Bioactive Compounds and Biological Properties of *Thunbergia laurifolia* Leaf Extract and Its Potential Use as Functional Drink

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ชื่อวิทยานิพนธ์ คุณสมบัติเชิงชีวภาพของสารสกัดใบรางจืด และความเป็นไปได้ในการนำไปใช้สำหรับผลิตภัณฑ์เครื่องดื่มเพื่อสุขภาพ

ผู้เขียน นางสาวมารศรี จันสี

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บทคัดย่อ

องค์ประกอบทางพฤกษเคมีและฤทธิ์ต้านอนุมูลอิสระของสารสกัดหยาบใบรางจืดด้วยน้ำ ประกอบด้วยสารกลุ่มฟีนอล ฟลาโวนอยด์ แทนนิน และสเตอรอล และมีฤทธิ์ต้านอนุมูลอิสระจากการตรวจสอบด้วยวิธี DPPH สูงที่สุด การศึกษาองค์ประกอบของสารกลุ่มฟีโนลิคและฟลาโวนอยด์ด้วยเครื่องลิควิดโครมาโทกราฟี แมสสเปกโทรมิเตอร์ พบว่าในสารสกัดหยาบใบรางจืดมีองค์ประกอบของ กรดคาเฟอิก กรดโรสแมรินิก คาเทชิน รูทิน ไอโสเควอซิทิน เควอซิทิน และอะพิจินิน การศึกษาผลของสารสกัดหยาบใบรางจืดต่อการเพิ่มฤทธิ์ของเอนไซม์ที่เกี่ยวกับการต้านอนุมูลอิสระในเซลล์ RAW264.7, HEK293 และ HepG2 พบว่าสารสกัดหยาบใบรางจืดสามารถช่วยส่งเสริมฤทธิ์ของเอนไซม์คะตาเลสและกลูตาไทโอนเปอร์ออกซิเดสในเซลล์ HEK293 และ HepG2 และลดปริมาณสารมาโลนไดอัลดีไฮด์ในเซลล์ทั้งสองลงได้ จากการทดลองแสดงให้เห็นว่าความเป็นพิษของสารสกัดหยาบใบรางจืดมีค่าต่ำเมื่อเทียบกับเซลล์ที่ทำการศึกษา (>100 ไมโครกรัมต่อมิลลิลิตร) นอกจากนี้ยังพบว่าสารสกัดหยาบใบรางจืดสามารถลดปริมาณการผลิตสารไนตริกออกไซด์จากการเหนี่ยวนำด้วยลิโปโพลีแซคคาไรด์ในเซลล์ RAW264.7 ลงได้ โดยมีค่าความเข้มข้นของสารสกัดหยาบใบรางจืดที่ออกฤทธิ์ยับยั้งได้ 50 เปอร์เซ็นต์ (IC_{50}) คือ 25.74±1.51 ไมโครกรัมต่อมิลลิลิตร

ผลจากการศึกษาการต้านความเป็นพิษของโลหะหนักแคดเมียม โดยการแบ่งการศึกษาออกเป็น 3 ชุดการทดลอง ประกอบไปด้วย การใช้สารสกัดหยาบใบรางจืดร่วมกันกับแคดเมียม การใช้สารสกัดหยาบใบรางจืดก่อนและหลังได้รับแคดเมียม ทดสอบในเซลล์ทั้ง 3 ชนิด และวัดค่าการอยู่รอดของเซลล์ด้วยวิธี MTT พบว่าสารสกัดหยาบใบรางจืดสามารถป้องกันเซลล์ทุกชนิดที่ทำการศึกษาจากความเป็นพิษของแคดเมียม และสามารถต้านความเป็นพิษจากแคดเมียมในเซลล์เมื่อใช้ร่วมกับสารคีเลต ($CaNa_2EDTA$) นอกจากนี้ยังพบว่าสารสกัดหยาบสามารถป้องกันและช่วยฟื้นฟูเซลล์ HepG2 และ HEK293 ตามลำดับ ได้ดีกว่าการใช้ยาอะโทรพีนจากพิษยาฆ่าแมลงคลอร์ไพริฟอส และเมโทมิล

การศึกษานี้ แสดงให้เห็นว่าสารสกัดหยาบใบรางจืดด้วยน้ำที่ระดับความเข้มข้นที่เหมาะสม สามารถต้านการอักเสบโดยการยับยั้งการผลิตไนตริกออกไซด์ และสามารถเพิ่มฤทธิ์ของเอนไซม์ที่เกี่ยวกับการต้านอนุมูลอิสระ รวมทั้งป้องกันและรักษาเซลล์จากความเป็นพิษของแคดเมียมและยาฆ่าแมลง ผลการศึกษานี้สนับสนุนภูมิปัญญาพื้นบ้านในการใช้ใบรางจืด อย่างไรก็ตามการใช้ใบรางจืดเพื่อรักษาหรือฟื้นฟูร่างกายจากพิษต่างๆ ต้องทำการตรวจสอบอย่างรอบคอบเพื่อความปลอดภัยต่อไป

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ABSTRACT

The phytochemical screening including phenolic and flavonoid profiling and antioxidant activity in the aqueous crude extract of *Thunbergia laurifolia* leaves were evaluated. The crude extract contained phenols, flavonoids, tannins and sterols groups and exhibited the highest in DPPH antioxidant activity. The liquid chromatography-mass spectrometry **(**LC-MS) analyses indicated the presence of caffeic acid, rosmarinic acid, catechin, rutin, isoquercetin, quercetin and apigenin compounds. The antioxidant enzyme improvement of the crude extract was tested with murine macrophage (RAW264.7), human embryonic kidney cells (HEK293) and human hepatocellular carcinoma (HepG2) as a tested cell model. The crude extract increased catalase (CAT) and glutathione peroxidase (GPx) activities and decreased malondialdehyde (MDA) levels in HEK293 and HepG2 cells. The results indicated that cytotoxicity of the crude extract when treated with those cell lines was quite low (>100 μg/ml). In addition, the crude extract could reduce nitric oxide production in RAW 264.7 cells induced by lipopolysaccharide (LPS) which 50% of inhibitory concentration (IC_{50}) value of the crude extract was 25.74±1.51 μg/ml.

Effect of the crude extract on anti-cadmium (Cd) toxicity in RAW264.7, HEK293 and HepG2 were investigated. The experiment was designed as 3 groups; combined, pre- and post-treatments and cell viability were determined with MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). It was found that the crude extract showed the protective against Cd agent in all cell types and more improved therapeutic efficacy when using combined with a chelating agent ($CaNa_2EDTA$). To evaluate the anti-insecticides toxicity (chlorpyrifos, CP, and methomyl, MT), the experiment were tasted in similar ways as Cd toxicity assay. The results indicated that the crude extract provided protection and healing effect on CP and

MT insecticides better than using atropine sulfate (AS) standard drug especially in HepG2 and HEK293 cells, respectively.

The outcome of this experiment indicated that the aqueous crude extract of *T. laurifolia* leaves at proper dose could be anti-inflammatory agent by NO inhibition process and improved antioxidant enzymes activity including healing and protection functions against Cd heavy metal, CP and MT insecticides. This results supported the Thai folklore wisdom for using *T. laurifolia* leaves, however, to be safe for health claims, the clinical trial still needed and more investigated.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEWS

1.1 Introduction

Recently, the perception of having food is changed to be not only concerning calorie and nutrition but also other functions including antioxidant, immune enhancing, blood lowering and so on. Besides, being the basic nutrients as carbohydrates, lipids, proteins, minerals and vitamins, many other constituents classified as bioactive compounds are more interesting. Chlorophylls, carotenoids, flavonoids and other phytochemicals are grouping in bioactive compounds (Oonsivilai, 2006). Actually, plant foods have been used as an important source of folk medicine since ancient age. Even now, the World Health Organization (WHO) estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their folk medicines (Tripathi and Tripathi, 2003). In addition, the numerous new pharmaceuticals, healthcare, nutraceutical and functional products are found in plant materials (Salawu *et al*., 2011).

Thunbergia laurifolia or Rang Jued, a local Thai plant belongs to the family of Acanthaceae which is commonly consumed as an herbal tea. A fresh and dried form of this plant leaves, bark, and root are used as anti-dote for insecticide, drug, and chemical toxic (Thongsaard and Marsden, 2002; Oonsivilai, 2006). Moreover, some scientific data have shown the rapid growth in used pesticide as a significant problem for Thailand, as found in many other developing countries with an intensifying agriculture (Grovermann *et al*., 2013). Thailand relies heavily on pesticide use as a powerful tool for crop protection in order to increase production levels, product quality and product appearance as a market requirement. The use of pesticides in Thailand is therefore necessary and has increased significantly over time (Panuwet *et al*., 2012). The heavy use leads to high levels of pesticide residues in the ecosystem (including soil, sediment, water, aquatic life and agricultural products). As well know that humans are placed at the top of the food chain; therefore, pesticides including the residues from heavy metal, are also found in the human body and consequently cause adverse health effects (Sapbamrer *et al*., 2011). Because heavy metal residues can enter the food chain

and can be bioaccumulation with endangering human health, then globally concerns as serious problem. The main threats to human health from heavy metals are associated with exposure to lead, cadmium, mercury and arsenic (Järup, 2003) especially, cadmium (Cd). The extremely long biological half-life of Cd as a cumulative toxin in the liver and kidney makes up the bulk of total body burden (Gonick, 2008).

Although, the *T.laurifolia* herb was reported to have detox and anti-dote effect for pesticides and heavy metals, recently this plant is prohibited to use or produce as food or drinking product by Thai FDA, Food and Drug Administration of Thailand. Because of a lack of scientific data about its safety thus it may have a negative effect on blood system, liver, and kidney (Thai FDA, 2015a; Thai FDA, 2015b), therefore, using of this plant leaf for drinking needs to be carefully for consumption.

This present work aims to determine the main bioactive compounds and some biological properties of *T. laurifolia* extracts on antioxidant, anti-inflammatory, and anti-dote toxicity to support of the scientific data for proving and or warning the utilization of *T. laurifolia* extract.

1.2 Literature Reviews

1.2.1 The Inflammatory reaction

Inflammation is a contributing factor to the muscle loss observed during cancer, chronic heart failure, chronic renal diseases, HIV/AIDS and bacterial infections (Apenten, 2010). The physical characteristics of inflammation were described by Celsus as redness (rubor), swelling (tumor), heat (calor), and pain (dolor). Rubor is the redness that occurs as a result of the increased blood flow to the inflamed area. The tumor is swelling of the inflamed tissue as a result of increased capillary permeability and fluid accumulation. Calor is the increase in temperature (heat) that occurs in the inflamed area as a result of increased blood flow and dolor is pain that occurs in the inflamed area as a result of stimulation of sensory neurons. It is usually categorized into acute and chronic inflammation.

1.2.1.1 Acute inflammation

The acute inflammation is the initial response to tissue injury, where chronic inflammation is the subsequent reaction that may occur if the stimulus cannot be removed (Davies *et al*., 2001; Zdanowicz, 2003). The acute inflammatory response may be divided into main two stages as followed (Figure 1.1) (Zdanowicz, 2003).

1. Vascular response

• Rapid vasoconstriction of blood vessels occurs in the injured area and is followed by rapid vasodilatation.

• An increase in capillary permeability occurs in the injured area leading to swelling and edema. The fluids that enter the injured area are useful for diluting out any bacterial toxins or irritants present in the tissue.

2. Cellular response

• Phagocytic neutrophils are the first white blood cells to arrive in the injured area. Leukocytes are attracted to the injured area by certain bacterial substances as well as by cellular debris and cytokines (chemotaxis).

• As fluid leaves, the capillaries, the viscosity of blood increases and leukocytes precipitate to the walls of the capillary. This process is called margination. Leukocytes undergo a change in shape and squeeze through the now more permeable capillaries into the tissues. The movement of leukocytes through the capillary wall is called diapedesis.

• Other white blood cells such as eosinophils and basophils also arrive at the injured area and release substances such as histamine that enhance the inflammatory reaction. Histamine is a powerful vasodilator that increases capillary permeability. Monocytes will also enter the inflamed tissues where they mature into phagocytic macrophages.

• Cytokines such as interleukin and tumor necrosis factor are released to enhance the inflammatory and immune response.

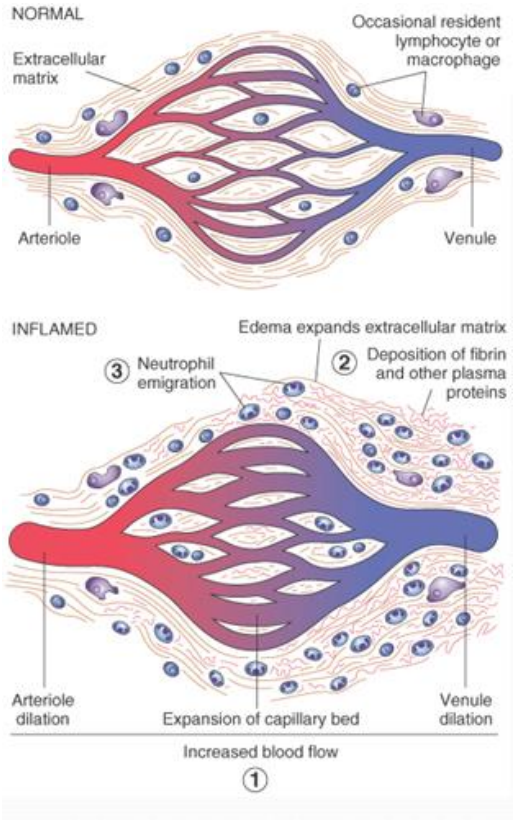


Figure 1.1 The vascular and cell response in acute inflammation

Source: Kumar *et al*. (2005)

1.2.1.2 Chronic inflammation

If the stimulus or causative agent is persistent, the inflammation becomes chronic in nature. Chronic inflammation is characterized by the infiltration of macrophages and lymphocytes into the inflamed area. Macrophages phagocytes and remove dead tissue cells and polymorphs but can also damage healthy cells via the production of free radicals such as $NO^{\bullet}$ and $O_2^{\bullet -}$. Macrophages and fibroblasts produce cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) and radicals such as $NO^{\bullet}$, which mediates cell proliferation and the inflammatory process. T-cells are involved in the removal of the antigen in a more specific way. Antigen-presenting cells (APC) are thought to uptake and process the antigen via their class II major

compatibility complexes. The antigen is presented to the T-cell by APCs and then specific antibodies can be produced by B-cells to remove the offending organism. The formation of a granule from the mass of immune cells attracted to the infected area is also a hallmark of chronic inflammation (Davies *et al*., 2001). The inflammatory action stage and their causes were shown in Table 1.1.

Table 1.1 Inflammatory status and their causes

Inflammatory Status	Causes
Acute inflammation	Induced by histamine, carrageenan, picryl chloride, inflammatory cytokines (IL-1, IL-6, IL-8, TNF-α), LPS administration
Chronic inflammation	Arthritic conditions Inflammatory bowel diseases Myocardial infarction
Systemic inflammation[a]	Sepsis Endotoxemia Respiratory distress syndrome

Source: Modified from Owusu-Apenten (2010)

Remark: [a] Typically induced by LPS injection.

1.2.1.3 Inflammatory mediators

Inflammatory mediators are chemical substances released from injured or activated cells co-ordinate the development of the inflammatory response. Such chemical mediators include histamine, prostaglandins, and interleukins produced by macrophages and mast cells increase vascular permeability and attract phagocytes and

lymphocytes to the site of infection (Owusu-Apenten, 2010). Mediators involved in the inflammatory response were listed in Table 2.2.

Table 1.2 Inflammatory and immune mediators

Compound	Function
Histamine	Histamine (H1) receptor produces vasodilatation, increased vascular permeability, contraction of other smooth muscles, increased gastric secretion
Eicosanoids	
Prostaglandins	Increased vasodilatation, contraction of bronchial smooth muscles
Leukotrienes	Increased vasodilatation, cell adherence and chemotaxis of polymorphonuclear cells, contraction of bronchial muscle
Platelet activating factor	Platelet aggregation, vasodilatation, increased vascular permeability, chemotaxis, and activation of leukocytes
Bradykinin	Increased vasodilatation, increased vascular permeability, stimulation of pain nerve endings, induces cough
Nitric oxide	Pro-inflammatory, increases vasodilatation, increases vascular permeability
Cytokines	Pro-inflammatory (TNF-α and IL-1) or anti-inflammatory (IL-4, IL-10, and IL-13)

Source: Owusu-Apenten (2010)

1.2.2 The cytotoxicity of pesticides and cadmium

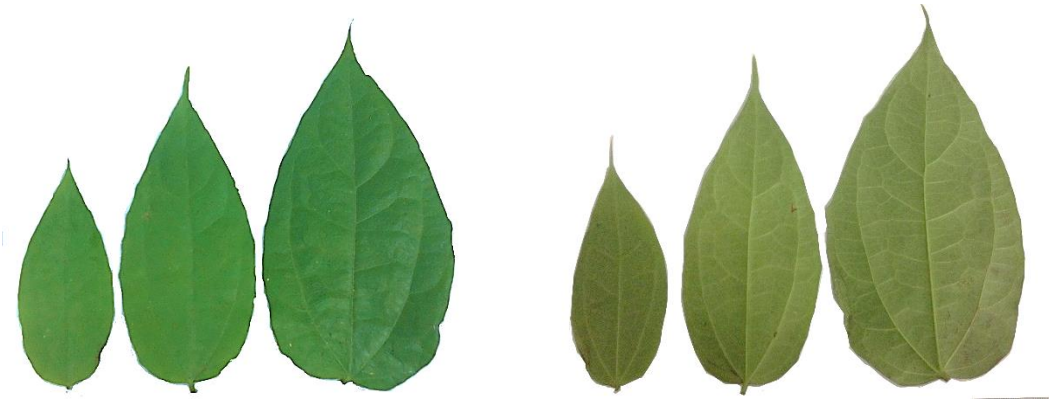
The main factors involved in environmental contamination of today's world are focusing on pesticides and cadmium particularly in the food chain. Pesticides can harm human life and can disturb the function of different organs in the body, including nervous, endocrine, immune, reproductive, renal, cardiovascular and respiratory systems (Mostafalou and Abdollahi, 2013). Ilboudo *et al*. (2014) reported the cytotoxic effect of five pesticides including Deltamethrin, Fenitrothion, Fipronil, Lambda-cyalothrine, and Teflubenzuron alone or in combination in human intestinal Caco-2 cells especially, Fipronil and Fenitrothion which were shown the most impacting on the cell. Moreover, Rana and Shivanandappa (2010) studied the cytotoxicity of the two pesticides, thiram, and endosulfan in Ehrlich ascites tumor and found the cytotoxic actions of the pesticides on the cells were characterized by glutathione depletion, induction of reactive oxygen species (ROS). In case of cadmium, Katsumiti *et al*. (2014) showed the mechanisms action of CdS quantum dots, a primarily utilized in the manufacturing of nanoparticles (NPs) were assessed at sub-lethal concentrations (0.31-5 mg Cd/L) in the hemocytes and gill cells of the mussel *Mytilus galloprovincialis* through a series of functional *in vitro* assays: production of reactive oxygen species (ROS), catalase (CAT) activity, DNA damage, lysosomal acid phosphatase (AcP) activity, multixenobiotic resistance (MXR) transport activity, Na-K-ATPase activity and phagocytic activity and damage to actin cytoskeleton and found cell-mediated immunity and gill cell function represent significant targets for CdS quantum dots toxicity. In addition, Chen *et al.* (2014) studied the effects of cadmium on cell proliferation, apoptosis and proto-oncogene expression in zebrafish liver cells (ZFL) and the results showed that Cd can be effectively accumulated in ZFL cells.

1.2.3 *Thunbergia laurifolia* leaves

1.2.3.1 Botanical description

In Thailand, *T. laurifolia* is called in several names including Rang Jued, Yaw Kaew, Kob Sha Nang, Gum Lung Chang Puak, or Krua Nan Nae in the North (Aritajat *et al*., 2004). It is a climbing plant with smooth opposed leaves

along the stem. The leaves are 8-10 cm long and 4-5 cm broad, broad-based, narrowing to a pointed tip, usually with scalloped lobes towards the base (Figure 1.2). The characteristic of *T. laurifolia* flowers are trumpet-shaped and the seed pod is cone-shaped, 1 cm long, with a round base and it can be divided into three types of flowers; white, yellow and purple flowers. The purple flower cultivar has been reported to have several distinct pharmacological properties particularly from extracts derived from stems, roots, and leaves (Oonsivilai, 2006).



(A) (B)

Figure 1.2 Characteristic of *Thunbergia laurifolia* leaves in Thailand; young leaves (left), developing leaves (middle), and mature leaves (right); (A) dorsal side and (B) ventral side of leaves.

1.2.3.2 Chemical composition and some active compounds

The content of fiber, ash, protein, fat, and carbohydrate on the dry basis weight of *T. laurifolia* leaves was 16.82, 18.79, 16.70, 1.68 and 46.01%, respectively (Jaiboon *et al.*, 2010). The phytochemistry of the leaves was classified into 5 groups (Chuthaputti, 2010) (1) sterols such as beta-sitosterol, stigmasterol, alphaspinasterol (2) phenolics such as apigenin, caffeic acid, gallic acid and protocatechuic (Chuthaputti, 2010; Oonsivilai *et al.*, 2007) (3) carotenoids such as lutein (Chuthaputti, 2010), (4) unclassified steroids

(Chuthaputti, 2010) and (5) glycosides such as 8-epi-grandifloric acid, 3'-O-β-glucopyranosyl-stilbericoside, grandifloric acid, benzyl β-glucopyranoside, benzyl β-(2'-O-β-glucopyranosyl)-glucopyranoside, 6-C-glucopyranosyl apigenin, 6,8-di-C-glucopyranosyl apigenin, (*E*)-2-hexenyl-β-glucopyranoside, and hexanol-β-glucopyranoside (Kanchanapoom *et al*., 2002; Chuthaputti, 2010; Chan *et al*., 2011). Though, the leaves of this plant are the main part of utilization, bark and root are also used (Thongsaard and Marsden, 2002). Some studies on *T. laurifolia* leaves in particular functional properties which are related to human health benefit are in section 1.2.3.3 as the following.

1.2.3.3 Biological effects of leaves

1. Anti-inflammatory effect

For more than 30 years, preclinical and clinical researches have been investigated to prove anti-inflammatory effects in *T. laurifolia* leaves (Chuthaputti, 2010). The anti-inflammatory efficiency dose of the aqueous leaves extract of *T. laurifolia* (ED_{50}=2.5 g/kg body weight) has been reported to be two folds compared with *Garcinia mangostanarind* rind extract (ED_{50}=5.5 g/kg body weight) using carrageenan-induced paw edema model in mice (Pongphasuk *et al*., 2005). Moreover, Wonkchalee *et al*. (2012) reported that *T. laurifolia* leaves possessed anti-inflammatory and antioxidant properties which improved liver function in hamsters treated with liver fluke infection or after administration of N-nitrosodimethylamine (NDMA, a potent hepatotoxin, carcinogen, and mutagen). The research found that fresh and dried aqueous extract from *T. laurifolia* leaves clearly reduced the inflammatory cells treated with *O. viverrini*, a human liver fluke which is the primary risk factor for cholangiocarcinoma, reduced NDMA-administered groups of Syrian hamsters. The anti-inflammatory activity of the plant extracts was well correlated with the total antioxidant capacity. Additionally, Boonyarikpunchai *et al*. (2014) reported rosmarinic acid, isolated from an ethanolic extract of *T. laurifolia* leaves (Suwanchaikasem *et al*., 2014) provided anti-inflammatory effects against acute and chronic inflammation.

2. Antioxidant effect

It was found that aqueous extraction of *T. laurifolia* leaves had higher total phenolic content (TPC) compared to ethanol and acetone extract (Oonsivilai, 2006). Moreover, the aqueous extract also yielded the highest activity to free radical scavenging (DPPH) with lowest EC_{50} value and FRAP compared to ethanol and acetone extracts (Oonsivilai, *et al.,* 2008; Chan *et al.,* 2011). Furthermore, Chan *et al*. (2013) reported that the developing leaves had the highest TPC of 513 mg GAE/100 g, followed by young and mature leaves with values of 407 and 298 mg GAE/100 g, respectively. High TPC and free radical scavenging activities of *T. laurifolia* leaves extract has been stated to relevant against human breast cancer cells (Jetawattana *et al*., 2015) and protects hemolysis in mice during *Plasmodium berghei* infection, cause of malaria, through the inhibition of oxidative stress (Khobjai *et al*., 2014).

3. Anti-microbial effect

The pharmacological property of *T. laurifolia* has also been reported to process antimicrobial activity including antibacterial, antifungal and antiviral. Pukumpuang *et al*. (2012) reported the ethanolic extracts from *T. laurifolia* leaves showed inhibition clear zone activity on *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis* and *Streptococcus pyogenes*. While, aqueous extract of this plant inhibited MRSA and *Streptococcus pyogenes*, ethanolic leaf extract, showed inhibition of *Bacillus subtilis* under UV light inducing but not for *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Aspergillus fumigatus* (Cheeptham and Towers, 2002; Chan *et al*., 2011). Wirotesangthong *et al*. (2009) reported that the aqueous leaves extract of the plant showed neuraminidase (NA) inhibition in the influenza viruses, including type A (H1N1 and H3N2) and B. Moreover, Tewtrakul *et al*. (2003) addressed that the ethanolic and water extracts of leaves showed mild anti-HIV virus by inhibited HIV-1 integrase even not strong effectively.

4. Hepatoprotective activity

Several data reported the aqueous extract of the leaves possessed hepatoprotective activity. In the rats treated with ethanolic extract can protect hepatic disorder when induced by ethanol. Moreover, the hepatoprotective activity of aqueous extract of the leaves against treated with rat hepatocytes had also been reported (Chanawirat *et al*., 2000; Pramyothin *et al*., 2005; Oonsivilai, 2006; Chan *et al.*, 2011).

5. Anti-diabetic effect

Taking the aqueous leave extract as 60 mg in 1 ml/day for 15-day showed a decreased level of blood glucose and recovery of some β-cells in diabetic rats. The result explained that the leaf contained insulin-like substance(s) which directly act as hypoglycemic agents, or some substances may induce the regenerative process of β-cells (Aritajat *et al*., 2004; Chan *et al*., 2011).

6. Detoxifying effect

Morkmek *et al*. (2010) reported the effects of the aqueous leaves extract on detoxification of cadmium in the rat. It was found that abnormal appearance and behavior was lesser in rats fed with the extract prior to cadmium exposure than in those fed with the extract after cadmium exposure. Furthermore, Phyu and Tangpong (2013) showed that the aqueous leaves extract significantly prevented Pb induced neurotoxicity in a dose-dependent pattern which was indicated by the comparatively better performance of treated mice. Amount of phenolic content, antioxidant and neuroprotective properties increased along with the concentration of *T. laurifolia* extract. The detoxifying effect of the aqueous leaves extract on paraquat was reported by Usanawarong *et al*. (2000) who addressed that the aqueous leaves extract can detoxify against paraquat-intoxicated rat by decreasing plasma malonaldehyde, an indicator of lipid peroxidation of the paraquat-intoxicated rat. Moreover, Chinacarawat *et al*. (2012) suggested that orally administered *T. laurifolia* capsule at the dose of 600 mg/day for 2 weeks continuously can reduce organophosphate and carbamate insecticide poisoning and had no side effects in a high-risk volunteer.

1.2.3.4 Quality factors affecting

Normally, *T. laurifolia* is used as herbal tea product. Drying process serves as an essential part of tea processing, influence to its antioxidant content and appearance which affects the commercial value of the tea (Chong and Lim, 2012). Chan and Lim (2006) reported that using a household microwave oven for drying *T. laurifolia* leaves possessed higher total phenolic content, and antioxidant activity measured by DPPH, FRAP and ferrous ion chelating (FIC) assays compared with conventional oven-dried and sun-dried as well as fresh leaves. Moreover, the finding of Chan *et al*. (2011) studied the effects of various thermal and non-thermal drying methods on the antioxidant properties of leaves and teas of *T. laurifolia* which the leaves (15 g) were shredded, and divided to microwave-dried (1.5 min), oven-dried (3 h), freeze-dried (overnight), and freeze-withered (2 h). Then the treated leaves were extracted by steeping in hot water (1 h) to obtain the tea infusions. The result revealed that using microwave was a great choice to process the *T. laurifolia* tea. Moreover, using temperature affect to the efficacy of extraction. Wonkchalee *et al*. (2012) reported that using boiled water for extraction of dried *T. laurifolia* leaves gave the highest FRAP assay values compared with using water at room temperature with both fresh and dried leaf extraction. Mahasarakul *et al*. (2013) found that free radical scavenging activity of *T. laurifolia* beverage which prepared from sun-dried leaves and vines boiled in hot water and stored at 4ºC in closed packaging decreased significantly when increased storage time.

1.2.4 Herb-drug interactions

Herbs have been used for medicinal purposes since the beginning of recorded time. Millions of people today still use herbal therapies along with prescription and non-prescription medications. In addition, many of drugs including digitalis, morphine, atropine and several chemotherapeutic agents were developed from plants. Although considered natural, many of these herbal therapies can interact with other medications, causing either potentially dangerous side effects and or reduced benefits from the medication (Yaheya and Ismail, 2009). Interactions between herbals and

medications can be caused by either pharmacodynamics or pharmacokinetic mechanisms. Pharmacodynamic interactions can occur when an herbal product produces additive, synergistic or antagonist activity in relation to the conventional drug. Pharmacodynamic interactions are related to pharmarcologic activity of the interacting agents and can affect organ systems, receptor sites or enzymes. Pharmacokinetic interactions occur when herbs change the absorption, distribution, metabolism, protein binding or excretion of a drug that results in altered levels of the drug or its metabolisms. Most of the current evidence of pharmacokinetic drug interactions involves metabolizing enzymes and drug transporters (Chanbasha, 2010). There were few reports of interactions between herbal medicines and toxic agents. However, Dumrongsakunchai *et al*. (2007) reported the *T. laurifolia* extract particularly the ethanolic extracts were potent inhibitors of cytochromes P450 (CYP) which are the main enzymes responsible for the metabolism of clinically used drugs and generally, interaction between herbs and drugs can occur via inhibition and induction of CYP. Moreover, Rocejanasaroj *et al*. (2014) suggested that *T. laurifolia* extract can reduce oxidative stress, minimize toxicity by regulating the expression CYP mRNAs for suitable production of CYP isoenzymes and may be beneficial for detoxification.

1.3 Objectives of the Research

1. To study the antioxidant activity and investigate the phenolics and flavonoids profile in the aqueous crude extract of *T. laurifolia* leaves.

2. To investigate the cytotoxicity of the crude extract on cells and study the effect of the crude extract on antioxidant enzymes, malondialdehyde (MDA) level and nitric oxide (NO) inhibition in the cell lines.

3. To study the anti-Cd toxicity of crude extracts in the cell lines.

4. To study the anti-insecticides toxicity of crude extract in the cell lines.

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CHAPTER 2

PHYTOCHEMICAL SCREENING AND ANTIOXIDANT ACTIVITY OF AQUEOUS CRUDE EXTRACT OF *THUNBERGIA LAURIFOLIA* (RANG JUED) LEAVES

2.1 Abstract

Thunbergia laurifolia or Rang Jued has been used in Thailand as folklore medicine since ancient times and has been proved to have a great antioxidant activity. The phytochemical screening including phenolic and flavonoid profiling and antioxidant activity in the aqueous crude extract of *T. laurifolia* leaves were evaluated. The phytochemical screening indicated that the crude extract contained phenols, flavonoids, tannins and sterols groups. Total extractable phenolic contents (TPC), total extractable flavonoid content (TFC), and antioxidant activity including, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, ferric ion reducing antioxidant power (FRAP) activity and ferrous ions chelating activity (FIC) were assays. The crude extract exhibited highest in DPPH radical scavenging followed by the FRAP and ABTS activity, respectively. The liquid chromatography-mass spectrometry **(**LC-MS) analyses indicated the presence of caffeic acid, rosmarinic acid, catechin, rutin, isoquercetin, quercetin and apigenin as bioactive compounds. The obtained results indicated that the crude extract contained several of antioxidant agents which should be supported for the healthy claim.

2.2 Introduction

Thunbergia laurifolia, with the Thai name 'Rang Jued', belongs to the family Acanthaceae (Morkmek *et al*., 2010) and has been used in Thai traditional medicine for many centuries. The extracts of fresh and dried leaves, barks and roots have been reported as an antidote for treating insecticide-, drug-, arsenic-, strychnine-, alcohol-, and food poisoning as well as other chemical toxins (Thongsaard and Marsden, 2002; Oonsivilai, 2006; Inta *et al*., 2013; Rocejanasaroj *et al*., 2014;

Maneenoon *et al*., 2015). Moreover, this plant has been reported to have antioxidant and anti-inflammatory properties (Chan *et al*., 2012; Wonkchalee *et al*., 2012). In general, Thais consumed *T. laurifolia* as tea, capsule and powder forms. To make an herbal tea, dried and ground leaves packed in the tea bag will be steeped in hot water (Chan *et al*., 2012; Singtonat and Osathanunkul, 2015). Actually, the content of chemical composition and active compounds depend on location, environmental and nutrition content in the soil of plant growth (Oonsivilai, 2006; Jaiboon *et al.*, 2010). Moreover, the plant leaves preparation and extraction influenced the composition type and quantity of active compound profile which leads to biological effect (Chan *et al*., 2011). Therefore, the objective of this study was taken an aqueous crude extract of *T. laurifolia* leaves to determine the phytochemical screening, antioxidant activity, and phenolic and flavonoid profiles by LC-MS.

2.3 Materials and Methods

2.3.1 Chemicals

Most of the chemicals used for the determination of antioxidant activity, phytochemical screening, high-performance liquid chromatographic (HPLC) and liquid chromatography-mass spectrometry (LC-MS) analysis were purchased from Sigma-Aldrich, Seelze, Germany otherwise from Merck, Darmstadt, Germany; Ajax Finechem, Auckland, New Zealand; QRAC, Selangor, Malaysia; Fisher Scientific, Leicestershire, England; and LAB-SCAN, Dublin, Ireland.

2.3.2 Raw materials

Developing or intermediate stage leaves of *T. laurifolia* (between dark green and bright green color, and without variegated) which can still be folded without breaking easily, were purchased (10 kg) directly from the contact farmer in Bangkok, Thailand and transported to the laboratory within 24 h after harvesting.

2.3.3 Methods

2.3.3.1 Plant preparation and extraction

According to the traditional folk medicine method, fresh leaves were removed from climber, washed with tap water, drained and air dried for 5-8 d to obtain a moisture content of 8-10 % (w/w), then ground to be a fine powder with 20-40 mesh, and stored in a dark bottle at room temperature. As this step, the dried leaves accounted for 57.81% from selected fresh leaves. Then the powder was extracted in hot water at 98±1ºC (1:10 w/v) for 1 h, and then filtered through three layers of gauze followed by Whatman No. 4 filter paper. The filtrate was freeze-dried and stored in a desiccator at 4°C for further study as dried crude extract (Ruangyuttikarn *et al*., 2013). The yield of freeze-dried crude extract was 0.87% from selected fresh leaves.

2.3.3.2 Proximate compositions and mineral contents

The powder of *T. laurifolia* was taken to the analyses of a proximate composition including of protein, ash, fat, fiber and carbohydrate according to the method of AOAC (2000) while mineral contents (magnesium, copper, zinc, and iron) were analyzed following the AOAC (2003).

2.3.3.3 Phytochemical screening assay

The crude extract was analyzed for phytochemical screening following the method of Sofowara (1993) and Harborne (1973). Briefly, the crude extract was dissolved in distilled water to obtain the concentration at 5 mg/ml as working solution before taken to further analysis as follows:

Phenol test: The crude extract solution, 2 ml was warmed up at 45-50ºC by a water bath. Then 2 ml of 3% iron (III) chloride ($FeCl_3$) was added. Formation of green or blue color was recorded for the presence of phenols.

Flavonoid tests: For flavonoid I test; 1 ml of the crude extract solution was added to 1 ml of 10% lead acetate then gently shaken. A muddy brownish precipitate represented the presence of flavonoid was noted.

For flavonoid II test; 1 ml of the crude extract solution was added with a few drops of 10% $FeCl_3$ before shaken. A wooly brownish precipitate has reported the presence of flavonoids.

Alkaloid tests: The crude extract solution, 1 ml was stirred with 5 ml of 1% hydrochloric acid (HCl) using a water bath (60ºC) for 15 min to complete the reaction before taken to filter. Thereafter, alkaloids (I) test was checked by adding 1 ml of Dragendorff reagent into 1 ml of the filtrate. The formation of cloudy orange was recorded. Test for alkaloid II was performed by adding 1 ml of Mayer reagent into 1 ml of the filtrate. A light yellow color was noted. Test for alkaloid III was evaluated by adding 1 ml of Wagner reagent into 1 ml of the filtrate. The observation of turbid brown color indicated the presence of alkaloids.

Tannin test: The crude extract solution, 1 ml was added into 1 ml of 3% $FeCl_3$. A greenish black precipitate represented the presence of tannins.

Saponin test: The crude extract solution, 0.2 ml was mixed with 5 ml of distilled water then shaken vigorously for 5 min. Persistence of foams was the indicator for saponins.

Terpenoid test: The crude extract solution, 5 ml was mixed in 2 ml chloroform. Then, 3 ml concentrated sulfuric acid (H_2SO_4) acid was carefully added to observe a reddish brown coloration between upper and lower layer.

Sterol test: Sterol (Salkowski's test) test was started by adding 2 ml of concentrated H_2SO_4 acid into 2 ml of the extract solution. A red precipitate indicated steroidal ring.

2.3.3.4 Total extractable phenolic and flavonoid contents and antioxidant activity assay

Total extractable phenolic content: The total phenolic content of the extracts were measured using a modified Folin-Ciocalteu method (Tan and Kassim, 2011). Briefly, 20 µl of 0.5 mg/ml of the extract was added to 96-well microplate. Then, an amount of 100 µl of Folin-Ciocalteu reagent (10% v/v) and 80 µl of sodium carbonate (7.5% w/v) were added and mixed thoroughly. After incubation for 30 min in the dark at ambient temperature, the absorbance was measured at 765 nm using the microplate reader. The

total extractable phenolic contents were expressed as mg of gallic acid, Trolox and caffeic acid equivalents (GAE, TE, and CAE/g extract) via the calibration curve.

Total extractable flavonoid content: Total extractable flavonoid content was measured by the colorimetric method (Kim *et al.*, 2003) with some modifications. Briefly, 800 µl of distilled water was added into 200 µl of 0.5 mg/ml of the extract followed by 60 µl of 5% (w/v) sodium nitrite solution and 60 µl of 10% (w/v) aluminium chloride solution. The mixture was allowed to stand at ambient temperature for 5 min then 400 µl of 1 M sodium hydroxide was added. Then, the volume of reaction mixture was made up to 2 ml with distilled water and mixed thoroughly. The absorbance of solutions was measured with spectrophotometry at 510 nm. Total extractable flavonoid contents were calculated from the standard curve of catechin and expressed as mg of catechin equivalent (CE)/g extract.

ABTS radical scavenging activity: ABTS assay, the procedure followed the method of Arnao *et al.* (2001) with a modification by using microplate reader. The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowed them to react for 12-14 h at room temperature in the dark. The solution was then diluted by mixing 1 ml of ABTS solution with 48 ml of distilled water in order to obtain an absorbance of 1.1±0.02 units at 734 nm. Fresh ABTS solution was prepared and used within 2 h. The prepared extract (15 µl) was mixed with 285 µl of ABTS solution and the mixture was kept at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm using the micro-plate reader. A standard curve of gallic acid, Trolox and caffeic acid were prepared. The activity was expressed as mg equivalent of GAE, TE and CAE/g extract.

DPPH radical scavenging activity: DPPH radical scavenging activity was determined by DPPH assay using the modified method from Shimada *et al.* (1992) by using micro-plate reader. Briefly, 150 µl of the extract was added to 150 µl of 0.2 to mM DPPH in 95% ethanol. The mixture was shaken lightly to mix well and stand at ambient temperature for 30 min in the dark. The absorbance of the sample was determined using the microplate reader by the blank control obtain an absorbance of 0.8±0.1 units at 517

nm. Standard curves were prepared using gallic acid, Trolox, and caffeic acid and reported as mg equivalent (GAE, TE, and CAE) /g extract.

FRAP reducing antioxidant power activity: The FRAP assay was done according to Benzie and Strain (1996) with a modification by using microplate reader. The stock solutions included 300 mM acetate buffer [3.1 g sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) and 16 ml acetic acid ($C_2H_4O_2$)], pH 3.6, 10 mM of 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM Iron(III) chloride hexahydrate ($FeCl_3.6H_2O$) solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed up at 37ºC before using. The extract at 0.5 mg/ml (15 µl) as allowed to react with 285 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then performed at 593 nm. The standard curves were prepared using gallic acid, Trolox, and caffeic acid and reported as mg equivalent (GAE, TE, and CAE) /g extract.

Ferrous ions chelating activity (FIC): The chelating activity on ferrous (Fe^{2+}) was determined using the method of Decker and Welch (1990). One milliliter of extract solution was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM iron (II) chloride ($FeCl_2$) and 0.2 ml of 5 mM ferrozine for 20 min at ambient temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. A standard curve was prepared using ethylene diamine tetra-acetic acid (EDTA). The activity was expressed as mg EDTA equivalent (EDTAE)/g extract.

2.3.3.5 Liquid chromatography-mass spectrometry (LC-MS) analysis for phenolic and flavonoid compounds

The crude extract of *T. laurifolia* leaves was dissolved with water (50 mg/ml) before subjected to quantitatively measure by LC-MS according to the methodology and equipment of the Central Laboratory (Bangkok, Thailand) Co., Ltd. LC-MS analysis was performed using an Agilent 1100 series liquid chromatograph/mass selective detector equipped with a quadrupole mass spectrometer

(Agilent Technologies, Waldrom, Germany). The liquid chromatographic system consisted of a quaternary pump, on-line vacuum degasser, and thermostatic column compartment, connected in line to a mass spectrometer. 20 µl of the sample was prepared and injected into the HPLC system using LiChroCART RP-18e column (150×4.6 mm, 5 µm). Acetonitrile (solvent A) and 10 mM ammonium formate buffer pH 4 with formic acid (solvent B) were used as mobile phase. The column was first equilibrated with 100% solvent B for 5 min and thereafter the ratio of solvent A was increased to 40% in 60 min with a flow rate of 1.0 ml/min and the column temperature was controlled at 40ºC. The mass spectrometer was fitted to an atmospheric pressure electrospray ionization (ESI) source, operated in positive ion mode. The electrospray capillary voltage was set to 4000 V, with a nebulizing gas flow rate at 13 L/min and a drying gas temperature of 320°C. Mass spectrometry data were acquired in the scan mode (mass range *m*/*z* 100-700). Phenolic standards consisted of caffeic acid, rosmarinic acid, and hydroquinone while, flavonoids were catechin, rutin, isoquercetin, eriodictyol, quercetin, apigenin, and kaempferol.

2.3.3.6 Statistical analysis

Completely randomized design (CRD) was used throughout in this experiment. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. Significance was declared at $p<0.05$ using the statistical software.

2.4 Results and Discussion

2.4.1 Proximate compositions and mineral contents

The yield of *T. laurifolia* leaves started from selected fresh leaves to dried leaves and crude extract was 57.81% and 0.87% respectively. The proximate compositions and several minerals contents of dried leaves were shown in Table 2.1. The results showed that the percentage of crude protein, crude fat, crude fiber, ash and carbohydrate of the dried leaf were 13.98±1.90%, 1.83±0.12%, 11.16±0.44%, 19.93±1.90 and 53.10±2.23% (dry weight basis) respectively. While, Jaiboon *et al.* (2010) reported that the *T. laurifolia* leaves contained crude protein, crude fat, crude fiber, ash, and carbohydrate as 16.70%, 1.68%, 16.82%, 18.79%, and 46.01% respectively based on dry basis weight. Regarding to the proximate composition, it was found that this present dried leaf was very high in ash content compared with the information of commercial black tea (4.94±0.70%) and green tea (4.57±0.82%) (Adnan *et al.*, 2013). Besides type of plants, the chemical compositions and mineral contents of leaf depend on several factors such as the maturing stage of leaf and its growth environment including local soil composition (Pyankov *et al*., 2001; Stein *et al.*, 2016).

The contents of mineral in *T. laurifolia* leaves indicated that the leaves were a good source of various macro elements such as potassium (K), phosphorus (P), and magnesium (Mg) (Table 2.1) which may be further utilized for some purpose. Mg is one of the most important elements which involves many enzyme activities and the structural stabilization of tissues (Guo *et al*., 2016). This element involves with plant leaf color or chlorophyll content. Without Mg, chlorophyll can not capture the needed sun energy for photosynthesis. This element is useful for carbohydrates metabolism and cell membrane stabilization in plants (Pasternak *et al*., 2010). P is required for ATP and nucleic acid synthesis (RNA and DNA), and protein production while, K has a profound effect on the profile and distribution of the primary metabolites in plant tissues such as organic acid, amino acid and soluble sugars particularly reducing sugars, in addition, K helps to protect from pathogens and insects invasion (Amtmann *et al*., 2008; Rashid *et al*., 2016) as a result of accumulation of inhibitory amino acid (GABA, glycine and taurine), phytoalexin, phenols and auxins which relates to plant defense mechanism

(Perrenoud, 1990; Amtmann *et al*., 2008). In addition, Troufflard *et al*. (2010) reported that K induced the jasmonate signal network to trigger of defense response toward necrotrophs and insects (Wasternack, 2007). A high content of K in this experiment may be a key explanation for intensive plant growth and less or even no evasion from insect and pathogen found in the plant. Moreover, K was reported to be the important cofactor of several enzyme systems such as Na^+/K^+-ATPase in Na^+- K^+ pump system to keep the suitable homeostasis of the animal cells and pyruvate kinase which an important enzyme in carbohydrate metabolism in plant cells (Sterner and Elser, 2002; Jin *et al*., 2011).

The microelement contents such as zinc (Zn), copper (Cu), iron (Fe) and selenium (Se) of *T. laurifolia* leaves ranged from highest to lowest content as following: Fe>Zn>Cu>Se, respectively. Microelements are required in small concentrations and importance for the growth, production and so on in the plant. Fe was reported is an essential microelement for almost all living organisms because it plays a critical role in numerous metabolic processes such as DNA synthesis, respiration, and photosynthesis (Rout and Sahoo, 2015). Furthermore, Zn is an essential element in all organisms particularly in form of Zn^{2+}. It acts as a catalytic or co-factor for a numerous of enzymes including a chelator nicotianamine, a non-proteinogenic amino acid acting as a high-affinity metal chelator in the plant for homeostasis balancing (Sinclair and Krämer, 2012). Moreover, Zn is one of the most relevant minerals to human health, because of its antioxidant and anti-inflammatory properties. Marreiro *et al*. (2017) suggested that Zn acts as a cofactor for important enzymes involved in the proper functioning of the antioxidant defense system, protects cells against oxidative damage and the stabilization of membranes by inhibits the pro-oxidant enzyme, nicotinamide adenine dinucleotide phosphate oxidase (NADPH-Oxidase). In addition, Zn can decrease inflammatory cytokines such as IL-1b and TNF-α (Prasad, 2014). Cu and Se, are also required for many enzymatic activities in plants and has influenced the vigor of plants and leaves including antioxidant balancing to offer the possibility of disease management (Feng *et al*., 2013; Brinate *et al*., 2015; Sutradhar *et al*., 2017). These trace elements were reported as essential components in antioxidant enzymes for many organisms, including plants, animals, and humans. For example, glutathione peroxidase

(GPx), cytoplasmic superoxide dismutase (SOD) and catalase (CAT) enzymes, require Se, Cu-Zn, and Fe metals as a cofactor, respectively (Vural *et al*., 2010). Therefore, there was hypothesized that these minerals would have an effect on the biological property including antioxidant and anti-inflammatory activity which would be explained later.

With high ash and mineral content found in this extract led to the assumption that this plant must have a specific biological property which related to not only phytochemical including phenolics and flavonoids but minerals also. In addition, it pointed out that with high ash and mineral contents in this plant may be used for sports drink and be an alternative drug for diarrhea patient, however, it needs to be further studied.

Table 2.1 Proximate composition and mineral content of *T. laurifolia* dried leaf

Contents	Dried-leaf *T. laurifolia*
Proximate compositions (% dry weight basis)	
- Crude protein	13.98±1.90
- Crude fat	1.83±0.12
- Ash	19.93±1.90
- Crude fiber	11.16±0.44
- Carbohydrate	53.10±2.23
Mineral contents (mg/kg dry leaf)	
- Potassium (K)	20,600.43
- Phosphorus (P)	2,500.75
- Magnesium (Mg)	4,548.30
- Iron (Fe)	84.14
- Zinc (Zn)	35.51
- Copper (Cu)	16.99
- Selenium (Se)	0.73

Values are represented as the mean ± standard deviation (n=3).

2.4.2 Phytochemical screening

The results showed that phytochemicals presented in *T. laurifolia* leaves extracted by water (high polarity media) were phenols, flavonoids, tannins, and sterols (Table 2.2). It pointed out that various active compounds contained in the leaves were water-soluble compounds. In addition, from our observations and according to folklore medical record, this plant is rarely invaded by insect and animals, maybe due to its terrible taste which is related to phenols including tannin content which was explained earlier. There has been reported that tannin provided many biological properties including anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (Saxena *et al.*, 2013). Putiyanan *et al*. (2008) reported that ethanolic extracts of the plant leaves showed only sterol but not tannin, alkaloid, and glucosides. While, Oonsivilai *et al.* (2007) and Chuthaputti (2010) reported that the leaves contained sterols, phenols, steroids, and glycosides. It pointed out that there were some different phytochemicals found in this plant extract. These differing result may be due to growth location and conditions, extraction preparation and leaf age (Oonsivilai, 2006; Chan *et al*., 2013). Alkaloids and terpenoids are well known as a toxic substance and associated with bitter flavors. Saponins are classified as the group of steroidal saponin, triterpenoid saponin and steroidal alkaloids (Solihah *et al*., 2012; Saxena *et al.*, 2013). Having no terpenoids, alkaloids, and saponins in this screening test may indicate low toxicity and less unpleasant taste which prone to have more marketable acceptability. However, actually terpenoids, alkaloids and saponins of many plants also promoted health benefit through the anti-carcinogenic, anti-microbial, anti-inflammatory and antioxidant activities (Dolan *et al.*, 2010; Solihah *et al.*, 2012; Saxena *et al.*, 2013; Yalavarthi and Thiruvengadarajan, 2013). Though, screening test could not identify a pure compound containing in the crude extract, at least it is a good guideline to narrow the group of a chemical compound which is easier to operate with LC-MS profiling further.

Table 2.2 Preliminary phytochemical screening of the aqueous crude extract of *T. laurifolia* leaves

Contents	***T. laurifolia* leaves extract**
Phenols	+
Flavonoids (Test I and II)	+
Terpenoids	-
Alkaloids (Test I, II and III)	-
Tannins	+
Saponins	-
Sterols	+

+ mean present; - mean absent

2.4.3 Total extractable phenolic and flavonoid contents and antioxidant activities

In the experiments, total extractable phenolic content (TPC), total extractable flavonoid content (TFC), ABTS radical scavenging, DPPH radical scavenging, FRAP reducing antioxidant power and ferrous ions chelating activity (FIC) were investigated and presented in Table 2.3. Three different standard agents i.e. Trolox, gallic acid and caffeic acid were used in TPC, ABTS, DPPH, and FRAP. The results showed that the value of TPC in this experiment was 123.68±2.94 mg GAE/g dry crude extract. Moreover, the result showed that caffeic acid expressed highest ability to react with Folin's phenol reagent (the lowest value). The higher value the lower ability, therefore, mg equivalent of each standard antioxidant was ranked as TE>GAE>CAE. Generally, gallic acid is used as a standard antioxidant for TPC determination based on its high functional groups, -OH group. In this experiment it was found that caffeic acid gave better ability which was in agreement with Stratil *et al*. (2006) who suggested that caffeic acid can react with Folin-Ciocalteu method better than gallic acid however, they reported that the antioxidant activity based on FRAP and DPPH assay when using gallic acid was higher than caffeic acid. Similarly, it was found that activity of gallic acid in this experiment was the highest ability when taken to evaluate antioxidant property based on ABTS, DPPH and FRAP assay. Therefore, the

antioxidant value in this present work was ranked as TE>CAE>GAE. Comparing to other findings, it pointed out that it was difficult to make a good conclusion for TPC determination in each plant when the used standard antioxidant was different. Generally, the difference of TPC and TFC contents including the antioxidant activity may depend on many variable factors such as planting location, extract preparation and stage of leaf development (Oonsivilai, 2006; Chan *et al*., 2012; Pukumpuang *et al*., 2012). In this experiment using developing leaves of *T. laurifolia* then extracted with boiling water which may help solubilization and extraction ability. Chan *et al*. (2013) reported that the developing leaves of *T. laurifolia* had the highest TPC compared with young and mature leaves. Moreover, Oonsivilai *et al*. (2008) reported that the sample extracted with water showed the highest of TPC followed by ethanol and acetone extractions, respectively. While Wonkchalee *et al*. (2012) reported that using boiled water for extraction the dried *T. laurifolia* leaves gave the highest FRAP assay values compared with using water at room temperature in both fresh and dried leaves extraction. In this study, the results showed that antioxidant activity was highest in DPPH assay followed by the FRAP and ABTS tests, respectively. As known that DPPH assay responds better to less polar antioxidants (Berker *et al*., 2013). As mentioned above using hot water can help dissolve and break down plant structure, which may be more efficiently extracted both high and less polar antioxidant compounds. Actually, using an aqueous solvent (water) should provide more high polarity antioxidant compounds which are easily determined by FRAP and/or ABTS assays (Martysiak-Żurowska *et al*., 2012). Masek *et al*. (2016) reported that caffeic acid, a member of the phenylpropanoid phenolic class, exhibited higher DPPH than ABTS activity. However, in this experiment high-temperature water (95±2°C), dried powder and a leaf-development stage which normally contains less wax cuticle, were used for extraction. These conditions may destroy heat sensitive and high polarity antioxidant compounds such as glucosides compounds and sugar (Sharma *et al.*, 2015), leading to a higher proportion of lesser polarity and high heat resistant compounds instead. Based on antioxidant activity and TPC, as well as TFC, it was confirmed that active compounds present in the extract were heat resistant compounds with not high polarity.

The result found in this experiment also revealed that the crude extract exhibited FIC which can act as a secondary antioxidant activity as 2.36±0.37 mg EDTAE/g dry extract (Lim *et al.,* 2007). In fact, the active chelating compound is a typical characteristic of flavonoids. However, Oonsivilai *et al.* (2007) reported that not only a flavonoid compound but also caffeic acid (not flavonoids) was assumed to be main compounds having metal chelating property were reported in the aqueous leaf extract (Psotová *et al*., 2003; Symonowicz and Kolanek, 2012).

Table 2.3 TPC, TFC and antioxidant activity of the aqueous crude extract of *T. laurifolia* leaves

Assay	Value
TPC	
- mg GAE/g dry extract	123.68±2.94[b]
- mg TE/g dry extract	603.41±21.38[a]
- mg CAE/g dry extract	77.79±1.89[c]
TFC (mg CE/g dry extract)	62.83±2.85
ABTS	
- mg GAE/g dry extract	41.66±1.63[c]
- mg TE/g dry extract	277.89±9.09[a]
- mg CAE/g dry extract	88.36±2.63[b]
DPPH	
- mg GAE/g dry extract	156.61±1.47[c]
- mg TE/g dry extract	238.02±1.72[a]
- mg CAE/g dry extract	187.21±1.48[b]
FRAP	
- mg GAE/g dry extract	39.08±0.26[c]
- mg TE/g dry extract	192.13±1.25[a]
- mg CAE/g dry extract	87.50±0.58[b]
FIC (mg EDTA /g dry extract)	2.36±0.37

[a-c] Mean within a column with different letters are significantly difference (p<0.05); TPC means total extractable phenolic content; TFC means total extractable flavonoid content; FIC means ferrous ions chelating activity; GAE means gallic acid equivalent; TE means Trolox equivalent; CAE means caffeic acid equivalent and CE means catechin equivalent. Values are represented as the mean ± standard deviation (n=3).

2.4.4 Phenolic and flavonoid compounds determined by LC-MS

In this present work, LC-MS was used for characterization of phenolic and flavonoid compounds. Ten standard compounds, including 3 phenolics, such as caffeic acid, rosmarinic acid and hydroquinone and 7 flavonoids, such as catechin, rutin, isoquercetin, eriodictyol, quercetin, apigenin, and kaempferol, were used (Table 2.5). The contents of caffeic acid, rutin, isoquercetin, rosmarinic acid, catechin, quercetin, and apigenin were 199.21±20.72, 132.26±11.45, 114.54±6.04, 90.28±14.51, 69.54±11.55, 61.19±8.23 and 41.32±4.16 mg/kg crude dry extract, respectively. While, constituents of *T. laurifolia* extract from other scientific reports were gallic acid, caffeic acid, protocatechuic acid, chlorogenic acid, catechin, rutin, apigenin and rosmarinic acid when identified by HPLC and NMR technique (Kanchanapoom *et al*., 2002; Oonsivilai, 2006; Mahasarakul *et al*., 2013; Boonyarikpunchai *et al*., 2014; Suwanchaikasem *et al*., 2014; Sultana *et al*., 2015). The similarly and differences in phenolic and flavonoid profiles of each researcher group may due to several factors such as variation of plant cultivars, location and environmental conditions of plant growth (Oonsivilai, 2006) including plant preparation and extraction. As mentioned in material preparation that the plant used in this experiment was slowly dried in ambient temperature (followed folklore medicine guideline) without any leaching processed which was different from other research groups. Then, the dried sample was extracted with soaking in hot water (95±2°C) which can destroy heat-labile compounds but help dissolve less polarity compound as earlier mentioned. Moreover, the results of this study showed the presence of caffeic acid, rutin, and isoquercetin as the major compounds. Caffeic acid was reported as a strong antioxidant and responsible for different biological activities such as anti-inflammatory and anti-tumor (Chang *et al*., 2010; Chao *et al*., 2010; Sato *et al*., 2011). While, rutin was reported to provide a stronger DPPH radical scavenging activity than butylated hydroxytoluene (BHT) and exhibited lipid peroxidation inhibition better than ascorbic acid at a concentration of 0.05 mg/ml (Yang *et al*., 2008). However, there was some unknown of phenolic and flavonoid compounds in this experiment which may provide antioxidant activity and other biological activities, therefore further analysis still needs to be carried out.

Table 2.4 Phenolic and flavonoid contents of *T. laurifolia* leaves from aqueous crude extract determined by LC-MS

Peak NO.	Compounds	Retention time (min)	$[M+H]^+$ (m/z)	References	Contents (mg/kg crude dry extract)
1	Catechin	12.54±0.02	291.0	Sun *et a*l. (2007); Vihakas (2014)	69.54±11.55
2	Caffeic acid	13.03±0.04	181.1	Sun *et al*. (2007); Falcão *et al.* (2013)	199.21±20.72
3	Rosmarinic acid	14.67±0.27	361.0	Mena *et al.* (2016)	90.28±14.51
4	Rutin	5.34±0.01	611.0	Bino *et al.* (2005); Bravo *et al.* (2007)	132.26±11.45
5	Isoquercetin	16.46±0.08	465.0	Simirgiotis *et al.* (2013)	114.54±6.04
6	Hydroquinone	23.80±0.28	328.3	Almodóvar *et al.* (2011)	ND
7	Eriodictyol	31.25±0.07	289.0	Fabre *et al*. (2001); Tsimogiannis *et al*. (2007)	ND
8	Quercetin	34.16±0.28	303.0	Fabre *et al.* (2001); Tsimogiannis *et al.* (2007)	61.19±8.23
9	Apigenin	43.35±0.03	271.0	Tsimogiannis *et al.* (2007); Simirgiotis *et al*. (2013)	41.32±4.16
10	Kaempferol	44.33±0.06	287.0	Tsimogiannis *et al*. (2007); Vihakas (2014)	ND

ND=not detected; Values are represented as the mean ± standard deviation (n=3).

2.5 Conclusion

Dried-leaves of *T. laurifolia* contained protein, fat, ash, fiber carbohydrate and some minerals particularly K, P, and Mg. Using hot water to extract the leaves provided 5 groups in the phytochemical screening test; phenols, flavonoids, tannins and sterols with the antioxidant activity based on TPC, TFC, ABTS, DPPH, FRAP and ferrous iron chelating property. Profiling of phenolics identified by LC-MS consisted of caffeic acid, and rosmarinic acid while, flavonoid compounds were catechin, rutin, isoquercetin, quercetin, and apigenin. However, there were some unknown compounds which needed to be future investigated.

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CHAPTER 3

IMPROVEMENT OF ANTIOXIDANT ENZYME ACTIVITY AND NITRIC OXIDE INHIBITION OF *THUNBERGIA LAURIFOLIA* (RANG JUED) LEAVES EXTRACT IN CELLS CULTURE MODEL

3.1 Abstract

Thunbergia laurifolia is a Thai herb and has been used in Thai folklore medicine for centuries. Generally, Thais consume *T. laurifolia* as an herbal tea because of its health claim. The aims of this study were to determine the influence of aqueous crude extracts of *T. laurifolia* leaves on cytotoxicity property, endogenous antioxidant enzyme activity, lipid peroxidation indicated by malondialdehyde (MDA) production using three type of cells, murine macrophage (RAW264.7), human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells and nitric oxide inhibition in RAW264.7 cells. The crude extract showed a low cytotoxicity (>100 μg/ml) in all cell types and increased catalase (CAT) and glutathione peroxidase (GPx) activities while decreased malondialdehyde (MDA) levels in HEK293 and HepG2 cells. In addition, the crude extract helped to reduce nitric oxide production in RAW264.7 cells induced by lipopolysaccharide (LPS) showing of the 50% of inhibitory concentration (IC_{50}) value of the crude extract was found at 25.74±1.51 μg/ml. It was concluded that the crude extract can improve antioxidant enzymes level and inhibit lipid peroxidation as well as provide an anti-inflammatory activity.

3.2 Introduction

Thunbergia laurifolia or Rang Jued, a local Thai plant belongs to the Acanthaceae family (Morkmek *et al*., 2010) is commonly consumed in the form of herbal tea. It is widely used by Thai folklore medicine and considered as household medicine. Fresh and dried leaves were reported to utilize as antioxidants and anti-inflammatory (Chan *et al*., 2012; Wonkchalee *et al*., 2012). Previous study showed that the aqueous crude extract of *T. laurifolia* leaves contained a high total extractable phenolic content (TPC) which demonstrated high antioxidant activity based on free radical scavenging, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric ion reducing antioxidant power assay (FRAP) (Chapter 2, section 2.4.3). Oxidative stress is a condition associated with an increased rate of cellular damage induced by ROS and oxidized active molecules (O_2, H_2O_2) via the formation of the lipid peroxidation product especially, malondialdehyde (MDA) (Roopha and Padmalatha, 2012) leading to the loss of balancing between ROS generation and scavenging activities. As a result, it can cause many diseases such as inflammation, diabetes, cardiovascular diseases and cancer (Arulselvan *et al*., 2016). Actually, in normal and healthy body condition, there is a balance between reactive oxygen species formation or free radical and endogenous antioxidant defense mechanisms (Arulselvan *et al*., 2016). In addition, ROS are mostly removed by endogenous antioxidants enzymes including catalase (CAT) and glutathione peroxidase (GPx) (Magalingam *et al*., 2013; Trchounian *et al*., 2016).

Therefore, the objective of this work was to investigate some biological activities of aqueous crude extracted from *T. laurifolia* leaves determined by cytotoxicity, endogenous antioxidant enzyme activities (CAT and GPx) and MDA production using RAW264.7 murine macrophage, HEK293 kidney and HepG2 liver cells as well as nitric oxide inhibition in RAW264.7 cells was also monitored.

3.3 Materials and Methods

3.3.1 Chemicals

Chemicals used for determination of cells culture were purchased from Gibco (Carlsbad, California). Most of the chemicals used for the determination of antioxidant activity, cytotoxicity, and nitric oxide (NO) production were purchased from Sigma-Aldrich, Seelze, Germany otherwise from Merck, Darmstadt, Germany; Ajax Finechem, Auckland, New Zealand; QRAC, Selangor, Malaysia; Fisher Scientific, Leicestershire, England; and LAB-SCAN, Dublin, Ireland.

3.3.2 Raw materials

T. laurifolia leaves at developing or intermediate stage (between dark green and bright green color, and without variegated) which can be folded without

break easily were directly (10 kg) purchased from the contact farmer in Bangkok, Thailand and transported to the laboratory within 24 h.

3.3.3 Methods

3.3.3.1 Plant preparation and extraction

The leaves were removed from a climber, washed with tap water, drained and air dried for 5-8 d followed a folk medicine method to obtain the moisture content as 8-10 % (w/w), ground to be a fine powder with 20-40 mesh, and stored in a dark bottle at room temperature. Then, the powder of the leaves was soaked in hot water, 98±1°C (1:10 w/v) for 1 h, and then filtered through three layers of gauze followed by Whatman No. 4 filter paper. The filtrate was freeze-dried and stored at 4°C for further study as dried crude extract (Ruangyuttikarn *et al*., 2013).

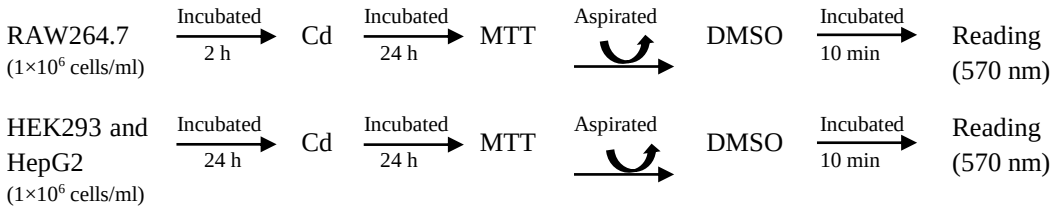
3.3.3.2 Cell Culture

Murine macrophage RAW264.7 cells and human embryonic kidney cells (HEK293) were purchased from American Type Culture Collection (ATCC, USA) and were all grown in RPMI–1640 medium and minimum essential medium (MEM) respectively, while human hepatocellular carcinoma cells (HepG2) kindly provided by Assoc. Prof. Dr. Teerapol Srichana (Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand) were cultured in Dulbecco's Minimal Essential Media (DMEM). The media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were culture at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in a fully humidified incubator. The cells were harvested with 0.25% trypsin-EDTA and suspended in a fresh medium. In this experiment, all cell types were used at passage 25-60 for testing.

3.3.3.3 Determination of the crude extract on cytotoxicity by MTT assay

The cells were seeded in a 96-well plate with 1×10^6 cells/ml by adjusting the cell density with culture medium and counted by a standard trypan blue cell counting technique before incubated RAW264.7 cells for 2 h and 24 h for HEK293 and HepG2 cells to allow cell adhere to the flask. The crude extract as the concentration of

0.01-2.00 mg/ml was added to the wells and incubated for 24 h. The solution was removed from the cell cultures and then cells were treated with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983). Briefly, 100 µl of MTT (0.5 mg/ml) was added to each well and incubate for 2 h for RAW264.7 cells and 3 h for HEK293 and HepG2 cells at 37°C. After incubation, the media was aspirated and 100 µl of DMSO was added to each well to dissolve the formazan. Cells were incubated for 10 min at 37°C before taken to read an absorbance at 570 nm with microplate spectrophotometer as followed steps;



To determine the effect of the crude extract on MTT assays, therefore, the experiment was set up as followed.

Normal control : Media + Media.

Tested sample : Media + Sample

The amount of cell viability was detected by MTT cytotoxicity assay and calculated percentage of cell viability as followed equation;

$$\% \text{ Cell viability} = [OD_{sample} / OD_{control}] \times 100$$

3.3.3.4 Determination of the crude extract on antioxidant enzyme activity and malondialdehyde value

Preparation of endogenous cellular extract: All cell lines were seeded at 1×10^6 cells/ml in 60 mm tissue culture dishes and incubated for 24 h to allow cells to grow and adhere to the dish. In each culture dish, the cells were washed twice with 2 ml of PBS (pH 7.2) before treating with the crude extract (0.01-1.00 mg/ml). The crude extract was added to the dishes and incubated for 24 h at 37°C before removed from cell cultures. Cells in each culture dishes were harvested by incubation (1 min for

HEK293, 5 min for HepG2 and 10 min for RAW264.7) with 0.5 ml of 0.25% trypsin-EDTA. Then, 1 ml of culture media was added and centrifuged at 1,000× g for 10 min. Cell pellets were washed with ice chilled PBS (500 ml, 2 times). Cell pellets were then lysed by sonication on ice for 1 min using a probe-type sonicator (Vibra-Cell, Sonics and Materials Inc., Newtown, CT, USA) pulsing at 15 s on and 10 s off cycles (Du *et al.*, 2016). The mixture was then centrifuged at 10,000x g for 10 min at 4°C and the supernatant (endogenous cellular extract, ECE) was assayed for enzyme activity. Protein contents were determined with a modified method of Bradford (1976), using bovine serum albumin as a standard and protein concentration.

Catalase (CAT) activity: The activity of catalase (CAT) was determined by monitoring the decreasing of the absorbance at 240 nm due to H_2O_2 consumption following the method of Aebi (1974) with minor modifications. The 1.9 ml reaction mixtures contained a 50 mM phosphate buffer (pH 7.0) and 0.25 mM H_2O_2 was mixed with 100 µl ECE in a UV cuvette and using an ultraviolet spectrophotometer for measure the absorbance.

Glutathione peroxidase (GPx) activity: The activity of glutathione peroxidase (GPx) was determined based on glutathione oxidation by GPx in the presence of DTNB (Flohe and Gunzler, 1984) with minor modifications. Briefly, 200 µl of ECE was added to 400 µl of 0.1 mM GSH, 200 µl of 0.067 M Na_2HPO_4, then incubated at 28 °C for 5 min before added 200 µl of 1.3 mM H_2O_2 and stored at room temperature for 10 min followed by added 1 ml of 1% TCA and stored in ice bath for 30 min. Then, 480 µl of the mentioned reaction was added to 2.2 ml of 0.32 M Na_2HPO_4, 320 µl of 1 mM DTNB and stored at room temperature before measured the absorbance at 412 nm. Both of enzyme activities were calculated as enzyme unite per mg protein according to the calibration curve of standard BSA by Bradford's assay (1976) and expressed as a unite per mg protein and percentage of the control.

Malondialdehyde (MDA) value: To measure of malondialdehyde (MDA) value, the modified method of Heath and Packer (1968) was used, 600 µl of ECE were mixed with 3 ml of 20% TCA containing 0.8% of TBA (w/v) and heated at 95° C for 60 min. The reaction was cooled down under running tap water and centrifuged at 3,000×g for 15

min at 4°C before measured the absorbance at 532 nm using MDA assay as an external standard and expressed as nmol per mg protein and percentage of the control.

3.3.3.5 Determination of the extract on nitric oxide (NO) inhibition in RAW264.7 cells

This method is used to measure the production of NO in a macrophage cell line. Briefly, RAW264.7 at the density of 1×10^6 cell/ml were cultured in RPMI-1640 medium and incubated at 37°C with 5% CO_2 for 2 h to allow cells adhered to the flask. Cells density were adjusted by culture medium and counted by a standard trypan blue cell counting technique. N^G-nitro-L-arginine (L-NA) was used as a positive control. The medium was replaced with fresh medium containing 0.4 µg/ml of lipopolysaccharide (LPS) to stimulate NO production and followed by the addition of the crude extract at various concentrations (10 to 500 µg/ml) and further incubated for 24 h. Then, 100 µl of supernatant was moved to another 96-well plate to measure NO production by Griess's assay and left cells were tested for cytotoxicity by MTT assay. To determine NO production, the 100 µl of Griess's reagent was added to the well plate which has 100 µl of the supernatant. Then, NO production was measured using a microplate reader at 570 nm. The inhibition (%) of NO production was calculated by using the following equation.

$$\% \text{ Inhibition of NO} = \frac{[(\text{Negative control-Normal control})-(\text{Tasted sample-Blank sample})]}{(\text{Negative control-Normal control})} \times 100$$

The effect of extract and L-NA on NO inhibition will be determined 50% of inhibitory concentration (IC_{50}) values by graphically according to National Cancer Institute guidelines for the extracts (Itharat *et al*., 2004). The experiment was set up as followed.

Normal control	: Media + Media + Media
Negative control	: Media + LPS + Media
Positive control	: Media + LPS + L-NA
Tested sample	: Media + Extract + LPS
Blank sample	: Media + Extract + Media

3.3.3.6 Statistical analysis

Completely randomized design (CRD) was used throughout in this experiment. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. Significance was declared at $p<0.05$ using the statistical software.

3.4 Results and Discussion

3.4.1 Cytotoxicity property

As well known, RAW264.3 is the immune cell which responses to the first defense against pathogens (Helali *et al*., 2016) and this cell is most sensitive compared with other cells. While, HEK293 and HepG2 cells from kidney and liver organs, a function for removing waste products and excess fluid from the body. The cytotoxicity is an essential measurement to ensure the potential toxicity of the crude extract and the result showed in Figure 3.1. The results showed that 50% cytotoxicity concentration (CC_{50}) of the crude extract of each cell type was higher than 100 µg/ml (Table 3.1) which was classified as low cytotoxicity (Oonsivilai, 2006; Ioset *et al*., 2009). This result was in agreement with finding of Oonsivilai *et al*. (2008) who found that the aqueous extract of *T. laurifolia* was judged as low cytotoxicity with CC_{50}>100 µg/ml after taken to test with mouse connective tissue (L929), baby hamster Syrian kidney (BHK(21)C13), human liver hepatocarcinoma (HepG2) and human colon adenocarcinoma (Caco-2) cell lines. Moreover, Chivapat *et al*. (2009) suggested that using the aqueous extracts at doses ranging from 20 to 2,000 mg/kg/day did not affect body weight, food consumption, behavior and general health on Wistar rats.

In addition, in this experiment using 10 µg/ml of the crude extract provided RAW264.7 cells viability more than the control condition. It was hypothesized that at the proper dose, the crude extract containing both nutrition (protein, fat carbohydrate, and minerals) and bioactive compounds (phenolic and flavonoid) may help cell proliferation or strength from mentioned nutritions, antioxidant and anti-inflammatory properties (Chattaviriya *et al.*, 2010; Ruangyuttikarn *et al*., 2013). Some scientific data reported that some minerals especially, Mg^{2+} and Ca^{2+} are the essential minerals for maintaining the functionality of cell line adhesion

(Attramadal, 1969; Takeichi and Okada, 1972). While, at higher concentration, the crude extract can reduce all cell types via bility which followed the nature rule that is too much of anything can cause an adverse effect (Galati and O'Brien, 2004; Rezk *et al*., 2015). It pointed out that too much such mentioned minerals may disturb cell adhesion leading to higher floating cells and less attached cell counts. Furthermore, phenolics and flavonoids can be not only antioxidant but also can be pro-oxidant under some certain conditions (Yordi *et al*., 2012). Higher phenolics may induce oxidative damage by reacting with various biomolecules, such as lipids, proteins and DNA instead besides radicals (Procházková *et al*., 2011; Yordi *et al*., 2012). As known that, *T. laurifolia* leaves was high in phenolic compounds (Oonsivilai *et al*., 2008; Chan *et al*., 2011; Wonkchalee *et al*., 2012) and other elements particularly potassium (K), phosphorus (P), magnesium (Mg), iron (Fe), zinc (Zn) and copper (Cu) etc. (chapter 2, section 2.4.1). However, it was noticed that the cell adhered to the plate was lesser when the crude extract concentration increased at some level. This may due to the disruption of cell adherence by minerals effect (Attramadal, 1969; Takeichi and Okada, 1972). In addition, the finding of some researchers also reported that apigenin and quercetin can induce cell apoptosis via pro-oxidant action (Galati and O'Brien, 2004; Kyselova *et al*., 2011) while, caffeic acid can induce mitochondrial apoptotic pathway (Chang *et al*., 2010).

However, closely focused on changing of cell viability or cytotoxicity of HEK293 and HepG2 test cells found that it changed as a small wave along the concentration increased. As understanding, living cells tend to adapt themselves to the environment when homeostatic swings in a small range or did not lose their balance (Brooks *et al*., 2011). Moreover, in dried *T. laurifolia* leaves contained some minerals such as K, P, and Mg which may play an important role in a large number of cellular processes including acting as a cofactor in enzymatic reactions and transmembrane ion movements especially in kidney and liver cells (Bara *et al*., 1993; Rose and Valdes, 1994; Blaine *et al*., 2015). Therefore, cell viability of HEK293 kidney and HepG2 cells did not decrease as the increasing of the crude extract concentration. However, as mentioned before that HepG2 liver cell in this experiment was carcinoma cell, it may can not directly compared with normal cells including RAW264.7 and HepG2 cells. On the other hand, it was hypothesized that the *T. laurifolia* extract may increase carcinoma

cell growth which may more harmful for cancer patients. Therefore, carcinoma cell lines must be needed to further studied to find out their mechanism response.

From the result, it pointed out that the sensitivity of the cell to the crude extract was type dependent. The most sensitive one was RAW264.7 cells compared with the other two. It indicated that this cell may contain some compounds different from other cells or it was possible that RAW264.7 may have sensitive trigger gene to send the signal to other cells to manage the foreign thing for example LPS, endotoxin and histamine etc. (Owusu-Apenten, 2010) or even other over elements or compounds. This result pointed out that using *T. laurifolia* needed to be caution in for healthy people while cancer patients should not take until more scientific data and strict dose are supported.

However, to be safe for all test cell types, the crude extract with not higher than 500 μg/ml which provided cell viability more than 80% was selected for next experiment to finding out the NO inhibition in RAW264.7 macrophage cells (section 3.4.3).

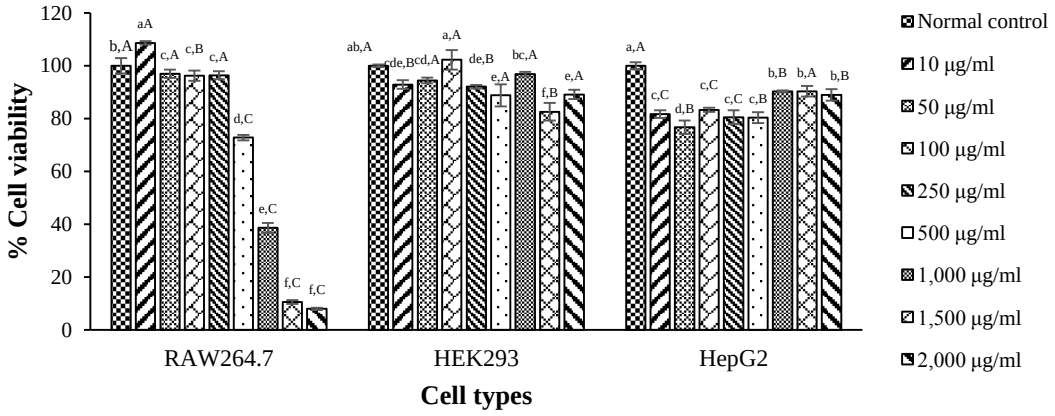


Figure 3.1 Effect of the aqueous crude extract of *T. laurifolia* leaves on cell viability value in RAW264.7, HEK293 and HepG2 cells.

Remark: $^{a\text{-}f}$ mean within cell types with different letters are significantly different (p<0.05). $^{A\text{-}C}$ mean within an extract concentration with different letters are significantly different (p<0.05). Values are mean ± standard deviation (n=3).

Table 3.1 50% cytotoxicity concentration (CC_{50}) values of the aqueous crude extract of *T. laurifolia* leaves on each cell type

Cell type	CC_{50} (μg/ml)
RAW264.7	>100
HEK293	>100
HepG2	>100

Values are mean ± standard deviation (n=3).

3.4.2 Antioxidant enzyme activity and malondialdehyde value

The effect of the crude extract on antioxidant enzyme activities (CAT and GPx) including MDA level in all cell types, RAW264.7, HEK293 and HepG2 cells showed in Table 3.1 and Figure 3.2. Regarding CAT antioxidant enzyme activity, it was revealed that HepG2 exhibited the highest value followed by HEK293 and RAW264.7 cells. While, GPx was highest to lowest from RAW264.7>HEK293>HepG2 respectively. In addition, Szymonik-Lesiuk *et.al* (2003) reported that the CAT was higher in the liver while GPx was lower in the kidney of Wistar rats. However, in this study, found highest GPx activity was found in RAW264.7 macrophage cell, and lowest in HepG2 carcinoma liver cell, Actually, GPx found widely spread in almost all tissues and was reported to be high in liver and commonly observed to be low in cancer cell (Jung *et al*., 2006; Magalingam *et al*., 2013). However, Hanif *et al*. (2005) found that no significant differences in GPx including CAT activities between the human liver cancer (HepG2) and human liver normal cell lines (WRL-68). In fact, CAT was reported the highest activity in liver, kidney, and red blood cells (Schriner and Linford, 2006). This enzyme is the first antioxidant enzyme to convert hydrogen peroxide into water and oxygen and its catalytic reaction is a one-step process while, GPx is the main scavenger of hydrogen peroxide by catalyze the oxidation of glutathione and use hydroperoxides as substrates (Magalingam *et al*., 2013). Moreover, the result showed that the activity of CAT and GPx (Figure 3.2A and 3.2B) in both of cell types (HEK293 and HepG2 cells) were higher compared with control. CAT and GPx are intracellular enzymes which form the primary defense system against oxidative

stress. In fact, both of antioxidant enzymes (CAT and GPx) are believed to play an important role in the prevention of oxidative stress-related diseases such as cancer, cardiovascular disease and protective effect from heavy metal-induced oxidative stress (Flora *et al.*, 2008; Bak *et al.*, 2012). Moreover, Ognjanović *et al*. (2003) reported that Cd-induced oxidative stress in rats affected to the antioxidant enzymes working for balancing oxidative stress system in cells, like CuZnSOD, CAT, GPx, glutathione reductase (GR) and glutathione-S-transferase (GSTs). The previous studied (chapter 2, section 2.4.4) stated that the crude extract of *T. laurifolia* leaves showed a various array of phenolic and flavonoid compounds including caffeic acid, rutin, isoquercetin, rosmarinic acid, catechin, quercetin, and apigenin. Generally, the phenolic compounds have been claimed as natural antioxidant agents by scavenging free radical and reducing the lipid peroxidation. Caffeic acid was reported to improve the redox balance in the liver of the rats by raising the activity of antioxidant enzyme including GPx and CAT with facilitating the removal of peroxide (Olayinka *et al*., 2017). While, Jayanthi and Subash (2010) found that caffeic acid can increase the activities of antioxidant enzymes against oxytetracycline induced hepatotoxicity in rats via the antioxidant defense system through the enhanced scavenging of free radical. Moreover, quercetin was reported to show the ability to improvement antioxidant enzyme including CAT and GPx in the renal tissue (Almaghrabi, 2015) and apigenin also up-regulated the gene expression of antioxidant enzymes (Jung, 2014). In addition, from a previous studied (section 2.4.1 in chapter 2) demonstrated that *T. laurifolia* dry leaves contained many essential elements (Fe, Zn, Cu, and Se) which were reported as a co-factor of the antioxidant enzyme including CAT and GPx. Then, using fine powder of *T. laurifolia* dried leaves to soak in high-temperature water (95±2°C for 1 h) may help the extraction ability of these essential minerals to be more released and played the role to enhance cell proliferation and antioxidant activity. However, to make a solid conclusion, minerals content in the extract should be analyzed and further studied.

Although, the increasing of CAT and GPx enzymes did not appear intthe RAW264.7 cell (Figure 3.2A and 3.2B) when increased the crude extract content. Moreover, the activity of both antioxidant enzymes from RAW264.7 cell decreased when the concentration of crude extract increased compared with control. It pointed out that the crude extract, especially at high concentration (1,000 μg/ml), may induce more

oxidative stress as the first line of defense against the pathogen (Navegantes *et al*., 2017) or foreign compound, therefore antioxidant enzyme may be used more for homeostasis balancing in this RAW264.7 cell. Regardless of cell types, CAT seemed to significantly change as changing the crude extract concentrations than GPx. This result was in agreement in finding of Kaynar *et al*. (1994) who reported that in the lesion segment, GPx and SOD activities were not significantly changed (p>0.05) but CAT activity was significantly high compared with rostral and caudal segment after experiment spinal cord injury in the rat. They concluded that neither H_2O_2 nor H_2O_2 derived radicals was not the main reason for tissue damage. There were several scientific data reported that phenolic and flavonoid compounds, as well as trace elements, can act as pro-oxidant and induce oxidative stress in the cells (Galati and O'Brien, 2004; Halliwell, 2008; Kyselova, 2011; Halliwell, 2014; Nanda and Agrawal, 2016). Therefore, more cytotoxicity property of the crude extract tested in the RAW264.7 cell well related to higher extract concentration, however, HEK293 and HepG2 cells did not exhibit that phenomenon may due to strong antioxidant enzymes activity (Figure 3.1). As known, both liver and kidney cells contain higher antioxidant enzymes activity (Schriner and Linford, 2006) which may help to better balance the homoeostasis of the cell from oxidative stress compared with other cells. However, needed to keep in mind that HepG2 in this experiment was carcinoma cell while other cell lines were a normal cell, therefore, it might be difficult to make a solid conclusion whether that antioxidant enzymes contents in what type was higher. Therefore, the normal liver cell line would be investigated and compared with carcinoma one further.

The level of MDA in this experiment was shown in Figure 3.1C. Generally, MDA is used as the end product of lipid peroxidation which is a widely accepted concept of cell damage leading to disease onset including Alzheimer's disease, rheumatic arthritis, and cancer (Magalingam *et al*., 2013). The results showed that MDA level significantly decreased in HEK293 while increased in RAW264.7 when the concentration of the crude extract increased which was related to the increasing of CAT and GPx enzyme activities. There were some scientific data stated both CAT and GPx enzymes involved with hydrogen peroxide elimination which directly related to MDA reduction (Haron, 1991; Jung *et al*., 2006). The reduction of MDA level, when treated with the crude extract may indicate oxidative stress

protection. This result was in agreement with Abdallah *et al*. (2011) who reported that the combination of caffeic acid and quercetin pretreatments significantly reduced the levels of MDA from Lambda-cyhalothrin, insecticide toxin inducing oxidative stress in rat erythrocytes. However, there was no such a kind phenomenon found in HepG2 since MDA increased then decreased at higher concentration of extract. Casalino *et al*. (2002) reported that there was not a good relationship between some antioxidant enzymes including CAT with MDA level in rat liver and kidney organs.

In addition, the result indicated that using the crude extract concentration at 100 μg/ml seemed to be the most proper based on the results of antioxidant enzyme activity and MDA level as well as cytotoxicity property in all cell types. As mentioned earlier that RAW264.7, a macrophage cell is the most sensitive cell to many trigger substances. It pointed out that the sensitive cell as RAW264.7 may have the special gene to fast response to foreign matter (Navegantes *et al*., 2017) or internal mediators. Therefore, any toxic substance including MDA even itself may not too dangerous compared with pesticides or heavy metal must be minimized by protecting mechanism or eliminating pathway. However, there was some scientific information reported that half-life of MDA in the human body was very short, hence measurement of plasma MDA did not provide reliable information outcome in patients who were acute paraquat intoxication (Gil *et al*., 2010)**.** Therefore, as investigation time and other oxidized parameters besides MDA including 4-hydroxynonenal (4-HNE) (Zhong and Yin, 2015) as well as animal trial need to be further studied before a conclusion will be made solidly. However, from the results of cytotoxicity including antioxidant enzymes and MDA level of the crude extract on HepG2 carcinoma cell indicated that the crude extract increased cell viability when the concentration of extract increased. Therefore, it pointed out that using this crude extract may need to pay more attention for healthy people who should not drink it much while cancer patients should avoid. However, for more detail, mechanism of supporting cancer cells growth needed to be further studied.

Table 3.2 Effect of *T. laurifolia* extract on antioxidant enzymes and malondialdehyde value in endogenous cellular extract (ECE) from RAW264.7, HEK293 and HepG2 cells

Treatments	**Cell types**		
	RAW264.7	**HEK293**	**HepG2**
CAT (unit/mg protein)			
- Control	3.18 ± 0.21^{aB}	3.46 ± 060^{bB}	7.21 ± 0.56^{bA}
- 10 μg/ml crude extract	3.81 ± 0.38^{aB}	3.47 ± 0.62^{bB}	11.69 ± 0.22^{aA}
- 100 μg/ml crude extract	2.12 ± 0.35^{bB}	5.54 ± 0.54^{aB}	11.53 ± 0.54^{aA}
- 1,000 μg/ml crude extract	13.51 ± 5.63^{cC}	5.13 ± 0.47^{aB}	11.79 ± 0.74^{aA}
GPx (unit/mg protein)			
- Control	27.83 ± 0.18^{bA}	9.40 ± 0.29^{dB}	5.62 ± 0.18^{cC}
- 10 μg/ml crude extract	28.88 ± 0.24^{aA}	9.99 ± 0.07^{cB}	8.12 ± 0.32^{bC}
- 100 μg/ml crude extract	23.19 ± 0.17^{dA}	10.65 ± 0.63^{bB}	8.75 ± 0.31^{aC}
- 1,000 μg/ml crude extract	24.94 ± 0.31^{cA}	11.85 ± 0.45^{aB}	8.72 ± 0.39^{aC}
MDA (nmol/mg protein)			
- Control	3.95 ± 0.059^{cC}	6.30 ± 0.117^{aB}	7.42 ± 0.059^{bA}
- 10 μg/ml crude extract	3.57 ± 0.050^{dC}	5.34 ± 0.086^{bB}	7.07 ± 0.037^{cA}
- 100 μg/ml crude extract	4.59 ± 0.108^{bB}	4.04 ± 0.053^{cC}	7.93 ± 0.146^{aA}
- 1,000 μg/ml crude extract	8.77 ± 0.073^{aA}	3.69±0.024dC	6.20 ± 0.100^{dB}

$^{a-d}$ mean within cell types with different letters are significantly different ($p<0.05$). $^{A-C}$ mean within an extract concentration with different letters are significantly different ($p<0.05$). Values are mean ± standard deviation (n=3).

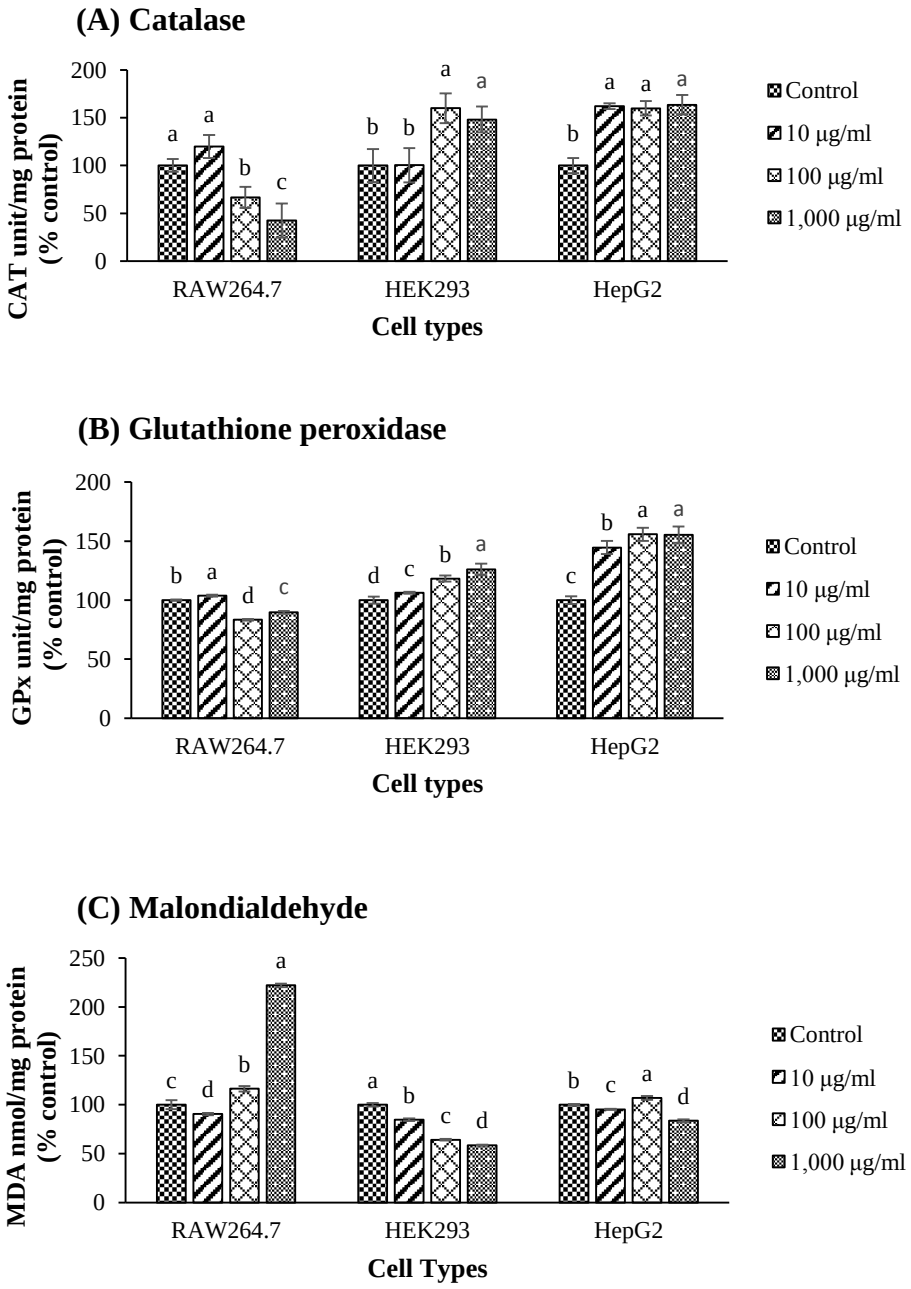


Figure 3.2 Antioxidant enzymes activities and malondialdehyde value of endogenous cellular extract (ECE) treated with *T. lauriforlia* extract in RAW264.7, HEK293 and HepG2 cells; (A) catalase; (B) glutathione peroxidase and (C) malondialdehyde.

Remark: [a-d] mean within cell types with different letters are significantly different (p<0.05). Values are represented as the mean ± standard deviation (n=3).

3.4.3 NO inhibition of the crude extract in RAW264.7 macrophage cells

It is accepted that NO production, an important molecule in the inflammatory response, is generally used for anti-inflammatory activity determination in RAW264.7 induced by LPS. However, it should keep in mind that the inflammatory effect involves various mediators including histamine, prostaglandins (PGs), leukotrienes (LTB4), platelet activation factor (PAF), bradykinin, serotonin, lipoxins, cytokines and growth factors (Nile and Park, 2013). In addition, the inflammatory effect can be generated through COX-2 (Lee *et al*., 2015a) not only the pathway of iNOS. In this experiment, the highest NO inhibition (96.80±2.46%) which was comparable to normal control was found when the extract was applied at 300 µg/ml ($p<0.05$). In fact, the crude extract seemed to significantly reduce NO production when the extract was applied at 10 µg/ml as shown in Figure 3.3A. The results also indicated that NO inhibition reached a saturation when the crude extract was used in the range 100 to 500 µg/ml. Surprisingly, cell viability of RAW264.7 induced by LPS and followed with the crude extract (tested sample) was significantly increased (Figure 3.3B) when compared with the treated cells with the crude extract without LPS induction (blank sample). In general, the crude extract would induce cell death at higher doses (>300 µg/ml) may be due to the crude extract toxicity. However, if the cell was already induced with LPS to get some stress then the crude extract would help the cell to survive. Therefore, this may support the claim of folklore medicine doctors who normally used the *T. laurifolia* leaves for the patient who approached the toxic compounds as pesticide or hangover. This may due to TPC function that has been proposed to play a major role in cytotoxicity and anti-inflammatory activity (Dolara *et al*., 2005; Saxena *et al.*, 2013).

In this study L-NA, iNOS inhibitor was used as a positive control, and the results showed that the crude extract exhibited IC_{50} at 25.74±1.51µg/ml while L-NA was 27.82±1.26 µg/ml (Table 3.2) with cell viability higher than 80%. It indicated that the crude extract provided NO inhibition better than the commercial chemical agent. Moreover, when taken caffeic acid and apigenin which was found in the crude extract as highest and lowest contents, respectively to check NO inhibition, it was found that NO inhibition with IC_{50} was 61.47±2.60 and 7.61±0.40, respectively. This pointed out that the activity of the composition found in the crude extract even low in content

may play a key role to NO inhibition. In addition, it was supported the concept that natural product containing mixed compounds is more safety compared with the synthetic single compound drug. According to the previous study, several phenolics and flavonoids were found in the crude extract of *T. laurifolia* leaves. Flavonoids have been reported to possess anti-inflammatory activity *in vitro* and *in vivo* (García-Lafuente *et al*., 2009; Rathee *et al*., 2009; Funakoshi-Tago *et al*., 2011). In addition, apigenin either aglycone or glucoside also have been reported to provide a good anti-inflammatory effect (Rathee *et al*., 2009; Lee, *et al*., 2015b) by protecting endothelial cell inflammation (Duarte *et al*., 2013). Recently, Lee *et al*. (2015b) reported that apigenin significantly suppressed production of NO in RAW264.7 stimulated with LPS through inducible nitric oxide synthase (iNOS) inhibition. In addition, Zhang *et al*. (2014) stated that LPS-induced macrophage inflammation was inhibited by applying apigenin through multiple mechanisms, for example; TNFα and IL-6 cytokines. Moreover, Lee *et al*. (2015a) suggested that rutin, a low molecular weight of flavonoid glycoside, reduced the inflammatory effect by suppressing the expression of COX-2 and iNOS in RAW264.7. Not only flavonoid apigenin but many phenolic compounds such as caffeic acid, gallic acid and protocatechuic acid obtained from the aqueous crude extract of *T. laurifolia* leaves as well as the rosmarinic acid from ethanolic extract also expressed their anti-inflammatory activity (Huang *et al*., 2009; Boonyarikpunchai *et al*., 2014). Actually, both caffeic acid and caffeic acid ester were reported to have both antioxidant and anti-inflammatory properties (Chao *et al*., 2010; Veres, 2012; Zhang *et al*., 2014) via multi pathways such as the inhibition of NF-κB, p38/ERK including iNOS expression (Song *et al*., 2002; Shin *et al*., 2004; Jung *et al*., 2008; Liu *et al*., 2014; Zhang *et al*., 2014).

As a whole, various chemical constituents found in the crude extract has its functional properties and played a role for biological activity through multi-pathway. The crude extract was safe to use with effective dose.

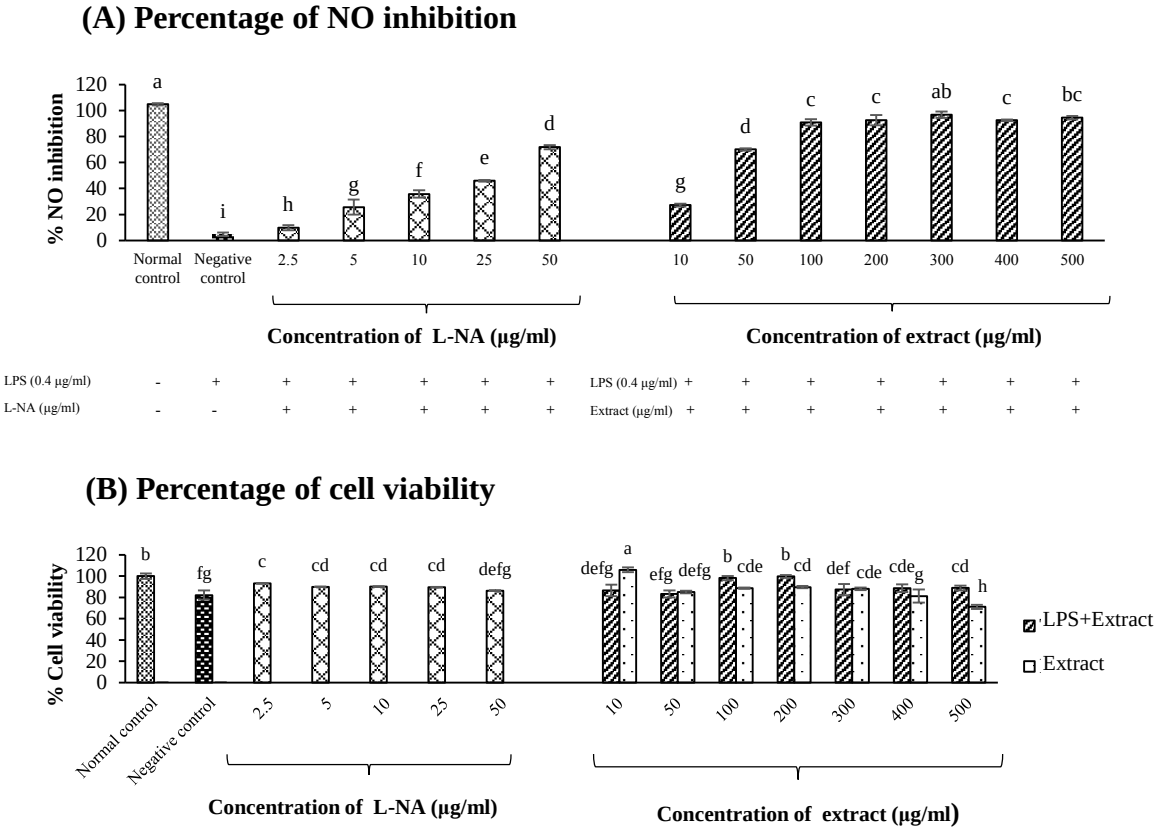


Figure 3.3 Effect of the aqueous crude extract of *T. laurifolia* leaves on NO inhibition and cell viability in RAW264.7 macrophage cells induced by LPS; (A) percentage of NO inhibition value; (B) percentage of cell viability value.

Remark: [a-g] mean within a figure with different letters are significantly different (p<0.05). + means added; - means not added. Values are mean ± standard deviation (n=3).

Table 3.3 IC_{50} values of the aqueous crude extract of *T. laurifolia* leaves compared with L-NA and some phenolic compounds on RAW264.7 macrophage cells

Contents	IC_{50} (µg/ml)
Crude extract	25.74±1.51[c]
L-NA	27.82±1.26[b]
Caffeic acid	61.47±2.60[a]
Apigenin	7.61±0.40[d]

[a-d] mean within a table with different letters are significantly different (p<0.05). Values are represent as mean ± standard deviation (n=3).

3.5 Conclusion

The present findings demonstrated that the aqueous crude extract of *T. larifolia* leaves showed a low cytotoxicity in all cell types, RAW264.7, HEK293 and HepG2 cells with CC_{50} more than 100 μg/ml. The crude extract significantly increased antioxidant enzyme activities, CAT and GPx while decreased MDA level in HEK293 kidney and HepG2 liver cells but not in RAW264.7 macrophage cells. The crude extract inhibited NO production in RAW264.7 cells and showed effective inhibition higher than L-NA. The obtained results demonstrated that using *T. laurifolia* leaves for herbal tea at appropriate concentration could support function of antioxidant enzymes and anti-inflammatory however, further study in animal and clinical for more safety detail still needs.

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CHAPTER 4

EFFECT OF THE AQUEOUS CRUDE EXTRACT OF *THUNBERGIA LAURIFOLIA* (RANG JUED) LEAVES ON ANTI-CADMIUM TOXICITY IN CELLS CULTURE MODEL

4.1 Abstract

Since ancient time, *Thunbergia laurifolia* (*T. laurifolia*) or Rang Jued has been used as herbal tea and folklore medicine as a detoxifying agent. Cd contamination is now world widespread and causes a serious public health problem. The aim of this study was to determine the anti-cadmium (Cd) toxicity of an aqueous crude extract of *T. laurifolia* leaves using murine macrophage (RAW264.7), human embryonic kidney cells (HEK293) and human hepatocellular carcinoma (HepG2) as a tested cell model. The experiment was designed as 3 groups including combined, pre and post-treatments, therefore, cell viability were determined with MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). It was found that the crude extract showed the protective against Cd-induced toxin in all cell types, in addition, the combination therapy effect was noticed when $CaNa_2EDTA$, a chelating agent was used. Therefore, it was concluded that the crude extract can protect Cd-induced oxidative stress in cells may due to antioxidant properties.

4.2 Introduction

Cadmium (Cd) is a hazard element to most living cell particularly in the higher living cell including animals and humans. Its half-life in the human body is about 30 years which is long and difficult to remove (Roopha and Padmalatha, 2012). Cd residues is a serious international concern due to its bioaccumulation which impacts to human health (Järup, 2003). Accumulation of Cd in the body can be found in almost all animal tissues, especially in the liver and kidney, which build up the total body burden because of no homeostatic mechanism to get rid of Cd to a constant safe level (Han *et al*. 2006; Gonick, 2008). Numerous scientific informations stated that Cd-induced harmful through different mechanisms involving disruption of cell adhesion, disruption in cellular signal transduction and apoptosis, inhibition of DNA repair including

disruption of the cellular antioxidant system and induction of reactive oxygen species (ROS) (Ercal *et al*., 2001; Fujiwara *et al.*, 2012; Zhu and Chan, 2012). Oxidative stress is a condition associated with an increased rate of cellular damage induced by ROS and oxidized active molecules (O_2, $O_2^{\bullet-}$, $OH^{\bullet}$, H_2O_2) via the formation of lipid peroxidation and protein oxidation product (Cecarini *et al*., 2007; Roopha and Padmalatha, 2012) leading to the loss of balancing between ROS generation and scavenging activities. As known that in the human body, the macrophage is a sensitive and first defense against pathogens and toxin while liver and kidney deal with toxic elimination and be major target sites of Cd-induced acute and chronic toxicity, respectively (Sabolić *et al*., 2010; (Helali *et al*., 2016)

Thunbergia laurifolia or Rang Jued, a local Thai plant belonging to the family Acanthaceae is commonly consumed as an herbal tea. Fresh and dried plant leaves, bark and roots are used traditionally as an antidote for insecticide, drug, heavy metal and toxic chemical exposure (Thongsaard and Marsden, 2002; Chuthaputti, 2010). Rang jued leaves were also reported as a source of bioactive compounds and have been utilized as antioxidants (Oonsivilai, 2006; Chan *et al*., 2013). In addition, our previous studies showed that *T. laurifolia* leaves can increase antioxidant enzyme. The crude extract contained a high total extractable phenolic content (TPC) and provided high antioxidant activity based on radical scavenging, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric ion reducing antioxidant power assay (FRAP) (chapter 2, section 2.4.3).

Therefore, the aim of this present work was to investigate the influence of aqueous crude extracts of *T. laurifolia* leaves on anti-Cd toxicity in murine macrophage (RAW264.7), human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells.

4.3 Materials and Methods

4.3.1 Chemicals

Chemicals used for determination of cells culture were purchased from Gibco (Carlsbad, California). Most of the chemicals used for the determination of antioxidant activity, cytotoxicity, and anti-inflammatory activity were purchased from

Sigma-Aldrich, Seelze, Germany otherwise from Merck, Darmstadt, Germany; Ajax Finechem, Auckland, New Zealand; QRAC, Selangor, Malaysia; Fisher Scientific, Leicestershire, England; and LAB-SCAN, Dublin, Ireland.

4.3.2 Raw materials

T. laurifolia leaves collected at developing or intermediate stage (between dark green and bright green color, and without variegated) which can be folded without break easily were directly purchased (10 kg) from the contact farmer in Bangkok, Thailand and transported to the laboratory within 24 h.

4.3.3 Methods

4.3.3.1 Plant preparation and extraction

T. laurifolia leaves leaves were removed from climber, washed with tap water, drained and air dried followed a folk medicine method for 5-8 d to obtain the moisture content as 8-10 % (w/w), ground to be a fine powder with 20-40 mesh, and stored in a dark bottle at room temperature before used within 6 mo. The powder was soaked in hot water, 98±1°C (1:10 w/v) for 1 h, and then filtered through three layers of gauze followed by Whatman No. 4 filter paper. The filtrate was freeze-dried and stored at 4°C for further study as crude dried extract (Ruangyuttikarn *et al*., 2013).

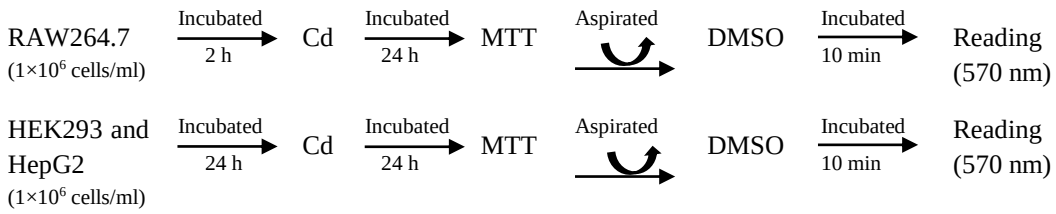
4.3.3.2 Cell Culture

Murine macrophage RAW264.7 cells and human embryonic kidney cells (HEK293) were purchased from American Type Culture Collection (ATCC, USA) and were all grown in RPMI–1640 medium and minimum essential medium (MEM) respectively, while human hepatocellular carcinoma cells (HepG2) kindly provided by Assoc. Prof. Dr. Teerapol Srichana (Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand) were cultured in Dulbecco's Minimal Essential Media (DMEM). The media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in a fully humidified incubator. The cells were

harvested with 0.25% trypsin-EDTA and suspended in a fresh medium. In this experiment, all cell types were used at passage 25-60 for testing.

4.3.3.3 Determination of the crude extract on anti-Cd property

Cytotoxicity of Cd was tested on RAW264.7, HEK293 and HepG2 cells by MTT assay (Mosmann, 1983). The concentration at 50% of cytotoxicity (CC_{50}) of Cd agent was calculated by plotting the percentage of cells survival versus the concentrations of Cd. Briefly, cells were plated in a 96-well plate at a density of 1×10^6 cells/ml (counted by a standard trypan blue cell counting technique) and incubate for 2 h for RAW264.7 cells and 3 h for HEK293 and HepG2 cells at 37°C for allowed cells to attach before treated with the Cd solution at various concentrations (20-120 µmol/L) which incubated for 24 h at 37°C. Then, 100 µl of MTT (0.5 mg/ml) was added to each well and incubated for 2 h for RAW264.7 cells and 3 h for HEK293 and HepG2 cells at 37°C. After incubation, the media were aspirated and 100 µl of DMSO was added to each well to dissolve the formazan for 10 min at 37°C before taken to read an absorbance at 570 nm with microplate spectrophotometer as followed steps;



The effect of the crude extract on the type of each cell induced by Cd at CC_{50} was investigated. Briefly, cells were plated in a 96-well plate at a density of 1×10^6 cells/ml and allowed cells attach to the culture plate (2 h for RAW264.7 and 24 h for HEK293 and HepG2 cells) before treated with the crude extract or chelating agent. The testing group was divided into three groups as together (combined-treatment), before (pre-treatment) and after treated with Cd at CC_{50} (post-treatment). The treatments were briefly explained as followed;

Group 1 (combined-treatment by adding the extract and Cd (CC_{50}) together)

- Normal control : Media (24 h)
- CC_{50} of Cd (negative control) : Media and Cd at CC_{50} (24 h)
- Tested sample : Extract and Cd at CC_{50} (24 h)

Group 2 (Pre-treatment by adding the extract before Cd (CC_{50}))

- Normal control : Media (24 h) + Media (24 h)
- CC_{50} of Cd (negative control) : Media (24 h) + Cd at CC_{50} (24 h)
- Tested sample : Extract (24 h) + Cd at CC_{50} (24 h)

Group 3 (Post-treatment by adding the extract after Cd (CC_{50}))

- Normal control : Media (24 h) + Media (24 h)
- CC_{50} of Cd (negative control) : Cd at CC_{50} (24 h) + Media (24 h)
- Tested sample : Cd at CC_{50} (24 h) + Extract (24 h)

The amount of cell viability was detected by MTT cytotoxicity assay and calculated percentage of cell viability as followed equation;

$$\% \text{ Cell viability} = [OD_{sample} / OD_{control}] \times 100$$

4.3.3.4 Determination of the chelating agent ($CaNa_2EDTA$) on anti-Cd property

To determine anti-Cd toxicity in this experiment, calcium disodium ethylenediamine tetraacetic acid ($CaNa_2EDTA$) was used as a chelating agent for a positive control. The testing group was divided into three groups as followed;

Group 1 (combined-treatment by adding the EDTA and Cd (CC_{50}) together)

- Normal control : Media (24 h)
- CC_{50} of Cd (negative control) : Media and Cd at CC_{50} (24 h)
- Tested EDTA : $CaNa_2EDTA$ and Cd at CC_{50} (24 h)

Group 2 (Pre-treatment by adding the EDTA before Cd (CC_{50}))

- Normal control : Media (24 h) + Media (24 h)
- CC_{50} of Cd (negative control) : Media (24 h) + Cd at CC_{50} (24 h)
- Tested EDTA : $CaNa_2EDTA$ (24 h) + Cd at CC_{50} (24 h)

Group 3 (Post-treatment by adding the CA after Cd (CC_{50}))

- Normal control : Media (24 h) + Media (24 h)
- CC_{50} of Cd (negative control) : Cd at CC_{50} (24 h) + Media (24 h)
- Tested EDTA : Cd at CC_{50} (24 h) + $CaNa_2EDTA$ (24 h)

The amount of cell viability was detected by MTT cytotoxicity assay and calculated percentage of cell viability as followed equation;

$$\% \text{ Cell viability} = [OD_{sample} / OD_{control}] \times 100$$

The highest activity of anti-Cd toxicity of chelating agent from each cell type was selected to test for finding the combination effect of the crude extract coupled with a chelating agent.

4.3.3.5 Determination of the crude extract coupled with a chelating agent ($CaNa_2EDTA$) on anti-Cd property

The combination protecting and healing of the crude extract and $CaNa_2EDTA$ on Cd toxicity was tested on RAW264.7, HEK293 and HepG2 cells by MTT assay. The testing groups were divided into two groups as followed;

Group 1 (Pre-treatment by adding the extract before CA mixed with Cd (CC_{50}))

- Normal control : Media (24 h) + Media (24 h)
- CC_{50} of Cd (negative control) : Media (24 h) + Cd at CC_{50} (24 h)
- CC_{50} of Cd + EDTA (positive control) : Media (24 h) + EDTA mixed with Cd at CC_{50} (24 h)
- Tested sample : Extract (24 h) + EDTA mixed with Cd at CC_{50} (24 h)

Group 2 (Post-treatment by adding the extract after CA mixed with Cd (CC_{50}))

- Normal control : Media (24 h) + Media (24 h)
- CC_{50} of Cd (negative control) : Cd at CC_{50} (24 h) + Media (24 h)
- CC_{50} of Cd + EDTA (positive control) : EDTA mixed with Cd at CC_{50} (24 h) + Media (24 h)
- Tested sample : EDTA mixed with Cd at CC_{50} (24 h) + Extract (24 h)

The equation for a calculated percentage of cell viability as followed;

$$\% \text{ Cell viability} = [OD_{sample} / OD_{control}] \times 100$$

4.3.3.5 Statistical analysis

Completely randomized design (CRD) was used throughout in this experiment. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. Significance was declared at $p<0.05$ using the statistical software.

4.4 Results and Discussion

4.4.1 Cytotoxicity of Cd on various cell types

Cytotoxicity of the $CdCl_2$ on RAW264.7, HEK293 and HepG2 cells were presented in Table 4.1. From the result, it pointed out that HepG2 cells provided the most resistance to $CdCl_2$ this may due to liver cells contain high of antioxidant enzyme substances such as glutathione (GHS), glutathione peroxidase (GPx) and catalase (CAT) (Jung *et al*., 2006; Schriner and Linford, 2006; Magalingam *et al*., 2013) which can protect cells from Cd-induced oxidative stress. Compared CC_{50} to cell viability of each cell type also confirmed that RAW264.7 was easily destroyed by Cd toxicity particularly when the cell was approached to Cd for 24 h. Actually, RAW264.7 is a macrophage cell which can move and kill microbe or toxin by phagocytosis (Petricevich *et al*., 2008). It pointed out that phagocytosis action of RAW264.7 macrophage cell was not strong and enough function to remove or eliminate Cd which is a serious toxic element. In addition, it was possible that since RAW264.7 act as a

first screener and messenger to send the information to other cells or system to support further, therefore, protection effect and defense was not strong (Ashley *et al*., 2012).

This finding out was in agreement with the recommendation of a physician who has to treat the patients who digested or exposed to Cd by only systemic symptom not curing or removal process because of the high toxicity of Cd.

Table 4.1 50% cytotoxicity concentration (CC_{50}) of $CdCl_2$ toxicity on cells viability determined by MTT assay

Cell types	CC_{50} of Cd (μmol/L)
RAW264.7	42.99
HEK293	64.09
HepG2	75.37

4.4.2 Anti-Cd toxicity property of crude extract

Effect of the crude extract on the anti-Cd toxicity of *T. laurifolia* leaves was showed in Figure 4.1. The results indicated that the crude extract can improve HepG2 and HEK293 cells survival higher than RAW264.7 cells. Moreover, the crude extract showed the protection and recovery function to cells as pre and post-treatments (Figure 4.1B and 4.1C) but not in combined-treatment. Generally, Cd-induced toxicity in the cell via several apoptotic pathways such as disruption of the cellular antioxidant enzyme, inhibition of cell adhesion, induction of ROS and inhibition of DNA repair (Ercal *et al*., 2001; Fujiwara *et al*., 2012; Zhu and Chan, 2012). In addition, Cd is claimed as a critical cause of imbalance between pro-oxidant and antioxidant homeostasis via oxidative stress by being a catalyst in the formation of ROS, increasing lipid peroxidation, reducing glutathione, and protein-bound sulfhydryl groups (Navas-Acien *et al*., 2004: Flora, *et al*., 2008).

From the previous studied, the results indicated that the crude extract containing high phenolics and flavonoids played an important role to increase antioxidant enzymes, CAT and GPx (chapter 2 and 3). It pointed out that the crude extract can protect and/or recover injured cells from the toxic of Cd. Mehta and Gowder (2015) reported that antioxidant enzymes act as ROS inhibitors. An increase of both

antioxidants including antioxidant enzymes and exogenous antioxidant via polyphenols in the crude extract improved the anti-Cd property. This result was in agreement of some researchers who reported that an aqueous extract of *T. laurifolia* leaves significantly prevented kidney damage induced by Cd exposure in Wistar rats (Chattaviriya *et al.*, 2010) and decreased the abnormal appearance and behavior in rat when it was administrated prior to Cd exposure (Morkmek *et al.*, 2010). However, the crude extract seemed to not help in all cell types survival in combined-treatment. This phenomenon may due to the Cd-flavonoids interaction because of its chelating property led to bigger chelation molecule then easier to cell membrane attachment as a bigger bullet. As known that when the membrane was leakage, it lost their barrier. Therefore, the flavonoid-Cd complex would get through the cell before lysis by some enzyme in the cell, leading to free Cd and induce cell death due to acute toxicity. Gonzales *et al.* (2015) reported that some lysosomal enzymes such as β-glucuronidase which found in liver and kidney cells can play a crucial role in the cellular metabolism of flavonoids by releasing the active flavonoids in the cell. Generally, flavonoids can form a complex with metal cations via chelating action (Lachman *et al*., 2005) and the complexation of flavonoids with metal cations can considerably change their lipophilicity and interaction with the lipid bilayer and easier to penetrate into the hydrophobic sites of membranes (Tarahovsky *et al*., 2014). Therefore, more cell death in combined-treatment compared with negative control occurred as a result of combined cytotoxicity effect. Moreover, cell viability of RAW264.7 was continuously reduced in post-treatment. With understanding Cd agent with high acute toxic made RAW264.7 cells injure (which contains low activity of CAT, 3.18±0.21 U/mg protein) even death in first 24 h, therefore, there was no help cell recovery after.

As known that until now, there is no medicine to directly cure Cd toxicity. Therefore, the best way to prevent Cd toxicity is to not contact or digest Cd-contaminated food. In addition, taking the crude extract or standard drug with suitable dose should be fastest after Cd exposure.

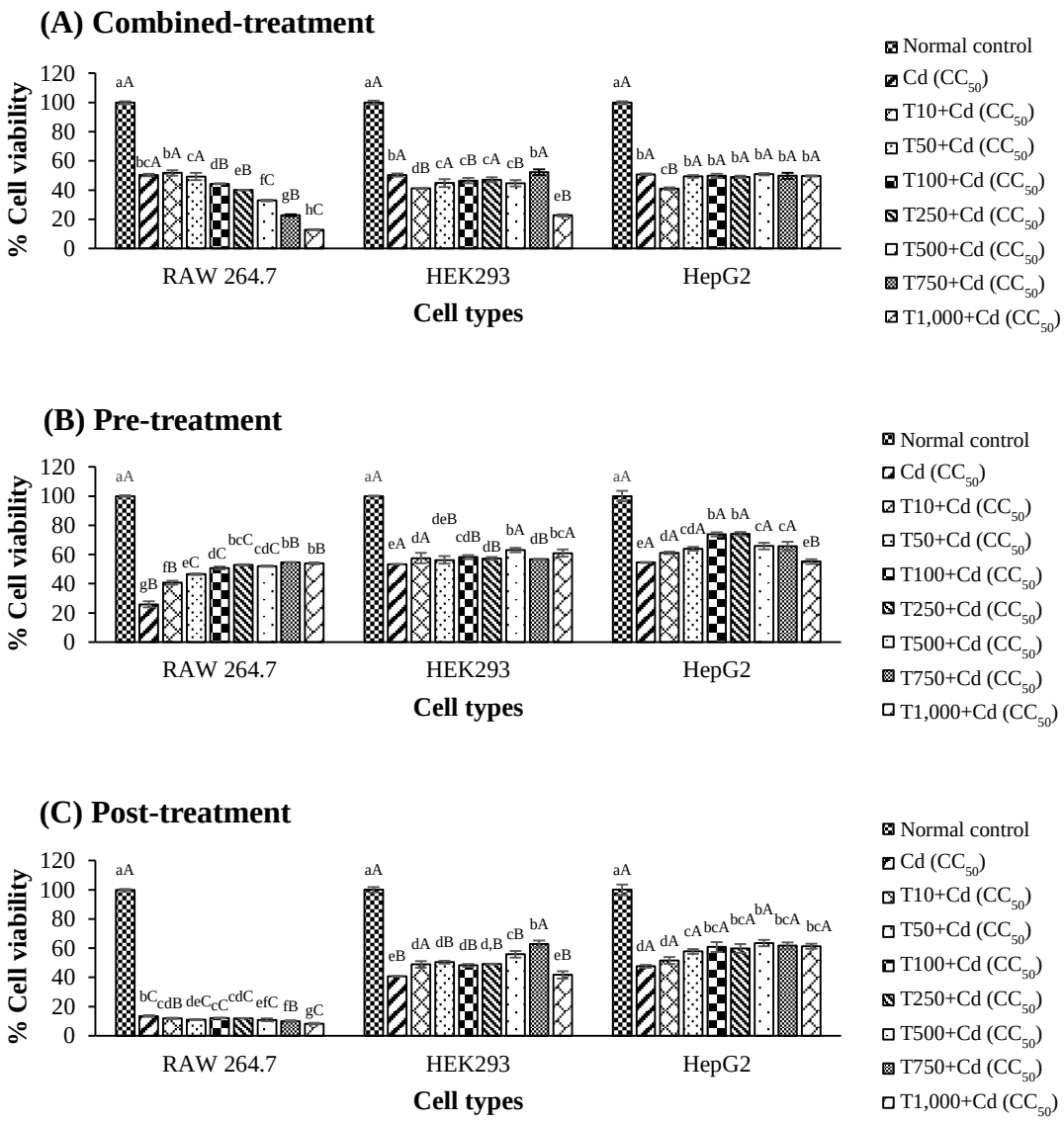


Figure 4.1 Anti-Cd toxicity of the aqueous crude extract of *T. laurifolia* leaves in RAW264.7, HEK293 and HepG2 cells determined by MTT assay; (A) combined-treatments; (B) pre-treatment and (C) post-treatment with crude extract.

Remark: $^{a\text{-}h}$ mean within cell types with different letters are significantly different (p<0.05). $^{A\text{-}C}$ mean within an extract concentration with different letters are significantly different (p<0.05). T means the crude extract of *T. laurifolia* leaves (µg/ml). Values are mean ± standard deviation (n=3).

4.4.3 Anti-Cd toxicity property of the chelating agent

In this study, $CaNa_2EDTA$ was used for the chelating agent as a positive control (Figure 4.2) and the concentration was selected from its cytotoxicity value of each cell type (Appendix B) with cell viability higher than 80%. The result showed that chelating agent can help cell survival in combined- treatment condition on all cell types, RAW264.7, HEK293 and HepG2 cells (Figure 4.2A). However, chelating agent seemed to help cells survival not much in pre and post-treatments (Figure 4.2B and 4.2C) when compared with combined-treatment. Comparison of the cytotoxicity value of the crude extract (Figure 4.1A) and $CaNa_2EDTA$ (Figure 4.2A) indicated that the active compounds containing in the crude extract may not use the same pathway compared with $CaNa_2EDTA$. In fact, $CaNa_2EDTA$ is a derivative of ethylenediamine tetraacetic acid (EDTA) and the main function of it is binding Cd ions to form complex ring-like structure or chelates and removes Cd toxic metal from the desired site in the body (Flora and Pachauri, 2010). This chelating property can decrease Cd toxin and help cell survival, particularly in combined-treatment when compared with pre and post-treatment which were treated with $CaNa_2EDTA$ before and after Cd agent for 24 h respectively. Actually, $CaNa_2EDTA$ is the most commonly used chelating agent for heavy metal curing especially for lead and cadmium (Flora and Pachauri, 2010; Blaurock-Busch and Busch, 2014). However, as well-known $CaNa_2EDTA$ did not have a specific activity for chelating with only Cd but also other essential minerals such as Zn, Cu, Fe, Co and Mn (Smith, 2013) which involves various enzyme function including cofactors. Moreover, the risks associated with $CaNa_2EDTA$ therapy was reported such as bone marrow depression, prolonged bleeding time, convulsions, respiratory arrest including renal failures (Knudtson *et al*., 2002). Furthermore, some researcher reported that EDTA provided cytotoxic effect and inflammation responses on animal cells (Ballal *et al*., 2006; Zaccaro Scelza *et al*., 2010).

In this present work, the result showed the percentage of cell viability in HEK293 cells which was smaller than HepG2 and RAW264.7 cells. As well known that HEK293, kidney cells deal with mineral balancing the homeostasis which related to Ca^{2+} content (Blaine *et al*., 2015). The excess Ca^{2+} from $CaNa_2EDTA$ dissociation may lead to Ca^{2+} toxicity which explained the less viability of HEK293 cell lines.

Similar to the finding of Flora *et al*. (2008) who suggested that the Ca salt of EDTA provided major toxic side effects on the renal system causing the necrosis of tubular cells. Closely focused on RAW264.7 cell viability in pre-treatment (Figure 4.2B) found that higher cell survival was observed when compared with negative control. This result relates to the EDTA residue on cell surface membrane after $CaNa_2EDTA$ solution was aspirated then, it can protect the cell from Cd by chelation effect. As well known, EDTA can insertion into the lipid membrane (Prachayasittikul *et al*., 2007) and RAW264.7 macrophage cell was reported as high of fatty acid on the membrane (Schoeniger *et al*., 2011). However, the higher concentration of $CaNa_2EDTA$ decreased RAW264.7 cell survival may from its cytotoxic effect which was shown in Appendix B.

From this result indicated that using $CaNa_2EDTA$ in the condition of combined-treatment was the most effective therapy. Therefore, the best concentration of $CaNa_2EDTA$ for each cell type in combined-treatment was selected for next experiment to studying the combined therapy with the crude extract on Cd toxicity. While, using the crude extract in pre and post-treatments could help cell survival from Cd toxicity. The difference result from using $CaNa_2EDTA$ and the crude extract may due to many reasons; anti-Cd mechanism both of them different, side effect or toxicity of chelating agent was higher compared with crude extract in the same concentration (the cytotoxicity of $CaNa_2EDTA$ on each cell type were showed in appendix B).

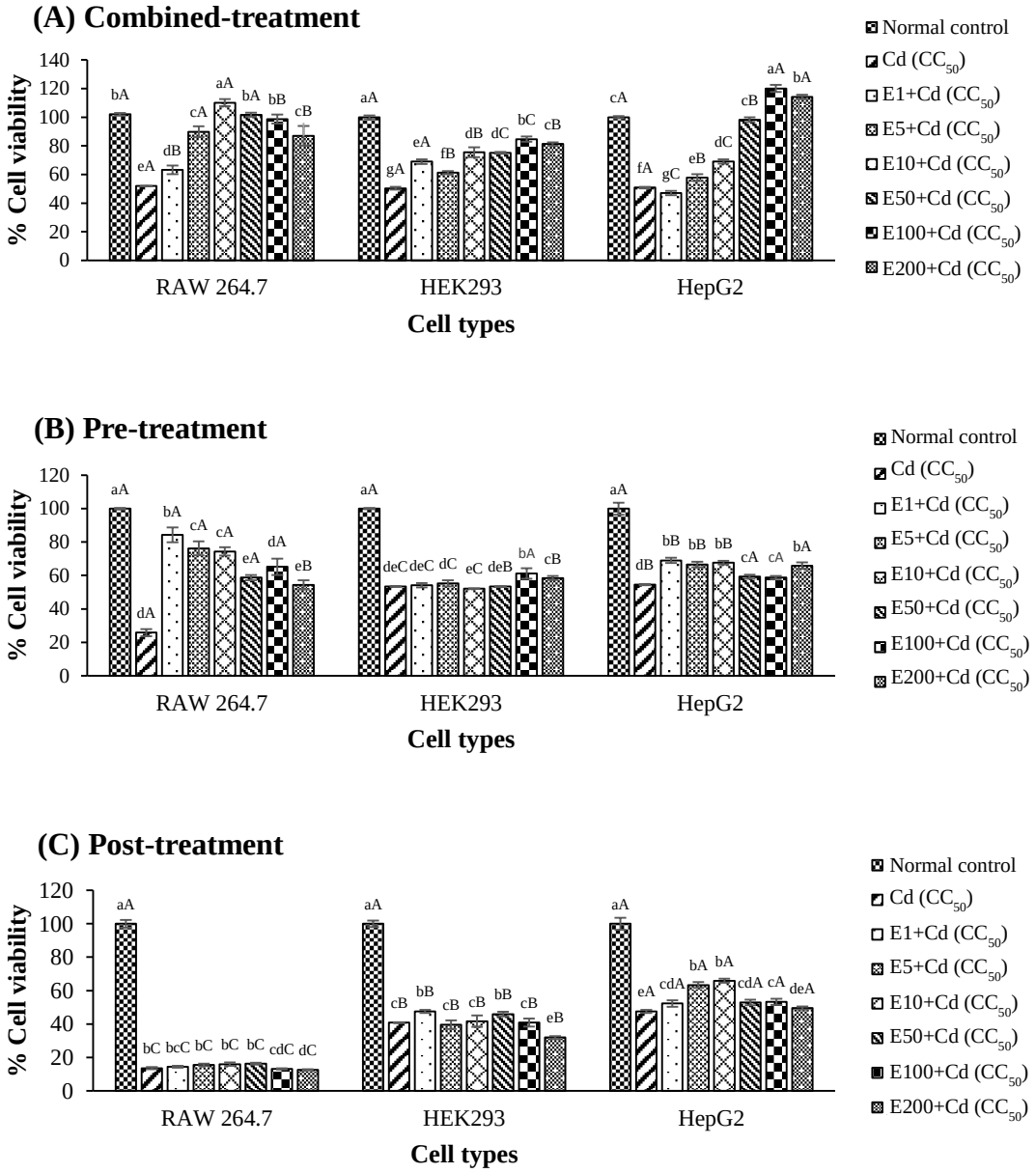


Figure 4.2 Anti-Cd toxicity of $CaNa_2EDTA$ in RAW264.7, HEK293 and HepG2 cells determined by MTT assay; (A) combined-treatments; (B) pre-treatment and (C) post-treatment with crude extract.

Remark: $^{a-h}$ mean within cell types with different letters are significantly different ($p<0.05$). $^{A-C}$ mean within an extract concentration with different letters are significantly different ($p<0.05$). E means $CaNa_2EDTA$ solution (µg/ml). Values are mean ± standard deviation (n=3).

4.4.4 Anti-Cd toxicity property of crude extract and chelating agent

To study the interaction between the crude extract and chelating agent, the experiment was designed as 2 groups; pre and post-treatments. Cd agent mixed with $CaNa_2EDTA$ was taken to treat on the cell lines before and after treated with the crude extract. The most effective concentration of $CaNa_2EDTA$ of each cell type in the previous part as 10 μg/ml for RAW264.7 and 100 μg/ml for HEK293 and HepG2 was used as positive control. The results showed that cell viability significantly increased when the concentration of the crude extract increased compared with negative control (Cd agent) and positive control (Cd agent mixed with $CaNa_2EDTA$). It pointed out that bioactive compounds from the crude extract can help and support the ability of $CaNa_2EDTA$ against Cd toxin via antioxidant properties by phenolic and flavonoid compounds. Moreover, the results showed that cell viability of HepG2 cells was higher when the crude extract was used at 500-1,000 μg/ml in pre-treatment (Figure 4.3A) and 1,000 μg/ml in post-treatment (Figure 4.3B) compared with normal control respectively. It was indicated that the extract may help cell proliferation or strength as a result of some nutrition or bioactive compounds as glucoside and phenolic compounds which were claimed as supplementary compound for cell proliferation and protecting cell death via its antioxidant and anti-inflammatory effect (Chattaviriya *et al.,* 2010; Ruangyuttikarn *et al*., 2013).

Besides, phenolic and flavonoid compounds, various phytochemical such as sterols, steroids, tannin, and glycosides (Chuthaputti, 2010) were also reported in the *T. laurifolia* which may also support cell survival via antioxidant activity, anti-inflammatory etc. (Saxena *et al.,* 2013). In addition, types and quantity of active compound were regulated by the stage of leaves, location, and method of extraction (Oonsivilai, 2006), which later may affect on cells survival or toxicity in different ways. For example, the leaves in developing with aqueous extraction provided the highest of TPC (Oonsivilai, *et al.,* 2008; Chan *et al.,* 2013). From this result, it was noticed that the abnormal shapes, loss of adhesion and cell merge when the cells were treated with Cd at a certain concentration (Figure 4.4). However, it was found that using the crude extract (50 μg/ml in RAW264.7 and HEK293; 1,000 μg/ml in HepG2) can better prevent or recover abnormal phenomenal compared with negative control and positive

one. This result indicated that the aqueous crude extract of *T. laurifolia* leaves can be used as an alternative therapy for Cd-induced disease coupled with the drug without any negative effect as antagonist action. However, further study is necessary to fully clarify the *T. laurifolia* extract in an animal model to prove the Cd therapy.

In conclusion, using the extract or drinking the herbal tea together with using a chelating agent for therapy should have more advantage compared with using the crude extract or chelating agent alone.

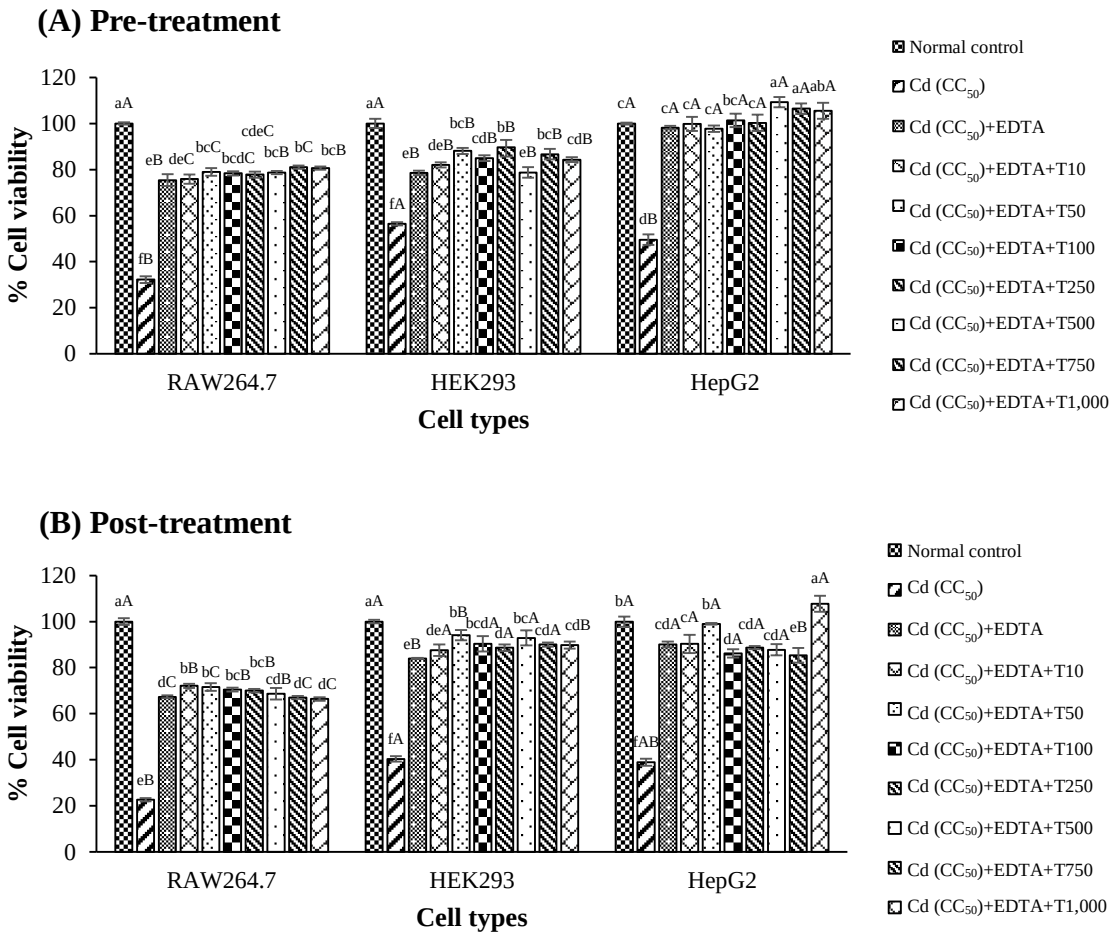


Figure 4.3 Anti-Cd toxicity of the aqueous crude extract of *T. laurifolia* leaves coupled with $CaNa_2EDTA$ on cell viability in RAW264.7, HEK293 and HepG2 cells determined by MTT assay; (A) pre-treatment and (B) post-treatment.

Remark: [a-m] Means within a figure with different letters are significantly different (p<0.05). T means the crude extract of *T. laurifolia* leaves (μg/ml). Values are mean ± standard deviation (n=3).

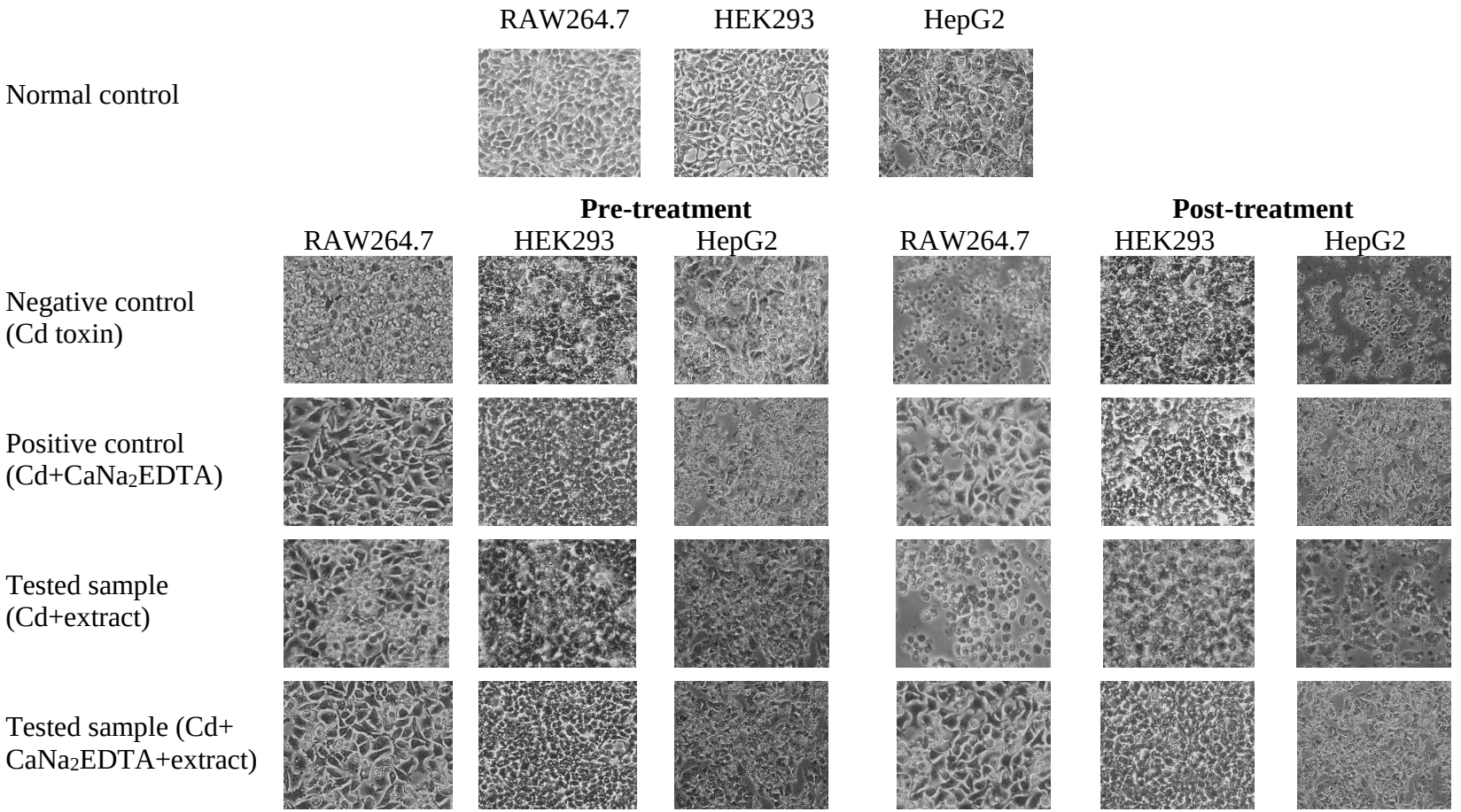


Figure 4.4 Morphology of RAW264.7, HEK293 and HepG2 cells treated with Cd-induced toxicity and crud extract coupled with $CaNa_2EDTA$.

4.5 Conclusion

The present findings demonstrated that Cd toxicity of each cell type was different. The most sensitive cell for Cd was RAW264.7 followed by HEK293 and HepG2 cells respectively. The aqueous crude extract of *T. larifolia* leaves significantly protect cells against Cd-induced toxicity in all cell types. Cd toxicity was more pronounced further when the cell was treated with the crude extract together with Cd. The $CaNa_2EDTA$ agent can help cell survival better in a combined-treatment way. However, anti-Cd toxicity was higher when the crude extract and $CaNa_2EDTA$ were used together. Therefore, clinical trial should be performed to guarantee before to apply.

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CHAPTER 5

EFFECT OF THE AQUEOUS CRUDE EXTRACT OF *THUNBERGIA LAURIFOLIA* (RANG JUED) LEAVES ON ANTI-INSECTICIDES TOXICITY IN CELLS CULTURE MODEL

5.1 Abstract

Thunbergia laurifolia (*T. laurifolia*) Rang Jued has been used for folklore medicine purposes and consumed as an herbal tea in Thailand since the ancient times. To evaluate the anti-insecticides toxicity, the middle stage of plant leaves was extracted with hot water at 95±2°C. Murine macrophage (RAW264.7), human embryonic kidney (HEK293) and human hepatocellular carcinoma (HepG2) cells, were treated with the crude extract either together (combined-treatment), before (pre-treatment),) and after (post-treatment) exposure to chlorpyrifos (CP) and methomyl (MT) and cell viability was determined by MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to find out anti-insecticides property. The results indicated that the crude extract improved cells viability in all cell types induced by CP and MT toxins. The crude extract provided protection and recover effect on CP and MT insecticides better than atropine sulfate (AS) standard drug especially in HepG2 and HEK293 cells respectively. Therefore, it was concluded that the extract can be used as anti-insecticides however, it needs more investigation to play safe for functional drinks processing.

5.2 Introduction

A rapid increase of insecticide usage is a hot issue for Thailand and other developing countries with an intensive agriculture (Grovermann *et al.*, 2013). Agriculture in Thailand is reported to be heavily dependent up on pesticide usage for crop protection in order to increase production levels, quality and appearance (Panuwet *et al*., 2012). As humans are at the top of the food chain, therefore, food containing toxic substances must be potentially accumulated in the human body (Sapbamrer *et al*., 2011). Organophosphate as chlorpyrifos (CP) and carbamate insecticide including

methomyl (MT) are very toxic and hazardous insecticides (Tamimi *et al*., 2009; Li *et al.*, 2015) and banned in many countries. Their toxicity has been reported to relate to acetyl cholinesterase inhibition, one of the important enzymes involving human nervous systems (Amritha and Kaliwal, 2016). Based on Thai folk medicine, *Thunbergia laurifolia*, is used to reduce various insecticides toxicity.

T. laurifolia, with the Thai name 'Rang Jued', belongs to the family Acanthaceae, (Morkmek *et al*., 2010) has been used in Thai traditional medicine for many centuries. The extracts of fresh and dried leaves, barks and roots are mainly used as an antidote for treating insecticide-, drug-, arsenic-, strychnine-, alcohol-, and food poisoning including chemical toxins (Thongsaard and Marsden, 2002; Oonsivilai, 2006; Inta *et al*., 2013; Rocejanasaroj *et al*., 2014; Maneenoon *et al*., 2015). Moreover, this plant has been reported to have antioxidant and anti-inflammatory properties (Chan *et al*., 2012; Wonkchalee *et al.*, 2012). Previous studies demonstrated that the aqueous crude extract of *T. laurifolia* leaves contained high phenolics and flavonoids and provided a protective effect against Cd-induced oxidative stress in all cell types including RAW264.7, HEK293 and HepG2 cells (chapter 2 and 4).

The objective of this study was to determine the effect of the aqueous crude extract of *T. laurifolia* on protecting effect against CP and MT insecticides, which are the most commonly used insecticides in fresh produce by using RAW264.7, HEK293 and HepG2 cells as test models.

5.3 Materials and Methods

5.3.1 Chemicals

Chemicals used for determination of cells culture were purchased from Gibco (Carlsbad, California). Most of the chemicals used for the determination of antioxidant activity, cytotoxicity, and anti-inflammatory activity were purchased from Sigma-Aldrich, Seelze, Germany otherwise from Merck, Darmstadt, Germany; Ajax Finechem, Auckland, New Zealand; QRAC, Selangor, Malaysia; Fisher Scientific, Leicestershire, England; and LAB-SCAN, Dublin, Ireland.

5.3.2 Raw materials

T. laurifolia leaves at developing or intermediate stage (between dark green and bright green color, and without variegated) which can be folded without break easily were directly purchased (10 kg) from the contact farmer in Bangkok, Thailand and transported to the laboratory within 24 h.

5.3.3 Methods

5.3.3.1 Plant preparation and extraction

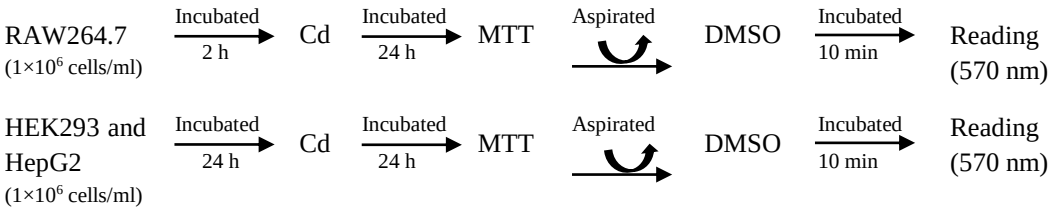
The leaves removed from climber, washed with tap water, drained and air dried followed a folk medicine method for 5-8 d to obtain the moisture content as 8-10 % (w/w), ground to be a fine powder with 20-40 mesh, and stored in a dark bottle at room temperature before used within 6 mo. The powder was soaked in hot water, 98±1°C (1:10 w/v) for 1 h, and then filtered through three layers of gauze followed by Whatman No. 4 filter paper. The filtrate was freeze-dried and stored at 4°C for further study as dried crude extract (Ruangyuttikarn *et al*., 2013).

5.3.3.2 Cell Culture

Murine macrophage RAW264.7 cells and human embryonic kidney cells (HEK293) were purchased from American Type Culture Collection (ATCC, USA) and were all grown in RPMI–1640 medium and minimum essential medium (MEM) respectively, while human hepatocellular carcinoma cells (HepG2) provided by Assoc. Prof. Dr. Teerapol Srichana (Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand) were culture in Dulbecco's Minimal Essential Media (DMEM). The media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in a fully humidified incubator. The cells were harvested with 0.25% trypsin-EDTA and suspended in a fresh medium. In this experiment, all cell types were used at passage 25-60 for testing.

5.3.3.3 Determination of the crude extract on anti-insecticide property

Cytotoxicity of both chlorpyrifos (CP), a representative of organophosphate and methomyl (MT), methyl carbamate insecticides were tested on RAW264.7, HEK293 and HepG2 cells by MTT assay Mosmann (1983). The concentration at 50% of cytotoxicity (CC_{50}) of each insecticide was calculated by plotting the percentage of cells survival versus the concentrations of insecticides. Briefly, cells were plated in a 96-well plate at a density of 1×10^6 cells/ml (counted by a standard trypan blue cell counting technique) and allowed to attach for 24 h before treated with the toxin solution at various concentrations which incubated for 24 h at 37°C. Then, 100 µl of MTT (0.5 mg/ml) was added to each well and incubated for 2 h in RAW264.7 and 3 h in HEK293 and HepG2 cells at 37°C. After incubation, the media were aspirated and 100 µl of DMSO was added to each well to dissolve the formazan for 10 min at 37°C before taken to read an absorbance at 570 nm with microplate spectrophotometer as followed steps;



The effect of the crude extract on each cell types induced by each insecticide at CC_{50} was investigated. Briefly, cells were plated in a 96-well plate at a density of 1×10^6 cells/ml and allowed cells attach to the culture plate (2 h for RAW264.7 and 24 h for HEK293 and HepG2 cells) before used in the experiments. The testing group was divided into three groups as together (combined-treatment), before (pre-treatment) and after treated with insecticide at CC_{50} (post-treatment). The crude extract was allowed to have contact time with the cells for 24 h as followed;

Group 1 (combined-treatment by adding the crude extract and insecticides (CC_{50}) together)

- Normal control : Media (24 h)
- CC_{50} of insecticides (negative control): Media and insecticides at CC_{50} (24 h)
- Tasted sample : Extract and insecticides at CC_{50} (24 h)

Group 2 (Pre-treatment by adding the crude extract before insecticides (CC_{50}))

- Normal control : Media (24 h)+Media (24 h)
- CC_{50} of insecticides (negative control): Media (24 h)+Insecticides at CC_{50} (24 h)
- Tested sample : Extract (24 h)+Insecticides at CC_{50} (24 h)

Group 3 (Post-treatment by adding the crude extract after insecticides (CC_{50}))

- Normal control : Media (24 h)+Media (24 h)
- CC_{50} of insecticides (negative control): Insecticides at CC_{50} (24 h)+Media (24 h)
- Tested sample : Insecticides at CC_{50} (24 h)+Extract (24 h)

The amount of cell viability was detected by MTT cytotoxicity assay and calculated percentage of cell viability as followed equation;

$$\% \text{ Cell viability} = [OD_{sample} / OD_{control}] \times 100$$

5.3.3.4 Determination of the atropine sulfate (AS) on anti-insecticide property

To determine anti-insecticide toxicities in this experiment, atropine sulfate (AS) was used for a positive control. The testing group was divided into three groups as followed;

Group 1 (combined-treatment by adding the AS and insecticides (CC_{50}) together)

- Normal control : Media (24 h)
- CC_{50} of insecticides (negative control): Media and insecticides at CC_{50} (24 h)
- Tested AS : AS and insecticides at CC_{50} (24 h)

Group 2 (Pre-treatment by adding the AS before insecticides (CC_{50}))

- Normal control : Media (24 h)+Media (24 h)
- CC_{50} of insecticides (negative control): Media (24 h)+Insecticides at CC_{50} (24 h)
- Tested AS : AS+Insecticides at CC_{50} (24 h)

Group 3 (Post-treatment by adding the AS after insecticides (CC_{50}))

- Normal control : Media (24 h)+Media (24 h)
- CC_{50} of insecticides (negative control): Insecticides at CC_{50} (24 h)+Media (24 h)
- Tested AS : Insecticides at CC_{50} (24 h)+AS (24 h)

The amount of cell viability was detected by MTT cytotoxicity assay and calculated percentage of cell viability as followed equation;

$$\% \text{ Cell viability} = [OD_{sample}/ OD_{control}] \times 100$$

5.3.3.5 Statistical analysis

Completely randomized design (CRD) was used throughout in this experiment. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests. Significance was declared at $p<0.05$ using the statistical software.

5.4 Results and Discussion

5.4.1 Cytotoxicity of CP

Chlorpyrifos (CP), an insecticide originated from organophosphate was treated on each cell type and reported in CC_{50} value (Table 5.1). It was found that CP insecticide could induce cell death from highest to lowest concentration as followed; RAW264.7>HepG2>HEK293 respectively. The result indicated that CP had the ability to induced HEK293, a kidney cell death with the lowest concentration compared with other cells while the result showed that RAW264.7 cell was most resisted to CP. This effect may due to the composition of the cell membrane and the polarity of CP. Schoeniger *et al*. (2011) reported that RAW264.7 macrophage cell contained high of

lipid composition on membrane while CP was non-polar or less water solubility (WHO, 2004). As known that cell culture system which was high polarity did not facilitate to CP toxin get through the cell which had fat content, then survival rate high of RAW264.7 was significantly noticed. Moreover, CP was eliminated primarily through the kidneys in urine (Extension Toxicology Network, 1993a). Kidneys are the major detoxification organ for many xenobiotic and CP is one of the nephrotoxicity (Rekha *et al*., 2013). Moreover, CP is known as an AChE inhibitor and Xing *et al.* (2013) reported that CP could higher decrease the AChE activity in kidneys than that of liver common carp (*Cyprinus carpio*). It pointed out that if this insecticide was tested in the animal subject the result of CC_{50} may different because the testing system and the animal body was not similar. Therefore, to make a solid conclusion animal trial and clinical trial needs to be confirmed. The CC_{50} of CP was taken to study on anti-CP toxicity property of crude extract and AS on cells later.

Table 5.1 50% cytotoxicity concentration (CC_{50}) of chlorpyrifos (CP) toxicity on cells viability determined by MTT assay

Cell types	CC_{50} of CP (µl/ml)
RAW264.7	0.25
HEK293	0.11
HepG2	0.13

5.4.2 Anti-CP toxicity property of the crude extract and AS on cells

The effect of the crude extract on CP toxicity property on RAW264.7, HEK293 and HepG2 cells were showed in Figure 5.1. It was found that the crude extract could increase HepG2 cell survival significantly then decreased at higher concentration of extract when the cell was treated with CP in all treatments, combined, pre and post-treatment. While, the crude extract as combined-treatment can improve RAW264.7 cells survival (Figure 5.1A) at 10 to 500 µg/ml of extract. CP toxicity was proposed to make cell death via oxidative stress (Verma and Srivastava, 2003; McCarthy *et al*., 2004) through cell membrane which composes of poly-unsaturated fatty acids, a primary target for reactive oxygen attack leading to cell membrane damage (Repetto *et*

al., 2012). However, there was reported that when used appropriate phenolic and flavonoid concentration, oxidative stress was reduced or even inhibited cell membrane damage (Nijveldt *et al*., 2001; Karimi *et al*., 2012). Therefore, it pointed out that the crude extract containing high phenolics with high antioxidant responded to oxidative stress reduction leading to high cell viability found in this experiment. Similar to the finding of Usanawarong *et al*. (2000) who showed that the aqueous leaf extract of *T. laurifolia* can decrease plasma malonaldehyde (MDA), an indicator of lipid peroxidation-derived from free-radical mediated reactions of a rat with paraquat. Moreover, Lertpongpipat and Chaiyakhun (2011) reported that drinking *T. laurifolia* tea could increase the level of cholinesterase, an enzyme located in the intercellular space that is responsible for acetylcholine degradation in the human nervous system. In fact, this important enzyme was inhibited by organophosphate and carbamate insecticides affecting the degradation of acetylcholine and unbalancing the equilibrium leading to a variety of short-term and chronic effects such as a headache, lowered heartbeat, visual disorders including cancer (Eleršek and Filipič, 2011). While, Chinacarawat *et al*. (2012) suggested that oral administration of the *T. laurifolia* extract, at the dose of 600 mg per day for 2 weeks, did not have any side effects in farmer volunteers who had a high risk of organophosphate and carbamate insecticides intake. However, in this experiment, the results revealed that the crude extract could not increase cell survival in HEK293 moreover, a high concentration of crude extract induced more cells death. It pointed out that biological activity of any active compound may depend on many variable factors such as quantity and quality of bioactive compounds as well as type and functionality of cellular target. Furthermore, the results showed that the CP was more toxic on HEK293 and HepG2 cells than RAW264.7 cells which related to the cytotoxicity property of CP on cells (Table 5.1). The cell viability of RAW264.7 cells in negative control (cell only contact with CP) was better resistance and recovery compared with the other cell types (Figure 5.1B and 5.1C).

AS was used for positive control in this experiment, and the results indicated that AS could decrease RAW264.7 cells death in combined-treatment (Figure 5.2.) but not much function in other cells and other treatments. It pointed out that AS can protect cells death from CP especially in combined-treatment, which AS and CP was added together on the cell. As well known, CP poisoning can be divided according

to the site of acetylcholine accumulation in the organism; central neural system, peripheral autonomic nerve system, nicotine receptors especially muscarine receptors which was found in kidney, liver and macrophage cells (Li *et al*., 2009; Eleršek and Filipič, 2011; Hussmann *et al*., 2011; Koarai *et al*., 2012). The mechanism of AS is an inhibition of muscarinic acetylcholine receptors, the cause of muscarinic cholinergic toxidrome such as indigestion, lowered heartbeat and visual disorders (Eddleston and Chowdhury, 2015). However, AS has a short half-life (3.0 ± 0.9 h in an adult) and easy to destroy by hydrolysis enzymatic, particularly in the liver organ (Drugbank, 2018). Moreover, cell viability of RAW264.7 in combined and pre-treatments (Figure 5.2A and 5.2B) seemed to decrease when the concentration of AS increased. This phenomenon may due to the combined cytotoxic effect of AS and CP on cells. Actually, AS showed cytotoxic by itself on the tested cells when the higher concentration was applied (0.0006-1 μg/ml) and the cytotoxicity of AS on each cell type was shown in Appendix C. Similar to the finding of Rodrigues *et al*. (2013) who reported that AS provided cytotoxic effect and inhibited cell proliferation in HepG2 liver cells.

From the results indicated that the crude extract provided anti-CP toxicity property better than AS standard drug in pre and post-treatments. Therefore, the crude extract might have both protection and curing effect even better compared with AS standard drug. This may due to multi-functions of various compounds including phenolics, flavonoids even other material compounds which support cell growth and inhibit oxidation process.

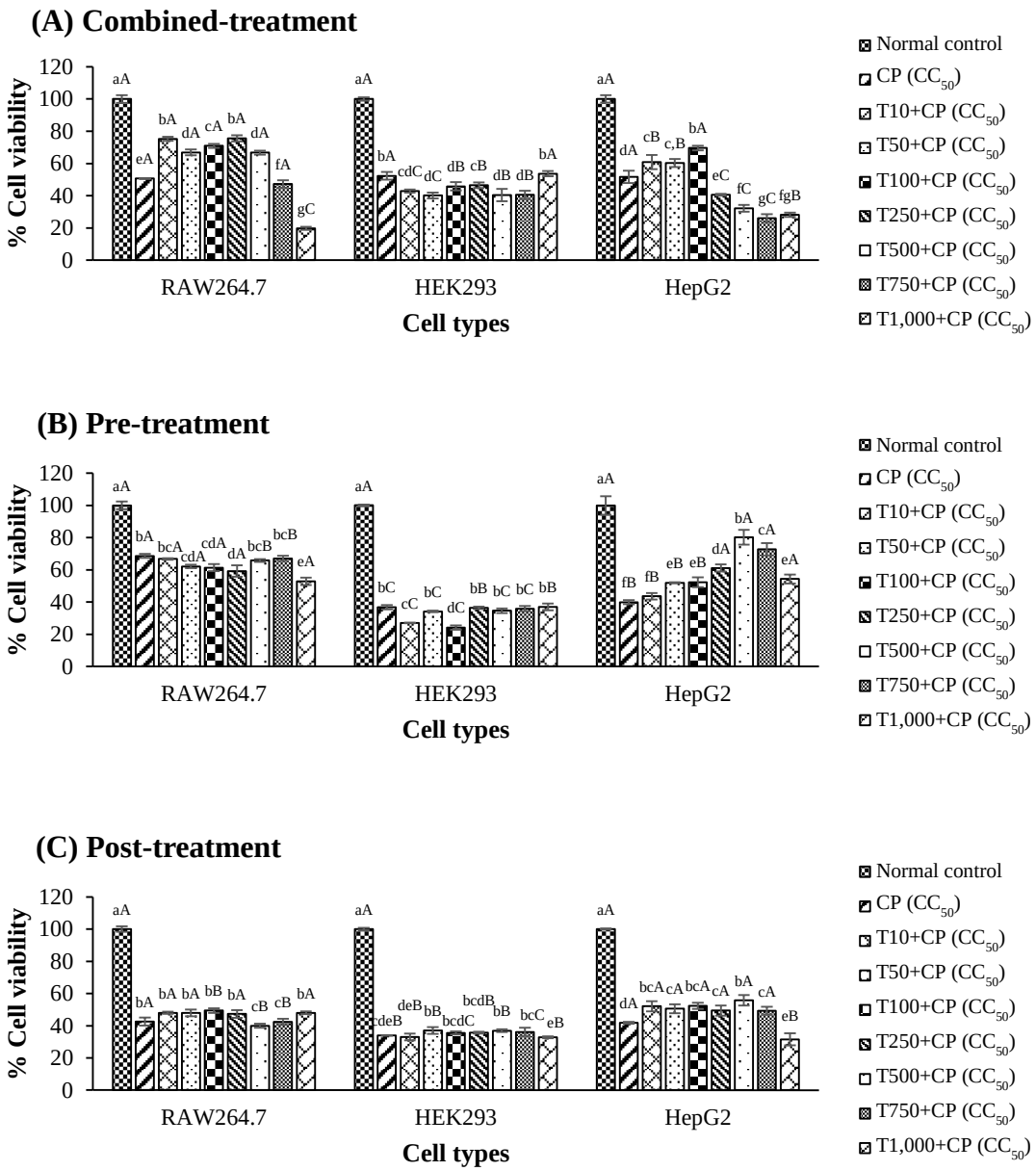


Figure 5.1 Anti-CP toxicity of the aqueous crude extract of *T. laurifolia* leaves in RAW264.7, HEK293 and HepG2 cells determined by MTT assay; (A) combined-treatments; (B) pre-treatment and (C) post-treatment with crude extract.

Remark: $^{a\text{-}g}$ mean within cell types with different letters are significantly different (p<0.05). $^{A\text{-}C}$ mean within an extract concentration with different letters are significantly different (p<0.05). T means the crude extract of *T. laurifolia* leaf (μg/ml). Values are mean ± standard deviation (n=3).

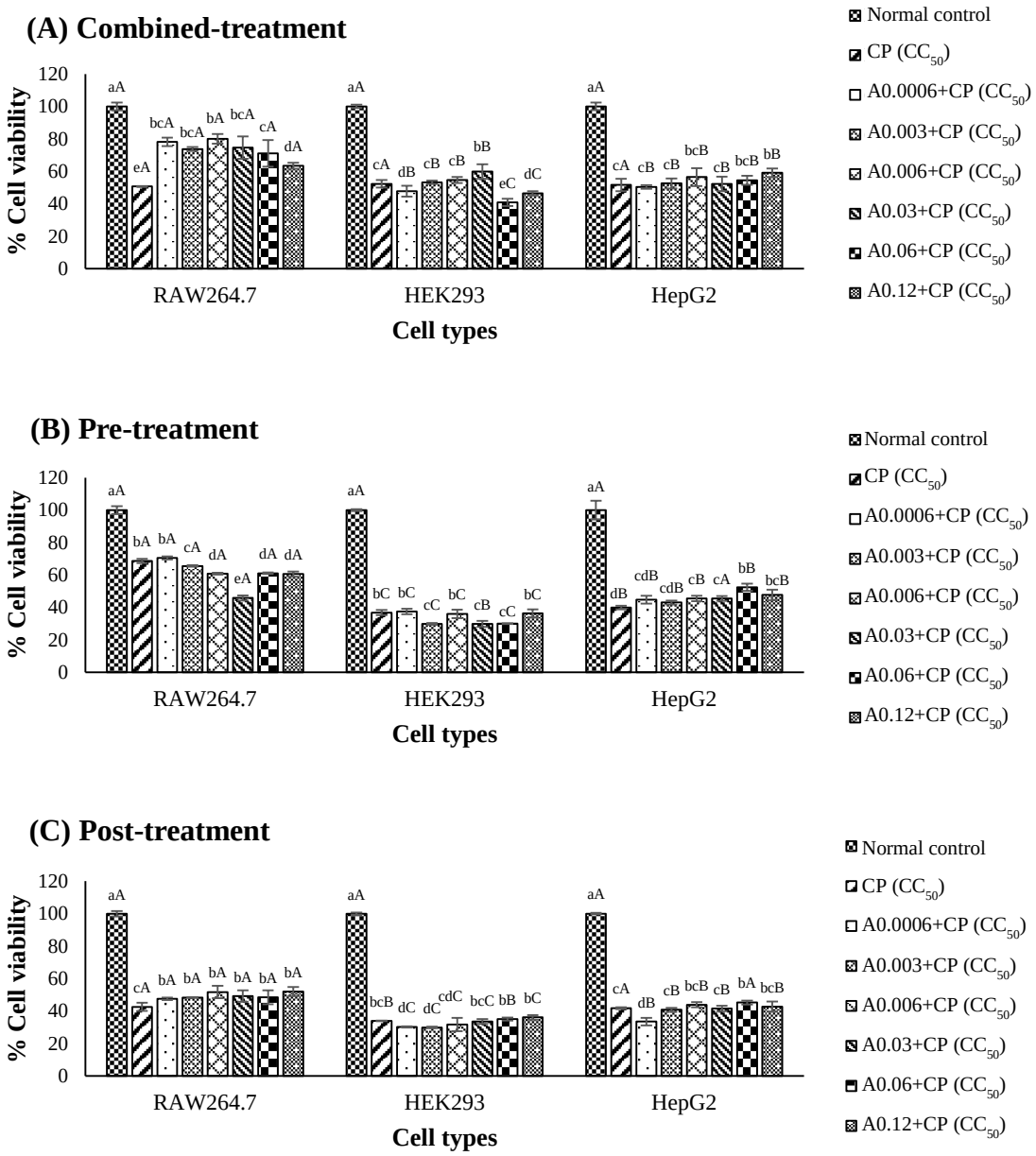


Figure 5.2 Anti-CP toxicity of AS in RAW264.7, HEK293 and HepG2 cells determined by MTT assay; (A) combined-treatments; (B) pre-treatment and (C) post-treatment with crude extract.

Remark: $^{a\text{-}e}$ mean within cell types with different letters are significantly different (p<0.05). $^{A\text{-}C}$ mean within an AS concentration with different letters are significantly different (p<0.05). A means atropine sulfate solution (μg/ml). Values are mean ± standard deviation (n=3).

5.4.3 Cytotoxicity of MT

The cytotoxicity at CC_{50} of MT, a carbamate insecticide was showed in Table 5.2. From the results indicated that MT significantly induced cells death on RAW264.7 compared with HEK293 and HepG2. This result was not in agreement with CC_{50} of CP (Table 5.1) which provided least induced toxin in the RAW264.7 cell. This may due to because MT has more polarity compared with CP therefore, it can dissolve in the system and easily to contact to the cell leading to induced toxic on cell more CP. In addition, CP has short half-life compared with MT (Extension Toxicology Network, 1993a; Extension Toxicology Network, 1993b) therefore toxicity of MT was more retained. Generally, MT is reported to strongly induce genotoxicity via chromosome aberrations, sister-chromatid exchanges and DNA damage including inducing oxidative stress by an increased level of lipid peroxidation (Fabre *et al*., 2001; Mansour *et al*., 2009; Trachantong *et al*., 2017). It pointed out that, RAW264.7 was the most sensitive cell compared with HEK293 kidney and HepG2 liver cells which actually function for removing waste products and excess fluid from the body leading to more tolerant to the toxin. But as mentioned in CP toxicity that cell line test may not responsive for real suitable as animal and the human body. The CC_{50} of MT was then selected as the working concentration for assessing the anti-MT toxicity property of the crude extract and AS.

Table 5.2 50% cytotoxicity concentration (CC_{50}) of MT toxicity on cells viability determined by MTT assay

Cell types	CC_{50} of MT (mg/ml)
RAW264.7	0.54
HEK293	3.88
HepG2	5.78

5.4.2 Anti-MT toxicity property of the crude extract and AS on cells

The effect of anti-MT toxicity property of the crude extract was tasted in similar ways as CP toxicity assay and the results showed in Figure 5.3. It was revealed that the crude extract could help HEK293 cells survival in all treatments, in addition, the crude extract showed an increasing of cell viability in combined and post-treatments in RAW264.7 cells. Moreover, AS provided a greater protection of cell viability especially, in RAW264.7 cells (Figure 5.4). However, the crude extract seemed to not help, particularly at higher concentration, the crude extract even induced cell death in HepG2 cells. It implied that more cytotoxicity could be found in some cell types due to nature of cell and dose-dependent which differ from one to another or others. Therefore, the animal trial is another step to verify and finally clinical trial is very important. Moreover, cell viability in RAW264.7 clearly decreased when the concentration of the extract exceeded 0.5 mg/ml in both the combined and post-treatment groups (Figure 5.3A and 5.3C). This phenomenon was in agreement with the cytotoxicity activity of the extract that was reported in a previous study where showed a dose-dependent reduction of cell viability when the concentration of the crude extract increased (chapter 3, section 3.4.2). Under some circumstances, there was reported that phenolics and flavonoids can induce oxidative stress in the cell (Halliwell, 2008; Halliwell, 2014) as pro-oxidant to react with various biomolecule including lipids, proteins, and DNA (Procházková *et al*., 2011; Yordi *et al*., 2012). Therefore, it implied that both phenolic and flavonoid compounds can have antioxidant and pro-oxidant properties depending on many factors including dosage, the structure of such a polyphenol, cellular redox status or the presence of metal ions in the system (Yordi *et al*., 2012; Kim *et al*., 2014) as well as tasted cell target. Moreover, some phenolic and flavonoid compounds such as gallic acid and epigallocatechin gallate (EGCG) were reported to induce H_2O_2 generation, a cause of oxidative stress either intracellular and/or in cell culture medium and toxic to cells (Halliwell, 2008; Halliwell, 2014). Some researchers reported that at a high concentration of EGCG as 100 µM could induce apoptosis in RAW264.7 macrophage cells by inhibiting autophagy, a lysosomal catabolic process that degrades accumulated and unnecessary intracellular materials (Hashimoto and Sakagami, 2008; Kim *et al*., 2014). While some flavonoids, such as

apigenin, quercetin, naringenin, fisetin and myricetin, also induced apoptosis via pro-oxidant action (Galati and O'Brien, 2004; Kyselova, 2011). Furthermore, caffeic acid was reported to induce apoptosis via the mitochondrial apoptotic pathway (Chang *et al*., 2010). This phenomenon may fellow the natural rule is that everything can be 'good' or 'bad' substances in certain circumstances which also found in bioactive compounds as phenolic and flavonoid. Moreover, too much or too less is not useful even badly.

In general, in the *in vitro* studies reported here, showed that CP and MT provided more toxic on HEK293 and HepG2 cells compared with RAW264.7 cells. This may be due to different toxicity mechanisms of toxins, individual half-life, and cellular targets as well as cell line test or animal trial even patients. In fact, MT is quickly absorbed from the skin, lungs and gastrointestinal tract and can be broken down and transformed in the liver (Extension Toxicology Network, 1993b) while CP is eliminated primarily through the kidneys in urine (Extension Toxicology Network, 1993a). Moreover, RAW264.7 is a macrophage cells type which was reported the most contain choline acetyltransferase, choline transporters, acetylcholinesterase (AChE), and acetylcholine (Kawashima and Fujii, 2004; Razani-Boroujerdi *et al*., 2008). Therefore, any component function inhibits system involving acetylcholine which related to AS action should have more protection effect in the RAW264.7 cell. However, the results suggesting the using of *T. laurifolia* extract is needed to be caution and further studied in both animal and clinical trials to ensure the safety is required.

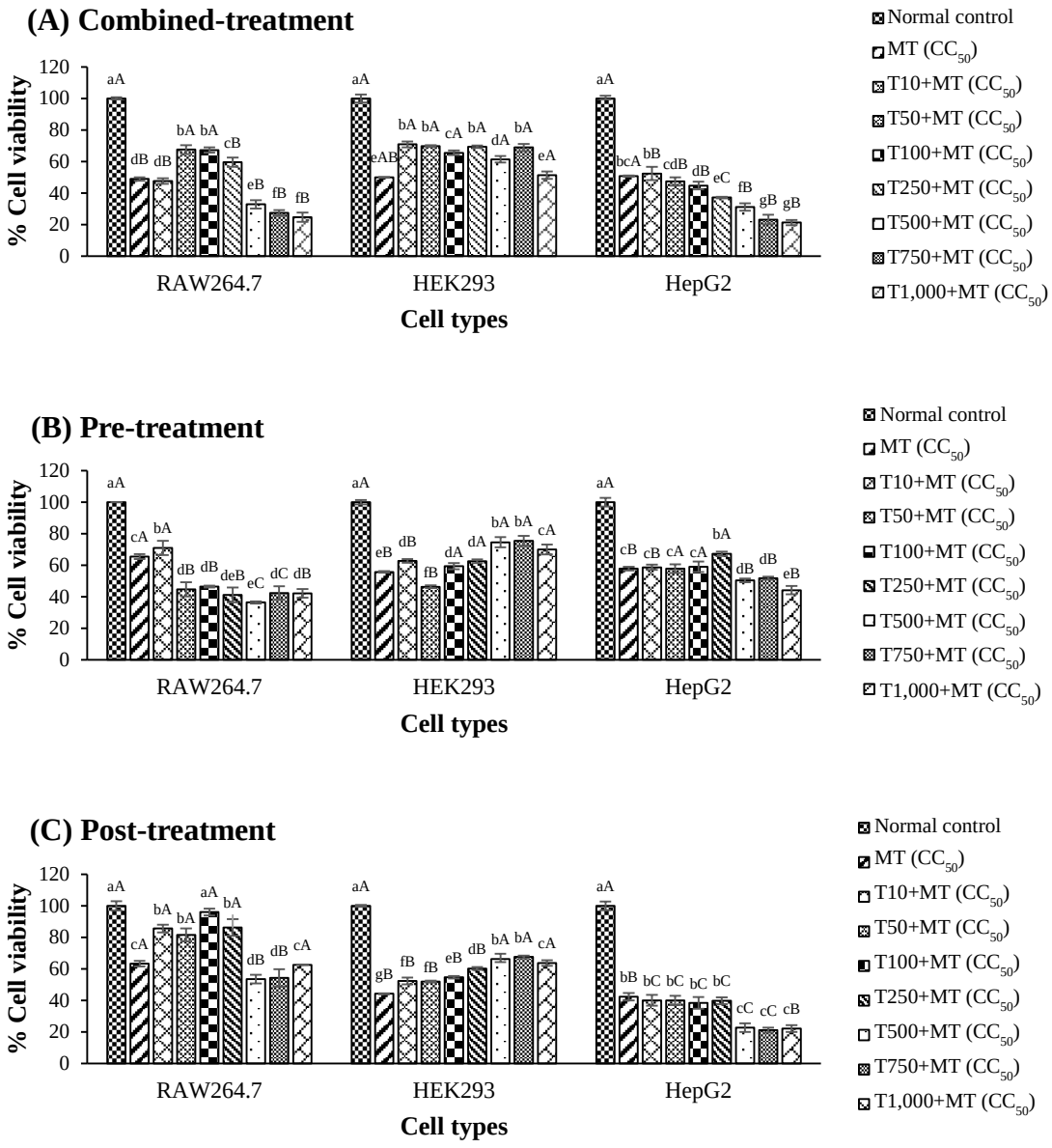


Figure 5.3 Anti-MT toxicity of the aqueous crude extract of *T. laurifolia* leaves in RAW264.7, HEK293 and HepG2 cells determined by MTT assay; (A) combined-treatment; (B) pre-treatment and (C) post-treatment with crude extract.

Remark: $^{a-g}$ mean within cell types with different letters are significantly different (p<0.05). $^{A-C}$ mean within an extract concentration with different letters are significantly different (p<0.05). T means the crude extract of *T. laurifolia* leaf (µg/ml). Values are mean ± standard deviation (n=3).

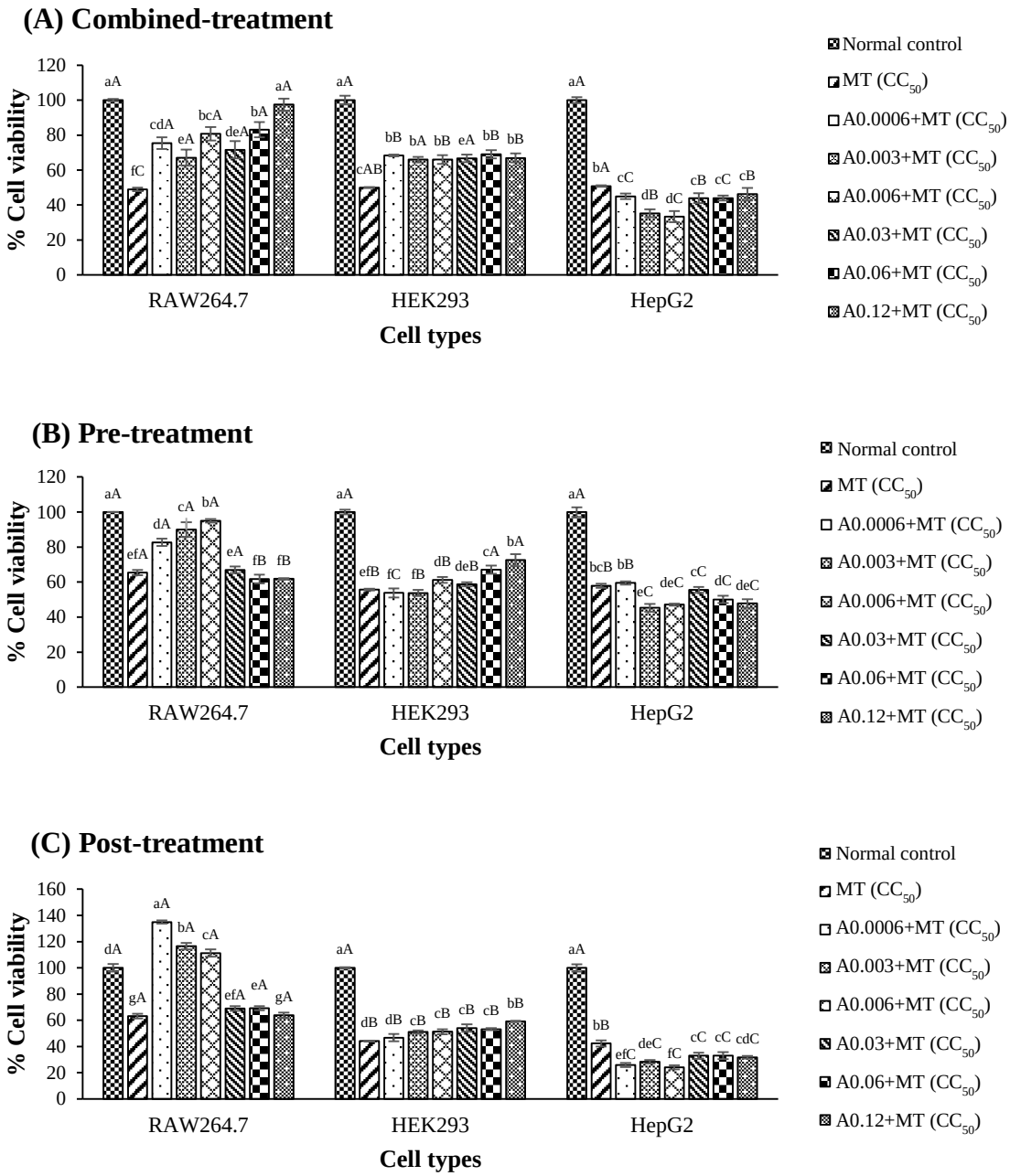


Figure 5.4 Anti-MT toxicity of AS in RAW264.7, HEK293 and HepG2 cells determined by MTT assay; (A) combined-treatments; (B) pre-treatment and (C) post-treatment with crude extract.

Remark: $^{a\text{-}f}$ mean within cell types with different letters are significantly different (p<0.05). $^{A\text{-}C}$ mean within an AS concentration with different letters are significantly different (p<0.05). A means atropine sulfate solution (μg/ml). Values are mean ± standard deviation (n=3).

5.5 Conclusion

The cytotoxicity effect of CP and MT insecticides was highest on HEK293 and RAW264.7 cells respectively. The aqueous crude extract of *T. laurifolia* leaves showed effective healing and protection functions for anti-insecticides on cell types in different sensitivity. The crude extract protected and/or recovered cells death especially in HepG2 cell from CP toxin and HEK293 cells from MT toxin. The AS standard drug protected cells death from CP toxin and MT, especially in RAW264.7 cells. This experiment supported the Thai folklore wisdom for using *T. laurifolia* tea as anti-insecticides agent but further study for more safety detail still needed.

5.6 References

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CHAPTER 6

SUMMARY AND FUTURE WORKS

6.1 Summary

Dried leaves of *Thunbergia laurifolia* contained all proximate component including protein, fat, ash, fiber carbohydrate and some minerals. phytochemical screening test indicated that the aqueous crude extract compost of phenols, flavonoids, tannins and sterols, in addition, the crude extracted showed the antioxidant activity based on TPC, TFC, ABTS, DPPH, FRAP, and iron chelating property. Phenolics profiling identified by LC-MS consisted of caffeic acid, and rosmarinic acid while, flavonoid compounds were catechin, rutin, isoquercetin, quercetin, and apigenin. However, there were some unknown compounds need to be future investigated.

The aqueous crude extract was taken to investigate some biological activities by using RAW264.7, HEK293 and HepG2 cells as a test model. The result showed that the crude extract significantly increased antioxidant enzymes and significantly decreased MDA level in HEK293 kidney and HepG2 liver cells but not in RAW264.7 macrophage cells. In addition, even the crude extract had a low cytotoxicity, it inhibited NO production in RAW 264.7 cells**.**

The effect of using crude extract on Cd toxicity property significantly protected cells against Cd-induced toxicity in all cell types. Cd toxicity was more pronounced further when the cells were treated with the crude extract together with Cd or in combined-treatment. The $CaNa_2EDTA$ agent can help cell survival the most in combined-treatment. The crude extract helped cells survival when the chelating agent was applied via antioxidant and chelation properties after treated with Cd.

The anti-insecticides of crude extract showed effective healing and protection functions for anti-insecticides on each cell type even with different sensitivity. The crude extract protects and/or recover cells death especially in HepG2 cell from CP toxin and HEK293 cells from MT toxin. The AS standard drug can protect cells death from CP toxin and RAW264.7 cells from MT, especially in combined-treatment. This experiment supported the Thai folklore wisdom for using *T. laurifolia* tea as anti-insecticides agent but further study for more safety detail still needed.

6.2 Recommendation for future works

The improvement of *T. laurifolia* for functional drinking product should be further investigated in animal or clinical trials and carefully considered.

Appendices

APPENDIX A

ANALYTICAL METHODS

A1. Moisture content (AOAC, 2000)

Method

1. Dry the empty dish and lid in the oven at 105 °C for 2-3 hr. and transfer to a desiccator to cool (30 min). Weigh the empty dish and lid.
2. Weigh about 1-2 g of the sample to the dish. Spread the sample with a spatula.
3. Place the dish with the sample in the oven. Dry for 4-5 hr. at 105 °C.
4. After drying, transfer the dish with a partially covered lid to the desiccator to cool. Reweigh the dish and its dried content.

Calculation

$$\% \text{ Moisture} = \frac{(W_1 - W_2)}{W_2} \times 100$$

where: W_1 = weight (g) of sample before drying.

W_2 = weight (g) of sample after drying.

A2. Ash (AOAC, 2000)

Method

1. The crucible and lid are first placed in the furnace at 550 °C for 3 hr. or ensure that impurities on the surface or crucible are burned off. Cool the crucible in the desiccator (30 min).
2. Weigh the crucible and lid to 4 decimal places.
3. Weigh about 1-2 g of sample into the crucible. Heat over low Bunsen flame with the lid half covered. When fumes are no longer produced, place crucible and lid in the furnace.
4. Heat at 600 °C for 3 hr. During heating, do not cover with the lid. Place the lid on after complete heating to prevent loss of fluffy ash. Cool down in the desiccators.

5. Weigh the ash with crucible and lid until turning to gray. If not, return the crucible and lid to the further ashing.

Calculation

$$\% \text{ Ash content} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

A3. Crud protein (AOAC, 2000)

Reagents

- Kjeldahl catalyst: Mix 9 parts of potassium sulphate (K_2SO_4) anhydrous, nitrogen-free with 1 parts of copper sulphate ($CuSO_4$)
- Sulfuric acid (H_2SO_4)
- 40% NaOH solution (w/v)
- 0.02 N HCl solution
- 4% H_3BO_3 solution (w/v)
- Indicator solution : Mix 100 ml of 0.1% methyl red (in 90% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Methods

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g of kjeldahl catalyst, and 20 ml of conc. H_2SO_4.
3. Prepare a tube containing the above chemical except for sample as a blank. Place flasks in an inclined position and heat gently until frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on the condenser, and with the tip of condenser immersed in standard acid and 5-7 indicator in the receiver. Rotate flask to mix content thoroughly; then until all NH_3 has distilled.
6. Remove receiver, wash tip of the condenser, and titrate excess standard acid in distillate with standard NaOH solution.

Calculation

$$\% \text{ Protein} = \frac{(A-B) \times N \times 1.4007 \times 6.25}{W}$$

where: A = volume (ml) of 0.02 N HCl used sample titration
B = volume (ml) of 0.02 N HCl used in blank titration
N = Normality of HCl
W = weight (g) of sample
14.07 = atomic weight of nitrogen
6.25 = the protein-nitrogen conversion factor for fish and its by-products

A4. Crud fat (AOAC, 2000)

Reagents

- Petroleum ether

Method

1. The bottle and lid are firstly placed in the incubator at 105 °C overnight to ensure that weight of bottle was stable.
2. Weigh about 3-5-g of the sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet.
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 hr. (heat rate of 150 drops/min).
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90 °C until the solvent was completely evaporated and the bottle was completely dry.
9. After drying, transfer the bottle with a partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

Calculation

$$\% \text{ Crude fat} = \frac{\text{Weigh of fat} \times 100}{\text{Weigh of sample}}$$

A5. Crud fiber

Reagents

1. 0.312 N NaOH solution (w/v)
2. 0.255 N H_2SO_4 solution (v/v)
3. Alcohol: Methyl, isopropyl, or 95% ethyl alcohol
4. Antifoam

Method

1. Grind the sample to be finer or size about 1 mm and dry the sample in the oven at 105 °C for 2-3 hr.
2. Dry the glass crucible in the furnace at 550 °C for 1 hr. or ensure that impurities on the surface or crucible are burned off. Cool the crucible in the desiccator (30 min).
3. Weigh about 2 g of the sample to the fiber bag and place it in the crucible, then attach the cylinder to the crucible. Pour 150 ml of boiling sulphuric acid into the assembled cylinder and crucible and if necessary add a few drops of anti-foaming agent.
4. Bring the liquid to the boil within 5±2 min and boil vigorously for exactly for exactly 30 min.
5. Open the tap to the discharge pipe under vacuum and filter the sulphuric acid through the filter crucible and wash the residue with three consecutive 30 ml portion of boiling water for ensuring the residue is filtered dry after each washing.
6. Close the outlet tap and pour 150 ml boiling potassium hydroxide solution to the assembled cylinder and crucible and add a few drops of anti-forming.

Then, bring the liquid to boiling point within 5±2 min and boil vigorously for exactly 30 min. Filter and repeat the washing procedure used for the sulphuric acid

7. After the final washing and drying and disconnect the crucible and its contents and reconnect it to the cold extraction unit. Apply the vacuum and wash the residue in the crucible with three consecutive 25 ml portions of acetone, ensuring that the residue is filtered dry after each washing.
8. Dry the crucible to constant weight in the oven at 105 °C for 2-3 hr. After each drying cool in the desicator and weigh rapidly. Place the crucible in the furnace at 550 °C for 3 hr. Cool down in the desiccators before weighting.

Calculation

$$\% \text{ Crude fiber } = \frac{(B-C)}{A} \times 100$$

A = Mass of sample (g)

B = Weight of crucible +sample after washing, boiling and drying (g)

C = Weight of crucible +sample of ash (g)

APPENDIX B

CYTOTOXICITY OF $CaNa_2EDTA$

Concentrations of $CaNa_2EDTA$ (μg/ml)	Cell viability (%)		
	RAW264.7	HEK293	HepG2
1	99.98±0.47	87.16±3.48	84.36±1.52
10	104.50±0.94	84.42±3.34	88.20±4.60
50	103.14±1.78	84.44±2.09	87.27±2.21
100	83.77±1.26	85.40±1.40	84.70±3.28
200	82.79±0.72	82.99±3.83	81.18±2.23
500	60.70±1.82	76.08±3.83	76.02±4.65
1,000	51.11±3.14	57.67±2.88	53.99±4.65

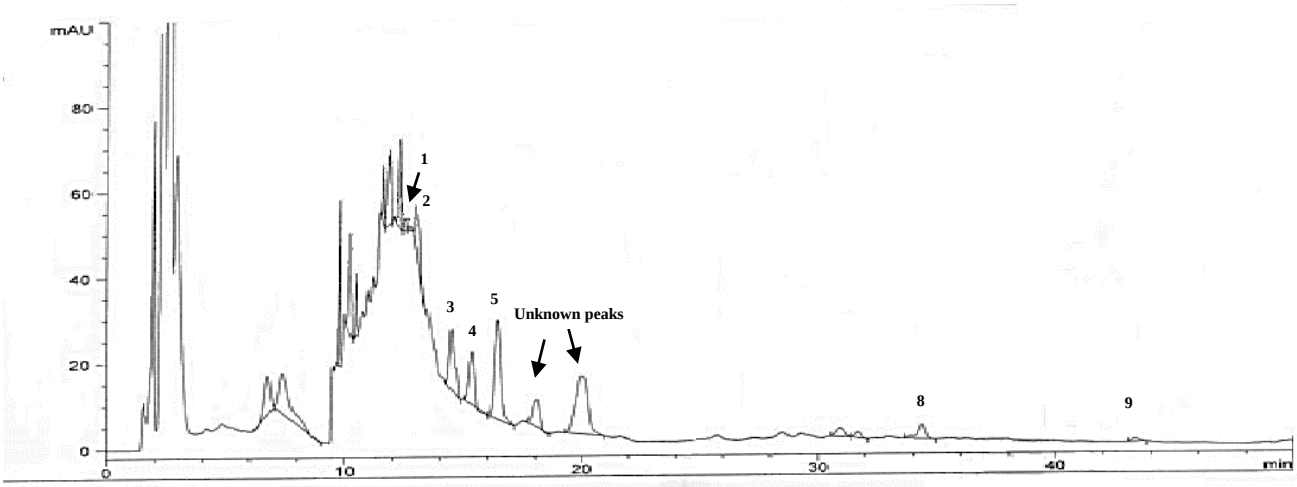
APPENDIX C

CYTOTOXICITY OF ATROPINE SULPHATE

Concentrations of Atropine sulphate (μg/ml)	Cell viability (%)		
	RAW264.7	HEK293	HepG2
0.0006	98.72±1.92	97.30±4.83	102.18±0.35
0.003	97.79±0.39	96.29±4.93	101.34±2.26
0.006	96.81±2.86	90.26±3.15	106.54±0.66
0.06	96.12±0.87	86.06±0.78	91.75±1.05
0.12	89.22±2.59	90.84±1.33	95.34±3.25
0.24	81.95±1.81	90.70±0.55	95.27±0.92
0.48	74.76±1.81	66.93±2.64	75.82±1.61
1	29.81±1.72	33.62±1.64	37.62±3.59

APPENDIX D

CHROMATOGRAME OF THE AQUEOUS CRUDE EXTRACT



Chromatogram of phenolic and flavonoid compounds in the aqueous crude extract of *T. laurifolia* leaves determined by LC-MS.

Remark: 1= Catechin, 2= Caffeic acid, 3= Rosmarinic acid, 4= Rutin, 5= Isoquercetin, 8= Quercetin, 9= Apigenin

VITAE

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Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biotechnology, Second Class Honor)	Prince of Songkla University	2005
Master of Science (Food Science and Technology)	Prince of Songkla University	2011

Scholarship Awards during Enrolment

- The Graduate School PSU-Ph.D. Scholarship from Prince of Songkla University.
- Graduate School Dissertation Funding for Thesis from Prince of Songkla University.
- Overseas Thesis Research Scholarship from Prince of Songkla University (Visited at RWTH Aachen University from 1st June 2017 until 30th September 2017 under the supervision of Prof. Dr. Alan Slusarenko at Plant Physiology Department, Aachen, Germany).

List of Publications

Junsi, M., Siripongvutikorn, S., Takahashi Yupanqui, C. and Usawakesmanee, W. 2017. Efficacy of *Thunbergia laurifolia* (Rang Jued) aqueous leaf extract for specific biological activities using RAW 264.7 macrophage cells as test model. Int. Food Res. J. 24(6): 2317-2329.

Junsi, M., Siripongvutikorn, S., Takahashi Yupanqui, C. and Usawakesmanee, W. 2017. Phenolic and flavonoid compounds in aqueous extracts of *Thunbergia laurifolia* leaves and their effect on the toxicity of the carbamate insecticide methomyl to murine macrophage cells. Funct. Food Health Dis. 7(7): 529-544.

Junsi, M. and Siripongvutikorn, S. 2016. Mini Review: *Thunbergia laurifolia*, a traditional herbal tea of Thailand: botanical, chemical composition, biological properties and processing influence. Int. Food Res. J. 23(3): 923-927.

List of Proceeding

Junsi, M., Siripongvutikorn, S., Takahashi Yupanqui, C. and Usawakesmanee, W. 2018. Rang Jued (*Thunbergia laurifolia*) leaves extract protects against cadmium toxicity in HEK293 kidney cells. International Conference on Food and Applied Bioscience (FAB 2018). Chiang Mai, Thailand.

List of Presentations

Oral presentation in the topic "Antioxidant and Anti-inflammatory Activities of Bioactive Compounds Derived from *Thunbergia laurifolia* Aqueous Leave Extract" in 18th International Conference on Food Security and Nutrition, Bali, Indonesia. 13-14 October 2016.

Poster presentation in the topic of "Rang Jued (*Thunbergia laurifolia*) leaves extract protects against cadmium toxicity in HEK293 kidney cells" Chiang Mai, Thailand. 1-2 February 2018.