CHAPTER III

RESEARCH METHODOLOGY

Instruments and materials

1. Instruments

1.1 Automated Tissue Processor (Shandon)

1.2 Centrifugation machine (HERMLE Labortechnik, CE)

1.3 Embedding (Kunz instruments, Shandon)

1.4 Hood

1.5 Light microscope (Olympus CX31,Olympus)

1.6 Microtome (cut 5062, SLEE mainz)

1.7 pH meter (Eutech instruments, WPA)

1.8 Slide warming plate (Kunz instruments, Shandon)

1.9 Tissue floating bath

1.10 Vernier caliper

1.11 Weighing machine (CP224S, Sartorius)

2. Materials & Reagents

2.1 Absolute Ethyl alcohol (Mallinckrodt Chemicals)

2.2 Atropine sulfate (T.P. Drug laboratories CO., LTD.)

2.3 Beakers

2.4 Cassettes

2.5 Centrifuge tube (Corning®)

2.6 Chromic catgut (Ehicon)

2.7 Disposable syringes (Nipro)

2.8 Distilled water (DW)

2.9 Eosin solution

2.10 Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) (Sigma- aldrich®)

2.11 Formaldehyde solution (UNIVAR, Ajax Finechem Pty Ltd.)

2.12 Gauze packs

2.13 Glycerol

2.14 Hypodermic needle (Nipro)

2.15 Microscope slides

2.16 Microtome blade

2.17 Microtube (Hycon)

2.18 Modified hematoxylin solution

2.19 Nembutal® (CEVA SANTE ANIMALE)

2.20 Normal saline solution

2.21 Permount®

2.22 Propylene glycol (TTK science, CO.)

2.23 Sodiumdihydrogen phosphate monohydrate (MERK)

2.24 Sodium hydroxide (Univar, APS)

2.25 Tissue embedding medium (Paraplast plus, McCormick scientific)

2.26 Xylene (RCI LabscanLimitted)

2.27 95% Ethyl alcohol (ETHAL 95)

2.28 17α-Ethynylestradiol, minimum 98% HPLC (Sigma-aldrich, CO.)

Methods

1. Plant material

In this study, the roots of AR were collected from Rayong province, Thailand. The voucher specimen (collection no. RKT 0005) was kept at Faculty of Pharmaceutical Sciences, Naresuan University, Phitsanulok, Th ailand and at PBM herbarium, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

2. Plant extraction

The roots of AR were air-dried at room temperature and then dried at 45 °C in a hot air oven for 24 hour before being milled into coarse powder. The dry powdered roots of AR were first macerated at room temperature with hexane for 3 days. Then, residue was macerated with 95% ethanol for 3 days. After that, it was filtered and the residue was extracted again using the same procedure. The filtrates were pooled and concentrated using a rotary evaporator to yield the crude defatted ethanolic extract.

During treatment, the crude of AR ethanolic extract was mixed with propylene glycol (PG) to a working suspension of 100 and 1000 mg/kg B.W. The suspension was administered to the rats during 0700-0800 h.

3. Animals

Sixty-day-old female Wistar rats were purchased from the National Laboratory Animal Center Mahidol University, Nakhon Prathom, Thailand. The rats were acclimatized for at least two weeks before the experiment. They were housed in the animal room with control lighting (lights on at 06:00-18:00 h) in which the temperature was maintained at 25 ± 2 °C. All rats were fed with a standard rat diet (C.P.082, LOT NO.9, S.W.T Co. Ltd, Thailand) and water ad libitum. The experiment protocol was approved by the Ethical committee for the Use of Animal, Naresuan University.

4. Experimental protocol

At the beginning of the experiment, ninety-day-old female Wistar rats with average weights of 250-300 g were randomly divided into five groups:

1. SHAM group (n = 6): the rats were gavaged daily with propylene glycol (PG) as sham operated control.

2. OVX group (n = 6): the OVX rats were gavaged daily with PG as the vehicle control. This group was an OVX control group.

3. OVX+AR100 group (n = 6): the OVX rats were gavaged daily with AR at the dose of 100 mg/kg B.W. as a treatment group.

4. OVX+AR1000 group (n = 6): the OVX rats were gavaged daily with AR at the dose of 1000 mg/kg B.W. as a treatment group.

5. OVX+17α-ethynylestradiol (EE) group (n = 6): the OVX rats were gavaged daily with EE at the dose level of 0.1 mg/kg B.W.. as a positive control.

The treatment continued (for 90 days) and throughout the experimental periods, all rats were weighed once a week.

Ninety-day-old adult female Wistar rats with regular estrous cycles (4-5 days) for three consecutive cycles before the study period were used. The ovariectomy was done during a diestrous phase to keep the consistent lowest levels of the hormones in rats. In all OVX groups, rats were bilaterally ovariectomized under pentobarbital anesthesia (50 mg/kg B.W.) in aseptic technique. Ovariectomy was performed by

ligation and excision of the ovaries. In the SHAM group, the ovaries were exposed and gently manipulated without excision.

The treatment schedule was separated into the following periods: recovery period for 15 days, treatment period for 90 days, and the end of treatment (Fig 16). After OVX, rats were kept for a fifteen days recovery period before submitting to the treatment. In the treatment period, the SHAM rats were gavaged daily with vehicle and the OVX rats were gavaged daily with vehicle, AR (100 and 1000 mg/kg B.W.) or EE (0.1 mg/kg B.W.), respectively.

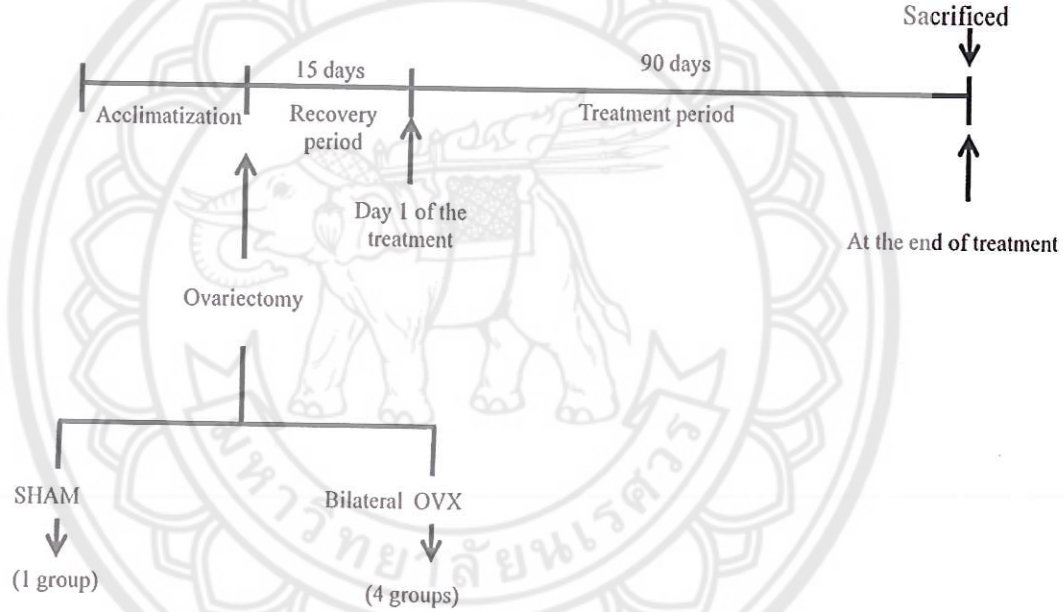

Figure 16 Experimental schedule

After 90 days of the treatment, all rats were anesthesized using pentobarbital. Blood samples were collected by cardiac puncture for biochemical analysis. The uterus and right femur of all rats were weighed. The femoral length, defined as the distance between the greater trochanter and the medial condyle, was measured in the right femurs using vernier caliper. The thickness of the same femur was measured at metaphyseal region using vernier caliper. The left femur, mammary gland and uterus

of all rats were fixed in 10% (w/v) neutral buffered formalin solution and manipulated for histological examination.

5. Vaginal cornification assay

The vaginal epithelium was checked daily between 0800 and 0900 h with the disposable pipette and soaked in 0.9 % normal saline solution before use. The disposable pipette was pushed gently into the entrance of the vagina to a depth of 2-5 mm and the fluid was flushed into the vagina and backed up into the pipette by gently squeezing. The vaginal cells were smeared onto a microscopic slide. Then, the vaginal epithelial cells were observed under a light microscope. The vaginal epithelial cells were classified into three cell types: leukocyte cells, nucleated cells, and cornified cells. The appearance of cornified cells was used as an indicator of estrogenic activity. The large numbers of leukocytes and small numbers of cornified cells were used as metoestrus phase. Di-estrus phase was consisted mainly of leukocytes. Pro-estrus phase was characterized by nucleated cells in low to moderate numbers.

6. Preparation of 17α-Ethynylestradiol solution

The 17α-Ethynylestradiol powder (Sigma, St. Louise, MO) was dissolved in a small volume of absolute ethanol. The powder was dissolved and then the DW was added. After that the solution was allowed to stand at room temperature to evaporate the ethanol. The stock solution was kept in the dark bottles at 4°C (Urasopon, et al., 2007).

7. Biochemical analysis

Blood samples from all rats were collected by cardiac puncture method at the end of 90-day experimental period to analyse blood biochemistry. Blood samples were prevented clotting by heparin and the serum was separated by centrifugation at 1000 rpm for 10 minutes at room temperature. Serum was kept at -80°C until analysis. The calcium (Ca), inorganic phosphorus (Pi), and alkaline phosphatase (ALP) were measured by assessing with automated analytical system at Central Laboratory Unit, Naresuan University Hospital. Serum estradiol (E_2) level was measured using electrochemiluminescence immunoassay (ECLIA) with Elecsys 2010 automate analyzer (Roche Diagnostics GM6H, Mannhein, Germany) at Faculty of Medicine, Chiang Mai University Hospital. The determination of β-CrossLaps (β-CTx) and total procollagen type 1 amino-terminal propeptide (P_1NP) were carried out by

immunoassay using autoanalyzer (Elecsys, Cobas®) at the Clinical Pathology Unit, Buddhachinaraj Hospital.

8. Histological analysis

At the end of treatment (day 90), all rats were sacrificed under anesthesia. The uterus, mammary gland and left femur were removed, kept in the fixative (10% neutral buffered formalin) at least 72 hours. Before tissue processing, bone specimens were cut to a small size and then decalcified with EDTA-G solution. The method of bone decalcification was slightly modified from Mio and Scutt (2002) and Urasopon et al. (2008). After completing the fixation, the bone tissues were treated with EDTA-G solution (pH 7.4) at 4 °C. Tissues were left standing for 3 weeks in EDTA-G to remove the calcium content. The EDTA-G solution was changed every week. The decalcified bones, formalin fixed uterus and mammary glands were dehydrated in an ethanol series and embedded in paraffin wax. All block was then cut into section of 5 µm thickness and stained with H&E according to standard protocol, for assessment of histological changes. The slides were analyzed by light microscope (Nikon Eclipse 80i, Nikon). The representative section was photographed. All images were captured with a digital photo camera that attached to the Nikon Eclipse 80i microscope.

9. Histomorphometric analysis

The histomorphometric analysis was carried out by using the image analysis software (Image J 1.44, Inc. Bethesda, MD USA). Parameters measured included the trabecular thickness, intertrabecular space, trabecular area and cortical thickness for bone histomorphometric analysis. The thickness of uterine layers and glandular area of mammary gland was measured as an index of proliferative effect.

9.1 Bone histomorphometric analysis

The sections were stained with H&E and examined for morphology under a light microscope. The bone tissue of the distal femur was photographed under low magnifications (4x). The bone tissue of the distal femur was determined at metaphyseal region. Measurements of trabecular bone microarchitecture were performed by four different methods; trabecular thickness, intertrabecular space, percentage of trabecular bone in relationship to the total area in distal femur and cortical thickness were measured and calculated.

Trabecular thickness: Trabecular thickness is one of the main morphometric parameters used to describe cancellous bone architecture. Thickness of trabecular bone has traditionally been measured using model based histomorphometric methods on sections. Trabecular thickness was defined as the average thickness of trabeculae on lines drawn orthogonal to the trabecular surface. Trabecular thickness was measured by line intercepts with gridlines. This parameter was measured according to Recker (1993).

Intertrabecular space: Intertrabecular space was also measured according to Recker (1993). Intertrabecular space was defined as the average distance between trabecular bones on lines drawn orthogonal to the marrow surface that measured by line intercepts with gridlines.

Trabecular area: The total area of bone comprises of fat, bone marrow and trabecular bone tissue. Through the image analysis software, the trabecular bone tissue was marked and measured with the assistance of a digital cursor pen. For the calculating the percentage of trabecular bone area, the total area was considered as 100%. The trabecular bone tissue area was converted into proportion of bone tissue according to the following formula: % of trabecular bone tissue = area measured x 100/ total area (Lucinda, et al., 2010). After the calculation of the proportion of bone tissue, the average of the proportions of the distal femur was established for further statistical processing.

Cortical thickness: Cortical thickness was measured as the orthogonal intercept from pericortical (outer) to the endocortical (inner) surface and was a measure of the mean cortical thickness of femur. The thickness was measured in the middle of the shaft. For each cortex three intercepts with gridlines were measured. This parameter measured for cortical bone has been described by Lind, et al. (1999).

9.2 Uterine histomorphometric analysis

The H&E stained sections were used to identify the location of each layer of endometrium and myometrium. Histomorphometric measurements of uterus included the thickness of endometrium and myometrium. Endometrium thickness was measured in the transverse plane at the point of greatest uterine diameter; the ventral and dorsal thickness of the endometrium were measured at an objective magnification of 4x, and an average thickness was derived for each animal

(Cline, et al., 2004). In each section, the endometrial thickness was determined by measuring the distance from the epithelium-endometrium junction to the endometrium -myometrium junction. Also, the thickness of myometrium was determined by measuring the distance from the endometrium-myometrium junction to the endometrium-perimetrium junction. The thickness measurement of endometrium and myometrium, which are the relative thickness these structures occupy in the tissue, were determined using image analysis software. Image analysis calculated the thickness of endometrium and myometrium.

9.3 Mammary gland histomorphometric analysis

The quantitative study of the mammary glandular area was described by Kapur, et al. (2008). All morphometric measurements were done on H&E stained slides, using a computerized image analysis system. For each animal, slide was selected and examined at a magnification of 4x. The mammary glandular area was accorded to this formula: % of mammary glandular area = area measured x 100/ total area.

10. Statistical analysis

The data was expressed as the mean ± standard error of the mean (S.E.M.). Comparison of the organs weights collected at the end of treatment, femoral parameters, and serum biochemical markers between the SHAM, OVX, AR and EE groups was analyzed by one-way analysis of variance (ANOVA) with post hoc test using LSD test. Significant differences were assumed at $p < 0.05$, $p < 0.01$ and $p < 0.001$.