CHAPTER III

MATERIALS AND METHODS

1. Animals

Two-month old female and male Sprague-Dawley rats were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand. They were housed at the Primate Research Unit, Chulalongkorn University, Bangkok, Thailand in a room at 23 ± 2 °C with 12-h light/12-h dark cycles. They were fed with a standard rodent diet (C. P. 082, Lot No.17, S. W. T. Co., Ltd, Thailand) for 4.5 months or until they were 6.5 months old. Two weeks in advance and during experimental period, rats were kept in three animals/cage and fed a soybean-free rodent diet (C. P. 082/SBF, Lot No. 050119, S. W. T. Co., Ltd, Thailand) to minimize the phytoestrogen content in the diet. The rodent diet (C.P. 082) contains 26% soybean meal and 8% full-fat soybeans in term of total raw weight. The other basic ingredients of C.P. 082 and C.P. 082/SBF diets were yellow corn, rice, rice by-products, fish meal, corn gluten meal, vegetable oil, salt, vitamins and minerals. The composition of the standard rodent diet and soybean-free diet are shown in Table 3.1.

To minimize the increase of body weight caused by OVX in female rats (Wronski et al., 1987), the food consumption of OVX rats was adjusted weekly to the level of sham-operated female rats. In case of male rats, feed was given *ad libitum*. Water was supplied *ad libitum*. The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for care and use of laboratory animals prepared by Chulalongkorn University.

Table 3.1 Composition of the standard rodent diet and soybean-free diet

Ingredients	Percentage of diet	
	standard rodent diet	**soybean-free diet**
Moisture	6.69	5.41
Protein	27.6	26.3
Fat (Ether extraction)	2.45	2.92
Fat (Acid hydrolysis)	8.36	7.98
Fiber	3.69	2.14
Ash	5.98	4.71
Calcium	1.17	0.94
Phosphorus	0.93	0.83
Sodium Chloride	0.51	0.44
Vitamin D	4,000 i.u./kg feed	4,000 i.u./kg feed

2. Experimental design

At 7 months of age, female and male rats were grouped by means of body weight (BW) in 7 groups (nine rats/group for each sex) as follows;

1) Initial control rats autopsied on day1 (IC)
2) Sham control rats fed with distilled water (SH)
3) OVX or ORX rats fed with 0.1 mg/kg BW/day of 17 α-ethinylestradiol (EE)
4) OVX or ORX rats fed with distilled water (P0)
5) OVX or ORX rats fed with 10 mg/kg BW/day of *P. mirifica* (P10)
6) OVX or ORX rats fed with 100 mg/kg BW/day of *P. mirifica* (P100)
7) OVX or ORX rats fed with 1,000 mg/kg BW/day of *P. mirifica* (P1000)

The experimental period was three months. In initial control group (IC), nine rats were killed on the first day (day1) of the experiment and their data were kept as a base line control. In sham control group (SH), nine rats were sham-operated one day (day0) before experiment and kept as the age matched control and gavaged daily with

1 ml distilled water during experimental period. To perform the sham-operation in female rats, ovaries were exteriorized and replaced to create a stress similar to that obtained by a bilateral OVX. In male, the scrotal skin was cut and sutured to create a stress similar to that obtained with a bilateral ORX. In EE, P0, P10, P100 and P1000 groups, rats were bilaterally ovariectomized or orchidectomized on day0 of experimental period. The OVX was done during a diestrous period to keep the consistent lowest levels of sex hormones. The ORX was done when the rats were 7 months old. After OVX or ORX, rats were divided into five groups: EE, P0, P10, P100 and P1000 (nine rats/group). In the EE group, rats were gavaged daily with 0.1 mg/kg BW/day of 17 α-ethinylestradiol during three-month experimental period. In the P0, P10, P100 and P1000 groups, rats were gavaged daily with 0, 10, 100 and 1,000 mg/kg BW/day of *P. mirifica* in 1 ml distilled water, respectively. The rats were gavaged at 01:00-03:00 PM.

During the experimental period, rats were kept 3 rats/cage. Body weights of rats were measured weekly and used to adjust the quantity of *P. mirifica* or 17 α-ethinylestradiol treatments. At the end of three-month experimental period, all rats were sacrificed. The uteri in females, and seminal vesicles and ventral prostate glands in males were removed and weighed immediately. Their tibia, femur and lumbar vertebra were defleshed from the adjacent tissues, wrapped in saline-soaked gauze bandages to prevent dehydration, and stored frozen at -20°C in small Ziploc freezer bags until bone mineral density (BMD) and bone mineral content (BMC) were measured (Järvinen et al., 2003).

2.1 Blood collection

Blood samples were collected at day0 and day90 between 10:00-11:00 AM by cardiac puncture under ether anesthesia. After sampling, the blood was chilled on ice. Other than the schedule of day0 and day90, blood collections in female rats were also done at the diestrous phase which was defined by the vaginal cytology assay (Malaivijitnond et al., 2006). In male rats, blood collections were similarly carried out as above. Blood samples were centrifuged immediately at 2500 rpm for 15 min at

4°C. The separated sera were kept at -20°C until assayed for estradiol or testosterone levels by radioimmunoassay technique.

2.2 Preparation of *P. mirifica* suspension

The same lot of fresh tuberous roots of *P. mirifica* Cultivar Wichai-III (Lot No. 990611) which were collected from the field in Chiang Mai Province, Thailand was used throughout this study. The voucher specimen of *P. mirifica* (No. BCU 11045) was deposited at the Herbarium of Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. *P. mirifica* suspension was prepared as described previously (Cherdshewasart et al., 2004; Malaivijitnond et al., 2006). In brief, the collected tuberous roots of *P. mirifica* were washed, sliced and completely dried in a hot-air oven at 70 °C. The dried materials was ground into a powder at a size of 100 Mesh. The powder was kept in desiccators until used. The suspensions of *P. mirifica* were freshly prepared by suspending the *P. mirifica* powder in distilled water. The rats were gavaged daily with the suspension of *P. mirifica* at the dose of 10, 100 or 1,000 mg/kg BW in 1 ml of the solution. The dose range followed that in the previous study in which the low dose (10 mg/kg BW/day) had no uterothrophic or vaginal conrnification effects, and the middle and high doses (100 and 1,000 mg/kg BW/day) had mild and strong effects, respectively, on reproductive organs (Malaivijitnond et al., 2006).

2.3 Preparation of 17 α-ethinylestradiol solution

The 17 α-ethinylestradiol powder with a minimum purity of 98% HPLC (Lot No. 024K1196, Sigma, St. Louis, MO) was dissolved in minimal volume of absolute ethanol and then diluted with distilled water to give a stock concentration of 100 mg/ml. The solution was left to evaporate ethanol overnight and the solution was kept in a dark bottle at -20 °C. The 17 α-ethinylestradiol suspension was adjusted with distilled water to a concentration of 0.1 mg/ml and kept in a dark bottle at 4 °C until feeding time.

The 17 α-ethinylestradiol is generally used as an oral contraceptive for women. It is well absorbed after an oral administration. The elimination half-life was 6 to 14 hours (Roy et al., 2005). The oral dose of 0.1 mg/kg BW/day of 17 α-ethinylestradiol used in this study was previously reported to prevent bone loss in OVX rats (Frolik et al., 1996).

3. Vaginal smear checks

Female rats with 4-5 days of estrous cycle before the onset of study were chosen. Estrous cycle was monitored daily by vaginal cytology assay between 08:00-09:00 AM. The glass-rod was inserted into the vagina of rats and gently touched against the vaginal wall. The vaginal cells were smeared into a drop of 0.9% normal saline on the glass slide. The vaginal epithelium cells observed under the light microscope (x100) were classified into three types: leukocyte cells (L), nucleated cells (O) and cornified cells (Co). The representative cell type was determined by choosing the majority of cells. Proestrus was identified when the vaginal cells were nucleated epithelia cell type, and occasionally contaminated with cornified cells. The estrous stage was predominated with large squamous cornified cells. Metestrus had a large number of leukocytes and cornified cells. Mostly leukocytes and occasional nucleated cells were found in diestrus (Malaivijitnond et al., 2006; Feltenstein and See, 2007). The appearance of cornified cells (or the majority of Co-cell type) was also used as an indicator of estrogenic activity of 17 α-ethinylestradiol and *P. mirifica.*

4. Uterine, seminal vesicle and ventral prostate gland wet weights

Uteri of female rats, and seminal vesicles and ventral prostate glands of male rats were excised, and all connective tissue was removed prior to wet weight recordings. To ease the individual differences on body weight, the relative organ weight (%) was used. The relative organ weight (%) was obtained by the division of the organ weight by the body weight x 100.

5. Bone measurement

With the high precision and accuracy analysis on small bones, especially for rat bones, a peripheral Quantitative Computed Tomography (pQCT) was used (Ferretti, 1995; Sato, 1995). The advantage of pQCT is that it can measure a three dimension image of bone and can provide a real volumetic density (Sato, 1995; Österman et al., 1998; Banu et al., 2004).

Bone mineral density and content (BMD and BMC, respectively) were measured using pQCT in the research M mode (XCT Research SA^+, Stratec Medizintechnik GmbH., Germany) (Figure 3.1). This system has a 50 kV X-ray tube with a current of 0.307 mA as the source of a 0.5 mm-thick beam. The determination has been performed both in axial and long bones, in cortical and trabecular compartments, at metaphyseal and diaphyseal sites as follows;

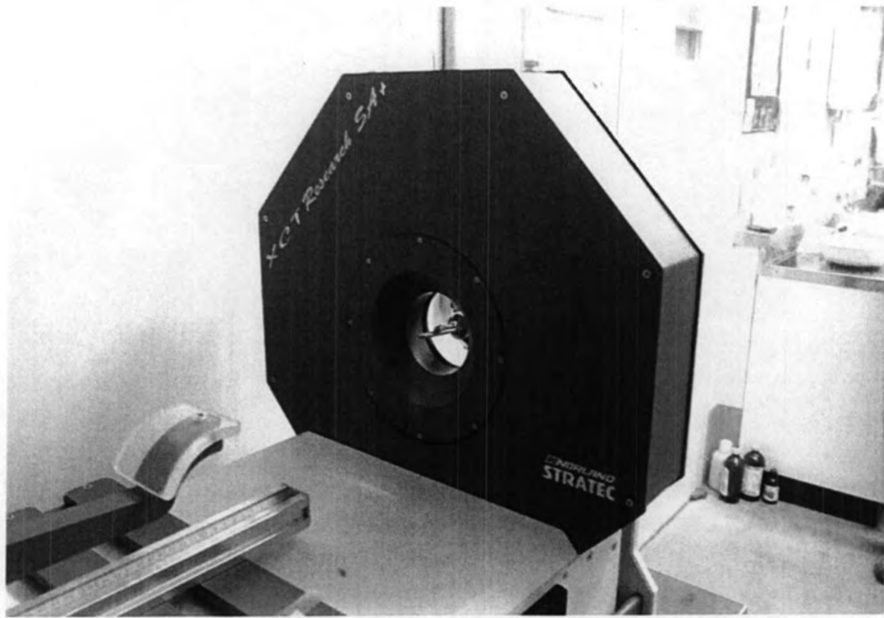
Tibia: Proximal tibial metaphysis (TM) was cross-sectionally scanned at 2, 2.5 and 3 mm below the growth plate of the tibia (Figure 3.2A and 3.3A). Tibial diaphysis (TD) was scanned at the midpoint (50% of the length of the tibia) and at both sides of the midpoint, 1 mm apart (Figure 3.2B and 3.3B).

Femur: Distal femoral metaphysis (FM) was cross-sectionally scanned at 2, 2.5 and 3 mm above the growth plate (Figure 3.2C) and femoral diaphysis (FD) was scanned at the midpoint (50% of the length of the femur) and at both sides of the midpoint, 1 mm apart (Figure 3.2D).

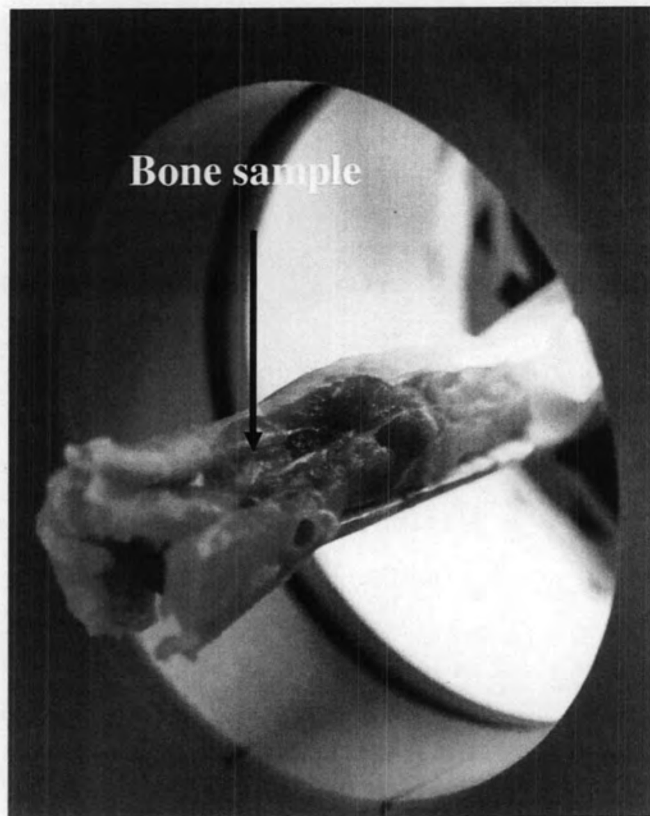
The fourth lumbar vertebra (L4): Fourth lumbar vertebra was cross-sectionally scanned at the midpoint of cranio-caudal longitudinal axis and at both sides of the midpoint, 1 mm apart from the midpoint along the longitudinal axis of the vertebra (Figure 3.2E).

The voxel size of each bone slice was 0.09 X 0.09 X 0.09 mm^3 and a slice thickness was 0.46 mm. Scan speed during CT scan was 3 mm/sec. The trabecular compartment was determined by the contour mode 2 and the peel mode 2 with the threshold value of 720 mg/cm^3, and the cortical compartment was determined by the

separation mode 2 with the threshold value of 900 mg/cm^3. Upon completion of the scanning, the following parameters were analyzed for each bone slice using XCT-5.50E software (Stratec Medizintechnik GmbH., Germany): trabecular bone mineral density (TbBMD), trabecular bone mineral content (TbBMC), cortical bone mineral density (CtBMD), and cortical bone mineral content (CtBMC) for the TM, FM and L4; and CtBMD and CtBMC for the TD and FD. The average of three scans made at all bone sites described above was analyzed. The procedure was slightly modified from Österman et al. (1998) and Banu et al. (2004).



(A)



(B)

Figure 3.1 Peripheral Quantitative Computed Tomography (pQCT) (A) and bone scanning by pQCT (B).

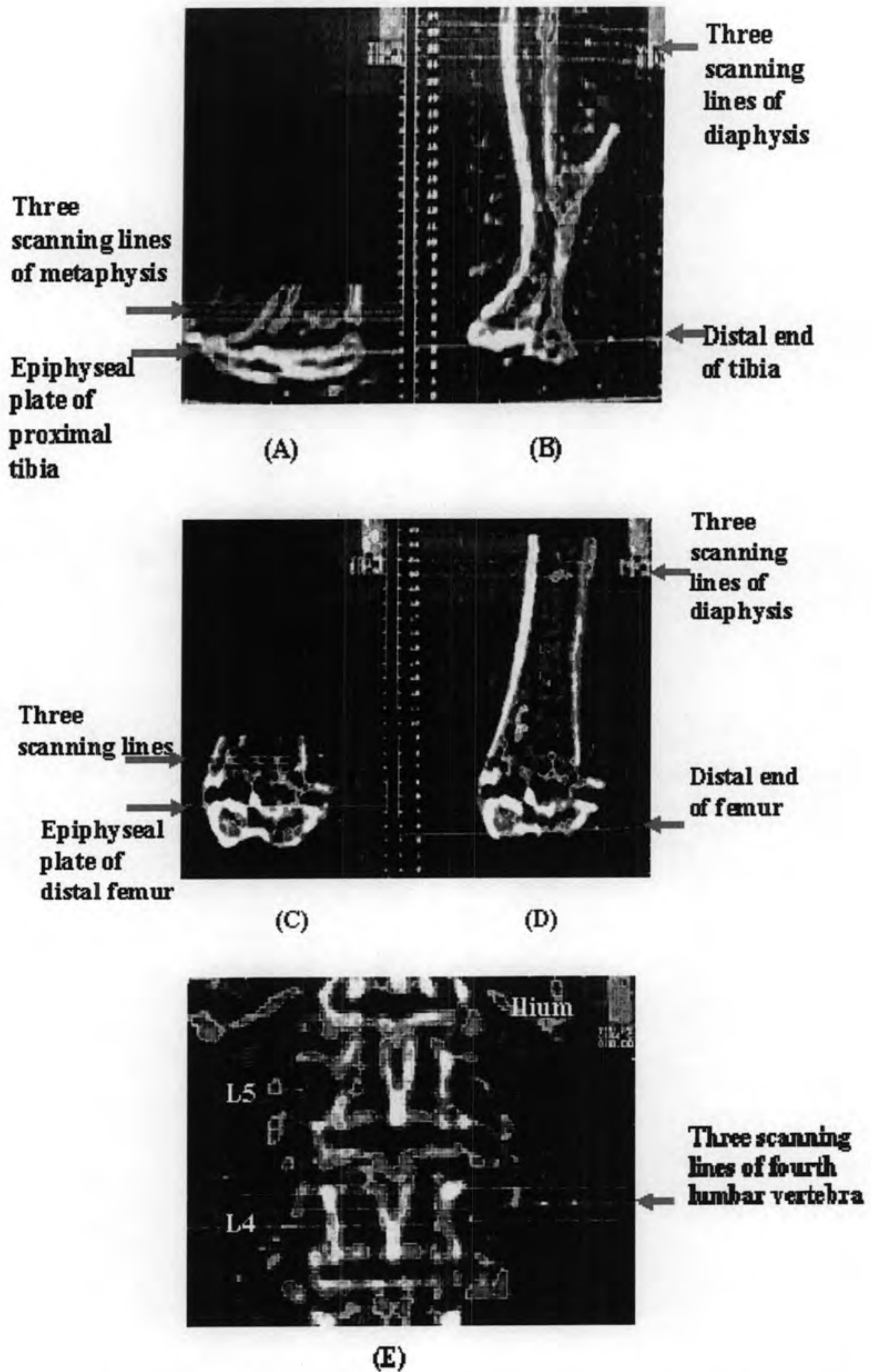
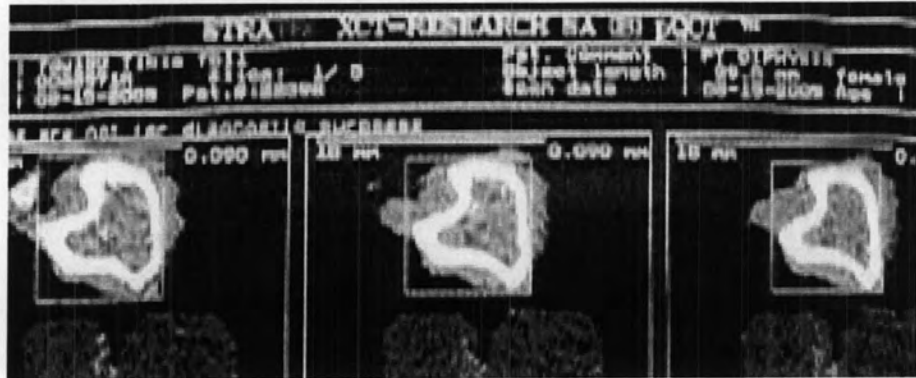
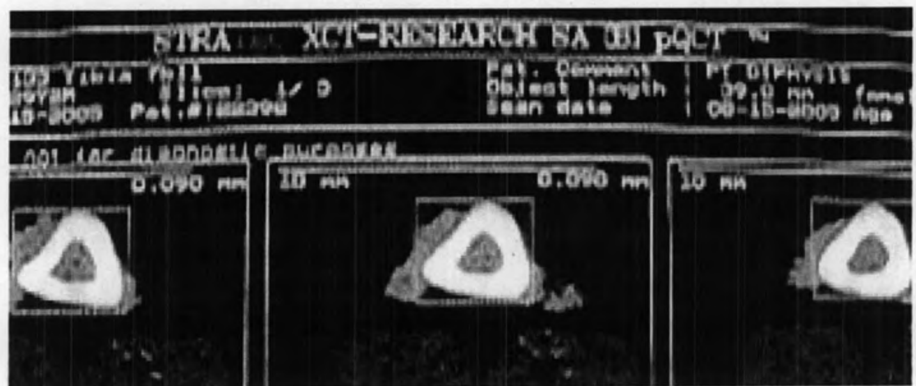


Figure 3.2 The longitudinal preview of bone scanning at metaphysis of proximal tibia (A), half in length of tibia (B), metaphysis of distal femur (C), half in length of femur (D), and half in length of fourth lumbar vertebra (E) using peripheral Quantitative Computed Tomography (pQCT).



(A)



(B)

Figure 3.3 A cross-sectionally scanned at the 2.0, 2.5 and 3.0 mm far from epiphyseal plate of proximal tibia (A) and the midpoint (50% of the length of the tibia) and both sides of the midpoint, 1 mm apart (B).

6. Bone histology

After rats were sacrificed, the left proximal tibiae were randomly selected for histopathological study. Bones were defleshed and placed in 10% phosphate-formalin buffer for at least 72 hours. Bones were cut to a small size and then decalcified in EDTA-G solution (EDTA disodium salt 14.50 g, NaOH 1.25 g, glycerol 15 ml and distilled water 100 ml) for 3 weeks by changing EDTA-G solution every week. After three weeks, the decalcified bones were dehydrated in series of ethanol gradient and clearing in xylene. They were then embedded in paraffin, cut into section of 5 μm thickness, and stained with Hematoxylin and Eosin (H&E). The slides were analyzed under the light microscope (ZEISS: Axiostar plus) and photographed using a digital camera (SONY: DSC-S85). The method of bone histopathological study was slightly modified from Miao and Scutt (2002).

7. Hormonal analysis

Serum estradiol (in female and male rats) and testosterone levels (in male rats) were analyzed by a double-antibody RIA system using I^{125}-labeled radioligands (estradiol RIA kit Lot No. 09305; testosterone RIA kit Lot No. 09305). The protocol was followed instruction provided by the Diagnostic Systems Laboratories, Inc, Texas, USA with a slight modification. After RIA processing, samples were counted for I^{125} –labeled radioligands using a gamma counter (model RAW-300, Shimadzu, Japan). To minimize the inter-assay variation, all samples in each group were run in a single assay. The intra-assay coefficients of variations of estradiol hormone were 8.50 and 8.59, respectively, for the high and low estradiol concentrations in female rats and 9.84 and 7.30, respectively, for the high and low estradiol concentrations in male rats. The intra-assay coefficients of variations were 7.65 and 5.52, respectively, for the high and low testosterone concentration.

8. Phytoestrogen analysis

Puerarin, daidzin, genistin, daidzein and genistein standards with purity of 80, 95, 95, 98 and 98%, respectively, were purchased from Sigma (St. Louis, MO, USA) and Fluka (Buchs, SG, Switzerland). Acetonitrile and acetic acid (High Performance Liquid Chromatography (HPLC) grade) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Absolute ethanol was purchased from Katayama Chemical (Osaka, Japan). Ultrapure distilled water was used.

The concentrations of five major isoflavone phytoestrogens mentioned above were determined in five lots of standard rodent diet and one lot of soybean-free rodent diet available in Thailand (S. W. T. Co., Ltd., Samutprakarn), and two lots of *P. mirifica*. Five lots of standard rodent diet were C.P. 082, Lot Nos. 2, 10, 18, 21 and 24 and one lot of soybean-free rodent diet was C.P. 082/SBF, Lot No. 050119. Two lots of *P. mirifica* Cultivar Wichai III were Lot No. 990609 (collected on June 9, 1999) and Lot No. 990611 (collected on June 11, 1999).

8.1 HPLC analysis

Contents of isoflavones, including puerarin, daidzin, genistin, daidzein and genistein were analyzed by the high performance liquid chromatography (HPLC) technique after samples were extracted as follows:

In the extraction step, samples were ground to be a homogenous powder (100 Mesh), and then 1 g of powder was mixed with 4 ml of 70% ethanol (Rostagno et al., 2004). The mixture was incubated in an incliner oven at 20 °C for 14 hours. After incubation, samples were centrifuged at 2800 rpm for 15 minutes and then the supernatant was collected and stored at -20 °C until analysis. The precipitation was again extracted with 4 ml of 70% ethanol in the same way as mentioned above, two more times, decreasing the incubation time to 6 hours. Supernatants collected from three extractions (200 µl each), were pooled together for a total of 600 µl and then dried up using centrifugal concentrator (Tomy, Japan) for 4 hours at room

temperature. Dry samples were re-dissolved in 200 μl of 0.4% acetic acid in ultrapure distilled water. The remaining supernatant from each extraction was used to evaluate the extraction efficiency.

In the HPLC analysis, a 5-ul injection volume of extracted solution was analyzed for five isoflavones using a high performance liquid chromatograph (HPLC, LC-9A, Shimadzu, Japan). Chromatography was performed in a column of 4.6 x 150 mm (ODS-80 TM, TOSOH, Japan) under an ambient temperature of approximately 17 °C. The mobile phase consisted of solution A (100 : 0.4 v/v of ultrapure distilled water : acetic acid) and solution B (100 : 0.4 v/v of acetonitrile : acetic acid). A linear gradient was maintained for 60 minutes from 20% to 100% of solution B in solution A with a flow rate of 1 ml/minute. The isoflavone content in the samples was analyzed by comparing the retention times and quantifying the amounts using standard curves of peak area of the isoflavone standards. Five isoflavone standards, puerarin, daidzin, genistin, daizein and genistein, were eluted at approximately 21.72, 24.77, 29.02, 34.58 and 39.06 minutes (Figure 3.4), as monitored by UV spectrophotometric detector at 260 nm (Shimadzu, Japan). Calibration curves were obtained for all isoflavones by plotting the standard concentrations as a function of peak area from HPLC analysis of a 5-μl injection volume using a Chromatopac machine (Shimadzu, Japan). The serial concentrations of standards at 0, 0.05, 0.1, 0.2 and 0.4 μg were chosen to cover the range of isoflavone concentrations in the samples. The analyses of the samples were run in duplicate for both extraction and HPLC analysis, and the data were averaged.

9. Statistical analysis

Results were reported as mean ± SEM for each group. The significant differences between groups were examined by one-way analysis of variance followed by Fisher's protected least significant difference test. The significant differences between each time point and the first time point in each group were examined by T-test. Differences were considered significance at the level of $p \leq 0.05$. The Statistical Packages for Social Science (SPSS) (version SPSS/PC 11.0, Chicago IL)

was used. Changes in bone parameters, BMD and BMC, with an advancing age were expressed by comparisons between IC and SH groups. The influence of OVX in female and ORX in male rats on bone parameters were evaluated by comparisons between P0 and SH groups. The effect of *P. mirifica* or EE treatments on bone parameters are expressed by 'the percent prevention' calculated using the following formula:

Percent prevention = (Mean value of treatment group - Mean value of P0 group) x 100 / (Mean value of SH group - Mean value of P0 group).

In case of non-significant difference between mean values of SH and P0 groups, changes of BMD and BMC in *P.mirifica* or EE treatment groups are expressed as 'the percent change' of the P0 group which was calculated using the following formula:

Percent change = (Mean value of treatment group – Mean value of P0 group) x 100/Mean value of P0 group

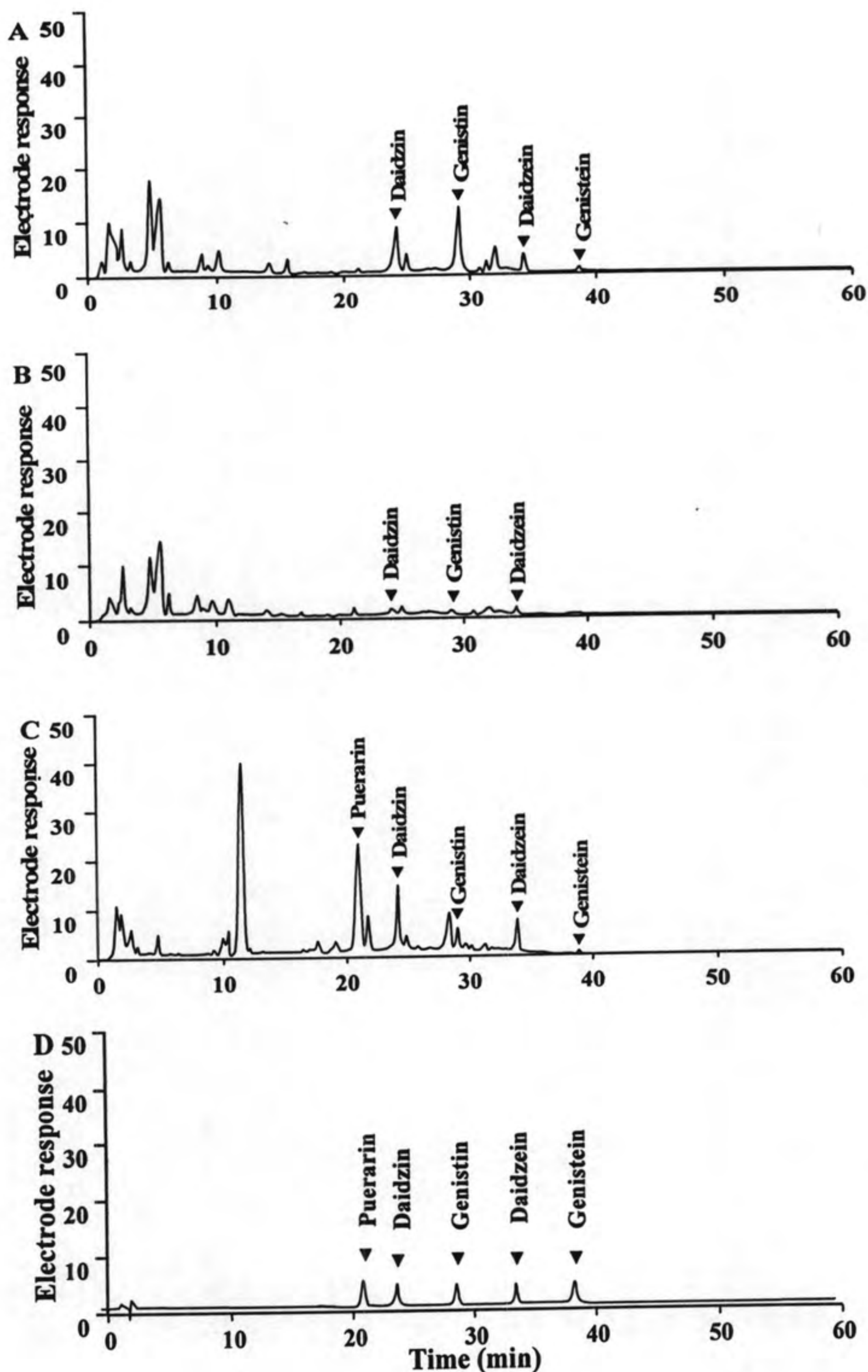


Figure 3.4 The HPLC fingerprint of isoflavone contents in standard rodent diet (C. P. 082 Lot No.3) (A), soybean-free rodent diet (C.P. 082/SBF) (B), *Pueraria mirifica* (Lot No. 990611) (C) and 0.1 μg of each isoflavone standard (D). The peaks were recorded by a UV spectophotometric detector at 260 nm.