The search for bioactive compounds in tropical plants to target hormone imbalance associated diseases.

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For my grandmother

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Abbreviations

5α-RII	5-alpha reductase type II
5-LOX	5-Lipo-oxygenase
ADT	Androgen deprivation treatment
AF	Activation function
AI	Androgen insensitive
APCs	Antigen presenting cells
Api	70% ethanolic *Alpinia oxyphylla* extract
Aquil	70% ethanolic *Aquilaria sinensis* extract
AR	Androgen receptor
ARA	Androgen receptor associated proteins
ARE	Androgen response elements
AS	Androgen sensitive
Astra	Aqueous *Astragalus membranaceus* extract
BCL-2	B-cell lymphoma 2
BPH	Benign prostatic hyperplasia
COX	Cyclo-oxygenase
CSS	Charcoal stripped serum
CZ	Central zone
DBD	DNA binding domain
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
EGF	Epidermal growth factor
ER	Estrogen receptors
EtOH	Ethanol
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GF	Growth factor
HSP	Heat shock proteins
IGF	Insulin growth factor
IL	Interleukin
KGF	Keratinocyte growth factor

KO	Knock-out
LBD	Ligand binding domain
LTB_4	Leukotriene B_4
LUTS	Lower urinary tract symptoms
MAPK	Mitogen-activated protein kinase
NSAID	Non-steroidal anti-inflammatory drug
P	Promoter
P9605	96% ethanolic *Piper cubeba* extract
PC	Prostate cancer
PGE_2	Prostaglandin E_2
PIN	Prostatic intraepithelial neoplasia
PKA	Protein Kinase A
PKC	Protein Kinase C
PSMA	Prostatic specific membrane antigen
PTEN	Phosphatase and tensin homolog
PZ	Peripheral zone
rmt	Room temperature
SHBG	Sex hormone binding globulin
SQM	Squamous metaplasia
TFA	Trifluoroacetic acid
TGF-β	B-Transforming growth factor
TNF	Tumor necrosis factor
TZ	Transitional zone

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1. Summary

Benign prostatic hyperplasia (BPH) and/or prostate cancer (PC) will affect at least 50% of the males once they have reached their fifties. However, despite the range of medical therapies available, effective treatment against BPH and PC still currently remains inadequate for some.

The annoying symptoms of BPH are mainly attributed to an enlarged prostate. Therefore, the current treatment strategy is to halt the androgen-dependent growth of the prostate and reduce its size. Several drugs have been employed with variable success to control prostatic growth. However, patients tend to self-medicate over a long period. As a result, this leads to another problem, unpleasant long-term side effects.

The treatment of PC in its early stages often warrants disease free survival for about 70-80% of the patients. Despite early aggressive therapy, 20% of the cases, unfortunately, experience disease progression to a state where the cancer no longer responses to therapy. At the moment, well-established medical options for this condition are limited and thus PC is one of the leading causes of cancer-associated deaths in western countries.

Evidence has supported the undoubted role of the androgen-signalling pathway in BPH, the pre cancerous prostatic hyperplasia and dysplasia that may progress to PC. The reduction of androgen-dependent prostatic growth has been the rational endocrine therapy for both BPH and PC. However, since the etiology of both diseases is multi-faceted, it is necessary to consider other contributing factors to develop more effective medication.

Medicinal plants are considered to be multi-component drugs (they contain numerous phytochemicals) and are thought to display a wide range of beneficial effects. They have been used therapeutically for centuries. Because of their historical place in medicine, they may have a better safety profile than synthetic drugs.

The objective of this thesis is to identify tropical medicinal plants, which could be used to target or support treatments for BPH and PC. Twenty herbal plants, with no known to date indications for both diseases, were selected. They were fractionated by using different ethanol (EtOH) concentrations. The initial screen (Chapter 4) aimed to identify plant extracts with the ability to inhibit the proliferation of LNCaP cells, an androgen dependent human prostate cancer cell line. All extracts were tested at a concentration of 30 μg/mL.

Four extracts, **Api**, (70% EtOH *Alpinia oxyphylla* extract), **Aquil** (70% EtOH *Aquilaria sinensis* extract), **Astra** (aqueous *Astragalus membranaceus* extract) and **P9605** (96% EtOH *Piper cubeba* extract) were selected for further investigations.

Recent research has demonstrated that androgens are not solely responsible for BPH and PC, estrogens, defective apoptosis and inflammation are, for example, also involved. An experimental test system using several methodological approaches was designed to test the above-mentioned extracts. The potential cytotoxicity of the extracts was investigated first to ensure that they did not attenuate LNCaP growth by inducing unspecific cell death. The extracts were also tested on HepG2 cells, a human hepatocarcinoma cell line, to identity any potential induction of liver-toxicity. Anti-androgenic and anti-estrogenic effects were determined by observing if the extracts 1) blocked the production of certain androgens and estrogens, 2) the steroid hormone receptor activation process, and 3) the actions of these sex hormones. The ability to induce apoptosis and the anti-inflammatory properties of the extracts were also tested. The methods employed were validated and synthetic controls were used whenever possible and compared with literature.

Api reduced the cellular viability of LNCaP and HepG2 cells at 20-30 µg/mL. It was not further investigated because the apparent reduced LNCap cell growth was most probably attributed to due to its cytotoxicity. The other extracts were non-cytotoxic on both cell lines at 30 µg/mL.

Astra inhibited androgen-dependent growth of LNCaP cells, however it did not show significant anti-androgenic, anti-estrogenic and anti-inflammatory properties. Unfortunately, it is beyond the scope of this project to discover its anti-proliferative mode of action.

The results of **Aquil** and **P9605** derived from the test system were more promising. **P9605** inhibited 5α-reductase type II and aromatase, which were involved in synthesising dihydrotestosterone (DHT) and estradiol respectively. It also antagonised the effects of DHT by several mechanisms. Furthermore, it inhibited the cyclo-oxygenases (COX) and 5-lipo-oxygenase which are involved in generating inflammatory mediators. **Aquil** possessed similar properties as **P9605**, except that it had no effects on the COXs.

In conclusion, we have identified some possible mechanisms of 2 tropical plants, *Aquilaria sinensis* and *Piper cubeba,* which could potentially be used to prevent/alleviate BPH and/or PC. This is the first time that these plants have shown to possess anti-androgenic and anti-estrogenic properties.

2. Introduction

2.1 General

Hormone imbalance associated diseases can orginate purely as a disorder of a gland or as a consequent of changing hormonal status of an organ due to factors such as age and environmental influences. Diseases, which fall into this category, range from mild cases of thyroid problems to life threatening illness such as diabetes. In this thesis, the focus will be on benign prostate hyperplasia (BPH) and prostate cancer (PC). The etiology of both pathologies is not well defined, however it is irrefutable that variations in the hormonal status of the prostate is involved.

Both of these dieases are extremely common in aging males; almost 90% of the men develop either BPH or PC between their fourth and ninth decades of life. Despite their high prevalence, current medical care is unable to eradicate or completely cure BPH and PC, at least for a subset of patients. With the unprecedented ageing poulation, there is a demand for more novel forms of treatment strategy or perhaps a shift to preventive medicine.

Plants are and hopefully will remain an essential source of therapeutic agents. They are being used to isolate bioactive compounds for direct use of drugs (e.g. digoxin, morphine, taxol) and for producing bioactive compounds of novel or known structures as lead compounds (e.g. metformin and verapamil are based on galegine and khellin respectively) [1]. Furthermore, since phytotherapy is becoming more popular amongst patients, plant-based medicine may have better patient compliance compared to synthetic drugs.

The search for bioactive components in tropical plants that may offer potential remedy, in one way or the other, to BPH and PC will be the centre of interest in this presented work.

The following chapters will provide an overview of the prostate, androgens, androgen receptor, BPH and PC.

2.2 The prostate

The human prostate is an androgen regulated exocrine gland surrounding the urethra just below the urinary bladder, in front of the rectum. The mature walnut-sized gland consists of branched alveolar-ductal structures embedded in a fibromuscular stroma [2]. Although its specific function is remains unclear, the prostate produces a clear, slightly alkaline fluid that constitutes 10-30% of the seminal fluid volume.

There are 4 distinct zones within the prostate (Table 2.2.1). These zones are derived from different embryonic origins, which may therefore explain the occurrence of BPH and PC in different areas of the prostate.

Table 2.2.1 Summary of information regarding the 4 different prostatic zones.

Name	***Proportion***	***Description***
The Peripheral Zone (PZ)	Comprises up to 70% of the total glandular mass.	The sub-capsular portion of the posterior aspect of the prostate gland surrounds the distal urethra. This is the site where more than 70% of PC originates.
The Central Zone (CZ)	Constitutes approximately 25% of the normal prostate gland.	This zone surrounds the ejaculatory ducts. It has more smooth muscle that the PZ. CZ tumours account for more than 25% of all PC.
The Transition Zone (TZ)	Responsible for 5% of the prostate volume. It consists of a pair of periurethral glands.	This zone is very rarely associated with carcinoma. It surrounds the proximal urethra and it's the region of the prostate gland responsible for BPH.
The Anterior Fibro-muscular zone	Accounts for approximately 5% of the prostatic weight.	This zone is usually devoid of glandular components and composed only of muscle and fibrous tissue.

(From *http://en.wikipedia.org/wiki/Prostate*)

The functional unit of the prostate composes of epithelium and stroma components. The epithelium consists mainly of secretory columnar epithelial cells, which arranges into a single cell layer, lining the epithelium. They synthesize proteins such as prostate specific antigens and prostate specific phosphatase and secrete them into the ductal lumen mucin. Notably, majority of PC arises from aberrantly functioning secretory epithelial cells. The prostate epithelium also composes of basal epithelial cells, neuroendocrine cells, non-epithelial fixed macrophages and intra-acinar lymphocytes [3].

The epithelium is physically separated from the stroma by a basement membrane. The composition of the stroma includes fibroblasts, smooth muscle cells, endothelial cells, nerve cells and infiltrating mast cells and lymphocytes. The prostatic epithelium and stroma interact with each other via various hormones and growth factors. The fibroblasts are stimulated by androgens to produce and secrete various growth factors such as epidermal growth factor (EGF), insulin growth factor (IGF) and keratinocyte growth factor (KGF), which could, in a paracrine fashion, induce epithelial cell growth and glandular development [4], [5].

The secretory epithelial cells express AR and they require continuous direct androgenic stimulation to maintain structural and functional viability. When the androgen level declines below a threshold, in the case of surgical or chemical castration, the secretory cells undergo apoptosis, causing glandular involution. Animal studies have also indicated that there was a ~90% loss of prostatic secretory epithelial cells through apoptosis after physical castration [6]. The basal cells remain after castration since most of them do not possess AR. On the other hand, a subset of basal cells is speculated to represent stem cells and although they do not depend on androgens for survival, they require androgens for proliferation and differentiation into secretory cells [7].

Under normal physiological conditions, these stem cells are stimulated by androgens to undergo proliferation and differentiation. Cells with accumulated damage are removed by apoptosis and a steady state balance is maintained between cell proliferation and apoptosis. However certain pathological assaults may trigger the hyper stimulation of androgen and/or growth factors, thus affecting the delicate balance of prostatic cell growth and death. Consequently, a subset of epithelial cells may evade the normal checkpoint control of cell cycle progression and proliferate aberrantly [3].

2.3. Hormones in the prostate

Hormones, in particularly the androgens, are essential for the development, growth and maintenance of the prostate. Besides androgens, several other hormones and/or their receptors have been detected in the prostate. These include estrogen, prolactin and growth hormone. Androgen is a term given to any steroid hormone that primarily influences the growth and development of the male reproductive system. Although there are other nature androgens (Table 2.3.1), testosterone is the primary circulating androgen.

Table 2.3.1 A list of androgens and their sources.

Androgens	***Source***	***Remarks***
Androstenedione	Produced by the testes, adrenal cortex, and ovaries	While androstenediones are converted metabolically to testosterone and other androgens, they are also the parent structure of estrone.
Androstenediol	Steroid metabolite	Is thought to act as the main regulator of gonadotropin secretion.
Androsterone	By-product of the breakdown of androgens, or derived from progesterone	Exerts minor masculinising effects, but with one-seventh the potency of testosterone.
Dehydroepiandrosterone (DHEA)	Produced in the adrenal cortex from cholesterol	A primary precursor of estrogen.
Dihydrotestosterone (DHT)	Potent metabolite of testosterone	Has 3-10 times greater affinity than testosterone to AR. It is synthesized mostly in peripheral tissues, such as the prostate.

(*Modified from http://en.wikipedia.org/wiki/Androgen*)

Testosterone is dominantly (>95%) synthesized in the Leydig cells of the testes. Only a small fraction of it is synthesised by the adrenal cortex. Testosterone produced is released into the bloodstream where a majority is complexed with a "carrier" protein, sex hormone binding globulin (SHBG) or albumin. SHBG is produced by the liver and plays an important role in regulating the amount of "free" testosterone circulating in the body at any one time. Only 1-3% of testosterone is free to diffuse from the blood stream into the prostatic cells. On the other hand, the prostate also possesses enzymes, which are involved in the biosynthesis of androgens and even estrogens (Fig. 2.3.2). This indicates that the prostate is capable of generating its own supply of sex hormones whenever it deems necessary.

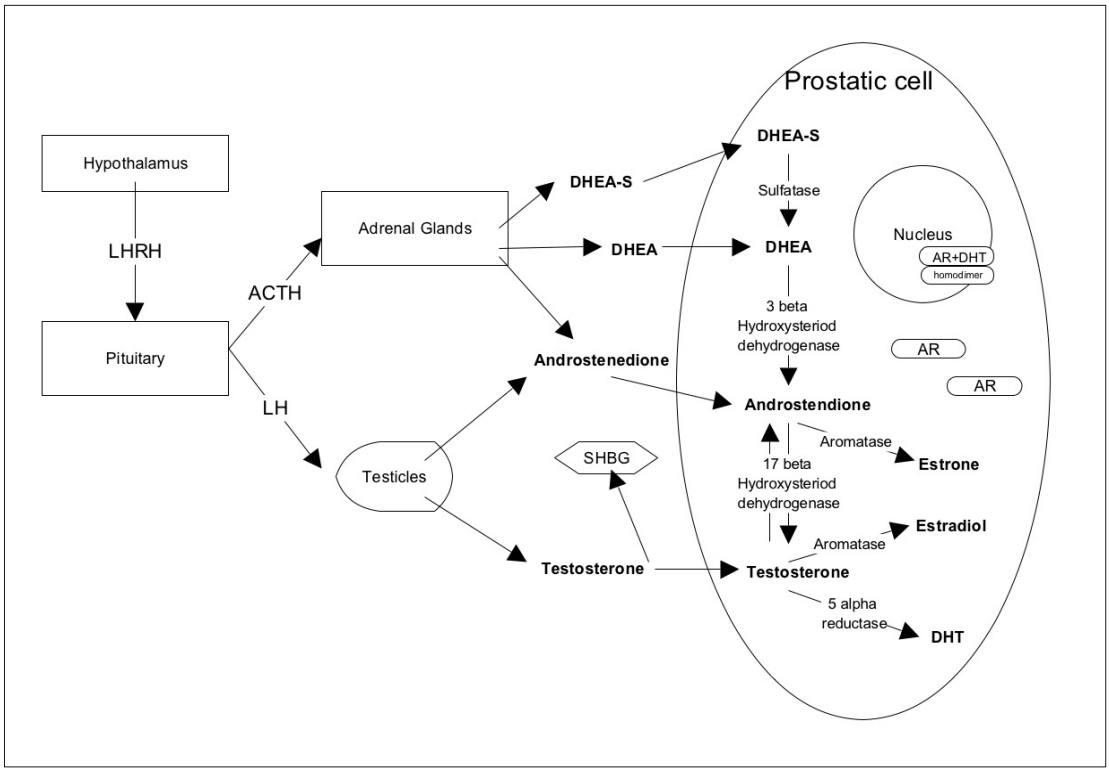

Fig. 2.3.1 Shows some possible intra-prostatic synthesis of androgens and estrogens.

2.4. Androgen receptor (AR)

Structure

The AR is a ligand dependent transcription factor and it belongs to the Type I steroid hormone receptors, which is one of the three functionally distinct subfamilies of the nuclear hormone gene superfamily. AR was first described in 1969 [8] and cloned in 1988 [9]. The gene is located on the X-chromosome at Xq11–12, contains 8 exons, and spans a length of approximately 90 kb of DNA [10].

Similar to other steroid receptor proteins, the full-length AR contains 4 domains: the amino-terminus regulatory domain, a highly conserved DNA-binding domain, a hinge region, and the ligand-binding domain [11]. Unlike the progesterone and estrogen receptors, the concept that another isoform of AR exists is not widely accepted due to lack of substantial evidence [12].

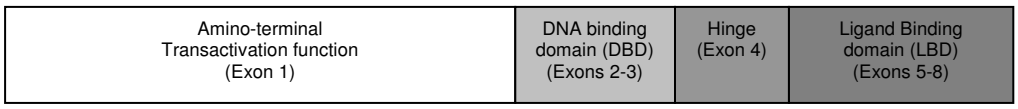

Fig. 2.4.1 A structural and functional map of a typical AR. It has approximately 900 amino acids and a molecular mass of ~110kDa. The amino-terminal consists of a constitutively active activation function (AF-1) and a ligand-dependent activation function (AF-2) arises in the LBD [13]. The DBD has 2 zinc fingers which that dictate the specific binding to the ARE.

Ligand dependent Activation

Unliganded ARs are sequestered in the cytoplasm as a multi-protein complex. They are associated with immunophilins and heat shock proteins (HSPs) 90, 70, and 56, which stabilize their tertiary structure and prevent them from constitutive activation [14]. When bound to a ligand, AR is phosphorylated, undergoes a conformation change and dissociates from HSPs. The activated AR forms a homodimer with another AR. This consequently exposes a nuclear localization signal within the dimer, where importins bind and facilitate the translocation of the ligand bound AR to the nucleus [15]. Once within the nucleus, they bind to canonical androgen response elements (ARE) on various androgen target genes. This can turn on or off transcription of the particular DNA. Co-regulatory proteins (co-activators/co-repressors) are recruited to form a mega-protein complex, which is poised to interact with other transcriptional mediators, cofactors and basal transcriptional machinery to modulate target gene transcription [11].

Ligand independent activation

Nuclear receptors are regulated by reversible phosphorylation and thus may also be activated by signalling pathways that originated at the cell surface. AR possesses a consensus phosphorylation site which indicates that it could be a substrate for protein kinase A & C (PKA & PKC), mitogen activated kinase and casein kinase II. This hypothesis is supported by the observation that PKA and PKC could enhance AR transactivation [16]. A number of other AR associated proteins (ARA) such as ARA 54, 55 and 70 also enhances AR transactivation.

Effects of AR activation

Testosterone and DHT bind with different affinities to the AR. This difference in binding affinity results in different levels of AR activation and therefore distinctive effects [17] (Table 2.4.1). Androgens modulate the synthesis of growth factors (GF) and their receptor availability.

Table 2.4.1 The different effects of androgens mediated by AR.

Effects of Testosterone	***Effects of DHT***
• Development of the internal accessory sexual organs • Regulation of FSH synthesis • Regulation of GF receptors • Maintenance of epithelium, microvilli, golgi secretory activity	• Development of the external sex organs • Increase DNA replication, cell growth • Induce SHBG and PSA production • Induce mesenchymal cells to secrete KGF and FGF • Downregulates TGF-β • Increasing angiogenesis due to upregulation of EGF and vascular endothelial growth factor • Inhibits apoptosis in LNCaP cells [18]. • Antiproliferative and PSA induction effects of 1α-25-dihydroxyvitamin $D_{3\ on}$ LNCaP are DHT dependent [19].

One possible explanation to account for these differences is that testosterone dissociates 3 times faster than DHT and is less effective in stabilizing the AR. The differences in dissociation rate of the two ligands to AR could be directly related to their different abilities in stimulating androgen responsive genes [17].

Degradation

Steroid hormone receptors have relatively short half-lives and they undergo systematic protein degradation. This is important in regulating the amount and duration of steroid receptor ligand effect. A study using green fluorescent protein technology demonstrated that AR migrated to the sub-nuclear compartment in the presence of the androgen within 15-60 mins. AR migrated rapidly back to the cytoplasm upon ligand dissociation and maintained its ability to re-enter the nucleus for at least four rounds of AR recycling after initial androgen treatment before degradation [20]. AR may be degraded by two independent pathways, Akt-proteasome and phosphatase and tensin homolog (PTEN) caspase-3 pathways [12].

Regulation

AR expression is regulated at several levels: AR mRNA translation, transcription, post-transcription, protein, half-life and degradation (Table 2.4.2). AR is the main instrumental tool in eliciting the effects of androgens. However androgens, in turn, play an immense role in regulating the action and levels of AR.

Table 2.4.2: Briefly describes the different possibilities to regulate the levels of AR

Levels	***Regulation mode***
AR mRNA transcription	**Androgens**: Results are controversial. Androgens decrease AR mRNA LNCaP cells and in rat ventral prostate [21], [22]. However other groups have shown an up-regulation of AR mRNA in rat and mouse prostate [23], genital skin fibroblasts [24]. **FSH**: Increases AR mRNA in Sertoli cells. **Growth hormone, Prolactin, and EGF**: Increase AR mRNA in prostatic cells.
AR protein expression	**Androgens**: Reported to modulate both stability and translation efficiency of AR mRNA [25].
AR nuclear import	**Androgens**: AR transfer is more efficient when bound to DHT then anti-androgens.
AR protein degradation	**Androgens**: Half-life of AR in LNCaP cells is ~ 3 hours but it longer than 10 hours in the presence of 10 nM of DHT [26].

2.5. Benign Prostatic Hyperplasia (BPH) and Prostate cancer (PC)

2.5.1. Introduction

BPH

BPH could be defined

1) Histologically: the microscopic benign proliferation of the prostatic stroma and epithelium in the transitional zone [27].

2) Clinically: the palpable enlargement of the prostate, which can be detected by digital rectal examination or ultrasonographic examination [27].

Microscopic nodular hyperplasia increases linearly with age in all ethnic groups and BPH is clinically identifiable in at least 50% of men over 50. However, only about 30% to 50% of the cases with clinical gland enlargement manifest lower urinary tract symptoms (LUTS) [28]. LUTS is a collection of annoying urinary symptoms associated with prostatic hyperplasia, which include urinary hesitancy, urinary retention and increased risk of urinary tract infections.

Functionally, the prostate reaches maturity at puberty. After achieving adult size, the prostate remains essentially the same size for several decades. Then, in midlife and beyond, prostatic growth occurs again in majority of the men. The explanation for this reawakening of the prostatic cells is still unclear [2].

A study done in 2004 identified certain risk factors for BPH and results have shown than Asian Americans have the lowest risk of clinical BPH. Alcohol and possibly cigarettes are related to a lower risk for BPH [29]. Other epidemiological studies have indicated that several risk factors associated with cardiovascular diseases apply for BPH as well. These include obesity, hypertension and diabetes type II [30], [31].

Introduction: PC

PC develops when prostate cells mutate and begin to multiply uncontrollably. 1 out of 6 men are now being diagnosed with PC [32]. Although in most cases, they are not clinically relevant, PC could be fatal for a proportion of the men. The current problem/challenge is to distinguish the nature of PC a man may have at a given time;

- Microscopic cancer that will never cause a problem.
- A clinically relevant cancer that will cause mortality if left untreated.
- Cancer that has already metastasized to distant organs hence incurable with localized therapy.

PC that metastasizes to other parts of the body, especially to the bones and lymph nodes, occurs in 2 general stages; androgen sensitive (AS) and androgen independent (AI). The initial PC usually arises from androgen-dependent epithelium, which requires androgens to grow, and is sensitive to androgen deprivation treatment (ADT). However, after prolonged ADT, the tumour progresses to an AI state where it no longer responses to ADT. It must be noted that although AI PC does not respond to ADT, androgens are still detected in these AI cancers [32].

2.5.2. Role of androgen/AR signalling pathway

Role of androgen axis in BPH

DHT stimulates glandular epithelium growth in the prostate and it is the major cause of rapid prostate enlargement that occurs between puberty and young adulthood. A study in 1974 observed that men deficient in 5α-reductase had hypoplastic prostates [33] and the relative success of Finasteride, a 5α-reductase type II blocker, in retarding prostatic growth by reducing DHT production both substantiate the role of DHT in BPH. It is well documented that as men age, their testosterone levels decline. Some researches have indicated that despite an overall decline in testosterone levels, the prostate is still able to synthesise similar quantities of DHT. It is therefore hypothesized that the changes in the equilibrium between testosterone and DHT may lead to an increase in prostatic growth [2].

Role of androgen axis in PC

Since the prostate is an androgen-dependent organ, it is rational to presume that prostate malignancy develops under abnormal androgen signalling. This hypothesis is, to some extent, supported by observations that eunuchs do not develop PC and that a higher incidence of PC is found in men who used androgens as anabolic agents or therapeutics [34].

Although patients show positive response initially to ADT, continuous treatment often results in PC progressing to AI states within 18-24 months [35]. There are several postulated theories explaining this development of resistance. Some of which, involve the AR or the development of alternative signalling pathways that bypass the function of AR [36].

Somatic Mutations of AR often bestow the receptor with hypersensitivity and promiscuous usage of ligands. The mutated receptor could be trans-activated by lower concentrations of androgens, by anti-androgens, and by non-androgenic ligands [37], [38]. About 50% of the mutations reported in ligand binding domain have been found to be associated with AI PC. T887A substitution in AR, which is found in LNCaP cells, allows it to be activated by other steroids and even by anti-androgens [39]. In addition, the R726L AR mutant is known to be activated by estradiol. PC may consist of clones with a range of different types of AR mutations [40].

AR amplification is rarely found in AS cancer but is common in recurrent therapy-resistant cancer [41], [42].

Ligand-independent activation of AR by growth factors such as IGF-I, KGF, EGF [43], and by cellular signalling regulators such as butyrate, interleukin-6 (IL-6), bombesin, and activators of the PKA signalling pathways are capable of transactivating AR [44].

Altered regulation or mutation of co-regulators is a potential mechanism for altered PC growth. Besides AR-specific co-regulators, more general steroid receptor co-regulators such as CBP, SRC-1, ARA70, and TRAM-1 as well as oncogenic molecules such as BRCA-1, RB, and Her2/neu have been demonstrated to influence AR trans-activation [45].

The progression of PC is likely to be the result of an abnormal AR status. Prolong ADT may contribute to the progression to an AI state by exerting selective pressure for clones expressing different AR phenotypes thus modifying the AR status of the tumour. In summary, the androgen axis is involved in both development of PC and the progression of the cancer to AI state.

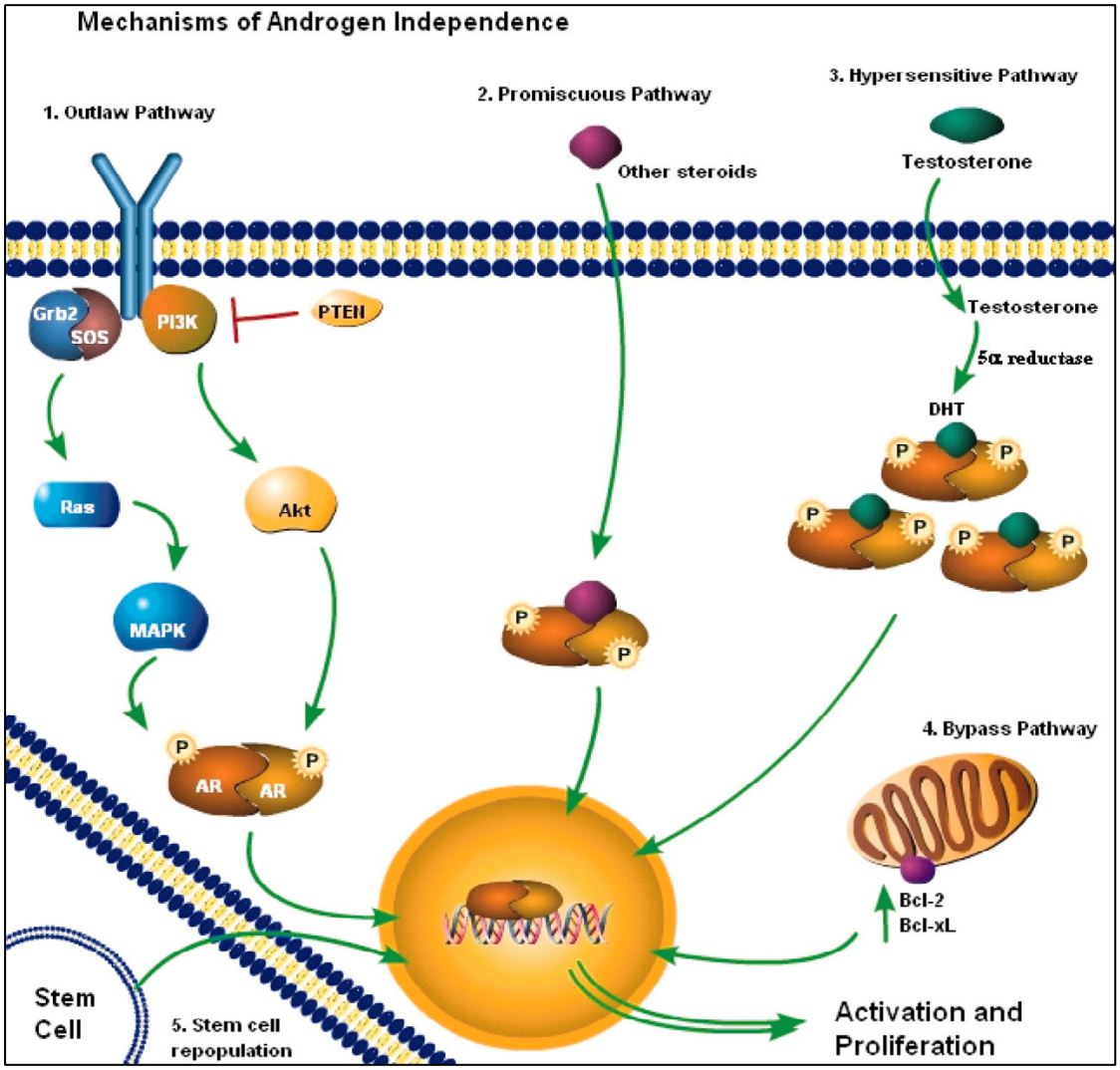

Fig. 2.5.2.1 Possible pathways leading to AI PC. (*Taken from [46]).*

1) In the outlaw pathway, receptor tyrosine kinases (RTKs) are activated, and the AR is phosphorylated by either the AKT (protein kinase B) or the mitogen-activated protein kinase (MAPK) pathway, producing a ligand-independent AR.

2) In the promiscuous pathway, the specificity of the AR is broadened so that non-androgenic steroid molecules normally present in the circulation can activate it.

3) In the hypersensitive pathway, more AR is produced (usually by gene amplification), AR has enhanced sensitivity to compensate for low levels of androgen, or more testosterone is converted to the more potent androgen, dihydrotestosterone (DHT), by 5α reductase.

4) In the bypass pathway, parallel survival pathways, such as that involving the anti-apoptotic protein BCL-2 (B-cell lymphoma 2), obviate the need for AR or its ligand.

5) In the stem-cell repopulation pathway, androgen-independent cancers stem cells are resistant to therapy and eventually become the primary population within the tumour.

2.5.3. Role of estrogen/ER signalling pathway

Role of estrogen axis in BPH

As men age, the intraprostatic estradiol concentration increases or remains constant while the androgen concentration decreases. There is a strong correlation between the increasing estradiol:DHT ratio and stromal hypertrophy [47]. Takase *et al.* have detected estrogen receptors and enzymes involved in estrogen metabolism in human prostates [48]. Although the role and mechanism of estrogens in the prostate is still unclear, there is growing evidence that estrogen could modify prostate growth and differentiation. An estrogen dominant environment is speculated to increase the production of androgen receptors and thus encouraging prostatic growth by sensitizing the prostate to androgen [49]. The current hypothesis is that the prostate locally produces estrogens to modulate the activity of epithelial and stromal cells.

Role of estrogen axis in PC

Evidence that estrogens are involved in the genesis and progression of prostate cancer came mainly from experiments with organ cultures of normal rat, human prostate or human prostate cancer samples. In these studies, estrogens were found to stimulate DNA synthesis and induce metaplastic epithelial morphology in both human [50] and rat prostate [51]. High doses of testosterone given together with estradiol, but not alone, stimulated carcinogenesis in adult male rats [52]. Aromatase knockout mice, which are estrogen deficient, did not develop PC [53]. Epidemiological data also showed that men with high serum levels of estrogens have a greater risk of PC [54], [55].

On the other hand, the putative beneficial effects of dietary estrogens are evident from laboratory and clinical studies. Dietary estrogens, which include phytoestrogens, lignans and flavonoids, have been promoted to reduce and prevent prostate diseases [56], [57]. Epidemiological studies have suggested a link between increased consumption of phyto-estrogens to a lower incidence of PC. This is particularly true when comparing men living in Asia with men in the West, where dietary estrogens intake is lower and PC incidence is higher [58].

These conflicting data indicate the diverse roles of estrogens. These differing actions of estrogens are mediated by two estrogen receptor (ER) subtypes; ER-α and ER-β. ER-α is associated with aberrant proliferation, inflammation and the development of malignancy, whereas ER-β is associated with anti-proliferation, differentiation and apoptosis [59].

Estrogens induce proliferation as mentioned earlier and the multi-layering of the prostatic epithelial cells. Squamous metaplasia (SQM), the proliferation stimulated by estrogens, is aberrant in contrast with the ordered and coordinated proliferation induced by androgens. This response is observed only in ER-β knockout (KO) mice and not ER-α KO mice by Risbridger *et al*. They could also demonstrate, using tissue recombination techniques, that stromal and epithelial ER-α expression is a prerequisite for the development of SQM [59], [60]. This aberrant proliferation may progress and lead to PC if left uncontrolled.

2.5.4. Role of apoptosis

The interactions of androgens with the prostate epithelium, stroma, other hormones and growth factors (GF) form a complex system, which regulates prostate growth. In normal tissues, homeostasis is maintained by a balance between cell proliferation and apoptosis. Apoptosis, also known as programmed cell death, is a regulated process, consisting of a series of molecular events that lead to cell death.

BPH may result from an over-activity of cell proliferative processes induced by hormones or from a reduced rate of apoptosis. For example, the changes in the balance between different hormones may up or down regulate growth factors or other proteins, which are involved in inducing apoptosis, thus leading to an overall increase in cellular growth.

Apoptosis is one of the most potent defences against cancer because it eliminates deleterious cells. Therefore, the pathogenesis of cancer is closely related with aberrantly regulated programmed cell death. The resistance to apoptotic cell death in response to radiation and chemotherapy is another property of recurrent prostate tumour cells besides androgen independence. Bcl-2 is an anti-apoptotic protein, and its over expression has been associated with resistance to androgen deprivation and poor outcome in some prostate cancer patients treated with radiotherapy [61]. Furthermore, Zhou *et al* have recently shown that prostatic epithelium-specific deficiency for p53 and Rb tumour suppressors, which are pro-apoptotic proteins, leads to metastatic cancer in mice [62]. One approach to combat PC would be to target some of these specific apoptotic regulators.

2.5.5. Role of inflammation

Role of inflammation in BPH

Prostatic inflammation is very common in BPH patients. Histological studies of BPH tissues have detected inflammatory cell infiltrates of varying densities in 30%- 50% of the cases [63]. Inflammatory infiltrate such as macrophages and lymphocytes are known to produce growth factors such as bFGF, cytokines IL-1 and IL-6. In situ studies have indicated that there is an elevated expression of pro-inflammatory cytokines in BPH. It is speculated that IL-6, IL-8, IL-17 may perpetuate chronic immune response and induce fibromuscular growth by an autocrine or paracrine loop or via induction of cyclo-oxygenase 2 (COX-2) expression [64]. COX-2 is a major enzyme that converts arachidonic acid to prostaglandins. Prostaglandins have various roles in mediating and moderating inflammation and are associated with the progression of BPH [64].

Moreover, aromatase gene (CYP19) is regulated by a promoter (PII), which is responsive to inflammatory cytokines [65]. An increase in aromatase expression increases local estrogen levels that may lead to an increase in prostatic proliferation.

A recent study has indicated that consumption of non-steroidal anti-inflammatory drugs (NSAIDs) is linked with lower risk of developing BPH and LUTS [66]. It is unclear if inflammation is the cause or result of BPH but its involvement indicates that anti-inflammatory drugs may help to retard development and worsening of the disease.

Role of inflammation in PC

There is much evidence that chronic inflammation leads to an increased cancer risk. Eicosanoids, generated by the cyclo-oxygenases (COXs) and lipoxygenases (LOXs), are believed to play important roles in tumour promotion, progression and metastasis besides being inflammatory mediators. Matsuyama *et al* have observed that while 5 and 12-LOX were present in low amounts in BPH and normal prostate tissues, marked increase in 5 and 12-LOX expressions were found in prostatic intraepithelial neoplasia (PIN) and PC tissues. Furthermore they also saw that LOX inhibitors could reduce the growth of PC cell lines via apoptosis dose dependently [67]. In addition, recent epidemiologic studies have suggested that the use of NSAIDs may also be associated with a reduced risk of prostate cancer. A large cohort study was done in 2005 to investigate aspirin and other NSAIDs and PC incidence. It was concluded that long term NSAID usage modestly reduced the risk of prostate cancer [68]. Thus targeting certain aspects of the inflammatory pathway may be another approach to treat PC.

2.6. Current Treatments

Current treatments in BPH

Many men with BPH are asymptomatic and many others are not bothered by their symptoms. Therefore watchful waiting is an appropriate management for these patients. When symptoms affect quality of life, the main objectives would be to provide fast and sustained relief of the symptoms and to control disease progression.

Conventional pharmacological options include α_1-blockers, 5α-reductase inhibitors, or for men with larger prostates, a combination of the two (Table 2.6.1). Alternative medicine, which includes phytotherapeuticals, is also very popular amongst BPH patients (Table 2.6.2). Surgical intervention was the golden standard treatment for several years. However, the incidence of after-surgery complications such as incontinence, impotence, urinary tract infections and the need for re-intervention is clinically significant. Moreover, most patients have been reported to prefer a less aggressive intervention.

Current treatments in PC

Although there are several PC treatments (e.g. prostatectomy, radiation therapy, watchful waiting, chemotherapy), androgen deprivation therapy (ADT) has been the cornerstone of therapy ever since its efficacy for treating prostate cancer was first demonstrated by Huggins and Hodges in the 1940s. The main strategy of ADT is to decrease the production or block the actions of testosterone on prostatic cells. ADT cannot eradicate PC but only slows down the cancer's growth and reduces the size of the tumour(s).

Table 2.6.1 Current prescribed pharmaceutical drugs for BPH.

Drug Class	***Mechanisms***	***Primary effects***	***Examples***	***Side effects***
α-adrenergic receptor blocker	Antagonises the α- adrenergic receptors, which cause the contraction of smooth muscles in the prostate and bladder.	Relaxes the bladder and prostate muscles, thus relieving the symptoms of BPH (difficulty in urination).	Terazosin, Doxazosin, Alfuzosin	fatigue, back pain, headache, weight gain, decreased sexual ability, blurred vision, oedema, rhinitis, upper respiratory tract infection, orthostatic hypotension
α_{1A}-adrenergic receptor blocker	More selective for α_{1A}-adrenergic receptor which are the dominant α-adrenoceptors in the prostate.	More specific for symptomatic treatment of BPH.	Tamsulosin	abnormal ejaculation, back pain, chest pain, diarrhoea, sinus problems, sleepiness
5α-reductase Type II inhibitor	Specific inhibition of the conversion of testosterone into DHT by 5α-reductase Type II, the main isoform in the prostate.	Halts the growth of the prostate.	Finasteride	Impotence, allergy to active ingredients (hypersensitivity), rash (allergic reaction), breast tenderness/swelling, ejaculation disorders, decreased sex drive
5α-reductase Type I & II inhibitor	General inhibition of the conversion of testosterone into DHT by targeting both isoforms of 5α-reductase.	Halts the growth of the prostate.	Dutasteride	similar to finasteride
Muscarinic antagonist	Inhibits M_2 and M_3 receptors which have roles in the control of urinary bladder function.	Relieves urinary difficulties, including frequent urination and inability to control urination.	Tolterodine	dry mouth, blurred vision, upset stomach, headache, constipation, dry eyes, dizziness

Table 2.6.2 List of popular phytotherapy used by BPH patients.

Plant	***Mechanisms***	***Active compounds***	***Results from literature***
Serenoa serrulata Saw Palmetto (Permixon®)	Inhibits 5α-reductase I&II Anti-proliferative effects Anti-inflammatory Apoptotic effects Inhibits aromatase Anti-androgenic Anti-estrogenic [64]	Sterols (β-sitosterol, campesterol, stigasterol) and flavonoids	• Serenoa serrulata extract inhibited >70% of the activities of 5α reductase I &II with 10 µg/mL [69]. • Inhibited aromatase with IC_{50} of 100 µg/mL [47]. • 100 µg/mL of the extract inhibited thymidine incorporation in LNCaP and PC-3 cell lines by more than 50% [70].
Pygeum africanum African plum tree (Tadenan®)	Prevents proliferation induced by PKC, bFGF, EGF, IGF of rat prostatic fibroblasts. Mild anti-inflammatory effects Antiandrogenic activity	Sterols, acidic phenols, triterpenoids	• 600 µg/mL of P. africanum extract inhibited androgen action by 40-60 fold [71]]. • Inhibited thymidine incorporation in LNCaP and PC-3 cell lines with an IC_{50} of 2.5 µg/mL [72].
Urtica dioica Nettle root	Inhibits aromatase Inhibits leukocytes Immuno-modulatory Anti-proliferative effects	Sterols, triterpenic acids, lignans, phenols	• Ethanolic extracts inhibited aromatase activity with IC50 of >100 µg/mL [47].
Epilobolium Willow herb	Anti-inflammatory Anti-androgenic Anti-proliferative effects [69]	Sterols, triterpenes, flavonoid glycosides.	• 75-100 µg/mL of *Epilobolium rosmarinifolium* extract inhibited thymidine incorporation in PZ-HPV-7 cell line [69]. • *Epilobolium parviflorum* extract inhibited 5α-R with IC_{50} of 160 µg/mL [73].
Vitex agnus Chaste tree	Reduces prolactin levels [71] Antiproliferative effects Apoptotic effects [72]	Flavonoids, iridoid glycosides, and terpenoids	• 10-30 µg/mL of Vitex agnus-castus fruit extracts inhibited proliferation of prostate cancer cell lines by 50% however at these concentrations there was an increase in cytotoxic effect by 2 folds compared to solvent controls [74].

Table 2.6.3 A list of common PC therapies that involve changing the hormonal status in the body.

Treatment	***Mechanisms***	***Effects***	***Examples***	***Side effects***
Orchiectomy	Surgical removal of testes.	Reduce androgen production.		Disfiguring, impotency, hot flashes. Side effects are permanent.
LHRH agonists and antagonists	Desensitize the pituitary to native GnRH stimulation.	Reduce androgen production.	Agonist: Zoladex, Lupron Antagonist: Abarelix	Impotency, hot flashes, altered lipid levels, decreased muscle strength GnRH agonists cause testosterone surge and flare initially
Anti-androgens	Bind to HSPs and prevent androgens from binding to AR.	Block actions of androgens.	Casodex, flutamide, nilandron	Gynecomastia (breast enlargement)
5α-reductase inhibitors	Block the conversion of testosterone to a more potent form, DHT.	Reduce DHT production.	Finasteride	Prostate cancer prevention trial have shown that although men taking finasteride had fewer prostate cancers overall (18 % of men in the finasteride group developed prostate cancer vs. 24% of men in the placebo group), the cancers in the finasteride group were of a higher grade (37% of cancers in the finasteride group were high-grade vs. 22% of the cancers in the placebo group). High-grade prostate cancers may be more aggressive and are more likely to spread outside the prostate. (http://www.cancer.gov/cancertopics/factsheet/pcptqa)
Combined anti-androgen blockade (CAB)	Therapy with an LHRH agonist and an anti-androgen.	Reduces androgen production and block androgen actions. Reduces testosterone surge and flare, 6 months survival advantage [75].		Small clinical benefit. Liver toxicity and additional cost may outbalance the benefit Long term CAB leads to sexual dysfunction, facial hair loss, muscle loss, osteoporosis and gynecomastia.
Intermittent Androgen blockade		Tumour cells surviving withdrawal are forced into normal pathways of differentiation by androgen replacement, apoptotic potential may be restored and disease progression may be delayed. Less toxicity and improved quality of life [75].		Difficult to decide when and how treatment should be carried out.

2.7. Inadequacy in present drug treatments and ongoing research.

Inadequacy in present in BPH treatments

At present, the two main pharmaceutical drugs prescribed by doctors are 1) finasteride to shrink the prostate and 2) α-blockers to relax smooth muscle tone. Both medications have enjoyed relative success with a large proportion of patients in relieving the disturbing LUTS symptoms. However, long-term application of these drugs leads to unpleasant side effects. Furthermore there are patients whose conditions were not improved by both 5α-reductase inhibitor and α-blockers. Currently, there is a lack of preventive medication for asymptomatic BPH against the possible enlargement of the prostate and LUTS development.

On going research for new BPH treatments

Besides conventional medicine, there are some popular alternative plant-based drugs. The most widely used herbal remedy in the United States and Europe is Saw palmetto. It is reported having actions similar to finasteride but with no major side effects. In 2006, a double blind, placebo-controlled, randomized clinical trial conducted by Bent *et al*, concluded that Saw palmetto did not improve symptoms or objective measures of BPH [76]. However, it must be pointed out that a specific preparation of Saw palmetto was tested.

To date, the other herbal remedies mentioned in table 2.6.2 have not undergone such rigorous clinical trials. Some interesting potential compounds against BPH, which are currently under investigation, include

- Extracts from the fruits from Brahea aramata [77] and Cuban royal palm [15], which also belong to the same Arecaceae family as Saw Palmetto.
- Lycopene, the primary carotenoid in tomatoes [15], [78].
- Silymarin, polyphenolic flavonoid from *Silybum marianum* [79].
- Indole-3-carbinol, a naturally occurring compound found in vegetables of the Brassica genus [80], [81].
- Isoflavones from Soya extracts [82].

Inadequacy in present in PC treatments

Reduction of androgen-dependent prostate growth is still the rational endocrine therapy for AS PC. Unfortunately, ADT is detrimental in the long run. When the disease has progressed to an AI status, well-established treatment options are limited.

On going research for new PC treatments

The current research includes

- Anti-angiogenesis therapy: Drugs to stop tumours from making new blood vessels, thus inhibiting their growth. The first anti-angiogenic drug, Bevacizumab (Avastin), approved by the FDA in 2004, blocks vascular endothelial growth factor receptor [32].
- Chemotherapy therapy: Docetaxel (Taxotere), which is an anti-mitotic drug, has shown to prolong the life span of PC patients [32], [46].
- Satraplatin: a platinum analogue that is being looked at for AI PC.
- Combination therapy: Compounds that enhance the effects of current drugs. Calcitrol, a vitamin D derivative, have shown promising result when combined with docetaxel.
- Vaccines: APC8015 (Provenge) uses autologous antigen presenting cells (APCs) loaded with the recombinant fusion of prostatic acid phosphatase linked to a molecule that specifically targets a receptor expressed on the surface of APCs. This approach aims to stimulate the body to develop an immune response to PC cells. Onyvax-P is another vaccine made from a cocktail of 3 irradiated allogeneic cell lines. Each cell line expresses antigens that represent a different stage of PC, therefore Onyvax-P contains a broad range of known and yet to be identified cancer-specific antigens [46].
- Radiolabeled monoclonal antibodies: Radiolabeled antibodies targeting prostate specific membrane antigens (PSMA) conjugated with various radiopharmaceuticals (e.g. lutetium) are being developed. PSMA is highly expressed in all PCs and on the tumour vascular endothelium of virtually all solid carcinomas but not on normal vascular endothelium. Therefore it may be possible to specifically kill PC tumour cells without harming normal cells [46].
- Targeting several intracellular cell-signalling pathways involved in cell growth such as MAPK pathway, raf proteins and mammalian target of rapamycin (mTOR) and receptors of growth factors (e.g. EGF, IGF) [32], [46].

2.8. Discussion

Although BPH and PC are inherently different pathologies, they share similar aetiology and certain treatments may be applied for both. Clearly BPH, in comparison to PC, could be controlled with more ease. The treatment strategy for BPH depends on the stage of the disease.

- Asymptomatic: Prevention against possible prostatic enlargement may be achieved through changing dietary habits. There are several compounds (e.g. lycopenes, isoflavones) in fruits and vegetables that may control prostate growth.
- Symptomatic: Besides the 2 main pharmacological approaches to BPH: α_1-blockers and 5α-reductase inhibitors, new medicine should also be developed to target other areas such as inflammation and the estrogen signalling pathways.

Now that we have a greater understanding of the molecular events involved in PC, the view that ADT is an effective therapy was simplistic. PC requires more individualized treatment and a systematic approach to target not only the cancer cells but also the microenvironment in which they proliferate. There are several novel approaches to tackle PC, especially AIPC (e.g. vaccines, antibodies, genetic therapy and inhibition of GFs). This project focuses on using plants that may offer some help against PC (prevention and treatment) by developing phyto-compounds that are cytostatic, downregulate AR levels, induce apoptosis and reduce inflammation.

2.9 References

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3. Aim of the thesis

The purpose of this project is to identify tropical plants with bioactive profiles that could be used to potentially prevent and/or provide additional supportive treatment for BPH and PC.

A bio-active profile of a plant extract should include properties that

- reduce proliferation induced by androgens and estrogens,
- possess apoptotic abilities to counter over proliferation,
- have anti-inflammatory effects,
- down regulate androgen receptors,
- are non cytotoxic

The present work involves screening several plant extracts for potential anti-proliferative effects on the human prostate cancer cell line, LNCaP. Potential candidates will then be further investigated to determine their mode of actions in reducing the cellular growth according to the flowchart below.

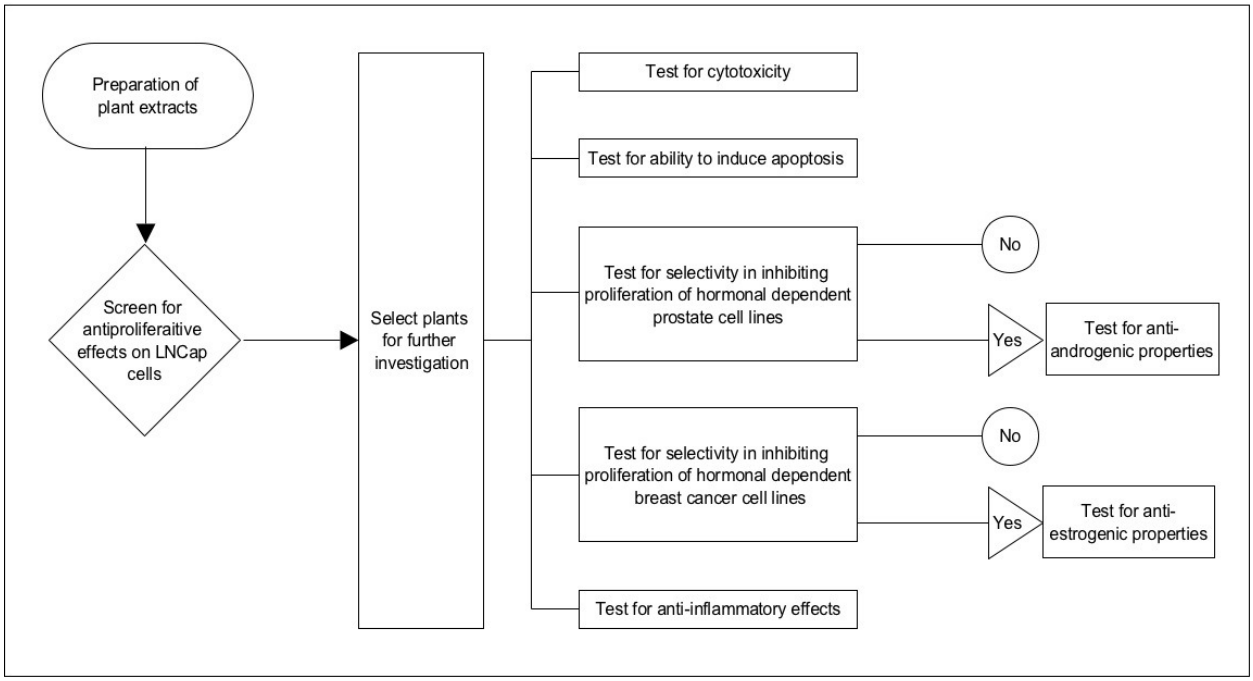

Fig. 3.1 Flowchart of the experimental setup, which would be implemented for the further investigation of plant extracts.

4. Primary screen

4.1 Introduction

There are several approaches to select plants as potential candidates;

1) Random selection followed by chemical screening and biological assays
2) Follow up of ethno-medical usage of plants.

Since this project is geared towards tropical plants, 20 plants from different Asian countries were selected (Table 4.1.1) for investigation based on their uses in various traditional medical systems.

The choice of solvents is unlimited. For this project, we have decided to use water, 30% (w/w), and 70% (w/w) ethanol (EtOH) for the initial extraction. Using different concentrations of ethanol, different groups of phytochemicals will be extracted depending on their polarity. For example, samples extracted with 70% EtOH will contain more lipophilic components than the aqueous extracts. The extracts, at 30µg/mL, were then tested for their potential antiproliferative effect on LNCaP cells.

Table 4.1.1 List of plants selected for the screen.

Plant	Family	Abbreviation	Part of Plant
Alpinia oxyphylla Miq.	Zingiberaceae	**Alp**	Fruit
Aquilaria sinensis Gilg.	Thymelaeaceae	**Aquil**	Heartwood
Astragalus membranaceus Fisch.	Leguminosae	**Astra**	Root
Curcuma aeruginosa Roxb.	Zingiberaceae	**Curum**	Rhizome
Epilobium parviflorum Schreber	Onagraceae	**Epi**	Leaves
Eucommia ulmoides Oliv.	Eucommiaceae	**Euco**	Bark
Fritillaria thunbergii Miq.	Liliaceae	**Frit**	Bulb
Gleditsia sinensis Lam.	Leguminosae	**Glet**	Spines
Kochia scoparia L.	Chenopodiacae	**Kosco**	Fruit
Leonurus japonicus Houtt.	Labiatae	**Leon**	Whole
Lindera aggregate Kosterm	Lauraceae	**Lind**	Root
Oldenlandia diffusa Roxb.	Rubiaceae	**Old**	Leaves
Patrinia scabiosaefolia Fisch.	Valerianaceae	**Pat**	Whole
Piper cubeba L.	Piperaceae	**Piper**	Fruit
Pueraria mirifica Airy Shaw & Suvat	Fabaceae	**Pueraria**	Tuber
Rubus chingii Hu	Rosaceae	**Rubi**	Fruit
Schisandra chinensis Baill.	Schisandraceae	**SchChi**	Fruit
Schisandra sphenanthera Rehder & E. Wilson	Schisandraceae	**SchSph**	Fruit
Sparganium stoloniferum Buch- Ham.	Sparganiaceae	**Sparg**	Rhizoma
Trichosanthes kirilowii Maxim.	Cucurbitaceae	**Tricho**	Root

4.2 Materials and methods

Materials

Fetal bovine serum (FBS) and the other reagents are of the highest quality available and were purchased from Sigma (Buchs, Switzerland) if not otherwise stated.

Preparation of the extracts

The dried plant materials were milled and extracted with 3 solvents; ultra pure water, 30% EtOH (w/w) and 70% EtOH (w/w). The mixtures were left overnight and the liquid fraction was separated from the solid residue by filtering through an AF-6 filter paper first, then through a 4-7 µm filter paper (Scleicher & Schuell, Dassel, Germany). The dried mass content of each liquid extract was then determined.

Cell culture

Human prostate adenocarcinoma cell line LNCaP-FGC was obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP cells were cultured with 1640 medium containing 2 mM L-glutamine and 10 mM HEPES, 1 mM sodium pyruvate, 2.5 g/L glucose, 3.5 g/L sodium bicarbonate and supplemented with 10% FBS. The cells were kept in a humidified incubator at 37°C and 5% CO_2 and passaged at 70-80% confluency.

Proliferation assay using WST

LNCaP cells were seeded at 5000 cells/well in 96-well plates. After 24 hours of pre-incubation, they were treated with 30 µg/mL of the extracts for 4-6 days. To detect changes in cellular numbers, WST-1 (Biovision, Mountain View, CA, USA) was added and incubated for 90 mins. This assay measures the ability of mitochondrial dehydrogenases in living cells to cleave tetrazolium salt WST-1 to formazan. The absorbance of the yellow formazan dye produced by viable cells was measured at 450nm with TECAN infinite 200 multifunctional microplate reader (Tecan Männedorf, Switzerland). The amount of formazan dye produced indicates the proportion of viable cells with respect to the solvent control (1% EtOH). However, it should be noted that it is assumed that the extracts do not directly interfere with the mitochondrial activity.

4.3 Results and Discussion

Results of screen

Twenty plants were screened for their potential antiproliferative effects on LNCaP cells. For each plant, 3 solvents, water, 30% EtOH and 70% EtOH were used to prepare the extracts. Extracts that could reduce the cell numbers by 50% in comparison to the solvent control were considered for selection.

Out of the 60 extracts, water extracts of *Alpinia oxyphylla* and *Astragalus membranaceus* (Fig.4.1A) and *Alpinia oxyphylla*, *Aquilaria sinensis*, *Epilobium parviflorum* and *Piper cubeba* 70% EtOH extracts (Fig.4.1C) could reduce LNCaP cell numbers quite significantly. *Epilobium parviflorum, which was used as a control for the screen,* is currently used as a phytotherapy against BPH. Therefore, it will not be further investigated.

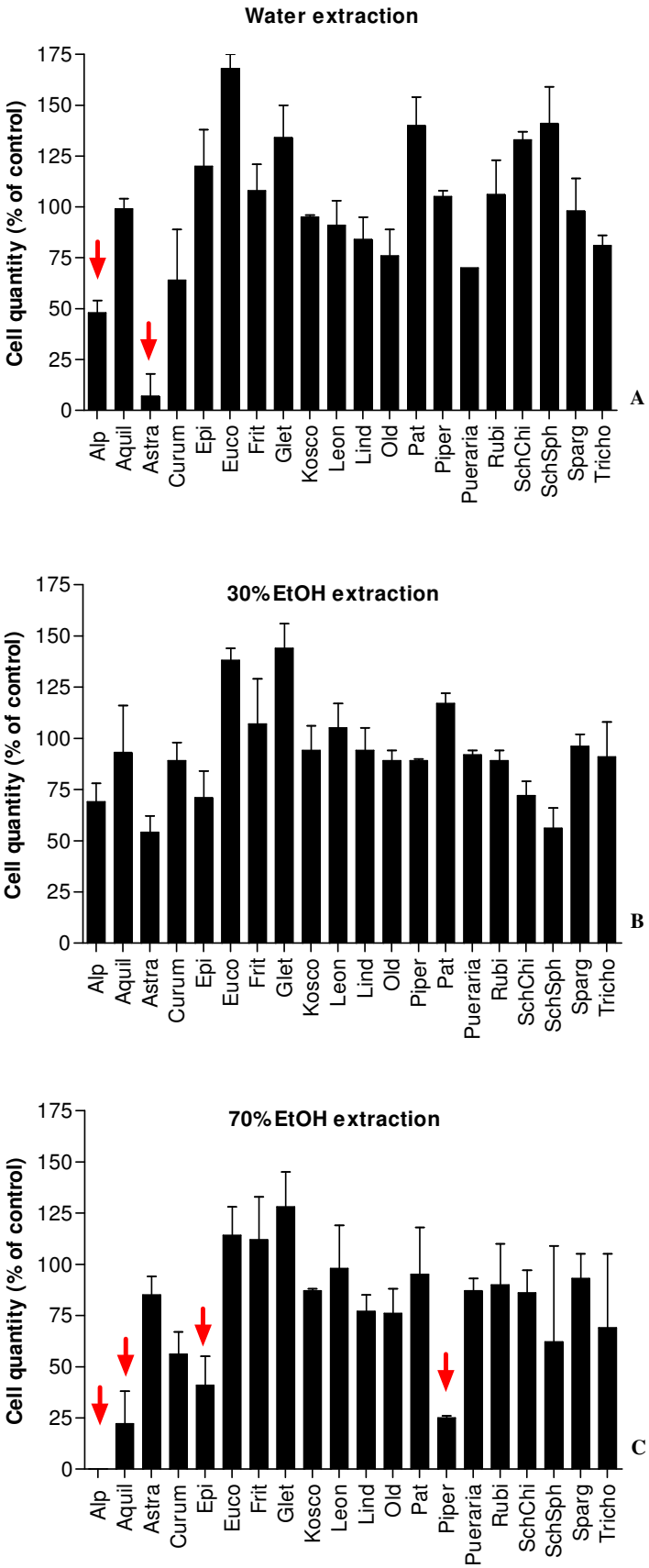

Fig. 4.1 Effects of plants extracted with water (A), 30% EtOH (B) and 70% EtOH on LNCaP cells.

Further Piper extractions

Piper cubeba was extracted using a wider range of ethanol concentrations. According to Fig. 4.2, extracts made from 50% EtOH onwards started to have antiproliferative effects on LNCaP cells. It suggests that the more lipophilic fractions contain more active compounds, which could be responsible for the reduced cellular growth.

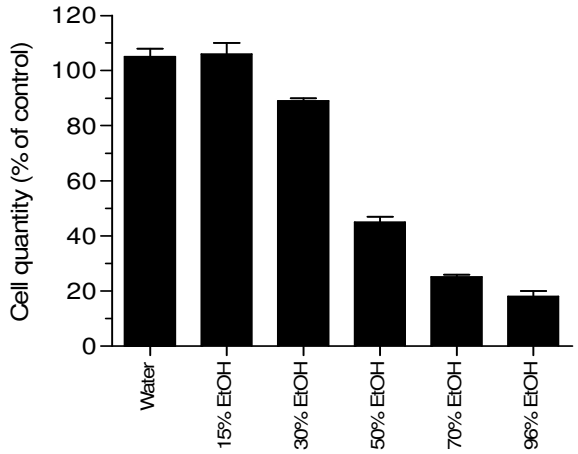

Fig. 4.2 The effects of different *Piper cubeba* extracts on the growth of LNCaP cells.

Selection of plant candidates

We have decided to use the following plant extracts for further research.

1. Astra, Water extract of *Astragalus membranaceus*
2. Alp, 70% EtOH extract of *Alpinia oxyphylla*
3. Aquil, 70% EtOH extract of *Aquilaria sinensis*
4. P9605, 96% EtOH extract of *Piper cubeba*

5. Potential hepatotoxicity

5.1 Introduction

Hepatotoxicity is a common side effect of phyto-medicine. Several medicinal plants (e.g. Kava kava) had to be withdrawal from the market because of their association to liver toxicity. We decided to test our extracts for potential hepatotoxicity first, before continuing with further investigations.

Activity of the mitochondrial electron transport chain and plasma membrane integrity are some of the cellular processes influenced by cytotoxic agents. The human hepatocarcinoma HepG2 cell line is frequently used as an *in vitro* model for studying hepatotoxicity. A simple hepatotoxicity test system measuring the effects of the extracts on the mitochondrial activity and plasma membrane integrity on HepG2 cells was therefore performed.

5.2 Materials and methods

Cell culture

The HepG2 cell line was obtained from the American Type Culture Collection. They were cultured in MEM medium containing 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate and 10% FBS. The cells were kept in a humidified incubator at 37°C and 5% CO_2 and passaged at 70-80% confluency.

Cytotoxicity assay

HepG2 cells were seeded 5000 cells/well. After 24 hours of pre-incubation, a range of several concentrations per extract was added. After 24 hours, WST-1 was added and incubated for 90 mins. The absorbance of the yellow formazan dye produced by viable cells was measured at 450nm with TECAN microplate reader. Terfenadine (100 µM, final concentration) served as a control. The mitochondrial activity was calculated as a % of the solvent control.

To investigate if the extracts damaged the plasma membrane, the supernatants were tested with the Lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Biovision; Mountain View, CA, USA). The principle of this method is based on the measurement of the activity of LDH released from cells with damaged plasma membrane. Equal volumes of assay reagent mixture (diaphorase/NAD^+ and tetrazolium salt INT) were incubated with the supernatant for 10 mins.

TECAN was used to measure the absorbance of the samples at 490 nm. Triton X, which destroys the plasma membrane, served as a positive control. Background effect of the extracts was also measured for both assays and taken into consideration when evaluating the results.

5.3 Results and Discussion

Potential acute toxicities of Api, Aquil, Astra and P9605 on HepG2 cells were examined after 24 hours incubated with the cells. All extracts did not disrupt the plasma membrane of the cells as indicated from the LDH results. Except for Api, none displayed any significant cytotoxic effects even at the highest concentrations tested. Api reduced mitochondrial activity by more than 50% between concentrations of 30 to 37 µg/mL.

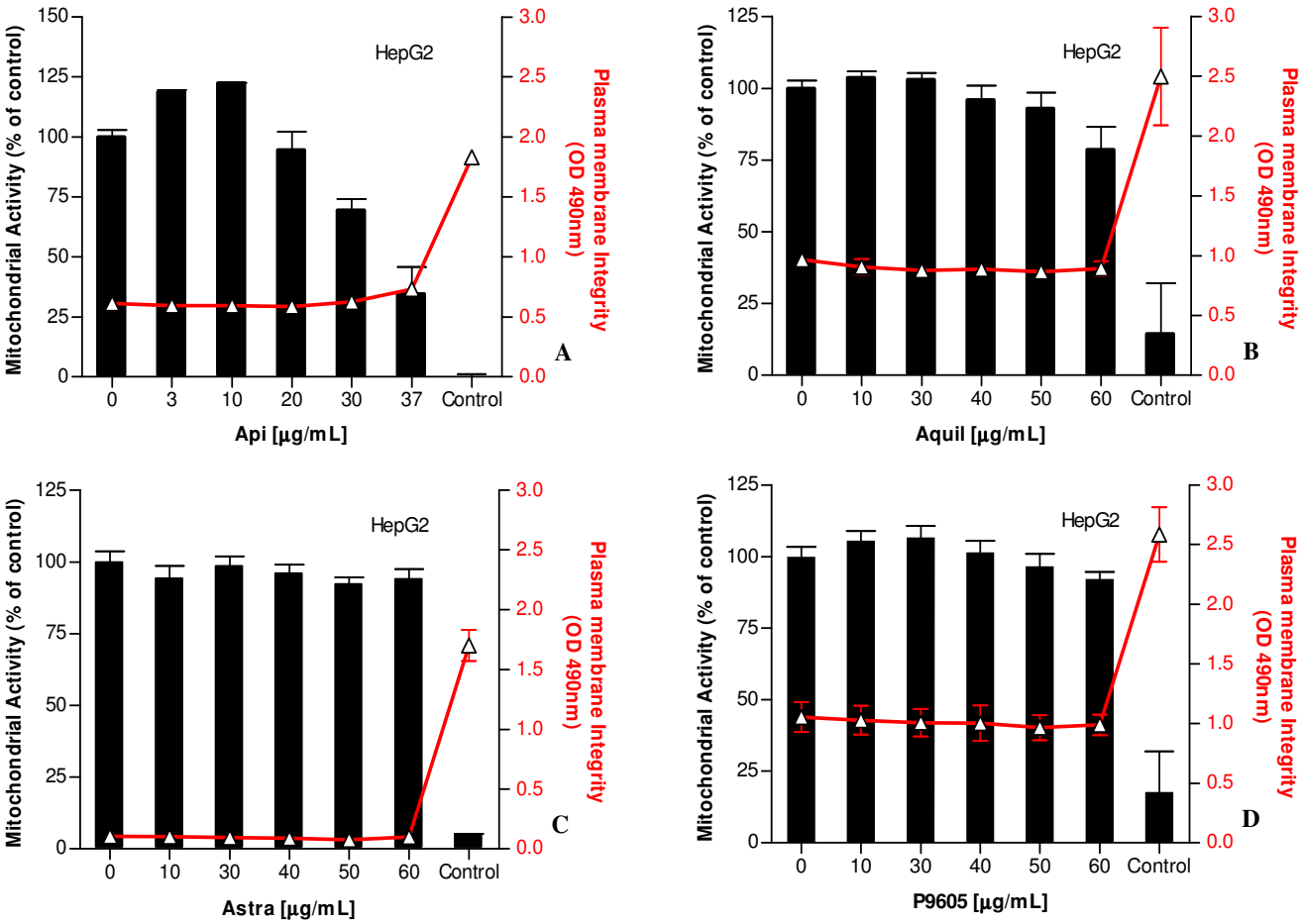

Fig. 5.1 Cytotoxicity data of Api (A), Aquil (B), Astra (C) and P9605 (D). Each graph shows the two parameters investigated; mitochondrial activity (Left Y-axis Bars) by WST assay and plasma membrane integrity (Right Y-axis, Line graph) by LDH assay. The control refers to terfenadine (WST assay) or Triton X (LDH assay). Data represent mean ± SD of n≥5.

6. *Piper cubeba* targets multiple aspects of the androgen-signalling pathway.

Original Paper

Piper cubeba targets multiple aspects of the androgen-signalling pathway. A potential phytotherapy against prostate cancer growth?

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Submitted to: Planta Medica

6.1 Abstract

Despite the high prevalence of prostate cancer (PC) in the Western world, there is a dearth of effective medication. Since the androgen-signalling pathway is very much involved in PC growth and development, we investigated the potential of *Piper cubeba* L. extract, P9605, in targeting multiple events simultaneously within this pathway. This may be more effective compared to an anti-androgen monotherapy. Our results indicated that P9605 inhibited proliferation in androgen-dependent LNCaP human prostate cancer cells by reducing DNA synthesis and inducing apoptosis. This anti-growth effect was less pronounced in the androgen independent PC-3 prostate cancer cell line. P9605 potently inhibited 5-α reductase II activity, which is responsible for converting testosterone to its active form, dihydrotestosterone (DHT), in the prostate. It also acted as an antagonist at recombinant wild type androgen receptors (AR). P9605 suppressed cell growth and prostate specific antigen (PSA) secretion stimulated by physiological concentrations of DHT in LNCaP cells. Interestingly, it down regulated AR levels. In conclusion, our findings suggest that P9605 may potentially retard the growth of androgen dependent PC via several mechanisms.

Key words: *Piper cubeba* (L.), Piperaceae, LNCaP cells, apoptosis, 5-α reductase II, androgen receptor, PSA.

6.2 Introduction

Prostate cancer (PC) is one of the leading causes of cancer-associated deaths among men in Western countries. According to the National Prostate Cancer Coalition, over 218,890 new cases of PC are forecasted for 2007 in the United States. Several risk factors, responsible for the development of PC, are age, race and diet. The early development stages of PC are often related to an uncontrollable proliferation of the prostate cells activated by androgens.

There are two major androgens, testosterone and dihydrotestosterone (DHT), in humans. Testosterone is the main secretory hormone while DHT is the active form of androgen in prostate cells. Androgen deprivation therapy (ADT) is one of the mainstream medical therapies [1] employed to treat locally advanced and advanced metastatic PC by drastically reducing the production and/or actions of androgens. Treatments include orchiectomy, the use of luteinizing hormone-releasing hormone (LHRH) analogues (e.g. leuprolide and goserelin) or LHRH antagonists (e.g. abarelix) and anti-androgens [2].

Although patients show positive response initially to treatment, continuous ADT often results in PC progressing to a hormone refractory state within 18-24 months [3]. There are several postulated mechanisms explaining the development of ADT resistance. These include somatic mutations of the androgen receptor (AR), AR amplification and the development of alternative signalling pathways that bypasses the growth and survival promoting function of AR [4]. Defects in apoptotic signalling pathways are also common in cancer cells, which enhance tumor progression, promote metastasis and therefore develop resistance to various forms of therapy [5].

Considering the essential roles of androgens and AR in PC, it is rational to identify novel agents that target multiple aspects of the androgen-signalling pathway. A compound that possesses anti-androgenic properties, reduces DHT production, induces apoptosis as well as down-regulates AR levels may reduce PC growth and its probability of progressing to a hormone refractory state.

Plants contain a rich horde of natural substances, which could provide promising bioactive candidates. There is some evidence that phytoestrogen (isoflavons, coumestans and lignans) may be useful in supporting the treatment of PC [6], [7].

Therefore, the aim of this project was to search for plant extracts with minimal acute and long-term toxicity, which may help to retard the growth and development of PC.

Piper cubeba L. is indigenous to South of Borneo and Indonesia (Java, Prince of Wales Island and Sumatra). The dried unripe fruits possess antiseptic, expectoral and diuretic properties. Powdered form or tinctures of the cubebs are used extensively in Indonesia for the treatment of gonorrhoea, dysentery, syphilis, chronic bladder inflammation, diarrhoea and asthma [8]. The important constituents of cubebs are volatile oil and lignans, which include cubebin, cubebic acid and cubeb-resin [9].

In this study, we investigated the effects of P9605, an ethanolic extract of *Piper cubeba* L., on the growth of 2 human prostate cell lines that represent two different hormonal states of PC and its potential anti-androgenic properties. We also hypothesized that cubebin could be an active compound (Fig.6.1A) so it was tested alongside with P9605 in several assays. For the first time, we report that P9605 has demonstrated the ability to retard androgen dependent cellular growth of a PC cell line via several different mechanisms.

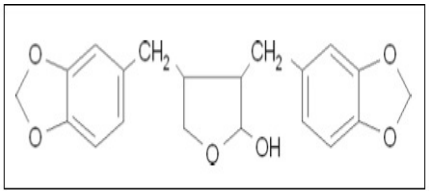

Fig. 6.1A Chemical structure of cubebin.

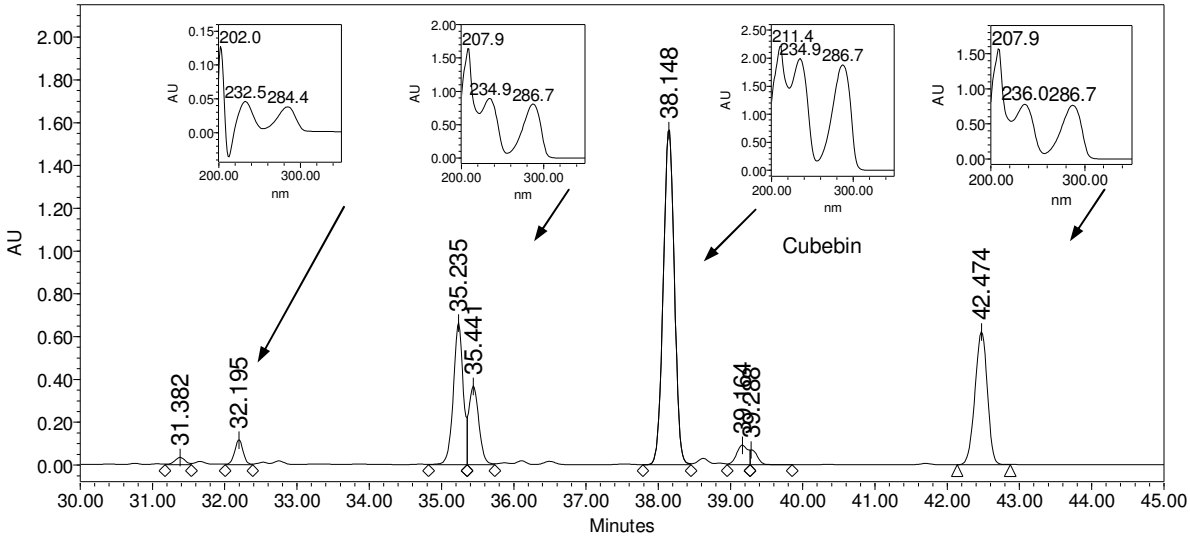

Fig. 6.1B A HPLC chromatogram of P9605. Peaks 1, 2 and 4 indicate the presence of lignans based on their typical UV spectra. Peak 3 was identified to be cubebin. Based on quantification of the different lignan peak areas, cubebin appeared to be the dominant lignan present.

6.3 Materials and Methods

Chemicals

All radioligands (purity > 97%) were purchased from Perkin Elmer (Boston, MA, USA). All cell culture mediums, fetal bovine serum (FBS) and the other reagents are of highest quality available and were purchased from Sigma (Buchs, Switzerland) if not otherwise stated. Charcoal stripped FBS (CSS) was obtained from HyClone (Logan, UT, USA)

Preparation of the extracts

Piper cubeba L. fruits were provided by Vitaplant AG, Witterswil, Switzerland. The milled seeds were de-fatted twice for an hour with hexane in a ratio of 1:5 (w:w) before filtering through an AF-6 filter paper (E. Begerow GmbH & Co; Langenlonheim, Germany). The residue was then oven dried before being extracted with 96% EtOH in a ratio of 1:5 (w:w).

HPLC analysis

An aliquot of the extract was analyzed by a Waters HPLC system with UV-VIS detection (280 - 600 nm) using a Nucleosil 120 – 3, C18 (250 × 4.6 mm) column with a pre-column of the same material (both Macherey Nagel; Oensingen, Switzerland) as stationary phase. The mobile phase consisted of two solvent systems {A: 0.1% trifluoroacetic acid (TFA) in water (v/v) and B: 100% acetonitrile in a gradient (0-30 min 90% A, 30-45 min 50% A, 45-46 min 10% A, 46-50 min 10% A)}. The column temperature was kept at 40 °C and the flow rate was set at 1.0 mL/min. The detection was carried out at 280 nm and quantification of the lignan in the extract was performed by the external standard method using cubebin (Sigma; St-Louis, MO, USA) as a reference substance.

Cell culture

The human prostate adenocarcinoma cell lines LNCaP-FGC and PC-3 were obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP cells were cultured with RPMI 1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and 10% FBS. PC-3 cells were kept in RPMI medium with 10% FBS. The cell lines were kept in a humidified incubator at 37 °C and 5% CO_2 and passaged at 70-80% confluency. Cultures used in subsequent experiments were passaged less than 25 times.

Cellular assays

Cells were seeded at 5000 cells/well in 96-well plates. After 24 hours of pre-incubation, they were treated with 3, 10, 30 µg/mL concentrations of P9605 for an indicated time period unless otherwise stated. Phenol red free medium was used for assays performed on LNCaP cells. Solvent control, 1% EtOH, was tested in all assays. Cubebin was also tested alongside for certain assays

Cytotoxicity assays

To investigate if the extracts damaged LNCaP cells' plasma membrane and affected their cellular viability, Lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Biovision; Mountain View, CA, USA) and WST-1 (Biovision; Mountain View, CA, USA) were performed respectively according to manufacturer's instructions. Background effects of the extracts were also measured for both assays and taken into consideration when evaluating the results.

DNA detection assay

Cells were treated with P9605 and cubebin for 4-6 days. DNA amount was quantified using CyQuant cell proliferation assay kit (Molecular Probes; Eugene, OR, USA) according to manufacturer's instructions. The CyQuant GR dye used in this assay exhibits strong fluorescence enhancement when bound to DNA. A range of DNA standards (0-1000 ng/mL) was included in every measurement.

^{3}H-Thymidine incorporation assay

After incubation with the samples, ^{3}H-Thymidine (3 µCi/mL) was added to each well and incubated at 37 ºC for 3 hours. The cells were then washed and harvested onto filter strips by a cell harvester (Brandel Inc; Gaithersburg, MD, USA), and radionucleotide incorporation was measured using Tri-Crab 1900 TR scintillating counter (Packard; Meriden, CT, USA).

Apoptosis assay

After 48 hours of incubation with samples, supernatants of LNCaP cells were assessed by Cell death detection ELISA^{PLUS} (Roche; Mannheim, Germany) according to supplier's instructions. The extent of apoptosis was computed as a ratio of the solvent control.

5α Reductase activity

This assay was preformed using homogenates of HEK293 cells over expressing 5α reductase type II. They were purchased from Dr. Hartmann (Department of Pharmaceutical and Medical Chemistry, University of Saarbrücken, Germany) [10]. cDNA encoding 5α reductase type II were inserted into a pRcCMV vector and expressed in these cells. The samples were pre-incubated with the cell homogenate (20 µg/mL per assay) for 5 mins at 37 ℃. After which, 80 nM ^{3}H-testosterone (substrate) was added to each well and incubated for 20 mins at 37 ℃. The remaining ^{3}H-testosterone and new steroids produced were extracted by ethyl acetate. Thin layer chromatography (TLC) (equal volumes of cyclohexane and ethyl acetate were used as an elution solvent) was preformed to separate the steroids. The TLC plate was then developed using a detection spray containing anisaldehyde, concentrated sulphuric acid, acetic acid glacial and methanol. To quantify the amount of ^{3}H-testosterone remaining and ^{3}H-DHT produced, bands on the TLC corresponding to the respective steroids were cut out and counted via the scintillating counter. The activity of the enzyme was determined by calculating the conversion of ^{3}H-testosterone to ^{3}H-DHT.

Androgen receptor binding

10, 30, 100 µg/mL of P9605 and 10, 30, 60 µg/mL of cubebin were incubated with recombinant androgen receptors (Invitrogen; Carlsbad, CA, USA) and ^{3}H- methyltrienolone (16 nM) at 4 ℃ overnight. To determine non-specific binding, 10 µM of non-radiolabelled methyltrienolone was used. After incubation, the receptor bound fraction in the assay mix was separated by centrifuging through MircoSpin G-25 columns (GE Healthcare; Piscataway, NJ, USA). The amount of radioligand bound to the receptors in the filtrate was quantified by the scintillating counter.

PSA detection

LNCaP cells were treated with P9605 and cubebin for 48 hours. After which, the supernatants of the cells were removed for PSA quantification. Human PSA ELISA kit (Alpha Diagnostic; San Antonio, TX, USA) was used according to supplier's instructions. The DNA contents of cells were determined by the CyQuant cell proliferation assay. The ratio of PSA produced versus DNA amount per well was calculated.

Western blot analysis

LNCaP cells were cultured in 25 cm^2 cell culture flasks until near confluency (80%) before the addition of the samples at concentrations 30, 15, 7.5 µg/mL. After incubation the cells were lysed. Equal amounts of cell lysates were mixed with 1x loading buffer (Laemlli Buffer, 5% 2-mercaptoethanol) and fractionated by electrophoresis on 8% SDS polyacrylamide gels. Proteins were electro transferred to nitrocellulose membranes (Millipore; Bedford, MA, USA). Blots were incubated overnight at 4 ℃ with primary mouse anti-human androgen receptor (Progen; Heidelberg, Germany) and anti-β-actin (Sigma; MO, USA). The membranes were then incubated for 1 hour with secondary goat anti-mouse IgGs conjugated with horseradish peroxidase (BioRad; Hercules, CA, USA). The blots were developed with chemiluminescent substrate and enhancer (BioRad, Hercules, CA, USA), followed by exposure to x-ray film. The images were scanned and analysed by Quantity One software programme (BioRad, Hercules, CA, USA).

Statistical analysis

Each data set represents the means ± standard deviation (SD) of at least 3 experiments. GraphPad Software Inc (Prism, version 4, San Diego, CA, USA) was used to calculate the IC_{50} values. For repetitive comparison of dose-response data with control values analysis of variance (ANOVA) with subsequent Dunnett multicomparison test was used (SPSS for windows, version 14.0, SPSS Inc., Chicago, Ill, USA). Statistical significance was established at values of $p< 0.05$. Asterisks (*), (**), (***) indicate $p<0.05$, $p<0.01$, $p<0.001$ respectively.

6.4 Results

The anti-proliferative effects of P9605 and cubebin were investigated on androgen dependent LNCaP cells and androgen insensitive PC-3 cells. After 4 days of treatment, both P9605 and cubebin produced a concentration-dependent reduction of LNCaP cells' DNA content as compared to solvent controls, with IC_{50} values of 26 µg/mL (Fig. 6.2A) and 18 µg/mL (Fig. 6.2B) respectively. At these concentrations, both did not affect the growth of PC-3 cells to the same extent.

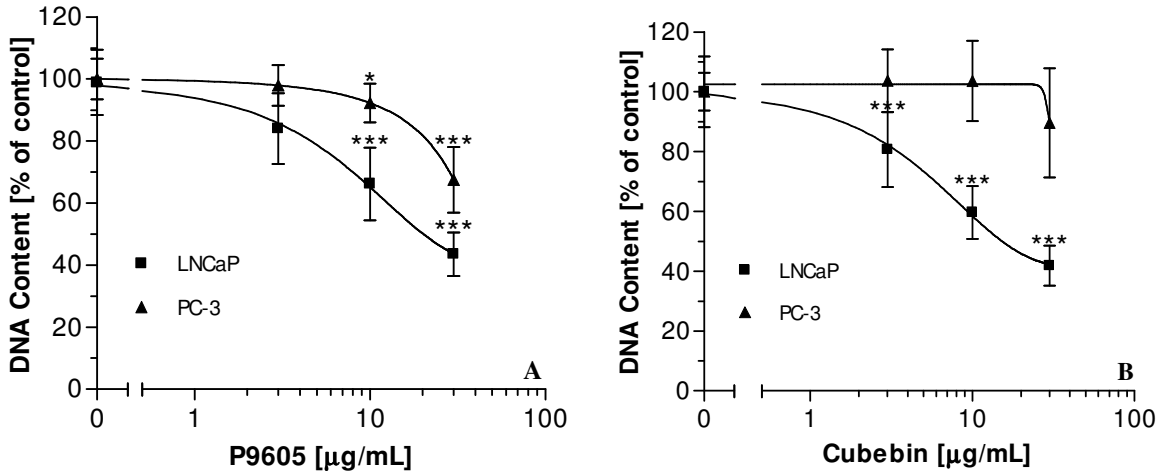

Fig. 6.2 Antiproliferative effects of P9605 (A) and cubebin (B) on LNCaP and PC-3 cells cultured in 10% FBS medium for 4 days. Data represent means±SD of n≥9. DNA content was quantified by CyQuant cell proliferation assay *p<0.05 vs control, **p<0.01 vs control, ***p<0.001 vs control.

Our HPLC analysis revealed that P9605 consisted of 16.53% of cubebin. We then compared the effects of P9605 with the calculated concentration of cubebin present in each dose of P9605 tested. We could show that 30 µg/mL of P9605 reduced LNCaP growth by 50%. Although in 30 µg/mL of P9605 there is approximately 5 µg/mL of cubebin, the application of the latter resulted in a significantly ($p<0.002$) lower extend of inhibition of about 25% (Fig. 6.3). Therefore, this indicates that there are other constituents in P9605 besides cubebin that may attribute to its antiproliferative properties. We have detected three other lignans in our HPLC chromatographic fingerprint of P9605 besides cubebin. Although they were present in lower amounts compared to cubebin, these lignans could very well be partly responsible for the reduced LNCaP growth (Fig. 6.1B).

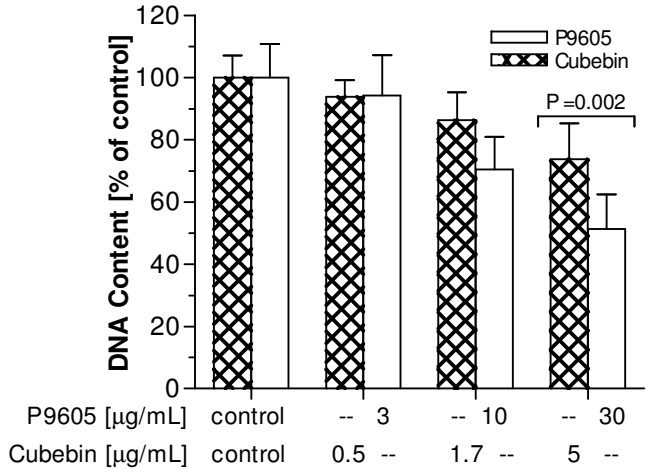

Fig. 6.3 The effect on LNCaP cellular growth induced by P9605 and its corresponding calculated quantity of cubebin present in each concentration were compared. DNA content of P9605 treated LNCaP cells were significantly ($p< 0.002$) different from cells treated cubebin at their highest concentrations.

To further confirm that P9605 antagonises androgen-dependent growth, P9605 was incubated with and without the addition of 1 nM DHT on LNCaP cells cultured in 10% charcoal stripped serum (CSS) medium. Our results showed that P9605 inhibited LNCaP cells' proliferation when cultured in androgen-free medium and P9605 at 10 µg/mL, could significantly ($p<0.001$) lower DHT induced growth by 2-folds (Fig. 6.4A). This proves that P9605 is not weakly androgenic, as it did not increase cell growth in the absence of DHT. In fact, it antagonises the proliferative effect of DHT.

In addition, we also examined if P9605 reduced DNA synthesis. It inhibited ^{3}H-thymidine incorporation with an IC_{50} value of 11.5 µg/mL (results not shown). 10 µg/mL of P9605 could reduce DNA synthesis induced by 1 nM DHT by 50% thus further validating the anti-androgenic property of P9605 (Fig. 6.4B).

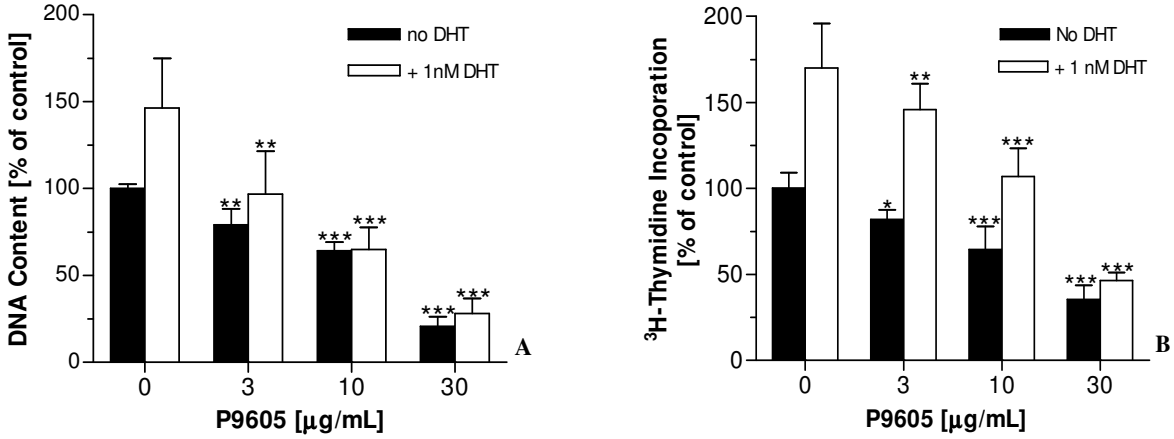

Fig. 6.4 LNCaP cells cultured in 10% CSS medium were treated with P9605 in the presence and absence of 1 nM DHT for 6 days. Changes in cell numbers were determined by quantifying the DNA content (A). ^{3}H-thymidine incorporation was also used to assess the effects of P9506 on DNA synthesis of LNCaP cells kept in 10% CSS medium with or without 1 nM DHT (B). Data represent means±SD of n≥5. All data points are expressed as% of the solvent controls. *p<0.05 vs control, **p<0.01 vs control, ***p<0.001 vs control.

The potential cytotoxicity of P9605 was investigated by observing the effects it had on LNCaP cells' mitochondrial activity and if it induced acute necrosis by destroying the cell membrane after 24 hours incubation. WST results showed that only concentrations greater than 40 µg/mL were toxic. The LDH assay, in contrast, indicated that no plasma membrane damage occurred even at 60 µg/mL. (Fig. 6.5)

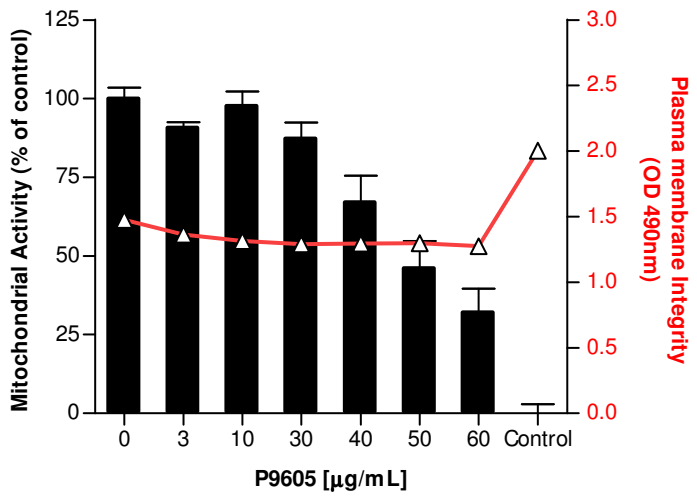

Fig. 6.5 Cytotoxicity data of P9605. The graph shows the two parameters investigated; mitochondrial activity (Left Y-axis, Bars) by WST assay and plasma membrane integrity (Right Y-axis, Line graph) by LDH assay. The control refers to terfenadine (WST assay) or Triton X (LDH assay). Data represents means±SD of n≥8.

We also investigated if the reduction of cell numbers by P9605 and cubebin could be partly due to apoptosis. At 30 µg/mL, P9605 and cubebin increased apoptosis by a factor of 5- and 2-folds respectively (Fig. 6.6A). On the contrary, even after 4 days of incubation, at 30 µg/mL, both samples did not induce necrosis (Fig. 6.6B).

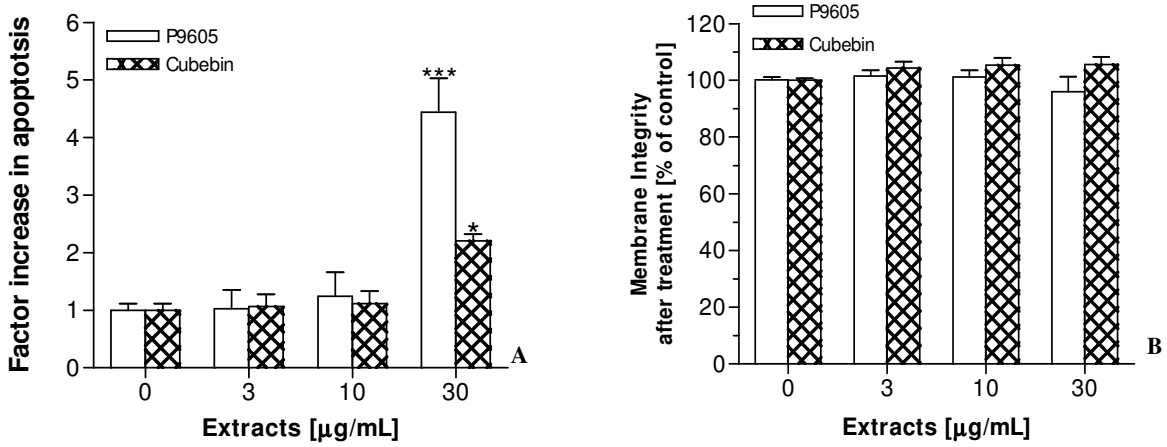

Fig. 6.6 Apoptotic effects of P9605 and cubebin were assessed on LNCaP cells lysates after 48 hours of treatment. DNA fragmentation, a common feature of the late stages of apoptosis was determined from the cell lysates. Values were calculated as a factor of the solvent control (A). The possible cytotoxic effects of prolonged incubation of P9605 and cubebin on LNCaP cells were assessed by LDH assay after 4 days of incubation. Membrane Integrity correlates inversely to the degree of necrosis caused by cytotoxicity of the extracts. Membrane Integrity was calculated as a % of the control (100% for solvent controls) (B). Data represent means±SD of n=6. *p<0.05 vs control, ***p<0.001 vs control.

TNF-α is one of the prime signals that induces apoptosis in a host of cells [11], [12]. We could observe this dose-dependent induction of apoptosis by TNF-α in LNCaP cells. We also saw that 100 nM DHT abolished TNF-α induced apoptosis and lowered the basal level of apoptosis. There was a trend that 10 µg/mL of P9605 diminished the anti-apoptotic effect of DHT. However, due to the high variability, this effect was not statistically significant (results not shown).

It was of interest, as well, to see if P9605 could inhibit DHT synthesis from testosterone. 5α Reductase II (5α-RII) is dominantly responsible for converting testosterone to DHT in prostate cells. Fig. 6.7A showed that both P9605 and cubebin inhibited 5α-RII activity with IC_{50} values of 3.6 µg/mL and 9.9 µg/mL respectively. Finasteride, a well-known 5α-RII synthetic inhibitor, served as a control. Its IC_{50} value of 3.7 nM, derived from our test system also corresponded to literature (data not shown) [10], [13].

Androgens mainly elicit their actions when bound to AR. The binding affinities of P9605 and cubebin to AR were therefore investigated. IC_{50} values of P9605 and cubebin derived from competitive binding assays using recombinant AR were 58 µg/mL and 25 µg/mL respectively (Fig. 6.7B).

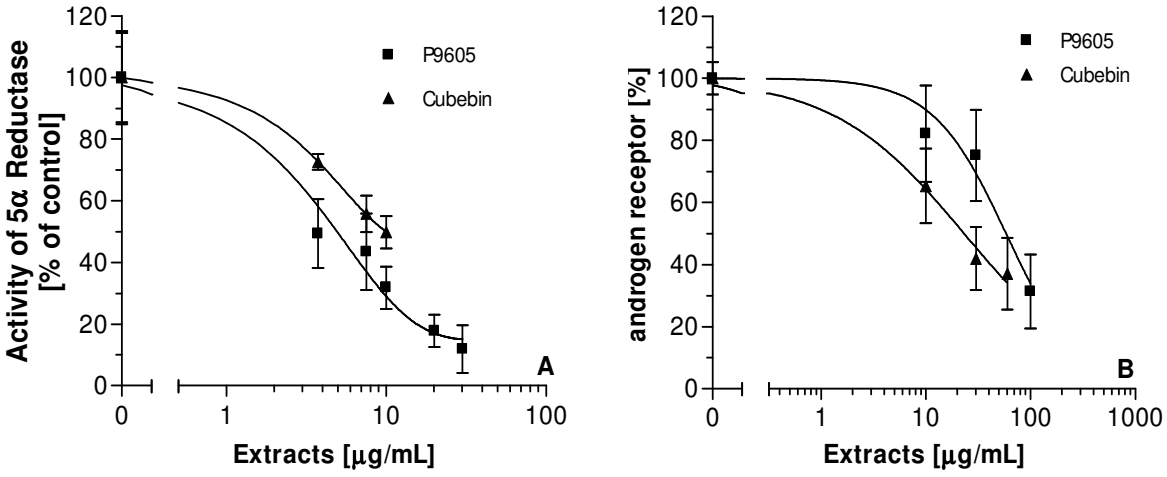

Fig. 6.7 Effects of P9605 and cubebin on 5α-RII enzyme activity (A). The relative binding affinities of P9605 and cubebin to recombinant AR were determined by competitive binding (B). Data represent means±SD of 2 experiments preformed in triplicates.

Transcription of the PSA gene is positively regulated by the AR [14]. PSA is produced and secreted when AR-DHT complex is bound to the specific androgen-responsive elements (ARE) on the PSA gene in LNCaP cells. 20 µg/mL of P9605 or cubebin could reduce the PSA levels by 50% after 48 hours treatment on LNCaP cells (Fig. 6.8A). The dose-dependent increase in PSA secretion induced by increasing DHT concentration conforms to literature [15], [16]. As we expected, 10 µg/mL of P9605 hindered this trend thus confirming that it antagonises DHT's action. (Fig. 6.8B)

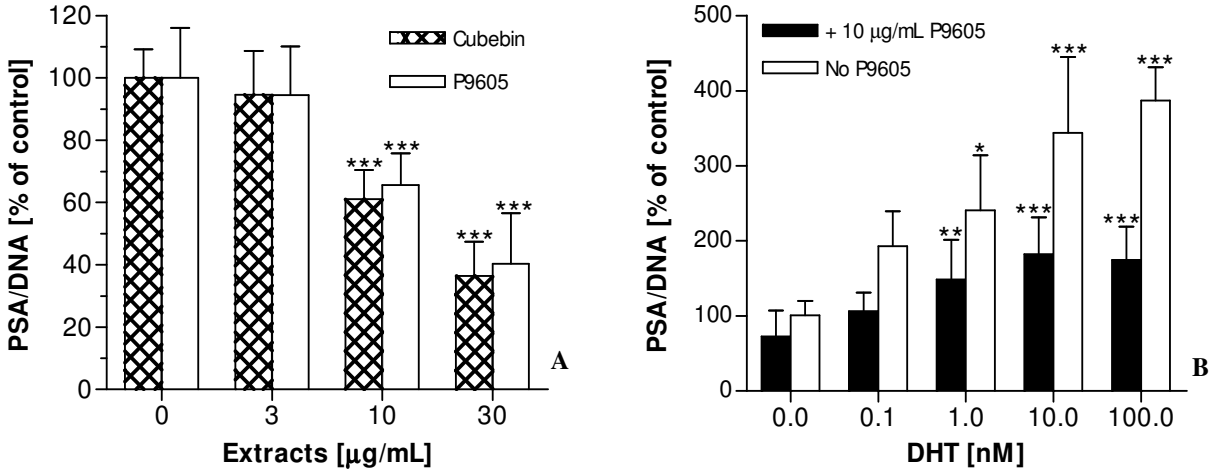

Fig. 6.8 P9605 and cubebin inhibited the secretion of PSA in LNCaP cells. The PSA levels in the cell supernatant were measured with the Human PSA ELISA kit after 48h treatment with different concentration of the samples (A). The DNA content of each well was determined by CyQuant. The data points were calculated as a ratio of PSA secreted to the DNA content of each well. Results represent means±SD of 3 experiments performed in triplicates. DHT induced PSA secretion dose-dependently in LNCaP cells and this trend was abrogated in the presence of 10 µg/mL of P9605. The cells were cultured in 10% CSS medium and treated for 48 hours (B). *p<0.05 vs control, **p<0.01 vs control, ***p<0.001 vs control.

The reduced PSA secretion could be attributed to several reasons such as P9605 competing with natural androgens for the AR or a general reduction in AR levels. To investigate if P9605 regulates AR levels, LNCaP cells were incubated with the samples before being harvested for western blot analysis. As indicated in Fig. 6.9, at 30 µg/mL, both samples reduced AR levels significantly after 48 hours.

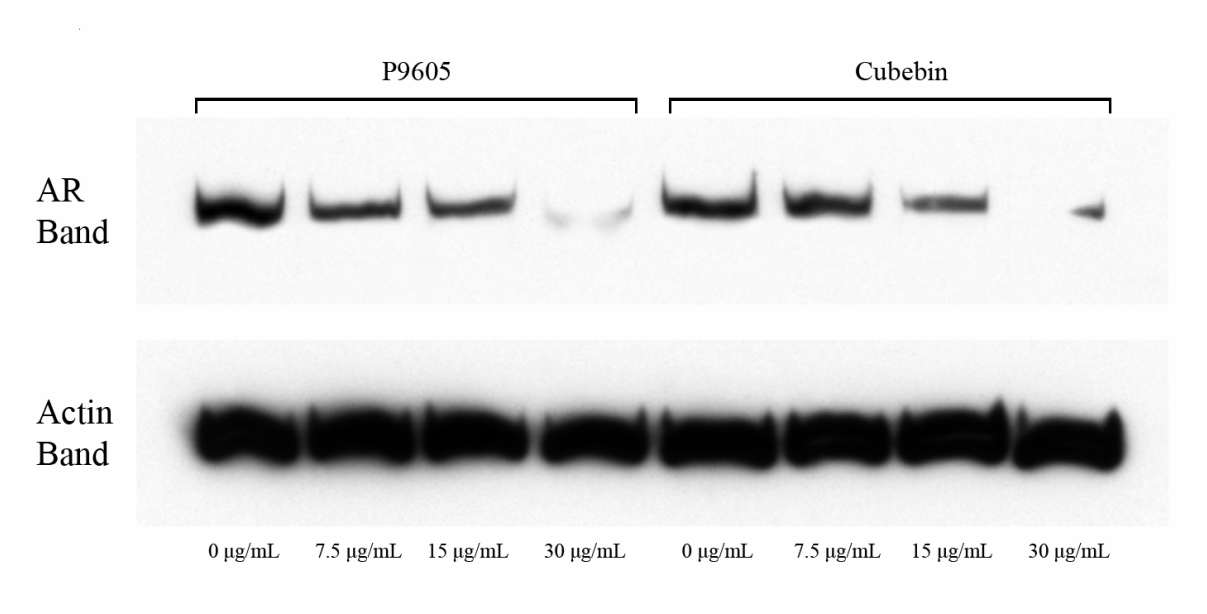

Fig. 6.9 Androgen receptor levels in LNCaP cells were reduced after 48 hours exposure to 7.5, 15, 30 µg/mL of P9605 and cubebin. β-Actin was used as a loading control. This change in AR quantity was detected by Western blot (n=3).

6.5 Discussion

Whilst ADT is a highly successful treatment against hormone-sensitive PC, the onset of hormone resistance happens in many cases. This clearly shows that ADT achieved surgically or chemically is inadequate. In fact, the treatment “forces” the prostate cancer cells to overcome their need for androgen and become androgen-refractory, thus resulting in greater risk of morbidity [14]. There are several molecular mechanisms underlying androgen independency. Mutated AR are responsible for 10-20% of PC patients [17], [18]. Others include the amplification of AR levels and the development of alternative signalling [19], [20]. Hence this present study investigates the various abilities P9605 possesses that may potentially help control the growth and development of PC more effectively.

LNCaP cells originated from a lymph node metastasis of a PC patient and are perhaps the best-studied *in vitro* model for androgen and AR signalling [21]. They express AR as well as androgen-inducible genes like PSA, which is a clinical marker for PC [14]. The PC-3 cell line was derived from a bone metastasis and they do not possess AR [22]. Both these cell lines are therefore quite adequate for our investigation purposes. P9605 suppressed the growth of androgen-dependent LNCaP cells more significantly than androgen-insensitive PC-3 cells. This suggests that the extract inhibits cell growth fuelled by androgens. Our results have shown that besides retarding proliferation by inhibiting DNA synthesis, P9605 also induces apoptosis. We also observed a trend where DHT reduced the basal level of apoptosis and that P9605 could reverse this anti-apoptotic nature of DHT. Induction of apoptosis is necessary to counter the over proliferation of PC cells.

Androgens elicit their various actions when bound to AR. Our competitive binding assays revealed that P9605 antagonised the binding of androgens to wild-type recombinant AR. This is reinforced by a functional assay. When DHT binds to AR in cells, the bound DHT and AR complex translocates to the nucleus and binds to specific ARE on the PSA gene. PSA is then produced and secreted. After treatment with P9605, there was a reduction in PSA secretion by LNCaP cells. Down-regulation of PSA secretion may also help counter PC growth. PSA has been reported to function as a growth factor in LNCaP cells [23], [24] and promote migration and metastasis of PC cells through several mechanisms, including the cleavage of insulin-like growth factor-binding protein and the degradation of extra cellular matrix proteins [25],[26].

It was also observed that DHT, at a physiological concentration of 1 nM, increased cellular numbers and DNA synthesis. DHT also dose-dependently increased PSA secretion in LNCaP cells. With 10 µg/mL of P9605, all these effects of DHT were abolished. Hence these evidence support the hypothesis that P9605 is anti-androgenic.

Besides antagonising the effects of DHT, P9505 reduced DHT synthesis by inhibiting 5α-RII. Reducing the availability of DHT to androgen-dependent prostate cancer cells would restrict its growth.

P9605 down regulated AR levels within 48 hours. This is an important observation. Since the AR is the main instrument through which androgens elicit their effects, this could explain the reduced proliferation levels and PSA secretion. As mentioned before, alterations (mutation, changes in quantity) to AR are possible explanations to why hormone resistance develops. Reduction of AR levels may therefore retard the growth and development of PC.

Cubebin, despite being a pure substance, proved to be less potent than P9605 in several experiments. This is not uncommon. The complex biogenic structure of plant extracts often leads to a broader spectrum of pharmacological activity. Interestingly, the concentrations of P9605 used in the assays were physiologically relevant. A man of average weight would have to consume 4 g of the plant material daily to obtain a blood concentration of roughly 30 µg/mL, assuming that absorption is maximum.

In summary, P9605 targets different aspects of the androgen-signalling pathway; reduces DHT production, competes with androgens for the AR, prevents DHT from eliciting its actions, inhibits PSA secretion and androgen dependent cellular growth, induces apoptosis and down-regulates AR levels. The simultaneous occurrence of these multiple actions may be a crucial factor in effective PC treatment; the rapid eradication of the cancerous cells by different tactics before they could transform and become androgen-independent.

There is still more to be investigated on the other mechanisms of P9605. The pathways, by which P9605 induces apoptosis, have not been elucidated yet for example. Further experiments should also address the question whether the extract lowers AR levels by reducing its mRNA production or speeding up its degradation. Although the initial results obtained in this study are very promising, further studies in animals and humans are still required to evaluate the potential properties of this extract *in vivo*.

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7. *Piper cubeba* demonstrates anti-estrogenic and anti-inflammatory properties.

Original Paper

Piper cubeba demonstrates anti-estrogenic and anti-inflammatory properties.

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7. 1 Abstract

We have recently shown that an ethanolic extract of *Piper cubeba* L, P9605, diminished the effects of androgens by targeting several aspects of the androgen/androgen-receptor signalling pathway. This present study aims to investigate if P9605 also exhibits other interesting features such as anti-estrogenic and anti-inflammatory properties.

P9605 significantly retarded growth induced by β-estradiol in MCF-7, a human breast cancer cell line. It inhibited aromatase activity, which is responsible for transforming androgens into estrogen. Our competitive binding assays also indicated that P9605 binds to both human recombinant estrogen α and β receptors. This same extract could prevent the production of certain eicosanoids which are inflammatory mediators, by inhibiting the activities of cyclo-oxygenases, COX-1, COX-2 and 5-lipo-oxygenase (5-LOX) as seen in our enzymatic assays. Furthermore, P9605 potently attenuated the induction of interleukin 6 (IL-6), a pro-inflammatory cytokine, in differentiated THP-1 cells, which were stimulated with lipo-polysaccharide (LPS).

Taken together with our previous results, P9605 has demonstrated to possess anti-androgenic, anti-estrogenic and anti-inflammatory properties. These results support the potential of P9605 as a phyto-therapy against benign prostatic hyperplasia (BPH).

Key Words: *Piper cubeba* (L.), Piperaceae, anti-androgenic, anti-estrogenic, anti-inflammatory, COX enzymes, 5-LOX, IL-6, aromatase, estrogen receptors, BPH.

7.2 Introduction

Benign prostatic hyperplasia (BPH) is the most common neoplasm in aging men. This benign proliferation of the stromal and epithelial cells in the prostate increases linearly with age in all ethnic groups [1]. The incidence of BPH rises sharply after age 40 and at least 50% of men over 50 years old suffer from it. 90% of men in their eighth decade of life are said to have histological evidence of BPH [2].

The prostate has four distinct zones: peripheral, central, transition and anterior fibro-muscular zone. BPH develops primary within the transition zone (TZ) that surrounds the urethra. The enlarging TZ presses against the urethra and bladder, which may lead to annoying lower urinary tract symptoms (LUTS) such as urinary hesitancy, urinary retention and increased risk of urinary tract infections.

Despite its high prevalence, the reasons why BPH develop remain elusive and it is suggested to be of heterogeneous etiology (hormones, age, and inflammation). Although it is still debatable if androgens are a causative factor for BPH, they undoubtedly play a role in this disease. Men castrated before puberty do not develop BPH. An observation in 1974 indicated that men deficient in 5-α-reductase (5α-RII) had hypoplastic prostates [3]. 5α-RII catalyzes the conversion of intracellular testosterone to a more active form, dihydrotestosterone (DHT). DHT is responsible for the rapid growth and development of the prostate during puberty thus it is a prime suspect in BPH.

As men age, the intraprostatic estradiol concentration increases. There is a strong correlation between the increasing estradiol:DHT ratio and stromal hypertrophy [4]. Takase *et al.* have detected estrogen receptors and enzymes involved in the estrogen metabolism in human prostates [5]. Although the role and mechanism of estrogen in the prostate are still unclear, there is growing evidence that estrogen could modify prostatic growth and differentiation. An estrogen dominant environment is speculated to increase the production of androgen receptors and thus encouraging prostatic growth by over-sensitizing the prostate to androgen [6]. The current hypothesis is that the prostate locally produces estrogen that modulates the epithelial and stromal cell activity.

Prostatic inflammation is an extremely common histological finding in BPH patients [7]. Approximately 5-20% of men diagnosed with BPH suffer from prostatitis-like symptoms [8]. A recent study has indicated that the consumption of non-steroidal anti-inflammatory drugs

(NSAIDs) is linked with lowered risk developing BPH and LUTS [9]. It is unclear if inflammation is the cause or result of BPH but its involvement indicates that anti-inflammatory drugs may help to retard development and worsening of the disease.

The current standard BPH medical management strategy is watchful waiting. Many men with BPH are asymptomatic, and many others are not bothered by their symptoms. When symptoms affect quality of life, pharmacological therapy would include a choice of an α-blocker (terazosin, tamsulosin) or/and a 5α-reductase inhibitor (finasteride, dutaseride). BPH patients also frequently use phytotherapy, such as saw palmetto, pumpkin seeds, nettle root and African plum tree. While some phytotherapy do have comparable efficacy when compared with both adrenoceptor blockers and 5α-RII inhibitors, their molecular mechanisms remain to be elucidated.

Presently, there is still a lack of preventive medication for asymptomatic BPH against possible enlargement of the prostate and development of LUTS. The purpose of this study is to identify novel agents that can be used to prevent and/or alleviate BPH.

Piper cubeba L. is indigenous to South of Borneo and Indonesia. The dried unripe fruits possess antiseptic, expectoral and diuretic properties. Powdered form or tinctures of the cubebs are used extensively in Indonesia for the treatment of gonorrhea, dysentery, syphilis and chronic bladder inflammation [10]. The important constituents of cubebs are volatile oil and lignans, which include cubebin, cubebic acid and cubeb-resin [11].

We have demonstrated that P9605, a 96% ethanolic extract of *Piper cubeba*, has potent anti-androgenic properties; it prevents the synthesis and multiple actions of DHT (unpublished results). This present study was undertaken to investigate if the same extract possesses anti-estrogenic effects and anti-inflammatory properties as well.

Fig. 7.1 Picture of the *Piper cubeba* plant (left) and its fruits (right)

7.3 Materials and Methods

Chemicals

All radioligands (purity >97%) were purchased from Perkin Elmer (Boston, MA, USA). All cell culture mediums, fetal bovine serum (FBS) and the other reagents are of highest quality available and were purchased from Sigma (Buchs, Switzerland) if not otherwise stated. Charcoal stripped FBS (CSS) was obtained from HyClone (Logan, UT, USA)

Plant Material

Piper cubeba L. fruits were purchased from Alfred Galka GmbH (Gittelde, Germany) and identified according to the Deutsches Arzneibuch 6 (DAB 6) by Dr. K. Berger Büter (Vitaplant AG, Witterswil, Switzerland). A voucher specimen (ViP_Pipc'03_2) is deposited at Vitaplant AG.

Preparation and analysis of the fluid extract

Sixty grams of fruits were milled and de-fatted twice with fresh hexane. After filtering through an AF-6 filter paper (E. Begerow GmbH & Co; Langenlonheim, Germany), the de-fatted residue was then vacuum-oven dried (40°C, 100 mbar) to remove all the hexane before being extracted at room temperature (rmt) for 2 hours with 96% (m/m) EtOH in a ratio of 1:5 (w:w).

An aliquot of the fluid extract was analyzed by a Waters HPLC system with UV-VIS detection (280 - 600 nm) using a Nucleosil 120 – 3, C18 (250 × 4.6 mm) column with a pre-column of the same material (both Macherey Nagel; Oensingen, Switzerland) as stationary phase. The mobile phase consisted of two solvent systems {A: 0.1% trifluoroacetic acid in water (v/v) and B: 100% acetonitrile in a gradient (0-30 min 90% A, 30-45 min 50% A, 45-46 min 10% A, 46-50 min 10% A)}. The column temperature was at 40°C and the flow rate was set at 1.0 mL/min. The detection was carried out at 280 nm and quantification of the lignans in the extract was performed by the external standard method using cubebin (Extrasynthese; Genay Cedex, France) as reference substance. The calculated extract yield of cubebin based on the weight of the de-fatted residue was approximately 8%.

Cell culture

The cell lines LNCaP-FGC, MCF-7, MDA and THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Human prostate adenocarcinoma LNCaP cells were cultured with RPMI 1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and 10% FBS. Phenol red free Minimum Essential Medium Eagle (MEM) containing 2 mM L-glutamine, 2.2 g/L sodium bicarbonate and 10% FBS was used to culture the breast cancer cell lines, MCF-7 and MDA cells. THP-1 cells were kept in RPMI 1640 medium containing 2 g/L $NaHCO_3$, 10 mM HEPES, 1 mM Na-Pyruvate, 30 µM mercaptoethanol and 10% FBS. Normal primary human prostate epithelial cells (PrEC) were purchased from CAMBREX Bio Science Walkersville, Inc. (Walkersville, MD, USA). These cells were cultured according to the supplier's instructions. HL-60 cells (DSMZ; Braunschweig, Germany) were cultured in complete RPMI 1640 medium supplemented with 10% FBS. 1% (v/v) penicillin/streptomycin solution was added to all cell cultures. The cell lines were kept in a humidified incubator at 37°C and 5% CO_2 and passaged at 70-80% confluency. Cultures used in subsequent experiments were passaged less than 15-25 times.

DNA detection assay

DNA amount was quantified using CyQUANT cell proliferation assay kit (Molecular Probes, Eugene, OR, USA). Cells (5000 cells/well in 96-well plates) were treated with P9605 (3, 10, 30 µg/mL) for 4 days. After incubation with the samples, the medium was discarded and the plates were frozen at -80°C. The CyQUANT GR dye used in this assay exhibits strong fluorescence enhancement when bound to DNA or RNA. After thawing the plate, the cells were incubated with 195 µL of CyQUANT lysis buffer containing DNA-free RNase (1.35 Kunitz units/mL) for 1 hour at room temperature to eliminate the RNA. 5 µL of CyQUANT GR dye reagent was then added to every well and incubated for 5 mins in the dark. TECAN infinite 200 multifunctional microplate reader (Tecan; Männerdorf, Switzerland) was used to measure fluorescence with the excitation wavelength set at 485 nm and the emission wavelength at 530 nm. The assay was linear over a range of 50 to 50,000 cells under these conditions. The DNA quantities were calculated using a DNA standard curve.

^{3}H-Thymidine incorporation assay

LNCaP and MCF-7 cells were seeded at 5000 cells/ well in 96-well plates. After 24 hours of pre-incubation, they were treated with a range of either DHT or β-estradiol concentrations and 10 µg/mL of P9605. After 72 hours incubation with the samples, ^{3}H-thymidine (3 µCi/mL) was then added to each well and incubated at 37°C for 3 hours. The cells were then washed and harvested onto filter strips by a cell harvester (Brandel Inc; Gaithersburg, MD, USA), and radionucleotide incorporation was measured using Tri-Crab 1900 TR scintillating counter (Packard; Meriden, CT, USA).

Aromatase activity

P9605 samples (3, 10, 30 µg/mL) and formestane (synthetic aromatase inhibitor) were incubated with an enzyme/substrate mixture of aromatase, CYP19, (BD Bioscience; Woburn, MA, USA) and ^{3}H-androstenedione in the presence of a NADPH regenerating system (BD Bioscience; Woburn, MA, USA). Aromatase catalyses the conversion of androstenedione into estrone, H_20 and formaldehyde. The H_20 by-product is radioactive. After 15 mins incubation at 37°C, the non-metabolised ^{3}H-androstenedione was extracted with dichloromethane for 5 mins. After centrifugation (3000 g, 5 mins), the water phase was removed and treated with 2% dextrane coated charcoal for 30 mins before centrifugation (4000 g, 10 mins). The supernatant was then measured with the scintillating counter. The activity of aromatase per sample was measured by determining the amount of radiolabel in the water phase and expressed as a percentage of the solvent control.

Estrogen binding

3, 10, 30, 123 µg/mL of P9605 were incubated with human recombinant estrogen α and β receptors (Invitrogen; Carlsbad, CA, USA) and ^{3}H-estradiol (2 nM) at rmt for 3 hours. To determine non-specific binding, 10 µM of non-radiolabelled β-estradiol was used. After incubation, the receptor bound fraction in the assay mix was separated by centrifuging through MircoSpin G-25 columns (GE Healthcare; Piscataway, NJ, USA). The amount of radioligand bound to the receptors in the filtrate was quantified by the scintillation counter.

Cyclo-oxygenase (COX) 1 & 2 assay

This enzymatic assay was performed on both ovine COX-1 and COX-2 enzymes (Cayman chemical; Ann Arbor, MI, USA). The samples were pre-incubated with the individual enzymes for 15 mins at rmt before starting the reaction with arachidonic acid (10 µM). Negative controls were carried out with denatured enzymes (destroyed by boiling). After 3 mins, the reaction was stopped by the addition of acetic acid (1 N). The samples were neutralized with NaOH (1 N) before quantifying the prostaglandin E_2 (PGE_2) produced with Enzyme Immuno Assay (EIA) Kits from Cayman. The optical densities (OD) were measured at 415 nm by TECAN reader. The quantities were calculated using a PGE_2 standard curve.

5-Lipoxygenase-Assay (5-LOX)

The assay was carried out as described by Bennet *et al* [12]. Human myeloid leukaemia HL-60 cells were differentiated for 6 to 8 days with DMSO (1.2% v/v) to induce the expression of 5-LOX. The activity of 5-LOX was measured by determining the quantity of leukotriene B_4 (LTB_4) produced. Briefly, the differentiated cells were suspended in PBS containing Ca^{2+} (1 mM) and glucose (1 mg/mL) and plated 1 x 10^6 cells/well in 96-well plates. After pre-incubation with the samples for 15 mins, the reaction was started by adding Ca^{2+} ionophore (4.8 mM) and arachidonic acid (9.8 M). Negative controls were carried out without Ca^{2+} ionophore stimulation. The assay mix was incubated for 15 mins at 37ºC and terminated by adding 100 µL methanol containing HCl (1 M, 3% v/v). After neutralization with 50 µL PBS and centrifugation (340 x g) for 10 mins, the LTB_4 concentration in the supernatant was determined with (LTB_4) EIA Kit from Cayman. The ODs were measured at 415 nm by TECAN reader. The quantities were calculated using a LTB_4 standard curve.

Il-6 Assay

IL-6 Cytokine-Assay was performed according to Golenbock *et al*. [13]. 50 pM/mL of phenol-12-myristate-13-acetate (PMA) was used to differentiate human THP-1 cells (5 x 10^5 cells/mL). After 3 days, the samples were pre-incubated for 30 mins at 37°C with the differentiated THP-1 cells before adding LPS (1 µg/mL) to induce IL-6 production. Negative controls were carried out with the assay mixture without LPS–stimulation. After 24 hours incubation, the supernatant was removed for the quantification of IL-6 with EIA Kit from Cayman. The ODs were measured at 415 nm by TECAN reader. The quantities were calculated using an IL-6 standard curve.

Adrenoceptor Binding

3, 10, 30, 60, 80 µg/mL of P9605 were incubated with human recombinant α_{1A} adrenergic receptors (Euroscreen S.A; Gosselies, Belgium) and ^{3}H-prazosin (2 nM) at 37°C for 30 mins. To determine non-specific binding, 10 µM of prazosin was used. After incubation, the receptor bound fraction in the assay mix was harvested onto filter strips by a cell harvester. The amount of radioligands bound to the filter strips was quantified by the scintillating counter.

Statistical analysis

Each data set represents the means ± standard deviation (SD) of at least 2-3 experiments ($n \geq 5$). The concentration response values were expressed as a percentage of the solvent control. GraphPad Software Inc (Prism, version 4, San Diego, CA, USA) was used to calculate the IC_{50} values. For repetitive comparison of dose-response data with control values analysis of variance (ANOVA) with subsequent Dunnett multicomparison test was used (SPSS for windows, version 14.0, SPSS Inc., Chicago, Ill, USA). Statistical significance was established at values of $p<0.05$. Asterisks (*), (**), (***) indicate $p<0.05$, $p<0.01$, $p<0.001$ respectively. Statistically insignificant data points were not denoted.

7.4 Results

The anti-growth effects of P9605 on the 2 hormone-dependent cancer cell lines, LNCaP (prostate) and MCF-7 (breast) were investigated. Estrogen receptor negative breast cancer cell line MDA and a human primary prostate epithelial cell line PrEC were also tested. 30 μg/mL of P9605 significantly ($p<0.001$) reduced the growth of all cell-lines by at least 50% (Fig.7.2A) except the MDA cells. Cytotoxicity tests also revealed that the P9605 concentrations tested were not toxic on these cell lines (data not shown).

The growth of both LNCaP and MCF-7 cells are fuelled by DHT and estradiol. We thus decided to test if P9605 could antagonise growth induced by these hormones. LNCaP cells exhibited a biphasic growth curve with maximum proliferation peaking at 1 nM DHT ($p<0.001$) and regressing at DHT concentrations greater than 1 nM (Fig.7.2B). This behaviour has been observed by several other groups [14], [15]. The growth of MCF-7 cells, on the other hand, was β-estradiol concentration dependent (Fig.7.2C). P9605, at 10 μg/mL, could reduce hormone-induced DNA synthesis of LNCaP cells at every concentration and even more potently at MCF-7 cells. The fact that P9605 did not affect the growth of MDA, which is estrogen–independent, further substantiates the possibility that extract counteracts the growth promoting effects of estrogens.

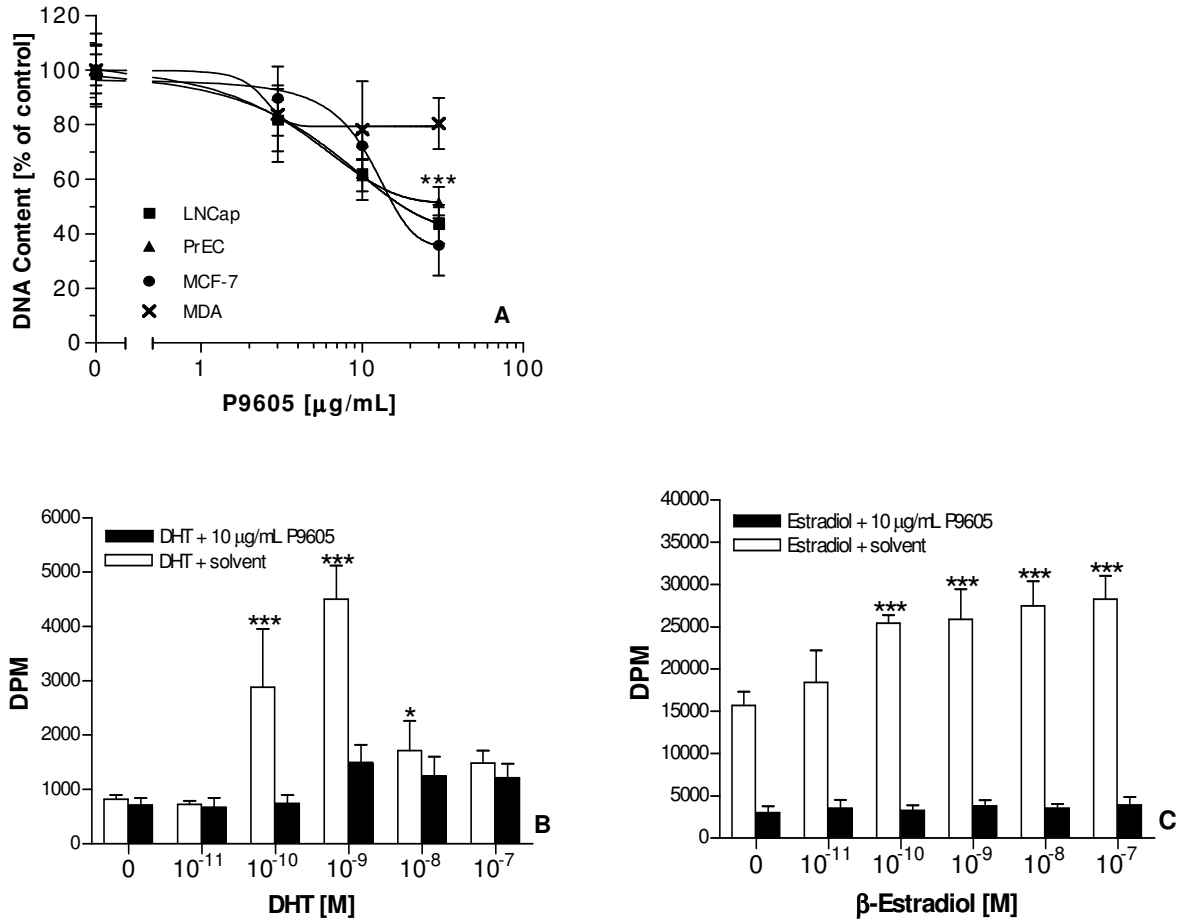

Fig. 7.2 Antiproliferative effects of P9605 on LNCaP, MCF-7, MDA and PrEC cells cultured for 4 days (A). DNA content was quantified by CyQUANT cell proliferation assay. The DNA content in all cell lines (except MDA) at P9605 concentration of 30 µg/mL was significantly reduced. *** indicates the statistical significant for all 3 cell lines (except MDA). ^{3}H-Thymidine incorporation was used to assess the effect of P9605 on the hormone-induced growth in LNCaP (B) and MCF-7(C) cells. The cells were cultured in medium containing 10% CSS in the presence of a range of hormone concentrations (DHT or β-estradiol) together with either 10 µg/mL of P9605 or solvent for 3 days. Data represent means±SD of 3 experiments. All data points are expressed as % of the solvent controls. *p<0.05 vs control, ***p<0.001 vs control.

Aromatase transforms androstenedione to estrone and testosterone to estradiol. This enzyme has been detected in the prostate and is a potential generator of estrogen. P9605 could inhibit aromatase activity with an IC_{50} value of less then 10 µg/mL (Fig. 7.3A). It was also of interest to observe if P9605 was able to bind to estrogen receptors. Competitive binding assays were performed on human recombinant estrogen α and β receptors and the IC_{50} values were <100 µg/mL for both receptors (Fig. 7.3B).

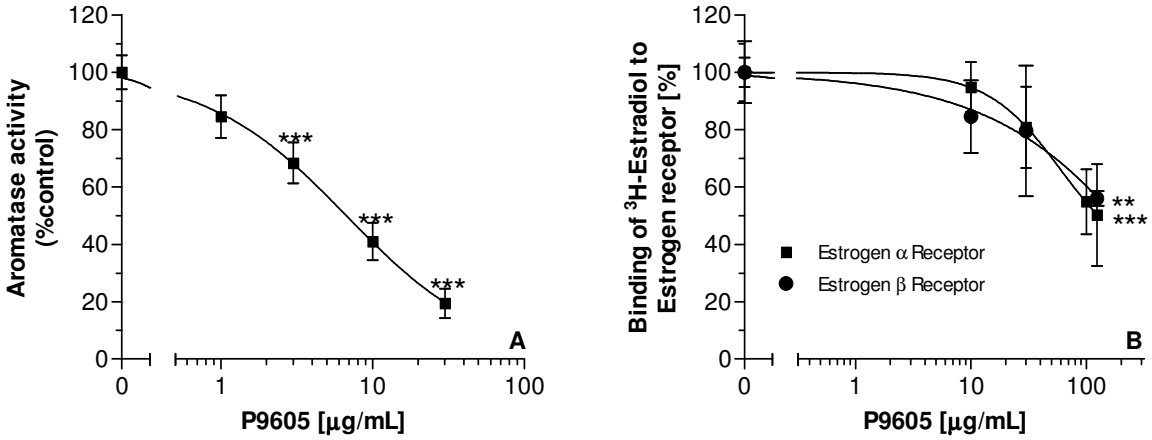

Fig. 7.3 Inhibitory action on aromatase by P9605 (A). Formestane, a well-known synthetic inhibitor, was a synthetic control for the enzymatic assay. The binding affinities of P9605 to estrogen receptors (ER) α and β were determined by competitive binding assay (B). The highest P9605 concentration significantly inhibited ER-α ($p<0.001$) and ER-β ($p<0.01$). Data represent means±SD of 3 experiments. *$p<0.05$ vs control, **$p<0.01$ vs control, ***$p<0.001$ vs control.

To examine its potential anti-inflammatory effects, we observed the inhibitory effect of P9605 on the activities of COX-1, COX-2 and 5-LOX enzymes. Their products, the eicosanoids, are involved in the inflammation process and are also implicated in the pathogenesis of a variety of human diseases, including cancer. P9605 potently inhibited all 3 enzymes (Fig. 7.4A, B and Table 7.1). P9605 was slightly more selectively for COX-2 than COX-1 according to their IC_{50} values. Comparing the IC_{50} values of P9605 on both arachidonic acid-metabolizing enzymes, it appeared that P9605 is more effective in reducing 5-LOX's activity then the cyclo-oxygenases'. It must be noted, however, that the activity of cyclo-oxygenases were performed on isolated enzymes while 5-LOX's activity in differentiated HL-60 cells was tested.

Cubebin, on the other hand, had no effects on the cyclo-oxygenases but it inhibited 5-LOX. (results not shown).

IL-6 is a pro-inflammatory cytokine. It has been detected in epithelial cells of BPH and thought to promote the growth and development of BPH by being a growth factor [16]. Differentiated THP-1 cells produce IL-6 when stimulated with LPS and our results have indicated that P9605 could drastically reduce this induction (Fig.7.4C).

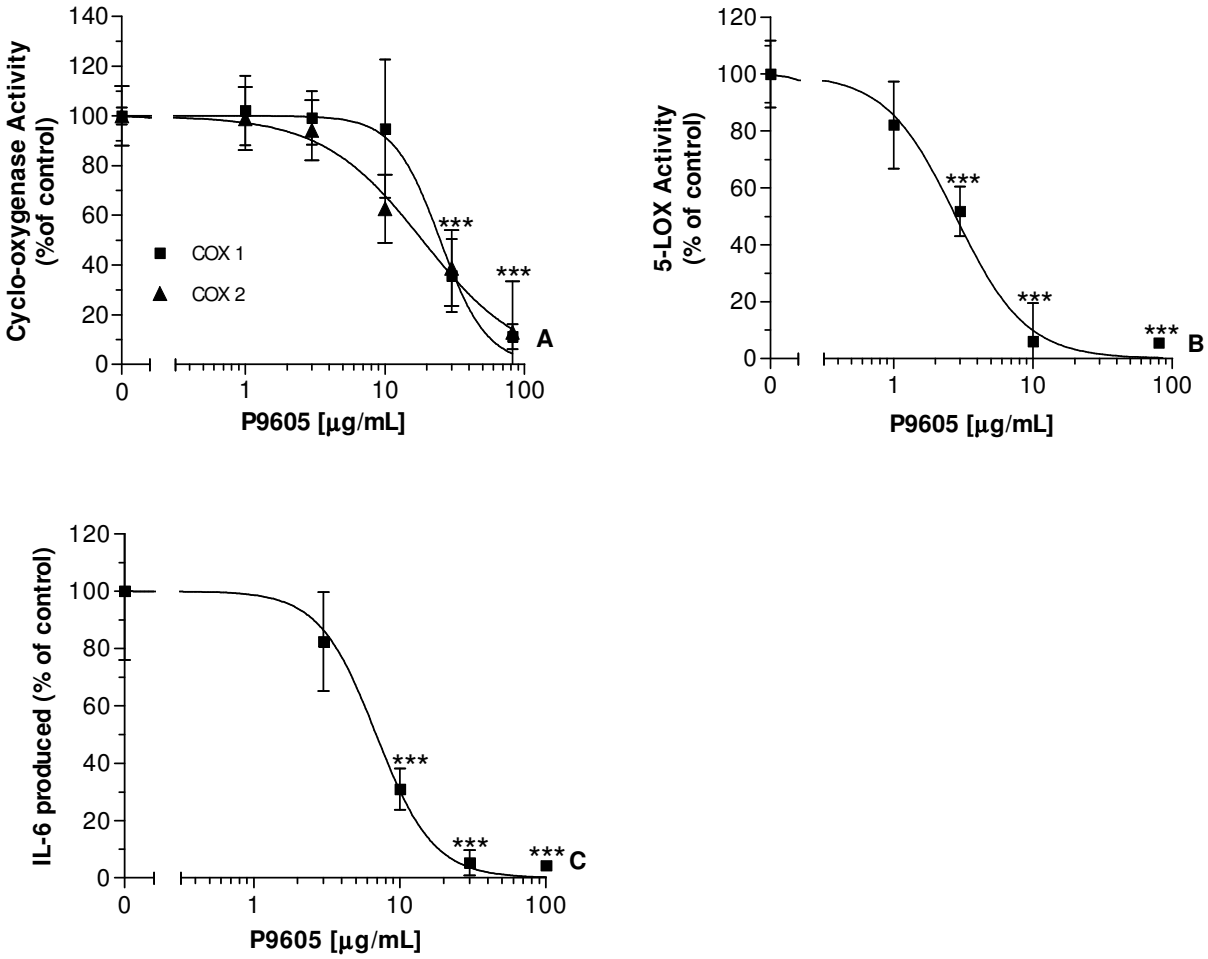

Fig. 7.4 Dose dependent inhibitory effect of P9605 on the activities of isolated COX 1 and 2 (A) *** indicates the statistical significant for both cyclo-oxygenases, 5-LOX in differentiated HL-60 cells (B) and the production of IL-6 by differentiated THP-1 cells (C). Their products were quantified by EIA kits. Data represent means±SD of at least 3 experiments. ***p<0.001 vs control.

Adrenoceptors are present in the prostatic stroma and are thought to influence the resting tone of the smooth muscle with the prostate and bladder neck. There are currently 3 distinct subtypes of α_1 adrenoceptors; α_{1A}, α_{1B} and α_{1D} have been cloned. The constriction of smooth muscle appears to be dominantly mediated by the α_{1A} adrenergic receptors. P9605 appears to prevent the binding of prazosin to this receptor with an IC_{50} value of ~100 µg/mL (Fig. 7.5).

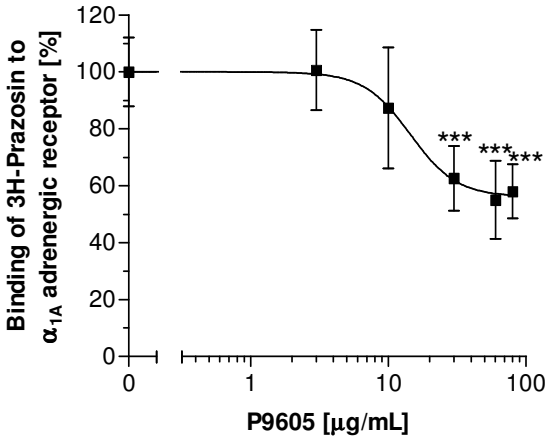

Fig. 7.5 The ability of P9605 to bind to human recombinant α_{1A} adrenergic receptor was determined by competitive binding assay. Prazosin was used as a synthetic control for the experiment. The data represent means±SD (n=6). ***p<0.001 vs control.

Table 7.1 IC_{50} values of P9605 in the various assays

Assay	IC_{50} of P9605	95% Confidence Intervals of IC_{50} values	Synthetic Control	IC_{50} of control
Aromatase	6.7 µg/mL	5.8 - 7.8 µg/mL	Formestane	22 nM
COX 1	25 µg/mL	20.5 - 30.2 µg/mL	Indomethacin	0.02 µM
COX 2	19 µg/mL	14.5 - 23.7 µg/mL	Indomethacin	4 µM
5-LOX	2.8 µg/mL	2.3 – 3.4 µg/mL	NDGA	0.1-0.2 µM
IL-6	6.9 µg/mL	5.0 – 9.4 µg/mL	Dexamethasone	0.1 nM

7.5 Discussion

In the present study, we have firstly demonstrated that P9605 reduced cellular growth in all the cell lines except MDA. LNCaP and MCF-7 cells possess androgen and estrogen receptors respectively and their proliferation is hormone-dependent. P9605 could reduce the enhanced DNA synthesis induced by DHT and estradiol in both LNCaP and MCF-7 cells respectively. Estrogen receptors (ER) are detectable in LNCaP cells [17], so it would have been possible to test if P9605 affected estrogen-induced growth. However, Mulder *et al* [18] have identified that the androgen receptor (AR) in LNCaP cells contains a point mutation in its steroid-binding domain (codon 868, Thr to Ala). This defect leads to a change in specificity of the AR. Estrogen and some anti-androgens can stimulate LNCaP cell growth rate through the AR activation [19]. MCF-7 cells contain both wild type ER α and β [20] therefore it is a better model.

We could only conclude that P9605 reduces estrogen-induced proliferation in MCF-7, inhibits aromatase and weakly binds to ERα and β. However, these effects could possibly occur in LNCaP cells. On the other hand, although PrEC cells are devoid of AR, their cellular growth was reduced by P9605. This indicates that, besides the androgen/AR signalling pathway, other mechanisms are involved in contributing to P9605's anti-proliferative effect. We have also recently tested another Piper extract (60% ethanolic extraction) on a well-established *in vivo* model for 5α-reductase inhibition in male rats. There was a dose response inhibition trend and a 14% inhibition of prostatic growth rate at the highest application dose of 200 mg/kg (results not shown).

According to our results, P9605 negatively interfered with the activities of COX-1, COX-2, 5-LOX and the production of IL-6. In literature, several *Piper* species have exhibited inhibitory activity against at least one of these 2 key enzymes of the arachidonic acid metabolism [21]. However this is the first time reported that *Piper cubeba* could inhibit these enzymes as well. We also tested if these anti-inflammatory properties could be attributed to cubebin, the dominant lignan present. Cubebin had no effects on the cyclo-oxygenases' activity but it could attenuate the actions of 5-LOX. It appears that other constituents in P9605 besides cubebin are responsible for the anti-inflammatory properties.

Several studies have indicated strong correlation between inflammation and BPH. COXs and LOXs are expressed in the prostate [22] and they generate eicosanoids, which are involved in numerous aspects of inflammatory responses and even in the differentiation of normal and tumor cells. Under abnormal circumstances, the over-activity or over-expression of these

enzymes could be partly responsible for the LUTS. Therefore, targeting these arachidonic acid-metabolizing enzymes may be another approach to tackle BPH.

Lymphoid cells such as macrophages and lymphocytes are often found within prostatic stromal nodules. They produce growth factors and cytokines such as bFGF, IL-6 and TNF-α [23], which may lead to an over-proliferation of prostatic cells. The ability of P9605 to reduce the production of IL-6 may help combat prostatic enlargement and inflammation.

The current medical strategy employed to tackle BPH is to relieve the symptoms and reduce the size of the prostate. P9605 has proven to possess anti-androgenic (unpublished data) and anti-estrogenic abilities, which could reduce the growth of the prostate. In addition, P9605's anti-inflammatory properties can serve to alleviate the painful symptoms associated with BPH. In conclusion, P9605 may be an interesting candidate for further studies in BPH patients.

Acknowledgements

We are grateful to Ursula Würgler and Frédéric Grandjean for the technical help and support.

7.6 References

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8. *Aquilaria sinensis*

8.1 Introduction

Aquilaria sinensis originates from the south of China. Its agarwood/resinous heartwood is not only a valuable source of incense wood but also used pharmaceutically as an anti-emetic, anti-tussive and sedative agent as a component of several oriental medical recipes [1].

One of its active compounds includes the sesquiterpenes such as baimuxinic acid and baimuxinal and 2-(2-phenylethyl) chromones [2], [3]. Sesquiterpenes are an important constituent of essential oils and they also function as pheromones and juvenile hormones in plants. Bioactive sesquiterpenes have been found to be anti-malarial [4] and anti-microbial [5]. Since Aquil, a 70% ethanolic extract *Aquilaria sinensis,* has shown promising results in the initial screen and hepatotoxicity experiment, it was further investigated in a series of tests for potential, anti-androgenic, anti-estrogenic and anti-inflammatory properties.

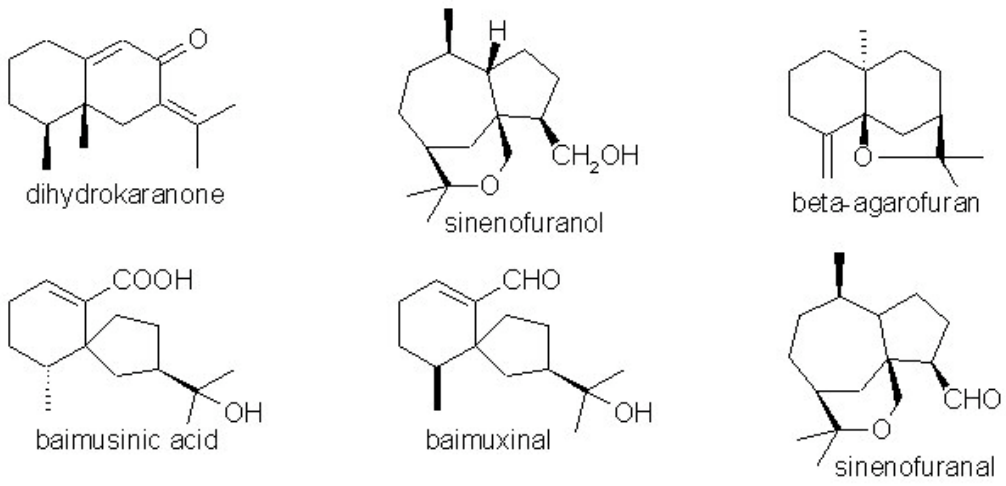

Fig. 8.1A Some of the sesquiterpenoids isolated from *Aquilaria sinensis.*

Fig. 8.1B Pictures of *Aquilaria sinensis* tree, agarwood and dried chips from the agarwood (From left to right)

8.2 Materials and Methods

Preparation of the extract

The dried plant material was milled and extracted with 3 different solvents; ultra pure water, 30% EtOH (w/w) and 70% EtOH (w/w) in a ratio of 1:10. The mixture was left overnight and the liquid fraction was separated from the solid residue by filtering through an AF-6 filter paper first and then through a 4-7 µm filter paper. The dried mass content of the liquid extract was then determined.

Other assays

Assays were performed according to methods described earlier. Please refer to chapters 6.3 and 7.3 for details.

8.3 Results

Aquil antagonises proliferative effects induced by sex hormones

Based on the results from the initial screen, Aquil, the *Aquilaria sinensis* extract made from 70% ethanol significantly reduced LNCaP cell numbers in comparison to the others. This anti proliferative effect was further validated by using different assays to assess cell growth; measuring the change in DNA content and the incorporation of radioactive thymidine. According to the results, Aquil specifically inhibited the growth of both hormone-dependent LNCaP (Fig. 8.2A) and MCF-7 (Fig. 8.2B) cell lines with IC_{50} values of ~11.5 µg/mL and 44 µg/mL respectively. It did not significantly reduce cellular growth of the other cell lines.

To substantiate the assumption that Aquil reduced proliferation by preventing the effects of sex hormones, LNCaP and MCF-7 cells were incubated with a range of DHT and estradiol respectively in the presence of either Aquil or solvent. 10 µg/mL of Aquil, in the absence of both hormones, did not increase cellular growth of both cell lines. However, it reduced hormone-induced DNA synthesis in LNCaP cells at every concentration (Fig. 8.2C) and even more potently at MCF-7 cells (Fig. 8.2D).

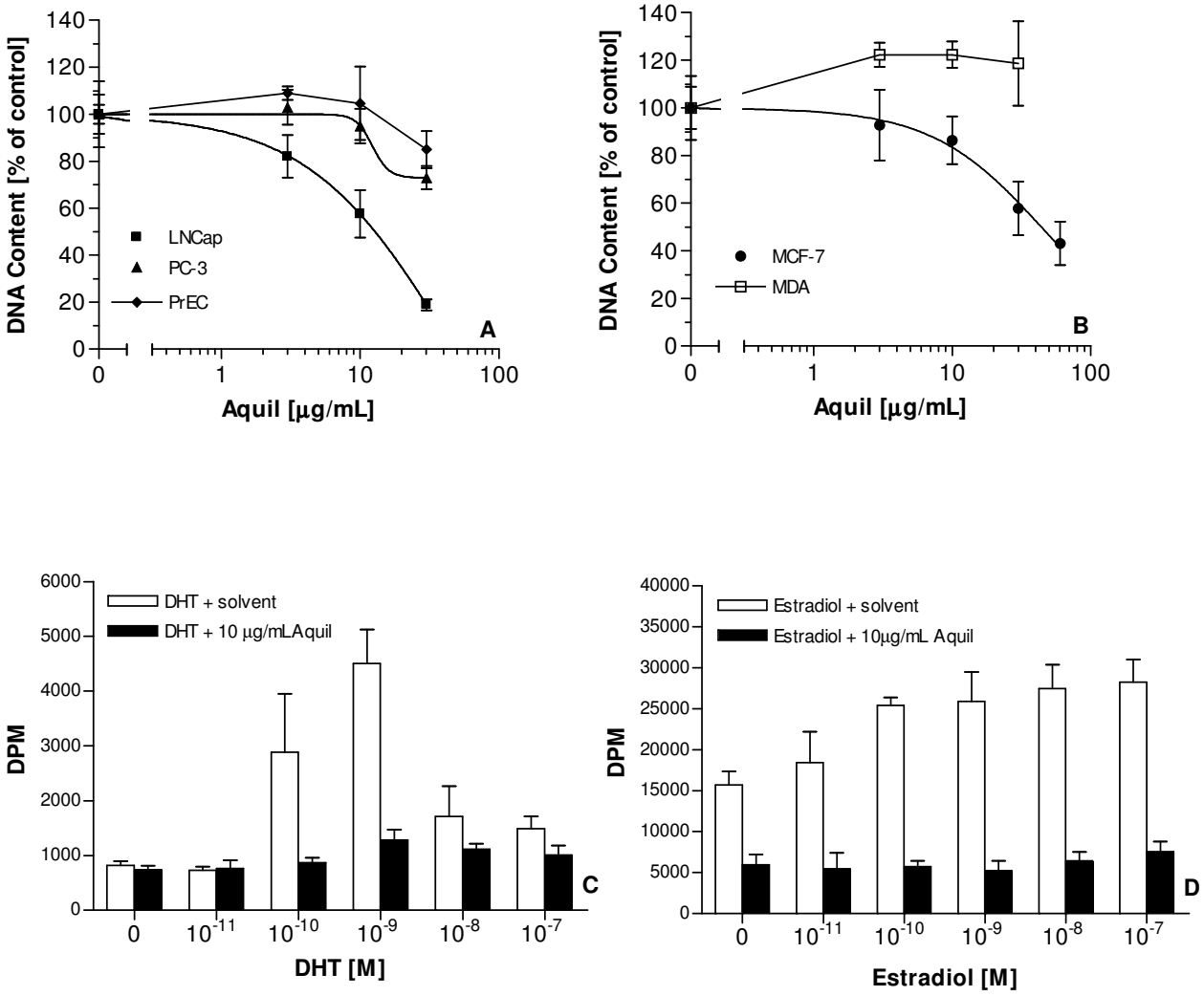

Fig. 8.2. Antiproliferative effects of Aquil on prostatic cell lines LNCaP, PC-3 and PrEC cells (A) and on breast cancer cell lines MCF-7 and MDA cells (B). The effects of Aquil on the hormone induced growth in LNCaP (C) and MCF-7 (D) cells. The cells were cultured in medium containing 10% CSS in the presence of a range of hormone concentrations (DHT or β-estradiol) together with either 10 µg/mL of Aquil or solvent for 3 days. Data represent means±SD of 3 experiments.

Cytotoxicity and apoptotic data of Aquil on LNCaP cells

Aquil exhibited no acute cytotoxic effects on mitochondrial activity and it did not induce necrosis even at 60 µg/mL (Fig. 8.3.A). On the other hand, programmed cell death was initiated by concentrations between 3 to 10 µg/mL (Fig. 8.3B). At 30 µg/mL, there is a 3-fold increase in apoptosis compared to the solvent control. There was a trend indicating that 10 µg/mL of Aquil may reverse the anti-apoptotic effect of DHT (Fig. 8.3C).

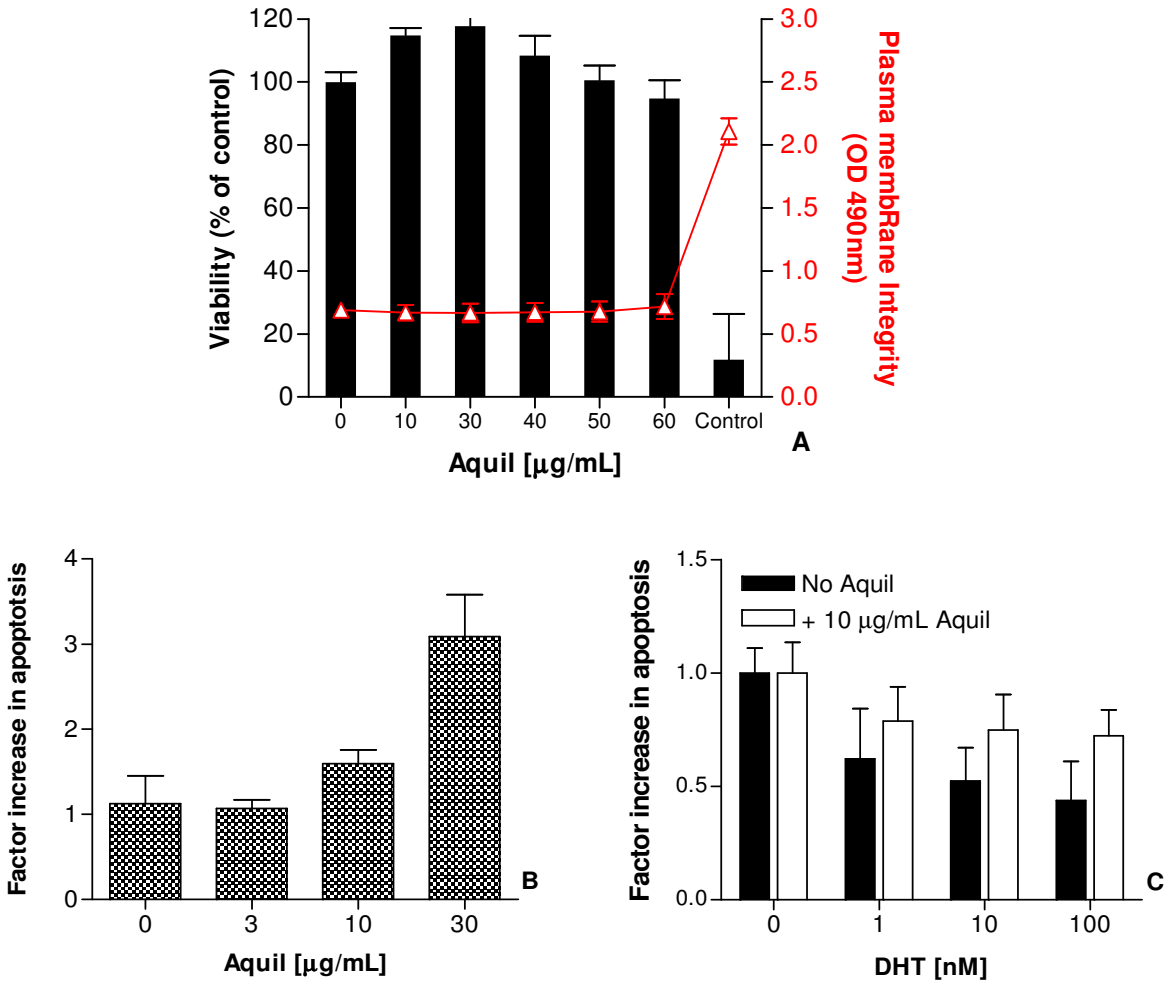

Fig. 8.3 Cytotoxicity data of Aquil (A). The graph shows the two parameters investigated; mitochondrial activity (Left Y-axis, Bars) by WST assay and plasma membrane integrity (Right Y-axis, Line graph) by LDH assay. The control refers to terfenadine (WST assay) or Triton X (LDH assay). Data represents means±SD of n≥8. Apoptotic inducing effects of Aquil were assessed on LNCaP cells lysates after 48 hours of treatment (B). The anti-apoptotic effect of DHT in LNCaP cells cultured in 10% CSS medium could be reversed by 10 µg/mL of Aquil (C). DNA fragmentation, a common feature of the late stages of apoptosis was determined from the cell lysates. Values were calculated as a factor of the solvent control.

Aquil reduces the synthesis of DHT and estrogen

As mentioned earlier, 5α-RII is responsible for the conversion of testosterone to DHT and aromatase transforms androgens to estrogen. Aquil was able to inhibit 5α-RII and aromatase with IC_{50} values of 20 µg/mL (Fig. 8.4A) and 35 µg/mL (Fig. 8.4B) respectively.

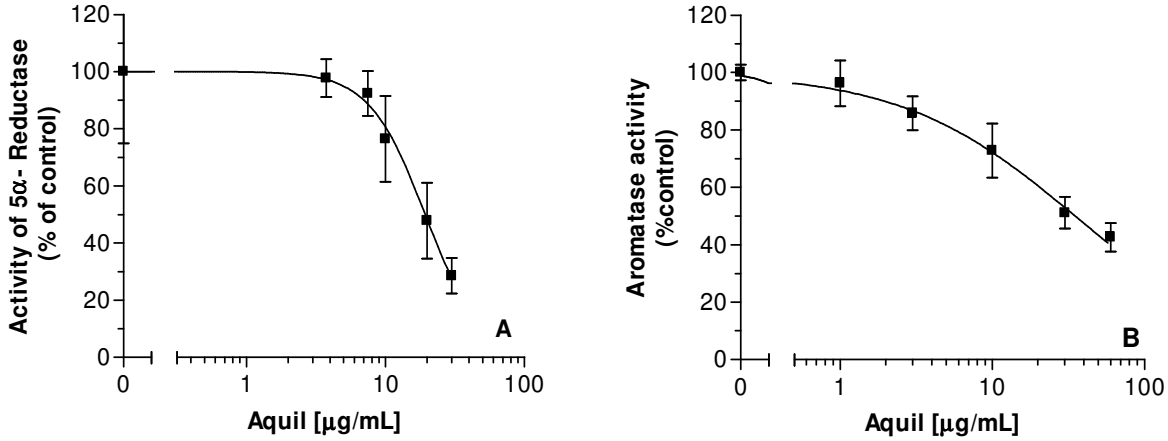

Fig. 8.4 Inhibitory action on 5α-RII (A) and Aromatase (B) by Aquil. Data represent means±SD of 3 experiments.

Aquil inhibits secretion of PSA in LNCaP cells

The PSA secretion levels were reduced 50% by 10 µg/mL of Aquil after 48 hours (Fig. 8.5A). DHT increased PSA secretion in a concentration-dependent fashion, which is greatly reduced in the presence of 10 µg/mL of Aquil (Fig. 8.5B)

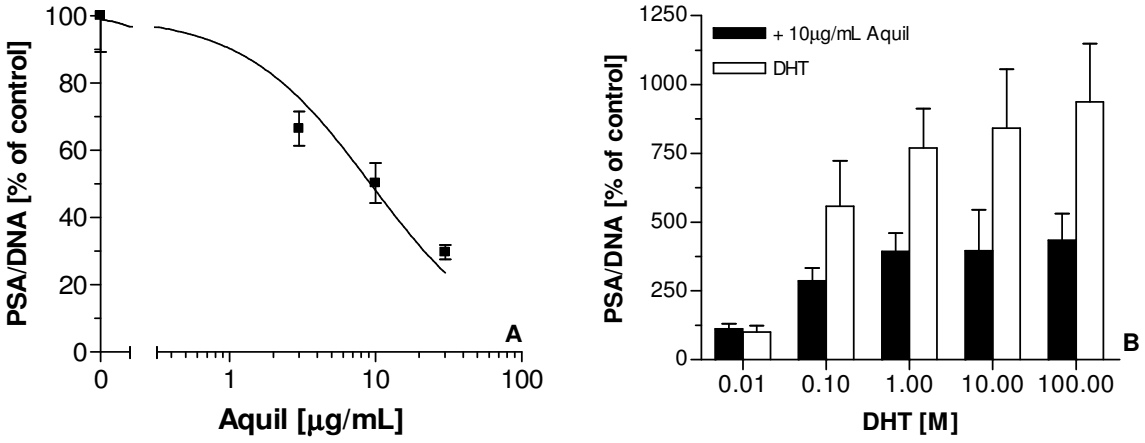

Fig. 8.5 Aquil inhibited the secretion of PSA in LNCaP cells (n=3) (A). DHT induced PSA secretion dose-dependently in LNCaP cells and this response was abrogated in the presence of 10 µg/mL of Aquil (B). The cells were cultured in 10% CSS medium and treated for 48 hours (n≥6). The data points are calculated as the ratio of PSA secreted to the DNA content of each well.

Aquil down regulates AR levels.

Aquil reduced the proliferative and PSA inducing effects of DHT. One postulated mechanism is via altering the levels of AR available. As Fig 8.6 indicates, there is a significant reduction of AR levels from 30 µg/mL onwards.

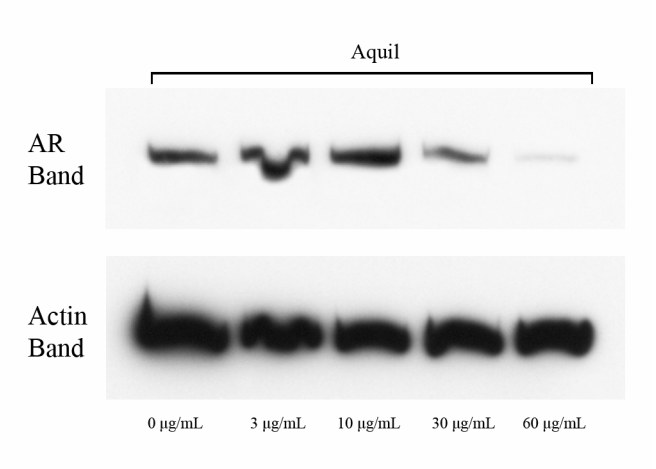

Fig. 8.6 Western Blot indicating the quantity of AR in LNCaP cells after 48 hours incubation with different concentrations of Aquil. β-Actin bands act as a loading control. (n=2)

Anti-inflammatory properties of Aquil

Although Aquil had no inhibitory effects on the activity of the cyclo-oxgenases, it specifically reduced 5-LOX's activity with an IC_{50} of ~30 µg/mL.

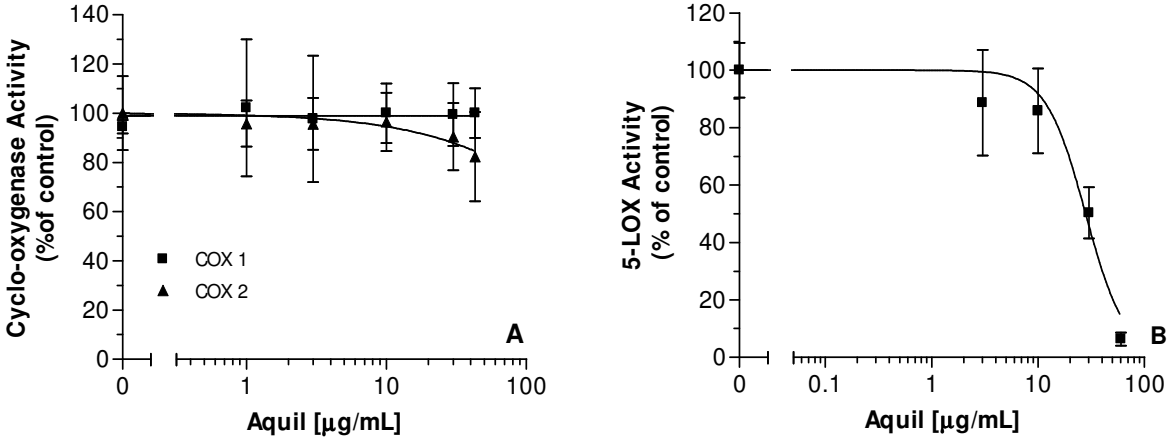

Fig. 8.7. Dose dependent inhibitory effect of Aquil on the activities of COX 1&2 (A) and 5-LOX in differentiated HL-60 cells (B). Data represent means±SD of n≥5.

8.4 Discussion

Similar to P9605, Aquil has shown to possess anti-androgenic, anti-estrogenic and anti-inflammatory properties.

In comparison to P9605,

Antiproliferative abilities:

Aquil specifically inhibited proliferation of the hormone-dependent cell lines LNCaP and MCF-7, while P9605, at 30 µg/mL, significantly ($p<0.001$) reduced cellular growth of AR-negative PC-3 and PrEC cells as well. By comparing IC_{50} values, Aquil proved to more potently exert anti-proliferative effects on LNCaP than P9605. However P9605 had a stronger effect on MCF-7 cells.

Cytotoxicity and Apoptotic effects:

Both extracts do not have any cytotoxic effects on the HepG2 cells, even at 60 µg/mL. While this result is also reflective of Aquil on LNCaP cells, P9605 reduces LNCaP cells' mitochondrial activity by 50% at 50 µg/mL. Aquil and P9605 induced apoptosis to a similar extent.

Other assays:

Aquil, although it inhibited both 5α-RII and aromatase with IC_{50} values of 20 µg/mL and 35 µg/mL respectively, was less superior then P9605 (P9605 had IC_{50} values of 3.6 µg/mL and 6.7 µg/mL in the 5α-RII and aromatase experiments respectively). On the other hand, Aquil reduced PSA secretion more effective then P9605 at the same concentration. Based on the western blot results, P9605 downregulated AR levels to a greater degree compared to Aquil at 30 µg/mL.

Anti-inflammatory properties:

P9605 inhibited the COX 1 & 2 and the 5-LOX activities. Although Aquil had no effects on the cyclo-oxygenases, it specifically inhibited 5-LOX.

Taken altogether, Aquil has demonstrated more dominant anti-androgenic than anti-estrogenic properties. Cellular assays have hinted that Aquil could more effectively antagonise the proliferative and PSA inducing abilities of DHT in comparison to P9605. Considering the ability of P9605 to down-regulate the AR levels more effectively than Aquil, it could be hypothesized that P9605 reduces the effects of DHT by this mechanism. Aquil, on the other hand, may antagonise the actions of androgens by 1) affect the binding affinity of DHT to AR, 2) prevent

HSPs from dissociating from the AR; 3) prevent the translocation of the ligand-bound AR to the nucleus, 4) affect the co-regulators, thus preventing the transcription process.

8.5 References

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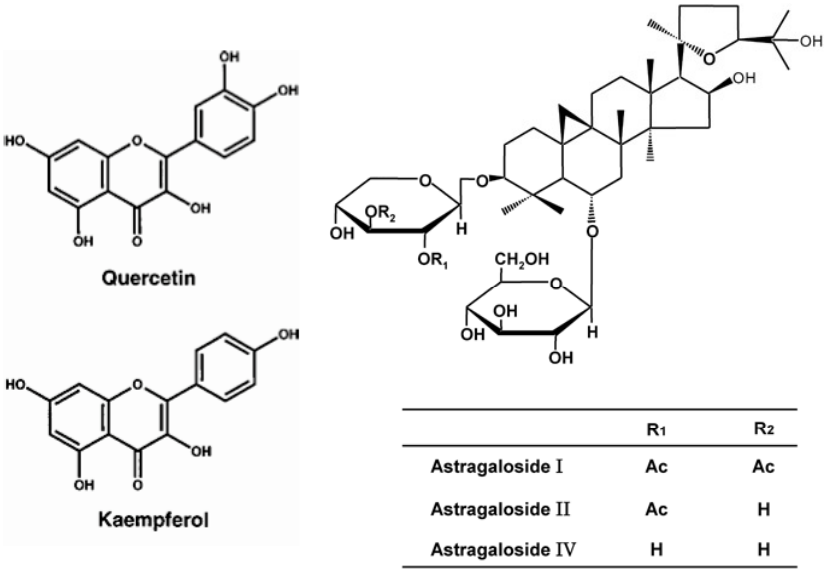
9. *Astragalus membranaceus*

9.1 Introduction

The genus *Astragalus* consists of about 2000 species of small shrubs. They are more commonly known as milk vetch. *Astragalus membranaceus* (*Astragalus M.*) is native to northern China and the elevated regions of the Chinese provinces, Yunnan and Sichuan. This Chinese astragalus species has been most extensively tested, both chemically and pharmacologically. According to traditional Chinese medicine, it acts as a tonic to protect the immune system [1]. Research has indicated that it enhances the immune response in vivo and in vitro [2], [3], [4].

Astragalus M. contains numerous components, including flavonoids [5] (e.g. quercetin, kaempferol), polysaccharides, triterpene saponins [6] (e.g. astragalosides I–VII), amino acids, and trace minerals.



	R_1	R_2
Astragaloside I	Ac	Ac
Astragaloside II	Ac	H
Astragaloside IV	H	H

Fig. 9.1A Structures of flavonoids (Quercetin, Kaempferol) and Astragalosides.

Fig. 9.1B Picture of *Astragalus membranaceus* plant (left) and slices of its dried roots (right).

9.2 Materials and Methods

Preparation of the extract

The dried plant material was milled and extracted with 3 different solvents; ultra pure water, 30% EtOH (w/w) and 70% EtOH (w/w) in a ratio of 1:5. The mixture was left overnight and the liquid fraction was separated from the solid residue by filtering through an AF-6 filter paper first and then through a 4-7 µm filter paper. The dried mass content of the liquid extract was then determined.

Other assays

Assays were performed according to methods described earlier. Please refer to chapters 6.3 & 7.3 for details.

9.3 Results

Astra inhibits LNCaP proliferation

The initial screen had indicated that Astra, the *Astragalus membranaceus* aqueous extract significantly reduced LNCaP cell numbers as quantified by the WST assay. This anti proliferative effect was further confirmed by measuring another parameter of proliferation, DNA content of LNCaP cells after treatment. According to Figs. 9.3A and B, Astra negatively influenced the growth of the LNCaP cells with an IC_{50} value of 20 µg/mL.

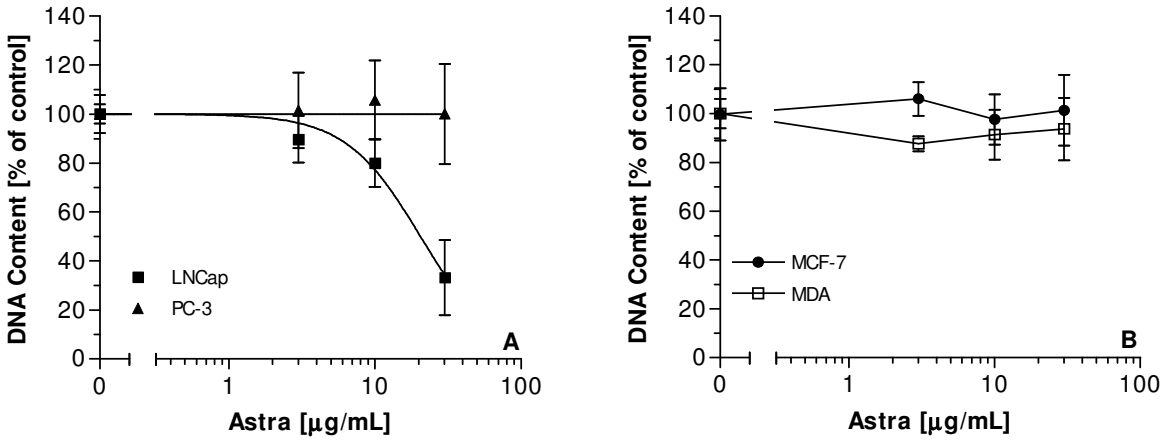


Fig. 9.2 Antiproliferative effects of Astra on prostate cell lines (A) and the breast cancer cell lines (B). The data represent means±SD of n≥6.

Cytotoxicity and apoptotic data of Astra on LNCaP cells

According to Fig. 9.3A, Astra is not cytotoxic to LNCaP cells even at the highest dose applied. At 30 µg/mL, LNCaP cells showed no significant increase in apoptosis (Fig. 9.3B).

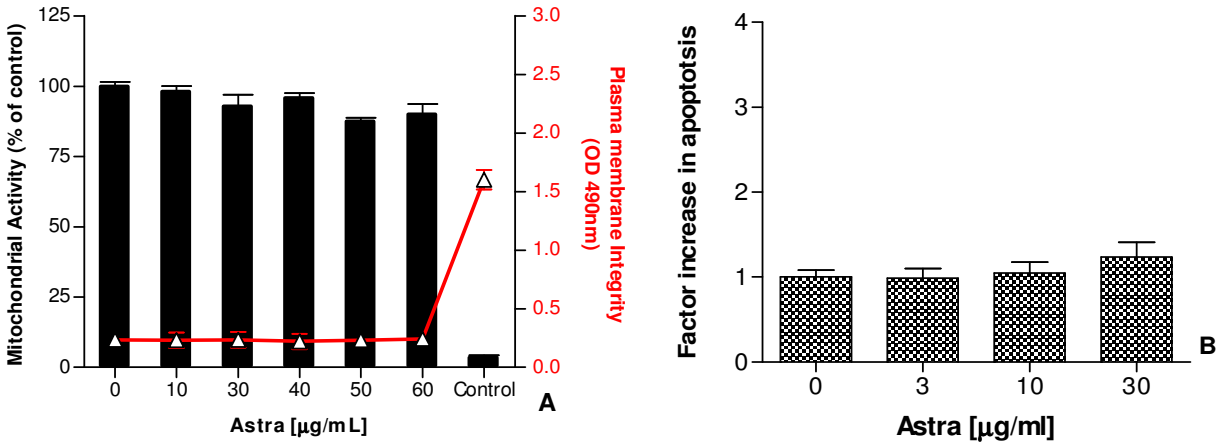


Fig. 9.3 Cytotoxicity data of Astra (A). The graph shows the two parameters investigated; mitochondrial activity (Left Y-axis, Bars) by WST assay and plasma membrane integrity (Right Y-axis, Line graph) by LDH assay. The control refers to terfenadine (WST assay) or Triton X (LDH assay). Data represent means±SD of n=5 (B). Apoptotic inducing effects of Aquil were assessed on LNCaP cells lysates after 48 hours of treatment. (n=6). Values were calculated as a factor of the solvent control.

Other assays

As Fig 9.4 indicates, Astra exhibited no effects on the activity of 5α-RII, aromatase, COXs, 5-LOX, PSA secretion and AR level even at very high doses ≥ 60 µg/mL.

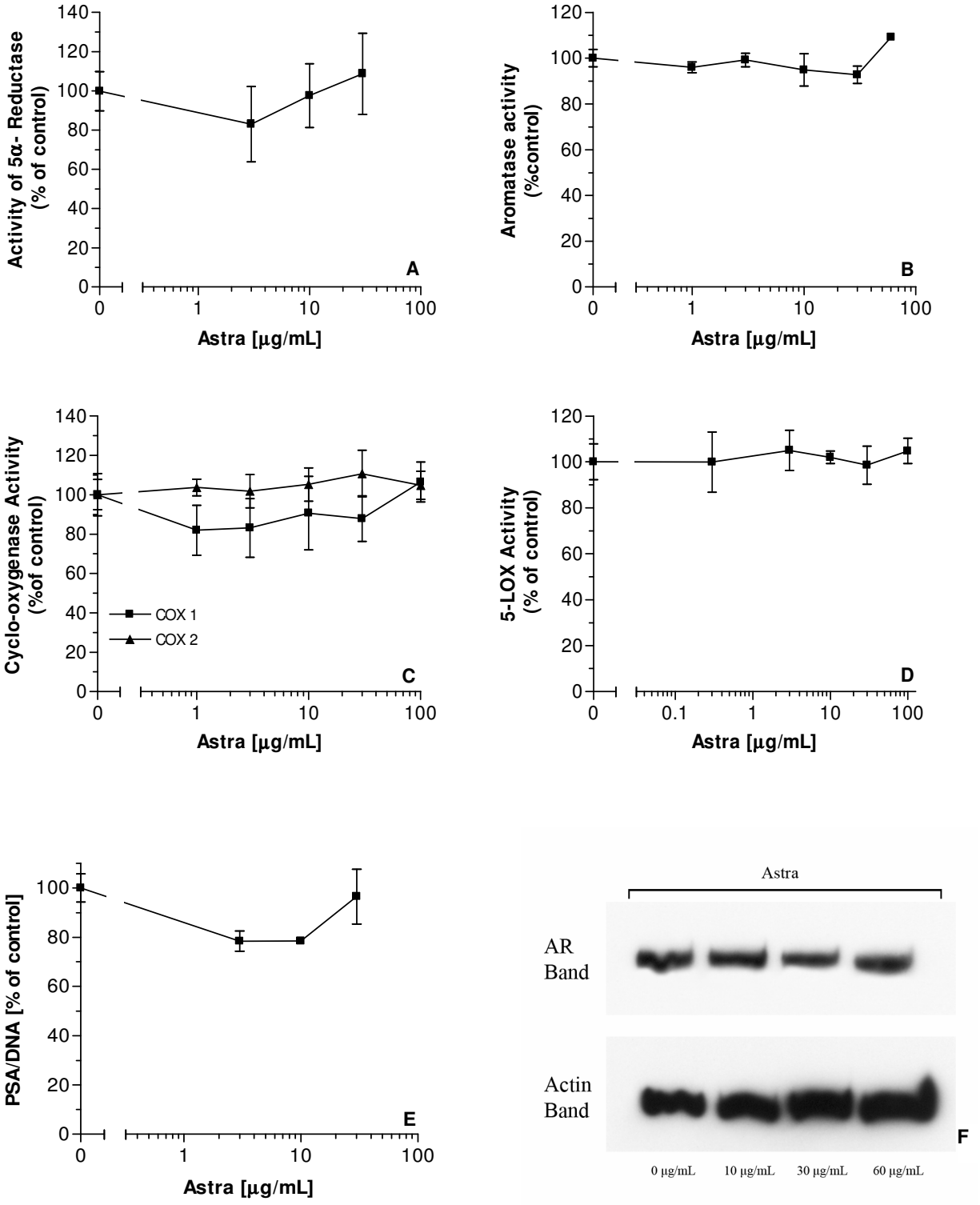


Fig. 9.4 Effects of Astra on 5α-RII (n= 6) (A), Aromatase (n=4) (B), COX 1&2 (n=6) (C), 5-LOX (n=6) (D), PSA secretion (n=3) (E) and western blot (n=1) (F)

9.4 Discussion

Although Astra inhibited LNCaP cell proliferation like its 2 other counterparts, P9605 and Aquil, it did not seem to do so via similar mechanisms. Net changes in the cell numbers depend on increase in cell growth or death. Astra is not a potent apoptosis inducer thus programmed cell death is not the explanation for the reduced cell quantity as compared to control. One hypothesis could be that LNCaP cells when treated with Astra, do not proliferate while those treated with solvent increased over time. It might be possible that Astra consists of phytochemicals that directly affect the cell-cycle regulation or induce cellular differentiation that could stop cells from proliferating.

9.5 References

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10. Conclusion & Outlook

Androgens are essential for normal prostate growth but they also have a permissive role in the genesis of both BPH and PC. Treatments that reduced androgen levels have demonstrated limited success in both pathologies. BPH and PC still continue to impose a major healthcare problem. It is clear now, that androgens are not solely responsible for these prostate diseases. Mounting evidence indicates that estrogens and inflammation are involved as well.
Our current knowledge of the complex interactions between the androgen, estrogen and inflammatory pathways is still in its infancy. However, should further research identify important cellular components involved in the cross talk among these pathways, they would certainly be potential targets for drug development.

Twenty tropical plants had been screened in this project and 2 plant extracts; *Aquilaria sinensis* (Aquil) and *Piper cubeba* (P9605), have demonstrated the potential to prevent and/or alleviate these two prostatic diseases. According to our investigations, their postulated mechanisms include

- reducing the availability of DHT by inhibiting 5α-RII
- reducing the availability of estrogen by inhibiting aromatase.
- preventing DHT actions (e.g. DHT induced proliferation and PSA secretion)
- competing with native ligands (e.g. DHT, estradiol) for the AR and ER.
- downregulating AR levels.
- inducing apoptosis
- reducing the production of inflammatory mediators by inhibiting COXs and/or 5-LOX.

In conclusion, P9605 and Aquil consist of bioactive components that could, not only, target the contributing role of the hormonal but also the inflammatory system involved in the etiology of BPH and PC.

The outlook for *Piper cubeba* is very promising; we have already started to initiate animal studies. Furthermore, epidemiological studies could also be performed to identify possible links between the *Piper cubeba* consumption and BPH. If results prove satisfactory, P9605 could potentially be used as a dietary supplement.

Aquilaria sinensis, however, is currently on the list of endangered species. Therefore the large-scale usage of the plant is understandably not acceptable unless it could be cultivated. However, Aquil possesses several active compounds especially the sesquiterpenes, a class of terpenes. More in depth research could focus on identifying if sesquiterpenes where responsible for the responses we had with Aquil.

Considering the anti-estrogenic properties of P9605 and Aquil, they may also be useful for diseases like breast cancer. Their anti-inflammatory properties could nevertheless be harnessed for inflammatory diseases such as asthma and arthritis. The possibility if both extracts could work in synergy could also be investigated.

There is no magic pill to treat BPH and PC, unfortunately. However, it is highly possible to prevent the development and progression of these two prostatic pathologies with adequate modifications to diet as observed from epidemiological studies. A plausible future possibility is to tailor-make one's diet together with supplements and medicine according to one's genetic and biochemical status in an attempt to reduce the risk of such diseases.

The aim of medicine is to prevent disease and prolong life,
the ideal of medicine is to eliminate the need of a physician.
William James Mayo (June 29, 1861 – July 28, 1939)

Curriculum Vitae

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Education

2004- current	PhD thesis (Phil Nat Sci) in Industrial Division of Clinical Pharmacology and Toxicology, University Hospital in Basel, Switzerland and Vitaplant AG (Witterswil, Switzerland) Topic: "The search for bioactive compounds in tropical plants to target hormone imbalance associated diseases." Supervision: Prof.Dr. Jürgen Drewe and Dr. Matthias Kreuter
1999- 2003	4 years B.Sc Hons in Pharmacology, University of Bristol (England) with Year- In- Industrial and a Home office license (Animal License).
1998-1999	National Junior College (Singapore)

Work Experience

June 2003- current	Pharmacologist at Vitaplant (50%). Have to routinely perform various bioassays on plant extracts according to GLP methods, establish, validate, create SOPs for new experiments as well as train and supervise trainees or new staff.
July 2001-2002	Year-in-industry with Novartis, Basel, with Dr. Jürg A.Gasser in the Bone Metabolism Laboratory. The project was to compare the accuracy of a novel in-vitro scanner with the existing models using rats of various osteo-conditions. Used various histological software programmes and techniques, use of fluorescence dyes, tissue preparation, handling live animals and analysing data produced. Results of my project were published.
Aug-Sept 2000	Worked in CIBA Vision, Novartis, Singapore as an administrator.

Publications:

- **Yam J**, Kreuter M, Drewe J. *Piper cubeba* targets multiple aspects of the androgen-signalling pathway. A potential phytotherapy against prostate cancer growth? *Planta Med* 2007, (In print).
- **Yam J**, Schaab A. Kreuter M, Drewe J. *Piper cubeba* demonstrates anti-estrogenic and anti-inflammatory properties *Planta Med* 2007, submitted.
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- J.A. Gasser, **J. Yam**, Non-invasive monitoring of changes in structural cancellous bone parameters over 20 weeks in rats with a novel prototype micro-CT. 3rd International Workshop on Musculoskeletal and Neuronal Interactions, Marbella Hotel, May 30th – June 3rd 2002, Corfu, Greece. Oral communication. J. Musculosk. Neuron. Interact., 2(4): 369,

Prizes/Awards Received

- Silver Award, 1998 Singapore Pre-University Science Fair (Research Project: “Haze and stomata”)