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THE EFFECT OF *CROTON MEMBRANACEUS*
ON DIHYDROTESTOSTERONE LEVELS IN BLOOD
AND ITS SYNTHESIS BY PROSTATIC
 5α -REDUCTASE IN THE RAT

A THESIS SUBMITTED TO THE DEPARTMENT OF
BIOCHEMISTRY, UNIVERSITY OF GHANA,
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF PHILOSOPHY



BY

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DECEMBER 1997.

DEDICATION

This work is dedicated to the memory of my son, David,
who died of cancer of the lymph nodes
on 26th October 1994, just when this work began.



DECLARATION

I declare that the experimental work described in this thesis was performed by me in the Department of Biochemistry, University of Ghana, National Nuclear Research Institute and the Centre for Scientific Research into Plant Medicine, under the supervision of Prof. Marian E. Addy. I certify that this work has not previously been accepted for any degree and is not being concurrently submitted in candidature for any other degree.

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ABSTRACT

Alcoholic extract of *Croton membranaceus* Mull. Arg is used at the Centre for Scientific Research into Plant Medicine at Mampong Akwapim to treat retention of urine due to benign prostatic hyperplasia (BPH). The effect of the extract on rat steroid 5 α -reductase activity *in vivo* was investigated using a radioimmunoassay technique to quantitate dihydrotestosterone (DHT) levels in blood. The effects of the extract and two unidentified alkaloidal isolates of the plant, provisionally referred to as CM-1 and CM-3, were investigated *in vitro* as potential inhibitors of 5 α -reductase activity using a thin layer chromatographic technique to quantitate DHT formed from testosterone.

The results show that the extract may not lower rat serum DHT levels significantly *in vivo* but both the extract and the isolated CM-1 inhibited the enzyme activity *in vitro* at the concentrations used. The other isolate, CM-3, did not inhibit the enzyme at the concentration used. The results obtained suggest that inhibition of 5 α -reductase activity may not be the major mechanism by which the extract exerts its action in BPH patients.

List of abbreviations

- A-dione = 5 α -androstane-3,17-dione.
- BPH = benign prostatic hyperplasia
- DHT = 5 α -dihydrotestosterone
- 3 α -diol = 5 α -androstane-3 α ,17 β -diol
- 3 β -diol = 5 α -androstane-3 β ,17 β -diol
- EDTA = ethylenediamine tetra acetic acid
- EGF = epidermal growth factor
- FGF = fibroblast growth factor
- GnRH = gonadotrophin releasing hormone
- HSD = hydroxysteroid dehydrogenase
- NGF = nerve growth factor
- POPOP = 1,4-di-2-(5-phenyloxazolyl)-benzene
- PPO = 2,5-diphenyloxazole
- 5 α -R = 5 α -reductase
- SD = standard deviation
- SEM = standard error of the mean
- TGF- β = transforming growth factor β

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Modern medicine has its roots in traditional medicine in which drugs were derived mainly from plant and animal sources. Divination sometimes formed part of the diagnostic process and treatment included various weird practices. As scientific knowledge about the causes and treatment of diseases increased, traditional medicine declined and in the developed countries was replaced with modern medical practice. In the developing countries however, traditional medical systems prevailed, with some modifications, to this day and continue to make significant contributions to health delivery. Often in such countries, patients first consult traditional medical practitioners for remedies to their ailments and only resort to modern clinical practitioners when the traditional remedies fail them.

Economic considerations may partly account for this reliance on traditional medicine. In many developing countries modern clinics are few and far between, so patients have to travel over long distances in order to consult qualified clinical medical practitioners. In areas where modern hospitals are nearby, the cost of consultation and treatment may be beyond the reach of many people. In contrast, within any

settlement in developing countries, it is not difficult to find a traditional medical practitioner in the neighbourhood, not to mention the relatively cheaper cost of treatment. This indicates that traditional medical practices may continue to play a useful role in many developing countries for many years to come.

The World Health Organisation, WHO, recognises this fact. In 1977, the World Health Assembly of the WHO decided that the main social target of governments and of WHO should be the attainment by all the people of the world by the year 2000 of a level of health that will permit them to lead a socially and economically productive life. This is popularly known as "Health for all by the year 2000". Primary health care was identified as the key to attaining this target and the international conference on primary health care held in Alma-Ata in 1978 declared among other things that "primary health care relies on health workers.... as well as traditional practitioners...to work as a health team and to respond to the expressed health needs of the community" (WHO, 1978). The WHO has, therefore, encouraged developing countries to utilise the services of traditional medical practitioners and to train traditional birth attendants to discharge their duties under more hygienic conditions and thereby contribute more effectively towards Primary Health Care (WHO, 1981). It has also encouraged scientific studies into medicinal herbs used

in developing countries including Ghana where the Organisation collaborates with institutions that deal with research into medicinal plants including the G.K. Noamesi laboratory at Hohoe and the Centre for Scientific Research into Plant Medicine (CSRPM) at Mampong. The Centre was established by the Government of Ghana in 1973 to undertake studies into the traditional medicine in order to establish the basis and efficacies of, and improve upon the plant-derived drugs used in the country.

One of the medicines being used at the CSRPM is derived from *Croton membranaceus*. Though this plant material has been used successfully to treat many cases of urine retention due to benign prostatic hyperplasia (BPH), there has been no investigation into the mode of action of the plant extract. This work on the effect of *C. membranaceus* on the metabolism of testosterone in the rat prostate may be the very first attempt to understand the mode of action of this medicinal plant.

1.2 LITERATURE REVIEW

1.2.1. THE PROSTATE GLAND

The prostate gland is one of the accessory male sex organs. The human adult prostate weighs approximately 20g and measures about 4cm at the base, which is near the bladder. Although no

anatomical demarcations are clearly visible on gross dissection or microscopic examination, the prostate is often regarded as having five lobes; the Posterior, the Middle, Anterior and two Lateral Lobes.

Microscopic examination of a section of the prostate reveals that it is composed of tubuloalveolar glands embedded in a stroma consisting of fibromuscular tissue (Figure 1). A well-defined basement membrane marks the transition from the stroma to the glands.

Two types of cells line the glands: secretory columnar cells and non-secretory basal cells. The columnar cells have well-developed Golgi apparatus and rough endoplasmic reticuli and are richly supplied with secretory vesicles and lysosomes that contain fibrinolysin, fibrinogenase and acid phosphatase. Prostate gland contains Prostate Specific Acid Phosphatase that is of considerable importance in the diagnosis of cancers of the prostate.

The prostate does not appear to be essential for the survival or reproductive ability of the male adult. Many patients survive after prostatectomy or Transurethral Resection of the Prostate (TURP). Also the case of several men who have rudimentary prostates but normal libido is mentioned by Kirby *et al.* (1992).

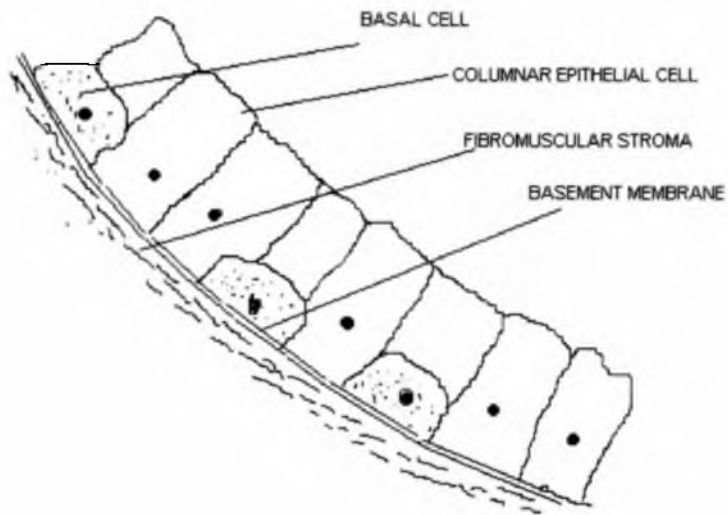


Figure 1: Cross section of the prostate showing types of cell lining the acinar.

1.2.2 THE METABOLISM OF TESTOSTERONE IN THE PROSTATE

About 65% of testosterone in the blood is bound to the α_2 -globulin, Sex Hormone Binding Globulin (SHBG); about 33% to albumin and 2% circulate free in plasma. When free testosterone enters the prostate, it may bind to the cytosolic steroid receptor and migrate into the nucleus where it stimulates RNA synthesis or may be transformed to polar catabolites along either of two possible pathways, the reductive or oxidative pathways.

The reductive pathway leads to the formation of 17β -hydroxy metabolites. The first step in that pathway involves the enzyme 5α -reductase which catalyses the reduction of testosterone to dihydrotestosterone (DHT). DHT formed may bind to the cytosolic steroid receptor and migrate into the nucleus where it also stimulates RNA synthesis. In the cytoplasm, DHT is further reduced to 5α -androstane- $3\alpha,17\beta$ -diol by the action of 3α -hydroxysteroid dehydrogenase (3α -HSD), or to the 3β analogue, 5α -androstane- $3\beta,17\beta$ -diol by the action of 3β -hydroxysteroid dehydrogenase (3β -HSD).

In the alternative oxidative pathway, testosterone is oxidised first to androstenedione by the action of the enzyme 17β -hydroxysteroid dehydrogenase (17β -HSD). Androstenedione is further transformed by 5α -reductase to 5α -androstenedione (A-dione). These and subsequent transformations are illustrated in Figure 2.

Whether testosterone will be metabolised by the oxidative or reductive pathway depends on the relative quantities of 17β -HSD and 5α -reductase respectively, and the relative amounts of oxidised and reduced cofactor available in the prostate. In the presence of more 5α -reductase and/or NADPH than 17β -HSD and/or NADP^+ , the reductive pathway predominates and more DHT than androstenedione is formed. When the cofactor is present mainly in the oxidised form, 17β -oxidation to androstenedione predominates (Morfin *et al.* 1978).

1.2.3 5α -REDUCTASE

5α -Reductase refers to the enzyme NADPH: Δ^4 -3-oxosteroid- 5α -oxidoreductase (E.C. 1.3.99.5.). It catalyses the reduction of testosterone to 5α -dihydrotestosterone (DHT) using NADPH as the cofactor. Other steroid substrates for the enzyme include androstenedione, progesterone, cortisol and corticosterone. The common feature of all these substrates is that they possess the

Δ^4 -oxo moiety in their structures.

During the 5α -reductase catalysed reaction, a pair of electrons is transferred from the cofactor, NADPH, to the steroid molecule such that the reduced steroid product has one hydrogen atom at the 5α position.

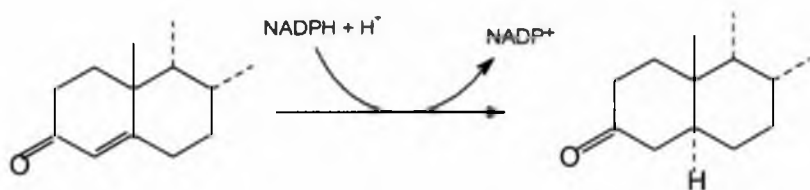


Figure 3: 5 α -Reductase-catalysed reaction.

The enzyme occurs in several androgen target as well as non-target tissues. It is present in testes, the prostate, epididymis, seminal vesicles and genital skin. It is also detectable in the liver, adrenal glands, hypothalamus, skin, pons, medulla oblongata and cerebellum (Thigpen *et al.* 1993).

The fact that inhibitors of 5 α -reductase activity successfully produce a reduction of prostate size in BPH patients (Kirby *et al.*, 1992) suggests that the enzyme plays a contributory role in the development of BPH. BPH involves both stromal and epithelial elements. Stromal cells are believed to initiate the process (McConnell, 1995; Lawson, 1993). Thereafter, however, stromal-epithelial interactions under the influence of androgens are believed to be responsible for the continued growth of the prostate. Of the androgens, the most potent in terms of prostatic growth is DHT (Bluestein and Oesterling, 1993; Morfin *et al.* 1978), which is formed by the action of

5 α -reductase. This makes the enzyme quite important in the development of BPH.

1.2.4 BENIGN PROSTATIC HYPERPLASIA

Benign Prostatic Hyperplasia (or Benign Prostatic Hypertrophy), BPH, refers to the non-neoplastic enlargement of the prostate gland often resulting in significant bladder outflow obstruction and therefore urine retention. Other symptoms that characterise the condition include:

- a. difficulty in initiating urinary flow,
- b. terminal dribbling,
- c. sensation of incomplete emptying of the bladder,
- d. impairment of the force of urine stream,
- e. intermittent streaming,
- f. urge incontinence and
- g. dysuria.

BPH is a universal disorder of elderly men. It afflicts the majority of men over the age of sixty and seems to know no racial boundaries. The condition is present in minor and asymptomatic form in most elderly men, but in about 5% it is exaggerated and causes urethral obstruction.

The pathogenesis of BPH is still incompletely understood but two factors - ageing and the presence of functional testes - are known to be prerequisites for its development. BPH occurs

mainly in adults above age forty-five and the incidence increases with age thereafter. Also, eunuchs such as the Skoptzys of Russia, who undergo castration at age 35 as part of ethnic ritual, do not develop BPH (Bluestein and Oesterling, 1993). Prostatic volume decreases after castration, suggesting a role for testicular hormones in the development of the condition.

Several hypotheses have been advanced over the years in an attempt to explain the pathogenesis of BPH. As early as 1935, Burrows suggested that the changes of the prostate in ageing men might be due to a relative excess of estrogens (Burrows 1935). Later, Zuckerman (1936) explained that the fibromuscular growth of the prostate was the result of an imbalance favouring an estrogenic substance over 'the male hormone proper'. Franks, (1954) developed this hormonal imbalance hypothesis further by explaining that the periurethral glandular region might be under the influence of estrogens, whereas the peripheral region is influenced by androgens. With age, there is a progressive decrease in the production of androgens while serum estrone and estradiol remain constant leading to a rise in the estrogen:androgen ratio. Consequently the glandular periurethral zone enlarges while the peripheral tissue is compressed into a capsule by the expanding glandular areas.

The hormonal imbalance hypothesis suggests that estrogens play a direct role in the development of BPH. A search for the probable, specific and direct role played by estrogen has revealed that estradiol-17 β administered together with DHT to previously castrated dogs can produce prostatic hyperplasia, that treatment of castrated dogs with estradiol-17 β produces 200% increase in cytosolic androgen-binding sites (Moore et al., 1979), and aromatase activity is higher in the periurethral and transition zones of human prostates with BPH than in the same region of normal human prostates.

The role of estrogens in the development of BPH in humans is, however, considered controversial because the dog model is different from human BPH, and there are reports that do not support a direct involvement of estrogens in the development of BPH. For instance, West et al. (1988) and Habenicht et al. (1987), noted that the administration of estrogen or aromatizable substrates to monkeys does not increase prostatic weight. Also, Levine et al. (1991), did not obtain any evidence of a direct stimulatory effect of estrogens on prostatic stroma or acinar cells in medically castrated men with established BPH. Instead, they noted that estrogens only induce squamous metaplasia in prostatic basal cells in the absence of androgens. Furthermore, Levine et al. (1992), observed that whereas Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) and DHT stimulate cultured

human prostatic fibroblast proliferation, estradiol-17 β has no such effect.

In spite of the controversy surrounding the direct involvement of estrogen in the development of BPH, there is evidence that the aromatase inhibitor, atamestane, produces some benefit in the treatment of human BPH by reducing prostatic volume significantly in patients receiving the drug (Tunn *et al.*, 1985). This suggests, at least, an indirect contribution by estrogens towards the pathogenesis of BPH.

The androgen (in particular DHT) accumulation hypothesis has also been in existence for sometime. It may have its origin in the work of Morfin *et al.* (1978) who suggested that "prostatic hyperplasia might only be due to 5 α -dihydrotestosterone, DHT, accumulation in the prostate". This followed earlier observations by Siiteri and Wilson (1970) that there is accumulation of DHT in human hyperplastic prostate, and by Robel *et al.* (1971), that DHT triggers epithelial cell growth and hyperplasia in rat prostate tissue cultures. However, it was realised later that there might have been an error in assigning values for DHT in normal prostate by the earlier workers. The hypothesis is thus no longer favoured (Walsh *et al.* 1983). There is, however, evidence that lowering prostate DHT levels results in

glandular atrophy and decrease in prostate size (McConnell, 1990).

The discovery of several growth factors as well as their receptors in various compartments of the prostate has provided support to the suggestion that prostatic enlargement might be due to internal secretions of the prostate. Recent evidence also seems to support this idea. Lubrano *et al.*, (1992), have obtained evidence which suggests that androgens might possibly act at the prostatic tissue level through their own receptors by modulating epidermal growth factor (EGF) production and/or secretion. Levine *et al.*, (1992), have also shown that EGF and fibroblast growth factor (FGF) directly stimulate cultured human prostatic fibroblast proliferation and that DHT has a similar but possibly indirect effect. Story *et al.*, (1994), have shown that three FGF's, (FGF-1, FGF-2, and FGF-7) that have been identified in the prostate, are potent mitogens.

Other growth factors that have been detected in prostatic tissue are nerve growth factor (NGF) and transforming growth factor-beta (TGF- β) (Story *et al.*, 1994; Pflug *et al.*, 1992). To account for the involvement of growth factors in the development of BPH, Lawson (1990) speculated that macro injury to the prostatic ducts from voiding, ejaculation or infection causes stromal proliferation as a healing response. In the process, growth factors are released at the site of injury

resulting in the formation of primitive mesenchyme with its inductive effect on epithelial growth. Lawson (1993) has reported that bFGF (FGF-2) occurs in higher concentration in BPH than in normal prostate, and that it is capable of inducing mesenchyme because it does the same in frog embryos.

The growth factor hypothesis is at present only speculative. More research is required to assess its merits as a plausible explanation for the pathogenesis of BPH.

Other hypotheses on the cause of BPH include the embryonic reawakening hypothesis (McNeal, 1983; Cunha *et al.*, 1980) and the Stem Cell hypothesis (Isaacs, 1987).

Though several hypotheses have been proposed, the pathogenesis of BPH is still not clearly understood. The situation is made even more complex by new cytogenetic evidence suggesting the possible involvement of chromosomal aberrations in the condition. Aly *et al.*, (1994) and Casalone *et al.*, (1993) observed that loss of chromosome Y was the most frequent and non-random chromosome abnormality in BPH. This abnormality occurs in prostate fibroblasts but not in the epithelial cells. Other abnormalities that have been found in BPH tissue are trisomy 7 and sporadic rearrangements involving chromosome 1. Presently, it is difficult to indicate which of the factors mentioned above predominates in the development of the

condition. However, the knowledge available now suggests useful ways of managing the condition.

1.2.5 DRUGS USED FOR MANAGING OR TREATING BPH

Acute BPH may be managed by catheterization and by surgical intervention. Drugs have also been used or tested for use in the treatment of BPH. These drugs may be sorted into two groups based on the mode of action. The first group includes those that are α -adrenergic blockers. Their use is based on the fact that there is a dynamic component to bladder outlet obstruction caused by BPH, which is regulated by α_1 -adrenergic receptors located in the prostatic stroma, the predominant one being α_{1c} subtype (Price *et al.*, 1993). Such drugs include terazosin, phenoxybenzamine and prazosin.

The second group includes those drugs that alter the hormonal status of the patient. Four types may be identified namely aromatase inhibitors, 5α -reductase inhibitors, antiandrogens and GnRH agonists. 5α -Reductase inhibitors block the conversion of testosterone to 5α -dihydrotestosterone, the most potent androgen in the prostate. They thereby deprive the prostate of DHT, which, as indicated above, is required for the development of BPH. Finasteride also referred to as Proscar (Figure 4) is a 5α -reductase inhibitor and has been approved for use in the treatment of BPH. It inhibits only one

of the two known isozymes of 5α -reductase (McConnell, 1995). Its drawbacks are that it may cause gynaecomastia and nodulation in the breast (Marandola *et al.*, 1997) and there is a risk of sexual dysfunction associated with its use (Gormley *et al.*, 1992). Others are 4MA (17β -N,N-diethylcarbamoyl-4-methyl-4-aza- 5α -androstane-3-one) (Mellin *et al.*, 1993) and 21,21-pentamethylene-4-aza- 5α -pregn-1-ene-3,20-dione (Figure 4).

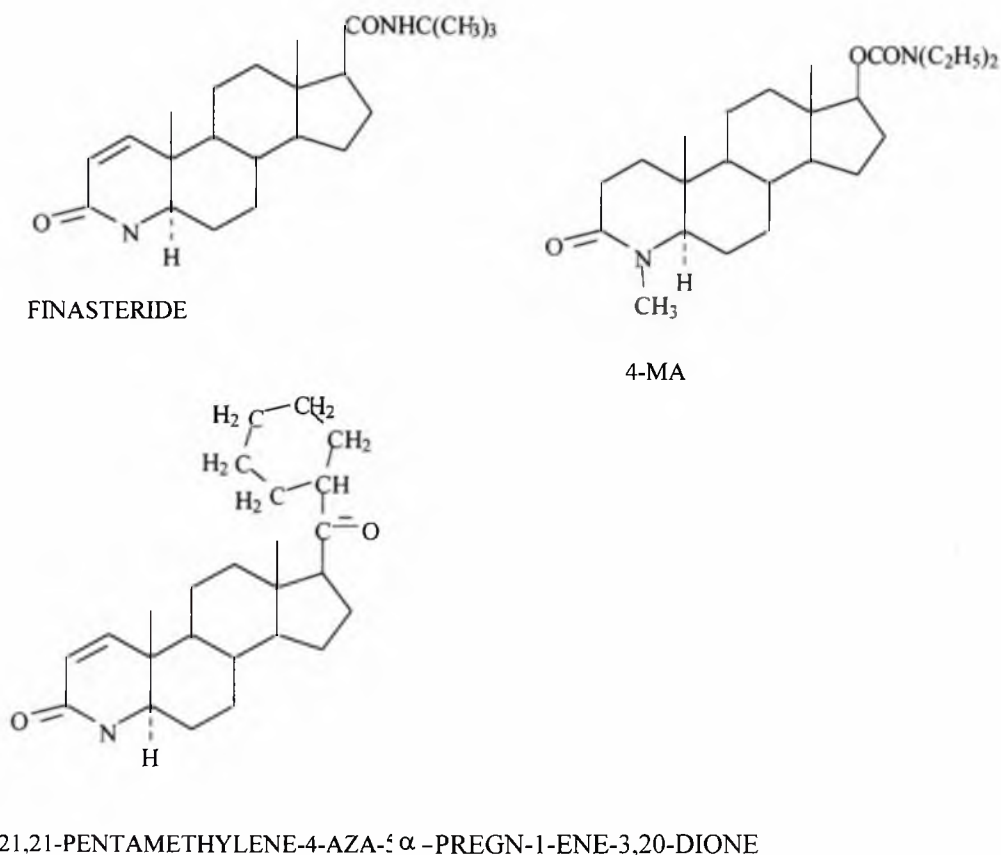


Figure 4 : Some 5α -reductase inhibitors.

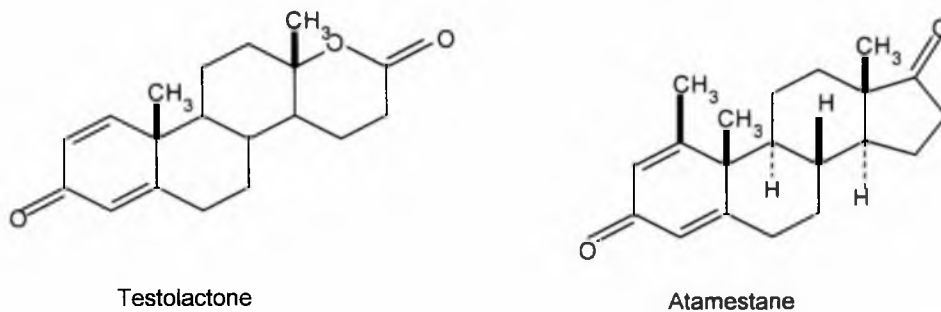
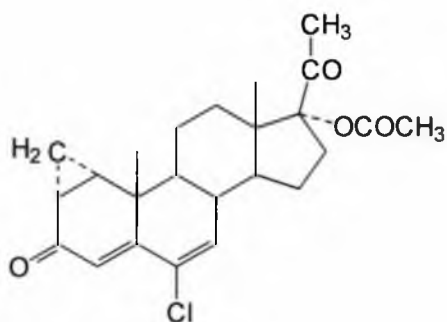


Figure 5 Some aromatase inhibitors.

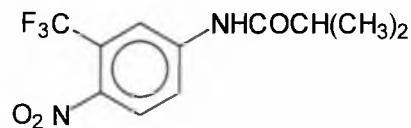
Aromatase complex converts circulating testosterone to 17β -estradiol, and androstenedione to estrone. Compounds that inhibit this enzyme complex produce significant reduction in prostate size but minimal improvement of peak urinary flow and reduction of residual urine volume. By inhibiting the enzyme, these compounds deprive the system of estrogens which, as indicated above, might be involved in the development of BPH. Such compounds include atamestane and testolactone (Figure 5).

The antiandrogens are those compounds that compete with the androgens for binding to the androgen receptors. In this way

they prevent biosynthesis of the mRNA of various growth factors (EGF and FGF's) required for the development of the prostate. The two best-known antiandrogens are cyproterone acetate and flutamide (Figure 6). The antiandrogens have serious side effects. They produce impotence and loss of libido as well as gastrointestinal disturbances. Their use may also result in gynaecomastia. For this reason, they have not been approved yet



Cyproterone acetate



Flutamide

Figure 6: Some antiandrogens

for use in treatment of BPH. Cyproterone acetate is, however, used to treat virilism in females. It has been found that when used in combination with 5 α -reductase inhibitors or aromatase inhibitors, antiandrogens produce greater decreases in prostatic volume than when used alone (Brinker, 1994; Martel et al., 1993).

Gonadotrophin releasing hormone (GnRH) acts on the pituitary to stimulate the secretion of luteinizing hormone (LH) which then induces the synthesis and secretion of steroid hormones in the testes. Several synthetic analogues of GnRH have been produced. Some of these analogues act as agonists of the GnRH receptor. Paradoxically, supra physiologic doses of these agonists tend to desensitise the receptor and consequently lead to a drastic fall in serum testosterone concentration, a form of medical castration. Some of these GnRH agonists have, therefore, been investigated for use in the treatment of BPH. They include buserelin (Bosch *et al.*, 1989), leuprolide (Levine *et al.*, 1991), and nafarelin acetate (Peters and Walsh, 1987). Although their use results in a decrease of about 30% of prostatic volume, these agonists have serious drawbacks. They are not only expensive but also produce hot flushes, hypogonadism with concomitant loss of libido and impotence, gynaecomastia and headaches.

1.2.6 HERBAL TREATMENT

Owing to the inconvenience of permanently wearing a catheter, the serious side effects of the various forms of chemotherapy available, and the non-desirability of surgical intervention, herbal treatment is considered a viable option by many people in some parts of the world for the treatment or management of BPH, using the following plants.

a *Serenoa repens*: Hexane extract of the berries of *Serenoa repens* (synonyms: *Sabal serrulata*; *Serenoa serrulata*) known commercially as Permixon, is reported to be effective in relieving urinary symptoms associated with BPH (Olle-Carellas, 1987; Chirillo-Marucco, 1983). Several studies have been carried out in an attempt to elucidate the mode of action of the extract. The extract inhibits the binding of the synthetic androgen, methyltrienolone, to its cytosolic receptors in rat prostate, (Carilla, 1984; Briley, 1983) and to androgen receptors in human foreskin fibroblasts (Sultan, 1984). It also inhibits the binding of DHT and testosterone to androgen receptors in human tissues obtained from myometrium, vaginal skin, abdominal skin and prepuce (Magdy El-Sheikh, 1988).

In a placebo controlled study of a number of BPH patients, nuclear estrogen, progesterone and androgen receptors were found to be significantly fewer in those who received *S. repens* extract than in the control group (Di-Silverio, 1992) suggesting that the extract may have antiestrogenic effect as well. More recently, Delos *et al.* (1994) indicated that the n-hexane lipid/sterol extract of the plant produces non-competitive inhibitory effect on the type 1 isozyme of 5 α -reductase of human DU 145 cells. (The DU 145-cell line was derived from a cerebral metastasis of human prostatic

epithelial cancer). This suggests that the extract might exert a 'regulatory inhibitory activity'.

It is believed that the activity of *S. repens* is in its steroid and/or fatty alcohol fractions. Rats given 50mg of the fatty alcohol fraction of the lipophilic extract produced greater decrease in the weight of both the ventral prostate and seminal vesicles than those of rats given 400mg of the total lipophilic extract (Jommi, 1989).

Docosanol and other fatty alcohols with more than 16 carbon atoms are reported to inhibit prostate and seminal vesicle enlargement (Debat, 1980). Docosanol is known to be present in *S. repens* extracts. In addition, the extract contains several phytosterols including β -sitosterol, stigmasterol and campesterol. The mode of action of *S. repens* is not well understood and is still the subject of scientific investigations.

b *Pygeum africanum*: Another plant used in the treatment of BPH is *Pygeum africanum* (syn: *Prunus africana*) Rosaceae. The commercial preparation is usually a chloroform extract of the bark and has the name 'Tadenan'. Constituents of this extract that have been identified include β -sitosterol, sitostenone, ursolic acid and n-docosyl trans-ferulate. The extract

contains fatty alcohols especially docosanol and up to 62% fatty acids.

P. africanum extract has little or no antiandrogenic effect (Briley, 1983) and exerts only a weak inhibitory effect on 5 α -reductase activity (Rhodes et al., 1993). It does not significantly affect serum levels of testosterone, LH, FSH, prolactin or estradiol (Carani, 1991) but enhances secretory activity of prostate and seminal vesicles in intact and castrated rats and improves prostatic acid phosphatase activity in men deficient in this function (Clavert et al., 1986). Recent evidence indicates that *P. africanum* extract inhibits the production of 5-lipoxygenase metabolites such as leukotrienes in human polymorphonuclear cells stimulated by calcium ionophore A23187 (Bombardelli and Morazzoni, 1997).

Clinical trials indicate that *P. africanum* extract provides symptomatic relief in urgency and frequency of urination but in most cases, no changes occur in prostate size and residual volume. Double blind placebo-controlled studies indicate a strong placebo effect ranging from 31% in one study to 50% in another (Brinker, 1994).

c *Other Herbs*

Apart from *S. repens* and *P. africanum* the following plants are also used in the treatment of BPH: *Cucurbita pepo* (Pumpkin), *Urtica dioica* and *Urtica urens* (Marandola et al., 1997;

Brinker, 1994). It is interesting to note that all these plants are rich in phytosterols and long chain fatty alcohols. In *C. pepo*, the seeds, which are used in the treatment, also contain abundant supply of tocopherol. As with *S. repens* and *P. africanum*, these produce symptomatic relief in both subjective and objective factors but no definitive mechanism of action may be provided owing to the very complex nature of the disease itself which can hardly be said to have been completely elucidated.

1.2.7 *CROTON MEMBRANACEUS*

Croton membranaceus grows wildly in West Africa where it seems to prefer areas close to the big rivers. In Nigeria, it is found near Wuru in the region of the confluence of the Niger and the Benue. It is also found near Onitsa in the south. In Ghana, it is found mainly in the Krobo-Gyakiti area near the Volta where the locals refer to it as Bokum.

The plant grows to a height of between 1m and 2m above ground. Its branches are slender and stellate-pubescent. The leaves are ovate and acutely acuminate. They grow to a size of between 2cm and 8cm long and 1cm to about 5cm broad. The slender, stellate-pubescent petioles may attain a length of 7cm and generally have a reddish-brown tinge. The leaves have entire margins and are covered with stellate hairs on both

surfaces. *C. membranaceus* bears only a few monoecious flowers on 5-6cm long racemes. Male flowers are borne on the upper part while female flowers occur at the lower part of the raceme. The flowers are very small and petals may be rudimentary or completely absent. The fruit is an ellipsoid capsule. *C. membranaceus* bears a characteristic pleasant odour in all parts including the roots. This fragrance may be useful for purposes of identifying the plant in the event of doubt. Workers at the CSRPM have managed, with great difficulty, to grow a few plants at the Centre's arboretum near Ayikuma. It has not been possible to cultivate the plant on a large scale because it appears most of the seeds formed are sterile.

Phytochemical screening of *C. membranaceus* at the CSRPM indicates the presence of alkaloids, terpenoids, fixed oil and polyuronides in the roots. Two of the alkaloids have been isolated and provisionally designated CM-1 and CM-3 pending structural elucidation and identification (see appendix B).

C. membranaceus extract has been used successfully to treat a number of BPH patients. In one case, a patient aged about 84, who had been wearing a catheter for about six months, was given an alcoholic extract of *C. membranaceus*, three dessertspoonfuls thrice daily. Three weeks after commencement of treatment, the catheter dropped off one night. The patient

lived for another two years, without wearing the catheter yet passing urine freely and painlessly, before dying aged about 86. Several BPH patients have been successfully treated with the extract prepared at the Centre (Daniel, 1997). For those patients, 500g of powdered root are macerated with 2.5L of 96% ethanol for seven days. The filtered extract is then diluted tenfold with water. A patient takes 30ml of this solution thrice daily.

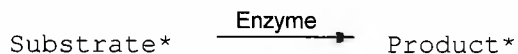
Though *C. membranaceus* has been used successfully to treat many patients, no scientific research has been conducted into its mode of action. The aim of this investigation is, therefore, to find out how the plant extract works by finding out first whether it inhibits the enzyme, 5 α -reductase, which is required to transform testosterone to the more potent androgen, DHT, which has been implicated in the development of BPH. In addition to the crude extract, which was evaluated both *in vivo* and *in vitro*, the isolated alkaloids, CM-1 and CM-3 were also evaluated in an *in vitro* assay of the enzyme.

1.3 THEORETICAL REVIEW OF METHODOLOGY

1.3.1 RADIOISOTOPES IN ENZYME ASSAYS

Radioisotopes may be incorporated into compounds as labels. Such labelled compounds may then be used as tools for research. They may be used for enzyme assays. Enzymes may be

assayed using radioisotope-labelled compounds as substrates. A labelled (*) substrate is converted into a labelled product as shown below.



Provided that the product and the substrate can be separated easily, it is possible to measure the rate of formation of the labelled product as an estimate of the enzyme activity. For example Mellin *et al.* (1993) assayed for 5 α -reductase activity by incubating ³H-labelled testosterone with tissue homogenate and separated the product DHT from the substrate by thin layer chromatography. Normington and Russell (1992) and Thigpen *et al.* (1993b) also assayed for 5 α -reductase activity using ¹⁴C-testosterone as the substrate and TLC as the method of separation. Gas liquid chromatography has also been used for separation of testosterone and DHT in 5 α -reductase assay (Bruchovsky and Wilson, 1968).

Particles and radiation emitted by radioisotope-labelled compounds can be counted and the counts used as a measure of substrate concentration. Gamma rays emitted by ¹²⁵I can be counted with a gamma counter while β -particles produced by ³H- and ¹⁴C-labelled compounds can be counted in a liquid scintillation counter. After separation and location with a

suitable reagent, the desired spot can be scrapped into a vial and prepared for counting.

Gamma counting can be done directly. Liquid scintillation counting, however, requires the use of scintillation cocktail. A typical scintillation cocktail is composed of primary and secondary fluors dissolved in a suitable solvent usually toluene or dioxane. Energy from the emitted particles excites solvent molecules, which therefore fluoresce usually at very short wavelength. Detection of such wavelength is not efficient

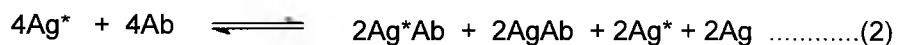
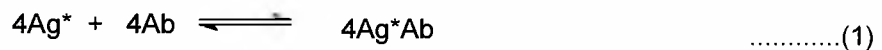
(Goulding, 1986). To improve the efficiency of detection, a primary fluor is dissolved in the solvent. The primary fluor molecules absorb the radiation from the solvent and fluoresce at longer wavelength. The efficiency of detection can be improved further by the addition of a secondary fluor. The secondary fluor shifts the fluorescence towards the longer wavelength region. The primary fluor, 2,5-diphenyloxazole (PPO), and the secondary fluor, 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP) are the most commonly used.

1.3.2 RADIOIMMUNOASSAY

Radioimmunoassay (RIA) originally applied to the measurement of insulin in plasma by Yalow and Berson (1960) provides one of the most important techniques for the determination of biologically important molecules and their metabolites.

Radioimmunoassay combines the specificity of the antigen-antibody reaction and the sensitivity of radioisotope techniques.

In theory, radioimmunoassay is based on competition between a radioisotope-labelled antigen or hapten and unlabelled antigen or hapten for a limited number of antibody binding sites in a fixed amount of antiserum. At equilibrium in excess antigen there will be both free and bound antigen. Under standard conditions the amount of labelled antigen bound to antibody will decrease as the amount of unlabelled antigen increases as illustrated in the chemical equations below.



Ab = Antibody; Ag* = Free labelled antigen;

Ag = Free unlabelled antigen, Ag*Ab = Bound labelled antigen and AgAb = Bound unlabelled antigen.

In equation 1 above, there is no unlabelled antigen so only labelled antigen binds to the antibody present. In equation 2, labelled and unlabelled antigen are present in equal amounts therefore half of the antigen-antibody complex formed is labelled. In equation 3, three quarters of the antigen

present is unlabelled therefore three quarters of the antibody binding sites are taken up by unlabelled antigen; only a quarter of the antigen-antibody complex contains labelled antigen.

In the RIA technique, the objective is to determine how much of the radioactive antigen is bound to antibody and how much is free. In order to do this, separation of bound and free, labelled antigen is necessary. Several different techniques to achieve separation have been developed and have led to several different versions of RIA. In the charcoal technique, dextran-coated charcoal is used to achieve separation while in the double antibody method, a second antibody is used to precipitate the antigen-antibody complex. Separation is much simplified in the coated-tube technique by immobilising the antibody on the inner surface of the test tube. In this way bound antigen remains attached to the test tube wall and separation is achieved by simply pouring off the supernatant fluid.

In all the various forms of RIA, a calibration curve is required. By using known amounts of unlabelled antigen and fixed amounts of antibody and labelled antigen, the amount of labelled antigen bound as a function of the total antigen added is measured and plotted as the calibration curve.

Radioimmunoassay may be used to determine the levels of small molecules such as DHT that are not immunogenic by conjugating them to macromolecules such as bovine serum albumin and raising antibodies to the complex. Levine *et al.* (1991) used RIA to determine serum DHT after selective inactivation of testosterone with KMnO_4 . Similarly, Puri *et al.* (1981) measured DHT levels in rhesus monkeys while Southan *et al.* (1992) determined DHT and other steroids in urine by radioimmunoassay techniques.

1.3.3 ADVANTAGES AND DISADVANTAGES OF RADIOISOTOPE TECHNIQUES

The main advantage of the radioisotope-based techniques described above lies in the high degree of sensitivity. It is possible using these methods to measure the low concentrations of metabolites present in biological materials. Often such metabolites may have concentrations of only a few nanograms per millilitre or less and cannot be measured by conventional chemical methods.

The most important disadvantage of the radioisotope techniques concerns the serious health hazards associated with their use. Ionisation due to radiation from radioisotopes can cause damage to DNA and lead to cancer if ingested or otherwise absorbed into the body.

The short lifespan of some radioisotopes limits their usefulness. ^{125}I has a half life of only 60 days. This means it cannot be stored for a longer period even if there is need to do so. Also the technology required for radioisotope work is expensive and may therefore not be within the reach of poor nations. Finally, owing to the health hazards associated with their use disposal of radioisotope waste creates serious environmental, social and political problems.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 REAGENTS

Radioimmunoassay kit using ^{125}I -labelled DHT was obtained from Diagnostic Laboratories Incorporated, USA.

Diethyl ether, n-hexane (HPLC grade), petroleum ether (boiling range, 50–70°C), and butanone were obtained from Fluka AG, CH.9470, Buchs.

Chloroform, calcium chloride, and magnesium chloride hexahydrate were obtained from Merck Laboratories Ltd.

Tris-HCl, β -mercaptoethanol, toluene, PPO and POPOP were obtained from BDH Chemical Ltd., Poole, England.

^{14}C -Testosterone was obtained from Amersham International.

Testosterone and 5 α -Dihydrotestosterone were obtained from Sigma Chemical Company, St. Louis, MO. USA.

2.1.2 *CROTON MEMBRANACEUS*

C. membranaceus was collected by staff of the production unit of the CSRPM from the wild. The identity was checked and confirmed by staff of the herbarium section of the Centre.

2.1.3. LABORATORY ANIMALS.

Adult male Wistar rats were either purchased from Ghana Medical School or obtained from the Centre's own animal house. Twenty rats were sorted into four groups A, B, C and D, of five animals each for use in the *in vivo* experiment. (Rats in each group were identified by marking them with picric acid solution at specific points on their bodies). All rats were kept under ordinary laboratory conditions and provided with feed and water *ad libitum*. In addition, rats in each group were given the daily dose of the corresponding *C. membranaceus* extract as indicated in the method section.

2.2 METHODS

2.2.1. *IN VIVO* EXPERIMENT:

a. Preparation of crude extract:

Roots of freshly harvested *C. membranaceus* were washed with water and air-dried for three days. The dry roots were cut into pieces and coarsely pulverised. One kilogram of the root powder was extracted for twenty-four hours with 96% ethanol in a soxhlet extractor. The alcoholic extract was concentrated

under reduced pressure to 200ml. This was labelled as stock solution.

The usual concentration of *C. membranaceus* extract administered to patients at the CSRPM was administered to the experimental rats. To prepare a solution with this concentration, 1ml of the stock solution was added to 24ml of 96% ethanol and then diluted tenfold with water to obtain 250ml of solution. This was regarded as the "normal" (1X) solution administered to the rats. Two other solutions, one 0.1 times (0.1X) and the other 10 times (10X) the "normal" solution were prepared using the appropriate volumes of the stock solution and 96% ethanol. Rats in the control group received a tenfold-diluted 96% ethanol, equivalent to vehicle.

b. Oral administration of extract

The volume of extract administered to the rats was based on the daily dose of 90ml of "normal" extract taken by human patients. Assuming an average weight of 70kg for man, the equivalent volume for each rat of 300g was approximately 0.39ml. Each rat was therefore given 0.4ml of appropriate solution daily according to the group it belonged to, i.e. Group A, control group, vehicle only; group B, 0.1X normal; group C, "normal"; group D, 10X normal.

c. Collection of blood samples

The tails of the rats were cut, about 2mm from the tips, and approximately 1.5ml of blood was withdrawn from the cut end by gently stroking the tails and collecting the blood into labelled Eppendorf tubes. Blood samples were centrifuged in a Beckman microfuge E. About 500 μ l of serum were removed carefully with a micropipette, using a fresh pipette tip for each sample, and placed in a new, labelled Eppendorf tube. Sera were stored at -20°C prior to use. Sera were obtained in this way fortnightly, for a period of 14 weeks beginning on the first day of the experiment prior to administration of *C. membranaceus* extract.

d. Determination of DHT by radioimmunoassay

Concentration of DHT was determined according to instructions on the kit as follows:

Four hundred microlitres of a serum sample were transferred into clean glass test tubes. Five hundred microlitres of oxidation solution provided in the kit were added and mixed thoroughly. After 15min incubation at room temperature, the oxidised sample was extracted with 4ml of n-hexane:ethanol (49:1) solution. Fifty microlitres of DHT sample buffer supplied by the manufacturer were added and mixed well. The tubes were centrifuged at 1500x g in a "Tomy CX-250"

centrifuge at 4°C. Two and half millilitres of the upper organic phase were transferred into a clean, labelled test tube and evaporated to dryness. The residue was reconstituted in 250µl of DHT sample diluent supplied with the kit.

Radioimmunoassay was conducted in accordance with the recommended protocol of the kit manufacturer. Two clean, plain polypropylene tubes (12 X 75mm) were labelled for total counts and arranged together with anti-DHT-coated tubes on test-tube racks. The first sixteen of the coated tubes were appropriately labelled in duplicates for standard DHT solutions containing 0, 25, 50, 125, 250, 600, 1200, and 2500pg/ml of DHT. One hundred microlitres of each of the above standard solutions were added to the bottom of appropriately labelled anti-DHT-coated tubes. Similarly, 100µl of each of the reconstituted rat serum extracts were added to the bottoms of appropriately labelled tubes in duplicates. Five hundred microlitres of [¹²⁵I]DHT were added to all tubes, including the total count tubes. After vortexing, all tubes, except the total count tubes, were arranged in a shaker water bath. The total count tubes were placed in a separate 100-ml beaker and placed in the shaker water bath.

All tubes were incubated, with shaking, for two hours at room temperature. After the period of incubation, the bound DHT was separated from the unbound by decanting the supernatant

fraction onto a thick wad of tissue paper. To avoid spilling, this was done by removing the base of a pipette-tip rack, inverting the hollow rack over the wad of tissue paper placed at the mouth of the beaker in which the samples are arranged and turning the whole arrangement upside down. A funnel placed beneath the rack was used to direct excess fluid into a labelled receptacle.

All samples were decanted except the total count tubes. The tubes were washed by adding 3ml of deionized water to all tubes, except the total count tubes, and decanting as described above. All tubes were allowed to drain onto fresh absorbent paper for one hour after which the mouths were wiped dry with tissue paper before returning them to the upright position. All tubes were counted in a gamma counter at the Chemistry Department of the National Nuclear Research Institute of the Ghana Atomic Energy Commission.

Rat serum DHT levels in response to administration of *C. membranaceus* extract were estimated with reference to a standard curve prepared using standard DHT solutions supplied with the radioimmunoassay kit as follows: %B/T for each standard DHT solution was calculated according to the formula $\%B/T = 100C_s / C_t$ where C_s = Mean sample counts per minute and C_t = Mean total counts per minute.

%B/T values were plotted against DHT concentrations in pg/ml on semilog graph paper. %B/T for the rat serum samples were also calculated and, from the values obtained, the corresponding DHT concentration in pg/ml were read from the graph.

2.2.2 *IN VITRO* EXPERIMENT:

a. **Demonstration of 5 α -reductase activity**

i **Isolation of nuclear fraction:**

The nuclear fraction isolated from prostates of adult male rats as described below, was used as a source of 5 α -reductase enzyme. Sixteen adult male rats, mean weight 215 ± 17 g, were castrated a day before they were killed by cervical dislocation. The prostate glands were removed and rinsed in ice-cold 0.2M sucrose solution. After removing the prostatic capsule, the tissue was chopped up with a pair of scissors to a pulpy mass on a clean glass plate. The pulpy mass was homogenised in 0.88M sucrose solution containing 1.5mM CaCl₂ in a clean hand-operated, glass homogeniser using a teflon pestle. To avoid excessive heating, the homogenizer was immersed in an ice-water bath at short intervals during the operation.

The homogenate was filtered through two layers of gauze and centrifuged at 100x g (1450 rpm, using the 87.2mm radius,

fixed angle rotor of the Denley-401 refrigerated centrifuge) at 4°C. The pellet was discarded and the supernatant fraction was centrifuged at 900x g (3050 rpm) to obtain the crude nuclear fraction as a pellet. This pellet was redispersed in 0.88M sucrose solution and centrifuged at 900x g. The pellet was dispersed in 7.0ml of 0.01M Tris buffer, pH 7.4, containing EDTA, $5.0 \times 10^{-5}M$; $MgCl_2$ $5.0 \times 10^{-3}M$; β -mercaptoethanol, $5.0 \times 10^{-4}M$ and NaCl, 0.05M. This nuclear fraction was kept in an ice-cold water bath prior to use.

ii. Enzyme assay

Seven test tubes, 15 x 150mm, were labelled as follows: substrate blank, enzyme blank, 5min, 10min, 20min, 30min, and 40min. Ten microlitres of ^{14}C -testosterone ($50\mu Ci/ml$) were measured into all the tubes except the one labelled substrate blank, and allowed to evaporate. One hundred microlitres of 96% ethanol and 1400 μl of tris buffer, pH 7.4, were added to all tubes except the enzyme blank tube. In addition, 500 μl of NADPH solution was added to all tubes. After vortexing briefly, all test tubes, as well as the nuclear fraction prepared as above, were placed in a shaker water-bath and preincubated for 10min at 37°C with gentle shaking. Five hundred microlitres of the nuclear fraction were added to all tubes except the enzyme blank tube, which received additional 500 μl of the buffer.

Timing from the moment of addition of nuclear fraction to the last test tube labelled 40min, the reactions were terminated after 5, 10, 20, 30 and 40min respectively, by the addition of 3ml of diethyl ether and vortexing. The reactions in the blank tubes were also stopped after 40min. The organic phase was separated from the aqueous phase by freezing and pouring the organic phase into appropriately labelled test tubes (15 X 75mm). The extraction was repeated with another 3ml of ether. The combined ether extract was left to evaporate.

In an earlier trial of the experiment, the reactions were carried out in duplicates and an additional pair of tubes for 50min incubation was included.

iii. Quantitative estimation of DHT formed

The residue obtained after the evaporation of the ether extract was redissolved in 200 μ l of a solution containing 1.5mg/ml DHT and 1.5mg/ml testosterone in chloroform and vortexed for 10s. Ten microlitres of the mixture as well as authentic testosterone and DHT were spotted on silica gel TLC plates and developed in petroleum ether/chloroform/butanone (17:1:2), the solvent front being allowed to travel 15cm from the origin. The spots were revealed by placing the plates in iodine vapour. Spots having the same R_f as authentic DHT were

marked out and the plate was left in the hood to allow the iodine to evaporate completely.

The DHT spots were then scrapped carefully into labelled scintillation vials. Fifteen millilitres of scintillation cocktail (Tpp) were added to the scrapings in the scintillation vials. Three one-minute counts were performed for each sample in a Packard liquid scintillation counter.

b. Effect of *C. membranaceus* extracts on 5 α -reductase activity

i Preparation of *C. membranaceus* extracts for incubation:

Two trials were carried out to determine the effect of the crude extract and the two isolated alkaloids on the enzyme. Twenty-five millilitres of *C. membranaceus* crude extract stock solution (see page 35) were evaporated under reduced pressure to obtain a semisolid mass. The crystalline alkaloids, CM-1 and CM-3, were prepared as described in appendix B.

For the initial trial, 0.1414g of the semisolid mass was dissolved in 10ml of 96% ethanol. One millilitre, of the resulting solution was diluted to 10ml to obtain 1.414mg/ml solution. Hundred and two hundred microlitres of this solution were used for incubation with the enzyme preparation. Similarly, 100 and 200 μ l of CM-1 solution (0.081mg/ml), and

100 and 200 μ l of CM-3 solution (0.20mg/ml) were used for incubation with the enzyme preparation.

For the second trial, 90mg of the semisolid material was dissolved in 9.0ml of 96% ethanol to obtain 10mg/ml crude extract solution. Fifty milligrams of crystalline CM-1 were dissolved in 10ml of 96% ethanol to obtain 5mg/ml CM-1 solution. Owing to non-availability of sufficient CM-3, that alkaloid was not used in the second trial.

ii Enzyme assay

Thirty-two rat prostates were used to prepare the nuclear fraction generally as described above. Following an accident resulting in the breakage of the tissue homogeniser, part of the homogenisation was done using mortar and pestle. The crude nuclear pellet was suspended in 20ml of buffer. Ten microlitres of crude extract solution (10mg/ml) were measured into tubes numbered 6-10, 100 μ l into tubes 11-15 and 1000 μ l into tubes 16-20. Similarly, 10ml of CM-1 solution (5mg/ml) were measured into tubes numbered 21-25, 100 μ l into tubes 26-30 and 1000 μ l into tubes 31-35. All the solutions were evaporated to dryness. Twenty microlitres of 14 C-testosterone solution in chloroform (approximately 7000cpm) were measured into these tubes and additional seven tubes numbered 1-5, 36 and 37 and allowed to evaporate to dryness. To dissolve the

residues in buffer, 100ml of 96% ethanol was added to all tubes followed by 1400ml of tris buffer pH 7.4.

Extra 500 μ l of tris buffer was added to tubes 36 and 37 (the enzyme blank tubes). Tubes 1-5 had no extract. Five hundred microlitres of NADPH solution were added to all tubes. After vortexing, all tubes were preincubated in a shaker water bath for ten minutes at 37°C together with the nuclear fraction suspension. Five hundred microlitres of nuclear fraction suspension were added to all tubes except the enzyme blank tubes numbered 36 and 37. The total volume in each reaction mixture was 2500 μ l giving 4000 μ g/ml and 2000 μ g/ml as the highest concentration of crude extract and CM-1 used respectively. Incubation was carried out as described previously. The reactions in all tubes were stopped by addition of 3ml of diethyl ether and vortexing briefly. The amount of DHT formed was quantitated as described in section **2.2.2a(iii)** above.

The same procedure was used for an initial trial during which the nuclear fraction was prepared from 25 rat prostates and approximately 250nCi of 3 H-testosterone (giving about 23000cpm) was used for the incubation which lasted 50min. The concentrations used were: crude extract, 56 and 112 μ g/ml; CM-1, 3 and 6 μ g/ml and CM-3, 8 and 16 μ g/ml of total incubation medium.

2.3 STATISTICAL ANALYSIS

In the *in vivo* experiments data was analysed by two-factor analysis of variance. The variation in the observed values of serum DHT was partitioned into two possible factors. These are variation due to the effect of different concentrations of *C. membranaceus* extract and that due the different days on which sera were taken from the rats. The variation due to different concentrations, representing different treatments, are shown as 'columns' under the heading 'source of variation' in the analysis of variance table. That due to the different days of drawing blood are shown as 'rows' under the same heading.

The F values for the data were calculated using the expression $F = \text{Mean sum of squares} / \text{Mean error sum of squares}$. The mean sum of squares for either the columns or rows were obtained by dividing the sum of squares for the columns or rows by the corresponding degree of freedom. To facilitate the calculations involved, the computer application Microsoft Excel was used for the analysis of variance.

Data for the *in vitro* experiments are presented as Mean \pm standard error.

CHAPTER THREE

3.0. RESULTS

3.1 IN VIVO EXPERIMENT: THE EFFECT OF *C. MEMBRANACEUS* ON SERUM DHT LEVELS

3.1.1. THE STANDARD CURVE

The mean counts per minute obtained for the standard DHT solutions are presented in Table 1. The values of %B/T, (percentage of radioactivity bound over total) were obtained by dividing the mean CPM by the mean total counts per minute, (34,563) and multiplying by 100. %B/T was plotted against DHT concentration on semilog graph paper (Figure 7). The concentration of DHT corresponding to the calculated %B/T (i.e. percent of bound radioactivity in the sample divided by mean total count per minute) were read off the standard curve and averaged. The results are presented in Table 2.

3.1.2. RAT SERUM DHT LEVELS.

The raw data for the *in vivo* experiment are presented in Table 2. The results for the two-way analysis of variance are shown in table 3. The F values calculated for the data are compared to the critical value of F at the 95% confidence level. Among the columns of data in table 2, the calculated value for the F statistic is 0.142, which is much lower than the critical value of 3.07 at the 95% confidence level. This indicates that there

are no significant differences between the DHT levels of rats given different concentrations of *C. membranaceus* extracts.

For

Table 1: Count per minute for the standard DHT solution.

DHT conc. in pg/ml	Mean count per min	%B/T
0	15470	44.70
25	15062	43.6
50	11194	32.4
125	9955	28.8
250	7366	21.3
600	4532	13.1
1200	3496	10.1
2500	1798	5.2

Mean total count per minute = 34,563

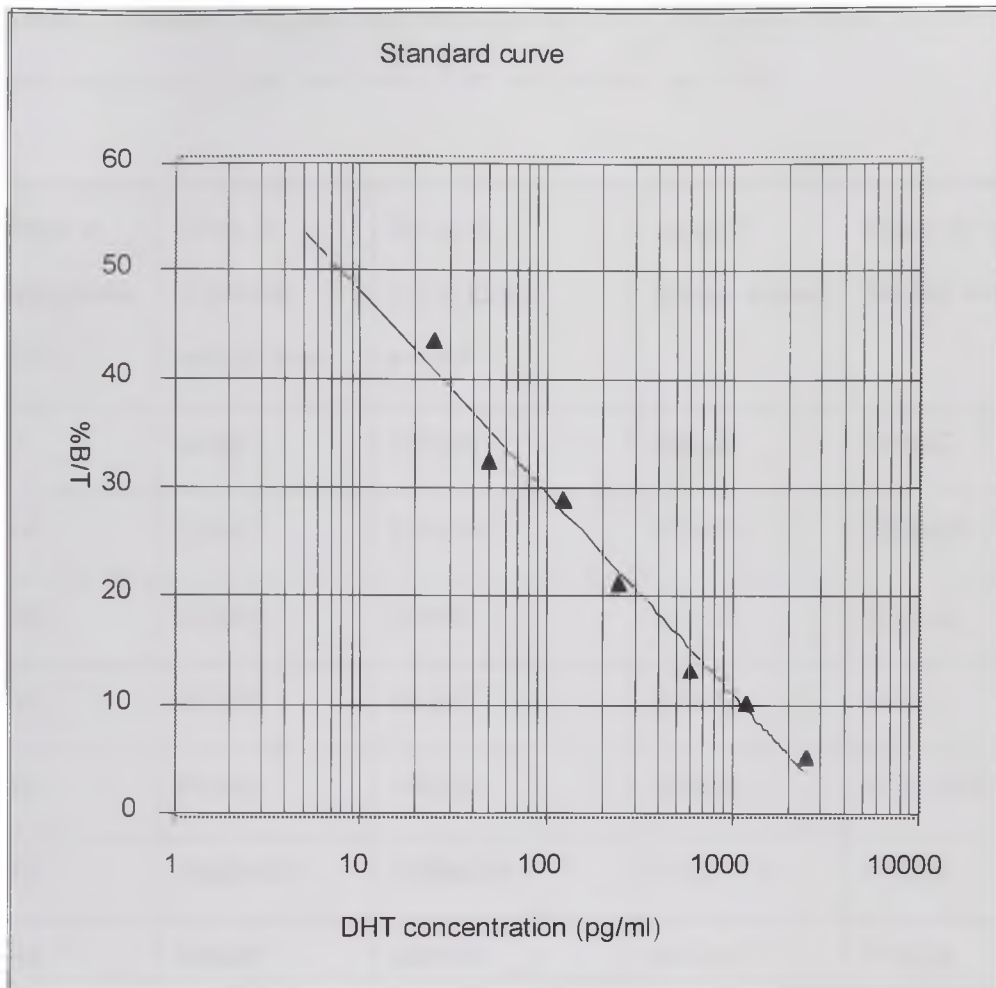


Figure 7: Effect of *C. membranaceus* on serum DHT levels. Standard curve for the estimation of DHT concentration in rat serum.

Table 2: Mean DHT concentration (pg/ml) of rats fed with *C. membranaceus* crude extract for different periods.

Days of administration	Group A: (Controls) vehicle only	Group B: 0.1 x Normal extract	Group C: Normal extract	Group D: 10x Normal extract
0	383±26	332±36	490±57	360±52
14	225±27	530±18	315±19	500±105
28	600±34	500±57	320±33	340±32
42	400±29	450±77	440±16	560±77
56	310±40	180±35	350±29	1130±253
70	1660±155	1100±129	1400±117	510±44
84	420±62	590±72	570±48	770±26
98	810±48	610±93	880±37	780±54

The values presented in Table 2 are the mean DHT level in pg/ml ± standard error of the mean.

Table 3: Results of analysis of variance of the data in table 2

Source of variation	Sum of squares	D. F	MS	F	Fcrit
Rows	1970599.2	7	28154.2	3.920	2.488
Columns	30599.6	3	10199.9	0.142	3.072
Error	1508132.2	21	71815.8		
Total	3509331	31			

Note: D.F. = Degrees of freedom;
 MS = Mean sum of squares;
 F = F statistic calculated for the data in Table 2
 F_{crit} = Critical value of F.

rows of data, since the calculated value of F (3.92) exceeds the critical value (2.49) at that level of confidence, there exist significant differences among the rows of data in table 2. In other words mean DHT levels changed significantly during the period of the experiment. Generally, the results indicate an increase in serum DHT levels during the period of the experiment. This increase is probably not due to the effect of the extract because even the control rats that did not receive any of the extract also showed similar increases. The differences could be due to physiological changes in the ageing rats.

3.2. IN VITRO EXPERIMENTS: THE EFFECTS OF CRUDE EXTRACT, CM-1 AND CM-3 ON 5 α -REDUCTASE ACTIVITY

3.2.1 5 α -REDUCTASE ACTIVITY IN NUCLEAR FRACTION

Table 4 shows the results of the tests for 5 α -reductase activity. The results, presented as counts per minute (cpm) of DHT formed at the times shown in minutes are also presented as line graphs in Figure 8A. The table also shows \approx radioactivity in DHT calculated based on total activity of testosterone added. Figure 8B is a graph of similar results obtained in a different experiment by incubating 14 C-testosterone with nuclear fraction for 40min.

3.2.2. EFFECT OF CRUDE EXTRACT ON 5 α -REDUCTASE ENZYME ACTIVITY:

The data showing the effect of the crude extract on the enzyme activity are shown in Tables 5 and 6. In the first trial of the experiment during which incubation with higher concentration of 14 C-testosterone (23000cpm) and a longer period of 50min were employed, no inhibition of 5 α -reductase activity was observed when 56 and 112 μ g/ml of crude extract were added to the incubation medium, Table 5. In the second trial, however, a comparison of the enzyme activity in the absence and presence of different concentrations of the crude

Table 4: Evidence of 5α -reductase activity: Incorporation of radioactivity into DHT as a function of time.

Time in minutes	CPM of DHT formed		Mean CPM of DHT formed	%Radio-activity in DHT
	A	B		
5	198.33	159.00	178.67	0.78
10	259.67	254.67	257.17	1.12
20	287.33	235.00	261.17	1.12
30	426.00	410.00	418	1.81
40	778.67	819.67	799.17	3.47
50	1068.33	1185.33	1126.83	4.89

Nuclear fraction was incubated with ^{14}C -testosterone (23000cpm), for 50min. A and B are replicates of the experiment.

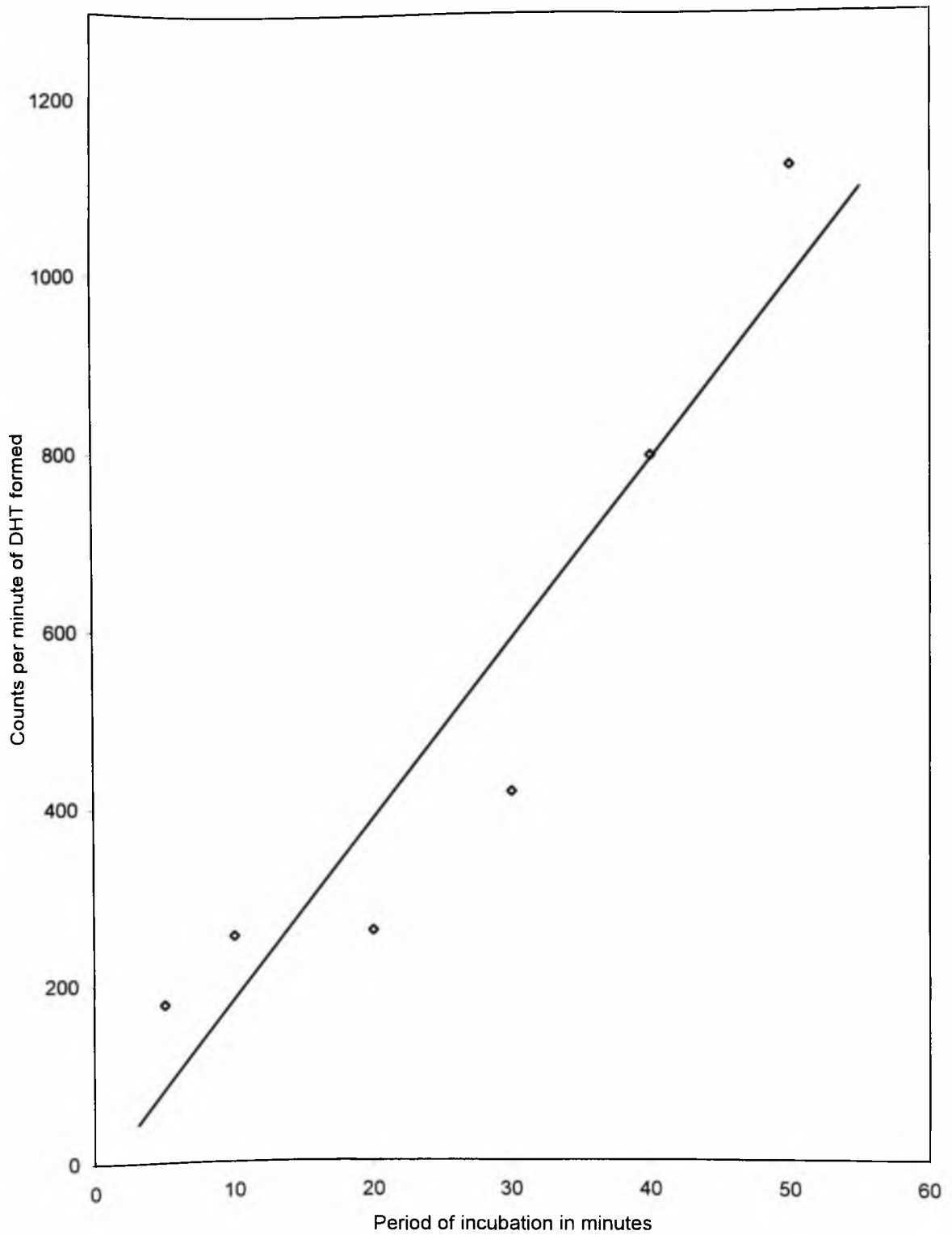


Figure 8A: Test for 5α -Reductase activity.

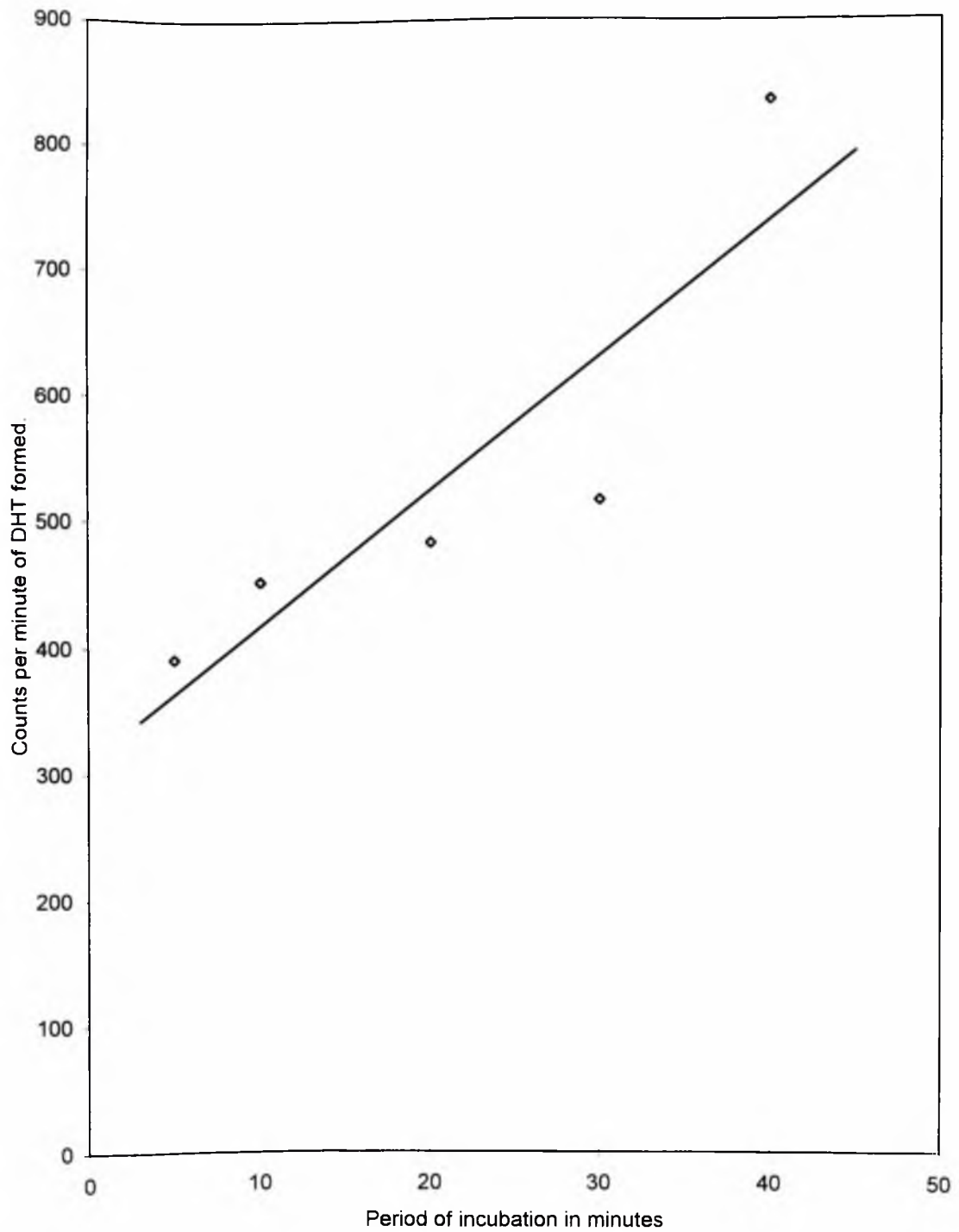


Figure 8B: Test for 5α -Reductase activity using 40min incubation period.

extract clearly indicates that the crude extract inhibited 5 α -reductase activity *in vitro*. The results indicate that the mean counts per minute of DHT formed decreased as the concentration of the crude extract in the incubation medium increased. The drop in the mean counts per minute of DHT formed for the 40 μ g/ml sample was not significant. There are significant differences, however, between the mean counts per minute for the 400 and 4000 μ g/ml samples and the controls. This suggests that the crude extract inhibited enzyme activity at these concentrations.

The inhibition was dose-dependent. This is revealed by the percentage decrease in the count per minute of DHT formed (Figure 9). At 40 μ g/ml, there was only 20% decrease. The decreases were 48% and 54% at 400 and 4000 μ g/ml respectively.

3.2.3 Effect of CM-1 and CM-3 on 5 α -reductase enzyme activity:

Tables 7 and 8 show the results obtained for the effect of CM-1 on 5 α -reductase activity. In table 7, the data indicates that inhibition of 5 α -reductase activity was not observed when 3 or 6 μ g/ml of CM-1 was included in the incubation medium. In table 8, the results indicate that CM-1 produced some inhibitory effect on 5 α -reductase activity in isolated prostatic nuclei.

Table 5: Results showing effect of crude extract on 5α -reductase activity.

SAMPLE	COUNTS PER MINUTE OF DHT FORMED		
	CONTROL	CRUDE EXTRACTS	
		56ug/ml	112ug/ml
1	1296	1271	1221
2	1185	1068	1269
3	953	1245	1115
4	1308	1311	1109
5	1322	1231	1216
Sum	6064	6126	5930
Mean	1213 \pm 69	1225 \pm 42	1186 \pm 32
SD	155	93	71

Nuclear fraction was incubated with 14 C-testosterone (23000cpm) for 50min.

Table 6: Results showing the effect of the crude extract of *C. membranaceus* on 5 α -reductase activity.

SAMPLE NUMBER	COUNTS PER MINUTE OF DHT FORMED			
	CONTROL	CRUDE EXTRACT		
		40 μ g/ml	400 μ g/ml	4000 μ g/ml
1	235	169	106	90
2	170	263	91	85
3	192	150	111	102
4	203	91	153	92
5	222	141	73	102
Sum	1022	814	534	471
Mean	204 \pm 11	163 \pm 28	107 \pm 13	94 \pm 3
S.D.	25	63	30	8
SEM	11	28	13	3
%Decrease	0	20	48	54

Nuclear fraction was incubated with 14 C-testosterone (7000cpm) for 40min.

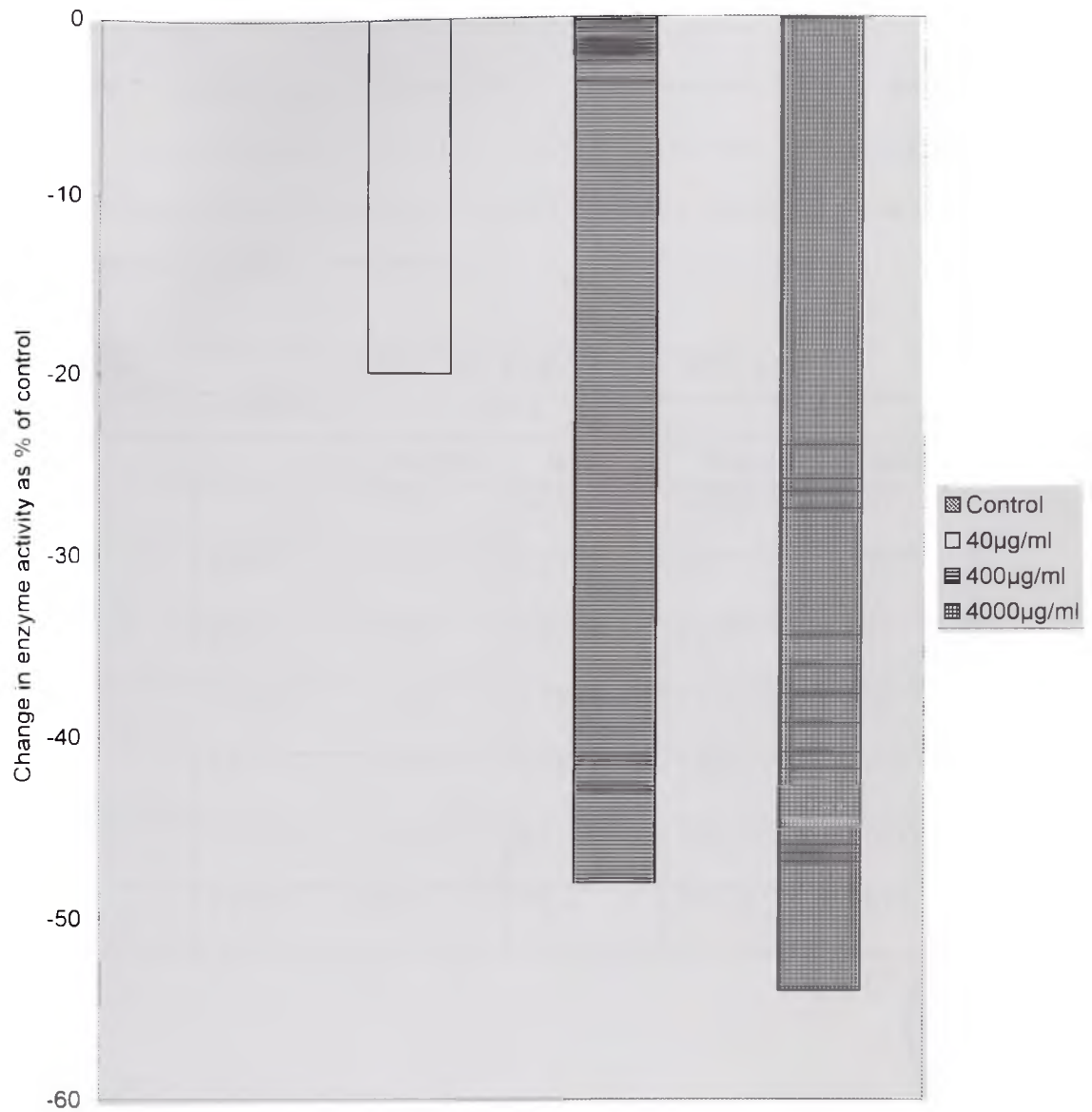


Figure 9: Bar chart showing the amount of inhibition produced by the crude extract on 5 α -Reductase activity.

Table 7: Effect of isolates of *C. membranaceus* (CM-1 and CM-3) on 5α -reductase activity: ^{14}C -testosterone (23000cpm) was incubated with prostatic nuclei in the presence and absence (control) of the isolates.

Sample	COUNTS PER MINUTE OF DHT FORMED				
	CONTROL	CM-1		CM-3	
		3 $\mu\text{g/ml}$	6 $\mu\text{g/ml}$	8 $\mu\text{g/ml}$	16 $\mu\text{g/ml}$
1	1296	1225	1184	1186	1048
2	1185	1257	1337	1157	1076
3	953	1185	1201	1649	1528
4	1308	1081	1210	746	1635
5	1322	1342	1145	1385	690
Mean	1213 \pm 69	1218 \pm 43	1215 \pm 32	1225 \pm 149	1195 \pm 172
SD	155.0	95.7	72.4	332.0	386.0

Percentage inhibitions of the control produced by various concentrations of CM-1 are shown as a bar chart in figure 10. At concentrations of 20 and 200 $\mu\text{g/ml}$ of CM-1, the inhibition, less than 20%, was not significant. A significant reduction

Table 8: Effect of CM-1 (an alkaloid isolated from *C. membranaceus*) on 5α -reductase activity *in vitro*.

Sample Number	COUNTS PER MINUTE OF DHT FORMED			
	CONTROL	CM-1		
		20 μ g/ml	200 μ g/ml	2000 μ g/ml
1	235	115	129.33	94
2	170	231	188	98
3	192	206	159	93
4	203	175	232	143
5	222	151	140	110
Sum	1022	878	848	538
Mean	204 \pm 11	176 \pm 20	170 \pm 19	108 \pm 9
SD	26	45	42	21
%Decrease	0	14.2	16.9	47.5

of enzyme activity was, however produced by 2000 μ g/ml of CM-1. The inhibition of the enzyme was 47.5% of the control value at that concentration.

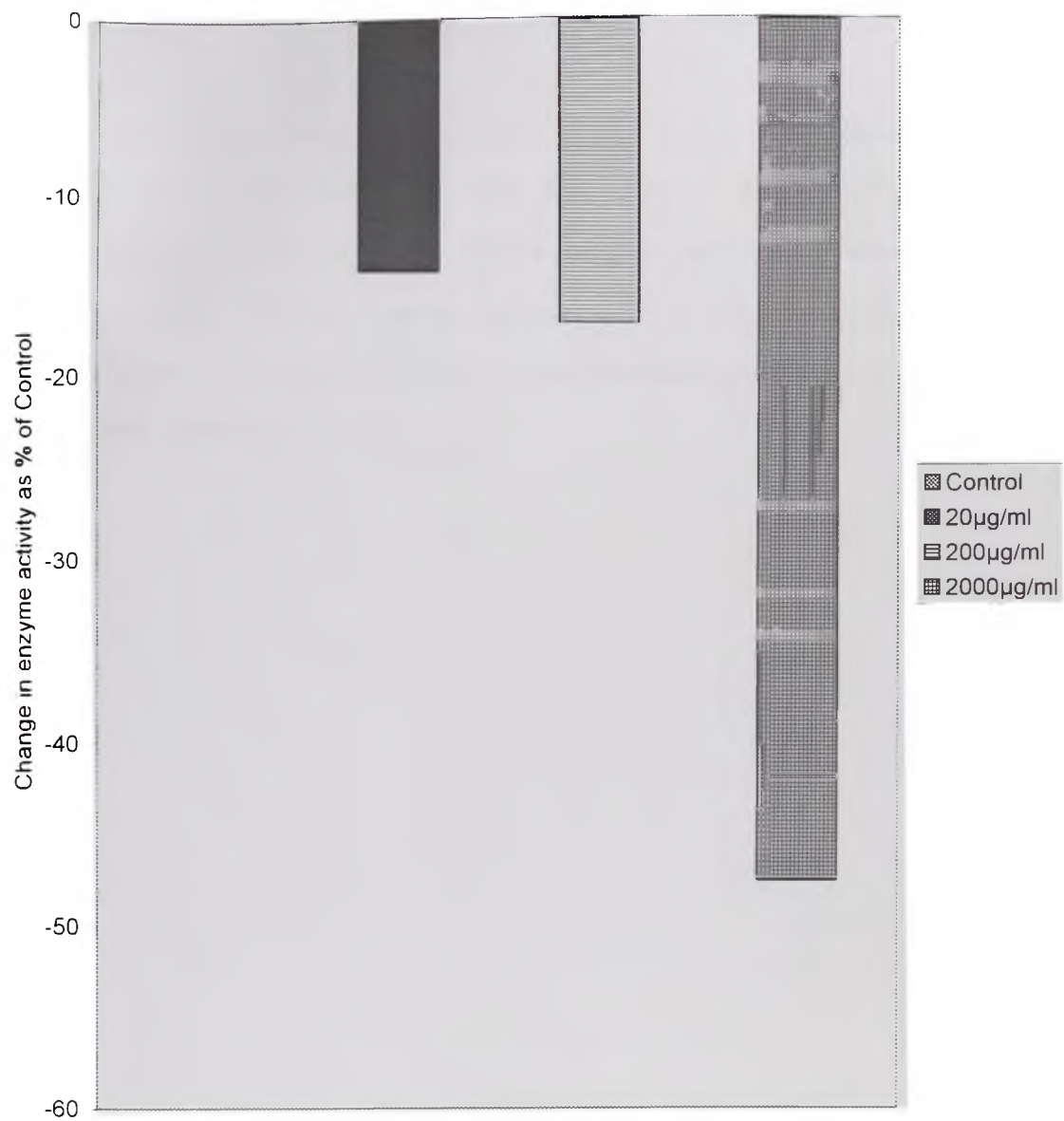


Figure 10: Bar chart showing the % inhibition of CM-1 on 5 α -Reductase activity

The effect of CM-3 on enzyme activity is also reported in table 7. It may be seen from the results that there was no observable inhibition of 5α -reductase activity when 8 and $16\mu\text{g/ml}$ of CM-3, were incubated with ^{14}C -testosterone (23000cpm) for 50 minutes in the presence of crude prostatic nuclear fraction.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

Infraversical obstruction resulting from Benign Prostatic Hyperplasia (BPH) is nowadays seldom a cause of mortality. However, its almost ubiquitous presence in elderly men makes it an important source of morbidity. Moreover, with the gradual demographic shift towards longevity world-wide, the economic burden of this disease seems certain to increase because of the fact that ageing appears to be a contributory factor in its development.

Surgery has been considered as the standard treatment for BPH patients with severe obstructive disorders of micturition that occur in the advanced stages of the illness. Surgical intervention is often accompanied by complaints of incontinence, epididymitis, impotence and urinary tract infections (Lepor, 1993). Pharmacological therapies capable of reducing the symptoms and delaying surgery, can therefore be of valid help in the management of the disease. Such therapies include plant extracts.

Plant extracts contain several active components including phytosterols, triterpenes, long chain fatty acids and alcohols, lignans, alkaloids and lectins. Some of these

Components of plant extracts possess various pharmacological properties including anti-inflammatory (Zarzuelo *et al.*, 1993; Gupta *et al.*, 1980), antioxidant (Morazzoni and Bombardelli, 1995), antimitotic (Wickramaratne *et al.*, 1993) as well as anticancer (Lenaz and De Furia, 1993) activities. Despite the long period of successful utilisation of these plant extracts in the management of diseases, the pharmacological modes of action of many of these plant extracts remain unknown.

Knowledge of the mode of action of medicinal plant extracts can not only lead to the discovery of the active components of such extracts but also permit their usage in treating other conditions for which they were originally not known to be applicable. Such knowledge may also provide a useful method for the standardisation and therefore improvement in the efficacy of medicinal plant preparations. These reasons prompted the desire to attempt to elucidate the mode of action of *Croton membranaceus* in alleviating the symptoms of BPH.

A plant extract that is capable of reversing the symptoms of BPH could possess antiestrogenic, antiandrogenic, 5 α -reductase inhibitory, aromatase complex inhibitory or α -adrenergic blocking activity, since all these activities are known to be involved in the development of BPH. It is also possible that the activity may be due to an effect of the extract on the rate of elimination of mitogenic growth factors or the

metabolites of DHT from the prostate. At least one of the metabolites of DHT, 3β -diol, (figure 2), is known to stimulate growth of rat prostatic explants maintained in culture (Baulieu and Robel, 1970).

In investigating the mode of action of this extract therefore, it became necessary to choose from among the various possibilities, only one possible mode of action for investigation since it would not be practicable to attempt all the possibilities in a single study. As no report of any previous study of the action of the extract existed, the decision to select the effect of the extract on the metabolism of testosterone and, in particular, on 5α -reductase, was based not only on the foreknowledge that 5α -reductase inhibitors do bring relief to BPH patients, but also on the literature suggesting that lowering prostate DHT levels results in glandular atrophy and decrease in prostate size (McConnell, 1990).

The *in vivo* procedures used in the first part of the study involved measurement of DHT levels in serum. As DHT levels in tissue fluids are very low, radioimmunoassay, a binding assay that relies on the specificity of the antigen-antibody reaction, has become the method of choice in the measurement of this hormone in the blood. The particular technique of radioimmunoassay used in the *in vivo* experiment, i.e. the

coated-tube technique, was, in addition to its sensitivity, simple and fast. However, the cost and short lifespan of the radioimmunoassay kit, coupled with initial experimental problems encountered with the 5 α -reductase enzyme activity in the isolated nuclear fraction during the *in vitro* experiments limited the use of the technique only to the *in vivo* experiments.

In the *in vivo* experiment, a sustained drop in serum DHT concentration below the initial value throughout the study period would have supported the hypothesis that the extract works by keeping levels of DHT low. However, on some days, the experimental group values were higher than those of the controls. This observation leads to the suggestion that the crude extract of *C. membranaceus* has no effect resulting in lowering of serum DHT levels *in vivo*. It is also possible that the result is due to the concentrations of extract used. A different concentration of extract could probably produce measurable differences in serum DHT levels.

To extrapolate this result to humans, that is, that *C. membranaceus* extract will have no effect on serum DHT, may not be valid. The effectiveness of a drug depends among other factors upon its bioavailability. For drugs administered orally, factors such as absorption from the gastrointestinal tract, metabolic transformation in the gut, portal blood

and/or liver, and elimination in the bile determine the bioavailability. As rats are different from humans, these factors could be different. Poor absorption of the active principles from the gut, rapid biotransformation and/or elimination from the rat could be responsible for the inability of the orally administered extract to produce a sustained depression of serum DHT levels.

The *in vitro* experiment designed to test for the effect of the extract on 5 α -reductase activity was performed first to establish the presence of the active enzyme in the nuclear fraction prepared and used for this investigation. Castration is known to induce 5 α -reductase activity in the degenerating prostate (Normington and Russell, 1992). That castration was required in order to produce detectable levels of DHT in this experiment suggests that the level of the enzyme in the prostates of the uncastrated rats might have been very low. In early experiments in which prostates from uncastrated rats were used, enzyme activity could not be demonstrated (results not shown). The results of the experiment following castration, however, positively demonstrated the presence of the enzyme in the nuclear fraction isolated and used for the *in vitro* investigations.

The crude extract caused 20, 48, and 54% decrease in enzyme activity at 40, 400, and 4000 μ g/ml respectively when the

extract was incubated with low substrate concentration (7000cpm). This suggests that inhibition of 5 α -reductase activity might explain or at least contribute to the mode of action of *C. membranaceus* in alleviating the symptoms of BPH. In the *in vivo* study, however, no effect on serum DHT levels could be inferred from the results obtained. This leads to the suggestion that the effect of the extract might be localised in the prostate. The extract might have had little or no significant effect on testosterone-metabolising tissues other than the prostate.

Depression of enzyme activity by substances administered to living organisms could result from inhibition at the level of mRNA synthesis or mRNA translation. It could also be the result of direct interaction between the enzyme molecule and the substance administered or its metabolite. *In vitro* enzyme inhibition, on the other hand, can only result from direct enzyme-inhibitor interaction unless the system is specifically designed to carry out protein synthesis. The system used in the *in vitro* assays in this work utilised isolated nuclear fraction. Isolated nuclear fractions can carry out very limited protein synthesis. In view of this, the fact that the crude extracts produced inhibition *in vitro* suggests that the active components of the extract interacted directly with the enzyme molecule.

Two unidentified alkaloid components (CM-1 and CM-3) of the extract were investigated for their effect on the 5 α -reductase activity. The decision to evaluate these components was based on the known pharmacological importance of alkaloids. Alkaloids are a heterogeneous group of naturally occurring nitrogenous organic compounds, other than the amino acids and nucleic acids, that are generally obtained from plants. Drugs such as reserpine, vinblastine, vincristine, atropine, scopolamine, ergonovine and ergotamine, which are used extensively in modern medical practice, belong to this group of compounds. Although they are not the only active compounds in plants, the activities of several plant extracts are traceable to their alkaloid components.

Based on the results of this investigation, it is evident that CM-1, just like the crude extract, also inhibits 5 α -reductase *in vitro*. However, the results suggest that this alkaloid is a less potent inhibitor than the crude extract. At a concentration of 200 μ g/ml, CM-1 produced less inhibition than 40 μ g/ml of crude extract. Previous study carried out at the CSRPM during the isolation of CM-1 from *C. membranaceus*, suggests that the crude extract contains about 0.08% by weight of CM-1. The amount of CM-1 in 40 μ g of extract is expected to be about 0.03 μ g. This is expected to make a negligible contribution to the effect of the extract. This suggests the presence of a more potent component in the extract.

Alternatively, the effect of the crude extract could be due to the cumulative and/or synergistic effect of CM-1 and some other component which may not necessarily be more potent than CM-1.

It is not possible at this stage to imagine that this other component is CM-3 since it did not produce any significant inhibition of the enzyme at the two low concentrations tested. Further work is necessary in order to identify the other component(s).

Whereas the crude extract at 40 μ g/ml produced some inhibition of 5 α -reductase, when a low concentration of substrate was used, there was no observable inhibition in the experiment in which 56 and 112 μ g/ml of the extract were included in the incubation medium with a high concentration of substrate giving 23000cpm of ¹⁴C-testosterone. Also both CM-1 and CM-3 did not produce any inhibition at the higher testosterone level. The analytical technique used for quantitation could possibly be responsible for the failure to observe any effects on the enzyme at the high concentration of substrate. The technique involved identification of DHT by R_f on TLC plates. Unlike radioimmunoassay, this technique does not discriminate between DHT and other metabolites such as 5 α -androstane-3 α -ol-17-one (androsterone) and 5 α -androstane-3 β -ol-17-one (see figure 2) that might co-migrate or have R_f's close to that of

DHT. The possibility of co-migration is inferred from the similarity of their chemical structures. At the lower substrate concentration equivalent to 7000cpm of ^{14}C -testosterone, and shorter period of incubation used for 40 $\mu\text{g/ml}$ experiment, the amount of these interfering substances could be insignificantly low. At higher substrate concentration (23000cpm) and longer period of incubation however their levels could be sufficiently high to mask the differences between the control and experimental levels of DHT formed.

In comparison with the experiment involving the lower substrate concentration, the initial trial, in which higher substrate concentration was used, seems to have converted a higher amount of substrate to DHT. In the initial trial, the mean control value of DHT formed is 5.3% of the total count of ^{14}C -testosterone used as substrate. In the later experiment, however, the mean control value formed only 2.9% of the 7000cpm of testosterone used. This discrepancy could be attributable to the quality of nuclear fraction used on the two different occasions on which the experiments were performed. The accident that resulted in the use of mortar and pestle instead of a proper tissue homogeniser, could probably be the cause of the difference in quality of the nuclear fractions used.

Excessive 5α -reductase activity and DHT production are linked to androgen-dependent, genetically determined skin disorders such as acne (Sansome and Reisner, 1971), male-pattern baldness (Schweikert and Wilson, 1974) and female hirsutism (Kuttann et al., 1977). The fact that the crude extract and CM-1 possess 5α -reductase inhibitory activity suggests that it could be used in treating the above conditions as well. This possibility needs to be investigated in view of its possible economic implications.

Although ethanol extract of *C. membranaceus* probably possesses inhibitory effect on steroid 5α -reductase activity *in vitro*, this may not be the only activity involved in the ability of the extract to reverse the symptoms of prostatism in BPH patients. At the usual concentration prescribed for BPH patients at the CSRPM at Mampong, the extract did not appear to lower serum DHT levels in rats. According to Daniel (1997), the action of the extract appears to be irreversible; on withdrawal of medication, the symptoms of prostatism do not recur. If the major action of the extract were inhibition of 5α -reductase, then on withdrawal of the extract, the enzyme activity should return to pre-treatment levels and this should be accompanied by relapse of the hyperplasia. That this does not occur indicates that the extract probably possesses other pharmacological properties too.

One possibility is that the extract could interfere with the production, secretion and/or receptor affinity of growth factors such as basic fibroblast growth factor (bFGF or FGF-2) and keratinocyte growth factor (KGF or FGF-7) which are thought to mediate the action of DHT in the prostate (Story et al., 1994; Lubrano et al., 1992). These growth factors are secreted by the stromal cells and act by both autocrine and paracrine mechanisms on stromal and epithelial cells of the prostate (Lawson, 1993). There is, therefore, the possibility that some components of *C. membranaceus* interfere with the binding of some of these growth factors to their receptors and thereby inhibit further proliferation of prostatic cells. According to the stem cell hypothesis (Isaacs, 1987), if the rate of cell proliferation falls below that of cell death, a decrease in size of the hyperplastic prostate could result. Investigations into the effect of the extract on synthesis, secretion and receptor binding of these growth factors are therefore suggested in order to increase the understanding of the mode of action of the extract.

In addition to growth factors, there is the need to investigate the effect of the crude extract on the aromatase complex, α -adrenoceptors, eicosanoid metabolism, Sex Hormone Binding Globulin (SHBG) and the androgen receptor since each of these factors is implicated in the development of BPH.

Finally, based on the results obtained and discussed in this report, it may be concluded that a total alcoholic extract of *C. membranaceus* inhibits 5 α -reductase enzyme activity present in rat prostatic nuclei *in vitro*. Part of this inhibitory activity is present in the unidentified, alkaloidal isolate, CM-1. The extract, as used at the CSRPM, however, does not lower rat serum DHT levels significantly *in vivo*. Furthermore, inhibition of the enzyme may not account fully for the observed effects of the extract. The activity of the extract, as used at the CSRPM, may therefore be due to a combination of localised 5 α -reductase inhibition in the prostate and some other pharmacological properties possessed by the extract. Further investigations are required in order to understand fully the mode of action of the plant extract in alleviating the symptoms of benign prostatic hyperplasia.

REFERENCES

- Aly M.S., Dal-Cin P., Van-den Voorde W., van-Poppel H., Ameye F., Baert L. and Van-den-Berge H. (1994). Chromosome abnormalities in benign prostatic hyperplasia. *Genes-Chromosomes-Cancer*, 9, 227-233.
- Baulieu E. E. and Robel R. (1970). Testosterone metabolites: their receptors, metabolism and action in the rat ventral prostate. In Some aspects of the aetiology and biochemistry of prostatic cancer, Proc. 3rd Tenovus Workshop. Eds. K Griffiths and C. G. Pierrepoint. Caerphilly: A.O.A. Publishing Co. pp 74-81.
- Bluestein D.L. and Oesterling J.E. (1993). Hormonal therapy in the management of benign prostatic hyperplasia. In Prostate diseases. Eds. H. Lepor and R.K. Lawson. Published by WB Saunders Co., Philadelphia. Chapter 16, pp 182-198.
- Bomberdelli E. and Morazzoni P. (1997). *Prunus africana* (Hook.f.) Kalkm. *Fitoterapia*, 68(3), 205-218.
- Bosch R.J.L.H., Griffiths D.J., Blom J.H.M. and Schroeder F.H. (1989). Treatment of benign prostatic hyperplasia by androgen deprivation: Effects on prostate size and Urodynamic parameters. *J. Urol.*, 141, 68-72.
- Briley M. (1983). Permixon, a new treatment for benign prostate hyperplasia, acts directly at the cytosolic androgen receptor in rat prostate. *Br. J. Pharmac.* 79, 327p.

- Brinker F.J. (1994).** An overview of conventional, experimental, and botanical treatments of non-malignant prostate conditions. *Brit. J Phytother*, 3, 154 - 176.
- Bruchovsky N. and Wilson J.D. (1968).** The conversion of testosterone to 5 α -androstan-17 β -ol-3-one by rat prostate *in vivo* and *in vitro*. *J. Biol. Chem.* 243, 2012 - 2021.
- Burrows H. (1935).** Pathological conditions induced by estrogenic compounds in the coagulation gland and prostate of the mouse. *Am J. Cancer*, 23, 490-512.
- Carani C. (1991).** Urological and sexual evaluation of treatment of benign prostatic disease using *Pygeum africanum* at high doses. *Arch Ital. Urol Nefrol Androl*, 63, 341-345.
- Carilla E. (1984).** Binding of Permixon, a new treatment for prostatic benign hyperplasia, to the cytosolic androgen receptor in the rat prostate. *J. Steroid Biochem*, 20, 521-523.
- Casalone R., Portentoso P., Granata P., Minelli E., Righi R., Meroni E., Pozzi E. and Chiaravalli A.M. (1993).** Chromosome changes in benign prostatic hyperplasia and their significance in the origin of prostatic carcinoma. *Cancer-Genet-Cytogenet*, 68, 126-130.
- Chirillo-Marucco E. (1983).** Extract of *Serenoa repens* (Permixon R) in the early treatment of prostatic hypertrophy. *Urologia*, 50, 1269-1277.
- Clavert A., Cranz C., Riffand J.P., Marquer C., Lacolle J.Y., and Bollack C. (1986).** Effects of an extract of the bark of

Pygeum africanum (V.1326) on prostatic secretions in the rat and in man. *Ann Urol.*, 20, 341-343.

Cunha G.R., Chung L.W.K., Shannon J.M. and Reese B.A. (1980). Stromal-Epithelial interactions in sex differentiation. *Biol. Reprod*, 22, 19-42.

Daniel H.E.A. (1997). Oral communication, Mampong-Akwapim.

Debat J. (1980). Higher alkanol compositions and the use thereof in treatment of prostate disorders. *Chem. Abs.*, 93, 7655w.

Delos S., Iehle C., Martin P.M. and Raynauld J.P. (1994). Inhibition of the activity of basic 5 α -reductase (type 1) detected in DU 145 cells and expressed in insect cells. *J. Steroid Biochem Mol Biol*, 48, 347-352.

Di Silverio F. (1992). Evidence that *Serenoa repens* extract displays an antiestrogenic activity in prostatic tissue of EPH patients. *Eur. Urol.*, 21, 309-314.

Franks L.M. (1954). Benign nodular hyperplasia of the prostate: A review. *Ann. R. Coll. Surg. Engl*, 14, 92-106.

Gormley G.J., Stoner E., Bruskevitz R.C., Imperato-McGinley J., Walsh P.C., McConnel J.D. (1992). The effect of finasteride in men with benign prostatic hyperplasia. *N. Engl J. Med.*, 327, 1185-1191.

Goulding K.H. (1986). Radioisotope techniques. In *A biologist's guide to principles and techniques of practical biochemistry*. Eds. K. Wilson and K.H. Goulding. Published by

Edward Arnold (Publishers) Ltd., London. Chapter 9, pp 314 - 344.

Gupta M.B., Nath R., Srivastava N., Shanker K., Kishor K. and Bhargava K.P. (1980). Anti-inflammatory and antipyretic activities of β -sitosterol. *Planta Medica*, 39, 157-163.

Habenicht U.F. Schwatz K., Neumann F. and El Etreby M.F. (1987). Induction of estrogen related hyperplastic changes in the prostate of the cynomolgus monkey (*Macaca fascicularis*) by androstenedione and its antagonization by the aromatase inhibitor 1-methyl-androsta-1, 4-diene-3, 17-dione. *Prostate*, 11, 313.

Isaacs J.T. (1987). Control of cell proliferation and cell death in the normal and neoplastic prostate: A stem cell model. In Benign Prostate Hyperplasia. Eds. G.H Rogers. D.S. Coffey, and G. Cunha. Washington DC: US Dept. of Health and Human Services, NIH Publication 87-2881, pp. 85-94.

Jommi G. (1989). Alcohols isolated from lipophilic *Serenoa repens* extracts and their use for the treatment of prostate pathologies. *Chem. Abs*, 111, 84073x.

Kirby R.S., Bryan Jerald I., Christmas T.J., Liu S., Holmes S.A.V., Vale J.A., Shanmuganathan K. and Webb J.A. (1992). Finasteride in the treatment of benign prostatic hyperplasia. A urodynamic evaluation. *Br J. Urol*, 70, 65-72.

Kuttann F., Mowszowicz I., Schaison G. and Mauvais-Jarvis P. (1977). Androgen production and skin metabolism in hirsutism. *J. Endocr*, 25, 83-91.

- Lawson R.K. (1993).** Etiology of benign prostatic hyperplasia. In Prostate diseases. Eds. H. Lepor and R.K. Lawson. Published by WB Saunders Co., Philadelphia. pp 89-95.
- Lawson R.K. (1990).** Benign prostatic hyperplasia and growth factors. *Urologe (A)*, 29, 5-7.
- Lenaz L. and De Furia M.D. (1993).** Taxol: a novel natural product with significant anticancer activity. *Fitoterapia*, 64 (suppl to No 1), 27-35.
- Lepor H. (1993).** The role of alpha-blockade in the therapy of BPH. In Prostate diseases. Eds. H. Lepor and RK Lawson. Published by WB Saunders Co., Philadelphia. Chapter 15, pp170-181
- Levine A.C., Ren M., Huber G.K. and Kirschenbaum A. (1992).** The effect of androgen, estrogen and growth factors on the proliferation of cultured fibroblasts derived from human fetal and adult prostates. *Endocrinol* 130, 2413-2419.
- Levine A.C., Kirschenbaum A., Droller M. and Gabilove J.L. (1991).** Effect of the addition of estrogen to medical castration on prostatic size, symptoms, histology and serum prostate specific antigen in four men with benign prostatic hypertrophy. *J. Urol*, 146, 790-793.
- Lubrano C., Sciarra F., Spera G., Pertrangeli E. and Toscano V. (1992).** Immunoreactive EGF in human benign prostatic hyperplasia: relationships with androgen and estrogen receptors. *J. Steroid Biochem Mol Biol*, 41, 683-687.

- Magdy El-Sheikh M. (1988).** The effect of Permixon on androgen receptors. *Obstet Gynecol Scand*, 67, 397-399.
- Marandola P., Jallous H., Bombardelli E. and Morazzoni P (1997).** Main phytoderivatives in the management of benign prostatic hyperplasia. *Fitoterapia*, 68(3), 195-204.
- Martel C., Trudel C., Couet J., Labrie C., Belanger A. and Labrie F. (1993).** Blockade of androstenedione-induced stimulation of androgen-sensitive parameters in the rat prostate by combination of Flutamide and 4-MA. *Mol Cell Endocrinol*, 91, 43-49.
- McConnell J.D. (1995).** Prostatic growth: New insights into hormonal regulation. *Br J. Urol*, 76, 1-5.
- McConnell J.D. (1990).** Medical management of benign prostatic hyperplasia with androgen suppression. *Prostate (suppl)* 3, 49-59.
- McNeal J.E. (1983).** The prostate gland: Morphology and pathobiology. *Monographs in Urology*, 4(1).
- Mellin T.N., Busch R.D. and Rasmusson G.H. (1993).** Azasteroids as inhibitors of testosterone 5 α -reductase in mammalian skin. *J. Steroid Biochem Mol Biol*, 44, 121-131.
- Moore R.J., Gazak J.M. and Wilson J.D. (1979).** Regulation of cytoplasmic dihydrotestosterone binding in dog prostate by 17 β -estradiol. *J. Clin Invest*, 63, 351-357.
- Morazzoni P. and Bombardelli E. (1995).** *Silybum marianum* (*Carduus marianus*). *Fitoterapia*, 66(1), 3-42.

- Morfin R.F., Di Stefano S., Bercovici J-P. and Floch H.H. (1978). Comparison of testosterone, 5 α -dihydrotestosterone and 5 α -androstane-3 β ,17 β -diol metabolisms in human normal and hyperplastic prostates. *J. Steroid Biochem*, 9, 245-252.
- Normington K. and Russell D.W. (1992). Tissue distribution and kinetic characteristics of rat steroid 5 α -reductase isozymes: Evidence for distinct physiological functions. *J. Biol Chem*, 267, 19548-19554.
- Olle-Carreras J. (1987). Our experience with hexane extract of *Serenoa repens* on the treatment of benign prostatic hypertrophy. *Arch Esp. Urol*, 40, 310-313.
- Peters C.A. and Walsh P.C. (1987). The effect of nafarelin acetate, a luteinising hormone releasing agonist, on benign prostatic hyperplasia. *N. Engl. J. Med*, 317, 599-604.
- Pflug B.R., Onoda M., Lynch J.H. and Djakiew D. (1992). Reduced expression of the low affinity nerve growth factor receptor in benign and malignant human prostatic tissue and loss of expression in four human metastatic prostate tumor cell lines. *Cancer Res.*, 52, 5403-5406.
- Price D.T., Schwinn D.A., Lomasney J.W., Allen L.F., Caron M.G. and Lefkowitz R.J. (1993). Identification, quantification and localization of mRNA for three distinct α_2 -adrenergic receptor subtypes in human prostate. *J. Urol*, 150, 546-551.

Puri V., Puri C.P. and Anand Kumar T.C. (1981). Serum levels of dihydrotestosterone in rhesus monkeys estimated by a non-chromatographic radioimmunoassay method. *J. Steroid Biochem.* 14, 877.

Rhodes L., Primka R.L., Berman C., Vergult G., Gabriel M., Malice M.F. and Cubelin B. (1993). Comparison of Finasteride (Proscar), a 5 α -reductase inhibitor and various commercial plant extracts in *in vitro* and *in vivo* 5 α -reductase inhibition. *Prostate*, 22, 43-51.

Robel P., Lasnitzki I. and Baullieu E.E. (1971). Hormone metabolism and action: testosterone and metabolites in prostate organ culture. *Biochimie*, 53, 81-97.

Sansome G. and Reisner R.M. (1971). Differential rates of conversion of testosterone to dihydrotestosterone in acne and normal human skin - a possible pathogenic factor in acne. *J. Invest Dermat*, 56, 366-372.

Schweikert H.U. and Wilson J.D. (1974). Regulation of human hair growth by steroid hormones, I. Testosterone metabolism in isolated hairs. *J. Clin Endocr Metab*, 38, 811-819.

Siiteri P.K. and Wilson J.D. (1970). Dihydrotestosterone on prostatic hypertrophy. I. Formation and content of DHT in the hypertrophic prostate of man. *J. Clin Invest*, 49, 1737-1745.

Southan G.J., Brooks R.V., Cowan D.A., Kicman A.T. Unnadkat N. and Walker C.J. (1992). Possible indices for the detection of

the administration of dihydrotestosterone to athletes. J. Steroid Biochem. Mol. Biol. 42, 87 - 94.

Story M.T., Hopp K.A., Molter M. and Meier D.A. (1994). Characteristics of FGF receptors expressed by stromal and epithelial cells cultured from normal and hyperplastic prostates. Growth Factors, 10, 269-280.

Sultan C. (1984). Inhibition of androgen metabolism and binding by a liposterolic extract of *Serenoa repens* in human foreskin fibroblasts. J. Steroid Biochem, 20, 515-519.

Thigpen A.E., Silver R.I., Guileyardo J.M., Casey M.L., McConnell J.D. and Russell D.W. (1993a). Tissue distribution and ontogeny of steroid 5 α -reductase isozyme expression. J. Clin Invest, 92, 903-910.

Thigpen A.E., Cala K.M. and Russell D.W. (1993b). Characterization of Chinese hamster ovary cell lines expressing human steroid 5 α -reductase isozymes. J. Biol. Chem. 268, 17404 - 17412.

Tunn U.W., Kaivers P. and Schweikert H.U. (1985). Conservative treatment of human prostatic hyperplasia. In Regulation of androgen action. Eds. N. Bruchovsky, A. Chapdelaine and F. Neumann. Published by R. Bruckner, Berlin. Pp87-90.

Walsh P.C., Hutchins G.M. and Ewing L.L. (1983). Tissue content of dihydrotestosterone in human prostatic hyperplasia is not supranormal. J. Clin Invest, 72, 1772-1777.

West N.B., Roselli C.E., Resko J.A., Greene G.L. and Brenner R.M. (1988). Estrogen and progesterin receptors and aromatase activity in rhesus monkey prostate. *Endocrinology*, 123, 2312.

Wickramaratne D.B.M., Pengsuparp T., Mar W. and Chai H-B. (1993). Novel antimitotic dibenzocyclo-octadiene lignan constituents of the stem bark of *Steganotaenia araliacea*. *J. Nat Prod*, 56, 2083-2090.

WHO (1981). Global strategy for Health for All by the Year 2000. Health for all series, No.3. Published by World Health Organisation, Geneva.

WHO (1978). Primary Health Care. Health for all series, No.1. Published by World Health Organisation, Geneva.

Yalow R.S. and Berson S.A. (1960). Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.*, 39, 1157.

Zarzuelo A., Garcia E., Jimenez J., Ocete M.A., Utrilla P. and Socorro O. (1993). Anti-inflammatory and anti-ulcerative activity of various species of the genus *Sideritis* from the Alpujarra region of Spain. *Fitoterapia*, 64(1), 26-30.

Zuckerman S. (1936). The endocrine control of the prostate. *Proc. Roy. Soc. Med.*, 29(2), 1557-1568.

APPENDIX A

Preparation of solutions

The following reagents and buffer solution used in the investigation were prepared as follows:

Tris-HCl buffer: pH 7.4

0.01M Tris-HCl buffer was prepared by dissolving 0.61g of Tris-HCl, 1.0ml of β -mercaptoethanol solution, 0.009g of disodium EDTA, 0.51g of $MgCl_2 \cdot 6H_2O$ and 1.46g of NaCl in 500ml of solution.

β -Mercaptoethanol solution:

β -Mercaptoethanol solution was prepared by dissolving 44 μ l of 99% pure β -mercaptoethanol, density 1.12g/ml, in 2.5ml of aqueous solution.

NADPH solution ($10^{-5}M$):

NADPH solution was prepared by dissolving 4.2mg of β -NADPH tetrasodium, in 50ml of aqueous solution.

Scintillation cocktail, (Tpp):

Scintillation cocktail was prepared by dissolving 5.0g of 2,5-diphenyloxazole and 0.05g of 1,4-di-2-(5-phenyloxazolyl)-benzene in 1000ml of toluene.

0.88M Sucrose, containing 1.5mM CaCl₂.

Sucrose solution, 0.88M, containing 1.5mM CaCl₂ was prepared by dissolving 75.31g of sucrose and 42mg of anhydrous CaCl₂ in 250ml of aqueous solution.

0.25M Sucrose

Sucrose solution, 0.25M, was prepared by dissolving 8.56g of sucrose in 100ml of aqueous solution.

APPENDIX B**ISOLATION OF CM-1 AND CM-3**

Thirteen kilograms of dried powdered roots of *C. membranaceus* were macerated overnight with 5% HCl. The acid extract was basified with ammonia to a pH of 10-11 and extracted with chloroform in a countercurrent extractor. The chloroform extract was concentrated under reduced pressure to 150ml and re-extracted with 10% tartaric acid solution (50ml x 4). The combined acid extract was basified again with ammonia to a pH of 9-10 and then extracted once more with chloroform (50ml x 3) to obtain fraction A. The chloroform solution containing tartaric acid-insoluble matter was dried with anhydrous sodium sulphate to obtain fraction B.

Fraction A was adsorbed onto alumina (type G II, Fluka 507C, neutral) and eluted successively with Petroleum ether (boiling range 40-60 C, 100ml), petroleum ether/diethyl ether 1:2 (60ml), and petroleum ether/ diethyl ether 1:9 (150ml). Elution with petroleum ether/ diethyl ether 1:9 afforded CM-3 as a white crystalline solid (Melting point 102 C).

Fraction B was adsorbed onto alumina (type G II, Fluka 507C, neutral) and eluted successively with petroleum ether and petroleum ether/ chloroform (9 : 1). Elution with the latter yielded CM-1 as a white crystalline solid (Melting point 108 C). CM-1 was recrystallized from diethyl ether.