CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

Background of androgens

Androgens were firstly discovered in 1936. Androgenic hormones are a class of steroid hormones that play an important role in the development and maintenance of male sex characteristics, anabolism and behaviors. They are produced in the testis, ovary, and adrenal glands. The major circulating androgen is testosterone which found in human tissues. In many androgen-sensitive tissues, testosterone is converted to the potent androgen, 5α-dihydrotestosterone (DHT) by the β-nicotinamide adenine dinucleotide phosphate (NADPH)-dependent enzyme 5α-reductase. Testosterone can also be converted to estradiol by P450-aromatase (Figure 1) [17].

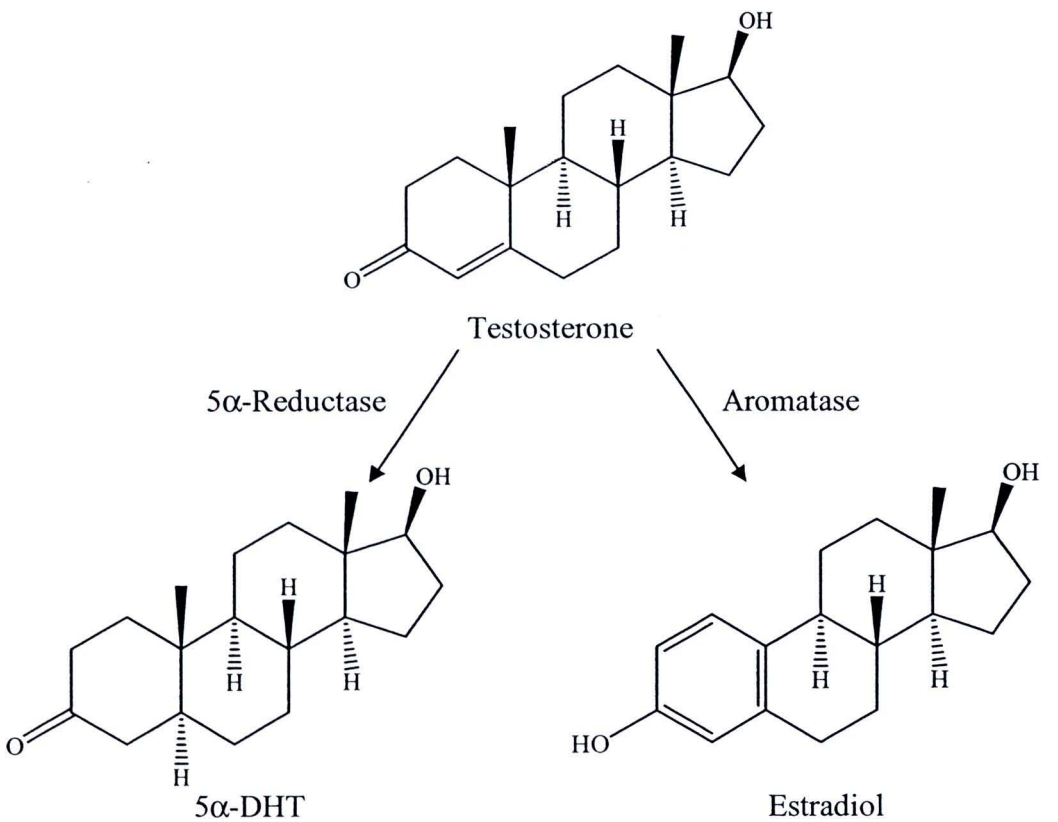


Figure 1 Enzymatic conversion of testosterone to biologically active metabolites, 5α-DHT and estradiol

The hormone testosterone is derived from cholesterol. It is the predominant circulating androgen and is produced mainly by the testis of males and the ovaries of females. The small amounts are also secreted by the adrenal glands. Testosterone circulates in the blood predominantly bound to carrier proteins such as sex hormone-binding globulin (SHBG), and the free circulating passively diffuses through the cell membrane (Figure 2).

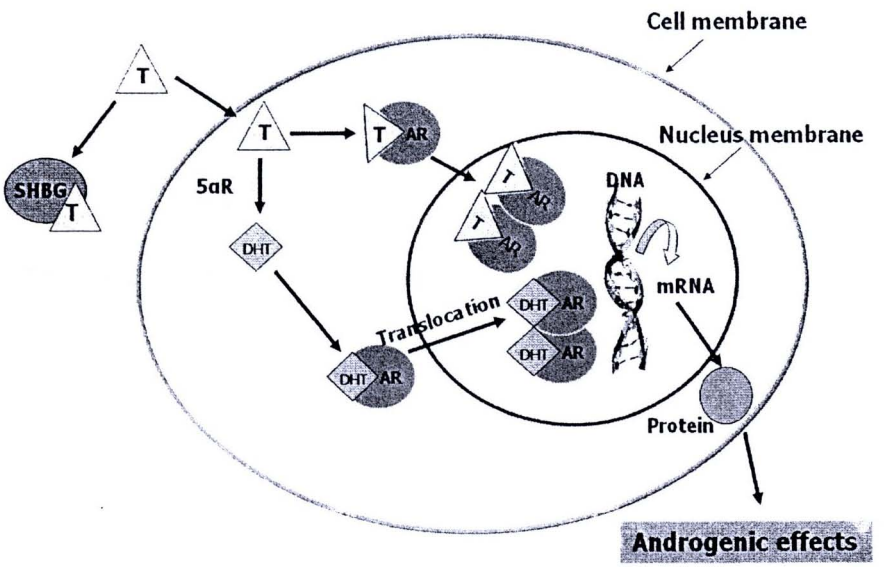


Figure 2 Mechanisms for target organ action of androgens (T = testosterone, SHBG = sex hormone-binding globulin, DHT = dihydrotestosterone, 5αR = 5α-reductase, AR = androgen receptor)

Note: modified from www.dermnet.com

Testosterone is converted to the more potent androgen, DHT by 5α-reductase or estradiol by aromatase pathway, and it may also act itself within the target cell. Both testosterone and DHT bind to the androgen receptor. DHT has a higher affinity for the receptor, and appears to be necessary for activity in some tissues such as prostate and hair follicles [18]. The androgen receptor is a member of the larger super family of ligand-activated nuclear hormone receptors. It acts as an intracellular membrane receptor localized in the cytoplasmic-nuclear portion of the target cell

(Table 1). The binding of these androgens to androgen receptor activates a steroid nuclear receptor complex resulting in the formation of homodimer. Then the homodimer interacts with androgen-responsive elements (AREs), the particular regions of the cellular DNA, and with various nuclear transcription factors. The transcription of the DNA sequence is initiates and produces the messenger RNA (mRNA). Finally, the levels of mRNA lead to an increase in protein synthesis resulting in the steroidal hormonal response regulating cell function, growth and differentiation [19].

Table 1 Tissue distribution of androgen receptor [20,21]

Category of response	Tissue
Androgenic effects	
Male reproductive tract	Testis, prostate, seminal vesicle, epididymus
Secondary sex characteristics	Skin, hair follicle, cockerel comb, and wattles
Brain	Hypothalamus, pituitary, preoptic area, cortex
Anabolic effects	*Levator ani* muscle, thigh muscle
Other responses	Sebaceous and preputial gland, androgen-sensitive tumor, kidney, uterus, liver

Biochemical properties of steroid 5α-reductase

Steroid 5α-reductase is a membrane-bound, NADPH-dependent enzyme that is responsible for the conversion of testosterone to DHT (Figure 3).

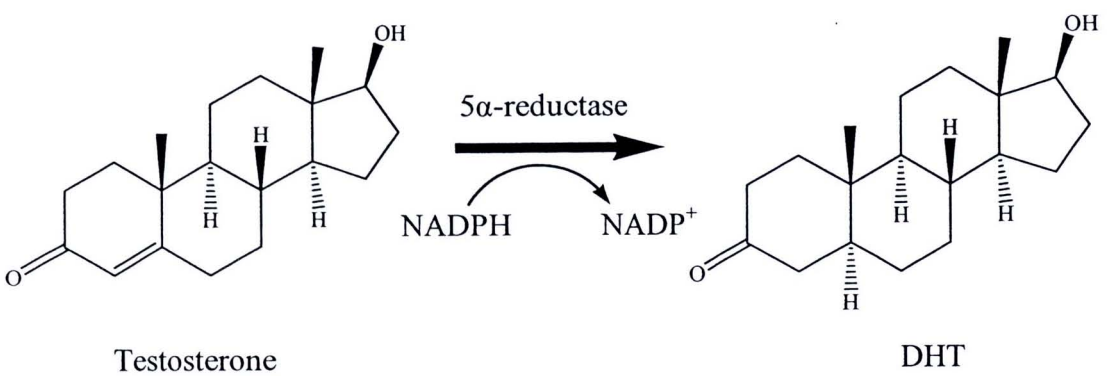


Figure 3 The enzymatic reaction catalyzed by steroid 5α-reductase [22]

There are two isozymes of 5α-reductase; type 1 and type 2. The two isozymes differ in chromosomal localization, constitution of amino acids as well as molecular weight, tissue expression patterns, enzyme kinetic parameters and pH optima. The gene responsible for the 5α-reductase type 1 isozyme resides on chromosome 5 while 5α-reductase type 2 is located on chromosome 2. The 5α-reductase type 1 presents predominantly in the skin and liver while 5α-reductase type 2 predominates in the prostate. The comparison of properties of two isozymes is summarized in Table 2.

Table 2 Comparison of properties of 5α-reductase isozymes [5]

Properties	Type 1	Type 2
Size	259 amino acids	245 amino acids
Molecular weight	29,462 Da	27,000 Da
Optimal pH	6-8.5	5.0-5.5
Biochemical properties	Hydrophobic	Hydrophobic
Gene location	SRD5A1, 5p15	SRD5A2, 2p23
Gene properties	5 exons, 4 introns	5 exons, 4 introns
In vitro inhibition by finasteride	K_i 300 nM	K_i = 3-5 nM
Localization (in tissues)	Sebaceous glands of the skin, sweat glands, dermal papilla cells, fibroblasts from all areas, epidermal keratinocytes, follicular keratinocytes, liver	Prostate, genitalskin, epididymis, seminal vesicles, liver
Selective to the inhibitors	Inhibitors with 4-methyl-4-aza functionality is very potent	4-aza,6-aza and charged 3-substituents derivatives are highly selective

The tissue distributions of steroid 5α-reductase isozymes have been reported. They found that one or more of 5α-reductase isozymes is detected in many tissues (Table 3).

Table 3 Tissue distribution of 5α-reductase isozymes [1]

Species	Tissue	5αR1[a]	5αR2[a]	Method of detection[b]
Rat	Ventral prostate	+	+	mRNA, protein
	Epididymis	+	+++	mRNA, protein
	Seminal vesicle, vas defense, testis	+	+	mRNA
	Liver	+++	-	mRNA, protein
	Adrenal, brain[c], colon, intestine, kidney	++	+	mRNA
	Heart	-	-	mRNA
	Lung	++	-	mRNA
	Muscle, spleen, stomach, ovary	+	-	mRNA
Human	Prostate, epididymis, seminal vesicle, genital skin	-	++	mRNA, protein
	Testis, ovary, adrenal, brain[d], kidney	-	-	mRNA, protein
	Liver	+	+	mRNA, protein
	Nongenital skin	++	-	mRNA, protein

Note: 5αR1 = 5α-reductase type 1, 5αR2 = 5α-reductase type 2

[a] + indicates that the mRNA or protein was detected. The number of + signs is an approximate indication of the amount of 5α-reductase isozymes detected.

[b] mRNA, isozyme mRNA detected by blot hybridization while protein is detected by immunobloting.

[c] whole brain

[d] cerebellum, hypothalamus, medulla oblongata, pituitary, pons

Each of species has the unique 5α-reductase expression patterns [1]. For example, 5α-reductase type 1 is present in the livers of mouse and rat while both isozymes are present in human liver. In the ventral prostate of rats, both isozymes are detected, whereas 5α-reductase type 2 is predominant. These differences highlight the fact that the presence in one species cannot always be broadly extrapolated.

Mechanism of 5α-reductase action

The chemical mechanism of testosterone reduction to DHT by 5α-reductase catalysis was investigated and proposed [5,18].

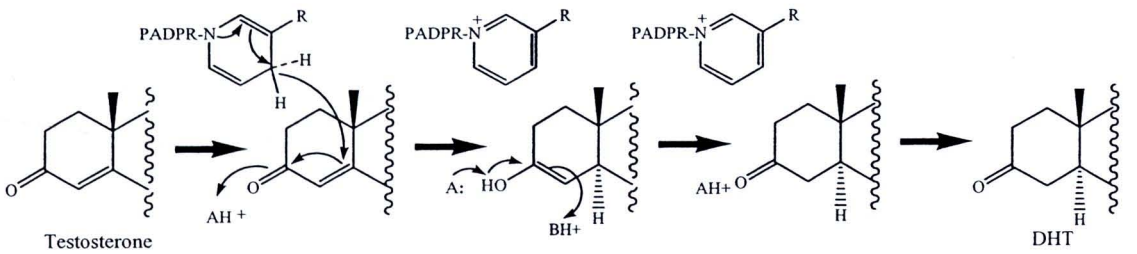


Figure 4 Mechanism of testosterone reduction by 5α-reductase [18]

The enzyme binds with NADPH to provide the binary complex, and then followed by binding of testosterone to afford a ternary complex (Figure 4). When the enone system is activated by a strong interaction with an electrophilic residue which present in the active site, the delocalized carbocation is formed. The direct hydride transfer from NADPH to the α face of delocalized carbocation provide the enolate of DHT which leads to a selective reduction at C-5. Protonation of the α-carbon of the enolate yields DHT, released from the active site. The oxidized cofactor (NADP) subsequently dissociates and the enzyme is ready for another turnover.

Hormonal effects

Androgens serve different functions in different stage of life. For example, they virilize the urogenital tract of the male embryo, and their action results in the development of the male phenotype. At puberty, the hormone act to transform the boy into a man. Minimal androgen secretion from the prepubertal testis and adrenal cortex suppress secretion of gonadotropins until, at a variable age, secretion of gonadotropins

becomes less sensitive to feedback inhibition and the testis start to enlarge. After that, the penis and scrotum begin to grow, and pubic hair appears. Early in puberty penile erections become frequent in most boys. The growth-promoting property of androgen causes an increase in height and the development of the skeletal musculature, leading to the quickly increase in body weight. The testis reach adult proportions before all the changes of puberty are completed [20].

The androgenic effects result in the development of secondary sex characteristics. The skin becomes thicker and tends to be oily owing to a proliferation of sebaceous glands and leading to acne in some individuals. Axillary hair grows, and hair on the trunk and limbs develops into the pattern typical of the male. Growth of the larynx, beard and body hair is developed. Androgen may also be responsible in part for the aggressive and sexual behavior of males and, in some species, for organizational effects in the brain during prenatal or early postnatal life [21].

Androgen-dependent disorders

Androgens play an important role in male sex differentiation and development. However, the abnormal production of DHT is associated with pathologies of the main target organs of this hormone; including benign prostate hyperplasia, prostate cancer, acne, hirsutism and androgenic alopecia.

1. Benign prostate hyperplasia (BPH)

Androgens play an important role in prostatic development and function. They are also the drivers of pathologic proliferation of prostate cells and reduced rates of apoptosis, resulting in gland enlargement in BPH and prostate cancer. BPH (it is also known as enlarged prostate) is one of the most common diseases of aging men. An enlarged prostate may squeeze the urethra and causes urinary problems. The expression of both 5α-reductase types 1 and 2 in BPH is increased due to the change in both the stromal and secretory area of the gland. These findings have led to investigation of the role of these isoenzymes in the pathogenesis and management of BPH [23].

2. Prostate cancer

Prostate cancer is major health concern among men and its incidence is increasing. The mechanisms underlying the development and progression of prostate cancer are poorly understood. Androgens play role in the initiation of prostate cancer and the balance between androgen-induced cell proliferation and apoptosis. The imbalance such as increased synthesis or decreased inactivation can lead to excessive androgen influence and increased cell proliferation. The majority of prostate tumors arise from the secretory, androgen-dependent epithelial cells. The 5α-reductase type 1 appears to be over expressed in tumour cells [23,24].

3. Acne

Many pathogenic factors of acne such as an androgen-stimulated increase in the production of sebum, obstruction of sebaceous follicles, proliferation of *Propionibacterium acnes* and inflammation have been identified [25]. The sebum production by sebaceous gland is the main cause for development of acne. The high levels of 5α-reductase type 1 which is presented in this tissue may play important role in sebum production [18].

4. Hirsutism

Hirsutism is the excessive hairiness on women in those parts of the body where terminal hair does not normally occur such as chest, abdomen, back and face. This symptom is caused by sensitization of androgen-dependent hair follicles converting vellus hair to darker and thicker terminal hair [26,27].

5. Androgenic alopecia

Androgenic alopecia (AGA) is a common type of baldness which referred to male-pattern hair loss or common baldness in men, and female-pattern hair loss in women. The multi-step molecular pathway of androgen can be involved in the pathogenesis of AGA. It is caused by vellus transformation of scalp hair [28,29].

Anti-androgen drugs

The tissue distributions of 5α-reductase and androgen receptor are mentioned above. Thus, the anti-androgens which exhibit inhibitory activity on 5α-reductase and/or block androgen receptor might be useful for the treatment of androgen-dependent disorders such as in the management of hyperplasia, prostate cancer, acne, male pattern baldness and varilizing syndromes in women. They are divided into three groups of drugs.

1. Inhibitor of androgen synthesis

Either gonadotropin-releasing hormone (GnRH) itself or an agonist such as *leuprolide* or *gonadorelin* showed the most effective inhibition of testosterone synthesis. They can decrease the plasma concentration of luteinizing hormone (LH) and testosterone. Antifungal agents of the imidazole class, such as ketoconazole and liarozole also showed secondary effect by blocking cytochrome P450 enzymes activity that involved in steroid hormone synthesis. It can be useful for therapeutic uses such as inducing androgen deprivation in patients with prostate cancer. In addition, spironolactone, an aldosterone antagonist, acts as a weak inhibitor of the binding of androgen to the androgen receptor, and it also inhibits the androgen biosynthesis. It can be useful for treatment hirsutism in some women by decreasing the growth rate and mean diameter of facial hair. This drug is commonly given together with an oral contraceptive [30].

2. 5α-reductase inhibitors

Many 5α-reductase inhibitors were pre-clinically and clinically investigated. For example, four clinically tested analogs (Figure 5), epristeride (SmithKline Beecham), finasteride (Merck), dutasteride or GG745 (Glaxo Wellcome) and MK-386 (Merck) were designed with different approaches, isozyme selectivities and mechanism of inhibition [18].

Epristeride (17-N-tert-butylcarboxamide androst-3,5-diene-3-carboxylic acid) is a noncompetitive 5α-reductase type 2 inhibitor under development by SmithKline Beecham for the treatment BPH and acne.

Finasteride (*N*-(1,1-dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide) is a member of 4-azasteroids. It is an orally active competitive inhibitor that acts by inhibiting 5α-reductase type 2. Finasteride was initially approved by the

U.S. Food and Drug Administration (FDA) in 1992 under the brand name Proscar for BPH treatment and later, it was approved under the brand name Propecia for male pattern baldness. However, finasteride causes possible adverse effects including gynecomastia, muscle growth impairment and severe myophathy [5,31].

Dutasteride (GG745), 17β-N-(2,5-bis(trifluoromethyl)-phenyl)-3-oxo-4-aza-5α-androst-1-ene-17-carboxamide, a competitive inhibitor of both 5α-reductase types 1 and 2. It has been approved by U.S. FDA in 2002 under the brand name Avodart for BPH treatment. It reduces DHT level results in decreasing the size of enlarge prostate and improve the urinary flow rate. The most common adverse reactions are impotence, decreased libido, breast disorders, and ejaculation disorders [5].

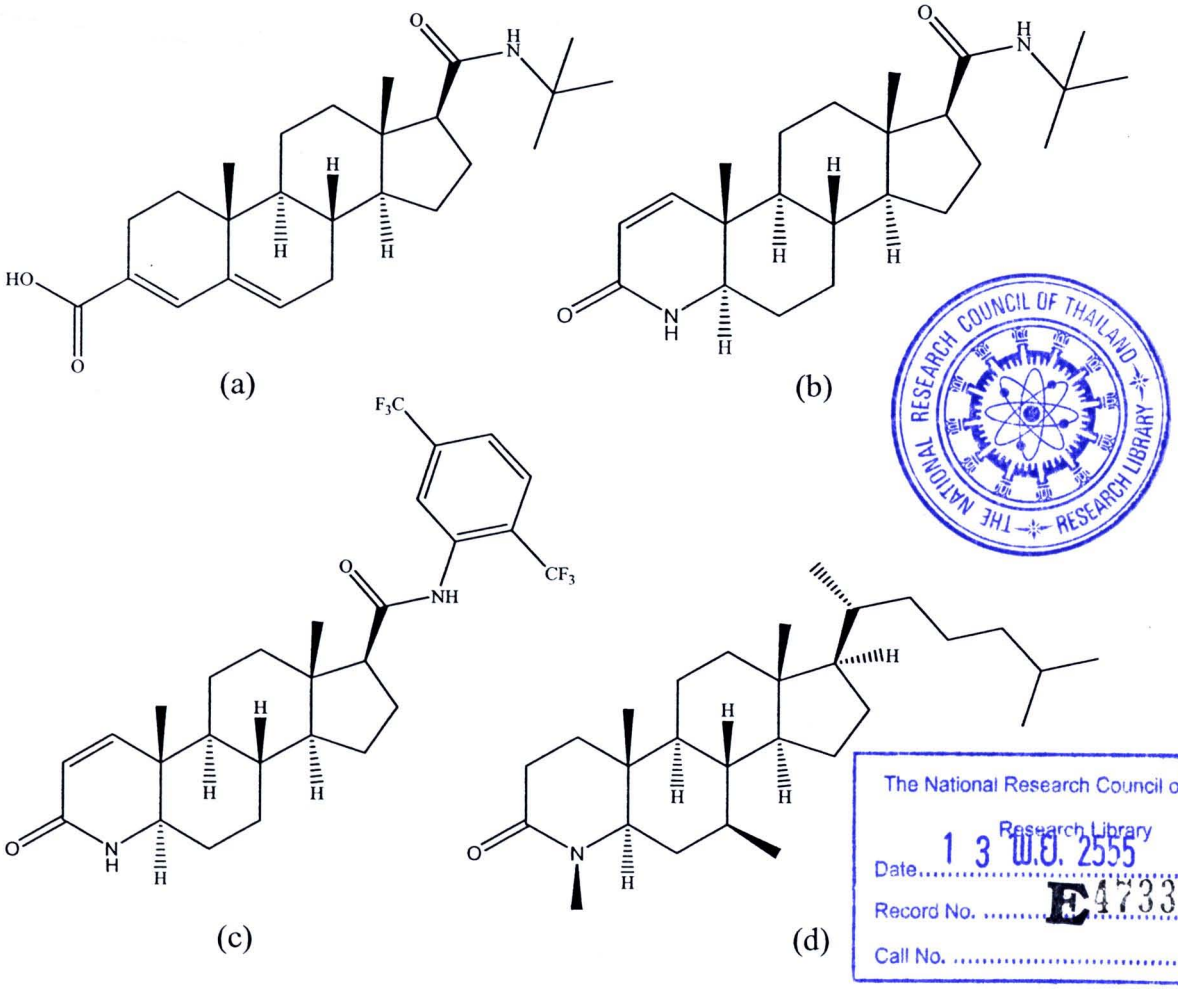


Figure 5 Inhibitors of 5α-reductase; (a) epristeride (b) finasteride (c) dutasteride (d) MK-386

MK-386 (4,7β-dimethyl-4-aza-5α-cholestan-3-one) is the 4-methyl-4-aza-steroid class of inhibitors. It is a selective 5α-reductase type 1 inhibitor and is associated with a suppression of sebum DHT without an influence on semen DHT [32].

3. Androgen receptor antagonist

Several drugs act as androgen antagonists such as cyproterone acetate, flutamide, bicalutamide and nilutamide [30]. The chemical structures of these compounds are shown on Figure 6.

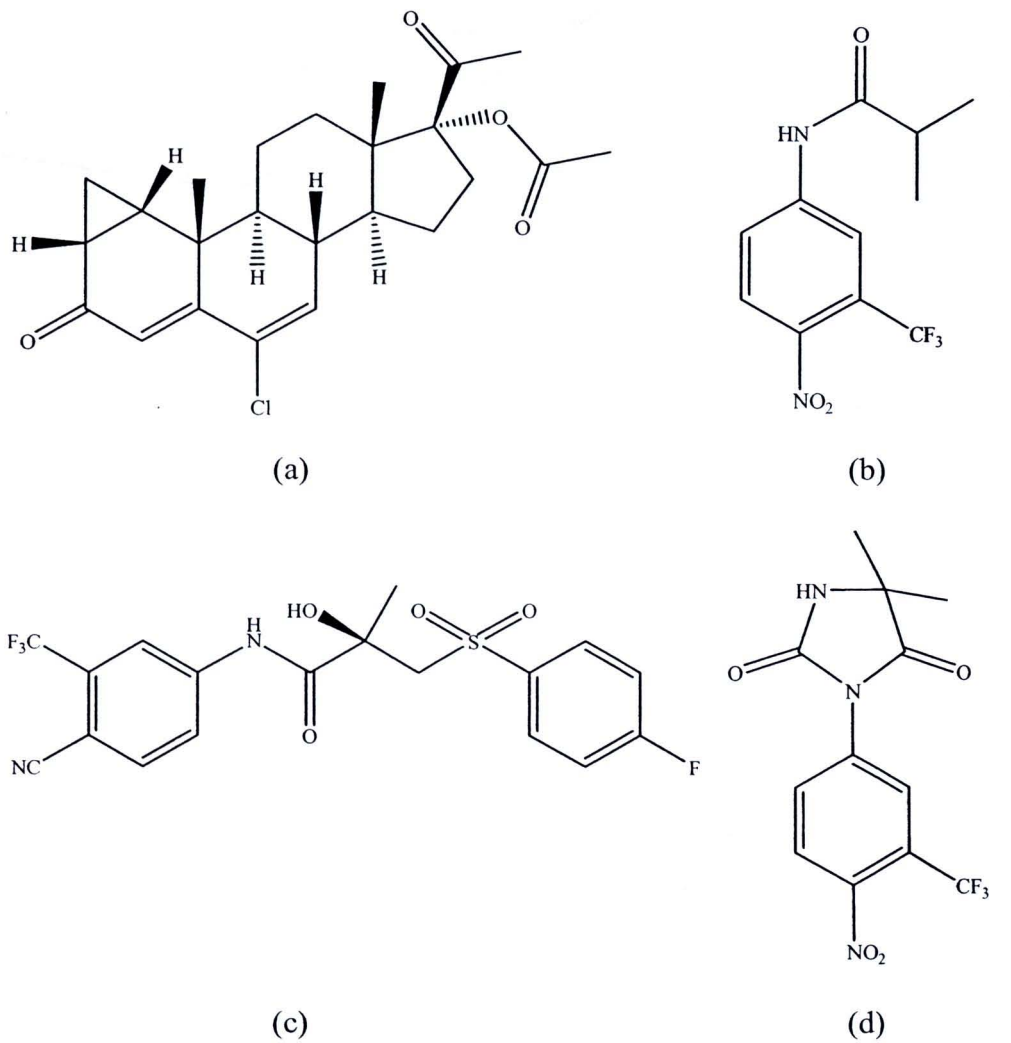


Figure 6 Androgen-receptor antagonists (a) cyproterone acetate (b) flutamide (c) bicalutamide and (d) nilutamide

Cyproterone acetate is a steroidal anti-androgen and found to be a potent androgen antagonist. It can be used to treat hirsutism, and is a common component in hormone therapy for male-to-female transsexual people.

Flutamide, an oral nonsteroidal anti-androgen drug, is used to treat prostate cancer. It competes with testosterone and DHT for binding to androgen receptors in the prostate gland. It prevents them from stimulating the prostate cancer cells to grow [33]. Flutamide has been replaced by a newer member of this class, bicalutamide, due to a better side effect profile. Flutamide may also be used to treat excess androgen levels in women. It is marketed by Schering-Plough under the brand name Eulexin. It is also known as flutamin [34].

Bicalutamide is an orally active nonsteroidal anti-androgen used in the treatment of prostate cancer and hirsutism. It has a four folds higher biding affinity to the androgen receptor, than flutamide.

Nilutamide, like bicalutamide, is an anti-androgen medication used in the treatment of advance stage prostate cancer. It can prolong life in men with prostate cancer. It is marketed under the name Nilandron in the Unites states and under the name Anandron in Canada.

Anti-androgens from the natural sources

Many natural products have been used in traditional medicines and they also have been reported for anti-androgenic activity and inhibition on 5α-reductase activity. Examples of these compounds are as follows:

Unsaturated aliphatic fatty acids including gamma-linolenic acid (GLA), alpha linolenic acid (ALA), linoleic acid, oleic acid and palmitic acid (Figure 7) were found to be 5α-reductase type 1 and 5α-reductase type 2 inhibitors. According to the study of Liang et al [35], the systemic effect after the topical application of GLA for 21 days was not found. Therefore, the compound may be potentially useful for treatment of androgen-dependent skin disorders.

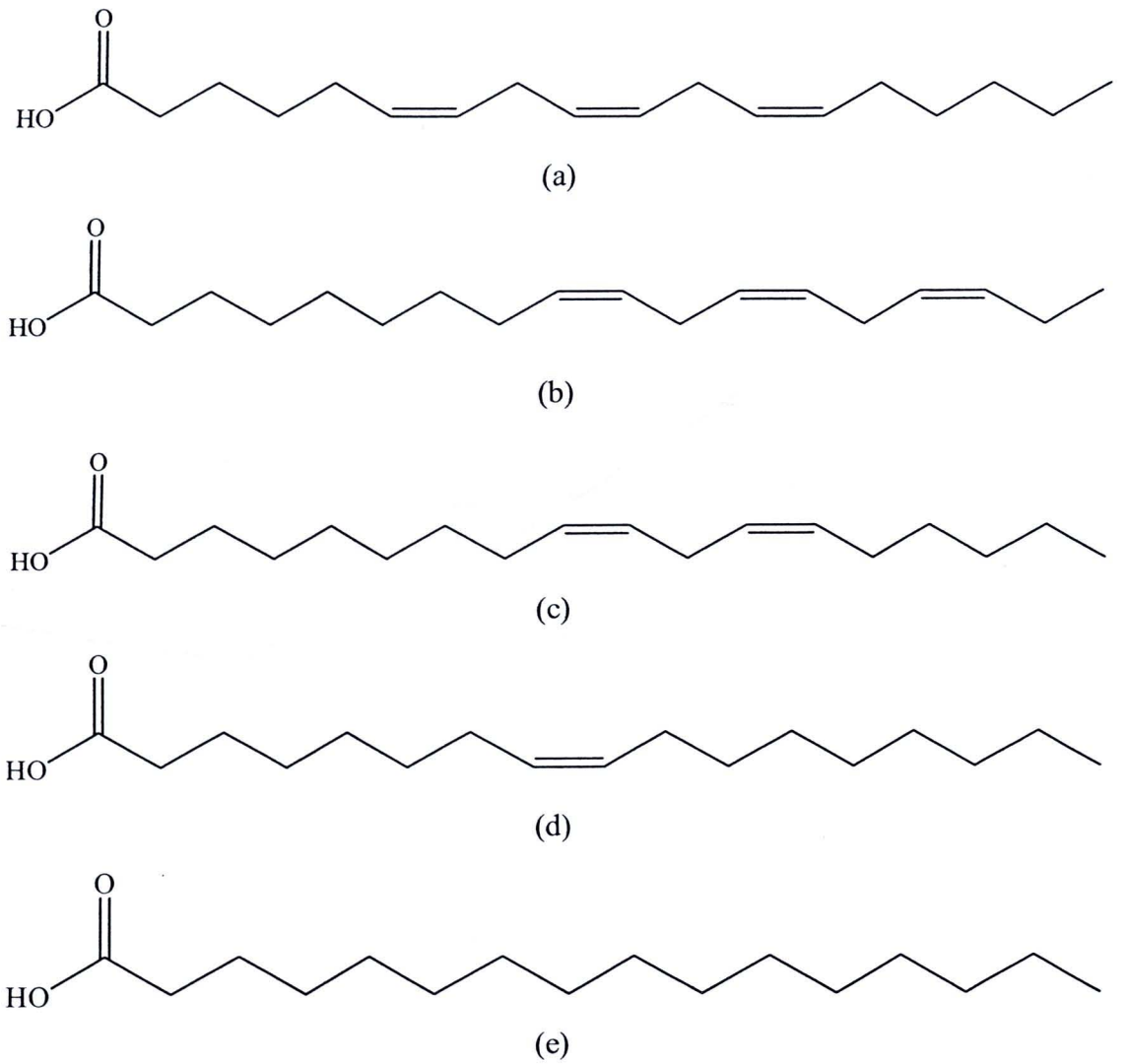


Figure 7 Chemical structures of (a) gamma-linolenic acid (b) alpha- linolenic acid (c) linoleic acid (d) oleic acid (e) palmitic acid

Three active compounds including myricanone, myricanol, myricetin (Figure 8) obtained from the aqueous ethanol extract of Myricae cortex (bark of *Myrica rubra* Sieb., Myricaceae) and lipophilic constituents (oleic, linoleic and palmitic acids) obtained from Lygodii Spora (spore of *Lygodium japonicum* Sw.) were also investigated on anti-androgenic activity. They showed *in vitro* testosterone 5α-reductase inhibitory activity and *in vivo* anti-androgenic activity by suppressing the growth of flank organ in castrated Syrian hamsters and/or hair regrowth after shaving in testosterone-treated C57Black/6CrSlc mice [36,37].

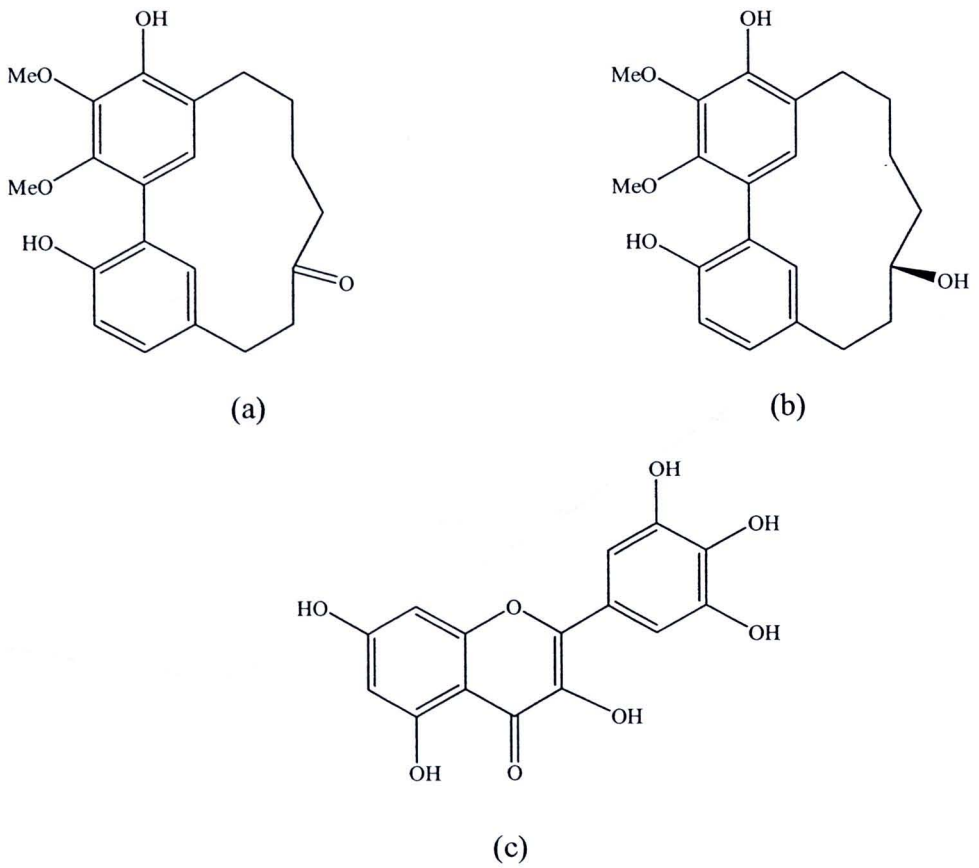


Figure 8 Chemical structures of (a) myricanone (b) myricanol and (c) myricetin

Azelaic acid is the saturated dicarboxylic acid (Figure 9) commonly found in wheat, rye and barley. It has been reported that the combination of azelaic acid and zinc sulphate could be an effective agent in the treatment of androgen related pathology of human skin [38].

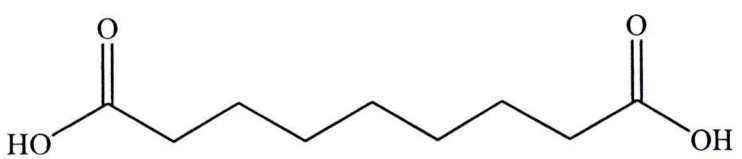
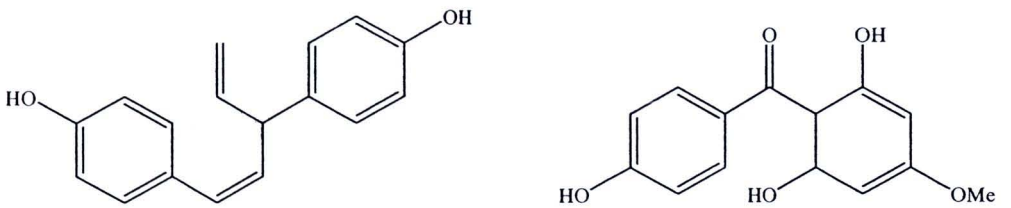


Figure 9 Chemical structure of azelaic acid

Two active compounds; cis-hinokiresinol and 2,6,4'-trihydroxy-4-methoxybenzophenone (Figure 10) from the diethyl ether extract of Anemarrhenae Rhizoma (rhizomes of *Anemarrhena asphodeloides* Bunge) showed testosterone 5α-reductase inhibitory activity. The inhibitory activity of cis-hinokiresinol was higher than that of ethinylestradiol [16].



(a) (b)

Figure 10 Chemical structures of (a) cis-hinokiresinol and (b) 2,6,4'-trihydroxy-4-methoxybenzophenone

Proanthocyanidins (Figure 11), or condensed tannins which are member of the flavonoid group has been studied. In 1998, Takahashi and colleagues investigated approximately 1000 plant extracts concerning for growth-promoting activity of hair follicle cells. They found that proanthocyanidins extracted from grape seeds showed proliferation effect on hair follicle cells. From *in vivo* test, they also found that proanthocyanidins converted the telogen phase into the anagen phase of hair growth cycle. The activity profile of proanthocyanidins was similar to minoxidil, the drug for treatment male pattern baldness. Proanthocyanidins were considered as the potential group of compounds for further application on androgenic alopecia treatment [39,40].

The 5α-reductase activity of major green tea catechins (Figure 12) including (-) epicatechin (EC), (-) epigallocatechin (EGC), (-) epicatechin-3-gallate (ECG), and (-) epigallocatechin-3-gallate (EGCG) were investigated [41]. The results found that ECG and EGCG are selective inhibitors of 5α-reductase type 1 while EC and EGC do not inhibit 5α-reductase. However, these catechins could suppress the growth of castrated hamster flank gland. The anti-androgenic effect might be through other mechanisms rather than inhibition of 5α-reductase activity.

EGCG also shows potent inhibition in cell-free but not in whole-cell assays of 5α-reductase. Interestingly, after replacing gallate ester of EGCG with long-chain fatty acids, 5α-reductase inhibitory activity increased. In addition, green tea seed extract also showed 5α-reductase inhibitory activity.

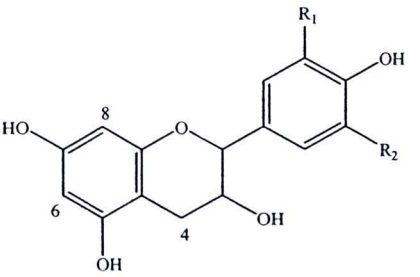


Figure 11 Composition of constituents units of proanthocyanidins; R1 and R2 represent a hydrogen atom or a hydroxyl group. Proanthocyanidins are oligomers and polymers composed of C-4 to C-8 or C-4 to C-6 linked flavan-3-ol units

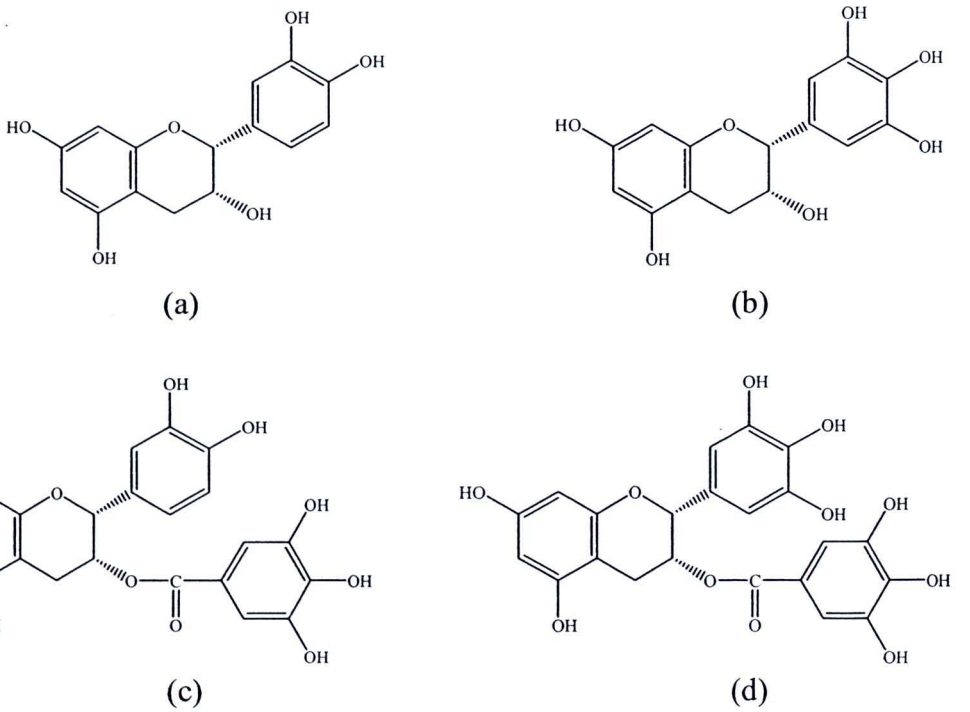


Figure 12 Chemical structures of green tea catechins (a) EC (b) EGC (c) ECG and (d) EGCG

The anti-androgenic activity of green tea seed extract hydrolysate containing kaempferol (Figure 13) was studied using HEK293 cell line. It showed good inhibitory effect on both 5α-reductase type 1 and type 2. The results also indicated that the inhibitory activity of a green tea extract hydrolysate on 5α-reductase type 2 increased in accordance with kaempferol content [42].

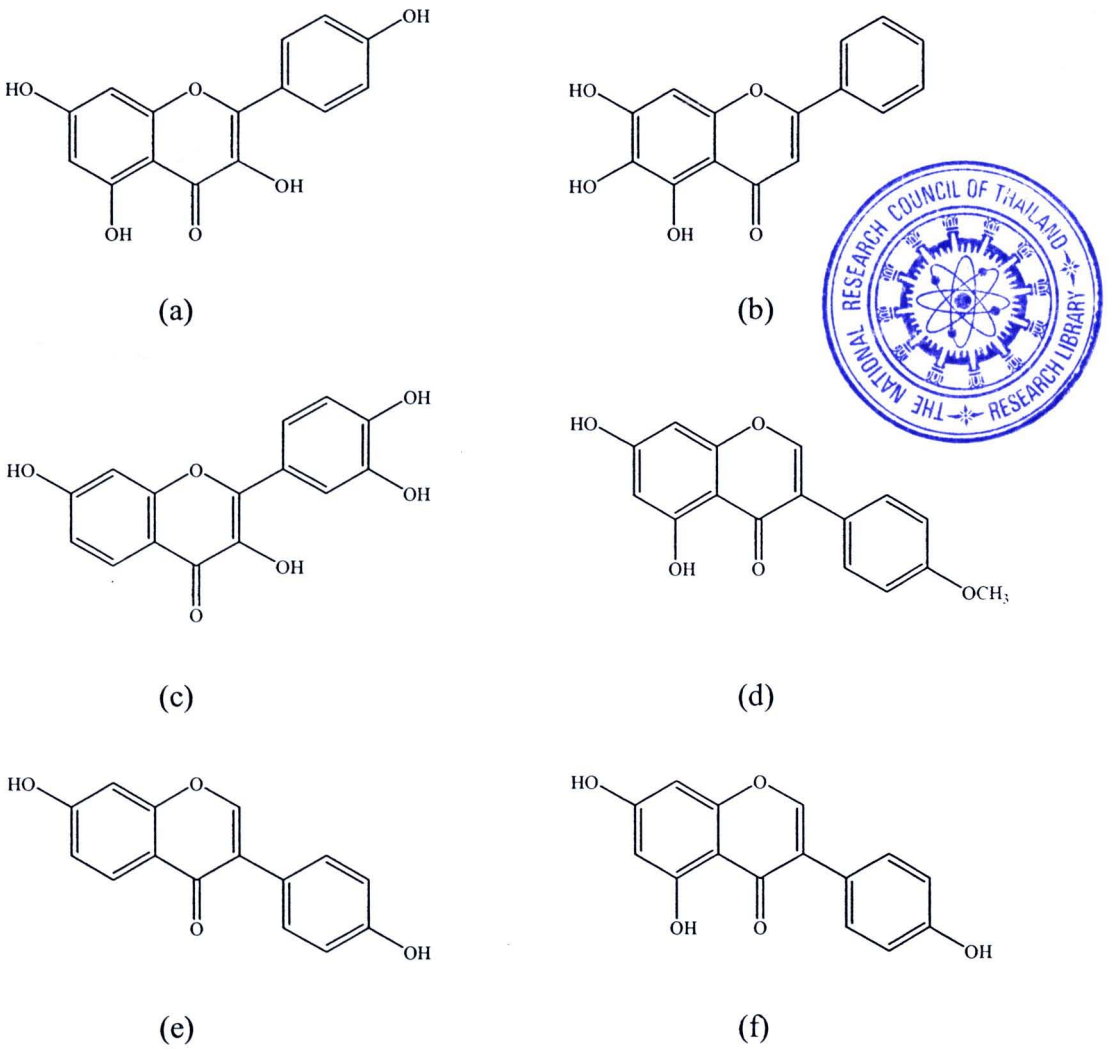


Figure 13 Structures of flavonoids tested for inhibitory activity against 5α-reductase; (a) kaempferol (b) baicalein (c) fisetin (d) biochanin A (e) daidzein and (f) genistein

Other flavonoids (Figure 13) including myricetin, quercitin, baicalein, and fisetin were reported as the potent inhibitors of 5α-reductase type 1 while biochanin A, daidzein, genistein showed potent inhibitors of 5α-reductase type 2 [43].

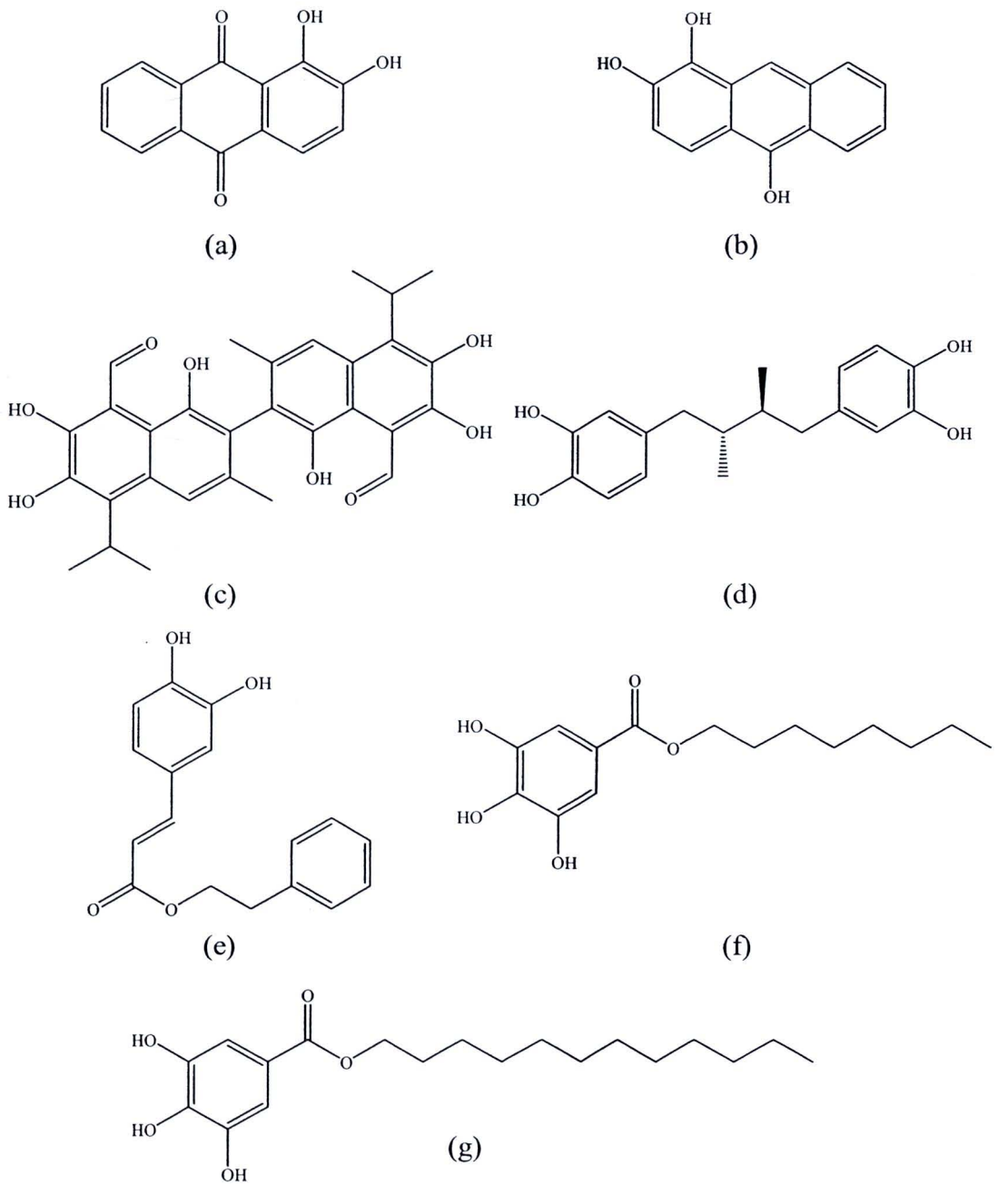


Figure 14 Structures of polyphenol tested for inhibitory activity against 5α-reductase; (a) alizarin (b) anthrarobin (c) gossypol (d) nordihydroguaiaretic acid (e) caffeic acid phenethyl ester (f) octyl gallates and (g) dodecyl gallates

Several other natural and synthetic polyphenol compounds showed higher inhibitory effect to 5α-reductase type 1 than type 2, including alizarin, anthrarobin, gossypol, nordihydroguaiaretic acid, caffeic acid phenethyl ester and octyl and dodecyl gallates (Figure 14). The catechol group plays important characteristic for selective inhibition of 5α-reductase type 1. Since some of these compounds are consumed as part of the normal diet or in supplements, they may be useful for the prevention or treatment of androgen-dependent disorders. However, the adverse effects of these compounds on male sexual differentiation were found [43].

Saw palmetto or *Serenoa repens* was mainly constituted of free fatty acids. Raynaud and colleagues recently indentified 5α-reductase type 1 and type 2 inhibitors from lipido-steric extract of this plant (LSESr, Permixon©). LSESr acts as non-competitive inhibition of 5α-reductase type 1 and uncompetitive inhibition of the 5α-reductase type 2 [44]. An *in vivo* effect of Saw palmetto on androgen-induced prostatic enlargement in rats was also studied. The results showed that Saw palmetto (whole berry and extract) reduced prostatic hyperplasia via androgen metabolism [45]. Saw palmetto also induced apoptosis of LNCaP cells in a time and dose-dependent manner [46].

Heartwood of *Artocarpus incisus* showed potent 5α-reductase inhibitory activity. Chlorophorin and artocarpin (Figure 15a and 15b) were responsible for the activity with the IC_{50} of 37 and 85 μM, respectively [47]. In addition, a geranylated chalcone (3'-geranyl-2',3,4,4'-tetrahydroxychalcone) (Figure 16) was isolated from leaves of this plant. It also showed 5α-reductase inhibitory activity with the IC_{50} of 104 μM [48].

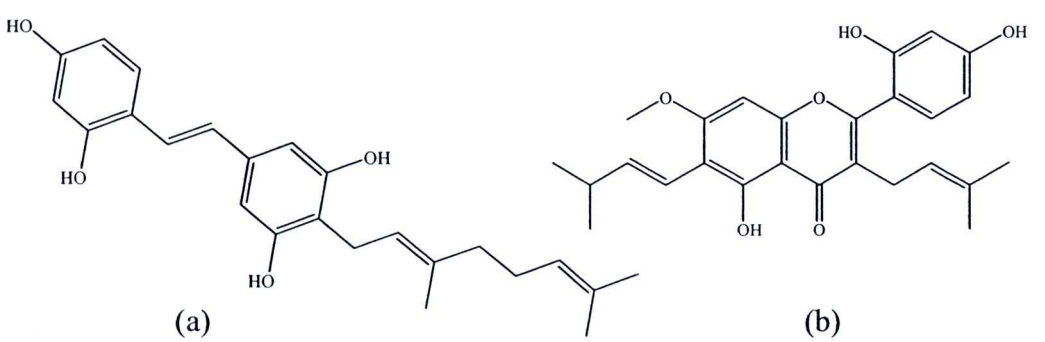


Figure 15 Chemical structures of (a) chlorophorin and (b) artocarpin

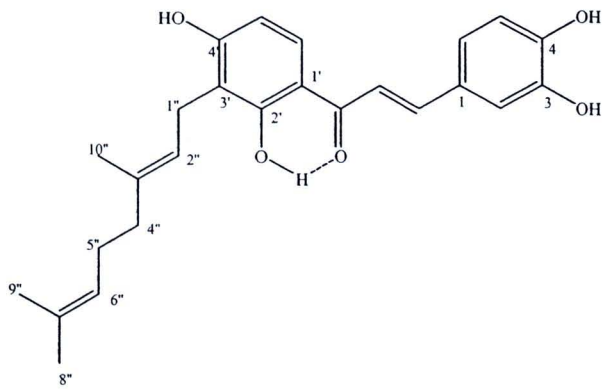


Figure 16 Chemical structure of 3'-geranyl-2',3,4,4'-tetrahydroxychalcone

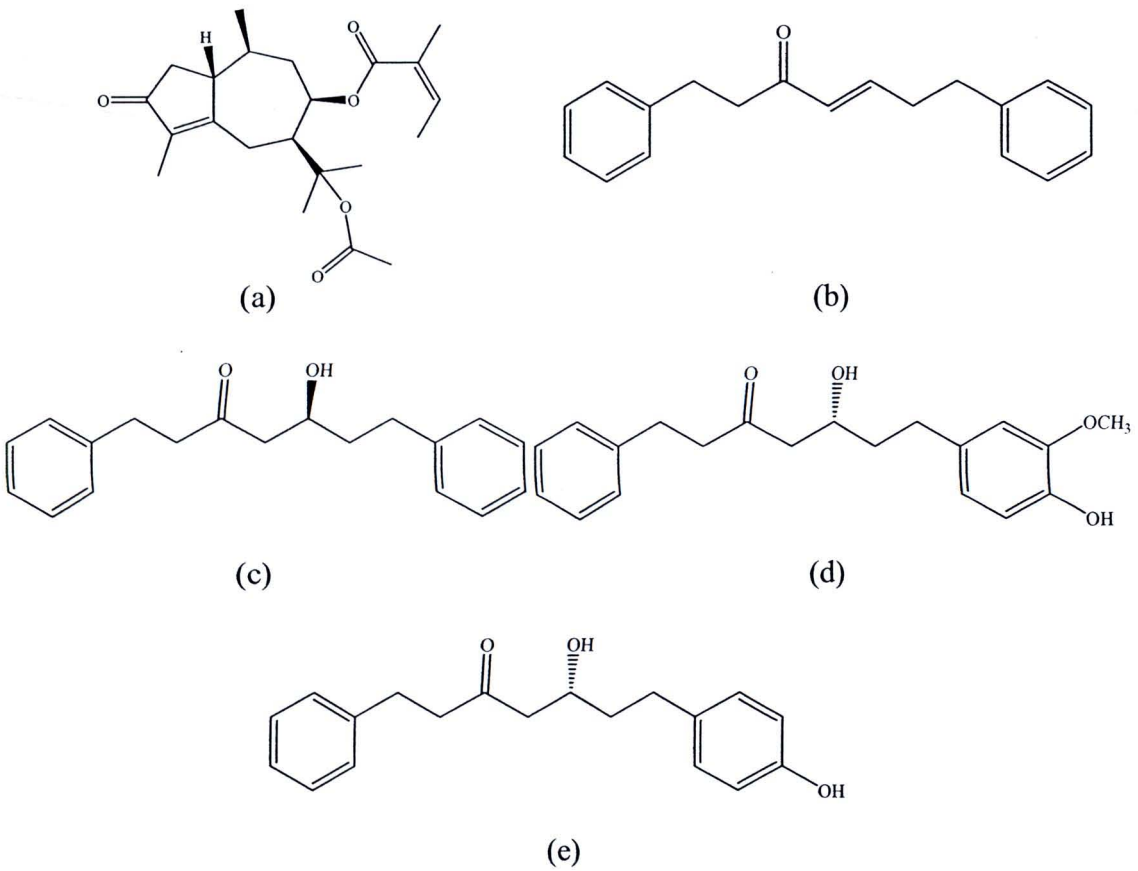


Figure 17 Chemical structures of (a) torilin and four diarylheptanoids tested for inhibitory activity against 5α-reductase; (b) 1,7-diphenylhept-4-en-3-one (c) dihydroyashabushiketol (d) 5-hydroxy-7-(4″-hydroxy-3″-methoxyphenyl)-1-phenyl-3-heptanone and (e) 5-hydroxy-7-(4″-hydroxyphenyl)-1-phenyl-3-heptanone

Torilin (Figure 17a) obtained from the methanolic extract of the fruits of *Torilis japonica* showed a potent *in vivo* inhibition against 5α-reductase activity. Its inhibition effect was stronger than α-linolenic acid but was weaker than finasteride [49]. In addition, diarylheptanoids (Figure 17b-17e) from *Alpinia officinarum* also showed inhibition against 5α-reductase activity which had been prepared from rat prostate [50].

The marine soft corals were also reported for their anti-androgenic effect. Steroidal compounds, PR-01-PR-04 (Figure 18), obtained from marine soft corals collected on the coasts of Andaman and Nicobar at Hori, Natkal and Kalipur Island showed inhibitory activity on the conversion of [^{3}H] T to [^{3}H] DHT [51].

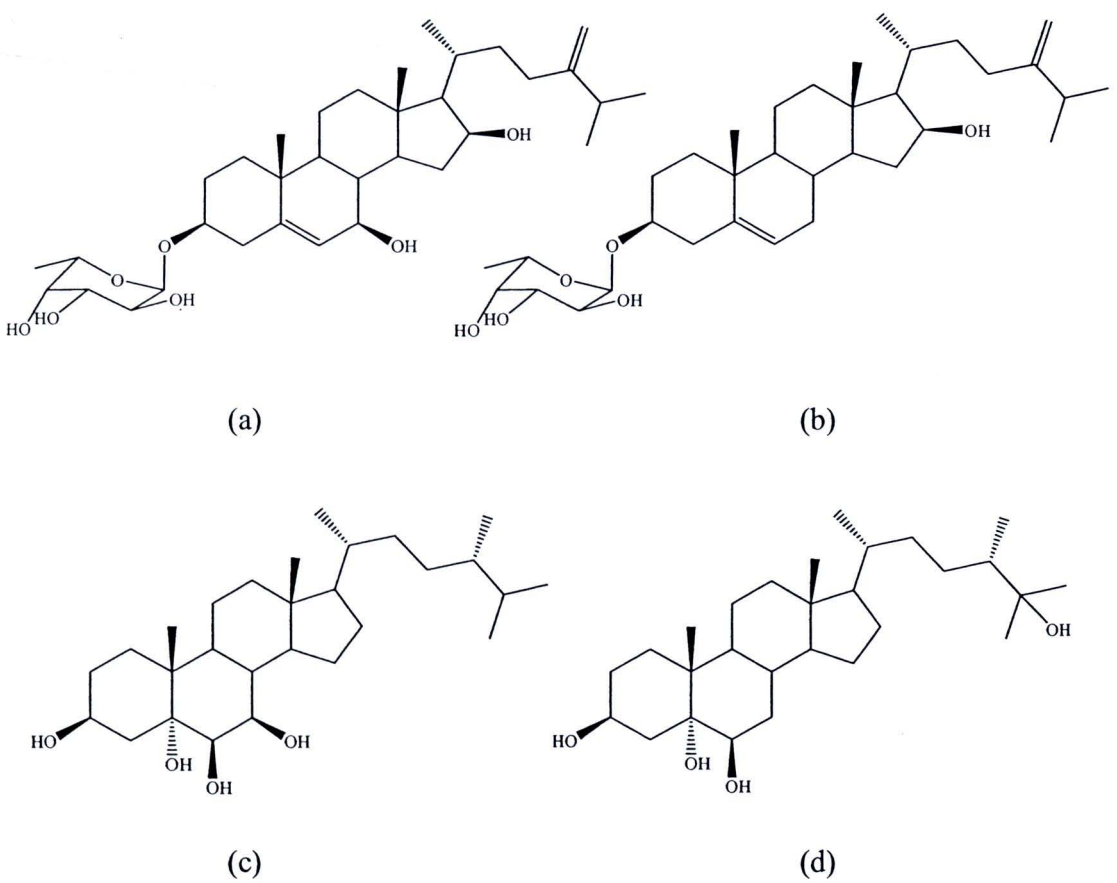


Figure 18 Structures of steroidal compounds extracted from soft corals; (a) PR-01 (b) PR-02 (c) PR-03 and (d) PR-04

The fruiting body of *Ganoderma lucidum* Fr. Krast (Ganodermataceae) which has long been used as folk medicine showed the strongest 5α-reductase inhibitory activity (on both 5α-reductase type 1 and type 2) among the extracts of 19 edible and medicinal mushrooms. It inhibited the growth of the ventral prostate in castrated rats [52]. The triterpenoid, ganoderol B (Figure 19), isolated from the fruiting body of this plant showed 5α-reductase inhibitory activity and androgen receptor binding activity [53,54]. Interestingly, it also showed inhibition effect on androgen-induced LNCaP cell growth and suppression effect on the regrowth of the ventral prostate induced by testosterone in rats. These results showed that *G. lucidum* might be a useful ingredient for the treatment of BPH [55]. Another plant, pumpkin seed is also claimed to be useful for the management of BPH [56].

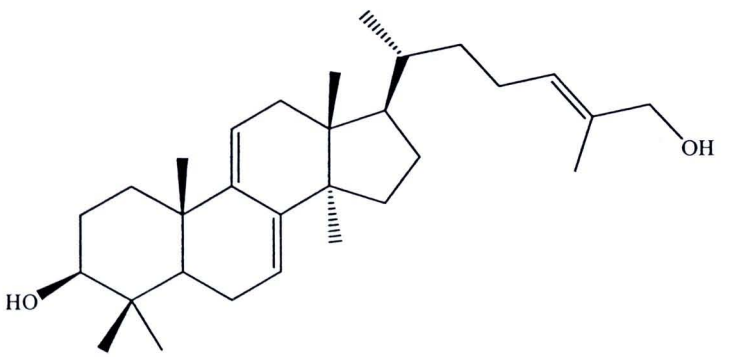
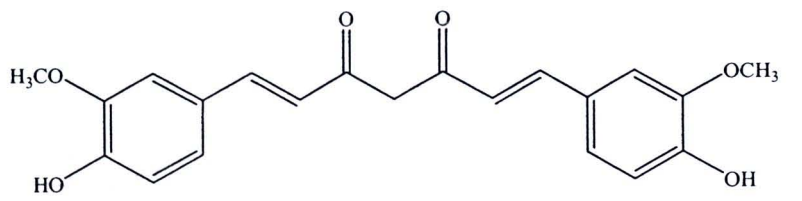


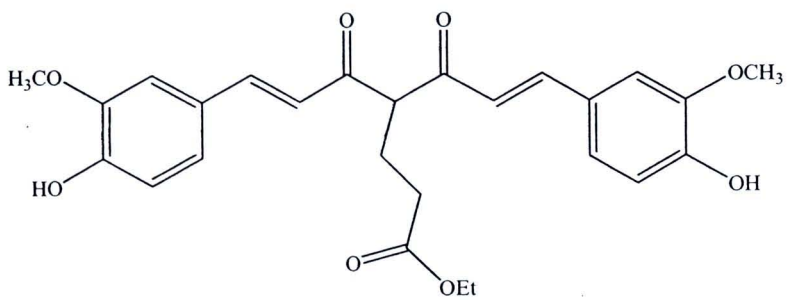
Figure 19 Structure of ganoderol B

The plants from different families were widely investigated the anti-androgenic activity. According to the study of Kumar et al. [57], various parts of 10 plants which were used for cooking or traditional medicine, were tested for 5α-reductase and evaluated the correlation between 5α-reductase inhibition effect and total phenolic content. The results showed that *Ocimum basilicum* L. showed highest finasteride equivalent 5α-reductase activity value. There was no correlation between 5α-reductase inhibitory activity and total phenolic content. Therefore, phytochemicals other than phenolic compounds might involve in the enzymatic inhibition. The extract might be useful for health promotion, prevention or treatment effects of androgenic related diseases.

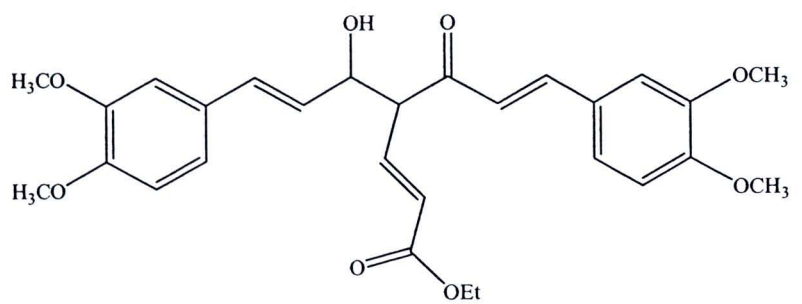
The plant extracts of Zingiberaceae, such as ginger or turmeric, have long been used as main ingredients of Thai herbal medicinal remedies for hair loss treatment. Curcumin (Figure 20a) showed the potent 5α-reductase inhibitory activity and suppression effect on the growth of castrated hamster flank gland [58]. To increase the inhibitory activity of curcumin, the new curcumin analogs such as 4-ethoxycarbonylethyl curcumin and 7-(3,4-dimethoxyphenyl)-4-[3-(3,4-dimethoxy phenyl)-acryloyl]-5-hydroxy-hepta-2,4,6-trienoic acid ethyl ester (Figure 20b and 20c) were developed as the lead anti-androgens for treatment of prostate cancer [59].



(a)



(b)



(c)

Figure 20 Structures of (a) curcumin (b) 4-ethoxycarbonylethyl curcumin and (c) 7-(3,4-dimethoxyphenyl)-4-[3-(3,4-dimethoxy phenyl)-acryloyl]-5-hydroxy-hepta-2,4,6-trienoic acid ethyl ester

Anti-androgen assays

The methods for evaluation of androgenic activity both *in vitro* and *in vivo* have been established.

1. *In vitro* assays

1.1 Assays for determination of 5α-reductase inhibitory activity

1.1.1 High performance liquid chromatography (HPLC) assay

This method has been used to detect testosterone remaining from testosterone reductase reaction. Testosterone and enzymes from rat's liver or prostrate were incubated with the tested samples. The reactions were started by addition of NADPH, and then the mixture solution was incubated. Finally, the reaction was stopped by addition of organic solvent such as dichloromethane. In the experimental, internal standard was added. After that the enzyme activity was calculated based on the amount of remaining testosterone analysed by HPLC. If the test compound shows inhibitory activity against the conversion of testosterone, the remained testosterone should not decrease. The advantages of this technique are its ease to operate, high sensitivity, high resolution and the column can be reused. However, the sample preparation can be tedious and sometimes the interfering peaks can hamper the analysis on the chromatogram.

1.1.2 Autoradiographic assay

This assay is based on the detection of the radio-labeled testosterone which is separated from DHT by thin layer chromatography (TLC) after the enzymatic reaction. The radioactivity profile is determined with an imaging analyzer. The inhibitory activity can be calculated from the percentage conversion of testosterone to DHT. The advantages of this technique are its sensitivity and high resolution. It shows higher specificity than HPLC because DHT can be detected. However, the hazardous radioactive reagent cannot be avoided in the experiment. The drawback of the two assays mentioned is low-throughput. Therefore, the high-throughput assay using immunoassay (IA) was developed.

1.1.3 Enzyme immunoassay (EIA)

An immunoassay is a biochemical test using an enzyme-bound antibody to detect antigen. The enzyme catalyzes a color reaction when exposed to substrate. The assay has advantage of the specific binding of an antibody to its antigen.

The quantity of antibody or antigen can be determined using either the antigen or antibody labeled with tracers. The label may consist of an enzyme (EIA), radioisotopes such as I-125, fluorescence substance. This technique is applied to measurement of total testosterone or DHT in samples after enzymatic reaction. For example, the testosterone EIA is based on the principle of competitive binding between testosterone in sample and testosterone–horseradish peroxidase (HRP) conjugate for a constant amount of rabbit anti-testosterone. During the incubation, a fixed amount of HRP-labeled testosterone competes with testosterone in the sample for a fixed amount of binding sited of the specific testosterone anti-body. Therefore, the amount of testosterone-HRP bound to the well decreases as the concentration of testosterone in samples increases. Unbound testosterone-peroxidase conjugate is then removed and the well washed, followed by addition of tetramethylbenzidine solution resulting in the development of color. This method can be used with microplates and absorbance is measured using spectrophotometer. Immunoassay has higher throughput than HPLC and autoradiography. It has high specificity and good sensitivity. However, it is a high cost method and it can cause a cross reactivity [60-63].

1.2 Assays for determination of androgen receptor binding

To study the mechanism of anti-androgenic activity, the test compounds were examined for their abilities to bind with androgen receptors. The fluorescence polarization (FP) technique was used to evaluate the binding ability based on the molecular orientation and mobility using polarized light and fluorescent tracer. It is a powerful tool to study molecular interaction. The binding of a fluorescence molecule to another molecule can be quantified by the change in its speed of rotation [55]. This assay provides a sensitive and efficient method for high throughput screening of potential androgen receptor ligand.

1.3 Cell proliferation assay

The anti-androgens and 5α-reductase inhibitor were tested for the ability to reduce the proliferation rate of the androgen-responsive cell such as PC3 and DU145 (both are isolated from metastatic foci of prostate carcinoma) [64]. However, PC3 has low testosterone 5α-reductase activity and expresses Prostate Specific Antigen (PSA). DU145 cells have moderate metastatic potential compared to PC3 cells and don't express PSA. Therefore, a cell line derived from androgen dependent

human prostate cancer cells (LNCaP) was established for the same general study. The main use of this cell is for studies of androgen-dependent growth, its androgen receptor and testing the anti-androgen compounds [65]. To study the anti-proliferation of substances on LNCaP cells, the cells were incubated with varying concentrations of test compounds with or without androgen for 3-7 days. Quantification of cell growth was determined by using several techniques such as counting cells under microscope, crystal violet staining assay, and MTT assay (3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [66].

Human skin may be considered as a target organ for androgens. Culture skin fibroblast is one of a valuable model for screening of compounds which might block the expression of androgen action by competing for the androgen receptor [67]. This model was also useful to assess the cytotoxicity of compounds.

2. *In vivo* assays

The well-established *in vivo* assays for evaluation of androgenic activity are the growth suppression of flank glands and growth suppression of the rat prostate model.

2.1 Growth suppression of hamster flank glands by topical application of test compounds

Hamster flank glands are highly sensitive to androgen stimulation. This gland composes of androgen-sensitive components including dermal melanocytes, sebaceous glands, and hair follicles. This animal model has been widely used for testing androgenic and anti-androgenic compounds [58,68]. Since the hamster has a pair of flank glands, a test compound can be applied topically to only one of flank gland while the other flank gland as a control. If a test compound has systemic effects, the size of both flank glands will be affected. The growth of hamster flank gland is androgen-dependent. It can be measured by an increase in the area of the pigmented macules. When the flank glands were treated topically with androgens (testosterone or DHT), the flank gland becomes larger and darker. If the test compound showed effective on testosterone treated group while ineffective on DHT treated group, it suggests that the mechanism might be involve the inhibition of 5α-reductase.

2.2 Growth suppression of rat prostate

Sprague-Dawley rats are used as a model. The enlargement of rat prostate is induced by subcutaneous injection of testosterone into the rat once daily. The test compound is orally administered to the rats at various concentrations. Then, their prostates are removed and their weights are determined in comparison with the control group [45,52,55].

Many plants have been reported for potent anti-androgenic activity. They might be potential sources for anti-androgenic compounds. A recent finding from our group reported that some Thai herbs in Zingiberaceae species including *Curcuma longa* L., *Kaempferia galanga* L., *Boesenbergia rotunda* (L) Mansf., *Zingiber officinale* Roscoe., and *Curcuma aeruginosa* Roxb. showed anti-androgenic activity. *C. aeruginosa* showed high anti-androgenic activity both *in vitro* and *in vivo* studies [6].

***Curcuma aeruginosa* Roxb.**

Curcuma aerugionsa Roxb. (Zingiberaceae) is a native plant of tropical areas. It is commonly known as Wan mahamek in Thai. It is a perennial with oblong tuber roots, leafy shoots are 45-60 cm high (Figure 21). Fresh rhizomes emit the ginger-like aroma. The plant blooms in rainy season [8,9,69].

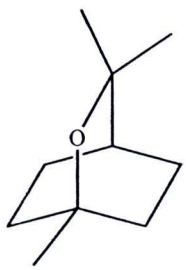
Pharmacological studies

In Thai traditional medicine, maceration of the rhizome with alcohol is used to treat uterine pain, uterine inflammation, postpartum uterine and perimenopausal bleeding [70]. Various pharmacological effects of this plant were scientifically proved such as postcoital contraception, anti-HIV actions, hepatoprotection, anti-microbial effects, antioxidation, reduce platelet-activation and antinociceptive effects [8,10-12].

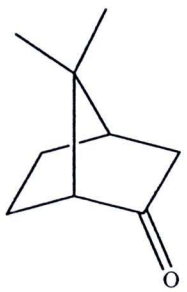
Figure 21 *C. aeruginosa* leaves, rhizomes and flowers

Chemical constituents

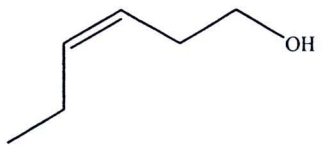
C. aerugnosa rhizomes and leaves contain essential oil of which 1,8-cineole, curzerenone, furanogermenone, camphor, (Z)-3-hexenol, zedoarol, furanodienone, curcumenol, isocurcumenol, beta-elemene, curzerene and germacrone are its composition [9,15,69,71,72]. Glucan and two components of starch (amylopectin and amylase) were isolated from the tuber of *C. aeruginosa* [14,73,74]. This plant also contains useful sesquiterpenes including zedoalactone A, zedoalactone B, furanodiene, furanodienone, dehydrocurdione, curcumenone, 13-hydroxygermacrone, zedoarondiol and zedoarol [13,14]. The structures of chemical constituents are shown in Figure 22.



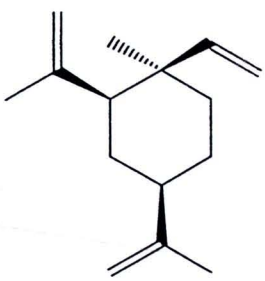
1,8-cineole



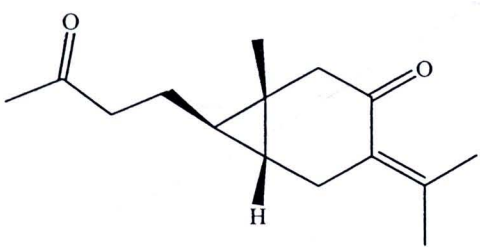
camphor



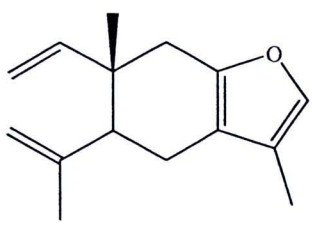
(Z)-3-hexanol



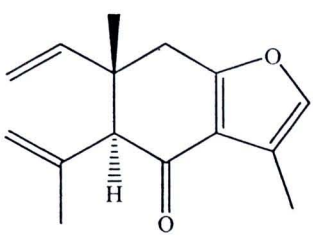
β-elemene



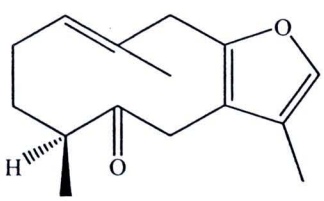
curcumenone



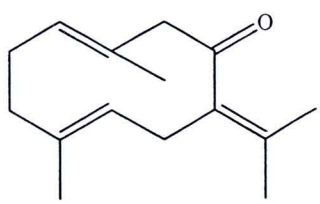
curzerene



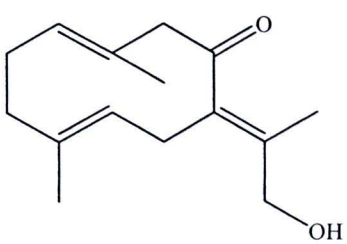
curzerenone



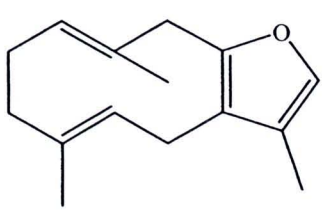
furanogermenone



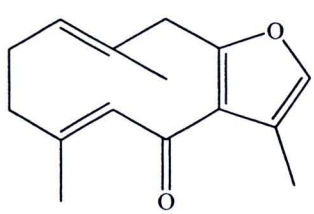
germacrone



13-hydroxy germacrone

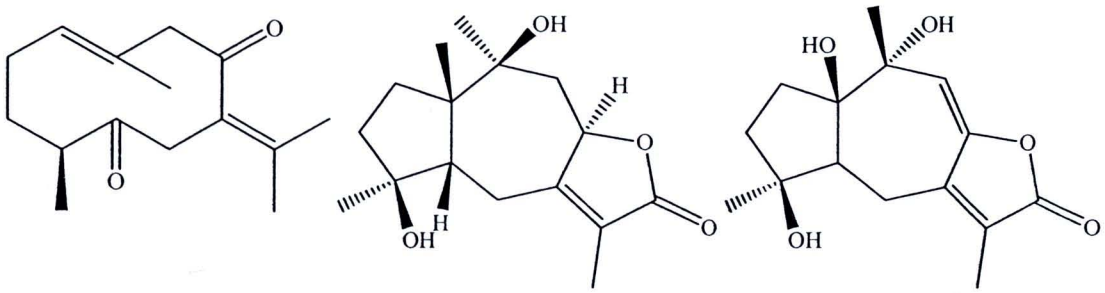


furanodiene



furanodienone

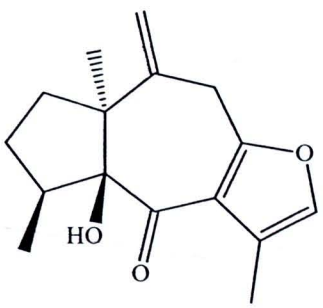
Figure 22 Some chemical constituents from *C. aeruginosa*



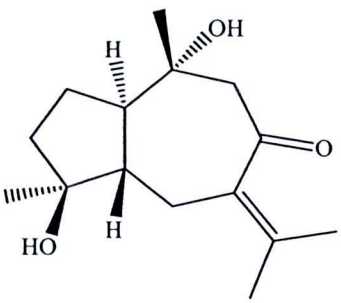
dehydrocurdione

zedoalactone A

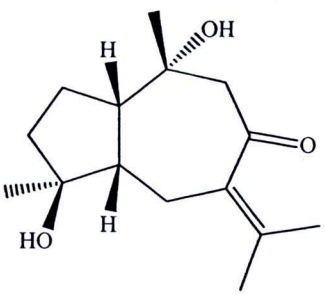
zedoalactone B



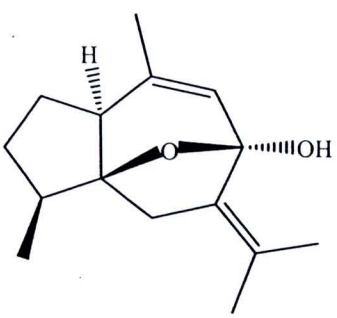
zedoarol



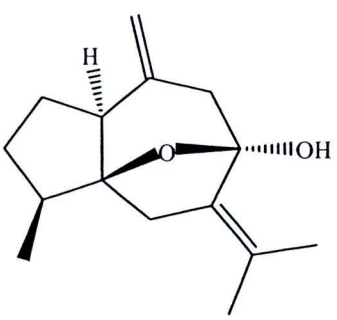
zedoarondiol



isozedoarondiol



curcumenol



isocurcumenol

Figure 22 (cont.)

Sesquiterpenes are a class of compounds which contain a skeleton of 15 carbons derived by the assembly of three isoprenoid units. The sesquiterpenoids can be classified as eudesmane, guaine, germacrane drimane etc. Germacrane sesquiterpenoids occur widely in nature. They are important constituents of essential oils, which have many applications in medical, soap and perfume formulations. They

have unique conformational flexibility. Biological activities of some sesquiterpenes are as follows:

Germacrone is a 10 membered ring monocyclic ketone sesquiterpenoid. This compound showed various biological activities including central nervous system depressant, antitumor, antiinflammation, antiulcer, antibacterial effects, antifungal effects, antitussive, vasodilator, choleretic, and hepatoprotection [75].

Dehydrocurdione, the major sesquiterpene in *Curcuma zedoaria*, has been reported that it showed the anti-inflammatory potency related to its antioxidant effect [76]. The effect of dehydrocurdione on smooth muscle was also investigated. The results suggested that the inhibitory effect of this compound on intestinal and vascular smooth muscle are mediated by blockade of Ca^{2+} entry from the extracellular space [77].

Curcumenol isolated from *C. zedoaria* was reported to inhibit the growth of S-180 sarcoma cells and mouse cervical U-14 cells [78]. Isocurcumenol was also characterized as the active compound of this plant and was found to inhibit the proliferation of cancer cells (human lung, leukemia, nasopharyngeal carcinoma and murine lymphoma cells) without inducing significant toxicity to the normal cells [79].

Zedoarondiol, a sesquiterpene lactone isolated from the rhizome of *Curcuma heyneana*, showed antiinflamtory activity by inhibiting iNOS, COX-2, and pro-inflammatory cytokine expressions [80].

From the literature reviews, sesquiterpenoids have various biological activities. Therefore, determination of these sesquiterpenes is very important for pharmacological study and quality control of plant materials. Several methods such as thin-layer chromatography (TLC), gas chromatography-flame ionization detector (GC-FID), gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) have been reported to quantify sesquiterpenes. Moreover, the stability profiles of these compounds might be useful for the further application on cosmetics and medicinal uses.

Recently, our group identified both *in vitro* and *in vivo* high anti-androgenic activity of the hexane extract of *C. aeruginosa* (Zingiberaceae) rhizomes [6]. The plant might be useful for the treatment androgen-disorders. However, the active compounds had not been determined yet. Therefore, the present studies are to do further

investigation on the isolation and identification of the chemical constituent responsible for anti-androgenic activity of *C. aeruginosa*. The 5α-reductase inhibitory activity of the isolated compounds was investigated using *in vitro* and *in vivo* assays. To study the possible mechanisms, the other potential mechanism of the active compound via binding to androgen receptor was also studied. The stability study of both the extract and the anti-androgen component were carried out. The data obtained from stability profiles study might be useful for the further application.