Effects of Seed Extracts from Traditional Nigerian Medical Plants on Prostate Cancer Cell Growth

BY

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THESIS

Submitted as partial fulfillment of the requirements for the degree of Master of Science in Pathology in the Graduate College of the University of Illinois at Chicago, 2013

Chicago, Illinois

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ACKNOWLEDGMENTS

Foremost, I would like express my most sincere gratitude to Dr. Maarten Bosland for his patience, motivation, enthusiasm, and immense knowledge while guiding me to complete my Masters. None of this would be possible if he had not taken me under his wing and helped me along the way.

I would like to thank Dr. Monday I. Akpanabiatu for providing me with the knowledge and materials essential for this study. My gratitude goes to Dr. Andre Kajdacsy-Balla who gave me a chance when I started as a non-degree graduate student and has provided guidance and the benefit of his knowledge throughout my Masters education.

A special thank you goes to professors Alan M. Diamond, Larisa Nonn, and Peter H. Gann who provided me with their immense knowledge and support. I would also like to thank Mike Schlicht, Nur Ozten, and Abeer Mostafa Mahmoud for all of their assistance in this project.

I would like to thank the United States Department of Veterans Affairs for providing the funding necessary to complete my Master of Science in Pathology.

Lastly, I would like to thank my family for all of their love and support. I would especially like to thank my parents Paul and Bonita Jagla for all of their encouragement throughout the course of my studies and my brother Michael Jagla for being there for me whenever I needed some help.

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LIST OF ABBREVIATIONS

AA     Atraric Acid
AR     Androgen Receptor
BPH    Benign Prostate Hyperplasia
CD     Cyclodextrin
Cdx    Casodex
ChIP   Chromatin Immunoprecipitation
CpdA   Compound A; 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride
CTD    C-terminal Domain
DBD    DNA Binding Domain
Dex    Dexamethasone
DHT    Dihydrotestosterone
ER     Estrogen Receptor
ERS    Endoplasmic Reticulum Stress
FA     Fluocinolone Acetonide
GFP    Green Fluorescent Protein
GR     Glucocorticoid Receptor
GRE    Glucocorticoid Transcriptional Response Element
HBD    Hormone Binding Domain
ISP    Isostrynopentamine
LBD    Ligand Binding Domain
MAO    Monoamine oxidase
ME-20  20% methanolic extract of U. dioica
MR     Mineralocorticoid Receptor
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<td>MS</td>
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<tr>
<td>NBBS</td>
<td>N-butylbenzenesulfonamide</td>
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<tr>
<td>NTD</td>
<td>N-terminal Domain</td>
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<td>pMTV</td>
<td>Mouse Mammary Tumor Virus Plasmid</td>
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<td>PR-B</td>
<td>Progesterone Receptor B</td>
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<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
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<td>R1881</td>
<td>Metribolone; Methyltrienolone</td>
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<td>shRNA</td>
<td>Small Hairpin RNA</td>
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<tr>
<td>siRNA</td>
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<tr>
<td>SRC1</td>
<td>Steroid Receptor Co-activator-1</td>
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<td>WHO</td>
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SUMMARY

Prostate cancer is a serious health problem worldwide and it is the second most common cause of cancer related mortality in men in Western countries. The world health organization (WHO) reports that 80% of the populations of some Asian and African countries rely on traditional medicine as their primary health care and even in developed countries 70% to 80% of the population has used some form of alternative medicine. A literature review conducted in this study pertaining to the effects of African plants on prostate cancer yielded few results highlighting the need for more research in this field.

In this study, the effect of ethanolic seed extracts from plants used in Nigerian traditional medicine on prostate cancer cell lines PC3 and LNCaP were investigated. Ethanolic extracts of *Carica papaya*, *Mucuna pruriens*, *Dennettia tripetala*, and *Piper guineense* in concentrations ranging from 0.01 µg/mL to 100 µg/mL were used to treat PC3 and LNCaP cells and cell viability was determined using trypan blue stain and hemocytometer counting.

At concentrations of 100 µg/mL, the extracts of *D. tripetala* and *P. guineense* were found to significantly inhibit cell growth in PC3 and LNCaP cells. Cytotoxic effects were also observed in LNCaP cells exposed to 100 µg/mL of *D. tripetala* and *P. guineense* extracts. A significant biphasic growth curve was observed in LNCaP cells exposed to *C. papaya* extract for 48 hours. *Conclusion*: *D. tripetala* and *P. guineense*, which are widely used in Nigerian traditional medicine, have significant growth inhibitory and cytotoxic effects on prostate cancer cells *in vitro*. These results warrant further investigation into the novel effects of *D. tripetala* and *P. guineense* on prostate cancer.
I. INTRODUCTION AND OBJECTIVES

I.A. **Introduction**

Prostate cancer is a serious health problem worldwide and it is the second most common cause of cancer related mortality in men in Western countries. In many countries around the world, including parts of Europe, Asia, and Africa, the first line of defense against benign prostate hyperplasia (BPH) and prostate cancer are natural remedies (Shenouda *et al.*, 2007). The world health organization (WHO) reports that 80% of the population of some Asian and African countries rely on traditional medicine as their primary health care and even in developed countries 70% to 80% of the population have used some form of alternative medicine (WHO, 2008). Unfortunately many traditional healers are not equipped to diagnose or treat prostate cancer. Another growing concern as the public becomes more aware of prostate cancer is the number of “snake oil dealers” or con artists that create remedies, such as herbal mixtures, that have no actual effect on prostate cancer and often cause patients more harm than good. The WHO reports that “scientific evidence from tests done to evaluate the safety and effectiveness of traditional medicine products and practices is limited” (WHO, 2008). For many the use of alternative treatments is mainly an issue of economic status.

In West Africa, like many developing parts of the world, people rely heavily upon traditional medicine for treatment of disease due to extreme poverty and a shortage of healthcare workers (Sawadogo *et al.*, 2012). According to Snyder *et al.* (2009) the average cost of treatment for prostate cancer can range between $10,804 for hormonal therapy and $17,795 for hormonal and radiation combined therapy. This is an unthinkable sum for many people such as those in
West Africa who spend an average of $10-$40 per year on healthcare and even for many in the United States the high cost of healthcare makes effective treatments inaccessible. Supplements such as Tadenan from the African plum tree (*Pygeum africanum*) and Saw Palmetto (*Serenoa repens*) are becoming increasingly popular alternatives for treatment of BPH and prostate cancer. There is a great need for more affordable treatments for prostate cancer worldwide. According to the WHO herbal remedies are the most popular form of traditional medicines and result in billions of dollars of revenue worldwide (WHO, 2008).

The WHO reports that 25% of pharmaceutical drugs are made from plants that were first used in traditional medicine (WHO, 2003). The scientific community recognizes the potential for cancer treatments from plant products and there is a vast body of work relating to possible treatments derived from plants. In Africa there is a wealth of diverse flora. Of the 300,000 plant species recorded in the world, more than 200,000 are found in the tropical countries of Africa and elsewhere (Sawadogo *et al*., 2012). However, the body of literature relating to African plants and their effects on prostate cancer is limited. Some African plants, such as *P. africanum*, have been studied for over 50 years and their effects on BPH are well established. However it has only been recently that research has begun to show scientific evidence of the effects of these plants on prostate cancer.

I.B. **Objectives**

The main objectives of this investigation were (1) to conduct a literature review on the effects African plants used in traditional herbal medicine had on prostate cancer, and (2) to determine the *in vitro* cytotoxicity and growth inhibitory properties of extracts from seeds of
Carica papaya, Mucuna pruriens, Dennettia tripetala, and Piper guineense, which are Nigerian plants used in traditional herbal medicine, on human prostate cancer cells.

I.C. **Plants Studied**

The leaves, seeds, and fruit of Carica papaya are used in Nigeria to treat a variety of ailments such as gonorrhea, malaria, and diabetes. There are many scientific studies that report on the beneficial effects of C. papaya due to its use worldwide, but not many studies relate to cancer. An epidemiological study conducted by Shahar *et al.* (2011) reported that intake of more than 22.7 g/day of papaya reduced prostate cancer risk by 2.7 times. Liew *et al.* (2012) reported that leaf extracts showed enhanced cytotoxic effect on hypoxic cancer cells by inhibiting HIF activities in Saos-2 human osteosarcoma cells. Another study by Li *et al.* (2012) reported that the compound benzyl glucosinolate found in seeds of C. papaya inhibited growth in human lung cancer H69.

*Dennettia tripetala* fruit is used in Nigeria typically as a stimulant, but other parts of the plant are used to treat ailments such as fever and toothache. There are few scientific studies relating to D. tripetala and no studies to date relating to cancer. Oyemitan *et al.* (2008) reported that the essential oil of *D. tripetala* possesses significant antinociceptive and antiinflammatory effects in the animal models. In another study, Milia´n *et al.* (2012) reported that the alkaloids synthesized from D. tripetala inhibited generation of reactive oxygen species in neutrophils.

*Mucuna pruriens* is used in Nigeria to treat intestinal worms and genito-urinary diseases. There are currently no studies relating the effects of *M. pruriens* to cancer. However, there are
scientific studies relating to diabetes, sexual performance, and various neurological conditions. A study by Shukla et al. (2009) reported that treatment with *M. pruriens* improved testosterone levels and semen quality in infertile men.

In Nigeria *Piper guineense* is used for a widely in traditional medicine for ailments such as mental illness, impotence, and fever to name a few. There are few studies pertaining to the effects of *P. guineense* in mammals. Agbonon et al. (2010) demonstrated that extracts of *P. guineense* strongly inhibited CYP3A4, CYP3A5 and CYP3A7, which effect drug metabolism and may have pharmacoenhancing potential. Studies by Agbor et al. (2007; 2012) reported the antioxidant properties of *P. guineense* both *in vitro* and *in vivo*.

Overall, there are few studies, if any, relating to these plants and their effects on prostate cancer. Most of the literature found relating to the plants in used in this study had only loosely implied anticancer activity. There is need for further investigation of the anticancer effects of these plants. This study is the first to study the effects of these plant’s effects on prostate cancer cells *in vitro*. 
II. LITERATURE REVIEW

There is a growing body of literature relating to the effects of African plants on prostate cancer, but many studies have been limited to initial results describing anti-androgenic effects or \textit{in vitro} growth inhibition of one or two prostate cancer cell lines. A great portion of the current literature involves fractionation of plant materials in an effort to discover the bioactive compounds that have effects on prostate cancer cell growth. These studies often begin with various preparations of powdered plant materials in aqueous or alcoholic solutions similar to those used in traditional medicine. These “crude extracts” will typically be fractionated into aqueous, methanolic, ethanolic, and hexane fractions according to solubility in an effort to isolate active compounds in activity guided assays. Mass spectrometry (MS) or spectral analysis have also been used to analyze the compounds resulting from these fractions. Unfortunately cancer research is still in its early stages in Africa due to the scarcity of technical facilities and limited financial resources (Sawadogo \textit{et al.}, 2012). The result is that much of the research exploring the effects of African plants on prostate cancer cells comes from collaborations with laboratories in more developed countries (Sawadogo \textit{et al.}, 2012). There is therefore a need to highlight some of the research involving African plants that have been reported to have beneficial effects on prostate cancer. The available published reports are described in detail in the following review.

II.A. \textbf{Phytochemicals with Anticancer Properties}

According to a review of literature by Sawadogo \textit{et al.} (2012) about 65\% of the phytochemicals from West African plants that held strong cytotoxicity against a panel of cancer cells were terpenes, while others included steroids and alkaloids. A brief overview of some of the
important phytochemicals and their functions will be given here based on the review of Sawadogo et al. (2012).

Sesquiterpenes according to Sawadogo et al. (2012) are found in the essential oils of vascular plants and have a large structural variety. These structural diversities lead to various pharmacological properties including antimalarial, antibacterial, antiviral, anti-inflammatory and anti-tumor activities. They may inhibit cell proliferation, exhibit cytotoxic effects, or even inhibit cancer cell migration.

Diterpenes are, according to Sawadogo et al. (2012), compounds found in essential oils and are known to have antibacterial, antiviral, antifungal, and expectorant properties. More recently they have been shown to also have chemotherapeutic and chemopreventive action. Such chemopreventive agents may inhibit the initiation, promotion, or progression stages of the carcinogenic process by blocking the formation and detoxification of carcinogenic chemicals. The chemotherapeutic effect of diterpenes is due to their cytotoxicity and their lipophilicity allows them to potentially target biological membranes.

Triterpenes are, according to Sawadogo et al. (2012), precursors to steroids in plants and animals that typically occur free, but can also occur as glycosides. They can have anticancer activities such as inhibition of cell growth and proliferation, exhibit cytotoxicity by altering proteins within a cell, and inhibit carcinogenesis. Some sequiterpenes exhibit their cytotoxic activity by interaction with triterpenes.
According to Sawadogo et al. (2012), plant steroids, including the phytosterol subclass, have been shown to have anticancer properties. Sterols have been shown to affect membrane structure and function of both tumor and host cells and they have been shown to affect signal transduction pathways that regulate growth and apoptosis (Awad and Fink, 2000).

Alkaloids are, according to Sawadogo et al. (2012), found mainly in stem and root bark or rhizomes of plants. They have been shown to inhibit multidrug resistance of cancer cells which may improve drug efficacy. Some alkaloids have cytotoxic effects that can cause DNA damage and some can even lead to apoptosis.

II.B. **Extensively Studied Plants**

Some African plants have been extensively studied for their medicinal effects. *Pygeum africanum* for instance has long been used in African traditional medicine and in Europe it has been used to treat prostate disorders since 1969 (Shenouda et al., 2007). There is extensive literature relating to the beneficial effects *P. africanum* has on prostate health with particular emphasis on BPH. Today supplements containing *P. africanum* such as Tadenan are used worldwide to treat BPH and other urinary tract complications. However, researchers are still unsure as to the compounds in *P. africanum* that induce these beneficial effects. It has only been relatively recently that researchers have begun to explore the effects of *P. africanum* on prostate cancer even though its beneficial effects on prostate health have long been studied. Many of these researchers tend to be biochemists attempting to find the beneficial compounds that make *P. africanum* effective against prostate cancer and BPH. *S. tuberculatiformis* is another medicinal plant that has long been used in Africa and has interesting biological activity in
pregnant sheep and is used as a contraceptive by women (Swart et al., 2003). Researchers have only recently begun to explore the anticancer effects of *S. tuberculatiformis*.

II.B.1. *Pygeum africanum*

As mentioned above the bark of the African plum tree (*P. africanum*) has long been used in African traditional medicine, has been popular in Europe for nearly 50 years, and is becoming more popular in the U.S. There is extensive research on the effects of *P. africanum* on BPH and there is strong evidence to support its role in relieving the symptoms of BPH. However, while these effects are well known, it is unknown as to what role *P. africanum* can play in prostate cancer. The novel effects of *P. africanum* have only begun to be explored and shown below are some studies pertaining to effects of *P. africanum* on prostate cancer.

II.B.1.a. **Atraric Acid**

Schleich *et al.* (2005) fractionated *P. africanum* bark in an effort to find compounds in the bark that may have anti-androgenic properties. Extracts were made using powdered *P. africanum* bark in n-hexane, dichloromethane, methanol, methanol-water (1:1), and water. The dichloromethane extract had anti-androgenic properties when exposed to CV1 cells transfected with human androgen receptor gene pSG-hAR. CV1 cells are a green monkey kidney cell line lacking endogenously expressed functional androgen, glucocorticoid, and progesterone receptors. The dichloromethane extract was evaporated and fractionated on a silica gel with a gradient system containing n-hexane, dichloromethane, methanol, and water. Schleich et al. (2005) discovered that the compound *atraric acid* (AA) was responsible for its anti-androgenic effects.
When CV1 cells transfected to express with human androgen receptor (AR) were incubated both with and without methyltrienolone (R1881), an AR agonist; AA significantly inhibited AR function in a concentration dependent manner starting at 1 µM. Further inhibition of function was shown in 10 µM and 100 µM. Similar inhibition was seen with commercially available AA. When AA is esterified further by two or three carbon atoms the inhibitory properties became much more potent.

Following these results, Papaioannou et al. (2009) used CV1, LNCaP, and C4-2 cells in order to test the effect of AA on the androgen receptor. They found that in the presence of AR agonists dihydrotestosterone (DHT) or R1881 at various concentrations, AA was able to reduce AR-mediated transactivation. To analyze the receptor specificity of AA, various members of the nuclear hormone receptor super family were used for reporter assays. Papaioannou et al. (2009) found that AA inhibits progesterone receptor-B (PR-B) significantly at a concentration of 100 µM, but not 10 µM. AA was also shown to weakly induce estrogen receptor (ER) activity in a dose dependent manner at higher concentrations of AA. Thus, AA at lower concentrations has specific effects on AR and at higher concentrations it can also affect PR-B and ER significantly. Using LNCaP cells and C4-2 cells Papaioannou et al. (2009) found that AA inhibits the expression of prostate specific antigen (PSA) when cells were grown in the presence of either DHT or R1881. Interestingly, AA at a concentration of 100 µM, inhibited growth of C4-2 cells in the absence of an agonist. This observation indicates that AA can inhibit PSA expression in both androgen dependent and androgen independent prostate cancer cells. Taken together, AA can inhibit the AR-mediated transactivation and endogenous androgen-induced gene expression. When cells were grown in the presence of 10 µM AA LNCaP cells and C4-2 cells exhibited
growth inhibition, but PC3 and CV1 cells which lack AR were not inhibited. These results indicate that AA works directly on the AR in a non-toxic manner (Papaionnou et al., 2009).

Using LNCaP cells in a cell invasion assay Papaioannou et al. (2009) discovered that AA inhibits LNCaP invasiveness through the extracellular matrix in the presence of R1881. However, AA had no effect on cell invasion by itself. This may indicate that AA is an AR antagonist even at the level of cell invasion.

In order to identify the molecular action of AA on the androgen receptor Papaioannou et al. (2009) created several AR mutants and found that a mutation in the ligand binding domain (LBD) rendered AA ineffective compared with wild type AR. Whereas N-terminal deletions exhibited no transactivation function and was not affected by AA. The SUMO-mutant of the AR, which lacks binding of corepressors Alien and SMRT, was also shown to be inhibited by AA. Further experiments showed that AA did not recruit corepressors as a mode of action for inhibiting the AR. Competitive hormone binding assays were performed and it was shown that AA was effective in competing for the hormone binding domain (HBD) at a concentration of 10 µM which was effective in both the inhibition of transactivation and growth proliferation. This finding strongly suggests that AA acts on the LBD of AR by binding to it and further assays showed that AA does not decrease the protein levels of AR or endogenous AR in cells as a mode of action (Papaionnou et al. 2009).

AA inhibits the ligand induced translocation of AR to the nucleus in LNCaP cells treated with R1881 and without or with AA prior to cell fractionation to yield nuclear and cytosolic
fractions. The results of Western blot analysis revealed that R1881 alone increased the nuclear fraction of AR, but in the presence of AA the AR remained mostly in the cytosolic fraction. This was confirmed by green fluorescent protein (GFP)-AR staining. Overall these data suggest that AA competes with androgens for the binding to AR and inhibits the nuclear translocation of AR (Papaionnou et al., 2009).

In summary, Schleich et al. (2005) discovered the bioactive compound atraric acid in *P. africanum* bark fractions. AA inhibited AR function in CV1 cells transfected to express human AR and this effect was became more potent when AA was esterified further by 1 or 2 carbon atoms. Papaionnou et al. (2009) found that AA is an AR antagonist that reduced AR-mediated transactivation in the presence of AR agonists, DHT and R1881, by competitive binding of the AR and inhibited nuclear translocation. AA effected PR-B and ER at high concentrations, but only effected AR at lower concentrations. AA inhibited LNCaP invasiveness in the presence of the agonist R1881, but not with AA alone. Lastly, AA inhibited PSA expression and cell growth LNCaP and C4-2 cells, which express AR, but did not inhibit cell growth in PC3 and CV1 cells, which do not express AR.

II.B.1.b. **NBBS**

Schleich et al. (2006) compared the anti-androgenic activity of diverse extracts of *P. africanum* stem bark and compared it with two other non-African plants *Serenoa repens* and *Cucurbita pepo*. They exposed CV1 cells transfected with human androgen receptor gene to the fractions in order to find the fractions that contained anti-androgenic activity. The two most potent androgen receptor inhibitors were the ethanolic and dichloromethane extracts which both
contained the compound N-butylbenzenesulfonamide (NBBS). When the activity of NBBS was compared with the major steroid of *P. africanum*, β-sitosterol, and triterpenic acids, oleonolic acid and ursolic acid, the most potent anti-androgenic effect came from NBBS which inhibited AR-mediated transactivation as well as hormone induction. This lead Schleich *et al.* (2006) to determine the other compounds do not act directly on androgen receptor. Tadenan, a commercially available *P. africanum* supplement, was also found to contain NBBS (Schleich *et al.*, 2006).

A follow up study by Papaioannou *et al.* (2010) studied the effects of NBBS derived from *P. africanum* bark samples and utilized several cell lines including LNCaP, C4-2, PC3, PC3-ARwt, and CV1 cells in an *in vitro* study similar to that of Papaioannou *et al.* (2009). To test for the potency of NBBS, a concentration series of androgen agonists, DHT and R1881, was applied to CV1 cells transfected to express human AR alongside a concentration series of NBBS with addition of 10 pM of R1881. Overall the tests strongly suggest that NBBS is able to repress the transactivation of human AR. Papaioannou *et al.* (2010) also found, using qRT-PCR normalized to beta-actin expression, that 100 µM NBBS was able to repress androgen induced PSA expression in both LNCaP and C4-2 cells (Papaioannou *et al.*, 2010).

Similar to their work with AA in 2009, Papaioannou *et al.* (2010) reported on the specificity of NBBS with other members of the hormone receptor superfamily. It was shown that NBBS targeted both PR-A and PR-B in addition to the AR. However, NBBS had no effect on the glucocorticoid receptor (GR), estrogen receptor (ER), or thyroid receptor. LNCaP cells were grown in the presence of 10 µM and 100 µM of NBBS. Significant growth inhibition was found
at days 5 and 8 days with 100 µM NBBS and day 8 with 10 µM of NBBS. The same growth
assays were run using C4-2 cells, PC3-ARwt cells, and PC3 cells, but only at the highest
concentration of 100 µM NBBS. The C4-2 and PC3-ARwt cells showed a similar inhibition of
cell growth as seen in the LNCaP cells which according to the authors may suggest that NBBS is
able to inhibit androgen-independent prostate cancer cells. Regular PC3 cells however were not
significantly affected by NBBS. This suggests that NBBS specifically inhibits the growth of
prostate cancer cells expressing the mutant or wild-type AR, further supporting its role as an AR-
antagonist. Western blot analysis revealed that NBBS did not reduce the amount of AR protein in
LNCaP cells exposed to even high, 100 µM, concentrations of NBBS for 3 to 7 days. This
suggests that NBBS does affect AR protein stability (Papaioannou et al., 2010).

To determine the specificity of NBBS for the human AR, Papaioannou et al. (2010)
created several AR mutants and found that NBBS was rendered inactive only when the HBD was
deleted. This suggests that the HBD is needed for NBBS mediated inhibition of AR
transactivation. A competitive whole cell binding assay with AR and radioactive labeled
mibolerone were performed. Treatment of AR with increasing amount of NBBS indeed resulted
in a decrease of mibolerone binding at 10 µM and 100 µM, which suggests that NBBS binds to
the HBD of AR (Papaioannou et al., 2010). The authors mention experiments indicating that
NBBS does not function by recruiting corepressors, although the actual data was not presented.
To determine whether NBBS could inhibit nuclear translocation of AR, LNCaP cells were
treated with R1881 and with or without NBBS prior to cell fractionation to yield nuclear and
cytosolic fractions analyzed by Western blot. R1881 alone increased the nuclear fraction of AR,
but in the presence of NBBS the AR remained mostly in the cystolic fraction. This was
confirmed by GFP tagged AR staining. Overall these data suggest that NBBS competes with androgens for the binding to AR and inhibits the nuclear translocation of AR (Papaioannou et al., 2010).

In summary, Schleich et al. (2006) found the bioactive compound NBBS in *P. africanum* bark fractions. NBBS expressed more potent anti-androgenic effects than β-siteosterol, oleonic acid, and ursolic acid. According to Schleich et al. (2006) NBBS inhibited nuclear translocation and hormone induction. Papaioannou et al. (2010) found that NBBS repressed androgen induced PSA expression and specifically inhibited growth of LNCaP, PC3-wt, and C4-2 cells which express AR, but did not inhibit growth in PC3 and CV1 cells, which do not express AR. NBBS was shown to be an AR antagonist that repressed transactivation of AR in the presence of AR agonist R1881 by competitive binding of the HBD and preventing nuclear translocation. Lastly, Papaioannou et al. (2010) showed that NBBS targets PR-A and PR-B in addition to AR.

II.B.1.c. **In vitro and in vivo studies with *P. africanum***

The most comprehensive study on the effects of *P. africanum* was published by Shenouda et al. (2007) who performed not only in vitro studies using PC3 and LNCaP cells, but an in vivo study using TRAMP mice as well. The major effects of *P. africanum* have been attributed to its main active ingredient, β-sitosterol which is present in high concentrations in *P. africanum* extract. In some of the in vitro experiments comparisons were made between an ethanolic extract of *P. africanum* and synthetic β-sitosterol from Sigma (St. Louis, MO). Shenouda et al. (2007) prepared ethanolic extracts (30% Weight/Volume) for cell culture
experiments and β-sitosterol from Sigma loaded on a cyclodextrin (CD) vehicle to improve bioavailability to the cultured cells.

In the in vitro studies, both cell lines, PC3 and LNCaP, experienced growth inhibition when exposed to the ethanolic extract with an IC_{50} of 2.5 µL/mL. Beta-sitosterol inhibited growth of LNCaP cells, but had no effect on PC3 cells. Cell proliferation of PC3 cells and LNCaP cells exposed to 2.5 µL/mL of extract using thymidine incorporation assay showed that from day 1 through day 3 of culture the *P. africanum* extract inhibited growth by 50% at a concentration of 2.5 µL/mL. Flow cytometry was used to analyze PC3 cells and LNCaP cells exposed to 2.5 µL/mL of the extract for 72 hours. Both cell lines had a significantly increased number of cells in S phase compared to control cells. They also showed a significant increase in apoptosis in both cell lines using a TUNEL assay.

Using the competitive [³H]-estradiol ligand-binding assay, the *P. africanum* extract and β-sitosterol were tested for their potential to displace bound estradiol in mouse uterine cytosol. Shenouda *et al.* (2007) showed that 5 µM of β-sitosterol did not compete for binding, but 5 µL/mL of the extract displaced >70% of the ³H-estradiol. Similar results were obtained when testing for their potential to displace bound DHT in LNCaP cells cytosol using a competitive [³H]-DHT ligand-binding assay. It was shown that 5 µM of β-sitosterol did not compete for binding, whereas 5 µL/mL of the extract displaced >60% of the ³H-DHT. Western immunoblot was used to quantify the expression levels of both ERα and PKC-α in PC-3 and LNCaP cells treated with 0, 1, and 2.5 µL/mL of the 30% *P. africanum* extract. PC3 cells displayed a dose dependent inhibition of ERα protein expression. LNCaP cells displayed an inhibition of PKC-α
protein at 2.5 μL/mL, but PC3 cells showed no inhibition of PKC-α. These results suggest that *P. africanum* extract may be an antagonist for the AR that works through competitive binding and contains other chemical components that are important for its inhibitory function in cells that do not contain an AR. These experiments also show that β-sitosterol is not the most important compound in *P. africanum* for prostate cancer cell inhibition or androgen displacement.

In the *in vivo* studies, Shenouda *et al.* (2007) used the TRAMP mouse model, in which 60-85% develops prostate cancer within 5 months. At 6 weeks male TRAMP mice were assigned to either casein-based diet (AIN 93G) or the same diet to which dry powdered *P. africanum* was added. The concentration of *P. africanum* was chosen in reference to the human consumption dose used for treatment of BPH. The formulation contained 0.128 grams *P. africanum*/kg diet and the mice were given continuous access to the diet. The mice were monitored weekly for body weight and tumor burden and were euthanized after 5 months. At termination of the experiment the reproductive tracts of the animals were harvested and testes and prostate were weighed. Prostate lesions were graded in a blinded fashion according to morphologic criteria established for mouse models of prostate cancer. Prostate cancer developed in fewer animals fed the *P. africanum* diet for 5 months (35%) in comparison to animals on the casein diet (62.5%) (Shenouda *et al.*, 2007). The largest effect was on the incidence of well differentiated carcinomas; only 20% of *P. africanum* fed mice presented this morphology compared to 50% of the casein fed mice. However, there was no significant effect on the incidence of poorly differentiated “neuro-endocrine like carcinomas”. There were no significant differences between the two groups in body weight, weight of the reproductive tract, testes, and prostate.
In the summary, *in vitro* Shenouda et al. (2007) study found that the growth of PC3 and LNCaP cells was inhibited when exposed to ethanolic extract of *P. africanum* with an IC$_{50}$ of 2.5 µL/mL. Flow cytometry showed that at 2.5 µL/mL of the ethanolic extract there was a significant increase in the number of PC3 and LNCaP cells in the S phase and TUNEL assays showed a significant increase in apoptosis. Ethanolic extract of *P. africanum* at 5 µL/mL showed >70% displacement in mouse uterine cytosol and >60% displacement in LNCaP cells of [³H]-estradiol and [³H]-DHT respectively in ligand-binding assays. These results suggest that *P. africanum* extract may be an antagonist for the AR and possibly work through other mechanisms. These results suggest that *P. africanum* extract may be an antagonist for the AR that works through competitive binding and contains other chemical components that are important for its inhibitory function in cells that do not contain an AR. These experiments also show that β-sitosterol is not the most important compound in *P. africanum* for prostate cancer cell inhibition or androgen displacement. *In vivo* experiments showed in TRAMP mice fed a diet containing *P. africanum* only 35% of the animals developed prostate cancer of which 20% had well differentiated carcinomas. Compare that with control animals in which 62.5% developed prostate cancer of which 50% had well differentiated carcinomas. These results demonstrate the anticancer effects of *P. africanum* both in vitro and in vivo are significant although further investigation is needed to determine the bioactive compounds contained in the extract.

II.B.2 *Salsola tuberculatiformis*

*S. tuberculatiformis* has long been used in Africa as an oral contraceptive. It is interesting biological activity in pregnant sheep, which causes extended gestation during pregnancy in a condition known as big lamb disease, and its use as a contraceptive for humans are what caught
researchers’ attention. Investigations into the activity of this plant have been conducted since the 1960’s and biochemical investigations lead to the isolation of 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammonium chloride also known as Compound A (CpdA) (Swart et al., 2003). CpdA, a non-steroidal compound, is a stable analog of a highly labile hydroxyphenyl aziridine precursor that is found in *S. tuberculatiformis* (Swart et al., 2003). CpdA has demonstrated a variety of effects such as inhibition of cytochrome p450c11 to displacing endogenous steroid from corticosteroid-binding globulin (Swart et al., 2003). Anti-androgenic effects of CpdA and effects on prostate cancer cells have been reported by Tanner et al. (2003) and Yamelyanov et al. (2008; 2012) and are summarized below.

II.B.2.a. **Studies by Tanner et al. (2003)**

Tanner et al. (2003) were the first to report that CpdA has anti-androgenic properties. They determined the ability of CpdA to repress ligand induced activation of specific and nonspecific androgen responsive reporter genes in CV1 cells transfected with human AR. They used luciferase reporter genes attached to plasmids; the nonspecific androgen regulatory plasmids were mouse mammary tumor virus (pMTV) and pC3(1)-TATA-luc which contains a 204-base pair PvuII/SstI fragment of the first intron of the C3(1) gene of prostate binding protein. The specific androgen regulatory plasmids contained the *slp* enhancer (pSLP-TATA-Luc), the *sc* enhancer (pSC-TATA-Luc), or the *pb* proximal promoter (pPB-Luc). When these reporters were induced by 1 nM of R1881, they were significantly repressed by 10 mM of CpdA and pSLP-TATA-Luc was even repressed by 1 mM of CpdA.
To determine the specificity of CpdA with other members of the hormone receptor superfamily, COS-7 cells were transfected with glucocorticoid transcriptional response element (GRE)-driven reporter constructs and the relevant receptor expression vectors. Ten mM of CpdA significantly repressed AR and PR in the presence of 0.1, 1, and 10 nM of R1881 for AR and progesterone for PR 10 mM of CpdA by 50% to 30%. However, CpdA had no effect on the ligand induced GR or mineralocorticoid receptor (MR).

Whole cell binding assays using COS-7 cells transfected with human AR were used to determine whether CpdA competes with [3H] – mibolerone for binding to the LBD of the AR. CpdA did not compete with [3H] – mibolerone when the cells were incubated for 24 hours in the presence of CpdA or in a cell free system. Interestingly there was a 10-20% decrease in specific binding of [3H] – mibolerone in the presence of 10 nM CpdA and higher concentrations.

A mammalian two-hybrid assay was used to determine whether CpdA can interfere with the N-Terminal Domain (NTD)/C-Terminal Domain (CTD) interaction of the human AR, which is essential for optimal AR function. COS-7 cells were transfected with a GRE-driven reporter construct and expression vectors encoding regions of the human AR including the DNA binding domain (DBD), LBD, and NTD fused to a V16 activation domain. CpdA alone did not induce transcription, but the N/C-interaction that occurs in the presence of 0.1 µM DHT was partially suppressed by the addition of 1 mM CpdA and completely repressed by 10 mM CpdA. This indicates that CpdA prevents the AR from adopting the stable conformation that is necessary to be transcriptionally active.
The p160 nuclear receptor co-activators, such as steroid receptor co-activator-1 (SRC1), interact with the NTD in a ligand independent manner and the LBD in a ligand dependent fashion and potentially play a role in bridging the N/C-interaction. To determine whether CpdA interferes with the recruitment of SRC1 to the AR a mammalian two-hybrid assay was used as described above with a SRC1 expression vector instead of the NTD of the human AR. Ten mM of CpdA weakly, but significantly inhibited the DHT induced activation of LBD by SRC1. To investigate the androgen independent interaction in the NTD, COS-7 cells were transfected with a reporter gene driven by five Gal4 response elements, Gal4-DBD and SRC1 fusion protein and NTD expression vectors. CpdA did not interfere with the interaction between SRC1 and the NTD. Similarly, COS-7 cells were transfected with the Gal4 reporter and a Gal4-DBD plus NTD fusion protein expression vector to determine whether CpdA inhibited the recruitment of the basal transcription machinery to the NTD, which allows the fusion protein to activate transcription. CpdA had no effect on transcription indicating, along with previous results, that CpdA does not interact with the NTD in any way that could interfere with the establishment of a transcriptionally competent complex.

After it was established that CpdA can repress androgen induced transcriptional activation in transfected cells, the effects of CpdA were studied in LNCaP cells, which have an endogenous AR. The effect of CpdA on PSA expression was studied using a Western immunoblot approach since the PSA gene can be stimulated by androgen. Ten mM of CpdA repressed even basal levels of PSA when added alone and in the presence of 1 nM of R1881; it significantly repressed PSA expression compared to R1881 alone. In the presence of R1881 alone, AR protein levels are repressed, but this effect was not seen when CpdA was added
alongside R1881. Also proliferation was significantly inhibited when LNCaP cells were grown in the presence of CpdA alone or CpdA plus R1881. However in COS-7, cells which lack AR, CpdA had no effect on growth under the same conditions, which indicates that the anti-proliferative effect can be solely contributed to the AR-mediated anti-androgenic action of CpdA.

In summary, Tanner et al. (2003) found that CpdA can repress non-specific and specific androgen regulatory plasmids when stimulated by R1881. CpdA repressed AR and PR by 50% and 30% respectively in the presence of R1881. Using mammalian two-hybrid assays, CpdA was found to inhibit N/C interactions that occur in AR stimulated by DHT which are needed for AR to be transcriptionally active. Further investigation revealed that CpdA inhibited LBD activation, but did not effect NTD in any way that would interfere with the establishment of transcriptionally competent complex. Lastly, Western blot revealed that CpdA suppressed PSA levels and significantly inhibited cell growth in LNCaP cells, which express AR, but did not suppress growth in COS-7 cells, which do not express AR.

II.B.2.b. Studies by Yemlyanov et al. (2008; 2012)

Subsequent to the Tanner et al. (2003) study, it was reported that CpdA may not only inhibit the AR, but also mediate gene inhibitory effects by activating the GR as well (Bosscher et al., 2005). Yemelyanov et al. (2008) addressed this question by comparing the docking of CpdA into the LBD of the AR and the GR with how steroid hormones dock into the binding pockets of these receptors. In addition, they determined the effects of CpdA on AR and GR function in prostate cancer cells and on growth and apoptosis of various prostate cancer cell lines that differ
in the presence of AR and GR. A panel of cell lines was used, including RWPE-1 cells (AR_{low}/
GR_{low}), PC3 and DU145 cells (GR^+/ AR^-), LNCAP cells (GR^-/ AR^+) and LNCaP-GR cells
(GR^+/AR^-). RWPE-1 cells are epithelial cells derived from the peripheral zone of a
histologically normal adult human prostate. DU145 is a human cell line, derived from a brain
metastasis of prostate cancer that is not hormone sensitive and does not express PSA.

Virtual docking comparisons were performed of CpdA with relevant steroids,
dexamethasone (Dex) for the GR and DHT for the AR, using crystal structures of GR and AR
LBDs obtained from the Protein Bank Database website and Molegro virtual docking software.
CpdA was found to share binding pockets within the LBDs of GR and AR with the
corresponding steroids and it also forms H-bonds with several amino acids that are involved in
generating bonds with the steroids. These results suggest that CpdA may compete with steroids
for binding to GR and AR, but Tanner et al. (2003) reported that competition for AR binding
between CpdA and mibolerone was only weak. To check these findings, Yemelyanov et al.
(2008) performed competitive binding assays using cystosol fractions from LNCAP cells (GR^-/
AR^+) and DU145 cells (GR^+/ AR^-). The cells were incubated for 24 hours at 4°C with 10 nMol/L
[^3]H-Dex binding to GR by 85% and[^3]H-DHT binding to AR by 30% compared with unlabeled
hormones, which inhibited binding by nearly 100%.

Next, experiments were performed to assess the effect of CpdA on the function of
endogenous GR and AR using all prostate cell lines listed above. Western blot analysis of whole
cell protein extracts was used to confirm the AR and GR protein levels in each cell line. Because
LNCaP-GR was the only cell line with a significant amount of both receptors, it was the most extensively studied for the effects of CpdA on GR and AR function.

LNCaP-GR cells were treated for 16 hours with 1 µMol/L DHT or 10 µMol/L CpdA for immunofluorescence staining and Western blot analysis of nuclear translocation of AR and GR. AR nuclear translocation appeared similar for CpdA and DHT. The same test was conducted for GR using DU145 cells and PC3 cells which have endogenous GR and LNCaP-GR cells which have an exogenous GR. When the cells were treated for 16 hours with 10 µMol/L fluocinolone acetonide (FA), which is a GR agonist, or CpdA; CpdA induced nuclear translocation of the GR, but to a significantly lesser extent than FA did.

LNCaP-GR cells transfected with MMTV-\textit{Firefly} luciferase and \textit{Renilla} luciferase reference reporter were treated with or without 0.1, 1, and 10 µMol/L Cpd and 10 µMol/L of the AR antagonist Casodex (Cdx) with or without 1 µMol/L DHT for 32 hours. CpdA inhibited AR-DNA binding and transcriptional activity in a concentration dependent manner both alone and in the presence of DHT and its effect was similar to that of Cdx. For GR tests, cells were transfected with TAT-\textit{Firefly} and \textit{Renilla} luciferase reference reporters and treated with 1 µMol/L FA with or without 0.1, 1, and 10 µMol/L Cpd and 10 µMol/L of the GR antagonist RU486 with or without 1 µMol/L FA for 32 hours. CpdA inhibited GR-DNA binding and transcriptional activity in a concentration dependent manner both alone and in the presence of FA similar to the effect of RU486.
The GR exhibits DNA independent mechanisms of gene regulation through negative interactions between GR and other transcription factors so Yemelyanov et al. (2008) next investigated how CpdA affects GR repression of other transcriptional factors. To do this, LNCaP-GR cells were transfected with luciferase reporters for a range of transcription factors. Of these, NF-κB, AP-1, Ets-1, Elk-1, SRF, CRE, and NFATc, were reduced in cells treated with CpdA for 32 hours when compared to untreated cells. Five of seven were inhibited by both FA and CpdA.

Because anti-inflammatory and possibly tumor suppressor effects can be caused by glucocorticoids through a negative interaction between the GR and NF-κB and AP-1, Yemelyanov et al. (2008) induced NF-κB by transfecting DU145, PC3, and LNCaP-GR cells with IκB kinase β (IKKβ), which is an upstream activating kinase. CpdA inhibited both basal and inducible NF-κB activity. When AP-1 was induced with either IL-1 or TNF-α in PC3 and LNCaP-GR cells, CpdA also inhibited both basal and induced levels of AP-1.

Nuclear translocation of NF-κB protein p65 and AP-1 protein c-Jun was analyzed in LNCaP-GR cells using immunofluorescence or Western blot. For NF-κB cells were treated with either 10 µMol/L CpdA or 1 µMol/L FA for 48 hours and stimulated them with 10 ng/mL TNF-α for 30 minutes. For AP-1 cells were stimulated with IL-1 instead. CpdA inhibited both NFκB and AP-1 nuclear translocation causing retention in the cytoplasm, a result that was not caused by FA. In aggregate, the results of Yemelyanov et al. (2008) indicate that CpdA possesses antiandrogenic properties and functions as a selective GR modulator that causes repression of transcription factors associated with GR activity.
Given these findings, Yemelyanov et al. (2008) wanted to examine the effects of CpdA on cell proliferation. All of the cell lines listed above were grown for 5 days in the presence of increasing concentrations of CpdA, ranging from $10^{-9}$ to $2\times10^{-5}$ mol/L. All cell lines exhibited growth inhibition at concentrations of 1 µMol/L and 10 µMol/L of CpdA. LNCaP-GR cells exhibited significantly more growth inhibition than LNCaP-V cells when grown in 10 µMol/L of CpdA for 12 days, indicating that GR plays an important role as a mediator of CpdA’s cytotoxic activity. The greatest effect was on PC3 and DU145 cells inhibiting growth by 70% - 90% compared with control cells, which was a much greater effect than FA ($10^{-6}$ mol/L) and RU846 ($10^{-5}$ mol/L). A colony forming assay using LNCaP, LNCaP-GR, and LNCaP-V cells, which are LNCaP cells infected with an empty virus, grown in 1% soft agar and treated with 10 µMol/L CpdA for 2 weeks. CpdA significantly reduced anchorage independent growth compared to control cells.

The effectiveness of CpdA against GR was tested using a short interfering RNA (siRNA) validated against a negative control inactive siRNA labeled with Cy3 in LNCaP-GR and PC3 cells. Blockage of the GR in both cell lines by the siRNA lead to a significantly loss of sensitivity to CpdA. To investigate the role of CpdA effects LNCaP cells were cloned that had a low AR expression and no GR. When exposed to 10 µMol/L CpdA for 10 days this clone exhibited almost complete resistance to CpdA. RWPE-1 cells which have low levels of both receptors also exhibited resistance to growth inhibitory effects of CpdA.

The proapoptotic potential of CpdA was evaluated using DU145 and PC3 cells which are resistant to several proapoptotic stimuli. The cells were treated with 1 µMol/L FA or 10 µMol/L
CpdA for 2 to 8 days at which time apoptosis was induced by exposing cells to 10 ng/mL TNF-α for 24 hours. Western blot analysis of PARP cleavage at that time revealed that long-term exposure to CpdA without TNF-α stimulation induced significant apoptosis. However CpdA treatment for only 2 or 3 days did not induce much apoptosis, but significantly increased the sensitivity of the cells to TNF-α induced apoptosis. When cells were treated for 3 and 6 days as described above to induce apoptosis and were analyzed using the ApoAlert Caspase Assay apoptosis induced by CpdA appeared to be caspase dependant and CpdA induced expression of several caspases, especially caspase-2 and caspase-3.

In summary, virtual docking comparisons and competitive binding assays revealed that CpdA competes with relevant steroids in both AR and GR binding pockets within the LBDs. CpdA was also shown to inhibit AR-DNA and GR-DNA binding and transcriptional activity in a dose dependent manner. There is reduced recruitment of several GR induced transcriptional factors in the presence of CpdA compared with control cells, most notably NF-κB and Ap-1. All cell lines experienced inhibited growth in the presence of CpdA with the greatest effect being on androgen independent cell lines, PC3 and DU145 cells, in which caspase pathways were activated by CpdA to induce apoptosis. Lastly, cell lines with low or no GR were not affected by CpdA. Overall, the findings of Yemelyanov et al. (2008) provided evidence that AR and GR-mediated signaling is critically involved in the cytotoxic effect of CpdA on prostate cancer cells in vitro.

In a follow up study, Yemelyanov et al. (2012) further investigated CpdA’s properties as a dual target steroid receptor modulator of GR and AR, its effects on prostate cancer cells, and
the effect of the proteasome inhibitor Bortezomib (BZ) on AR and GR stability and function in the presence of CpdA. First, they validated their proposed concept of treating prostate cancer through dual targeting of AR/GR by analyzing the expression of these receptors in the tissues of prostate cancer patients. Previous studies had revealed that AR is highly expressed in prostate cancer regardless of stage and that prostate cancer GR expression is lost in many untreated patients. Because the GR expression patients treated with androgen ablation or chemotherapy is unknown they analyzed benign prostate tissue, prostate carcinoma from untreated patients, and prostate carcinoma from patients treated with androgen ablation or chemotherapy using immunostaining. GR was expressed at high levels in treated patients with nuclear translocation in 60% of treated tumors regardless of Gleason score and both receptors were co-expressed in 56% of treated patients. This observation indicates considerable potential of therapy targeted at both AR and GR for advanced PC and demonstrated that this approach is feasible.

Next the regulation of AR and GR by BZ and its proapoptotic effects were assessed. LNCaP, PC3, DU145, and LNCaP-GR cells were exposed to 1, 0.1, and 0.01 µM of BZ for 24 hours and whole cell lysates were analyzed using Western blot. BZ significantly downregulated AR and upregulated GR in a concentration dependent manner. LNCaP and LNCaP-GR cells were treated with 1, 0.1, 0.01, 0.001 µM of BZ for 32 hours and analyzed for PARP cleavage using Western blot. In both cell lines downregulation of AR was found and in LNCaP-GR cells there was significant upregulation of GR in a dose dependant manner. The upregulation of GR in LNCaP-GR cells was directly correlated with levels of BZ induced apoptosis.
To test the significance of the GR for BZ-induced apoptosis LNCaP-GR cells were transfected with a small hairpin RNA (shRNA) that knocked down GR expression (LNCaP-GR-shRNA cells) using as a control a LNCaP-GR cell line in which GR expression is not silenced (LNCaP-GR-shNS cells). When LNCaP-GR-shRNA and LNCaP-GR-shNS cells were treated with 0.01 µM BZ for 32 hours and PARP cleavage was analyzed using Western blot BZ-induced apoptosis was significantly attenuated when GR was downregulated in the LNCaP-GR-shRNA cells. Yemelyanov et al. (2012) concluded that the results suggest that both AR and GR are critical to the therapeutic effect of BZ.

Since both CpdA and BZ affect AR and GR in a similar way, Yemelyanov et al. (2012) combined the two to evaluate whether BZ could enhance the effects of CpdA and whether combining them would allow decreasing their effective doses. To determine the optimal concentrations for combined treatment they assessed dose dependent cytostatic and growth inhibitory effects on LNCaP-GR cells exposed to concentrations of CpdA or BZ between 10 µM and 0.0001 µM. A 20-25% inhibition of growth was caused by 0.1 µM CpdA and 0.01 µM BZ.

A series of experiments were then performed assessing the combined effects of 0.5 µM CpdA and 0.01 µM BZ in LNCaP-V (AR+/GR−), LNCaP-GR-shRNA, and LNCaP-GR-shNS cells exposed for 4 days. The cells that expressed GR experienced greater growth inhibition than those that expressing no GR. LNCaP-GR-shRNA cells which experienced 50-60% GR knockdown displayed less growth inhibition than LNCaP-GR-shNS cells.
Experiments were next carried out by Yemelyanov et al. (2012) to assess growth inhibition and apoptosis where LNCaP-GR, DU145, PC3, LNCaP-GR-shRNA, and LNCaP-GR-shNS cells were exposed to 0.5 µM CpdA, 0.01 µM BZ, 0.1 µM FA, or combinations of BZ plus FA and BZ plus CpdA for 24 hours. Apoptosis was measured in three ways: 1) PARP cleavage using Western blot, 2) immunofluorescent microscopy with antibodies against cleaved PARP, and 3) quantitative analysis of cleaved PARP staining and propidium iodide (PI) incorporation using a Celligo cell analyzer. Growth inhibition and apoptosis in cells exposed to the CpdA plus BZ combination was much greater than with the FA plus BZ combination. The knockdown of GR by LNCaP-GR-shRNA not only decreased the growth inhibition of these cells, but also diminished the apoptosis caused by CpdA plus BZ combination. Yemelyanov et al. (2012) concluded that these results suggest that GR strongly contributes to the cytotoxic and cytostatic effects of this combined treatment. When caspase activity in LNCaP-GR cells exposed to the compounds as described above for 16 hours was analyzed using the ApoAlert Assay, CpdA plus BZ activated caspases more efficiently than FA plus BZ. Taken together these data of Yemelyanov et al. (2012) indicate significant anticancer effects of combined CpdA and BZ at concentrations that had minimal or no effects when these treatments were applied alone.

Yemelyanov et al. (2012) next investigated whether BZ enhances the effects of CpdA as an AR inhibitor and a selective modulator of GR. Luciferase assays and electrophoretic mobility shift assays revealed that BZ downregulated AR activity in LNCaP cells and LNCaP-GR cells which was reflected in AR protein decreases in Western blot assays. However, DHT protected AR from down regulation by BZ and restored most of its functional activity whereas CpdA did not prevent AR degradation or restore AR function. These same assays showed that BZ weakly
induced GR nuclear translocation although the increase of GR activity was only modest and still significantly lower than caused by FA. Treatment of LNCaP and LNCaP-GR cells with FA plus BZ combined caused nuclear accumulation of fully functional GR with high DNA binding and transcriptional activity whereas CpdA plus BZ resulted in low DNA binding and transactivation.

To determine how BZ combined with different GR ligands affects the expression of GR target genes promoter screening for GR binding sites was used for the AR regulator genes FKB5, pro-survival kinase SGK1, and BIP/HSPA5 and CHOP/GADD153 which are endoplasmic reticulum stress (ERS) regulators important for BZ induced apoptosis. Semiquantitative RT-PCR analysis indicated that expression of SGK1 and FKB5 was not affected or only minimally induced by CpdA or CpdA plus BZ, whereas FA or FA plus BZ combined markedly induced the expression of these genes. They found that BIP was activated by BZ and further induced by the FA plus BZ combination and CHOP/GADD153, which is a known target of BZ, was activated by CpdA. The combination of CpdA plus BZ increased CHOP expression whereas the combination of FA with BZ decreased its expression. GR binding to SGK1, BIP, and CHOP promoter regions was shown in treated LNCaP-GR cells using chromatin immunoprecipitation (ChIP) assays. GR loading in the presence of CpdA with or without BZ was weak at the SGK1 promoter, whereas at the CHOP/GADD153 promoter the opposite effect was found and GR loading was greatly increased in the presence of FA with or without BZ.

Because Yemelyanov et al. (2012) had shown that CpdA induces GR transrepression of other transcription factors such as AP-1 and NFκB in their previous study. They used a luciferase reporter assay to evaluate the combined effects of CpdA plus BZ in LNCaP-V, PC3, and
LNCaP-GR cells and found that CpdA plus BZ greatly inhibited basal and IL-6 induced AP-1 and NFκB dependent transcription. The suppression of transcription factor activity was greater in LNCaP-GR cells than LNCaP or LNCaP-V cells suggesting that GR plays an important role in the inhibition of AP-1 and NFκB by CpdA plus BZ.

Yemelyanov *et al.* (2012) next evaluated the GR dependent ERS response which is critical for the anticancer effects of BZ. Using Western blot analysis of LNCaP-GR cells, they showed that CpdA plus BZ induced ERS as indicated by upregulation of BIP/HSPA5 and ATF2 phosphorylation and nuclear translocation, and by stimulation of CHOP/GADD153. Western blot also revealed an upregulation of nuclear and cytoplasmic CHOP in LNCaP-GR, LNCaP, and PC3 cells treated with BZ with or without CpdA with the GR positive cells (LNCaP-GR and PC3) having the greatest increase in CHOP expression. However, when the cells were treated with FA plus BZ or DHT plus BZ there was a downregulation of CHOP in comparison with CpdA plus BZ. These results indicate the importance of GR in CHOP regulation and show that androgens and glucocorticoids can offset ERS in prostate cancer cells (Yemelyanov *et al.*, 2012).

To show the importance of CHOP for the combined CpdA plus BZ effects on prostate cancer cells, Yemelyanov *et al.* (2012) ran a series of experiments with LNCaP-GR, LNCaP-GR-CHOP, and LNCaP-GR-shCHOP cells, because LNCaP-GR-CHOP cells have an increased CHOP expression whereas LNCaP-GR-shCHOP have an shRNA that inhibits CHOP expression. Immunostaining of GR and CHOP/GADD153 and Western blot analysis for CHOP expression and PARP cleavage were used to analyze cells treated with CpdA plus. Growth inhibition and induction of apoptosis was significantly higher in LNCaP-GR-CHOP cells compared with
LNCaP-GR cells and these effects were greatly reduced in LNCaP-GR-shCHOP cells (Yemelyanov et al., 2012).

In summary, Yemelyanov et al. (2012) validated this study in prostate cancer patients in which 56% expressed both GR and AR. BZ was found to upregulate GR, which is directly correlated with BZ induced apoptosis, and downregulate AR. BZ and CpdA combination exhibited greater growth inhibitory effects and caspase inhibition than either compound alone and the combined effects were greater in cells that express GR than those that do not express GR. CHOP which is important for growth inhibition and apoptosis was upregualted cells exposed to BZ plus CpdA and the upregulation was even higher in cells expressing GR. Overall, this study shows that GR and AR are important for the cell regulatory effects of BZ and CpdA.

II.C. Plants that require further investigation

While some African plants such as those described above have been the focus of extensive research, some promising results have been obtained about lesser well-known plants. The plants described below have shown to have some effects on prostate cancer cells and may have potential to be novel treatments for prostate cancer. Unfortunately many of these studies do not have any follow-up studies that further investigated their potential to be anti-cancer agents.

II.C.1. Ocimum gratissimum

Ocimum gratissimum has been used in Nigeria for not only food, but also as a medicine for treatment of diarrhea, upset stomach, and hemorrhoids (Ekunwe et al., 2010). Ekunwe et al. (2010) had reported previous studies with aqueous extracts made from O. gratissimum on
bladder, colon, liver, and prostate cancer cells (data could not be found) that prompted them to hypothesize that it may harbor cancer fighting compounds. In the study summarized here partially purified extracts made from *O. gratissimum* were investigated for their anti-proliferative activity *in vitro* using PC3 cells. An aqueous extract and a crude ethanol extraction of dried, powdered *O. gratissimum* leaves were studied. The crude ethanol extraction was fractionated into four parts P1-4. P3 and P4 were further fractionated into P3-1, P3-2, P4-1, and P4-2.

Ekunwe *et al.* (2010) determined the anti-proliferative effects of these extracts on PC3 cells using a [*³H*]-thymidine incorporation assay. Cells were seeded in 6-well tissue culture plates and extracts were added when the cells reached 60-65% confluence at concentrations of 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 12.0, and 16.0 mg/mL for 18 hours. Cells were then labeled with [*³H*]-thymidine and incubated for 4–6 hours. Untreated cells grown in control medium and control medium containing 0.8% ethanol served as negative controls for aqueous and crude ethanol extracts respectively.

The aqueous extract of *O. gratissimum* leaf inhibited proliferation of PC3 cells in a concentration dependent manner. All but one (P1-2) of the partially purified fractions from the crude ethanol fraction yielded significant inhibitory effects at 1.6 mg/mL. The fractions P2, P3-2, and P4-2 exhibited the most significant inhibition of PC3 cell growth. When the aqueous extract, crude ethanolic extract, P2, P3-2, and P4-2 were compared at a concentration of 1.61 mg/mL, the partially purified fractions all appeared to be significantly more effective than the aqueous extract and the crude ethanolic extract exhibited the poorest results.
While these results indicate that *O. gratissimum* exhibits anti-proliferative effects on PC3 cells, they only provide limited information regarding this herb. Although Ekunwe *et al.* (2010) discovered several fractions that had significant anti-proliferative activity and recorded their spectral peaks, but they did not actually identify the compounds present in those fractions. They also only used the PC3 cell line, an androgen insensitive cell line, without comparing the results to an androgen sensitive cell line such as LNCaP cells. In most studies that involve African plants only one cell line is used, unfortunately, a common, but limiting trend found in this kind of research as will be discussed later.

II.C.2. *Strychnos usambarensis*

*S. usambarensis* is a small tree that is widespread in Africa and has been shown to have strong antimalarial effects as well as cytotoxic effects on several cancer cell lines, but it is not used in Africa as a medication due to its toxicity (Alvarez Cruz, 2008). Medical research is being conducted with various compounds found in the bark, roots, leaves, and seeds of *S. usambarensis* that have shown to have these interesting effects against cancer (Alvarez Cruz, 2008). While the focus of the following study by Saidou Balde *et al.* (2010) was not prostate cancer, it yielded interesting results relating to growth inhibition of PC3 cells when exposed to a compound found in *S. usambarensis*.

The effects of isostrychnopentamine (ISP) derived from the *S. usambarensis* has been shown by Frédérich *et al.* (2003, 2004) to have anti-malarial properties that are very potent and be capable of activating caspase-3 and -9 in human colon cancer cells thereby inducing apoptosis. Saidou Balde *et al.* (2010) studied the effects of ISP on apoptosis resistant
glioblastoma cells (U373 GBM) and non-small cell lung cancer cells (A549 NSCLC), using PC3 cells lines as a positive control because they claimed that PC3 cells are more susceptible to apoptosis. Using the MTT colorimetric assay to assess growth, they grew the cells in the presence or absence of ISP for 3 days at concentrations ranging between 1 nM and 10 μM and found that the IC$_{50}$ inhibitory concentration was 0.8 μM for U373 GBM and A549 NSCLC cells and 2.5 μM in PC-3 cells. They then used computer assisted phase-contrast microscopy (aka quantitative videomicroscopy) to confirm the antitumor effects of ISP. PC3 cells were incubated at their IC$_{50}$ concentration of ISP (2.5 μM) while the U373 GBM cells and A549 NSCLC cells were incubated at five times their IC$_{50}$ (4 μM). Cell growth was determined by the ratio between the number of cells counted in the last and first frames. The cells were counted at 6, 12, 24, and 48 hours of exposure. Increasing the concentration of ISP completely inhibited growth of U373 GBM cells at 12 hours and A549 NSCLC cells at 24 hours, whereas growth inhibition in PC3 cells did not take place until after 24 hours of exposure. It had previously been shown that U373 GBM cells are resistant to pro-apoptotic compounds even when in the presence of the compound at 10 times its IC$_{50}$, but apoptosis was induced by ISP in these cells at just 4 μM killing nearly 100% of the U373 GBM cells.

To determine whether ISP is a pro-apoptotic compound in U373 GBM and A549 NSCLC cells these cells were exposed to the IC$_{50}$ concentration of ISP and analyzed using flow cytometry at 48 and 72 hours of exposure. Most U373 GBM cells underwent apoptosis, while most A549 NSCLC cells did not undergo apoptosis which lead the investigators to hypothesize this may be related to much higher energy consumption by U373 GBM cells compared to A549 NSCLC cells.
In summary, isostrychnopentamine (ISP) is a bioactive constituent of *S. usambarensis* that has been shown to have proapoptotic and growth inhibitory effects on human PC-3 prostate cancer cells.

II.C.3. *Solanum erianthum* and *Solanum macranthum*

*S. erianthum* is a bush found in West Africa and worldwide in tropical areas and has a variety of medical applications while *S. macranthum* is a mostly ornamental tree (Modise and Mogotsi, 2008; Essien *et al.* 2012). Leaves and other parts of this bush are used in West Africa for their diuretic and purgative effects that may be beneficial to treat venereal diseases and other ailments and the leaves are even used to induce abortion (Modise and Mogotsi, 2008; Burkill, 2000). Essien *et al.* (2012) analyzed the chemical constituents and biological activities of the volatile oils from the leaves and fruits of *S. erianthum* and *S. macranthum*. The volatile oils were extracted and analyzed using gas chromatography-mass spectrometry. The major components of *S. erianthum* leaf oil were monoterpenes while sesquiterpenoids were the major component of the fruit oil. The major components of *S. macranthum* leaf oil were the diterpenoid, (E)-phytol, and fatty acids while sesquiterpenes made up the major portion of the fruit oil.

Once the composition of the volatile oils was known, Essien *et al.* (2012) tested the oils on Hs578T human breast ductal carcinoma and PC3 cells to assess their cytotoxic effects. Hs578T cells were exposed to 250 μg/mL of the oils and PC3 cells to 100 μg/mL of the oils for 48 hours and then the kill rates were established using the cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay. *S. erianthum* leaf oils induced ≥90% lethality in both cell lines, whereas the fruit oil produced only a limited effect on Hs578T cells and none on PC3 cells.
The fruit oil of *S. macranthum* had considerable cytotoxic effect on Hs578T cells inducing ≥90% lethality, but the leaf oil did not show any effects. Essien *et al.* (2012) also showed that the leaf oil of *S. erianthum* and fruit oil of *S. macranthum* had antimicrobial and antifungal activities.

In summary, *S. erianthum* leaf oils, but not fruit oils, have been shown to have growth inhibitory effects related to cytotoxicity on human PC-3 prostate cancer cells.

**II.C.4. *Urtica dioica***

*U. dioica* also known as stinging nettle is found in northern Africa and other parts of the world and has been used since the 1950s as a treatment for BPH. Based on a previous study which showed the proliferation reducing effects of *U. dioica* on BPH in mice Konrad *et al.* (2000) further tested a 20% methanolic extract of *U. dioica* (ME-20) which exhibited the most potent effect. LNCaP prostate cancer cells and hPCP cells, which are a benign prostatic stromal cell line, were grown in the presence or absence ME-20 in concentrations between 1.0E-3 pg/mL through 1.0E4 pg/mL for 7 days and cell proliferation was assessed using an MTT assay. The LNCaP cells experienced growth inhibition in a concentration dependent manner, whereas proliferation of the hPCP cells was not inhibited at any concentration.

Durak *et al.* (2004) investigated the effects of aqueous extract of *U. dioica* on adenosine deaminase (ADA). ADA is a key enzyme in DNA turnover and nucleotide metabolism which has been used as a target for chemotherapy for some forms of cancer. Prostate cancer tissue from ten patients who exhibited Gleason scores between 4 and 7 was homogenized in a saline solution and centrifuged to yield a supernatant which was incubated with *U. dioica* extract in
concentrations of 0, 25, 50, and 100 µL for 30 minutes after which the ADA activity was measured. ADA activity was significantly inhibited by the extract in a dose dependent manner, suggesting that *U. dioica* can lead to improvement in prostate cancer patients through inhibition of ADA since ADA inhibitors have been successful in some other cancer types (Durak *et al.*, 2004).

In summary, a methanolic extract of *U. dioica* had growth inhibitory effects on human LNCaP prostate cancer cells and an aqueous extract of *U. dioica* inhibited *ex vivo* activity of adenosine deaminase in human prostate cancer tissue.

II.D. **Conclusions**

The body of work relating to African plants and their effects on prostate cancer is limited. There have been a few promising studies as summarized above that have yielded some interesting results, although no follow-up work for many of these studies has been published. However, the design of most of these studies is not ideal. Many of the studies used only one prostate cancer cell line, either PC3 or LNCaP cells, instead of utilizing both of these cell lines to contrast effects on androgen independent and androgen dependent cells. As mentioned in the introduction, many of these studies focused more on the composition of extracts from plants to ascertain their bioactive compounds than their anticancer effects. In reviewing the literature, the need for more cooperation between African researchers and researchers from more developed countries becomes apparent. While the there was excellent elucidation of bioactive compounds in some of the cell culture studies, results could be improved by utilizing more than one prostate cancer cell line and extending the research to *in vivo* studies in animal models of prostate cancer.
The studies involving *P. africanum* and *S. tuberculatiformis* are great examples of what can be accomplished when researchers that biology methods to study and chemical analysis these plant extracts.
III. MATERIALS AND METHODS

III.A. **Plant Material**

*Piper guineense, Mucuna pruriens, Dennettia tripetala,* and *Carcica papaya* seed extracts were received from Dr. M.I. Akapanabiatu (University of Uyo), Ekpene Obo, Esit Eket Local Government, Nigeria, Africa.

III.B. **Preparation of Plant Extracts**

The seed extracts were dissolved in 7mL of ethanol and transferred into borosilicate glass (15x85 mm) disposable culture tubes (Fisher Scientific, Pittsburgh, PA, USA). The extracts were then placed into a heating block at 37°C and the ethanol was evaporated off using nitrogen gas in order to attain dry weight of extracts. The extracts were re-suspended in ethanol at 100 mg/mL, heated to 50°C, and vortexed every 10-25 minutes until the extract went into solution. Insoluble materials were saved in -20°C freezer. Once the plant materials were in solution at the 100 mg/mL concentration, the solution was diluted to 10 mg/mL, 1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL solutions in ethanol. All concentrations were stored in a -20°C freezer until used in the study.

III.C. **Cell Cultures**

LNCaP and PC3 prostate cancer cells were maintained in 75cm² Cell Flasks (Corning Inc, Corning, NY). Medium used for PC3 cells consisted of RPMI-1640 + L-Glutamine with phenol red (Gibco BRL Life Technologies, Grand Island, NY, USA), 10% Certified Fetal Bovine Serum (Gibco BRL Life Technologies, Grand Island, NY, USA), and 5% Antibiotic-Antimycotic, 100X (Gibco BRL Life Technologies, Grand Island, NY, USA). Medium used for
LNCaP cells consisted of RPMI-1640 + L-Glutamine with phenol red, 10% Certified Fetal Bovine Serum, 5% Antibiotic-Antimycotic, 100X, and 15mM HEPES buffer solution (Gibco BRL Life Technologies, Grand Island, NY, USA). Prior to treatment LNCaP cells were cultured at 300,000 cells/well and PC3 cells were cultured at 100,000 cells/well for 24 hours in 6-well multiwell plates (BD Falcon, Franklin Lakes, NJ, USA).

III.D. **Cell Viability**

LNCaP and PC3 cells were treated 24 hours after culturing on 6-well plates. The cells were cultured for 24-48 hours in the presence of 2 μl ethanol (0 μg/mL) or 2 μl of the ethanolic plant extracts in final concentrations of 100 μg/mL, 10 μg/mL, 1 μg/mL, 0.1 μg/mL, and 0.01 μg/mL. Absolute control 6-well plates, in which cells were cultured only with medium, were grown alongside treated cells. Cell viability was determined at 0, 24, 48 hours using 0.4% Trypan Blue (Lonza, Walkersville, MD, USA) staining and cell counting on a hemocytometer.

III.E. **Statistical Analysis**

All cell counts were made in triplicate as follows. For experiments with the PC-3 cell counts, triplicate counts were taken from a single well containing untreated cells, ethanol, or a specific concentration of ethanolic seed extract, while for LNCaP experiments single counts were taken from triplicate wells containing ethanol or a specific concentration of ethanolic seed extract. The number of cells per well were calculated from these counts and means ± standard error of the mean (SEM) were computed. The percent growth from the mean count at baseline over 24 and 48 hours was calculated for the ethanol controls and difference in growth of treated cells was computed as the percent of the mean ethanol control values. These data were analyzed
with one way analysis of variance (ANOVA) followed by a Dunnett *post hoc* multiple comparison test, comparing treated cells with the corresponding ethanol controls, and a test for linear trend was also conducted. In cases of apparent biphasic effects of an extract on cell growth, an ANOVA and trend analysis was carried out for each segment of the growth curve to obtain an estimate of the statistical significance of such non-linear effects. In cases of two sample comparisons, a two-sided t-test was carried out. A *p* value of $\leq 0.05$ was considered statistically significant. The analysis was carried out using GraphPad Instat software.
IV. RESULTS

IV.A. Preliminary Experiments

Preliminary exposures were performed in which PC3 and LNCaP cells were exposed to *Piper guineense, Mucuna pruriens, Dennettia tripetala,* and *Carcica papaya* extracts in concentrations ranging from 0.01 µg/mL - 100 µg/mL for 48 hours (data not shown). These results gave an initial insight into the effects the extracts would have on the different cell lines. During these experiments some interesting results were seen. The LNCaP cells in the 100 µg/mL concentration of *D. tripetala* extract were found to be swollen as shown in Figure 1. The medium in both the 10 µg/mL and 100 µg/mL concentrations of *D. tripetala* extract were found to be yellowish-orange and yellow indicating acidity. We theorized this may be caused by acid byproducts from the LNCaP cells when treated with the *D. tripetala* extract. To cope with the increased acidity a medium containing 15 mM HEPES buffer was made. LNCaP cells were exposed to ethanol, 10 µg/mL, and 100 µg/mL concentrations of *D. tripetala* extract in medium with or without 15 mM HEPES buffer. The results showed that 15 mM HEPES buffer had no significant effect on LNCaP cell growth in ethanol and 10 µg/mL wells, but there was a slight difference in the swelling of LNCaP cells between the wells containing 100 µg/mL of *D. tripetala* extract with HEPES buffer and those without (Figure 2 and 3). Since the LNCaP cells can grow in the medium containing 15 mM HEPES buffer without significant effect it was determined that medium containing 15 mM HEPES buffer would be used for all LNCaP cell cultures to temper potential effects of acidic byproducts.
Figure 1. The effect on LNCaP of exposure to *D. tripetala* extract. a) Swollen LNCaP cells 48 hours after exposure to 100 µg/mL of *D. tripetala* extract. b) LNCaP cells 48 hours after exposure to ethanol.
Figure 2. The effect on LNCaP of adding HEPES buffer to the medium when exposed to *D. tripetala* extract viewed at a 10x magnification. a) LNCaP cells exposed to ethanol in buffer with no HEPES. b) LNCaP cells exposed to ethanol in buffer with 15mM HEPES. c) LNCaP cells exposed to 100 µg/mL of *D. tripetala* extract in buffer with no HEPES buffer. d) LNCaP cells exposed to 100 µg/mL of *D. tripetala* extract in buffer with 15mM HEPES buffer.
Figure 3. The effect on LNCaP of adding HEPES buffer to the medium when exposed to *D. tripetala* extract at a 20x magnification. a) LNCaP cells exposed to ethanol in buffer with no HEPES. b) LNCaP cells exposed to ethanol in buffer with 15mM HEPES. c) LNCaP cells exposed to 100 µg/mL of *D. tripetala* extract in buffer with no HEPES buffer. d) LNCaP cells exposed to 100 µg/mL of *D. tripetala* extract in buffer with 15mM HEPES buffer.
IV.B. *Carica papaya*

The growth of PC3 cells exposed to *C. papaya* unaffected by the extract (Figure 5a). A slight decrease in growth was observed after 48 hours in wells that received the highest concentration (100 µg/mL) and a slight increase in growth was observed in wells exposed to ethanol (0 µg/mL) – 10 µg/mL of *C. papaya* when compared with untreated cells (Figure 4a). However these were shown to be statistically irrelevant when compared to vehicle control. There was no observable increase in PC3 cell death (Figures 6a, 7a).

LNCaP cells exposed to *C. papaya* showed an unexpected biphasic growth curve at 48 hours (Figure 9a). The overall ANOVA was significant (p = 0.02) and when analysis was limited to 1 µg/mL – 100 µg/mL concentrations the differences were significant (p < 0.01) as was the ANOVA (p = 0.001). At concentrations if ethanol (0 µg/mL) – 1 µg/mL concentration of *C. papaya* was significant (p < 0.05), but ANOVA results were not significant (p = 0.074). A slight decrease in the number of cells was observed in wells treated with 100 µg/mL of *C. papaya* and a slight increase in all other concentrations, including ethanol, compared to untreated cells (Figure 8a). There was no observable increase in LNCaP cell death (Figures 10a, 11a).

IV.C. *Dennettia tripetala*

There was significant decrease in cell growth at 48 hours, but not at 24 hours in PC3 cells treated with 100 µg/mL of *D. tripetala* extract compared with ethanol control (p < 0.01). However, this effect was not observed in other concentrations of *D. tripetala* extract (Figure 5b). This effect was also observed in a linear representation of the pooled data (Figure 4b). However, there was no observable increase in PC3 cell death (Figures 6b, 7b).

In LNCaP cells there was a significant decrease in cell growth in cells treated with 100 µg/mL of *D. tripetala* extract compared with ethanol control at both 24 hours (p < 0.05) and 48
Figure 4. Line graphs representing pooled data from experiments run in triplicate. a) *C. papaya* b) *D. tripetala* c) *M. pruriens* d) *P. guineense*
Figure 5.a) Effect of treatment of PC-3 cells with *Carica papaya* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 8-9). The results of ANOVA were not significant for either the 24 or the 48 hour time point.
**Denettia tripetala** in PC-3 cells (24 h)

![Graph showing the effect of treatment on cell numbers](image)

**Denettia tripetala** in PC-3 cells (48 h)

![Graph showing the effect of treatment on cell numbers](image)

Figure 5.b) Effect of treatment of PC-3 cells with *Denettia tripetala* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 8-9). The results of ANOVA were significant for the 48 hour time point (p = 0.0007), but not the 24 hour time point. The difference between the 100 μg/mL concentration and the ethanol (EtOH) group was significant (p < 0.01; **) as was the overall linear trend (p = 0.0003). When limiting the analysis to the 1.0 - 100 groups, the difference between the 1.0 and 100 μg/mL concentrations was significant (p < 0.05) as was the overall linear trend (p = 0.018) and the ANOVA was borderline significant (p = 0.058).
Figure 5.c) Effect of treatment of PC-3 cells with *Mucuna pruriens* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 8-9). The results of ANOVA were not significant for the 24 and 48 hour time points, but the overall linear trend was borderline significant for the 24 hour time point (p = 0.053). When limiting the analysis to the 1.0 - 100 groups, the difference between the 1.0 and 100 μg/mL concentrations was significant (p < 0.05) as was the overall linear trend (p = 0.018) and the ANOVA was borderline significant (p = 0.058).
Figure 5.d) Effect of treatment of PC-3 cells with *Piper guineense* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 8-9). The results of ANOVA were significant for the 48 hour time point (p < 0.0001), but not for the 24 hour time point. The difference with the control for the 100 μg/mL concentration was significant (p < 0.01; **) as was the overall linear trend (p = 0.0006).
Figure 6. Bar graph representing pooled data from triplicate experiments with PC-3 cells 24 hours post exposure. a) *C. papaya* b) *D. tripetala* c) *M. pruriens* d) *P. guineense*
Figure 7. Bar graph representation of the pooled data from experiments run in triplicate with PC-3 cells 48 hours post exposure. a) *C. papaya* b) *D. tripetala* c) *M. pruriens* d) *P. guineense*
hours (p < 0.01) (Figure 9b). The results of ANOVA were also significant at 24 hours (p = 0.0002) and 48 hours (p = 0.002). The effect of 100 µg/mL concentration of *D. tripetala* extract is observable in a linear representation of the pooled where as early as 24 hours the cell number dropped below baseline (Figure 8d).

An increase in necrotic cell death was observed for LNCaP cells exposed to 100 µg/mL of *D. tripetala* as indicated by uptake of trypan blue stain at 48 hours, but not at 24 hours (Figure 10b, 11b). Visual inspection of LNCaP cells under a light microscope showed a significantly higher number of dead cells at 48 hours than were observed with trypan blue stain (Figure 12).

### IV.D. *Mucuna pruriens*

The ANOVA results were not significant at 24 and 48 hours in PC3 cells, however at 48 hours the analysis of the difference between 1 µg/mL and 100 µg/mL concentrations of *M. pruriens* was significant (p < 0.05) (Figure 5c). No significant differences in growth were observed in LNCaP cells exposed to *M. pruriens* extracts at all concentrations (Figure 8c, 9c). There was no observable increase in PC3 and LNCaP cell death (Figures 6c, 7c, 10c, 11c).

### IV.E. *Piper guineense*

In PC3 cells, the results of ANOVA were significant at 48 hours (p < 0.0001), but not at 24 hours (Figure 5d). The difference between ethanol and 100 µg/mL concentration of *P. guineense* extract was significant (p < 0.01). The effect of 100 µg/mL concentration of *P. guineense* extract is observable in a linear representation of the pooled where as early as 24 hours the cell number dropped below baseline (Figure 4d).

In LNCaP cells, the results of ANOVA were highly significant for both 24 hours and 48 hours (p < 0.0001) (Figure 9d). The difference between 100 µg/mL concentration of *P. guineense* extract and ethanol were significant (p < 0.01) for both 24 and 48 hours.
difference between 0.01 µg/mL concentration of *P. guineense* extract and ethanol at 24 hours was significant (*p* < 0.05). The effect of 100 µg/mL concentration of *P. guineense* extract is observable in a linear representation of the pooled where as early as 24 hours the cell number dropped below baseline (Figure 8d).

LNCaP cells exposed to 100 µg/mL of *P. guineense* extract had an observable increase in the number of necrotic cells as indicated by uptake of trypan blue stain at both 24 and 48 hours (Figure 10d, 11d). Visual inspection of LNCaP cells under a light microscope also showed an increase in the number of dead cells at 48 hours (Figure 13).
Figure 8. Line graphs representing pooled data from triplicate experiments with LNCaP cells. a) *C. papaya* b) *D. tripetala* c) *M. pruriens* d) *P. guineense*
Figure 9.a) Effect of treatment of LNCaP cells with *Carica papaya* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 9). The results of ANOVA and linear trend analysis were not significant for the 24 time point. When limiting the analysis to the 1.0 - 100 μg/mL groups, the difference between the 1.0 and 100 μg/mL concentrations was significant (p < 0.05) as was the overall linear trend (p = 0.01) and the ANOVA was borderline significant (p = 0.063). A similar analysis for the EtOH – 1.0 μg/mL groups was not significant. For the 48 hour time point, the overall ANOVA was significant (p = 0.02), but there were no significant differences between treatment groups and the EtOH control and the linear trend was also not significant. When limiting the analysis to the 1.0 - 100 μg/mL groups, the difference between the 1.0 and 100 μg/mL concentrations was significant (p < 0.01) as were the overall linear trend (p = 0.0004) and ANOVA (p = 0.001). In a similar analysis for the EtOH – 1.0 μg/mL groups, the difference between the 1.0 μg/mL concentration and the EtOH group was significant (p < 0.05) as was the linear trend analysis (p = 0.01), while the results of ANOVA was borderline significant (p = 0.074).
Figure 9.b) Effect of treatment of LNCaP cells with *Denettia tripetala* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 9-11). The results of ANOVA and linear trend analysis were significant for the 24 time point (p = 0.0002 and p = 0.002, respectively) and also for the 48 hour time point (p < 0.0001 for both). The difference between the 100 µg/mL concentration and the EtOH control was significant at both time points (p < 0.05, *; and p < 0.01, **, respectively).
*Mucuna pruriens* in LNCaP cells (24 h)

**Mucuna pruriens** in LNCaP cells (48 h)

Figure 9.c) Effect of treatment of LNCaP cells with *Mucuna pruriens* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 9). The results of ANOVA and linear trend analysis were not significant for either time point and there were no significant difference between treatment and EtOH control groups.
Figure 9.d) Effect of treatment of LNCaP cells with *Piper guineense* for 24 and 48 hours presented as mean (± SEM) percent of ethanol (EtOH) control (n = 9). The results of ANOVA and linear trend analysis were highly significant for both time points (p < 0.0001). The difference between the 100 μg/mL and EtOH control groups were significant at p < 0.01 (***) for both points and the difference between the 0.01 μg/mL and EtOH control group was significant at 24 hours (p < 0.05; *).
Figure 10. Bar graph representation of the pooled data from experiments run in triplicate with LNCaP cells 24 hours post exposure. a) *C. papaya* b) *D. tripetala* c) *M. pruriens* d) *P. guineense*
Figure 11. Bar graph representation of the pooled data from experiments run in triplicate with LNCaP cells 48 hours post exposure. a) *Carica papaya* b) *Dennettia tripetala* c) *Mucuna pruriens* d) *Piper guineense*
Figure 12. The effects on LNCaP cells of exposure to 100 µg/mL of *D. tripetala* extract. All images taken 48 hours post exposure. The white bars represent 100 µm at 40x, 200 µm at 20x, and 400 µm at 10x. a) 10x magnification of LNCaP cells after exposure to ethanol. b) 10x magnification of LNCaP cells after exposure to 100 µg/mL of *D. tripetala* extract. c) 20x magnification of LNCaP cells after exposure to ethanol. d) 20x magnification of LNCaP cells after exposure to 100 µg/mL of *D. tripetala* extract. e) 40x magnification of LNCaP cells after exposure to ethanol. f) 40x magnification of LNCaP cells after exposure to 100 µg/mL of *D. tripetala* extract.
Figure 13. The effects on LNCaP cells of exposure to 100 µg/mL of *P. guineense* extract. All images taken 48 hours post exposure. The white bars represent 100 µm at 40x, 200 µm at 20x, and 400 µm at 10x. a) 10x magnification of LNCaP cells after exposure to ethanol. b) 10x magnification of LNCaP cells after exposure to 100 µg/mL of *P. guineense* extract. c) 20x magnification of LNCaP cells after exposure to ethanol. d) 20x magnification of LNCaP cells after exposure to 100 µg/mL of *P. guineense* extract. e) 40x magnification of LNCaP cells after exposure to ethanol. f) 40x magnification of LNCaP cells after exposure to 100 µg/mL of *P. guineense* extract.
C. papaya extract had no impact on growth in PC3 cells, but a biphasic growth curve was observed at 48 hours in LNCaP cells exposed to C. papaya extract (Figure 9a). There was a significant increase in LNCaP cell growth and a significant linear trend between ethanol control (0 µg/mL) – 1 µg/mL concentration of C. papaya extract at 48 hours. There was also a significant decrease in LNCaP cell growth and a significant linear trend between 1 µg/mL and 100 µg/mL concentrations at both 24 hours and 48 hours. Differences between the cell lines may explain why a significant growth pattern was observed in LNCaP cells, which express a mutant AR, but not in PC3 cells, which do not express AR, when the cells were exposed to C. papaya extract. Since these extracts are complex mixtures containing thousands of compounds one possible explanation for the biphasic growth curve observed in LNCaP cells that there could be two or more compounds active in C. papaya extract. One compound may be active at low concentrations up to 1 µg/mL which caused an increase in LNCaP cell proliferation before a secondary compound or compounds reached high enough concentrations to effectively inhibit the fist compound’s function in the 10 µg/mL and 100 µg/mL concentrations. However, this is only speculation and cannot be determined until fractionation of the C. papaya extract has been completed to isolate the bioactive compounds. Another explanation may be that the biphasic growth curve was caused by environmental conditions. However, this seems unlikely since the same effect was not seen in LNCaP cells exposed to D.tripetala extract (Figure 9b) which was tested alongside the C. papaya extract. Unfortunately there is not enough literature relating to the effects of C. papaya extracts on cancer to make a more informed discussion of these results.
The only observable effects in cells exposed to *M. pruriens* extract were in PC3 cells. An overall significant linear trend was observed at 24 hours and a significant decrease in growth and a significant linear trend was observed between the 1 μg/mL and 100 μg/mL concentrations of *M. pruriens* extract at 48 hours (Figure 5c). The decrease in growth at 48 hours between 1 μg/mL and 100 μg/mL concentrations of *M. pruriens* extract may have a relatively simple explanation. An increase in the concentration of a compound or multiple compounds may exert a greater effect on PC3 cells as dose increases from 1 μg/mL to 100 μg/mL causing a decrease in growth. Unfortunately there is no literature relating to the effects of *M. pruriens* extracts on cancer to that may explain these effects.

A significant decrease in PC3 cell growth was observed at 48 hours when cells were exposed to 100 μg/mL *D. tripetala* extract (Figure 6b). This was also observable in a line graph representation of the pooled data at 48 hours (Figure 4b). However, there were no significant effects in any other concentration of *D. tripetala* extract in PC3 cells. A significant decrease in cell growth in LNCaP cells exposed to 100 μg/mL *D. tripetala* extract was also observed as early as 24 hours after exposure (Figure 9b). The number of cells in LNCaP cells treated with 100 μg/mL *D. tripetala* extract dropped below baseline as early as 24 hours after treatment as seen in Figure 8b. The effects of the extract in LNCaP cells appear to be much more pronounced than in PC3 cells.

In LNCaP cells, clear cytotoxic effects were observed when exposed 100 μg/mL of *D. tripetala* extract as the cell growth drops below baseline as early as 24 hours after administration. At 48 hours there was a noticeable increase in necrotic cell death as shown by trypan blue
staining (Figure 11b). However, visual inspection with a light microscope indicated a greater amount of cell death than could be quantified with trypan blue staining (Figure 12). This may indicate that a compound or compounds present in the *D. tripetala* extract activated apoptotic pathways to initiate programmed cell death. According to Anaga and Asuzu (2011) glucose uptake in adipocyte cells exposed to *D. tripetala* extract is on par with insulin. This could mean that *D. tripetala* extract may act via the glucocorticoid receptors, which would explain its effects on both PC3 cells, which lack an androgen receptor, and LNCaP cells, which have a mutant androgen receptor that is also activated by glucocorticoid receptors. However, the cell growth inhibition and cell death is more pronounced in LNCaP cells than PC3 cells which may indicate that the *D. tripetala* extract also acts via the mutated androgen receptor.

Swelling of the LNCaP cells was observed 48 hours post exposure to 100 μg/mL of *D. tripetala* extract in preliminary experiments (Figures 1,2,3), but this was largely unnoticeable at earlier time points. In the preliminary experiments, LNCaP cell death was only seen after 72 hours post exposure to 100 μg/mL of *D. tripetala* extract, but not at 48 hours. The difference in timeline for swelling and cell death could be attributed to several factors. First, the preliminary experiments were carried out 8 months prior to definitive daily count experiments. During this time changes in the extract may have occurred that could explain the more rapid effects of the extract. Since cells were only inspected at baseline, 24 hours, and 48 hours the swelling of cells prior to death could have been easily missed due to a shift in the cell death time line. Second the technique for plating cells on the 6-well plates was changed between the preliminary experiments and the definitive daily count experiments. In preliminary experiments an estimation of LNCaP cell density in each well was made by estimating the percent confluence of each well
through visual inspection only. In the definitive daily counts experiments, the LNCaP cells were plated 300,000 cells/well to improve consistency. Thus, cell density could have been different between the preliminary experiments and definitive daily count experiments. This could impact the amount of *D. tripetala* extract taken up by each cell which could explain the changes in cell death and swelling changes seen between preliminary experiments and definitive daily count experiments. Lastly, a change in the LNCaP cells over time may have occurred and may also explain the differences seen between the preliminary experiments and definitive daily count experiments. The LNCaP line used in the preliminary experiments was lost due to a technical problem a few months prior to the definitive daily count experiments. LNCaP cells for the definitive daily count experiments came from another stock in the laboratory to replace the lost LNCaP cells. Differences in the cells could conceivably account for the differential effects of 100 µg/mL of *D. tripetala* extract. It could be that the cell line used in the definitive daily count experiments had a higher sensitivity to the *D. tripetala* extract which would account for a quicker cell death.

At 48 hours PC3 cells exposed to 100 µg/mL of *P. guineense* extract exhibited a significant inhibition of cell growth compared with ethanol control and a significant linear trend overall (Figure 5d). The growth inhibitory effect of 100 µg/mL of *P. guineense* extract was also noticeable in a linear representation of the pooled data (Figure 4d). In LNCaP cells, at 48 hours no significant differences in growth were observed in cells treated with *P. guineense* extract with exception of the cells treated with the 100 µg/mL concentration where a highly significant inhibition of growth was observed (Figure 9d). However, it is interesting to note that at 24 hours there was a significant increase in growth at 0.01 µg/mL of *P. guineense* extract and a significant
decrease in cells treated with the 100 µg/mL concentration (Figure 9d). The number of cells in LNCaP cells treated with 100 µg/mL *P. guineense* extract dropped below baseline as early as 24 hours after treatment as seen in Figure 8d.

Cytotoxic effects were observed in LNCaP cells exposed to 100 µg/mL of *P. guineense* extract as indicated by the rapid decline of cell number at 24 and 48 hours compared to baseline (Figure 8d) as well as the great number of necrotic cell deaths observed at 24 and 48 hours using trypan blue stain (Figure 10d, 11d). Visual inspection using a light microscope showed a great number of dead cells exposed to *P. guineense* extract confirming the cytotoxicity (Figure 13). In cells treated with 100 µg/mL of *D. tripetala* extract a great number of dead cells could be observed using a light microscope, but not counted using trypan blue stain. In cells treated with 100 µg/mL of *P. guineense* extract a great number of dead cells could be observed using a light microscope and also counted using trypan blue stain indicating *P. guineense* extract induced necrotic cell death rather than apoptotic cell death. However, it is worth noting that there was not an observable increase in the number of dead PC3 cells indicating that *P. guineense* extract is not cytotoxic to PC3 cells.

Flamand *et al.* (2010) found that inhibition of monoamine oxidase A (MAOA) in VCaP cells, a human prostate metastasis cell line, caused an inhibition of growth of these cells as well as downregulation for of Src, β-catenin, and MAPK oncogenic pathways. Lee *et al.* (2008) showed that a chemical found in *P. guineense* extracts called guineensine inhibited MAO activity. The inhibition of MAOA by guineensine may account for the decrease cell growth exhibited in PC3 cells. In LNCaP cells, there may be other compounds besides guineensine
present in *P. guineense* extract that interact with the androgen receptor pathways. A combination of MAOA inhibition cased by the compound guineensine and another compound could possibly cause the necrotic cell death so evident in LNCaP cells 48 hours after exposure to 100 µg/µL of *P. guineense* extract.

In summary, the *M. pruriens* extract exhibited mostly inconsequential results in both PC3 and LNCaP cell lines whereas the *D. tripetala* and *P. guineense* extracts had significant inhibition of both cell lines and a clear cytotoxic effects on LNCaP cells. *C. papaya* exhibited significant effects in LNCaP cells causing a biphasic growth curve. At this time there is not enough evidence to conclusively suggest the mechanisms of action in *D. tripetala* and *P. guineense* extracts. The *D. tripetala* extract may act through the glucocorticoid receptors in PC3 cells to inhibit cell growth and act through both the androgen and glucocorticoid receptors in LNCaP cells to activate apoptotic pathways to cause cell death. *P. guineense* extract causes inhibition of cell growth in PC3 cells through some unknown mechanism and caused massive necrotic cell death observed in LNCaP cells. Okwute *et al.* (1984) reported that *P. guineense* contains the chemical constituent guineensine and Lee *et al.* (2008) reported that guineensine inhibited MAO. Flamand *et al.* (2010) reported that MAOA is a mitochondrial enzyme that degrades neurotransmitters and is highly expressed in high grade prostate cancer. Studies by Agbor *et al.* (2007; 2012) suggest that *P. guineense* extracts have antioxidant effects and Agbonon *et al.* (2010) reported that *P. guineense* strongly inhibited CYP3A4, CYP3A5 and CYP3A7, which effect drug metabolism and may have pharmacoenhancing potential. There could potentially be multiple compounds present in *P. guineense* extract that cause a combined
effect to induce necrotic cell death. However, further investigation is needed to establish these mechanisms of cell inhibition and death in *D. tripetala* and *P. guineense*. 


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