# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Chemical Materials

Chemical used in this study were analytical and biotechnological grade purchasing from Bio Basic INC (Toronto, Canada), Fisher Scientifics (Manchester, UK), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Research Organics (Ohio, USA), Seikagaku Corporation (Tokyo, Japan), and Sigma (St. Louis, USA).

All of them were adenine sulfate, diisopropyl ether, dimethylsulfoxide, dextrose, 95% ethanol, 17β-estradiol, glucose, magnesium sulfate, β-mercaptoethanol, *o*rtho-nitrophenyl β-D-galactopyranoside, pentane, potassium hydroxide, potassium chloride, sodium carbonate, sodium dihydrogen phosphate, tamoxifen, yeast nitrogen base without amino acids, and Zymolyase 100T.

## 3.2 Plant Material

*Vanilla siamensis* Rolfe ex Downie growing in Khun Wang Royal Project Development Center, Mae Wang District, and Chiang Mai, Thailand was used in this work.. Mature vanilla beans were collected during the ninth month after pollination. The aerial parts were collected locally and the morphological characteristics were kindly identified by Mr. James F. Maxwell at Department of Biology, Faculty Science, Chiang Mai University, Thailand.

### 3.2.1 Preparation of Vanilla extracts

Fresh green beans of *Vanilla siamensis* Rolfe ex Downie (150 g) were cut to small pieces before they were extracted by Soxhlet extraction with pentane (500 mL, 16 h.). After removal of solvent by using rotary evaporator, the nonsaponifiable fraction was obtained by using the Itoh *et al*. procedure (Itoh, Tamura, & Matsumoto, 1973). The extracts were saponified with alcoholic potassium hydroxide (9 g of KOH in 100 mL 95% ethanol). The saponification was carried out at refluxing temperature under nitrogen for 1 h. The resulting solution (10 mL) was then diluted by distilled water (40 mL) and extracted with diisopropyl ether (30 mL, three times). The combined ether extract fractions were washed with a 10% aqueous sodium carbonate solution (4 mL, three times), then washed with distilled water to neutrality, dried, and evaporated. Stock solutions of the plant extracts were freshly prepared in dimethylsulfoxide (DMSO) at doses ranging from 1 µg to 1 mg/mL. The final concentration of DMSO in the test did not exceed 0.02%. The extracts were tested in both YES systems.

### 3.2.2 Gas Chromatography/Mass Spectrometry (GC/MS)

The extraction of *Vanilla siamensis* Rolfe ex Downie was performed using Gas Chromatography-mass Spectrometry Analysis (GC/MS). The separation of volatile compounds was accomplished using an Agilent 6890 GC (Agilent Technologies, Santa Clara, CA, USA) equipped with DB-Wax (30 mm length, 0.25 mm i.d., 0.50 µm film thickness; Agilent Technologies) columns, coupled with a 5973 NMS detector (Agilent Technologies) under electron impact ionization energy 70eV. The MS scan range was 30–500 atomic mass units (AMU). The carrier gas used was

helium at a flow rate of 1.0 mL/min. Samples were analyzed with the column held initially at 50˚C for 3 min and then increased to 230˚C with 10˚C/min heating rate. Finally temperature was increased to 230˚C and hold for 10 min. The injection was performed in split less mode at 230˚C. Generally, one must split the sample injection onto the column with an inlet injector splitter system. Split ratios of 100:1 are used for 0.2 microliter injections of 1 mg/mL diluted samples in DMSO. Compounds were identified on the basis of linear retention index, and EI mass spectra from the literature or from reference compounds. The identification of the individual components was done by Wiley mass spectral library on the basis of the mass fragments and e/Z values of each component.

### 3.3 Yeast strain and growth

The yeast *Saccharomyces cerevisiae* strain Y190 (*MATa, ura3-52, his3-200, ade2-101, trp1-901, leu2-3,112, URA3::GAL1-LacZ, LYS2::GAL1-HIS3 cyh*$^{r}$,; Clontech®, USA) harboring plasmid pGBT9-hERαLBD (*TRP1*), and pGAD424-hTIF2 (*LEU2*) coactivator, namely YES-hERα, and harboring plasmid pGBT9-hERβLBD (*TRP1*) and pGAD424-hSRC1(*LEU2*) coactivator , namely YES-hERβ, were kindly provided by Assoc. Prof. Chuenchit Boonchird, Department of Biotechnology, Faculty of Science, Mahidol University (Boonchird, Mahapanichkul, & Cherdshewasart, 2010).

#### 3.3.1 Incubation of yeast with estrogen compounds

Yeasts were maintained on synthetic dextrose minimal medium (SD) agar (0.67% yeast nitrogen base without amino acids, 2% glucose, and 1.5% agar). Yeast

strains were grown overnight at $30^0$C in a shaking incubator set at 200 rpm in 10 mL of SD broth supplemented with 0.002% adenine sulfate. To assay estrogenic activity of *V. siamensis*, the overnight culture YES-hERα or YES-hERβ was diluted in the appropriate SD broth to an $OD_{660}$ value of 0.1, and 50 µL of the diluted culture was incubated with 2.5 µL of the crude plant extract dose ranging from 1 µg to 1,000 mg/mL or 17β-estradiol ($E_2$, positive control) dissolved in DMSO a 1.5 ml-microtube containing 200 µL fresh SD broth supplemented with adenine. After incubation at 30°C with shaking for 4 hours, 150 µL of treated cells was dispened to each well of a 96-well microplate for the measurement of cell density at 660 nm using a microplate reader (Tecan Sunrise, Austria). Another 100 µL treated cells were centrifuged at 10,000 *g* and then resuspended in 200 µL Z-buffer (0.1 M sodium phosphate, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, and 3.5 mM β-mercaptoethanol) containing 0.2 mg/mL Zymolyase 100T. After yeasts were incubated at 37°C for 15 min, β-galactosidase activities were determined.

**3.3.2 β-galactosidase assay**

Zymoyase treated cell lysate was incubated with 40 µL substrate (4 mg/mL *o*rtho-nitrophenyl β-D-galactopyranoside in 0.1 M sodium phosphate buffer, pH 7.0) at 30°C for 30 min. When the yellow color of ortho-nitrophenol developed, 100 µL 1 M $Na_2CO_3$ was added to stop the reaction. To remove all cell debris, the reaction tube was centrifuged at 10,000 *g* for 5 min. The 150-µL supernatant was transferred to each well of a 96-well microplate and absorbance at 420 and 550 nm were measured with a microplate reader. β-galactosidase activity is reported in Miller units (Miller, 1972), using the following equation:

$$\text{Miller Unit} = 1000x\ ([OD_{420}]-[1.75x\ OD_{550}]/\ ([t]x[v]x\ [OD_{660}])$$

Where *t* was the length of incubation of reaction time (min), and *v* was the volume of culture used in assay (ml). $OD_{420}$ was the absorbance of the yellow *o*-nitrophenol at the end of reaction. $OD_{550}$ was the scatter from cell debris, which, when multiplied by 1.75 approximate the scatter observed at 420 nm. $OD_{660}$ was the cell density at the start of the assay. β-galactosidase activity of the liquid yeast cultures are expressed as the means and standard deviations of the results from three independent transformed yeast colonies.

### 3.3.3 Calculation of $EC_{50}$

Data of β-galactosidase unit and concentration of test compounds were fitted using a four-parameter logistic dose-response model with the aid of the SigmaPlot software, Version 9 for Windows (Systat Software, Inc., USA). The function was described as sigmoidal dose - response (variable slop) and $EC_{50}$ was calculated by using equation:

$$Y = (\text{BOTTOM} + [\text{TOP} - \text{BOTTOM}])/\ 1+10^{(\text{LogEC50-X})\text{-HillSlope}}$$

Where, Y was the response β-galactosidase unit value, which starts at Bottom and goes to Top with a sigmoid shape. X was the logarithm of compound concentration in the test, Top was the maximum response of β-galactosidase activity (ligand efficiency), and HillSlope was the relative slope of the middle region of the curve as estimated from a linear/log regression of the linear part of the dose-response curve. $LogEC_{50}$ was the sample logarithm concentration that causes 50% efficiency,

and Bottom was the detection limit. $EC_{50}$ value in the equation represents the ligand potency. By using this response curve, the estrogenic activity of the sample was expressed in equivalents of 17β-estradiol.

### 3.3.4 Calculation of Relative Potency

In order to compare each assay directly, the relative potency (Coldham *et al.*, 1997) were employed. The estrogenic relative potency (RP) of samples was computed by dividing the $EC_{50}$ of $E_2$ by the $EC_{50}$ of test samples, and then multiplying the values by 100 (the RP value of $E_2$ was 100 in the definition). The β-galactosidase unit, $EC_{50}$, and relative potency value compared with $E_2$ are reported as the mean ± SEM of at least three experiments.

## 3.4 Human fetal osteoblastic cell culture

Human fetal osteoblast cell line (hFOB 1.19 ATCC CRL-11372) was purchased from American Type Culture Collection (ATCC) .The hFOB1.19 cells were expanded in DMEM: F-12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 34$^{o}$C in a humidified incubator containing 95% air 5% $CO_2$. The culture medium was changed every 3 days. The confluent cells were subcultured to next passage using 0.25% trypsin-EDTA. *V. siamensis* was dissolved in dimethylsulfoxide (DMSO) and the final concentration of DMSO added to cells was lower than 0.1% (71). To evaluate the effects of *V. siamensis*, various concentrations of *V. siamensis*, (0.5, 1.0, 1.5, 2.5, 5.0 and 10.0 μg/mL) were added 24-h-starvated confluent cells. Control cells were cultured in

serum-free-DMEM and cells were harvested after 24-h-tratment. All treatments were performed in two different experiments done in duplicate.

### 3.4.1 Cell viability by MTT assay

Cell viability was assessed by 3-(4, 5-dimethlthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT). The hFOB1.19 cells were plated with the density 1.0 x $10^4$ cells per well into 24-well plates. After overnight culture, cells were treated with various concentrations of *V. siamensis* as described above for 24 and 48 h. At the end of treatment, 1.0 mL MTT solutions (0.5 mg/mL MTT dissolved in PBS) were added to each containing cells. The plate was incubated in a $CO_2$ incubator at 37$^o$C for 2 h. The medium was removed and formazan crystals dissolved in 1 mL of DMSO was added. After incubation for 10 minutes, absorbance was measured at 540 nm wavelength (reference wavelength 630 nm) using an automated microplate reader (Tecan Sunrise, Austria) (Ahmad *et al.*, 2006).

### 3.4.2 Mineralization Alizarin red-S staining

Approximately 1 x $10^4$ hFOB1.19 cells/cm$^2$ were plated in per well into 24-well plates (Nunc, Denmark) and incubated at 34°C for 24 h in normal culture medium. The 80% confluent cells were starved for 24 h. After starvation, the medium was then replaced with fresh medium containing the concentrations of 8 μg/mL of *V. siamensis* extract for 11 days. The degree of mineralization was determined in the dish using Alizarin Red staining. Briefly, medium was aspirated from the wells, and the cells were rinsed twice with PBS. The cells were fixed with ice-cold 70% (v/v) ethanol for 1 h. The ethanol was removed, and the cells were rinsed twice with deionized water. The cells were then stained with 40 mM Alizarin Red S in deionized

water (adjusted to pH 4.2) for 10 min at room temperature. The Alizarin Red S solution was removed by aspiration, and the cells were rinsed five times with deionized water. The water was removed by aspiration, and the cells were incubated in PBS for 15 min at room temperature on an orbital rotator. The PBS was removed, and the cells were rinsed once with fresh PBS. The cells were then distained for 15 min with 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0). The extracted stain was then transferred to a 96-well plate, and the absorbance at 562 nm was measured using an automated microplate reader (Tecan Sunrise, Austria).

### 3.4.3 Statistical analysis

The data were analyzed using Student's *t* test and significance was assigned at $p < 0.05$.