



UNIVERSITY OF GHANA

INVESTIGATING THE BINDING-TARGETS OF THE AQUEOUS ROOT EXTRACT OF
CROTON MEMBRANACEOUS IN HYPERPLASTIC PROSTATE TISSUE.

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BY
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DEDICATION

This thesis is dedicated to Jesus Christ, my saviour and my help.

DECLARATION

I, Theophilus Owusu Manu of the Department of Medical Laboratory Sciences of the University of Ghana, do hereby declare that, with the exception of the cited 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 Prof. George A. Asare and Dr. Mahmood Seidu.

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ABSTRACT

Background: Benign prostatic hyperplasia (BPH) is a condition that affects aging men with accompanying lower urinary tract symptoms and bladder outlet obstruction. Several drugs, both allopathic and phytotherapeutic, have been developed to manage BPH. *Croton membranaceus* is a popular Ghanaian plant that has been used to manage BPH and its associated symptoms. Several studies have indicated that the aqueous root extract of *Croton membranaceus* (CMARE) shrinks prostate in men with BPH. However, the mechanism of action of CMARE is not known. Research has proven that CMARE does not act using any of the mechanisms of action of the allopathic counterparts and this places a limitation on its recognition and patronage. This research will therefore exploit the next most probable mechanism of action of CMARE.

General aim: The study was aimed at investigating whether the aqueous root extract of *Croton membranaceus* shrinks hyperplastic prostate tissue by binding to androgen and/or estrogen receptors on the prostate tissue.

Methodology: Twenty five (25) adult male Sprague-Dawley (S-D) rats weighing between 200 g and 250 g were acclimatised to laboratory conditions and randomly grouped into five (5) groups of five (5). With exception of the control group, BPH was induced in all rats. Each of the castrated rats was administered 5mg/kg testosterone propionate seven (7) days after castration for 28 consecutive days to induce BPH. After successful induction of BPH, one group served as pathological model group whilst the other three served as drug treated groups. The first two drug treated groups received 30mg/kgb.wt. [low dose (LD)] and 300mg/kgb.wt. [high dose (HD)] respectively *Croton membranaceus aqueous* root extract (CMARE) *per os* by oral gavage for 28days. The last group served as a positive control group and received 0.5 mg/kgb.wt. finasteride *per os* for 28 days. Their prostate tissues were harvested, fixed with formalin and processed into slides. After antigen retrieval, the tissues were incubated with specific monoclonal antibodies raised against androgen and estrogen receptors. Slides of the tissues were observed under the fluorescence and light microscopes and results reported based on the intensity of fluorescence or colour development using a grading system of 1 to 4. Data obtained was analyzed by the Kruskal-Wallis H Test using the Statistical Package for Social Sciences (SPSS), version 24. The hypothesis was tested at a significance level of 0.05.

Results: There was a significant difference between the two groups with respect to estrogen receptor beta immunostaining ($p=0.015$). A significant difference was also observed between the control and the CMARE treated groups (i.e. low and high dose groups) regarding androgen receptors immunostaining ($p=0.023$ in either case). For estrogen beta staining, a significant difference ($p=0.015$) was observed between staining intensities of the control group and the CMARE treated groups. Comparing the low dose CMARE group to the high dose CMARE group, there was no significant difference between mean staining intensities in all three immunoassays ($p=1.000$).

Conclusion: The aqueous root extract of *Croton membranaceus* (CMARE) does not bind to androgen receptors in its action. However, the extract may inhibit the binding of intraprostatic androgens to the androgen receptors. Furthermore, CMARE may act as an estrogen beta receptor agonist whilst it does not interact with estrogen alpha receptors.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The prostate is a very important organ in the adult male. Hence diseases of this organ always draw the attention of researchers to find remedies to subdue the disease condition. Benign prostatic hyperplasia and prostate cancer are amongst several diseases of the prostate gland (Bartoletti *et al.*, 2009). Benign prostatic hyperplasia (BPH) can be defined as the progressive enlargement of the prostate gland commonly seen in older men. It is also defined as a non-malignant enlargement of prostate gland characterized by proliferation of the cellular elements such as its epithelial and stromal cells into a discrete mass or nodules (Mbaka *et al.*, 2017). The urethra passes through the prostate gland and as the gland enlarges, it causes the compression of the urethra and obstruction of urinary flow in men with BPH. Men with BPH may complain of difficulty initiating urination, incomplete bladder emptying, urinary urgency, weak urinary stream, dribbling or nocturia (Dhingra & Bhagwat, 2011). Estimates of BPH prevalence range from 40-50% at 50 years of age to as high as 80% for men aged 70 years and above (Kristal *et al.*, 2008). BPH therefore, seems to be a scare for aging men globally.

Although the actual cause of BPH remains incompletely understood, it is clear that androgens have a central role to play in its development. Dihydrotestosterone (DHT), an androgen derived from testosterone through the action of 5-alpha reductase and its metabolite, 3-alpha androstenediol, seem to be the major hormonal stimuli for stromal and glandular proliferation in men with nodular hyperplasia. Experimental work has also identified age-related increases in estrogen levels that may increase the expression of DHT, the progenitor of BPH (Bosland, 2005). The enzyme aromatase has been found to convert estrogens to testosterone thereby contributing

to the development of BPH. The incrimination of DHT in the pathogenesis of BPH forms the basis for the current use of 5-alpha reductase inhibitors in the treatment of symptomatic nodular hyperplasia. Several drugs have been synthesized and others extracted to shrink hyperplastic prostate; mainly based on knowledge of the pathogenesis of the condition. Some of the drugs act as receptor antagonists whilst others have inhibitory effects on key enzymes. Commonly used allopathic drugs for the treatment of BPH and its symptoms include finasteride, dutasteride and tamsulosin amongst others (McConnell *et al.*, 2003).

Interestingly, several complementary and alternative medicines (CAMs) have been used in the treatment of BPH and its symptoms. The use of CAMs is on the increase and they have been found to be effective in the treatment of BPH with less adverse effects. White peony, green tea, spearmint, black cohosh, chaste tree and saw palmetto are amongst the plants used in treating BPH (Grant & Ramasamy, 2012). In Ghana, a popular medicinal plant used for the management of BPH is *Croton membranaceus* (CM). The plant extract has been found to shrink hyperplastic prostate in an animal model (Afriyie *et al.*, 2014) and also in humans (Asare *et al.*, 2015). CM has been found to be effective and devoid of the side effects of reduced libido and sexual function as well as severe headaches as experienced with other drugs (finasteride and dutasteride) (Asare *et al.*, 2015).

1.2 Problem Statement

The aqueous root extract of *Croton membranaceus* has been proven to shrink hyperplastic prostate in adult males (Asare *et al.*, 2015). The extract has an added advantage of improving sexual function in subjects whilst improving lower urinary tract symptoms. The extract is also relatively inexpensive until recently and readily available compared to allopathic drugs used in managing BPH.

However, unlike allopathic drugs, CMARE lacks a lot of vital information which serves as a setback to its international recognition and patronage. A toxicological study has been performed on CMARE and proven to be safe (Asare *et al.*, 2014). On the other hand, several attempts have been made in investigating the mechanism of action of CMARE in shrinking hyperplastic prostate but the actual mechanism of action of CMARE has not been found out.

Allopathic drugs used in the management of BPH are either 5-alpha reductase inhibitors, aromatase inhibitors or alpha adrenergic receptor antagonists (alpha blockers). It is therefore expected that CMARE will act in one or more of these mechanisms. Surprisingly, several works done by scientists have proved that CMARE does not act in any of the mechanisms described above (Asiedu *et al.*, 2017). This creates a vacuum for investigating the exact mechanism of action of CMARE in shrinking hyperplastic prostate tissues.

1.3 Justification

Several studies have established that androgen/androgen receptors and estrogen/estrogen receptors have vital roles to play in the development of benign prostatic hyperplasia and progression of prostate cancer (Bonkhoff & Berges, 2009; Bonkhoff *et al.*, 1999; Bosland, 2005; Leav *et al.*, 2001; Liang *et al.*, 1993; Roberts *et al.*, 2004). It is therefore eminent that this area be explored to find out if CMARE acts by interacting with these receptors.

Androgens (mainly testosterone and dihydrotestosterone) may bind to their receptors on prostate tissues to induce hyperplasia in prostate cells. Similarly, estrogens may bind to estrogen alpha receptors to induce hyperplasia in prostate cells. On the other hand, estrogens may bind to estrogen beta receptors to induce apoptosis in prostate cells (Bonkhoff & Berges, 2009). As indicated earlier, CMARE does not inhibit 5-alpha reductase activity. The enzyme catalyzes the conversion of testosterone to dihydrotestosterone which binds to androgen receptors. Also CMARE does not inhibit aromatase activity. Aromatase catalyzes the conversion of testosterone to estrogen which binds to estrogen receptors.

If CMARE does not inhibit these enzymatic processes as indicated by research, and yet is able to shrink hyperplastic prostate tissues, there is a possibility that the extract may bind to androgen receptors on the prostate tissue to prevent androgen – androgen receptor binding, thereby preventing the onset of hyperplasia. Or CMARE may bind to estrogen alpha receptor on the prostate tissue to prevent estrogen - estrogen alpha receptor binding; thereby preventing the onset of hyperplasia. Or CMARE may up-regulate the binding of estrogen to estrogen beta receptor to induce apoptosis of prostate cells.

1.4 Hypothesis

H₀: The aqueous root extract of *Croton membranaceus* does not bind to androgen and estrogen receptors of prostate tissues.

1.5 Aim of Study

The study aimed at investigating whether the aqueous root extract of *Croton membranaceus* shrinks hyperplastic prostate tissue by binding to androgen and/or estrogen receptors on the prostate tissue.

1.6 Specific Objectives

The objectives of the study were;

1. To determine the binding of specific monoclonal antibodies to androgen receptors of prostate tissues.
2. To determine the binding of specific monoclonal antibodies to estrogen receptors, alpha and beta, of prostate tissues.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 THE PROSTATE

The human prostate is a walnut-sized organ found in males only. With a ductal-acinar histology, this organ lacks discernible lobular organization. The prostate gland surrounds the urethra just below the urinary bladder. As a gland, the prostate produces a clear, slightly alkaline fluid which contributes 10-30% of seminal fluid volume. The pH, ions and proteins of this secretion facilitates sperm motility through the acidic environment of the female vagina. In both normal and diseased conditions, human prostates secrete prostate specific antigens (PSA). The prostate is exclusive to mammals and is not essential for fertility. However, the prostate has received attention mainly because of the diseases associated with it (Pirkko, 2004).

The prostate gland like any other organ of the human body is confronted with disease conditions which tend to alter the anatomy and physiology of the prostate. Due to various disturbances to prostatic cells, several disease conditions confront the prostate. These include benign prostatic hyperplasia, prostatitis, high grade prostatic intraepithelial neoplasia (HGPIN) and prostate cancer (Bosland, 2005; Pirkko, 2004; Bonkhoff *et al.*, 2001).

In each of the disease conditions, the physiology and/or anatomy of the prostate gland is/are affected and histological changes are observable under the microscope. Therefore, in considering diseases of the prostate, such as benign prostatic hyperplasia, knowledge of the anatomy and histology of the prostate gland is very necessary.

2.1.1 Anatomy of the Human Prostate

The prostate gland is located in the sub-peritoneal compartment between the pelvic diaphragm and the peritoneal cavity. It is located behind the symphysis pubis, anterior to the rectum, and inferior to the urinary bladder. The prostate is conical in shape and surrounds the urethra as it exits from the bladder. The prostate gland is composed of a base, an apex, anterior, posterior, and inferior lateral surfaces. The base is attached to the neck of the bladder and is narrow and convex (Weinreb *et al.*, 2015).

The human prostate is composed of glandular and stromal cells. These cells are tightly fused within a capsule-like structure. The prostate capsule is composed of smooth muscles and covered with a layer of collagen. Nerve supply to the prostate is derived from the prostatic plexus and arterial supply by the branches of the internal iliac artery. The whole length of the prostatic urethra is enclosed by sphincteric muscles. The urethra is walled by smooth muscles. This ensures closure of the proximal urethra during ejaculation thereby preventing retrograde ejaculation in men (Fine *et al.*, 2012). The distal urethral sphincter, made of striated muscle fibers is located distal to the apex of the prostate gland. Damages to this particular sphincter during surgery, may lead to urinary incontinence.

The human prostate is divided into three (3); the central zone (CZ), transition zone (TZ) and peripheral zone (PZ). These compartments differ in their embryologic origins, histologies, anatomic landmarks, functions and susceptibility to diseases. Prostate cancers mostly arise from the PZ, which mainly of urogenital sinus origin (Selman, 2011). However, the CZ, which is derived from the Wolffian duct, is less susceptible to prostate cancer. The same can be said about the seminal vesicles (Mcneal & Hospital, 2001). The embryologic origins of the CZ and the PZ, is perhaps responsible for the vast difference in terms of their susceptibility to carcinogenesis.

Interestingly, though the TZ has similar embryologic origins to the cancer-prone PZ, the percentage of prostate cancer (PCa) arising from the TZ is lower. This may be explained by differences in the stromal component of these two zones. (Lee *et al.*, 2011).

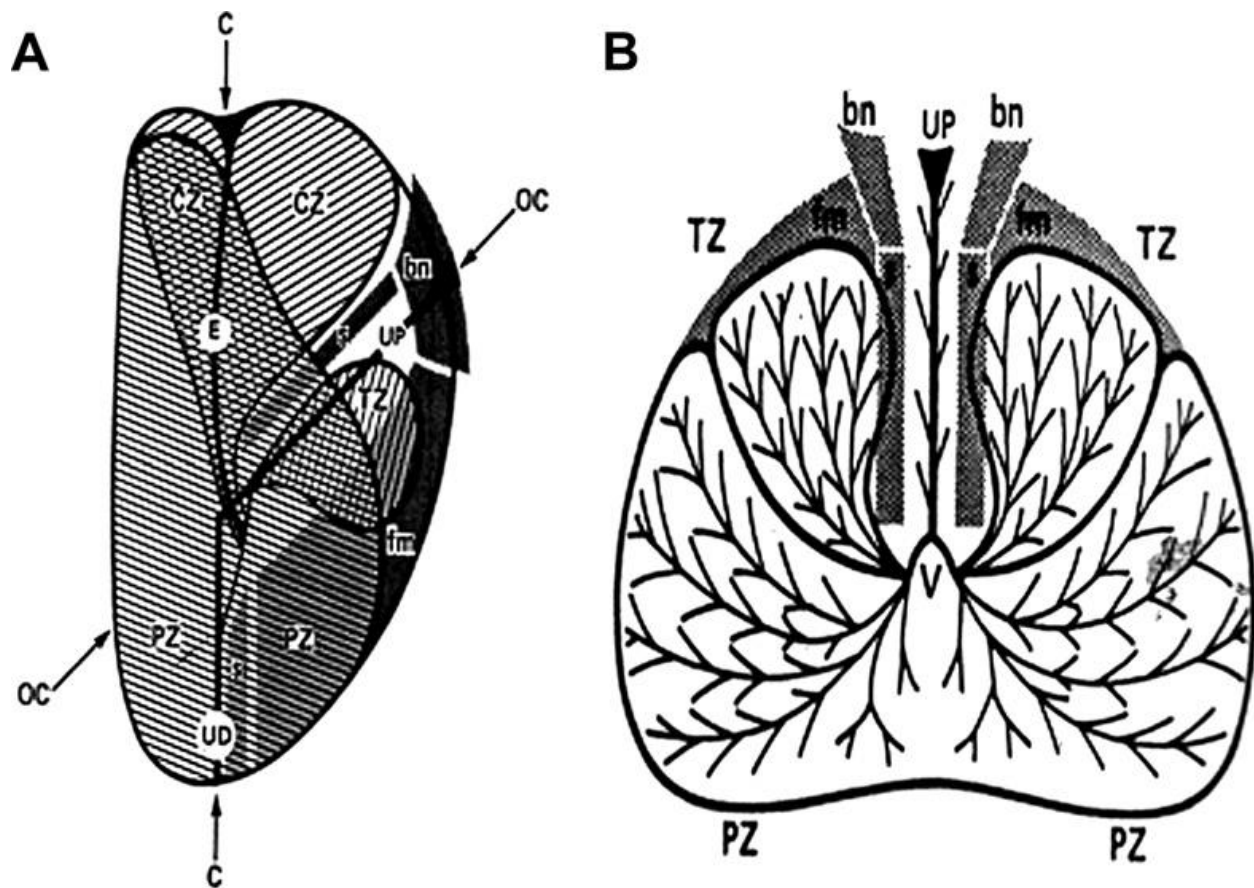


Figure 2.1: Zonal anatomy of the human prostate. A (coronal view); B (Transverse view)

2.1.2 Anatomy of the Mouse Prostate

Several mouse models of benign prostatic hyperplasia have been developed, which attempt to recapitulate the human disease. The mouse prostate consists of 4 distinct lobes contrary to three zones in humans. These lobes are the ventral, dorsal, lateral, and anterior lobes. The dorsal and lateral lobes are frequently grouped together as the dorsolateral prostate. In effect, the mouse

prostate can be said to have three main divisions as found in the human prostate. The mouse prostate surrounds the urinary bladder and the urethra. Lying against the urogenital diaphragm, the anterior lobe of the mouse prostate rests directly adjacent to the seminal vesicles. The mouse prostate is covered within a thin mesothelial-lined connective tissue which allows for a distinction between all of the different lobes (Raghow *et al.*, 2002). The stroma surrounding the glands of each lobe is embedded in collagen fibers with nerve bundles along the dorsolateral lobes. The different lobes of the mouse prostate differ in function and histology. However, they all appear as grossly clear and gelatinous. The ventral lobe of the murine prostate has leaf-like structure incompletely surrounds the urethra. The ventral lobe is composed of simple columnar epithelial cells, with basally located nuclei. This lobe does not present infolding or tufting that is typically observed in some of the other lobes. There is no anatomic and histologic match to the ventral lobe of the mouse prostate in humans (Foster *et al.*, 1997). The dorsal lobe consists of simple columnar epithelial cells with basophilic granular cytoplasm. The lateral lobe together with the dorsal lobe surrounds the urethra ventrally and bilaterally. Similar to the ventral prostate, the lateral lobe characteristically exhibits very little infolding. The dorsolateral prostate is most comparable to the PZ of the human prostate (Cunha, 2018).

The anterior lobe is also known as the coagulating gland and is directly adjacent to the seminal vesicles bilaterally. It is lined by simple columnar epithelium. The anterior prostate is most analogous to the CZ of the human prostate (Krege *et al.*, 1998).

2.2 Pathophysiology of benign Prostatic Hyperplasia

Benign prostatic hyperplasia (BPH) is a progressive condition characterised by prostate enlargement usually accompanied by lower urinary tract symptoms (LUTS) (Roehrborn *et al.*, 2009). Benign prostatic hyperplasia originates from the periurethral and transition zones of the

prostate and represents an expected occurrence for majority of the ageing male population (Briganti *et al.*, 2009). Although BPH is not common before age 40, approximately 50% of men develop BPH-related symptoms at age fifty (50). The incidence of BPH increases by 10% per decade and reaches 80% at about 80 years of age (Irani *et al.*, 2003). An estimated 75% of men older than age 50 have symptoms arising from BPH, and 20–30% of men approaching 80 years of age require surgical intervention for the management of BPH (Roehrborn *et al.*, 2009).

Several mechanisms are speculated to be involved in the development and progression of BPH. Although ageing represents the principal mechanism implicated, recent findings have also emphasised the roles of hormonal alterations (androgens and estrogen), metabolic syndrome and inflammation (Schenk *et al.*, 2010; Bartoletti *et al.*, 2009). Receptor gene polymorphisms have also been implicated in the pathophysiology of BPH (Roberts *et al.*, 2004). For the purpose of this study, the roles of androgens and estrogens as well as hormone receptors in the development of BPH are elaborated below.

2.2.1 The Roles of Androgen/Androgen Receptors And Estrogen/Estrogen Receptors In The Pathogenesis Of Benign Prostatic Hyperplasia And Prostate Carcinogenesis

An intact androgen system (androgens and their receptors) has been implicated in several studies to be the main factor responsible for prostate cancer and other pathologies of the prostate including benign prostatic hyperplasia and High Grade Prostatic Intraepithelial Neoplasia (HGPIN). Androgens, mainly testosterone and dihydrotestosterone bind to their receptors on the prostate tissue to signal the growth of prostatic cells (Roberts *et al.*, 2004). Hence an intact androgen system can result in the continual excessive growth of prostate cells and hence the prostate gland as a whole. This signaling has been used in part to explain the development of benign prostatic hyperplasia and prostate carcinogenesis. Androgens exert their effects by

binding to a single cytoplasmic androgen receptor, and their potency is determined by the binding affinity to the androgen receptor. Dihydrotestosterone binds five times more strongly than testosterone (Hoffman and Happle, 2000). The enzyme 5- α reductase, converts testosterone to its active form, dihydrotestosterone. Dihydrotestosterone is implicated not only in the development of benign prostatic hyperplasia but also in the pathogenesis of prostate cancer (Giles *et al.*, 2011). In view of this, several drugs have been synthesized to either block the androgen receptors or inhibit the action of the 5- α reductase enzyme. Dutasteride and finasteride are 5- α reductase inhibitors whereas tamsulosin, silodosin, doxazosin, alfuzosin and prazosin are alpha-adrenergic - receptor antagonists (alpha-blockers) (McConnell *et al.*, 2003).

As a remedy to treat pathologies of the prostate, synthetic estrogens were administered to patients with such conditions primarily. The therapeutic effect of estrogen in preventing prostate cancer growth was mainly obtained indirectly by feedback inhibition of the hypothalamic release of the luteinizing releasing hormone (LRH) leading to lowered serum androgen levels and castration like effects (Pirkko, 2004). However, over the years, research has proven that estrogens also have a role to play in the development of benign prostatic hyperplasia and prostate carcinogenesis (Bonkhoff & Berges, 2009).

The human prostate is equipped with a dual system of estrogen receptors, ERs. These are; estrogen receptor alpha and estrogen receptor beta which are localized in the stroma and epithelium respectively. These undergo profound remodeling during prostate cancer and tumour progression (Leav *et al.*, 2001). Studies have found the estrogen receptor alpha to be an oncogene whilst the beta receptor is a tumour suppressor (Prins & Korach, 2007). Estrogen receptor alpha has been found to be highly expressed in patients with prostate cancer, high grade

prostatic intraepithelial neoplasia and benign prostatic hyperplasia. Conversely, estrogen receptor beta has been observed to be markedly reduced in patients with the above conditions (Bonkhoff & Berges, 2009).

2.2.2 The Androgen receptor

The androgen receptor (AR) is a transcription factor involved in normal male sexual development and maintenance of the function of accessory sexual organs. In the etiology of BPH, androgen receptors get bound to androgens to initiate prostatic cell growth. However, in the absence of androgens, ARs are bound to heat-shock proteins thereby deactivating them. The AR is composed of three main parts: (1) central DNA-binding domain, (2) a ligand-binding domain which are separated by a hinge region and (3) an N-terminal region, which contains a variable number of poly-glutamine and poly-glycine repeats. These poly-amino acid repeats are involved in the regulation of the transcriptional activity of the AR. Racial differences in the repeat lengths have been investigated in molecular studies (Culig *et al.*, 2002).

Variations in the androgen receptor gene influence androgenic actions in males. These variations influence prostatic growth and other BPH-related outcomes. Two main polymorphisms in the androgen receptor gene have been identified; a variable CAG repeat that encodes a poly-glutamine repeat and a variable GGN repeat that encodes a poly-glycine repeat. A short CAG repeat length has been associated with increased androgen receptor transcriptional activity whereas a long CAG repeat length is associated with reduced androgen receptor expression (Ikonen *et al.*, 1997, He *et al.*, 1999). Interactions between the N-terminal (N) and carboxyl-terminal (ligand-binding) (C) domains of AR greatly affect its functional activity (Kemppainen *et al.*, 1999). Loss of AR function might occur because of the presence of mutations that disrupt

the N/C interaction. This may however, not affect AR-binding affinity (Langley *et al.*, 1998, Thompson *et al.*, 2001).

Structurally, the human AR resembles the human glucocorticoid or progesterone receptors (Schoenmakers *et al.*, 2000). However, glucocorticoid hormones are not able to stimulate growth of prostate cancer LNCaP (Cleutens *et al.* 1997). In contrast, dexamethasone stimulates the expression of the prostate specific antigen (PSA) gene in LNCaP sublines generated by stable transfection of glucocorticoid receptor cDNA.

2.2.3 Estrogen Receptors

Estrogen receptors (ER) are members of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors. Estrogen receptors, ER alpha and ER beta, contain DNA binding and ligand binding domains and are critically involved in regulating the normal function of reproductive tissues. They are located in the nucleus, though some estrogen receptors associate with the cell surface membrane and can be rapidly activated by exposure of cells to estrogen (Walz *et al.*, 2016). ER alpha and ER beta have been shown to be differentially activated by various ligands. Receptor-ligand interactions trigger a cascade of events, including dissociation from heat shock proteins, receptor dimerization, phosphorylation and the association of the hormone activated receptor with specific regulatory elements in target genes. Evidence suggests that ER α and ER β may be regulated by distinct mechanisms even though they share many functional characteristics (Montt-guevara *et al.*, 2015).

2.3 Diagnoses of BPH

On a daily basis, Urologists and Physicians diagnose several men with BPH. This is done by taking a complete medical, urologic and neurologic history of the patients in order to eliminate causes of LUTS other than BPH or bladder dysfunction. Most men with BPH visit the clinic only when symptoms begin to affect their quality of life (QoL). Symptoms of LUTS are assessed by a standardized questionnaire known as the International Prostate Symptom Score (IPSS). IPSS is useful in the quantification of the symptoms and severity of LUTS. This enables Physicians to make informed decisions on therapy. LUTS is graded from 0-35, where 0-7 indicates mild symptoms, scores of 8-19 and 20 -35 indicate moderate and severe symptoms respectively (Nunes *et al.*, 2017).

A physical examination of the supra-pubic area, external genitalia and testes may be examined by the physician. The presence of a palpable nodular usually demands prostatic biopsy. Digital rectal examination (DRE) can be used to assess the size, shape, nodularity and consistency of the prostate gland. Prostate Specific Antigen (PSA) assay is very useful in the diagnosis and monitoring of the progression of BPH and prostate cancer (Shum *et al.*, 2017). PSA values greater than 4.0 ng/ml are considered abnormal. Measuring PSA velocity (change over time), levels of free and protein-bound PSA and PSA density (PSA level divided by prostate volume) help improve the diagnostic accuracy of the biomarker. The use of cutoff values for PSA levels that are specific to the patient's age and race or ethnic group is also recommended. Following an abnormal PSA test result, a biopsy is advised to be taken for histological examinations (Pisco, 2012).

Urinalysis can be requested to screen for urinary tract infections and haematuria in order to eliminate urolithiasis or cancers of the kidney, bladder or prostate. LUTS are treated before

initiating other therapies. It is advisable for patients with complicated LUTS to be referred to a urologist. Other laboratory tests including urine culture, serum creatinine and glucose may be requested depending on the patient's history. Non-invasive optional tests may be performed on the patient. These include urinary flow rate and transrectal ultrasound (Mehraban, 2017).

2.4 Treatment for BPH

Treatment of BPH and its associated symptoms are done either through the medical or surgical means. Surgery produces a far greater improvement in symptoms and urodynamic parameters than drug therapy (Eri, 1997). This benefit is obtained, however, at the cost of lost work time, the need for hospitalisation and the performance of an invasive surgical procedure with a well described set of surgical complications. Patients with chronic urinary retention, substantial post-voiding residual urine, recurrent urinary tract infections, bladder stones or renal insufficiency are, as a rule, not treated medically. Usually, the severity of the urinary symptoms plays a key role in deciding treatment. Patients with severe symptoms of prostatism (AUA-SIIPSS score 20 to 35) are unlikely to gain sufficient relief from medical therapy, and are normally best treated with surgery (Macey & Raynor, 2016).

At the other end of the spectrum are men with mild symptoms of BPH (IPSS <7) and satisfactory bladder emptying. These men are generally considered to be not sufficiently bothered by their symptoms to require medical nor surgical therapies, but they might be re-evaluated after 1 year (Fakhrudin *et al.*, 2017). Men with moderate symptoms of BPH are generally considered the best candidates for medical treatment. However, the distinction between mild and moderate symptoms is arbitrary and there are wide differences in patient tolerance of symptoms. Some men with mild symptoms of BPH are treated medically (Pisco, 2012). Conversely, a large proportion of men with moderate symptoms prefer watchful waiting or surgery (Foo, 2017).

2.4.1 Surgical Treatment

2.4.1.1 Transurethral Resection of the Prostate (TURP)

Transurethral resection of the prostate (TURP) is considered the gold standard for surgical therapy for LUTS secondary to BPH. The procedure for TURP involves the circumferential resection of the prostate tissue (Macey & Raynor, 2016). TURP improves the average symptom score by 15 points and also increases maximum urinary flow rate (Q_{max}) in men treated with this procedure (Eri, 1997). Men treated by the TURP procedure may complain of complications such as postoperative bleeding (which may require blood transfusion), erectile dysfunction, bladder neck contracture, hematuria and urinary tract infections (Mehraban, 2017).

2.4.1.2 Transurethral Incision of the Prostate

Transurethral incision of the prostate (TUIP) is also useful when treating men with moderate-to-severe LUTS as a result of BPH when the prostate size is less than 30 millilitres. TUIP is typically an outpatient procedure involving one or two incisions in the prostate. (Macey & Raynor, 2016).

2.4.1.3 Transurethral Laser Surgical Management

Transurethral laser therapies for LUTS owing to BPH involve the use of a laser light to resect or vaporize the prostate tissue. The call for this approach is usually based on the patient's clinical presentation, surgeon's competence and experience, and risks versus benefits of the procedure. In comparison to TURP, laser therapies result in a better improvement in symptoms of voiding, with a reduced risk of bleeding, shorter postoperative catheterization time and reduced duration of stay in the hospital (Shergill, 2004).

2.4.1.4 Simple Prostatectomy

Simple prostatectomy is recommended for men with large prostate glands (prostate volumes greater than 80 millilitres) and also for men who cannot tolerate a transurethral procedure. Surgical approaches include a suprapubic, retropubic, or perianal approaches. The suprapubic approach is the most common for patients with very large prostate glands (Pisco, 2012). When compared with TURP, prostatectomy is associated with an increased risk of blood loss, transfusion and longer hospital stay. Simple prostatectomy shows a reduction in prostate symptom score by 10 points (on the average) and approximately 14 mL/s improvement in Qmax (Macey & Raynor, 2016).

2.4.2 Complementary and Alternative Medicines for LUTS Secondary to BPH

2.4.2.1 Alpha-Antagonists

Alpha-antagonists have been used in the treatments for patients with moderate-to-severe LUTS secondary to BPH. These drugs have been found to relax smooth muscles in the bladder and prostate as a result of inhibition of alpha-1 adrenergic receptors of the tissues. When alpha-1 adrenergic receptors are activated, the urethra contracts and impaired flow of urine occurs (Bairy, 2009). This mechanism contributes largely to the pathophysiology of LUTS secondary to BPH. Therefore, alpha-antagonists are thought to have the greatest influence on prostatic smooth muscle tone. These medications act in a dose-dependent manner and cause improvements in urinary symptom score and maximum urinary flow rate (Qmax). One main disadvantage with the use of these drugs is the adverse effect of retrograde ejaculation which happens as a result of relaxation of the smooth muscles in the prostate, bladder, seminal vesicles and vas deferens. Terazosin, doxazosin, and alfuzosin are examples of non-selective. Tamsulosin and silodosin are however selective for alpha-1 antagonists. Amongst these drugs, silodosin has the highest

affinity for alpha-1 adrenergic receptors. Improvements can occur within a few days after medication. However, maximal improvement generally occurs within 1 to 3 months after medication. (Dhingra & Bhagwat, 2011).

Other mild side effects of alpha-antagonists are dizziness, fatigue, nasal congestion and orthostatic hypotension. Men treated with these drugs may develop Intraoperative floppy iris syndrome.

2.4.2.2 5-alpha reductase inhibitors (5-ARIs)

The conversion of testosterone to dihydrotestosterone is catalyzed by the enzyme 5-alpha-reductase. Dihydrotestosterone is the main active androgen within the prostate and has a five-fold affinity for binding to androgen receptors on the prostate, compared to testosterone. Dihydrotestosterone induce prostatic cell growth when bound to androgen receptors and its effects on the prostate cells are higher than testosterone. Therefore, 5-alpha reductase inhibitors prevent the conversion of testosterone to the more potent form, dihydrotestosterone thereby reducing prostatic growth. 5-alpha reductase inhibitors include finasteride and dutasteride. Finasteride inhibits type II 5-alpha-reductase, whereas dutasteride inhibits both types I and II 5-alpha-reductases. Both drugs reduce serum levels of dihydrotestosterone. However, finasteride can reduce serum concentrations of the hormone by 70% whereas dutasteride reduces the serum concentrations of the hormone by 95% (Macey & Raynor, 2016). Symptom improvement may begin within several weeks of initiation of medication; however, it usually takes 6 to 9 months to achieve a very significant change in symptoms after starting 5-ARIs. There are however some disadvantages associated with these drugs. Men who take 5-ARIs may develop erectile dysfunction, decreased libido, decreased volume of ejaculate and gynecomastia. These adverse effects are reversible and uncommon after the first year of therapy. 5-ARIs may decrease DHT levels and reduce prostate volume by 15 to 25%. They may also increase Qmax by

approximately 10% and thus improve symptom score by 20 to 30%. A reduced risk of urinary retention and surgical therapy is also attained when 5-ARIs are used in the management of BPH and LUTS (Briganti *et al.*, 2009). Particularly, these medications are less effective than alpha-antagonists in improving LUTS when used as monotherapy (Eri, 1997).

2.4.2.3 Phosphodiesterase-5 Inhibitors

Tadalafil (Cialis) is the only Phosphodiesterase-5 inhibitors have been found useful in the treatment of BPH related LUTS as well as in the treatment of erectile dysfunction. Tadalafil and sildenafil are the two most common phosphodiesterase-5 inhibitors. Daily tadalafil therapy (5 mg) significantly improves erectile function, voiding symptoms and Quality Of Life. Tadalafil works better when used together an alpha-antagonist. The combined therapy significantly improves Qmax, voiding symptoms, and erectile dysfunction compared with an alpha-antagonist used as a monotherapy. (Gacci *et al.*, 2012; Nunes *et al.*, 2017).

2.5 *Croton Membranaceus*

Croton membranaceus belongs to the family Euphorbiaceae and is commonly found in West Africa, close to rivers. *Membranaceus* is the commonest species of the genus *croton* (Asiedu *et al.*, 2017). *Croton membranaceus* is common in Nigeria, Niger and Ghana. In Ghana, the plant grows near river Volta. The plant is mostly found in the Krobo-Gyakiti forest reserve area in the Eastern Region and the indigenes refer to it as “Bokum”(Asare *et al.*, 2011). The plant has been reported to be of various medicinal uses and it has been used by the indigenes for the treatment of several medical conditions.

The plant has become an important subject for investigations by scientists. The aqueous root extract of the plant has been found be nontoxic according to a study by Asare *et al.* 2011. The

study examined toxicity markers such as full blood count, bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, albumin, globulin, alkaline phosphatase (ALP), gamma-glutamyltranspeptidase (GGT), urea, creatinine and creatine kinase amongst a control group and two other groups treated with CMARE. Measured parameters of test groups were lower than those of the control group. Taking all factors into consideration, it was concluded that *Croton membranaceus* ingestion does not produce general acute toxicity (Asare *et al.*, 2011).

Furthermore, the anti-proliferative activity of *Croton membranaceus* on BPH-1 cells has been demonstrated by experimentation (Afriyie *et al.*, 2015). In the study, the aqueous root extract of *Croton membranaceus* was found to show a mitochondria-dependent apoptogenic activity against human BPH-1 cells. The study realised a dose-dependent inhibition in the proliferation of BPH-1 cells ($P < 0.05$). An alteration in the morphology and reduction in the densities of these cells was also observed. Additionally, CMARE was found to significantly up-regulate mRNA and protein levels of Bax, a regulator of apoptosis. However mRNA and protein levels of Bcl2 did not change significantly. The study then concluded on the note that, induction of mitochondria-dependent apoptosis of BPH-1 cells may be a possible mechanism of action of CMARE (Afriyie *et al.*, 2015). Another study has shown the cytotoxic activity of the methanolic extract of *C. membranaceus* roots against DLD-1 and MCF-7 cells.

The antimicrobial activity exhibited by the *Croton membranaceus* root extract supports its usefulness in treating secondary bacterial infection in measles as recently reported (Bayor *et al.*, 2009). The methanolic root extract and compounds isolated from *Croton membranaceus* were tested against the bacteria; *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* and the fungi; *Aspergillus niger* and *Candida albicans*, using the agar

diffusion and broth dilution techniques. The methanolic extract showed a significant ($p < 0.01$) antibacterial and antifungal activity against the test organisms used with minimum inhibitory concentrations (MICs) ranging from 0.53 - 1.43mg/ml (Bayor *et al.*, 2009). Furthermore, the root extract has been reported to exhibit markedly high cytotoxic activities particularly against human cancer cell lines (Bayor *et al.*, 2007). *Croton membranaceus* has also been found to possess anti-atherogenic and anti-ischaemic properties (Afriyie *et al.*, 2013) as well as improving biomarkers of cardiovascular disease and diabetes in animal models (Asare *et al.*, 2015).

Finally, the root extract is used in formulations for the treatment and management of prostate and its related cancer in Ghana (Asare *et al.*, 2015). Afriyie *et al.* (2014) conducted a study that investigated the treatment of benign prostatic hyperplasia with *Croton membranaceus* in an animal model. After the study, *Croton membranaceus* aqueous root extract (CMARE) was found to reduce stromal and epithelial cell growth and subsequently shrunk enlarged prostate. Further studies were done in a cohort of human subjects in which enough statistics were obtained to confirm the fact that CMARE shrinks prostate in men with BPH as well as improving lower urinary tract symptoms and quality of life in men (Asare *et al.*, 2015a; Asare *et al.*, 2015b). The study aimed at validating the use of freeze-dried *Croton membranaceus* ethanolic root extract for BPH management. Thirty-three patients were observed before and after 3-month administration of 20mg t.i.d orally. The International Prostate Symptom Score (IPSS) and the International Index of Erectile Function (IIEF) questionnaires were used to assess the quality of life of participants. Total/free PSA (tPSA, fPSA), renal, liver function, lipid tests and ultrasonographic imaging were performed. At the end of the study, Quality of life (QoL) improved ($P = 0.001$) in men treated with the extract. Mean tPSA reduced from 27.9 ± 19.0 to 16.2 ± 11.8 ng/mL ($P = 0.002$); fPSA from 6.1 ± 4.8 to 3.9 ± 2.9 ng/mL ($P = 0.045$); and prostate volume from $101.8 \pm$

41.3 to $54.5 \pm 24.8 \text{ cm}^3$ ($P = 0.023$). Based on the above stated statistics, the study concluded that *C. membranaceus* shrinks the prostate and improves quality of life of men.

The root extract is said to contain an alkaloid, a coumarin, diterpenoids, and phytosterols. Compounds isolated from the root extract include julocrotine, scopoletin, crotomembranafuran, gomojoside H, β -sitosterol, β -sitosterol-3-*O*-glucoside, stigmasterol and campesterol. Recently, N[N-(2-methylbutanoyl) glutaminoyl]-2-phenylethylamine has also been isolated (Bayor *et al.*, 2009). Gomojoside H has been found to have antimicrobial activities similar to gentamycin whereas β -sitosterol is known to have a structural analogy to estradiol, an estrogen as shown in figures 2.1 and 2.2. This explains in part the antimicrobial activity exhibited by the *C. membranaceus* root extract and supports its usefulness in treating secondary bacterial infection in measles (Bayor *et al.*, 2009).

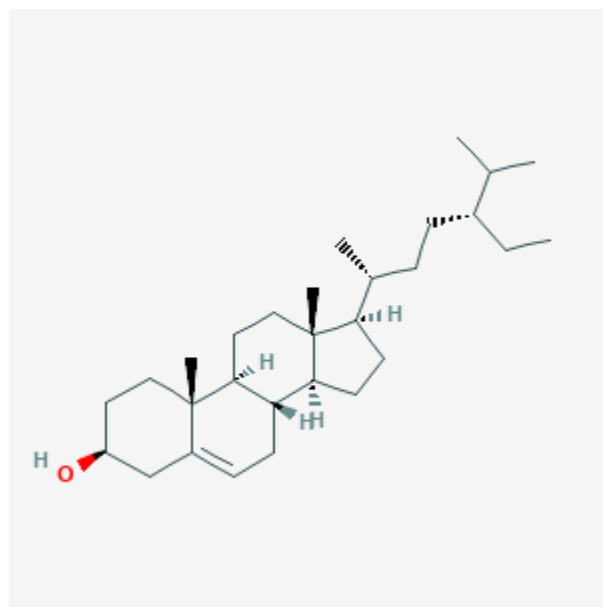


Figure 2.2: Structure of Beta sitosterol.

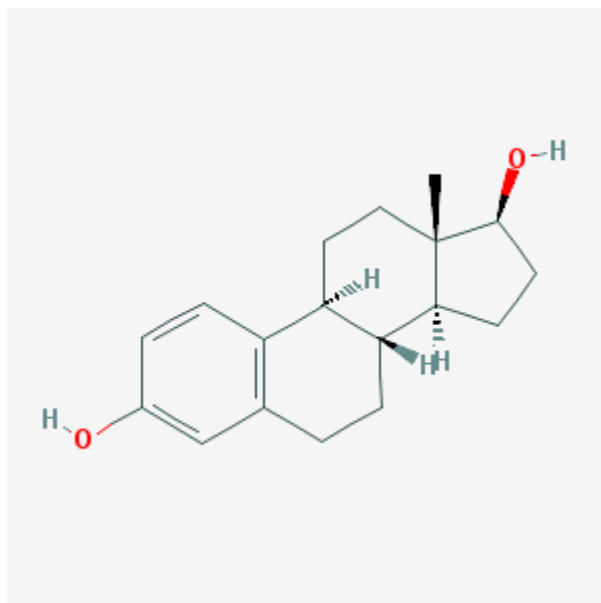


Figure 2.3: Structure of Estradiol.

2.6 Immunohistochemistry As An Investigative Tool

Immunohistochemistry (IHC) is a research tool that combines anatomy, histology, immunology and biochemistry. It has been adapted in so many areas of research including cancer studies, cell signaling and the development of drugs (Atkins *et al.*, 2004; Surng *et al.*, 2012). Developed from the antigen-antibody binding reaction, immunohistochemistry can be considered as a method that visualizes distribution and localization of specific antigens or cellular components in separated tissues or tissue sections. Compared to other bio-techniques that are based on the antigen-antibody reaction such as immunoprecipitation or western-blot, immunohistochemistry provides *in situ* information which promises a more convincing experimental result (Kumar *et al.*, 2000). Major components in a complete immunohistochemistry experiment include; a primary antibody binding to a specific antigen. The antibody-antigen complex is formed by incubation with a secondary, enzyme-conjugated antibody; with presence of substrate and chromogen, the enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding (Conklin *et al.*, 2013). Immunohistochemistry uses different staining procedures.

The **direct method** is a one-step staining method and involves a labeled antibody reacting directly with the antigen in tissue sections. This technique utilizes only one antibody and the procedure is short and quick (Tsumiyama *et al.*, 2013). The **indirect method** involves an unlabeled primary antibody (first layer) which reacts with tissue antigen, and a labeled secondary antibody (second layer) reacts with primary antibody (The secondary antibody must be against the Immunoglobulin G of the animal species in which the primary antibody has been raised) (Hsu *et al.*, 1981). This method is more sensitive due to signal amplification through several secondary antibody reactions with different antigenic sites on the primary antibody. The second layer antibody can be labeled with a fluorescent dye such as Fluorescein 5(6)-isothiocyanate (FITC), rhodamine or Texas red, and this is called indirect immunofluorescence method (Kalra, 1991). The second layer antibody may be labeled with an enzyme such as peroxidase, alkaline phosphatase or glucose oxidase, and this is called indirect immunoenzyme method (Mason & Sammons, 1978).

The **peroxidase anti-peroxidase (PAP) method** is a further development of the indirect technique and it involves a third layer which is a rabbit antibody to peroxidase, coupled with peroxidase to make a very stable peroxidase anti-peroxidase complex. The complex, composed of rabbit gaba-globulin and peroxidase, acts as a third layer antigen and becomes bound to the unconjugated goat anti-rabbit gaba-globulin of the second layer (Tsumiyama *et al.*, 2013). The **Avidin-Biotin Complex (ABC) method** is a standard IHC method and one of widely used technique for immunohistochemical staining. The technique involves three layers. The first layer is unlabeled primary antibody. The second layer is biotinylated secondary antibody. The third layer is a complex of avidin-biotin peroxidase (Hsu *et al.*, 1981).

CHAPTER THREE

3 MATERIALS AND METHODOLOGY

3.1 Study Design

The study was an experimental design. The earlier parts of this work had been done by Afriyie *et al.* (2014) and Asare *et al.* (2015). This study sought to continue from findings of the earlier parts done and it is therefore prudent to revisit steps that led to the findings.

3.2 Ethical Issues

Ethical clearance was obtained from the Ethics and Protocol Review Committee of The School of Biomedical and Allied Health Sciences, College of Health Sciences, University of Ghana. The ethics identification number was given as SBAHS-MD//AA/SA/2016-2017.

3.3 Preparation of The Aqueous Root Extract of *Croton Membranaceus*

The roots of *Croton membranaceus* were harvested in December 2012 and authenticated by the Center for Scientific Research into Plant Medicine (CSRPM), Mampong, Akwapim. The sample of the plant was deposited at the herbarium of CSRPM with a voucher specimen number CSR2110. The aqueous root extract was obtained as described below by Afriyie *et al.* (2013).

After collection, the roots were air-dried in a solar dryer for one week before milling. About 1 kg of milled *Croton membranaceus* was extracted with water for 24 hours on a shaker at room temperature. Each extract was filtered and re-extracted with the same solvent for another 24 hours. The pooled extracts were concentrated *in vacuo* at 50–55 °C before being transferred onto a freeze dryer to remove traces of water (Asare *et al.*, 2011). One thousand grams (1000 g) of dry powdered *C. membranaceus* root was macerated for 24 hours with 4000 mL of distilled water

and heated for 1 hour. The extract was filtered through medical gauze to separate it from the residue. Another 3000 mL of distilled water was added to the residue, macerated for a further 24 hours, and the above procedure repeated to obtain a second extract. The extracts were pooled and freeze-dried using Freeze Dryer Gamma 1-16/2-16 LSC (New York, 2004) (Afriyie *et al.*, 2013). The freeze-dried extract was weighed (20.6g) and stored in a sealed container in a refrigerator at a temperature of 2 to 8°C until use (Afriyie *et al.*, 2013).

3.4 Animals

Twenty-five (25) specific pathogen free (SPF) grade adult male Sprague-Dawley (S-D) rats weighing between 200g and 250g were purchased from Shanghai Si-Lai-Ke Experimental Limited (Shanghai, China) for the study. The rats were acclimatized to laboratory environment (21–23°C) with a 12 hour light - darkness cycle for 7 days prior to experimentation. The rats had access to standard laboratory diet and water *ad libitum*. All experimental procedures were carried out in accordance with international ethical guidelines and the National Institutes of Health Guide for the care and use of Laboratory Animals and were approved by Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine (Afriyie *et al.*, 2014). Previous studies had been done using 30, 150 and 300 mg/ kg b.wt of the aqueous root extract of *Croton membranaceus*. Therapeutic application is in the region of 30 mg/kg b.wt. Authors decided to examine the extremes (30 and 300 mg/kg b.wt dosage)(Asare *et al.*, 2015).

3.5 BPH Model Development In S-D Rats

The rats were randomly assigned into five (5) groups of five (5) rats and with exception of the control group; the other four groups of rats were anesthetized with phenobarbital (50mg/kgb.wt.i.p) and aseptically castrated to bilaterally remove testes. Each of the castrated rats was administered 5mg/kg testosterone propionate (Shanghai GM Pharmaceutical Company

Limited) seven (7) days after castration for 28 consecutive days to induce BPH (Wu *et al.*,2003; Zhou *et al.*, 2012). After successful induction of BPH, one group served as pathological model group whilst the other three served as drug treated groups.

The first two drug treated groups received 30mg/kgb.wt. [low dose (LD)] and 300mg/kgb.wt. [high dose (HD)] respectively *per os* *Croton membranaceus aqueous* root extract (CMARE) by oral gavage for 28days. The last group served as a positive control group and received 0.5 mg/kgb.wt. finasteride (HangzhouMerck, China) *per os* for 28 days. With the exception of the negative control which received normal saline throughout the experimental period, all other groups (pathological model, extract treated and positive control) received repeated testosterone injections (5mg/kg b.wt.) for the next 28 days after BPH induction. Body weights of rats were measured weekly throughout. BPH development was confirmed in each rat through microscopic examination of prostate tissues after an Eosin-hematoxylin staining procedure (Afriyie *et al.*, 2014). As recommended by manufacturers of the antibodies, testicular tissue from the control group were harvested and employed in the study to serve as a check on the study. This was designated the testicular control in the study. The testicular tissues were expected to give high intensity staining with all three antibodies.

3.6 Treatments

The negative control group was fed with normal chow and water only. The low-dose test group was also fed with normal chow and water and also administered 30 mg of CMARE/kg body weight daily for 28 days *per os* by oral gavage. The high-dose test group was also fed with normal chow and water and administered 300 mg of CMARE/kg body weight daily for 28 days *per os* by oral gavage (Afriyie *et al.*, 2014). The last group served as a positive control group and received 0.5 mg/kgb.wt. finasteride *per os* for 28 days. At the end of the experimental period, the

rats were fasted for 24hours after administration of last dose. Rats were anesthetized with light ether ketamine–diazepam to obtain blood from the abdominal aorta before being sacrificed (for future possible investigations). The prostate gland was then freed from connective tissues and immediately fixed with buffered formalin and transported to the histopathology laboratory. Testes from the control group were also harvested and isolated to be used as testicular controls

3.7 Histopathological Examination

Samples of the prostate tissues were cut up and accurately processed (dehydrated, cleared and paraffinized) using standard operating procedures with the aid of an automated tissue processor. The tissues were then embedded in paraffin and tissue blocks made. Afterwards, sections were made from the tissue blocks with the aid of a microtome at 5 micron thickness. Hematoxylin and eosin staining procedures were carried out according to standard staining procedures. The sections were evaluated microscopically for histological changes under a light microscope (Olympus,Tokyo). Tissue blocks were stored in a safe place. Testicular tissues obtained were treated in a similar manner.

3.7.1 Cutting of Sections

The actual work in this study began with the cutting of tissue sections. Stored tissue blocks were obtained and made ready by placing their surfaces on ice. Five (5) micron thick tissue sections were cut from the blocks using a leica rotary microtome (Berlin, 2008) and applied to electrostatically-charged micro slides. Three slides were prepared from each block and incubated at 60°C overnight.

3.7.2 Bringing Sections Down To Water

The slides were deparaffinized in two changes of xylene for 5 minutes each. The sections were gradually hydrated through decreasing grades of alcohols. The slides were washed in two changes of 100% ethanol for 5 minutes each, then into a change of 70% ethanol for 10 minutes. The slides were then washed in deionized water for 10 minutes. Excess liquid was blotted from the slides.

3.8 Antigen Retrieval/Unmasking

Certain antigenic determinants are masked by formalin fixation and paraffin embedding. Antigen unmasking helps expose these antigenic determinants. This was achieved by the heat treatment method. In this method, slides were placed in a glass jar and covered with 200mls of 10 mM sodium citrate buffer, pH 6.0 and heated at 120° C for 5 minutes in a pressure cooker. The slides were allowed to cool in the buffer for approximately 20 minutes and washed in distilled water three times for 2 minutes each (Kalra, 1991). Antigen retrieval solution was obtained from Leica Biosystems, Newcastle, UK. Manufacturer's instructions were followed in this procedure.

3.9 Antibodies

Anti-androgen receptor antibodies, anti-estrogen receptor alpha antibodies and anti-estrogen receptor beta antibodies were obtained from Santa Cruz Biotechnology, Germany. They were obtained in the concentrated liquid forms and transported on ice. On arrival, the antibodies were stored in a refrigerator at 4°C as provided by manufacturer's instructions. The antibodies were monoclonal (all from the IgG cell lines) and were raised from mice. The antibodies were all reactive to mouse, rat and human tissues according to manufacturer. The antibodies were

designed for the immunohistochemistry technique even though they supported the immunofluorescence and fluorescence in situ hybridization (FISH) techniques.

For all three antibodies, the dilution range was 1:50 – 1:500. On trial experimentation the 1:100 dilution gave the best staining and was chosen for the study. This concentration was achieved by dissolving 50 microlitres (ul) of antibody in 5 millilitres (ml) of 1.5% bovine serum albumin (BSA) in tris buffered saline (TBS). This was done for all three antibodies and the diluted antibody solutions were kept in sterile containers and accurately labeled. The anti-androgen receptor and anti-estrogen alpha receptor antibodies were directly labeled by the manufacturer, with fluorescein isothiocyanide (FITC), a fluorescence dye. The anti-estrogen beta receptor on the other hand was not labeled. It was a primary antibody. On the day of experimentation, the antibodies were brought to room temperature and made ready for use.

3.10 Immunohistochemistry Staining

Immunohistochemical staining was done using the direct and indirect methods. Determination of antibody binding to androgen and estrogen alpha receptors was achieved by the direct method whereas the binding of antibodies to estrogen beta receptor was determined by the indirect method. The choice of staining method was solely on the availability of reagents but not any other peculiar interest.

3.10.1 Direct Immunofluorescence Staining

In this procedure, firstly, specimens were incubated for 30 minutes with 10% bovine serum albumin (BSA). The slides were then washed with three changes of TBS for 2 minutes each and incubated with FITC conjugated anti-androgen receptor and anti-estrogen alpha receptor antibodies for 90 minutes at room temperature in a dark room. The slides were then washed with

three changes of TBS for 2 minutes each. Excess liquid was blotted from the slides without drying the specimen. Immediately, the slides were mounted with an aqueous mountant (Aquatex, Germany). The slides were examined in a dark room using a fluorescence microscope with appropriate filters. All steps involving FITC-conjugated antibodies were performed in the dark.

3.10.2 Indirect Immunohistochemistry Staining

The alkaline phosphatase-anti alkaline phosphatase (APAP) method was used. In this method, specimens were incubated for 30 minutes with 10% bovine serum albumin (BSA). They were then washed with three changes of TBS for 2 minutes each and incubated with primary antibody (anti-estrogen beta receptor antibody) for 60 minutes at room temperature. Slides were subsequently washed with three changes of TBS for 2 minutes each. The slides were again incubated with the secondary antibody which is a rabbit anti mouse antibody for 30 minutes. The slides were then washed in three changes of TBS for 2 minutes each. The slides were subsequently incubated with alkaline phosphatase anti phosphatase (APAP) for 30 minutes. The slides were then washed again in one change of TBS for 5 minutes. The slides were again incubated with the chromogenic dye (fast red) for 10 minutes. The slides were then washed with water for 5 minutes and counterstained in hematoxylin for 2 minutes. The slides were then blued under scott's tap water for 30 seconds. The slides were then taken out of the water and mounted with an aqueous mounting medium (Aquatex, Germany). The following precautions were observed in the course of immunohistochemical staining;

1. Excess reagents/liquid was blotted after each step, but drying of specimens between steps was avoided.
2. Sufficient reagent (approximately 100–200 μ l per slide) was used to cover the specimen.

3.11 Slides Examination and result reporting

Slides treated with fluorescence immunohistochemistry, were examined using a fluorescence microscope obtained from the Parasitology Department of the Noguchii Memorial Institute for Medical Research (NMIMR), University of Ghana. The microscope was inspected for optimum function and found out to be in a very good shape for the examination of slides. The slides were subsequently made ready for examination. Receptor-antibody binding was observed as a sharp green fluorescence. It is worth noting that all slides were examined within a maximum of 2 hours after immunofluorescence staining.

Slides treated with chromogenic immunohistochemistry, were examined using a light microscope obtained from the Histopathology laboratory of the Department of Medical Laboratory Sciences, University of Ghana (Olympus CX 41, Japan) with a camera (DP 20). The microscope was inspected for optimum function and found out to be in a very good shape for the examination of slides. The slides were subsequently made ready for examination. Receptor-antibody binding was observed as a red colour development. A colour/fluorescence intensity rating system of 1 to 4 was used to report the results observed as follow;

No staining/fluorescence	-	1
Low intensity staining/fluorescence	-	2
Moderate intensity staining/fluorescence	-	3
High intensity staining/fluorescence	-	4

3.12 Statistical analysis

Data were analyzed with the Kruskal-Wallis H Test using the Statistical Package for Social Sciences (SPSS), version 24, to determine whether the distribution of AR, ERa, and ERb were the same across the following study groups: control group, model group, low dose CM group, high dose CM group, finasteride treated group, testicular control group. This test statistic is similar in nature to the Mann-Whitney U Test, but allows for the comparison of scores on some continuous variables (stratified as ranks) for three or more groups. It is the non-parametric alternative to the One-way between groups analysis of variance test. The assumptions that need to be satisfied when selecting the Kruskal-Wallis test as a test statistic include the following: the samples must be randomly selected; observations must be independent, that is, each person or case must be counted only once, cannot occur in more than one category or group, and the data from one subject must not influence the data from another. All these assumptions were met, making the Kruskal-Wallis test an appropriate test statistic for the evaluation.

CHAPTER FOUR

4 RESULTS

A total of 30 slides were examined for each of the three assays; 90 slides were examined in all. For each assay, five (5) slides were examined for each group i.e. Five (5) slides for the control group, five (5) slides for the BPH model group, five (5) slides for the low dose CMARE treated group, five (5) slides for the high dose CMARE treated group, five (5) slides for the finasteride treated group and five (5) slides for the testicular control group. This is summarized in tables 4.1 to 4.3 below;

Table 4-1: Case Processing Summary for Androgen Receptor immunoassay

AR status	Group	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
	Control Group	5	100.0%	0	0.0%	5	100.0%
	Model Group	5	100.0%	0	0.0%	5	100.0%
	Low Dose CM Group	5	100.0%	0	0.0%	5	100.0%
	High Dose CM Group	5	100.0%	0	0.0%	5	100.0%
	Finasteride treated Group	5	100.0%	0	0.0%	5	100.0%
	Testicular control	5	100.0%	0	0.0%	5	100.0%

Table 4-2: Case Processing Summary for Estrogen Receptor Beta (ERb) immunoassay

ERb status	Group	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
	Control Group	5	100.0%	0	0.0%	5	100.0%
	Model Group	5	100.0%	0	0.0%	5	100.0%
	Low Dose CM Group	5	100.0%	0	0.0%	5	100.0%
	High Dose CM Group	5	100.0%	0	0.0%	5	100.0%
	Finasteride treated Group	5	100.0%	0	0.0%	5	100.0%
	Testicular control	5	100.0%	0	0.0%	5	100.0%

Table 4-3: Case Processing Summary for Estrogen Receptor Alpha (ERa) immunoassay

ERb status	Group	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
	Control Group	5	100.0%	0	0.0%	5	100.0%
	Model Group	5	100.0%	0	0.0%	5	100.0%
	Low Dose CM Group	5	100.0%	0	0.0%	5	100.0%
	High Dose CM Group	5	100.0%	0	0.0%	5	100.0%
	Finasteride treated Group	5	100.0%	0	0.0%	5	100.0%
	Testicular control	5	100.0%	0	0.0%	5	100.0%

4.1 Anti- Androgen Receptor (AR) Immunoassay

For the anti-androgen receptor antibody immunostaining, no stainings were observed for the control, BPH model and finasteride treated groups. However, stainings were observed for the CMARE treated groups (low and high dose groups) as well as the testicular control group. The average staining intensities for the three groups were of high degree. This is summarized in figure 4.1 below;

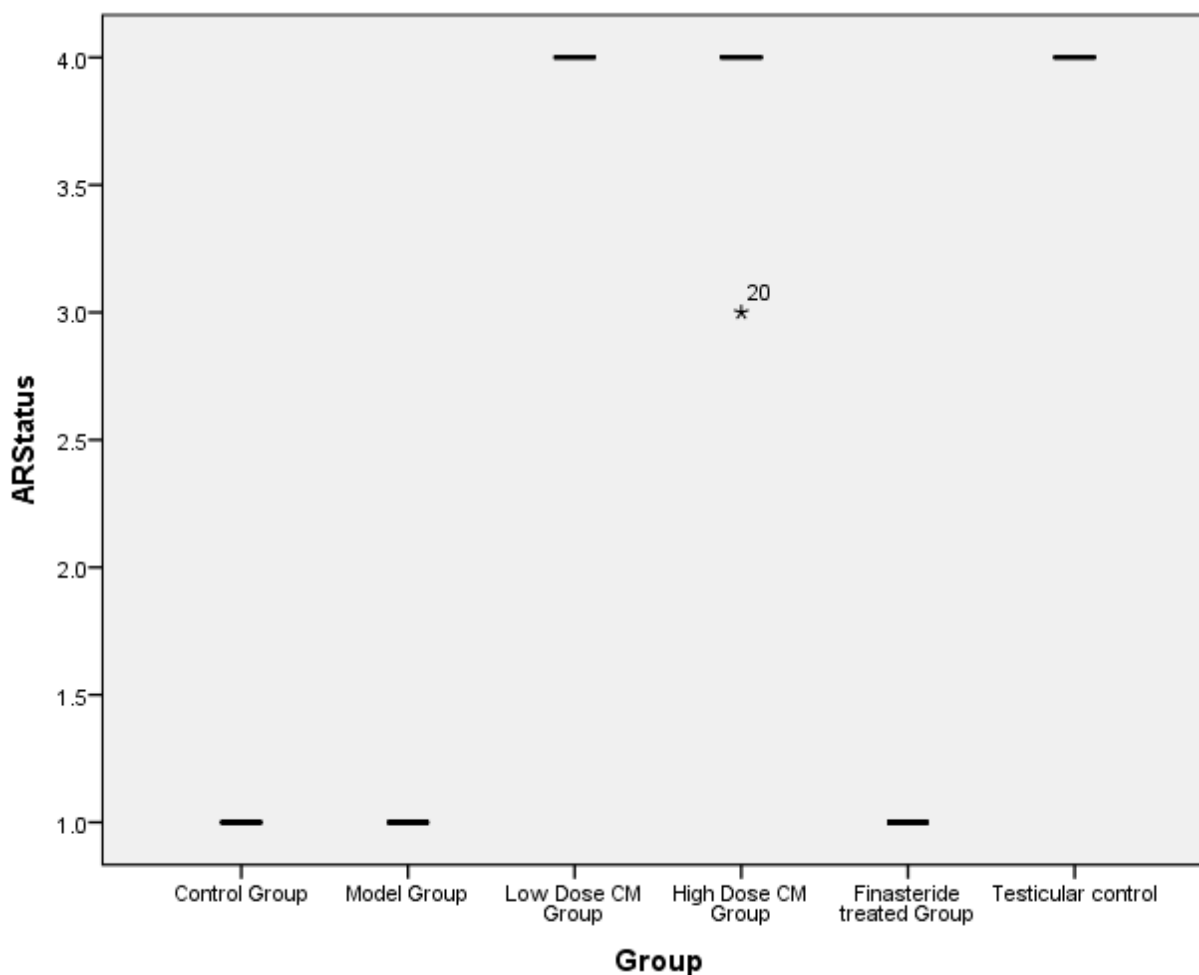


Figure 4.1: Stem and leaf plot representing the distribution of intensity of staining with anti-androgen receptor antibody amongst study groups.

4.2 Anti-Estrogen Receptor alpha (ERa) Immunoassay

For the anti-estrogen receptor alpha antibody immunostaining, all other groups apart from the testicular control group showed no staining. However, the staining intensity for the testicular control group was of a high degree. This is summarized in figure 4.2 below;

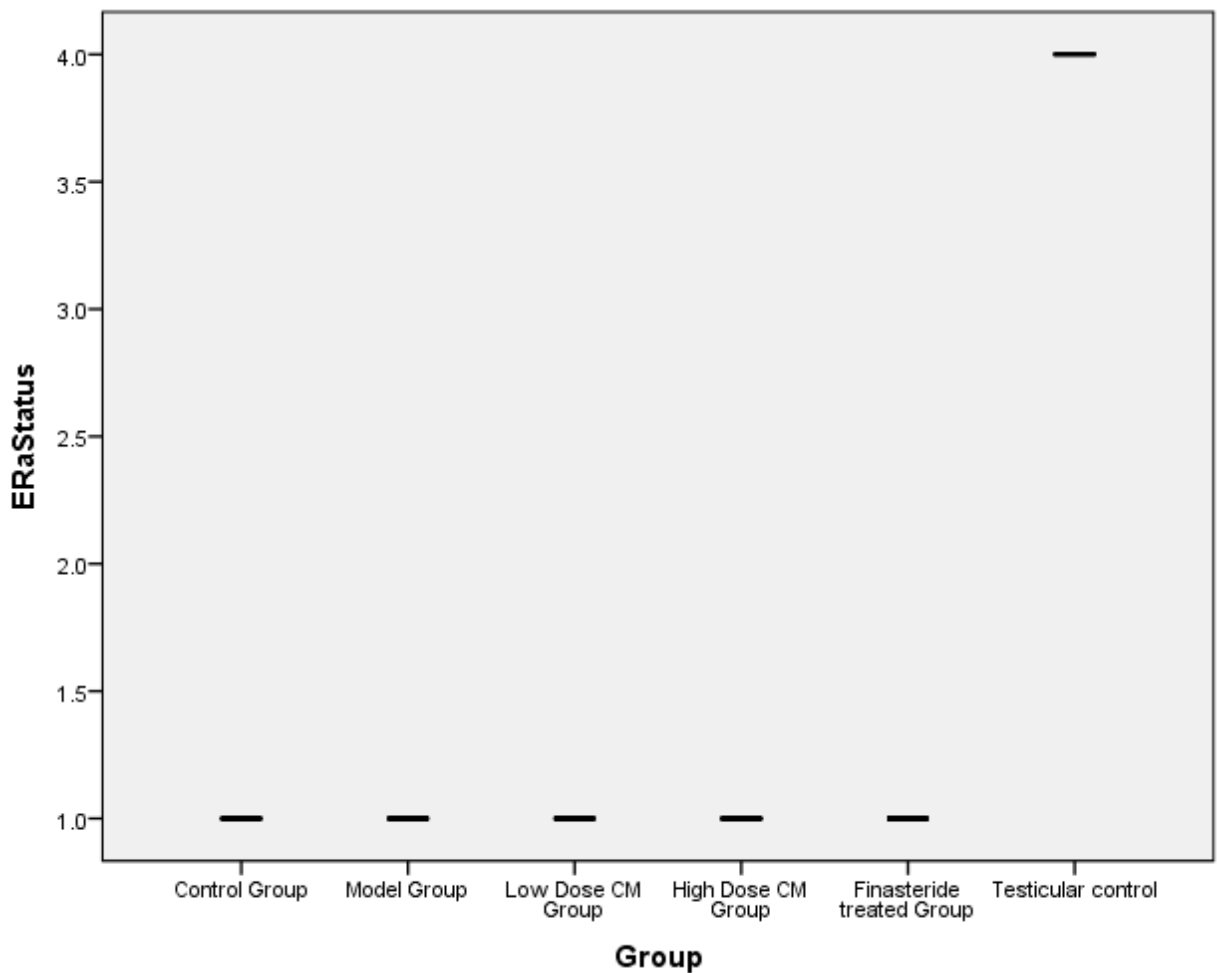


Figure 4.2: Stem and leaf plot representing the distribution of intensity of staining with anti-estrogen receptor alpha antibody amongst study groups.

4.3 Anti-Estrogen Receptor Beta (ERb) Immunoassay

For anti-estrogen receptor beta antibody immunostaining, the control group together with the testicular control group showed high intensity staining whilst the remaining four (4) groups showed no staining at all. This is summarized in figure 4.3 below;

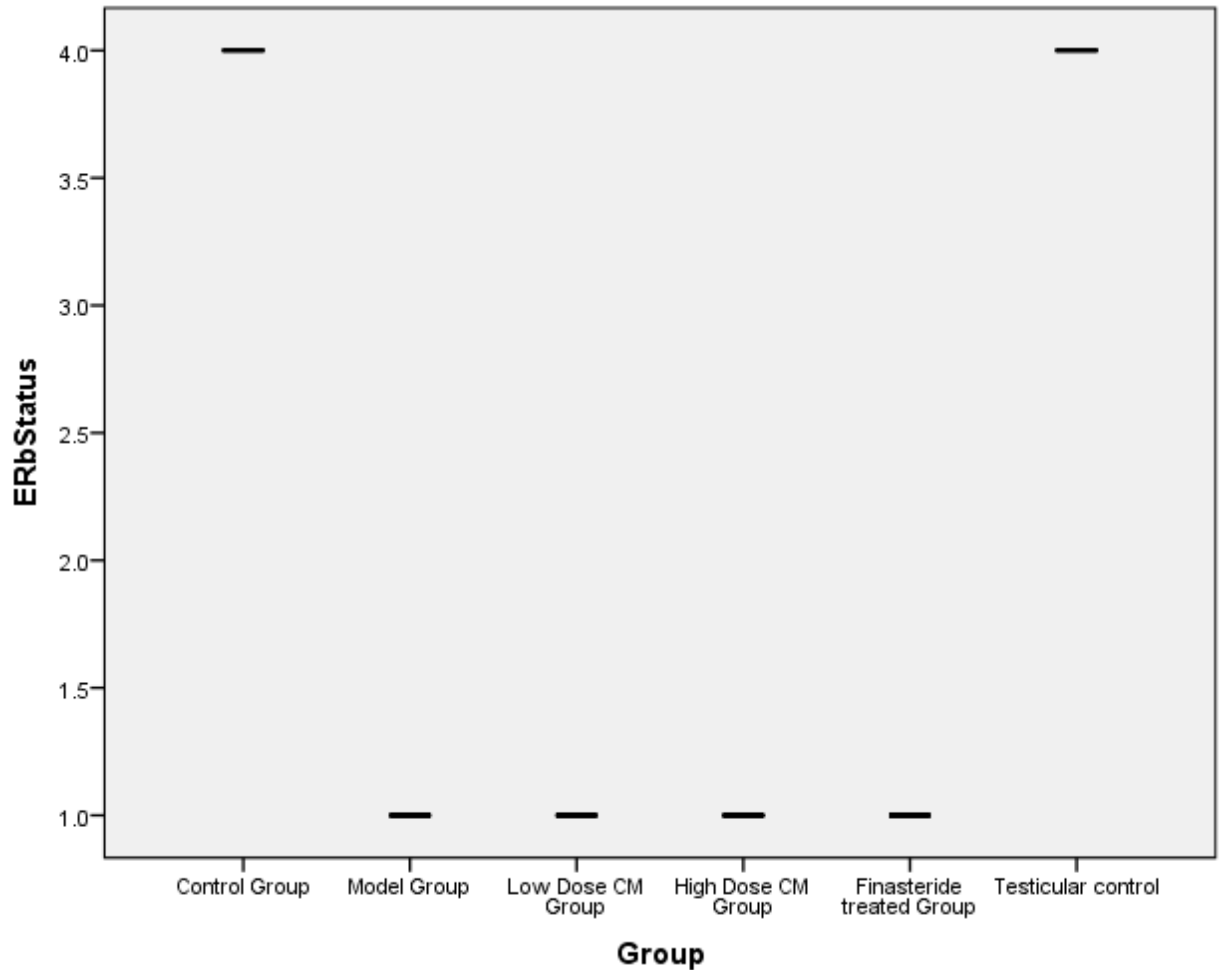


Figure 4.3: Stem and leaf plot representing the distribution of intensity of staining with anti-estrogen receptor beta antibody amongst study groups

The distribution of the various attributes (AR status, ERa status, and ERb status) observed in the study subjects within each of the groups were diverse. A summary of the distribution is presented in Table 4-4.

Table 4-4: Distribution of AR status, ERa status, and ERb status across the study groups

Study groups	Mean ranks		
	AR status	ERa status	ERb status
Control group	1.000	1.000	4.000
Model group	1.000	1.000	1.000
Low dose CM group	4.000	1.000	1.000
High dose CM group	4.000	1.000	1.000
Finasteride treated group	1.000	1.000	1.000
Testicular control group	4.000	4.000	4.000

AR = Anti-androgen receptor immunostaining ; ERa = Anti-estrogen receptor alpha immunostaining; ERb =Anti-estrogen receptor beta immunostaining ; CM = CMARE treated groups.

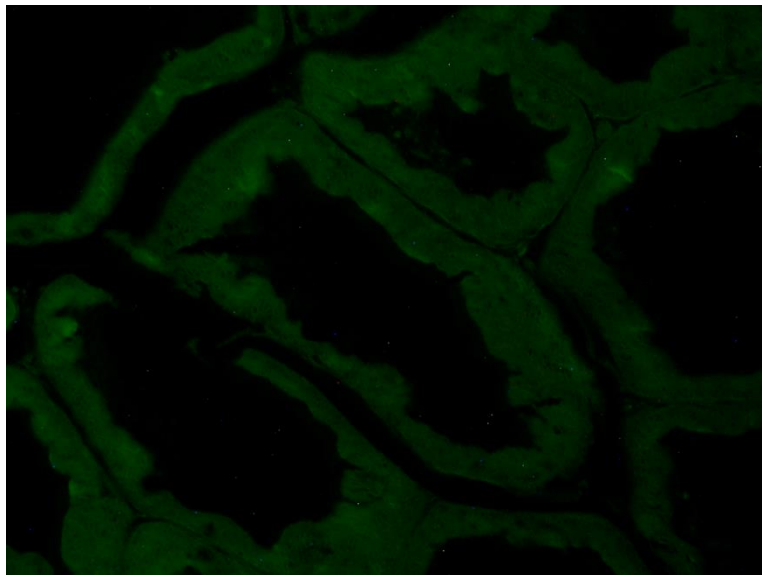


Figure 4.4: A fluorescence micrograph showing no fluorescence for anti-ERa immunostaining on rat prostate tissue (x10).

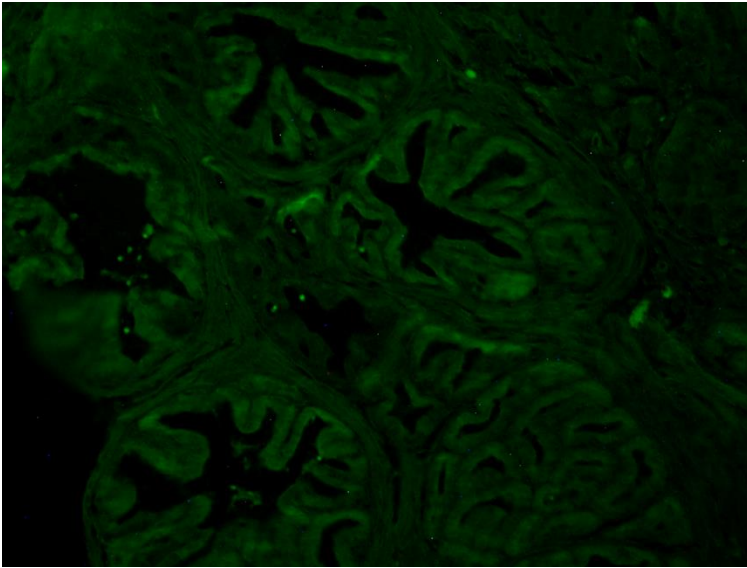


Figure 4.5: A fluorescence micrograph showing no fluorescence for anti-AR immunostaining on rat prostate tissue (x20).

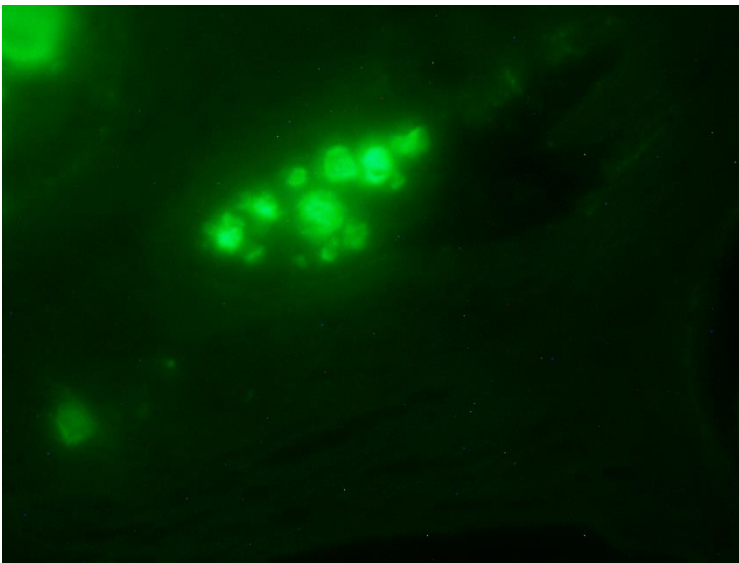


Figure 4.6: A fluorescence micrograph showing fluorescence for anti-ERa immunostaining on rat tissue (x10)

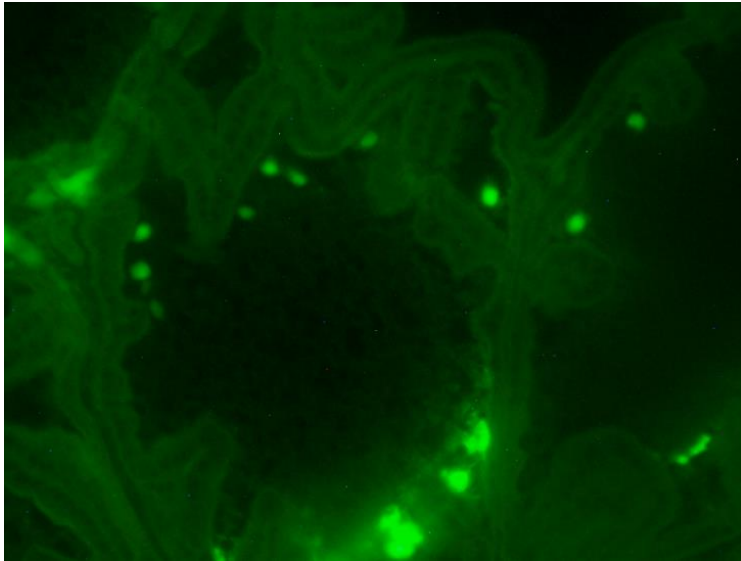


Figure 4.7: A fluorescence micrograph showing fluorescence for anti-AR immunostaining on rat prostate tissue (X10).

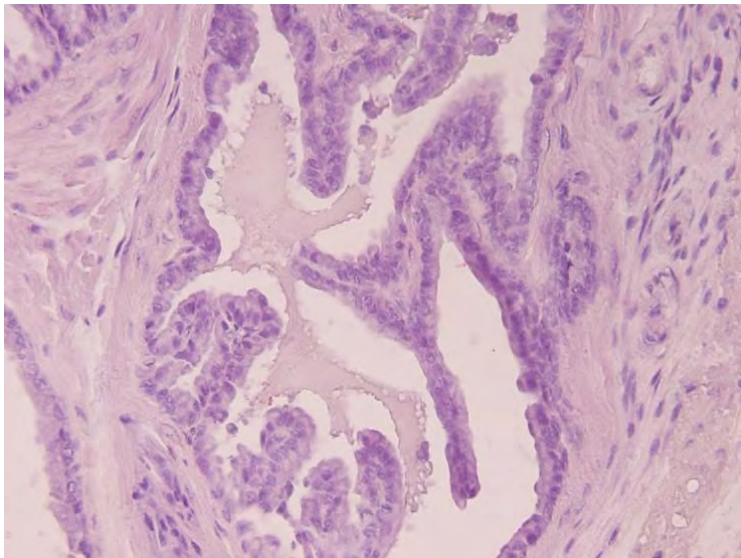


Figure 4.8: A chromogenic micrograph showing negative staining for anti-androgen beta receptor immunochemistry on rat prostate tissue (x10)

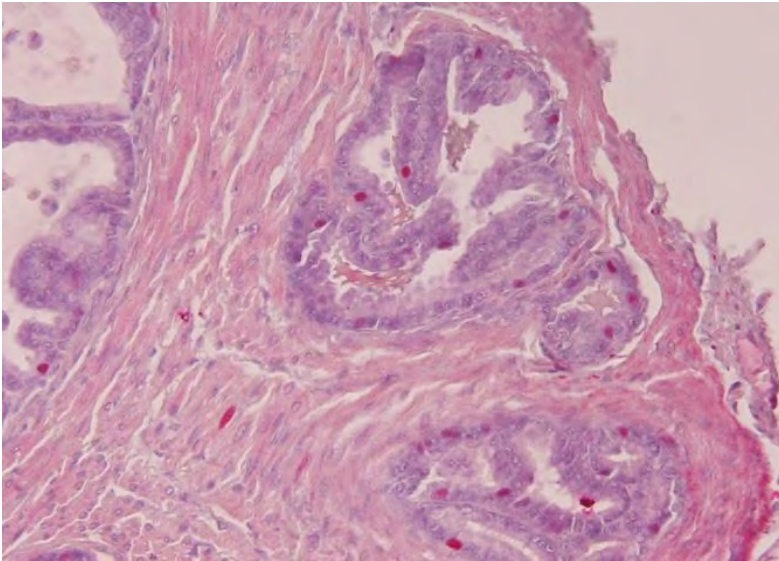


Figure 4.9: A chromogenic micrograph showing positive staining for anti-androgen beta receptor immunocytochemistry on rat prostate tissue (x10).

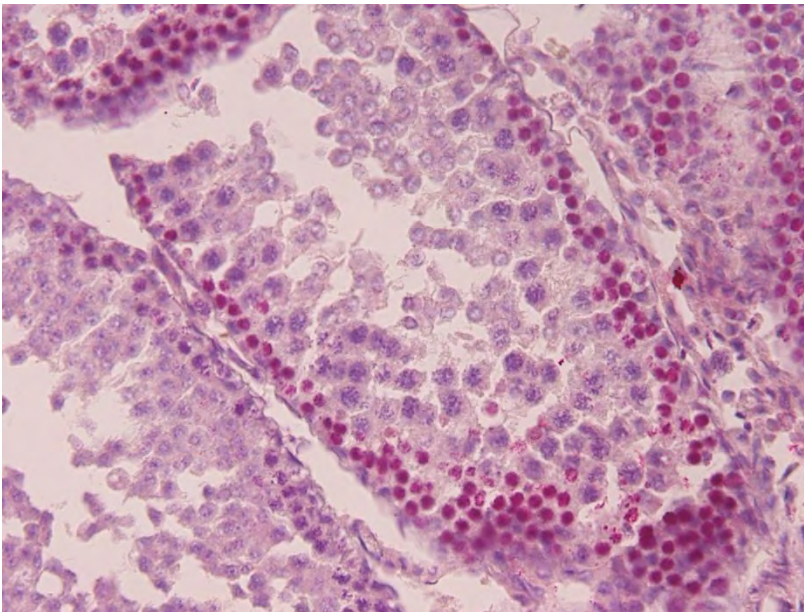


Figure 4.10: A chromogenic micrograph showing positive staining for anti-androgen beta receptor immunocytochemistry on rat testes (x10).

The Kruskal-Wallis Test revealed statistically significant differences in each of AR status [$\chi^2 (5, n = 30) = 28.250, p = 0.000$], ERa status [$\chi^2 (5, n = 30) = 29.000, p = 0.000$], and ERb status [$\chi^2 (5, n = 30) = 29.000, p = 0.000$] across the six different study groups (control group, $n = 5$; model group, $n = 5$; low dose CM group, $n = 5$; high dose CM group, $n = 5$; finasteride treated group, $n = 5$; testicular control group, $n = 5$). A summary of these differences are presented in Table 4.5.

Table 4-5: Summary of differences in attributes across the study groups

Attributes	<i>n</i>	χ^2	df	Sig
AR status	30	28.25	5	0.000
ERa status	30	29.00	5	0.000
ERb status	30	29.00	5	0.000

n = Total number; df = Degrees of freedom; Sig = Significance. AR = Anti-androgen receptor immunostaining; ERa=Anti-estrogen receptor alpha immunostaining; ERb =Anti-estrogen receptor beta immunostaining.

Even though the Kruskal-Wallis test revealed statistically significant differences in attributes for the study groups, the observed differences were not present in each of the pairs of groups.

Table 4.6 presents the differences in attributes between each pair of groups.

Table 4-6: Differences in attributes between the study groups

Differences between groups	AR Status		ERa Status		ERb Status	
	χ^2	Sig	χ^2	Sig	χ^2	Sig
Control group vs. Model group	0.000	1.000	0.000	1.000	15.000	0.015
Control group vs. Finasteride treated group	0.000	1.000	0.000	1.000	15.000	0.015
Control group vs. High dose CM group	-15.50	0.023	0.000	1.000	15.000	0.015
Control group vs. Low dose CM group	-15.50	0.023	0.000	1.000	15.000	0.015
Control group vs. Testicular control group	-15.50	0.023	-15.000	0.000	0.000	1.000
Model group vs. Finasteride treated group	0.000	1.000	0.000	1.000	0.000	1.000
Model group vs. High dose CM group	-15.500	0.023	0.000	1.000	0.000	1.000
Model group vs. Low dose CM group	-15.500	0.023	0.000	1.000	0.000	1.000
Model group vs. Testicular control group	-15.500	0.023	-15.000	1.000	-15.000	0.015
Finasteride treated group vs. High dose CM group	15.500	0.023	0.000	1.000	0.000	1.000
Finasteride treated group vs. Low dose CM group	15.500	0.023	0.000	1.000	0.000	1.000
Finasteride treated group vs. Testicular group	-15.500	0.023	-15.000	0.000	-15.000	0.015
High dose CM group vs. Low dose CM group	0.000	1.000	0.000	1.000	0.000	1.000
High dose CM group vs. Testicular control group	0.000	1.000	-15.000	0.000	-15.000	0.015
Low dose CM group vs. Testicular control group	0.000	1.000	-15.000	0.000	-15.000	0.015

AR=Anti-androgen receptor immunostaining; ERa= Anti-estrogen receptor alpha immunostaining; ERb =Anti-estrogen receptor beta immunostaining ; Std. Error (AR = 4.899; ERa = 3.596; ERb = 4.549)

Table 4.6 compares the staining intensities between experimental groups for the three immunoassays. It can be observed that there was no significant difference between the control and BPH model groups as far as immunostainings for androgen and estrogen alpha receptors are concerned. However, there was a significant difference between the two groups as far as immunostaining for estrogen receptor beta is concerned ($p= 0.015$). A significant difference ($p= 0.015$) was observed between the control group and the finasteride group as far as staining for estrogen receptor beta is concerned. However, no significant difference was observed between the two groups for androgen and estrogen alpha receptor immunostainings.

A significant difference was also observed between the control and the CMARE treated groups (i.e. low and high dose groups) as far as immunostaining for androgen receptors is concerned ($p=0.023$ in either case). For estrogen beta staining, a significant difference ($p=0.015$) was observed between staining intensities of the control group and the CMARE treated groups. There was however no significant difference between the control group and the CMARE treated groups as far as immunostaining for estrogen alpha receptor is concerned ($p=1.00$ in each case). Between the control and testicular control groups, significant differences were observed as far as stainings for androgen and estrogen alpha receptors are concerned ($p=0.023$ and 0.000 respectively). There was no significant difference between the two groups for the estrogen beta receptor immunosaining. Comparing the low dose CMARE group to the high dose CMARE group, there was no significant difference between mean staining intensities as far as the three immunoassays are concerned ($p=1.000$).

CHAPTER FIVE

5 DISCUSSION

Benign prostatic hyperplasia (BPH) represents a great scare for most men. Principally, it causes a lot of discomfort because of its associated lower urinary tract symptoms and bladder outflow obstructions. Men with BPH present with symptoms such as difficulty initiating urination, incomplete bladder emptying, urinary urgency, weak urinary stream, dribbling or nocturia (Dhingra & Bhagwat, 2011). BPH seems to be an inevitable occurrence for ageing men as its risks of occurrence increase with age. Although BPH is not common before age 40, approximately 50% of men develop BPH-related symptoms at age fifty (50). The incidence of BPH increases by 10% per decade and reaches 80% at about 80 years of age (Irani *et al.*, 2003). BPH if not properly managed, can also progress to prostate carcinoma and this increases the fear of men with the condition.

Several treatment options are available to men with BPH. Finasteride, dutasteride and tamsulosin are common allopathic medications used in the management of BPH and its associated symptoms. Complementary and alternative medicines are also available treatment options. *Croton membranaceus* is a famous medicinal plant in Ghana and the aqueous root extract of this plant has been found to shrink prostates of men with BPH. This study aimed at investigating its mode of action. It was suspected that the plant extract had an effect on the hormone-dependent pathway. Studies by Asiedu *et al.* (2018) however, has disproved this hypothesis and suggested that the plant extract may interact with hormonal receptors of the prostate tissue. Prostate tissues are equipped with androgen receptors as well as estrogen receptors, alpha and beta. These receptors bind to their respective ligands to initiate either proliferation or apoptosis. Androgens bind to androgen receptors of prostate tissues to initiate

proliferation of cells. A similar effect occurs when estrogen binds to estrogen receptor alpha, thereby leading to prostatic hyperplasia. On the contrary, apoptosis occurs when estrogen binds to estrogen beta receptors, leading to shrinkage of tissue thereby maintaining prostate size and volume (Nicholson *et al.*, 2013). Therefore, interactions between these receptors are very necessary as far as the etiology and treatment of BPH are concerned. It is of importance to state that, an antagonist for both AR and ER α as well as an agonist for ER β will be reliable treatment options for BPH. The aqueous root extract of *croton membranaceus* in its action is suspected to act as a receptor agonist or antagonist.

Due to the effect of estrogen receptor beta in apoptosis from *in vivo* and *in vitro* studies, an ER β agonist is been sought for the management of BPH. LY500307 was tried in a clinical trial but the study was ended abruptly as the agonist failed to improve BPH symptoms in subjects (Gacci *et al.*, 2012). This study sought to find out if CMARE is an ER β agonist, thus to investigate whether the plant extract interacts with ER β to initiate apoptosis in prostatic cells. The study sought to investigate the availability of receptor binding sites with monoclonal anti-estrogen beta receptor antibody, such that if the receptors are occupied by estrogens or ER β agonists, there will be no binding to the monoclonal antibodies and vice versa. From the study, no stainings were observed for the CMARE treated groups. This suggests that the ER β binding sites were occupied by estrogens and possibly an ER β agonist and thus there was no room for binding to monoclonal antibodies. No study has been done to this effect. However, *croton membranaceus* is known to contain beta-sitosterol, a compound with structural resemblance to estrogen. Due to this structural analogy, beta-sitosterol can fit into ER β binding sites, thereby causing CMARE to act as an ER β agonist. There was a significant difference in staining intensities between the CMARE treated group and the control group ($p=0.015$). However, no significant difference was observed between the CMARE treated group and the BPH model group ($p=1.000$).

The control group was observed to exhibit high intensity staining for estrogen beta receptor immunostaining, suggesting high expression of the receptors with accompanying availability of receptor binding sites. This finding is consistent with previous studies. Estrogen receptor beta has been established by several studies to be highly expressed in normal non-malignant tissues due to their induction of apoptosis (Pirkko, 2004; Bosland, 2005). Apoptosis has been found to be one of the mechanisms used by normal prostate tissues in maintaining normal prostate size and volume. It is therefore not surprising to have observed high intensity staining for the anti-estrogen receptor beta. It can be discussed that the receptors were highly expressed in these tissues and thus even after possible binding of endogenous estrogen, enough receptors were still available for binding with the anti-estrogen receptor beta antibodies hence the high intensity staining observed.

Estrogen alpha receptors bind to their ligands to induce proliferation of prostatic cells. Studies have shown that, as BPH progresses, estrogen alpha receptors are highly expressed. These receptors are also highly expressed in prostate cancers (Bonkhoff *et al.*, 1999). Therefore, in the treatment of BPH, a receptor antagonist that blocks the ERa and prevents it from interacting with estrogens will be an ideal treatment option. This study aimed at investigating whether CMARE acts as an ERa antagonist. No work has been previously been done to this effect with particular reference to *Croton membranaceus*. From the study, the CMARE treated groups were found not to show any stainings for the ERa immunoassay. Studies establish a high expression of ERa receptors in BPH (Bonkhoff *et al.*, 1999; Pirkko, 2004). Therefore, it was expected that much estrogen alpha receptors will be expressed but the findings from the study seems not to suggest so. However, a strong case can be made for high expression of the receptors with accompanying binding to intraprostatic estrogens or ERa antagonists leaving few or no receptor binding sites for the monoclonal antibodies.. However, there was no significant difference in staining between the

CMARE treated groups and the control as well as the BPH model groups. This finding therefore suggests that the absence of staining for ER α cannot be wholly attributed to the action of CMARE as similar results were seen in groups that were not treated with the extract. Therefore, CMARE cannot be thought of as acting as an ER α agonist in this regard.

Androgens and androgen receptors have been implicated in the etiology and progression of BPH as well as prostate cancers. Testosterone, the main male androgen and its more potent form, dihydrotestosterone (DHT) bind to androgen receptors to induce proliferation of prostatic cells (Culig *et al.*, 2002). It is on this principle that 5-alpha reductase inhibitors have been used in the treatment of BPH. These drugs inhibit the conversion of testosterone to dihydrotestosterone thereby reducing the interaction between AR and androgens, specifically DHT which has a higher affinity of binding to androgen receptors. Another way of reducing androgen-AR interactions is to employ the use of AR antagonists which block androgen receptors and prevent their interactions with androgens. Bicalutamide is a common non-steroidal androgen receptor antagonist. This study aimed at investigating whether CMARE shrinks prostate by acting as an AR antagonist.

From the study, CMARE and androgens did not bind to the androgen receptors making the receptors available for binding to the monoclonal anti-androgen receptor antibodies. Endogenous androgens not binding to the receptors may be as a result of low concentrations of the hormones in the tissue and probably in the serum as well. However, this study fails to address that concern. Furthermore, CMARE not binding to the free receptors *in vivo* suggests that the extract does not bind to the androgen receptor in its action and thus CMARE is not an androgen receptor antagonist. Binding of the monoclonal antibodies really suggests that the androgen receptors were expressed. This is consistent with findings that androgen receptors are highly expressed in

benign hyperplastic prostate tissues (Prins & Korach, 2007).

The control group was observed to exhibit no staining for the anti-androgen receptor and anti-estrogen receptor alpha immunostainings. However, a high intensity staining was observed for immunostaining with anti-estrogen receptor beta antibodies. Studies have shown that androgen and estrogen alpha receptors are normally expressed in normal prostate tissues but highly expressed in benign hyperplastic prostate tissues as well as prostate carcinomas (Nicholson *et al.*, 2013). These two receptors have been found to induce prostatic hyperplasia when they bind to their respective ligands (Bosland, 2005). And thus normal prostate tissues are expected to have reduced expressions of these ligands and thus the absence of hyperplasia in normal prostate tissues. Findings are consistent with the above cited studies. Therefore the absence of staining observed in the control group for these two receptors may be attributed to the very low expression of these receptors in the control group which have normal prostate tissues. Thus not much androgen receptors as well as estrogen alpha receptors were expressed by the tissues. Besides, the few receptors that might have been expressed are suspected to have been bound to endogenous hormone ligands. In effect, the absence of staining can be attributed to reduced receptor expression coupled with unavailable receptor binding sites for the antibodies to bind and exhibit visible stainings.

The BPH model group was observed exhibiting no staining as far as all the three immunoassays are concerned. This suggests that all three receptors; androgen, estrogen alpha and beta receptors were unavailable for binding with their respective monoclonal antibodies. Literature establishes that androgen and estrogen alpha receptors are highly expressed in hyperplastic prostate tissues due to their induction of hyperplasia when they get bound to their respective hormonal ligands (Bosland, 2005). Findings from the study seem to suggest that these receptors are rather less

expressed in the BPH model group, contrary to findings from various studies (Bonkhoff & Berges, 2009; Leav *et al.*, 2001). A very important point to consider is the availability of the binding sites of the receptors supposed they are well expressed. This brings to mind the possible occurrence that these receptors might be highly expressed but were however, bound by endogenous ligands i.e. androgens and estrogens. A shortfall of this study is the inability to assay serum and tissues concentrations of these hormones in the study animals simultaneously. This would give a clearer picture on this issue, helping in concluding as to whether these receptors were not highly expressed or they were highly expressed but were bound by endogenous hormonal ligands leaving the binding sites of these receptors unavailable for binding to monoclonal antibodies raised against these receptors.

The BPH model group exhibiting no staining for the estrogen receptor beta suggests that not much of the receptor was expressed. This is consistent with studies which have established that the estrogen beta receptors are less expressed in hyperplastic tissues (Prins & Korach, 2007). The receptors bind to their ligands to induce apoptosis in cells, ensuring the maintenance of tissue size and volume. Therefore it is not out of order to realize no staining for estrogen receptor beta immunostaining as few or no receptors were available for binding with the monoclonal antibodies.

From the results, it can be observed that there were no stainings for the two estrogen receptors being investigated within the low dose group. Two inferences can be made from this finding. Firstly, the expression of these receptors may be reduced. Secondly, the receptors might be expressed but may be bounded by intraprostatic estrogens *in vivo*. With respect to the former, estrogen beta receptors have been found to be negatively expressed in benign prostatic hyperplasia and thus not much receptors are expected to be expressed (Fixemer *et al.*, 2003). In view of this, few or no estrogen beta receptors were available for staining with the monoclonal

antibodies. This is consistent with findings from Pirkko (2004) which established the fact the estrogen beta receptors are reduced in BPH.

The high dose CMARE treated group exhibited same staining statuses with the low dose treated group as far as the three immunoassays are concerned. No significant difference was realised between their staining intensities as far as the three receptor assays are concerned ($p=1.000$). This brings to mind the fact that the binding of CMARE, if any, is not dose dependent.

Finasteride is a famous drug used in the treatment of BPH and its associated LUTS. As a famous drug for the treatment of BPH, a group was treated with this drug. Findings from tissues of rats treated with this drug indicated no staining for all the three anti-receptor immunoassays. This is consistent with the mechanism of action of the drug, finasteride. The drug has been found to be a 5-alpha reductase inhibitor and not a receptor agonist nor antagonist (Nunes *et al.*, 2017). Thus in its action, finasteride does not interact with these receptors being investigated.

There was a significant difference between the CMARE treated groups and the control group for anti-androgen receptor staining ($p=0.023$). The CMARE treated groups exhibited high intensity staining whereas no staining was observed for the control group. The control group had no BPH induction and the prostates of rats in this group are of normal anatomy. The CMARE treated groups had BPH and as literature confirms, androgen receptors are highly expressed in BPH (Gregory *et al.*, 2001).. The availability of AR binding sites for binding to monoclonal anti-AR antibodies suggest that the AR binding sites were occupied by neither intraprostatic androgens nor CMARE. This adds to the claim that in the action of the extract, CMARE does not bind to androgen receptors.

However, compared to the BPH model, the CMARE treated group exhibited a significant difference in anti-AR binding intensity ($p=0.023$). The BPH model is equally equipped with enough androgen receptors as in the CMARE group. However, no anti-AR staining was observed in this group. This suggests that the AR binding sites in this group might be occupied by intraprostatic androgens. Therefore, a case can be made for CMARE that it did not bind to the androgen receptors whilst ensuring that intraprostatic androgens do not bind to the androgen receptors. Therefore, it can be argued that, CMARE in its action to shrink prostate in BPH rat models, does not bind to androgen receptors whilst playing an inhibitory role in the binding of intraprostatic androgens to androgen receptors of the prostate tissue. This is consistent with a study by Asiedu *et al.* (2017) which concludes that *Croton membranaceus* is not a 5 alpha reductase inhibitor but impacts on the hormonal pathway by inhibiting the signaling between dihydrotestosterone and androgen receptors.

For anti-estrogen receptor alpha (anti-ERa) immunostaining, no significant difference was observed between the CMARE treated groups and the control groups ($p=1.000$). No staining was observed for the two groups. A similar result was seen for the BPH model group with corresponding insignificant difference when compared to the CMARE treated groups ($p=1.000$). In all three groups, the ERa receptors were unavailable for binding to the anti-ERa antibody used. This cannot be attributed to CMARE binding as no significant difference was observed when compared with the two groups. Common to the three groups would be the presence of estrogen. However, this study did not assay for intraprostatic estrogen concentration. This would clarify whether the estrogen alpha receptors were engaged by intraprostatic estrogens. However, a clear inference that can be made is the fact that CMARE does not interact with the estrogen alpha receptors in its action.

For estrogen beta receptor (ERb) immunostaining, no significant difference in staining intensity was observed amongst the BPH induced groups ($p=1.000$). However, there was a significant difference between the BPH induced groups and the control group ($p=0.015$). This is consistent with studies that suggest that estrogen beta receptors are highly expressed in normal tissues and negatively expressed in hyperplastic and malignant tissues. Estrogen beta receptors bind to their ligands to induce apoptosis, a mechanism used by normal prostate cells in maintaining prostate size and volume. Therefore, a reduction in the population of ERb is directly related to hyperplastic and malignant conditions. A suggested study on the effect of CMARE on ERb is to prolong administration of CMARE in BPH rat models and monitor the increase in ERb population with time.

The testicular control group was suggested by the manufacturers of the antibodies to ascertain the ability of the antibodies to bind to the receptors being investigated i.e. androgen receptor, estrogen receptors alpha and beta. Once staining in this group is confirmed, the ability of the antibodies to bind to these receptors becomes established and that becomes the basis for the investigations. The testes (of humans, rats and mouse) are equipped with androgen receptors as well as estrogen alpha and beta receptors (Lubahn *et al.*, 1988). These receptors are highly expressed in the adult mouse and thus are available for binding with ligands, whether endogenous or exogenous. In the absence of binding to endogenous ligands, these receptors are available for binding with exogenous ligands. Thus, in this group, receptor binding sites were available for binding with exogenous ligands. Antibodies raised against these specific receptors are capable of binding to these receptors. From the study, it was observed that, the testicular tissues exhibited high degree staining after incubation with the respective antibodies. This is consistent with literature and confirms the fact that the testis is equipped with these receptors (Pelletier *et al.*, 2000). More importantly, the high intensity staining connotes that, given the

presence and availability of free receptors, antibodies used in this study are capable of binding to these receptors.

Table 4-4 summarises the distribution of staining intensities amongst all groups with respect to all three immunoassays.

CHAPTER SIX

6 CONCLUSION

CONCLUSION

It can be concluded from this study that the aqueous root extract of *Croton membranaceus* (CMARE) does not bind to androgen receptors in its action. However, the extract may inhibit the binding of intraprostatic androgens to the androgen receptors. Furthermore, CMARE may act as an estrogen beta receptor agonist and does not interact with estrogen alpha receptors.

LIMITATION

The study did not measure serum and intraprostatic estrogens and androgens concentrations to rule out or otherwise, the possibility of receptor binding to their natural hormone ligands or hormonal analogues (more probably, beta-sitosterol). Unavailability of receptor binding sites to monoclonal receptor antibodies may be as a result of engagement of receptor binding sites by intraprostatic hormones.

RECOMMENDATIONS

1. The study should be repeated and serum and intraprostatic hormonal concentrations evaluated alongside to rule out the possibility of receptor engagement by these hormones. Metabolites of these hormones can also be evaluated.
2. The possibility of CMARE acting by upregulating the function of ERb in initiating apoptosis should be further investigated. The possibility of CMARE increasing ERb population can be determined by a prolonged administration of CMARE in BPH rat models and monitoring the increase in ERb population with time.

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