กลไกการออกฤทธิ์ของสารสกัดจากแก่นแสมทะเลและสาร avicequinone C ต่อเซลล์รากผมของผู้ป่วยโรคผมบางจากพันธุกรรม

นางสาวสุกัญญา นำสวัสดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

MECHANISM OF ACTION OF *AVICENNIA MARINA* HEARTWOOD EXTRACT AND AVICEQUINONE C ON HUMAN DERMAL PAPILLA CELLS ISOLATED FROM ANDROGENIC ALOPECIA PATIENTS

Miss Sukanya Numsawat

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biomedicinal Chemistry
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Faculty of Pharmaceutical Sciences
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Thesis Title	MECHANISM OF ACTION OF *AVICENNIA MARINA* HEARTWOOD EXTRACT AND AVICEQUINONE C ON HUMAN DERMAL PAPILLA CELLS ISOLATED FROM ANDROGENIC ALOPECIA PATIENTS
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สุกัญญา นำสวัสดิ์ : กลไกการออกฤทธิ์ของสารสกัดจากแก่นแสมทะเลและสาร avicequinone C ต่อเซลล์รากผมของผู้ป่วยโรคผมบางจากพันธุกรรม (MECHANISM OF ACTION OF *AVICENNIA MARINA* HEARTWOOD EXTRACT AND AVICEQUINONE C ON HUMAN DERMAL PAPILLA CELLS ISOLATED FROM ANDROGENIC ALOPECIA PATIENTS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. วันชัย ดีเอกนามกูล, 68 หน้า.

โรคผมบางจากกรรมพันธุ์ (Androgenic alopecia, AGA) เป็นโรคผมร่วงที่มีสาเหตุมาจากความผิดปกติของพันธุกรรมควบคู่กับการผลิตฮอร์โมน 5-แอลฟา-ไดไฮโดรเทสโทสเตอโรน (5-alpha-Dihydrotestosterone) เพิ่มมากขึ้น จากฮอร์โมนเทสโทสเตอโรน (testosterone,T) ซึ่งเป็นผลมาจาการทำงานของเอนไซม์ 5-แอลฟา-รีดักเทส (5-alpha-reductase) ในการศึกษาก่อนหน้าพบว่าสารสกัดจากแก่นแสมทะเล (*Avicennia marina* heart wood extract, AM extract) และสารสำคัญอวิซีควิโนนซี (avicequinone C; AC) มีความสามารถในการยับยั้งการทำงานของเอนไซม์ 5-แอลฟา-รีดักเทส ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษากลไกที่เป็นไปได้ในเชิงชีวเคมีของสารสกัดจากแก่นแสมทะเล (AM extract) และสารอวิซิควิโนน ซี (AC) ต่อการเจริญเติบโตของเส้นผมในเซลล์รากผมชนิด dermal papilla ที่มีหน้าที่เกี่ยวข้องกับการสร้างเส้นผม ซึ่งแยกได้จากผู้ป่วยผมบางจากพันธุกรรม จากการศึกษาพบว่าสารสกัดจากแก่นแสมทะเลที่ความเข้มข้น 10 ไมโครกรัมต่อมิลลิลิตร (10 µg/ml) และสารสำคัญ AC ที่ความเข้มข้น 10 ไมโครโมลาร์ (10 µM) สามารถยับยั้งการทำงานของเอนไซม์ 5-แอลฟา-รีดักเทส ในการลดการสร้างฮอร์โมน 5-แอลฟา-ไดไฮโดรเทสโทสเตอโรน ในเซลล์รากผมได้อย่างมีนัยสำคัญถึง 74% และ 68% ตามลำดับ นอกจากนี้ยังพบว่าสารสกัดจากแก่นแสมทะเลและสารสำคัญ AC สามารถยับยั้งการเคลื่อนที่ของตัวรับแอนโดรเจน (androgen receptor, AR) ที่เกิดการจับกับฮอร์โมนแอนโดรเจน (T and 5-alpha-Dihydrotestosterone) ไปยังนิวเคลียสซึ่งเป็นบริเวณที่ฮอร์โมน แอนโดรเจน (T and 5-alpha-Dihydrotestosterone) ทำงานและแสดงออกทางชีวภาพต่อไป ผลของการยับยั้งการทำงานของเอนไซม์ 5-แอลฟา-รีดักเทส และการยับยั้งการเคลื่อนที่ของตัวรับแอนโดรเจน (AR) มีผลต่อการแสดงออกของยีน (mRNA expression) ของสารที่เรียกว่า growth factors ที่ช่วยควบคุมและกระตุ้นให้เกิดการเจริญของเส้นผมในวัฏจักรของการเจริญเติบโตของเส้นผม โดยเฉพาะการเพิ่มการแสดงออกของยีน vascular endothelial growth factor (VEGF) และ Hepatocytes growth factor (HGF) ซึ่งมีบทบาทสำคัญในกระบวนการสร้างเส้นเลือดใหม่ (angiogenesis) และเกี่ยวข้องกับการกระตุ้นการเจริญของเส้นผม จากผลการศึกษาทั้งหมดของสารสกัดแสมทะเลและสารสำคัญอวิซีควิโนนซี (avicequinone C; AC) ในงานวิจัยนี้ทำให้ทราบถึงกลไกต่อการเจริญของเส้นผมในเซลล์รากผมซึ่งจะนำไปใช้ประโยชน์เพื่อเป็นข้อมูลสนับสนุนในการพัฒนานำสารสกัดแสมทะเลและสารสำคัญอวิซีควิโนนซี (avicequinone C; AC) ไปใช้ป้องกันและรักษาโรคผมบางจากพันธุกรรม (AGA)

ภาควิชา ชีวเคมีและจุลชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเวชเคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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SUKANYA NUMSAWAT: MECHANISM OF ACTION OF *AVICENNIA MARINA* HEARTWOOD EXTRACT AND AVICEQUINONE C ON HUMAN DERMAL PAPILLA CELLS ISOLATED FROM ANDROGENIC ALOPECIA PATIENTS. ADVISOR: ASSOC. PROF. WANCHAI DE-EKNAMKUL, Ph.D., 68 pp.

Androgenic alopecia (AGA), is a genetically determined disorder associated with an increased production of the hormone 5-alpha -dihydrotestosterone which is formed from testosterone (T) by the enzyme activity of 5-alpha-reductase. Previously, crude extracts prepared from heart wood of *Avicennia marina* (AM extract) and its active constituent, namely avicequinone C (AC) were found to be able to inhibit the enzyme activity of 5-alpha-reductase. In this study, the possible mechanism of the AM extract and AC compound on the biochemical actions of hair growth was evaluated by using dermal papilla cells (DPCs) isolated from AGA patients. The studies showed that AM extract (10μg/ml) and AC compound (10μM) could inhibit the activity of 5-alpha-reductase enzyme causing the reduction of 5-alpha -dihydrotestosterone formation by 74% and 68%, respectively. Independently, both AM extract and AC compound could also significantly inhibit the translocation of the bound-androgen receptor complex into the nucleus which is the site of T and 5-alpha -dihydrotestosterone action. Both inhibitory actions of 5alpha-reductase and nuclear translocation by AM and AC appeared to have subsequent effects on the increase in the mRNA expression of VEGF and HGF which play important roles in the formation of new blood vessels associated with the hair growth (angiogenesis). In summary,the results from this study revealed the mechanism of action of AM and AC on hair growth in DPCs which is useful for supporting the development of *Avicennia marina* extract and pure AC compound as a potential product to prevent and treat AGA patients.

Department: Biochemistry and Microbiology Student's Signature .....
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List of Abbreviations

%	percentage
µg	microgram
µl	microliter
µM	micromolar
5α-DHT	5α-dihydrotestoseterone
5α-R	5α-reductase
5α-R1	5α-reductase type 1
5α-R2	5α-reductase type 2
AC	aviciquinone C
AGA	androgenic alopecia
AM	*aivicennia marina*
AR	androgen receptor
bp	base- pair
DNA	deoxyribonucleic acid
dNTP mix	dinucleotide triphosphate mixture
DPC(s)	dermal papilla cell(s)
EDTA	ethylenediaminetertraacetic acid
et. al.,	Et Alii (latin), and others
FGF-7	fibroblast growth factor-7
Fi	finasteride
Flu	flutamide
HaCat	keratinocyte Cells
HF	hair follicle
HGF	hepatocytes growth factor
hr	hour
i.e.,	that is
IC_{50}	50% inhibitory concentration

IGF-1	insulin like growth factor 1
M	Molar
mg	milligram
$MgCl_2$	magnesium chloride
min	minutes
ml	milliter
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
nM	nano-molar
°C	degree Celsius
PCR	polymerase chain reaction
pH	negative log of hydronium ion
R^2	correlation coeffient
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
T	testosterone
TAE	tris-acetate-EDTA
TLC	thin layer chromatography
UV	ultra—violet
V	volts
VEGF	vascular endothelial growth factor
α	alpha
β	beta

CHAPTER I

Introduction

Background information

Scalp hair is a type of hair follicle that undergoes cycles to produce new hairs for supplying throughout lifetime of human. The hair cycle is controlled by numerous factors such as genetics, hormones including cytokines and some of growth factors. When the cycle become disorder, it will lead to the pre-maturation of hair loss (1, 2).

Androgenic alopecia is the most common type of hair loss in both men and Women who have genetically predisposition couple with overproducing of 5α-dihydrotestosterone (5α-DHT) (3-5). In men, there is atypical pattern of baldness that initiates loss of hair from frontal hairline of scalp. This is followed by diffusion over the vertex of scalp eventually to become completely baldness of this scalp area. In women, hair loss begins diffusion thinning over the frontal and central of scalp hair (6). The prevalence of this condition is growing problem although there are not in serious health condition, but the loss of scalp hair can affects self-esteem (7-9). The pathogenesis of androgenic alopecia is known to be the result of 5α-DHT activity which is derived from the conversion of testosterone (T) by 5α-reductase (5α-R) enzymes in dermal papilla cells (DPCs). The 5α-DHT is more potency than T which is a major circulating androgen. Both of androgens bind to androgen receptor (AR) in DPCs that are believed to be only direct site of androgen action in hair follicle. Thereafter, the ligand-receptor complex translocate into the nucleus and bind on the androgen response elements in the regions of androgen-regulated genes to initiate the signaling cascade in DPCs (10-12).

The 5α-DHT affects the hair follicle by causing premature entering of the cells into the catagen phase (apoptosis and regression phase), resulting in shortening the actively growth phase (anagen phase) and causing miniaturization event that changes from terminal hairs to no-growing vellus hairs. Moreover, it has an effect on alteration of some growth factors that are secreted by DPCs which are involved in the regulation of hair growth cycle, such as inhibiting insulin-like growth factor-I (IGF-1) production that has an effect on hair growth (13) or induces the expression of transforming growth factor-β_1 (TGF-β_1) mRNA in balding DPCs to inhibit the epithelial cell growth. In contrast, there has been a study showing that 5α-DHT-induced epithelial cell death by dickkop-f WNT signaling pathway inhibitor1 (DKK-1) can reverse this effect by neutralizing with its antibody (14, 15). These findings suggest that DPCs mediate the signals of androgen to follicular epithelial cells in a paracrine fashion. Thus, one target for treating androgenic alopecia is to inhibit 5α-R activity within the hair follicle (16).

To date, there are two popular methods for treating AGA. First, drug therapy and other is transplantation. For the drug therapy, there are limited to minoxidil and oral finasteride USFDA-approved synthetic drugs for treating AGA.

The oral- administration of finasteride is a selective inhibitor of 5α-reductase type2 (5α-R2) that decreases serum 5α-DHT as well as scalp 5α–DHT up to 70% compared to baseline. Topical Minoxidil, it was originally oral medication for hypertension that has common side effect, hypertrichosis which lead to the treatment of androgenic alopecia as topical solution but its mechanism of action remains unclear. Some studies have shown that minoxidil promotes DPCs cell survival and further inhibit cell death (17) and prolong anagen phase which result from the effect on potassium channel opener (18). Whatever, the efficacy of minoxidil is temporary and it shows side effects such as irritate scalp, itchiness, redness including hypertrichosis.

The oral finasteride increases sexual side effects such as libido, abnormal erection including gynecomastia that has a great impact on a patient's quality of life and lead to the termination of these treatment. The limtitation of hair transplantation are expensive cost and invasive treatment (9, 19). Thus, a search for new substances to prevent hair loss or stimulate hair growth in AGA is necessary. Natural compounds are of interest due to their potential source for drug developments. *Avicennia marina* (AM) is a species of mangrove trees belonging to the family Avicenniaceae. It has been used as traditional medicine in Egypt to cure skin diseases (20). In previous studies, it has been shown to have many biological activities such as antimicrobial (21, 22) antioxidant and antitumor (23-25). AM has been reported to be the source of many compounds such as terpenoids, anthraquinones, steroids, naphthalene, flavones, glucosides, xanthones (20, 26) which are present in different plant parts (barks, leaves, twigs, etc.) including its endophytes.

Recent studies have shown that the crude extract of AM has inhibitory activity on 5α-reductase type1 (5α-R1). They used the preparative TLC technique to fractionate the AM extract into fractions. Then, they found the target compound that exhibited similar 5α-R1 inhibitory activity in AM extract as avicequinone C (27). However, they did not investigate the possible effect of AM extract and its avicequinone C on hair growth. The aim of this study is to investigate the effect of AM extract and avicequinone C on the expression of growth factors: IGF-1, FGF-7, HGF, VEGF that involve in hair growth. Moreover, the effect of AM and avicequinone C on the nuclear translocation of androgen receptor will also be carried out to confirm the possible mechanism.

Conceptual framework

According to one of the pathogenesis in AGA, is genetically predisposed coupled with increasing the production of 5α-DHT which converses from T by 5α-R activity. In addition, androgen hormone also changes the expression of some growth factors that control hair growth. and result in premature termination of anagen (growth phase) associated with premature entry into catagen (resting phase) (28). And would be changed the vellus hair to terminal hair, resulting in scalp hair become hair loss. Thus, this study hypothesized that treatment with the *Avicennia marina* extract and avicequinone C as a 5α-reductase inhibitor might modulate the expression of some growth factors which play important role to promote hair growth or decrease premature hair loss (29-32). Furthermore, AM and AC maybe inhibit the translocation of androgen-receptor complex to the nucleus. That are believed to protect the effect of androgen on responsible for growth factor genes produced by the DPCs. And affects to the other cells such as epithelial cells, melanocytes via a paracrine pathway.

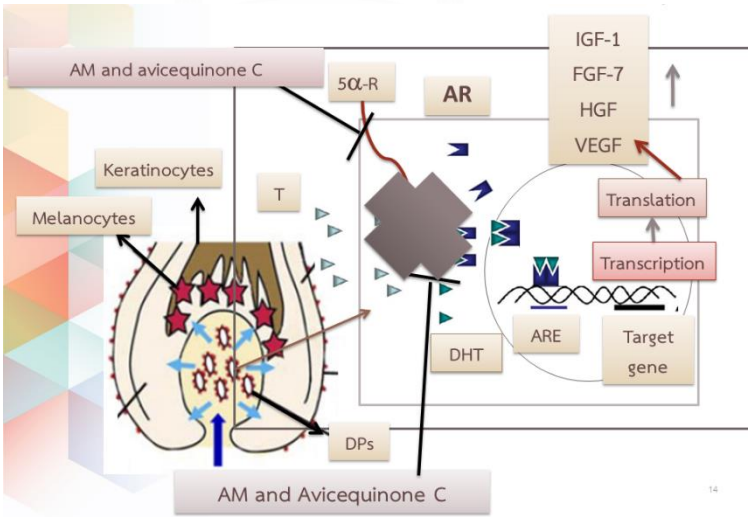

Figure 1. Conceptual framework of 5α-reductase inhibitor of the AM and AC in DPCs

Objectives

1. To investigate the 5α-reductase inhibiotory activity of *Avicennia marina* extract and avicequinone C on human dermal papilla cells isolated from androgenic alopecia patients.
2. To investigate the effect of *Avicenna marina* extract and avicequinone C on the expression of growth factors: IGF-1, FGF-7, HGF, and VEGF in human dermal papilla cells isolated from androgenic alopecia patients.
3. To investigate the effect of *Avicennia marina* extract and avicequinone C on the translocation of androgen receptor in human dermal papilla cells isolated from androgenic alopecia patients.

Scope of study

In this study, The effect of AM extract and its constituents, AC on the 5α-reductase inhibiotory were invesigated by using both of isolated human dermal papilla cells-based assay and human keratinocytes cells-base assay couple with HPTLC. To determine the expression of growth factor HGF, VEGF, IGF-1 and FGF-7 were determine by RT-PCR. The inhibition of the translocation of AR of isolated human dermal papilla cells was performed by immunocytochemistry analysis.

Contribution of the study

1. Obtain the Information about mechanism of AM and AC on the expression of growth factors such as IGF-1, FGF-7, HGF, VEGF including the inhibition translocation of androgen receptor in isolated DPCs from patients.
2. Information for supporting the AM and AC to develop as alternative products for treating AGA.

CHAPTER II
LITERATURE REVIEW

Introduction of hair follicle development

Hair follicle (HF) is skin appendages which have the most distinct function. It produces hair shaft and serves other many functions, such as thermal insulation, sensory perception, skin protection, especially its important roles in sex and social communication in human (2, 33). The development of hair follicle begins at the end of third gestational month as result of interaction between epithelial-mesenchymal compartments. This interaction plays a vital role in controlling hair follicle morphogenesis via signaling molecules (29, 30, 32, 34). The first signal from dermal fibroblasts, which are below the epidermis, induces the epidermal keratinocytes to form hair placode, and then the second signal from the epidermal placode acts on dermal fibroblast condensation to form a cluster which develops further into dermal papilla. Dermal papilla initiates the next signal to induce the descending growth of epidermal placode into the dermis, resulting in formation of the hair peg. Thereafter, the dermal papilla that is now located in the cavity become incorporated into the epithelial hair bulb. The epidermal keratinocytes that rapidly proliferate and differentiate into specific cell population, then form the structure of hair follicle such as hair shaft, inner root sheath which seperates the hair shaft from outer root sheath and appearance of epithelium bulges are along the external wall of outer root sheath. At around lasting stage of development, specific epithelial cells form a sebaceous gland that secrete sebum to coat the hair follicle including melanocytes that derived from neural crest migrate into HF. Then, the melanocytes differentiate and produce melanin. The formed melanin is transferred to the hair shaft where it determines the color of HF. The developing HF continues to both down-growth deeper into the skin until its proximal is located within subcutis and hair shaft moves up until it comes out free from the skin surface which eventually becomes the fully developed HF. The matured HFs that have similar anatomy of anagen hair follicle in adult HF will entry to hair growth cycle after finishing their developmental period (1, 2, 29, 35).

Hair growth cycle.

After its complete development, the HF undergoes the cycles of hair growth during postnatal life for maintaining and re-producing the new hair. The hair growth cycle is typically divided into three main phases. Anagen phase; an active growth and hair shaft production, catagen phase, apoptosis-driven regression period and telogen phase or resting phase shown in **Figure 2** (36). The first cycle begins after fully-formed hair shaft produced in anagen phase and entry to the catagen phase. In the beginning of catagen, proliferation and differentiation of epidermal keratinocytes are reduced and melanocytes stop activity to produce the pigment. While the DPCs are being resistant to apoptosis and out from the hair bulb thereafter to move up to reside near the bulge where are hair follicle stem-cell niche but still contact with epidermal compartment for epithelial-mesenchymal interaction. This is critical for continuing the cycling. The mechanism of HF regression is driven by apoptosis and involves cell remodeling event. Transition of anagen to catagen is associated with down-regulation of factors that promote cell proliferation in anagen phase such as IGF-1, FGF-7, HGF, VEGF (2, 30). In contrast, the catagen onset is induced by the up-regulation of some growth factors such as fibroblast growth factor-5 (FGF-5), transforming growth factor-β (TGF-β) and epidermal growth factor (EGF). In addition, previous studies have shown that apoptosis of follicular keratinocytes is induced by TGF-β_1 which is secreted via androgen stimulation (2, 14). In this phase, the HF length is reduce around 70% and last for 1-2 weeks before entering to the rest phase called telogen. The telogen phase or resting phase that are no activity, no molecular markers of the telogen follicle have been identified and morphological changing are still not clear. The HF becomes lack of melanin and short in length and is in the dermis, epithelial cells are above of tight cluster of papilla fibroblasts. However, it remains vascularization and contains all the cell types that are needed to produce fully HF in the next anagen phase. During this period, there are HF stem cells reside in the bulge that essential for telogen-anagen transition. The stem cells in the bulge are activated after activation of the hair germ stem cells (31, 34). Furthermore, sonic hedgehog (Shh) and noggin are up-regulated for induction of anagen phase. The hair follicle remains in this phase for

approximately three months until they receive a signal that initiates the new growth/anagen phase.

The new anagen begins with the hair bulb emerging from the secondary germ at the base of the telogen HF. It involves the complete re-growth or regeneration of the lower or cycling portion of HF. There are some signals that are sent by dermal papilla to the epithelial stem cells which may lead to initiate and regulate of the proliferation of the lower portion of hair follicle such as hair bulb keratinocytes and melanocytes in outer root sheath. During this anagen development, the morphological changes might be observed during hair follicle morphogenesis. There are many growth factors that up-regulated in this period such as IGF-1, HGF, FGF-7, and VEGF within DPCs and play important role as paracrine factors on the epithelial cell and melanocyte. The anagen phase last for 3-8 years and it has an effect on the HF length of stay in this phase. This means the normal scalp follicles are in anagen (80–85%), with the rest either in catagen (2%) or in telogen (10–15%) (30).

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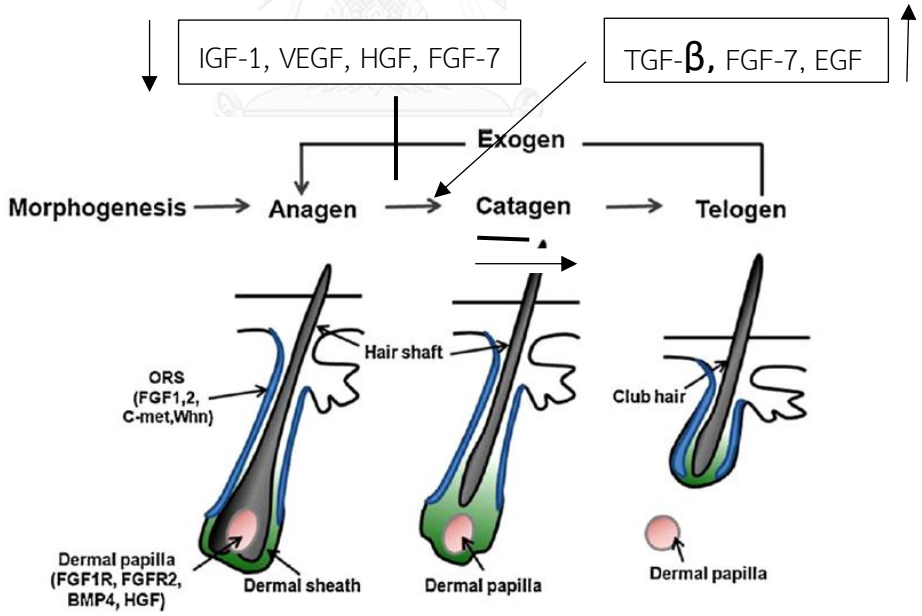


Figure 2. Schematic illustration of the hair cycles and some of growth factors and cytokines that regulate hair follicle transition between distinct hair cycle stages. down-regulation or up-regulation of different cytokines or growth factor driven the transition from active growth (anagen) phase into involution (catagen) phase (36).

Androgenic Alopecia

Androgenic alopecia (AGA), or male pattern baldness (MAGA) in men or female-pattern hair loss in women (FPHL), is a progressive disorder that is the most common type of hair loss that affects 60-70% of population. Both MAGA and FPHL develop after puberty, with variable progression. However, there are a slightly difference between men and women in the hair loss pattern. The MAGA has been described by Hamilton and Norwood who have classified the stage of AGA progression in 8 stages (37). In men, hair loss start from bi-temporal of the frontal hairline followed by diffusion thinning over the vertex until two area join together. It then becomes visible the scalp hair loss region. In women, hair loss begins diffusion thinning over the frontal and central of scalp hair and use of different scale to classify the stage of progression. Sinclair and Ludwig scales are two scales that generally describe the stage. Sinclair scale consists of 5 grades but Ludwig scale that has been used traditional it consists only 3 grades (4, 38-42). However, both sex are caused by genetically predisposed coupled with increasing the production of 5α-DHT which converses from T by 5α-R activity and result in premature termination of anagen associated with premature entry into catagen. The HF in androgenic alopecia shows the distinct characteristic of miniaturization of hair shaft, that transform the large, thick pigment terminal hairs to small, fine, short and lightly pigmented vellus hair (43, 44).

Androgens in androgenic Alopecia

Androgen, a steroid hormone, is formed through the pathway of steroidogenesis. This androgen metabolism pathway begins after the cholesterol substrate is converted to pregnenolone, followed by α-hydroxylation to make a group of weak androgens; dehydroepiandrosterone (DHEA). DHEA can be converted to more potent androgens such as testosterone. At the first step, DHEA is converted to androstenedione by 3β-hydroxysteroid dehydrogenase (3β-HSD) and then a further step, androstenedione is converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD) (10, 11, 45). Testosterone is the main androgen in blood circulating which normally is present only 1-2% in free form. The others are bound to the sex hormone binding globulin (SHBG) 90% and 19% to albumin.

Only the free form is capable to enter to the target cells and exerts its androgenic action (45). It affects several functions in human skin such as sebaceous gland growth and differentiation, wound healing, including affecting on hair growth cycle (43, 46, 47). The mechanism of androgen action is predominantly through the androgen- receptor (AR) which is a member of the steroid hormone receptor family of ligand-activated nuclear transcription factors (48). In most target cells, including DPCs where the androgen bind to its receptor, testosterone can be metabolized to 5α-DHT by the 5α-reductase enzyme (49). Testosterone and 5α-DHT are only two major types of androgens that cause AGA (40, 50, 51). Both of them bind to the androgen receptor. However, it is different from each other on their affinity for the androgen receptor, 5α-DHT is five-fold more potent than testosterone. After the ligand-receptor forms complex, the complex is translocated into nucleus where it will bind to androgen response elements in the regions of androgen-regulated genes to initiate the signaling cascade in DPCs. The androgen-regulated genes are responsible for growth factor genes produced by the DPCsand affects to the other cells such as epithelial cells, melanocytes via a paracrine pathway (12, 52). Moreover, the previous studies have shown that 5α-DHT has an effect on alteration of some growth factors which are secreted by DPCs and involved in the regulation of hair growth cycle such as inhibiting insulin-like growth factor-I (IGF-1) production that affects hair growth, induces the expression of **TGF-**β_1 mRNA in balding DPCs to inhibit the epithelial-cell growth, and induces epithelial cell death by DKK-1 (13, 15, 46, 53, 54).

5α-reductase enzyme

The steroid-5-reductases (5α-R) family consist of 3 subfamilies; 5α-reductases type1 and 5α-reductases type2, 5α-reductases type3, but only 5α-R1 and 2 isozymes are NADPH-dependent, membrane associated (microsomal) enzymes. These enzymes are irreversibly reduce the delta 4,5 bond (double bond between carbons 4 and 5; $\Delta^{4,5}$) of C-19 and C-21 steroids substrate (10). The essential role of 5α-R in mammalian male are involved in the prostate and external genitalia development. It also causes of some skin disorder such as overproduction of more potent 5α-DHT from testosterone is a pathology of AGA.

Biochemical Properties

The primary structures of human 5α-R isozymesare hydrophobic proteins. They are composed of 254-260 amino acids with predicted molecular weights of 28,000-29,000 Dalton. Furtermore, 5α-R1 shows a broad pH optimum. ranging between 6.0-8.5, It has a low substrate affinity for testosterone, (*Km;* 1–5 μM). On the other hand, 5α-R2 exhibits a narrow acidic pH optimum (pH 5–5.5) and a high substrate affinity for testosterone (*Km* ;0.004–1 μM). Therefore, under the same optimal conditions, 5α-R2 has a higher 5α-reducing activity than 5α-R1 (10, 49).

Tissue Distribution

The expression of 5α-R1 and 5α-R2 within human tissues are different. 5α-R1 is found in chest and back skin (nongenitai skin), liver, adrenal glands, sebaceous glands while 5α-R2 is found in the prostate, epididymis, seminal vesicle, genital skin, and slightly in liver. It has been shown that dermal papilla cells of balding scalp contains more 5α-R2 than the occipital area (10, 45, 55)**.**

Dermal papilla cell (DPCs)

The hair follicle contains both of epidermal (epithelial) and dermal (mesenchymal) compartment (34, 52). effective cross-talk between these two compartment is to be key for hair reconstitution and hair growth (29). Dermal part of hair follicle could be divided into two compartments, the dermal papilla (DP) and Dermal sheath (DS) (**Figure 2**.) (56, 57). A cluster of cells in DP is dermal papilla cells (DPCS) which are specialized fibroblast of mesenchymal origin. DPCs are located in the bottom of hair follicle. It is an essential for hair induction, hair follicle regeneration, hair growth and form a particularly sized and pigmented hair shaft. DPCs is to be a source of various cytokines and growth factors that involve in the regulation of the hair growth and melanogenesis. DPCs may be directly influence the surrounding cells by releasing some factors. These factors include insulin-like growth factor -1 (IGF-1), found in DPCs and serve as morphogen in hair growth (37, 48, 49). Hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) that involve in angiogenesis (58, 59). In the previous study of Goldman and colleagues demonstrated that the

expression of VEGF in human alopecia follicles significantly decreased comparing to the normal follicles (60). Furthermore, minoxidil which is an approved pharmaceutical drug to treat AGA could promote hair growth via increasing the expression of VEGF (61). Fibroblast growth factor-7 [FGF-7], it is also one of growth factors that effect on hair follicle growth and differentiation. Furthermore, previous study has been shown that FGF-7 inhibit the transition to catagen phase (62, 63). The DPCs are not only essential for hair growth and function, but are also a primary direct target site of androgen action (45). Androgen do cause such as alteration some cytokines and growth factors resulting in reduction of hair diameter, growth rate, length, pigmentation (12, 64). Furthermore, DPCs are reservoir of cells with the potential to differentiate into a range of cell types that are of potential therapeutic importance (65, 66). There are many studies to use DPCs as a model for studying of their role in hair growth including as a cell-based assay for searching new products to treat hair loss (67).

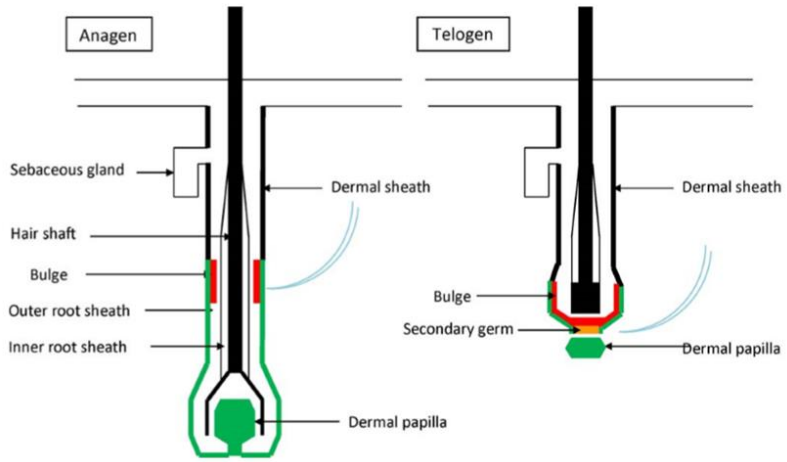


Figure 3. The Schematic illustration of a hair follicle. The hair follicle dermal papilla cells (green) are located in the bottom of hair follicle. The dermal sheath (black) reside above the DPCs where the hair follicle stem cells (red) are located. In telogen, the DPCs resides adjacent to the secondary germ (yellow).

Isolation and culture of DPCs

There are two methods widely used for isolation of DPCs, simple surgical microdissection and surgical microdissection with enzymatic digestion. However, isolation of DPCs are time consuming because it depends on manual microdissection skilled of the operator (68). The DPCs outgrowth from explant can be expected in five days to several weeks in culture medium. Many characteristics of cultured DPCs have been discovered. These includes the flattend, spread-out growth morphology, a low proliferative activity and tend to grow into multi-layered aggregates when culture DPCs become to be confluence. Which are differently from skin fibroblast as show in **Figure 4** (45, 65, 69).

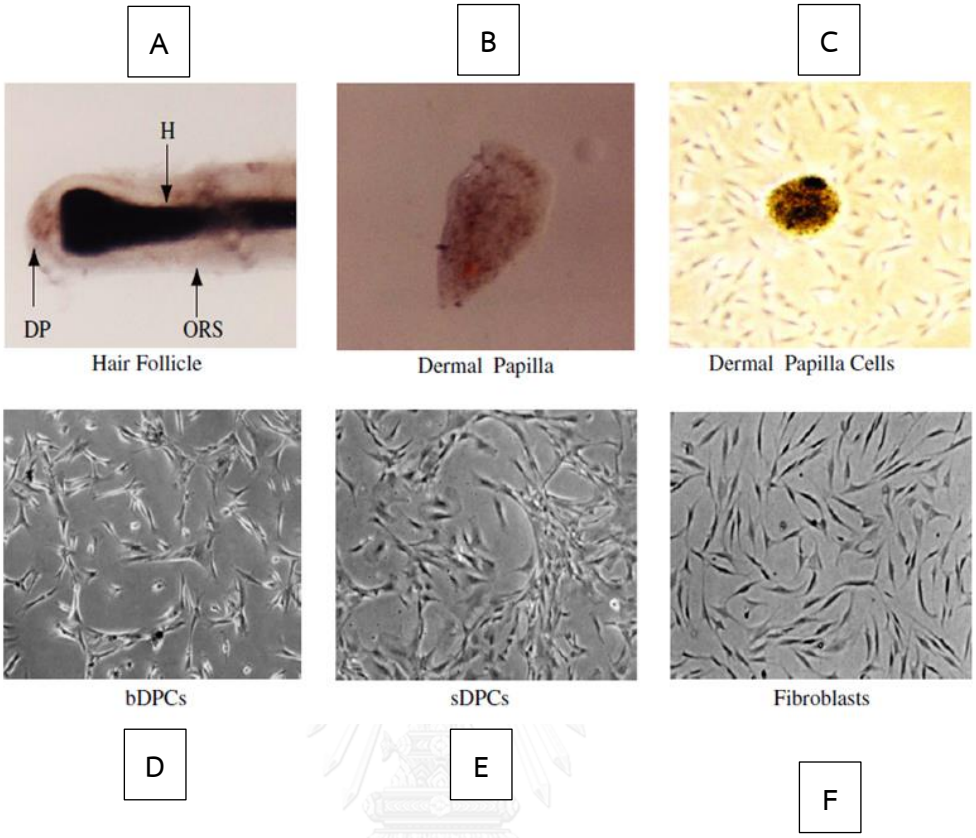


Figure 4. The picture show morphological characteristics of DPCs in literature. **A:** Intact hair follicle bulb with dermal papilla (DP), hair fiber (H) and outer root sheath (ORS). **B:** A pear shape of typical isolated follicular dermal papilla. **C:** The isolated papillae formed a clump of cells at the periphery of the outgrowth after 3 weeks of culturing. **D:** DPCs from beard, **E:** scalp and **F:** skin at the fourth passage (45).

Avicennia marina

Avicennia marina (AM) is a species of mangrove trees that is belonging to the family Avicenniaceae. Typically, the plant is widely distributed along tropical and subtropical coastlines. AM has been used as traditional medicine in Egypt to cure skin diseases (22) and also used as against fish stings, ringworms, sores, boils, skin ulcers and scabies. In the previous study, it has been shown to contain many biological activities such as antimicrobial antioxidant antitumor (21, 23-25). In the literature, AM has been reported to be a source of many compounds such as

terpenoids, anthraquinones, steroids, naphthalene, flavones, glucosides, xanthones (20, 26) which are present in the different parts (barks, leaves, twigs, etc.) including its endophytes (mainly endophytic fungi) .

Recently, Jain et al (27). found that the methanolic crude extract of AM from barks has inhibitory activity of 5α-reductase type 1 (5α-R1). The 5α-R1 which is one of the enzymes present in the steroidogenesis pathway converts testosterone to a much more potent androgen 5α-DHT. Overproduction of 5α-DHT causes androgen-dependent diseases such as benign prostate cancer, acne including androgenic alopecia. Therefore, they used the preparative TLC technique for activity-guided fractionation of the compound. Finally. they found that the active compound in AM is avicequinone C which is a furanonaphthaquinone type of structure.

CHAPTER III

MATERIALS AND METHODS

Cell cultures and equipment

- Cell culture hood: ESCO Class II BSC, streamline®, USA
- Centrifuge: Eppendorf 5417R, Germany
- Gel electrophoresis: Takara Mupid® - exU, Japan
- Gel documentation system: Bio-rad's Gel-Doc, USA
- Incubator: NUAIRE™ NU-8500, USA
- Inverted fluorescent microscope: Olympus IX51, USA
- Stereomicroscope: optica Co.,Ltd., Thailand
- Thin layer chromatography developing tank: CAMAG, Switzerland
- Thin layer chromatography dip tank: CAMAG Immersion device III, Switzerland
- Thin layer chromatography heater: CAMAG TLC PLATE HEATER III, Switzerland
- Thin layer chromatography image detector: CAMAG REPOSTAR 3, Switzerland
- Thin layer chromatography sample loader: CAMAG LINOMAT 5, Switzerland
- Thin layer chromatography scanner: CAMAG TLC SCANNER 3, Switzerland
- Polymerase chain reaction system: Bio-Rad MyCycler™ thermal cycler, USA
- Rotary Evaporator: Buchi Rotavapor R-210, Switzerland
- Spectrophotometer: Beckman Coulter® Multimode detector DTX880, USA
- Spectrophotometer: Evolution™ 600 UV-Vis Spectrophotometer, Thermo Fisher Scientific, USA
- Speed Vacuum: Savant Speed Vac SC-100, USA
- Weighing balance: Mettler Toledo Excellence Plus, Thailand

Materials

- Autopipette: BIOHIT, Finland
- Cell culture conical tubes, tips and flask: Corning, USA
- Cell culture dish 35 mm: Corning, USA
- Insulin needle: Terumo, USA
- Surgical ophthalmic blades: Alcon surgical ophthalamic knife® Novartis, USA
- Thin layer chromatography plates: Merck TLC Silica gel 60 F_{254}, Germany
- 6-well plate: Corning, USA
- 24-well plate: Corning, USA
- 96-well plate: Corning, USA

Enzymes, reagents and kits

- 4% paraformaldehyde: Sigma-Aldrich, USA
- 5α-DHT: Sigma-Aldrich, USA
- Acetic acid: RCI Labscan, Thailand
- Acetone: RCI Labscan, Thailand
- Acetonitrile: RCI Labscan, Thailand
- Agarose-LE: Affymetrix, USA
- Avicequinone C: we was obtained by chemical synthesis in our laboratory.
- Chloroform: Sigma-Aldrich, USA
- Dimethyl sulfoxide (DMSO): Amresco®, USA
- Dutasteride: BDG Synthesis, New Zealand
- Ethidium bromide: Invitrogen, USA
- Ethyl acetate: RCI Labscan, Thailand
- Finasteride: Sigma-Aldrich, USA
- Flutamide: Sigma-Aldrich, USA
- Isopropanol: Sigma-Aldrich, USA

- Methanol: RCI Labscan, Thailand
- Phosphoric acid: RCI Labscan, Thailand
- Testosterone: Sigma-Aldrich, USA
- Toluene: RCI Labscan, Thailand
- 2% (v/v) bovine serum albumin: Sigma-Aldrich, USA
- 0.25% Trypsin-EDTA: Invitrogen, USA
- 0.2% TritonX-100: Sigma-Aldrich, USA
- 6X DNA loading dye: Thermo Fisher Scientific, USA
- Antibiotic-antimycotic solution (100X): Invitrogen, USA
- Deoxyadenosine triphosphate (dATP): Fermentas, USA
- Deoxycytidine triphosphate (dCTP): Fermentas, USA
- Deoxyguanosine triphosphate (dGTP): Fermentas, USA
- Deoxythymidine triphosphate (dTTP): Fermentas, USA
- Dnase I enzyme: Fermentas, USA
- Fetal bovine serum (FBS): Invitrogen, USA
- First strand cDNA synthesis kit: Fermentas, USA
- GeneRuler 1-kb DNA ladder: Thermo Fisher Scientific, USA
- Mesenchymal Stem Cell Growth Supplement: Science cell, USA
- Dulbecco's Modified Eagle Medium (DMEM): Thermo Fisher Scientific, USA
- Hanks' balanced salt: Thermo Fisher Scientific, USA
- PrestoBlue® cell viability reagent (10X): Invitrogen, USA
- phosphate buffer solution (PBS): Thermo Fisher Scientific, USA
- Platinum® *Taq* polymerase: Invitrogen, USA
- RMPI medium: Invitrogen, USA
- Monoclonal rabbit AR antibody: Cell signaling technology, USA
- Tris-acetate-EDTA (TAE) buffer (50X): Amresco®, USA
- Toluidine blue: Sigma-Aldrich, USA

- Trypan blue solution: Sigma-Aldrich, USA
- TRIzol reagent: Sigma-Aldrich, USA
- Williams' media E: Thermo Fisher Scientific, USA
- Secondary Alexa Flur488 labeled donkey anti-rabbit IgG antibody:
- Cell signaling technology, USA

Plant material

The heartwood extract of *Avicenna marina* (AM) was prepared according to the previous method used in our laboratory by Dr. Ruchy Jain (27). Practically, the heartwood part was ground into powder and then extracted by maceration using 100% methanol at room temperature for two days. The methanolic extract was then evaporated to dryness at 45 °C using a rotary evaporator and kept at -20 °C until being used. The extract samples were dissolved in dimethyl sulfoxide (DMSO) for preparing stock solution and kept at 4°C until being diluted for experiment testing.

Isolation of human hair follicle DPCs and cell culture establishment

The hair biopsy specimens were obtained from patients with AGA.
During hair transplantation surgery. The patients were informed and written consent. The protocol was approved from the Ethics Review Committee for Research Involving Human Research Subjects, Health Sciences Group, Chulalongkorn university.

The hair biopsy specimens were transferred into Williams' E serum-free medium with 1% antibiotic–antimycotic at 4 °C and then washed three times with Hanks' balanced salt solution. DPCs were isolated and cultured by the method described previously (68, 70, 71) with slightly modification using simple micro-dissection technique. Briefly, the DPCs from each hair follicle obtained by simple dissection at the level of the dermal papilla. Opthalmic surgical blade and insulin needle were used for isolating the part of dermal papilla that encapsulate with dermal sheath under the stereomicroscope. After the simple dissection, the isolated dermal papilla were cultured in a six-well plate using Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% mesenchymal stem cell growth factor, 10% FBS and 1% antibiotic–antimycotic (complete medium) at 37 °C under a humidified

atmosphere of 95% air and 5% CO2. The obtained primary cultures were left untouched for 15 days to 30 days or until the dermal papilla were attached and the first cell migration becomes apparent. Thereafter, the medium were changed every 3 days. Once they become confluent, the cells were subcultured by using 0.25% trypsin /EDTA. The dermal papilla in the third to ten passages were be used for experiments (72, 73).

Culturing of human keratinocyte cells (HaCat cells)

HaCat cells obtained from ATCC were cultured in DMEM containing 10% FBS, 1% antibiotic–antimycotic at 37 °C under a humidified atmosphere of 95% air and 5% CO2 in T-25 flask. The medium was changed every 2 days. The HaCat cells at passages between 50-80 were used in this study.

Characterization of isolated DPCs

Toluidine blue O-staining

DPCs have many characteristics in form of cultured cells. They contain and excrete acid and neutral mucopolysaccharides into extracellular matrix. When being stained with low pH toluidine blue, DPCs show deep blue. With increasing pH, they become purple color (72, 74). Therefore, we used these specific characteristics for identifying the isolated DPCs. The method for specific staining of DPCs was followed the one previously reported by Wujj, 2005. First, DPCs were grown in a 24-well plate and cultured for 24 hr and washed with cold PBS. The cells were then fixed with 4% paraformaldehyde for 30 minutes and rinsed three times with PBS. Then the cells were treated with 0.2%Triton-100 for 1 hr at room temperature and rinsed again with PBS, followed by staining steps of toluidine blue O (0.1% toluidine blue O, pH 1 and pH 8.0) for 1 hr

Transcription level

RT-PCR technique was used for checking the target genes that are expressed in DPCs and their involvement in the pathology of androgenic alopecia. These include 5α-reductase type1, 5α-reductase type2 and androgen receptor. Previous studies (55)

have shown that these genes are expressed in DPCs of scalp hair follicle (75). In this study, the forward and reverse primers used for gene amplification are listed in Table1.

Total RNA was extracted by using TRIzol reagent. First, the culture cells were washed with cold PBS and then, lysed by adding TRIzol reagent and resuspending to obtain cell lysates by pipetting several times. The lysate was transferred to an eppendorf tube and chloroform was added to the isolate RNA in the aqueous phase. Before the sample was centrifuged at 13,000 rpm, 4°C 15 min, the tube was shaken and left standing at room temperature for 5 min. After centrifugation, the solution in the tube was separated into 3 phases, the upper aqueous phase that contains the RNA fraction, the interphase and the lower phenol-chloroform phase. The RNA fraction was collected and precipitated by isopropanol for 10 min at room temperature. The RNA fraction was centrifuged again at 13,000 rpm, 4°C 10 min and form a gel-like pellet in the bottom of the tube. Thereafter, discarded the supernatant and washed the pellet 2 by using 75% ethanol then centrifuged at 13,000 rpm 4°C 5 min, and then opened the tube to dry the RNA pellet until almost complete. Finally, the pellet was dissolved in RNase free water. The concentration of RNA was measured with a spectrophotometer at OD260 and OD280. The ration of OD260/280 values was calculated for checking the purity of the RNA extract. cDNA synthesis was then performed by using reverse transcriptase enzyme that according to the manufacturer's protocol (26), One microgram of total RNA was subjected to reverse transcription (RT) reaction using random oligonucleotide primers and reverse transcriptase. RT reaction product was then amplified by PCR using Taq DNA polymerase under the optimize conditions. The PCR reaction comprised 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPmix, 0.4 µM each of forward (F) and reverse (R) primer and 2.5 unit platinum® Taq polymerase. The PCR -thermal profile is shown in Table 2. The PCR products were mixed with 6x DNA loading dye and run on 1% agarose gel electrophoresis in 1X TAE buffer at 100V, with 1-kb DNA ladder. Then, the agarose gel was stained with ethidium bromide, de-stained with water and visualized under UV transilluminator using gel documentation system.

Table 1. Forward and reverse primers and the expected sizes of 5α-reductase enzymes, androgen receptor and β-actin.

Name	Primer pair	Expected size (bp)
5α-reductase type 1 Genbank: NM_001047.2	F: 5' CTGATGCGAGGAGGAAAGCCTATGC3' R: 5' GTCCAGATGCCTTTGCCTCACCTTG 3	1025
5α-reductase type 2 Genbank: NM_000348.3	F: 5' TGCCTTCTGCACTGGAAATGGAGTC3' R: 5' GGAGTGGGTTTGCTCTGGGTCTTTG3'	801
Androgen receptor Genbank: NM_000044.3	F: 5' CGTGCGCGAAGTGATCCAGAA 3' R: 5' TGCGCTGTCGTCTAGCAGAGAA 3'	811
β-actin (internal control) Genbank: NM_001101.3	F: 5' ATGATGATATCGCCGCGCTC 3' R: 5' GCGCTCGGTGAGGATCTTCA 3'	584

Table 2. The PCR -thermal profile for amplifying 5α-reductase enzymes type 1, and type 2, androgen receptor and β-actin gene.

	Temp.	Time (minutes)	cycle
Innitial denature	92 °C	0.30	1
Denaturation	92 °C	0.30	1
Annealing	48 °C 50 °C 55 °C	0.30 0.30 0.30	10 10 15
Extension	72 °C	1.0	1*
Final extension	72 °C	5.0	1

*For 5α-1 gene, extension

Protein expression of androgen receptor (AR)

Immunofluorescence staining of AR was used for determining the AR expression in cytoplasm of DPCs (76). The method for detection was performed as described by Kim A.R et al, 2014. First, the DPCs were seeded onto chambered slides and cultured for cell attachment. After 24 hr, the cells were washed three times with ice-cold PBS and fixed with absolute methanol in PBS for 15 min at 20°C. After fixation, the cells were washed three times in ice-cold PBS and incubated with 2% (v/v) bovine serum albumin in PBS-T (PBS with 0.5% (v/v) TritonX-100, and nonionic detergent for 1 h to permeabilize the cells in order to allow antibodies to access to intra-organell antigens and to block nonspecific antiserum binding. Thereafter, the resultant was incubated with a monoclonal rabbit AR antibody diluted in blocking buffer for overnight at 4°C. After washing off the primary antibody with PBS-T, the secondary Alexa Flur488 labeled donkey anti-rabbit IgG antibody (1:600; Molecular Probes), diluted in blocking buffer, was incubated for 4 hr in the dark. After removing the secondary antibody, the cells were washed three times with PBS-T and nuclei were counterstained with 1 μg/ml Hoechst stain for 15 minutes. The cells were then imaged by using fluorescence spectroscopy.

Determination of cell viability

The optimal non-toxic concentration used in this study was determined by using PrestoBlue® reagent. Both of DPCs and HaCat cells were seeded at a cell density of 1 × 105 cells/ml onto 96-well plates (100 μl of 10,000 cells/well) and cultured for cell attachment for 24 hr. After that, the cells were separately by treating with various concentrations of 100 μl of AM or AC using 0.1% DMSO as a control. The concentration of AM in the range of 40, 20, 10, 5 and 2.5 μg/ml and of AC in the range of 40, 20, 10, 5 and 2.5 μM. Cell viability was measured after being incubated at 37°C for 48 hr. Then, the cells were incubated with PrestoBlue® in RPMI medium for 2 hr. If there were viable cells, PrestoBlue® would be changed from blue to a purple-pink color, which could be detected using

the spectrophotometer at 570 nm. The percentage of cell viability was calculated from following equation:

$$\% \text{ cell viability} = A_{sample} / A_{control} \times 100$$

A_{sample} = The absorbance of treated group

$A_{control}$ = The absorbance of control group

Determination of 5α-reductase inhibitory activity

The 5α-reductase inhibitory activity of AM and AC was carried out according to the previously described method (27). Briefly, The cells (DPCs and HaCat) were seeded at a cell density of 2× 105 cells/ml onto 6-well plates (1 ml, 200,000 cells/well). After 24 hr, the cells were separately treated with 1 ml of 30 μM testosterone and 1ml of 0.1% DMSO (internal control), 1 ml of 30 μM testosterone and 1 ml of 10 μg/ml AM and 10 μM of AC, including 2 ml of 0.1% DMSO (negative control). The cells were treated for 36 hr. Then, the cell culture medium was collected in conical tubes and the attached cells were tested for cell viability using the 1× PrestoBlue® reagent in RPMI medium. Testosterone and its product, 5α-DHT, were extracted from the cell culture medium by using an equal volume of ethyl acetate. The ethyl acetate fraction was dried, reconstituted with 20 μl of methanol and spotted on a TLC Silica gel 60 F_{254} aluminum plate. The TLC plate was developed using toluene : acetone : acetic acid at a ratio of 8:2:0.2 as the mobile phase. The developed TLC plate was then dipped in a solution of 42.5% phosphoric acid and heated at 120 ℃ for 20 min, for the visual detection of 5α-DHT at 366 nm using a TLC reprostar imager and the amount is quantified using an image analyzing program. The inhibitory activity is determined through the decrease in 5α-DHT production relative to the internal control .

For determine the half maximal inhibitory concentration (IC50) value of AM, HaCat cell were use as cell-based and seeded at a cell density of 2× 105 cells/ml onto 6-well plates (1 ml, 200,000 cells/well). Followed the protocol that mentioned above, the cells were separately treated with 1 ml of 30 μM testosterone and 1ml of 0.1% DMSO (internal control), 1ml of 30 μM testosterone and 1 ml of 40, 20, 10, 5 and 2.5 μg/ml AM including 2 ml of 0.1% DMSO (negative control).

Determination for gene expression of growth factors

The expression of all the target growth factors (IGF-1, HGF, VEGF, FGF-7) was determined by RT-PCR for transcriptional level. Before the analysis, DPCs were seeded in culture dish for 24 hr until they were attached in the bottom. The DPCs were treated with various concentrations of AM and AC for 6, 12, 24 and 48 hr. RT-PCR analysis for growth factor mRNA expression. Total RNA was extracted by using TRIzol reagent. First, the cultured cells were washed with cold PBS and then, lysed by adding TRIzol reagent and resuspended by pipetting several times using a micropipetter. The cell lysate was transferred to an eppendorf tube and chloroform was added to the isolate RNA in the aqueous phase. Before the sample was centrifuged at 13,000 rpm, 4°C 15 min, the tube was shaken and left standing at room temperature for 5 min. After centrifugation, the solution in the tube was separated into 3 phases, the upper aqueous phase that contains the RNA fraction, the interphase and the lower phenol-chloroform phase. The RNA fraction were collected and precipitated by isopropanol for 10 min at room temperature. The RNA fraction was centrifuged again at 13,000 rpm, 4°C 10 min and form a gel-like pellet in the bottom of the tube. Thereafter, the supernatant was discarded and the pellet was washed 2 times by using 75% ethanol followed by centrifuge at 13,000 rpm at 4°C for 5 min. The tube was open to dry the RNA pellet until almost complete. Finally, the pellet was dissolved in RNase free water. The concentration of RNA was measured with a spectrophotometer at $OD_{260.}$. Moreover, the preparation was determined for the ration of $OD_{260/280}$ values for checking the purity of the RNA extract.

cDNA synthesis were then performed by using reverse transcriptase enzyme according to the manufacturer's protocol , One microgram of total RNA was subjected to reverse transcription (RT) reaction using random oligonucleotide primers and reverse transcriptase. RT reaction product was then amplified by PCR using *Taq* DNA polymerase under the optimize conditions (the same protocol as the expression of target genes protocal). The PCR -thermal profile is shown in Table 4. The primer of all interesting growth factors were designed using the NCBI primer design tool from the full length mRNA sequence obtain from the NCBI GenBank, and were made to order at 1st Base Laboratories (Selangor, Malaysia).

The forward and reverse primers for amplifying are listed in Table 3. The PCR products were run on a 1% agarose gel electrophoresis and visualized by ethidium bromide staining, de-stained with water and visualized under UV trans-illuminator using gel documentation system.

Table 3. Forward and reverse primers and the expected sizes of growth factors and β-actin.

Name	Primer pair	Expected size bp
Insulin-like growth factors 1 (IGF-1) Genbank: NM_000618.3	F: 5' CTCCTCGCATCTCTTCTACC 3' R: 5' GTTTCCTGCACTCCCTCTAC 3'	361
Fibroblast growth factors-7 (FGF-7) Genbank: NM_002009.3	F: 5' ATGAACACCCGGAGCACTAC 3' R: 5'AAATCTCCCTGCTGGAACTG 3'	653
Hepatocyte growth factors (HGF) Genbank: NM_000601.4	F: 5'CAGAGGTACGCTACGAAGTC3' R: 5'GATGTGCCACTCGTAATAGG 3'	1320
Vascular endothelial growth Factore (VEGF) Genbank: AAA35789.1	F: 5'ACCCATGGCAGAAGGAGGAG 3' R: 5'CCTTGCAACGCGAGTCTGTG 3'	440
β-actin (internal control) Genbank: NM_001101.3	F: 5'ATGATGATATCGCCGCGCTC 3' 584 R: 5' GCGCTCGGTGAGGATCTTCA 3'	

Table 4. The PCR -thermal profile for amplifying growth factors gene and β-actin gene.

	Temp.	Time (minutes)	cycle
Innitial denature	92 °C	0.30	1
Denaturation	92 °C	0.30	1
Annealing	48 °C 50 °C 55 °C	0.30 0.30 0.30	10 10 15
Extension	72 °C	1.0	1*
Final extension	72 °C	5.0	1

*For HGF gene, extension time

Determination of the inhibitory effect on the translocation of androgen receptor complex

Androgen signaling is mediated by the androgen receptor (AR). Generally, AR is located in the cytoplasm bound to the heat shock protein (HSP) which stabilizes the AR. When the androgens (as ligand) bind to androgen receptor, it changes conformation followed by the complex of ligand-androgen translocate to the nucleus. So, this potential to be a target to inhibit the androgen activity (48, 77). In this study, the immunofluorescence staining of AR was used for determining the possible effect of AM and AC on inhibitory nuclear translocation of AR complex. The method for detection was performed as described by Kim A.R et al, 2014 (78). First, the DPCs were seeded onto 24 wells-plate and cultured for cell attachment for 24 hr. Then, the cells were treated with AM (10 or 20 μg/ml) or AC (10 μM) in the presence of 30μM T or 20nM 5α-DHT except the control group. The control group were added only with 30μM T or 20 nM 5α-DHT and used 20 nM flutamide (androgen receptor inhibitor) as a positive control. After the treatment, the cells were washed three times in ice-cold PBS and fixed with absolute methanol for 15 min at 20°C. After fixation, the cells were washed three times in ice-cold PBS

and incubated with 2% (v/v) bovine serum albumin in PBS-T (PBS with 0.5% TritonX-100, nonionic detergent) for 1 hr to permeabilization and blocking of the cells in order to allow antibody access to intra-organell antigens and blocking nonspecific antiserum binding. Thereafter, incubated with a monoclonal rabbit AR antibody diluted in blocking buffer, overnight at 4°C. After washing off the primary antibody with PBS-T, the secondary Alexa Flur488 labeled donkey anti-rabbit IgG antibody (1:600; Molecular Probes), diluted in blocking buffer, was incubated for 4 hr in the dark. After removing the secondary antibody, the cells were washed three times with PBS-T and nuclei were counterstained with 1 µg/ml Hoechst for 15 minutes. The cells were imaged by using fluorescence spectroscopy at room temperature.

Statistical analysis

All of the experiments were performed in triplicate, and the data was presented as the means ± SD. Statistical analysis will be performed using One-way ANOVA followed by Dunnett's post hoc mean comparison. A level of $P< 0.05$ was considered statistically significant. The SPSS program was used for statistical analysis.

CHAPTER IV
RESULTS AND DISCUSSION

Isolation of dermal papilla cells (DPCs) from AGA patients

Dermal papilla cells (DPCs) were isolated from the bulb of dissected hair follicle obtained from occipital scalp region of patient undergoing hair transplant by simple micro-dissection method (68, 70, 71). The DPC as membrane-bound, pear-shaped structures were isolated from intact hair- follicle as shown in **Figures 5A and 5B**. It was found that the membrane-bound DPC was attached very hard to cultured dish when freely floating. It needed to scratch into the of bottom cultured dish and leave it untouched until the first cells migration from explant were observed (65, 68, 72). The initial outgrowth of DPCs from the explant occurred around 14 days to a month. The cells migrated from the explant in form of spread-out growth or spoke-like pattern as shown in **Figure 5C.** The DPCs exhibited many characteristics that include flattened morphology, irregular spindle shape and their ability to form multi-layered aggregation at cell confluence as shown in **Figure 6** (69, 73, 79-82).

Characterization of isolated DPCs

Toluidine blue O-staining

Toluidine blue O staining was used for demonstration of metachromasia property of extracellular matrix (ECM) where DPCs embedded in (81). There are previous published studies showing that there are different basement-membrane components expressed by DPCs. These include acid and neutral mucopolysaccharides (glycosaminoglycans) which can be classified into sulfated acid mucopolysaccharides (chondroitin sulphate) and non-sulfated acid mucopolysaccharides (hyaluronic acid) (81, 83). It is essential for cellular differentiation or proliferation including to bind to growth factors.

The positive result of metachromasia is dependent on the present of acid groups of the mucopolysaccharide (74, 84). At acidic pH, pH≤2, only strong acid components, sulfated acid mucopolysaccharides such as chondroitin sulphate were showed metachromasia. At pH 3-8, non-sulfated acid mucopolysaccharides such as hyaluronic acid were showed metachromasia. Our results in **Figure 7.** show that the cultured DPCs displayed a positive to toluidine blue O staining which changed the color reaction when its pH value was increased from pH2 to pH8.

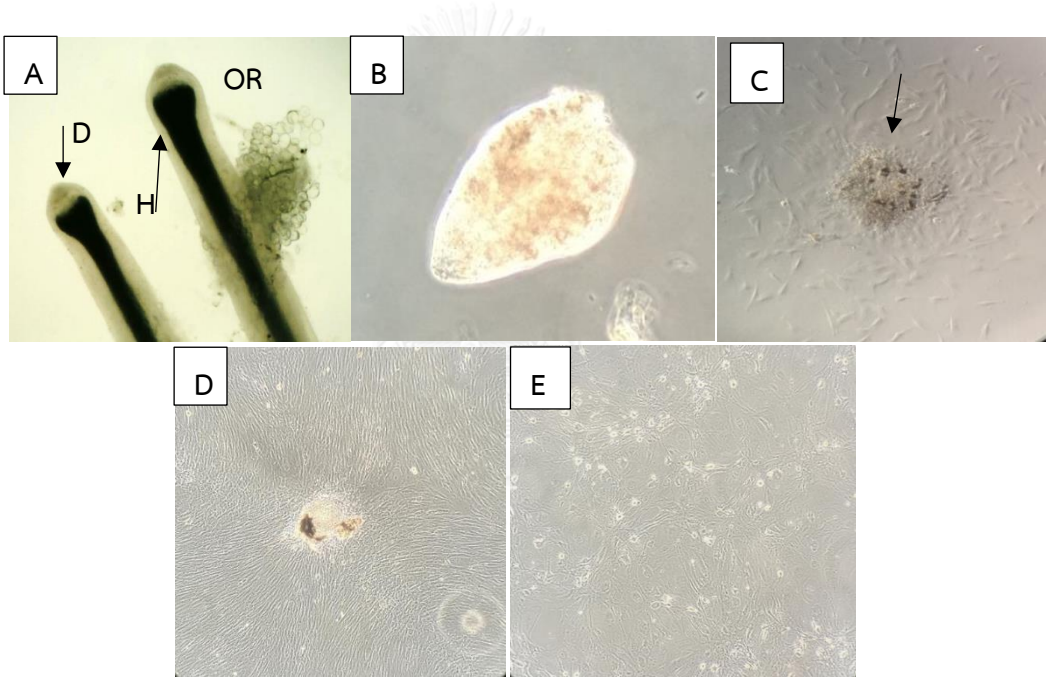


Figure 5. The morphological characteristics of the isolated DPCs.

A. Intact hair follicle bulb with dermal papilla cells (**DPCs**),
hair fiber (**H**) and outer root sheath (**ORS**).

B. A pear shape of typical membrane-bound DPCs.

C. The initial outgrowth of isolated DPCs after 3 weeks of culturing,
showing spoke-like out growth.

D. The passage 2 of isolated DPCs before cell confluence.

E. The isolated DPCs at cell confluence.

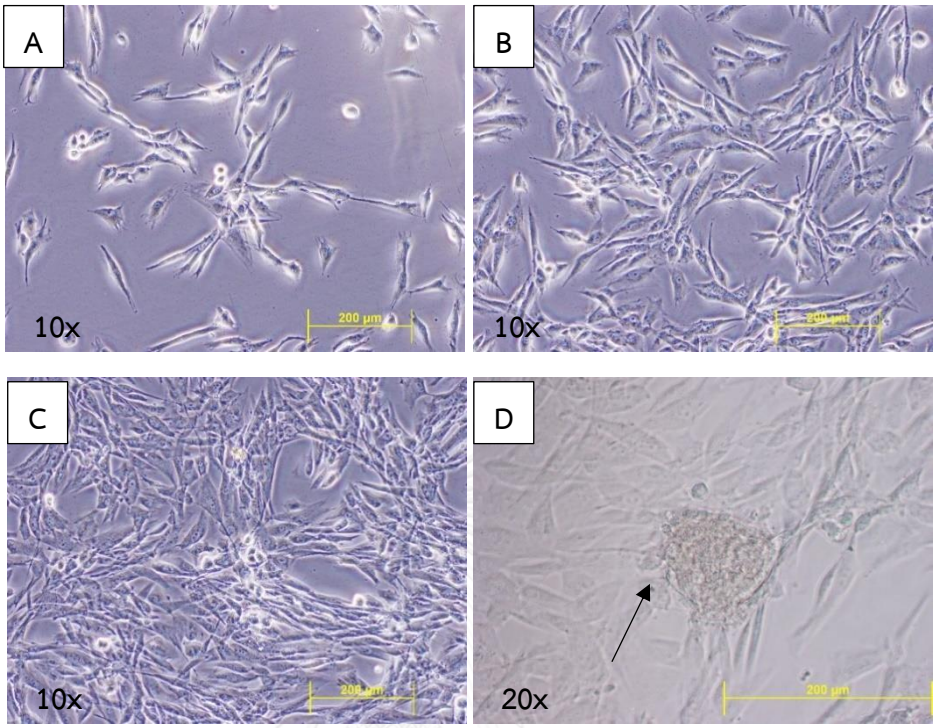


Figure 6. A. and B. The isolated DPCs at low cell density after culturing 1 and 2 days, show flatted and spindle-shape morphology. **C. and D.** As their number increases, the isolated DPCs formed into multi-layered aggregation before confluence.

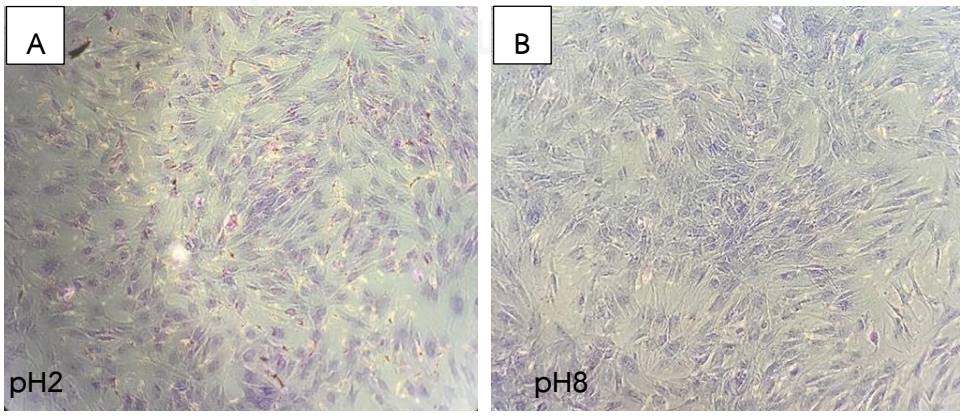


Figure 7. The DPCs were stained deep blue with toluidine blue O at pH2 and became purple when pH went up to 8.

Gene expression of 5α-R1, 5α-R2 and AR

It is well-known that androgens are the cause of hair loss in AGA. Their effect on AGA is exerted through the androgen receptor (AR) in the DPC (28, 46, 52) and the over-activity of 5α-reductase enzyme (5α-R). DPCs are believed to be only a direct site of androgen action where the androgen bind to its receptor and 5α-R could be exert its activity (45, 55, 75). In determining the effect on 5α-R and AR in DPCs, their expression levels in the cells were evaluated. RT-PCR was performed with specific primers for two isoforms of the enzyme, 5α-R1, 5α-R2, and AR. The result revealed that AR and 5α-R1, 5α-R2 were all expressed in the isolated DPCs. (**Figure 8.**)

The expression of all target genes were reported previously (55). Our results confirmed the suitability of DPCs as a cell-based model to investigate of the mechanism of AM and AC as 5α-R inhibitor for treating AGA.

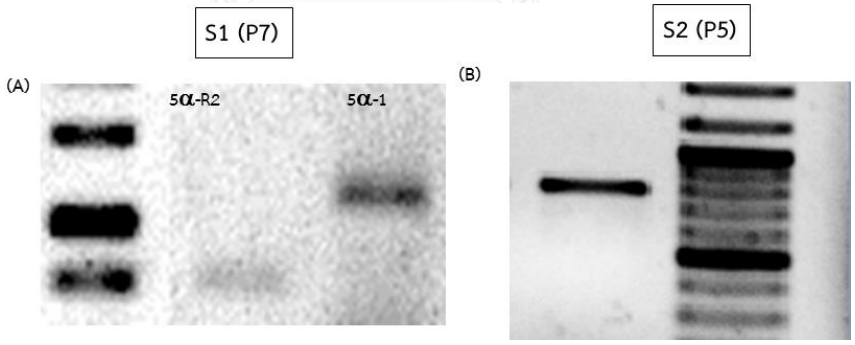


Figure 8. RT-PCR showing the expression of target gene. **(A)** The expression of 5α-R1**,** 1025 bp and 5α-R2, 801 bp in P7. **(B)** The expression of AR**,** 801 bp in P5.

Protein expression of androgen receptor (AR).

AR is a member of the steroid hormone receptor family of ligand-activated nuclear transcription factors. It is translocated into nucleus after forming of ligand-receptor complex and bind to androgen response elements in the nucleus to initiate the signaling cascade in the target cell (43). Therefore, we evaluated the protein-expression of AR by using immunocytochemical technique with AR antibody which displays in green fluorescence. The immunostaining showed that the DPCs expressed AR up to the translational level. Moreover, by using 20nM DHT as active androgen hormone to stimulate the nuclear translocation of AR for 12 h, the translocation of AR to the nucleus was detected. As shown in **Figure 9**, AR staining (green fluorescence) was detected at the same level in both the cytoplasm and nucleus under normal condition (**Figure 9A**). In contrast, the activated condition by DHT stimulation showed the AR staining mainly in nucleus (**Figure 9B**). These results confirmed that the androgen receptors in the DPCs are still functionable.

In summary, our results suggest that the isolated DPCs from occipital area could be used as a model for searching new substances to treat AGA. They showed properties that are still functional, such as expressing 5α-R1 and 5α-R2 including AR at both the transcriptional and translational levels. These are consistent with other publications that used isolated human dermal papilla cell as a model for investigation on hair growth (3, 66).

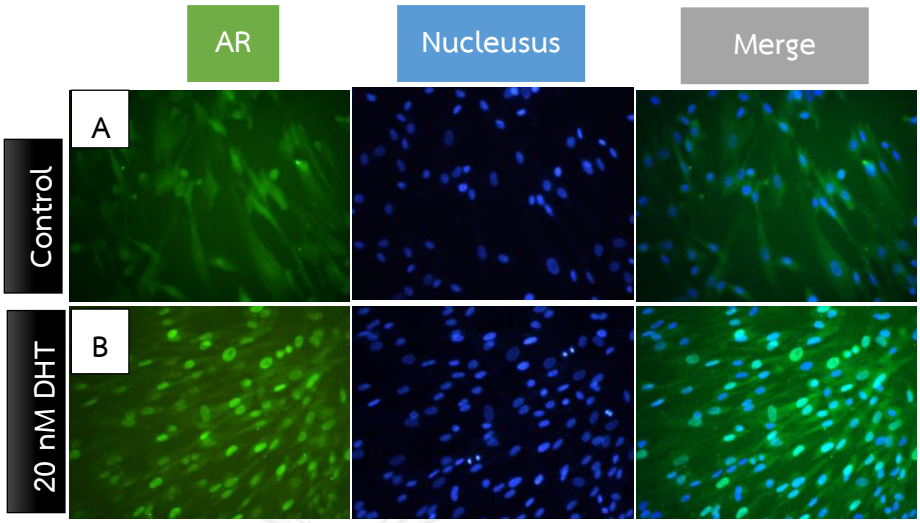


Figure 9. Androgen receptor (AR) expression. The isolated DPCs were immune-stained with AR antibody (green fluorescence) and counterstained with Hoechst nuclear staining (blue fluorescence). **A.** Cells were without treatment under normal condition (without 20nM DHT treatment), showed the same level intensity of AR staining in both cytoplasm and nucleus (top panel). **B.** Cells were treated with 20nM DHT for 12 hour, AR staining was more detected in nucleus when compared with cytoplasm (bottom panel).

Determination of cell viability

Before studying the effect of AM extract and AC compound, the optimal non-toxicity concentrations of AM and AC on DPCs and kearatinocyte (HaCat) cells were evaluated. The cell viability test was performed by using PrestoBlue® reagent. The cells were treated with various concentrations of AM and AC using 0.1% DMSO as a control. The concentration of AM ranging from 40, 20, 10, 5 and 2.5 µg/ml and 40, 20, 10, 5 and 2.5µM of AC. Cell viability were then measured after incubated at 37°C for 48 hr. The results of cell viability test on DPCs showed that the AM exhibited non-toxicity at the concentration less than 40 µg/ml ($p< 0.05$) (2.5,5,10 and 20 µg/ml) (**Figure 10.**) while AC exhibited more toxicity than AM Its non- toxicity of was observed at the concentration of less than 20 µM ($p< 0.05$) (2.5,5 and 10 µM) (**Figure 11.**),

In addition, the cytotoxicity test was also evaluated on HaCat cells. The results showed that the non-toxicity of AM on HaCat cells was observed at the concentration of less than 40 µg/ml ($p< 0.05$) which was also the same as that for DPCs (**Figure 12.**). AC also showed more toxicity than AM in HaCat cells, the non-toxicity of AC at concentration less than 20 µM ($p< 0.05$) (**Figure 13.**).

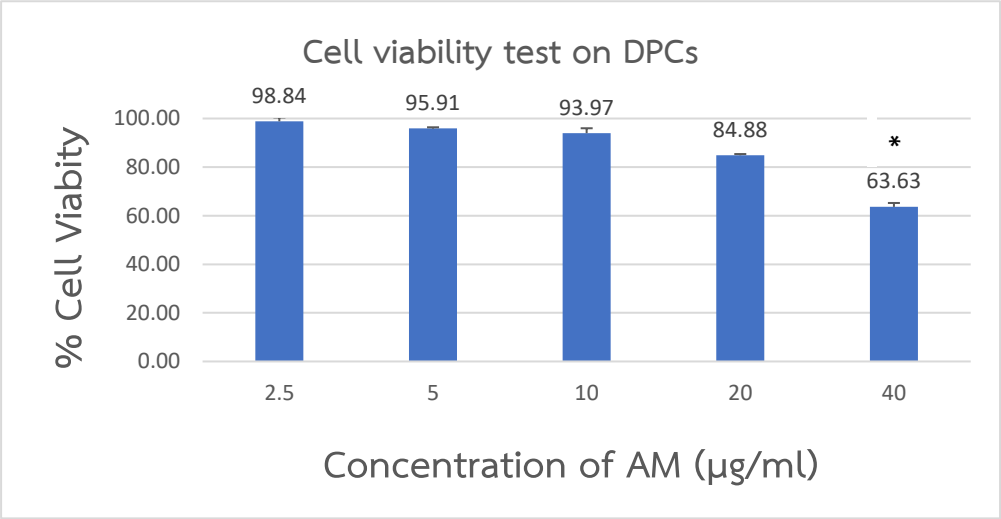


Figure 10. The cell viability test of AM on DPCs

* indicated the significant difference in at compared to other groups (p<0.05).

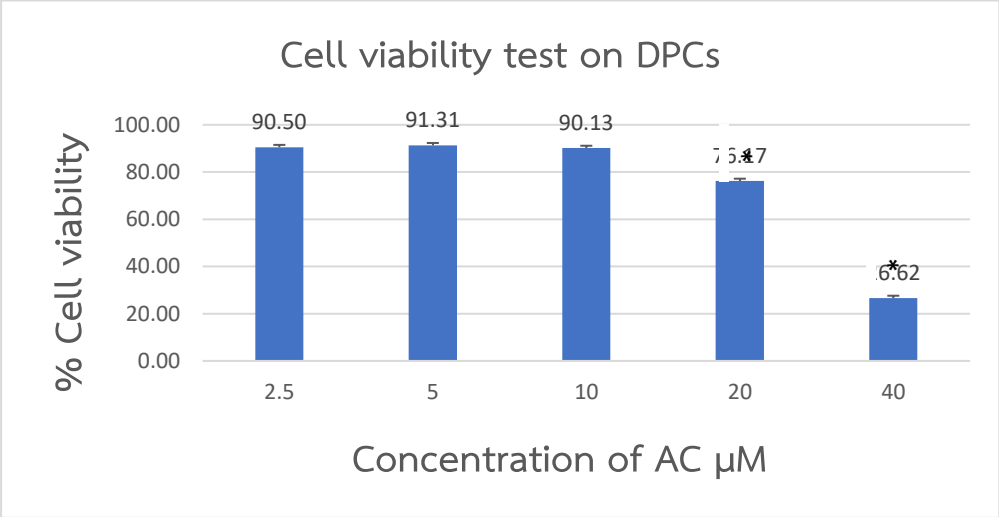


Figure 11. The cell viability test of AC on DPC

* indicated the significant difference in at compared to other groups (p<0.05)

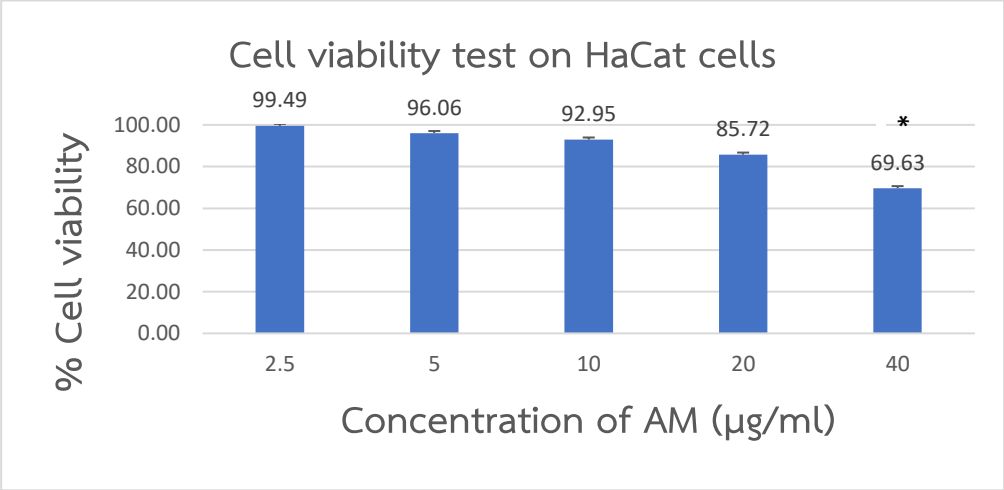


Figure 12. The cell viability test of AM on HaCat cells

* indicated the significant difference in at compared to other groups (p<0.05)

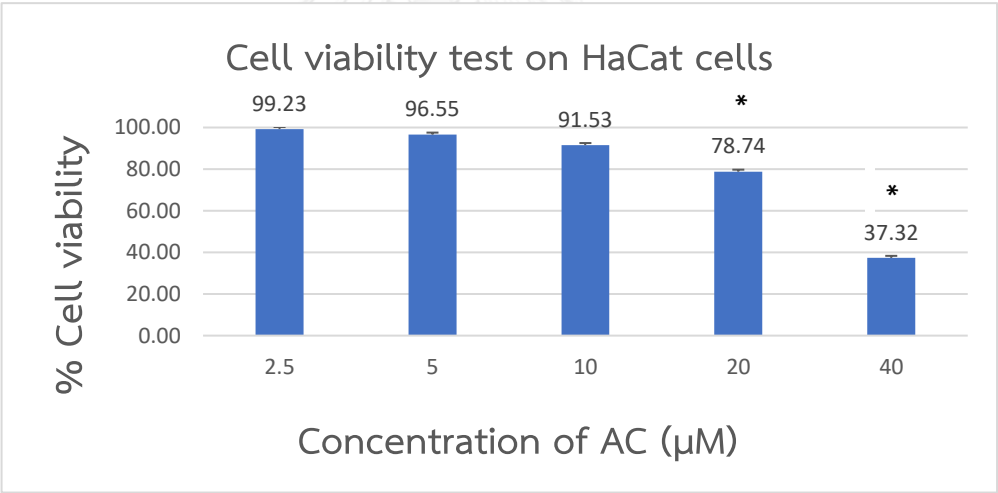


Figure 13. The cell viability test of AC on HaCat cells

* indicated the significant difference in at compared to other groups (p<0.05)

Determination of the 5α-reductase inhibitor activity

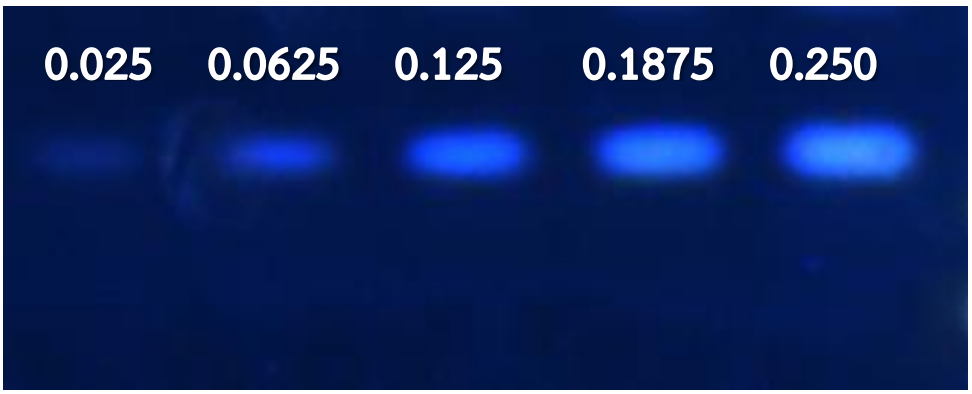
Based on the pathology of AGA, the over-production of 5α-DHT by 5α-R action is believed to be the cause (10, 75). In general, **5α**-R plays a major role in the reduction of testosterone (T) into a more potent androgen, 5α-dihydrotestosterone (5α-DHT). Many studies have shown that plant extracts or substances with anti-**5α**-R activity can promote hair growth (85-89). Thus, one of the targets for treating AGA is to reduce the activity of 5α-R enzyme. In this study, the 5α-R inhibitory activity was performed by using cell-based assay coupled with a non-radioactive TLC detection technique which was developed previously (27). In to visualizing the results of 5α-R inhibitory activity on TLC plate. Various concentrations of 5α-DHT were first spotted on a TLC plate. Then, quantitative analysis of the product 5α-DHT was examined by analyzing the resulting a images of developed and derivatized plate which was taken under 366 nm. The 5α-DHT band on the TLC plate was quantitated by a specific using image analysis program, (Image Processing and Analysis in Java or ImageJ software) which was conducted to obtain a standard curve as shown in **Figures 14A** and **14B.**

In terms of cell treatment, the isolated DPCs were treated seperated with AM and AC at their highest non-toxic concentrations and cell viability more than 85% (10μg/ml AM, 10μM AC) for 36 hr. As shown in **Figures 15A** and **15B,** each lane represents the inhibitory activity of each substance. The lane "4" is the internal control group representing the conversion of substrate T to product 5α-DHT, while the lane "Cell-T" was the negative control. For the results, AM at 10μg/ml (lane 1) showed 73.40 ± 8.40 % inhibition while AC at 10μM (lane 2) showed slightly lower with 67.71 ± 3.81 % inhibition (**Figure 15B**). Finasteride (Fi) which is a competitive inhibitor of 5α-R2 at 1μM was used as a positive control (90, 91), showing Fi 100% inhibition. These results confirmed that DPCs contained both type 1 and 2 of 5α-R, and AM and AC could inhibit the activity of 5α-R in DPCs. Moreover, HaCat cells which are part of hair follicle and hair shaft (92, 93) It was found that 5α-R1 was also expressed and the RT-PCR were also used for comparison results shown in **Figure 18.**, also revealed the expression of 5α-R1 in HaCat cells,

just like the isolated DPCs. By treating the HaCat with AM and AC under the same conditions, it was found that AM at 10μg/ml (lane 5) showed 74.52 ± 8.20 % inhibition while AC at 10μM (lane 3) showed only 41.12 ± 16.29 % inhibition (**Figure 16B**). Comparing between 10μg/ml AM and 10μM AC (which are a highest non-toxic concentrations), AM showed highest inhibitory activity on 5α-R1 with the IC_{50} as 2.28 μg/ml (**Figure 17**) in HaCat cells.

In summary, the crude extract AM and its active constituent AC could inhibit the 5α-R activity causing the reduction in 5α-DHT production by more than 50% in both DPCs and Hacat cells. Both cells play important roles to control hair growth through their interaction. DPCs could stimulate the proliferation and differentiation of keratinocyte cells (HaCat cells) by producing some of growth factors or cytokines while Hacat cells produce the major protein of hair or keratin that affect on length and thicken of hair follicle (94, 95).

(A)



(B)

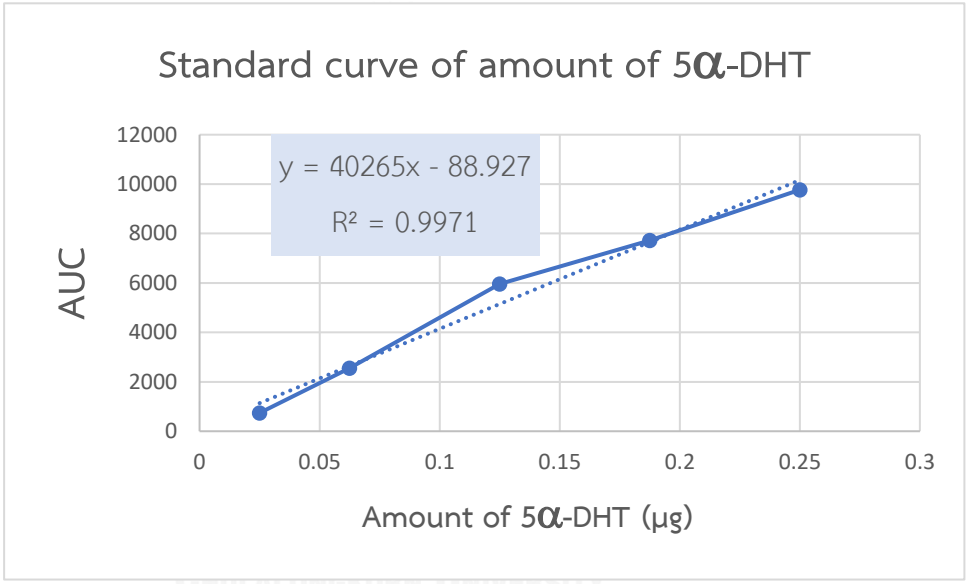
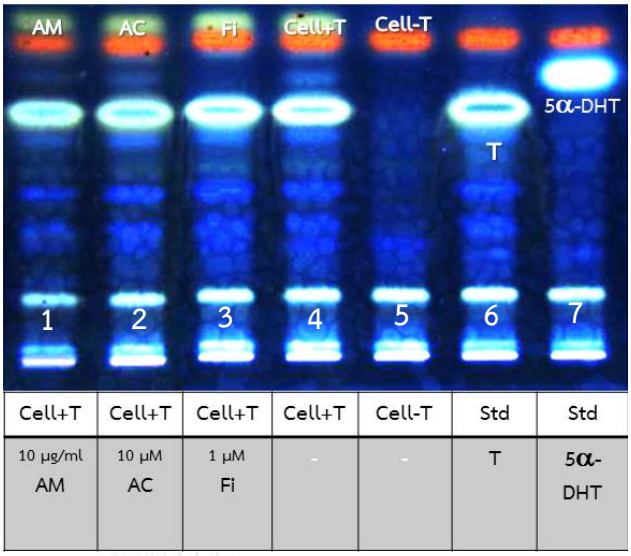


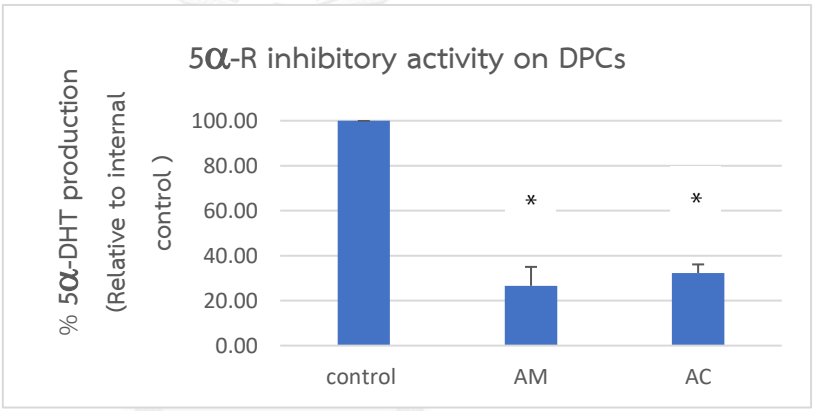
Figure 14. Standard curve of 5α-DHT, **A**. An image of developed and derivatized plate of 5α-DHT which was taken at 366 nm. **B.** Average standard curve of 5α-DHT was generated by using image analyzing program, (Image J software).

(A)



Cell+T	Cell+T	Cell+T	Cell+T	Cell-T	Std	Std
10 µg/ml AM	10 µM AC	1 µM Fi	-	-	T	5α-DHT

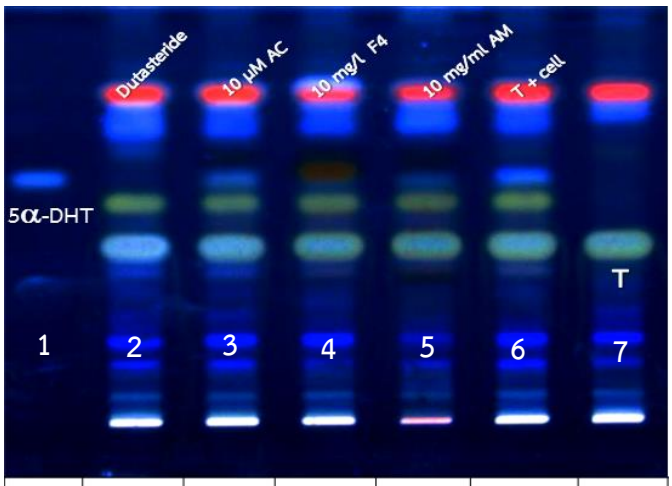
(B)



% Inhibition on 5α-R activity on DPCs (Relative to internal control)	
Control	100
AM (10 µg/ml)	73.40 ± 8.40
AC (10 µM; 2.56 µg/ml)	67.71 ± 3.81

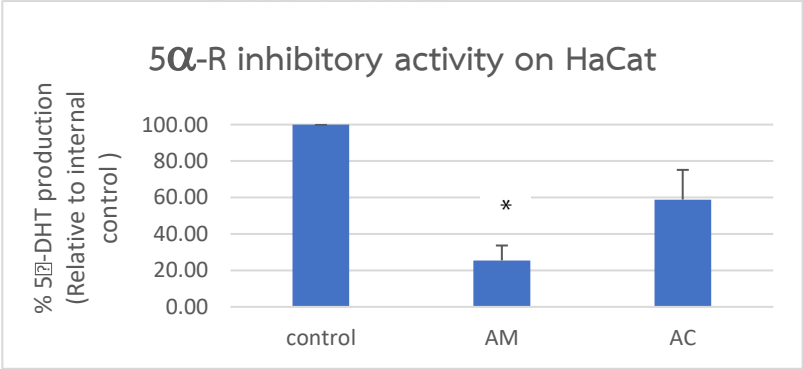
Figure 15. (A) A TLC plate showing 5α-R inhibitory activity of 10 µg/ml AM, 10 µM AC and 1µM finasteride (lane1,2,3 respectively) by isolated DPC cell-based assay using non-radioactive TLC detection technique. The internal control (cell without inhibitor) is shown in lane 4, standard DHT and T are shown in 6,7. The bar graph showed the % 5α-DHT production of AM and AC relative with internal. control. **(B)** The table showed % inhibition of 10µg/ml AM, 10µM AC in DPCs. * indicated the significant difference in at compared to control group ($p<0.05$).

(A)



Std	Cell+T	Cell+T	Cell+T	Cell+T	Cell+T	Std
5α-DHT	1 µM DU	10 µM AC	10 µg/ml F4	10 µg/ml AM	- Control	T

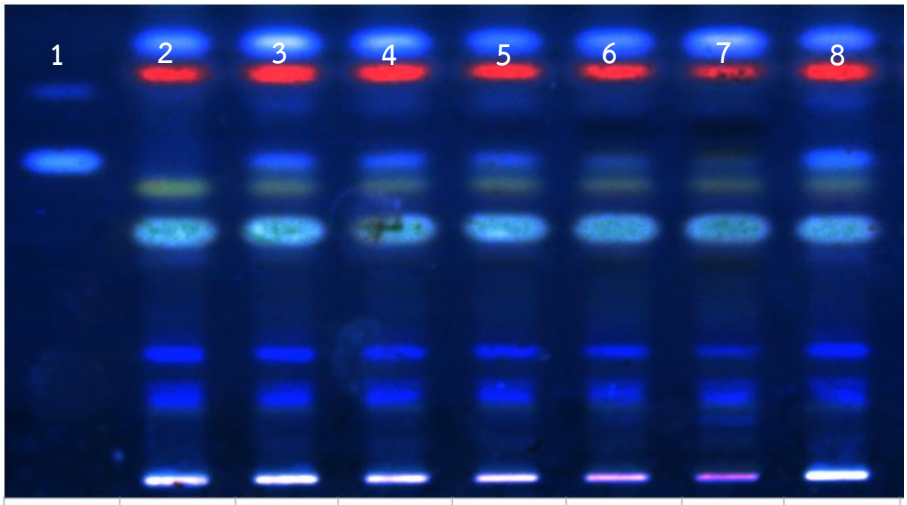
(B)



% Inhibition on 5α-R activity on HaCat (Relative to internal control)	
Control	100
AM (10 µg/ml)	74.52 ± 8.20
AC (10 µM; 2.56 µg/ml)	41.12 ± 16.29

Figure 16. (A) TLC plate showing 5α-R1 inhibitory activity of 10 µg/ml AM, 10 µM AC and 1µM dutasteride (lane 2,3,4 respectively) in HaCat cell-based assay using non-radioactive TLC detection technique. The internal control (cell without inhibitor) is shown in lane 6, standard DHT and T are shown in 1,7 respectively. The bar graph showed the % 5α-DHT production of AM and AC relative with internal control. **(B)** The table showed % inhibition of 10 µg/ml AM, 10 µM AC in HaCat. * indicated the significant difference in at compared to control group ($p<0.05$).

(A)



Std	Cell+T	Cell+T	Cell+T	Cell+T	Cell+T	Cell+T	Cell+T
5α-DHT	1 µM Du	1.25 µg/ml AM	2.5 µg/ml AM	5.0 µg/ml AM	10.0 µg/ml AM	20.0 µg/ml AM	30 µM T

(B)

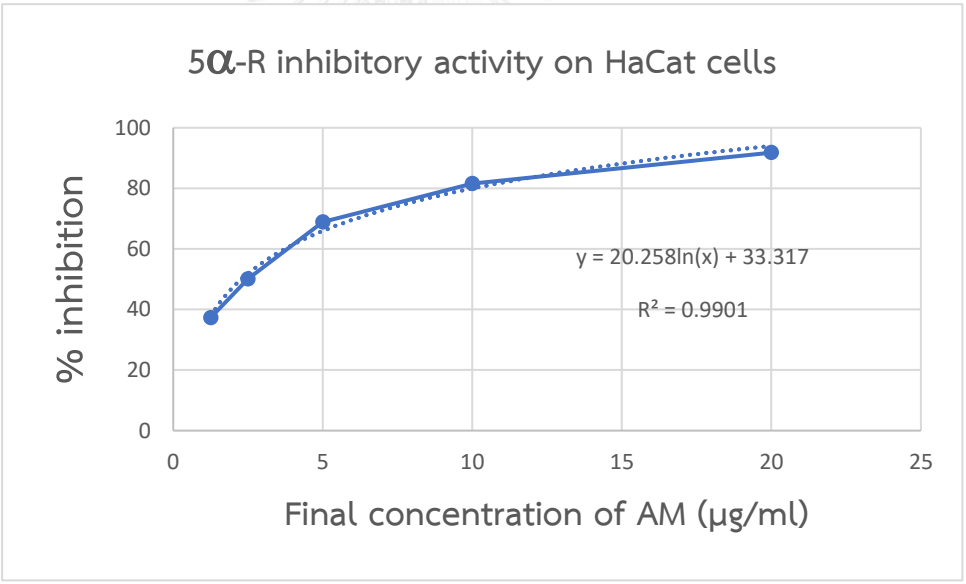


Figure 17. (A) TLC plate showing 5α-R1 inhibitory activity of AM at 20, 10, 5, 2.5, 1.25 µg/ml on 5α-R1. The internal control (cell without inhibitor) is shown in lane 8 and standard 5α-DHT is shown in lane 1. **(B)** IC_{50} of AM in HaCat cells.

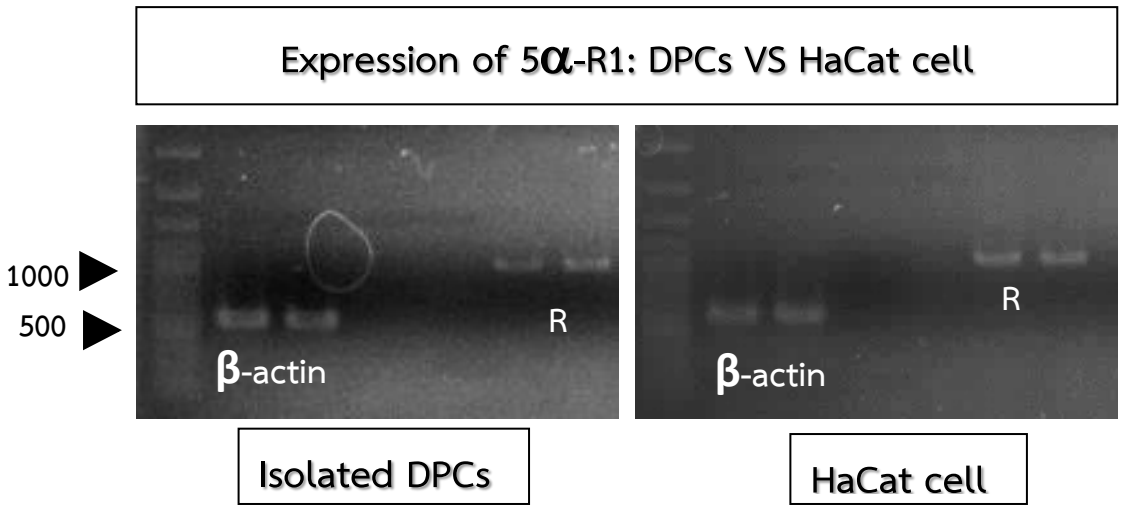


Figure 18. RT-PCR showing the expression of 5α-R1**,** 1025 bp and β-actin, 584 bp in isolated DPCs and HaCat cells.

Determination of the inhibitory effect on the translocation of AR

Because AGA is an androgen-mediated disorder in which the mechanism involves nuclear translocation of the AR upon binding of androgens that result in activation their activity. This results in the vellus transformation of scalp hair to change from the thicken scalp hair to thin scalp hair . Therfore, we examined whether AM and AC treatments inhibited nuclear translocation of the AR in DPCs after stimulation by T and 5α-DHT.

In this study, 30μM T and 20nM 5α-DHT were used as the active androgen to stimulate the translocation of AR and nonsteroidal antiandrogen drug flutamide as a positive control (Flutamide competes with testosterone and 5α-DHT for binding to the AR in target cell) (96). The immunostaining for AR are shown in **Figures 19, 20, 21 and 22.** The first row shows the green fluorescences staining the AR using Ab against AR, the second row; shows the blue fluorescence nuclear staining by Hoechst dye and the third row shows the merged image. Under normal conditions (non-stimulation of T and 5α-DHT), AR staining (green fluorescence) was detected the same level in both cytoplasm and nucleus as shown in **Figures 19A., 20A., 21A. and 22A.** The experiment was divided into two groups. First, we examined the effects of AM and AC on nuclear translocation of the AR in DPCs after stimulation by T.

The second is 5α-DHT stimulation group. Both T and 5α-DHT stimulated DPCs lead to nuclear translocation of the AR from the cytoplasm, as observed by the intensity of green fluorescences in nucleus more than cytoplasm as in **Figures 19B., 20B., 21B., and 22B.** The results revealed that in both groups, 10 μg/ml AM and 10 μM AC could decrease nuclear translocation of the AR stimulated by T and 5α-DHT (as evidenced by the merging of green and blue fluorescences at the nucleus) (**Figures 18C., 19C., 20C., and 21C.**) as much as that of 10 μM flutamide (positive control) in DPCs (Figure **18E., 19D., 20E., and 21D.**). In addition, we increased the concentration of AM to 20μg/ml, the results showed more lower the level of intensity of green fluorescence in nuclease when compared with 10 μg/ml AM (**Figure 18D., 20D.**). However, we observed that the nuclear inhibition effect of AM and AC on DPCs stimulated with 20nM 5α-DHT lower than the inhibition on DPCs stimulated with 10 μM T.

In summary, these results suggested that both AM and AC could lower the effect of T and 5α-DHT in DPCs by reducing the nuclear translocation of AR. These finding was in agreement with the previous studies. Kim AR. et al, and Ishikura N. et al (77, 78), have reported that inhibition of nuclear translocation of AR from cytoplasm into nucleus could decrease the androgens activity. Furthermore, the inhibition of androgen activity is a potential method to have preventive AGA.

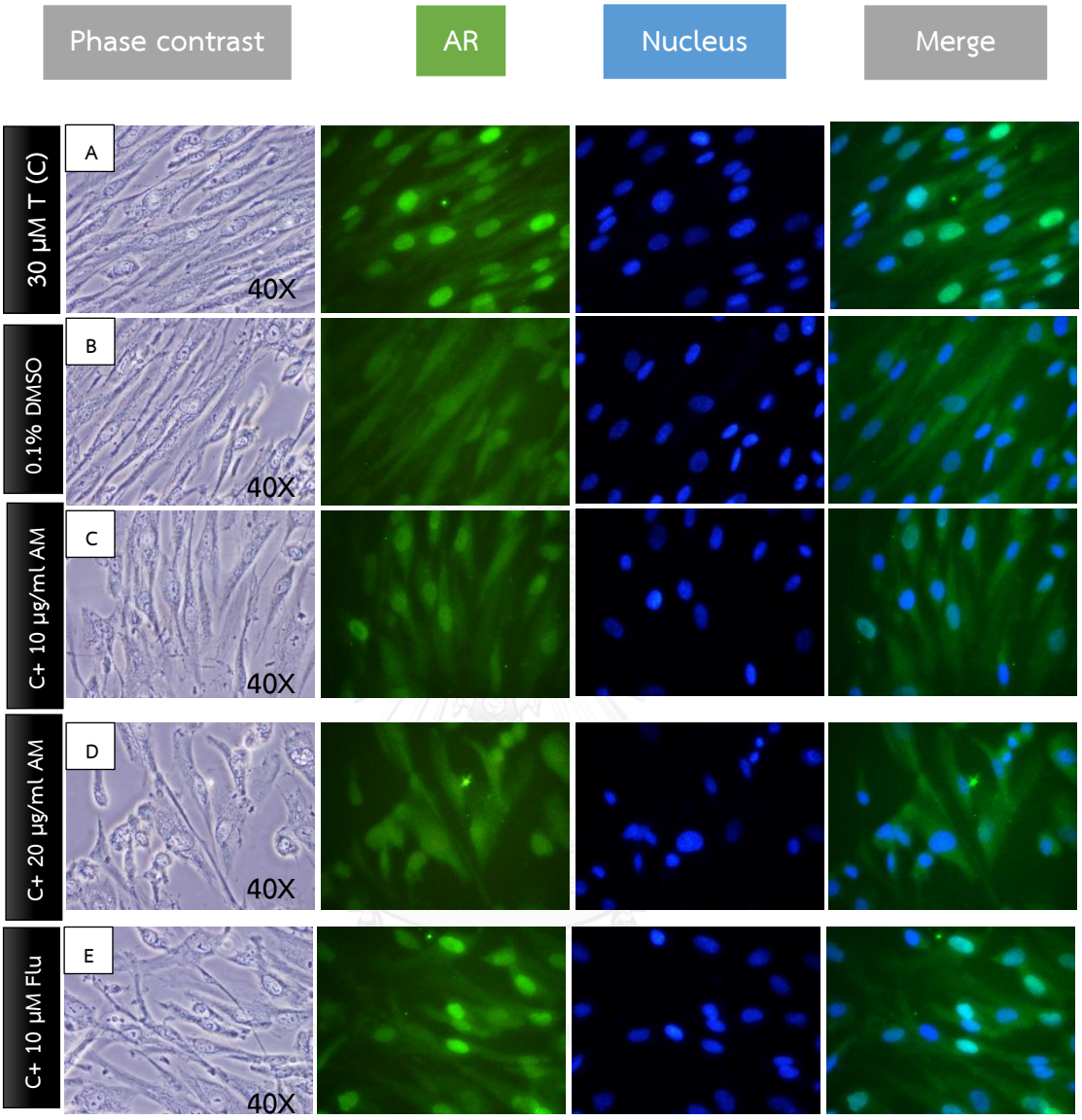


Figure 19. The immunofluorescent staining of the androgen receptor (AR) and observed under a Fluorescences-microscope (40×). For each experimental condition, the first column shows the phase contrast of DPCs, the second column shows the AR, the third column shows the nucleus, and the fourth column shows the merged image. **A.** DPCs were be under normal condition (without androgens stimulation). **B.** DPCs were treated with 30 µM T for 12 hour as control group (C) , AR staining was detected more intensity in nucleus. **C. and D**. DPCs were treated with 10µg/ml AM and 20µg/ml AM and stimulated by 30µM T **E.** DPCs were treated with 10 µM Flutamide and stimulated by 30 µM T as a positive control.

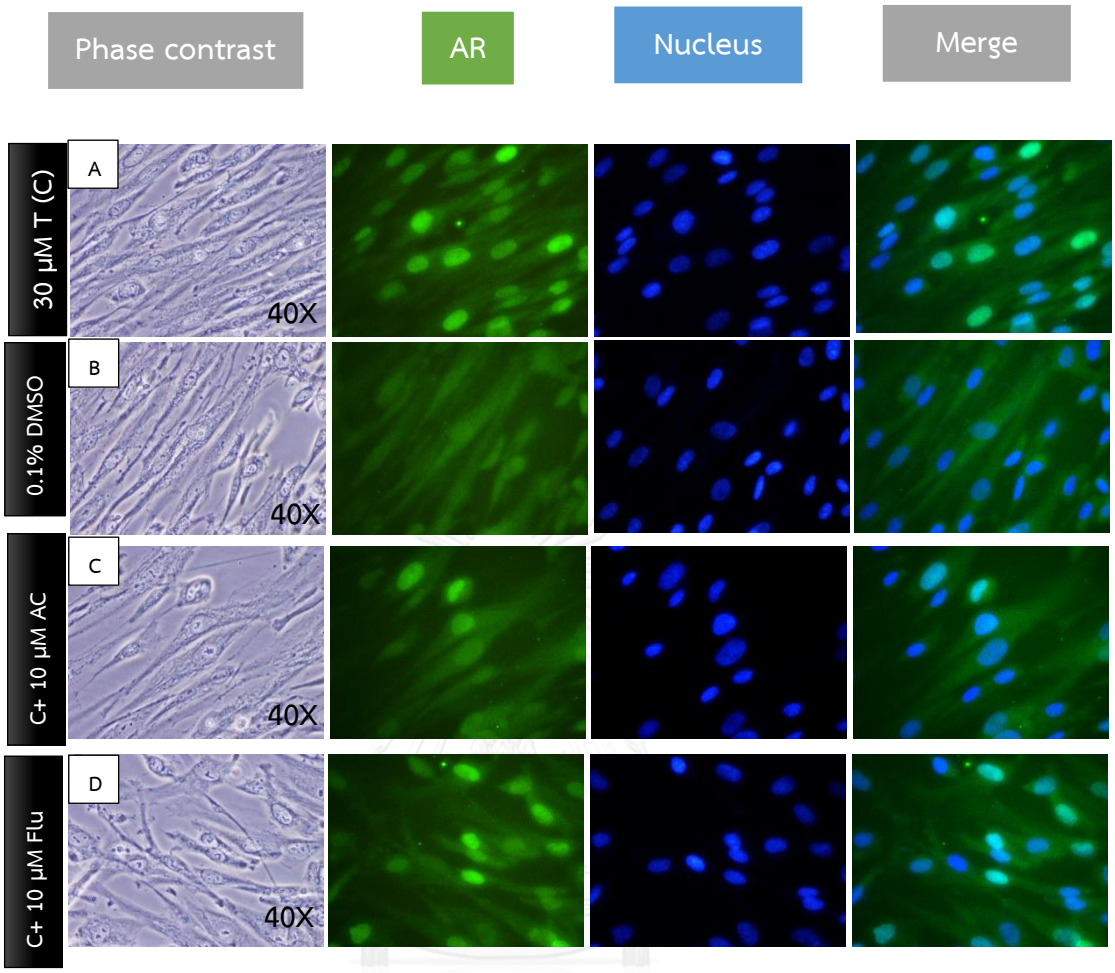


Figure 20. The immunofluorescent staining of the androgen receptor (AR) and observed under a Fluorescences-microscope (40×). For each experimental condition, the first column shows the phase contrast of DPCs, the second column shows the AR, the third column shows the nucleus, and the fourth column shows the merged image. **A.** DPCs were be under normal condition (without androgens stimulation), showed the same level intensity of AR staining in both cytoplasm and nucleus. **B.** DPCs were treated with 30 µM T for 12 hour, AR staining was detected mainly in nucleus **C.** DPCs were treated with 20 µM AC and stimulated by 30 µM T. **D.** DPCs were treated with 10 µM Flutamide and stimulated by 30 µM T as a positive control.

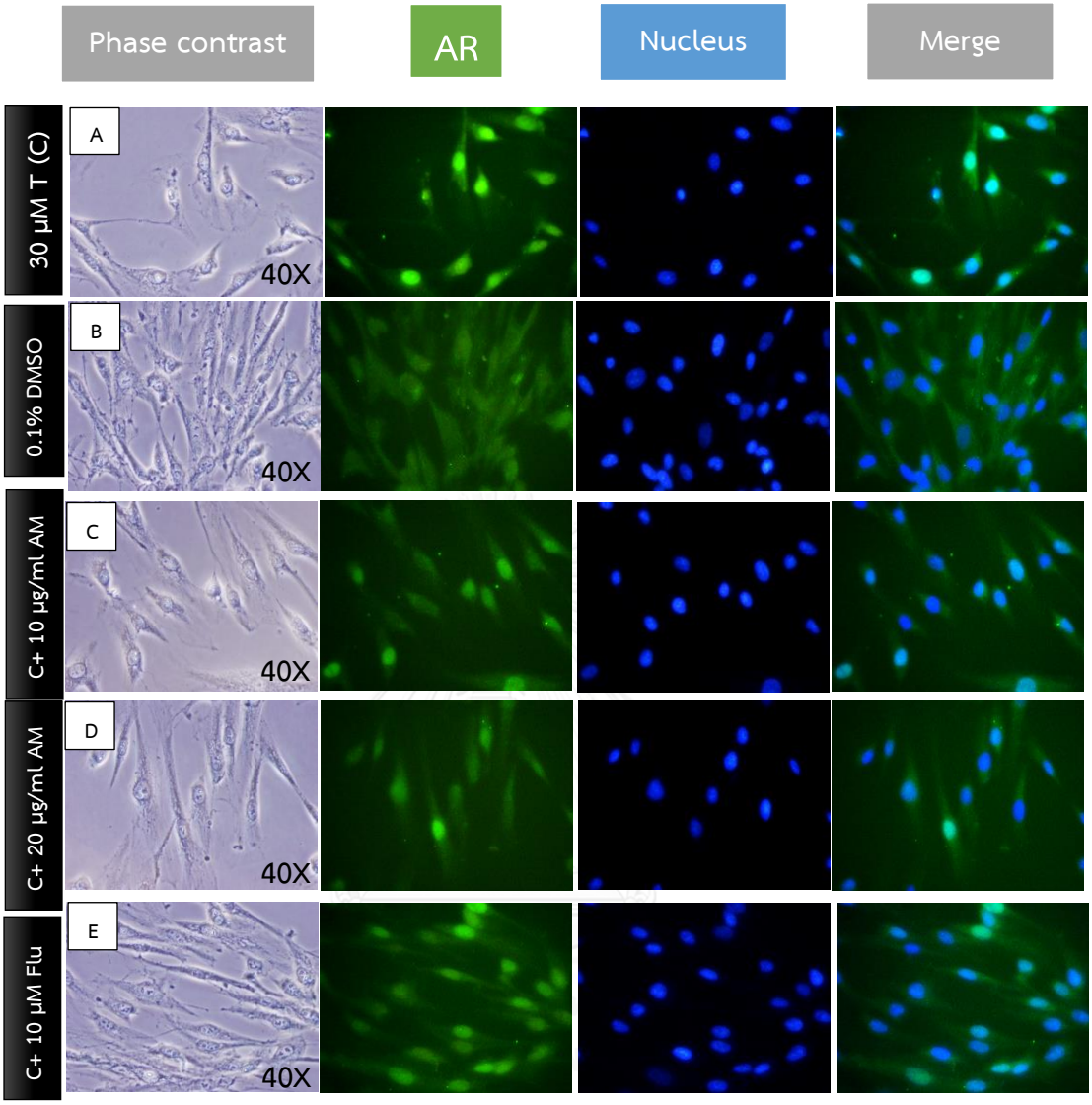


Figure 21. The immunofluorescent staining of the androgen receptor (AR) and observed under a Fluorescences-microscope (40×). For each experimental condition, the first column shows the phase contrast of DPCs, the second column shows the AR, the third column shows the nucleus, and the fourth column shows the merged image. **A.** DPCs were be under normal condition (without androgens stimulation), showed the same level intensity of AR staining in both cytoplasm and nucleus. **B.** DPCs were treated with 20 nM 5α-DHT for 12 hour, AR staining was detected mainly in nucleus. **C. and D.** DPCs were treated with 10 µg/ml AM and 20 µg/ml AM and stimulated by 20 nM 5α-DHT. **E.** DPCs were treated with 10µM Flutamide and stimulated by 20 nM DHT as a positive control.

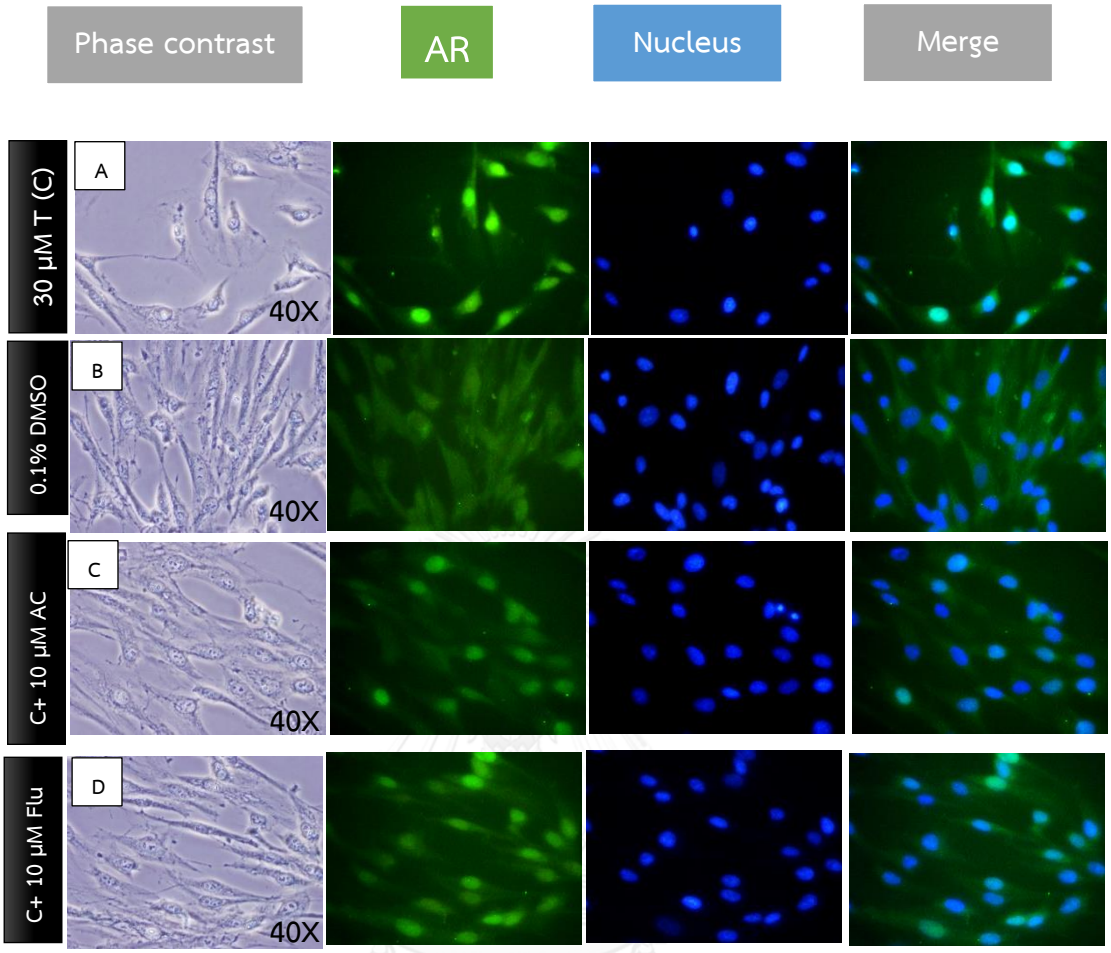


Figure 22. The immunofluorescent staining of the androgen receptor (AR) and observed under a Fluorescences-microscope (40×). For each experimental condition, the first column shows the phase contrast of DPCs, the second column shows the AR, the third column shows the nucleus, and the fourth column shows the merged image. **A.** DPCs were be under normal condition (without androgens stimulation), showed the same level intensity of AR staining in both cytoplasm and nucleus. **B.** DPCs were treated with 20 nM 5α-DHT for 12 hour, AR staining was detected mainly in nucleus. **C.** 10 µM AC and stimulated by 20nM 5α-DHT. **D.** DPCs were treated with 10µM Flutamide and stimulated by 20 nM 5α-DHT as a positive control.

Determination for gene expression of growth factors

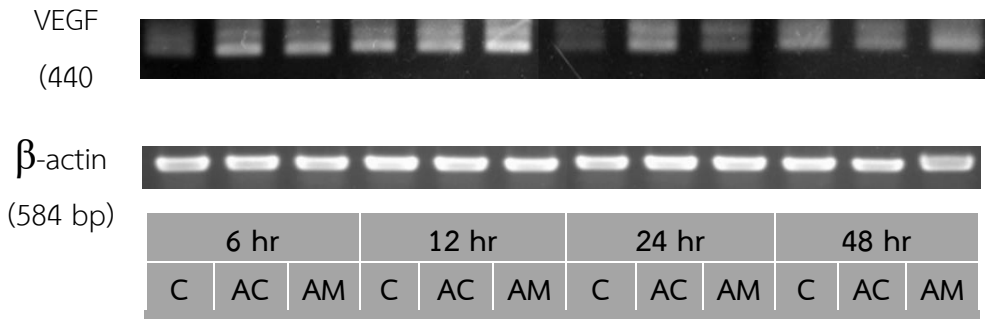
Based on the pathogenic mechanism of AGA, androgens are first bound to their receptors, gene expression in DPCs is then altered so that various cytokines and growth factors are produced to regulate and promote hair growth. These growth factors include IGF-I, FGF-7 (KGF), HGF and VEGF which are found in DPCs of the hair follicle (30, 53, 54, 62, 63, 97, 98). These growth factors have many functions such as stimulation of proliferation, differentiation of epidermal keratinocytes. In addition, some growth factors are essential for maintaining of anagen or growth phase in hair cycles (54, 58, 66). Thus, AM and AC which was shown to act as potential 5α-R inhibitors might also have an effect on the decrease nuclear translocation of AR causing the expression of these growth factors.

To prove this assumption, the effect of AM and AC on the mRNA expression of these growth factors was studied by RT-PCR. The results of the mRNA expression were visualized on 1% agarose gels and intensity of the bands indicated the expression level of each growth factor which were analyzed by using ImageJ software as shown in **Figures 23 and 24**. The level of mRNA expression was quantitated relative to the control group (30μM T treated group), the RT-PCR results showed that AM and AC significantly increased the level of VEGF and HGF expression in the presence of 30 μM T. AM at 10μg/ml significantly increased the mRNA expression of VEGF after 6 and 12 hr of incubation, up to 3.53 ± 0.14 times and 2.06 ± 0.17 times, respectively (**Figure 23A.**), but after 12 hr, AM slightly increased the mRNA expression of VEGF when compared with control group. Similarly, 10μM AC also significantly increased the mRNA expression of VEGF after 6 and 12 hr incubation time, up to 3.55 ± 0.34 times and 1.51 ± 0.09 times, respectively. Moreover, it was found that AC could up-regulation the mRNA expression of VEGF to 1.99 ± 0.24 times after 24 incubation time. A similar overcoming effect of AM and AC was also observed on the mRNA expression of HGF, AM at 10μg/ml significantly increased the mRNA expression of HGF up to 2.17 ± 0.63 and 4.81 ± 0.47 after 24 and 48 hr of incubation, respectively. Also, 10μM AC showed the induction of the mRNA expression of HGF up to 3.22 ± 0.17, 3.84 ± 0.25 respectively, while the effect of AC and AM on up-regulation of the mRNA expression of FGF-7 and IGF-1 was not observed (**Figures 25 and 26)**.

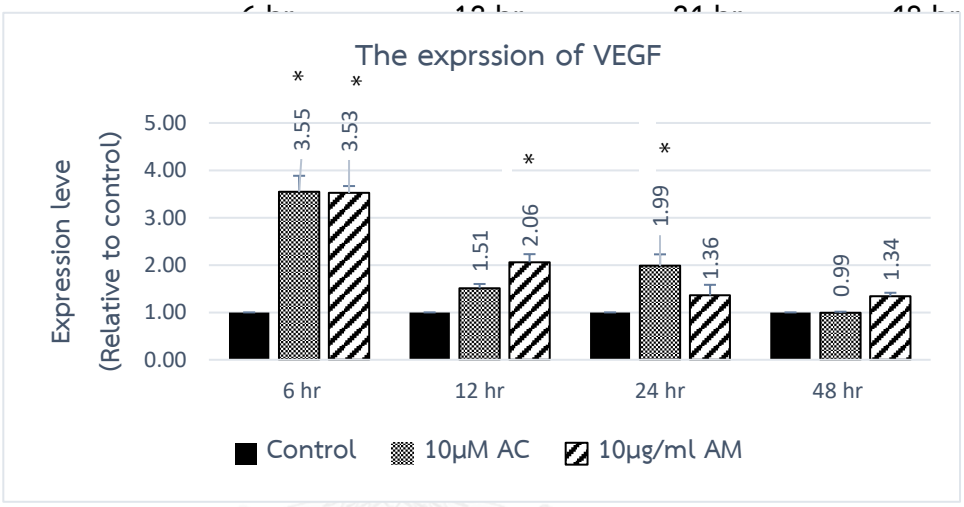
For IGF-1 and FGF-7, only slightly increase or decrease of their mRNA expressions were observed when compared with the control group, but the difference was not significant. From these mRNA expression results, it should be noted that AM and AC might promote hair growth by stimulating the expression of VEGF and HGF which are the growth factors involved in the regulation and stimulation hair growth (93, 99, 100). Vascular endothelial growth factor (VEGF) plays important roles in angiogenesis and stimulating the new blood vessels that are associated with the hair growth and hair cycling (59-61, 101, 102). In addition, hepatocytes growth factor (HGF) is also involved in the formation of new blood vessels around the hair follicles.

Furthermore, HGF is essential for regulating the interaction between the DPCs and HaCat cells (epithelial keratinocytes cells) (103, 104). Proliferation, differentiation and apoptosis of keratinocytes and epithelial-mesenchymal interaction that important of hair follicle morphogenesis and hair growth (29, 105).

(A)



(B)



AM

The DPCs were treated with 10μM AC against the effect of 30 μM T.

The DPCs were treated with 10μg/ml AM against the effect of 30 μM T.

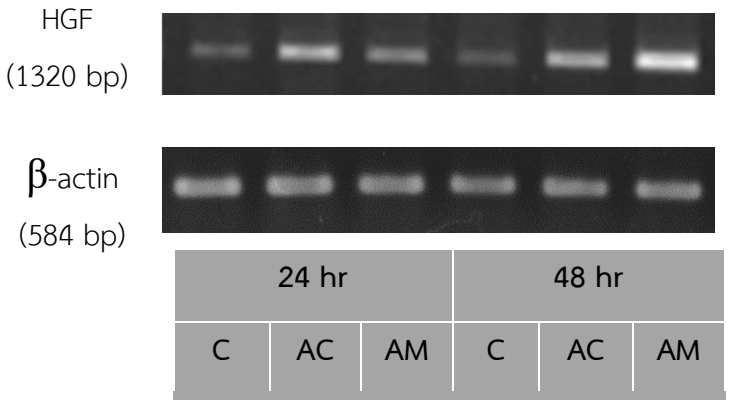
Figure 23. VEGF expression affected by 10μg/ml AM and 10μM AC. DPCs were incubated with 30 μM T and treated with AM and AC at various times (6, 12, 24, 48 hour). **(A)** RT-PCR showing the mRNA expression of VEGF in each condition.

(B) Quantitative analysis of mRNA expression of VEGF using ImageJ software.

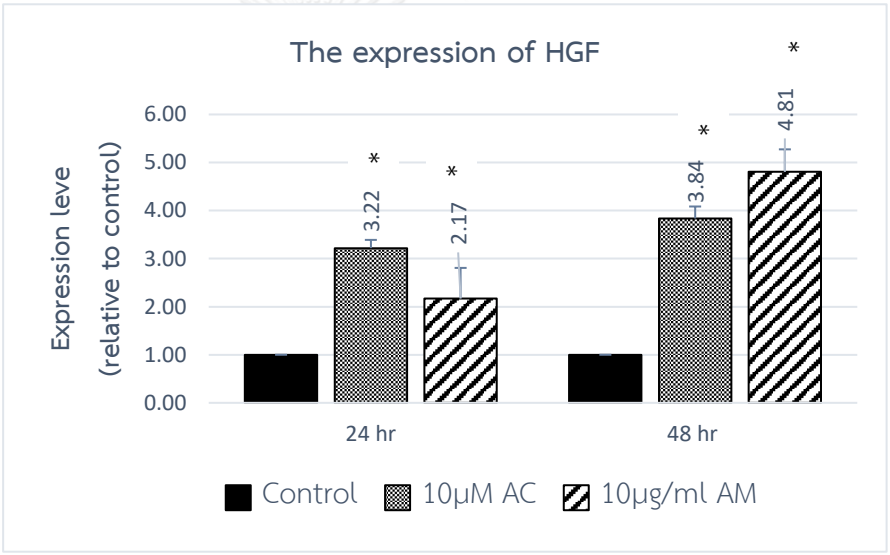
DPCs were incubated only with 30 μM T as a control.The histograms represented the relative level of the mRNA expression of VEGF (relative to control group).

* indicated the significant difference in at compared to control group ($p<0.05$).

(A)



(B)



The DPCs were treated with 10μM AC against the effect of 30 μM T.

The DPCs were treated with 10μg/ml AM against the effect of 30 μM T.

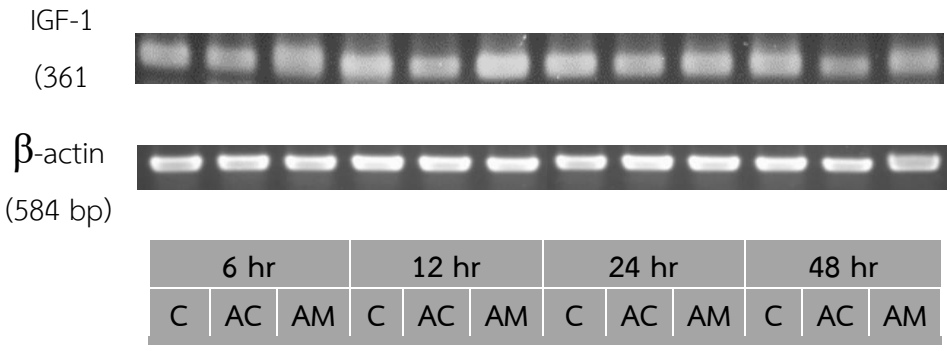
Figure 24. HGF expression affected by 10μg/ml AM and 10μM AC. DPCs were incubated with 30 μM T and treated with AM and AC at various times (24, 48 hour).

(A) RT-PCR showing the mRNA expression of HGF in each condition.

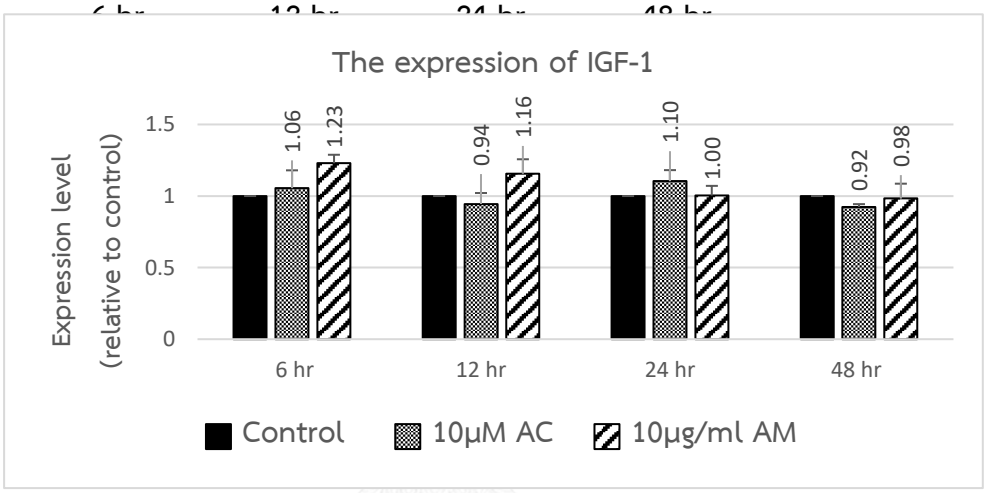
(B) Quantitative analysis of mRNA expression of HGF using ImageJ software. DPCs were incubated only with 30 μM T as a control. The histograms represented the relative level of the mRNA expression of HGF (relative to control group).

* indicated the significant difference in at compared to control group ($p<0.05$).

(A)



(B)

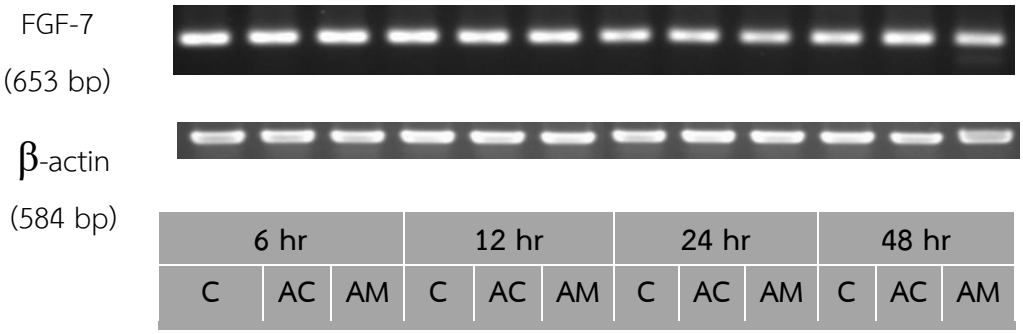


The DPCs were treated with 10μM AC against the effect of 30 μM T.

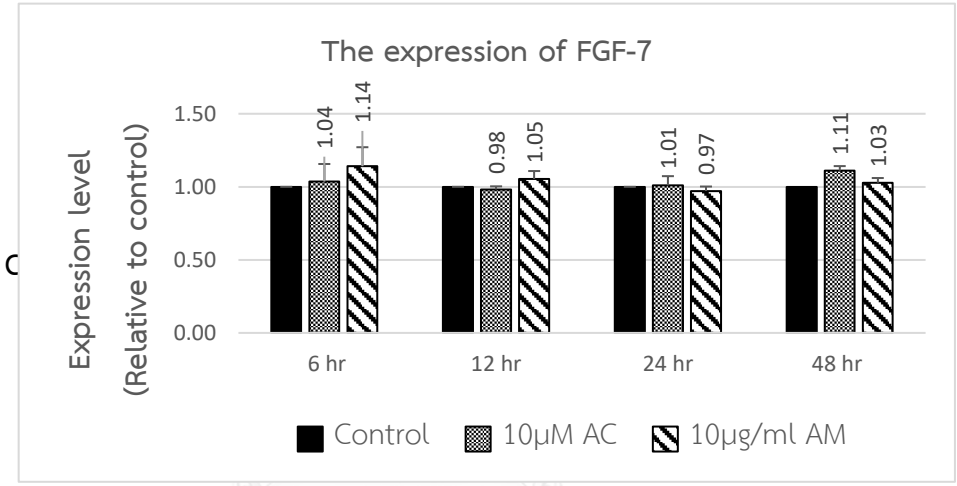
The DPCs were treated with 10μg/ml AM against the effect of 30 μM T.

Figure 25. IGF-1 expression affected by 10μg/ml AM and 10μM AC. DPCs were incubated with 30 μM T and treated with AM and AC at various times (6, 12, 24, 48 hour). **(A)** RT-PCR showing the mRNA expression of IGF-1 in each condition. **(B)** Quantitative analysis of mRNA expression of IGF-1 using ImageJ software. DPCs were incubated only with 30 μM T as a control. The histograms represented the relative level of the mRNA expression of IGF-1 (relative to control group).

(A)



(B)



C

The DPCs were treated with 10μM AC against the effect of 30 μM T.

The DPCs were treated with 10μg/ml AM against the effect of 30 μM T.

Figure 26. FGF-7 expression affected by 10μg/ml AM and 10μM AC. DPCs were incubated with 30 μM T and treated with AM and AC at various times (6, 12, 24, and 48 hour). **(A)** RT-PCR showing the mRNA expression of FGF-7 in each condition. **(B)** Quantitative analysis of mRNA expression of FGF-7 using ImageJ software. DPCs were incubated only with 30 μM T as a control. The histograms represented the relative level of the mRNA expression of FGF-7 (relative to control group).

CHAPTER V
CONCLUSION

AGA is a common type of scalp hair loss in both men and women. it is a genetically determined disorder associated with the increased production of 5α-DHT which is formed from testosterone (T) by the enzyme activity of 5α-R. Androgens and its receptor (AR) cause signaling and biochemical reactions which result in the alteration of cytokines and growth factors, leading to premature hair loss. Therefore, potential drug to be used for preventing or curing AGA should be demonstrated to be able to inhibit the enzyme activity 5α-R to reduce the androgen formation or blocking the AR activation in DPCs.

We have shown in this study that DPCs could be isolated by simple microdissection. The isolated DPCs clearly exhibited the expression of target genes such as 5α-R1, 5α-R2 and AR receptor. These expressed characteristics convinced us that the isolated DPCs are suitable to be a model for investigating the mechanisms of action of AM extract and AC compound. These include 5α-R inhibitory activity, inhibition of nuclear translocation of androgen AR complex, and the expression of some involved growth factors.

The AM extract (10μg/ml) and AC compound (10μM) exhibited clearly as 5α-R inhibitor which could significantly reduce the formation of potent 5α-DHT in both DPCs and HaCat cells. The pure compound of AC at 10μM exhibited its highest inhibitory activity, causing the reduction the 5α-DHT production by 67.7% in DPCs and 41.1% in HaCat cells, whereas AM at 10μg/ml could reduce the formation of 5α-DHT by 73.5% in DPCs and 74.5% in HaCat cells. Itis well-known that the androgens exert their activity through binding with AR. The reduction of 5α-DHT would lead to lowering of receptor-ligand complex resulting in decreasing the nuclear translocation of AR and androgen activity (**Figure 26**). Therefore, AM and AC were further tested for their potential to inhibit the nuclear translocation of AR in the presence of androgens. It was found that AM (10μg/ml) and AC (10μM) could in deed decrease the nuclear translocation of AR in presence of 30μM T as a stimulator.

Similarly, in the presence of the more potent 5α-DHT as a stimulator, AM (10μg/ml) and AC (10μM) also showed stronger nuclear translocation inhibitory activity. This indicates that both AM and AC exhibit their inhibitory activities not only at the step of 5α-R enzyme (thus, lowering the availability of receptor-ligand complex for nuclear translocation) but also at the step of nuclear translocation process of the receptor complex in DPCs. Moreover, the treatment with AM or AC in the presence of T showed a significant increase in the mRNA expression of growth factors, including VEGF and HGF. These results suggest that AM and AC can promote hair growth by stimulating the expression of VEGF and HGF which are signal transduction molecules that proceed with intercellular signal transduction and regulate the hair follicle cyclical growth. Both VEGF and HGF play important roles in the angiogenesis involving in the formation of new blood vessels that are associated with the hair growth and hair cycling. The perifollicular capillaly network is coupled to the hair cycle, increasing during the anagen phase and then regressing during the catagen and telogen phase. These have been further supported by the study of the activity of minoxidil which has been used to treat AGA. Several supports have been demonstrated by showing the mechanism of action of minoxidil, suggesting that it stimulates hair growth via the expression of VEGF.

In summary, our results suggest that AM extract and its active AC compound possess a potent *in vitro* 5α-R inhibitory activitiy coupled with inhibition of the androgen effect in DPCs causing the decrease of the androgen-dependent activation of the AR. Furthermore, they increases the mRNA expression of some growth factors which are generally believed to stimulate hair growth. These finding might support the use of AM extract and AC as an ingredient in hair growth-protective hair loss in AGA.

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