Chapter III

Materials and Methods

Contents

3.1 Equipment

Autopipette: BIOHIT, Finland

Carbon nuclear magnetic resonance: Varian AS-400 spectrometer (100 MHz), USA

Cell culture hood: ESCO Class II BSC, Streamline®, USA

Cell culture conical tubes, tips and flask: Corning, USA

Centrifuge: Eppendorf 5417R, Germany

Gel electrophoresis: Takara Mupid®- exU, Japan

Gel documentation system: Bio-Rad's Gel-Doc, USA

High performance liquid chromatography column: Tosho Bioscience TSK gel ODS (2x25cm, 5µm), Capcell Pak ODS (0.46x25cm, 5µm), Japan

High performance liquid chromatography pump: Jasco® 887-PU, 880-PU and PU-2089, Thailand

High performance liquid chromatography detector: Jasco® 875-UV UV-VIS detector, Thailand

High performance liquid chromatography recorder: Sekonic SS-250 F, Japan

Incubator: NUAIRE™ NU-8500, USA

Inverted fluorescent microscope: Olympus IX51, USA

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 REPROSTAR 3, Switzerland

Thin layer chromatography sample loader: CAMAG LINOMAT 5, Switzerland

Thin layer chromatography scanner: CAMAG TLC SCANNER 3, Switzerland

Thin layer chromatography plates: Merck TLC Silica gel 60 F_{254}, Germany

Polymerase chain reaction system: Bio-Rad MyCycler™ thermal cycler, USA

Preparative thin layer chromatography plates: Merck PLC Silica gel 60 F_{254}, Germany

Proton nuclear magnetic resonance: Varian AS-400 spectrometer (400 MHz), USA

Rotary Evaporator: Buchi Rotavapor R-210, Switzerland

Spectrophotometer: Beckman Coulter® Multimode Detector DTX880, USA.

Speed Vacuum: Savant Speed Vac SC-100, USA

Weighing balance: Mettler Toledo Excellence Plus, Thailand

3.2 Chemicals

5α-DHT: Sigma-Aldrich, USA

Acetic acid: RCI Labscan, Thailand

Acetone: RCI Labscan, Thailand

Acetonitrile: RCI Labscan, Thailand

Agarose-LE: Affymetrix, USA

Dimethyl sulfoxide (DMSO): Amresco®, USA

Dutasteride: BDG Synthesis, New Zealand

Ethidium Bromide: Invitrogen, USA

Ethyl acetate: RCI Labscan, Thailand

Methanol: RCI Labscan, Thailand

Phosphoric Acid: RCI Labscan, Thailand

T: Sigma-Aldrich, USA

Toluene: RCI Labscan, Thailand

3.3 Enzymes, reagents and kits

0.25% Trypsin-EDTA: Invitrogen, 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: Invitrogen, USA

First strand cDNA synthesis kit: Fermentas, USA

GeneRuler 1-kb DNA ladder: Thermo Fisher Scientific, USA

Hoechst 33342: Invitrogen, USA

Mesenchymal stem cell medium and supplements: Sciencell Research Laboratories, USA

Platinum® *Taq* polymerase: Invitrogen, USA

PrestoBlue® cell viability reagent (10X): Invitrogen, USA

Propidium iodide: Invitrogen, USA

RNeasy® Mini kit: Qiagen, USA.

RPMI medium: Invitrogen, USA

Tris-acetate-EDTA (TAE) buffer (50X): Amresco®, USA

3.4 Plant materials and extraction

The list of the Thai plants and plant parts used in this study is shown in **Table 5**. The plant parts used for each species were ground into powder and extracted through maceration using 100% methanol at room temperature for two days. The methanolic extracts were then evaporated to dryness at 45°C using rotary evaporator and kept at -20°C until used.

Table 5

List of Thai medicinal plants used in this study

Medicinal plant	Abbreviation	Family	Plants part
Afgekia sericea	AS	*Fabaceae*	Aerial
Alpinia galangal	AG	*Zingiberaceae*	Fresh Rhizome
Alternanthera sessilis	ASC[b]	*Amaranthaceae*	Whole plant
Alternanthera sessilis	ASHE[c]	*Amaranthaceae*	Whole plant
Alternanthera sessilis	ASHO[a]	*Amaranthaceae*	Whole plant
Avicennia marina	AM	*Acanthaceae*	Heartwood
Bacopa monnerii	BM3	*Plantaginaceae*	Aerial
Balanophora abbreviate	BA	*Balanophoraceae*	Aerial
Barleria cristata	BC	*Acanthaceae*	Root

Table 5 (Cont.)

List of Thai medicinal plants used in this study

Medicinal plant	Abbreviation	Family	Plants part
Bauhinia malabarica	BM2	*Fabaceae*	Leaves
Butea monosperma	BM	*Fabaceae*	Stem
Caesalpiria digyna	CD	*Fabaceae*	Stem
Centella asiatica	CA	*Mackinlayaceae*	Aerial
Chrysanthemum indicum	CI	*Asteraceae*	Dried Flower
Citrus hystix	CH	*Rutaceae*	Fresh Peel
Citrus limonum	CL	*Rutaceae*	Fresh Peel
Citrus reticulata blanco	CRB	*Rutaceae*	Dried Peel
Clitoria ternatea	CT	*Fabaceae*	Dried Flowers
Crotalasia retusa	CR	*Fabaceae*	Root
Curcuma longa	CLO	*Zingiberaceae*	Dried Rhizome
Dalbergia parviflora	DP	*Leguminosae*	Heartwood
Derris elliptica	DE	*Leguminosae*	Stem
Diospyros mollis	DM	*Ebenaceae*	Stem
Kaempferia galangal	KG	*Zingiberaceae*	Dried Rhizome
Leersia hexandra	LH	*Poaceae*	Stem
Maclura cochinchinen	MC	*Moraceae*	Stem
Micromelum minutum	MM	*Rutaceae*	Stem
Myristica fragrans	MF	*Myristicaceae*	Aerial
Ochna integerrima	OI	*Ochnaceae*	Stem
Olendra musifolia	OM	*Oleandraceae*	Stem
Pterygota alata	PA	*Malvaceae*	Stem
Randia horrida	RH	*Rubiaceae*	Root
Salacia verrucosa	SV	*Celastraceae*	Stem
Scoparia dulcis	SD	*Plantaginaceae*	Stem
Seena garrethiana	SG	*Fabaceae*	Heartwood
Senna timoriensis	ST	*Fabaceae*	Stem
Tarenna fragans	TF	*Rubiaceae*	Leaves
Tarenna hoaensis	TH	*Rubiaceae*	Stem
Telosma minor	TM	*Asclepiadaceae*	Stem
Zanthoxylum limonella	ZL	*Rutaceae*	Stem
Zingiber officinale	ZO	*Zingiberaceae*	Fresh Rhizome

a- Partitioned with water

b- Partitioned with dichloromethane

c- Partitioned with hexane

3.5 Culturing of human hair dermal papilla cells

HHDPCs, obtained from Sciencell Research Laboratories (Carlsbad, USA), were grown in mesenchymal stem cell medium containing 5% fetal bovine serum (FBS), mesenchymal stem cell medium supplement, and 1X antibiotic-antimycotic solution at 37°C in 5% CO_2 in a T-25 flask. The culture medium was changed every three days until it reached 70% confluency and then the medium was changed every day. The cells grown between passages 2 to 6 were used in this study.

3.6 Checking for the presence of androgen receptor and enzymes involved in the steroidogenesis pathway in human hair dermal papilla cells

RT-PCR was used to check the presence of AR and various enzymes involved in the steroidogenesis pathway in passages 2, 4, 5 and 6 of HHDPCs. This includes 5α-R1, 5α-R2, aromatase and HSD17β2. Firstly, the total RNA was extracted from HHDPCs according to the manufacturer's instructions of the RNeasy® mini kit. The RNA was subsequently treated with DNase I to remove any genomic DNA contamination. cDNA was synthesized from the treated RNA using the first strand cDNA synthesis kit. The cDNA obtained from this reaction was used as a DNA template for PCR reactions. The PCR reaction comprised of 1X PCR buffer, 2 mM $MgCl_2$, 0.4 mM dNTP mix of dATP, dCTP, dGTP, dTTP, 0.4 µM each of forward (F) and reverse (R) primer and 2.5 units of Platinum® *Taq* polymerase. The F and R primers, shown in **Table 6**, were designed based on the protein region of the full-length sequence obtain from the NCBI GenBank using Clone manager (Scientific & Educational Software, USA) and made to order at 1st Base Laboratories (Selangor, Malaysia). The PCR-thermal profile started with an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products was mixed with 6X DNA loading dye and was run on 1% agarose gel electrophoresis in 1X TAE buffer at 100V, with 1-kb DNA ladder. The agarose gel was then stained with ethidium bromide, de-stained with water and visualized under UV transilluminator using gel documentation system.

Table 6

Forward and reverse primers and the expected sizes of androgen receptor, enzymes and β-actin

Name	Primer pair (F - □ - R)	Expected size (bp)
Androgen receptor (AR)	F: 5' CGTGCGCGAAGTGATCCAGAA 3'	811
GenBank:NM_000044.3	R: 5' TGCGCTGTCGTCTAGCAGAGAA 3'	
5α-reductase type 1 (5α-R1)	F: 5' ACTGCATCCTCCTGGCCATGTTC 3'	380
GenBank:NM_001047.2	R: 5' GGCATAGCCACACCACTCCATGA 3'	
5α-reductase type 2 (5α-R2)	F:5' AAGCACACGGAGAGCCTGAA 3'	450
GenBank:NM_000348.3	R: 5' GCCACCTTGTGGAATCCTGTAGC 3'	
Aromatase	F: 5' AGTGCCTGCAACTACTACAACC 3'	1000
GenBank:NM_000103.3	R: 5' CGAGTCTGTGCATCCTTCCAAT 3'	
17β-Hydroxysteroid dehydrogenases type 2 (HSD17β2)	F: 5' GTCCTGGTGACAGGTGGTGATT 3'	804
GenBank: NM_002153.2	R: 5' GTGAGCAAGGCAGATCCACAAG 3'	
β-actin (internal control)	F: 5' ATGATGATATCGCCGCGCTC 3'	584
GenBank:NM_001101.3	R: 5' GCGGTCGGTGAGGATCTTCA 3'	

3.7 Development of human hair dermal papilla cell-based assay system

3.7.1 Cytotoxicity of testosterone on human hair dermal papilla cells

HHDPCs were seeded at a cell density of $1x10^5$ cells/ml onto 96-well plates (100 µl of 10,000 cells/well). After 24 hours, the cells were separately treated with 100 µl of $2x10^{-4}$ M, $2x10^{-5}$ M, $2x10^{-6}$ M, $2x10^{-7}$ M or $2x10^{-8}$ M T and 100 µl of 1% DMSO (control). The final concentrations of T (i.e., half the concentration treated) were 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M, respectively. Cell viability was measured 24 hours after the treatment using the 1X PrestoBlue® reagent in RPMI medium. In the presence of viable cells, PrestoBlue® changes from a non-fluorescent blue color to a fluorescent purple-pink color, which is detected using the Multimode Detector DTX 880 (Beckman Coulter®, Indianapolis, USA), a bottom-read fluorospectrophotometer with an excitation/emission of 535/615 nm. The highest final non-toxic concentration was then used as the starting concentration for optimizing the amount of T needed to observe enzyme activity.

3.7.2 Optimization of the incubation time and testosterone concentration

HHDPCs were seeded at a cell density of $1x10^5$ cells/ml onto a 96-well plate (100 µl of 10,000 cells/well). After 24 hours, the cells were separately treated with various concentrations of T, starting from 50 µl of four times the highest final non-toxic concentration with 50 µl of 2% DMSO; and 100 µl of 2% DMSO (negative control). The cells were treated for 24 and 48 hours, before the cell culture medium was collected in Eppendorf tubes, and the attached cells were tested for cell viability using the 1X PrestoBlue® reagent in RPMI medium. T and its product, 5α-DHT, were extracted from the cell culture medium using an equal volume of ethyl acetate. The ethyl acetate fraction was then dried using speed vacuum, 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 at a ratio of 8:2 as the mobile phase [44]. The developed TLC plate was dipped in a solution of 42.5% phosphoric acid and heated at 120°C for 20 min, for the visual detection of 5α-DHT at 366 nm using a TLC reprostar imager (Camag, Switzerland), and the amount was quantified using an image analyzing program, Quantity One (Bio-Rad, USA). The final concentration of T and the incubation time suitable for detecting the 5α-DHT production were used for screening methanolic plant extracts for 5α-R inhibitory activity.

3.7.3 Optimizing the concentration of methanolic plant extracts

To obtain an optimum non-toxic concentration of each plant extract, its cytotoxicity on HHDPCs was first tested. HHDPCs were seeded at a cell density of $1x10^5$ cells/ml onto 96-well plates (100 µl of 10,000 cells/well). After 24 hours, the cells were treated with 100 µl of each plant extract ranging from 5-40 µg/ml to obtain the final concentration ranging from 2.5-20 µg/ml. Cell viability was measured 24 hours after treatment using the 1X PrestoBlue® reagent in RPMI medium. The highest final non-toxic concentration for each compound was then used for the 5α-R inhibitory activity test.

3.8 Screening of methanolic plant extracts for 5α-reductase inhibitory activity using the optimized assay system

HHDPCs were seeded at a cell density of $1x10^5$ cells/ml onto 96-well plates (100 µl of 10,000 cells/well). After 24 hours, the cells were separately treated with 50 µl of four times the optimized final concentration of T and 50 µl of 2% DMSO (internal control); 50 µl of four times the optimized final concentration of T and 50 µl of four times the highest final non-toxic concentration of methanolic plant extracts; and 100 µl of 2% DMSO (negative control). After treating the cells for the optimized incubation time, T and 5α-DHT were extracted from the cell culture medium and analyzed as described above. The inhibitory activity was determined through the decrease in 5α-DHT produced by HHDPCs relative to the internal control. In order to avoid false positive results, cell viability of the attached treated cells was also tested. In addition, the IC_{50} value of the most potent methanolic plant extract was also determined.

3.9 Studies on the cytotoxicity of 5α-dihydrotestosterone on human hair dermal papilla cells

HHDPCs were seeded at a cell density of $1x10^5$ cells/ml onto 96-well plates (100 µl of 10,000 cells/well). After 24 hours, the cells were separately treated with 100 µl of $2x10^{-4}$ M, $2x10^{-5}$ M, $2x10^{-6}$ M, $2x10^{-7}$ M or $2x10^{-8}$ M 5α-DHT and 100 µl of 1% DMSO (control). The final concentrations of 5α-DHT (i.e., half the concentration treated) were 10^{-4} M, 10^{-5} M, 10^{-6} M, 10^{-7} M and 10^{-8} M, respectively. Cell viability was measured 24 hours after the treatment using the 1X PrestoBlue® reagent in RPMI medium. The highest final non-toxic concentrations of 5α-DHT were then used for further studies.

3.10 Determination of the anti-androgenic activity of the 5α-reductase inhibitory plant extract

HHDPCs were seeded at a cell density of $4x10^5$ cells/ml onto 24-well plates (250 µl of 100,000 cells/well). After 24 hours, the cells were separately treated with 125 µl of four times the optimized final T concentration and 125 µl of 2% DMSO; 125 µl of four times the highest final non-toxic concentrations of 5α-DHT and 125 µl of 2% DMSO; 125 µl of four times the final 5α-R inhibitory concentration and 125 µl of

2% DMSO; 125 µl of four times the optimized final T concentration and 125 µl of four times the final 5α-R inhibitory concentration; 125 µl of four times the highest final non-toxic concentrations of 5α-DHT and 125 µl of four times the final 5α-R inhibitory concentration; and 250 µl of 2% DMSO (control). After 24 hours of treatment, the culture medium was removed, and the cells were trypsinized using 0.25% trypsin-EDTA and collected in Eppendorf tubes. Total RNA was extracted from the collected cells and converted to cDNA using the first strand cDNA synthesis kit. The cDNA was used as a template for RT-PCR to observe the up- or down-regulation of various growth factors, including IGF-1, FGF-7, HGF and VEGF, involved in hair growth. The PCR reaction comprised of 1X PCR buffer, 2 mM $MgCl_2$, 0.4 mM dNTP mix of dATP, dCTP, dGTP, dTTP, 0.4 µM each of F and R primer and 2.5 units of Platinum® *Taq* polymerase. The F and R primers for these growth factors were designed based on the protein region of the full-length sequence obtain from the NCBI GenBank and are shown in **Table 7**. The PCR-thermal profile started with an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 49°C for 30 sec, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products were analyzed using 1% agarose gel electrophoresis in 1X TAE buffer, and the mRNA expression of the growth factors was quantitated using ImageLab software (Bio-Rad, USA).

Table 7

Forward and reverse primers and the expected sizes of growth factors

Name	Primer pair (F - R)	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' AAATCTGCCTGCTGGAACTG 3'	653
Hepatocyte growth factors (HGF) GenBank:NM_000601.4	F: 5' CAGAGGTACGCTACGAAGTC 3' R: 5' GATGTGCCACTCGTAATAGG 3'	1320
Vascular endothelial growth factors (VEGF) GenBank:AAA35789.1	F: 5' ACCCATGGCAGAAGGAGGAG 3' R: 5' CCTTGCAACGCGAGTCTGTG 3'	440

3.11 Effectiveness of androgens and 5α-reductase inhibitory plant extract on human hair dermal papilla cell's morphology

HHDPCs were seeded at a cell density of $4x10^5$ cells/ml onto 24-well plates (250 µl of 100,000 cells/well). After 24 hours, the cells were separately treated with 125 µl of four times the optimized final T concentration and 125 µl of 2% DMSO; 125 µl of four times the highest final non-toxic concentration of 5α-DHT and 125 µl of 2% DMSO; 125 µl of four times the final 5α-R inhibitory concentration and 125 µl of 2% DMSO; 125 µl of four times the optimized final T concentration and 125 µl of four times the final 5α-R inhibitory concentration; 125 µl of four times the highest final non-toxic concentration of 5α-DHT and 125 µl of four times the final 5α-R inhibitory concentration; and 250 µl of 2% DMSO (control). After 24 hours of the treatment, the cells were co-stained with 10 µg/ml of Hoechst 33342 and Propidium iodide (PI) and then their morphology were analyzed using Olympus IX51, an inverted fluorescent microscope.

3.12 Thin layer chromatography profile of the 5α-reductase inhibitory plant extract

TLC was used to observe the complexity of the 5α-R inhibitory plant extract. The TLC Silica gel 60 F_{254} aluminum plate was spotted with 10 µl of the 5α-R inhibitory plant extract at a concentration of 3.5 mg/ml and developed using toluene: acetonitrile: ethyl acetate: acetic acid in the ratio of 7:1:3:0.03 as the mobile phase. The developed plate was visualized under the wavelengths of 254 and 366 nm.

3.13 Isolation and structural analysis of the active compound(s) from the 5α-reductase inhibitory plant extract

Preparative thin layer chromatography (PLC) was used to separate and isolate compounds from the most potent 5α-R inhibitory plant extract. The extract was developed, using the same system as mentioned in 3.12, on the PLC Silica gel 60 F_{254} glass plate and each band/fraction were isolated through scratching. The bands/fractions were then tested for the 5α-R inhibitory activity using the developed

assay system. The purity of active compound(s) were checked using HPLC before structure elucidations were conducted using proton (^{1}H-NMR) and carbon nuclear magnetic resonance (^{13}C-NMR). In addition, the purified 5α-R inhibitory compound(s) were also tested for the changes in the mRNA expression of the growth factors, as mentioned in 3.10.

3.14 Statistical analysis

All of the experiments were performed in triplicate, and the data are shown as the means ± SD. One-way ANOVA statistical analysis was used, and a *P-value* <0.05 was considered to be statistically significant.