KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY,

KUMASI

ANTIPROLIFERATIVE ACTIVITY OF AQUEOUS LEAF EXTRACT OF *ANNONA MURICATA* (LINN.) ON RAT PROSTATE, *BPH-1* CELLS AND SOME TARGET GENES.

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

(CHEMICAL PATHOLGY)

In the

Department of Molecular Medicine,

School of Medical Sciences

By

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DECLARATION

I, Derek Amartey Doku, do hereby declare that, with the exception of the works that have been duly acknowledged and cited as belonging to others, this study is my original research and has not been presented for another degree elsewhere, either in part or as a whole. The study for this thesis was carried out at the department of Molecular Medicine, KNUST.

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ABSTRACT

Annona muricata (Linn.) is an evergreen tropical tree of the Annonaceae family that posseses phytotherapeutic bioactive compounds known as annonaceous acetogenins effective for the treatment of cancers and other conditions. This study aimed at investigating the effect of the aqueous leaf extract of *A. muricata* on human BPH-1 cells, the prostate organ as well as certain target cellular genes of proliferative activity. Dried *A. muricata* leaves were pulverized, and the aqueous crude extract obtained. HPLC was used for monitoring various batches of the *A. muricata* leaf extract (AMLE). The MTT assay was performed on BPH-1 cells (1 x 10^5 per well) using AMLE concentrations of 0.5, 1.0 and 1.5 mg/mL for 24, 48 and 72 hours. Microscopic examination of proliferation as well as morphology of the cells was carried out. RT-PCR was used to examine possible target genes, Bax and Bcl-2, using mRNA extracted from cells. Fifteen (15) F344 male rats (150-200 g) were placed in three groups of five (5). The low dose (LD) and high dose (HD) groups were gavaged 30 mg/mL and 300 mg/mL respectively and fed *ad libitum* alongside five (5) control (ctrl) group rats. Rats were sacrificed after 60 days. Whole blood was sampled by cardiac puncture and processed for biochemical assay. Prostate, seminal vesicles and testes were harvested, weighed and stored for histological examination. HPLC chromatographic fingerprint monitoring of different batches of AMLE yielded eight peaks. *In vitro* cell viability analysis showed a dose dependent growth inhibitory effect of AMLE on BPH-1

cells. The IC_{50} obtained for AMLE was 1.36 mg/ml. Increasing doses of AMLE directly up-regulated the levels of the proapototic protein Bax, whiles down-regulating the levels of the anti-apoptotic protein Bcl-2. *In vivo* studies showed statistically significant seminal vesicle indices recorded for both (LD) and (HD) groups (p=0.004 and p=0.009 respectively) compared with control (ctrl) group. There was no statistically significant difference between PSA levels of test groups compared with control groups. Histological examinations of the prostatic and seminal vesicle tissues however showed dose dependent morphological changes with AMLE treatment. The acinii were empty of secretions and there was marked atrophy with increased cellularity observed. AMLE exerts decreased secretory activity on prostate with flattening of acinar epithelial linings being demonstrated. The prostate epithelial and stromal cells were flattened with scanty prostatic secretions in the lumen. Thus, AMLE has antiproliferative activity against BPH1 cells.

DEDICATION

This research is dedicated to my entire family, especially Florence Kumadoe and to the memory of Victor Doku.

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I am very grateful to Jesus Christ for giving me the breath, strength and ability to pursue this Master of Science programme. I am also immensely thankful to my supervisors Dr. George Asare and Dr. Robert Ngala for their thorough work of supervision, guidance, encouragement and enormous contributions towards this work. I also wish to thank Mr. Daniel Afriyie of Accra Police Hospital, Dr. Samuel Adjei and the staff of the Animal Experimentation Unit of NMIMR, Prof. Rahman, Dr. Mahmood and Janet Ocloo of the Department of Pathology, UG. I am thankful to all the members of faculty of the Department of Molecular Medicine, as well as my colleagues. I am also appreciative of all my workcolleagues from the School of Biomedical and Allied Health Sciences, UG.

I am also highly thankful to my family for all the prayer, guidance and support.

May the Lord Jesus Christ bless you all richly.

LIST OF ABBREVIATIONS

3D-PMB		Three-dimensional prostate mapping biopsy
ACE		Acetonitrile
ADT	-	Androgen deprivation therapy
ALT	-	Alanine Aminotransferase
AM	-	*Annona muricata*
AMACR	-	α-methylacyl-CoA racemase
AMLE	-	*Annona muricata* leaf extract
AP-1	-	Activator Protein-1
Apaf-1	-	Apoptotic protease activating factor – 1
AR	-	Analytical reagent
ASAP	-	atypical small acinar proliferation
AST	-	Aspartate aminotransferase
ATP	-	Adenosine triphosphate

AUA	-	American Urological Association
AUR	-	Acute urinary retention
Bad		Bcl-2-associated death promoter
Bak		BCL2-Antagonist/Killer 1
Bax		BCL2-Associated X Protein
Bcl-2		B-Cell Leukemia-2
Bcl-xL	-	B-cell lymphoma-extra large
BPE	-	Benign prostatic enlargement
BPH	-	Benign prostatic hyperplasia
BPH-1	-	Benign prostatic hyperplasia epithelial cell line
CAB	-	Complete androgen blockade
CAM	-	Complementary and alternative medicine
CAT	-	Catalase
cDNA	-	Complementary deoxyribonucleic acid

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COMBAT	-	Combination of Avodart and Tamsulosin
CZ	-	Central zone
DHT	-	Dihydrotestosterone
DMSO	-	Dimethyl sulfoxide
DRE	-	Digital rectal examination
EBRT		External beam radiotherapy
EGCG		Epigallocatechin-3-gallate
EGF		Epithelial growth factor
EGFR		Epithelial growth factor receptor
ELISA	-	Enzyme-linked immunosorbent assay FBS
	-	Fetal bovine serum
fPSA	-	Free prostate specific antigen
FSH	-	Follicle stimulating hormone
GAPDH	-	Glyceraldehyde-3-phosphate dehydrogenase
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H&E - Hematoxylin and eosin

HDR - High-dose rate

HeLa - Henrietta Lacks (human immortal cell line)

HIFU - High intensity frequency ultrasound

hK2 - Human glandular kallikrein 2

HL-60 - Human promyelocytic leukemia cells

HoLEP - holmium laser enucleation of the prostate

HPLC - High performance liquid chromatographic

HRP Horseradish peroxidase

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IGF-1		insulin-like growth factor 1
IGRT		Intensity guided radiation therapy
IMRT		Intensity modulated radiation therapy
IPSS	-	International Prostate System Score
KGF	-	Keratinocyte growth factor
LDR	-	Low-dose rate
LH	-	Luteinizing hormone
LHRH	-	Luteinizing hormone releasing hormone
LRP	-	Laparoscopic radical prostatectomy
LUTS	-	Lower urinary tracts symptoms
MAC	-	mitochondrial apoptosis-induced channel
MDA	-	Malondialdehyde
MDR	-	Multi-drug resistant

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MRI - Magnetic resonance imaging

MTOPs - Medical Therapy of Prostatic Symptoms

MTT - 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide

NAD(P)H - Reduced Nicotinamide adenine dinucleotide phosphate
NADH Reduced Nicotinamide adenine dinucleotide

NHT Neo-adjunctive hormonal therapy

OECD Organisation for Economic Co-operation and Development

PACA-2 - Human pancreatic carcinoma

PBRT - Proton beam radiation therapy

PC-3 - Human prostate cancer

PCa - Prostate cancer

PCA-3 - prostate cancer antigen 3

PI - Prostatic index

PIN - prostatic intraepithelial neoplasia

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PREDICT	-	Prospective European Doxazosin and Combination Therapy
PSA	-	Prostate specific antigen
PSMA	-	Prostate-specific membrane antigen
PUG	-	Posterior urethral glands
PUMA	-	p53-Upregulated Modulator of Apoptosis
PVR	-	post-void residual volume
PZ	-	Perpiheral zone
RALP		Robot-assisted laparoscopic prostatectomy
RP		Radical prostatectomy
RPMI		Roswell Park Memorial Institute
RPP	-	Radical perineal prostatectomy
RRP	-	Radical retropubic prostatectomy
RT-PCR	-	Real Time Polymerase Chain Reaction
SBRT	-	Stereotactic body radiation therapy

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SNPs - Single nucleotide polymorphisms

SOD - Superoxide dismutase

SV - Seminal vesicle

SVI - Seminal vesicle index TGF-β1 -

Transforming growth factor beta 1

tPSA - Total prostate specific antigen TRUS

- Transrectal ultrasonography

TUIP - Transurethral incision of the prostate

TUMT - Transurethral microwave thermotherapy

TUNA - Transurethral needle ablation

	-	
TUNEL		Terminal deoxynucleotidyl transferase dUTP nick end labeling
TURP	-	transurethral resection of the prostate
TZ	-	Transitional zone
VEGF	-	Vascular endothelial growth factor

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CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Prostate cancer (PCa) is a disease of the prostate in which the cells differentiate, become malignant and rapidly proliferate to form a tumor mass. PCa comes second only to lung cancer with respect to male cancer deaths. It however has the highest incidence among all male cancers. PCa accounted for approximately 29% of newly reported cases of cancer and 9% of all reported cancer deaths among men in the USA in 2012 (American Cancer Society, 2012). Furthermore, it was reported as the most frequently occuring male cancer in the UK making up to a quarter of all newly reported male cancer cases in 2012 (Cancer Statistics Registration, 2012). It accounts for approximately 30% of cancers diagnosed each year (Australian Institute of Health and Welfare Cancer Incidence Projections, 2012) in Australian men. This makes it follow lung cancer closely as the second highest cause of male cancer deaths (Smith, 2012). In Ghana, it is reported that prostate cancer is responsible for 17.35% of all male cancer deaths making it second only to liver cancer (Wiredu and Armah, 2006).

The treatment of this common condition has however been the major point of controversy. Conventional treatment regimens have produced adverse effects (Steineck *et al.*, 2002). Apart from being expensive, causing urinary and erectile problems, some of the adverse effects of conventional therapy include toxicity and growth inhibition to normal cells, (Singh *et al.*, 2006). It is reported also that a lot of people have prostate cancer without they and their doctors knowing initially because

it does not present with any deleterious symptoms, thus interventional therapy is unneeded (Johansson *et al.*, 2004; Albertsen *et al.*, 2005). Therefore, there is increasing advocacy for what is termed "watchful waiting" (Johansson *et al.*, 2004; Andren *et al.*, 2006), or the even newer alternative called "active surveillance" in early cases of prostate cancer. Radical prostatectomy, radiation therapy, cryotherapy and high intensity frequency ultrasound are all employed in treating localized prostate cancer. Hormonal therapy and chemotherapy are the main remedies in advanced cancer treatment regimen.

Benign prostatic hyperplasia (BPH), also known as nodular hyperplasia of the prostate, is an abnormally increased growth in volume of the prostate gland causing urethral compression by the proliferating cells that are found in the periurethral area of the prostate gland. The prevalence of BPH is very high among older men. As high as 90% incidence is reported for men between the age range of 80 to 90 (Nickel, 2006). As many as 4.5 million visits to the clinic in the United States in 2000 were related to BPH (Wei *et al.*, 2005). Among elderly Ghanaian men (aged 50 – 74 yrs), the prevalence of DRE-detected enlarged prostate was reported to be 62.3%, and that of PSA≥1.5 ng/ml was 35.3% (Chokkalingam *et al.*, 2012).

Open surgery and procedures such as the transurethral needle ablation (TUNA) and transurethral microwave thermotherapy (TUMT) which are less severely invasive are among the major means of treatment.

Chemotherapeutic agents used in the treatment of BPH are mainly alpha blockers and 5 α-reductase inhibitors. There is an ever increasing shift towards patronizing complementary and alternative medicine (CAM) all

over the world for a wide number of ailments. And the use of medicinal plants is one of the major access avenues of CAM for various ailments.

Annona muricata (Linn.), referred to commonly in English as soursop or "Apre" in Akan, is a member of the family Annonaceae. It is an evergreen tropical fruit tree that grows to about 5-6 metres in height and produces a green edible heart-shaped fruit of about 15-20 cm in diameter with a white flesh and dark seeds inside. The leaves, stem, bark, root and seeds of *A. muricata* possesses several of a bioactive group of substances known as annonaceous acetogenins. A number of these acetogenins have been isolated and their biological activities have

been well documented. The monotetrahydrofuran annonaceous acetogenins, cis-corossolone, annocatalin, annonacin, annonacinone, solamin, and corossolone have been isolated from *A. muricata* leaves. Acetogenins **1** (annoreticuin-9-one) and **2** (*cis*-annoreticuin) isolated initially from other species, *A. reticulata* and *A. Montana* respectively, have both been reported to show significant cytotoxic activity *in vitro* against two human hepatoma cell lines namely Hep G2 and 2,2,15. Acetogenin **1** targets the human pancreatic tumor cell line (PACA-2), human prostate adenocarcinoma (PC-3) (Kim *et al.*, 1998; Woo *et al.* 1999; Ragasa *et al.*, 2012) and human lung carcinoma (A-549) (Zhao *et al.*, 1993; Ragasa *et al.*, 2012). The dichloromethane extract of the seeds of *A. muricata* yielded annoreticuin-9-one (**1**), while the flesh of the fruit yielded *cis*-annoreticuin (**2**) (Ragasa *et al.*, 2012). Kim *et al.* (1998) demonstrated the presence of the Annonaceous acetogenins – muricoreacin as well as murihexocin C (mono-tetrahydrofurans) in *A. muricata,* particularly in the leaves showed significant cytotoxic activities

that targets PCa cell line PC-3, and pancreatic carcinoma cell line PACA-2. *A. muricata* (Linn.) leaf extracts of ethyl acetate showed a higher death rate to HeLa cells than the ethanol and distilled water extracts. The chloroform extract also showed a higher death rate in HeLa cells than ethyl acetate extract. The chloroform extracts seems to have a superior preference for cancer causing viruses (Astirin *et al.*, 2013). The extract was moderately cytotoxic to normal cells (WRL-68 normal human hepatic cells), compared to cancerous cells of human breast carcinoma, (MDA-MB-435S) as well as human immortalized keratinocyte cells (HaCaT). This may be due to the presence of antineoplastic substances obtained in therapeutically active amounts from n-butanolic leaf extract of *A. muricata* (George *et al.*, 2012). The aqueous leaf extract is said to contain general glycosides, condensed tannins, saponins and flavonoids, and did not show any toxicity on systemic organs in an acute toxicity study (LD_{50}<5000 mg/kg b.wt) (Arthur *et al.*, 2011). Phenol, flavonoid and flavonol levels contained in the extracts of *A. muricata* are reported differ in different parts of the plant (Pieme *et al.*, 2014).

However, the use of an aqueous infusion of about 140 µg/cup was said to have caused neurotoxicity related to atypical parkinsonism in Guadeloupe (Champy *et al.*, 2005). The *A. muricata* ethanolic leaf extract has been shown to have hypoglycaemic and antidiabetic effects (Gupta *et al.*, 2005), as well as a protective effect on the lipid profile (Adewole and Ojewole, 2009).

Apoptosis is also called programmed cell death and describes the normal and controlled growth phemenon of generally seen in eukaryotic cells

which involves cellular death. It is a genetically controlled process that may produce neoplasm in the event of an alteration or defect in the process (Berges *et al.*, 1995). Among the several proteins involved in the cellular apoptotic pathway, the Bcl-2 family of proteins have emerged as vital regulators involved in the intrinsic pathway (mitochondriamediated apoptosis). The proteins of this group function either as apoptosis-promoting proteins such as Bax and Bak, or as apoptosisinhibiting proteins such as Bcl-2 and Bcl-xL (Isaacs and Coffey, 1989; Chao and Korsmeyer, 1998).

Studies have shown an excessive proliferation of the stromal (37-fold) and epithelial (9- fold) cells in some BPH cases (Claus *et al.*, 1993). The likely imbalance of molecular mechanisms of proliferation and apoptosis underpins the development of BPH and related cancers. Too little apoptosis in malignant cells is a major culprit for the development of cancers (Wong, 2011). It is crucial that research is geared at evaluating a cell-population's response to chemotherapeutic agents and growth factors in terms of cell viability, proliferation and apoptosis in conducting BPH and related cancer drug development studies (Kyprianou *et al.*, 1998; Duan *et al.*, 2012). Due to the widely held perception that phytotherapeutic agents are more cost-effective, safer and have fewer side effects compared with conventional therapy for the management of ailments including BPH and its related cancers, there is a growing interest in their development (Wilt *et al.*, 1998; Thompson, 2003).

1.2 PROBLEM STATEMENT

BPH affects more than 42% of men from age 51 to 60, 70% of men from

61-70 and as many as 90% of men in their 80s and above (Nickel, 2006). Prostate enlargement often results in lower urinary tract symptoms (LUTS) and this adversely affects the quality of life of such patients (Gharaee-Kermani and Macoska, 2013).

There is as yet no medicinal cure for BPH and the related cancers. Management of BPH has been mainly to provide relief-treatment for the symptoms of the condition. PCa treatment is of great concern because there is no more than a 15-year survival window predicted for it (Johansson, 2004) with current treatment options. However, recurrence of the condition once treated is very common.

Current conventional treatment regimens for both BPH and its related cancer have produced very adverse effects (Steineck *et al.*, 2002). Apart from being expensive, causing urinary incontinence and erectile dysfunction, some of the adverse effects of conventional therapy include toxicity and growth inhibition to normal cells, (Singh *et al.*, 2003). Indigenous African medicines are reported to be in very wide use in spite of the apparent lack of scientific evidence to back their quality, efficacy and safety (Muhammad and Awaisu, 2008). This lack of scientific data to back the anecdotal evidence poses a limitation to the use of phytochemicals even though they may be beneficial.

1.3 JUSTIFICATION

Medicinal plant therapy affords its users a relatively cheaper alternative to orthodox medicine. Herbal medicines are apparently considered relatively safer compared with conventional therapy (Gurib-Fakim, 2006). There is a surge in the interest and use of plant medicine. The world over,

an estimated 75-80% of the people access medicinal plants as the foremost treatment option (Monteagudo *et al.*, 2006). The increase in demand for herbal medicine is fostered by the increase in the attention it is being given (Crook, 2006). With more people shying away from conventional medicinal therapy for the treatment of conditions bordering on sexual function and fertility, as well as cancers, medicinal plant therapy proves to be a very indispensable resort. Currently, there is widespread interest in developing phytotherapeutic agents for the management of BPH and its cancers due to the perception that, they are safer, more cost-effective and have fewer side effects than their conventional alternatives (Wilt *et al.*, 1998; Thompson, 2003). Therapeutic agents that can influence apoptosis have emerged as potential targets for optimizing treatment of BPH and its related cancers. The likely imbalance of molecular mechanisms of proliferation and apoptosis underpins the development of BPH and related cancers (Wong, 2011). However, studies have shown an excessive proliferation of the stromal (37-fold) and epithelial (9- fold) cells in some BPH cases (Claus *et al.*, 1993). Therefore, studies related to cell viability and proliferations are vital for evaluating a cell-population's response to chemotherapeutic agents in BPH drug development studies (Kyprianou *et al.*, 1998; Duan *et al.*, 2012).

Acetogenins, the bioactive chemical found in *A. muricata* have been reported by some researchers as being only moderately cytotoxic to normal cells compared to cancerous cells (George *et al.*, 2012), and by others, as being toxic to cancer cells and non-toxic to non-cancerous cells (Rieser *et al.*, 1991; Zeng *et al.*, 1996; Ragasa *et al.*, 2012). However,

there is a dearth of information concerning the usefulness of acetogenic plants for the treatment of BPH. There is hardly any literature on studies employing acetogenins in BPH cell lines. Although acetogenins have been shown to have cytotoxic activity against the prostate PC-3 cell line (Kim *et al.*, 1998), there is no information on an *in-vivo* study with regards to its effect on the prostate.

In the development of anti-proliferative or anti-tumor drugs, including antineoplastic drugs, exploiting biological agents that address the issue of making a distinction between granting greater specificity for abnormally proliferating cells while doing no or minimal harm to healthy cells is still the ultimate goal (Adams, 2001).

1.4 AIM

The aim of the study was to investigate the effect of AMLE on the prostate hyperplastic cells (*BPH-1*), the prostate organ as well as target cellular apoptotic genes.

1.4.1 Objectives

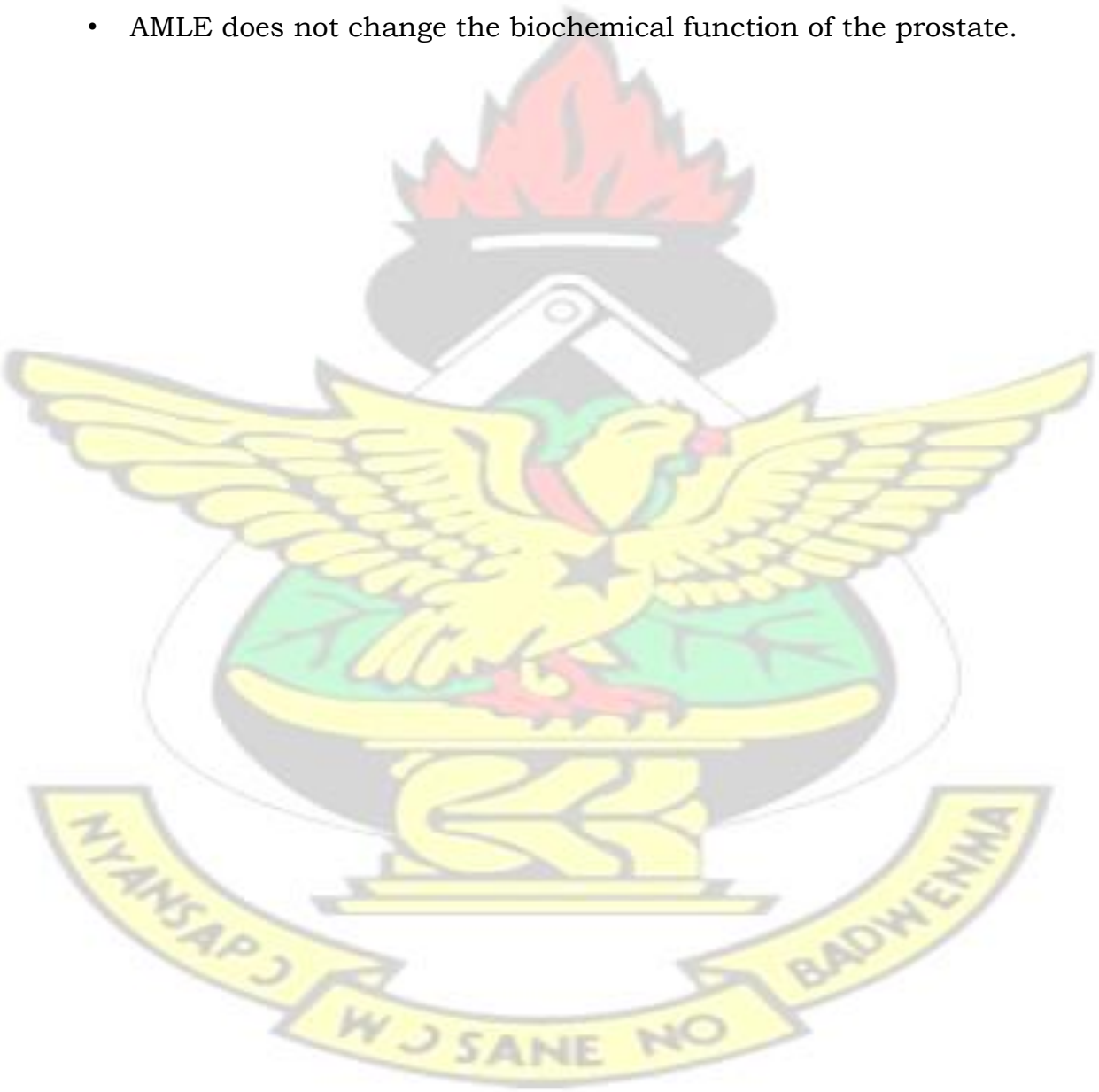
1. To establish the fingerprint guideline of *Annona muricata* leaf extract (AMLE).
2. To determine the antiproliferative-impact of AMLE on the growth of BPH-1 cells.
3. To investigate the effect of AMLE on the expression of apoptotic and anti-apoptotic regulatory genes.
4. To evaluate the effect of AMLE on biochemical function in prostate.

5. To determine the effect of AMLE on prostate and accessory organs.

1.4.2 Hypotheses

1.4.2.1 Null Hypothesis

- AMLE does not inhibit abnormal proliferative activity in BPH tissue via apoptosis.
- AMLE does not inhibit prostatic tissue growth *in vivo*.
- AMLE does not change the biochemical function of the prostate.



CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. THE PROSTATE AND OTHER ACCESSORY GLANDS OF THE URINO-REPRODUCTIVE SYSTEM.

2.1.1 The Prostate

The prostate is an androgen regulated exocrine gland of about 3 cm long and 4 cm across that is sited at the neck of the bladder exiting it as it conjoins the ductus deferens at the ejaculatory duct. It surrounds the beginning of the urethra and is anterior to the rectum.

It is made of mostly exocrine glandular tissue and fibromuscular tissue. The fibromuscular tissue is a made up of two kinds of tissue namely smooth muscle tissue and dense irregular connective tissue. The connective tissue has numerous collagen fibers covering the outermost layer of the prostate and the urethra.

The prostate is generally partitioned in two different ways namely as zones and as lobes (thevisualmd, 2014).

The Peripheral Zone (PZ) is the lower capsular area of the posterior segment of the prostate gland. This encompasses the distal urethra and it is also the location of origin for approximately 70–80% of all PCas. The Central Zone (CZ) is the zone that surrounds the ejaculatory ducts and accounts for about 2.5% of prostate cancers. The Transition Zone (TZ) is the site of origin for approximately 10–20% of prostate cancers. It encompasses the proximal urethra and it is the area of the prostate gland that keeps growing for the entirety of a man's life. It also represents the

main region for the occurrence of benign prostatic enlargement (BPE) occurs (PCTG, 2010). Anterior fibro-muscular zone (or stromal) is made up of fibrous and muscular tissue only and not comprised of glandula tissue.

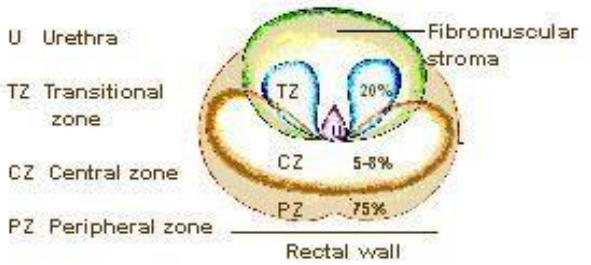


Figure 2.1: Partitioning of prostate gland showing the four zones and the proportion of prostate comprised per zone.

Source: training.seer.cancer

The lateral lobes form the largest segment of the lobes. They are a pair of rounded lobes situated at the anterior end of the prostate and meet at the midline. It spans the entirety of the prostate zones. The anterior lobes (isthmus) are a much smaller-sized triangle made up of fibromuscular tissue and located anterior to the urethra. They contract to expel semen during ejaculation. The lateral, the median, posterior lobes virtually corresponds with TZ, CZ and PZ respectively (thevisualmd, 2014).

The prostate produces above 30% of the non-cellular constituent of semen that provides the prime conditions necessary for the survival and motility of sperm within the reproductive organ of females (Gat *et al.*, 2008). It secretes a milky fluid that contains simple sugars (fructose and glucose) that nourishes sperms; produces proteins such as PSA, prostatic

acid phosphatase, β-microseminoprotein, proteolytic enzymes, and also produces alkaline chemicals that neutralizes acidic vaginal secretions to ensure sperm survival in the female body. It has high zinc levels and contains appreciable levels of sodium, potassium and calcium (Quizlet, 2015).

Dysfunction of the prostate is commonly associated with dysfunction in surrounding organs such as the seminal vesicles and the testes because of the close interplay of their biochemical functions. The seminal vesicles secrete the seminal fluid. This carries the sperm through the two ejaculatory ducts narrowing and converging in the center of the prostate where it merges with the urethra. Normal function of the prostate is also reliant on mainly testicular androgens.

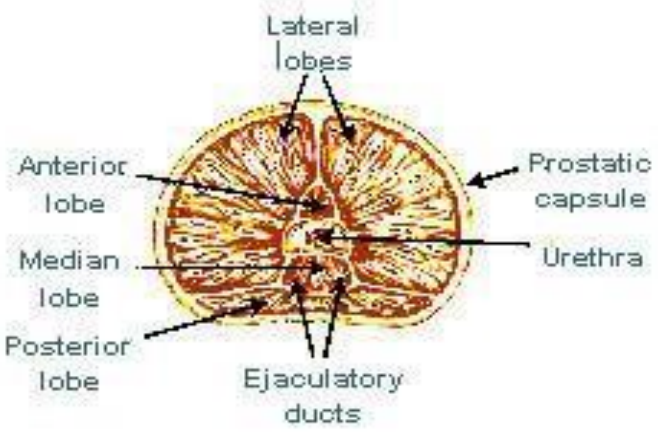


Figure 2.2: Partitioning of prostate gland showing the four lobes and the prostate-area span of lobes.

Source: training.seer.cancer

2.1.2 Seminal Vesicles

The seminal vesicles (SV) are a pair of folded and coiled tubular glands that are postero-inferior to the bladder and lateral to the ductus deferens.

The SV has a mucosa that is made up of a lining of interspersed columnar cells and a laminar propria; and a heavy muscular wall. The secretions of the vesicular glands are stored in lumen (Young, 2006). The SV epithelia are composed of mainly stratified columnar cells whose height and activity is reliant on plasma testosterone.

The dense secretions produced by the SV is an alkaline fluid that contain proteins, enzymes, fructose, mucus, prostaglandins, phosphorylcholine, vitamin C and flavins (Coward and Wells, 2013). SV possesses a high 5-alpha reductase activity. This is the enzyme catalyzes the formation of dihydrotestosterone (DHT) from its precursor androgen, testosterone. DHT is an essential androgen for the development of the prostate. The size and activity of the SVs are dependent on these androgens.

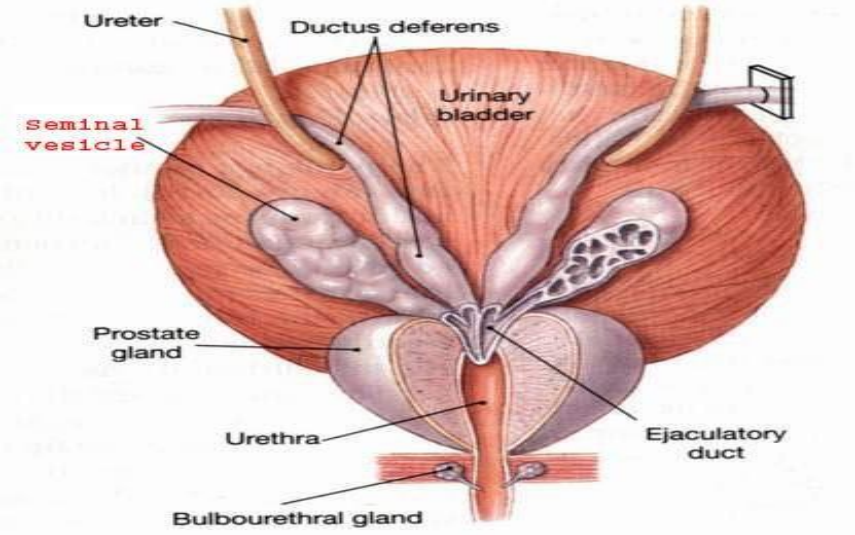


Figure 2.3: Seminal vesicle showing position relative to prostate gland.

Source: studyblue.com

2.1.3 The Testes

The testes are a pair of oval-shaped organs contained in scrotal sacs located directly behind the penis and in front of the anus. They produce the sperm and androgens.

The testis spreads into lobules that contain tightly coiled tubings known as the seminiferous tubules. These seminiferous tubules are the storehouses of the germ cells, Leydig cells as well as the the Sertoli cells. The germ cells differentiate progressively into the spermatocytes. The Leydig cells are the producers of the testosterone whiles the Sertoli cells support and protect the spermatocytes (Walker and Cheng, 2005).

Via the vas deferens, the testes carry sperms through the spermatic cord toward the prostate and the urethra.

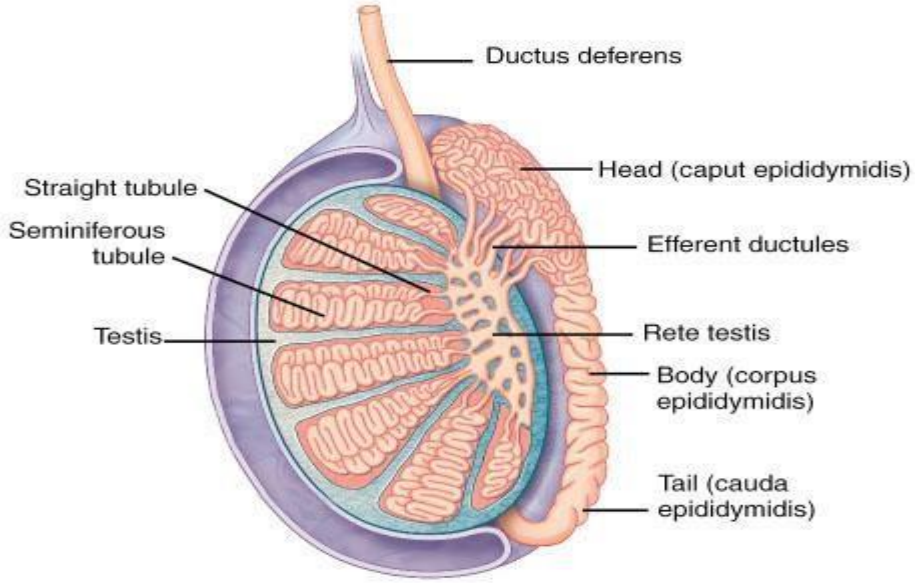


Figure 2.4: Longitudinal section of testis showing epididymis

Source: slideshare.net

2.2 CLINICAL CONDITIONS OF THE PROSTATE

A number of clinical conditions affect the prostate gland. They include prostatism, prostatitis, prostalgia, BPH and prostate cancers. Prostatism is any condition of the prostate that interfers with the bladder urine-flow. Prostatitis is a condition where the prostate gland is inflamed and may be accompanied by discomfort, pain, frequent or infrequent urination, and fever. Prostalgia is a painful condition of the prostate gland. Prostate cancer is any cancer that develops within the prostate. It is probably the prostate condition of utmost concern. BPH is the most common non-cancerous prostate problem. It is conceivably a greater health burden than PCa because many more people with

indolent PCa die with the condition than from it (Smith *et al.*, 2012).

2.2.1 Prostate Cancer (PCa)

In prostate cancer (PCa) disease, the prostate cells differentiate, become malignant and rapidly proliferates to form a tumor mass. Almost all PCas

are adenocarcinomas. The development of the cancer is most common at the peripheral zone. Non-adenocarcinomas like transitional cell carcinoma, small cell carcinoma and sarcoma rarely occur (Nutting *et al.*, 1997). In most parts of the world, PCa ranks as one of the most prevalent male cancers. Generally in Europe, PCa is the solid neoplasm with the highest incidence (Boyle and Ferlay, 2004). The West African incidence is reported to stand at 4.7–19.8 per 100,000 men per year (Chu *et al.*, 2011). In Ghana, male cancer deaths attributable to prostate cancer was 17.35% making it the second highest cancer killer after cancer of the liver (21.15%) (Wiredu and Armah, 2006). In the GLOBOCAN 2002 database, it was estimated that, in Ghana, the 1-year prevalence is 734; and the 5-year prevalence is estimated at 2,451; and the number of deaths per year being 758 (Ferlay *et al.*, 2004).

Several factors may cause or contribute to the development of PCa. They include genetic, lifestyle and medical factors. However, a full understanding of the causes of PCa is still elusive (Hsing and Chokkalingam, 2006).

Certain common symptoms of PCa are mostly absent in the inital phase in up to two-thirds of the population (Miller *et al.*, 2003). The symtoms most commonly experienced in PCa are mainly problems of urinary dysfunction and include polyuria or urinary incontinence, nocturia, dysuria, hematuria, and difficulty starting and maintaining a constant urinary stream or decreased force of stream. It also presents with problems of sexual dysfunction and mal-performance such as difficulty achieving erection, painful ejaculation and blood in semen (Miller *et al.*, 2003). Extracapsular or metastasized PCa may invade the bones and

lymph nodes and present problems such as discomfort in the pelvic area, vertebral pain, leg weakness from compressed spine and faecal discharge problems (Van der Cruijsen-Koeter *et al.*, 2005).

The main tools employed for the diagnosis of PCa include digital rectum examination (DRE), transrectal ultrasonography (TRUS), PSA levels of blood serum and PSA variants. However, the definite diagnosis is made when the adenocarcinoma has been substantiated histopathologically surgically obtained samples or samples obtained by biopsy. Several probing biochemical and molecular candidate markers for PCa keep emerging. They include p53, a marker of growth control and apoptosis, which has long been associated with advanced stages of PCa, metastases and an overall worse prognosis (Bookstein *et al.*, 1993; Navone *et al.*, 1993; Osman *et al.*, 1999; Inoue *et al.*, 2005) and Bcl-2, a protein expressed in primary PCa, which has been shown along with p53, to have the ability to prognosticate a relapse of the condition following radical prostatectomy and radiotherapy (Heidenberg *et al.*,
1995; Raffo *et al.*, 1995; Apakama *et al.*, 1996). Others include Chromogranin A (CGA or GRN-A) (Deftos and Abrahamsson, 1998), αmethylacyl-CoA racemase (AMACR) (Luo *et al*, 2002), human glandular kallikrein 2 (hK2) (Vickers *et al.*, 2010, Vickers *et al.*, 2011), nuclear factor-κB (NF-κB) (Domingo-Domenech *et al.*, 2005), Ki-67 (Chang *et al.*, 2005; Rubio *et al.*, 2005; Revelos *et al.*, 2005), Vascular endothelial growth factor (VEGF) and transforming growth factor (TGF-β1) (Barre *et al.*, 2003; Shariat *et al.*, 2004; George *et al.*, 2005), epithelial growth factor receptor (EGFR) and the oncogene c-erbB2/HER-2 (Di Lorenzo *et al.*, 2002). Other tests like urinalysis and urine culture tests are

performed to check for urinary tract infections that might be the cause of the symptoms. Urinary cytology may be performed to rule out bladder cancer.

The main treatment regimens for PCa are the expectant management methods namely watchful waiting and active surveillance which are used mainly for low risk cancers; while surgery (mainly radical prostatectomy), radiation therapy, and cryotherapy are applied to more aggressive cancers. Hormonal therapy and chemotherapy are mainly used for cancers that have spread beyond the prostate. These have emerged as treatment options for PCa (Beerlage *et al.*, 2000; Fahmy and Bissada, 2003; Han and Belldegrun, 2004; Rees *et al.*, 2004) that may be used singularly or in various combinations.

2.2.2 Benign Prostatic Hyperplasia (BPH)

Benign Prostatic Hyperplasia (BPH) is also referred to as nodular hyperplasia of the prostate, is an abnormal proliferation of periurethral cells causing a growth in the size of the prostate gland (epithelial and stromal cells) and urethral compression. It involves the formation of discrete nodules in the periurethral region, compressing the urethral canal leading to a decrease in the normal flow of urine. It normally affects the stromal cells predominantly. It also affects the glandular epithelial cells and it is associated mainly with the posterior urethral glands (PUG) as well as the transitional zone (TZ), and to a lesser extent the peripheral zone (PZ) (Wasserman, 2006). Following embryonic reawakening, there is a preliminary rise in the small stromal periurethral as well as the TZ glandular nodule numbers (McNeal, 1990).

Subsequent to this is the phase characterized by a surge in the numbers of larger nodules as well as an increase in the stromalepithelial ratio (Bartsch 1979; McNeal 1990). BPH may be defined classically as a histological development of prostatic hyperplasia that is typically characterized by urinary retention, with a frequent need to urinate and other urinary symptoms termed lower urinary tracts symptoms (LUTS).

2.2.3 Epidemiology – BPH

In 2006, the global prevalence of BPH among men was reported with respect to age to stand at over 42% in those in their 50s; 70% in those between the ages of 60 and 70; and 90% in those from their 80s into 90s (Nickel, 2006). However, histologic BPH in men is reported to be present in about 50% of those in the age range of 51 to 60, 70% in the range of 61 to 70 years, and 90% in the range of 81 to 90 years (Bushman, 2009; Paolone, 2010). In a study among Ghanaian men, the prevalences reported, for DRE-detected enlarged prostate, PSA≥1.5 ng/ml (prostate volume ≥ 30 cm^3), moderate-to-severe LUTS (IPSS≥8), and an enlarged prostate on DRE plus moderate-to-severe LUTS
(IPSS≥8) were 62.3%, 35.3%, 19.9% and 13.3% respectively
(Chokkalingam *et al.*, 2012).

2.2.4 Causes of BPH

2.2.4.1 Hormonal and Growth Factors

Dihydrotestosterone (DHT), produced by the reduction of testosterone circulating in the prostate by the enzyme 5α-reductase, is the principal androgen that influences prostate volume. The androgen hormones testosterone and DHT promote prostate cell proliferation (Feldman and Feldman, 2001) whiles estrogen indirectly regulates the development of

BPH by the local conversion of androgens to estrogen (Ho *et al.*, 2008). At the molecular level, the underlying cause for BPH development is not exactly well elucudated. However, there is an intricate interplay of androgens, stromal epithelial interactions, growth factors like epidermal growth factor (EGF), insulin-like growth factor (IGF), transforming growth factor-β (TGF-β), keratinocyte growth factor (KGF), neurotransmitters and oestrogens are implicated, (Gillenwater *et al.*, 2002). Vitamin D receptors which regulate cell growth proliferation, as well as Cytochrome P45017 which mediates sex steroid hormone synthesis are possible factors that can influence BPH risk (Thorner, 2009).

2.2.4.2 Lifestyle Factors

Lifestyle factors such as diet, exercise, smoking, having multiple sex partners (Parsons, 2011), and oxidative stress are believed to influence prostate growth (Suzuki *et al.*, 2002). Wein *et al.* (2007) categorizes age, androgen and functional androgen receptors, genetics, obesity, dyslipidaemia and diabetes as risk factors associated with BPH whiles diet, BMI, smoking, hypertension and sexual mal-function are categorized as possible risk factors. Although former smokers, heavy alcohol consumers, people with histories of hypertension and heart disease are all reported to be positively associated with developing LUTS (Joseph *et al.*, 2003), another study has reported that high consumption of alcohol is associated with a reduced risk for BPH whiles high fat diet and high red meat intake pose an increased risk (Kristal *et al.*, 2007).

2.4.4.3 Disease Conditions

Some studies posit that metabolic syndrome could very likely be implicated may both the pathogenesis as well as the progression of BPH and PCa (Alcaraz *et al.*, 2009; De Nunzio *et al.*, 2012).

2.2.5 Diagnosis of BPH

2.2.5.1 LUTS and Symptoms of BPH

The terminology LUTS was proposed by Abrams in 1994, later endorsed by the 5th International Consultation on BPH in the stead of such vague expressions as "clinical BPH," "symptomatic BPH," and "prostatism" (Chatelain *et al.*, 2001). The Consultation recommended that the use of BPH should be limited to histologically diagnosed cases whereas benign prostatic enlargement (BPE) should be used where there is no pathological evidence. It was submitted also that bladder outlet obstruction (BOO) should be used where a high detrusor pressure is linked with a low rate of urine-flow because of the rather there is no strong connection established yet for urinary pointers like symptoms and urodynamics (Chatelain *et al.*, 2001).

Current and former smokers, heavy- alcohol consumers, and people with history of hypertension and heart disease are all positively associated with developing LUTS (Joseph, 2003). The abnormal growth in prostate size may not always present with clinical symptoms (Donovan *et al.*, 1997); the traditionally known symptoms associated with BPH include terminal dribbling, difficulty in initiating urinary flow, lowered force of urine stream, intermittent streaming, urge incontinence and nocturia. Acute urinary retention or prostate surgery and deterioration (increase)

in symptom score are prostatic events that have also been associated with BPH (Muruganandham *et al.*, 2007).

Although the name suggests a benign disposition of the condition, 40–50% of men who show a histological evidence of BPH develop a clinically significant hyperplasia (Rubenstein and McVary, 2008). Progressively, BPH symptoms may develop into urinary retention associated complications like hypotonia, obstructive uropathy and urinary tract infections resulting from bladder bacterial stasis and bladder stones may result from crystallized salts in residual urine.

2.2.5.2 Digital Rectal Examination (DRE)

DRE is a technique for inspecting the prostate for abnormalities via inserting the finger through the rectum. The prostate is felt for any hard or irregular areas and examined whether it is larger than expected for a particular age. DRE is also able to detect cancer that has progressed beyond early stage, with a sensitivity of about 60% and 90% specificity (Chodak et al., 1989).

2.2.5.3 Biochemical Markers

Like PCa, BPH is associated with high PSA and may cause urinary problems but is not associated with cancer. BPH however co-exists with occult PCa in 10-30% of cases (Alcaraz *et al.*, 2009). PSA is a kallikreinlike serine protease that is normally produced by the prostatic epithelial cells. Serum levels may be elevated in the presence of benign prostatic hypertrophy (BPH), prostate cancer, prostatitis and other nonmalignant conditions like. PSA levels are very highly used as an index for detecting the development of BPH because of its direct relationship with prostate

volume. PSA is related to prostate volume in an agedependent, log-linear fashion (Roehrborn, 1999). Both PSA and prostatic volume also show long-term changes with respect to symptom scores as well as with the rate of urinary flow. There is no universally set threshold for considering PSA level as abnormal. Men below age 50 are generally expected to have levels of less than 2.5 ng/ml; less than 3.5 ng/ml for men aged between 50 to 60; less than 4.5 ng/ml for men between the ages of 60 to 69; and < 6.5 ng/ml for men from age 70 upwards. PSA measurement has low specificity in that levels between 410 ng/ml has only a 25% chance for detecting cancer; and 20% of people with PCa have normal PSA levels (< 4.0 ng/ml) (Catalona *et al.*,
1997; McGarty, 2010). The measurement of the prostate cancer antigen 3 (PCA-3) has emerged as one of the best markers for differentiating cancer-related from non-specific PSA elevation (Vlaeminck-Guillem *et al.*, 2008).

Discriminating BPH from PCa mostly uses the investigative concept of free PSA (fPSA) to total PSA (tPSA) ratio (f/t PSA). Epidemiological studies have shown that elevated fPSA level can predict clinical BPH independently of tPSA levels (Meigs *et al.*, 2001). The fPSA/tPSA can be used to rule out the suspicion of having PCa if the ratio is lower than normal at PSA concentrations of 2.6–10 ng/ml (Ito *et al.*, 2003). ProPSA, the precursor form of PSA, also serves as a valuable indicator for the differentiation of PCa from benign processes (Oh *et al.*, 2003).

The hK2 expression has been found to be increased in high-grade PIN,

PCa and in lymph node metastases. A cohort of specimen obtained via radical prostatectomy showed that in the development of the condition, hK2 expression increased progressively through the stages of benign epithelium, primary cancer to lymph-node metastases (Darson *et al.* 1999). It has proven a better diagnostic marker compared to free PSA ratio in the "grayzone" to discriminate PCa from BPH (Mao *et al.*, 2010). Delta-catenin transcripts are highly expressed PCa but not expressed at all or expressed to a very low level in the BPH tissue (Burger *et al.*, 2002), Prostate-specific membrane antigen (PSMA), a discriminatory marker is shown to be up-regulated in PCa tissue but not in BPH tissue; an overlap between the two conditions is absent.

Other tests like urinalysis and urine culture tests are performed to check for urinary tract infections that might be the cause of the symptoms. Urinary cytology may be performed to rule out bladder cancer.

Ultrasound techniques have also been used to detect the size of the prostate as well as the bladder to determine oversized age-related prostate. Transrectal ultrasound (TRUS) is an invasive procedure that uses high-frequency sound waves to scan the prostate by a probe through the rectum to determine whether it is enlarged. The reverberating sound-waves are collected via a transducer unto a computer that uses those sound waves to create a real-time image that displays the structure of the internal organs of the body, their movement as well as blood-flow in their vessels.

2.2.5.4 Symptom Score Tools

Aside the biochemical markers, score-tools have been developed for the quantification of the severity of LUTS as well as response to treatment in BPH patients. These tools include the Boyarski score and the Madsen-Iversen score which are presently considered obsolete; and the widely used American Urological Association (AUA-SI) score, or its enhanced alternative the International Prostate System Score (IPSS). Symptom score is adjudged as one of the more powerful predictors of symptomatic outcome (Roehrborn, 2001).

The IPSS is structured to make a set of seven queries that pertains to urinary symptoms and one pertaining to the quality of life. The seven questions are scored from 0 to 5 each, making a total ranging between 0 and 35. The questions bordering on urinary symptoms probe the occurrence of the following symptoms withinin the immediate past month: (**1**) Incomplete emptying (sensation of not emptying the bladder); (**2**) Frequency (number of urinations in two hours); (**3**) Intermittency (stoppages of flow whiles passing urine); (**4**) Urgency (difficulty in postponing urination); (**5**) Weak stream (having a weak urinary stream); (**6**) Straining (forcing to start urine flow). (**7**) Nocturia (frequency of getting up to pass urine at night). A total score of 1-7 is adjudged mild, a score of 8-19 is adjudged moderate and a score between 20-35 is considered severe. The question concerning the quality of life enquires about how pleased one would be to spend the rest of his life with the present urinary condition.

However, the International Scientific Committee (SCI) recommends that physicians consider history, physical examination, appropriate

laboratory tests, DRE and other pertinent evaluations to rule out prostate cancer. Cystoscopy could be used to look directly in the urethra and/or bladder. The study of urodynamics of the pressure-flow can be useful for testing interior-pressure whiles passing urine. Uroflowmetry gives a measure of how fast urine flows, and post-void residual volume (PVR) measures the post-urination bladder residual urine volume. Ultrasound of the prostate to view for enlargement is also regularly used in the diagnosis of BPH.

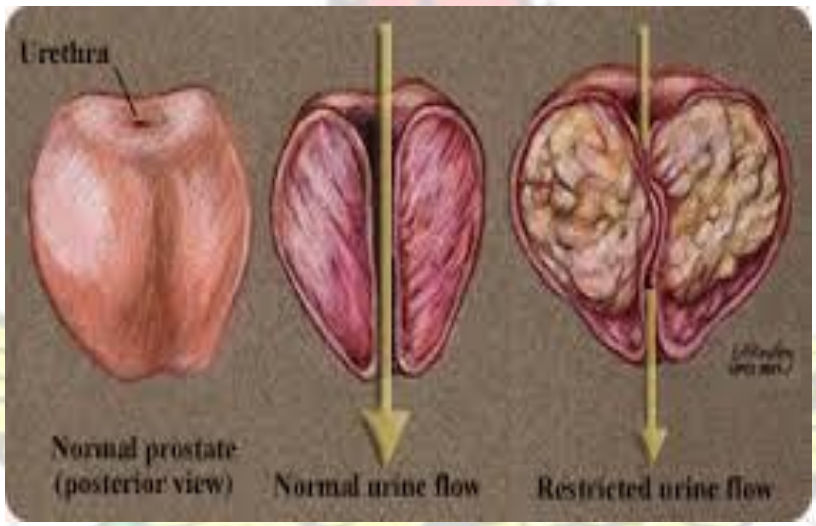


Figure 2.5: Posterior view of normal prostate, and longitudinal sectional view of normal and benign hyperplastic prostates.

Source: ping of health.com

2.2.5.5 Treatment of BPH

About 90% of BPH sufferers (over 50 years) do not need medical or surgical intervention for treating the condition (medicinenet, 2013). Depending on the severity of the condition, the conventional treatment regimens for BPH may involve non-interventional, invasive and chemotherapeutic procedures. The non-interventional treatments involve watchful waiting and sometimes active surveillance.

2.2.5.6 Invasive Procedures

Open prostatectomy and surgical treatments with varying levels of invasiveness are options employed in the BPH treatment. Open prostatectomy entails the excising areas of the interior surface of the prostate via a suprapubic or retropubic incision. The incision is made in the lower abdominal or peritoneal area. It is prescribed mainly for men whose prostate glands are significantly enlarged (NIH, 2014).

Transurethral resection of the prostate (TURP) is the standard for BPH treatment. In TURP, the inner part of the prostate gland that surrounds the urethra is removed (Madersbacher and Marberger, 2003). A *resectoscope* is introduced via the penile opening through the urethra to the prostate and a heated wire, electrical current or laser beam is used in vaporizing the tissue. It is sometimes used in men with advanced prostate cancer to help relieve symptoms, such as urinary problems (Urosurgery, 2010).

Transurethral incision of the prostate (TUIP) is also referred to as bladder neck incision. It is similar to TURP, but is commonly applied to prostates of relatively small volumes. As in TURP, a scope is inserted via the penile opening to the prostate and a small incision is made in the tissue to expand the urethra to allow for less painful and better flow of urine rather than removing prostate tissue (Urosurgery, 2010).

In the transurethral needle ablation (TUNA) of the prostate, a transurethral catheter is inserted into the prostate to deliver low level radio frequency via deployable needles to kill the prostate tissue thereby reducing prostate size and alleviating BPH symptoms (emedicine, 2014).

Transurethral Microwave Thermotherapy (TUMT) uses a transurethral antenna to deliver heat via microwave radiation to injure prostate tissue and provide relief from bladder obstruction (Rubeinstein and Mcvary, 2003).

Laser surgery employs two laser-based surgical methods for the killing the residual prostate tissue. The first, photosensitive vaporization of the prostate, is performed mainly in men with whose prostate can only be described as moderately enlarged; wheras the second, holmium laser enucleation of the prostate (HoLEP) which shows comparable outcomes with TURP (Heidenrich *et al.*, 2011) is used for men with severely enlarged prostates. An end-firing pulsed solid state laser with a wavelength of 2140 nm completely removes the adenoma.

There are several severe adverse side effects and complications to these treatment procedures including impotence, erectile dysfunction, loss of libido, ejaculatory problems, urinary incontinence, urethral stricture and gynaecomastia.

2.2.5.7 Conventional Medical Therapy

The medications for treating BPH are mainly α-blockers and 5αreductase inhibitors. The most common α-blockers used include alfuzosin (Uroxatral), terazosin (Hytrin), doxazosin (Cardura) and tamsulosin (Flomax). These smooth muscle relaxing drugs are used for reducing BOO but not prostate size. Finasteride (Proscar), dutasteride (Avodart) and Botolunim toxin (Botox) are 5α-reductase inhibitor oral medications used to treat BPH. They relieve BPH symptoms and actually cause prostate shrinkage by means of lowering the conversion of testosterone to DHT for

prostate growth (Andriole et al., 2004). These must however be used indefinitely to prevent symptoms from recurring. These drugs however present adverse side effects to users. The side effects of alpha blockers include dizziness, asthenia, retrograde ejaculation, orthostatic hypotension and intraoperative floppy iris syndrome in cataract surgery (Chang, 2005; Brogden *et al.*, 2007), and those of 5α-reductase inhibitors are lowered libido, erectile dysfunction as well as ejaculation abnormalities (Heiderich *et al.*, 2011).

The possible effects of using combination therapy as well as their possible challenges have been explored by several studies. A combination therapy made up of the α-blockers together with the 5αreductase inhibitors has shown superior effect in men with larger prostates and prevents the progression of disease as well as improve symptoms (Lepor *et al.*, 1996). The Prospective European Doxazosin and Combination Therapy (PREDICT) trial however verified that statistically, monotherapy and combination therapy outcomes specifically urine flow rate and symptom score (IPSS) are not different (Kirby *et al.*, 2003).

However, these were short-term studies and longer-term studies like the Medical Therapy of Prostatic Symptoms (MTOPs) trial have shown a relatively lower risk of clinical progression with combination therapy which is statistically significant in comparison with monotherapy and placebo spanning an average of 4.5 years of follow-up (Hunter *et al.*, 1996). Post-hoc analysis however showed that as prostate volume decreased (prostate volume < 25 ml); the levels of reduction observed between treatment and placebo groups were not statistically significant. In the Combination of Avodart and Tamsulosin (COMBAT) trial using in

which subjects had prostate volumes of > 30 ml with IPSS of ≥ 12, it was established that the combination therapy improved both the symptom scores and urinary flow rates significantly, and furthermore lowered the relative risk of acute urinary retention (AUR) compared with monotherapy.

The use of anticholinergics has also demonstrated an high efficacy as well as an option for BPH treatment as well as for managing its symptoms (Heidenreich *et al.*, 2013). Medical therapy is reported to cause BPH sufferers to present with complications including infection (<1 - 12%), bladder calculi (0.3 - 3.4%), renal failure (<2.5%), incontinence (<1%) and urinary retention (1 - 2%/yr). These negatively influence the quality of life of the sufferers of the condition to a grave extent (Mebust *et al.*,, 1989; Grosse, 1990; Wasson, 1995; Hunter *et al.*, 1996; McConnell, 2003).

2.3 PHYTOTHERAPY

The medicinal uses of plants have been described in ancient Egyptian and Chinese writings since 3000 BC. The practice is however believed to have preceded recorded history.

Early records describe how Ancient Egyptians used absinth, cassia, cumin, fennel, garlic, myrrh, juniper and many others for medicinal purposes (Sneader, 2005). Medical sytems for example the Indian Ayurveda and the Traditional Chinese Medicine were fundamentally established as herbal-centered therapeutic systems. Africans and Americans have long been known to use herbal medicine for their healing rituals (Sneader, 2005). Traditional medicine has long been considered an important resort for the development of orthodox drugs. Examples of

such drugs are morphine, atropine, digoxin, codeine, quinine, reserpine, vincristine and taxol (Duke *et al.*, 1985; Bruneton, 1999). In 2001, Phillipson reported more than half of the 20 topmost were developed with natural products as basis for their studies. It is also reported that the active ingredient of about 25% - 30% of prescriptions in modern medicine are derivatives of medicinal plants

(Kumar *et al.*, 2012).

2.3.1 Phytotherapy for the treatment of BPH

Due to the severe adverse effects of BPH and generally conditions of the prostate, including related cancers on the quality of life, all alternatives to treat or ameliorate symptoms of these conditions are incessantly being explored. Traditional plant therapies are one of such alternatives being researched for treatment especially because the conventional therapies may not stop prostate growth or prevent recurrence of prostate disease.

Although there is some evidence backing the claim that phytotherapeutic drugs have effects towards treating prostate conditions, there is scarcely any convincing evidence by clinical trials proving their efficacy for treatment. It has been reported that none of the pica guidelines have recommended their use (Wehrberger *et al.*, 2012). Genistein has demonstrated an ability of inhibiting 5-alphareductase (Evans *et al.*, 1995) as well as the growth of LNCaP, DU-145 and PC3 PCa cell lines, and also to induce apoptosis in these cell lines (Onozawa *et al.*, 1998; Bektic *et al.*, 2004; Ouchi *et al.*, 2005).

Curcumin (*Curcuma longa*) inhibited NF-κB activation and activator Protein-1 (AP-1) whiles inducing apoptosis. This correlated with the

down-regulation of Bcl-2 and Bcl-xL expression and the activation of procaspase-3 and expression of procaspase-8 (Mukhopadhyay *et al.*, 2001).

Epigallocatechin-3-gallate, (EGCG), the polyphenolic fractions of green tea, induced apoptosis, cell-growth inhibition and cell-cycle dysregulation (Adhami *et al.*, 2003). Treatment of DU-145 and LNCaP cells by EGCG induced apoptosis, and caused a G0/G1 cell cycle arrest and cyclin kinase inhibition of WAF1/p21 independently of the p53 status of the cells (Gupta *et al.*, 2000).

Lycopenes are present in organs with hormonally regulated tissues such as the prostate, where the highest concentrations of lycopene are found (Stahl *et al.*, 1992). Some serum- or plasma-based studies have supported a 25-30% reduction in the risk of PCa by lycopene (Giovannucci, 2002; Etminan *et al.*, 2004).

The phytotherapeutic agents *Serenoa repens* (saw palmetto), *Pygeum africanum* (African prune tree), *Urtica dioica* (nettle) *and Cucurbita pepo* (pumpkin seed) have all been claimed to help prevent and treat BPH or its symptoms (Barnes, 2002; Silvestri *et al.*, 2013; Levy *et al.*, 2014). A systematic review and meta-analysis as well as general consensus from randomized controlled trials (RCTs) all decried the claim of *Serenoa repens* as being more efficacious than placebo (Wilt *et al.*, 1998).

However, some other randomised clinical trials as well as a number of meta-analyses have provided evidence in favour of the efficacy of *Serenoa repens* in boosting urinary flow rate and improving IPSS score (Boyle *et al.*, 2004; MacDonald *et al.*, 2013). There is documented record stating

that the actual clinical benefit of *Serenoa repens* have yet to be determined (Franklin, 2009) inspite of the fact that it has no debilitating effect on the male sexual function (Zlotta, 2005) which is one of the commonest adverse effects of the orthodox treatment of BPH.

In a double-blind trial which spanned a period of about a year, the efficacy of a herbal preparation made up of a mixture of saw palmetto and nettle comparable to that of finasteride in alleviating BPH symptoms. Furthermore, it showed superior tolerance in terms of adverse side effects compared to the drug group (Sokeland and Albrecht, 1997).

Phellodendron amurense (cork tree), a plant from the family Rutaceae has the ability to impede prostatic contractility, thus suggesting it may potentially relieve urethral obstruction in managing urological disorders experienced by men who are suffering from BPH (Xu and Ventura, 2010). The fruit-body extract of *Ganoderma lucidum* (reishi mushroom) posseses a significantly strong 5 α-reductase inhibitory activity. It has been proven in castrated rats that this extract repressed the testosterone-induced enlargement of the ventral prostate normally caused by testosterone-induction, suggesting its usefulness for the treatment of BPH (Fujita *et al.*, 2005).

Lepidium meyenii (red maca) was specific to reducing prostate weight but not seminal vesicle weight. It is suggested that this could very well pass for a treatment option for conditions of the prostate, particularly BPH (Gasco *et al.*, 2007). *Hypoxis rooperi* and *Secale cereal* have demonstrated preliminary evidence of improving symptom score and urological symptoms in BPH (Wilt *et al.*, 2000).

Phytotherapeutic drug research has proven promising in revealing natural anti-prostatic growth and anti-prostate cancer activities possessed by herbal plants.

2.4 *ANNONA MURICATA*

Annona muricata (Linn.) refers to a tropical fruit tree that is commonly called soursop in English. It is several other common names including guanábana among Spanish-Speaking North and South Americans, and graviola in the Portuguese-Speaking country Brazil where it is very commonly found in the Amazon. Locally, it is referred to as "Apre" (Akan).

2.4.1 Description and Distribution

It is an evergreen tree of the Annonacea family that produces a green edible heart-shaped fruit of about 15-20 cm in diameter that has a white flesh with dark seeds inside. It grows to about 5 to 6 metres in height and has large, glossy, dark-green leaves.

Annona muricata is claimed to be native to Central America (Wele *et al.*, 2004). Among the various indigenes of the tropical world, the diversity of the conditions *A. muricata* is used for treating is largely dependent on the part of the plant used.

The root, bark, seeds, fruit and leaves are all put to different use in different places. Generally, the leaves, the roots as well as the bark, are widely accepted to have sedative, hypotensive, and antispasmodic effects. The traditional administration of *A. muricata* towards these effects is usually via tea preparations.

Certain traditional folks in Guyana use the leaf and or the bark as a sedative and make liquid formulations from them for cardiovascular health. In other Caribbean countries, particularly in Jamaica, the West Indies and Haiti, the fruit or its juice are used for treating parasites, diarrhea and fever; whiles the bark or the leaf is used as a sedative, an antispasmodic, a nervine; and for treating heart conditions, parasitic infections, hypertension, asthma, coughs and flu, as well as helping with difficult childbirths. In the Amazonian region of Brazil, the oil extracted from the *A. muricata* leaves as well as the immature fruit are blended together with olive oil and applied topically for the treatment of rheumatism, arthritis pain and neuralgia; the leaf itself is administered as tea for treating liver problems, diabetes, and catarrh. In Peru, the leaf is also used as a sedative and an antispasmodic whiles the crushed seed is used for killing parasites.

In tropical Africa, *A. muricata* is widely considered an anticancer agent and also extensively used as an insecticide, piscicide, vermifuge, sedative, antiparasitic, astringent, hypotensive agent, as well as used in the treatment of skin diseases, pain, fevers and coughs. In India, the leaf and root bark are generally considered as antihelminthic and antiphlogistic agents; and the flowers and fruit pods are use for treating catarrh (Watt and Breyer-brandwijk, 1962). In Cameroon, the leaves of *A. muricata* are used to manage diabetes and its complications.

Figure 2.6: *Annona muricata* plant showing stem with leaves and fruit. Source: commons.wikimedia.org

2.4.2 Biochemical Composition

A number of phytochemicals from differents parts of the *Annona species* have been isolated as well as characterized. The key compounds of medicinal importance in these plants are the annonaceous acetogenins. As far back as 1940, Tattersfield and co-researchers reported that *A. muricata* posseses different alkaloid chemicals in the seeds and roots in addition to antitumor acetogenins. The presence of alkaloids in the leaf has also been reported (Leboeuf *et al.*, 1982).

Later studies named some of the isolated and characterized phytochemicals include the annonaceous acetogenins (Wu *et al.*, 1995; Zeng *et al.*, 1996; Kim *et al.*, 1998; Chang *et al.*, 2003) and others such as lactones and isoquinoline alkaloids; cardiac glycosides, tannins, coumarine, procyanidins, flavonoids, pentacyclic terpenoid saponins;

pcoumaric acid, stearic acid, myristic acid, β stepharine, reticuline, ellagic acid, gamma-amino butyric acid (GABA); phytosterols (sitosterol, stigmatsterol), sugars, alcohol, aldehydes, organic and inorganic acids, metals, inorganic salts, vitamins B and C (Watt and Breyer-brandwijk, 1962; Taylor, 2002; Gavamukulya *et al.*, 2014). Seven isoquinoline alkaloids namely reticuline, coclaurine, coreximine, atherosperminine, stepharine, anomurine and anomuricine were isolated from the leaves, root and stem barks of *A. muricata* (Rieser *et al.*, 1991). More recent reports confirm that *A. muricata* posseses glycosides, condensed tannins, saponins, (Arthur *et al.*, 2011) flavonoids, alkaloids and acetogenins (Pandey and Barve 2011; Gajalakshmi *et al.* 2012).

A proximate analysis study showed that the seed has a high fat and carbohydrate content and its oil contains copious amounts of unsaturated fatty acids with oleic acid making up to 41% and linoleic acid making up to 30%. The dominant saturated acid is palmitic which makes up to 20%. It also has crude protein and fiber as well as minerals such as potassium, calcium, phosphorus, sodium and magnesium (Kimbonguila *et al.*, 2010).

The sour-acidic tasting pulp of the fleshy fruit consists of 80% water, 18% carbohydrates, 1% protein, and some amount of vitamins B1, B2 and C, potassium and dietary fiber (Lim, 2012).

2.5 ANNONACEOUS ACETOGENINS

Most of the studies that sought to explore the medicinal property of *A. muricata* (Linn.) focused on exploring the phytotherapeutic activity and

mode of action of the major bioactive compounds known as annonaceous acetogenins.

Only members of the Annonaceae family possess annonaceous acetogenins. Several diverse acetogenins have been isolated from different parts in plants of the family. In 2004 alone, nine monotetrahydrofuranic Annonaceous acetogenins namely montalicins A to E (1-5), cis-annoreticuin (6), montalicins F (7), I (8), and J (9), together with eight known acetogenins 10-17, were isolated from the seeds of *Annona montana* by high performance liquid chromatographic (HPLC) (Liaw *et al.*, 2004). The dichloromethane extract of the seeds of *A. muricata* produced annoreticuin-9-one (**1**), while the flesh of the fruit yielded cis-annoreticuin (**2**) and sabadelin (**3**). Acetogenins **1** and **2** were first isolated from *A. reticulata* and *A. montana*, respectively (Ragasa *et al.*, 2012). A recent study that employed a bioassay-guided fractionation of the fruit powder also yielded three novel C35 Annonaceous acetogenins: muricins J, K, and L which all demonstrated an antiproliferative effect against human PCa cell line PC-3 (Sun *et al.*,
2014).

There are several hundreds of these acetogenins and many of them have been documented to show bioactivity. It was observed that in 27 years, over 500 acetogenins were isolated from various parts of different Annonaceus species (Liaw, 2010). Out of about 80 plants of the Annonaceae family screened, about half of them were described as showing significant bioactivity and worthy of fractionation (Alali *et al*, 1999). A list of the annonaceous acetogenins from *A. muricata* discovered

by the year 2002 were published in the Technical Data Report for Graviola (Taylor, 2002).

Several acetogenins from various parts of Annonaceous plants are still being unearthed. Some of the most recently published ones include annonacin, bullatacin, annonin VI, goniothalamin, sylvaticin. Quite recently, seven isoquinoline alkaloids including reticuline, coclaurine, coreximine, atherosperminine, stepharine, anomurine and anomuricine have been isolated from the leaves, root and stem barks of *A.muricata* (Sulaiman *et al.*, 2012). A more recent report identified for the first time the presence of an acetogenin murisolin, as well as stigmasterol and triglyceride in the seeds (Ragasa *et al.*, 2014).

2.5.1 Chemical Structure of Acetogenins

Annonaceous acetogenins are waxy polyketide compounds that consist of C32 or C34 long chain fatty acids bearing a 2-propanol unit on the C2 thus forming lactone derivatives of long-chain fatty acids. Most acetogenins contain one or more tetrahydrofuran rings while some rather have tetrahydropyran rings or epoxy rings, or are linear (Bermejo, 2005). Although the carbon skeletons of the fatty acid derivatives bear close semblance, they are distinguished in the relative as well as absolute configuration observed in their different stereogenic oxygen functions.

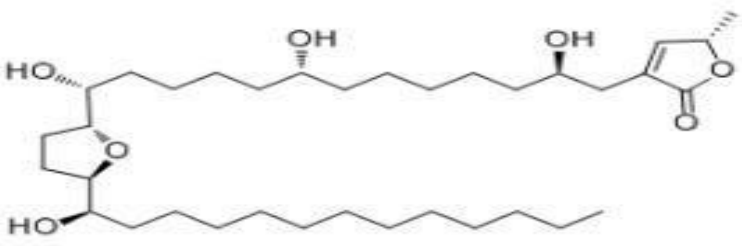


Figure 2.7: Chemical structure of an Acetogenin (Annonacin)

Source: albtechnology.com

The difficulty in developing chemotherapeutic drugs from these naturally occurring acetogenins has been the challenge of producing a novel chemical that is able to retain the full potency of the natural product. Just to synthesize the major acetogenin, annonacin, took researchers about ten years. The toxicity of pure annonacin in cortical neurons was reportedly enhanced in the presence of crude plant extract (Potts, 2012). Even at low doses, it has been reported to be significantly toxic against breast, cervical, ovarian, skin and bladder cancer cell lines (Yuan, 2003). An annonacin concentration of 10 mg/kg showed a 57.9% inhibition of Lewis lung cancer *in vivo* compared to Adriamycin which showed a 54.6% (Wang *et al.*, 2002). Along with accummulating mannose and glucose levels, Annonacin prevents neuronal death whiles preventing non-dopaminergic neurons from surviving (Lannuzel *et al.*, 2003).

2.5.2 Mechanism of Antitumor Action of Acetogenins

Acetogenins have been reported to be very effective against tumors with proven anticancer agent resistance (Purdue News, 1997).

Annonacin has been shown to be able to induce apoptotic cell death via a Bax and caspase-3 related pathway by enhancing the expression of the apoptosis-promoting proteins Bax and Bad, as well as the p53 and p21 but not the anti-apoptotic Bcl-2 or Bcl-xL at the G1 phase of the cell cycle (Yuan, 2003). By such a mechanism, acetogenins generally are able to decrease cytosolic ATP and deprive cancer cells of energy resulting in apoptosis (Oberlies *et al.*, 1997; Alali *et al.*, 1999).

The mechanism by which acetogenins generally exhibit their potent bioactivities is by the depletion of ATP levels via the inhibition of complex I of the mitochondrial electron transport systems and NADH oxidase of plasma membranes of the tumor cells. They cause oxidative phosphorylation in the mitochondria by inhibiting the complex I enzyme NADH: ubiquinone oxidoreductase (Oberlies *et al.*, 1997; Alali *et al.*, 1999; Tormo *et al.*, 2000). The inhibitory action has been shown to occur at the ubiquinone-catalytic sites in complex I and in microbial glucose dehydrogenase (Friedrich *et al.*, 1994). The acetogenins are reported to interact with the NADH: ubiquinone oxidoreductase (complex I) in mammalian and in the insect mitochondrial electron transport systems; and with ubiquinone NAD(P)H oxidase in the cytoplasmic membranes of cancer cells (Konno *et al.*, 2008).

Acetogenins have a special ability to inhibit ubiquinone-linked NADH oxidase processes which occur only in the membranes of cancerous tumor cells thereby making them mostly toxic to cancer cells but nontoxic to non-cancerous cells (Rieser *et al.*, 1991; Zeng *et al.*, 1996).

Cancer cells, especially those undergoing chemotherapy develop an intracellular P-glycoprotein mediated pump that is dependent on ATP energy to push out anticancer agents before such agents can kill them (Alali *et al.*, 1999; Tormo *et al.*, 2000). This mechanism makes them develop multi-drug resistance (MDR). The ability to foil the ATP dependent resistance mechanisms in cancer cells particularly make acetogenins not only toxic against cancer cells, but also demonstrate an exceptional affinity for killing MDR cancer cells (Alali *et al.*, 1999) as against normal

cells that do not possess this pump, and therefore have less need of such ample amounts of ATP energy for metabolism and survival.

The cell death achieved through the depletion of ATP levels via the inhibition of the complex I of mitochondrial electron transport systems and NADH oxidase of plasma membranes of the tumor cells is by the mechanism of apoptosis.

2.5.3 Apoptosis

Apoptosis, also known as programmed cell death denotes the normal and controlled growth phenomenon process of cell death that is generally seen in eukaryotic cells which proceeds as part normal cellular growth. It is genetically controlled and neoplasms may result from an alteration or defect in this process (Berges *et al.*, 1995).

Apoptosis in mitochondria is referred to as` the intrinsic apoptosis pathway. Intrinsic apoptosis is activated in response to different kinds of intracellular stress and injury stimuli including growth factor withdrawal, oncogenes, direct DNA damage, hypoxia, survival factor deprivation, unfolding stresses in the endoplasmic reticulum and death receptor stimulation, viral infections or cellular damage by toxins, free radicals, or radiation.

These stimuli induce a loss of transmembrane potential in the inner mitochondrial membrane resulting in a release of apoptosis-promoting proteins into the cytosol. The cellular stress sensor p53 then initiates apoptosis. It upregulates the p53-Upregulated Modulator of Apoptosis (PUMA) and activates pro-apoptotic B-Cell Leukemia-2 (Bcl2) family

members like Bax whiles repressing anti-apoptotic Bcl-2 proteins like bcl2.

Cytochrome c is produced via the mitochondrial apoptosis-induced channel (MAC), in the outer mitochondrial membrane, and binds with Apoptotic protease activating factor – 1 (Apaf-1) and ATP, which then binds to pro-caspase-9 to form a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form, caspase-9, which sequentially activates the effector caspase-3 to carry out cellular degradation.

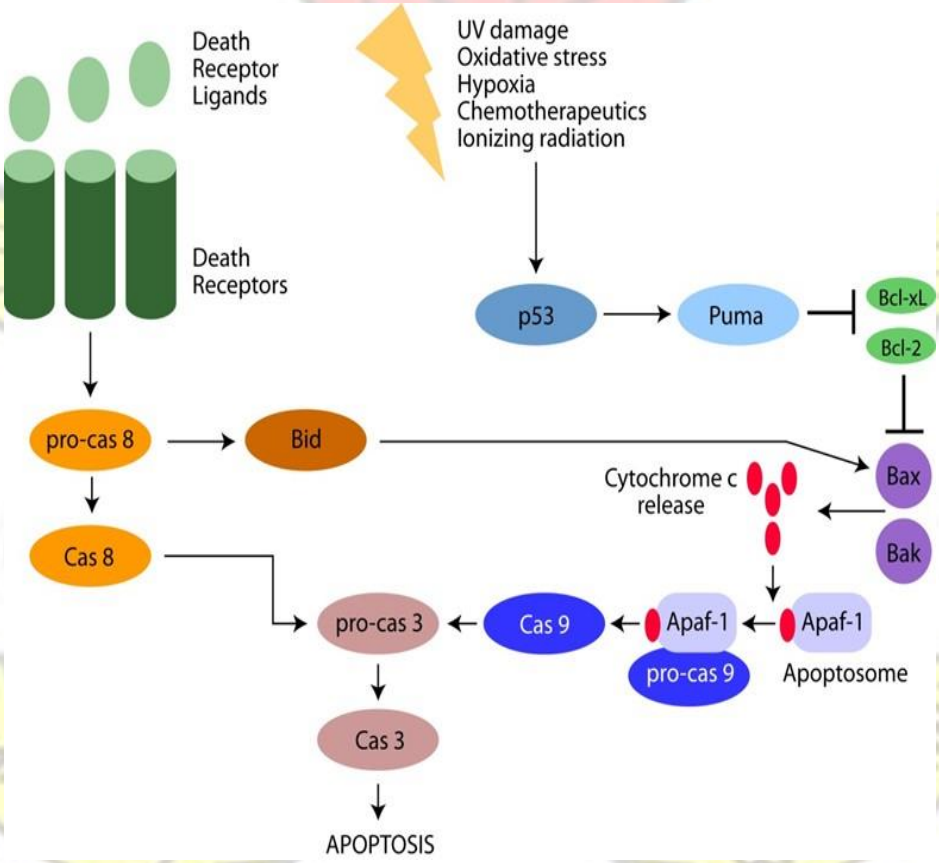


Figure 2.8: Pictorial representation of the intrinsic process of apoptosis showing the initiation by stressors to final stage apoptosis by caspases.

Source: intechopen.com

A. muricata has been shown to induce apoptotic cell death in the G1 phase of the cell cycle by enhancing the expression of the pro-apoptotic proteins Bax and Bad, as well as the p53 and p21 but not the antiapoptotic Bcl-2 or Bcl-xL (Yuan, 2003). A recent study showed that the leaf, roots and twig extracts of *A. muricata* induced apoptosis on HL-60 cells via a G0/G1 phase cell cycle arrest mechanism (Pieme *et al.*, 2014).

2.6 THE *A. MURICATA* LEAVES

2.6.1 Medicinal Properties

The leaves are lanceolate and glossy dark green in color and traditionally used as an antispasmodic, a sedative, a nervine for heart conditions, and for the treatment of headaches, hypertension, cough and asthma (Lans, 2006).

From the leaves of *A. muricata* have been isolated several acetogenins. In a screening program in 1976, the National Cancer Institute showed that the leaves and stems of *A. muricata* actively showed toxicity against cancer cells (National Cancer Institute, 1976). Acetogenins **1** (annoreticuin-9-one) and **2** (*cis*-annoreticuin) isolated initially from other species, *A. reticulata* and *A. Montana* respectively, have both been reported to show significant cytotoxic activity *in vitro* against two human hepatoma cell lines namely Hep G2 and 2,2,15. Acetogenins also targets the human pancreatic tumor cell line (PACA-2), human prostate adenocarcinoma (PC-3) (Kim *et al.*, 1998; Woo *et al.* 1999; Ragasa *et al.*, 2012) and human lung carcinoma (A-549) (Zhao *et al.*, 1993; Ragasa *et al.*, 2012). Annocatalin (**5**) is also reported to have a high selectivity for the Hep 2,2,15 cell line (Liaw *et al.*, 2002; Chang *et al.*, 2003).

2.6.1.1 General Medicinal Properties

Studies have shown the leaf extract to be effective against malaria parasites (Gbeasor, 1990; Antoun, 1993). Its protective effect on lipid profile and its antioxidant-enzymes activity have been documented (Adewole and Ojewale, 2009). A recent study reported that A. muricata leaf extract reduced blood glucose, serum creatinine, MDA, nitrite, LDLcholesterol levels; AST and ALT activities. It also improved total cholesterol and triglycerides levels as well as the activities of SOD and CAT (Florence et al., 2014).

The leaves contain essential oils that possess rheumatological, parasitic, anti-diarrhoeal, and antineuralgic properties (Gleye et al., 1998; Kossouoh et al., 2007). Their boiled water infusions have antispasmodic and astringent properties (Khan et al., 1998), and useful for the treatment of kidney ailments (Duke, 1985), jaundice (Mshana et al., 2000), diabetes and gastric upset (Adewole and Caxton-Martins, 2006).

Alcoholic extracts of *A. muricata* leaf were reported non-toxic and had no adverse impact in mice intraperitoneally at a concentration of 100 mg/kg. Explorative behavior in these mice was however reduced and mild abdominal constrictions were reported at a dosage of 300 mg/kg (N'Gouemo, 1997).

2.6.1.2 Antitumor Activity

Acetogenins from Annonaceous leaves have been reported to show activity against human tumor cell lines (Ye *et al.*, 1996). The leaf demonstrated antitumor activity in the *in vitro* anticrustacean assay system (Wu *et al.*, 1995). In other studies, the leaf showed cytostatic activity by inhibiting

tumor cell growth including Adriamycin resistant human mammary adenocarcinoma MCF-7/Adr cells *in vitro* (Oberlies *et al.*, 1997). *A. muricata* leaf is 10,000 times stronger than Adriamycin in killing colon cancer cells (Astirin *et al.*, 2013).

The ethanolic leaf extract demonstrated cytotoxic activity against the human kidney carcinoma CA-A498 (Zeng *et al.*, 1996), human breast carcinoma MCF-7 (Kim *et al.*, 1998), bovine kidney cell line MDBK (Betancur-Galvis *et al.*, 1999) and human hepatoma hep G 2,2,15 (Liaw *et al.*, 2002).

In HeLa cells, the ethanol and distilled water extracts showed a lower death rate compared to ethyl acetate extract which also showed a lower death rate compared to chloroform extract. The chloroform extract is apparently a better alternative for cancer causing viruses (Astirin *et al.*, 2013). The ethanolic extracts of the leaf and even the stem have been reported to show activity against human oral epidermoid carcinoma CA9KB (Leaman *et al.*, 1995). The methanolic, hexane and ethyl acetate extracts have shown activity against human histiocytic lymphoma U937 (Jaramillo *et al.*, 2000).

Therapeutically active antineoplastic compounds are purportedly present in the n-butanolic leaf extract of *A. muricata* (George *et al.*, 2012). This extract has been shown to be moderately cytotoxic to normal cells (WRL-68 normal human hepatic cells), when compared with those obtained for the cancerous cells human breast carcinoma MDA-MB-435S and human immortalized keratinocyte HaCaT (George *et al.*, 2012).

Although there have been a number of in vitro studies showing the impact of *A. muricata* on prostate cancer lines, there is scarcely any literature on its effect on the *BPH-1* cell lines.

2.6.2 *In Vivo* Studies

There is a critical dearth of *in vivo* studies involving the use of an *A. muricata*. Antinociceptive and anti-inflammatory activities have been reported in both mice and rat models treated with ethanolic leaf extract of *A. muricata* (de Sousa *et al.,* 2010). The ethanolic leaf extract also showed hypoglycaemic and antidiabetic effects in streptozotocin (STZ)induced diabetic rats and alloxan-induced diabetic rabbit treated with (Gupta *et al.*, 2005). Extracts of *A. muricata* were shown to promote regeneration of the pancreatic β-cells of islets in Wistar rats (Adeyemi *et al.*, 2007). AMLE has reportedly shown protective effects on serum lipid profiles and oxidative stress in hepatocytes of streptozotocin-treated diabetic rats (Adewole and Ojewole, 2009). The leaves of A. muricata has also been suggested to possess tumor suppression potential in that it reduced chemically induced skin papillomagenesis in mice (Hamizah *et al.*, 2012).

High-level consumption of soursop has furthermore been reported to distort the cytoarchitecture of adrenal glands in Wistar rats (Ezejindu *et al.*, 2014).

There is as yet no literature on both *in vivo* and *in vitro* studies with regards to *A. muricata* and BPH.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY DESIGN

The study design was an experimental study where acqueous extracts of *Annona muricata* leaves were obtained, and various batches of the extract (AMLE) analyzed by HPLC fingerprint-profiling guidelines. *In vitro* analyses were also made to determine the effect of the AMLE on BPH-1 cell viability and on the cell proliferation regulatory genes, Bcl-2 and Bax. In an *in vivo* study, the F344 rats were administered the extract, and the histological effects of the extract on the prostate determined. An analytical estimation of the biochemical marker PSA was performed.

3.2 STUDY SITES

The *A. muricata* leaf extracts were freeze-dried at the Ghana Atomic Energy Commission. High performance liquid chromatography (HPLC) analysis was performed at the Department of Chemical Pathology of the Noguchi Memorial Institute of Medical Research. Rats were housed at the Animal Experimentation Unit of the Microbiology Department, College of Health Sciences, Korle Bu. Laboratory analyses were performed at the Animal Experimentation Unit Laboratory of the

Noguchi Memorial Institute of Medical Research and the Medical Laboratory Science Department of the School of Biomedical and Allied Health Sciences, Korle-Bu.

3.3 SAMPLING TECHNIQUE

Plants were conveniently sampled from the outskirts of Accra. The households from where plants were sampled were randomly selected.

Simple random sampling technique was also used to assign rats to various groups.

3.4 PLANT MATERIAL EXTRACTION.

The leaves of *A. muricata* were collected from the outskirts of Accra between July and August 2013, and authenticated at the Ghana Herbarium of the University of Ghana. Specimens were identified by Mr. J.T. Mensah and deposited with voucher number UG 00178.AM.215/13 issued. The leaves were hand-washed by rubbing the surface gently under running-water. They were later sun-dried for 3 days. The dried leaves were milled and soaked in the proportion of 1 kg of milled substance to 4000 mL of water for 24 hrs. The mixture was then boiled for 1 hour and filtered through fine linen gauze. The resulting marc was then soaked in another 3000 mL of water for 24 hrs. The mixture was again boiled for 1 hour and filtered. The two filtered solutions were pooled together and freeze-dried with the CHRIST Freeze Dryer Gamma1-16/2- 16LSC (2004 version) at the Ghana Atomic Energy Commission. The aqueous yield from 1 kg of ground substance was 25.2 g.

3.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) ANALYSIS

Different batches of the extract were taken and monitored by chromatographic fingerprint. A Shimadzu HPLC system (Kyoto, Japan) with Diode Array Detector and Ultimate XB-C18 column (150 x 4.6 mm, 5 □m) was used to analyze the samples. The absorbance was measured

at 208 nm. The mobile phase solvent A was water and solvent B acetonitrile (ACE) at a flow rate of 1 mL/min and an injection volume of 1 □L. The gradient run ACE: H_2O was as follows: from 10%:90% to 10%:90% (0 – 10 min); from 10%:90% to 85%:15% (10 – 30 min); from 85%:15% to 85%:15% (30 – 40 min). The sample injection volume was 10 µL. This optimum easily controlled and reproducible procedure of extraction described previously was established from the fingerprint results. The identification of the various peaks was outside the scope of this study.

3.6 *IN VITRO* STUDIES

3.6.1 BPH-1 Cell Viability Studies

3.6.1.1 Principle of Assay

Metabolically active cells incubated in the tetrazolium compound MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) reduce it to insoluble purple formazan dye crystals with an absorbance maximum near 570 nm. Dimethylsulfoxide (DMSO) is then added to solubilize the crystals. The rate of tetrazolium reduction is proportional to the level of cell viability.

3.6.1.2 Assay Procedure

Cell viability assays were performed on BPH-1 cells. Into each of the wells of a 96-well plate was seeded 1 x 10^5 cells in a 0.1 mL RPMI 1640, 10% fetal bovine serum (FBS) medium.

Primary cell lines were sub-cultured twice.

Cells were treated with 0.5, 1.0 and 1.5 mg/mL extract in phosphate buffer solution (PBS) and incubated at 37°C for 24, 48 and 72 hrs. At the end of treatment time for various plates, the medium was replaced by 100 µL 3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA) per well and incubated for an additional 4 hrs at 37°C. The reaction was stopped by adding 100 µL DMSO, AR grade (Sigma, USA) to each well to dissolve the purple-blue MTT formazan precipitate. The absorbance was read at 570 nm on an ELISA microplate reader (BioTek, Elx800, VT, USA). The growth inhibition by AMLE was assessed in terms of percent viability where vehicle treated cells was considered as having a viability of 100%.

Growth inhibition = $\frac{\textbf{A} \text{ (untreated sample)} - \textbf{A} \text{ (treated sample)}}{\textbf{A} \text{ (untreated sample)}} \times 100\%$

A – Absorbance

3.6.1.3 Determination of IC_{50}

IC_{50} was determined from a graph of percentage cell viability against AMLE concentration, as the concentration of AMLE that resulted in 50% growth inhibition of seeded cells. IC_{50} was determined for the incubation periods of 24, 48 and 72 hrs. Vehicle treated cells were considered as being 100% viable.

3.6.2 RNA Extraction and RT-PCR Analysis

BPH-1 cells were seeded into 6 well plates at a density of 1 x 10^4 per well in 2 mL 10% FBS medium and treated with 0.5, 1.0 and 1.5 mg/mL plant extract in PBS for 48 hrs. Total RNA was isolated using TriZol reagent (Invitrogen) (California, USA). Purity of the RNA isolated was tested by the

use of the Thermo Scientific Nanodrop™ 1000 spectrophotometer (Wilmington, DE, USA). The ratio of absorbance at
260 nm and 280 nm ranged from 1.75 to 1.92. Oligo (dT)-primed RNA (1 g) was reverse-transcribed using the SuperScript II transcriptase kit (RR047A) (Bio Inc, Takara, Japan) according to the manufacturer's instructions.

TaqDNA polymerase (Fermentas) (Burlington, Canada) was used in the amplification of the cDNA obtained. The levels of target genes, Bax and Bcl-2 present were determined using the cDNA obtained and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control. The following sequence of primers was used for amplification: Bcl-2 forward – 5'-GG TGGTGGAGG AACTCT TCA-3' and reverse 5'-GAGCAGCGTCT TCAGAGACA-3'; Bax – forward 5'CCAAGAAGCTG AGCGAG TGT-3' and reverse 5'-TC ACGGAG GAAGTCCAG TGT-3'; GAPDH forward 5' TGCTGAGTATGTCGTGGAG-3' and reverse 5'-GTGTTCTGAGTGGCAGTGAT-3'.

Thermal cycler (GeneAmp® PCR System 9700, Applied Biosysytems) profiles were as follows: For Bcl-2; the initial denaturation was performed at 94°C for 5min, followed by 35 cycles at 94°C, annealing was then done at 58°C for 1 min and polymerization at 72°C for 1 min. The final polymerization step was for carried out for 10 min at 72°C. For Bax; the initial denaturation was performed at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, annealing was done at 55°C for 30 sec and polymerization at 72°C for 45 sec. The final polymerization step was for 10 min at 72°C. For GAPDH; initial denaturation was performed at 94°C

for 30 sec, followed by 35 cycles at 94°C for 30 sec, annealing was done at 58°C for 1 min and polymerization at 72°C for 1 min. The final polymerization step was for 10 min at 72°C. Electrophoretic sizing of the PCR products were done by 1.5% agarose and visualized under UV light using a Bio-Rad 2000 gel documentation system (CA, USA). PCR band sizes were approximately 268 bp, 248 bp and 240 bp for Bcl-2, Bax and GADPH respectively.

3.7 *IN VIVO* STUDIES

3.7.1 Inclusion and Exclusion Criteria

3.7.1.1 Inclusion Criteria

1. Matured pathogen-free male F344 rats.
2. Rats weighing 150-200g.

3.7.1.2 Exclusion Criteria

1. Female rats.
2. Non-F344 male rats.
3. Unhealthy rats (non-pathogen free).
4. Rats weighing under 150g or over 200g.

3.7.1 Animals

The Organisation for Economic Co-operation and Development (pb) protocol on the use of laboratory animals was adopted (OECD, 1998). This was approved by the Scientific and Technical Committee of the Noguchi Memorial Institute for Medical Research (STC – 2009-02-03). A total of fifteen (15) male F344 rats aged (8 weeks old) and weighing

between 150-200g were obtained from the Noguchi Memorial Institute for Medical Research.

Rats were randomly divided into three groups of five (5) rats each, namely Group I (control), group II [low dose (LD)] and group III [high dose (HD)]. All animals were housed in stainless steel cages at the animal husbandry facility of the University of Ghana Medical School in Korle-Bu where they were maintained under a controlled environment of 12:12 light:dark cycle, 50 ± 5% humidity and 25 ± 2°C temperature, and adequate ventilation. The animals were allowed a 14-day acclimatization period prior to the start of experiment, and all were fed *ad libitum* a standard chow diet (AIN-93G Fomulation by GAFCO – Ghana Ltd) and water throughout the duration of the experiment. Control group rats were given no drug treatment. LD group rats were administered *A. muricata* extract at a dose of 30 mg/kg b.wt and HD group rats were administered the extract at a dose of 300 mg/kg b.wt. via oral gavage for 60 days. During the 14-day acclimatization period, there were weekly measurements of body weight and daily observations for clinical toxidromes and mortality during each period of dosing and at 30 mins, 1 hr, 3 hrs and 6 hrs post-administration.

After the sixty (60) days of extract administration, all animals were asphyxiated with chloroform and cardiac puncture was performed to draw about 5 ml of whole blood into gel separator tubes. The blood samples were centrifuged at 5000 rpm and the serum collected into eppendorf tubes and kept at -20°C until use. The terminal body weights of animals were measured; prostate, seminal vesicles and testes were harvested and weighed. Prostate and seminal vesicles were preserved in

10% buffered formaldehyde solution while testes were preserved in Bouin's solution for histological examination.

3.7.2 Biochemical Determination of Total Prostate-Specific Antigen (t-PSA)

3.7.2.1 Principle of the Assay:

A monoclonal anti-PSA antibody and PSA horseradish peroxidase HRP conjugate system was used; where sample assay were incubated together with PSA-HRP conjugate in pre coated plate. PSA from samples and PSA-HRP conjugate compete for the anti PSA antibody binding site. A substate for HRP is therafter complexed to form a blue coloured product, and the reaction stopped. The yellow colour imparted by the stop solution is measured spectrophotometrically at 450nm. PSA concentration in the sample is determined from a standard curve as inverse of the intensity of the coloured product formed from the binding of PSA HRP conjugate to anti PSA antibody binding sites because the more binding sites occupied by sample PSA, the lesser the remaining sites to bind PSA HRP conjugate.

3.7.2.2 Procedure

This was a competitive enzyme immunoassay technique assay performed by an ELISA method according to the kit manufacturer's instruction (My BioSource – San Diego, CA, USA).

A monoclonal anti-PSA antibody and PSA-HRP conjugate system was used. The assay sample and buffer were incubated together with PSAHRP conjugate in pre-coated plate for an hour. After the incubation period, the wells were decanted and washed five times. They were thereafter incubated with a substrate for HRP enzyme which produced a blue coloured complex. Finally, a stop solution was added to end the reaction.

The stop solution turned the product yellow. The intensity of yellow-colored product was measured spectrophotometrically at 450nm in a microplate reader. A standard curve relating the intensity of the color (optical density) to the concentration of standards was plotted. The PSA concentration in each sample was interpolated from this standard curve.

3.7.3 Histopathological Analysis

Fat and connective tissue-freed prostate, seminal vesicles and testes were harvested from rats, blotted with clean tissue, examined and weighed to obtain organ to body weight ratios. Thereafter, the tissues were processed by a slight modification of the protocol used by (Adeteye *et al.*, 2011). The steps involved in tissue processing include fixation, dehydration, clearing, infiltration, embedding, blocking, sectioning, and staining. Testes were fixed in Bouin's solution, whiles prostate and seminal vesicle were immediately fixed in 10% buffered formaldehyde solution and subsequently transferred to a graded series of absolute alcohol (50%, 70%, 90%), and cleared in xylene.

The cleared tissues were infiltrated in molten paraffin wax in the oven at 58^{o}C. Three changes of molten paraffin wax were made at one-hour intervals, after which the tissues were embedded in wax and made into blocks of wax. Three micrometer (3μm) sectioned slides of the tissues were hematoxylin and eosin (H&E) stained and examined microscopically for histological changes using Olympus BX 51TF (Olympus Corporation) (Tokyo, Japan) light microscope connected to a digital camera. Images of selected sections were captured at 100x and 400x magnifications.

3.8 DATA ANALYSIS

Graph Pad Prism Software (Version 6.0 for windows) (California, USA) was used to perform statistical analysis of data. The results were expressed as means ± standard error of mean (SEM), n=5. A one way analysis of variance (ANOVA) was performed to test the significance of differences between control group and dose groups mean outcomes. *Post hoc* analysis was performed with Bonferroni multiple comparison test where ANOVA showed significant differences. *P* values ≤ 0.05 were considered statistically significant.

3.9 ETHICAL ISSUES

Ethical clearance for this study was obtained from the Scientific and Technical Committee of the Noguchi Memorial Institute for Medical Research, University of Ghana and assigned the ethics number STC – 2009-02-03.

CHAPTER FOUR

4.0 RESULTS

In this study, the 1000 g of *A. muricata* leaf yielded 25.2 g of the extracted powder.

4.1. HPLC ANALYSIS

From figure 4.1, areas of peaks 3, 4, 6, 5, 2, 1, 8, 7 corresponded to the ratios 27.1:18.8:15.6:12.5:11.4:7.8:3.5:3.4. Results from the study are reproducible if fingerprint and its ratios are the same.

A Shimadzu HPLC system (Kyoto, Japan) with Diode Array Detector and an Ultimate XB-C18 column (150 x 4.6 mm, 5 □m) was used.

Chromatographic profile of the aqueous extract of AMLE is shown in the figure 4.1.

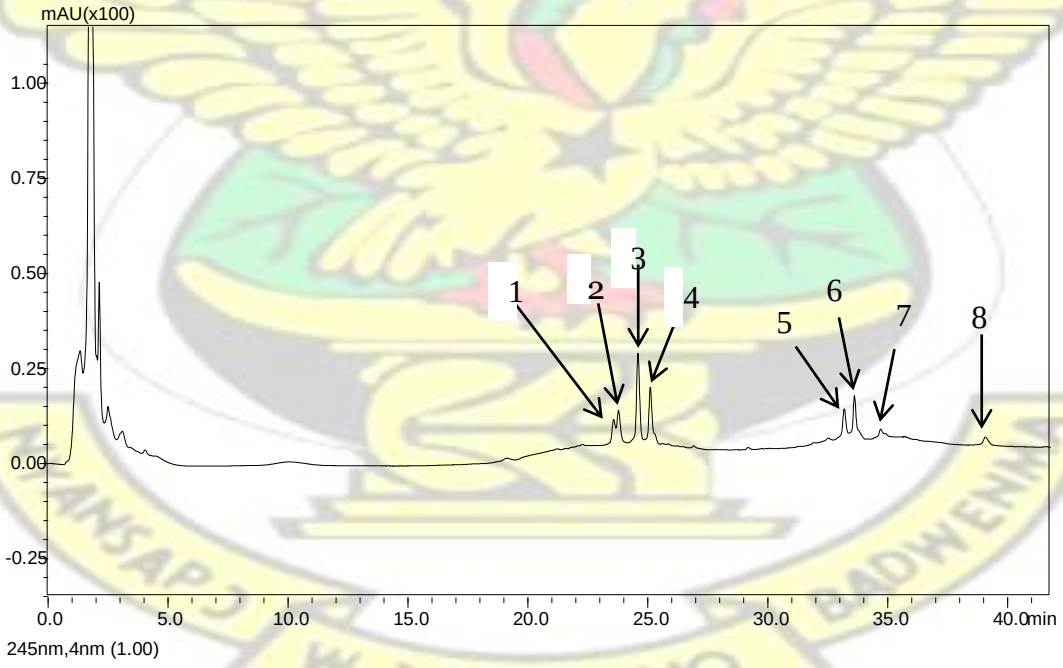


Figure 4.1: Area under curve of HPLC peaks for AMLE measured at an absorbance of 208 nm. The mobile phase solvent A (water) and solvent B[acetonitrile (ACE)] had a flow rate of 1 mL/min and an injection volume of 1 □L and showed a gradient run ACE: H2O was as follows: from 10%:90% to 10%:90% (0 – 10 min); from 10%:90% to 85%:15% (10 – 30

min); from 85%:15% to 85%:15% (30 – 40 min). The sample injection volume was 10 µL and the column temperature set at 30C.

Table 4.1: Area under curve from HPLC with corresponding peak ratios of 8 peaks isolated for AMLE.

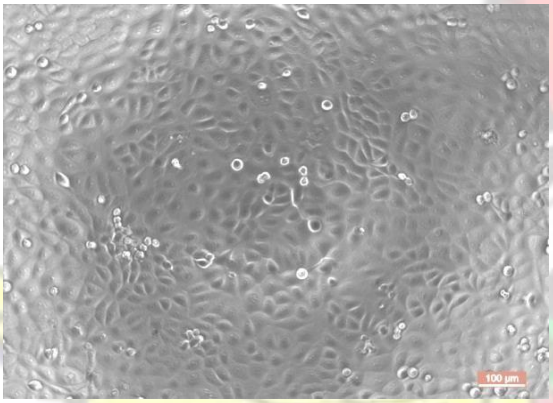
Peak	Area Under Curve (AUC)	Peak Ratio
1	1.1492	7.8
2	1.6811	11.4
3	3.9991	27.1
4	2.7795	18.8
5	1.8501	12.5
6	2.298	15.6
7	0.5016	3.4
8	0.5138	3.5

In order to control the quality of *A. muricata* extract, the optimum ratio determined for the peak areas of 1-8 were of 7.8, 11.4, 27.1, 18.8, 12.5, 15.6, 3.4 and 3.5, respectively. The order of magnitude (highest to lowest) for the peak areas 1-8 are 3> 4> 6> 5> 2> 1> 8> 7 with corresponding optimum ratios of 27.1> 18.8> 15.6> 12.5> 11.4> 7.8> 3.5> 3.4.

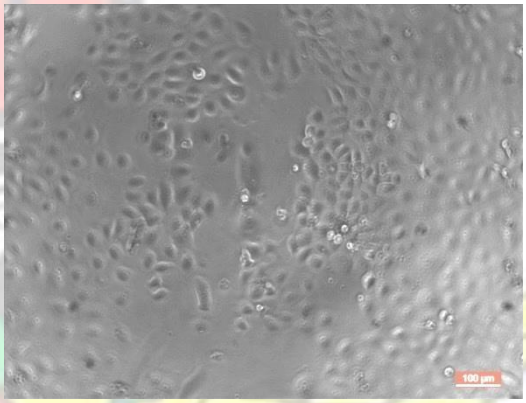
4.2. *In Vitro* Assays

4.2.1 BPH-1 Cell Viability

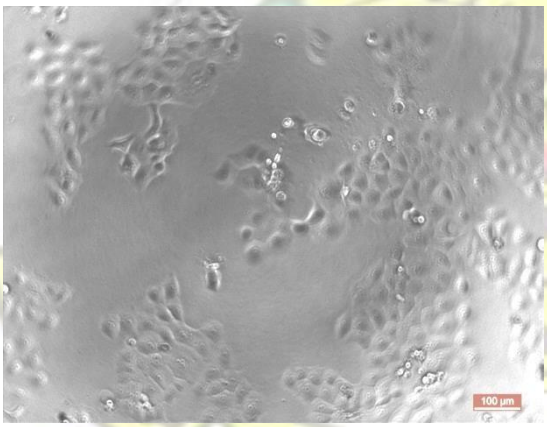
BPH-1 cell viability using MTT assay demonstrated significant dosedependent decrease as the concentration of the plant extract increased from 0 mg/ml to 1.5 mg/mL (Figure 4.2). The morphology of the cell remains the same in terms of disruption of cell. Thus, the cell growth inhibition seen is a direct effect of the anti-proliferative effect of *A. muricata*.



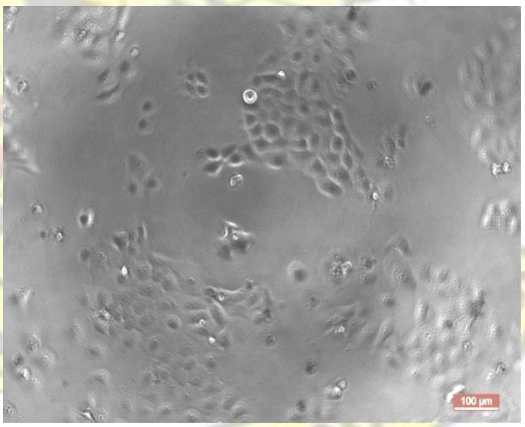
a) Cell viability (0 mg/mL)



b) Cell viability (0.5 mg/mL)



c) Cell viability (1.0 mg/mL)



d) Cell viabilty (1.5 mg/mL)

Figure 4.2: BPH-1 cell proliferation at various concentrations. From the figure, there was obsereved a progressively decreasing cell density with increasing AMLE concentrations 0, 0.5, 1.0 and 1.5 shown in figures a, b, c and d respectively. Cell morphology remained unchanged for all concentrations.

4.2.2. MTT Assay

The BPH-1 cell viability using MTT assay demonstrated significant dosedependent decrease. Statistical differences of each dose (0.5, 1 and 1.5 mg/mL) compared to the control (0mg/mL) was significant ($p < 0.05$). The IC_{50} recorded was 1.36 mg/mL for 48 hrs.

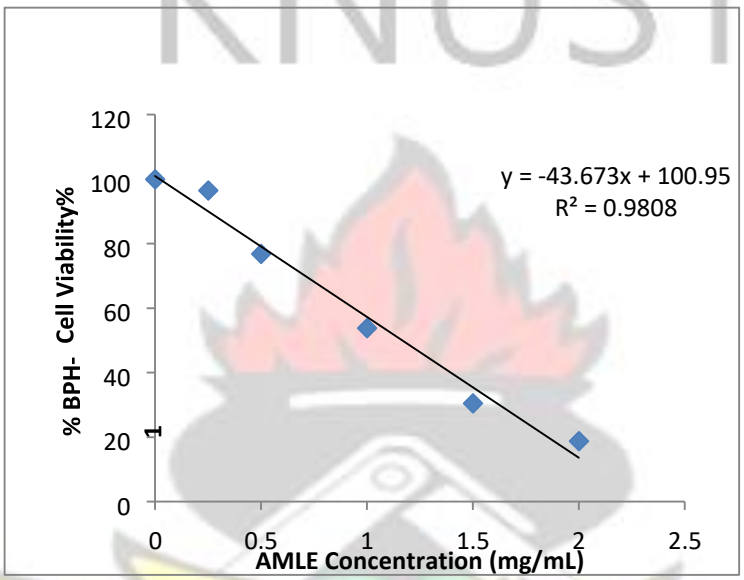


Figure 4.3 Growth inhibition of 50% was recorded at an AMLE concentration of 1.36 mg/mL.

Similar patterns were obtained for MTT viability test at 24, 48 and 72 hrs. At the end of the first 24 hrs of AMLE treatment, the BPH-1 cell viability recorded a dose-depedent decrease from 100 to 85.5%, then to 71.9% and finally to 41.0% for 0, 0.5, 1.0 and 1.5 mg/mL respectively. The reduction at 0.5 mg/mL AMLE concentration compared to control (0 mg/mL) was close to showing a statistically significant difference ($p = 0.0577$). However, there were statistically significant differences obtained for the decrease in cell proliferation at 1.0 mg/dL and 1.5 mg/dL compared to control ($p = 0.0024$ and $p < 0.0001$ respectively).

Overall, ANOVA showed a statistically significant difference ($p < 0.0001$) for the changes in various concentration means compared with control at 24hrs of AMLE treatment.

BPH-1 cell viability test at 48 hrs of treatment with AMLE also showed a dose dependent decrease in cell proliferation as the dose increased from 0 to 1.5 mg/mL. Cell viability reduced from 100% to 80.0%, 64.7% and 48.4% as the dose increased from 0 to 0.5 to 1.0 and to 1.5 mg/mL, respectively (figure 4.4). At the highest dose of 1.5 mg/mL BPH-1 cell viability was almost 50%. Statistically significant differences were obtained for doses 0.5, 1.0 and 1.5 mg/mL compared to the control were significant ($p = 0.0027$, $P < 0.0001$ and $P < 0.0001$, respectively). There was a statistically significant difference in the differences between means of various concentration proliferation values as indicated by ANOVA ($p < 0.0001$).

The 72 hr post AMLE treatment BPH-1 cell viability test showed a dose dependent decrease in cell proliferation as seen in dose increases from 0 to 1.5 mg/mL. Cell viability reduced from 100% to 76.9%, to 53.9% and then to 30.5% as the dose increased from 0, 0.5, 1.0 to 1.5 mg/mL, respectively (figure 4.3). The cell viability at all doses showed a highly significant difference from the control ($p < 0.0001$).

At the highest dose of 1.5 mg/mL, it was observed that the decrease in cell growth was near cell extinction. It had reached 30.5% from an initial of about 100% at 72 hrs. ANOVA showed a statistically significant difference between the various concentrations ($p < 0.0001$).

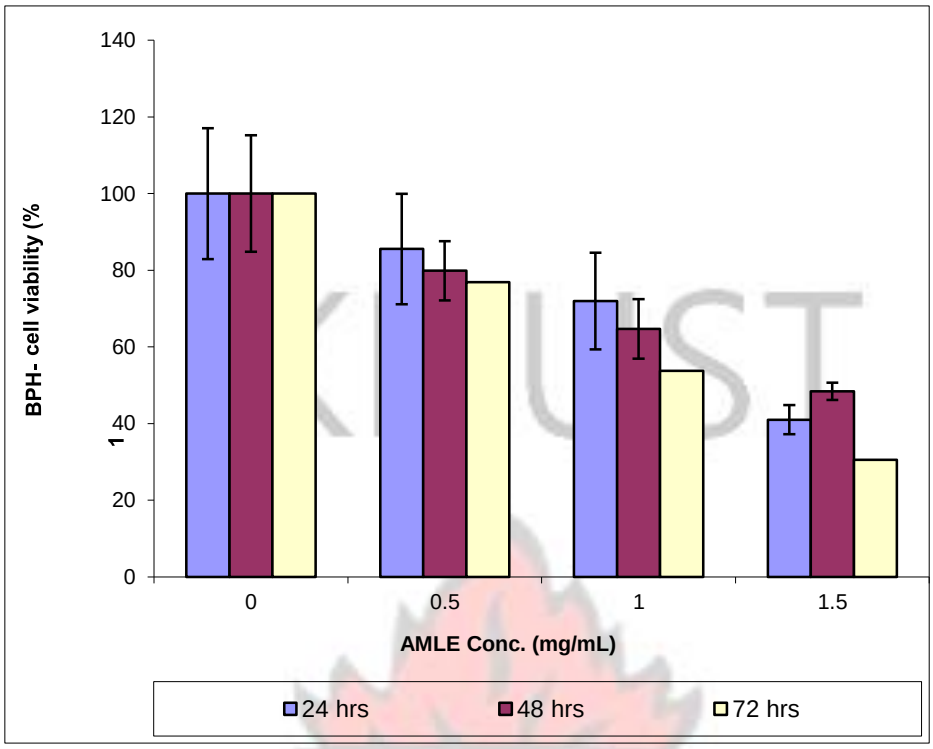


Figure 4.4: BPH-1 cell viability at various concentrations for 24 hrs, 48 hrs and 72 hrs. An overall decrease in cell viability achieved with increasing concentrations of AMLE.

4.2.3. RT-PCR Analysis

At the different concentrations of AMLE, Real Time Polymerase Chain Reaction (RT-PCR) analysis expressed distinct bands. A decrease in band intensity for Bcl-2 gene expression was observed as the concentration of AMLE increased. Conversely there was an increase in Bax expression in a dose dependent manner.

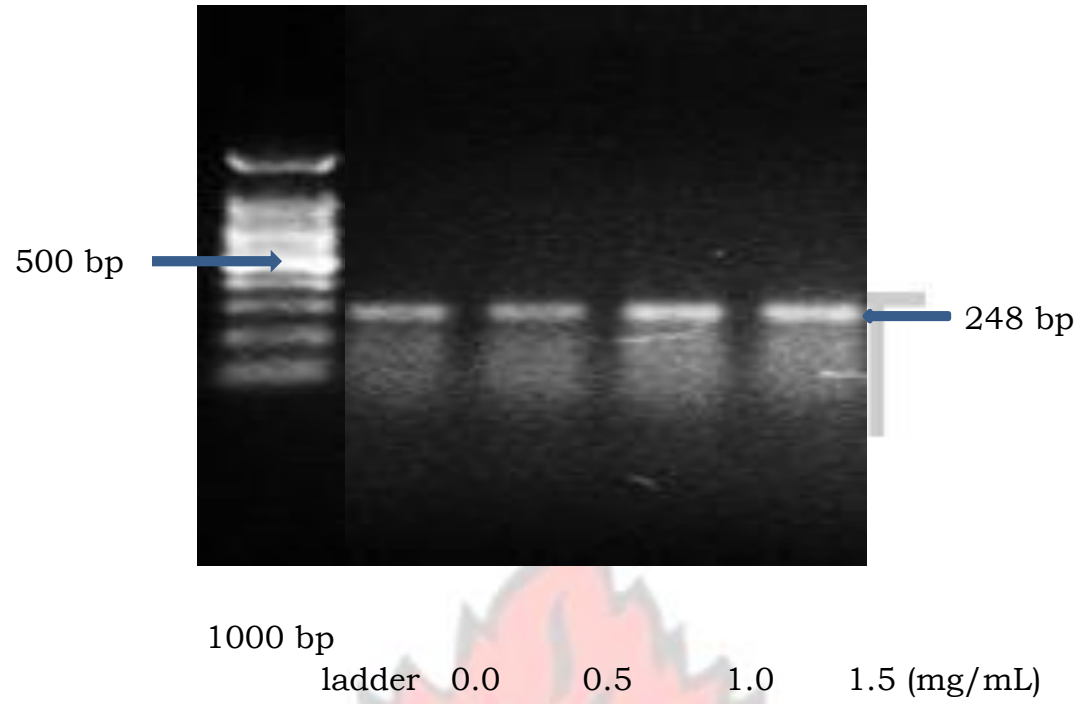
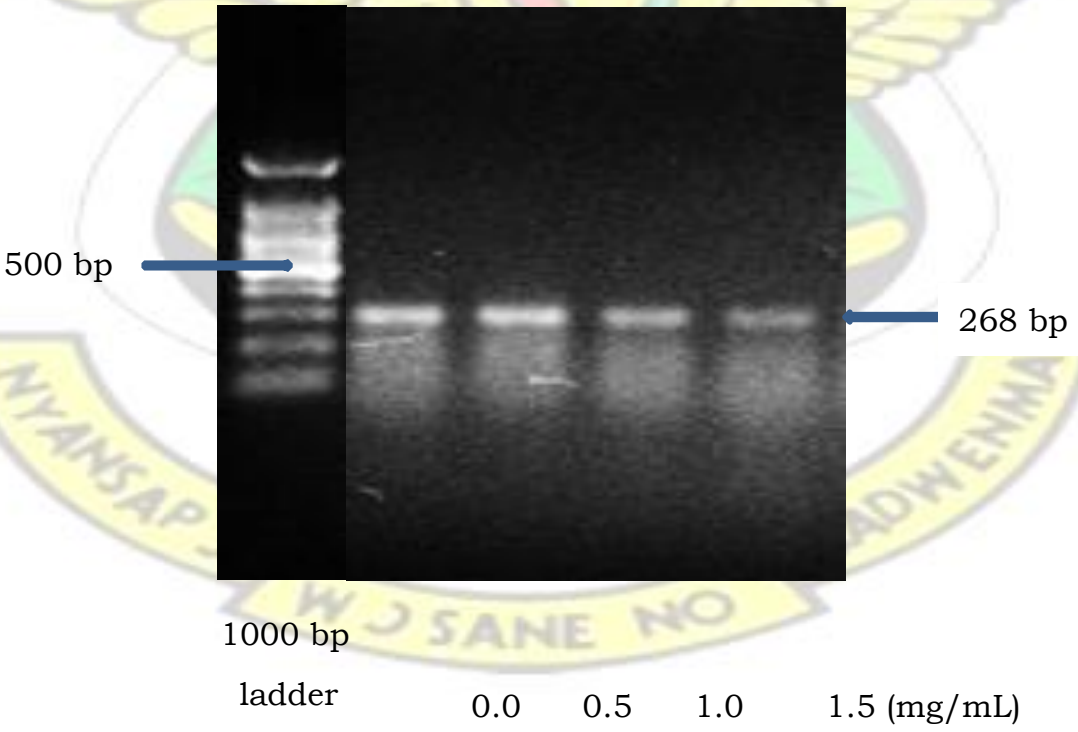


Figure 4.5: Electrophoretic gel image showing increasing band intensity for Bax protein with increasing concentrations of AMLE, vis-à-vis a 1000 bp ladder. Bax band was measured at 248 bp.



Electrophoretic gel image showing decreasing band intensity for Bcl2 protein with increasing concentrations of AMLE

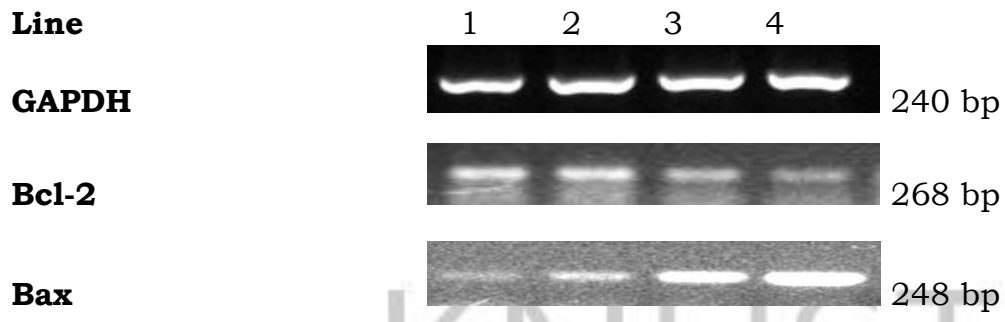


Figure 4.7: Electrophoretic bands of Bax and Bcl-2 at various concentrations of AMLE. Bax band intensity showing an increase with increasing AMLE concentration whiles Bcl-2 band intensity decreased with increasing AMLE concentration. The positive internal control, GAPDH, showed intense strong bands.

4.3 *IN VIVO* ASSAYS

4.3.1 Biochemical Assay (PSA)

Table 4.2: Total Prostate Specific Antigen concentrations for the AMLE treated rat groups compared with untreated control group.

	Control (ng/mL)	**Low Dose Group (ng/mL)**	**High Dose Group (ng/mL)**
1	0.405	0.401	0.404
2	0.409	0.411	0.399
3	0.415	0.403	0.395
4	0.412	0.396	0.397
5	0.394	0.407	0.398
Mean ± SEM	**0.407 ± 0.004**	**0.404 ± 0.003**	**0.399 ± 0.002**
P-value		**0.467**	**0.066**

There was a decrease in the levels of PSA concentration between both low dose and high dose AMLE treated groups as compared to the control

group. However, these were not statistically significant. There was an observed greater decrease in the high dose group than the low dose group compared with the control group.

4.3.2 Macroscopic: Prostatic Index

AMLE reduced the mean size of the prostate in the test groups. Prostatic index (PI) (wet wt. of prostate gland / total b. wt. x 100) for the Control, LD and HD groups were 0.178 ± 0.086, 0.152 ± 0.075, 0.157 ± 0.061, respectively (Figure 4.8). Although there was a slight reduction in PI, these differences did not prove statistically significant. The LD group and HD group recorded p-values of 0.1735 and 0.1781 respectively compared with the control group.

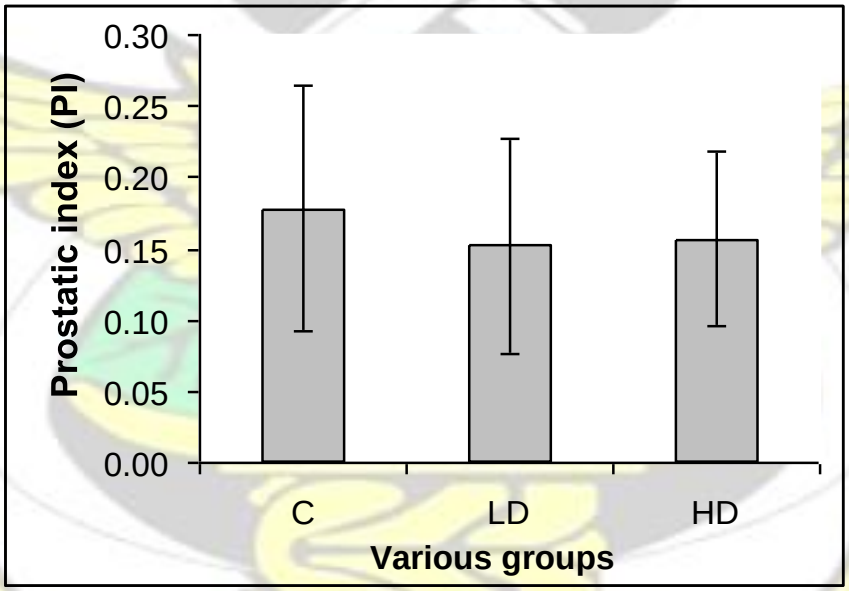


Figure 4.8: Prostatic indices for control (C), low dose (LD) and high dose (HD) rat groups. This figure demonstrates the reduction in prostatic index (PI) at day 60 of *A. muricata* administration. There was a statistically insignificant decrease in PI of control group compared with both the LD (30 mg/kg b.wt) and HD (300 mg/kg b.wt).

4.3.3. Macroscopic: Seminal Vesicle Index

The "seminal vesicle index" (SVI) (wet wt. of seminal vesicle / total b. wt. x 100) for the control, LD and HD were 0.437 ± 0.069, 0.184 ± 0.041 and 0.227 ± 0.052, respectively. The differences between SVI of control and treated groups were significant (Figure 4.9).

SVI was significantly reduced from 0.437 ± 0.069 (Control group) to 0.184 ± 0.041 (LD) and 0.227 ± 0.052 (HD). Thus, *A. muricata* caused about 50% relative reduction in the seminal vesicle size. Differences between control and LD as well as control and HD were statistically significant (*P = 0.004 and †P = 0.009, respectively).

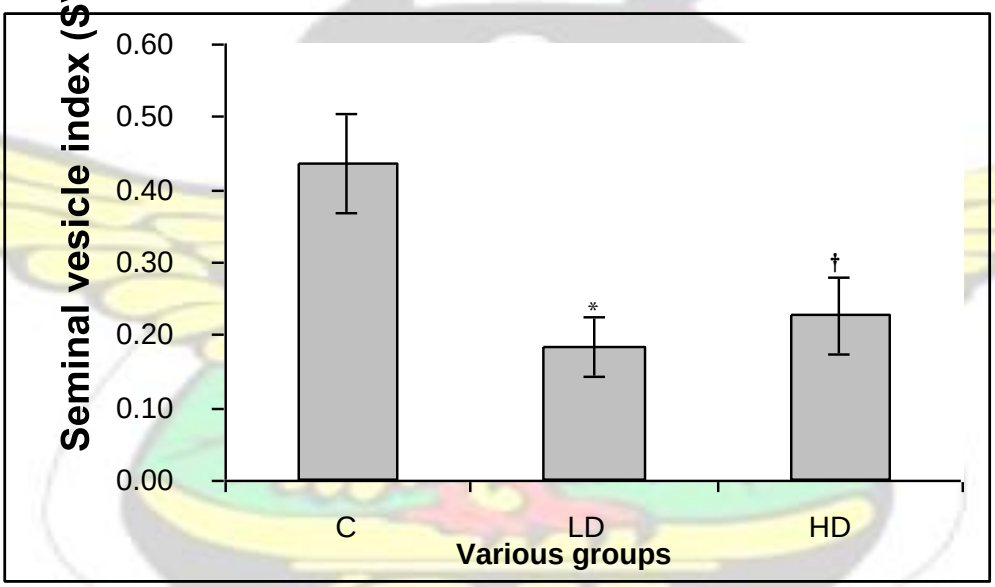


Figure 4.9: Seminal vesicle indices for control (C), low dose (LD) and high dose (HD) rat groups. Statistically significant decreases shown for both low and high dose groups compared with control group.

4.3.4 Microscopic: Histology of Prostate

The prostate of control rats fed on standard chow showed normal histological structures (Figures 4.6a & d). The alveoli showed tall columnar epithelial cells with an apparent high ratio of cytoplasm to nucleus. However, representative sections of the prostate tissues

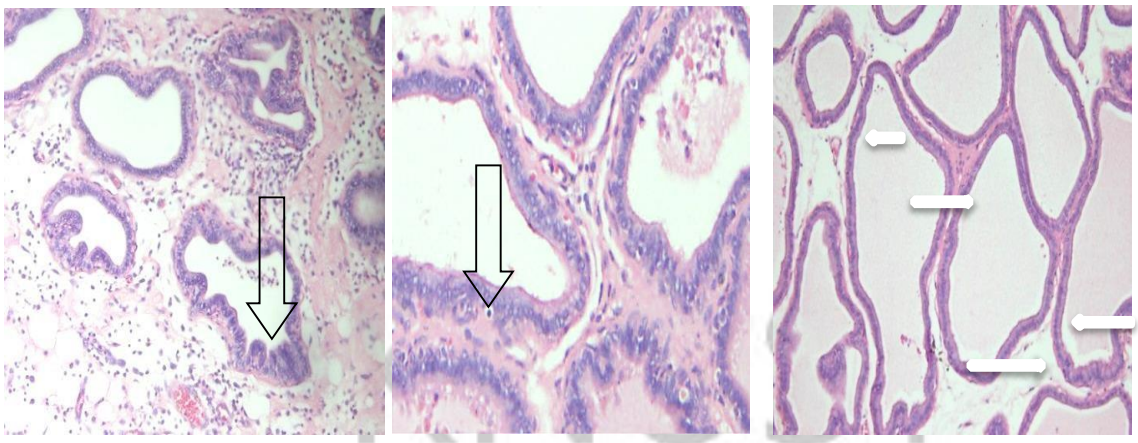
obtained from rats that were administered the low dose of AMLE showed apoptosis in the epithelium of the glandular acini (Figures 4.8b & e). The apoptotic processes involved discrete condensation of the chromatin, to finely outlined granular masses bordering the nuclear envelope, shrinkage of the cells, twisting of the cellular and nuclear outlines, and fragmentation of the nucleus. The affected cells disintegrated into membrane-bound apoptotic bodies that remained as a ghost space along with its neighboring cells. Furthermore, the cell membrane and the membrane encasing the apoptotic fragments

retained their integrity. However, there was no associated inflammation in the apoptotic areas. Rats that received low dose of the leaf extract showed marked reduction in cytoplasm and secretory activity of the acini. Compared to those of control prostate glands, representative sections of prostrate obtained from high dose group, showed dramatic decrease in prostatic fluid in the acinar lumens (Figures 4.8c & f). The lumens were empty and there were flattening of the internal lining of the acinar lumen. This led to an apparent decrease in the average cell number per unit area and this decreased the thickness of the prostate epithelium. Treatment with high dose of leaf extract for 60 days caused reduction of the stromal, acini size and shrinking of the epithelium, but it appeared flat with a decrease in the thickness of the fibro-muscular

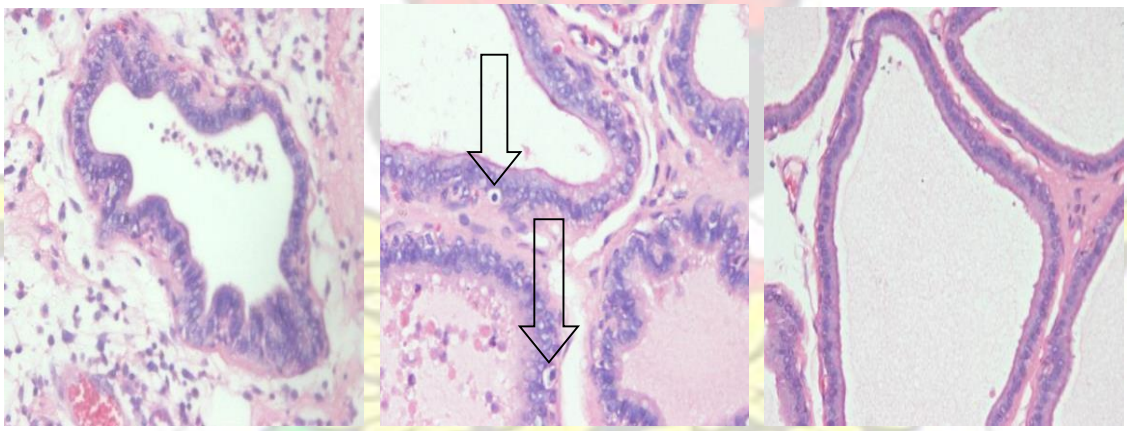
layer.

a. Ctrl (0 mg/kg b.wt) b. LD (30mg/kg b.wt) c. HD (300mg/kg b.wt)

Figure 4.10: (H&E x100). Sections of rat prostates post-60-day administration of: (a.) Control (Ctrl) group, no AMLE, but normal rat chow and water only. Photomicrograph shows normal glandular structure of the prostate. Microvilli are prominently conspicuous; (b.) 30 mg/kg body weight of AMLE/day (Low dose); condensation of nuclear material (apoptosis) of the glandular epithelium was noted (c.) 300 mg/kg body



weight of AMLE/day (High dose); scanty prostatic secretion with flattening of the acinar epithelial lining was noted. There is a significant reduction in the epithelial thickness.



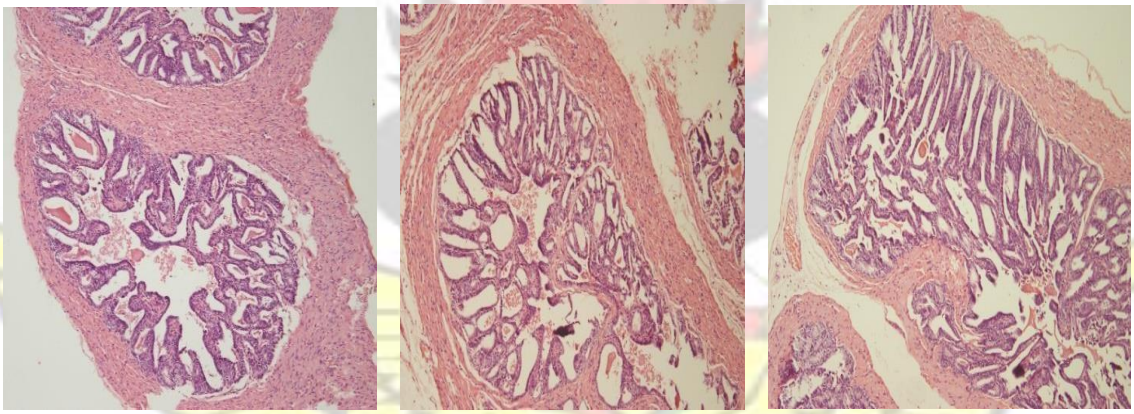
d. Ctrl (0mg/kg b.wt) e. LD (30mg/kg b.wt) f. HD (300mg/kg b.wt)

Figure 4.10: (H&E x400). Sections of rat prostates post-60-day administration of: (d.) no AMLE, but normal rat chow and water only. Conspicuous microvilli projection with an apparent high ratio of cytoplasm to nucleus was observed (e.) 30 mg/kg body weight of AMLE/day (Low dose); condensation and marginalization of the nuclear material was detected as indicated by arrows. (f.) 300 mg/kg body weight of AMLE/day (High dose) demonstrates pyknotic nuclei and decreased secretion in acini with flattening of the epithelial linings.

4.3.5 Microscopic: Histology of seminal vesicle

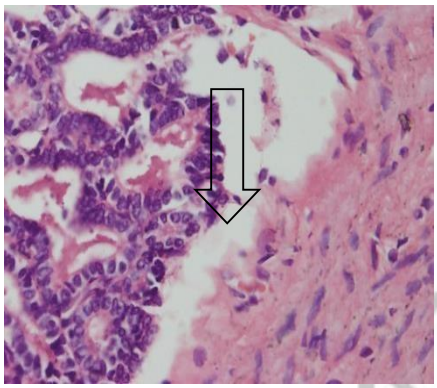
Microscopically, the acini of the seminal vesicle of the control rats had the normal structure where nuclei are basal and the cytoplasm appeared prominently eosinophilic (Figure 4.9a). Many of the cells showed

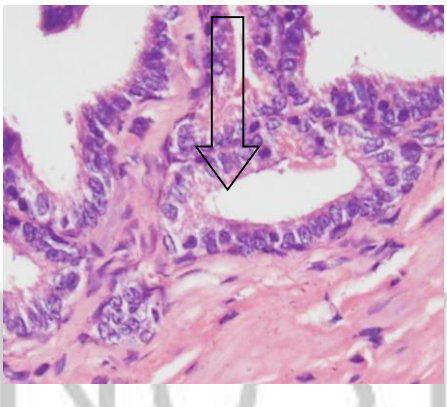
vacuolation in the cytoplasm which is the indication of maturity. However, both low and high dose levels of leaf extract resulted in atrophy and loss of secretion in the seminal vesicle (Figures 4.9b & c). The nuclei of the acinar cells appeared to be smaller, and structureless eosinophilic substances were found in the acini. The central lumen of the gland showed occasional pyknotic nuclei as seen in the lining epithelium, characteristic of apoptosis. Nonetheless, rats from either low and or high dose groups showed no evidence of inflammatory changes in the seminal vesicle (Figures 10d & e).



a. Ctrl (0 mg/kg b.wt) b. LD (30 mg/kg b.wt) c. HD (300 mg/kg b.wt)

Figure 4.11: (H&E x100). Sections of rat seminal vesicles post 60-day administration of: (a.) no AMLE, but normal rat chow and water only. Normal histology, with presence of pseudostratified epithelium of low cylindrical cells that were identified in the base line was seen; (b.) 30 mg/kg body weight of AMLE/day (Low dose). Thickening of the basal epithelium of connective tissue and reduction of secretion in the acini were apparent; (c.) 300 mg/kg body weight of AMLE/day (High dose). There was observed an increased cellularity and the acini empty of secretion. Marked atrophy of the seminal tissues was also noticed.





d. LD (30 mg/kg b.wt) e. HD (300 mg/kg b.wt)

Figure 4.11: (H&E x400). Sections of rat seminal vesicles post 60-day administration of: (d.) Fluid was perceived to have separated the epithelium from the submucosa (arrow). The nuclei are densely colored and packed; (e.) Epithelial lining was observed to show pyknotic nucleus (arrow).

4.3.6 Microscopic: Histology of Testes

In comparison with the control, representative sections of testes examined from rats fed on both low and high level of leaf extracts showed active spermatogenesis. Representative sections of tissues showed normal histological structures of the testes, expressed seminiferous tubules containing different kinds of germ cells; somatic Sertoli cells, spermatogonia, spermatozoa, spermatids and spermatocytes. The interstitial tissues found between seminiferous tubules showed interstitial cells and Leydig cells. There were no evidences of adverse effects of any kind including degenerative, inflammatory and or atrophic alterations in the testicular tissues.

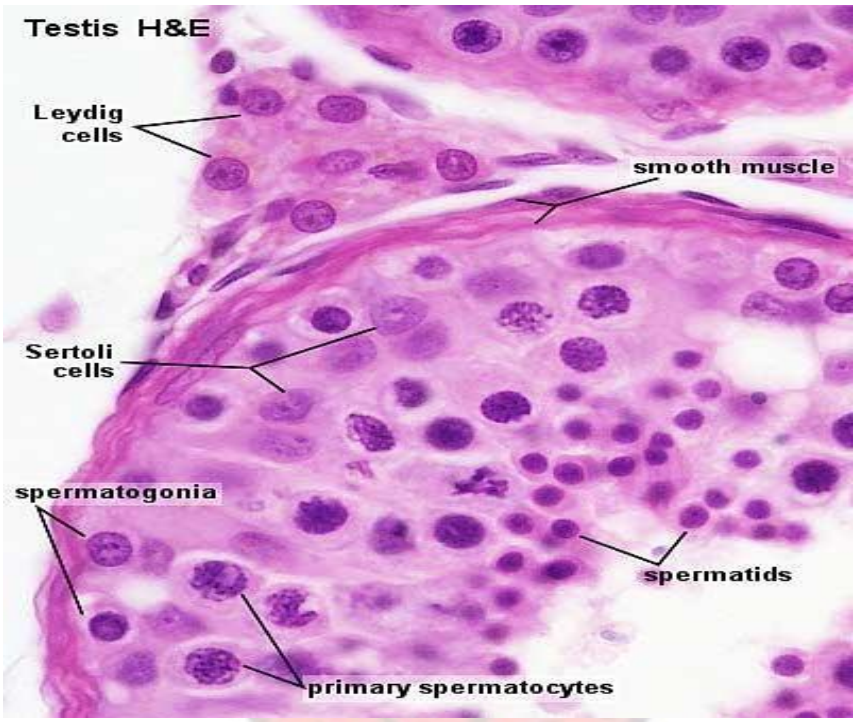


Figure 4.12: (H&E, x400) Hematoxylin and eosin stained histological sections of rat testes showing fully active spermatogenesis.

CHAPTER FIVE

5.0 DISCUSSION

5.1 DISCUSSION

The aqueous crude extract yield of *A. muricata* obtained in this study was 2.52 % (w/w) which is comparable to the yield of 2.62 % (w/w) obtained by Adewole and Caxton-Martins in 2006. A study using nbutanol as extraction solvent produced a 5.4 g yield from 50 g of leaf powder (George *et al.*, 2012). Among other factors, the yield is apparently higher with polar solvents. However, the total phenolic concentration in AMLE was reported to be nearly twice as high as that in the ethanolic extract. And the IC_{50} for *in vitro* antioxidant activity of that study was over two times

lesser in the aqueous extract (0.9077 mg/mL) compared with the ethanolic extract (2.0456 mg/mL) (Gavamukulya *et al.*, 2014).

The Rf values recorded for finger-printed HPLC for AMLE revealed 8 peaks within the range of 0.1 to 0.3. The HPLC fingerprint analysis is reported to be a diagnostic tool for the correct identification of plants (Seasotiya *et al.*, 2014). HPLC fingerprinting technique can be employed to provide semi-quantitative information about the major active phytoconstituents in a plant extract. This makes it useful for the assessment of plant extract quality. In this study, however, the technique was employed solely for the qualitative purpose of the authentication of the plant extract as well as monitoring the purity profile of various batches, and not for the chemical identification of peaks. Medicinal plant extract profiling is necessary and critical for establishing the authenticity and quality of the plant. This ensures results produced are truly representative of the medicinal plant being verified (Chatterjee *et al.*, 2010). The adopted practice of re-evaluating any new batch of extract received, serves as a reliable indicator of identifying genetic variability in the plant populations and as a marker for the active phytochemicals of the plant (Maier, 2010). Other researchers have employed similar practices for other species including DNA finger printing of *Annona squamosa* Linn. (Abhishek *et al.*, 2009) and four *Annona species* (Ahmad *et al.*, 2010; Elhawary *et al.*, 2013).

Both the cell viability and MTT assays produced similar patterns where BPH-1 cell proliferation was reduced with increasing doses of AMLE (0 mg/mL <0.5 mg/mL <1.0 mg/mL <1.5 mg/mL) (figures 4.2 and 4.3). The decrease in cell density with increasing concentration of AMLE as revealed by the cell viability study is a direct effect of the antiproliferative

effect of *A. muricata.* Furthermore, the morphology of the cell remained unchanged, thus suggesting that the cell growth inhibition is attributable to direct cytostatic rather than cytotoxic effect of AMLE (Oberlies *et al.*, 1997). However, the antiproliferative effect of *A. muricata* on HL-60 cells was reported to both exhibit loss of cell viability as well as change cell morphology (Pieme *et al.*, 2014). High-level consumption of soursop has furthermore been reported to distort the cytoarchitecture of adrenal glands in Wistar rats (Ezejindu *et al.*, 2014).

The MTT assay confirmed the dose dependent decrease of BPH-1 cell proliferation by *A. muricata* with the largest decrease by the highest dose of 1.5 mg/mL through 1.0 mg/mL and 0.5 mg/mL from the nontreatment (control) level of 0 mg/mL. At 0.5 mg/mL level of AMLE, the reduction was very close to showing statistical significance by the first 24hrs of incubation ($p = 0.0577$). The high potency of AMLE against cell proliferation was still established by the very high level of significance showed for the dose of 1.0 mg/ml and 1.5 mg/mL ($p = 0.0024$ and $p < 0.0001$ respectively). The doses of 1.0 mg/mL and 1.5 mg/mL showed a significant reduction at all incubation times. The IC_{50} measured for this experiment was 1.36 mg/mL. The high IC_{50} value of *A. muricata* reveals why *A. muricata* is considered as not being cytotoxic to normal cells (Ragasa *et al.*, 2012). An unexpected greater reduction was obtained at 24 hrs (41%) than at 48 hrs (48%) at the highest dose of 1.5 mg/mL. The statistical difference found for this unexplainable observation was rather highly significant ($p = 0.0002$). Nonetheless, at the same concentration (1.5 mg/mL), the lowest reduction in cell density was obtained at the highest incubation time of 72hrs (30.5%). This study thus provides

evidence that *A. muricata* leaf extract significantly inhibits BPH-1 cell growth *in vitro* by 48hrs.

The growth inhibition of the BPH-1 cell line may indicate a potential capability of AMLE to exert an inhibitory effect to human prostate tumour cell proliferation due to the presence of acetogenins. Such cytostatic activity of acetogenins in the leaves of *A. muricata* has been verified in tumor cell growth. This includes an *in vitro* demonstration against Adriamycin resistant human mammary adenocarcinoma MCF7/Adr cells *in vitro* (Oberlies *et al.*, 1997). *A. muricata* has been reported to induce apoptosis in the PCa cell lines LNCaP, DU-145 (Onozawa *et al.*, 1998; Bektic *et al.*, 2004; Ouchi *et al.*, 2005). Acetogenins in *A. muricata* leaves have been shown to also exhibit cytotoxic activities in the human prostate adenocarcinoma cell line, PC-3 among others (Kim *et al.*, 1998). Varying amounts of the biologically active agent present in Annonaceous acetogenins have been reported in the leaf, stem, bark and seeds of *A. muricata* (Tormo *et al.*, 2003; Kojima, 2004).

Other aqueous plant extracts have been reported to exert inhibitory effects on cell growth and colony formation of prostate cancer *PC-3* cells. *Artemisia vulgaris* demonstrated 8-65% inhibition at similar doses as *A. muricata* for 24 hrs. Correspondingly, an increased cell growth inhibition after incubation of these cells from 24 to 72 hrs at various doses was observed (Nawab *et al.*, 2011). Both the purified annonacin and crude ethanolic extract have been shown to reduce the relative viability of cortical neurons by 50% at 48 hours post treatment. And the toxicity of annonacin was reportedly enhanced in the presence of crude extract (Potts, 2012).

MTT assays of various solvent extracts of *Lasienthera africanum* have demonstrated anti-proliferative effect on prostate *PC-3* epithelial cell lines. The aqueous leaf extract produced an 18.8% more survival ability compared to acetone extract. The crude compound was furthermore shown to exert a greater effect on prostate epithelial cells (Matheen *et al.*, 2012). The peel extract of *Punica granatum* L. var. spinosa (pomegranate) dose dependently suppressed the proliferation of *PC-3* cells at an IC_{50} of 0.25 mg/mL by an apoptotic process that causes DNA strand breaks that have been detected qualitatively through TUNEL assay (Sepehr *et al.*, 2012). The ethanolic neem leaf extract (ENLE) was also shown to produce 50% inhibition at an effective dose of 0.1 mg/ml in both PC-3 and LNCaP cells. It has been postulated that ENLE causes apoptotic induction by impeding cell proliferation via the PI3K/Akt pathway in LNCaP and PC-3 (Gunadharini *et al.*, 2011). Extracts of Dandelion leaf (DLE) (genus: *Taraxacum*) are also reported to block the invading LNCaP cells into collagen type I (Sigstedt *et al.*, 2008). The viability of LNCaP C4-2B cells in an MTT assay showed a significant decrease to 60.5±2.1% at 0.05 g/ml within 24 hrs. However, DLE did not affect the increase in LNCaP C4-2B cells. Other acqueous extracts that were prepared from dandelion root as well its flowers also did not increase the viability of LNCaP C4-2B cells (Sigstedt *et al.*, 2008). *A. muricata* has been widely acclaimed in most areas where it is found as showing potency for cancer treatment.

Several studies have established that the expression of the antiapoptotic protein Bcl-2 is inhibited whereas the expression of the proapoptotic proteins Bax and Bak are enhanced in cell lines undergoing apoptosis. PCR results in this study demonstrated a decrease in band intensity for

the Bcl-2 gene whereas band intensity for Bax gene was increased with increasing concentrations of AMLE from 0 mg/mL to 1.5 mg/mL. Imbalance of molecular mechanisms of proliferation and apoptosis has been found to be associated with the development of BPH and its related cancers, with evidence of reduction of apoptosis as a major underpinning factor. In this study the aqueous leaf extract of *A. muricata* induced an up-regulation of Bax but a down-regulation of Bcl2 in BPH-1 cells (Figure 4.6). The up- and down-regulations of Bax and
Bcl-2 respectively suggest that the acetogenins in AMLE may effect the killing of cells via an apoptotic mechanism. It also suggests that *A. muricata* may possess anticancer properties purportedly observed in some studies (Yuan *et al.*, 2003; Moghadamtousi *et al.*, 2015). *A. muricata* leaf has indeed been reported to have acetogenins that induce apoptosis (Astirin *et al.*, 2013).

Apoptosis involves a cascading sequence of cellular events including: condensation of the chromatin, fragmentation of DNA, blebbing of the cytoplasmic membrane and then shrinkage of the cell (Bøe *et al.*, 1991). Various initiator and executor caspases are involved. Anticancer agents with the capability of inhibiting poly ADP-ribose polymerase (PARP) and other substances are reported to make them susceptible to the cleaving action of caspase-3 in response to DNA strand breaks leading to apoptosis (Nicholson *et al.*, 1997; Mancini *et al.*, 1998). A study involving the use of *Artemisia vulgaris* indicated that its extracts caused caspase-3 activation that resulted in PARP cleavage (Nawab, 2011).

Failure of the regulation of apoptosis is said to be the key principle to the success of carcinogenesis (Mayer and Bukau, 2005). Annonacin,

considered the main *A. muricata* acetogenin, is documented as being able to cause cell death by apoptosis in the G1 phase of the cell cycle. The mechanism involves promoting pro-apoptotic protein (Bax and Bad) expression, as well as the p53 and p21 whiles inhibiting the antiapoptotic protein (Bcl-2 or Bcl-xL) expression (Yuan, 2003). A recent study using an ethyl acetate extract of *A. muricata* leaf produced a higher p53 expression indicating a greater apoptotic activity; and a lower hsp70 expression indicating a more stable homeostatic regulation in Raji cells (Astirin *et al.*, 2014). Another study employing immunofluorescence analysis using the same ethyl acetate leaf-extract revealed an increase in Bax expression with a decrease in Bcl-2 expression as the underlying mechanism of the anticancer properties of the extract against the HT-29 and HCT-116 colon cancer cell lines (Moghadamtousi *et al.*, 2014).

The cell death via apoptosis is purportedly achieved generally by the diminution of the amount of ATP through inhibiting complex I in the mitochondrial electron transport systems and NADH oxidase of plasma membranes of the tumor cells, causing oxidative phosphorylation at the ubiquinone-catalytic sites in complex I (Yuan *et al.*, 2003; Kojima *et al.*, 2010). The ubiquinone-linked NADH oxidase processes are only found in the membranes of cancerous tumor cells thereby making acetogenins, particularly annonacin, mostly toxic to cancer cells, including multi-drug resistant (MDR) cancer cells, but non-toxic to noncancerous cells (Rieser *et al.*, 1991; Zeng *et al.*, 1996; Tormo *et al.*, 2000). By 1995, it was reported that fourteen (14) different acetogenins tested demonstrated ATP-blocking activity as well as potency against proven MDR cancer cells (Oberlies *et al.*, 1995). Recent studies still support the claim that the

apoptotic effects are attributable to acetogenins. *A. muricata* ethyl acetate leaf extract was reported to cause an attenuation of mitochondrial membrane potential (MMP) via upregulation of Bax and down-regulation of Bcl-2 in human lung A549 cell lines (Moghadamtousi *et al.*, 2014). However, another recent study posits the principle that underlies the chemotherapeutic activity of *A. muricata* is the synergism found in its phytochemicals including the acetogenins (Pieme *et al.*, 2014).

Aside acetogenins, other phytotherapeutic agents have been shown to induce apoptosis through the Bax/Bcl-2 regulation. The natural cyanide-containing substance, Amygdalin, abundantly found in the seeds of plants of the prunasin family, is reported to induce cell death via apoptosis in the PCa cell lines DU145 and LNCaP by caspase-3 activation via up-regulating the Bax whiles down-regulating the Bcl-2 proteins (Chang *et al.*, 2006). *Commiphora mukul*, used in Indian Ayurvedic medicine possesses the extract gugulipid which induces the proapoptotic proteins Bax and Bak, and decreases the apoptosisinhibiting protein Bcl-2 in the PCa cell LNCaP (Xiao *et al.*, 2011). The medicinal plant *Withania somnifera* contains Withaferin A (WFA), a withanolide that reportedly generates apoptotic activity in human melanoma cells by producing reactive oxygen species as well as inhibiting Bcl-2 (Mayola *et al.*, 2011). Neem leaf extract (NLE) is reported to possess a tetranorterpenoid limonoid, nimbolide, which induces significant apoptosis by preferentially binding to the apoptosisinhibiting protein Bcl-2 as the putative target. This was shown in preclinical models of Waldenströms macroglobulinemia (Chitta *et al.*, 2014).

The observed decrease in the size of the prostate of rats treated with

AMLE compared to controls did not prove to be statistically significant (P = 0.1735 and 0.1781 for statistical differences between control group, LD and HD groups respectively). Post-hoc analysis following the Medical Therapy of Prostatic Symptoms (MTOPs) trial demonstrated that decreasing prostate volumes to levels of less than 25 ml in humans causes the difference in prostate volume between treatment and placebo groups to also decrease; howbeit, to a level where there was no statistically significant difference observed.

However, in the Combination of Avodart and Tamsulosin (COMBAT) trials using men with prostate volume greater than 30 ml and IPSS of 12 and above, it was concluded that therapy had significantly improved symptom scores and urinary flow rates. Compared with monotherapy, COMBAT also reduced the relative risk of acute urinary retention (AUR) (Heidenreich *et al.*, 2013).

In this study, there was no significant difference shown between the prostate volumes of treated and control groups presumably because the normal-sized, non-hyperplastic prostates of rats used were not large enough to show significant difference in the level of decrease in prostate volumes resulting from AMLE treatment. Several studies have reported the selectivity in cytotoxicity of *A. muricata* against cancerous cells whilst being non-toxic to normal cells (Rieser *et al.*, 1991; Zeng *et al.*, 1996; Ragasa *et al.*, 2012). The chloroform extract of *A. muricata* was reported to be only moderately cytotoxic to normal cells (WRL-68 normal human hepatic cells) (George *et al.*, 2012). It can be inferably postulated that the decrease in prostate volume may be more pronounced or demonstrated

more significantly in larger-sized hyperplastic prostates and probably cancerous prostate cells. Besides, acetogenins in *A. muricata* have been shown to act by a mechanism that foils ATP dependent resistance in cancerous cells; the absence of this resistance mechanism in normal cells makes *A. muricata* exert a lesser effect on them (Alali *et al.*, 1999; Tormo *et al.*, 2000).

Microscopically, representative sections of tissues of prostate obtained from rats that received low dose of leaf extract showed apoptosis in the epithelium of the glandular acini (Figures 4.7c & d). Representative sections of high dose group rats showed dramatic decrease in prostatic fluid in the acinar lumens with flattening of the internal lining of the acinar lumen. This could possibly be due to a reduction of gonadotropin output as a result of alterations in prostate gland, function and form of seminal vesicle and accessory organ consequent to apoptosis. It has already been verified that changes in those organs could result from reduced gonadotropin output which is vital to steroidogenesis (Hagras *et al.*, 2008).

From this study, representative sections of prostate tissue of AMLE treated rat groups also showed characteristics including discrete condensation of the chromatin, to finely outlined granular masses bordering the nuclear envelope, shrinkage of the cells, twisting of the cellular and nuclear outlines, and fragmentation of the nucleus. Evidently, the tissue features emerged from apoptotic events in the epithelium of the glandular acini (Figures 4.7c & d). The affected cell disintegrated into membrane-bound apoptotic bodies that remained as a ghost space along with its neighboring cells. Furthermore, cellular

membranes as well as the membranes surrounding the apoptotic fragments were unchanged, and there was no associated inflammation in the apoptotic areas. The histological changes observed in the prostate of rats given 30 and 300 mg of leaf extract of *A. muricata* apparently agree with findings from previous studies where methanolic and or ether extracts of *C. colocynthis* Schrad (Dhanotiya *et al*,. 2009) and *Citrullus lantatus* (Olamide *et al.*, 2011) were used and epithelial cells showed a less involuated histo-architecture. The decreased epithelial height and secretory activity also agrees with the findings of Londonkar *et al.* (2000). Similar minimization of spaces between prostate acini as observed in this study was seen by Ghlissi *et al.* (2012) using *Habbatus sauda* seeds. Another study that reported an improvement of acini in cancer–induced male rats using *Crateva nurvala* also demonstrated a reduction in the spaces between prostatic acini (George *et al.*, 2012).

The seminal vesicle index (SVI) recorded for the control, LD and HD were 0.437 ± 0.069; 0.184 ± 0.041 and 0.227 ± 0.052, respectively.

Thus, *A. muricata* achieved about 50% relative reduction in the SV size. Statistical differences between control and LD, and control and HD were significant (P = 0.004 and 0.009, respectively) (Figure 4.8). The observed greater decrease in SVI of the low dose group than that of the high dose group could not be explained. However, a highly increased cellularity was observed microscopically for the high dose group, with reduced acini secretions seen in both groups. The weight of the vesicles is the combined weight of the organ as well as the secretions in it.

Microscopically, representative sections of SV tissue obtained from rats that received low dose of leaf extract showed marked reduction in cytoplasm and secretory activity of the acini (Figure 4.8). Both low and high dose levels of AMLE resulted in atrophy and loss of secretion in the seminal vesicle (Figures 4.8b & c). The characteristic features of reduced acinar cells with structureless eosinophilic substances, occasional pyknotic nuclei seen in the epithelium lining as well as the absence of evidence of inflammatory changes in the seminal vesicles of both low and high dose rat groups affirm the assertion of an apoptotic related cellular activity, a direct effect of AMLE treatment.

The changes in the seminal vesicle showed accumulation of fluid in the submucous layer. In the case of the seminal vesicle, the immediate cause of apoptosis observed is likely due to accumulation of fluid deep into the sub-mucous layer and thus isolated epithelial cells must have been deprived of nutrients. A similar effect was produced in the vas deferens of mouse by the administration of oestrogen (Harsh *et al.*, 1939). In rats, increased secretory activity of the seminal vesicle is associated with increased testosterone production followed by increased seminal vesicle weight (Higgins and Burchell, 1978; Zanato *et al.*, 1994).

Indeed, the enzyme that catalyzes the conversion from testosterone to dihydrotestosterone (DHT) – 5-alpha reductase – has a very high activity in the seminal vesicle. DHT is important for the development of the prostate. However, it is also responsible for the pathologic growth of the prostate. DHT binds to androgen receptors with subsequent modulation of target genes causing BPH and its related cancer (Bartsch *et al.*, 2002). To arrest BPH and the development of cancer, 5 alpha-reductase

inhibitors are administered to act as pathologic substrates of the disease, thereby arresting the disease, reducing the prostate volume and improving symptoms (Andriole *et al.*, 2004).

Although, in the present study, androgen levels in the rats were not measured, it is however expected that it would highly influence the development of the reproductive organs. It has been long established that estrogen may act closely with androgens in the development of accessory sex organs. Androgens stimulate the epithelia, and estrogens, the connective tissue (Clegg, 1953). An increased estrogen:androgen ratio that induces proliferation of prostatic stromal cells had previously been demonstrated. This has been adduced as a key explanation for BPH development (King *et al.*, 2006). Inferentially, the significant decrease in SVI in the treatment groups may indicate a reduction in DHT thus the shrinkage of the prostate as seen in the reduced PI of the treated groups.

Cell apoptosis from prostate and seminal vesicle do not appear to have been described for rats fed plant extracts. However, this study showed apoptosis in the glandular epithelium in both prostate and seminal vesicles. It would seem that the onset of apoptotic effect in the seminal vesicle may not necessarily correlate with the appearance of prostatic cell apoptosis, although the nature of induction of the effect may well be changed in the latter organ, a matter that needs a more rigorous investigation. It is worth noting that an effective BPH treatment should be aimed at realizing a decrease in the stromal as well as the glandular components (Habenicht *et al.*, 1993).

In comparison with the control, representative sections of testes examined from rats fed on both low and high level of leaf extracts showed active spermatogenesis (not shown in the figure). Representative sections of tissues revealed unchanged histology with respect to structures of the testes. They expressed seminiferous tubules containing different kinds of germ cells; spermatocytes, spermatogonia, spermatozoa, spermatids, and somatic Sertoli cells. The interstitial tissues found between seminiferous tubules showed interstitial cells and Leydig cells. There was no evidence of adverse effects of any kind including degenerative, inflammatory and or atrophic alterations in the testicular tissues. Indeed the anti-inflammatory activity of *Annona muricata* leaves has been evaluated and reported (Foong and Hamid, 2012). In addition, the absence of ill effects toward spermatogenesis in the rats so gavaged, suggests that *A. muricata* would have an advantage over other plant extracts so far used to prevent prostate hyperplasia (Torres, 2012).

The difference obtained in the biochemical analyte PSA, between the AMLE treated rats and controls was not statistically significant. PSA is produced by normal prostate cells. *A. muricata* is known not to appreciably affect normal cells. PSA is normally localized in the prostate region and only minute levels are found in the blood. An elevated plasma PSA level would often result from conditions such as prostatitis, BPH and PCa that can cause a disruption of prostate cell architecture thereby allowing for an abnormally high release of the protein into circulation (Wang *et al.*, 1979).

In this study rats with normal prostate were used. *A. muricata* is well known to be very benign in its effect towards normal cells (George *et al.*,

2012; Ragasa *et al.*, 2012). Thus the insignificant decrease in PSA levels for both LD and HD rats as compared to those of controls adds credence to the claim that *A. muricata* is not appreciably deleterious to normal cells even at high doses.

CHAPTER SIX

6.0 CONCLUSION, LIMITATION & RECOMMENDATION

6.1 CONCLUSION

AMLE exerts a cytostatic effect on BPH-1 cell proliferation. The unchanged morphology seen in the cell suggests that the cell growth

inhibition is attributable to direct cytostatic rather than cytotoxic effect of AMLE.

AMLE has anti-proliferative and pro-apototic effects on BPH-1 cells. The study revealed that it up-regulates the pro-apototic protein Bax whiles it down-regulates the anti-apoptotic protein Bcl-2. Its antiproliferative property in the prostate earmarks it as a plausible candidate drug for the treatment of both BPH and related cancers. The evidence presented here on the aqueous leaf extract of *A. muricata* provide the basis for further studies using BPH animal models and possibly prostate cancer induced animals to ascertain its anticancer activity *in vivo*.

The biochemical function of the prostate to produce PSA was minimally affected by the administration of AMLE. Furthermore, normal prostate volume is not significantly reduced by AMLE administration. Howbeit, the epithelia and stroma of prostate cells are susceptible to damage, and the cell susceptible to killing by AMLE treatment.

6.2 LIMITATION

1. BPH-induced rats were not used in this study, thus the effect of AMLE on BPH-1 cells was not shown in-vivo.
2. The levels of other biochemical markers like testosterone, dihydrotestosterone and 5-alpha reductase which could be correlated with prostatic and seminal vesicle indices were not measured.

6.3 RECOMMENDATIONS

1. Another study using BPH-induced rats should be performed to show directly the effect of AMLE on BPH-1 cells in-vivo.
2. The levels of biochemical markers such as testosterone, dihydrotestosterone and 5-alpha reductase should be analyzed along PSA in rats, and correlated with prostatic and seminal vesicle indices to further elucidate the likely mechanism of the apoptotic effect of AMLE.

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APPENDIX

IN VITRO DATA

MTT ASSAY

24 hrs

	Concentrations			
	0mg/mL	**0.5mg/mL**	**1mg/mL**	**1.5mg/mL**
1	93.136	81.473	72.413	39.690
2	98.482	99.837	82.758	40.419
3	119.341	83.013	63.480	40.685
4	87.093	85.747	65.543	39.971
5	103.499	77.427	75.575	44.319
Mean	**100.310**	**85.499**	**71.954**	**41.017**

48 hrs

	Concentrations			
	0mg/mL	**0.5mg/mL**	**1mg/mL**	**1.5mg/mL**

1	108.298	85.433	72.513	46.620
2	102.153	79.017	64.411	48.565
3	84.570	72.053	59.304	46.868
4	99.136	81.926	62.130	50.216
5	104.860	80.939	65.134	49.875
Mean	**99.803**	**79.873**	**64.698**	**48.429**

72 hrs

	Concentrations			
	0mg/mL	**0.5mg/mL**	**1mg/mL**	**1.5mg/mL**
1	104.989	76.115	53.315	30.770
2	99.221	77.867	56.127	29.997
3	99.039	75.521	52.587	29.424
4	100.534	78.739	51.646	31.541
5	98.122	76.023	55.236	30.812
Mean	**100.381**	**76.853**	**53.782**	**30.509**

***IN VIVO* WEIGHT MEASUREMENTS**

Control (C)

	Terminal	**Prost Wt**	**Sem Ves**	**PI**	**SVI**
C1	270	0.35	1.41	**0.159**	**0.522**
C2	270	0.4	0.96	**0.160**	**0.356**

C3	265	0.43	1.18	**0.179**	**0.445**
C4	278	0.29	1.18	**0.130**	**0.424**
C5	268	0.36	1.15	**0.155**	**0.429**
Avg	**270.2**	**0.366**	**1.176**	**0.157**	**0.44**

Low (L) Dose

	Terminal	Prost Wt	Sem Ves	PI	SVI
L1	230	0.42	0.3	**0.183**	**0.130**
L2	210	0.29	0.39	**0.138**	**0.186**
L2	260	0.29	0.5	**0.112**	**0.192**
L4	240	0.42	0.55	**0.175**	**0.229**
L5	235	0.36	0.44	**0.153**	**0.187**
Avg	**235**	**0.356**	**0.436**	**0.152**	**0.18**

High (H) Dose

	Terminal	Prost Wt	Sem Ves	PI	SVI
H1	220	0.61	0.59	**0.226**	**0.268**

H2	250	0.42	0.43	**0.156**	**0.172**
H3	240	0.45	0.66	**0.170**	**0.275**
H4	223	0.45	0.43	**0.162**	**0.193**
H5	233	0.48	0.53	**0.179**	**0.227**
Avg	**233.2**	**0.482**	**0.528**	**0.178**	**0.23**