CHAPTER 2

MATERIALS AND METHODS

2.1 Apparatus and chemicals

Apparatus

1. Analytical balance (three digits), OHAUS, U.S.A
2. Analytical balance (four digits), OHAUS, U.S.A
3. Atomic absorption spectrophotometer with Zeeman background correction equipped with hollow Cd cathode lamp and autosampler, Varian SpectraA800Z, Australia
4. Autopipettes (1-10, 5-50, 10-100, 20-200 and 100-1,000 μL), Socorex, Switzerland
5. Centrifuge tube 15 mL, Corning Incorporated, Mexico
6. Clinical Chemistry Analyzer, Hitachi 917, Japan
7. Disposable needle No.20, Nipro, Japan
8. Disposable syringe (5, 10 mL), Nipro, Japan
9. Disposable tuberculin syringe plus needle, Nipro, Japan
10. EDTA tube, Cellab, Australia
11. Erlenmeyer flask 50 mL, Kimax, U.S.A
12. Erlenmeyer flask 125 mL, Pyrex, Japan
13. Filter paper No.4, Whatman, UK
14. Glass column chromatography, Thailand

15. Glass pasteur pipette 150 mm, Marienfeld, Germany
16. Glass pasteur pipette 225 mm, Isolab, Germany
17. Laboratory bottles (250, 500 and 1000 mL), Duran, Germany
18. Light microscope, Olympus, Japan
19. Lyophilizer, CHRIST®, Germany
20. Microcentifuge tube 1.5 mL, Hycon Plastic Inc., U.S.A
21. Metabolic cage, TECHNIPLAST®, Italy
22. Nuclear magnetic resonance spectroscopy (NMR), Bruker Analytische GmbH, Rheinstetten, Germany
23. Pipette tips (1-200 and 1000 µL), Axygen, U.S.A
24. Rotary evaporator, BUCHI, Japan
25. Round bottom flasks (50, 100 and 500 mL), Duran, Germany
26. TLC aluminium sheet 60F254 20×20 cm, Merck, U.S.A
27. Vortex mixer, model VM-300, Taiwan

Chemicals

All solvents used in the isolation, identification and characterization of the *T. laurifolia* leaf extract were distilled and all glassware was cleaned with acetone to prevent contamination before use.

All solvents used in Cd quantification were AAS grade. Cd standard was purchased from Sigma-Aldrich, Germany. All plastic ware and glassware were cleaned up with 20% HNO_3 to prevent contamination.

Other chemicals and reagents were analytical grade and purchased from local agency. A list of chemicals and solution preparation were shown in Appendix A.

2.2 Obtaining of *T. laurifolia* Lindl. leaves

Fresh, mature leaves of *T. laurifolia* were collected from Ob Khan National Park, Hangdong District, Chiang Mai Province, Thailand. Taxonomy of the *T. laurifolia* leaf was identified at the Queen Sirikit Botanic Garden, Mae Rim District, Chiang Mai Province. The leaves were washed with tap water, dried and ground to course powder and stored in amber glass bottles at room temperature before extraction.

2.3 Extraction of *T. laurifolia* Lindl. leaves

T. laurifolia leaf powder was soaked in boiled distilled water (1:10 W/V) for 1 hour then filtered through three layers of gauze followed by Whatman No.4 filter paper. The filtrate was lyophilized and stored in a dessicator at 4°C. The extract was redissolved in distilled water to desired concentrations just prior to use.

2.4 Isolation of the *T. laurifolia* Lindl. leaf extract by column chromatography

Column chromatography is a method used to purify individual chemical compounds from mixtures of compounds. The classical preparative glass chromatography column (diameter 50 mm and 1 m height) with a glass stop cock at the bottom was ordered from local made and used in this experiment.

Firstly, the column was filled with approximately 200 g of dry silica gel 60 (0.063-0.2 mm). It was used as an absorbent. The column was added with 95% ethanol extract of *T. laurifolia* leaf (prepared by dissolved *T. laurifolia* leaf extract 15 g with 95% ethanol 500 mL and mixed with silica gel 60 approximately 1 g and

dried the extract by an evaporator) on top of the adsorbent and eluted the constituents in the plant extract with 800 mL of hexane:ethyl acetate at the ratio 100:0, 80:20, 60:40 and 20:80 respectively followed by 800 mL of ethyl acetate:methanol 0:100, 80:20, 60:40, 40:60 and 20:80, respectively. Each fraction was collected and evaporated before running on TLC and visualized under ultraviolet light at the wavelength of 254 nm. Each fraction was characterized by ^{1}H NMR.

2.5 Characterization of *T. laurifolia* Lindl. leaf extract constituents by NMR

Sample preparation

Each fraction of *T. laurifolia* leaf extract was dissolved in deuterated solvent. Fraction 1, 3, 4, 5, 6 and 7 used $CDCl_3$ and fraction 13, 14 used DMSO-d_6 before transferring to a 5-mm NMR tube for analyzing in the ^{1}H NMR spectroscopy.

Procedure of analysis

The principle of NMR involved two steps. First, the machine is aligned the polarization of the magnetic nuclear spins in an applied and constant magnetic field (H_0). Second, the alignment of the nuclear spins is purturbed by employing an electro-magnetic, usually radio frequency (RF) pulse. The required perturbing frequency is dependent upon the H_0 and the nuclei of an observation.

In this study, the ^{1}H NMR spectra was acquired on a Bruker Advance 400 MHz spectrophotometer. The spectra of each fractions were acquired with zg30 or water suppression from the Bruker pulse program. Data was obtained over 8,278 Hz sweep width and digitized with 60 k complex data points. Spectra was primarily

processed in the frequency domain using XWINNMR (Bruker GmBH). The free induction decays (FIDs) were multiplied by an exponential function corresponding to 0.3 Hz line broadening.

2.6 Animal experiments

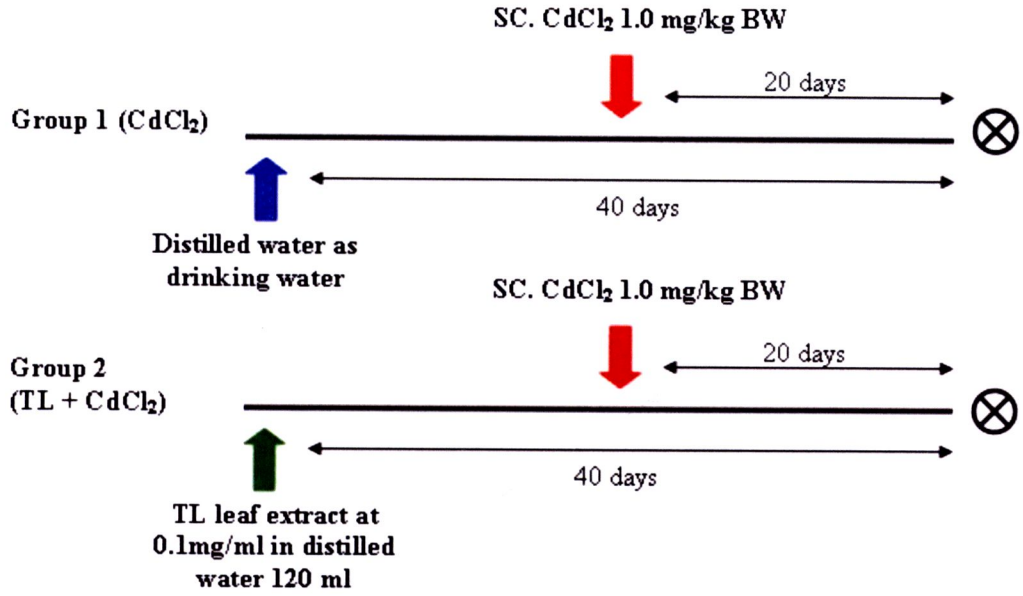
Twenty eight adult male Wistar rats (200-250 g) were used in this study. The rats were acclimatized under controlled experimental conditions at room temperature of 25 ± 2°C with 12 hr light and 12 hr dark cycle and humidity of 50 ± 10% for one week before experiments. They had free access to drinking water and standard rodent pellets throughout the experiment.

T. laurifolia Lindl. leaf extract preparation

T. laurifolia leaf crude extract and the major or selected fraction of *T. laurifolia* leaves dosages were calculated and prepared presumably as if the dosage of human in daily drinking tea for one cup a day. Therefore, approximately 0.1 mg/mL for the studying effect of *T. laurifolia* leaf crude extract or 0.02 mg/mL of the *T. laurifolia* leaf isolated fraction were prepared and feeding via drinking water to rats before and during $CdCl_2$ administration.

Animal experiment 1: Studying the effect of T. laurifolia Lindl. leaf crude extract on cadmium induced hepatorenal toxicities

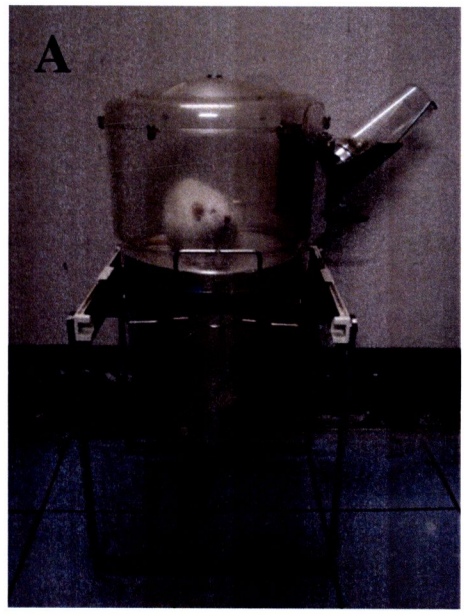
Twelve rats were randomized into two groups of six and treated as the following scheme;



SC. = Subcutaneous injection, TL = *T. laurifolia* leaf extract, ⊗ = Termination

Rats in group 1 were positive control and provided distilled water without *T. laurifolia* leaf extract for 20 days, then treated with daily subcutaneously injection of $CdCl_2$ solution (1.0 mg/kg) in isotonic saline for 20 more days. The treatment group (group 2) was provided *T. laurifolia* leaf extract (0.1 mg/mL) in drinking water for 20 days prior to the commencement of the $CdCl_2$ treatment (same dose) for 20 more days and the *T. laurifolia* leaf extract supply in drinking water was continued throughout the experiment.

The body weight and water consumption of each rat were measured daily. Twenty four hour urine samples were collected using metabolic cages from each rat on three occasions; Day 0 or 1; Day 20; and Day 40 (Figure 4A) for determined urinary creatinine and urinary Cd concentrations. At the end of experiment (day 40) all rats were anesthetized with sodium thiopenthal solution and blood sample was collected by cannulated via jugular artery (Figure 4B).



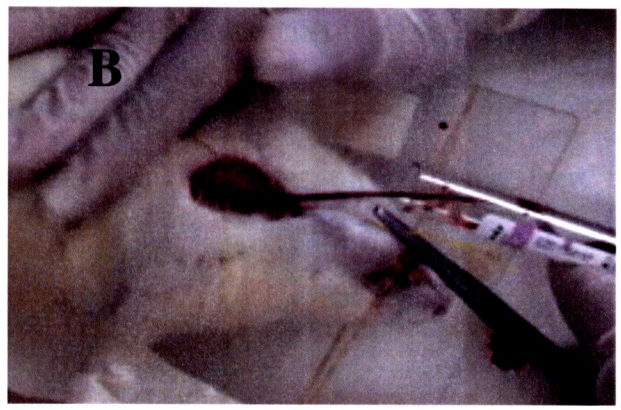
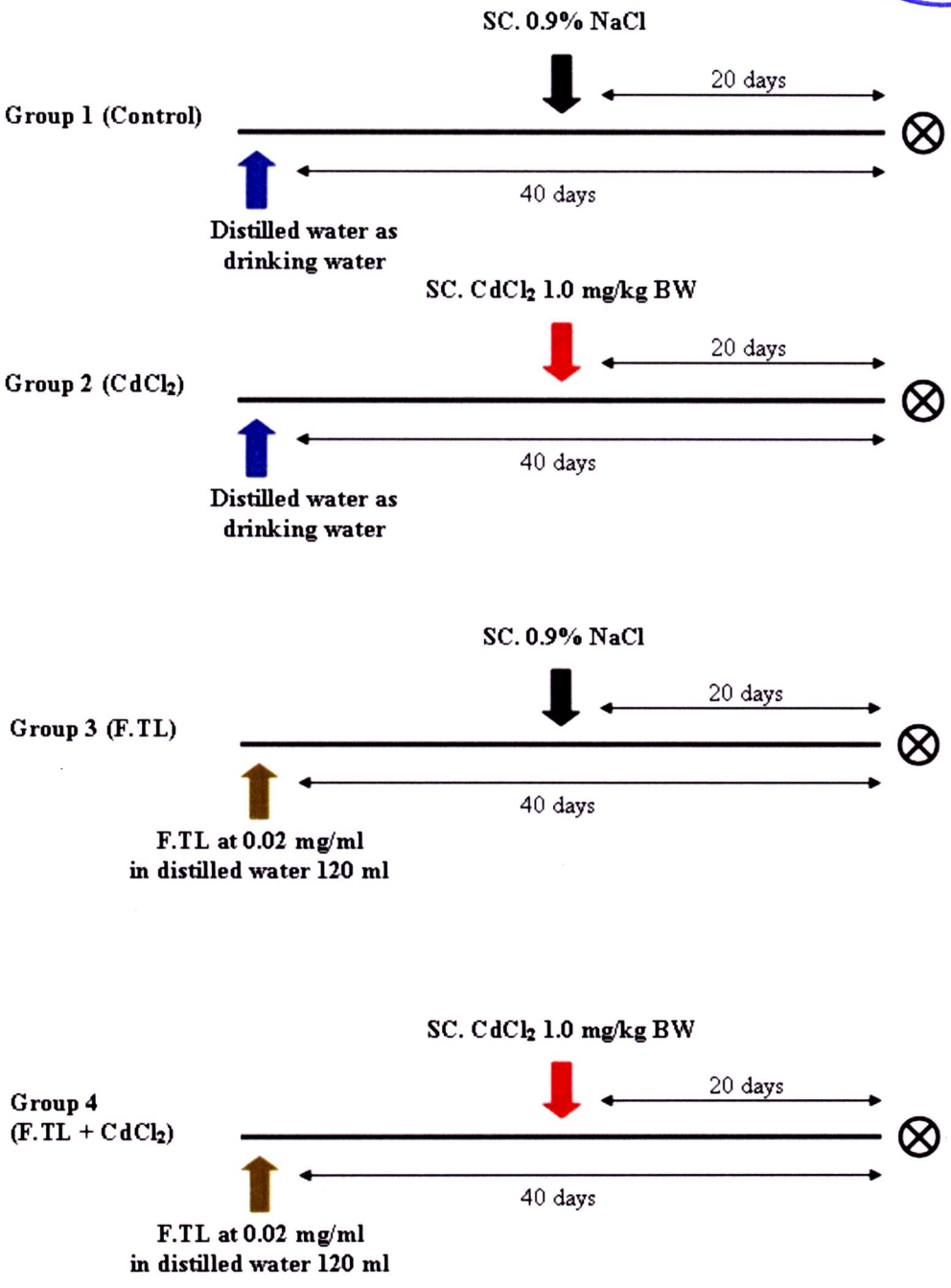


Figure 4 Collection of rats urine performed by using a metabolic cage **(A)** and blood was collected by cannulated via jugular artery **(B)**.

Blood sample was kept in EDTA tube for determining blood Cd concentrations. The remained blood was washed out from rat's body by perfusion with normal saline solution. The kidneys and livers were removed and washed with normal saline solution, weighed and kept in 10% neutral-buffered formalin solution for histopathological examination.

Animal experiment 2: Studying the effect of the selected fraction of T. laurifolia Lindl. leaf extract on cadmium induced hepatorenal toxicities

Sixteen rats were randomized into four groups; group 1 and group 3 had three rats in each group, group 2 and group 4 had five rats in each group. Rats in group 1 were control and provided distilled water for 20 days, then treated with daily subcutaneously injection of normal saline for 20 more days. The positive control group (group 2) was provided distilled water for 20 days and treated with daily subcutaneously injection of $CdCl_2$ solution (1.0 mg/kg) in isotonic saline for 20 more days. Group 3 was provided the selected fraction of *T. laurifolia* leaf extract (F.TL) (0.02 mg/mL) supply in drinking water for 40 days, then treated with daily subcutaneously injection of normal saline for 20 more days. The treatment group (group 4) was provided F.TL (same dose) in drinking water for 20 days prior to the commencement of the $CdCl_2$ treatment (same dose) for 20 more days and the F.TL supply in drinking water was continued throughout the experiment as summarized in the following scheme:



SC. = Subcutaneous injection, F.TL = The selected fraction of *T. laurifolia* leaf extract, ⊗ = Termination

The body weight and water consumption of each rat were measured daily. Twenty four hour urine samples, blood samples, the kidneys and livers were collected as the same as the animal experiment 1.

2.7 Quantification of urinary creatinine and cadmium

Determination of urinary creatinine

Creatinine concentrations were determined by kinetic test without deproteinization according to the Jeffe's method using a clinical chemistry analyzer (Hitachi 917, Japan).

Creatinine was formed a colored orange-red complex in an alkaline picrate solution after adding 50 μL of urine sample into each tube and followed by 1,000 μL of 0.16 mol/L sodium hydroxide and incubated for 5 minutes. Two hundred and fifty microliters of 4 mmol/L picric acid was added, mixed and measured the sample at the absorbance of 492 nm. The difference of absorbance at fixed time was proportionally to the concentration of creatinine in the urine.

Determination of urinary cadmium

Urinary Cd concentrations were measured by graphite furnace atomic absorption spectrometer (GFAAS) with Zeeman-GFAAS background correction using 5% monobasic ammonium phosphate solution as a modifier. The urinary Cd concentration was corrected by urinary creatinine concentration.

The urine sample was diluted in 0.1% nitric acid solution before analysis. Cd standard solution was diluted with 0.1% nitric acid to a concentration of 1 part per

billion (ppb). Cd concentrations at 0.2, 0.4, 0.6, 0.8 and 1.0 ppb were prepared and used to establish a standard curve.

The principle of the GFAAS technique was based on formation of free atoms which would absorb light at specific frequency or wavelength of the element of interest (Cd was 228.8 nm) within certain limits. The amount of light absorbed was linearly correlated to the concentration determined from a standard curve after calibrating the instrument with standard of known concentrations of the element. The free atoms of Cd could be produced from samples by the application of high voltage to induced specific temperature in order to get free atom for measurement. There were three stages of the temperature program; including drying stage (removing solvent), ashing stage (removing sample matrix) and atomizating stage (decomposing sample molecule to be Cd atom.

The urine samples (500 μL) were transferred to the cups of the GFAAS and put into an autosampler and run with the furnace temperature program as previously described in Morkmek, 2011. Briefly, the run time for one sample was 67.8 seconds with the temperature program for drying stage at 120 °C for 55 seconds, ashing stage at 250 °C for 8 seconds and atomizing stage at 1,800 °C for 4.8 seconds, respectively.

Determination of blood cadmium

Whole blood Cd concentrations were measured by GFAAS with Zeeman-GFAAS background correction using 5% monobasic ammonium phosphate solution as a modifier.

Five hundred microliters of whole blood was digested in 1,000 μL of 5% nitric acid solution, mixed for 30 seconds, left in room temperature for an hour and then centrifuged twice at 12,000 rpm for 5 minutes. The supernatant was removed for analysis. Stock Cd standard solution was diluted with 5% nitric acid to a concentration of 10 ppb. Cd standard at the concentrations of 2 and 5 ppb were used to set up a standard addition curve.

The supernatant (500 μL) from blood sample was transferred to the cup of the GFAAS and put into an auto sampler and run with a furnace temperature program as the program used for determination of the urinary Cd.

2.8 Histopathological examination

The kidney and liver were dissected for being representative sections for histopathological processing. The tissue was embedded in a paraffin block and cut at five microns sections, stained with hematoxylin-eosin and examined under light microscope to determine the morphological changes. The microscopic examination was performed under an expert pathologist's supervision at the Department of Pathology, Faculty of Medicine, Chiang Mai University.

2.9 Statistical analysis

Data analysis was evaluated by Student's t-test using SPSS version 16.0. Statistical significant was considered when p value was less than 0.05.

2.10 Ethical approval

The study protocol was approved by the Animal Ethics Committees, Faculty of Medicine, Chiang Mai University (Approved protocol No.16/2010, August 10th, 2010).