104-111.

Dudev, T. and Lim, C. (2007). Effect of carboxylate-binding mode on metal binding/selectivity and function in proteins. *Chemical Research*. **40** (1): 85-93.

Exley, C., Charles, L.M., Barr, L., Martin, C., Polwart, A., Darbre, P.D. (2007). Aluminium in human breast tissue. *Journal of Inorganic Biochemistry*. **101** (9): 1344-6.

Figueras, M.J. and Guarro, J. (1997). X-ray microanalysis of black piedra. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology*. **72**: 275-281.

Gad El-Rab, S.M.F., Shoreit, A.A.F. and Fukumori, Y. (2006). Effects of cadmium stress on growth, morphology, and protein expression in *Rhodobacter capsulatus* B10. *Bioscience, biotechnology, and biochemistry*. **70** (10): 2394-2402.

Grohmann A. (1991). Additives for the disinfection of drinking water (chlorine, chlorine dioxide, ozone and silver). In: Aurand K, Hasselbarth U, Lange-Asschenfeldt H, Steuer W, editors. The drinking water regulation. Berlin, Erich Schmidt Verlag, German.

Grohmann A. and Carlson S. (1977) Assessment of treatment and disinfection of swimming pool water using dichloroisocyanurate. *A B Arch des Badew*. **30**: 197-200.

Hahn, S., Koppenhöfer, J., Wibbertmann, A. and Mangelsdorf, I. (2006). Resorcinol: concise international chemical assessment. *World Health Organization*. **71**: 223.

Hfaiedh, N., Murat, J.C. and Elfeki, A. (2012). A combination of ascorbic acid and α-tocopherol or a combination of Mg and Zn are both able to reduce the adverse effects of lindane-poisoning on rat brain and liver. *Journal of Trace Element and Medical Biology*. **26** (4): 1-6.

Holmes, A.R., Cannon, R.D., Shepherd, M.G. (1991). Effect of calcium ion uptake on *Candida albicans* morphology. *Federation of European Microbiological Societies Microbiology Letters*. **61** (2-3): 187-93.

Janausch, I.G., Zientz, E., Tran, Q.H., Kroger, A. and Unden, G. (2002). C4-dicarboxylate carriers and sensors in bacteria. *Biochimica et Biophysica Acta*. **1553** (1-2): 39-56.

Jing, N.H. and Huyop, F. (2007). Dehalogenation of chlorinated aliphatic acid by *Rhodococcus sp*. *Asia Pacific Journal of Molecular Biology and Biotechnology*. **15** (3): 147-151.

Khan, T., Ahmad, M., Khan, H. and Ahmad, W. (2006). Standardization of crude extracts derived from selected medicinal plants of Pakistan for elemental composition using SEM-EDX. *Asian Journal of Plant Sciences*. **5** (2): 211-216.

Krovacek, K., Eriksson, M.L., Gonzalez- Rey, C., Rosinsky, J. and Ciznar, I. (2000). Isolation, biochemical and serological characterization of *Plesiomonas shigelloides* from freshwater in Northern Europe. *Complimentary Immunology, Microbiology and Infectious Diseases*. **23**: 45-51.

Kumar, A., Kumar, A., Thakur, P., Patil, S., Payal, C., Kumar, A. and Sharma, P. (2012). Antibacterial activity of green tea (*Camellia sinensis*) extracts against various bacteria

isolated from environmental sources. *Recent Research in Science and Technology*. **4** (1): 19-23.

Le, T.K.V., Jeong, J.J. and Kim, D.H. (2012). Clionosterol and ethyl cholestan-22-enol isolated from the rhizome of *Polygala tenuifolia* inhibit phosphatidylinositol 3-kinase/akt pathway. *Biological and pharmaceutical bulletin*. **35** (8): 1379-1383.

Ling, T., Poupon, E., Rueden, E.J., Kim, S.H. and Theodorakis, E.A. (2002). Unified synthesis of quinone sesquiterpenes based on a radical decarboxylation and quinone addition reaction. *Journal of American Chemical Society*. **124**: 12261-12267.

Lokhande, R., Singare, P. and Andhale, M. (2010). Study on mineral content of some ayurvedic Indian medicinal plants by instrumental neutron activation analysis and AAS techniques. *Health Science Journal*. **4** (3): 157-168.

Masoko, P.J. and Eloff, J.N. (2006). Bioautography indicates the multiplicity of antifungal Compounds from twenty-four southern African *Combretum* species. *African Journal of Biotechnology*. **5**: 1625-1647.

Meds-cape, (2011). Aspirin: more evidence that low dose is all that is needed. *Cme.medscape.com*. 1-2.

Miceli, H.M., Diaz, J.A. and Lee, S.A. (2011). Emerging opportunistic yeast infections. *The Lancet Infectious Diseases*. **11** (2): 142-151.

Muijselaar, P.G.H.M., Claessens, H.A. and Cramers, C.A. (1995). Parameters controlling the elution window and retention factors in micellar electrokinetic capillary chromatography. *Journal of Chromatography A*. **696**: 273-284.

Muruganantham, S., Anbalagan, G. and Ramamurthy, N. (2009). FT-IR and SEM-EDS comparative analysis of medicinal plants, *Eclipta Alba* Hassk and *Eclipta Prostrata* Linn. *Romanian Journal of Biophysics*. **19** (4): 285-294.

Ndip, R.N., Ajonglefac, A.N., Wirna, T., Luma, H.N., Wirmum, C., Efange, S.M.N. (2009). *In-vitro* antimicrobial activity of *Ageratum conyzoides* on clinical isolates of *Helicobacter pylori*. *African Journal of Pharmacy and Pharmacology*. **3**: 585-592.

Nic, M., Jirat, J. and Kosata, B. (2006). Quinones, IUPAC Compendium of Chemical Terminology. *Gold book.* **1351**: 8-10.

Okeleye, B.I., Bessong, P.O. and Ndip, R.N. (2011). Preliminary phytochemical screening and *in vitro* anti-*Helicobacter pylori* activity of extracts of the stem bark of *Bridelia micrantha* (Hochst., Baill., Euphorbiaceae). *Molecules*. **16** (8): 6193-6205.

Okeleye, B.I., Samie, A., Bessong, P.O., Mkwetshana, N.T., Green, E., Clarke, A.M. and Ndip, R.N. (2010). Crude ethyl acetate extract of the stem bark of *Peltophorum africanum* (Sond, Fabaceae) possessing *in vitro* inhibitory and bactericidal activity against clinical isolates of *Helicobacter pylori*. *Journal of Medicinal Plants Research.* **4**: 1432-1440.

Otang, W.M., Grierson, D.S. and Ndip, R.N. (2011). The effect of the acetone extract of *Arctotis arctotoides* (asteraceae) on the growth and ultrastructure of some opportunistic fungi associated with HIV/AIDS. *International Journal of Molecular Sciences*. **12**: 9226-9235.

Rashid ch, A., Qureshi, M.Z., Raza, S.A., William, J. and Arshad, M. (2010). Quantitative determination of antioxidant potential of *Artemisia persica*. *Analele UniversităŃii din Bucuresti – Chimie (serie nouă)*. **19** (1): 23-30.

Silver, J.E., Bellinghausen, P., Fowler, N., Pipes, R. (2010). Method development strategies for amine bonded phase columns for medium pressure liquid chromatography. *American Chemical Society*. **239**: 79.

Skibinski, G. (2003). Thin-Layer Chromatography. *Phung Phamarceuticals*. **236**: 77-86.

Smith, I.C. and Carson, B.L. (1977). Trace elements in the environment (Thallium). *Ann Arbor Michigan*. **1**: 394.

Sorensen, H.T., Mellemkjaer, L. and Blot, W.J. (2000). Risk of upper gastrointestinal bleeding associated with use of low-dose aspirin. *American Journal of Gastroenterology*. **95** (9): 2218-2224.

Tolouee, M., Alinezhad, S., Saberi, R., Eslamifar, A., Zad, S.J., Jaimand, K., Taeb, J., Rezaee, M.B., Kawachi, M., Shams-Ghahfarokhi, M. (2010). Effect of *Matricaria chamomilla* L. flower essential oil on the growth and ultrastructure of *Aspergillus niger* van Tieghem. *International Journal of Food Microbiology*. **139** (3): 127-133.

Turos, E., Revell, K.D., Ramaraju, P., Gergeres, D.A., Greenhalgh, K., Young, A., Sathyanarayan, N., Dickey, S., Lim, D., Alhamadsheh, M.M. and Reynolds, K. (2008). Unsymmetric aryl-alkyl disulfide growth inhibitors of methicillin-resistant *Staphylococcus aureus* and *Bacillus anthracis*. *Bioorganic & Medicinal Chemistry*. **16**: 6501-6508.

Valentine, J.S., Doucette, P.A. and Potter, S.Z. (2005). Copper-zinc superoxide dismutase and amyotrophic lateral sclerosis. *Annual Review of Biochemistry*. **74**: 563-593.

Walker, G.M., Sullivan, P.A., Shepherd, M.G. (1984). Magnesium and the regulation of germ-tube formation in *Candida albicans*. *Journal of General Microbiology*. **130** (8): 1941-1945.

Wei, D., Zhengyuo, L., Guomin, W., Yingwu, Y., Yingguo, L. and Yuxian, X. (2006). Separation and purification of natural pyrethrins by reversed phase high performance Liquid chromatography. *China Journal of Analytical Chemistry*. **34** (1): 1776-1778.

Yrjönen, T., Peiwu, L., Summanen, J., Hopia, A. and Vuorela, H. (2003). Free radical-scavenging activity of phenolics by reversed-phase TLC," *Journal of the American Oil Chemists' Society*. **80** (1): 9-14.

Zhang, J.P., Zhang, Y.B., Lin, J.B. and Chen, X.M. (2012). Metal azolate frameworks: from crystal engineering to functional materials. *Chemical Reviews*. **112**: 1001-1033.

ZiAmons, E., Goffin, E., Lejeune, R., Angenot, L. and Thunus, L. (2004). FT-IR measurement of tagitinin C after solvent extraction from *Tithonia diversifolia*. *Talanta*. **62** (2): 383-387.

CHAPTER FIVE

Polyphenolic content and *in vitro* antioxidant evaluation of the stem bark extract of *Peltophorum africanum* (Sond, Fabaceae)

ABSTRACT

The study evaluated the *in vitro* antioxidant activities of *Peltophorum africanum* stem bark extract by means of radical scavenging and ferric reducing power analysis using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2`-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) kit, hydrogen peroxide (H_2O_2), iron (iii) chloride (Fe^{3+}) and nitric oxide (NO). To assess the likely effects of secondary metabolites on the activities observed; total proanthocyanidins, phenolics, flavonols, and flavonoids were determined using standard phytochemical methods. Data was analyzed by ANOVA test and the *p*-value < 0.05 was considered significant. Extract scavenging activity of 88.73± 6.69 % (25 µg mL^{-1}), 53.93±1.09 % (25 µg mL^{-1}), 87.293±6.64 % (25 µg mL^{-1}), 10.55±2.16 mM (0.42 mM) and 3.8115±0.06 (25 µg mL^{-1}) were recorded for H_2O_2, NO, DPPH, ABTS and Fe^{3+} reducing power respectively. These values were comparable to the standard compounds; DBPC*BHT, L (+) - Ascorbic acid and Trolox™ ($p < 0.05$). Proanthocyanidins (92.18±4.68 mg/g) occurred more ($p < 0.05$) in the extract compared to all other compounds tested: phenolics (60.53±1.46 mg/g) > flavonoids (18.37± 2.11 mg/g), > flavonols (11.20±3.90 mg/g). However the difference between flavonols and flavonoids was not significant ($p > 0.05$) at 95% confidence interval. The results of this study validate the folkloric use of *P. africanum* which could be exploited as an easily available source of natural antioxidants, in the pharmaceutical sector or as a food supplement.

5.1. INTRODUCTION

Antioxidants are molecules that are capable of neutralizing the harmful effects of the reactive oxygen species (ROS) through an enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non enzymatic defence system (vitamin E, vitamin C and glutathione) (Paz´dzioch-Czochra and Widen ska, 2002). However, with the growing damaging environmental factors such as smoke, radiation and toxic chemicals; the natural defence system is often weakened (Valko *et al*., 2006). The ROS consist of free radicals ($OH^{\cdot}$, $O_2^{\cdot -}$, $^{\cdot}NO$, $RO_2^{\cdot}$ and $LOO^{\cdot}$) and non-free radical species (H_2O_2, O_2^{-1}, O_3, and LOOH). Radicals are chemically unstable atoms that cause oxidative damage to various biomolecules including lipid cells, proteins and DNA. They are known to be the underlying cause of oxidative stress which is grossly implicated in the pathogenesis of various diseases such as cancer, diabetes, cardiovascular diseases, aging and metabolic syndrome (Mandal *et al*., 2009).

Some synthetic antioxidant agents including butylated hydroxyanisole (BHA) and the related compound, butylated hydroxytoluene (BHT) are phenolics compounds that are often added to foods to preserve fats. These are commercially available; on the other hand, they have been considered to be toxic to living cells (Mbaebie *et al*., 2012). In recent years, there is an increasing interest in the discovery of natural antioxidants from medicinal plants. These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS, and therefore can enhance the immune defence and lower the risk of cancer and degenerative diseases. This activity is mainly due to the presence of compounds such as flavonoids, phenols, flavonols, carotenoids and proanthocyanidins (Ebrahimzadeh *et al*., 2010).

Peltophorum africanum (Fabaceae), also known as Weeping Wattle, is a semi-deciduous to deciduous tree, widespread in South Africa. Leaves and bark have been conventionally used to

clear intestinal parasites and relieve stomach problems; stem bark used to treat colic, diarrhoea, human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS), venereal diseases and infertility; and roots are used to heal sore throat and toothache (Bizimenyera *et al*., 2005 (A); Theo *et al*., 2009). It has also been reported to be active against *E. coli, S. aureus, C. jejuni*, HIV-1 (Betulinic acid) and the acetone, hexane, ethanol and dichloromethane extract to contain scavenging property (Samie *et al*., 2005; Bizimenyera *et al*., 2005 (B); Bizimenyera *et al*., 2005 (A); Theo *et al*., 2009). Other species of this plant common in other countries such as *Peltophorum pterocarpum* (DC) Backer ex K. Heyne (family Leguminosae) has been reported to contain phytochemicals (Nathan *et al*., 2012; Taiwo *et al*., 2013), high antioxidant activity (ethyl acetate and methanol) with known compounds (Jain *et al*., 2012).

To the best of our knowledge, there is no scientific report on the antioxidant potential of the ethyl acetate extract of this plant despite the marked susceptibility reported in our previous study against *Helicobacter pylori*, an organism implicated in gastritis, ulcer and gastric cancer (Okeleye *et al*., 2010; Tanih *et al*., 2010). Therefore, the present study was aimed to assess the qualitative and quantitative compositions of the phytochemicals and antioxidant potential of the ethyl acetate stem bark extract of *P. africanum* in order to provide scientific basis to justify its therapeutic usage.

5.2. MATERIALS AND METHODS

5.2.1. Preparation of plant extract

Peltophorum africanum (stem bark) was selected based on ethnobotanical survey. The plant was collected and identified in Limpopo Province, South Africa in partnership with a botanist at the University of Venda (voucher specimen number BP01). The extraction and the sterility test were done as previously reported (Okeleye *et al*., 2013).

5.2.2. Total polyphenolics content

5.2.2.1. Phenolics

Folin-Ciocalteu method was used to determine the total phenolics content of the extract (Wolfe *et al*., 2003). One millilitre of the extract (10 mg mL^{-1}) was mixed with 5 mL of Folin-Ciocalteu reagent (10% v/v) and 4 mL of Na_2CO_3 (7.5% w/v). Samples of extract were kept at a final concentration of 1 mg mL^{-1}. The mixture was vortexed and allowed to stand for 30 min at 40°C for colour development. Absorbance was measured at 765 nm (Helios Epsilon Thermo Spectronic; USA) and expressed in terms of Gallic acid equivalent (mg/ g of dry mass) using the calibration curve equation: $Y = 0.1216x$, $R^2 = 0.936512$, where; x was the absorbance and Y was the Gallic acid equivalent (mg/g). The experiment was conducted in triplicate and the results reported as mean ± SD values.

5.2.2.2. Flavonols

Total flavonols was estimated using the method of Kumaran and Karunakaran (2007). To 2 mL of a sample (3.5 mg mL^{-1}), 2 mL of $AlCl_3$ ethanol (2% w/v) and 3 mL $NaNO_2$ (5% w/v) solutions were added. Samples were maintained at a final concentration of 1 mg mL^{-1}. After 2.5

h at 20^{o}C, absorbance was read at 440 nm. Total flavonols were calculated as quercetin (mg/g) using the equation: Y= 0.0255x, R^2 = 0.9812, where; x was the absorbance and Y the quercetin equivalent (mg/g).

5.2.2.3. Proanthocyanidins

The procedure reported by Mbaebie *et al.* (2012) was used to determine the total proanthocyanidins. A volume of 0.5 mL of 10 mg mL^{-1} extract solution was mixed with 3 mL of vanillin-methanol (4% w/v) solution and 1.5 mL HCl. Extracts were at a final concentration of 1 mg mL^{-1}. The mixture was allowed to stand for 15 minutes and then measured at 500 nm (Helios Epsilon Thermo Spectronic; USA). The total proanthocyanidins was expressed as catechin equivalents (mg/g) based on the following curve equation: Y = 0.5825x, R^2 = 0.9277, where; x was the absorbance and Y the catechin equivalent (mg/g).

5.2.2.4. Flavonoids

Total flavonoid was determined using the method of Ordonez *et al*. (2006). A volume of 0.5 mL of $AlCl_3$ ethanol solution (2% w/v) was added to 0.5 mL of sample solution (2 mg mL^{-1}). Extracts were evaluated in triplicate at a final concentration of 1 mg mL^{-1}. The resultant mixture was incubated for 1 h for yellow colour development which indicated the presence of flavonoid. The absorbance was measured at 420 nm using Helios Epsilon Thermo Spectronic (USA). Total flavonoid was calculated as quercetin equivalent (mg/g) and was quantified on the basis of the following equation: Y = 0.255x, R^2 = 0.9812, where x was the absorbance and Y the quercetin equivalent (mg/g).

5.2.3. *In vitro* antioxidant capacities

5.2.3.1. Ferric-reducing power (FRAP) assay

The reducing power of the extract was evaluated according to the method of Kumar and Hemalatha (2011). Exactly 1.0 mL of the extract prepared in distilled water (25 – 500 µg mL^{-1}) was added to the mixture containing 2.5 mL of phosphate buffer (0.2 M; pH 6.6) and 2.5 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1% w/v). Standard compounds used were L (+) - Ascorbic acid ($C_6H_8O_6$) and 2, 6 – Di – *tert* – butyl – 4 – methyl phenol [$(CH_3)_3C]_2C_6H_2(CH_3)OH$]. The resulting mixture was incubated at 50°C for 20 min, followed by the addition of 2.5 mL of CCl_3COOH (10% w/v) and then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1 %, w/v). The experiment was conducted in duplicate and absorbance measured at 700 nm (Helios Epsilon Thermo Spectronic, USA) against a blank sample of only phosphate buffer. Higher reducing power of the extract was indicated by the increased absorbance.

5.2.3.2. Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the plant extract was estimated using the method of Nabavi *et al*. (2009). Four millilitres of extract prepared in distilled water (25 – 500 µg mL^{-1}) was mixed with 0.6 mL of H_2O_2 solution (4 mM) prepared in phosphate buffer (0.1 M; pH 7.4) and incubated for 10 min. A negative control containing H_2O_2 and methanol was included in the experiment. The absorbance of the solution was taken at 230 nm (Helios Epsilon Thermo Spectronic, USA) against a blank solution containing the phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenged by the extract and standard compounds (DBPC*BHT and L (+) - Ascorbic acid) were calculated as follows: % Scavenged

$[H_2O_2] = [(Ao - A_1)/Ao] \times 100$ where Ao was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard.

5.2.3.3. Scavenging activity of nitric oxide

The method of Ebrahimzadeh *et al*. (2010) was used with some modifications. A volume of 2 mL of sodium nitroprusside (10 mM) prepared in phosphate buffer saline (0.5 mM; pH 7.4) was mixed with 0.5 mL of plant extract and standard compounds; $C_6H_8O_6$ and $[(CH_3)_3C]_2C_6H_2(CH_3)OH$ at different concentrations (25 – 500 µg mL^{-1}). Sodium nitroprusside mixed with methanol was used in the procedure as a negative control. The mixture was incubated at 25°C for 2 $^1/_2$ hrs and 0.5 mL of incubation solution was withdrawn and mixed with 0.5 mL of Griess reagent [1.0 mL sulfanilic acid reagent (0.33 % dissolved in 20 % glacial acetic acid) mixed with 1.0 mL of naphthylethylenediamine dichloride (0.1% w/v)]. The absorbance was measured at 540 nm (Analytical & Diagnostic Product Gen™ BioTek, USA) after incubation at room temperature for 30 min. The percentage of nitric oxide scavenged was calculated as follows: % Scavenged [NO] = $[(Ao - A_1)/Ao] \times 100$ where Ao was the absorbance of the control and A_1 was the absorbance in the presence of extract and standards.

5.2.3.4. DPPH radical scavenging assay

The total antioxidant capacity of the extract was carried out using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as previously described by Shen *et al*. (2010). A solution of 0.135 mM DPPH in methanol was prepared and 1.0 mL of this solution mixed with 1.0 mL of extract (25 – 500 µg mL^{-1}) dissolved in methanol. DPPH in methanol was used as a negative control. The combination was thoroughly mixed and left in the dark at room temperature for 30 min before

the absorbance was measured at 517 nm (Helios Epsilon Thermo Spectronic, USA). L (+) - Ascorbic acid and DBPC*BHT were used as the references. All the tests were run in duplicate. The ability to scavenge DPPH radical was calculated using the following equation: % Scavenged [DPPH] = $[(Ao - A_1)/Ao] \times 100$ where Ao was the absorbance of the control and A_1 was the absorbance in the presence of extract and standards.

5.2.3.5. ABTS radical scavenging assay

ABTS (2, 2`-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging assay was carried out using antioxidant assay kit (Sigma, USA) and manufacturer's instructions were followed. Briefly, 10µL of each sample (0 – 0.42 Mm); ethyl acetate extract and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox^{TM}); a water-soluble vitamin E analog, served as a standard or control antioxidant), was added to 20µL of myoglobin (from horse heart) in 96 well plate. Exactly 150µL of ABTS was added and the mixture incubated at room temperature for 5 minutes. Stop solution (100µL) was added to each well and the endpoint absorbance read at 405 nm using Analytical & Diagnostic Product Gen™ BioTek, USA. Concentration was varied at 0 to 0.42 mM and assay buffer (500 µL) without the samples (0 mM) was included as negative control. ABTS scavenging capacity was expressed as total antioxidant concentration in relation to the Trolox™ standard concentration calculated using the equation: X (mM) = $[y(A_{405}) - \text{Intercept/Slope}] \times$ dilution factor; obtained from the linear regression of the standard curve ($y = -1.3066x + 1.3235$, $R^2 = 0.9477$), where $y(A_{405})$ was the average absorbance of the test sample. The experiment was conducted in duplicate and the results reported as mean ± SD values.

5.2.4. Statistical analysis

Statistical analysis was performed using MINITAB, version 12 for windows and the SPSS Version 17.0 (Illinois USA, 2011). Regression analysis was used to determine effective concentration ($EC_{50;}$ MINITAB). The one way ANOVA test was used to determine if there was any statistical difference in the activity of the ethyl acetate extract and the standard compounds (DBPC*BHT, L (+) - Ascorbic acid and Trolox™) against DPPH, ABTS, NO, H_2O_2, ferric reducing agent and polyphenolics (SPSS). *P*-values < 0.05 were considered significant.

5.3. RESULTS

5.3.1. Total polyphenolics content

Polyphenolic compounds may contribute directly to anti-oxidative activities. Higher quantity of proanthocyanidins (92.18±4.68 mg/g of catechin equivalent) was observed to be present in the extract compared to all other compounds tested ($p < 0.05$), followed by phenolics (60.53± 1.46 mg/g Gallic acid equivalent); flavonoids (18.37± 2.11 mg/g of quercetin equivalent) and flavonols (11.20±3.90 mg/g quercetin equivalent) respectively as represented in Figure 5.1. However the difference between flavonols and flavonoids was not significant ($p > 0.05$) at 95% confidence interval.

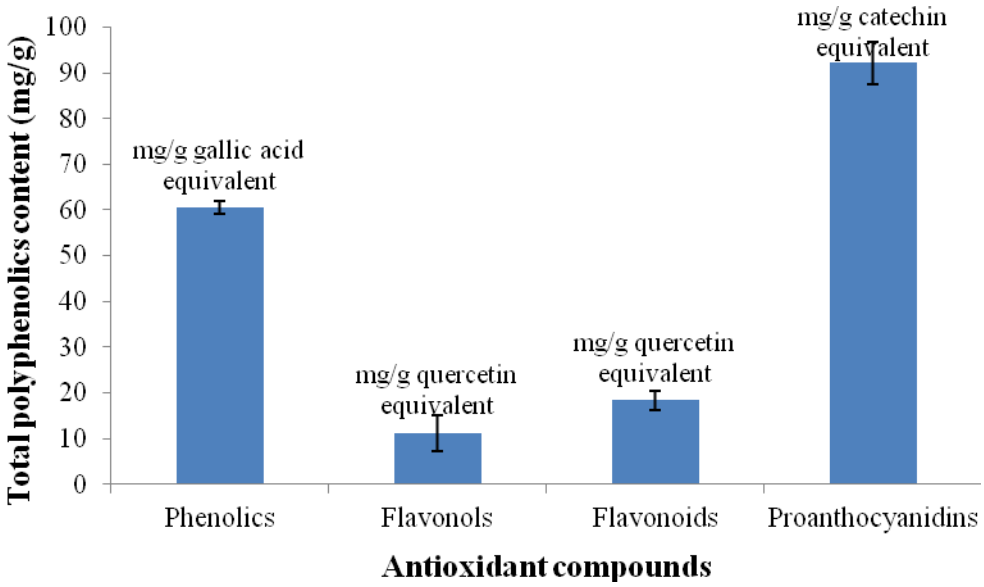


Figure 5.1: Total polyphenolic content of ethyl acetate extract of *P. africanum*.

5.3.2. Antioxidant capacities

5.3.2.1. Ferric-reducing power activity

An important mechanism of phenolic antioxidant action is Fe^{3+} reduction which is often used as an indicator of electron donating activity. The antioxidant potentials of the plant extract was estimated from their ability to reduce Fe^{3+} to Fe^{2+} with observable colouration from lemon to green after the addition of ferrous chloride. Ferric reducing activity of 3.8115±0.06 and 3.828±0.05 were observed at 25 µg mL^{-1} and 500 µg mL^{-1} concentration of the extract. The extract (3.828±0.051) and 2, 6 – Di – *tert* – butyl – 4 – methyl phenol (DBPC*BHT; 3.828± 0.040) were higher in reducing power activity than L (+) - Ascorbic acid (3.446±0.070; $p < 0.001$), however; there was no significant difference observed between the extract and DBPC*BHT ($p > 0.05$) as presented in Figure 5.2.

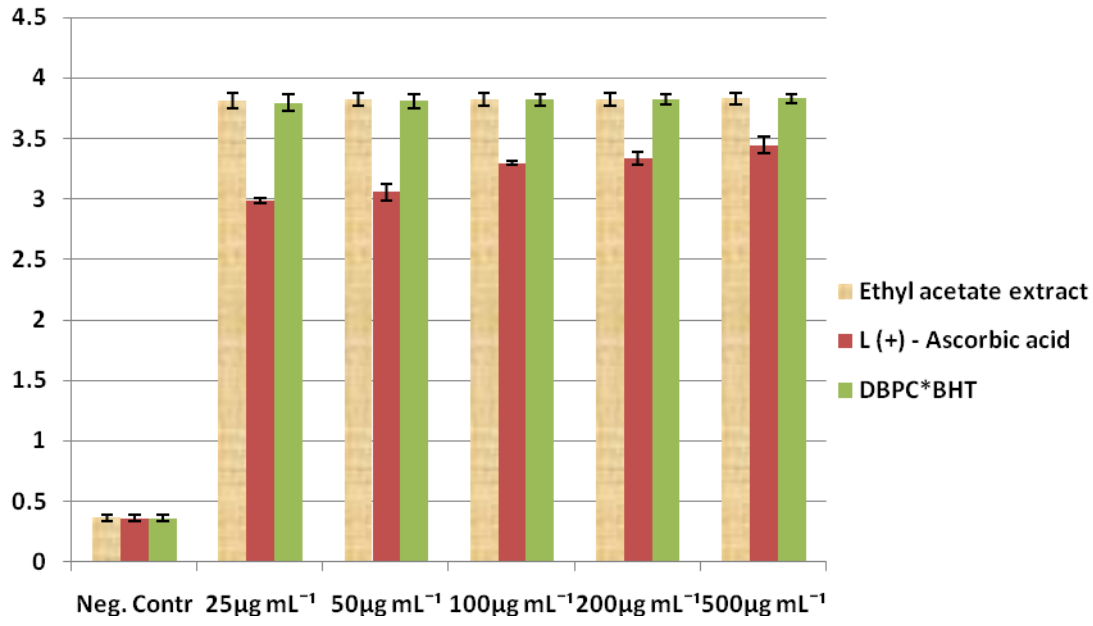


Figure 5.2: Reducing power of the ethyl acetate extract of *P. africanum* in comparison to L (+) - Ascorbic acid and DBPC*BHT.

5.3.2.2. Hydrogen peroxide scavenging activity

The measurement of H_2O_2 scavenging activity is one of the useful methods to determine the ability of antioxidants to decrease the level of prooxidants. At concentrations of 25 μg mL^{-1} & 500 μg mL^{-1}, ethyl acetate extract exhibited 88.73± 6.69 and 92.86±2.39 percent hydrogen peroxide scavenging activity, respectively, while, at the same concentration, higher activity was observed for L (+) - Ascorbic acid (99.54±0.42 & 99.62±0.42 %) and DBPC*BHT (98.81±0.83 & 98.69±0.39 %) respectively (Figure 5.3). There was no statistically significant difference observed in activity of the extract and the standard compounds ($p > 0.05$).

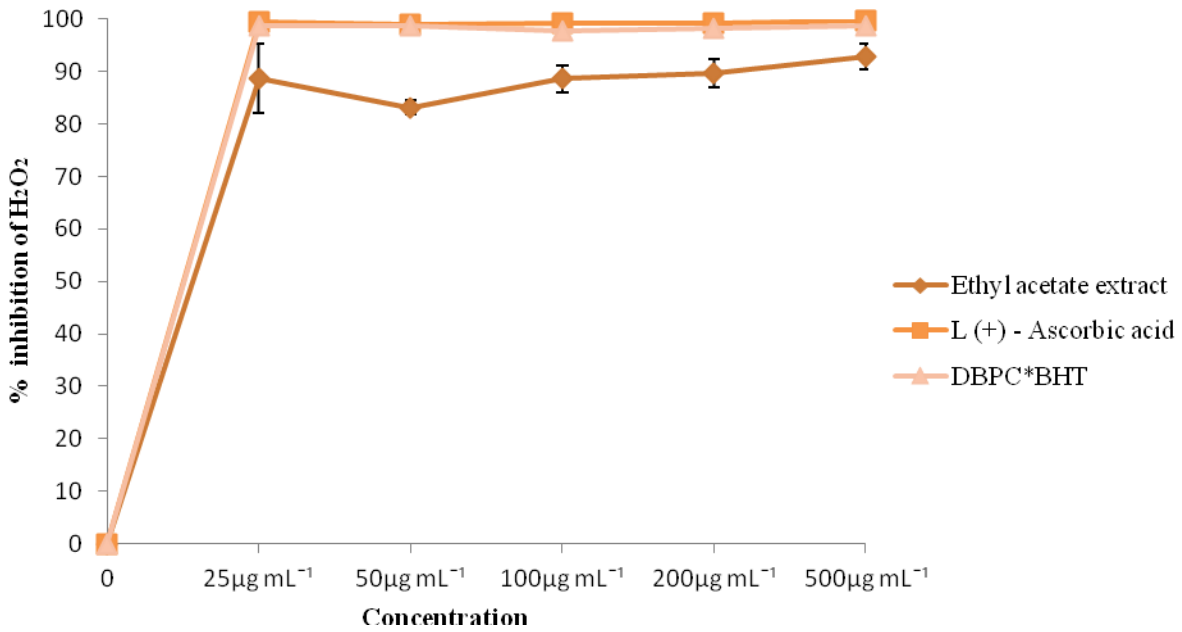


Figure 5.3: Percentage inhibition of hydrogen peroxide scavenging activity of ethyl acetate extract of *P. africanum*, L (+) - Ascorbic acid and DBPC*BHT.

5.3.2.3. Scavenging activity of nitric oxide

The highest percentage inhibitory activity of the extract, L (+) - Ascorbic acid and DBPC*BHT against nitric oxide radical was 53.93±1.09 (25µg mL^{-1}), 51.37±9.85 (50µg mL^{-1}) and 49.97±0.49 (50µg mL^{-1}) percent (Figure 5.4). The inhibitory effect of the extract was relatively similar to the standard drugs, as no statistical difference was observed in their activities ($p > 0.05$).

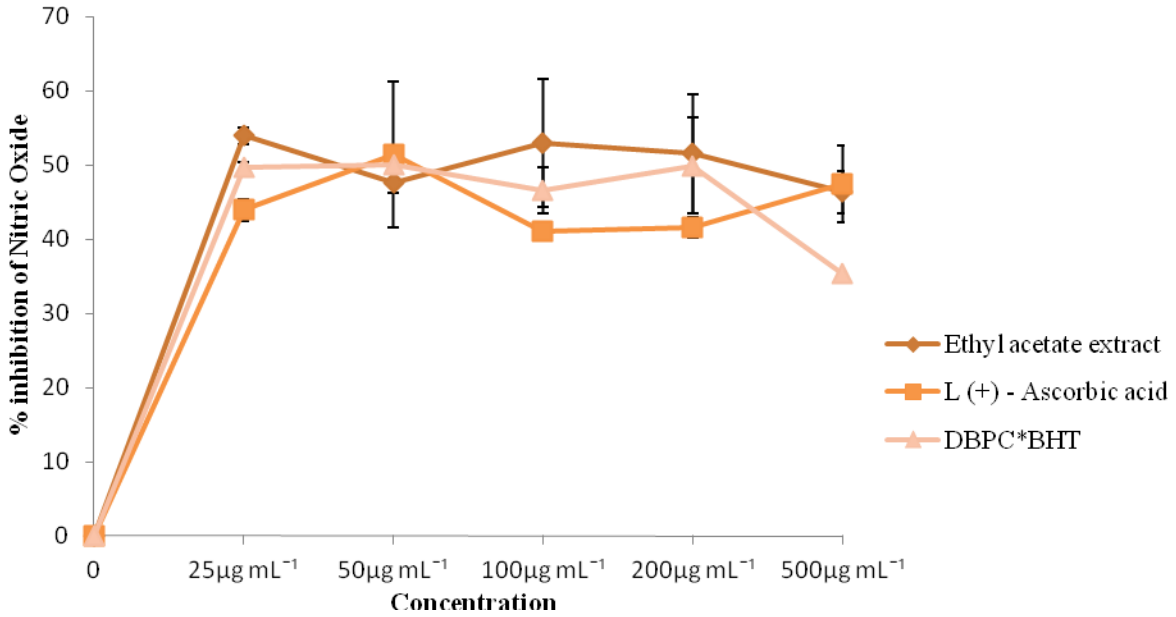


Figure 5.4: Nitric oxide scavenging activity of the ethyl acetate extract of *P. africanum* compared to L (+) - Ascorbic acid and DBPC*BHT.

5.3.2.4. DPPH radical scavenging assay

The stable DPPH radical is a commonly used substrate for relatively quick evaluation of free radical scavenging activity. Exactly 87.293±6.64 and 89.503±0.39 % inhibition of DPPH was noted at 50 µg mL^{-1} and 25µg mL^{-1} of the extract respectively. The scavenging effect of DBPC*BHT increased distinctively with concentration. At the highest dose of 500 µg mL^{-1}, L (+) - Ascorbic acid showed high scavenging effect of 92.40±0.59 percent, followed by the extract (89.50±0.39 %) and DBPC*BHT (82.60±1.56 %) respectively as shown in Figure 5.5. The EC_{50} (effective concentration at 50 %) of Ethyl acetate extract, (+) - Ascorbic acid and DBPC*BHT that scavenged H_2O_2, NO and DPPH were analysed and presented in Table 5.1.

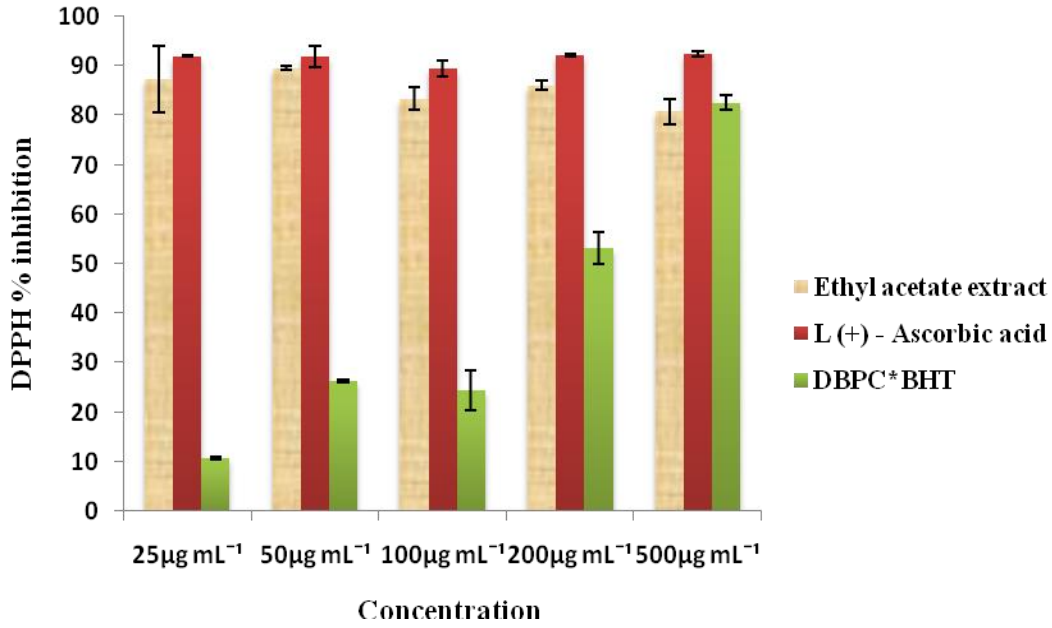


Figure 5.5: DPPH radical scavenging activity of the ethyl acetate extract of *P. africanum* in comparison to L (+) - Ascorbic acid and DBPC*BHT.

Table 5.1: Effective concentration (EC) of the scavengers using regression analysis

	Ethyl acetate extract		(+) - Ascorbic acid		DBPC*BHT	
	R. Eq.*	**EC_{50}* ($\mu g\ mL^{-1}$)**	**R. Eq.**	**EC_{50} ($\mu g\ mL^{-1}$)**	**R. Eq.**	**EC_{50} ($\mu g\ mL^{-1}$)**
H_2O_2	Y = 86.2 + 0.0138X	131.16±1.65	Y = 99.2 + 0.000790X	88.61±0.17	Y = 98.5 + 0.00010X	100±0.31
NO	Y = 52.4 - 0.0110X	204.55±1.89	Y = 44.6 + 0.0025X	276±3.15	Y = 51.5 - 0.0296X	298.99±1.84
DPPH	Y = 87.9 - 0.0142X	193.66±1.46	Y = 91.2 + 0.00228X	109.65±0.80	Y = 14.4 + 0.143X	225.80±5.27

*R. Eq., Regression Equation; *EC_{50}, Effective Concentration at which 50% of radicals were scavenged.

5.3.2.5. ABTS radical scavenging assay

ABTS radical assay, can be used in both organic and aqueous solvent systems. Our result showed the scavenging activity of *P. africanum* stem bark extract against ABTS radical in a concentration dependent manner. At 0.42 mM, higher antioxidant activity was observed for the extract (10.55±0.45 mM) than in Trolox™ (6.07±0.62 mM; Figure 5.6). Comparison was made based on a calibration graph generated using Trolox™ and it was noted that the ABTS scavenging activities between the extract and the Trolox™ were statistically insignificant ($p > 0.05$).

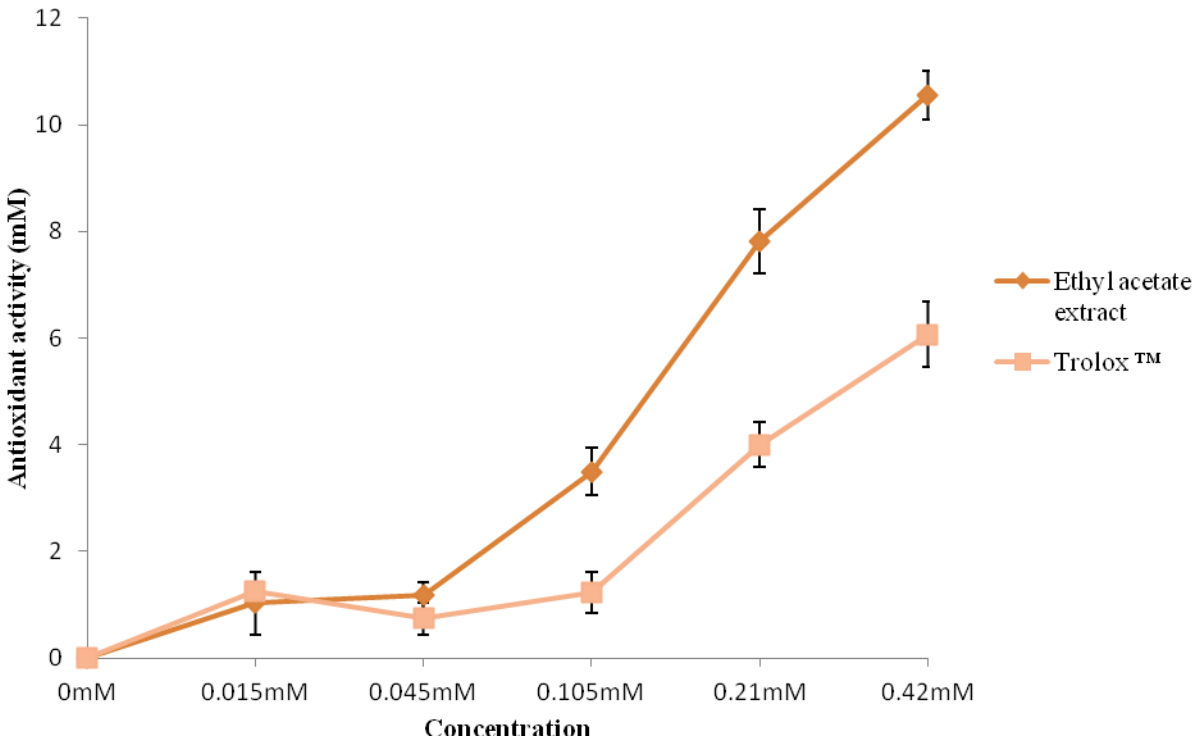


Figure 5.6: ABTS radical scavenging antioxidant activity in relation to the Trolox™ standard concentration.

5.4. DISCUSSION

Free radicals are constantly generated in living system and they can cause extensive damage to tissues and biomolecules leading to various disease conditions, especially degenerative diseases. Many synthetic drugs protect against oxidative damage but most often with adverse side effects (Bizimenyera *et al*., 2005 (B); Ndip *et al*., 2009). An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines (Yazdanparast *et al*., 2008). This study established the total proanthocyanidins, phenolics, flavonols, flavonoids and high antioxidant activity in the ethyl acetate extract of *P. africanum*. Exactly 89.503±0.39 % inhibition of DPPH was noted at 50 µg mL^{-1}, this with known compounds such as; Hexadecanoic acid, β-Sitosterol, Cholesta-4,6-dien-3-ol,benzoate and other several monoterpenes and sesquiterpenes components identified in our previous study justify its valuable pharmacological activities (Okeleye *et al*., 2013). This is in line with other study on *Peltophorum pterocarpum* (DC) Backer ex K. Heyne (family Leguminosae) reported to contain phytochemicals and antioxidant activity of 96.70 ± 0.22 % DPPH inhibition at 80 µg mL^{-1} with similar identified compounds (Nathan *et al*., 2012; Jain *et al*., 2012; Taiwo *et al*., 2013).

Proanthocyanidins (92.18±4.68 mg/g) occurred more ($p < 0.05$) in the extract compared to all other compounds tested: phenolics (60.53±1.46 mg/g) > flavonoids (18.37± 2.11 mg/g), > flavonols (11.20±3.90 mg/g). However the difference between flavonols and flavonoids was not significant ($p > 0.05$) at 95% confidence interval. The differences in polarity of the antioxidant components are notable reason why phenolics compounds and antioxidant activity of the extract differ (Benzie and Strain, 1996; Meyer *et al*., 1997; Chang *et al*., 2002). Proanthocyanidins help to defend the body from tissue damage and to improve blood distribution by reinforcing the blood vessels, while flavonoids in the human diet reduce the risk of different cancers and have

been shown to exhibit their actions through effects on membrane permeability, and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase A2 (Benzie and Strain, 1996; Meyer *et al*., 1997; Mbaebie *et al*., 2012). The reducing capacity of the compounds in the extract (3.828±0.051) serve as a significant indicator of its potential antioxidant activity. Antioxidant activities have been ascribed to various mechanisms such as prevention of chain initiation, disintegration of peroxides, reducing capacity and radical scavenging (Benzie and Strain, 1996; Yildirim *et al*., 2000). The reducing power of the plant extract (3.828±0.051) was compared with the standard DBPC*BHT (3.828±0.040) and no significant difference was observed in their potency ($p > 0.05$), while for L (+) - Ascorbic acid it was 3.446± 0.070 ($p < 0.001$) which is similar to the findings reported by Jain *et al*. (2012).

Scavenging of H_2O_2 by extract (EC_{50} = 131.16±1.65µg mL^{-1}) is credited to it's phenolics (60.53±1.46), which can offer electrons to H_2O_2, thus neutralizing it to H_2O. Even though H_2O_2 itself is not very reactive, it can occasionally cause cytotoxicity by formation of hydroxyl radicals in the cell (Ebrahimzadeh *et al*., 2010). This demonstrated similar results with the standard compounds; L (+) - Ascorbic acid (EC_{50} = 88.61±0.17µg mL^{-1}), and DBPC*BHT (EC_{50} = 100±0.31µg mL^{-1}). Fe3+ was reduced to Fe2+ in the presence of extract and the reference compounds which corroborates significant effect on the activities against nitric oxide (NO) observed [NO: extract (EC_{50} = 204.55±1.89µg mL^{-1}) in comparison to L (+) - Ascorbic acid (EC_{50} = 276±3.15µg mL^{-1}) and DBPC*BHT (EC_{50} = 298.99±1.84µg mL^{-1})], a finding which is similar to the result of other reports (Balakrishnan *et al*., 2009; Ebrahimzadeh *et al*., 2010). NO is lipophilic and produced by phagocytes and endothelial cells from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases. It is a strong pleiotropic inhibitor of physiological processes such as smooth muscle

relaxation, neuronal signaling and can significantly be inhibited through direct competition with plant extract for oxygen (Jagetia *et al*., 2004; Balakrishnan *et al*., 2009).

Mineral nutritional status and physical properties of soil greatly influence the presence of secondary metabolites in different parts of a plant (Mandal *et al*., 2009). Plant phenolics constitute one of the major groups of compounds acting as primary antioxidant or free radical terminators (Igbinosa *et al*., 2011). High antioxidant activities observed (ABTS, 10.55±0.45 mM; DPPH, EC_{50} = 193.66±1.46µg mL^{-1}) have been contributed by the combined effect of phytochemicals and polyphenolic compounds. As the reaction between antioxidant molecules and radical progressed, there was a decrease in absorbance of DPPH radical (reverse is the case in ABTS radical) with an increase in concentration. This results in the scavenging of the radical by hydrogen donation; hence, a noticeable change in colour from purple to yellow (Elmastas *et al*., 2006). High antioxidant activity of 89.503±0.39 % DPPH inhibition at 50 µg mL^{-1}; 53.93±1.09 %, 88.73± 6.69 % inhibition of NO and H_2O_2 respectively at 25 µg mL^{-1} observed in this study is similar to other investigators (Bizimenyera *et al*., 2005 (B); Balakrishnan *et al*., 2009; Sridharamurthy *et al*., 2012; Jain *et al*., 2012), therefore adding to its credibility as a likely template or adjunct of an antioxidant compound.

5.5. CONCLUSION

The findings of this study demonstrated high *in vitro* antioxidant activity of the ethyl acetate extract of *P. africanum* which is considerably aided by the high quantity of secondary metabolites; polyhenolics, and phytochemicals. It can therefore be useful in preventing or slowing down the progress of various oxidative stress induced diseases. Furthemore it could be exploited as an easily available source of natural antioxidant, as a food supplement or in the

pharmaceutical sector. Further work is however ongoing to isolate and identify the antioxidant compounds and evaluate *in vivo* antioxidant activities of this extract.

REFERENCES

Balakrishnan, N., Panda, A.B., Raj, N.R., Shrivastava, A. and Prathani, R. (2009). The evaluation of nitric oxide scavenging activity of *Acalypha indica* Linn root. *Asian Journal of Research Chemistry*. **2** (2): 148-150.

Benzie, I.F.F. and Strain, J.J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of 'Antioxidant power': The FRAP assay. *Analytical Biochemistry*. **239** (1): 70-76.

Bizimenyera, E.S., Aderogba, M.A., Eloff, J.N. and Swan, G.E. (2005). Potential of Neuroprotective Antioxidant-Based Therapeutics from *Peltophorum Africanum* Sond (Fabaceae). *African Journal of Traditional and Complementary, and Alternative Medicine*. **28** (1): 99-106.

Bizimenyera, S.E., Swan, G.E., Chikoto, H. and Eloff JN. (2005). Rationale for using *Peltophorum africanum* (Fabaceae) extracts in veterinary medicine. *Journal of South African Veterinary Associatio*n. **76** (2): 54-58.

Chang, C., Yang, M., Wen, H. and Chern, J. (2002). Estimation of total flavonoid content in Propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis*. **10** (3): 178-182.

Ebrahimzadeh, M.A., Nabavi, S.M., Nabavi, S.F., Bahramian, F. and Bekhradnia, A.R. (2010). Antioxidant and free radical scavenging activity of *H. officinalis, L. Var. angustifolius, V. odorata, B. hyrcana and C. speciosum*. *Pakistan Journal of Pharmaceutical Sciences*. **23** (1): 29-34.

Elmastas, M., Gulcin, I., Isildak, O., Kufrevioglu, O.I., Ibaoglu, K. and Aboul-Enein, H.Y. (2006). Radical scavenging activity and antioxidant capacity of bay leaf extracts. *Journal of the Iranian Chemical Society*. **3** (3): 258-266.

Igbinosa, O.O., Igbinosa, H.I., Chigor, V.N., Uzunuigbe, O.E., Oyedemi, S.O., Odjadjare, E.E., *et al*., (2011). Polyphenolic contents and antioxidant potential of stem bark extracts from *Jatropha curcas* (Linn). *International Journal of Molecular Sciences*. **12** (5): 2958-2971.

Jagetia, G.C., Rao, S.K., Baliga, M.S. and Babu, K.S. (2004). The evaluation of nitric oxide scavenging activity of certain herbal formulations *in vitro*: A preliminary study. *Phytotherapy Research*. **18** (7): 561-565.

Jain, S.C., Pancholi, B. and Jain, R. (2012). Antimicrobial, free radical scavenging activities and chemical composition of *Peltophorum pterocarpum* Baker ex K. Heyne stem extract. *Der Pharma Chemica*. **4** (5): 2073-2079.

Kumar, R.S. and Hemalatha, S. (2011). *In vitro* antioxidant activity of alcoholic leaf extract and subfractions of *Alangium lamarckii* Thwaites. *Journal of Chemical and Pharmaceutical Research*. **3** (1): 259-267.

Kumaran, A. and Karunakaran, R.J. (2007). *In vitro* antioxidant activities of methanol extracts of five *Phyllanthus* species from India. *Lebens Wiss Technology - Food Science and Technology*. **40** (2): 344-352.

Mandal, M., Misra, T.K. and Ghosal, M. (2009). Free-radical scavenging activity and phytochemical analysis in the leaf and stem of Drymaria diandra Blume. *International Journal of Integrative Biology*. **7** (2): 80-844.

Mbaebie, B.O., Edeoga, H.O. and Afolayan, A.J. (2012). Phytochemical analysis and antioxidants activities of aqueous stem bark extract of *Schotia latifolia* Jacq. *Asian Pacific Journal of Tropical Biomedicine*. **2** (2): 118-124.

Meyer, A.S., Heinonen, M. and Frankel, E.N. (1997). Antioxidant interactions of catechin, cyanidin, caffeic acid, quercetin, and ellagic acid on human LDL oxidation. *Food Chemistry*. **61** (1-2): 71-75.

Nabavi, S.M., Ebrahimzadeh, M.A., Nabavi, S.F., Fazelian, M. Eslami, B. (2009). *In vitro* antioxidant and free radical scavenging activity of *Diospyros lotus* and *Pyrus boissieriana* growing in Iran. *Pharmacognosy Magazine*. **5** (18): 122-126.

Nathan, V.K., Antonisamy, J.M., Gnanaraj, W.E. and Subramanian, K.M. (2012). Phytochemical and bio-efficacy studies on methanolic flower extracts of *Peltophorum pterocarpum* (DC.) Baker ex Heyne. *Asian Pacific Journal of Tropical Biomedicine*. **2** (2): 641-645.

Ndip, R.N., Ajonglefac, A.N., Wirna, T., Luma, H.N., Wirmum, C. and Efange, S.M.N. *In-vitro* antimicrobial activity of Ageratum conyzoides on clinical isolates of *Helicobacter pylori*. *African Journal of Pharmacy and Pharmacology*. **3** (11): 585-592.

Okeleye, B.I., Mkwetshana, N.T. and Ndip, R.N. (2013). Evaluation of the Antibacterial and Antifungal Potential of *Peltophorum africanum*: Toxicological Effect on Human Chang Liver Cell Line. *TheScientificWorldJournal*. 2013:878735.doi:10.1155/2013/878735.

Okeleye, B.I., Samie, A., Bessong, P.O., Mkwetshana, N.F., Green, E., Clarke, A.M., *et al*. (2010). Crude ethyl acetate extract of the stem bark of *Peltophorum africanum* (Sond,

Fabaceae) possessing *in vitro* inhibitory and bactericidal activity against clinical isolates of *Helicobacter pylori*. *Journal of Medicinal Plant Research*. **4** (14): 1432-1440.

Ordonez, A.A.L., Gomez, J.D., Vattuone, M. and Isla, M.I. (2006). Antioxidant activities of *Sechium edule* (Jacq). *Food Chemistry*. **40** (2): 452-458.

Paz´dzioch-Czochra, M. and Widen ska, A. (2002). Spectrofluorimetric determination of hydrogen peroxide scavenging activity. *Analytica Chimica Acta*. **452** (2): 177-184.

Samie, A., Obi, C.L., Bessong, P.O. and Namrita, L. (2005). Activity profiles of fourteen selected medicinal plants from Rural Venda communities in South Africa against fifteen clinical bacterial species. *African Journal of Biotechnology*. **4** (12): 1443-1451.

Shen, Q., Zhang, B., Xu, R., Wang, Y., Ding, X. and Li, P. (2010). Antioxidant activity *in vitro* of selenium-contained protein from the se-enriched *Bifidobacterium animalis*. *Anaerobe*. **16** (4): 380-386.

Sridharamurthy, N.B., Ashok, B. and Yogananda, R. (2012). Evaluation of Antioxidant and Acetyl Cholinesterase inhibitory activity of *Peltophorum pterocarpum* in Scopolamine treated Rats. *International Journal of Drug Development and Research*. **4** (3): 115-127.

Taiwo, O.E., Efere, M.O., Joseph, M.A and Saburi, A.A. (2013). Acetyl and buteryl cholinesterase inhibitory effect of *Peltophorum pterocarpum* (DC) Backer ex K. Heyne (family Leguminosae). *Journal of Pharmacognosy and Phytotherapy*. **5** (5): 77-82.

Tanih, N.F., Okeleye, B.I., Ndip, L.M., Clarke, A.M., Naidoo, N., Mkwetshana, N., *et al*. (2010). *Helicobacter pylori* prevalence in dyspeptic patients in the Eastern Cape Province-race and disease status. *South African Medical Journal*. **100** (11): 734-737.

Theo, A., Masebe, T., Suzuki, Y., Kikuchi, H., Wada, S., Obi, L.C., *et al*. (2009). *Peltophorum africanum*, a traditional South African medicinal plant, contains an anti HIV-1 constituent, betulinic acid. *Tohoku Journal of Experimental Medicine*. **217** (2): 93-99.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T.D., Mazur, M. and Telser, J. (2006). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry and Cell Biology*. **39** (1): 44-843.

Wolfe, K., Wu, X. and Liu, R.H. (2003). Antioxidant activity of apple peels. *Journal of Agricultural and Food Chemistry*. **51** (3): 609-614.

Yazdanparast, R., Bahramikias, S. and Ardestani, A. (2008). *Nasturtium oficinale* reduces oxidative stress and enhances antioxidant capacity in hypercholesterolaemic rats. *Chemico-Biological Interactions*. **172** (3): 176-184.

Yildirim, A., Mavi, A., Oktay, M., Kara, A.A., Algur, O.F. and Bilaloglu, V. (2000). Comparison of antioxidant and antimicrobial activities of tilia (*Tilia argentea* Desf Ex DC), sage (*Savia triloba* L.), and black tea (*Camellia sinensis*) extracts. *Journal of Agricultural and Food Chemistry*. **48** (10): 5030-5034.

CHAPTER SIX

In-vitro assessment of the antiproliferative and apoptotic potential of the ethyl acetate extract of *Peltophorum africanum* on different cancer cell lines: ultrastructural characterization of treated cells

ABSTRACT

Peltophorum africanum, a member of the family Fabaceae (Sond) is an African weeping wattle used in traditional medicine in South Africa. The study evaluated the *in vitro* antiproliferative and apoptotic potential of the ethyl acetate extract (EAE) of this plant in order to validate its pharmacological use. Antiproliferation of human breast (MCF-7), colon (HT-29) and cervical (HeLa) cancer cell lines by EAE was investigated using the Cell Titer-Blue viability assay and the mechanism of action delineated using the Nucleic Acid and Protein Purification Nucleospin® Tissue Kit, Scanning Electron Microscope (SEM), propidium iodide (PI) and acridine orange (AO) double-staining techniques. Significant reduction in cell viability of the cells was noted as the MCF-7 cells were reduced from 100 - 54.33±1.84 % after 72 hrs of treatment with 5 µg/mL of EAE (*P. value* < 0.05). Internucleosomal DNA of MCF-7, HT-29 and HeLa cells were randomly fragmented into an uninterrupted spectrum of sizes, complemented by the intercalation of nucleic acid-specific fluorochromes by PI and AO spotting two phases of apoptosis; early (EA) and late (LA) apoptosis. Distinctive ultramorphological changes observed include; cell shrinkage, membrane blebbing, and typical cell induced death. The ethyl acetate extract of *P. africanum* has the potential to induce apoptosis and could be further studied *in vivo* as a likely template for new anticancer therapy.

6.1. INTRODUCTION

Cancer is the uncontrolled growth of cells coupled with malignant behavior: invasion and metastasis; resulting from genetic and environmental interactions (Hirsch, 2006). It has been reported that about 80-90% of all cancers is associated with environmental factors, and about 35% of them may be due to the effects of dietary factors (Doll and Peto, 1981). More than 10% of all deaths worldwide are caused by cancers. The most recently calculated age adjusted incidence rates (AAR) of breast cancer among women in India range from 25 to 31 cases per 100,000 (Curado *et al*., 2007). Cervical cancer is the most common cancer in Thailand with the age standardized incidence rate (ASR) of 19.5 per 100,000 person-years (Pengsaa *et al*., 2003). Preserved and red meat is directly linked to an increased risk for colorectal cancer (Key *et al*., 2004).

Acute myeloid leukaemia and other metastases have been reported to result from the drugs such as irinotecan used in the treatment of other cancer (Merrouche *et al*., 2006; Shapiro *et al*., 2007). Over time, cancer cells become more resistant to chemotherapy treatments; recently small pumps on the surface of cancer cells that actively pump drugs from inside the cell to the outside were identified (Hirsch, 2006; Joensuu, 2008). Momentous research efforts have focused on novel chemotherapeutic drugs from medicinal plants in search of cancer inhibitors and therapy (Wu *et al*., 2002). Such plants are valuable sources of bioactive compounds and phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer activities which play a major role in human health care, as about 80 % of the world population relies on the use of alternative medicines (Pezzuto, 1997).

P. africanum, a locally used medicinal plant common in South Africa has been widely studied for its cytotoxicity, antioxidant and antimicrobial, with profound activities reported (Okeleye *et*

al., 2013). To the best of our knowledge, the anticancer activity of this plant has not been evaluated. In view of this, we therefore aimed at investigating the antiproliferative effect of the ethyl acetate extract of the plant on human breast, cervical and colon cancer cell lines and its likely mechanisms of actions, in an effort to find a relatively cheap source of bioactive lead which could serve as a useful template for the synthesis of an anticancer agent.

6.2. MATERIALS AND METHODS

6.2.1. Preparation of the extract

P. africanum was obtained, identified, archived and prepared as reported in our previous study (Okeleye *et al*., 2013).

6.2.2. Cancer cell culture and maintenance

Cancer cell lines; MCF-7 (breast), HT-29 (colon) and HeLa (cervical) used in this study was a kind donation from Prof. Maryna Van De Venter of Nelson Mandela Metropolitan University, South Africa. Briefly, a vial containing cells (1 mL) was thawed in a water-bath regulated at 37 ^{O}C for 2 - 5 minutes and diluted with 9 mL pre-warmed (10 – 15 minutes in water bath at 37 ^{O}C) Dulbecco's minimum essential medium (DMEM) containing 10 % fetal bovine serum (FBS). The cells were incubated in a 37 ^{O}C humidified incubator (Shel Lab, USA) at 5 % CO_2 for multiplication and adherence. Maintenance of cells was achieved as the old medium was aspirated, washed with 10 mL phosphate buffer saline (PBS), trypsinized (0.5 - 1 mL trypsin) and then split into a fresh medium until the desired cell number and confluence was attained.

6.2.3. Cell Titer-Blue viability assay

Anticancer activity of the EAE of *P. africanum* was evaluated on three cancer cell lines (MCF-7, HT-29 and HeLa cell) using microculture CellTiter-Blue viability (Promega, USA) assay as previously described (Gupta *et al*., 1996; Merghoub *et al*., 2009). The 96-well microplates were seeded with 200 μL DMEM + high glucose, L-glutamine and sodium pyruvate (Thermo Sceintific, South Logan, Utah) containing 6.0 x 10^3 cells in suspension and incubated in CO_2 (5 %) incubator at 37 ^{o}C. After 24 hrs incubation and attachment, the cells were treated with 1000,

500, 250, 125, 75, 25 and 5 µg/mL concentration of the extract. Each plate included untreated cell controls and a blank cell-free control. Exactly 60 µM of curcumin (Sigma-Aldrich, South Africa) was used as positive control and 0.5% DMSO as negative control. After 24, 48 and 72 hrs of incubation, cell viability was determined by adding cell titer blue as an indicator and further incubated for 4 hrs. Fluorescence was read at 570/620 nm using Analytical & Diagnostic Product Gen™ spectrophotometer (BioTek, Highland Park, USA). All experiments for the extract were carried out in triplicates and the concentration which inhibited 50 % of cellular growth (IC_{50} value) was determined.

6.2.4. Mechanism of action

6.2.4.1. DNA fragmentation analysis

Fragmentation of the DNA was analyzed in line with a previously described method (Herrmann *et al*., 1994). The cell lines; MCF-7, HT-29 and HeLa cell at a concentration of 1×10^6 mL^{-1} each, were treated with EAE in a 6-well tissue culture plate after 24 hrs of attachment at IC_{50}, $IC_{50} \times 2$, $IC_{50} \times 4$ and $IC_{50} \times 8$ (in duplicate), including a negative control (untreated cells) and positive control (60 µM curcumin and 10 µg/mL actinomycin D). After 24 hrs of treatment, cells were harvested using sterile scraper and washed with PBS prior to DNA isolation. DNA extraction was carried out using Nucleic Acid and Protein Purification Nucleospin® Tissue Kit (Macherey-Nagel, Germany) according to the manufacturer's manual instructions. Briefly, 10 µL of the DNA in TEA buffer was loaded onto 1.8 % agarose gel containing 0.5 µg/mL (5 µL in 100 mL of gel) ethidium bromide. Electrophoresis was conducted progressively at 35, 67 and 100 V for 4 hrs and the DNA fragments were visualized and photographed under UV

illumination XD – 79, WL/26 MX, 230 V ~ 50/60 HZ (Alliance 4.7, Taiwan, France). Based on the results obtained, only the MCF-7 cell line was studied further.

6.2.4.2. Quantification and stages of apoptosis using intercalation of nucleic acid-specific fluorochromes by propidium iodide and acridine orange

Morphological changes resulting in the leakage and fragmentation of DNA by EAE were assessed under fluorescence microscope using propidium iodide (PI) and acridine orange (AO) double-staining according to the method of Mohan *et al*. (2011), with few modifications. MCF-7 cells were seeded in a 6-well tissue culture plate at a concentration of 1×10^6 mL^{-1} and treated after 24 hrs with EAE at $IC_{50,}$ $IC_{50} \times 4$ and $IC_{50} \times 8$ respectively. Untreated cells were used as negative control and cells treated with 60 µM of curcumin as positive control. The experiment was performed in duplicate. The cells were collected after 24 hrs of treatment using a sterile scraper, centrifuged at 300 × g for 10 minutes and the cellular pellet washed twice with PBS. Fluorescent dyes; PI (10 µL) and AO (10 µL) were added to the cell (20 µL) at equal volumes and promptly observed under UV-fluorescence microscope. The percentages of viable, early apoptotic, late apoptosis and secondary necrotic cells were determined (Mohan *et al*., 2011).

6.2.4.3. Morphological characterization of treated cells

The morphological characteristics of the cells (MCF-7) were determined using scanning electron microscope (SEM; JSM-6390LV, Jeol, Japan). Approximately 1×10^6 mL^{-1} of the MCF-7 cells were seeded in a 6-well tissue culture plate and treated after 24 hrs with EAE at $IC_{50,}$ $IC_{50} \times 4$ and $IC_{50} \times 8$. Untreated cells and cells treated with 60 µM of curcumin were included as negative and positive control respectively. Cells were collected, washed twice in phosphate buffered

saline (PBS) and then centrifuged at 1000 rpm for 5 minutes; after which it was fixed in 2.5 % gluteraldehyde prepared in 0.1 M PBS. After washing, the cells were post fixed on poly-L-lysine-coated glass coverslip, with 1 % osmium tetroxide (OsO_4) in 0.2 M PBS for 30 minutes, and then washed with PBS. Cells were mounted onto stubs and coated using IB3 Ion Coater (EIKO, Japan) after dehydrated through graded ethanol (30, 50, 70, 85 and 95 %) and critical point dried (CPD). Different sections of the cells were micro-analyzed and the representative spectra presented (Xin *et al*., 2001).

6.2.5. Statistical analysis

Statistically significant differences among the three cell line and the apoptosis phase percentage values of MCF-7 compared with the control were determined by one way analysis of variance (ANOVA) and *P. value* < 0.05 was considered significant, while IC_{50} was determined using the regression analysis test. All analyses were performed with Minitab statistical software (student version 12 for windows).

6.2.6. Ethical consideration

This study which is a continuation of our line of studies on microbial pathogens and anti-infective from medicinal plants had been approved by the institutional review board of the University of Fort Hare.

6.3. RESULTS

6.3.1. Anti-proliferation activity of the extract (EAE) against the cancer cell lines

The EAE exhibited inhibitory effect against the MCF-7, HT-29 and HeLa cell lines. The viable cell of MCF-7 was significantly reduced from 100 % (Negative control) to 62.20±9.41 %, 75.92±2.14 % and 54.33±1.84 % after treatment with 5 µg/mL of EAE at 24, 48 and 72 hrs respectively (*P. value* < 0.05). Meanwhile reduction of viable cell to 32.55±1.55 %, 33.11±2.92 % and 22.00±0.80 % were noted after treating with 500 µg/mL of EAE at 24, 48 and 72 hrs respectively. At 5 µg/mL - 1000 µg/mL, the HT-29 cell proliferation decreased from 65.74±4.36 %, 64.75±2.23 % and 73.82±0.27 % to 33.57±3.17 %, 21.21±0.57 % and 14.80±2.21 % after 24, 48 and 72 hrs respectively. For HeLa, decline in viable cells ranged from 78.22±3.71 (5 µg/mL, 24 hrs) to 19.74±1.60 (1000 µg/mL, 72 hrs). The percentage reduction in proliferation of all the treated cancer cell lines was conspicuous and statistically significant (*P. value* < 0.05). Curcumin (Positive control) was observed to be active at 60 µM as the cells were reduced from 100 % (Negative control) to 36.15±0.60 (24 hrs), 35.93±1.57 (48 hrs) and 22.23±6.86 (72 hrs) for MCF-7; 76.44±.20.34 (24 hrs), 23.48±1.70 (48 hrs) and 41.78±3.34 (72 hrs) for HT-29; and 45.99±3.32 (24 hrs), 30.19±2.58 (48 hrs) and 24.98±0.93 (72 hrs) for HeLa cell (Figure 6.1). Inhibitory concentration at 50 % (IC_{50} in µg mL^{-1}) of MCF-7 (IC_{50} = 389.05±7.56, 24 hrs; 312.84±6.55, 48 hrs; 321.38±5.91, 72 hrs), HT-29 (IC_{50} = 281.16±4.389, 24 hrs; 331.19±5.67, 48 hrs; 330.86±8.56, 72 hrs) and HeLa cell line (IC_{50} = 310.16±6.33, 24 hrs; 375.0±4.71, 48 hrs; 325.23±8.03, 72 hrs) were recorded using regression analysis (Minitab Program, Version 12 for Windows).

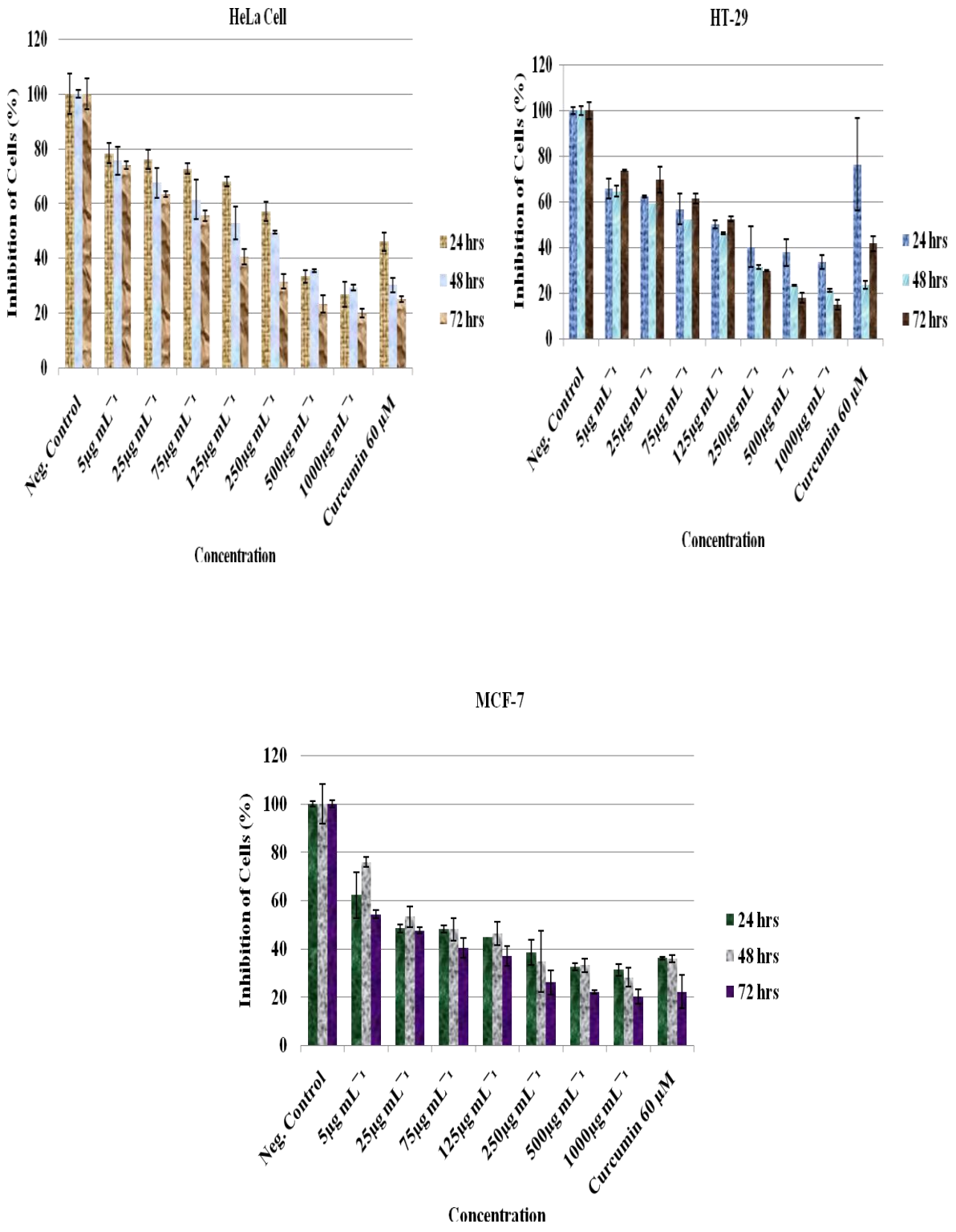


Figure 6.1: *In vitro* cell (HeLa, HT-29 and MCF-7) inhibitory profile of the EAE.

6.3.2. Apoptotic analysis

6.3.2.1. Internucleosomal DNA fragmentation

The DNA of the treated MCF-7, HT-29 and HeLa cell lines exhibited a random degradation with a non-specific and continuous spectrum of sizes during the cellular degeneration after 24 hrs treatments. Prominent random fragmentation of the DNA was observed at IC_{50}×8 for all the three treated cells (MCF-7, HT-29 and HeLa) compared to the positive control (curcumin and actinomycin D) indicating early apoptosis. Moderate degradation was observed at IC_{50}, IC_{50}×2 and IC_{50}×4 for HT-29 and IC_{50}×4 for MCF-7 signifying late apoptosis (Figure 6.2).

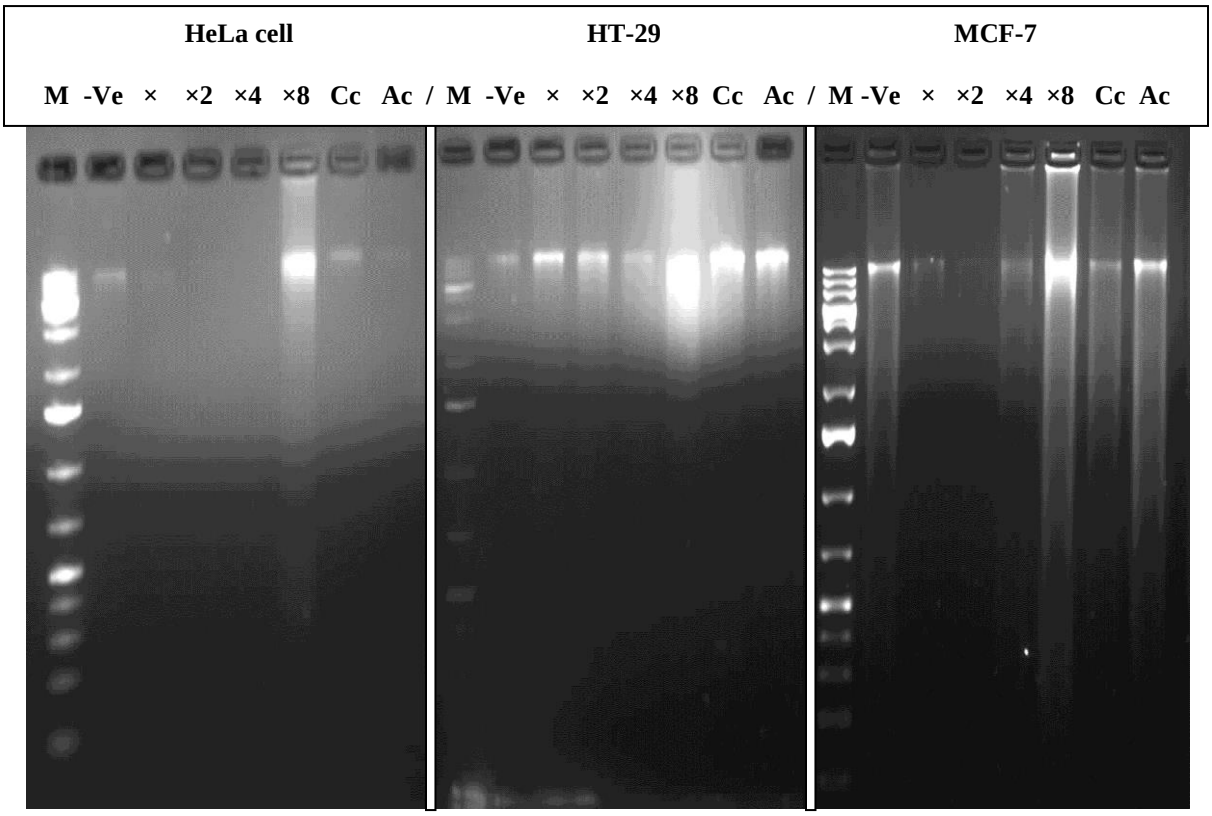
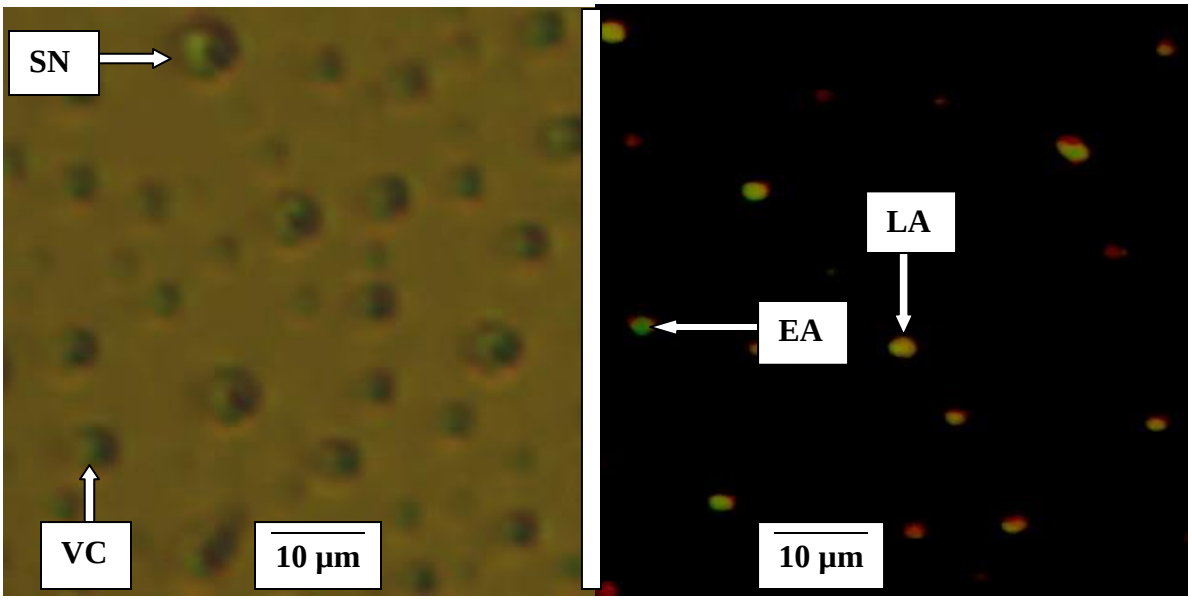


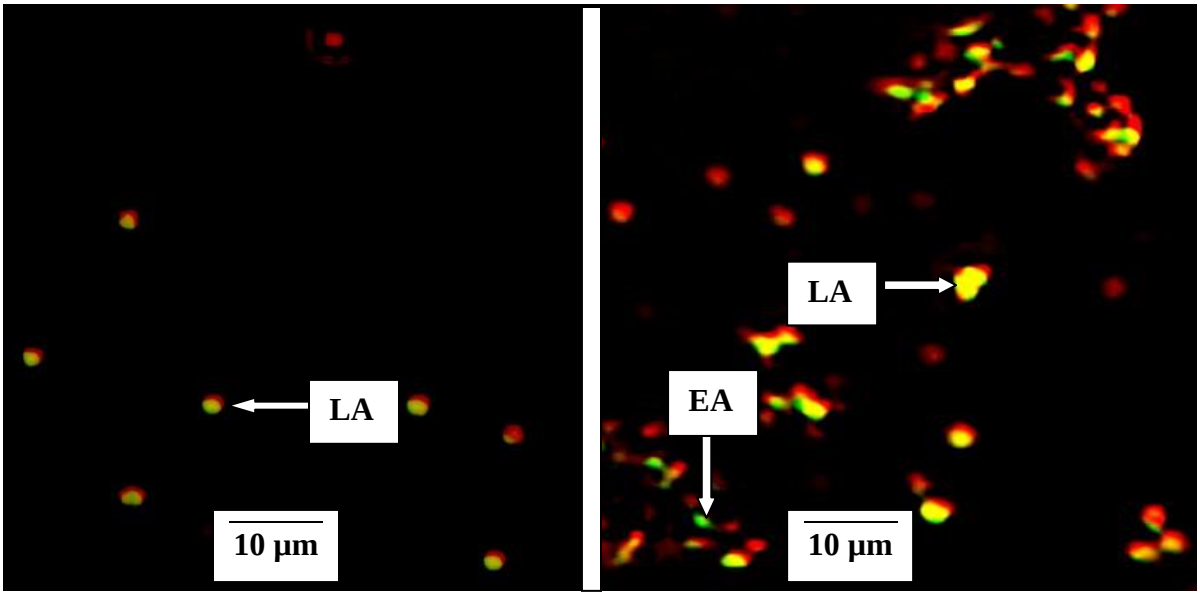
Figure 6.2: Cellular degradation of cells resulted in random fragmentation of DNA. M=Marker, -Ve= Negative control (untreated cell), X=IC_{50}, X2=IC_{50}×2, X4=IC_{50}×4, X8=IC_{50}×8, Cc=Curcumin (60 µM), Ac=Actinomycin D (10 µg/mL).

6.3.2.2. Phases and degree of apoptosis

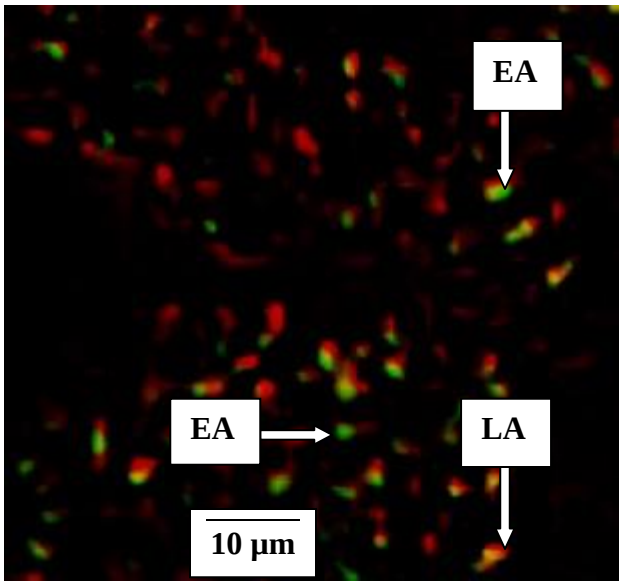
Propidium iodide (PI) and acridine orange (AO) double-staining analysis revealed phases of morphological changes and the degree of induction of apoptosis. Intercalation of AO within the fragmented DNA was observed in early apoptosis (EA) as bright-green colour in treated cells (MCF-7) compared to untreated cells with a green normal structure (Figure 6.3). Approximately 75.5 ± 2.1, 31 ± 1.4, 24.4 ± 0.7 and 4.5 ± 2.1 % expression of EA were observed in IC_{50}×8, IC_{50}×4, curcumin and IC_{50} respectively (Figure 4). Late apoptosis (LA) intercalated PI and AO, appeared as reddish orange and secondary necrosis (SN) as orange nucleus with intact structure (Figure 3). The expression of LA was 93 ± 1.4, 64 ± 1.4, 60 ± 0.9 and 16 ± 1.4 % for IC_{50}, IC_{50}×4, curcumin and IC_{50}×8 respectively. Apoptosis (EA) of MCF-7 increased significantly ($p < 0.05$) in a concentration dependent manner; however, no significant ($p > 0.05$) difference was observed in the SN cell (Figure 6.4).



(a) **(b)**



(c) **(d)**



(e)

Figure 6.3: MCF-7 stages of apoptosis. **(a)** Untreated cell (negative control), **(b)** Curcumin (60 µM), **(c)** EAE IC_{50}, **(d)** EAE IC_{50}×4, **(e)** EAE IC_{50}×8. The criteria for identification: 1. Viable cells (VC) observed to have green normally shaped nuclei, 2. Early apoptosis (EA) appeared to have light-green nucleus showing aggregation and condensation of chromatin, 3. Dense orange area of chromatin condensation showing late apoptosis (LA), 4. Orange intact nucleus portraying secondary necrosis (SN).

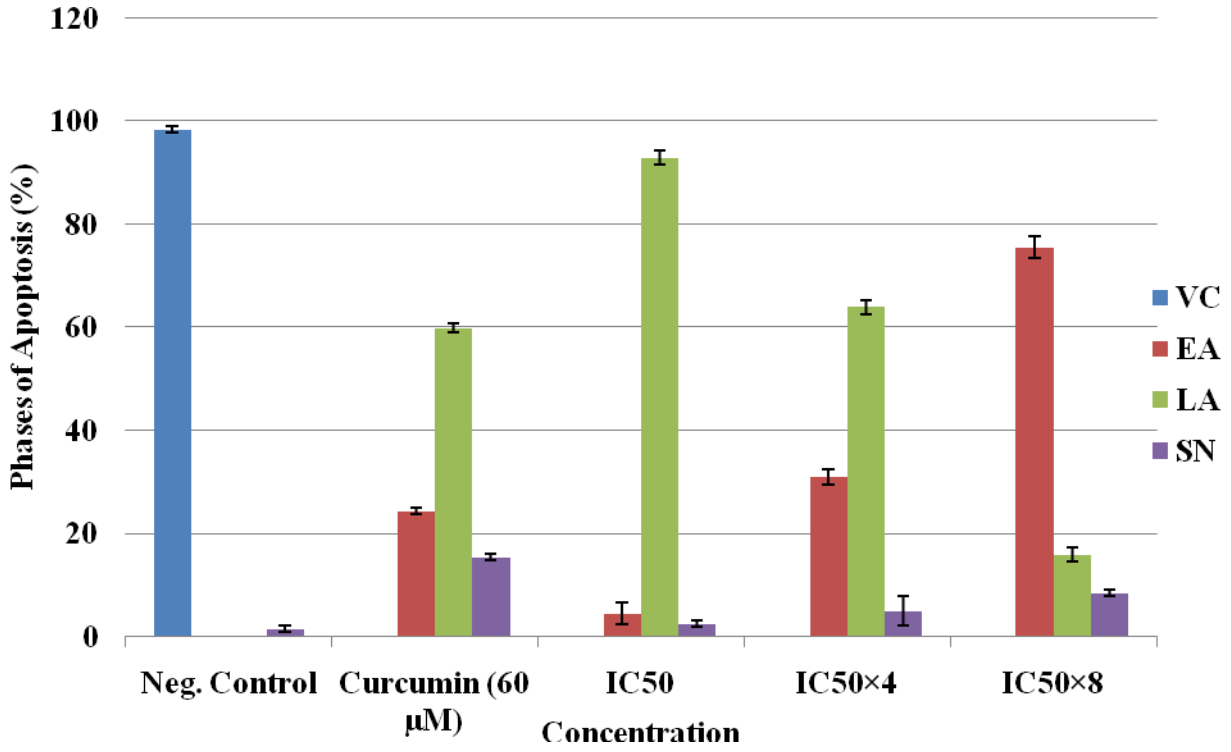
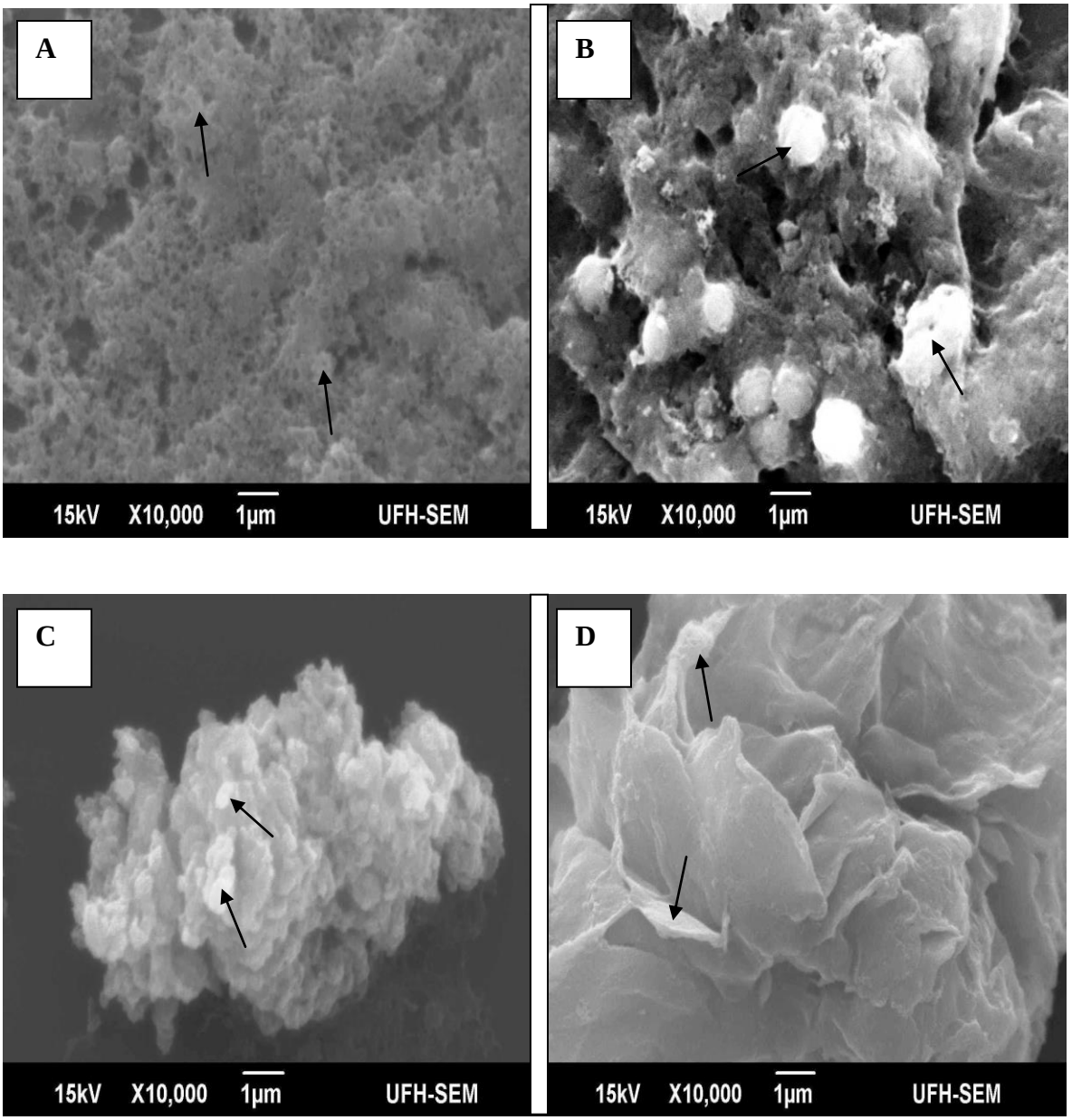


Figure 6.4: Quantification of stages of human breast cancer cell apoptosis induced by EAE, compared with untreated and positive control (Curcumin). VC, Viable cell; EA, Early apoptosis; LA, Late apoptosis; SN, Secondary necrosis.

6.3.2.3. Ultrastructural characterization of treated cells

Distinctive morphological changes corresponding to characteristic cellular apoptotic stages were observed in SEM analysis with the degree and the rate of morphological changes become greater as extract concentrations increased, reaching the maximal (typical apoptosis) responses at IC_{50}×8 of EAE. Visible changes observed include, decrease in cell volume, cell shrinkage, cell retraction, formation and separated of apoptotic bodies, membrane blebbing and typical cell induced death (Figure 6.5). At IC_{50}, EAE treated MCF-7 extensively demonstrated cell shrinkage, membrane blebbing and separated apoptotic bodies (Figure 6.5 c) compared to flat, smooth and confluent negative control (Figure 6.5 a).


A
15kV X10,000 1µm UFH-SEM
B
15kV X10,000 1µm UFH-SEM
C
15kV X10,000 1µm UFH-SEM
D
15kV X10,000 1µm UFH-SEM

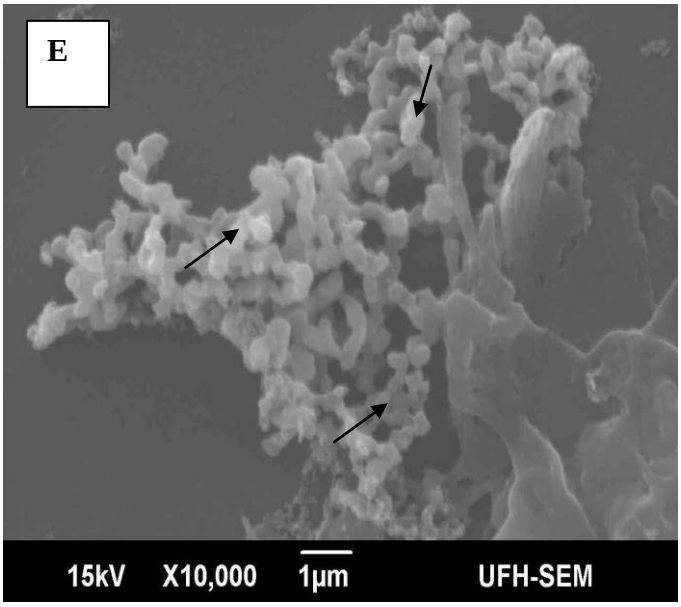


Figure 6.5: Ultrastructural changes of EAE treated MCF-7 in a concentration dependent manner. **(A)** The characteristic of untreated cells was observed as flat and with smooth surface, a typical morphological feature of a cancer cell; **(B)** Curcumin treated MCF-7 (60µM), showed different form of rounding and cell shrinkage; **(C)** EAE treated MCF-7 (IC_{50}) demonstrated cell shrinkage, membrane blebbing and separated apoptotic bodies; **(D)** EAE treated MCF-7 (IC_{50}×4) exhibited surface blebbing and cell retraction and; **(E)** EAE treated MCF-7 (IC_{50}×8) showed signs of typical apoptosis (induced cell death).

6.4. DISCUSSION

Cell proliferation analysis using microculture CellTiter-Blue viability assay revealed notable apoptotic effect of EAE in a concentration dependent manner against human breast (MCF-7), colon (HT-29) and cervical (HeLa) cancer cell. Exactly 25 µg/mL of EAE reduced MCF-7 viable cell to 48.38 ± 1.56 % after 24 hrs of treatment. Approximately 62.36 ± 0.42 and 76.10 ± 3.54 % reductions were observed against HT-29 and HeLa cell respectively, at the same concentration and time of treatment. Apoptosis is a highly regulated systematic form of programed cell death, functioning as a regulator of biological homeostasis; often associated with the cells that are advancing towards the cell cycle (Compton, 1992). It is an energy consumed process involving loss of cell to cell contact, cell shrinkage, condensation of nuclear chromatin, and finally endonucleolytic fragmentation of genomic DNA (Bortner *et al*., 1995). Necrosis on the other hand is initiated by severe toxic stress, characterized by deterioration of ATP concentrations, intracellular damage of organelles and induction of an acute inflammatory response (Fernandez *et al*., 1994). Apoptosis has been reported to be initiated by natural products which are similar to the observations noted in this study (Szliszka *et al*., 2011; Herrmann *et al*., 1994; Lee *et al*., 2008; Giessrigl *et al*., 2012; Leong *et al*., 2011; Okeleye *et al*., 2013).

This study demonstrated that treatment of MCF-7, HT-29 and HeLa cancer cells with EAE caused alteration in genetic material (DNA fragmentation) and morphological changes showing a random degradation with a continuous spectrum of sizes accompanying cellular degeneration. Internucleosomal fragmentation of genomic DNA which has been reported as one of the leading biochemical markers of apoptosis (Williamson, 1970; Burgoyne and Burgoyne, 1973) results in the production of oligonucleosomal fragments of different molecular weight which is generated by chromatin and enhanced by the Ca/Mg-dependent endonuclease cascade. Though the role of

caspase 3 was not investigated in this study, it has been reported that DNA fragmentation is set off by caspase 3 activation of inactive CAD (caspase activated deoxyribonuclease) through exclusion of its inhibitors; a biochemical characteristic of inherent apoptotic cell death (Dahlgren, 1980; Frederick and Xinbo, 2002). It is speculated that this might have been responsible for the observed changes.

Breast cancer is one of the most common cancers in women with an increasing global prevalence (Yip *et al*., 2006). Cervical cancer is considered as a serious public health problem; second to breast cancer in women worldwide (Monsonego, 2006; Xian *et al*., 2007). As shown by the SEM analysis, EAE-treated MCF-7 breast cancer cell lost its flattened morphology, became shrunk with membrane blebbing and separated apoptotic bodies due to cytoplasm retraction around the nucleus compared to the negative control that still appeared flat and well attached to the substrate. This is similar to the observations reported by other investigators (Panagopoulou *et al*., 2002; Tanih and Ndip, 2013). Cytological observations by AO and PI double staining and SEM analysis further showed multinucleation, holes and abnormalities of mitochondrial cristae of the apoptotic cells.

A continuous spectrum of DNA fragmentation was observed in this study as opposed to a distinct internucleosomal DNA cleavage. Extracellular Ca^{2+} has been reported to enhance internucleosomal DNA cleavage (Williamson, 1970; Burgoyne and Burgoyne, 1973; Choi and Rothman, 1990). Reports in the literature holds that compounds which protect cells or elevate K^{+} suppresses DNA fragmentation while any agent(s) which activates a variety of cellular protein kinases (PKC) are capable of supporting cell survival, but not necessarily needed to inhibit internucleosomal DNA fragmentation (Rukenstein *et al*., 1991). However, zinc significantly inhibits specific internucleosomal and nonspecific DNA fragmentation (Martin *et al*., 1991;

Brown *et al*., 1993). A loss of intracellular Cl^- is normally associated to cell volume reduction, which is a late apoptotic event common to most apoptotic pathways. A high extracellular NaCl medium creates an unfavourable electrochemical gradient for Cl^- efflux and could alter the cytosolic ionic composition that inhibits nucleosomal DNA cleavage during the apoptotic process (Sarkadi and Parker, 1991); Cl^- efflux coupled with the activation of K^+ channels sustains electroneutrality, particularly when cytosolic volume diminishes. Obstruction of a class of K^+ pathway has been reported to deter apoptosis associated cell shrinkage; hence, demonstrating the related mechanisms of cell contraction in late apoptotic phases (McCarthy and Cotter, 1997). In a separate study conducted in our laboratory (Please, see chapter 4), we demonstrated the presence of several metals/ions in this extract, which had a remarkable effect on the morphology of bacterial and yeast cells. Bearing in mind that most known proteins contain metal (Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, and Mn^{2+}) cofactor(s), which carry out an array of tasks ranging from protein structure stabilization to enzyme catalysis, activating many fundamental life processes (Dudev and Lim, 2007) we are constrained to speculate that these ions could have been responsible for the observed continuous spectrum of DNA fragmentation as opposed to specific or distinct DNA fragmentation in line with the cited reports.

Previous studies have reported that defects in apoptotic pathways or repression of apoptosis contribute to expansion of cancer development (Miyashita and Reed, 1993). Natural products that are able to eliminate abnormal cells by induction of apoptosis rather than inhibit or repress malignant growth; that are less toxic and mutagenic than current treatment regimens, will produce new therapies against cancer chemoprevention and cure (Sun, 2001; Zahri *et al*., 2009). With the multitude of compounds reported to be present in the EAE of *P. africanum* in our

previous study (Okeleye *et al*., 2013); it is therefore probable that a mixture of therapeutic agents are needed for utmost therapeutic benefits.

6.5. CONCLUSION

This study demonstrated that EAE significantly inhibited the proliferation of MCF-7, HeLa and HT-29 treated compared to untreated cells. However, a stronger cytotoxic effect on MCF-7 cell was noted through ultrastructural disruption and early internucleosomal DNA fragmentation via membrane leakage; indicating its valuable marker for investigating the mechanisms of cell viability and apoptosis. The current findings therefore call for further studies using animal models to investigate possible antitumor leads from *Peltophorum africanum*.

REFERENCES

Bortner, C.D., Oldenburg, N.B.E. and Cilowski, J.A. (1995). The role of DNA fragmentation in apoptosis. *Trends in Cell Biology*. **5**: 21-26.

Brown, D.G., Sun, X.M. and Cohen, GM. (1993). Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to nucleosomal fragmentation. *The Journal of Biological Chemistry*. **5**: 3037-3039.

Burgoyne, L.A. and Burgoyne, L.A. (1973). Chromatin sub-structure. The digestion of chromatin DNA at regularly spaced sites by a nuclear deoxyribonuclease. *Biochemical and Biophysical Research Communications*. **52**: 504-510.

Choi, D.W. and Rothman, S.M. (1990). The role of glutamate neurotoxicity in hypoxicischemic neuronal death. *Annual Review of Neuroscience*. **13**: 171-182.

Compton, M.M. (1992). A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer and Metastasis Reviews*. **11**: 105-119.

Curado, M.P., Edwards, B., Shin, H.R., Storm, H., Ferlay, J., Heanue, M. and Boyle, P. (2007). eds: Cancer Incidence in Five Continents. France: Lyon. *International Agency for Research on Cancer Scientific Publication.* **9**: 1-10.

Dahlgren, R.M.T. (1980). A revised system of classification of the angiosperms. *Botanical Journal of the Linnean Society*. **80**: 9-124.

Doll, R. and Peto, R. (1981). The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *Journal of the National Cancer Institute*. **66**: 1191-1308.

Dudev, T. and Lim, C. (2007). Effect of carboxylate-binding mode on metal binding/selectivity and function in proteins. *Chemical Research*. **40**: 85-93.

Fernandez, P., Rotello, R., Rangini, Z., Doupe, A., Drexler, H.C. and Yuan, J. (1994). Expression of a specific marker of avian programmed cell death in both apoptosis and necrosis. *Proceedings of the National Academy of Sciences USA*. **91**: 8652-8655.

Frederick, L.K. and Xinbo, Z. (2002). Apoptosis: biochemical aspects and clinical implications. *Clinica Chimica Acta*. **326**: 27-45.

Giessrigl, B., Yazici, G., Teichmann, M., Kopf, S., Ghassmi, S., Atanasov, A.G., Dirsch, V.M., Grusch, M., Jager, W., Ozmen, A. and Krupitza, G. (2012). Effects of *Scrophularia* extracts on tumor cell proliferation, death and intravasation through lymphoendothelial cell barriers. *International Journal of Oncology*. **40**: 2063-2074.

Herrmann, M., Lorenz, H.M., Voll, R., Grunke, M., Woith, W. and Kaide, J.R. (1994). A rapid and simple method for the isolation of apoptotic DNA fragment. *Nucleic Acids Research*. **22**: 5506-5507.

Hirsch, J. (2006). An anniversary for cancer chemotherapy. *Journal of the American Medical Association*. **296**: 1518-1520.

Joensuu H. (2008). Systemic chemotherapy of cancer: from weapon to treatment. *Lancet Oncology*. **9**: 304.

Key, T.J., Schatzkin, A., Willett, W.C., Allen, N.E., Spencer, E.A. and Travis, R.C. (2004). Diet, nutrition and the prevention of cancer. *Public Health Nutrition*. **7**: 187- 200.

Lee, S.M., Kwon, J.I., Choi, Y.H., Eom, H.S. and Chi, G.Y. (2008). Induction of G2/M arrest and apoptosis by water extract of Strychni Semen in human gastric carcinoma AGS cells. *Phytotherapy Research*. **22**: 752-758.

Leong, O.K., Muhammad, T.S.T. and Sulaiman, S.F. (2011). Cytotoxic activities of *Physalis minima* L. chloroform extract on human lung adenocarcinoma NCI-H23 cell lines by induction of apoptosis. *Evidence-Based Complementary and Alternative Medicine*. 2011:185064, doi:10.1093/ecam/nep057.2011.

Martin, S.J., Mazdai, G., Strain, J.J., Cotter, T.G. and Hannigan, B.M. (1991). Programmed cell death (apoptosis) in lymphoid and myeloid cell lines during zinc deficiency. *Clinical & Experimental Immunology*. **83**: 338-343.

McCarthy, J.V. and Cotter, T.G. (1997). Cell shrinkage and apoptosis: a role for potassium and sodium ion efflux. *Cell death and differentiation*. **4**: 756-770.

Merrouche, Y., Mugneret, F. and Cahn, J.F. (2006). Secondary acute promyelocytic leukemia following irinotecan and oxaliplatin for advanced colon cancer. *Annals of Oncology*. **17**: 1025-1026.

Miyashita, T. and Reed, J.C. (1993). Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood*. **81**: 151-157.

Mohan, S., Bustamam, A., Ibrahim, S., Al-Zubairi, A.S., Aspollah, M., Abdullah, R. and Elhassan, MM. (2011). *In vitro* ultramorphological assessment of apoptosis on CEMss induced by linoleic acid-rich fraction from *Typhonium flagelliforme* Tuber. *Evidence-Based Complementary and Alternative Medicine* 2011:421894. doi: /2011/10.1093/ecam/neq010.

Monsonego J. (2006). Cervical cancer prevention: the impact of HPV vaccination. *Gynecology Obstetrics Fertility*. **34**: 189-201.

Okeleye, B.I., Mkwetshana, N.T. and Ndip, R.N. (2013). Evaluation of the Antibacterial and Antifungal Potential of *Peltophorum africanum*: Toxicological Effect on Human Chang Liver Cell Line. *Scientific World Journal*. 2013:878735.*doi*:10.1155/2013/878735.

Panagopoulou, E., Kouloukoussa, M., Voloudaki-Baltatzi, I., Kittas, C. and Marinos, E. (2002). The effect of 12-O-tetradecanoylphorbol-13-acetate on Sertoli cell morphology and cytoskeletal organization: an *in vitro* study by means of cryo-SEM and fluorescent techniques. *Biology of the Cell*. **94**: 117-125.

Pengsaa, P., Jindavijak, S. Cervix uteri. In: Sriplung, H., Sontipong, S., Martin, N., Wiangnon, S., Vootiprux, V., Cheirsilpa, A. *et al*. (2003). eds: Cancer in Thailand. Bangkok: *Medical Publications*. **3**: 49-50.

Pezzuto, J.M. (1997). Plant-derived anticancer agents. *Biochemical Pharmacology*. **53**: 121-133.

Rukenstein, A., Rydel, R.E. and Greene, L.A. (1991). Multiple agents rescue PCI2 cells from serum-free cell death by translation- and transcription independent mechanisms. *Journal of Neuroscience*. **11**: 2552-2563.

Sarkadi, B. and Parker, J.C. (1991). Activation of ion transport pathways by changes in cell volume. *Biochimica et Biophysica Acta*. **1071**: 407-427.

Shapiro, S., Hughes, G., Al-Obaidi, M.J., O'Reilly, E., Ramesh, S., Smith, J., Ahmad, R., Dawson, C., Riddle, P. and Sekhar, M. (2007). Acute myeloid leukaemia secondary to

treatment with capecitabine for metastatic colorectal cancer. *European Journal of Haematology*. **78**: 543-544.

Sun, S.Y. (2001). Apoptosis induction by chemopreventive agents. *Drug News & Perspectives*. **14**: 75.

Szliszka, E., Czuba, Z.P., Bronikowska, J., Mertas, A., Paradysz, A. and Krol, W. (2011). Ethanolic extract of propolis augments TRAIL-induced apoptotic death in prostate cancer cells. *Evidence-Based Complementary and Alternative Medicine*. 2011:535172. doi: /2011/10.1093/ecam/nep180.

Tanih, N.F. and Ndip R.N. (2013). The Acetone Extract of *Sclerocarya birrea* (Anacardiaceae) *possesses* antiproliferative and apoptotic potential against Human Breast Cancer Cell Lines (MCF7). *Scientific World Journal*. 2013:956206. *doi:* 10.1155/2013/956206.

Williamson R. (1970). Properties of rapidly labeled deoxyribonucleic acid fragments isolated from the cytoplasm of primary cultures of embryonic mouse liver cells. *Journal of Molecular Biology*. **51**: 157-168.

Wu, J., Wu, Y. and Yang, B.B. (2002). Anticancer activity of *Hemsleya amabilis* extract. *Life Sciences*. **71**: 2161-2170.

Xian, M., Ito, K., Nakazato, T., Shimizu, T., Chen, C.K., Yamato, K., Murakami, A., Ohigashi, H., Ikeda, Y. and Kizaki, M. (2007). Zerumbone, a bioactive sesquiterpene, induces G2/M cell cycle arrest and apoptosis in leukemia cells via a Fas- and mitochondria-mediated pathway. *Cancer Science*. **98**: 118-126.

Xin, L., Yu-Jun, C., Zhi-Xiao, L., Zhong-Li, L., Shao-Fu, Y. and Jin-Chang, ZH. (2001). *Cladonia furcata* polysaccharide induced apoptosis in human leukemia K562 cells. *Acta Pharmacologica Sinica*. **22**: 716-720.

Yip, C.H., Nur Aishah, M.T. and Ibraham, M. (2006). Epidemiology of breast cancer in Malaysia. *Asian Pacific Journal of Cancer Prevention*. **7**: 369-74.

Zahri, S., Razavi, S.M., Niri, F.H. and Mohammadi, S. (2009). Induction of programmed cell death by *Prangos uloptera*, a medicinal plant. *Biological Research*. **42**: 517-522.

CHAPTER SEVEN

In-vitro evaluation of the anticancer potential of TEt10 against human cervical cancer cell (HeLa cell): implication of caspase-3 and sub-G1 DNA cell cycle analysis

ABSTRACT

The anticancer potential of BEtA2 and TEt10 among five fractions were tested against cervical (HeLa cells) and breast (MCF-7) cancer cells using the microculture cell titer blue viability assay. The TEt10 mechanism of action on HeLa cells was established using the Scanning Electron Microscope (SEM), Propidium iodide (PI) and Acridine orange (AO) double-staining techniques, the Cleaved Caspase 3 (Asp 175) Alexa Fluor® 488 Antibody and the Coulter® DNA Prep^{TM} Reagents Kit. TEt10 was potentially cytotoxic against human normal cells (chang liver cell) at EC_{50} of 37 µg/mL and 74 µg/mL after 24 and 48 h of treatment respectively. Marked antiproliferative activity of 13.2 µg/mL (EC_{50}) was observed when HeLa cells were treated for 48 h indicating the selectivity of TEt10 to cancer cells when compared to normal cells. Both early (59 ± 1.41 %) and late stages of apoptosis (28.5 ± 2.12 %) were noted with distinctive morphological changes corresponding to a typical cellular surface morphology of apoptosis, including cell shrinkage, membrane blebbing and formation of typical apoptotic bodies. The study recorded 705.102 ± 28.56 % TEt10 caspase-3 activity compared to curcumin 592.857 ± 165.76 % (positive control) and untreated (negative control; 100 ± 15.81 %) cells. Percentage HeLa cell with Sub-G1 DNA phase increased from 0.13 ± 0.06 % (negative control) to 13.8 ± 3.04 % compared to curcumin (8.17 ± 2.20 %) after treatment with TEt10. It is therefore concluded that the compounds identified in the TEt10 including Colchicine, N-(trifluoroacetyl)methyl-N-deacetyl-, Lupeol and .gamma.-Sitosterol or beta.-Sitosterol may be

responsible for the activity observed and these could be further studied *in vivo* as a potential template for new anticancer treatment.

7.1. INTRODUCTION

Cancer is one of the major health problems worldwide and its current treatments have a number of undesired a side effects (Alabsi *et al*., 2012). Cancer is a generic term for a group of more than 100 diseases that can affect any part of the body. The risk of cancer is increased by mutations in cancer-related genes or post-translational protein modifications by nitration, nitrosation, phosphorylation, acetylation, poly(ADP-ribosyl)ation or lipid peroxidation by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Hussain *et al*., 2003). Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen species as by-products. Overproduction of such free radicals can cause oxidative damage to biomolecules (e.g. lipids, proteins, DNA), eventually leading to many chronic diseases, such as, cancer, diabetes and other degenerative diseases (Niki, 1997; Poulson *et al*., 1998).

Cervical cancer is a malignant neoplasm of the cervical area and is the second most common cancer as well as one of the leading cause of cancer-related death for women worldwide and an important women's health problem in developing countries, causing 270,000 deaths in women each year (Widowati *et al*., 2013). It has been demonstrated that human papillomavirus (HPV) infection is a necessary cause; and smoking a risk factor for cervical cancer (Walboomers *et al*., 1999; Plummer *et al*., 2003). Breast cancer in women is the second leading cause of mortality (Zienolddiny *et al*., 2013). The etiology of breast cancer involves several other risk factors, such as environmental factors, increased dietary fat intake; hormonal factors, ionizing radiation exposure and alcohol consumption. Genetic factors, including rare high-risk mutations (BRCA1 and BRCA2) and more moderate susceptibility variants (CHEK2, ATM) are also involved in the etiology of breast cancer (Boyd *et al*., 1993; Zienolddiny *et al*., 2013).

Nature has long been an important source of medicinal agents and the use of plants in traditional medicine systems of many other cultures has been extensively documented (Arvigo and Balick 1993; Gupta, 1995; Ndip *et al*., 2009). Identification of bioactive compounds present in diet with potential anticancer efficacy has been an important area of research in recent years and cancer prevention and treatment using traditional South Africa medicines have attracted increasing interest (du Plessis-Stoman *et al*., 2011; Tanih and Ndip, 2013).

The ability of an agent to trigger caspase activation appears to be a critical determinant of sensitivity or resistance to anticancer therapies. Caspases are a family of cysteine proteases and known to play a central role in various models of cell death. The activation of caspases such as caspase-3 leads to downstream cleavage of various cytoplasmic or nuclear substrates which mark many of the morphological features of apoptosis (Brauns *et al*., 2005). Apoptosis is a form of programmed cell death controlled by the activation of caspase and regulation of apoptotic proteins such as p53, Bax, Bad, Bcl-2, Bcl-xl and other molecules (Jourdain and Martinou, 2009).

The genome integrity is tightly regulated by cell cycle to stop any genetic alteration from being passed through to subsequent generations. However, deregulations of cell cycle checkpoints including those of the G1/S and G2/M phases have been documented to be associated with malignancy. Cell cycle arrest provides an opportunity for DNA repair to occur, hence inhibiting replication of the damaged template. It is regarded as one of the effective strategies for eliminating cancer cells (Nunez, 2001; Foster, 2008; Lam *et al*., 2012).

The present study therefore aimed at assessing the anti-proliferative effects and mechanism of action of the fractions of the ethyl acetate extract of *Peltophorum africanum*, a plant commonly used traditionally in South Africa in the treatment of diarrhoea, human immunodeficiency virus/

acquired immune deficiency syndrome (HIV/AIDS) and venereal diseases, in order to ascertain its therapeutic potential in cancer care.

7.2. MATERIALS AND METHODS

7.2.1. Sample preparation

P. africanum was collected, identified and the extract and fractions prepared as reported in our prior study (Okeleye *et al*., 2013).

7.2.2. Cell lines

Human chang liver (normal) cell was used to determine the cytotoxicity while three human cancer cell lines, MCF-7 (breast cancer) and HeLa (cervical cancer) were used to determine the antiproliferative potential of the fractions. Cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 10 % fetal bovine serum (FBS) and incubated in a 37 ^{o}C humidified incubator (Shel Lab, USA) at 5 % CO_2.

7.2.3. Cell growth inhibition assay

Cytotoxicity of T1, BEtA2, TEt10, TEt1, T2 and antiproliferative potential of the BEtA2 and TEt10 fractions were determined using microculture CellTiter-Blue viability (Promega, USA) assay as previously described with modifications (Gloeckner *et al*., 2001). Cells were seeded in flat-bottom 96 well culture plates (Nunc) with 200 µL DMEM + high glucose, L-glutamine and sodium pyruvate (Thermo Sceintific, South Logan, Utah) at 30 000 cells per milliliter and incubated overnight at 37°C in a humidified incubator containing 5% CO_2. After 24 hrs of incubation and attachment, the cells were treated with the fractions at the concentration range from 24 to 6250 µg/mL. Exactly 60 µM of curcumin (Sigma-Aldrich, South Africa) was used as positive control and 0.5 % DMSO as negative control. After 24, 48 and 72 hrs of treatment, cell viability was determined by adding cell titer blue as an indicator and further incubated for 4 hrs.

Fluorescence was read at 570/620 nm using Analytical & Diagnostic Product Gen™ spectrophotometer (BioTek, Highland Park, USA). All experiments were carried out in duplicates and the EC_{50} (Effective concentration at which 50 % of cellular growth is inhibited) values were calculated using the GraphPad Prism4 software package. Percentage inhibition of cell growth was calculated as: % Cell inhibition = [(Cell growth (control) − Cell growth (treatment) / (Cell growth (control))] × 100 (Gloeckner *et al*., 2001; Das *et al*., 2010).

7.2.4. Propidium iodide and acridine orange double staining analysis of the treated HeLa cell

Morphological changes were assessed using propidium iodide (PI) and acridine orange (AO) double-staining according to the method of Ali *et al*. (2001) and Ciapetti *et al*. (2002). Cells were seeded in a 6-well tissue culture plate at 1, 000 000 cells per milliliter and treated after 24 hrs (EC_{50} of TEt10) and then spun down at 300 × g for 10 minutes. Cellular pellet was washed twice with PBS and 20 µL fluorescent dyes; PI (10 µL) and AO (10 µL) were added to the cell at equal volumes and promptly observed under UV-fluorescence microscope. The percentages of early apoptotic, late apoptosis and secondary necrotic cells were determined (Ciapetti *et al*., 2002).

7.2.5. Scanning electron microscope analysis of cells

Cervical cancer cell line was analysed further based on the marked activity of TEt10 observed against it. Approximately 1, 000 000 cells per milliliter of the HeLa cells were seeded in a 6-well tissue culture plate and treated with TEt10 after 24 hrs at EC_{50}. Exactly 60 µM curcumin (Sigma-Aldrich, South Africa) was included as a positive control. Cells were washed in PBS, centrifuged at 1000 rpm for 5 minutes and then fixed in 2.5 % gluteraldehyde prepared in 0.1 M PBS. Cells

were post fixed with 1 % osmium tetroxide (OsO_4) in 0.2 M PBS for 30 minutes and coated using IB3 Ion Coater (EIKO, Japan) after dehydrated through graded ethanol (30, 50, 70, 85 and 95 %) and critical point dried (CPD). The morphological characteristics of the cells were examined using JSM-6390LV scanning electron microscope (SEM; Jeol, Japan) (Talib *et al*., 2013).

7.2.6. Assay for caspase 3 activity

Caspase 3 activity was determined using the Cleaved Caspase 3 (Asp 175) Alexa Fluor® 488 Antibody (Beckman Coulter). Cells were seeded into 10 cm cell culture dishes (Nunc) at $1.15x10^5$ cells per ml and incubated in a 37 ºC incubator supplemented with 5 % CO_2 for 24 hours before 20 µg/ mL of TEt10 and 60 µM curcumin (Sigma-Aldrich, South Africa; positive control) were added and then incubated for 48 hrs. The assay was carried out as per the manufacturer's instructions and analyzed on a Beckman Coulter FC500 flow cytometer (du Plessis-Stoman *et al*., 2011).

7.2.7. DNA cell cycle analysis

DNA cell cycle analysis of TEt10, EAE (ethyl acetate extract), curcumin (Sigma-Aldrich, South Africa; positive control) was performed using the Coulter® DNA PrepTM Reagents Kit (Beckman Coulter). Cells were seeded and treated as described for the caspase 3 assay. The assay was performed as per the manufacturer's instructions and the results analyzed on a Beckman Coulter FC500 flow cytometer (du Plessis-Stoman *et al*., 2011).

7.2.8. GC-MS analysis of TEt10

Agilent 6890N GC with CTC Combi-PAL Auto-sampler and Agilent 5975B MS with Rtx®-5MS (30 m, 0.25 mm ID, 0.5 µm film thickness) were used for the analysis. Exactly 1 µL volume of TEt10 was injected onto a GC column with helium as the carrier gas. Injection temperature and MS transfer were set at 280 °C with acquisition mode scanning mass range of 40 to 550 m/z (electron ionization at 70 Ev) (Adams, 1989).

7.3. RESULTS

7.3.1. Cytotoxicity and antiproliferative effect of the fractions against normal and cancer cell lines

The cytotoxicity and antiproliferative results are as shown in figure 7.1 and 7.2 with the EC_{50} (Effective concentration at 50 %) corresponding to those in table 7.1 and figure 7.3. Fraction T1 at 24 µg/mL and 98 µg/mL caused 18.26 ± 1.37 % and 24.995 ± 0.3 % (n=2) inhibition against normal human (Chang liver) cell, meanwhile 1.58 ± 0.58 % and 21.385 ± 1.53 % were observed for TEt1 at the same concentration in 24 h of treatment respectively. For T2 low activity of 0.115 ± 0.08 % and 5.185 ± 1.45 % inhibition were noted at 24 µg/mL and 98 µg/mL concentration. In consideration of the cytotoxicity, the extracts could be classified into; potentially cytotoxic (TEt10 EC_{50} = 37 µg/mL), moderate cytotoxic (T1 EC_{50} = 193 µg/mL and BEtA2 EC_{50} = 197 µg/mL) and low cytotoxic (T2 EC_{50} = 391 µg/mL and TEt1 EC_{50} = 781 µg/mL) activities (24 h; table 7.1). The viability of MCF-7 and HeLa cell lines was reduced in a concentration dependent manner across the period of treatment (24, 48 and 72 h), with a potential antiproliferative activity of 13.2 µg/mL (EC_{50}) when HeLa cells were treated for 48 h with TEt10. This is remarkable (figure 7.3 and table 7.1) as it shows the selectivity of TEt10 to cancer cells when compared to normal cells (EC_{50} = 74 µg/mL; 48h). As similar trend was observed for BEtA2 with 35.64 ± 1.6 % and 44.31 ± 1.44 % (Chang liver cell); 23.05 ± 0.24 % and 66.325 ± 1.11 % (MCF-7); 37.93 ± 2.6 % and 55.655 ± 3.02 % (HeLa cell) rate of inhibition within 48 h of treatment at 49 µg/mL and 98 µg/mL concentration respectively (figure 7.1 and 7.2).

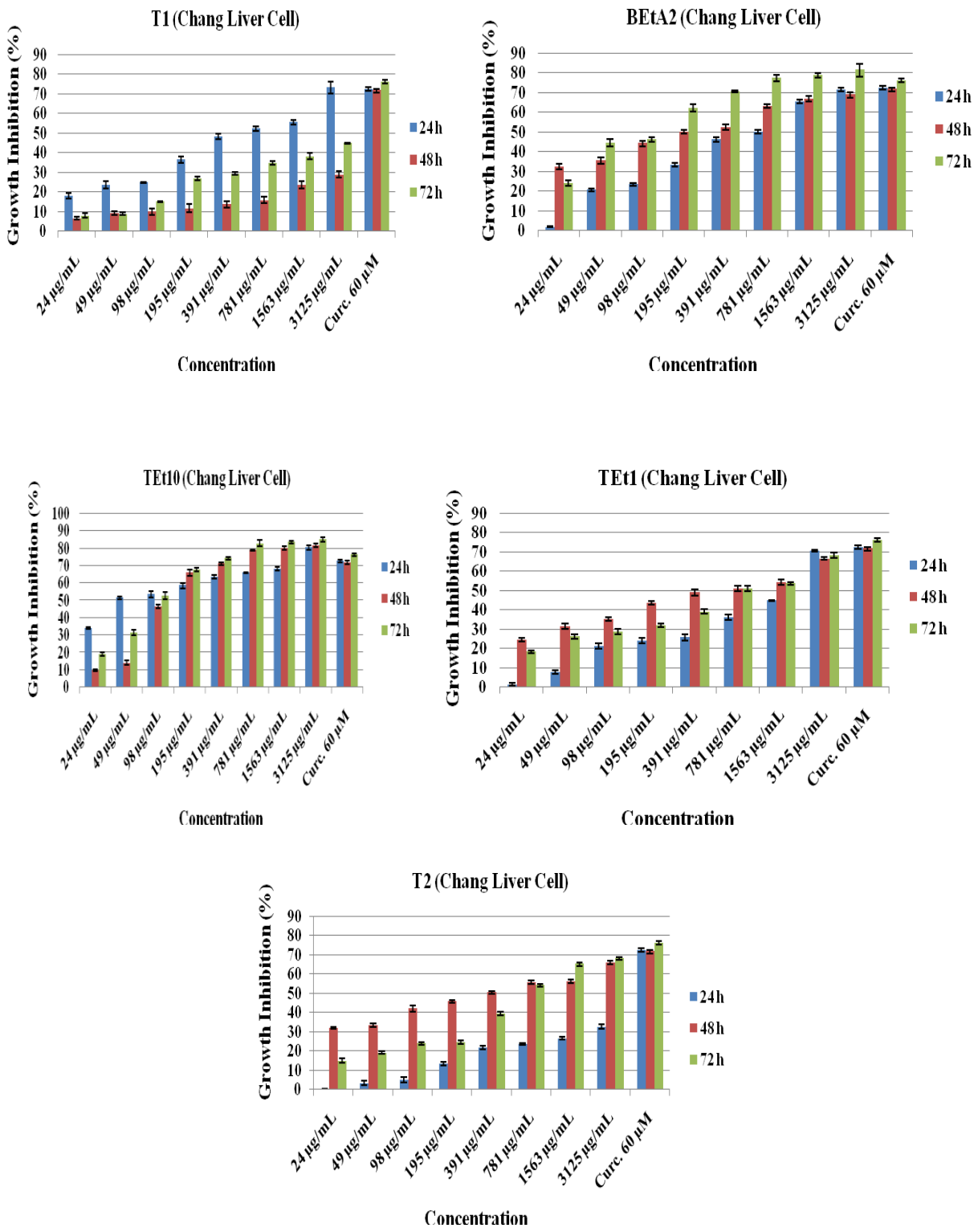


Figure 7.1: Percentage growth inhibition showing the rate of toxicity of the fractions

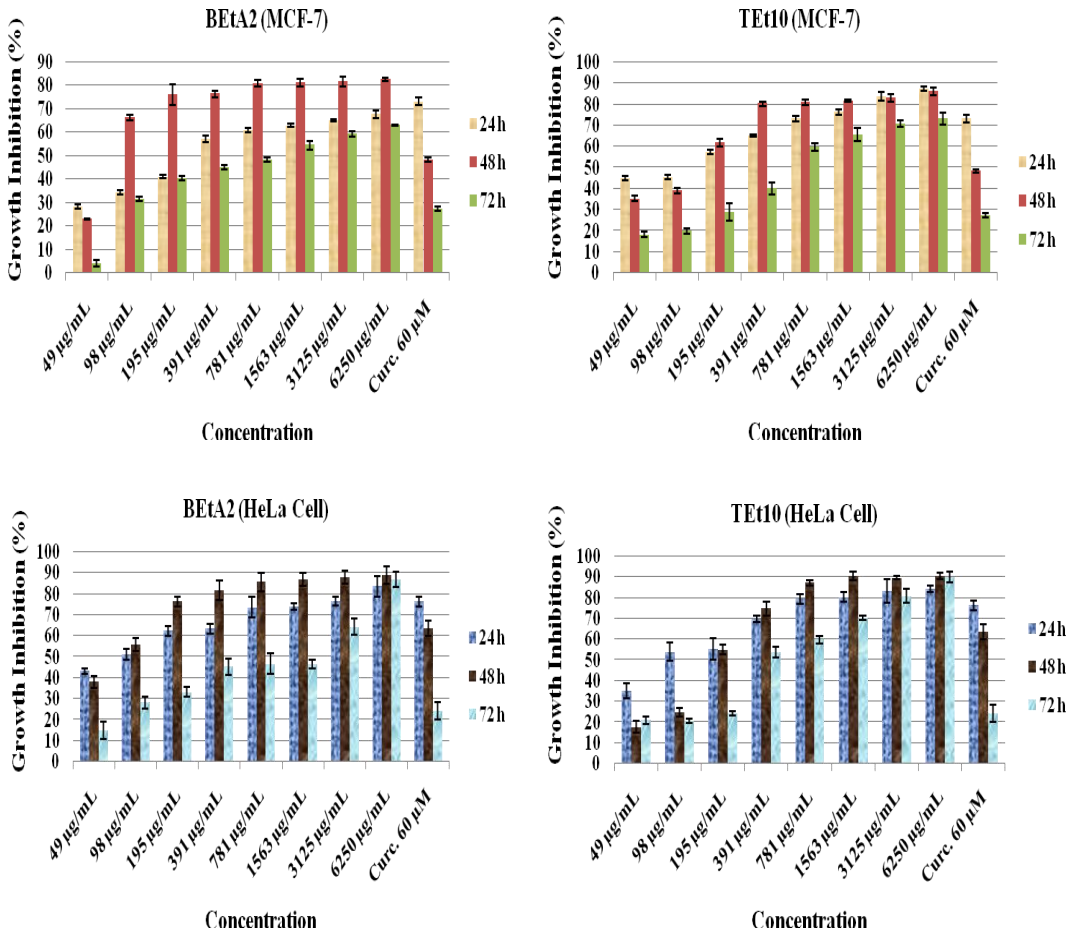


Figure 7.2: Percentage growth inhibition of the cancer cells by the fractions

Table 7.1: Effective concentration of the fractions against Chang liver cell, MCF-7 and HeLa cell

Fractions/ Cell Type	**EC_{50} (µg/mL)***		
Cytotoxicity (Chang Liver Cell)	**24 h**	**48 h**	**72 h**
T1	193	781	193
BEtA2	197	49	40
TEt10	37	74	67
TEt1	781	98	291
T2	391	74	284
Anticancer (MCF-7)			
BEtA2	98	57	98
TEt10	49	137	230
(HeLa Cell)			
BEtA2	49	70	391
TEt10	69	13.2	207

***,** Effective concentration at 50 % of inhibition

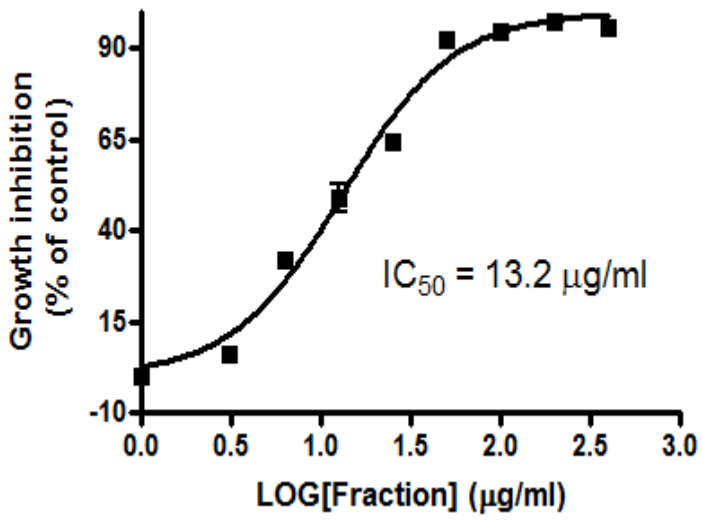


Figure 7.3: Marked effective concentration at 50 % (EC_{50}) inhibition of HeLa cell by TEt10 after 48 h of treatment

7.3.2. Stages of apoptosis by intercalation of nucleic acid-specific fluorochromes

Fluorochrome staining was carried out to examine whether the cells death are due to excess TEt10 in HeLa cell culture and their toxicity to cells, leading to apoptosis and necrosis. The study revealed that TEt10 triggered morphological features that relate to apoptosis (Figure 7.4). Early apoptosis (59 ± 1.41 %) was obvious by intercalated AO within the fragmented DNA showing bright-green colour, while late stages of apoptosis (28.5 ± 2.12 %) were indicated by the presence of orange colour due to the binding of AO to denatured DNA. Secondary necrosis (12.5 ± 3.54 %) appeared as bright red (Figure 7.5).

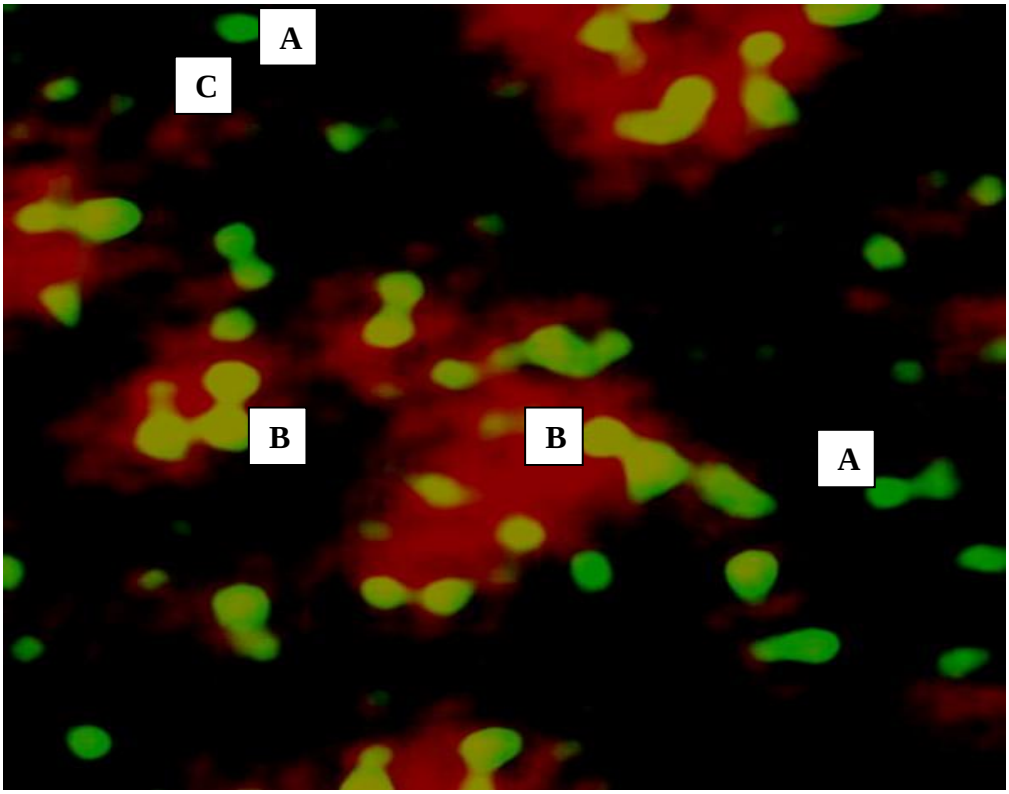


Figure 7.4: HeLa cell stages of apoptosis. The criteria for identification: A. Early apoptosis (EA) appeared to have intercalated acridine orange (bright green nucleus), showing aggregation and condensation of chromatin, amongst the fragmented DNA, B. Dense orange area of chromatin condensation with blebbing and nuclear margination indicated late apoptosis (LA), C. Bright red colored secondary necrosis.

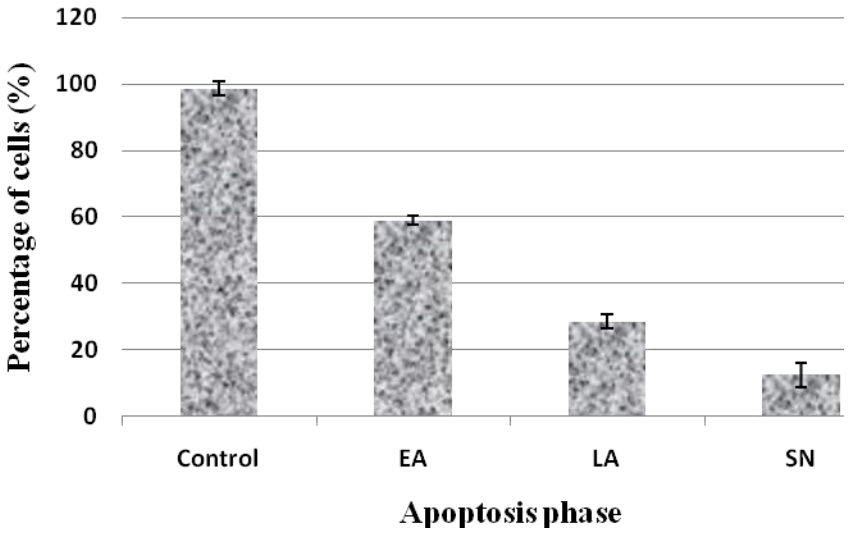
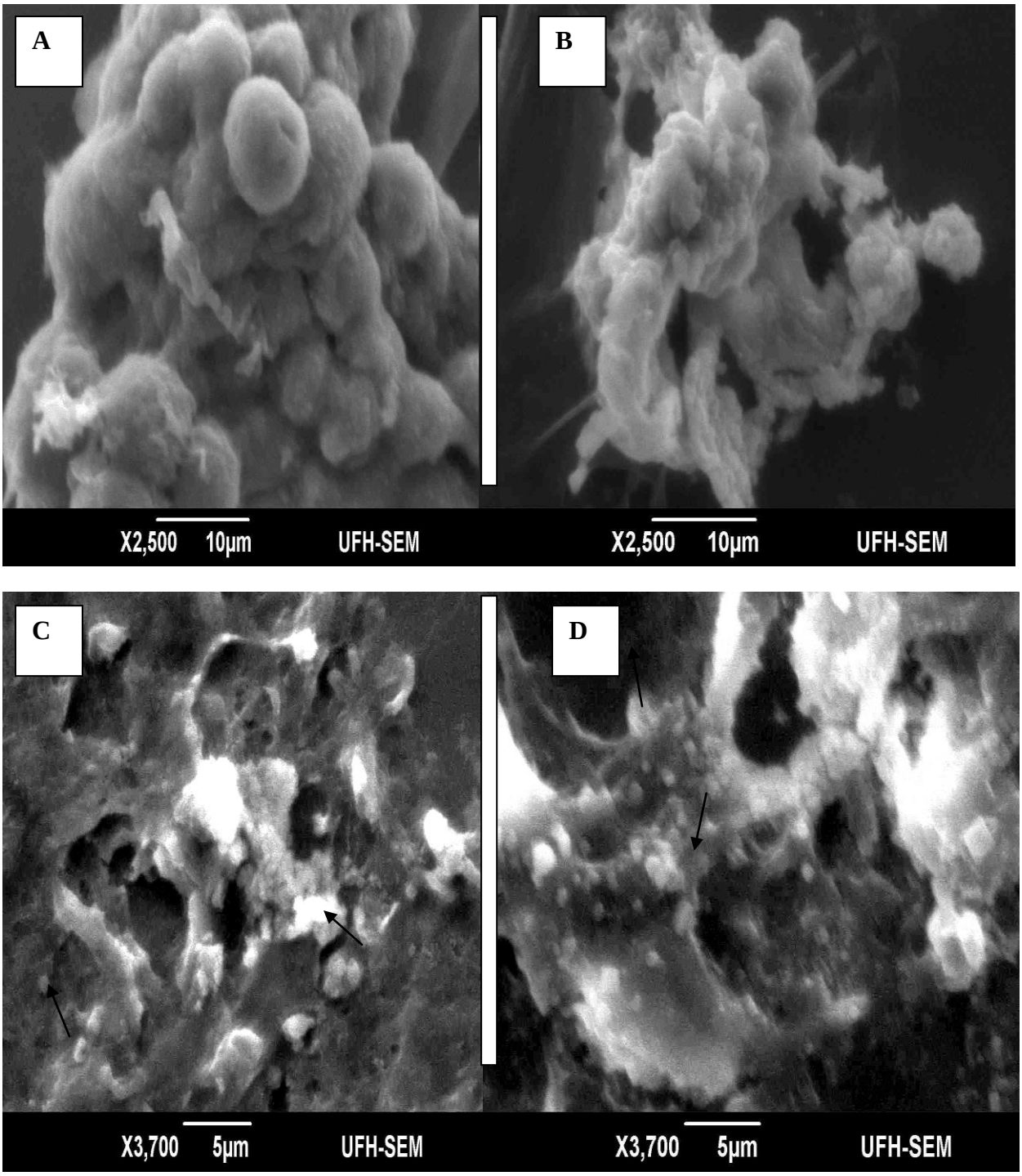


Figure 7.5. Percentages of early apoptosis (EA), late apoptosis (LA) and secondary necrotic (SN) cells after TEt10 treatment.

7.3.3. Ultramorphological characterization of cells

Morphological analysis of treated HeLa cells was carried out to observe the morphological and ultrastructural alteration caused by TEt10. The extracellular structure analysis was carried out by SEM and the interpretation of electromicrographs showed distinctive morphological changes corresponding to a typical cellular surface morphology of apoptosis, including cell shrinkage, membrane blebbing and formation of typical apoptotic bodies (Figure 7.6 C, D and E). This is similar to curcumin treated cell (Figure 7.6 B), but on the contrary, untreated HeLa cells showed well-preserved morphology (Figure 7.6 A). These apoptotic effects were found to be concentration correlated and this was noticed when considering the apoptotic bodies and membrane blebbing formation as an indicator of cell death via apoptosis.


A
B
X2,500 10µm UFH-SEM
X2,500 10µm UFH-SEM
C
D
X3,700 5µm UFH-SEM
X3,700 5µm UFH-SEM

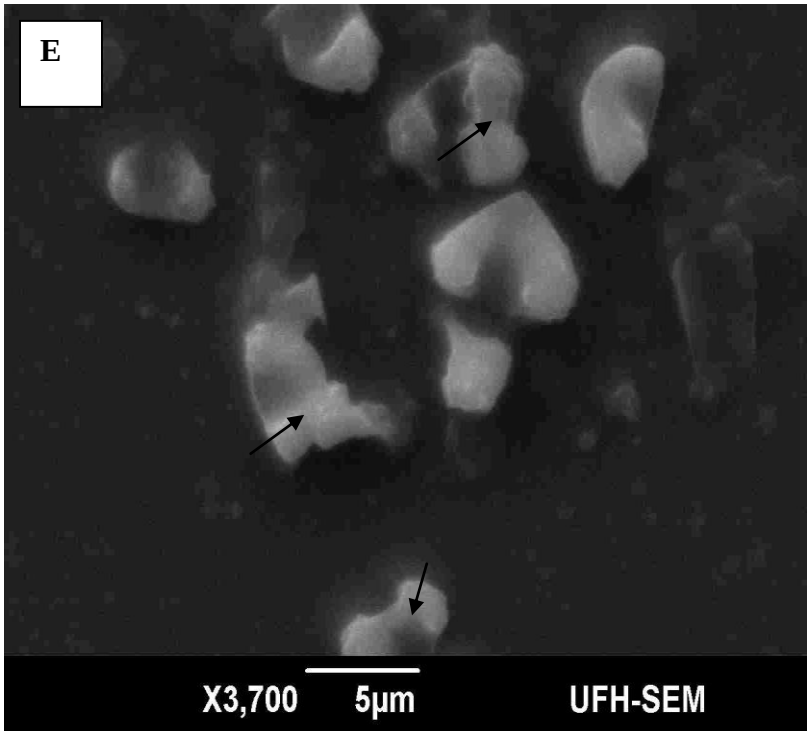


Figure 7.6: Morphological appearance of TEt10 treated HeLa cell. **(A)** Untreated cells observed as round and smooth, a typical ultrastructural feature of a cancer cell, **(B)** Curcumin treated cell (60µM/6000 cells/200 µL) appeared muggy and shrink, **(C)** TEt10 treated cell (IC_{50}/6000 cells/200 µL) demonstrated membrane blebbing and separated apoptotic bodies, **(D)** TEt10 treated cell (IC_{50}×2/6000 cells/200 µL) exhibited surface blebbing, cell retraction, fragment of apoptotic cells and **(E)** TEt10 treated cell (IC_{50}×4/6000 cells/200 µL) showed apoptotic cells.

7.3.4. Activity of caspase-3 in TEt10-induced apoptosis

The study explored the possibility of whether the TEt10-induced cell death was mediated by caspases. As shown in Figure 7.7, the treatment of HeLa cells with 20 μg/mL TEt10 resulted in the cleavage of caspase-3. Results indicated 705.102 ± 28.56 % and 592.857 ± 165.76 % of caspase-3 activity in TEt10 and curcumin (positive control) treated HeLa cells respectively compared to untreated (negative control; 100 ± 15.81 %) cells. These results point towards involvement of caspase-3 pathways in execution of TEt10-induced apoptosis.

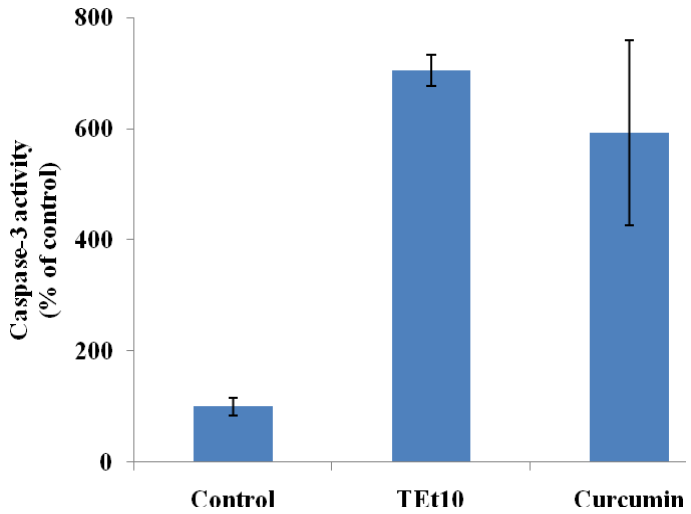


Figure 7.7: Percentage increase in activated caspase 3 of the treated and untreated cells.

7.3.5. Cell cycle distribution by flow cytometry analysis

Flow cytometry enables the identification of the cell distribution during the various phases of the cell cycle. Impact of the TEt10 on different phases of cell cycle in HeLa cells was evaluated and the three phases observed in a proliferating cell population as shown in figure 7.8 were the sub-G1, G1-, S- and G2M- phases. For HeLa cells, G1- phase takes the order of 37.67 ± 2.00 % (negative control) > 28.4 ± 0.7 % (TEt10) > 26.5 ± 0.71 % (Curcumin) followed by G2M- phase of 48.6 ± 2.26 % (Curcumin) > 36.93 ± 0.64 % (negative control) > 30.8 ± 0.17 % (TEt10) and S- phase of 23 ± 1.41 % (Curcumin) > 21.7 ± 2.09 % (TEt10) > 20.63 ± 0.38 % (negative control) respectively. For MCF-7, G1- phase takes the order of 46.57 ± 1.53 % (negative control) > 43.4 ± 2.5 % (ethyl acetate extract, EAE) > 29.4 ± 1.56 % (Curcumin) followed by G2M-phase of 42.83 ± 2.30 % (EAE) > 41.8 ± 2.69 % (Curcumin) > 31.4 ± 1.6 % (negative control) and S- phase of 26.1 ± 0.85 % (Curcumin) > 19.6 ± 0.17 % (negative control) > 10.63 ± 0.57 % (EAE) respectively.

Percentage of G1- phase and G2M- phase of the MCF-7 treated with EAE (43.4 ± 2.5 % and 42.83 ± 2.30 %) is higher than that of the HeLa cells treated with TEt10 (28.4 ± 0.7 % and 30.8 ± 0.17 %) respectively. Meanwhile S- phase of HeLa cells treated with TEt10 (21.7 ± 2.09 %) is higher than that of the MCF-7 treated with EAE (10.63 ± 0.57 %). Percentage HeLa cell with Sub-G1 DNA phase was remarkably increased from 0.13 ± 0.06 % (negative control) to 13.8 ± 3.04 % compared to curcumin (8.17 ± 2.20 %) after treatment with TEt10, meanwhile for MCF-7, 0.3 ± 0.1 % (negative control), 1.57 ± 0.25 % (EAE) and 2.45 ± 0.35 % (curcumin) were observed respectively.

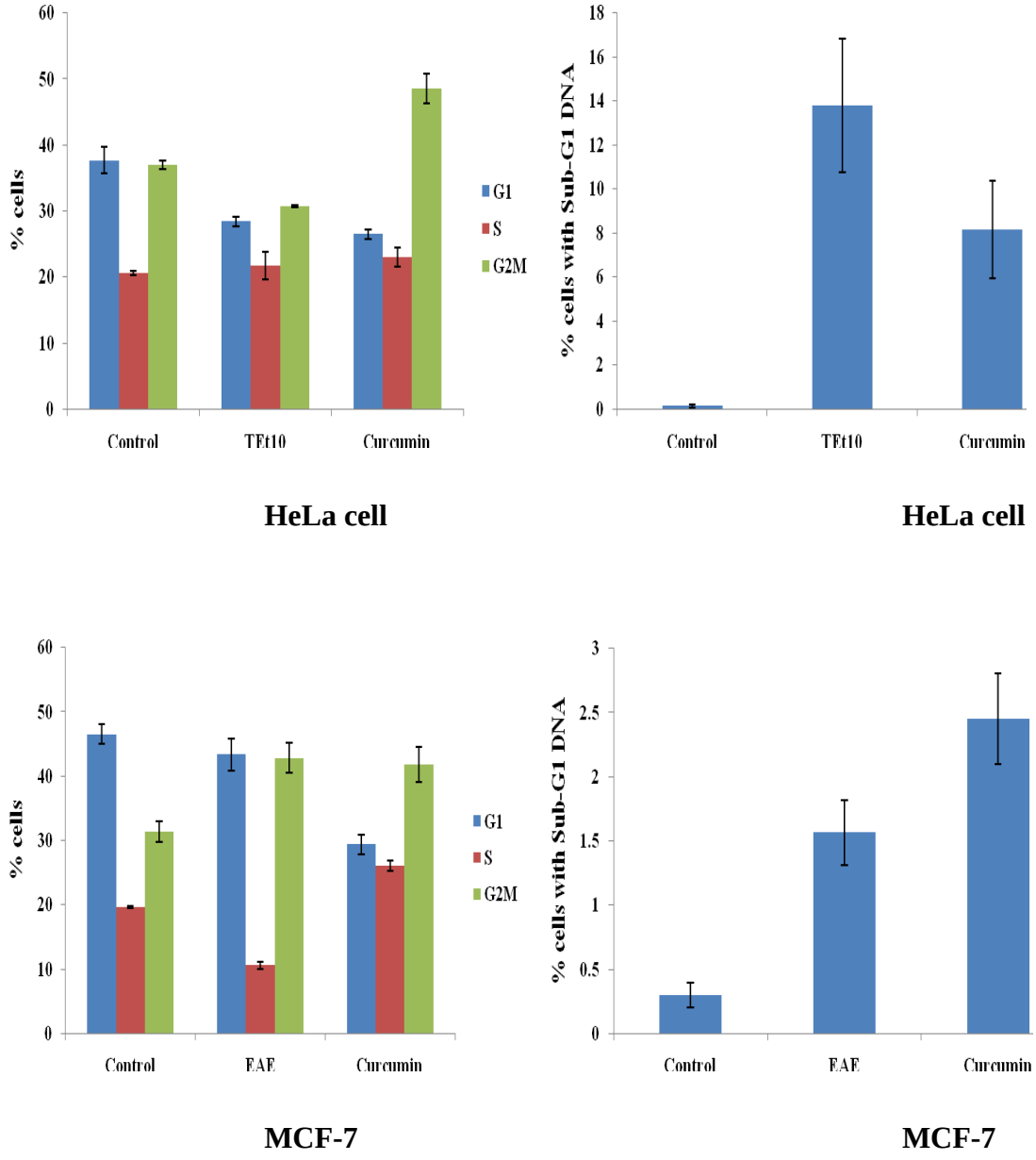


Figure 7.8: The percentage of cells in the different DNA cell cycle phases of the treated and untreated.

7.3.6. Identified compounds from the TEt10 fraction of ethyl acetate extract of *P. africanum*.

The fraction TEt10 demonstrated marked activity compared to other fractions. Volatile compounds identified in the fraction are those typically found in food and pharmaceutical products as shown in table 7.2 and figure 7.9. Lupeol, with the retention time (RT) of 20.7715 and 59 % quality match (QM) has the highest quantity (Q) of 48.4 %, followed by .gamma.-Sitosterol or beta.-Sitosterol, 23.9 % (19.9054 RT; 99 % QM) and Stigmast-4-en-3-one (Sitostenone), 7.3 % (21.0281 RT; 91 % QM) among others. Other compounds identified are n-Hexadecanoic acid, Stigmasterol, Campesterol, Stigmastanol, Colchicine, N-(trifluoroacetyl)methyl-N-deacetyl- and 1-Docosanethiol.

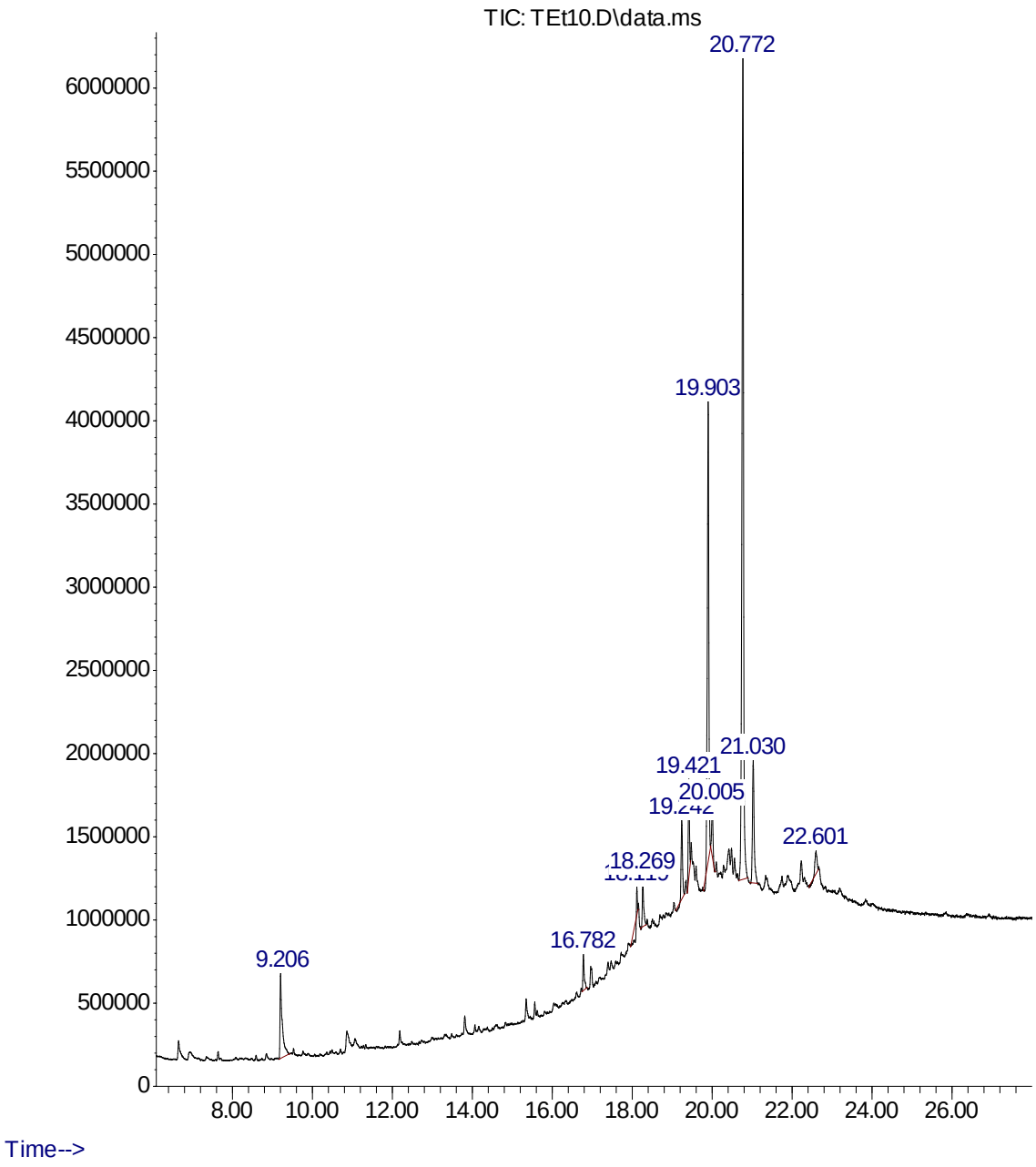


Figure 7.9: Total ion chromatogram (TIC) showing the compounds identified from TEt10.

Table 7.2: Identified compounds from the TEt10 fraction

	TEt10			
Peak	**Retention Time**	**Library Identification**	**Quality Match**	**Area Percent**
1	9.2047	n-Hexadecanoic acid	99	6.4
2	16.7811	1-Docosanethiol	70	1.7
4	18.2695	Colchicine, N-(trifluoroacetyl)methyl-N-deacetyl-	49	2.3
5	19.2446	Campesterol	53	3.5
6	19.4178	Stigmasterol	64	4.0
7	19.9054	.gamma.-Sitosterol or beta.-Sitosterol	99	23.9
8	20.0016	Stigmastanol	49	2.4
9	20.7715	Lupeol	59	48.4
10	21.0281	Stigmast-4-en-3-one (Sitostenone)	91	7.3

7.4. DISCUSSION

Cancer is one of the most prominent human diseases which have stimulated scientific and commercial interest in the discovery of new anticancer agents from natural sources (Sowemimo *et al*., 2009). Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Sasidharan *et al*., 2011). In this study five fractions (T1, BEtA2, TEt10, TEt1, T2) purified from the ethyl acetate extract of *P. africanum* were tested for cytotoxicity while two (BEtA2 and TEt10) were further investigated for their anticancer potential. Local medical practitioners use *P. africanum* for treating a wide variety of ailments and the fractions tested induced cytotoxicity in normal human cell (Chang liver), breast cancer (MCF-7) and cervix cancer (HeLa) cells. TEt10 was able to reduce the proliferation of the chang liver (cytotoxicity) and HeLa (anticancer) cell lines by up to 50 % **(**EC_{50}), when tested at 74 µg/mL and 13.2 µg/mL respectively which shows its selectivity. Cytotoxic agents are known to induce DNA damage in normal cells as well as cancer cells. However, fast growing cells are more susceptible to DNA damaging agents due to the greater probability of more sites being exposed on DNA within replicative cycles and in addition, cancer cells frequently have defective repair pathways resulting in DNA damage being sustained (Lam *et al*., 2012).

Since the cytotoxicity was found to be selective, the study further sought to determine the mechanism of antiproliferation. Fluorescent dyes, acridine orange (AO) and propidium iodide (PI) double staining techniques and scanning electron microscope (SEM) analysis were used to determine morphological characteristics. Various stages of apoptosis were observed, starting from chromatin condensation; apoptotic body formation, membrane blebbing and cell shrinkage, grouped into early and late apoptosis depending on the dye intercalation of the cell DNA

fragmented induced by TEt10. Apoptosis is one form of physiological or active cell death, or a key pathway for regulating homeostasis and morphogenesis of mammalian cells and is connected with several diseases, especially cancer. Much emphasis has been placed on discovering new compounds that target tumour cells more efficiently and selectively with minimal toxic effects on normal cells (Edris, 2009).

Pharmacologic inhibitor of caspases was used to confirm caspase-3 involvement in TEt10-induced apoptosis. Exactly 705.102 ± 28.56 % (TEt10) and 592.857 ± 165.76 % (curcumin) of caspase-3 activity were observed after cervix cancer cells treatment (HeLa cells), compared to untreated (negative control; 100 ± 15.81 %) cells. Activation of both extrinsic and intrinsic caspase pathways has been well established to be the major mechanisms of apoptotic cell death in most cellular systems (Fadeel and Orrenius, 2005). Caspases are aspartate-specific cysteine proteases that play critical roles in execution of apoptosis program. Activation of caspases results in cleavage and inactivation of key cellular proteins (Thornberry and Lazebnick, 1998; Wolf and Green, 1999). Fourteen mammalian caspases have been identified, three of which (caspase-3, -6, and -7) are thought to coordinate the execution phase of apoptosis by cleaving multiple structural and repair proteins. Caspase-3 is required for typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation and DNA fragmentation (Salim *et al*., 2013).

In addition to determining the relative cellular DNA content, flow cytometry also enables the identification of the cell distribution during the various phases of the cell cycle as hyper-proliferation is a characteristic common to tumour cells, which as a result are more susceptible to cell cycle modulation (Lam *et al*., 2012). Sub-G1 DNA phase was remarkably increased after treatment of the HeLa cells with 20 μg/mL of the TEt10 for 48 h (Figure 7.8) from 0.13 ± 0.06 % (negative control) to 13.8 ± 3.04 %. This result indicates that the TEt10 fraction induces sub-G1

phase arrest in the HeLa cells. However, the selected concentrations slightly altered the cell cycle (sub-G1) distribution in the MCF-7 cell line after treatment with the ethyl acetate extract (EAE) from 0.3 ± 0.1 % (negative control) to 1.57 ± 0.25 %. These effects were also compared with a known therapeutic reagent (Curcumin: HeLa cells, 8.17 ± 2.20 %; MCF-7, 2.45 ± 0.35 %). Meanwhile, percentage HeLa cell with S- phase followed the order of 23 ± 1.41 % (Curcumin) > 21.7 ± 2.09 % (TEt10) > 20.63 ± 0.38 % (negative control) respectively. Salim *et al*. (2013), arguably reported that the most important phases are the S phase, when DNA replication occurs, and the M phase, when the cell divides into two daughter cells. Four distinct phases could be recognized in a proliferating cell population: the G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). However, G2- and M-phase, which both have an identical DNA content, could not be discriminated based on their differences in DNA content (Nunez, 2001).

TEt10 were able to reduce the proliferation of the HeLa cell lines by up to 50 % **(**EC_{50}) at 13.2 µg/mL. This is similar to colchicine and its derivative (3-demethyl-N-formyl-N-deacetylcolchicine) reported by Itharat and Ooraikul (2007) that showed activity against HuCCA-1 cells (IC_{50}) at 0.2 and 0.625 µg/mL, respectively. A number of the compounds isolated from TEt10 are previously known products from plants which must have contributed to the activity observed. For example, Colchicine, N-(trifluoroacetyl)methyl-N-deacetyl- (18.2695 RT, 49 % QM, 2.3 % Q) also known as the alkaloid colchicine, is used medicinally in the treatment of gout, a painful disease that results from inflamed joints. Colchicine works by interrupting the animal and plant cells mitosis process (both *in-vitro* and *in-vivo*) especially in the division of cancerous cells (Itharat and Ooraikul, 2007). Lupeol (20.7715 RT, 59 QM, 48.4 Q), the highest compounds in TEt10 has been reported to perform a variety of activities including antiinflammatory, antiulcerogenic and antioxidant (Lewis and Hanson, 1991).

The mechanism of action for many currently used anticancer agents have been specifically targeted to regulate the apoptotic pathway, further stressing the role of programmed cell death in maintaining normal homeostasis. A major concern of cancer chemotherapy is the side effects caused by the non-specific targeting of both normal and cancerous cells by therapeutic drugs. Therefore, several researchers have carried out anti-cancer studies on plant extracts, their fractions as well as natural compounds based on their biochemical properties of apoptosis (Edris, 2009; Kaseb *et al*., 2010), similar to this study.

7.5. CONCLUSIONS

This study established that the fractions from the ethyl acetate extract of *Peltophorum africanum* are cytotoxic against chang liver cell and inhibited the proliferation of HeLa and MCF-7. However, a stronger selectivity effect of TEt10 on HeLa cell **(**EC_{50} = 13.2 µg/ml) was confirmed by high activity of caspase-3 (705.102 ± 28.56 %), percentage HeLa cell with Sub-G1 DNA phase (13.8 ± 3.04 %), ultrastructural disruption and stages of apoptosis by intercalation of fragmented nucleic acid-specific fluorochromes observed. The present findings therefore call for advance studies using animal models to investigate potential antitumor leads from TEt10 compounds such as Lupeol and .gamma.-Sitosterol or beta.-Sitosterol.

REFERENCES

Adams, P. R. (1989). *Identification of Essential Oil Components by Ion Trap Mass Spectroscopy*. Academic Press, New York, NY: USA.

Alabsi, A. M, Ali, R., Ali, A. M., Al-Dubai, S. A., Harun, H., Abu Kasim, N. H. and Alsalahi, A. (2012). Apoptosis induction, cell cycle arrest and *in vitro* anticancer activity of gonothalamin in a cancer cell lines. *The Asian Pacific Journal of Cancer Prevention*. **13**(10):5131-5136.

Ali, A. M., Umar, N. T., Mohamed, S. M. et al., (2001). Induction of apoptosis in leukaemic cells by goniothalamin. *Journal of Biochemistry, Molecular Biology and Biophysics*. **5**(3):227-235.

Arvigo, R. and Balick, M. (1993). Rainforest Remedies. Lotus Press, Twin Lakes, Wisconsin.

Boyd, N. F., Martin, L. J., Noffel, M., Lockwood, G. A. and Trichler, D. L. (1993). A meta-analysis of studies of dietary fat and breast cancer risk. *British Journal of Cancer*. **68**(3):627-36.

Brauns, S. C., Dealtry, G., Milne, P., Naude, R. and Van de Venter, M. (2005). Caspase-3 activation and induction of PARP cleavage by cyclic dipeptide cyclo(Phe-Pro) in HT-29 cells. *Anticancer Research*. **25**:4197-4202.

Ciapetti, G., Granchi, D., Savarino, L., Cenni, E., Magrini, E., Baldini, N. and Giunti A. (2002). *In vitro* testing of the potential for orthopedic bone cements to cause apoptosis of osteoblast-like cells. *Biomaterials*. **23**(2):617-627.

Das, K., Tiwari, R.K.S. and Shrivastava, D.K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. Journal of Medicinal Plants Research. **4** (2): 104-111.

du Plessis-Stoman, D., du Preez, J. G. H. and van de Venter, M. (2011). Combination treatment with oxaliplatin and mangiferin causes increased apoptosis and downregulation of NFKB in cancer cell lines. *African Journal of Traditional, Complementary and Alternative medicines*. **8**(2):177-184.

Edris, A. E. (2009). Anti-cancer properties of nigella spp. essential oils and their major constituents, Thymoquinone and-Elemene. *Current Clinical Pharmacology.* **4**:43-46.

Fadeel, B. and Orrenius, S. (2005). Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. *Journal of Internal Medicine*. **258**:479-517.

Foster, I. (2008). Cancer: A cell cycle defect. *Radiography*. **14**:144-149.

Gloeckner, H., Jonuleit, T., and Lemke, H. D. (2001). Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dye Alamar Blue. *Journal of Immunological Methods*. **252**: 131-138.

Gupta, M. P. (1995). 270 Plantas Medicinales Iberoamericanas. Talleres de Editoral Presencia Ltda., Bogota, Colombia.

Hussain, S. P., Hofseth, L. J., Harris, C. C. (2003). Radical causes of cancer. *Nature Reviews Cancer*. **3**:276-86.

Itharat, A. and B. Ooraikul. (2007). Research on Thai medicinal plants for cancer treatment. *Advances in Medicinal Plant Research*. **81**:287-317.

Jourdain, A. and Martinou, J. C. (2009). Mitochondrial outermembrane permeabilization and remodelling in apoptosis. *The International Journal of Biochemistry & Cell Biology*. **41**(10):1884-1889.

Kaseb, A. O., Chinnakannu, K., Chen, D., Sivanandam, A., Tejwani, S., Menon, M., Dou, Q. P., Reddy, B. S., Padhye, S., Azmi, A., Wang, Z., Philip, P. A., Kucuk, O., Sarkar, F. H. and Mohammad, R. M. (2010). Review on molecular and therapeutic potential of thymoquinone in cancer. *Nutrition and Cancer*. **62**:938-946.

Lam, M., Carmichael, A. R. and Griffiths, H. R. (2012). An aqueous extract of *Fagonia cretica* induces DNA damage, cell cycle arrest and apoptosis in breast cancer cells via FOXO3a and p53 expression. *PLoS ONE*. **7**(6): e40152. doi:10.1371/journal.pone.0040152.

Lewis, D. A. and Hanson P. J. (1991). 4 anti-ulcer drugs of plant origin. *Progress in Medicinal Chemistry*. **28**:201-231.

Ndip, R. N, Ajonglefac, A. N., Wirna, T., Luma, H. N., Wirmum, C., Efange, S. M. N. (2009). *In-vitro* antimicrobial activity of Ageratum conyzoides on clinical isolates of *Helicobacter pylori*. *African Journal of Pharmacy and Pharmacology*. **3**:585-592.

Niki, E. (1997). Free radicals, antioxidants, and cancer. In: Ohigashi, H., Osawa, T., Terao, J., Watanabe, S., Yoshikawa, T. (Eds.), Food factors for cancer prevention. Springer, Tokyo, pp. 55-57.

Nunez, R. (2001). DNA measurement and cell cycle analysis by flow cytometry. *Current Issues in Molecular Biology*. **3**(3):67-70.

Okeleye, B. I., Mkwetshana, N. T. and Ndip, R. N. (2013). Evaluation of the Antibacterial and Antifungal Potential of *Peltophorum africanum*: Toxicological Effect on Human Chang Liver Cell Line," *ScientificWorld Journal*, vol. 2013, no. 878735.*doi*:10.1155/2013/878735, pp. 1-9.

Plummer, M., Herrero, R., Franceschi, S., Meijer, C. J., Snijders, P., Bosch, F. X., de Sanjosé, S. and Muñoz, N. Smoking and cervical cancer: pooled analysis of the IARC multi- centric case--control study (2003). *Cancer Causes Control*. **14**(9):805-14.

Poulson, H. E., Prieme, H., Loft, S., (1998). Role of oxidative DNA damage in cancer initiation and promotion. *European Journal of Cancer Prevention*. **7**(1)9-16.

Salim, L. Z. A., Mohan, S., Othman, R. and Abdelwahab, S. I., Kamalidehghan, B., Sheikh, B. Y. and Ibrahim, M. Y. (2013). Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro. *Molecules*, **18**, 11219-11240; doi:10.3390/molecules180911219.

Sasidharan, S., Chen, Y., Saravanan, D., Sundram, K. M. and Latha, L. Y. (2011). Extraction, isolation and characterization of bioactive compounds from plants' extracts. *The African Journal of Traditional, Complementary and Alternative medicines*. **8**(1):1-10.

Sowemimo, A., van de Venter, M., Baatjies, L. and Koekemoer, T. (2009). Cytotoxic activity of selected Nigerian plants. *The African Journal of Traditional, Complementary and Alternative Medicines*. **6**(4):526-528.

Talib, W. H., Issa, R. A., Kherissat, F. and Mahasneh, A. M. (2013). Jordanian Ducrosia flabellifolia Inhibits Proliferation of Breast Cancer Cells by Inducing Apoptosis. *British Journal of Medicine & Medical Research*. **3**(3):771-783.

Tanih, N. F. and Ndip, R. N. (2013). The Acetone Extract of *Sclerocarya birrea* (Anacardiaceae) *possesses* antiproliferative and apoptotic potential against Human Breast Cancer Cell Lines (MCF7). *The Scientific World Journal. 2013:*956206. *doi:* 10.1155/2013/956206.

Thornberry, N. and Lazebnick, Y. (1998). Caspases: enemies within. *Science*. **281**:1312-1316.

Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bossch, F. X., Kummer, J. A., Shah, K. V., Snijders, P. J., Peto, J., Meijer, C. J. and Muñoz, N. (1999). Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *Journal of Pathology*. **189**:12-19.

Widowati, W., Wijaya, L., Teresa, L. W., Bachtiar, I., Yelliantty, Y., Laksmitawati, D. R. (2013). Antioxidant, anticancer, and apoptosis-inducing effects of Piper extracts in HeLa cells. *Journal of Experimental and Integrative Medicine*. **3**(3):225-230.

Wolf, B. B. and Green, D. R. (1999). Suicidal tendencies: apoptotic cell death by caspase family proteinases. *Journal of Biological Chemistry*. **274**:20049-20052.

Zienolddiny, S., Haugen, A., Lie, J. A. S., Kjuus, H., Anmarkrud, K. H. and Kjærheim, K. (2013). Analysis of polymorphisms in the circadian related genes and breast cancer risk in Norwegian nurses working night shifts. *Breast Cancer Research*. **15**:53.

CHAPTER EIGHT

General discussion, Conclusions and Recommendations

8.1. GENERAL DISCUSSION

Advancement in science has made the isolation and identification of compounds of medical importance from plants that are used in traditional medicinal practices achievable. Plant parts such as leaves, roots, stems, barks and rhizomes are often extracted using different solvents. In most cases, extracts have been shown to be biologically active both in the *in vitro* and *in vivo* test systems while some plant derived compounds are effective in combination or as single entities (Van Wyk and Wink, 2004; Balunas and Kinghorn, 2005). The plants consumed as fruits, teas, wines, vegetables and medicines may contain a large quantity of antioxidant compounds which scavenge free radicals and thought to reduce the risks of degenerative diseases such as, diabetes, cancer and diseases caused by virus, bacteria, molds and parasites (Rice-Evans *et al*., 1996; Sun *et al*., 2002; Cui *et al*., 2005; Pieters and Vlietinck, 2005). Medicinal plants have played important roles in the treatment of cancer. Cancer is a major public health problem in both developed and developing countries and since 1990, there has been a 22 % increase in cancer incidence and mortality with the four most frequent cancers being lung, breast, colorectal, and stomach (Parkin *et al*., 2001; Balunas and Kinghorn, 2005).

Marked potency of the ethyl acetate extract was observed against *Plesiomonas shigelloides* ATCC 51903 showing 24.5 ± 3.5, 26.5 ± 2.1 and 32 ± 2.8 mm zones of inhibition at 25, 50, and 100 mg/mL concentration of the extract, with MIC and MLC values of 0.15625 and 0.3125 mg/mL, respectively. This is more potent compared to the study of Kalirajan *et al*. (2013). *Plesiomonas shigelloides* is a Gram-negative; thermotolerant, motile and pleomorphic

microorganism, weakly related to species of Enterobacteriaceae and Vibrionaceae and shown to cause meningitis, osteomyelitis, sepsis and other non-diarrhoeal diseases (Farmer *et al*., 1992; Hernandez and Rodriguez de Garcia, 1992). Lethal dose at 50 (LD_{50}) showed 82.64 ± 1.40 μg/mL of cytotoxicity after 24 h of treatment with the crude extract. This is similar in activity with the result of Razak *et al*., (2011) that reported EC_{50} less than 100 µg/mL. However both were less in activity compared to fraction TEt10 with EC_{50} of 37 μg/mL observed in the current study. Cytotoxicity screening model provide important preliminary data to help select plant extracts and fractions with potential antitumoral properties for future studies. The ability to measure early indicators of toxicity is an essential part of drug discovery. *In vitro* cytotoxicity assays involving tissue specific cell cultures are considered as valuable predictors of human drug toxicity. As a primary organ for drug metabolism, the liver is often subject to toxic effects, consequently *in vitro* cellular cytotoxicity studies focus on human hepatocytes (Cardellina *et al*., 1999).

Fractions BEtA2 and BEtA4 inhibited 50 % of test organisms (IC_{50}) at 1.0 ± 0.5 and 1.0 ± 0.6 mg/mL, meanwhile 90 % were inhibited (IC_{90}) at 2.5 ± 0.8, 3.1 ± 2.3 mg/mL respectively. At highest concentration of T1 (3.125 mg/mL), 57.8 and 96.2 % inhibition of *C. albicans* and *S. aureus* were observed respectively with significant morphological changes caused by the fractions, such as disintegration, visible perforation of the cell wall (bacterial), deformation of the germ tubes (*C. albicans*) and the formation of apoptotic bodies in HeLa cell by TEt10 fraction. These visible effects of the extract and fractions have been attributed to the reduction or inhibition of the extracellular material that includes some elements that give them their characteristic virulence, hence, enhance their pathogenicity by affecting the permeability and subsequent invasion of hosts' membranes (Figueras and Guarro, 1997; Das *et al*., 2010). For

example, *P. aeruginosa* treated with EAE had a reduction of sodium from 5.55 % - 1.50 %, while for *C. albicans* pottasium was reduced from 4.16 % - 0.76 % by fraction T1.

Compounds indentified in the fractions have been reported to have a number of medicinal benefits. For example palmitic acid or n-hexadecanoic acid, is a saturated fatty acid. Many fatty acids are known to have antinflamatory, antibacterial and antifungal properties, and can modulate immune responses by acting directly on T cells (Agoramoorthy *et al*., 2007). Stigmasterol is used as a precursor in the manufacture of vitamin D_3 and semisynthetic progesterone, a valuable human hormone that plays an important physiological role in the regulatory and tissue rebuilding mechanisms related to estrogen effects, as well as acting as an intermediate in the biosynthesis of androgens, estrogens, and corticoids (Kametani and Furuyama 1987; Batta *et al*., 2006). Campesterol is a precursor of anabolic steroid boldenone, used in veterinary medicine to induce growth in cattle (Draisci *et al*., 2007). Campesterol molecules also compete with cholesterol and thus reduce the absorption of cholesterol in the human intestine (Choudhary and Tran, 2011). Vegetables, fruits, nuts and seeds containing campesterol include; banana, pomegranate, pepper, coffee, grapefruit, cucumber, onion, oat, potato, and lemon grass (Gul and Amar, 2006). Campesterol has anti-inflammatory effects and is mostly used as a treatment for benign prostate hyperplasia (Gabay *et al*., 2010).

Extract scavenging activity of 88.73±6.69 % (25 µg mL^{-1}), 53.93±1.09 % (25 µg mL^{-1}), 87.293±6.64 % (25 µg mL^{-1}), 10.55±2.16 mM (0.42 mM) and 3.8115±0.06 (25 µg mL^{-1}) were recorded for H_2O_2, NO, DPPH, ABTS and Fe^{3+} reducing power respectively which is similar to the results reported by other investigators (Bizimenyera *et al*., 2007; Chauke *et al*., 2012; Shahwar *et al*., 2012). In nature, Antioxidants (AO) are grouped into endogenous and exogenous, with promising therapeutic effects which occur in the cytosol, mitochondria or in plasma

(Berger, 2005). The interest in polyphenolic antioxidants has increased remarkably in the last decade because of their elevated capacity in scavenging free radicals associated with various diseases. Plants antioxidants contribute in the protection of the body from free radical deterioration by retardation of lipid peroxidation which is usually linked with the presence of anthocyanins, phenolic acids, flavonoids, and tannins (Rimm *et al*., 1996; Huang *et al*., 2002).

Significant reduction in cell viability of the cells was noted as the breast cancer (MCF-7) cells were reduced from 100 - 54.33±1.84 % after 72 hrs of treatment with 5 µg/mL of EAE (*P. value* < 0.05). The cytotoxic potential of TEt10 against human normal cells (chang liver cell) was observed at EC_{50} to be 37 µg/mL and 74 µg/mL after treatment for 24 and 48 h respectively with antiproliferative activity of 13.2 µg/mL (EC_{50}) against HeLa cells (48 h). There are 458,000 deaths per year from breast cancer worldwide making it the most common cause of female cancer death in both the developed and developing world (Ferlay *et al*., 2010). Cancer of the uterine cervix is the second most common cancer among women in less-developed countries. Infection with the human papillomavirus (HPV) is the most important cause of cervical cancer, but other factors, including smoking, parity and oral contraceptive use have been reported (Green *et al*., 2003; Appleby *et al*., 2009). Disruption of the circadian rhythm has been hypothesized to increase cancer risk through its impact on the production of the circadian hormone melatonin (Stevens, 2005; Blask *et al*., 2011). The circadian clock is regulated by several proteins, such as aryl hydrocarbon receptor nuclear translocator-likes (ARNTL or BMAL1, ARNTL2 or BMAL2) also known as Mop3, circadian locomotor output cycle kaput (CLOCK), neuronal PAS domain protein 2 (NPAS2), periods (PER1, PER2 and PER3), cryptochromes (CRY1 and CRY2), TIMELESS, ROR-a and ROR-b, that interact with each other in various transcriptional and translational feedback loops (Griffett and Burris, 2013).

Considering the mechanism of action investigated in this study, it could be deduced that the extract (EAE) and fraction TEt10 caused apoptosis of breast cancer and cervical cancer cells respectively. An observation of G2M- phase of 42.83 ± 2.30 % (EAE; MCF-7), Sub-G1 DNA phase increased from 0.13 ± 0.06 % (negative control) to 13.8 ± 3.04 % (TEt10; HeLa cell) of cell cycle analysis and 705.102 ± 28.56 % (TEt10; HeLa cell) caspase-3 activities. Cell-cycle analysis is a method in cell biology that employs flow cytometry to distinguish cells in different phases of the cell cycle. Before analysis, the cells are permeabilised and treated with a fluorescent dye usually propidium iodide (PI) that stains DNA quantitatively (Van Dilla *et al*., 1969). The fluorescence intensity of the stained cells at certain wavelengths therefore correlate with the amount of DNA they contain. The cell-cycle (aka, cell division cycle) consists of a series of processes involved in the growth, replication, and division of cells. The cell-cycle can be divided into two major stages, interphase (a phase between mitotic events) and mitosis. There are three distinct, successive stages within interphase, called G1, S, and G2 phases (Van Dilla *et al*., 1969; Juan *et al*., 1997). Meanwhile caspase-3 is the most widely studied of the effector caspases. Caspase-3 is produced as an inactive 32-kDa proenzyme, which is cleaved at an aspartate residue to yield a 12-kDa and a 17-kDa subunit. Two 12-kDa and two 17-kDa subunits combine to form the active caspase 3 enzyme (Stennicke and Salvesen, 1998). Caspase 3 cleaves a wide range of cellular substrates including structural proteins (e.g., lamins) and DNA repair enzymes [e.g., poly(ADP-ribose) polymerase]. It also activates an endonuclease caspase-activated DNAse, which causes the DNA fragmentation that is characteristic of apoptosis. Several studies have shown that caspase-3 activation is required for apoptosis induction in response to chemotherapeutic drugs (Stennicke and Salvesen, 1998; O'Donovan *et al*., 2003).

8.2. CONCLUSIONS

Based on this study, the following conclusions can be drawn;

1. The ethyl acetate extract (EAE) and fractions were both bactericidal (MICindex ≤ 2) and bacteriostatic/fungistatic (MICindex > 2) in activity with significant ultramorphological alterations.
2. EAE was 50 % cytotoxic at lethal dose (LD_{50}) of 82.64 ± 1.40 μg/mL after 24 hrs of treatment. This cell-based lethality assay is an indication that EAE possesses bactericidal and fungicidal activities, pesticidal effects, and various pharmacologic actions.
3. A high antioxidant activity of 87.293±6.64 % (DPPH), 88.73± 6.69 % (H_2O_2) at 25 µg mL^{-1} and phenolic content of 60.53±1.46 mg/g were observed indicating the likely exploitation of the extract as an easily available source of natural antioxidants.
4. EAE was apoptotic against human breast (MCF-7), colon (HT-29) and cervical (HeLa) cancer cell lines as delineated via antiproliferation, ultrastructural disruption and early internucleosomal DNA fragmentation.
5. Fraction TEt10 was cytotoxic (EC_{50}; 74 µg/mL) and highly antiproliferative against cervical cancer cell (EC_{50}; 13.2 µg/mL) indicating the selectivity of TEt10 to cancer cells when compared to normal cells (Chang Liver cells).
6. Evaluation of caspase-3 activity (705.102 ± 28.56 %) and cell cycle analysis (sub-G1 DNA phase; 0.13 ± 0.06 % to 13.8 ± 3.04 %) of TEt10 against cervical cancer, with remarkable activities showed that it induced apoptosis and could be a potential template for new anticancer treatment.
7. The compounds present in the fractions of the EAE such as Colchicine, N-(trifluoroacetyl)methyl-N-deacetyl-, Lupeol, Gallic acid, [(benzoic acid, 3,4,5-

tris(trimethylsiloxy), trimethylsilyl)], silane,[[(3.beta.,24R)-ergost-5-en-3-yl]oxy]trimethyl, ferulic acid, Hop-22(29)-en-3.beta.-ol and .gamma.-Sitosterol or beta.-Sitosterol may be responsible for the activity observed.

8.3. RECOMMENDATIONS

Based on these findings the following can be recommended;

1. Since the ethyl acetate extract (EAE) was toxic to the human Chang liver cells, this plant extract should be used with caution, and further studies using *in vivo* (animal model) approach should be conducted to confirm this finding.
2. EAE could be exploited as an easily available source of natural antioxidant, as a food supplement or in the pharmaceutical sector. Further work is however recommended to evaluate *in vivo* antioxidant activities of the extract.
3. The present findings also call for advance studies using animal models to investigate potential antitumor leads from TEt10 compounds such as Lupeol and .gamma.-Sitosterol or beta.-Sitosterol in single or combined.

REFERENCES

Agoramoorthy, M., Chandrasekaran, V. and Venkatesalu, M. J. H. (2007). Antibacterial and antifungal activities of fatty acid methyl esters of the blind-your-eye mangrove from India. *Brazilian Journal of Microbiology*. **38**:739-742.

Appleby, P., Beral, V., Berrington, de G. A., Green, J. and Sweetland, S. (2009). Cervical carcinoma and sexual behaviour: collaborative reanalysis of individual data on 15461 women with cervical carcinoma and 29164 women without cervical carcinoma from 21 epidemiological studies. *Cancer Epidemiology, Biomarkers & Prevention*. **18**(4):1060-9.

Balunas, M. J. and Kinghorn, A. D. (2005). Drug discovery from medicinal plants. *Life Sciences*. **78**:431-41.

Batta, A. K., Xu, G., Honda, A., Miyazaki, T. and Salen, G. (2006). Stigmasterol reduces plasma cholesterol levels and inhibits hepatic synthesis and intestinal absorption in the rat. *Metabolism*. **55**(3):292-9.

Berger, M. M. (2005). Can oxidative damage be treated nutritionally? *Clinical Nutrition*. **24**:172-183.

Bizimenyera, E. S., Aderogba, M. A., Eloff, J. N. and Swan, G. E. (2007). Potential of Neuroprotective Antioxidant-Based Therapeutics from *Peltophorum Africanum* Sond. (Fabaceae). *African Journal of Traditional, Complementary and Alternative Medicines*. **4**(1):99-106.

Blask, D. E., Hill, S. M., Dauchy, R. T., Xiang, S., Yuan, L., Duplessis, T., Mao, L., Dauchy, E., Sauer, L. A. (2011). Circadian regulation of molecular, dietary, and metabolic signaling mechanisms of human breast cancer growth by the nocturnal melatonin signal

and the consequences of its disruption by light at night. *Journal of Pineal Research.* **51**:259-269.

Cardellina, J. H., Fuller, R. W., Gamble, W. R., Westergaard, C., Boswell, J., Munro, M. H. G., Currens, M. and Boyd, M.P. (1999). Evolving strategies for the selection dereplication and prioritization of antitumor and HIV inhibitory natural products extracts. (Eds. Bohlin, L. and Bruhn, J.G.) *Bioassay Methods in Natural Product Research and Development, Kluwer Academic Publishers, Dordrecht*. pp 25-36.

Chauke, A. M., Shai, L. J., Mphahlele, P. M. and Mogale, M. A. (2012). Radical scavenging activity of selected medicinal plants from Limpopo Province of South Africa. *African Journal of Traditional, Complementary and Alternative Medicines*. **9**(3):426-430.

Choudhary, S. P. and Tran, L. S. (2011). Phytosterols: Perspectives in human nutrition and clinical therapy. *Current Medicinal Chemistry*. **18** (29): 4557-67.

Cui, Y., Kim, D. S. and Park, K. C. (2005). Antioxidant effect of *Inonotus obliquus*. *Journal of Ethnopharmacology*. **96**:79-85.

Das, K., Tiwari, R. K. S. and Shrivastava, D. K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*. **4**(2):104-111.

Draisci, R., Merlanti, R., Ferretti, G., Fantozzi, L., Ferranti, C., Capolongo, F., Segato, S., Montesissa, C. (2007). Excretion profile of boldenone in urine of veal calves fed two different milk replacers. *Analytica Chimica Acta*. **586** (1-2): 171-176.

Farmer, J. J., Arduino, M. J. and Hickman-Brenner, F. W. (1992). The genera *Aeromonas* and *Plesiomonas*. In *The Prokaryotes. A Handbook on the Biology of*

Bacteria: Ecophysiology, Isolation, Identification, Applications, Vol. **1**, (Balows, A., Truper, H. C., Dworkin, M., Harder, W. & Schleifer, K. H., Eds) pp. 3012-3045.

Ferlay, J., Shin, H. R., Bray, F., Forman, D., Mathers, C. and Parkin, D. M. (2010). Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *The International Journal of Cancer*. **127**:2893-2917.

Figueras, M. J. and Guarro, J. (1997). X-ray microanalysis of black piedra. *Antonie van Leeuwenhoek International Journal of General and Molecular Microbiology*. **72**:275-281.

Gabay, O., Sanchez, C., Salvat, C., Chevy, F., Breton, M., Nourissat, G., Wolf, C., Jacques, C. et al. (2010). Stigmasterol: A phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis and Cartilage*. **18** (1): 106-16.

Green, J., Berrington, de G. A., Sweetland, S., Beral, V., Chilvers, C., Crossley, B., Deacon, J., Hermon, C., Jha, P., Mant, D., Peto, J., Pike, M. and Vessey, M. P. (2003). Risk factors for adenocarcinoma and squamous cell carcinoma of the cervix in women aged 20-44 years: the UK National Case-Control Study of Cervical Cancer. *The British Journal of Cancer*. **89**:2078-2086.

Griffett, K. and Burris, T. P. (2013). The mammalian clock and chronopharmacology. *Bioorganic & Medicinal Chemistry Letters*. **23**:1929-1934.

Gul, M. K. and Amar, S. (2006). Sterols and the phytosterol content in oilseed rape (Brassica napus L.). *Journal of Cell and Molecular Biology*. **5**: 71-9.

Hernandez, P. and Rodriguez de Garcia, R. (1997). Prevalence of *Plesiomonas shigelloides* in surface water. *Archivos Latinoamericanos de Nutricion*. **47**:47-9.

Huang, D., Ou, B., Hampsch-Woodill, M. F, Judith, A., Prior, R. L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry*. **50**:4437-4444.

Juan, G., Traganos, F., James, W., Ray, J., Roberge, M., Sauve, D., Anderson, H. and Darzynkiewicz, Z. (1997). Histone H3 Phosphorylation and Expression of Cyclins A and B1 measured in individual cells during their progression through G2 and Mitosis. *Cytometry*. **32**:71.

Kalirajan, A., Narayanan, K. R., Ranjitsingh, A. J. A., Ramalakshmi, C. and Parvathiraj, P. (2013). Bioprospecting medicinal plant *Aerva lanata* Juss. Ex Schult. Flowers for potential antimicrobial activity against clinical and fish-borne pathogens. *Indian Journal of Natural Products and Resources*. **4**(3):306-311.

Kametani, T. and Furuyama, H. (1987). Synthesis of vitamin D3 and related compounds. *Medicinal Research Reviews*. **7** (2): 147-171.

O'Donovan, N., Crown, J., Stunell, H., Hill, A. D. K., McDermott, E., O'Higgins, N and Duffy, M. J. (2003). Caspase-3 in Breast Cancer. *Clinical Cancer Research*. **9**:738-742.

Parkin, D. M., Bray, F., Ferlay, J. and Pisani, P. (2001). Estimating the world cancer burden-Globocan2000. *International Journal of Cancer*. **94**:153-6.

Pieters, L. and Vlietinck, A. J. (2005). Bioguided isolation of pharmacologically active plant components, still a valuable strategy for the finding of new lead compounds. *Journal of Ethnopharmacology*. **100**:57-60.

Razak, F. A., Majid, Z. M., Rahman, M. A. and Zuraiza, M. (2011). Cytotoxic activity of selected plants extracts on normal and cancerous oral mucosal cells. *Planta Medica*. 77 - PM206. DOI: 10.1055/s-0031-1282964.

Rice-Evans, C. A., Miller, N. J., Bolwell, P. G., Bramley, P. M. and Pridham, J. B. (1995). The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research*. **22**:375-383.

Rimm, E. B., Ascherio, A., Grovannucci, E., Spielgelman, D., Stampfer, M. J. and Willet, W. C. (1996). Vegetable, fruits, and cereal fiber intake and risk of coronary heart disease among men. *Journal of the American Medical Association*. **275**:447-451.

Shahwar, D. and Raza, M. A. (2012). Antioxidant potential of phenolic extracts of *Mimusops elengi*. *Asian Pacific Journal of Tropical Biomedicine*. **2**(7):547-550.

Stennicke, H. R. and Salvesen, G. S. (1998). Properties of the caspases. *Biochimica et Biophysica Acta*. **1387**:17-31.

Stevens, R. G. (2005). Circadian disruption and breast cancer: from melatonin to clock genes. *Epidemiology*. **16**:254-258.

Sun, J., Chu, Y. F., Wu, X. and Liu, R. H. (2002). Antioxidant and antiproliferative activities of common fruits. *Journal of Agricultural and Food Chemistry*. **50**:7449-7454.

Van Dilla, M. A., Trujillo T. T., Mullaney, P. F. and Coulter, J. R (1969). Cell Microfluorometry: A Method for Rapid Fluorescence Measurement. *Science*. **163**(3872):1213-1214.

Van Wyk, B. and Wink, M. (2004). Medicinal Plants of the World. Briza Publications.

APPENDICES

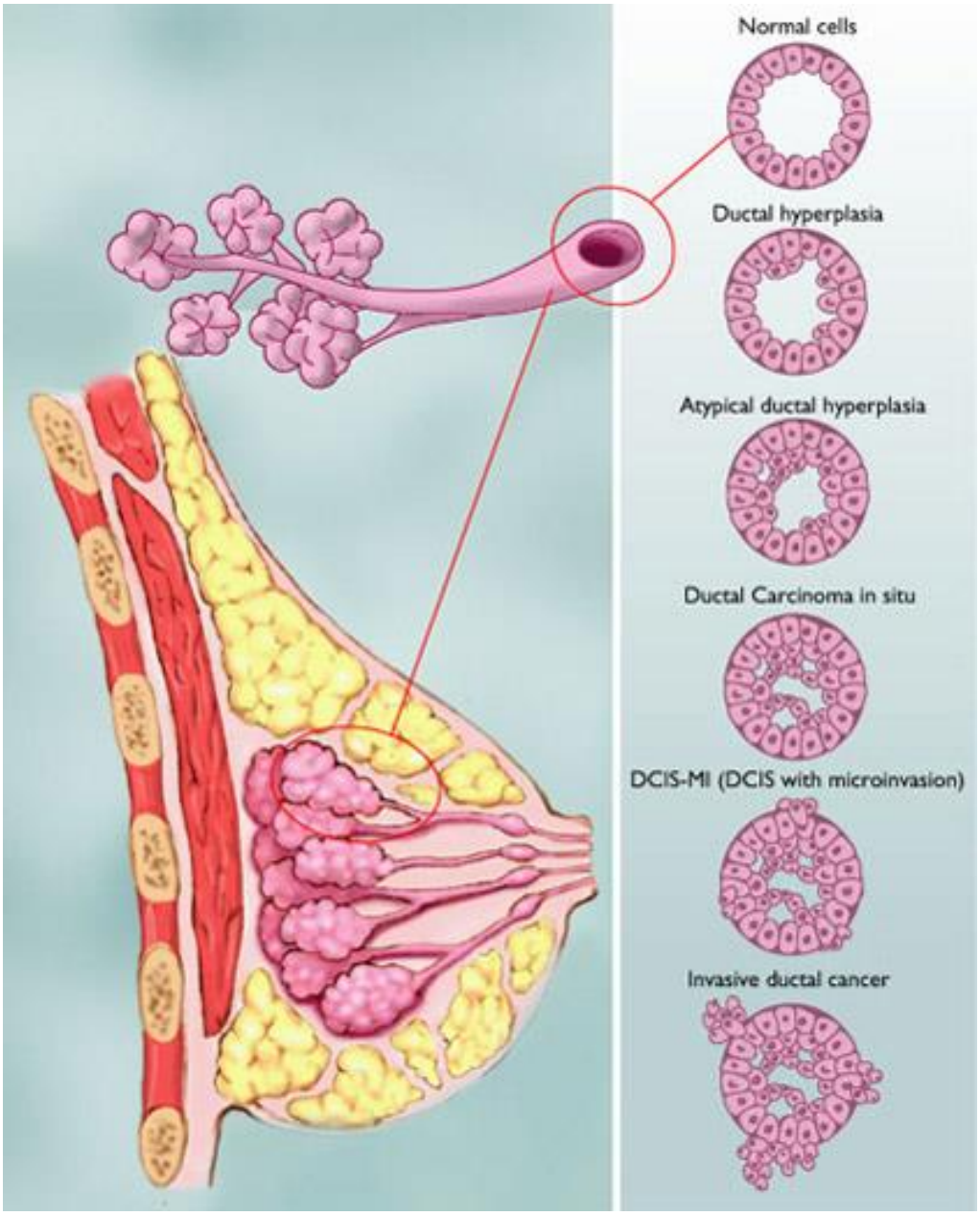
APPENDIX 1:

PLANT USED IN THE STUDY

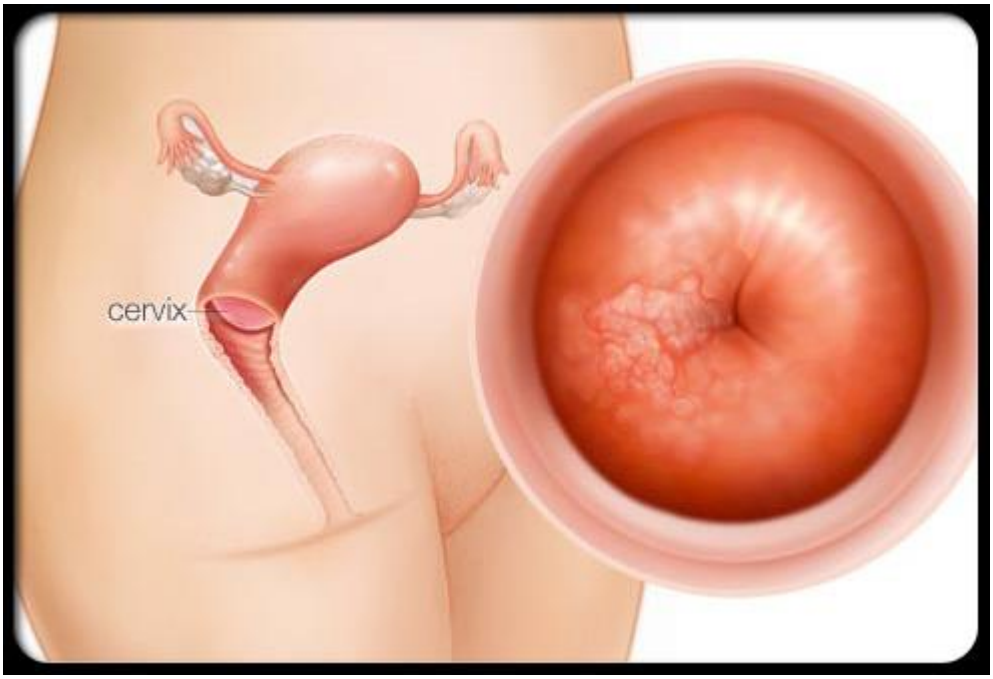
Stem bark, leaves and flowers of *Peltophorum africanum*

APPENDIX 2:

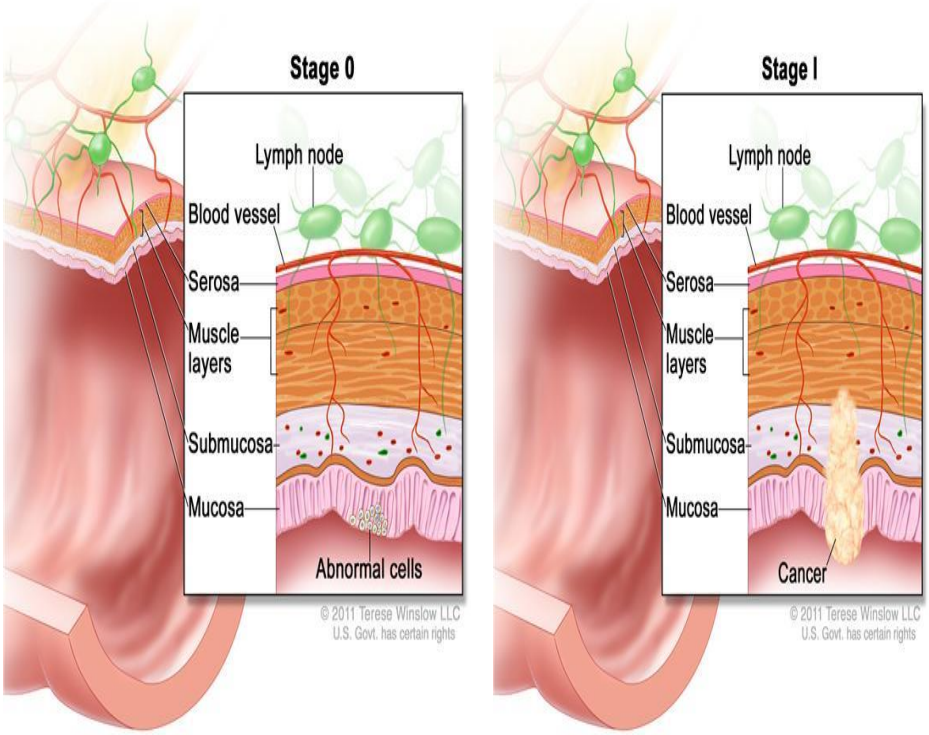
CANCER DEVELOPMENT



Breast Cancer



Cervical Cancer



Colon Cancer

APPENDIX 3:

PUBLICATIONS, CONFERENCE PRESENTATION, MANUSCRIPTS UNDER REVIEW & IN PREPARATTION

LIST OF PUBLICATION

1. **Okeleye, B. I.,** Mkwetshana, N. T. and Ndip, R. N. (2013). Evaluation of the Antibacterial and Antifungal Potential of *Peltophorum africanum*: Toxicological Effect on Human Chang Liver Cell Line," *Scientific World Journal*, vol. 2013, no. 878735.*doi*:10.1155/2013/878735, pp. 1-9.

CONFERENCE PRESENTATION

1. **Okeleye, B. I.,** Mkwetshana, N. T. and Ndip, R. N. (2013). Phytochemical analysis, polyphenolic content and *in vitro* antioxidant evaluation of the stem bark extract of *Peltophorum africanum* (Sond, Fabaceae). 5th WSU International Research Conference. Conference theme: "Implementation science in green economy." International Convention Centre, East London, Eastern Cape, South Africa. 22-24 August 2012.

MANUSCRIPTS UNDER REVIEW

1. **Okeleye, B. I.,** Nongogo V., Mkwetshana, N. T. and Ndip, R. N. (2013). Polyphenolic content and *in vitro* antioxidant evaluation of the stem bark extract of *Peltophorum africanum* (Sond, Fabaceae). *Indian Journal of Pharmaceutical Sciences*.
2. **Okeleye, B. I.,** Mkwetshana, N. T. and Ndip, R. N. (2013). *In-vitro* assessment of the antiproliferative and apoptotic potential of the ethyl acetate extract of *Peltophorum africanum* on different cancer cell lines: ultrastructural characterization of treated cells. *Scientific World Journal*.

MANUSCRIPTS IN PREPARATION

1. Evaluation of compounds and heavy metals in the ethyl acetate extract of *Peltophorum africanum*: Morphological effects on pathogenic bacteria and yeast cell.

2. *In-vitro* evaluation of the anticancer potential of TEt10 against human cervical cancer cell (HeLa cell): implication of caspase-3 and sub-G1 DNA cell cycle analysis.