KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

THE EFFECT OF *CROTON MEMBRANACEUS* ON THE HORMONE-DEPENDENT PATHWAY IN BENIGN PROSTATIC HYPERPLASIA

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY IN CHEMICAL PATHOLOGY

In the

Department of Molecular Medicine,

School of Medical Sciences

By

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DEDICATION

To mum, dad and to all the men who dedicated themselves to this project

. DECLARATION

I, Bernice Asiedu of the Department of Chemical Pathology of the Kwame Nkrumah University of Science and Technology, do hereby declare that, with the exception of the quoted articles and references, this project work was duly carried out by me and the results obtained herein are a true reflection of the work done under the supervision of Dr. George Asare and Dr. Robert Ngala

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LIST OF ABBREVIATIONS

AED	_	Androstenedione
AR	–	Androgen receptor
5 ARI	–	5 Alpha reductase inhibitors
5 AR	–	5 Alpha reductase
ARE	_	Androgen response element
3α-adiol	_	Androstanediol adiol
3α-adiol-G	_	3 Alpha-adiol glucuronide

3β-diol	_	Androstanediol diol
AKRIC1	_	aldo-keto reductase family 1, member C1
AKRIC2	_	aldo-keto reductase family 1, member C2
AUR	_	Acute urinary retention
BPH	–	Benign prostatic hyperplasia
CAM	–	Complementary and Alternative Medicine
CM	–	*Croton membraneceus*
DHT	–	Dihydrotestosterone
DRE	–	Digital rectal examination
EGF	_	Epithelial growth factors
EIF	_	Epithelial inhibitory factors
ER α	–	Estrogen alpha receptor
ER β	–	Estrogen beta receptor
ESR	_	Estrogen response element
fPSA	_	Free prostate specific antigen
FT	_	Free testosterone
GPR 30	_	Intracellular G-protein coupled receptor
HSD	_	Hydroxysteroid dehydrogenase
IPSS	–	International Prostrate Symptom Score
IGF	_	Insulinlike growth factors
LUTS	–	Lower urinary tract symptoms
MTOPS	_	Medical therapy of prostatic symptoms studies
PCA	–	Prostate cancer

PSA	–	Prostate specific antigen
SERM	–	Selective estrogen receptor modulators
SHBG	_	Sex hormone binding globulin
SRD5A1	_	Steroid 5-alpha-reductase type 1
T	_	Testosterone
t.i.d.	-	"ter in die" (Latin) 3 times a day).
TMPRSS 2	_	Transmembrane protease serine 2
TNFA	_	Tumour necrosis factor α
tPSA	_	Total prostate specific antigen
TURP	–	Transurethral resection of the prostate

ABSTRACT

Current pharmacotherapeutic strategies modulate the active hormones involved in androgen biosynthesis as androgen estrogen imbalance has been implicated in the aetiology of benign prostatic hyperplasia (BPH). However, their usage has recorded adverse side effects even after discontinuation. Attention has therefore shifted to complementary and alternative medicine (CAM). *Croton membranaceus* (CM) has been in use for the management of benign prostatic (BPH) in Ghana for decades, yet its precise mechanism of action in humans is yet to be proven. This study therefore sought to elucidate the effect of CM on the sex steroid hormone dependent pathway.

The study population consisted of 30 BPH patients between the ages of 46-87 years and 30 non-BPH men between the ages of 46-72 years served as controls. BPH patients on CM were observed for 12 weeks. Data were collected at baseline and after 3 months of treatment with the drug. Urologic parameters such as lower urinary tract symptoms

(LUTS) and prostate volume (PV) were assessed via the International Prostate Symptom Score and ultrascan, respectively. Hormones [testosterone (T), free testosterone (FT), androstenedione (AED), dihydrotestosterone (DHT), estrone (E1) and estradiol (E2)] and their metabolites [androstanediol Adiol (3α-adiol) and androstanediol diol (3βdiol)] as well as total and free prostate specific antigens (tPSA and fPSA)

were assayed by enzyme immunoassay (ELISA) techniques. Non-

parametric analyses were performed as data were not normally distributed. At baseline, median concentrations of T, FT, DHT, 3α-adiol and 3β-diol did not differ between BPH patients and controls. However, AED (pvalue=0.027) and E2 (p-value = 0.029) levels were significantly decreased and increased, respectively, in controls. Enzyme indices for aromatase activity (E1/AED) and aldo-keto reductase family 1, member C1 (AKR1C1) which was calculated as 3β-diol/DHT were significantly lower (p = 0.014 and 0.036, respectively) in controls. In BPH patients, age correlated positively with tPSA (p=0.049, r=0.348). Prostate volume and fPSA associated negatively with FT (p=-0.01, r=-0.514). IPSS had an inverse relationship with 3β-diol (p=0.00, r=-0.656) and with AKRIC1 (p=-0.519, p=0.02). Treatment with CM significantly increased DHT (p-value =0.005.) and decreased E1 (p-value =

0.009). Median concentrations of AED, T, 3αadiol and E2 decreased while FT increased non-significantly3α-adiol/DHT reflecting aldo-keto reductase family 1, member C2 (AKRIC2) activity decreased significantly. Other enzyme indices DHT/T, E1/AED, E2/T, 3βdiol/DHT were unchanged. CM significantly decreased IPSS, PV, tPSA and fPSA by 28.3%, 31.4%, 31.3% and 17.6%, respectively. Percentage change observed in IPSS correlated negatively with increment in 3β-diol, while that observed in tPSA and free PSA associated with the change induced in the level of FT and DHT by the plant extract. CM is slow acting but effective for the management of BPH. CM modulates the androgens in the sex steroid pathway of BPH patients by inhibiting the activity of enzymes involved in the conversion of DHT into its androgenic metabolite (3α-adiol).

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND INFORMATION

Benign prostatic hyperplasia (BPH) is histologically defined as progressive stromal and epithelial cell proliferation in the transition zone of the prostate (Lepart, 2014). Clinically, it presents as enlarged prostate associated with lower urinary tract symptoms (LUTS) such as unfinished emptying, urinary hesitancy, weak stream, frequency and urgency, and may have a significant impact on the quality of life (Lephart, 2014). The incidence of the condition appears to have a linear relationship with age. It occurs in about 10% of men below the age of 40 and increases to about
80% by 80 years of age (Garg *et al.*, 2013). It is estimated that by age 60, 30% will need surgical intervention to avoid obstructive uropathy (Toshiro *et al*, 2004). The cause of BPH and LUTS remains unknown; however, the sex steroid hormone-dependent pathway is implicated.

Prostate development and function are both controlled by testicular hormones. Testosterone (T) is the major androgen secreted by the fetal and adult testis. It serves as a precursor for the formation of the potent metabolite in target tissues, dihydrotestoterone (DHT) via 5α reductase (5AR) enzymes. DHT is the mediator of most androgen effects in the male physiology (Wilson, 2011) and the signaling between DHT and androgen receptor (AR) induces prostatic growth. The management of BPH via

modulation of this hormone as well as its enzymes has yielded remarkable results (Barkin, 2011; Loke *et al.*, 2013; Mathur *et al.*, 2014).

Androgens are considered to play a 'permissive' role in the induction of BPH as several studies have demonstrated the condition occurs only in the presence of both estrogen and androgens (Risbridger *et al.*, 2003; Ho *et al.*, 2008; Ricke *et al.*, 2008). It is argued that reduction in testosterone is a catabolic process and does not stimulate growth or endothelial dysfunction suggesting that androgens cannot be responsible for the condition (Nicholson and Ricke, 2011; Williams, 2012). Estrogen levels remain stable with aging but plasma testosterone falls due to testicular activity/ function (Prezioso *et al.*, 2007). The consistent level of estrogen has been attributed to aromatase activity of adipose and muscle tissue converting estrogen to testosterone. Estrogen-responsive tissues contain aromatase p450 in their endoplasmic reticulum, adjacent to genomic estrogen receptors indicating that compounds that increase the activity of aromatase will eventually upregulate the intracellular biosynthesis of estradiol (Williams, 2012). Imbalance in estradiol-17β is generally accepted as a principal cause of clinical BPH (Prezioso *et al.*, 2007). Estrogens exert their effect by binding to specific intracellular estrogen receptors (ERs), ER α and β. ER α is known to stimulate proliferation of prostate cells while the β induces apoptosis (Garg *et al.*, 2013). Receptors are able to modify cellular metabolism and/or growth after binding to appropriate ligand, usually a distinct entity like hormones (Farnsworth, 1999).

Based on this knowledge, drug formulation to inhibit androgen and estrogen signaling (hormone and receptor activity) has been developed [eg. 5-alpha reductase inhibitors (5ARI), selective estrogen receptor modulators (SERM) etc.] and the results of these drugs have produced remarkable results in the treatment of the condition. These drugs are the mainstay for pharmacotherapy. However, recent reports on the side effects have caused scientists to investigate further for novel and better treatment regimens for the condition. Side effects include low libido, low potency, and ejaculatory dysfunction. Furthermore, cardiovascular and thrombo-embolic events have been recorded with the use of some of these drugs suggesting a negative impact on the quality of life (Prezioso *et al.*, 2007; Kumar *et al.*, 2012; Carrasquillo *et al.*, 2014). These undesirable changes have therefore brought complementary and alternative medicines (CAMs) into the lime light.

The use of CAMs is on the increase, due to the fact that it appears to be effective in preventing and treating disease conditions with seemingly less adverse effects (Appiah *et al.*, 2013; Afriyie *et al.,* 2014; Asare *et al.*, 2015). The use of medicinal plants for the treatment of BPH has seen remarkable improvement. In an update on plant derived anti-androgens, several plants including white peony, green tea, spearmint, black cohesh, chaste tree and saw palmetto were described as effective in BPH treatment by inhibiting the production of androgens and estrogens; inhibiting the activities of aromatase, 5AR, gonadotrophins; inducing apoptosis and activating

caspases among others (Grant and Ramasamy, 2012). Most of these findings were *in vitro* or *in vivo* studies, requiring well-designed, randomized, placebo-controlled studies, adequately powered with adequate follow-up time. This is almost impossible in patients as patients' most likely will not opt for this regimen as a first line treatment option.
Furthermore, the ill-effect on the placebo group has ethical considerations.

In Ghana, the popular medicinal plant for the management of BPH is *Croton membranaceus* (CM). *In vivo* studies have demonstrated that the CM can markedly improve biomarkers of BPH, as both low and high doses of
30 and 300 mg/kg b.wt significantly reduced prostate specific antigen (PSA) levels and prostatic index comparable to the control model and those on the standard drug finasteride. It subsequently shrunk the enlarged prostate (Afriyie *et al.*, 2014). That study was recently demonstrated in humans, and similar results were observed. BPH patients who opted for phytotherapy with CM (20 mg t.i.d.) were observed for 3 months, and the quality of life, free and total PSA along with prostate volume were markedly improved without any recorded effect on sexual function (Asare *et al.*,
2015).

Marked improvement of BPH has been observed by reduction of androgen and inhibition of 5AR enzymes as well as the use of aromatase inhibitors and anti-estrogens that regulate the androgen and estrogen signaling. One of the proposed mechanisms of action of CM is androgen inhibition (Appiah

et al., 2013). Additionally, in *in vivo* experiments, CM yielded similar results as finasteride, a 5-ARI. Whether CM is also a 5-ARI is unknown. It is therefore necessary to explore the effect of CM on the hormones implicated in the initiation and development of the disease, in order to substantiate the plants extract's influence on the related endocrine pathway.

1.2 AIM

The aim of the study was to determine the effect of *Croton membranaceus* on major sex hormones, metabolites and enzyme indices in benign prostate hyperplasia patients.

1.2.1 Objectives

The objectives of the study were as follows:

1. To determine the effect of BPH on sex steroid hormones and metabolites (testosterone, dihydrotestosterone, androstenedione, estrone, estradiol, androstanediol adiol and androstanediol) and the enzyme indices for 5αreducatse, aromatase, aldo-keto reductase family 1, member C1 (AKRIC1) and aldo-keto reductase family 1, member C2 (AKRIC2) activities.
2. To determine longitudinally the effect of CM on sex steroid hormones, metabolites and enzyme indices after 12 weeks of treatment.

3. To determine the association of sex steroids with; age, prostate volume, and prostate specific antigen (PSA) as predictive indices of BPH

1.3 HYPOTHESIS

H_0 : *Croton membranaceus* will not significantly influence the levels of sex steroids, their metabolites and associated enzyme activities.

1.4 RATIONALE

The ambiguity of the definition of BPH makes it difficult for population studies to predict the prevalence of BPH. Hence, authors apply as many metrics as are available or known to them. Chokkalingam *et al.* (2012) determined the prevalence of BPH in Ghanaian men by digital rectal examination (DRE), International Prostate Symptom Score (IPSS), PSA ≥ 1.5 ng/ml and symptomatic BPH as 62.3%, 19.9%, 35.3% and 13.3%, respectively. BPH causes considerable morbidity and affects the quality of life of aging men. Pharmaceutical interventions include the use of alphablockers, 5-alpha reductase inhibitors (5-ARIs), combination of αadrenergic–receptor blockers and 5-ARIs, and in severe cases

transurethral resection of the prostate (TURP). CAM provides an option to replace and improve existing medicines, and new avenues for the treatment

of BPH as pharmaceutical interventions have adverse side effects in the form of reduced libido, reduced potency, ejaculatory dysfunction, and hypotension among others. About 70 % of Ghanaians depend on traditional medicine for their health care. There is approximately one traditional medicine practitioner for every 400 people, compared to one allopathic doctor for every 12 000 people (Yarney *et al.*, 2013).

In Ghana, CM is widely used for the treatment of prostate associated diseases especially BPH. CM is multi-targeting naturally, inexpensive and safe with no recorded side-effects, compared to synthetic agents. It improves total international prostate symptom score (IPSS) with consequent shrinkage of prostate volume, and speculated to employ several mechanisms to induce its cytotoxic activity in BPH cells (Appiah *et al.*, 2013). Yet not much work has been done to establish its mechanism(s) of action and those done were either *in vivo* or *in vitro*. Since antiandrogenic activity is suggested to be a possible mechanism, it is rationally justifiable to explore if it has any effect on the hormones involved in androgen and estrogen signaling; and therefore an ideal therapeutic approach for the management of BPH.

CHAPTER 2

LITERATURE REVIEW

2.1 THE PROSTATE

2.1.1 Anatomy and Physiology

The human prostate is a walnut-sized organ, with ductal–acinar histology, that lacks discernible lobular organization. The human prostate can be divided into four distinct zones and the significance of this architecture is based upon the relationship of these zones to prostatic disease (Yam, 2007). Benign prostatic hyperplasia (BPH), a nonmalignant overgrowth that is common among aging men, occurs mainly in the transition zone, and prostate carcinoma arises primarily in the peripheral zone. The prostate gland surrounds the urethra just below the urinary bladder and produces a clear, slightly alkaline fluid constituting 10-30% of seminal fluid (Owen and Katz, 2005). The pH, proteins and ions of this secretion enables sperm motility and protect sperms in the passage through the acidic environment of the female vagina. An essential protein secreted by prostate in both normal and diseased state is the prostate specific antigen (PSA), also known as Kallikrein III (Balk *et al.*, 2003). The prostate is exclusive to mammals and is not essential for fertility hence the prostate has received attention mainly because of the diseases associated with it.
(Abate-Shen and Shen, 2000).

2.1.2 Formation and morphology

The functional unit of the prostate comprises of epithelium and stromal components. The glandular duct of the epithelium is lined with different cell types; secretory luminal cells, basal cells and neuroendocrine cells. The predominant cell type is the secretory luminal cell. The luminal cells are terminally differentiated and characterized by expression of androgen receptors (AR). They require continuous and direct androgenic stimulation to maintain structural and functional viability. They synthesize proteins such as PSA and prostate specific phosphatase. The basal cells are poorly differentiated and express low/ undetectable levels of AR. Furthermore, they do not have secretory function and do not depend on androgens for survival although it is speculated that a subset of basal cells require androgens for proliferation and differentiation into secretory cells as they may be intermediate between basal and luminal cells (Lateef, 2013). Neuroendocrine cells are not dependent on androgen and provide paracine signals that support the growth of the secretory luminal cells (Abate-Shen and Shen, 2000).

A basement membrane separates the epithelium from the stromal. The composition of the stromal includes fibroblasts, smooth muscle cells, endothelial cells, nerve cells and infiltrating mast cells and lymphocytes. The prostatic epithelium and stromal interact with each other via various hormones and growth factors. The fibroblasts express AR and are androgen responsive. They produce and secrete various growth factors such as

epidermal growth factor (EGF), insulin growth factor (IGF) and keratinocyte growth factor (KGF), which could, in a paracrine fashion, induce epithelial cell growth and glandular development (Abate-Shen and Shen, 2000; Balk *et al.*, 2003).

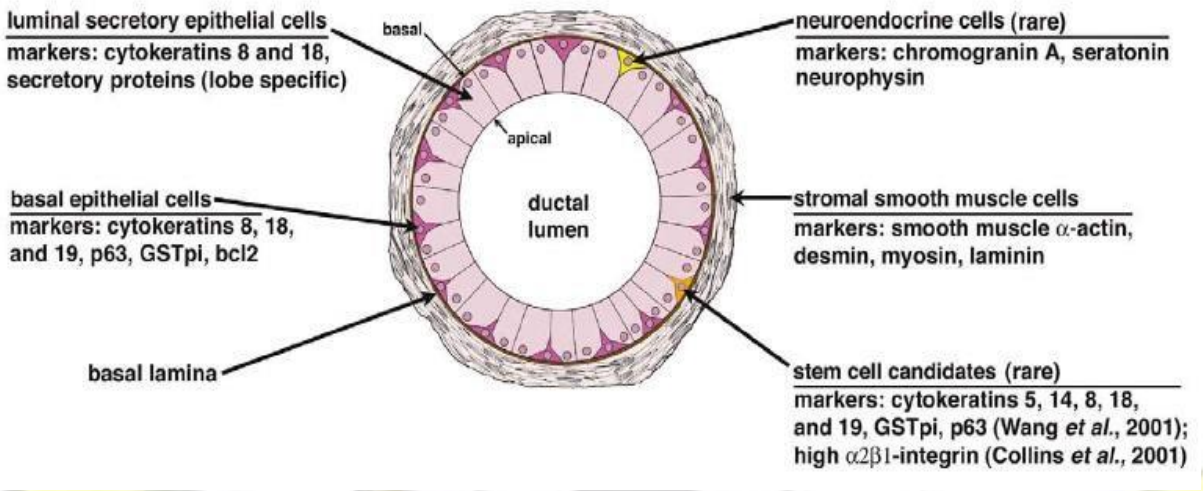

Figure 2.1.The prostate gland: *This diagram presents the cell types and its secretory products of the various cells in the prostate gland* (Marker *et al.*, 2003).

2.1.3 Formation of the prostate gland

During embryogenesis, the prostate forms through epithelial budding from the urogenital sinus, a hindgut derivative of endodermal origin. In midgestation, the cloaca divides, separating primitive urogenital sinus from the terminal region. Formation of the urinary bladder is from the most rostral part (vesiculo-urethral part) while the penile part originates from the most caudal part (phallic part). The prostate gland stems from the

intermediate region known as the pelvic part commonly referred to as the urogenital sinus (Abate-Shen and Shen, 2000).

The fetal testis secretes testosterone into the circulation at sufficient levels to stimulate the differentiation and growth of the urogenital sinus tissues. However after birth, plasma testosterone decreases to a low baseline level and the prostate does not grow until puberty. At puberty, large amounts of androgens are secreted from the testes to stimulate prostatic cells to undergo morpho-functional maturation, giving rise to the various histological zones and functional tubule alveolar glands. The human prostate grows to a maximum size of about 20 g and mature morphology at 18-20 years of age (Ho and Habib, 2011).

Prostate formation and development relies heavily on the interaction between the stromal and the epithelial. At the initial stage of prostate development, only the prostate stromal bear AR, hence the androgens acted only on stromal cells. This produces the epithelium-stimulating factor which stimulates epithelial growth. Stimulation of epithelial cells produces the epithelial growth factors (EDGF) which in turn stimulate stromal growth hence a positive feedback loop. The loop is broken by the action of androgens on the epithelial cells to produce epithelial inhibitory factors (EDIF). When the EDIF is high enough corresponding to enough epithelial cells, prostate growth cells ceases. This quiescent stage is maintained by the constant level of EDIF production. A reduction of EDIF or drop in the sensitivity of the

stromal growth leads to renewed stromal growth hence renewed epithelial cell growth. Eventually, it is the interaction between the stromal and epithelium that maintains the growth quiescence (Desgrandchamps and Teillac, 1994).

2.1.4 Regulation of prostate development

The development and growth of the prostate gland is androgen dependent. The hypothalamus regulates the production of androgens via secretion of gonadotrophin-releasing hormone (GnRH) which acts on the pituitary gland. In response, the pituitary gland secretes luteinizing hormone (LH), LH then induces the secretion of testosterone from the leydig cells of the testis. This pathway releases about 95% of testosterone. The remaining 5% is obtained when corticotrophin-releasing hormone induces adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH induces the adrenal glands to produce testosterone and other weak androgens (Figure 2.2) (Jennbacken, 2009).

2.1.5 Diseases of the prostate

Prostate disease is a general term that describes a number of medical conditions that can affect the prostate gland. There are three (3) pathologic processes that affect the prostate gland with sufficient frequency; prostatitis, benign prostate hyperplasia and prostate cancer.

In recent times, the incidence and prevalence of these conditions are soaring either as a result of better diagnostic markers or longevity of life in general. Prostatitis makes up about 2% of prostatic diseases, while BPH and prostate cancer constitute 80% and 18%, respectively (Bock-Oruma *et al.*, 2013). BPH and prostate cancer coexist, and about 10 -20% of men who undergo transurethral resection of the prostate (TURP) for BPH are found to have cancer. Likewise 20% of prostate cancer patients have BPH. However, the debate on the relatedness of these two conditions remain unresolved (Schenk *et al.*, 2011).

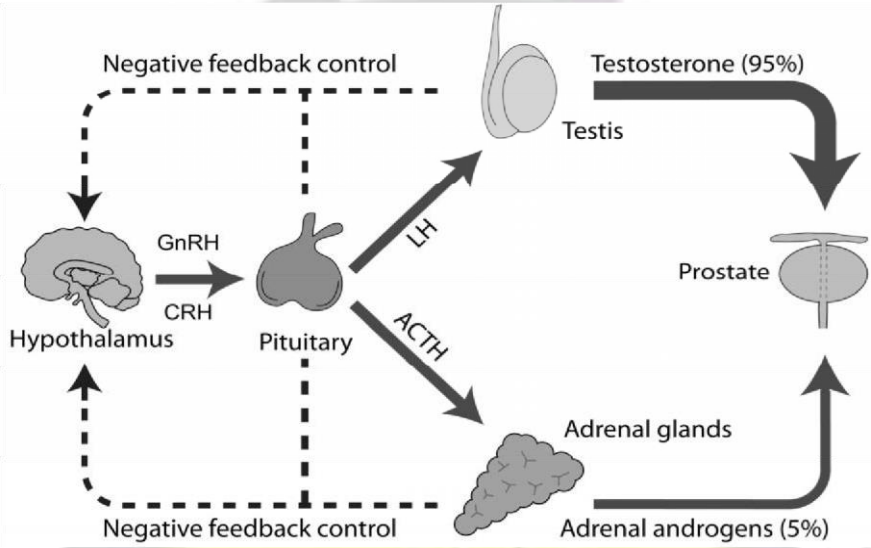

Figure 2.2. Regulation of the prostate gland: *The hypothalamus regulates the prostate gland via the pituitary through a negative feedback system* (Jennbacken, 2009)

African-Americans have 2.2 times greater risk of receiving a prostate cancer diagnosis than Caucasians (Pettaway *et al.*, 2011). However, Africans have recorded a low risk compared to their African-American counterparts and

yet at distant-stage of the condition, rates are comparable for Africans and Black Americans (Chu *et al.*, 2011). Among Africans, those in the East recorded the highest prevalence, while those in the West recorded the least (Chu *et al.*, 2011). On the other hand, the risk of the development and progression of BPH is similar, or possibly higher, in African-Americans compared with Caucasians (Roehrborn and Ray, 2006). However, to the best of my knowledge there are no direct studies that compared the prevalence of BPH in Africans with African-Americans and there appears to be paucity of data on the prevalence of the condition among individual African countries. In Ghana, only one population-based study exists in literature. In that study, the prevalence of BPH in Ghanaian men by DRE detection, IPSS, PSA ≥ 1.5 ng/ml and symptomatic BPH was
62.3%, 19.9%, 35.3% and 13.3%, respectively (Chokkalingam *et al.*, 2012).

2.2 BENIGN PROSTATIC HYPERPLASIA

2.2.1. Definition

BPH is defined by two systems, histological findings and clinical presentation. In a normal prostate, the ratio of the stromal to the epithelial components is 2:1 whereas BPH is characterized by a four-fold increase and a nearly doubling of the glandular elements of the prostate within the transition zone. It is therefore suggested that BPH is basically a proliferative stromal hyperplasia (Alonso-Magdalena *et al.*, 2009). Clinically, a consensus seems to have been reached on three

characteristics for the condition to manifest: bladder outflow obstruction, lower urinary tract symptoms and prostate enlargement. The ideal cutoff for each of these parameters to determine case definition for population studies and early detection of the condition, is yet to be determined (Kok *et al.*, 2006).

2.2.2 Symptoms of BPH

BPH is a pathologic process that contributes to LUTS but is not solely responsible for LUTS. Recent evidence has shown that conditions such as polyuria, sleep disorders among other medical conditions could also be involved in the development of LUTS. Voiding symptoms in BPH are largely due to bladder outlet obstruction (BOO) associated with prostatic enlargement (BPE) and failure to empty bladder has been attributed to obstruction or detrusor under-activity or a combination of both. Post micturition symptoms like post voiding dribbling is also a significant cause of interference in quality of life. BPH patients also suffer storage symptoms known as overactive bladder (OAB) syndrome in the form of urgency, frequency, nocturia and urgency incontinence and these symptoms are more bothersome than the voiding symptoms when accompanied by incontinence, although these symptoms may be associated with other medical conditions either than BPH. It is noteworthy that it is not automatic for men with BPH to have LUTS and vice versa (Roehrborn and McConnell, 2007).

2.2.3 Diagnosis

BPH diagnosis is made by taking a complete medical, urologic and neurologic history of the patients in order to rule out causes of LUTS other than BPH or bladder dysfunction. More often than not patients present to the clinic when symptoms begin to affect their quality of life. Symptoms of LUTS are assessed by a standardized questionnaire known as the International Prostate Symptom Score (IPSS). This allows the physician to quantify the symptoms and severity of the LUTS in order to make an informed decision on therapy. LUTS is graded from 0-35, where 0-7 indicates mild symptoms, a score of 8 to 19 and a score of 20 to 35 suggest moderate and severe symptoms, respectively (Tanguay *et al.*, 2009).

This is then followed by a physical examination of the supra-pubic area, external genitalia and testes. However, DRE is the most important aspect:- size, shape, symmetry, quality, nodularity and consistency of the prostate are assessed. The presence of palpable nodular usually calls for prostatic biopsy. When combined with PSA, the diagnostic accuracy is improved for PCa (Tanguay *et al.*, 2009).

PSA values above 4.0 ng/ml are considered abnormal, though lower cutoff levels have been proposed because normal PSA values do not necessarily

rule out PCa. Instigating the need to improve the diagnostic accuracy of the biomarker include measuring PSA velocity (change over time), levels of free and protein-bound PSA, PSA density (PSA level divided by the prostate volume), and the use of cutoff values for PSA levels that are specific to the patient's age and race or ethnic group. The clinical usefulness of these strategies remain unproved (Hoffman, 2011; Zeliadt *et al.*, 2012). Further to an abnormal PSA value, a biopsy is advised. Cases that tend out to be prostate cancer are referred to the urologist.

Urinalysis is also ordered to screen for urinary tract infection and hematuria in order to rule out urolithiasis or cancer of the kidney, bladder, or prostate. LUTS are treated before initiation of other therapies. For patients who cannot completely empty their bladder or have palpable bladder on abdominal examination, a post voiding residual urine measurement is done to rule out "silent" urinary retention (normal residual urine volume, <100 ml). It is advisable for patients with complicated LUTS to be referred to a urologist (Sarma and Wei, 2012). Other laboratory tests including urine culture, serum creatinine and glucose may be ordered depending on the patient's history. Non-invasive Optional tests include urine flow rate, pressure flow studies, cytoscopy, renal and transrectal ultrasound.

2.2.4 Etiology

The molecular mechanism underlying the development of BPH remains unresolved. However, the concept of 'embryonic reawakening of stromal cell inductive potential' of McNeal is widely accepted among other theories (McNeal, 1990). It is noteworthy that androgens and aging remain essential components of the various theories.

Embroyonic reawakening theory: According to McNeal, BPH develops as a result of inductive effect of local stromal. That study observed a grandular budding of a new aveoli in the periurethral zone of the prostate and this was an expression of the re-awakened inductive potential of the stromal (McNeal, 1990: Ho and Habib, 2011). The inductive effect of the stromal induced hyperplastic changes in the epithelium. When primary human BPH stromal fibroblasts and epithelial cells cultured separated were significantly decreased compared to cultured together , indicating that only the mesenchyme had an inductive potential. On the other hand, the release and expression of growth factors that act on the epithelial can also be induced by the stromal androgen/AR signaling (Izumi *et al.*, 2013). The reawakening of the embryonic cellular growth potential could also be mediated by abnormal levels of growth factors like epidermal growth factor, basic fibroblast growth factor and transforming growth factor β (tGF-β) from either the epithelial or stromal compartment (Ho and Habib, 2011).

The stem cell theory: The stem cell theory proposes that BPH could result from changed properties of stem cells. The number of stem cell within the prostate is unknown but believed to be very low. Normally, stem cells are relatively undifferentiated, with preserved numbers, have unlimited proliferating potential, easily adapt to the environment and are pluripotent, allowing them to give rise to a number of different cell types. When the proliferating cells reach maturity, they undergo programmed cell death. However, aging reduces the rate of cell death by inducing a blockage in the maturation process to terminally differentiated cells (Roehrborn and McConnell, 2007). It is noteworthy that BPH men with large prostate have been found to have high levels of epithelial cells [senescence-associated βgalactosidase (SA-β-gal)] suggestive of cell accumulation (Castro *et al.*, 2003).

There is a rapid proliferation of cells in the early phase of the condition. However, the disease appears to be established in the face of an equal or reduced rate of cell replication. An up regulation of the proliferation and a down regulation of apoptosis have been observed in BPH (Claus *et al.*, 1993; Claus *et al.*, 1997). Increased expression of anti-apoptotic pathway genes (e.g., BCL2) supports this hypothesis (Kyprianou *et al.*, 1996). Androgens affect the activity of growth factor-β (TGF-β) which modulate apoptosis (Nicholson *et al.*, 2013).

The Hormonal theory: As males age, there is a shift in prostatic androgen metabolism which leads to an abnormal accumulation of the more potent DHT in the prostate which promotes its growth and survival. However, there are conflicting results on the levels of DHT. Some studies have reported higher levels in BPH tissues, while others reported no change relative to normal prostate tissues (Kristal *et al.*, 2008; Marks *et al.*, 2008; Jarvis *et al.*, 2015). In BPH tissues androgen-responsive genes, ELL associated factor 2 (EFEA), elongation factor, RNA polymerase II, FK506 binding protein 5 (FKBP5), phosphorin aminotransferase 1 were found to be significantly elevated compared to normal tissues (Briganti *et al.*, 2009), indicating that androgen signaling forms a big part of the disease process. Relative to testosterone, DHT binds to AR with a more potent affinity. Following complex activities described in detail hereafter, target genes called the androgen response elements (AREs) are recruited to initiate transcription of genes regulating growth, differentiation and survival (Tan *et al.*, 2014). Hence the more DHT in the prostate, the more cells are formed increasing cell number and thus increasing the size of the prostate.

Additionally, is the testosterone-estrogen imbalance. This theory suggests that an age-associated imbalance between circulating estrogens and testosterone plays a role in the pathogenesis of BPH. In humans, the serum testosterone and free-testosterone levels decrease with age, but the serum estradiol level is constant throughout life (Prezioso *et al.*, 2007). Ageing creates an estrogen-dominant status. It is suggested that androgen cannot

exclusively induce the condition but estrogen may play a critical role although report on the association between estrogen and BPH development is diffused (Coffey and Walsh, 1990; Kristal *et al.*, 2008; Schauer *et al.*, 2009). Endogenously derived estrogens have been known to induce prostatic proliferation via its receptors, while ERα is known to induce proliferation, ER β inhibits proliferation of stromal cells (Chen *et al.*, 2012).

2.3 HORMONES

2.3.1 Hormones in the prostate

Androgens are particularly essential for the development, growth and maintenance of the prostate however, besides androgens, several other hormones and/or their receptors have been detected in the prostate. These include estrogen, prolactin and growth hormone among others. Androgen is a term given to any steroid hormone that primarily influences the growth and development of the male reproductive system. Although there are other natural androgens, testosterone (T) is the primary circulating androgen. Sex hormones can be easily distinguished by the carbon numbers, C-19 being androgens, C-18 being estrogens, and C-21 being progestenoids (Chen *et al.*, 2002).

2.3.2 Androgen metabolism

Testosterone synthesis is primarily performed by the leydig cells of the testes, while metabolism of adrenal androgens contribute to less than 5% of

circulating T in eugonadal men (Jennbacken, 2009). T released into the bloodstream is complexed with a 'carrier protein', sex hormone binding globulin (SHBG) or albumin. SHBG regulates the amount of "free" testosterone circulating in blood. Only 1-3% of free testosterone diffuses into the prostatic cells (Yam, 2007). However, the prostate possess proteins and enzymes which are capable of making its own androgens and even estrogens. Free testosterone is thought to constitute the active hormone and provides a better measure of testosterone status in the body than total testosterone level. Ideally, free testosterone is measured by the gold standard method of equilibrium dialysis. It is laborious and impractical for routine laboratory practice. Scientists have therefore proposed many equations to estimate plasma free testosterone levels in men using the measured concentrations of total testosterone, albumin and SHBG (Ho and Habib, 2011). However, there are now enzyme linked immunosorbent assay (ELISA) techniques that can measure free testosterone (FT).

The first step of testosterone biosynthesis primarily occurs in the testis, either through the D5 route or the D4 route (Figure 2.3). The processes are mediated by several P450 and non-P450 enzymes including StAR, CYP11A1, CYP17A1, HSD3B2, and HSD17B3 (Fukami *et al.*, 2013). Humans preferentially ply the D5 route especially in the fetal life (Fluck¨ *et al.*, 2003). Human CYP11A1 encodes 17α-hydroxylase and 17/20 lyase. Pregnenlone and progesterone are catalyzed by 17α-hydroxylase with equal efficiency but 17/20 lyase has a relatively higher affinity for 17-OH

pregnenolone (Fukami *et al.*, 2013). T is then converted to DHT via 5AR. This occurs in androgen-target tissues such as the genital skin and the prostate. Alternatively, studies of *Macropus eugenii* young testis and immature mouse testis suggest androstanediol as the major source for the DHT production (Shaw *et al.*, 2000; Wilson *et al.*, 2003). This pathway bypasses T, allowing cholesterol to be metabolizsed to DHT. This route has been named "backdoor pathway". The 'backdoor' is mediated by androstanediol produced from 17-OH progesterone, 17-OH dihydroproges- terone, 17-OH allopregna- nolone (Fukami *et al.*, 2013).

5AR activity is the core requirement for the backdoor pathway and 17-OH progesterone and progesterone serves as excellent substrate. The relative function of CYP17A1 compared with SRD5A1 determines whether 17-OH progesterone and progesterone are used in the "frontdoor" or "backdoor". The "frontdoor" or "backdoor" pathway may also be determined by the degree of the 17/20 lyase activity of CYP17A1 (Fukami *et al.*, 2013).

In humans, the backdoor pathway has been shown to be an essential source of androgens for male sex development, operating in the fetal testis under physiological conditions. The human fetal testis contains sufficient steroid 5-alpha-reductase type 1 (SRD5A1) and variable degrees of the remaining enzymes required for the backdoor pathway such as AKR1Cs, CYP17A1, HSD17B3, and HSD17B6 (Flück *et al.*, 2011). Hence humans use both the 'frontdoor' and the 'backdoor'.

The transformation of T to DHT is performed by 3 or 5 17βHSDs in tissues and cells in which the level of expression of 5AR is low or absent, such as in the testicles and the muscle. In tissues that have 5AR, type 5 17β-HSD is involved in the formation of DHT via the backdoor pathway. Hence in tissues like prostate, skin and liver, DHT is probably formed by the backdoor pathway (Luu-The and Labrie, 2010). A number of studies have been conducted to support this "backdoor" theory. In aging men, the influence of andrenal androgens acting as precursors becomes pronounced. AKR1C3 is the principal 17-ketosteroid reductase responsible for testosterone production and has been expressed at RNA, Protein and functional level. The conversion of 5α-DHT to androstanediol adiol (3αadiol) is dependent on 3-ketosteroid reductase (AKR1C2); it allows or prevents it from binding to its receptors. AKR1C1 converts 5α-DHT to androstanediol (3β-diol) where 3β-diol is a proapoptotic ligand for estrogen receptor (Penning, 2010). In spite of these, it is suggested that in low grade cancer and possibly in BPH, it is the classical androgenic stimulation that is required whereas, it is hypothesized that progression to high grade tumors may at least in some cases require the 'backdoor' androgenic stimulation (Hoque *et al.*, 2015).

2.3.3 The enzymes

In the prostate where 5α-reductases are expressed, 5α-reductases together with type 5 17β hydroxysteroid dehydrogenase (5 17β-HSD) are involved in

the conversion of T to DHT (Luu-The and Labrie, 2010). Three 5AR (5AR1, 5AR2, 5AR3) have been identified and are ubiquitously expressed; however two (5AR1 and 5AR2) have been extensively studied and found to be involved in steroid 5α reduction. 5AR3 is important for *N*-glycosylation of nascent proteins (Azzouni and Mohler, 2012). In adults, 5AR1 is expressed in non-genital skin, the liver and certain brain regions, and also at lower levels in the prostate, genital skin, epididymis, seminal vesicles, testis, adrenal gland and kidney. 5AR2 is expressed at relatively high levels in the prostate, genital skin, epididymis, seminal vesicles and liver (Wang *et al.*, 2014). In the prostate, type 2 predominate in the stromal while type 1 predominant in the epithelium (Goldenberg *et al.*, 2009).

All three 5AR enzymes (5AR1, 5AR2, 5AR3) are microsomal nicotinamide adenine dinucleotid phosphate (NADPH) dependent enzymes with 259, 254 and 318 amino acid residues and molecular weights of 29.5, 28.4 and 29.9kDa, respectively. 5AR1 operates at an optimum pH of 6-8.5, while that of 5AR2 is 5-5.5. They have similar gene structure with five exons and four introns located on different chromosomes with SRD5A1 on 5p15, SRD5A2 on 2p23 and SRD5A3 on 4q12.11(Wang *et al.*, 2014).

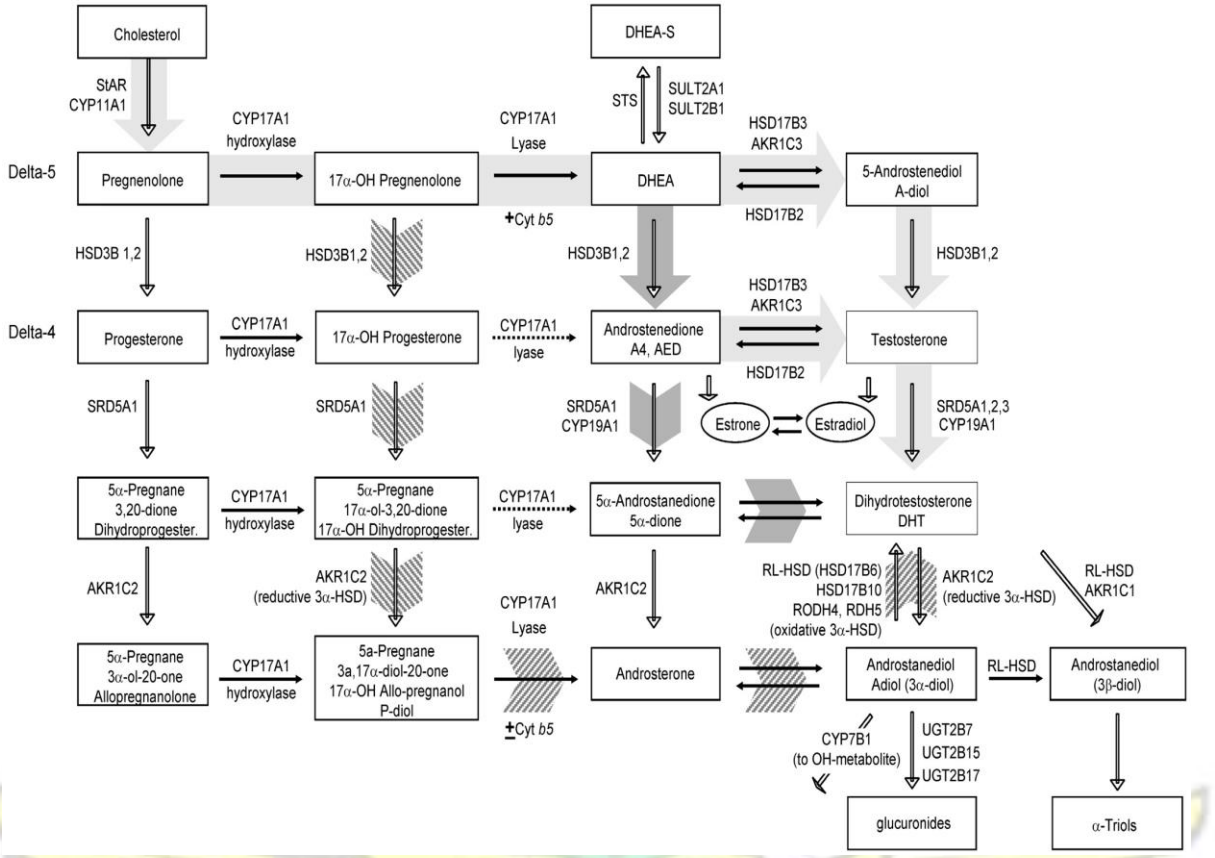

Figure 2.3: Androgen biosynthesis pathway: *Androgens are synthesized via a classical and non-classical pathway. Classical pathway is shown in light gray arrows while the backdoor pathway is the hatched arrows (Mostaghel, 2014).*

The conversion of DHT from testosterone relies heavily on the 5AR enzymes. While 5AR1 is mainly responsible for the conversion of androstenedione (AED) to 5α-androstanedione, 5AR2 converts T to DHT. AED and T are inter-convertible. In the prostate, DHT is transformed to several metabolites. Two important metabolites are androstanediol adiol

(3α-adiol) and -androstanediol (3β-diol), which are formed by the action of 3α-hydroxysteroid dehydrogenase (3α-HSD), type 3 AKR1C2 and 20αhydroxysteroid dehydrogenase (AKR1C1) (Stanczyk *et al.*, 2013). androstanediol adiol is a peripheral metabolite of DHT, and its glucuronide,

3α-androstanediol gluconide (3α-adiol-G) has been described as a marker of local androgen excess due to the increased activity of testosterone metabolism (Pavičić *et al.*, 2013). Overall, serum levels of 3αadiol-G appears to represent either adrenal androgen production or skin 5 α-reductase activity (Wu *et al.*, 2001; Chen *et al.*, 2002). The role of 3αadiol-G as a marker of peripheral 5α-reductase has recently been criticized. It seems that 3α-adiol-G largely reflects adrenal androgen secretion and does not reflect primary peripheral 5α-reductase activity. Therefore, the measurement of 3α-adiol-G may only be useful in monitoring therapy with 5α-reductase inhibitors (Pavičić *et al.*, 2013). On the other hand, serum DHT to T may be used as an indirect indicator of 5AR activity (Parsons *et al.*, 2010; Liao *et al.*, 2012) and may be used to monitor treatment with 5ARI (Mohler *et al.*, 2004; Shiraishi *et al.*, 2008). The activity of AKRIC1 and AKR1C2 may also be represented by 3αadiol/DHT and 3β-diol/DHT respectively (Geller *et al.*, 1976).

2.3.4 Role of Androgens in the prostate

Functionally, T stabilizes the Wolffian ducts to develop into epididymis, vas deferens and seminal vesicles while DHT is a prerequisite for androgen induced-differentiation of the external genitalia. DHT stimulates the development of the prostate as well as the differentiation of urogenital swellings, the genital tubercle and the urethral folds into penis and scrotum (Hiort, 2013). The biological effects of T and DHT are directed to androgen receptors to induce transcriptional activity (Heinlein and Chang, 2004).

However, DHT binds AR with an affinity about 2-5 times that of T and also induces androgen signaling with about ten times potency than T (Azzouni and Mohler, 2012).

Binding of androgens to AR stimulate the receptor to form complexes with chaperone proteins. The complex then undergoes post-translational modifications including phosphorylation. Simultaneously, AR dissociates from heat shock proteins, binds with another AR to form a homodimer. The homodimer is subsequently translocated into the nucleus from the cytoplasm via binding with importin-α. In the nucleus, receptor dimers bind to androgen response elements (AREs) in the promoter regions of target genes, such as prostate specific antigen (PSA) and transmembrane protease serine 2 (TMPRSS2), etc., to which they recruit various coregulatory proteins to facilitate transcription, leading to responses such as growth and survival (Tan *et al.*, 2014). Further to this classical AR signaling, non-genomic signaling of the AR has also been reported. Extranuclear signaling induces extremely rapid changes in cellular function and are likely to be important mediators of androgen action (Nicholson and Ricke, 2011). In effect, DHT stimulates several growth factors that drive cellular proliferation in the human prostate, including growth-stimulatory epidermal growth factor (EGF), keratinocyte growth factor (KGF) and insulinlike growth factors (IGFs).

Recent studies indicate the importance of DHT to be two-fold: either acting as an androgen and or metabolized into androstanediol (3β-diol), an androgen which is also a ligand for ERβ (Nicholson and Ricke, 2011). 3βdiol to ERβ binds with equivalent potency as 17β-estradiol (E2) to activate transcription (Pak *et al.*, 2005), this binding is sufficient to regulate growth in the prostate gland (Weihua *et al.*, 2001).

2.3.5 Estrogen metabolism and signaling

The prostate is commonly thought of as an androgen target tissue, but it is also an important target of estrogens as well. It has been realized that males are more "reactive" to estrogens than females (Prezioso *et al.*, 2007). In men, 75–90% of the circulating estrogens are believed to be derived from peripheral conversion of testosterone and androstenedione to estradiol (E2) and estrone (E1), respectively (Figure 2.3.), in adipose, brain, bone and other tissues. Conversion in the testes contributes 10–25% of circulating estrogens (Ho and Habib, 2011). The total E2 production rate in the human male has been estimated to be 35-45 μg (0.130-0.165 μmol) per day (de Ronde and de Jong, 2011).

Estrogens within circulation can be endogenous or exogenous. Commonly found endogenously derived estrogens include estrone (E1), estradiol (E2) and estriol (E3). However, E2 has been shown to be a potent estrogen and a powerful inducer of prostatic proliferation. In men, the aromatization of T to

E2 in fat and muscle, forms the bulk of circulating E2 while up to 20% is secreted by leydig cells of the testes (Nicholson and Ricke, 2011). However, in addition to endogenously produced estrogens, there are those acquired outside the body. Xenoestrogens can be derived from plant sources (e.g., phytoestrogens) or synthetic such as the many endocrine disruptors or environmental estrogens (Wynder *et al.*, 2015).

Estrogens exert both direct and indirect effects on the prostatic organ. The indirect estrogen action is mediated at the hypothalamic level to suppress circulating androgens and produce a 'castration-like' effect. Estrogen has an inhibitory effect on the secretion of luteinizing hormone (LH) in the pituitary. As indicated previously (figure 2.2), LH stimulate the secretion of T by the testicular leydig cells, hence the negative feedback effect results in a decreased secretion of T which in turn affect the production of DHT (Loci *et al.*, 2013).

The direct actions of estrogens are mediated by two distinct receptors, estrogen receptor (ER) α and β in the prostate (Chen *et al.*, 2012). These two classical receptors located in the cytosol translocate to the nucleus and act as transcription factors upon ligand binding (ERα and ERβ, encoded by the estrogen response elements (ESR) genes *ESR1* and *ESR2*, respectively). The classical mechanism of ESR action involves estrogen binding to receptors located in the nucleus. After binding estrogen, ESR dissociates from its chaperone proteins, phosphorylates, and dimerizes. Hormone binding also

induces a conformational change within the ligand binding domain of the receptors, and this conformational change allows for the recruitment of coactivator proteins;- for example, amplified in breast cancer-1 (AIB1), nuclear-receptorcoactivator-1 (NCoA-1/SRC1) and the p300 and CREB-binding protein (CBP)-associated factor (PCAF) (Lazari *et al.*, 2009). The activated ESR-dimer complexes bind directly to specific ESR or to promoters of target genes (Nicholson and Ricke, 2011). Extranuclear signaling (i.e. non-genomic signaling) of ERs results in rapid biochemical effects, such as increased intracellular calcium or nitric oxide, or induction of enzymes, especially by phosphorylation (Nicholson and Ricke, 2011). In addition, non-genomic signaling mechanisms include plasma membrane-associated estrogen receptors α and β, and an intracellular G-protein coupled receptor localized to the endoplasmic reticulum (gpER, also known as GPR30) (Villablanca *et al.*, 2013). ER*α* and GPR30 promote proliferation, whereas ER*β* has proapoptotic and pro-

differentiating functions (Lee *et al.*, 2014).

Males are exposed to high estrogens *in utero* and during aging. High estrogen exposure *in utero* has been found to induce squamous metaplasia contributing to the high incidence of BPH. Also, maternal exposure to diestilbesterol during pregnancy has been found to induce squamous metaplasia (SQM) (Prins and Korach, 2008). Also in aging when the gland is completely matured, exposure to estrogen induces squamous metaplasia (Risbridger *et al.*, 2003). SQM induced by estrogen is aberrant and mediated by ERα in the stromal and epithelium of the gland.

2.3.6 The enzyme

Estrogens are synthesized from androgens by the aromatase complex, which contains the cytochrome P450 enzyme encoded by the CYP19 gene on chromosome 15q21.1 (Boon *et al.*, 2010). It is localized at microsomal organelles of estrogen-producing cells. It has a high substrate (androgens) specificity that is conferred by the hydrophobic and polar residues lining the androstenedione cleft, which complements the steroid backbone. Aromatization occurs when the aromatase complex converts C19 androgen substrates to C18 estrogens in three consecutive reactions: (1) hydroxylation (2) oxidation and (3) demethylation resulting in the C19 Aring being converted into a phenolic ring characteristic of estrogens (Boon *et al.*, 2010). Aromatase is widely expressed in a large number of tissues such as testis (sertoli and leydig cells, spermatocytes, spermatids and spermatozoa), ovary (granulosa cells and luteal corpus), brain (including hypothalamus), hair follicles, and fibroblasts (Rochira *et al.*, 2015).

Many researchers have estimated aromatase activity in cells and tissues along with its gene however to the best of my knowledge only one study determined aromatase activity in serum and seminal fluid of infertile men (AL-LNaqeeb and Fakhrildrin, 2015). The study observed significant decreases of both serum and seminal aromatase activity for males with normozoospermia compared with males with oligozoospermia and

azoospermia, resulting in the conclusion that high aromatase activity was associated with infertility in men. For the purpose of observing aromatase activity in serum/plasma, aromatase activity was inferred from the ratios of E1/AED and E2/T (Slater *et al.*, 2001).

2.3.6 The role of sex steroid hormones and their enzymes in BPH

The role of androgen in the pathogenesis stems from the fact that antiandrogen therapy causes rapid reduction in prostate size emphasizes androgen necessity. Also, in castrated animals, treatment with androgen induced prostatic re-growth, proliferation and increased prostate size. However, the facts that androgen supplementation does not increase the risk of BPH, and serum androgen levels decline with age indicate that other factors may be involved (Nicholson and Ricke, 2011). Hence, it is well accepted that androgens are not the main cause of BPH, but rather they play a permissive role in the disease development and progression. In the prostate, the total level of testosterone is 0.4 g/g and the total level of DHT is 4.5 ng/g. The total concentration of T in the blood which is 18.2 nmol/L, is 10 times higher than that of DHT and the fact that DHT circulates in low concentrations bound to plasma proteins, lends support to the fact that DHT has a minor effect on prostatic growth (Saad *et al.*, 2011). It has been proven that estrogen/androgen balance is needed for the disease development (Xiang-Yun *et al.*, 2010). As males age, serum androgens decline while

serum levels of E2 remain relatively constant resulting in a net effect of an increased serum E2 to T ratio associated with BPH
(Nicholson and Ricke, 2011).

It is argued that aging results in an increase in intra-prostatic DHT levels associated with BPH in both animal and human studies (Berry *et al.*,1986; Sasagawa *et al.*, 1990; Jarvis *et al.*, 2015). However, data on the influence of androgens and estrogens on the prostate is diffused. It has been demonstrated in a group of older males (mean age 59.8 years) that there was no significant correlation of serum testosterone levels (total, free or bioavailable) with either prostate volume or IPSS (Jarvis *et al.*, 2015). However, higher DHT levels have been found to be associated with prostate enlargement (Marks *et al.*, 2008) and men with low levels of androgens are at a lower risk of BPH (Saad *et al.*, 2011; Trumble *et al.*, 2015). The prostate volume of BPH patients correlated with serum free T, E2 and E1 in men who underwent radical prostatectomy for low volume prostate cancer (Schauer *et al.*, 2009). Estradiol-17β, together with DHT, or a 5αandrostanediol, induced a fourfold increase in prostate weight and DNA content, bringing to bear the synergistic effects of estrogens and androgens on the gland (Coffey and Walsh, 1990). On the other hand, In a nested case-control study, high testosterone levels, estradiol levels, and testosterone:17β-diol-glucuronide ratio reflecting decreased activity of 5αreductase were associated with reduced BPH risk (Kristal *et al.*, 2008).

5AR1 and 5AR2 are expressed in both epithelial and stromal cells of BPH tissues supporting the hypothesis that both enzymes are involved in the pathogenesis of the condition. Relative to normal prostate, these enzymes are over-expressed and 5AR2 is the predominant form (Wang *et al.*, 2014). It has been illustrated by immunostaining that the expression of 5AR1 was low in BPH and increased with intensity from prostatic intraepithelial neoplasia (PIN) through to metastatic PCa. This was however not the case with 5AR2 as the highest immnuostaining was rather found in BPH and decreased along the trend (Thomas *et al.*, 2005; Thomas *et al.*, 2008). Contrary to this, high grade PCa has demonstrated greater expression of 5-AR1 and 5-AR2 relative to low grade PCa (Titus *et al.*, 2005).

Nicholson *et al.*(2013) concluded that there were more sex steroid hormone responsive cells in BPH compared to normal prostate as they found an increased percentage of AR positive cells and increased AR intensity in both epithelial and stromal cells in BPH compared to normal prostate.
Other studies have supported the fact that there is abundant AR expression in BPH epithelium and stromal (Tsurusaki *et al.*, 2003; AlonsoMagdalena *et al.*, 2009; Nicholson *et al.*, 2013). On the other hand, AR intensity in BPH compared to normal prostate have been reported to be similar (Hetzl *et al.*, 2012).

Changes in the CAG tandem-repeats in the exon 1 of the AR gene lend support to the role of AR in BPH. Exon-1 of the AR, is needed for the

transcription of AR target genes. Polymorphism in the CAG tandem in BPH is conflicting (Nelson *et al.*, 2002; Nicholson and Rick, 2011; Kizilay *et al.*, 2014). It has been demonstrated that men with shorter CAG repeats in the exon-1 are susceptible to enlarged prostate, exhibit moderate to severe obstructive voiding symptoms and need for BPH surgery, as it inversely correlate with AR's activity of transcription. However, a cohort study of Finnish men showed an association between shorter CAG repeats in the AR gene and a rather small prostate volume. These conflicting results may be due to genetic differences in population hence suggesting a relevant role for AR polymorphism in BPH (Nicholson and Ricke, 2011; Kizilay *et al.*, 2014). Promoter regions of the PSA gene is a target site of AR, hence increased AR activity is likely responsible for the increase in serum PSA that often accompanies BPH. Although the role of PSA in prostate cancer screening remains controversial, it has emerged as a prominent biomarker for BPH (Malati *et al.*, 2006; El Melegy *et al.*, 2010).

It has been a matter of debate as to whether or not the prostate has the capacity to locally synthesis estrogen. This matter was laid to rest with aromatase expression from homogenous prostate samples obtained from patient tissues with Laser capture micro-dissection (LCM) (Ellem *et al.*, 2004). In that study a low aromatase activity was determined hence the ability of the prostate to convert testosterone to estrogen, is undoubted. Besides detecting aromatase expression, it was further observed that all BPH and PCa tissue samples expressed aromatase (Ellem *et al.*, 2004; Ho *et al.*,

2008). Hitherto, it had been demonstrated that in BPH, an abnormal gene regulation occurs resulting in overexpression of aromatase (Hiramatsu *et al.*, 1997) although according to another study, no aromatase transcripts were found either in BPH or LNCap cells (Negri-Cesi *et al.*, 1998).

The conversion of testosterone to estrogen is dependent on the aromatase enzyme. Once formed the estrogens binds to the Estrogen receptors α or β. While 3β-diol binds ERβ, estradiol appears to act primarily through ERα to augment HPA reactivity (Handa *et al.*, 2009). It is well accepted that the ERα induces proliferation while ERβ induce apoptosis. ERβ effect its action of cellular apoptosis via the extrinsic pathway, mediated by tumour necrosis factor (TNFA) (Nelson *et al.*, 2014). ERα possibly aid in setting a glycolytic profile required for the proliferation of rapidly dividing cells in normal tissues with high-energy demand and tumors consequently promoting cellular proliferation (Inoue and Misawa, 2015). High expression of ERs has been observed in BPH (Song *et al.*, 2012). Compared to ERα, β has been observed to be reduced in BPH (Yang *et al.*, 2009) as well as with age (Kim *et al.*, 2015).

2.3.7 Hormones: Therapeutics and Rationale

Basic and translation research in the disease condition has led to more differentiated therapy for the aged male with BPH. Three options are now available: watchful waiting, pharmacological treatment and surgical treatment. Treatment options are usually based on whether or not patients

are not bothered by symptoms. Watchful waiting is recommended when patients are not bothered by the conditions and patients may be in any stage of the condition. Pharmacological treatment, phytotherapy, surgery and minimal invasive procedures are recommended as symptoms become bothersome. It is however noteworthy that many patients are unwilling to undergo any invasive procedure (Morlock *et al.*, 2013). The present medical therapies focus mostly on relief of LUTS and have some impact on benign prostatic enlargement and prevent progression of the condition.

The fact that DHT is a more potent androgen in the prostate has led to the development of drugs that inhibit the activities of its enzymes 5AR to reduce the formation of DHT and its metabolites that binds to AR to induce prostatic growth. Common 5α-reductase (5AR) inhibitors are dutasteride and finasteride. Dutasteride inhibits both enzymes while finasteride acts only on the type II (Loke *et al.*, 2013).

Bladder outlet obstruction (BOO) is in part caused by BPH mediated by α1-adrenergic receptors associated with prostatic smooth muscle. 1adrenergic agonist norepinephrine contracts human prostate and activation of α1–adrenergic receptors results in increased prostatic smooth muscle tone with urethral constriction and impaired flow of urine is a major factor in the pathophysiology of symptomatic BPH (Mathur *et al.*, 2014). The alpha blockers operate by relaxing the muscles at the bladder neck and within the prostate for easy, strong and complete emptying of urine. In spite

of the fact that it is quick acting, it does not prevent progression of the condition (Briganti *et al.*, 2009).

Both of these drug formulations have yielded very desirable results as monotherapy, however some or most physicians employ the use of both for effective treatment outcome. The medical therapy of prostatic symptoms (MTOPS) studies conducted on men with BPH symptoms, PSA< 4 compared monotherapy of doxozasin, finasteride or placebo to the combination. The combination yielded a 67% risk reduction in progression of disease at 4.5 mean year although there was no significant response in any of the arms compared with placebo at 1 year with a 66% risk reduction in developing acute urinary retention or the need for surgery in the combined arm compared to the placebo. Prior to this, dutatstride monotherapy trial yielded an 80% better symptom response, 56% reduction in PSA, 27% volume reduction and a 70% risk reduction in either acute urinary retention (AUR) or the need for surgery (Barkin, 2011).

Significant barriers to the use of 5ARI have been well documented, especially its effects on sexual function even after drug discontinuation. Among these are impotence, decreased libido and abnormal ejaculation that affect about 10% and gynecomatia affect about 1%. Besides these, hypersensitivity, association with PCa and teratogenicity of the male fetus has also been recorded (Carrasquillo *et al.*, 2014). On the other hand, therapies involving α-adrenergic blockers are frequently associated with potential cardio-

vascular consequences (including postural hypotension, syncope, asthenia, fatigue, cardiovascular events, headaches and dizziness) (Kumar *et al.*, 2012).

Based on knowledge of the role of estrogen in the development and progress of BPH, it is conceivable that aromatase inhibition would reduce estrogen levels and eventually influence disease process and symptomatology of the condition. An aromatase inhibitor atamestane has been demonstrated to reduce estradiol, and estrone in serum with a concomitant rise in androgen. However, improvement in clinical symptoms were not particularly different from the placebo group although another study reported a 15% decrease in prostatic volume after treatment for 3 months (Ho and Habib, 2011). Although aromatse inhibitors have virtually been abandoned for therapy, there is yet renewed interest because selective estrogen receptor modulators (SERM) seem to offer better results (Nicholson and Ricke, 2011).

SERMs are non-steroidal compounds that can affect the ER signaling and affect tissues that are estrogen sensitive. They act either as agonist or antagonist depending on the site of action. As ERβ induce apoptosis and ERα aberrant proliferation, a rationale mechanism to neutralize the illeffect of increased estrogen signaling by up regulating ERβ and down regulating ERα receptor is desirable. SERMs have been demonstrated to induce such an effect of up-regulating ERβ and down-regulating ERα in BPH stromal

cells hence SERMs have the potential of managing BPH. In fact various natural and synthetic SERM have been identified (Garg *et al.*, 2013). Among SERMs drugs developed are raloxifen and toremifene. These drugs are able to bind with ERβ more effectively and with a higher affinity to induce its beneficial effect on the diseased prostate while acting as selective ERα antagonist (Yang *et al.*, 2010). Most of these evidence stems from *in vivo* and *in vitro* studies. There are however very few human clinical studies with these drugs. When tamoxifen was tried among 10 men before going on TURP, AR and progestrone receptors reduced drastically (Nicholson and Ricke, 2011) requiring the need for more clinical trials and with a better study power.

In a double-blind phase III study, a combination of toremifene and androgen deprivation significantly reduced the incidence of vertebral fractures in PCa patients, although it did not affect the incidence of PCa at 20 mg in high-grade prostatic intraepithelial neoplasia (PIN) patients (Garg *et al.*, 2013). In a multicentre trial, BPH patients on mepatrin produced significant differences in the mean IPSS compared to control. However, ill effects such as cardiovascular and thromboembolic events have been recorded for the use of some of these drugs (Prezioso *et al.*, 2007).

It is therefore suggested that molecules/drugs that can modulate estrogen and estrogen signaling when used as an adjunct to existing androgentargeting drugs may effectively manage BPH with decreased dosage and side effects (Kumar *et al.*, 2012).

2.4 COMPLEMENTARY AND ALTERNATIVE MEDICINE (CAM)

The national Institutes of Health in North America defines complementary and alternative medicine (CAM) as a constellation of diverse medical and healthcare system and products that are not part of conventional medicine. CAM is broadly categorized into natural products, mind-body medicine, and manipulative and body-based practices. Phytotherapy is the use of plants and plant extracts for medicinal purposes (Kim, 2012).

2.4.1 Phytotherapy for BPH

An increasing number of people are drifting towards the use of CAM. It has been reported that in the US about a third of patients on pharmacological treatment for BPH used herbal preparations alone or in combination with prescribed drugs (Grant and Ramasamy, 2012; Gharaee-Kermani and Macoska, 2013) because they believe it is safer than synthetic drugs. What is more, this belief is founded on personal sentiment and information from friends and media (Kim, 2012). In most countries, patients do not require medical prescription to obtain herbal preparations. In Italy however, some herbal preparation are registered and require a prescription. In fact, in Germany and Austria, phytotherapy is the first line of treatment for patients with mild to moderate urologic problems representing about 90% of all drug prescription for BPH treatment (Allkanjari and Vitalone, 2015). Informing the urologist about the use of phytotherapy is important as some of these drugs

may interact with prescribed drugs or increase risk of complications (Kim, 2012). It is therefore imperative that thorough investigations are carried on some of these plant and plant extracts meant for medicinal purposes.

For the treatment of BPH, several plants have been found to be useful. Popular amongst them are white peony, green tea, spearmint, black cohesh, chaste tree and saw palmetto. The efficacy of most of these were examined via *in vitro* or *in vivo* studies requiring a well-designed, randomized, placebo-controlled studies adequately powered with adequate follow-up time (Grant and Ramasamy, 2012). This is mostly impossible as patients most likely will not opt for this procedure as first line treatment.

Furthermore, the ill-effect on the placebo group is an ethical issue.

2.4.2 Phytotherapy: mechansim of action

Several phytotherapies for the treatment of BPH have been found to exhibit mechanisms analogous to those of pharmacotherapy. Several plant extracts have been found to inhibit the activity of 5AR as well as block AR signaling. Among those that possess such properties include saw palmetto (*Serenoa repens*), gingsen, green tea, reishi, cucurbitapepo, African plum. For example, green tea contains epigallocatechins that inhibit 5AR conversion of T to DHT as well as degrading of AR protein (Pagano *et al.*, 2014). On the other hand, cucurbitapepo competes with DHT for AR binding. Further to this, some phytotherapies have been found to be antagonistic to

alpha adrenergic blockers. Cernilton and saw palmetto have demonstrated this property *in vitro* (Allkanjari and Vitalone, 2015;
Keehn and Lowe, 2015).

The use of P9605, an ethanolic extract of *Piper cubeba* has been demonstrated to exhibit anti-estrogenic properties using human breast cancer cell lines. According to that study, it effectively inhibited aromatase activity and bound effectively to both ERα and ERβ, promoting the use of P9605 in phytotherapy for BPH (Yam *et al.*, 2008).

In vitro tests have demonstrated that some plant extracts induce apoptosis in BPH cells. Saw palmetto induces apoptosis by inhibiting signal transduction pathways involved in apoptosis (Keehn and Lowe, 2015);

Black cohesh, Nettle roots as well as Cernilton also induce apoptosis.

2.4.3 *Croton membranaceus*

Croton belongs to the family of Euphorbiaceae established by Carolus Linnaeus in 1767. Over 1223 species of the Croton genus have been identified and accepted in The World Checklist and Bibliography of Euphorbiaceae. The genus name "Kroton" is greek representing ticks because the seeds look like ticks (Nath *et al.*, 2013). The most common species of Croton in West Africa is the *membranaceus* and it grows close to rivers**.** In Ghana, it grows near the Volta river and the indigenes refer to it

as Bokum. It is difficult to cultivate the plant because it appears the seeds are sterile. However, the Centre for Plant Medicine Research (Mampong, Akwapim) has managed to cultivate the plant at the Centre's arboretum (Aboagye, 1997). The part of the plant commonly used for medicinal purposes is the root. The leaves are used to aromatize tobacco and as a tonic and bitter to improve digestion. Essential oil from the bark is used to treat cough, fever, flatulence, diarrhea and nausea. Root extract is used for the management of secondary bacterial infection in measles and extensively for prostate associated conditions (Mshana, 2000; Bayor *et al.*, 2007; Ayim *et al.*, 2008; Asare *et al.*, 2011; Afriyie *et al.*, 2014; Asare *et al.*, 2015). The Croton genus contains variable phytochemicals. terpenoids, chiefly diterpenoid are the predominant secondary metabolite constituents in the genus (Nath *et al.*, 2013). For the species *membranaceus*, a phytochemical screening has identified a new furano-clerodanediterpeniod, crotomembranafuran, N[N-(2-methylbutanoyl) glutaminoyl-2-phenylethylamine (Bayor, 2008; Sarkodie *et al.*, 2014). Prior to this, glutarimide alkaloid, julocrotine; beta-sitosterol; beta- sitosterol-3-Dglucoside; labdanediterpioid, gomojoside H, and DL-thrietol had already been isolated (Aboagye *et al.*, 2000; Bayor, 2008).

2.4.4 Preclinical and clinical evidence for the management of BPH

Bayor *et al.* (2007), investigated ten plant species used in Ghana for the treatment of various cancers and found two, *Zanthoxylum xanthoxyloides*

bark extract and *Croton membranaceus* root extracts to exhibit markedly high cytotoxic activities against D-Lactate dehydrogenase (DLD-1) and Michigan Cancer Foundation-7 human breast (MCF-7), lending support for the use of both plants in the treatment of cancer. Biochemically, the extract significantly reduces triglyceride (TG) and very low density lipoprotein (LDL) as well as creatinine kinase and lactate dehydrogenase supporting the fact that it is non-toxic (Afriyie *et al.*, 2013). *In vivo* studies have demonstrated that root extract of CM target specifically the prostate. Furthermore, Afriyie *et al.* (2014) examined the liver, heart, kidney, testes and prostate of 6-8 weeks male Sprague-Dawley after 90 days of treatment with the *Croton membranaceus* aqueous root extract and found only the prostate to have changed compared to the control group, whilst thickness and infoldings of epithelial cells shrunk with increasing dosage along with a reduction in PSA. Following this, the group proceeded to investigate the efficacy of the plant extract in BPH-induced rats and found after 28 days of treatment that CM (30 and 300 mg/kg b.wt.) reduced the prostate volume to a size equivalent to the use of finasteride. CM and finasteride attenuated prostatic growth with a resultant thin layer of stromal and epithelial cells similar to the control and significantly decreased the PSA concentration (Afriyie *et al.*, 2014), thus validating it for the treatment of BPH. This informed the decision for clinical studies where the prostate shrunk remarkably with a corresponding improvement in the men's quality of life. Furthermore, free and total PSA, renal, liver function, lipid tests were also assessed in these patients. Significant decreases were observed with both free and total PSA

levels. There were no significant changes in renal, lipid and liver function tests except for total and indirect bilirubin (Asare *et al.*, 2015).

Several mechanisms have been proposed for the effect of CM in BPH. Inhibition of cholesterol absorption, acetylcholinestrase (AChE), antioxidant action, α- adrenergic antagonism and 5AR inhibition caused by the presence of phytosterols, β-sitosterols, stigmasterol, campesterol, scoleptin manganese and julocrotine (Appiah *et al.*, 2013) are assumed to be the principle underpinning the mechanism of action.

In an attempt to elucidate the mechanism of action, only one of these pathways has been explored. Using BPH-1 cell lines, CM induced a significant dose-dependent inhibition in the proliferation of BPH-1 cells with a concomitant up regulation of the mRNA and protein levels of Bax, which is a promoter for apoptosis. It was proposed that CM might ply the mitochondria-dependent apoptosis pathway (Afriyie *et al.*, 2015). Other mechanisms proposed remain to be elucidated.

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY DESIGN

Observational studies are studies that allow the researcher to observe natural relationships between factors and outcomes without interceding as part of the study design. Case-control study designs fall under observational studies. In this study, participants were identified based on their BPH status. On the other hand, a pre-post study design that falls under interventional studies, allows for the measurement of outcomes after a particular treatment. Based on the time of data collection, experimental studies could be retrospective or prospective. Retrospective data is obtained from past records or by asking clients to recall past exposure and experience whereas, prospective studies follow participants through time, collecting data in the process (Thiese, 2014).

This study was an observational prospective clinical study employing, both case-control and pre-post study designs. The case-control study constituted cases of newly diagnosed BPH patients and controls without the disease condition, while the pre-post study used only the BPH patients.

Patients on *Croton membranaceus* root extract marketed as URO 500 (10 mg extract per capsule) from the Center for Plant Medicine Research in Ghana (Mampong , Akwapim) were observed (Dosage: 2 tablets t.i.d).

Baseline and after treatment urologic outcome characteristics (Prostate volume and Total IPSS score) and serum/plasma samples for biochemical analyses were taken.

3.2 STUDY SITE

The study was conducted at the Ghana Police Hospital. This is one of the few hospital approved by the ministry of Health to offer CAM to patients who wish to opt for such alternative treatment models.

3.3 ETHICAL ISSUES

This study was part of a bigger study for which consent had been sought from the University of Ghana School of Biomedical and Allied Health Sciences, with ethics clearance number:

SAHSET/SAHS/PSM/ML/09/AA/26A/2012-2013 (Appendix II). The study complied with the Helsinki Declaration of 1964 (revised in October 2008). Informed consent form (Appendix I) was administered to all subjects who agreed to participate in the study.

3.4 SAMPLE SIZE

Sample size was determined by Cochrane's formula:

$$N = \frac{Z^2 * P(1-P)}{E^2} = \frac{1.96^2 * 0.13(1-0.13)}{0.10^2} = 43.45$$

N(sample size)

Z(z-score associated with confidence interval) = 1.96

P(Prevalence rate within the population, 13.3%)= 0.13

E(acceptable error margin)=0.10

Although the calculated sample size was 44, 30 BPH patients were observed. This was an appropriable number considering the fact that some well-established instructions like the FDA accepts few dozen to 300 diseased participants for phase II drug trials (FDA, 2014).

3.4 PARTICIPANTS

3.3.1 Inclusion Criteria

The inclusion criteria were as follows:

- Clinically diagnosed men with BPH who opted for treatment with CM as study group
- Apparently healthy men as controls

3.3.2 Exclusion Criteria

The exclusion criteria were as follows:

- All women were excluded
- Men with conditions other than BPH
- Men with BPH but on therapies other than CM
- Men on combined therapies

3.4 PROCEDURE FOR DATA COLLECTION

A simple purposive sampling technique was employed for this study. Three study tools were used; questionnaire, abdominapelvic scan and laboratory assays.

3.4.1 Questionnaire for demographic and IPSS

Symptoms of LUTS were assessed by a 7 standardized questionnaire known as the International Prostate Symptom Score (IPSS).The answers were assigned points from 0 to 5, with 5 representing worsening symptoms. The total score therefore ranged from 0 to 35 (asymptomatic to very symptomatic) (Appendix III).

3.4.2 Scan of prostate volume

Prostate volumes were obtained by abdominapelvic scan using the Sonoscape Digital Colour Doppler Ultrasound system 551-6000 (Shenzhen, China). Prior to scanning, patients had to drink about 1.5 L of water and wait for 1-2 hours. This was to enable better visibility of the prostate. Patients were then laid on an ultrasound couch and the pelvic area exposed. To bridge acoustic impedance between patient skin and probe surface, a liquid gel was applied and the prostate and bladder volumes at full capacity was obtained. Post void residual volume was obtained on a second scan after patients had voided urine.

3.4.3 Laboratory assays

3.4.3.1. Sample collection

Venous blood samples (5 mls) were taken from those who met the criteria and consented, into gel separator tubes and EDTA tubes. This was done before 9 am as early morning sampling is recommended for androgens. Samples were then centrifuged at 3500 rpm for 5 minutes, using Heal Force centrifuge [Shanghai Lishen Scientific equipment Co (Shanghai, China)] to obtain serum and plasma. Buffy coat was also obtained after centrifugation at 2200 rpm for 10 minutes. Buffy coat samples were stored at -70^{0}C for another project. Plasma and serum were aliquotted into microcentrifuge tubes and stored at -20^{0}C until ready for analyses. All

samples obtained from a subject were assayed in the same run for each parameter to exclude inter-assay variation from changes in hormone levels within subjects.

The following biochemical assays were performed: tPSA, fPSA, androstenedione (AED), testosterone (T), Free testosterone (FT) dihydrotestosterone (DHT), androstanediol adiol (3α-adiol), androstanediol (3β-diol), estrone (E1) and estradiol (E2)

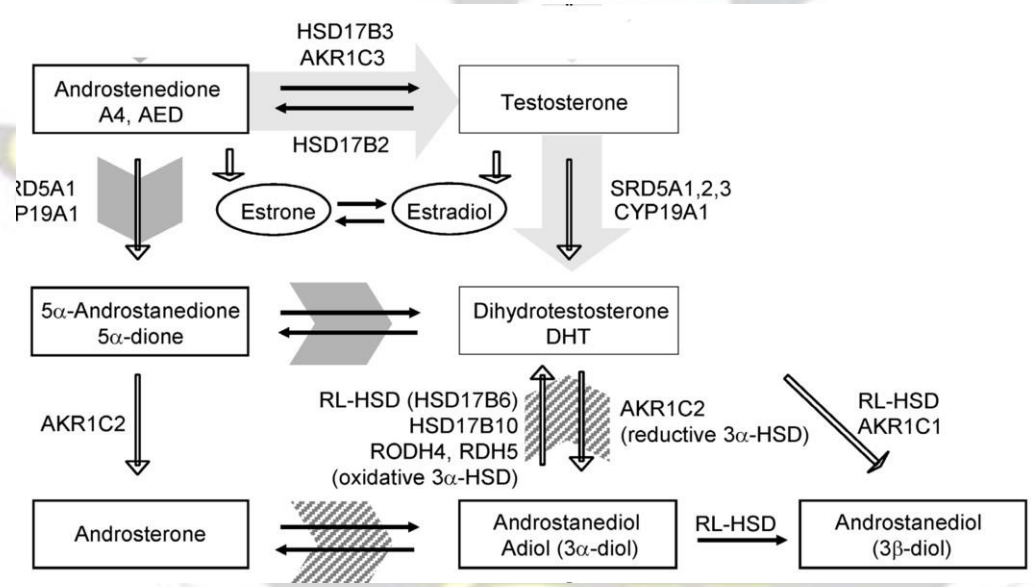

Figure 3.1: Overview of Hormones Assayed

The following hormones, AED, E1, E2, T, DHT, 3a-Diol and 3β-diol were assayed. E1, E2 and 3β-diol binds either ERa or ERβ while T, DHT and 3adiol binds AR (Mostaghel.et al., 2014)

3.4.3.2 Total and free PSA Principle:

Both free and total serum PSA were performed using ELISA kits from Monobind Inc. (California, USA). The test employed a sandwich method. A soluble sandwich complex forms at the surface of the microplate through the interaction of streptavidin and biotinylated monoclonal anti-PSA to form an enzyme-labeled antibody that binds with the native antigen in the sample without stearic hindrance. The reaction is allowed to stabilize. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. A standard curve is drawn from known reference samples provided in the kits to extrapolate the concentrations of the unknown samples.

Procedure:

1. An amount of 50 µl of the appropriate reference standards, controls and samples were pipetted into assigned wells.
2. An amount of 100 µl of the PSA (Total and free) enzyme reagent were added to each well, covered and incubated for 60 min at 20-27^{o}C.
3. Content of the microplate was discarded followed by washing with 350 µl of wash buffer 5x. Wash buffer was also discarded.
4. An amount of 100 µl of working substrate solution (tetramethylbenzidine and hydrogen peroxide in buffer) was added and incubated for 15 min.
5. To stop the reaction, 50 µl of the stop solution (1N HCL) was added.

6. Reaction was read bi-chromatically using 450 nm as the main, and 630 nm as the reference wavelength.
7. A standard curve was drawn with standards and used to extrapolate the concentration of the samples.

3.4.3.3 Hormonal assays Principle:

All hormones and metabolites; [androstenedione (AED), testosterone (T), dihydrotestosterone (DHT), androstanediol adiol (3α-adiol), androstanediol (3β-diol), estrone (E1) and estradiol (E2)] were assayed using ELISA kits from Sunlong Biotech Co. Ltd (Hangzhou, China). All the hormonal assays used the Sandwich-ELISA method. The micro-ELISA strip plate was precoated with antibody specific to a particular hormone. Corresponding antigens in the plasma bind to form antigen-antibody complex. This antigen-antibody complex binds a secondary antibody, horseradish peroxidase (HRP)-conjugated antibody specific to the hormone, to form a stable sandwich complex. The enzyme activity of the antibody-antigen-HRP complex obtained after washing and further binding to a substrate. The chromogen developed is proportional to the antigen concentration of the hormone.

Procedure:

The assays were performed according to the manufacturer's instructions for the following hormones: androstenedione (AED), testosterone (T), dihydrotestosterone (DHT), androstanediol adiol (3α-adiol), androstanediol (3β-diol), estrone (E1) and estradiol (E2). The procedure was the same throughout. However, each hormone had its own ELISA plate coated with the corresponding antibody.

1. Standards were diluted to produce the required concentration for each hormone. An amount of 50 µl of each standard was added to wells B1-F1, leaving A1 empty for blank.

2. In sample wells, 40 µl sample dilution buffer was added first, followed by 10 µl sample and sealed, thus creating a dilution factor of 5.

3. The reaction was then incubated for 30 min at 37℃.

4. Excess components were washed with 350 µl wash solution (5×).

5. An amount of 50 µl of secondary antibody (HRP-Conjugate reagent) was then added to each well except the blank control well and incubated for another 30 min at 37°C and washed as in step 4.

6. Chromogen A (tetramethylbenzidine) and B (hydrogen peroxide) (50 µl, one after the other) were added to each well, mixed gently and incubated at 37°C for 15 min avoiding exposure to light during the incubation period.

7. The reaction was then terminated with 50 µl of stop solution

(1N HCL) to each well. The color changed from blue to yellow.

8. The absorbance was read at 450 nm using multiscan microplate reader (Buckingham, UK) within 30 min of final colour development.

3.5 DATA ANALYSIS

Nonparametric methods Mann-Whitney U test and Wilcoxon sign rank test were employed for independent and related samples, respectively, as normality check performed with Kolmogorov-Smirnove test with Lilliefor's correction showed deviation from Gaussian distribution. Results are therefore presented in percentiles. Correlation analyses were computed with spearman correlation. Regression analyses were avoided as assumption for its use could not be applied to the data. All statistical significance levels reported are 2-tailed. P value less than 0.05 was considered significant. Statistical analyses were performed using SPSS software, Version 21.0 (Chicago, USA).

CHAPTER 4

RESULTS

4.1 EFFECT OF BPH ON ANDROGEN BIOSYNTHESIS

For the cross-sectional study, the study population consisted of thirty (30) BPH patients and thirty (30) men without BPH as controls. The age range of the BPH patients was 46-87 years while the controls aged between 4672 years. The mean difference between their ages were significant (P=0.000) (Figure 4.1).

Total PSA (tPSA) concentration between them was also significantly different ($p<0.001$). The median tPSA estimated for patients and controls were 18.15 (9.22-42.2) ng/ml and 0.90 (0.50-1.40) ng/ml, respectively. All controls recorded tPSA values < 4.0 ng/ml, while 5 of the cases recorded values below 4.0 ng/ml.

Table 4.1 presents the interquartile concentrations of the hormones, metabolites and enzyme indices. The controls had a significantly higher androstenedione concentration (AED) than the BPH patients (p=0.027). AED level was 336 (299-380) pg/ml for BPH patients while that of the controls was 386 (343-477) pg/ml. Median concentrations of T and DHT of BPH patients were relatively higher in BPH patients compared to controls. Median concentrations of T and DHT of patients versus controls were 6.72 (4.64-8.23) ng/ml versus 6.52 (4.42-8.97) ng/ml and 302 (256334) versus 295

(264-357) pg/ml. Estimated FT, 3α-adiol and 3β-diol concentrations for BPH patients were 93.9 (71.3-122) pg/ml, 9.71 (8.7014.0) ng/ml and 3.82 (3.29-5.14) ng/ml, respectively. The controls recorded relatively higher values of 101(74.8-130) pg/ml, 11.8 (9.50-16.3) ng/ml and 4.8 (3.38-8.36) ng/ml for FT, 3α-adiol and 3β-diol respectively. These differences were not significant. Both estrogens, E1 and E2 were higher in BPH patients. Median E1 concentration for patients was 71.5 (56.3-95.6) pg/ml whiles that of the controls was 61.0 (33.1-88.8) pg/ml. E2 concentration was significant (p=0.029). Estimated medians for cases and controls were 29.9 (23.5-33.6) pg/ml and 23.8 (20.4-28.6) pg/ml,
respectively.

Enzyme index DHT/T reflecting 5AR activity was slightly higher in patients compared with controls 0.050 (0.035-0.061) versus 0.049 (0.034-0.077). Similarly, E1/AED and E2/T both representing aromatase activity were also higher in patients than controls. E2/T was significant (p=0.014) while that of E1/AED was not. 3α-adiol/DHT and 3β-diol/DHT showing the activity of AKIRC2 and AKIRC1, respectively, were both reduced in the BPH group. AKIRC2's activity estimated for cases against controls was 34.2 (27.3-47.1) and 40.0 (30.5-48.6). AKIRC1's activity was significantly higher in controls (p=0.036). Median enzyme activity of AKIRC1 was 13.2 (10.5-
16.8) and 16.5 (11.7-23.6) for patients and controls, respectively.

4.2 EFFECT OF CM ON ANDROGEN METABOLISM IN BPH PATIENTS

To observe changes in BPH characteristics and hormones, pre-post analysis was employed. An initial number of 46 BPH patients were recruited for the study; however, not all could follow through to the end of the study. Thirty (30) matched blood samples could be retrieved by the end of the study. Seventeen (17) patients allowed for their prostate to be scanned at the start of the study, 8 of these patients came back for re-scan after treatment with the drug.

Treatment with the plant extract markedly improved BPH symptoms. IPSS scores of 0-7, 8-19 and 20-35 were graded as mild, moderate and severe, respectively. The median IPSS for patients at baseline and at the end of the 3 months treatment were 15.5 (6.50-22.5) and 12.0 (6.00-16.0), respectively ($p<0.00$) (Table 4.2). There was a percent mean decrease of 28.3% in IPSS. Prostate volume decreased significantly from 99.3 cm^3 to 55.8 cm^3 after treatment with CM ($p=0.01$).

Total PSA levels reduced drastically from 18.15 (9.22-42.42) ng/ml to 11.25 (4.80-24.4) ng/ml ($p=0.00$) (Table 4.2) with plant extract in the BPH patients. Two of the patients had their tPSA dropping below 4.0 ng/ml. Patients fPSA dropped from 2.8 (1.80-8.20) ng/ml to 2.1 (1.40-3.40) ng/ml ($p<0.00$). However, %fPSA increased from 17.87 (14.1-40.0)% to 23.34 (14.1-42.50) which was not significant (Table 4.2).

Table 4.3 presents the percentiles and statistical significance between hormones and enzyme indices estimated in this study. AED and 3α-adiol decreased non-, while T, FT, DHT and 3β-diol increased respectively. All of these changes were not significant except for DHT (p=0.005). Both E1 and E2 decreased. E1 decreased significantly by (p=0.009) while E2 decreased non-significantly(p=0.088).

5AR (DHT/T) activity increased while aromatase (E1/AED & E2/T), AKIRC1 (3β-diol/DHT) and AKIRC2 (3α-adiol/DHT) activities all decreased.
All of these changes were non-significant. Median difference observed in 3α-adiol/DHT was significant (p=0.009).

4.3 CORRELATION ANALYSIS

Table 4.4 represents the correlation analysis in BPH patients between clinical parameters and age at baseline. Age was found to associate only was tPSA (r=0.348, p=0.049). The association between age, clinical parameters, hormones and enzyme indices at baseline follows in Table 4.5.
There was a weak positive correlation between age and T (r=0.268, p=0.04). However, all other hormones and enzyme indices did not associate with age. There was no significant association between hormones and prostate volume except with FT. Prostate volume correlated negatively with FT (r=-0.514, p=0.01). Similarly, fPSA correlated significantly only with FT (r=-

0.419, p=0.03). IPSS correlated negatively with both 3β-diol and 3βdiol/DHT at (r=-0.656, p=0.00) and (r= -0.519, p=0.02). IPSS had no significant association with any other variable.

After treatment, percent change (improvement) in clinical parameters correlated with percent changes observed in hormones and enzyme indices (Table 4.6). The study observed that decreases observed in IPSS, tPSA, fPSA associated with increases in 3β-diol, FT and DHT. IPSS had a significant moderate correlation with 3β-diol (r= -0.498, p=0.026), fPSA had a significant moderate correlation with FT and DHT (r=-0.392,p=0.043) and (r=-0.401, p=0.028), respectively. tPSA also correlated with FT (r=-0.467, p=0.014). All other associations were not significant.

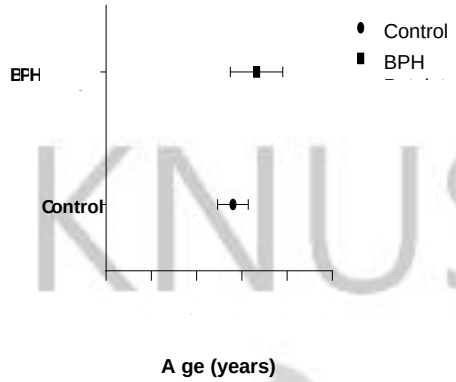

Figure 4.1: Age profile of study participants. Age is presented as the mean with error bars. Age was higher in patients with BPH compared to the controls ($p < 0.001$)

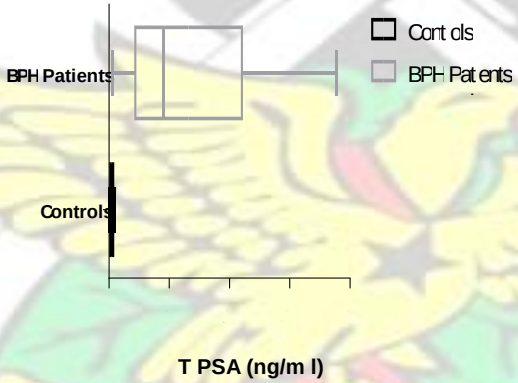

Figure 4.2: This diagram depicts the median total PSA (tPSA) concentrations of the study groups ($p<0.001$).

Table 4.1: Comparison of hormone concentrations and enzyme indices in BPH patients and Controls

	Case Group (baseline)			Control			
Variables	25th Percentile	Median	75th Percentile	25th Percentile	Median	75th Percentile	**P-value**
AED (pg/ml)	298	336	379	342	386	477	0.027*
T (ng/ml)	4.64	6.72	8.23	4.42	6.52	8.97	0.722
FT (pg/ml)	71.3	93.9	121	74.8	101	130	0.560
DHT (pg/ml)	255	302	334	263	295	357	0.761
3α-adiol(ng/ml)	8.70	9.71	14.0	9.51	11.8	16.3	0.310
3β-diol (ng/ml)	3.29	3.82	5.14	3.38	4.80	8.36	0.135
E1 (pg/ml)	56.3	71.5	95.6	33.1	61.0	89.0	0.206
E2 (pg/ml)	23.5	29.9	33.6	20.4	23.8	28.6	0.029*
DHT/T	0.035	0.050	0.061	0.034	0.049	0.077	0.863
E1/AED	0.186	0.228	0.269	0.110	0.146	0.221	0.014*
E2/T	0.004	0.005	0.006	0.003	0.004	0.006	0.088
3α-adiol/DHT	27.3	34.2	47.1	30.5	40.0	48.6	0.444
3β-Dio/DHT	10.5	13.2	16.8	11.7	16.50	23.6	0.036*

Data are presented as median with interquartile ranges (25th and 75th percentile).*P-values <0.050 was considered significant. Differences in medians (50th) are computed by Mann-Whitney test. Abbreviations [AED- androstenedione, T-Testosterone, DHT-diydrotestosterone, FT-free testosterone, 3αadiol- androstanediol adiol (3α-adiol), 3β-diol- androstenediol (3β-diol): DHT/T indicates 5AR activity, 3α-adiol/DHT and 3β-diol/DHT represent indices for AKR1C2 and AKR1C1 while E1/AED and E2/T represent aromatase activity].

4.2. General Characteristics of BPH Patients and the effect of treatment

		Baseline			Treatment				
Variable	N	25th Percentile	Median	75th Percentile	25th Percentile	Median	75th Percentile	% Mean difference	**P-Value**
IPSS	20	6.50	15.50	22.50	6.00	9.50	16.00	-28.34	0.00*
Prostate volume ($cm^{3)}$	8	66.10	99.25	127.80	33.52	55.80	68.95	-31.39	0.01*
tPSA	30	9.22	18.15	42.20	4.80	11.25	22.40	-31.27	0.00*
fPSA	30	1.80	2.80	8.20	1.40	2.10	3.40	-17.65	0.00*
%fPSA	30	14.11	17.87	40.00	14.10	23.34	42.50	14.12	0.10

*Data are shown as percentiles (25^{th}, 50^{th} and 75^{th} percentiles). *P-Values <0.050. Median differences are compared by* **Wilcoxon sign rank test.**

Mean differences are expressed as $(\exp(\text{mean}[\log(xi) - \log(xo)]) - 1) \times 100$. *Abbreviations: [IPSS- Total International prostate symptom score, tPSA-total prostate specific antigen, fPSA-free prostate specific antigen, %fPSA -Ratio of free to total PSA expressed as percentage].*

Table 4.3: Comparison of hormone concentrations and enzymes indices at baseline and after treatment

		Baseline			After treatment			
Variable	N	Percentile 25	Median	Percentile 75	Percentile 25	Median	Percentile 75	**P-Value**
AED (pg/ml)	30	299	337	380	269	313	394	0.309
T (ng/ml)	30	4.64	6.72	8.23	4.52	6.13	8.85	0.746
FT (pg/ml)	29	71.3	93.8	122	74.8	125	185	0.340
DHT (pg/ml)	30	256	302	334	311	340	361	0.005*
3α-adiol (ng/ml)	30	8.70	9.71	14.03	8.49	9.57	10.86	0.073
3β-diol (ng/ml)	29	3.29	3.82	5.14	3.66	4.15	4.55	0.831
E1 (pg/ml)	29	56.3	72.8	95.6	28.9	54.8	71.4	0.009*
E2 (pg/ml)	30	23.5	29.9	33.7	20.3	25.5	30.1	0.088
DHT/T	30	0.035	0.050	0.061	0.041	0.055	0.072	0.440
E1/AED	29	0.186	0.224	0.269	0.133	0.179	0.217	0.059
E2/T	30	0.004	0.005	0.006	0.003	0.004	0.006	0.229
3α-adiol/DHT	30	27.3	34.2	47.1	23.3	27.7	33.8	0.009*
3β-diol/DHT	30	10.5	13.2	16.8	10.56	12.4	14.0	0.626

Data are presented as median, 25th and 75th percentiles. Statistical significance was determined by Wilcoxon sign rank test. *P<0.05. Abbreviations [AED: Androstenedione, T-Testosterone, DHT-diydrotestosterone, FT-Free Testosterone, 3α-adiol- Androstanediol adiol (3α-adiol), 3β-Diol- Androstanediol (3β-diol), DHT/T reflects 5AR activity. 3α-adiol/DHT and 3β-Diol/DHT represent indices for AKR1C2 and AKR1C1 while E1/AED and E2/T represent aromatase activity].

Table 4.4: Correlation between Age and clinical parameters at baseline

		Age	T IPSS	Prostate volume (cm3)	tPSA (ng/ml)	fPSA (ng/ml)	%fPSA
Age	r	1	0.140	-0.326	0.348	0.158	-0.328
	P-Value		0.557	0.201	0.049*	0.404	0.077
T IPSS	r		1.000	-0.242	0.000	-0.103	0.191
	P-Value			0.426	0.999	0.665	0.419
Prostate volume (cm3)	r			1.000	-0.177	-0.270	0.077
	P-Value				0.497	0.295	0.768
tPSA (ng/ml)	r				1.000	.552	-.623
	P-Value					0.002**	0.000**
fPSA (ng/ml)	r					1.000	0.124
	P-Value						0.513
%fPSA	r						1.000
	P-Value						

**P-Values were computes by spearman analysis. *P<0.05 were considered significant.*

Table 4.5: Correlation analysis between age, clinical outcome and biomarker and hormones levels at baseline

	Age		IPSS		Prostate volume (cm^3)		tPSA (ng/ml)		fPSA (ng/ml)		%fPSA	
	r	P-Value	r	P-Value	r	P-Value	r	P-Value	r	P-Value	r	P-Value
AED (pg/ml)	0.01	0.96	-0.23	0.34	0.34	0.18	-0.13	0.49	-0.14	0.46	-0.03	0.88
T (ng/ml)	.268	0.04*	-0.10	0.66	-0.06	0.83	-0.09	0.64	-0.07	0.73	-0.03	0.87
FT (pg/ml)	0.14	0.58	0.22	0.41	-.514	0.01*	-0.33	0.08	-.419	0.03*	0.02	0.93
DHT (pg/ml)	0.02	0.89	-0.28	0.23	0.03	0.91	-0.15	0.42	-0.10	0.61	0.01	0.96
3α-adiol (ng/ml)	0.08	0.56	-0.24	0.31	0.00	0.99	0.00	0.98	-0.02	0.92	-0.10	0.59
3β-diol (ng/ml)	0.00	0.99	-.656	0.00*	0.16	0.54	-0.07	0.73	-0.14	0.45	-0.11	0.57
E1 (pg/ml)	0.10	0.43	-0.38	0.10	-0.18	0.50	-0.16	0.40	-0.25	0.17	-0.13	0.48
E2 (pg/ml)	0.17	0.19	-0.28	0.23	0.34	0.18	0.11	0.57	0.28	0.13	0.10	0.60
DHT/T	-0.33	0.08	-0.06	-0.82	0.05	0.84	0.06	0.76	0.56	0.77	0.037	0.85
E1/AED	0.04	0.76	-0.30	0.19	-0.29	0.26	-0.14	0.46	-0.21	0.27	-0.02	0.92
E2/T	-0.13	0.33	-0.05	0.85	0.19	0.46	0.23	0.22	0.31	0.10	0.07	0.70
3α-adiol/DHT	0.04	0.76	-0.05	0.84	0.00	0.99	0.12	0.51	0.09	0.64	-0.12	0.53
3β-diol/DHT	-0.06	0.66	-.519	0.02*	0.21	0.43	-0.03	0.87	-0.06	0.74	-0.06	0.75

**P-Value < 0.05. Spearman correlation was used for the analysis*

Table 4.6 Correlation between Percent Change in clinical outcome and hormonal levels after treatment with CM

	IPSS		PV		tPSA		fPSA	
% Change/12 weeks	r	P-Value	r	P-Value	r	P-Value	r	P-Value
AED	0.101	0.672	0.024	0.955	-0.172	0.362	0.050	0.793
T	-0.026	0.912	0.571	0.139	0.013	0.946	-0.105	0.581
FT	0.305	0.219	0.048	0.911	-.467	0.014*	-.392	0.043*
DHT	-0.257	0.273	0.143	0.736	-0.222	0.238	-.401	0.028*
3α-adiol	0.151	0.526	0.381	0.352	-0.080	0.675	-0.051	0.789
3β-diol	-.498	0.026*	-0.262	0.531	-0.068	0.726	-0.055	0.775
E1	0.251	0.286	0.452	0.260	-0.229	0.232	-0.249	0.192
E2	0.031	0.897	0.214	0.610	0.105	0.579	0.294	0.115
DHT/T	-0.261	0.289	-0.571	0.139	-0.033	0.864	-0.120	0.534
E1/AED	0.084	0.726	0.190	0.651	-0.105	0.587	-0.216	0.260

E2/T	0.031	0.897	0.214	0.610	0.105	0.579	0.294	0.115
3α-adiol/DHT	0.203	0.390	0.238	0.570	-0.019	0.922	0.077	0.686
3β-Diol/DHT	-0.291	0.214	-0.143	0.736	-0.017	0.931	0.127	0.512

***P<0.05 was considered significant. Percent change was estimated as change in after treatment and baseline level expressed as a percentage of the baseline value*.**

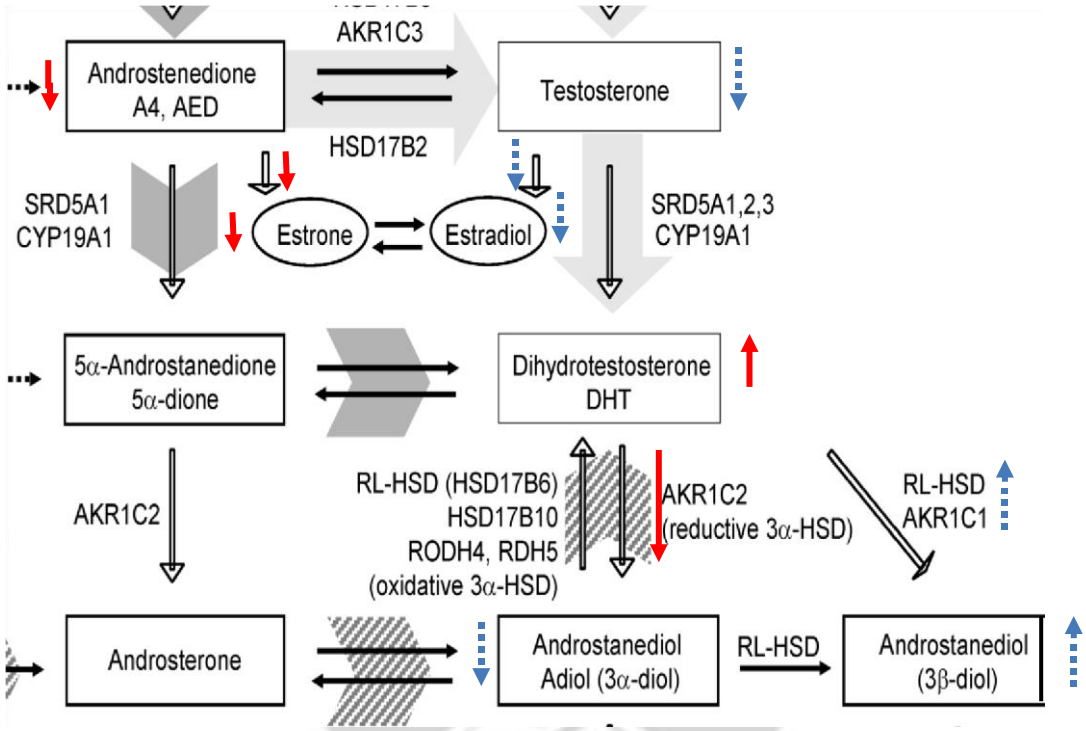

Figure 4.2: The effect of CM on the androgen metabolic pathway in BPH patients. *Red arrows indicate significant increase (P<0.05) and decrease while blue dashed arrows are non-significant increase and decrease. Significant decreases were observed in AED and E1 concentrations. Also, there were significant decreases in aromatase (E1/AED) and AKRIC2 (3aadiol/DHT). On the other hand, a significant increase was observed in DHT levels. Decreases were observed in T, E2 and 3a-adiol as well as aromatase (E2/T). Furthermore 3β-diol and AKRIC1 increased.*

CHAPTER 5

DISCUSSION

5.1 DISCUSSION

The development of BPH has become a natural phenomenon among aged people, affecting their quality of life and impacting also on a country's economy as well its health section. Three factors mark the presence of BPH; LUTS, enlarged prostate and bladder outlet obstruction. Of these three, this study assessed two, LUTS by IPSS questionnaire form and prostate enlargement by taking an ultraurethral scan of the prostate.

Age represents a singular most important factor that independently predicts the condition (Briganti *et al.*, 2009; Garg *et al.*, 2013). It has been proven that as males age, the prevalence of BPH increases. Age correlates with urologic outcomes (Zhang *et al.*, 2013; Egan *et al.*, 2015; Shim *et al.*, 2015). Briganti *et al.* (2009) explains that in aging men, tissue remodeling processes occur in the transition zone of the prostate disrupting the delicate balance of interacting growth factor signaling pathways, stromal-epithelial interactions leads to increase prostate volume and these usually occur in basal cells, changing their metabolic
rate, and making them become enlarged and hypertrophic.
Unfortunately, this study could not properly assess this fact probably due to the small sample power. Age did not correlate with IPSS and prostate volume. Agrawal *et al.* (2008) and Vesely *et al.* (2003) also reported no significant correlation between age and prostate volume. Additionally, the relationship between age and prostate volume in men with LUTS has been

reported to be nonlinear (El Din *et al.*, 1996). LUTS may arise from neurophysiological changes in the lower urinary tract
(Berry *et al.*, 1984; Vesely *et al.*, 2003).

This study reports a high level of tPSA levels among BPH patients. An observation consistent with other studies (Ceylan *et al.*, 2013; Stanczyk *et al.*, 2013; Yalçınkaya *et al.*, 2013). As with other studies, tPSA correlated with age (Battikhi, 2003; Mochtar *et al.*, 2003; Nnabugwu *et al.*, 2015). It is established that as one ages, prostate volume increases; so does PSA.

Several theories have been propounded for the etiology of BPH and three of these seem to gain grounds; hormone imbalance, embroyonic reawakening and stem cell theory. The hormonal imbalance has received much attention due to the dramatic response of androgen scrotal ablation (castration) and the effect of hormone-based pharmacotherapy in diseased patients.

The influence of BPH on hormones was assessed at baseline. Testosterone (T) level between patients and controls were insignificant, consistent with findings from other cross sectional studies (Rył *et al.*, 2015; Usoro *et al.*, 2015). On the other hand, other studies reported a significant difference in baseline T between BPH and controls (Kristal *et al.*, 2008; Stanczky *et al.*, 2013). However, free testosterone (FT) was not significant. This is in line with the study of Ryl *et al.* (2015). This study observed that the level

of androstenedione (AED) was significantly higher in BPH patients compared with controls, thus supported by the study of Stanczyk *et al.* (2013). The plasma dihydrotestosterone (DHT) level between the BPH patients and the controls was also not significant in this study. Intraprostatic DHT was found to be significantly increased in BPH, however serum DHT levels was found to be relatively higher in treatment group compared to placebo groups at baseline (Wurzel *et al.*, 2007). In a recent study the odds of a higher baseline level of DHT was found to increase the risk of BPH (Parsons *et al.*, 2010).

This study reports a higher level of 3α-adiol and 3β-diol in controls compared to BPH patients. Geller *et al.* (1976) found the levels of intraprostatic 3α-adiol and 3β-diol in tissues of BPH patients to be significantly lower. To the best of my knowledge 3α-adiol and 3β-diol have not as yet been assayed in serum/plasma samples of BPH patients, although it is clearly accepted to play androgenic and estrogenic roles. In fact, tissue levels of 3α-adiol correlate with that of DHT (Monti *et al.*, 1998).

This study reports a significant higher E2 in patients compared with controls as was reported by Roberts *et al.* (2004). Estrone though higher in BPH patients, was not significant. Studies report a significant higher E2 level in controls compared with BPH patients (Liao *et al.*, 2012; Rył *et al.*, 2015). Kristal *et al.* (2008) recorded no significant difference in

estradiol as did Uroso *et al.* (2015) between BPH and controls. However, Skoldofors *et al.* (1978) reports a significant higher level of estrone in BPH.

5AR activity was relatively higher in the BPH patients compared to the controls. Parson *et al.* (2010) and Liao *et al.* (2012) found a higher 5AR activity to associate with increased risk of BPH and an association with increasing prostate volume. Aromatase converts estrogen to testosterone and its activity was represented by the ratios of E2/T and E1/AED. Aromatase activity defined as E1/AED was significantly higher in BPH patients. Other studies however documented a rather increased activity of E2/T in BPH (Kristal *et al.*, 2008; Liao *et al.*, 2012) although the present study did not make this observation. AKRIC1 was significantly higher in controls compared to cases however AKRIC2 was not significant. No study has reported this finding in serum/plasma except for Geller *et al.* (1976) who reported a similar trend in prostate tissues.

Hormone concentrations seem to correlate with urologic outcome. This study found a significant inverse relationship between 3β-diol and IPSS and FT and prostate volume. Prostate volume correlates positively with LUTS (Bosch *et al.*, 2008; Wang *et al.*, 2008; Lim and Buchan, 2014; Haghsheno *et al.*, 2015) while one other study observed a weak association between them (Sciarra *et al.*, 1998). Although this study did not find this association due to the sample size, it is agreeable that if

3β-diol induces apoptosis and it is down regulated, IPSS would be high and vice versa.

A weak positive relationship was found between age and T in this study. In BPH, this association has not been established (Liu *et al.*, 2007; Favilla *et al.*, 2010), yet it is suggested that men with high T are likely to experience BPH (Trumble *et al.*, 2015; Kristal., *et al.*, 2008). That T decreases by 2-3% (Leifke *et al.*, 2000; Harman *et al.*, 2001) annually in healthy men has been contradicted by the study of Mustafa *et al.* (2014). In a meta-analysis by Saad *et al.* (2011), additional androgens do not influence prostatic growth once androgen receptor binding capacity is reached. High T translates into more FT and subsequently more DHT that saturate the binding sites of AR for prostatic growth. In the formation of the massive prostate volume, high androgenic activity may be needed to stimulate prostatic growth, hence the use of FT, which forms the active component needed for the formation of the more potent androgen DHT. Therefore less FT use gives room for prostatic growth and the release of PSA. It is noteworthy that this study recorded T concentrations of both patients and controls at values lower than the saturation point for human prostate tissues (Khera *et al.*, 2014).

Much as androgens form part of the development of BPH, this occurs in the presence of estrogens. Results from this study strongly support the estrogen hypothesis. In this study the high amount of E2 along high activity of aromatase and reduced AKRIC1 activity are likely to be involved

in the development of BPH. Increased aromatase activity increases the formation of estrogen. Estrogens bind to ERα and ERβ to induce proliferation or apoptosis in prostatic cells, respectively. In BPH, ERα is up regulated (Royuela *et al.*, 2001; Yang *et al.*, 2009) and the presence of an appropriate ligand E2 is likely to activate it to induce proliferation of the stromal cells. To substantiate this, AKRIC1 which is supposed to convert androgens into 3β-diol, known to stimulate ERβ is down regulated. The plasma androgen levels were increased in the BPH group in this study, although this did not produce statistical significance probably due to the fact that although testosterone increases with age, DHT levels are not affected by age (Carson and Rittmaster, 2003; Saad *et al.*, 2011). Controls were significantly younger. BPH is thought to evolve from reduced apoptosis, increased proliferation and also high androgenic activity (Claus *et al.*, 1993; Claus *et al.*, 1997; Kristal *et al.*, 2006; Alonso-Madgalene *et al.*, 2009).

Hormonal modulation influence androgen and estrogen signaling by blocking receptors or down-regulating active hormones that drive hyperproliferation of the prostatic cells or inducing apoptosis to ensure an equal rate of proliferation to apoptosis seem rationale. The goal of this study was to assess the influence of CM, a common medical plant used in Ghana for the management of BPH on the hormone dependent pathway.

This study found the plant extract to increase %fPSA by 14.1% and decreased IPSS, prostate volume and tPSA by 28.3%, 31.4% and 31.3%,

respectively. Low %fPSA has been found to be a strong predictor of later detection of PCa (Sasaki *et al.*, 2014) and drugs that seek to help in the management of prostate associated disease are expected to lift this ratio higher.

Finasteride treatment for BPH over 9 months increased %fPSA by about 10% in BPH patients (Matzkin *et al.*, 1996) while another study reports 13-17% improvement over the same period of time (Pannek *et al.*, 1998). Treatment with 0.5 mg dutaseride once daily for 12 weeks yielded a significant 28.4% decrement in IPSS score (Desgrandchamps *et al.*, 2006). In a recent Chinese randomized, double-blind, parallel-group, placebo-controlled study of 253 BPH patients with prostate volume ≥ 30 cm^3, 0.5 mg dutasteride daily for six (6) months significantly reduced prostate volume by 17.14% (Na *et al.,* 2012). Choi *et al.* (2012) examined the effect of combination therapy α-blockers and 5ARI (dutasteride and finasteride) to elicit a 28.2 versus 20.5%, 43.6 versus 39.2 % 4.6 versus 3.5 decrease in prostate volume, PSA and IPSS score over 12 months. Similarly, Xu *et al.* (2016) reported a 40% reduction in PSA after 6-12 months of treatment with finasteride.

BPH patients on Himplasia, a polyherbal formulation for 3 months had their IPSS improved by 64% (Sahu and Kulkarni, 2003). On the other hand, the widely accepted *Serenoa repens* produced no significant effect in urologic outcome after a randomized control trial over 12 weeks (Willetts *et al.*, 2003). However, combined *Serenoa repens* and *Urtica dioca*

(160 mg/120 mg) extract induced an improvement in IPSS comparable to finasteride (Sökeland, 2000). It has recently been demonstrated that improvements in quality of life and prostate symptoms were equivalent across medical regimens used in clinical practice for the management of the condition. The study compared αadrenergic blockers, phytotherapy (*Serenoa repens* formed 95.2%), 5ARI and combination therapy of α-blockers and 5ARI over six months in patients after watchful waiting (Alcaraz *et al.*, 2016).

TURP has been the definitive treatment option for BPH and PCa but the rate of these surgeries have declined due to the advent of effective medical therapy. This study however, examined the entire hormones and metabolites in the androgenic pathway. CM increased FT and DHT with DHT being significant and yet decreased AED, AKRIC2 significantly. Widely used medical therapies are the α-blockers and the 5ARI. Of the 5ARI, dutasteride and finasteride are in common use (Harkaway and Issa, 2006). The 5ARIs effect their actions via inhibition of the 5AR enzyme involved in the conversion of testosterone to DHT which is purported to be involved in prostatic growth in adults. In a randomized, double-blinded placebo-control, dutasteride (0.5 mg) and finasteride (5 mg) significantly suppressed the production of serum DHT by 94% and 73% with transient rise in T after 52 weeks of treatment (Amory *et al.*, 2007). In a rather shorter study of 24 weeks, 5.0 mg and 0.5 mg dutasteride suppressed DHT production by 98.4% and 94.7%, respectively, while finasteride decreased DHT by 70 .8 %. Furthermore, T levels increased but not significantly (Clark *et al.*, 2004). However,

another study reports a rather lower decrease in DHT as 67.3% and 30.3% for dutatsteride and finasteride, respectively, for the same length of treatment with the drugs (Botto *et al.*, 2005). Hoque *et al.* (2015) demonstrated a significant 22% increment in AED in 317 PCa patients who had been on finasteride for 3 years.

Contrary to results from the 5ARI, 5ARI activity was unaffected by CM; a Clear indication that the enzymes involved in the formation of DHT are not influenced by CM. DHT continued to form either from free testosterone or 5α-androstanediol. *In vitro* and *in vivo* studies conducted showed a similar trend where DHT was unaffected by CM (Aboagye, 2000: Afriyie *et al.*, 2014). However, there seems to be a blockage in the transformation of DHT into its metabolite (3α-adiol) hence the observed accumulation.

There was a reduction in 3α-adiol concentration. CAG repeats in AR gene have been associated with BPH (Nelson and Witte, 2002). Studies in apparently healthy men have shown that African-Americans have the highest prevalence of short CAG repeat length (Kizilay *et al.*, 2014). It has been demonstrated that 3α-adiol is a stronger activator of mutant AR receptor in LNCap cells, and fuels more cell proliferation, prostatespecific antigen (PSA) mRNA expression, and PSA promoter than DHT.

It is therefore suggested that selective inhibition of 3α-adiol may be useful to extend the time when PCa is sensitive to androgen deprivation therapy (Mizokami *et al.*, 2004).

In this study, CM reduced both E1 and E2. However indices representing aromatase activity did not reduce significantly. 3β-diol increased and AKRIC1 represented by 3β-diol/DHT did not differ after treatment. There have been few clinical trials with anti-estrogens and aromatase inhibitors. An aromatse inhibitor anastrozole (1 mg daily) introduced in hypogonadal men for 3 months increased testosterone levels with consequent rise in DHT and decreased estrogen levels, significantly. Additionally, T levels remained within the normal range with further treatment. Estrogen levels did not revert to baseline between 3 and 12 months of treatment in that study (Burnett-Bowie *et al.*, 2009). Similarly, Atamestane at doses of 100 and 300 mg for 48 weeks significantly decreased estrogens concentrations with concomitant rise in androgens (Radlmaier *et al.*, 1996). The authors concluded that the drug had no effect on BPH yet the study did not assess any BPH characteristic apart from the androgens. Raloxifene, a SERM, binds selectively with ERβ to induce apoptosis in stromal cells while tamoxifene binds with a higher affinity to inhibit prolieferation in stromal cell (Yang *et al.*, 2010). The ethanolic extract of *Piper cubeba* known to demonstrate anti-estrogenic properties has not yet been tried for the treatment of BPH (Yam *et al.*, 2008).

It can be inferred that given time, CM could have caused a significant change in 3β-diol which will eventually translate into an increased activity of AKRIC1. Due to evidence of the effect of ERβ in apoptosis from *in vivo* and *in vitro* studies, an ERβ agonist is being sought for the management

of BPH. LY500307, was tried recently in a clinical trial, but the study was ended abruptly as the agonist failed to improve BPH symptoms (Roehrborn *et al.*, 2015). It will be of interest to establish whether CM is a ERβ agonist or ERα antagonist.

This study further explored the association between the significant improvement in PSA and urologic outcome (IPSS and PV). The study found changes in IPSS to result from changes in the concentration in 3β-diol. Increases in 3β-diol imply that CM most likely exerts it effect on LUTS by making more 3β-diol which binds to ERβ to induce apoptosis in the prostatic cells. The anti-proliferative and apoptotic effects of CM have been recently demonstrated in BPH-1 cell (Afriyie *et al.*, 2015). Changes in prostate volume however did not correlate with changes in any of the hormones and this could be due to the small sample power. Changes in PSA associated with changes in FT. As explained earlier the more FT formed the more unlikely it is transformed into the more potent androgen DHT for it to elicit androgenic effect needed for prostatic growth. This will allow for the saturation point for AR binding activity to be reached and then subsequent F T and DHT formed may not elicit any further androgenic activity. It is postulated that, CM down-regulates the enzyme (AKRIC1) to prevent DHT from binding to AR hence the accumulation of DHT and FT observed. Subsequently, 3β-diol was upregulated; this metabolite binds ERβ to induce apoptosis. This postulate is in line with *in vitro* studies where CM was demonstrated to elicit apoptogenic properties via the mitochondrion-dependent pathway

(Afriyie *et al.*, 2015).

CHAPTER SIX

CONCLUSION

6.1 CONCLUSION

This study supports the estrogen hypothesis implicated in the etiology of BHP due to the high levels of estrogens found in BPH patients. CM is slow acting but effective for the management of BPH as it improves LUTS and reduces prostate volume and PSA concentration. CM is not a 5AR inhibitor but impacts on the hormonal pathway by inhibiting the signaling between DHT and AR as it prevents the formation of androgenic DHT metabolite (3α-adiol) while up-regulating the formation of DHT metabolite (3β-diol).

6.2 LIMITATION

1. Controls could only be determined as BPH-negative by PSA levels. Ultrasonographic imaging could not be done as volunteers would not consent.
2. BPH cases could not be matched with a placebo as ethical concerns were involved considering the patients' condition of LUTS as well as the duration of the study. Hence, the study was observational.

6.3 RECOMMENDATIONS

1. Animal studies using tissues instead of serum/plasma to ascertain suggested pathway.

2. Estrogen receptors (ERα and β) may be assessed to determine whether the drug blocks the ERα or up-regulate the ERβ in animal studies.
3. AR polymorphism may be determined to ascertain whether this contributed to the efficacy of the drug in human studies.
4. Anthropometeric parameters (Body Mass Index, Waist to hip ratio) and other fat parameters like visceral fat that correlates with estrogens must be taken into account.
5. To assess whether withdrawal of the drug will revert the situation in animal studies.
6. To assess the effect of CM combination therapy with α-blockers.
7. To specifically target the enzyme activities (AKRIC1 and AKRIC2) in tissues and also determine if the enzymes involved in converting 3α-adiol to DHT might be involved.

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APPENDIX I

PARTICIPANT INFORMATION SHEET

Title: The effect of the aqueous root extract of *Croton membranaceus* on the treatment of Benign Prostate Hyperplasia.

You are being invited to take part in a research study with the above study title. Before you make a decision, it is important that you understand why the research is being doen and what it will involve. Please take time to the following carefully.

The purposive of the research is to examine the effect of the root extract of *Croton membraneceous* on the androgen biosynthetic pathway in an attempt to elucidate its mechanism of action.

You are under no obligation to take part in the study. If you do decide to part take you are free to withdraw at any time without reason.

If you would like to take part, you will be asked to sign a consent form indicating that you are happy to take part in the study. You will asked be asked to give 5 mls of blood and answer some questions (for those who fit the criteria).

We do not expect any specific benefits for the individual taking part. However, information obtained will help in validation the drug for use.

We would like to reassure you that your personal details would be kept strictly confidential and entitled to you if you are interested in the results.

No one, except the research would have access to these details and no identifying details would appear in our published results.

Counting on your co-operation
Thank you.

CONSENT FORM

Title of Research: The effect of the aqueous root extract of *Croton membranaceus* on the treatment of Benign Prostate Hyperplasia.

Name of Candidate .....

Address .....

Declaration

I ..... wish to state that the procedures involved in this research have been fully explained to my understanding and agree to participate in this study. I was not coerced or induced and I have freely consented to be a participant.

Signed ..... Date .....

Or

Patient's thumbprint

Date .....

Investigator's statement

I confirm that I have carefully explained the nature, demands and foreseeable risk of the proposed study to the volunteer,

Signed …… Date ……

APPENDIX II

ETHICAL CLEARANCE

SCHOOL OF ALLIED HEALTH SCIENCES
COLLEGE OF HEALTH SCIENCES
UNIVERSITY OF GHANA
ACADEMIC AFFAIRS

Phone: +233-0302-687974/5
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P. O .Box KB 143
Korle Bu
Accra
Ghana

My Ref. No. SAHS/PSM/ML/09
Your Ref. No.

RECEIVED
02 NOV 2012

24th October, 2012

Dr. George Awuku Asare,
Dept. of Med. Lab. Sci.,
SAHS.
Korle Bu.

Dear Dr. Asare,

ETHICS CLEARANCE

Ethics Identification Number: SAHS – ET./SAHS/PSM/ML/09/AA/26A/2012-2013.

Following a meeting of the Ethics and Protocol Review Committee of the School of Allied Health Sciences held on Tuesday 23rd October, 2012, I write on behalf of the Committee to approve your research proposal as follows:

TITLE OF RESEARCH PROPOSAL: **"Observational Study: The effect of the aqueous root extract of Croton membranaceus on the treatment of Benign Prostate Hyperplasia and Prostate cancer".**

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Committee on completion of the research. The Committee may observe the procedures and records of the research during and after implementation.

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

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

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

APPENDIX III

International prostate symptom score (IPSS)

Name: Date:

	Not at all	Less than 1	Less than half	About half the	More than half	Almost always	**Your score**
Incomplete emptying Over the past month, how often have you had a sensation of not emptying your bladder completely after you finish urinating?	0	1	2	3	4	5	
Frequency Over the past month, how often have you had to urinate again less than two hours after you finished urinating?	0	1	2	3	4	5	
Intermittency Over the past month, how often have you found you stopped and started again several times when you urinated?	0	1	2	3	4	5	
Urgency Over the last month, how difficult have you found it to postpone urination?	0	1	2	3	4	5	
Weak stream Over the past month, how often have you had a weak urinary stream?	0	1	2	3	4	5	
Straining Over the past month, how often have you had to push or strain to begin urination?	0	1	2	3	4	5	

	None	time	times	times	times	times or more	**Your score**
Nocturia Over the past month, many times did you most typically get up to urinate from the time you went to bed until the time you got up in the morning?	0	1	2	3	4	5	

Total IPSS score	

Total score: 0-7 Mildly symptomatic; 8-19 moderately symptomatic; 20-35 severely symptomatic.