CHAPTER 2

Materials and Methods

Part I Plant extraction

1. Materials

Glassware

- 0.5-L Glass bottle (SiamPack, Chiang Mai, Thailand)

Instruments

- Blender (Matsushita Electric Industrial Co., Ltd., Kadoma, Osaka, Japan)
- Hot air oven (Memmert GmbH, Memmert, Germany)
- Rotary evaporator (Eyela®, Rikakikai Co. Ltd., Tokyo, Japan)
- 20-mesh sieve (W.S. Tyler, Inc., Cleveland, OH, USA)

Chemicals

- Ethanol (AR grade) (Union Science, Chiang Mai, Thailand)
- Ethyl acetate (AR grade) (Merck Darmstadt, Germany)
- Hexane (AR grade) (Merck, Darmstadt, Germany)

2. Methods

2.1 Plant preparation

Plant materials (Appendix A) were weighted and then washed in distilled water to remove the dust and other foreign materials. After that, dried in hot air oven at 50 °C until the constant weight was achieved. The dried samples were blended, powdered to 20-mesh size and kept in desiccator until used.

2.2 Plant maceration and fractionation

Plant powders were submerging in absolute ethanol at room temperature accompanied by liquid-solid ratio of 10 mL/g as the described by Chaiyana (2013) with some modified. The extraction process was performed in 0.5-L jar with stirring during 24 hours in triplicate. After filtration by filtration apparatus connected to vacuum pump, the solvent was removed from extract by rotary evaporator and then measuring the mass of dry residue. The solvent was also removed from the rest of the extract by hot air oven at 50 °C until the constant mass was achieved and then kept in glass vial at 4 °C until used.

The process of selected plant fractionation is the same maceration but using different polarity of solvent as described by Tachakittirungrod (2007) with some modified. The plant powder was macerated with n-hexane for 24 hours, at room temperature. After filtration, n-hexane was added to the residual at the same volume and stirred frequently. The process was repeated until a clear solution is obtained. The solutions obtained were collected and pooled in the same bottle subsequently evaporated by rotary evaporator. The residue was further macerated consecutively using the same procedure with ethyl acetate and ethanol, respectively. The dried extract was kept in glass vial at 4 °C until used.

Part II Screening of aromatase inhibitory activity from 22 Thai plants

1. Materials

Glassware

- Corning PYREX® Tissue Grinder, Potter-Elvehjem PTFE Pestle (Corning, Tewksbury, MA, USA)
- Nunc™ F96 MicroWell™ Black microplate (Thermo Fisher Scientific, Waltham, MA, United States)

Instruments

- Agilent 1260 infinity HPLC system (Agilent Technologies, Inc., Waldbronn, Germany)
- Beckman Coulter Optima™ L-100XP UltraCentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA)
- Beckman Coulter fluorescence multimode detector DTX 880 (Beckman Coulter, Inc., San Diego, CA, USA)
- NanoDrop™ 2000. (Thermo Fisher Scientific, Waltham, MA, USA)
- Virtis Genesis 12 EL Console freeze dryer (Canberra Parkard, Toronto, ON, Canada)

Chemicals

- Acetonitrile, Methanol (Merck Darmstadt, Germany)
- Antibiotic antimycotic, DMEM, Fetal Bovine Serum, Trypsin-EDTA 0.25% (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA)
- Chrysin, Hesperitin, Morin, Quercetin, Quercetrin, and Rutin (Sigma-Aldrich, St. Louis, MO, USA)
- Dibenzylfluorescein, (Sigma-Aldrich, St. Louis, MO, USA)
- HEPES and DMSO (Amresco LLC, Solon, OH, USA)
- KCl (Fisher Chemical, Fair Lawn, NJ)
- Liquid nitrogen (Faculty of Veterinary Medicine, Chiang Mai, Thailand)
- NADPH (Tocris Bioscience, Bristol, UK)

2. Methods

2.1 Microsome preparation

Nile tilapia hepatic microsome were prepared according to Iwata (1996) with some modified. Fish were anesthetized by hypothermic shock in the ice bucket and then killed by decapitation. Liver was quickly removed in to chilled 1.15% KCl solution, chopped into small pieces and then snapshotted in liquid nitrogen. The three volumes (w/v) of chilled 1.15% KCl solution was added into the homogenate of liver in Potter-Elvehjem homogenizer and homogenized by hand homogenizer in the ice bucket for 30 minutes. The homogenate was pooled prior to centrifugation at 10,000 x g, 4 ^{o}C, for 30 minute. The supernatant was collected and then was ultracentrifuged at 105,000 x g, 4 ^{o}C, for 1 hour. The pellet of microsome was obtained as indicated in Figure 2.1. Eventually, Microsomal fractions were divided into two parts. One part was quickly frozen at -20 ^{o}C and another one part was lyophilized before keeping in -20 ^{o}C refrigerator.

2.2 Fish microsome characterization

2.2.1 Protein determination

Protein concentration was determined by using NanoDrop™ 2000. Firstly, the instrument was calibrated by distilled water subsequently applied 2 µL of 2-fold serial dilution of samples on the pedestal. The protein concentration, contain aromatic side chains that influenced light absorption at 280 nm, were automatically calculated by the NanoDrop™ 2000 software.

2.2.2 SDS-PAGE analysis

Microsomal proteins were analyzed on 10% and 12% resolving acrylamide gel according to Laemmli (1970) containing 10% (w/v) SDS, 1.5 M Tris–HCl (pH 8.8), 10% (w/v) ammonium persulphate and TEMED. The stacking gel (4.8%) was madeby using 10% SDS, 0.5 M Tris–HCl (pH 6.8), 10% (w/v) ammonium persulphate and TEMED. The electrophoresis was accomplished at 100 Volt with running buffer (pH 8.3). The BenchMark^{TM} Protein Ladder (Cat. No. 10747-012) was used as molecular weight standards. The gel was stained with 0.5% coomassie Brilliant Blue R-250 in 45% (v/v) methanol, 10% (v/v) acetic acid for overnight and then distained in 20% (v/v)

methanol until the blue color in background was removed. The gel was washed twice with deionized water prior analyzing with Gel Doc EZ system (Bio-Rad Laboratories Inc., Hercules, California, United States).

2.2.3 SEM examination

The morphology of lyophilized microsome was visualized under vacuum lens of the desktop SEM, Phenom ProX (Crest Nanosolutions (M) Sdn Bhd, Kuala Lumpur, Malaysia).

2.2.4 Aromatase assay

Aromatase inhibition was quantified by measuring the fluorescent intensity of fluorescein, dibenzylfluorescein (DBF) product in black 96 well microplate as described by Stresser (2000). The 10 µl of extract or standard quercetin was pre-incubated with 100 µl of tilapia microsome for 60 minutes at room temperature and then, 10 µl of 20 µM DBF was added and incubated for 30 minute at room temperature. The 10 µl of 0.3 mM NADPH was added to the mixture and allow DBF generated fluorescence product. Finally, the signal was measured at 485 nm (excitation) and 530 nm (emission). The sample was done in triplicate and averaged to generate dose response curves. The extract or standard were used at least four different concentrations for IC_{50} values calculation. All extracts were dissolved in dimethyl sulfoxide (DMSO) and diluted to final concentration. The equal volume of DMSO was added to control wells (Endringer, 2008).

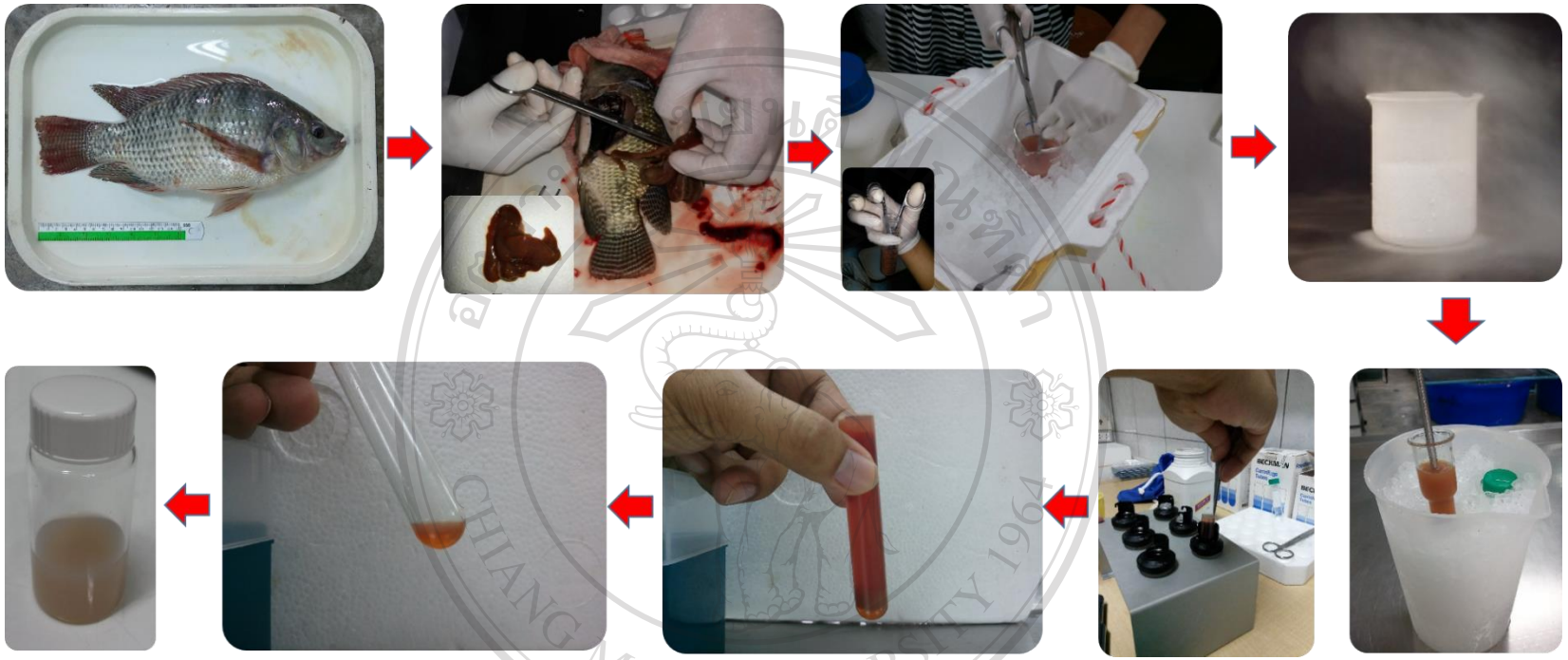

Figure 2.1 Microsome preparation process. Fish were anesthetized by hypothermic shock and then killed by decapitation. Liver were quickly removed in to 1.15% KCl solution, chopped and then snapshotted in liquid nitrogen. The chilled 1.15% KCl solution was added into the homogenate of liver in Potter-Elvehjem homogenizer and homogenized for 30 min in the ice. The homogenate was pooled and centrifuged at 10,000 x g, 4 ^{o}C, for 30 minute and then ultracentrifuged at 105,000 x g, 4 ^{o}C, for 1 hour. The pellet of microsome was obtained.

2.2.5 Evaluation of male and female microsomal enzymes.

Both freshly prepared and lyophilized microsomes of male and female were optimized in various concentration of aromatase inhibitor, quercetin, for checking the response of aromatase in the same conditions. The protocol was performed as described in 2.2.4 Aromatase assay.

2.2.6 Evaluation of the concentration of microsomal protein

The lyophilized microsome was dissolved in 10 mM HEPES buffer, pH 7.2-pH 8.0, to final concentration of protein at 700 µg/mL and prepared 5 serials by 2 fold dilution for examination of the effect of concentration of microsomal protein on various pH. The protocol was perform as described in 2.2.4 aromatase assay.

2.2.7 Evaluation of inhibitors on aromatase activity

The 1 mg of chrysin, hesperitin, morin, quercetin, quercetrin, and rutin were dissolved in DMSO and diluted 6 serials 2-fold dilution. The protocol was perform as described in 2.2.4 Aromatase assay. The inhibition of aromatase was calculated and expressed as percentage of inhibitory effect.

2.2.8 Determination of V_{max} and K_m values

The kinetic parameters K_m and V_{max} of aromatase activity were determined from Lineweaver-Burk plot optimal assay conditions (Lineweaver, 1934). The Lineweaver–Burk plot was widely used to examine enzyme kinetics by using non-linear regression software. The y-intercept of the graph a graph represents 1/ Vmax and the x-intercept represents $-1/K_m$. The reaction of aromatase on DBF in various concentration were examined every 60 seconds and generated curve by using Microsoft Excel 2013 software (Microsoft corporation, Redmond, Washington, United States)

2.3 Determination of plant ethanolic extract

2.3.1 Inhibitory activity effect of plant extracts on aromatase

The 1 mg of plant extract was prepared in 1 mL of DMSO. The protocol was perform as described in 2.2.4 Aromatase assay. The 5 serial 2- fold dilution was performed in triplicate and then generated inhibition curve with linear equation by Microsoft excel 2013 software.

2.3.2 Flavonoid content determination

The total flavonoids content of plant ethanolic extracts were determined by the aluminum chloride colorimetric method as described by Chang (2002). The crude extract were mixed with distilled water and then added 5% $NaNO_2$ solution, 10% $AlCl_3$ solution, respectively. After incubation for 5 min, NaOH solution was added, and adjusted the final volume to 10 mL with distilled water. The mixture was incubated at room temperature for 20 minutes and then measured the absorbance at 510 nm. The total flavonoid content was performed in triplicate and then calculated from the equation of quercetin calibration curve.

2.4 Cytotoxicity of fraction against HepG2 and MCF-7 cell lines

HepG2 (Human hepatoma; ATCC: 77400) and MCF-7 (Human breast carcinoma; ATCC: HTB-22) cell lines were kindly provided by Assist. Prof. Dr. Ariyapong Wongnoppavich (Department of Biochemistry, Chiang Mai University, Thailand). The cancer cells were maintained in DMEM medium added 10% of fetal bovine serum (FBS) in the presence of antibacterial and antifungal in 75 mL sterile flask and incubated at 37 ± 0.5 °C with 5% CO_2. Cytotoxicity was assayed by MTT technique. Briefly, Exponentially growing cells were harvested by trpysinized and washed in DMEM medium. The single cells were counted under microscope were seeded at 5 x 10^5 cell/well on 96-well plate. After 24 hr, various concentrations of extract were added and then incubated at the same condition for 20 hr prior replacing with FBS-free DMEM medium. The 50 μL of MTT were added and incubated for an additional 4 h. Finally, the 150 μL of DMSO were added to dissolve formazan crystals. The absorbance was measured in 570 nm by Beckman Coulter fluorescence multimode detector DTX 880. The IC_{50} values were determined by plotting the concentration of extract or fraction against cell viability. The cytotoxicity of standard chrysin on HepG-2 and MCF-7 was revealed in appendix B.

2.5 HPLC analysis

Chrysin concentration in medium was quantitated on Agilent 1260 infinity HPLC system (Agilent Technologies, Inc., Waldbronn, Germany). The separation could be achieved with Hypersil™ ODS C18 HPLC Columns (4.0 mm x 250 mm, 5-micron) (Agilent Technologies, Inc., Santa Clara, California, USA), using a mobile phase comprising methanol and 1M acetic acid in water (80:20). The separation was performed by injected 10 µL of sample with flow rate of 1.0 mL/minute at 25 °C, 270 nm. The chrysin content was calculated from the equation of chrysin standard curve in concentration range of 31.25-500.00 µg/mL as indicated in Appendix C.

Part III Development of Nanochrysin entrapment

1. Materials

Glassware

- Quartz cuvette (Malvern Zetasizer, Malvern Instruments, Malvern, United Kingdom)

Plastic ware

- Folded electrophoretic cells (Malvern Zetasizer, Malvern Instruments, Malvern, United Kingdom)

Instrument

- Malvern Zetasizer Nano ZS (Malvern Zetasizer, Malvern Instruments Ltd., Worcestershire, United Kingdom)
- Vortex genie-2 (Scientific industries, Inc., Bohemia, NY, USA)

Chemicals

- Acetic acid, Acetone, Span 60, Span 80 (AR grade), (RCI Labscan, Bangkok, Thailand)
- CelluSep T1 nominal MWCO 3500 (Membrane Filtration Products, Inc., Seguin, USA)
- Chrysin, Cholesterol, Deoxycholic acid, (Sigma-Aldrich Co., St. Louis, USA)
- Ethanol (Scharlau, Barcelona, Spain)
- HPβCD (Roquette America, Inc., Waterloo, Ontario, Canada)
- Methanol (Merck Darmstadt, Germany)
- PLGA (Purasorb® PDLG 5002, Purac Biomaterials, Gorinchem, Netherlands)
- Pluronic F-68, Pluronic F-127 (O-BASF Co., Ludwigshafen, Germany)
- Tween 20, Tween 80 (Namsian Co. Ltd., Bangkok, Thailand)

2. Methods

2.1 HPβCD entrapment

Chrysin loaded polymeric micelles were prepared by dissolving chrysin in ethanol. The drug solution was added dropwise with HPβCD solution to obtain the mixture of chrysin-polymer at the weight ratios of 1:1 (mole:mole). After that, Tween 80 was added. Deionized water was added to volume and the mixture was frozen at -20 °C and subsequently lyophilized under vacuum for 24 h. After lyophilization, the obtained dry chrysin loaded HPβCD micelles was re-suspended in deionized water to the desired drug concentration for further studies.

2.2 Poloxamer entrapment

Two types of poloxamers; Pluronic F-68 and Pluronic F-127 were used in this study. Chrysin loaded polymeric micelles were prepared by dissolving chrysin separately in two different organic solvents; acetone or ethanol. The drug solution was added dropwise with Pluronic F-68 or Pluronic F-127 solution to obtain the mixture of chrysin-polymer at the weight ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 1:10, and 1:15. After that, Tween 80 was added. Deionized water was added to volume and the mixture was frozen at -20 °C and subsequently lyophilized under vacuum for 24 h. After lyophilization, the obtained dry chrysin loaded Pluronic F-68 and Pluronic F-127 micelles namely CS-P68 and CS-P127, respectively, were re-suspended in deionized water to the desired drug concentration for further studies.

2.3 PLGA entrapment

Chrysin loaded polymeric micelles were prepared by separated dissolving chrysin and PLGA in acetone. The drug solution was added PLGA solution to obtain the mixture of chrysin-polymer at the weight ratios of 1:1, 1:2.5, 1:5, 1:7.5, 1:10, and 1:12.5 (w/w). After that, Tween 80 was added. Deionized water was added to volume and then top-up 2-fold volume before evaporation by hot air oven incubation, the obtained dry chrysin loaded PLGA micelles was re-suspended in deionized water to the desired drug concentration for further studies.

2.4 Bilosome entrapment

Vesicle Bilosomes were prepared by thin film hydration technique referred to Arunothayanun (1999). A molar ratio of non-ionic surfactants, cholesterol, deoxycholic acid and chrysin was used (as indicated in Table 2.1) referred to the method of Waddad (2013). The non-ionic surfactants (Span 60, Span 80, Tween 20 or Tween 80) and cholesterol were dissolved in an organic phase of chloroform-methanol (1:1 ; v/v). The organic solvent was eliminated by using rotary evaporator at 45 °C. Dried thin film was re-suspended with deionized water and swirling by hand shaking for 60 minutes. Bilosome was obtained after using bath sonicator for 10 minutes.

Table 2.1 Attribution of levels to factors in experiments

factors	**Level**		
	1	**2**	**3**
Amount of chrysin (mole)	1	1	1
Amount of non-ionic surfactant (mole)			
Span60 (for F1-F3)	1	2	3
Span80 (for F4-F6)	1	2	3
Tween80 (for F7-F9)	1	2	3
Tween20 (for F10-F12)	1	2	3
Amount of cholesterol (mole)	0.5	1.0	1.5
Deoxycholic acid (mole)	0.1	0.2	0.3

2.5 Entrapment efficiency

The nanoparticles were centrifuged at 12,500 rpm and collected the supernatant containing free drug for further analyzed by HPLC. The amount of drug value was unentrapped chrysin in the nanoparticles. The total amount of drug added to the formulation was subtracted by amount of drug found in the supernatant which gives the amount of drug entrapped in the nanoparticles (Govender, 1999). The formulations were evaluated for entrapment efficiency by the following formula: (Mass of drug in nanoparticle/ Mass of chrysin in formulation) x100.

2.6 PCS analysis

Size and size distribution (PDI) of nanoparticles were measured using Zetasizer Nano ZS working on the principle of photon correlation spectroscopy (PCS). A portion of 1 mL of the micelle dispersion in water was transferred into a quartz cuvette and exposed to laser light diffraction at an angle of 173°. The intensity of the peak that showed the highest population of the micelles of that size was recorded. Zeta potential of the micelles was measured using the same instrument by electrophoretical mobility in folded capillary cells and determined three times for each sample. Results were automatically calculated by the analyzer.

2.7 Chrysin releasing analysis

The release of chrysin was examined by a dialysis-diffusion method. Releasing of chrysin from nanoparticles was performed in 10mM HEPES buffer added 0.1% (w/v) tween 80 in 10 mM HEPES Buffer at different pH values (Yallapu, 2010), simulating the pH conditions of the fish blood pH (pH 7.6-8.0) and from the habitat water (pH 7.0), without enzymes. In detail, 0.5 mL of nanoparticles was taken into dialysis bag (regenerate cellulose membrane with a molecular weight cut-off and immersed into 25 mL of buffer and stirred at 100 rpm at room temperature. Every hour, Aliquots, 3 mL, of recipient solution was sampling and replaced with fresh buffer to volume. The concentration of chrysin in the different samples was measured by using HPLC.

2.8 *In vivo* toxicity study

The in vivo toxicity experiment was done using a method described by (Pamanji, 2015) with some modification. Briefly, 4-h of age fresh zebrafish eggs were gently filled into a series of 10 mL aqueous clear mixtures containing nanopaticles with chrysin concentrations of 1, 10, 100, 1,000 and 10,000 ng/mL. The incubation temperature was 28°C. The number of zebrafish eggs was 15 for each system. Water without any polymeric micelles was used as a control. The mortality of the embryos in each system was observed every 24 hours for a period of 72 hours under a stereo microscope (Nikon, Tokyo, Japan).

2.9 Statistical analysis

The preparation, size measurement, and toxicity study were done in triplicate and the results are expressed as mean ± SD Statistical analysis was done by using ANOVA and P-value at a level of 95% confidence limit.

Part IV Zebrafish Treatment

1. Materials

Glassware

- Pyrex ® petri dishes (Fisher Scientific, Pittsburgh, PA, USA)
- 1-L Aquaria with oxygen apparatus (Chiang Mai, Thailand)

Fish food

- TetraMin flake food (Tetra, Germany)

Chemicals

- MS222, Haematoxylin, and Eosin (Sigma-Aldrich, St. Louis, MO, USA)

2. Methods

2.1 Fertility check

The female zebrafish was allowed to mate with normal males individually. Each mating pair was housed in a chamber from the evening until the next day morning. The eggs was check for fertility by incubated for 24 hours at room temperature. Sexually mature females were used for treatments after checking for maturity. The 6-selected healthy zebrafish were put into the aquaria for further study on treatment for masculinization.

2.2 The masculinization process

The mature adult female zebrafish was performed as described by Takatsu (2013). Selected zebrafish were immersed into the water containing nanoparticles with chrysin concentrations of 10 or 100 ng/mL. The free vehicle was also used as the negative control in female group. The normal male and female zebrafish were housed at the same condition without nanoparticle. Each group was housed in separate small aquaria with a filtration system. All zebrafish were fed twice a day until 60 days at 28 ± 1 ^{o}C and a 14-hour light/ 10-hour dark cycle. All fish were terminated by using MS-222 prior gonad examination.

2.3 Histological study

Gonads were removed and fixed in formalin immediately. Fixation was allowed to proceed for a minimum of 24 hours before processed to wax embedded blocks as described by Feist (2004). Sections were cut into 3-5 μm by using rotary microtome, and then mounting on glass slides before drying and staining with haematoxylin and eosin (H&E), Stained sections were examined and captured under stereo microscope (Nikon, Tokyo, Japan).