CHAPTER 1

INTRODUCTION

1.1 *Thunbergia laurifolia* Lindl.

General description

Thunbergia laurifolia Lindl. is a vine widely distributed in Southeast Asia belonging to the larger family of *Acanthaceae* (Wagner *et al*., 1999). The common name is "Laurel Clock Vine" or "Blue trumpet vine" in Thai it is known as "Rang juert". The characteristic of *T. laurifolia* is a shrub, the leaves are opposite, heart-shaped with serrated leaf margin, taper to a pointed tip and glabrous surface (Figure 1). Flowers are not scented and borne on pendulous inflorescences. The hermaphrodite flower is trumpet-shaped with a short broad tube, white outside and yellowish inside (Putiyanan *et al*., 2008) (Figure 2). The full bloom of flower has 3 inch diameter and blooms from November to January. Once the flower has withered, it becomes fruit, which is a pod of shaped. The end of the pod is pointed like a bird's bill. The corolla is pale blue in color with 5–7 petals, one larger than the others. It can be divided into three types designated by flower color: white, yellow, or purple. Purple varieties are believed to possess compounds that deliver health benefits particularly from materials of the stem, root and leaves (Oonsivilai *et al*., 2007). The plants are common in tropical and mixed forest throughout the country, often cultivated as an ornamental plant or trained over walls or trellises.

Figure 1 *T. laurifolia* Lindl. leaves

(http://gooherb.tarad.com/shop/g/gooherb/img-lib/con_20071007210415_i.jpg)

Figure 2 *T. laurifolia* Lindl. flower

(http://www.jittraflorist.com/images/column_1258297489/1258298069.jpg)

Pharmacology and toxicology studies of T. laurifolia Lindl.

Different parts of the plant are used for various medicine purposes. In example; fresh leaves has been commonly used in Thai traditional medicine as an antipyretic or an antidote for insecticide and several toxin intoxication. The dried leaves used as an antidote for arsenic, strychnine, ethanol and insect's toxin. The dried stem used as antipyretic agent. In addition, the dried root used as anti-inflammatic, heal swelling and antipyretic agent (Panichayupakaranunt, 2011).

The aqueous extract of dried *T. laurifolia* leaves could antagonize the toxic effect of Folidol-E605 in rats. The rats were fed with single dose of 2 ml/100 g BW via intragastric intubation of the extract immediately after intraperitoneal injection of 20 µl/kg BW of Folidol. It was found that the extract could decrease mortality rate in rats from 56.67 ± 3.33% to 5.00 ± 2.87% by either the peritoneal or subcutaneous injection (Ruangyuttikarn, 1980).

T. laurifolia leaves extract had no effect on the induction of micronucleus formation in human lymphocyte and rats bone marrow cells. However, it was an effective antimutagenic substance for methomyl (Boonyarat, 2004). Moreover, it could also be a protective agent for methomyl induced cholinesterase inhibition by increasing the numbers of acetylcholinesterase-positive neurons and color intensity of nerve fibers in rat's duodenum myenteric plexuses after reducing both numbers and color intensity of the neurons and nerve fibers induced by methomyl insecticide (Chaiyasing, 2005).

The aqueous extract of *T. laurifolia* leaves in the massive single oral dose of 10 g/kg BW did not produce toxic effects and did not affect the behavioral patterns in rats. In addition, oral administration of the extract at the dose of 500 mg/kg BW for 28 days continually did not produce mortal effect or change in gross morphology of the internal organs and no histological changes of visceral organs of rats (Wisitpongpan *et al*., 2003). Consistently, oral administration of the extract 2,000 mg/kg BW for six months did not produce any histological alterations of the visceral organs of rats. However, the result suggested that hematological and clinical chemistry values should be monitored during prolonged use of the *T. laurifolia* leaf extract (Chivapat *et al*., 2009).

A primary study on effect of *T. laurifolia* leaves on glucose blood levels and reproductive system for diabetic rats has shown that oral administration of *T. laurifolia* leaves extract at the concentration of 60 mg/ml/day to diabetic rats for 15 days decreased levels of blood glucose. However, this extract did not exert an improvement of reproductive system which was deteriorated by diabetes (Aritajat *et al*., 2004).

Oonsivilai *et al*. (2007 ; 2008) found water extract of *T. laurifolia* leaves had high content of total phenolic acids. Acetone and ethanol extract had rich of chlorophyll. They suggested that the detoxificated effect of *T. laurifolia* extract do to the potential role of both phenolic acids and natural chlorophyll constituents. Moreover, water crude extract of *T. laurifolia* leaves had been used to test for the IC50 and summarized as low cytotoxicity at the concentration of over 100 µg/mL and expressed high antioxidant activities.

Praditsathawong (2009) reported four cases of horseshoe crab poisoning and were treated with *T. laurifolia* leaves juice. All patients were given the extract after going to be coma for forty minutes. The patients regained consciousness and gradually recovered. However, the dose of *T. laurifolia* should be studied to use for decreased time to treat the patients and decreased complicated symptoms.

A recent report of the aqueous extract of *T. laurifolia* leaves showed that *T. laurifolia* could alleviate symptoms of lead poisoning in mice (Tangpong and Satarug, 2010). Co-treatment with *T. laurifolia* at the concentration of 100 mg/kg BW and 200 mg/kg BW was found to alleviate adverse effects of lead on learning deficit and memory loss, increased activity of enzyme caspase-3, maintained total anti-oxidant capacity and anti-oxidant enzymes in the brain.

Chemical constituents of T. laurifolia Lindl.

Flavonoids such as apigenin, casmosiin, delphinidin-3-5-di-*O*-β-D-glucoside and chorogenic acid have been found in both flowers and leaf materials of *T. laurifolia*. It also contains other bioactive phenolic constituents including delphinidin 3:5-di-*O*-β-d-glucopyranoside and apigenin-7-*O*-β-D-glucopyranoside (Purnima and Gupta 1978; Thongsaard and Marsden, 2002).

Jitpuwngam (1997) reported that water and methanol *T. laurifolia* leaves extracts consisted of four amino acids including methionine, glycine, serine and unidentified amino acid. There were eight types of steroids and carotenoid in the petroleum ether extract of the leaves. Charumanee *et al*. (1998) and Krairachareon *et al*. (1999) also found *T. laurifolia* leaves consisted six types of steroids from ethanol extract and also two types of steroid from hexane extract.

Kanchanapoom *et al*. (2002) reported two iridoid glucosides, 8-epi-grandiforic and 3'-*O*-β-glucopyranosyl-stibericoside isolated from the aerial part of *T. laurifolia* with seven known compounds; benzyl β-glucopyranoside, benzyl β(2'-*O*-β–glucopyranosyl), glucopyranoside, grandifloric acid, (*E*)-2-hexenyly-β-glucopyranoside, hexanol-β-glucopyranoside, 6-*C*-glucopyranosylapigenin and 6,8-di-*C*-glucopyranosylapigenin.

Recently, the report of Morkmek *et al*. (2010) found that the major constituents of *T. laurifolia* leaf extract were benzyl, hexyl and hexenyl glucoside compounds identified by proton nuclear magnetic resonance spectroscopy.

1.2 Cadmium

Cadmium is a naturally occurring minor element, one of the metallic components in the earth's crust and oceans, and present everywhere in our environment. It was first discovered in Germany in 1817 by German chemist F. Strohmeier (Nordberg, 2009). It is a chemical element in the periodic table that symbolized as Cd and its atomic number is 48 and atomic mass is 112.4 g/mol. It is a soft, bluish-white metal which easily cut with a knife and usually present in the environment as an organic salt such as cadmium oxide (CdO), cadmium chloride ($CdCl_2$) or cadmium sulfate ($CdSO_4$) (WHO,1992; ATSDR, 1998). Cd metal is produced as a by-product from the extracting, smelting and refining of the nonferrous metals; zinc, lead and copper. Cd metal and Cd compounds are used as pigments, stabilizers, coatings, specialty alloys, electronic compounds, but, most of all used in rechargeable nickel-cadmium batteries.

Exposure to cadmium

Most human Cd exposure comes from food and water as well as cigarette smoke and air contaminations (Chowdhury *et al*., 1987; WHO, 1992; Kocak and Akcil, 2006; Jihen *et al*., 2008). Cd is transferred to human via food chain; soil to plant; plant to animal and animal to human (Chaiwong *et al*., 2009). Various plants can accumulate Cd from soil, e.g. crops and radish bulb (Haouem *et al*., 2007), especially rice and tobacco because of its high rates of soil to plant transference.

Toxicokinetics of cadmium

Absorption: Human is generally exposed to Cd by two main routes, inhalation and ingestion. Data from experimental animals and humans have shown that pulmonary absorption is higher than gastrointestinal absorption; approximately 50% of inhaled Cd compound is absorbed to the circulation, but the gastrointestinal absorption of Cd has been reported to be approximately 3-8% of the ingested load (Chowdhury *et al*., 1987). The absorption of Cd depends on physiological status such as age, dietary intake, iron storage, gender and smoking.

Distribution: After uptake from the lung or gastrointestinal tract (Figure 3), Cd transports in blood plasma initially bind to albumin. Cd bound albumin is preferentially taken up by the liver. In the liver, Cd induces the synthesis of metallothionien (MT) and, a few days after exposure; MT-bound Cd appears in the blood plasma. Because of its low molecular weight, Cd-MT is efficiently filtered through the glomeruli and thereafter taken by the tubules. Cd accumulates in the liver and kidney and has long biological half-life about 30 years in human (Haouem *et al*., 2007; Nordberg, 2009).

MT is an important transport and storage protein for cadmium and other metals. They are low molecular weight proteins. Cd induces MT synthesis in many organs including the liver and kidney. The binding of intracellular cadmium to MT in the tissues protects against the toxicity of Cd. Cd that is not bound to MT may therefore play a role in the pathogenesis of Cd-related tissue injury (Klaassen *et al*., 2009).

Excretion: Cd absorbs enterically and eliminates very slowly by urinary and fecal mechanisms (0.01-0.02 % of the body burden per day). Several studies have shown that in the general population urinary cadmium excretion increased with age and when cadmium induced renal tubular damage occurred it was related to body burden and recent exposure. Cd in feces is a good indicator of recent daily intake from food in the absence of inhalation exposure and related to body burden same as urine (WHO, 1992).

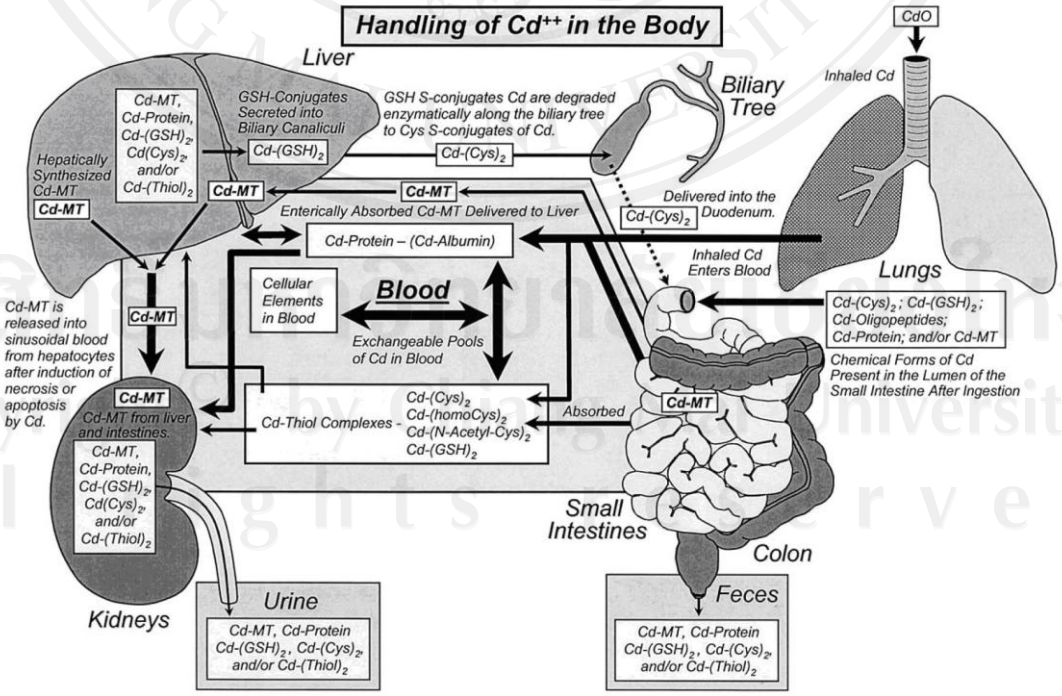

Figure 3 Toxicokinetics of Cd (Zalups and Ahmad, 2003)

Toxicities of cadmium

Acute toxicities: The toxicities occurred mainly due to high concentrations uptakes of Cd by inhalation via fumes and ingestion via food and water contamination. High inhalation of Cd (about 5 mg/m^3) causes acute pneumonitis, pulmonary edema and fetal. High ingestion of Cd (more than 15 mg Cd/kg) causes severe nausea, vomiting and abdominal pain (ATSDR, 1998).

Chronic toxicities: Lower Cd concentration with longer periods of exposure will cause chronic Cd toxicities. The kidney is most frequently the critical organ. In addition, Cd also affects hepatic, bones, respiratory system, hematopoietic system, cardiovascular system, reproductive system, neuron, and carcinogenicity.

Nephrotoxicity: The mainly accumulation of Cd in the kidney; the target organ of Cd, in renal cortex leads to renal tubular dysfunction (WHO, 1992; Griffin *et al*., 2000; Thijssen *et al*., 2007; Nordberg, 2009; Prozialeck *et al*., 2009) with proteinuria, glucosuria, hypercalciuria, aminoaciduria, hyperphosphaturia, polyuria and decrease ability to buffer acid load (Jarup, 2002) as well as histopathological changes in animal experiments including proximal tubular cell degeneration, interstitial inflammation and fibrosis, glomerular swelling, atrophic and pyknotic nuclei, interstitial edema, glomerular basement membrane swelling, mitochondria swelling, apoptosis, necrosis, occasional segmental sclerosis and death of mesangial cells (Aughey *et al*., 1984; Jihen *et al*., 2008; Prozialeck *et al*., 2009; Yang *et al*., 2009).

Hepatotoxicity: The liver is a major storage and metabolic organ for the in *vivo* handling of Cd. It is a large reserve capacity and has the ability to induce anti-

oxidant systems. In animal experiment, hepatic toxicity assessed changes in histopathology of liver cells including necrosis with pyknotic nuclei, dilation of sinusoid (Jihen *et al*., 2008; Tarasub *et al*., 2008) and levels of the liver anti-oxidants such as superoxide dismutase (SOD), reduced glutathione (GSH) and nitric oxide (NO) (ATSDR, 1998).

Osteopathy: Cd can affect calcium, phosphorus and bone metabolism. It can cause osteomalacia and/or osteoporosis. The renal tubular dysfunction associated with osteomalacia and/or osteoporosis causes severe manifestation is called Itai-Itai disease (Bhattacharyya, 2009).

Respiratory system: In upper respiratory system, Cd causes chronic inflammation of the nose, pharynx and lyrynx. Lower respiratory system, Cd impairs lung functions and causes chronic obstructive lung disease. The effects on the lung increase risk to lung cancer and mortality (ATSDR, 1998).

Hematopoietic system: Anemia is a common finding in animals after exposure to Cd because of decreasing gastrointestinal absorption of iron. Altered immune function has also been reported (ATSDR, 1998).

Cardiovascular system: Cd can increase the blood pressure and the risk of hypertension. In long-term high-exposure, the hypertension has been understood to arise secondary to the loss of kidney function. A number of active genes in the kidney are related to the control of blood pressure in physiologic state, however, including those affecting salt excretion and reabsorption, vascular tone, and volume homeostasis and it is plausible that even low-level exposure to cadmium may affect the blood

pressure control of human body. However, few studies supported the role of cadmium-induced nephropathy including tubular damage, which might induce increase of blood pressure in part (Eum *et al*., 2008).

Reproductive system: Cd can cause many reproductive effects such as failure of maturation of spermatozoa, failure of developmental progression in pre-implantation embryo and failure of implantation (Thompson and Bannigan, 2008).

Neurotoxicity: Small amounts of Cd reach the brain because of the selective permeability of the blood brain barrier (BBB), but higher concentrations can be reached if a vehicle such as ethanol is employed, due to its ability to diffuse across all biological membranes, thus allowing Cd penetration through BBB. Neuronal damage and the possible mechanisms involved in that damage. Oxidative stress, interference with calcium, and zinc-dependent processes and apoptosis induction are the main processes involved in Cd neurotoxicity (M´endez-Armenta and R´ıos, 2007).

Carcinogenicity: Cd has been classified as a human carcinogen group I by the International Agency for Research on Cancer (IARC). Both human and animal experiments reported Cd can cause lung and prostate cancer. The major mechanisms involved in Cd carcinogenesis can be broadly categorized into four groups, aberrant gene expression, inhibition of DNA damage repair, inhibition of apoptosis, and induction of oxidative stress (Joseph, 2009).

1.3 Contamination of cadmium in Thailand

Recently, environmental pollution of Cd has been discovered in Mae Sot district, Tak province (Simmon *et al*., 2005; Teeyakasem *et al*., 2007;

Swaddiwudhipong *et al*., 2007). In 1977 zinc mining was established in Phrathat Phadaeng which is a sub-district located upstream of Mae Tao creek, the main source of water supply used for agriculture in Mae Sot district. Cd was assumably released from the zinc mining into the environment; water, soil and rice in the polluted area.

A Mae Sot General Hospital team has conducted a large-scale health impact survey for residents who live in 12 villages of 3 sub-districts; Phrathat Phadaeng, Mae Tao and Mae Ku. The team investigated urinary Cd concentration levels in 2004 and reported (Swaddiwudhipong *et al*., 2007) a high rate of Cd exposed subjects, with 9.2% of urinary Cd between 5 to 10 µg/g Cr, and 2.5% of the subjects having urinary Cd higher than 10 µg/g Cr whereas the recommended acceptable level of urinary Cd is 2 µg/g Cr (WHO, 1992).

Furthermore, 7,697 people, aged between 15 to 60 year old lived in the contaminated areas had been surveyed for urinary Cd. The result illustrated the 3 levels of concentrations; less than 5 µg Cd/g Cr was 92.80%; 5-10 µg Cd/g Cr was 4.90% and more than 10 µg Cd/g Cr was 2.30% (Swaddiwudhipong *et al*., 2007). Moreover, High urinary Cd concentrations and urinary renal biomarkers (β2-microglobulin and N-acetyl-β-D-glucosaminidase) in Mae Sot residents were found high risk of renal dysfunction, especially the farmers who daily eat their own grown rice (Teeyakasem *et al*., 2007; Honda *et al*.,2010).

Chaiwong *et al*. (2009) also reported that Mae Tao people had the highest concentration of urinary Cd compared to people in Phrathat Phadaeng and Mae Ku and women had higher urinary Cd than men due to an increased gastrointestinal uptake of cadmium at low iron store.

Panyamoon *et al*. (2009) developed the new biomarker for renal tubular dysfunction, kidney injury molecule-1 (KIM-1) using ELISA technique and showed that it was a very sensitive and specific biomarker for early detection of renal tubular dysfunction and the KIM-1 levels correlated well with Cd concentrations in both blood and urine of the Mae Sot population.

Nambunmee *et al*. (2010) have reported that the excretion of bone resorption markers was positively correlated to the ratio of excreted calcium and the urinary Cd and bone resorption was accelerated by an impaired Ca reabsorption in the renal tubules. In addition, Swaddiwudhipong *et al*. (2010) have found Cd might increase the risk of hypertension. They found positive correlation of urinary Cd and hypertension in persons living in Cd-contaminated villages in northwestern Thailand. Recently, Nambunmee *et al*. (2011) reported that high Cd exposure in Mae Sot's population was also associated with high anemia prevalence.

Thus, Cd exposed population in Tak province has been a public concern for chronic Cd toxicity.

1.4 Detoxification of cadmium

Several natural substances have been reported to be used for detoxification of Cd toxicities such as onion, garlic, naringinin, quercetin, selenium, zinc and *Thunbergia laurifolia*.

***Onion and garlic*:** Ola-Mudathir *et al*. (2008), Suru (2008) and Obioha *et al*. (2009) reported that the aqueous extracts of onion and garlic could protect against Cd

induced testicular oxidative damage, spermiotoxicity, renal damage in rats and oxidative damage in rat's liver by possibly reducing lipid peroxidation and increased the antioxidant defence mechanism.

***Naringinin*:** Naringinin is a naturally occurring citrus flavonone which has been reported to have a wide range of pharmacological properties. Administration of naringenin at a dose of 50 mg/kg markedly reduced the toxicity of Cd and preserved the normal histological architecture of the renal tissue (Renugadevi and Prabu, 2009). This study suggested that the nephroprotective potential of naringenin in Cd toxicity might be due to its antioxidant and metal chelating properties. Moreover, at the same dosage of naringinin, the histopathological studies in the liver of rats also showed a reduction of Cd toxicity and preserved the normal histological architecture of the tissue (Renugadevi and Prabu, 2010b).

***Quercetin*:** Quercetin is bioflavonoid in the class of flavonols according to it's presence in high concentration in fruits and vegetables and it's a very potent antioxidant, thus, it was frequently used to detoxify several toxic agents. Morales *et al*. (2006) reported that quercetin help prevent renal tubular damage and increase oxidative stress induced by chronic Cd administration in rats. Renugadevi and Prabu (2010a) also found that quercetin treatment markedly attenuated the Cd-induced biochemical alterations in serum, urine and renal tissue. Moreover, quercetin also ameliorated the Cd-induced pathological change in rat's kidney.

***Selenium and zinc*:** Ji Hen *et al*. (2008) reported that selenium and zinc could have a cooperative effect in the protection against Cd-induced structural damage in the rat liver but not in the rat kidney. However, combination of selenium and zinc could

have a protective effect on Cd-induced oxidative stress in rat kidney (Imed *et al*., 2009).

***T. laurifolia Lindl*.:** The leaf extract could detoxify some effect of Cd toxicity reported by Morkmek *et al*. (2010). They found that the leaf extract given to rats orally did not prevent mortality in rats exposed to high dose of Cd. However, abnormal appearances and behaviors were observed less in rats fed the leaf extract prior to Cd exposure than in those rats fed with leaf extract after Cd exposure. The constituents of the extract were identified as benzyl, hexynyl and hexyl glucoside compounds by nuclear magnetic resonance spectroscopy (NMR). They concluded that the *T. laurifolia* leaf extract might reduce some effects of Cd toxicity, but the conclusion was uncertain due to the high mortality rate of the rats in the experiments.

Therefore the hypothesis of this thesis was *T. laurifolia* leaf extract, especially its interested fraction, could effectively reduce Cd toxicity if giving the extract as tea instead of gavaging directly to rats stomach, as in Morkmek *et al*. (2010)'s study, before and during treated with a low dose of $CdCl_2$ solution at the suitable dosage, not as high dosage as in Morkmek *et al*.'s study.

1.5 Objective of the study

It has been quite clear that people who live in Mae Sot district, Tak province have high urinary and blood Cd and some of them show high renal dysfunction and bone metabolic disorder. However, there is no any specific treatment for minimizing Cd toxicity in this population. If *T. laurifolia* leaf extract could help reduce Cd toxicity in animals it might be valuable to apply or implement the results to unavoidable Cd exposed population. Therefore, the objectives of this study were to

- isolate or fractionate the constituents in *T. laurifolia* leaf extract using column chromatographic technique
- identify and characterize the interested compounds in the fractionated leaf extract using nuclear magnetic resonance spectroscopy technique
- investigate the effect of the selected compounds in isolated fraction on Cd induced hepatorenal toxicities using rat's model compared to the crude extract by giving *T. laurifolia* leaf extract in drinking water before subcutaneously injecting an optimal concentration of $CdCl_2$ solution to the rats