CHAPTER II

MATERIALS AND METHODS

Apparatus and chemicals

Apparatus

1. Atomic absorption spectrometry (Varian SpectraA800Z, Australia) with Zeeman background correction equipped with a hollow cadmium cathode lamp and auto sampler was used for cadmium quantification.

2. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy (Bruker Analytische GmbH, Rheinstetten, Germany) was used for characterization of *T. laurifolia* leaf extract.

3. Metabolic cage (TECHNIPLAST® Italy) was used to collect urinary sample.

4. Lyophilizer (CHRIST®, Germany) was used for preparation of *T. laurifolia* leaf extract before characterization by NMR.

5. Light microscope (Olympus, Japan) was used for histopathological examination of rats' kidneys and livers.

6. Auto pipettes (10-100, 100-1,000 µl) (Socorex, Switzerland) and all plastic ware and glassware used for quantification of cadmium were cleaned with 20% HNO_3 before use to prevent contamination.

Chemicals

All solvents used in cadmium quantification were AAS grade. The cadmium standard used in atomic spectroscopy was purchased from Sigma-Aldrich, Germany. Other chemicals and reagents were analytical grade and purchased from a local agency. Lists of chemicals and solution preparations are shown in Appendix A and B, respectively.

Establishment of a rat model for renal and hepatic injuries induced by cadmium exposure

Rats

Male Wistar rats purchased from the National Laboratory Animal Centre, Mahidol University, weighing between 275-300 g were acclimatized for one week in standardized laboratory conditions (temperature 22 ± 2 ˚C, 12/12 hr light/dark cycle). They were allowed free access to drinking water and commercial standard rodent food pellets. The study protocol was approved by the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University on 20 May 2009.

Induction of renal and hepatic injuries by $CdCl_2$

Six male Wistar rats were randomized into two groups each of three rats. The control group (n = 3) was injected subcutaneously with normal saline solution (0.9% NaCl). The treated group (n = 3) was dosed with $CdCl_2$ solution (1.2 mg Cd/kg BW) by subcutaneous injection. The treatment protocol was for 20 doses of $CdCl_2$ at a frequency of one dose per day, 5 days per week for 4 weeks. The dose of $CdCl_2$ and the period of treatment were modified from that used by Prozialeck *et al*., 2009.

Collection of rat's urine and blood

A urine sample was collected from each on day 0 and on day 26 using a metabolic cage over a 24 hr period (Figure 8) and stored at −20 °C before analysis.

A blood sample was collected by cardiac puncture (Figure 9) on the last day of the experiment. The rats were anesthetized by intraperitoneal injection of sodium phenobarbital and the blood samples were stored in EDTA preservative vacutainers at −20 °C before analysis.

Collection of rat's kidneys and livers

After blood was collected, each animal was perfused with physiological saline via the portal vein. The kidney and liver was removed, washed with physiological saline solution to remove excess blood and then fixed in 10% neutral-buffered formalin for 48 hr for histopathological examination.

Histopathological study

The kidneys and livers were dissected and representative tissue blocks were embedded in paraffin. Four micron thick sections were cut and stained with hematoxylin-eosin dye then examined under a light microscope for morphological changes. The microscopic examination was performed under the supervision of an expert pathologist at the Department of Pathology, Faculty of Medicine, Chiang Mai University.

Figure 8 Metabolic cage used to collect rat urine. The rat was allowed to drink water and eat pellet food throughout. Urine and feces were collected separately in individual containers.

Figure 9 Cardiac puncture, a technique to obtain a single large volume of blood from a rat under terminal anesthesia.

Determination of urinary creatinine

Creatinine concentration was determined by a kinetic colorimetric assay based on the Jaffe reaction (Lauhachinda and Heuseugcharern, 2004) with automated clinical chemistry analyzer (ModularP800, Roche Diagnostics, Switzerland).

Principle

The principle of this reaction is that creatinine forms a yellow-orange complex with picric acid in alkaline solution. The rate of dye formation is proportional to the creatinine concentration in the urine. The chemical reaction is as follows:

$$\text{Creatinine + Picric acid} \xrightarrow{\text{Alkaline}} \text{Yellow-orange complex}$$

Procedure

A urine sample 10 µl was mixed with 180 µl of 0.2 N sodium hydroxide. Then 36 µl of 25 mM picric acid was added to the mixture. After mixing, the solution was incubated for 15 minutes and absorbance at 505 nm measured by spectrophotometer.

A calibration curve of urinary creatinine standards was prepared before testing the rat's urine samples. Quality control materials were also used to test the accuracy and precision of the method. All values obtained had to fall within the defined limits.

Urinary cadmium concentration was corrected by the urinary creatinine concentration.

Determination of urinary cadmium

Urinary cadmium concentrations were measured by graphite furnace atomic absorption spectrometer (GFAAS) with Zeeman-GFAAS background correction using 5% monobasic ammonium phosphate as a modifier.

Principle of GFAAS

The principle of GFAAS is to place the sample in a high temperature environment (burner or furnace) and compare absorbance measurements at the ground excited states of the element. A lamp with the cathode made from pure metal or an alloy of the desired element, used as an external source to emit the line spectrum of the element. Absorbance was measured at the cadmium line (228.8 nm). The intensity of absorbance provides information on the quantity of the element in the sample. The stages of GFAAS were drying, ashing and atomizing stages. The sample is dried to completely remove solvent. The ashing stage further removes unwanted components of the sample matrix. In the atomizing stage, the temperature and rate of heating are optimized to atomize the element. A flow of argon gas through and around the graphite tube removes optimum volatile sample components from the atomizer at each stage and protects the graphite tube from oxidation. During the drying and ashing stages of the analysis, the total gas flow through the atomizer was programmed for the maximum rate of 3.0 L/min, since the most efficient removal of the products of these steps would occur with the highest flow rate.

Sample preparation

Each sample was diluted 1:1 (v/v) with 0.1% HNO_3 before analysis. The stock cadmium standard solution was diluted with 0.1% HNO_3 to 1 part per billion (ppb). This secondary standard was used to prepare standards at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 ppb for establishing a standard concentration curve.

Procedure

The urine samples were transferred to the cups of the GFAAS, put into auto sampler and run with a furnace temperature program as follows:

Table 1 Temperature program of Zeeman-graphite furnace atomic absorption spectrometer for determination of urinary cadmium

Step No.	Temperature (°C)	Time (Seconds)	Gas flow (L/minutes)	Read command
1	85	5	3.0	No
2	95	40	3.0	No
3	120	10	3.0	No
4	250	5	3.0	No
5	250	1	3.0	No
6	250	2	0	No
7	1,800	0.8	0	Yes
8	1,800	2	0	Yes
9	1,800	2	3.0	No

(Ref: Analytical Methods for Graphite Tube Atomizers, Varian, Australia)

Determination of whole blood cadmium

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

Sample preparation

Each blood sample was digested with 5% HNO_3 at a ratio of 1:3 (v/v), then mixed stood for an hour then and centrifuged at 12,500 rpm for 5 minutes. The supernatant was removed for GFAAS analysis. The stock cadmium standard solution was diluted with 5% HNO_3 to a concentration of 10 ppb. Cadmium standard at concentrations of 2 and 5 ppb were used to set up a standard addition curve.

Procedure

The supernatant from each blood sample was transferred to the cups of GFAAS, put into auto sampler and run with the same furnace temperature program as used for determination of urinary cadmium (Table 1).

Detoxification effects of *T. laurifolia* leaf extract on cadmium induced renal and hepatic injuries

Plant extract

T. laurifolia leaves were collected from the Ob Khan National Park, Hang Dong District, Chiang Mai Province in July and October 2009. The taxonomic identification of the plant was confirmed at the Queen Sirikit Botanic Garden, Mae Rim District, Chiang Mai Province. The leaves were washed with tap water, dried at

room temperature for 5 days, ground to a powder and then stored in an amber glass bottle at room temperature before extraction.

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

^{1}H NMR characterization of the constituents of *T. laurifolia* leaf extract

The leaf extract constituents were characterized using ^{1}H NMR spectroscopy on a Bruker AVANCE-400 spectrometer with water suppression technique.

Principle of NMR

The basic arrangement of an NMR spectrometer is to position the sample in a magnetic field and excite it by pulsations in the radio frequency input circuit. The realigned magnetic fields induced a radio signal in the output circuit which generates an output signal. Fourier analysis of the complex output produced the spectrum. The pulse was repeated as many times as necessary to identify the signal from the background noise.

Sample preparation

The lyophilized residue of *T. laurifolia* leaf extract was redissolved in deuterated water (D_2O) then placed in a 5 mm NMR tube for analysis by ^{1}H NMR spectroscopy.

Procedure

The ^{1}H NMR spectra were acquired on a Bruker Avance 400 MHz spectrometer. The spectra of the extract were acquired using a pulse sequence with water suppression from the Bruker pulse program. Data were obtained over a 8,278 Hz sweep width and digitized with 60 k complex data points. The number of scans was 256. Total acquisition time was 20 minutes. Spectra were 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.

***T. laurifolia* dose preparation**

The dose of *T. laurifolia* leaf extract fed to the rats was 125 mg/kg BW. This was the saturation concentration for the leaf extract in water.

Experiment to study the antagonistic effect of *T. laurifolia* on cadmium toxicities

Eighteen rats were randomized into three groups of six rats. Rats in the positive control group (group 1) were injected subcutaneously with 1.2 mg/kg BW of $CdCl_2$ solution for 5 days a week for 4 weeks followed by water orally for another 26 days. Rats in the leaf extract treatment group (group 2) was injected with the same $CdCl_2$ dose as the positive control group and then fed a daily dose of leaf extract for another 26 days. Rats in group 3 were injected with the $CdCl_2$ solution as for groups 1 and 2 and then fed leaf extract twice a day for another 26 days. The body weight, appearance and behavior of the rats in all three groups were observed and recorded daily.

Experiment to study the prophylactic effect of *T. laurifolia* on cadmium toxicities

Eighteen rats were separated into three equal groups. Rats in the positive control group (group 1) were fed orally with distilled water 5 days a week for 4 weeks. This treatment was followed by subcutaneous injection of 20 doses of 1.2 mg/kg BW of $CdCl_2$ solution over the next 26 days. Group 2 (pre-treatment) was fed the leaf extract 5 days a week for 4 weeks and then $CdCl_2$ injection for another 26 days. Group 3 (co-treatment) was fed the leaf extract the same as group 2 and then fed a daily dose of leaf extract and $CdCl_2$ injection for another 26 days. The body weight, appearance and behavior of the rats were observed and recorded daily.

Statistical analysis

All data are expressed as the mean ± standard error of mean (SE). The statistical significance was evaluated by one-way analysis of variance (ANOVA) using SPSS version 12.0. Values are considered statistically significant when $p<0.05$.