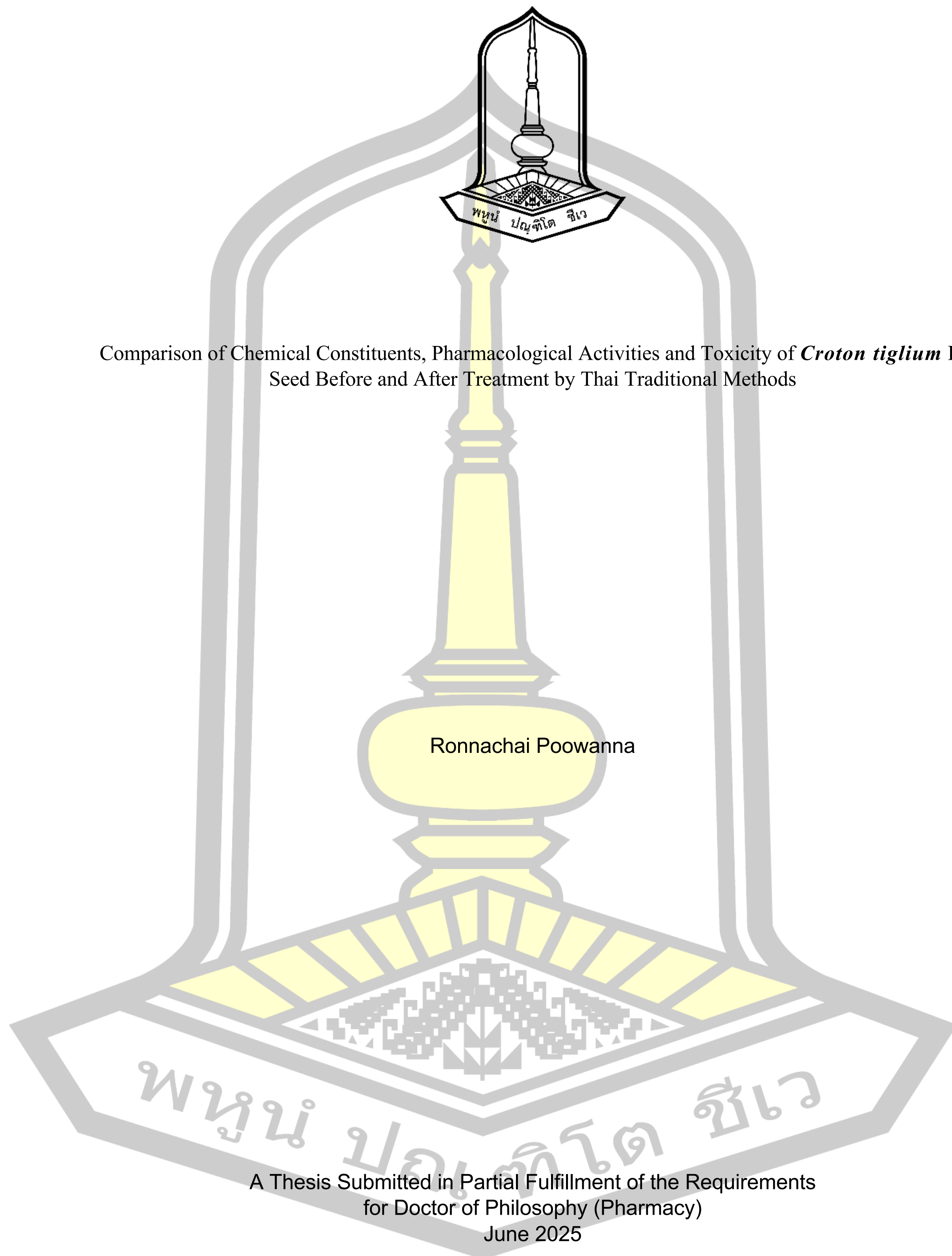

Comparison of Chemical Constituents, Pharmacological Activities and Toxicity of ***Croton tiglium*** L. Seed Before and After Treatment by Thai Traditional Methods

Ronnachai Poowanna

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy (Pharmacy)
June 2025

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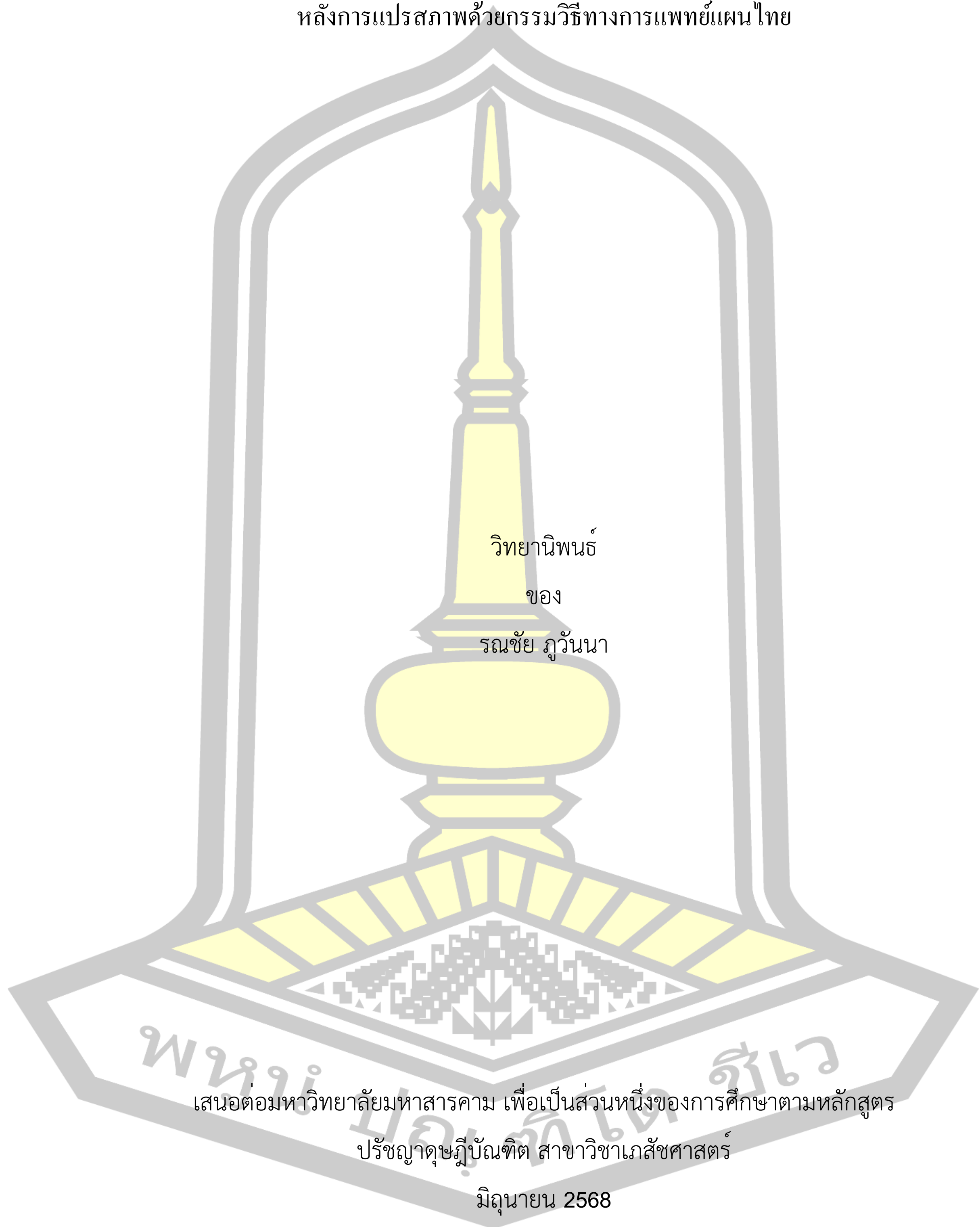

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ของ

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มิถุนายน 2568

ลิขสิทธิ์เป็นของมหาวิทยาลัยมหาสารคาม

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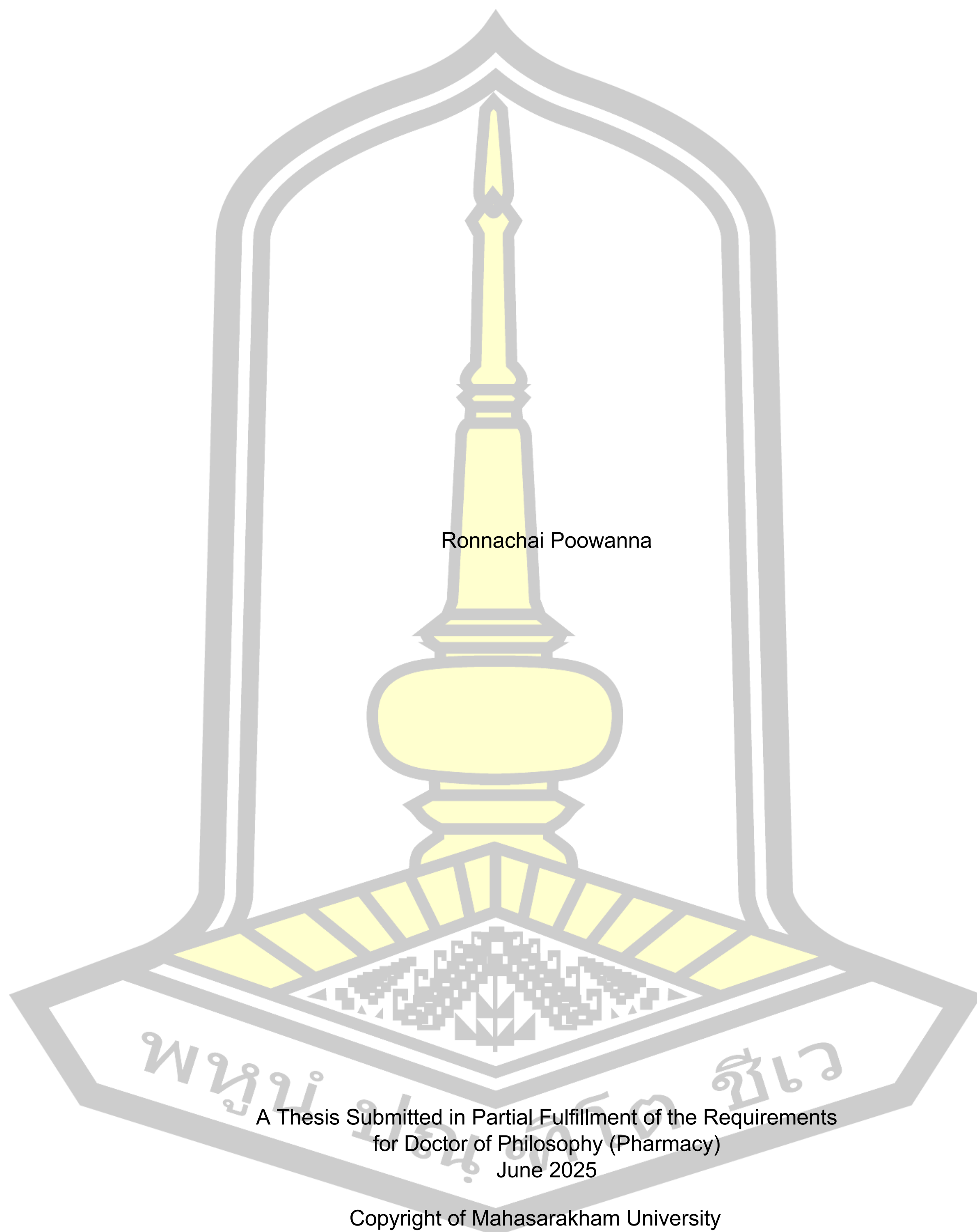

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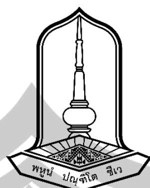

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TITLE Comparison of Chemical Constituents, Pharmacological Activities and Toxicity of ***Croton tiglium*** L. Seed Before and After Treatment by Thai Traditional Methods

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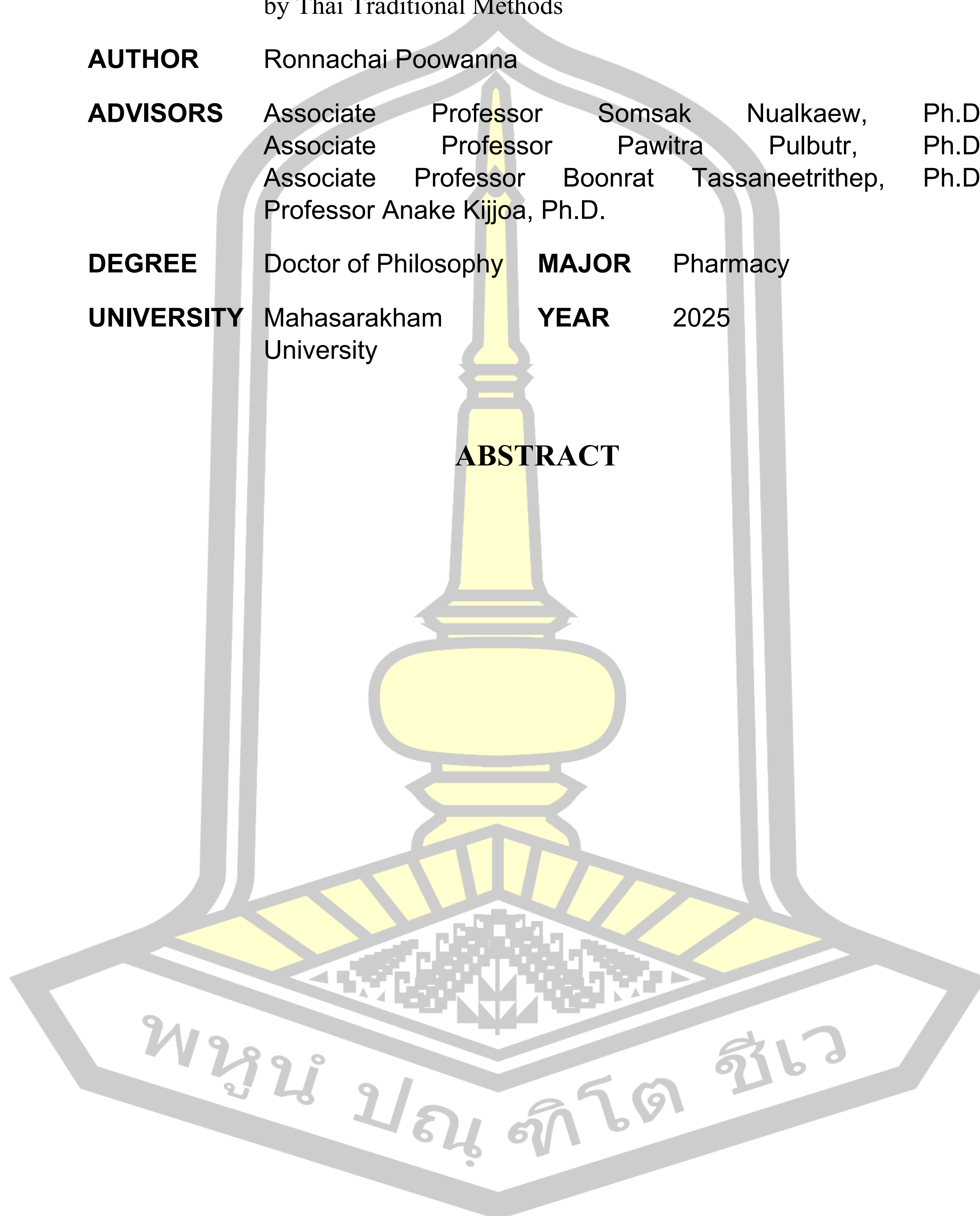
UNIVERSITY Mahasarakham University **YEAR** 2025

ABSTRACT



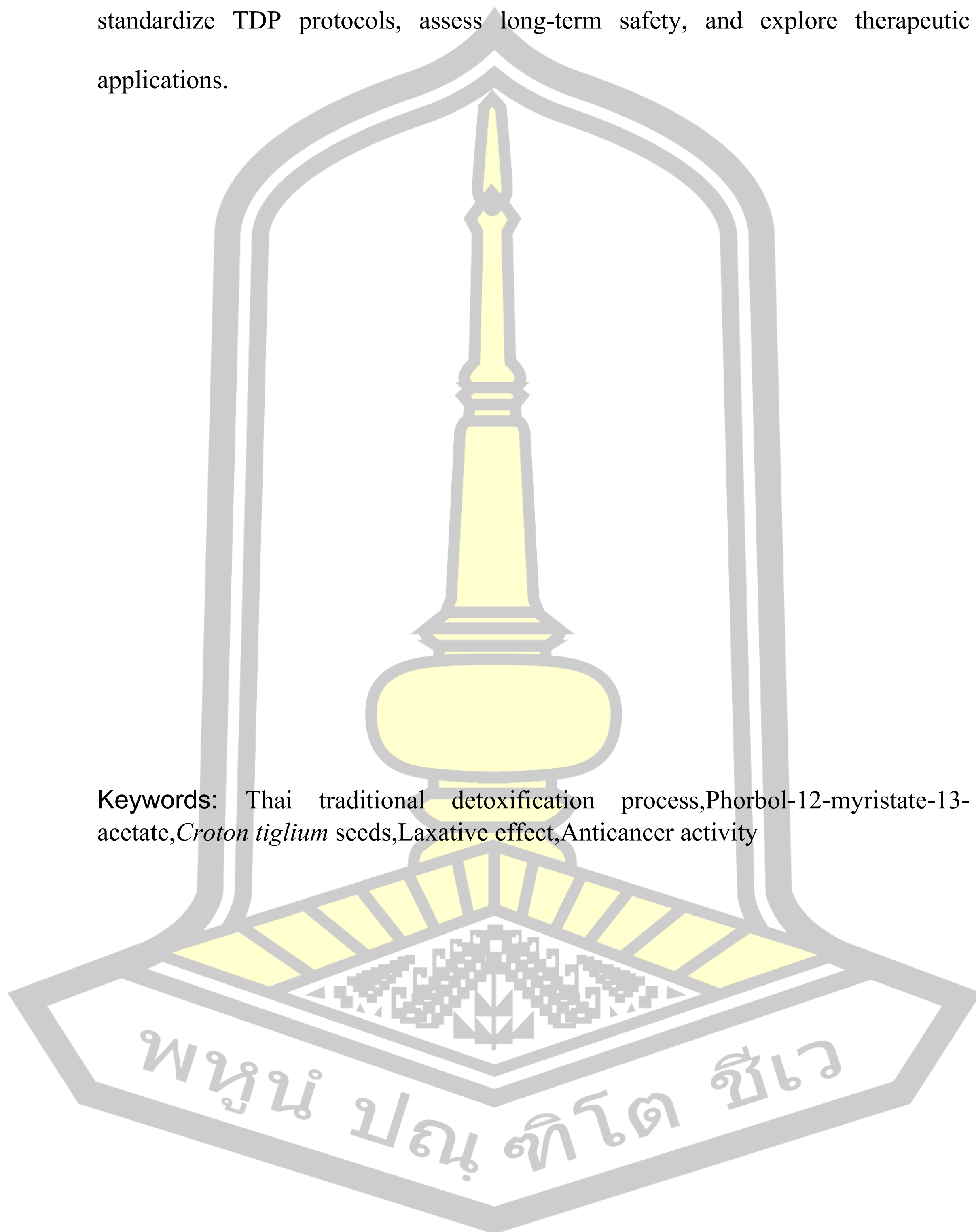
The Ministry of Public Health in Thailand has declared *Croton tiglium* seeds a hazardous herb strictly prohibited in Thai traditional medicine due to their toxic properties since 1976, despite their continued use in various countries. In Thai traditional medicine, the traditional detoxification process (TDP) is employed to reduce the seeds toxicity, though these methods lack scientific validation.

This study aimed to investigate the effects of Thai TDP on *C. tiglium* seeds by comparing the chemical, acute toxicity, laxative action, and anticancer activity of *C. tiglium* seeds before and after treatment. Crotonic acid and phorbol 12-myristate 13-acetate (PMA), two major irritants in *C. tigilium* seeds, were measured by using HPLC. The analysis revealed a reduction in crotonic acid (from 0.001 mg/g to undetectable levels) and PMA (from 1.59 to 1.22 mg/g) after Thai TDP. Acute toxicity tests (up to 2,000 mg/kg) showed no mortality or significant abnormalities. The purgative effect was reduced in detoxified extracts, as evidenced by lower fecal weight and water content compared to non-detoxified samples. Histopathological examination revealed inflammation in rats treated with non-detoxified extracts, but not in those receiving detoxified ones.

In vitro cytotoxicity assays showed the extract was non-toxic to normal cells (Hex293T) but induced significant apoptosis in cancer cells, especially at 200 µg/mL, with increased Bax expression and caspase 3 and caspase 9 activation, indicating a caspase-dependent apoptotic pathway.

In conclusion, Thai TDP effectively reduces the toxicity of *C. tiglium* seeds, supporting their potential safe reintroduction into Thai traditional medicine. These findings highlight the importance of validating traditional detoxification methods to

ensure the safety and efficacy of herbal medicines. Further research is needed to standardize TDP protocols, assess long-term safety, and explore therapeutic applications.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Keywords: Thai traditional detoxification process,Phorbol-12-myristate-13-acetate,*Croton tiglium* seeds,Laxative effect,Anticancer activity

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Ronnachai Poowanna

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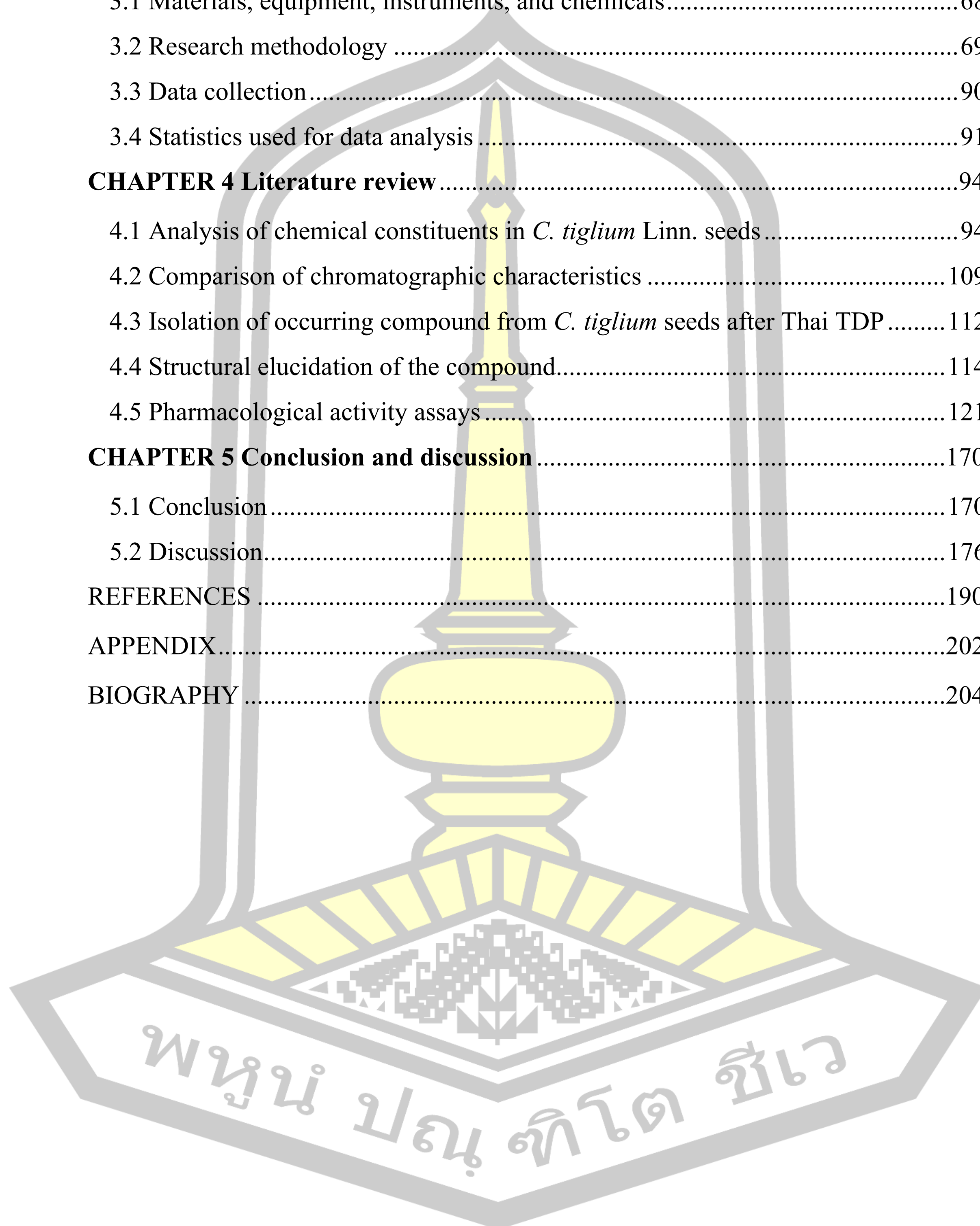

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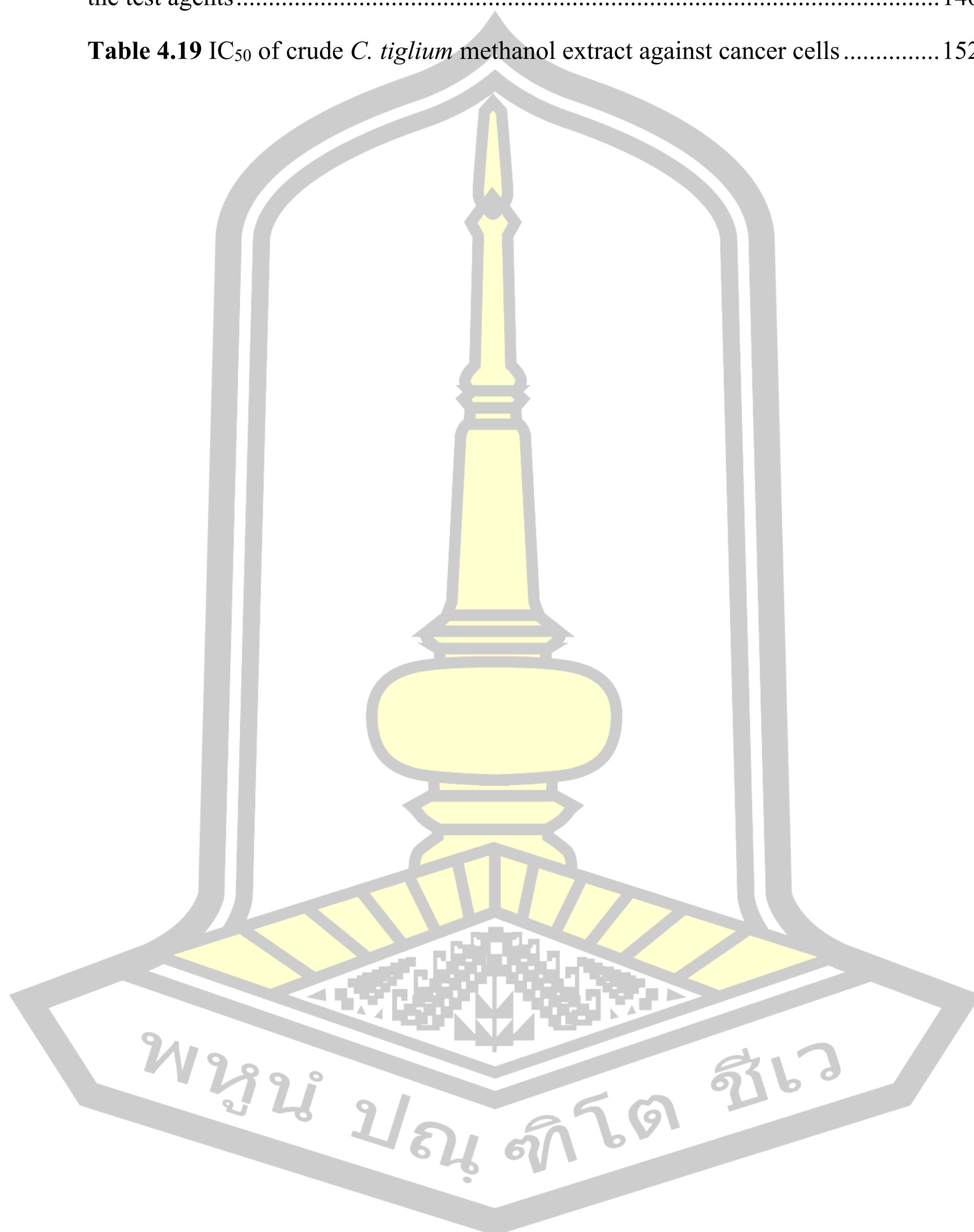

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CHAPTER 1

Introduction

1.1 Background

Croton tiglium Linn., commonly known as the purging croton, belongs to the Euphorbiaceae family. This medicinal plant is widely distributed across tropical countries, including Thailand, India, Sri Lanka, China, and Malaysia. In Thai traditional medicine, its seeds, known as "Salod seed", have been used as a potent purgative to alleviate abdominal pain and for treatment of wind disorders, cough, phlegm, lymphatic issues, blood impurities, and parasitic infections[1]. Salod seed or *C. tiglium* seed is a common ingredient in several Thai traditional medicine formulations, such as Ya Tud Rak Krasai, Ya Praju Krasai Pla Moh, Ya Ka Krasai Lin Kra Bue, and Ya Ka Krasai Krawn Thao, among others[1]. Many of these formulations have been historically used to treat conditions equivalent to cancer in modern medicine[2]. The traditional use of *C. tiglium* seed aligns with pharmacological studies, which have demonstrated its potential in inhibiting the growth of various cancer cells, such as liver cancer[3], leukemia[4], lung cancer[4, 5], and gastric cancer[4].

However, previous research has identified phorbol-12-myristate-13-acetate[6] in *C. tiglium* seed, a compound known to promote tumor development[7]. Due to this finding, the Ministry of Public Health of Thailand has classified Salod seed and its oil as hazardous herbal medicines, and has prohibited their use in Thai traditional medicine formulations since 1976[8]. As a result, despite its long history of use, *C. tiglium* seed ceased to be utilized in Thai traditional medicine practices. In Thai traditional medicine,

C. tiglium seed was not used directly but had to undergo detoxification processes to neutralize its toxicity. These methods have not been thoroughly investigated, raising concerns among practitioners about the adequacy of evidence supporting its prohibition.

Recent studies have reported various pharmacological properties of *C. tiglium* seed extracts, including antifungal[9], antibacterial[10], anti-HIV[11], anti-inflammatory[12], and anticancer activities[3-5].

Chemical studies comparing before and after Thai Traditional detoxification processed (Thai TDP) of *C. tiglium* seed demonstrated significant changes in its chemical composition. For instance, four Thai traditional detoxification processed methods were investigated: 1) Boiling the seeds with rice husks, salt, and water until the rice husks fully expanded. 2) Boiling the seeds with *Tamarindus indica* leaves and *Siamese cassia* leaves. 3) Soaking the seeds in fermented fish brine overnight and roasting them put in kaffir lime until charred. 4) Crushing and roasting the seeds until the oil is removed.

The results showed that methods 1, 3, and 4 significantly reduced PMA levels by 44.47%, 7.33%, and 79.76%, respectively, while method 2 increased PMA levels by 13.91%. Additionally, new compounds were detected after these processes, although their pharmacological effects remain unexplored[13]. In India, a different detoxification method was used. This method consists of soaking the seeds overnight, grinding them into powder, wrapping them in cotton cloth, and boiling them in cow's milk at 120°C for three hours. This method reduced PMA levels from 5.2 mg/100 g to 1.8 mg/100 g and eliminated crotonic acid[14].

These findings indicate that traditional practitioners in Thailand and in India recognized the toxic nature of *C. tiglium* seed, and the need to detoxify them prior to medicinal use. However, there are no comprehensive studies comparing its toxicity, chemical composition, and pharmacological activities before and after processing. Therefore, the present research aims to fill this gap by comparison of the chemical composition, anticancer properties, tumor promoting effects, laxative activities, and toxicity of the raw and processed *C. tiglium* seed. This study also seeks to provide evidence to clarify its safety and potential for medicinal use, particularly in cancer treatment, which is a pressing global health issue.

1.2 Research objectives

1.2.1 To compare the HPLC chromatogram of *C. tiglium* seed before and after Thai TDP.

1.2.2 To develop a method for isolation of new compounds formed *C. tiglium* seed after Thai TDP.

1.2.3 To evaluate the laxative, anticancer, tumor-promoting activities, and toxicity of *C. tiglium* seed before and after Thai TDP.

1.2.4 To investigate the anticancer and tumor-promoting activities of newly identified compounds after detoxification process.

1.3 Research hypotheses

1.3.1 The HPLC chromatogram of *C. tiglium* seed before and after Thai TDP differ significantly.

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1.3.2 The toxicity and pharmacological effects of *C. tiglium* seed before and after Thai TDP differ significantly.

1.3.3 The laxative, anticancer, tumor-promoting activities, and toxicity of *C. tiglium* seed before and after Thai TDP differ significantly.

1.3.4 Newly identified compounds after detoxification process exhibit distinct anticancer and tumor-promoting activities.

1.4 Significance of the research

This research aims to validate the mechanisms of Thai TDP applied to *C. tiglium* seed. It seeks to clarify whether the detoxification process is intended to reduce toxicity or diminish the potency of the seed. The findings will enhance the understanding of herbal detoxification principles in Thai traditional medicine, enabling the establishment of more detailed, high-quality, and effective standard operating procedures. Additionally, if the detoxified *C. tiglium* seed or the newly derived compounds exhibit anticancer properties, this may offer a future alternative for cancer treatment.

1.5 Scope of the research

The study is divided into three main experimental sections:

1.5.1 Chemical composition analysis of *C. tiglium* seed before and after detoxification process by two experimental procedures:

1.5.1.1 Quantification of PMA and crotonic acid using high-performance liquid chromatography.

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1.5.1.2 Isolation and structure elucidation of new compounds after detoxification process.

1.5.2 Toxicity and pharmacological activity of *C. tiglium* seed before and after detoxification process

1.5.2.1 Anticancer activity assays, including cell cycle inhibition, apoptosis induction, colony formation inhibition, and anti-proliferative effects.

1.5.2.2 Carcinogenic potential assessment, focusing on p53, p51 Bax, Caspase3, Caspase9, PARP, and Cytochrome c protein levels.

1.5.2.3 *In vivo* laxative effect testing in animal models.

1.5.2.4 Toxicity testing in animal models.

1.5.3 Toxicity and pharmacological studies of newly formed compounds after detoxification process.

1.5.3.1 Anticancer activity assays, which are similar to those described in section 1.5.2.1.

1.5.3.2 Carcinogenic potential assessment, focusing on p53, p51 Bax, Caspase3, Caspase9, PARP, and Cytochrome c protein levels.

1.5.4 Data collection and analysis

Experimental results were collected, analyzed, summarized, and presented to demonstrate the observed changes.

1.5.5 Variables in the study

1.5.5.1 Comparison of the chemical compositions of *C. tiglium* seed before and after detoxification process.

1) Independent variables:

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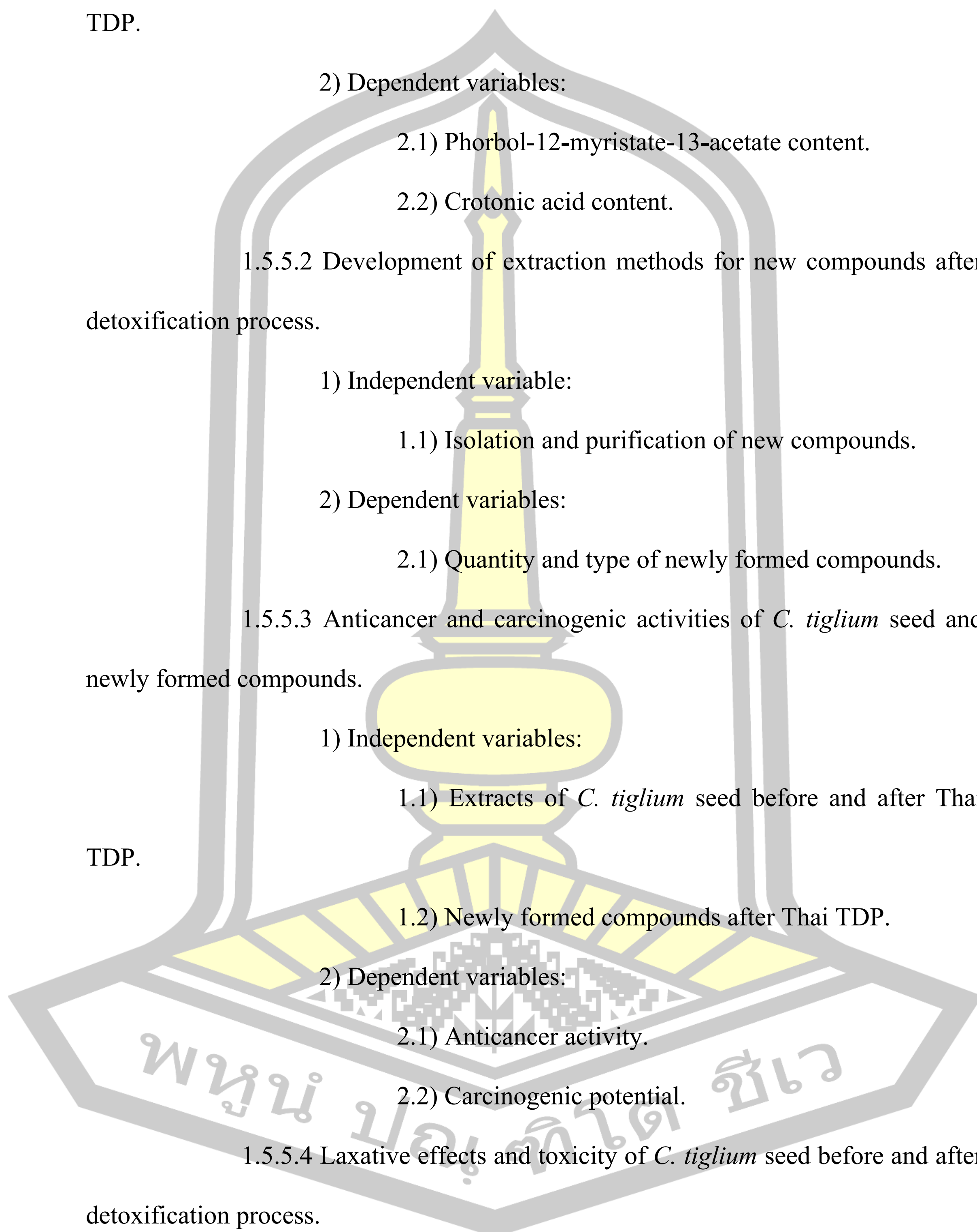


1.1) Extracts of *C. tiglium* seed before and after Thai TDP.

2) Dependent variables:

2.1) Phorbol-12-myristate-13-acetate content.

2.2) Crotonic acid content.

1.5.5.2 Development of extraction methods for new compounds after detoxification process.

1) Independent variable:

1.1) Isolation and purification of new compounds.

2) Dependent variables:

2.1) Quantity and type of newly formed compounds.

1.5.5.3 Anticancer and carcinogenic activities of *C. tiglium* seed and newly formed compounds.

1) Independent variables:

1.1) Extracts of *C. tiglium* seed before and after Thai TDP.

1.2) Newly formed compounds after Thai TDP.

2) Dependent variables:

2.1) Anticancer activity.

2.2) Carcinogenic potential.

1.5.5.4 Laxative effects and toxicity of *C. tiglium* seed before and after detoxification process.

1) Independent variable:

1.1) *C. tiglium* seed before and after detoxification process.

2) Dependent variables:

2.1) Laxative effects in animal models.

2.2) Toxicity in animal models.

1.6 Research locations

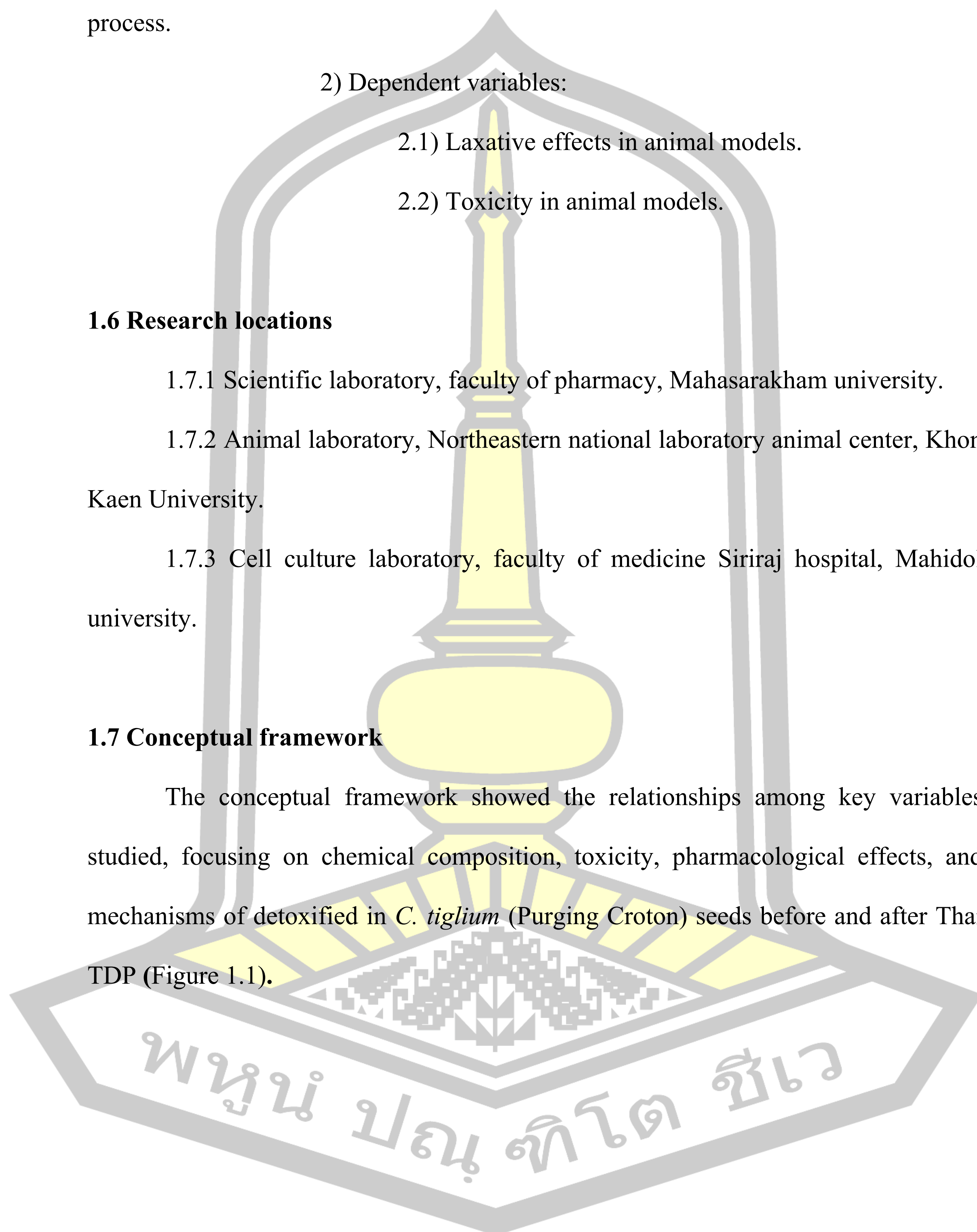
1.7.1 Scientific laboratory, faculty of pharmacy, Mahasarakham university.

1.7.2 Animal laboratory, Northeastern national laboratory animal center, Khon Kaen University.

1.7.3 Cell culture laboratory, faculty of medicine Siriraj hospital, Mahidol university.

1.7 Conceptual framework

The conceptual framework showed the relationships among key variables studied, focusing on chemical composition, toxicity, pharmacological effects, and mechanisms of detoxified in *C. tiglium* (Purging Croton) seeds before and after Thai TDP (Figure 1.1).



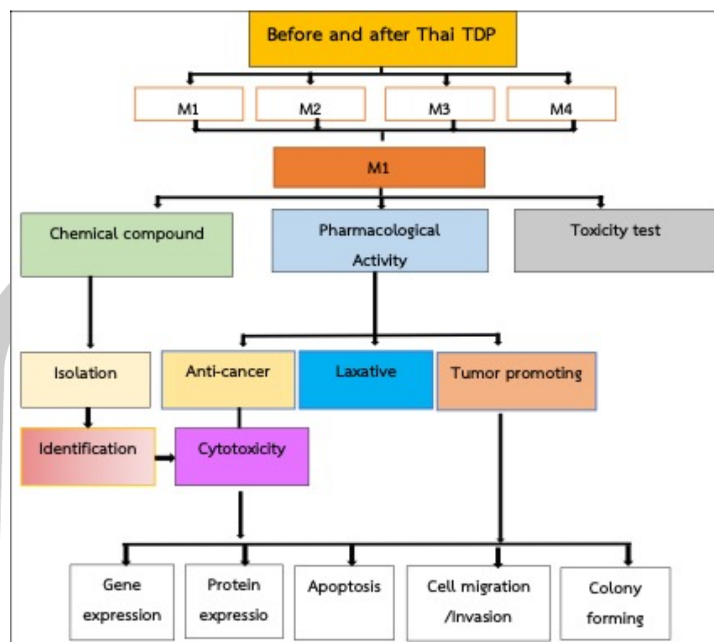


Figure 1.1 Conceptual framework

1.8 Expected benefits

1.9.1 Understanding the mechanisms: Clear insights into the mechanisms behind the detoxification process of *C. tiglium* seeds, ensuring accurate evaluation whether the detoxification process reduces toxicity or diminishes efficacy.

1.9.2 Evidence-based decisions: Data supporting the prohibition of *C. tiglium* or reevaluation of its use if detoxified seeds are proven safe and exhibit anticancer activity.

1.9.3 New compounds identification: Discovery of new compounds formed after the detoxification process.

1.9.4 Extraction methods: Development of effective methods for isolation of newly formed compounds from detoxified seeds.

1.9.5 Toxicity insights: Comprehensive data on the carcinogenic potential of newly formed compounds after detoxification process.

1.9.6 Pharmacological effects: Information on the pharmacological properties, such as anticancer activity and laxative effects, of the new compounds formed after detoxification process.

1.9.7 Hematological toxicity data: Detailed analysis of the hematological.

CHAPTER 2

Literature review

The comparison of chemical composition, pharmacological effects, and toxicity of *C. tiglium* seeds before and after Thai TDP is discussed. The literature review and relevant studies are categorized in the following sections:

1) Botanical aspects of *C. tiglium*

2) Use of *C. tiglium* in traditional medicine

3) Chemical composition of *C. tiglium*

4) Pharmacological effects of *C. tiglium*

5) Toxicity of *C. tiglium*

6) Laxatives and purgative effects of *C. tiglium* seed

7) Herbal processing principles in Thai

8) Cancer

9) *In vitro assays* for assessing anticancer and carcinogenic activities

10) Acute oral toxicity testing based on the OECD 420 guideline

11) Other related studies

2.1 Botanical aspects of *C. tiglium*

The general and scientific details of *C. tiglium*[15, 16].

Common names: Salod, Salod Ton (Central region), Mak Lod (Northeastern region), Ma Khang, Ma Tang, Ma Tod, Mak Thang, Hasakhuen, Luk Phan Satru (Northern region), Mak Yong (Mae Hong Son).

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Scientific name: *C. tiglium* Linn.

Taxonomy

(United States Department of Agriculture, 2020)

Kingdom Plantae

Subkingdom Tracheobionta

Superdivision Spermatophyta

Division Magnoliophyta

Class Magnoliopsida

Subclass Rosidae

Order Euphorbiales

Family Euphorbiaceae

Genus *Croton* L.

Species *tiglium*

C. tiglium is a shrub that grows 3-6 meters in height. It has simple, ovate leaves, 2-7 cm wide and 5-14 cm long, with a rounded base and pointed tip. The leaf margins are serrated, and the leaves exhibit 3-5 veins with petioles up to 4 cm long. The flowers are small, greenish-white, and may appear solitary or as clusters at the branch tips. The species is monoecious or dioecious, with male flowers having 4-6 petals and multiple stamens. Female flowers lack petals or have very small ones. The fruit is broadly oblong to triangular, measuring 1-1.5 cm wide and 2 cm long. It splits open when dry, releasing seeds that are oblong, light brown, and measure 0.6-1 cm[17]. (Figure 2.1)

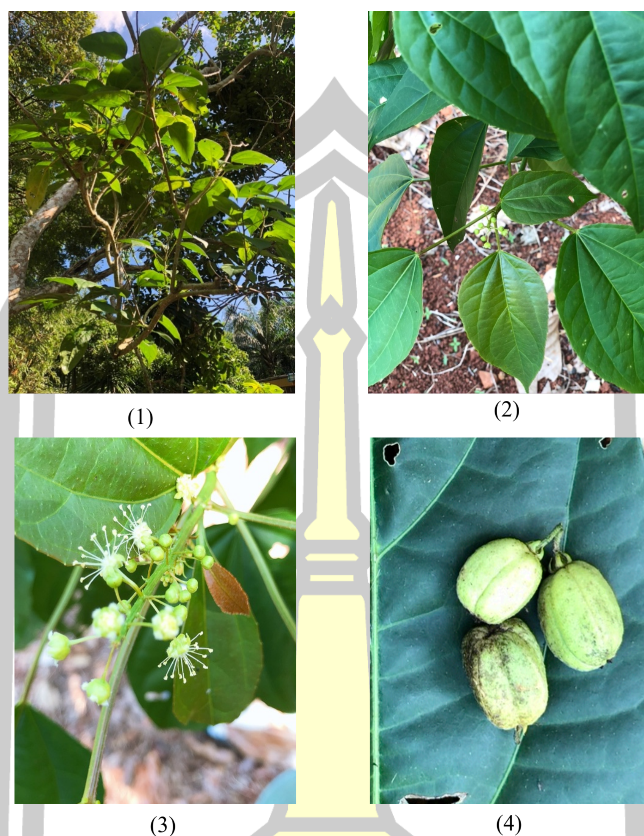


Figure 2.1 The characteristics of various parts of the *C. tiglium* plant are as follows: 1.Stem and leaves 2.Flowers 3.Fruit 4.Immature fruit (Source: Sawi, Chumphon Province)

2.2 Utilization of *C. tiglium* in Thai Traditional Medicine[1]

C. tiglium Linn. (familiy Euphorbiaceae), commonly known as "Salod" in Thai, is a medicinal plant found in tropical regions such as Thailand, India, Sri Lanka, China, and Malaysia. In Thai traditional medicine, the seeds of *C. tiglium* are used as a potent purgative and are prescribed for treatment of abdominal pain, wind disorders, asthma, phlegm, lymphatic discharge, and parasites. It is also incorporated into several herbal formulations aimed at managing symptoms resembling cancer. Examples include remedies like "Yatad Rak Krasai (give a Thai languge here)," "Yapraju Krasai Pla Moh (give a Thai languge here)," "Yaka Krasai Lin Krapue (give a Thai languge here),"

"Yapraju Krasai Dan (give a Thai languge here)," and other formulations used for treatment of various conditions like tumors, boils, and abscesses.

The medicinal uses of *C. tiglium* in Thai traditional medicine are as follows:

- Leaves: Used for treatment of skin diseases and sexually transmitted infections.

- Flowers: Used for treatment of skin diseases.

- Seeds: used as a powerful laxative.

- Bark: used as an expectorant for phlegm.

- Roots: used for treatment of leprosy and to expel phlegm.

2.3 Chemical composition of *C. tiglium*

The seeds of *C. tiglium* contain 7.5% moisture, 15% total ash[18], 20% protein and 30–60% croton oil[19, 20]. Additionally, carbohydrates, flavonoids, and alkaloids are present. The chemical components can be classified as follows[18].

2.3.1 Proteins: Examples include croton globulin and croton albumin.

2.3.2 Fatty Acids

Fatty acids isolated from croton oil include decanoic acid, dodecanoic acid, hexadecanoic acid, tetradecanoic acid, 15-methyl-11-hexadecenoic acid, linoleic acid, oleic acid, eicosenoic acid[5, 21]. Other fatty acids identified include 9-tetradecenoic acid, 9,11-octadecadienoic acid, and 5,9-heptacosadienoic acid, among others[18].

2.3.3 Enzymes

Enzymes such as lipase, invertase, amylase, raffinase, and proteolytic enzymes are presented.

2.3.4 Amino Acids

Studies by Aboulthana et al. (2019)[18] have identified essential amino acids such as threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, and tryptophan, as well as non-essential amino acids including aspartic acid, serine, glutamic acid, glycine, proline, alanine, tyrosine, histidine, and arginine.

2.3.5 Diterpenes

Croton oil contains various diterpenes such as 13-*O*-acetylphorbol-20-linoleate, 13-*O* -tigloyl phorbol-20-linoleate, 12-*O*-acetylphorbol-13-tigliate, 12- *O*-decanoylphorbol-13-(2-methylbutyrate), 12- *O*-tigloylphorbol-13-(2-methylbutirate),12-*O*-acetylphorbol-13-decanoate, 12-*O*-tetradecanoylphorbol-13-acetate, 12-*O*-(2-methylbutiroyl) - phorbol - 13 – dodecanoate[22], phorbol acetate methylbutenoate, deoxyphorbol acetate methylbutenoate, phorbol methylbutanoate isobutyrate, phorbol decanoate acetate, phorbol acetate butyrate[12, 23] 12-*O*-tiglylphorbol-13-(2-methyl)butyrate, 12-*O*-acetylphorbol-13-Isobutyrate, 12-*O*-benzoyl phorbol-13-(2-methyl)butyrate, 12-*O*-tiglyl-7-oxo-5-ene-phorbol-13-(2-methylbutyrate) and13-*O*-(2-metyl) butyryl-4-deoxy-4α-phorbol[4] 12-*O*-(2-Methyl)butyryl-4α-deoxyphorbol-13- isobutyrate, 20-formyl-4α-deoxyphorbol -13-acetate, 12-*O*-acetyl-4α-deoxyphorbol-13-(2-methyl) butyrate, 12-*O*-tiglyl-4α-deoxy phorbol-13-acetate, 12-*O*-tiglyl-4α-deoxyphorbol-13-(2-methyl) butyrate, 12-*O*-tiglyl-4α-deoxyphorbol-13-isobutyrate, 12-*O*-(2-methyl)butyryl- 4α-deoxyphorbol-13-acetate, 12-*O*-tiglylphorbol-13-(2-methyl)butyrate, 12-*O*-tiglylphorbol -13-acetate, 12-*O*-tiglyl-7-oxo-5-ene-phorbol-13-(2- methyl)butyrate, 12-*O*-acetylphorbol-13-tigliate, 13-*O*-Acetylphorbol-20-linoleate[5], 12-*O*-decanoyl-7-hydroperoxy-phorbol-5-ene-13 acetate, 12-*O*-tiglyl-4-deoxy-4α -phorbol-13- acetate, 12-*O*-tiglylphorbol-13-(2-methyl) butyrate, 12-*O*-isobutyryl phorbol-13-acetate, 12-*O*-tiglylphorbol-13-propionate, 12-*O*-tiglylphorbol-13-isobutyrate, 12-*O*-(2-methyl) butyrylphorbol-13-acetate, 12-*O*- tiglylphorbol-13-

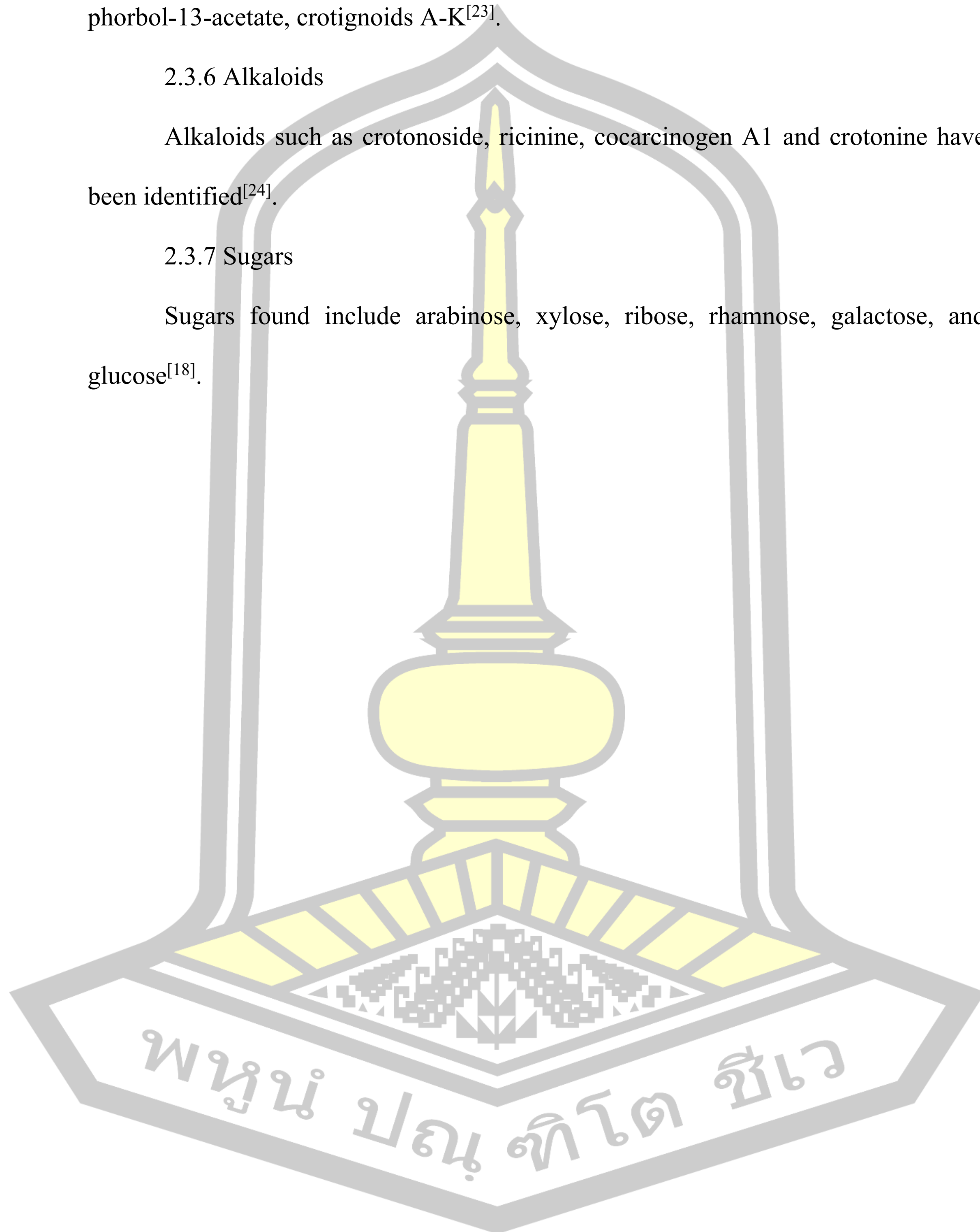
acetate, 12-*O*-(2-methyl)butyryl phorbol-13- isobutyrate, 12-*O*-tiglyl-4-deoxy-4α-phorbol-13-acetate, crotignoids A-K[23].

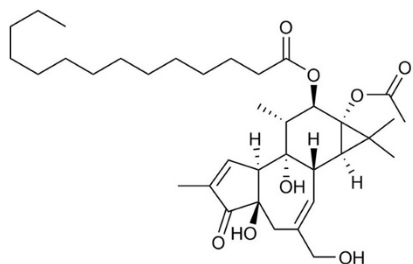
2.3.6 Alkaloids

Alkaloids such as crotonoside, ricinine, cocarcinogen A1 and crotonine have been identified[24].

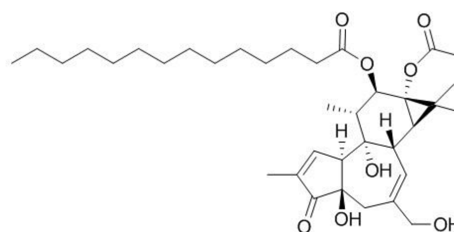
2.3.7 Sugars

Sugars found include arabinose, xylose, ribose, rhamnose, galactose, and glucose[18].

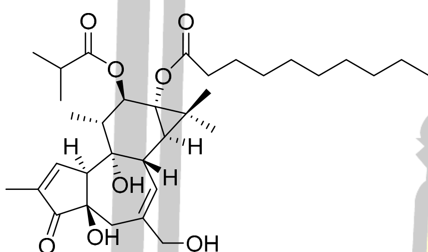




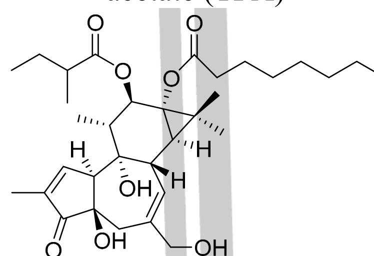
Phorbol-12-myristate-13-acetate (PMA)



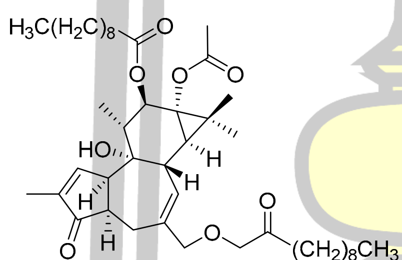
12-*O*-Tetradecanoylphorbol-13-acetate (TPA)



12-*O*-isobutyrylphorbol-13-decanoate



12- *O* -(2-methyl)butyrylphorbol-13-octanoate



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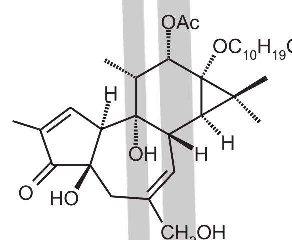

12-*O*-acetylphorbol-13-decanoate

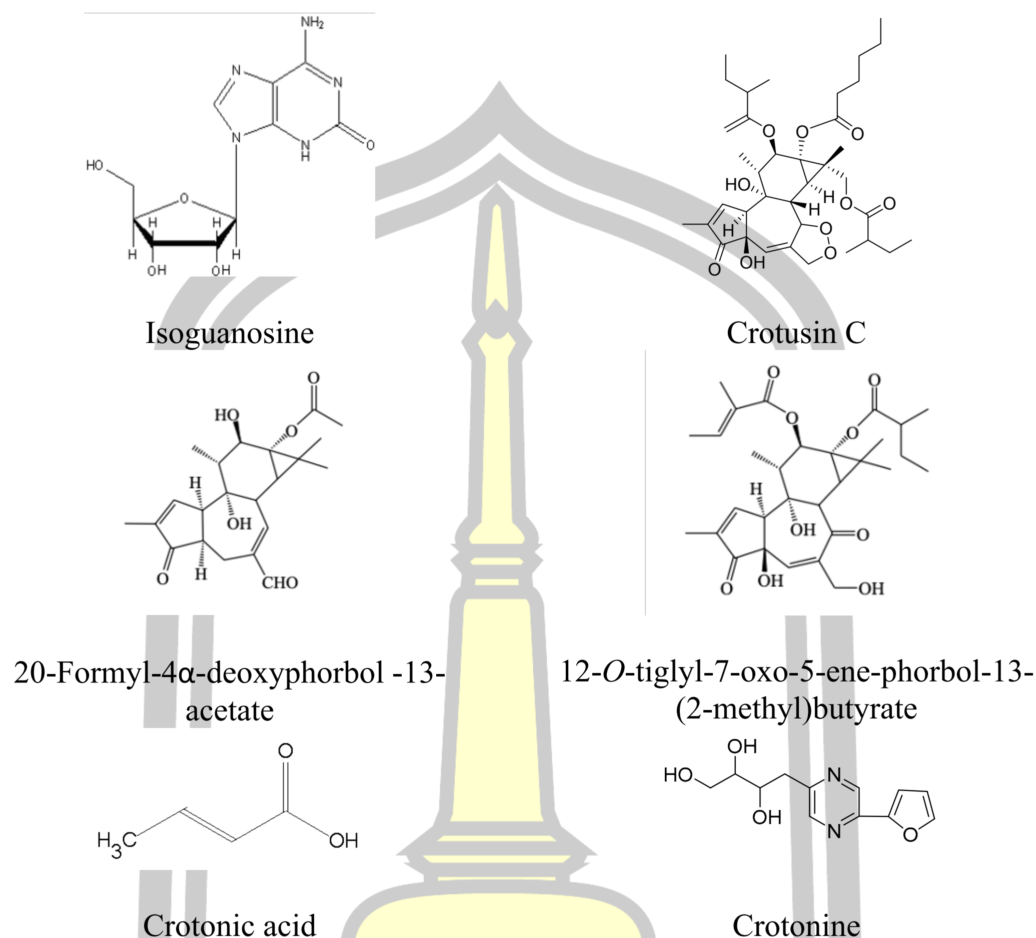

Figure 2.2 Structures of some compounds found in *C. tiglium*

2.4 Pharmacological effects of *C. tiglium*

2.4.1 Anticancer activity

2.4.1.1 Liver cancer

A study of the anticancer properties of *C. tiglium* seeds was carried out by grinding the seeds (7.1 kg) into powder and extracting them with methanol (3 cycles) for 72 hours. After evaporation, a crude methanol extract (957.9 g) was obtained, which was further partitioned with petroleum ether and n-butanol to yield 740.4 g of crude extracts. These were 9 fractionated using ethyl acetate-petroleum ether (0–100%). Fraction 4 was analyzed and found to contain:

- 12-*O*-tiglylphorbol-4-deoxy-4β-phorbol-13-acetate (1)
- 12-*O*-tiglylphorbol-4-deoxy-4β-phorbol-13-acetate (2)
- 13-*O*-acetylphorbol-4-deoxy-4β-phorbol-20-oleate (3)
- 13-*O*-acetylphorbol-4-deoxy-4β-phorbol-20-linoleate (4)

These compounds were tested against two liver cancer cell lines, SNU387 and SNU398, using the MTT assay. Cell viability was measured with a microplate reader at 570 nm, with paclitaxel serving as a positive control. The results are summarized in Table 2.1. Compounds 3-O-acetylphorbol-4-deoxy-4β-phorbol-20-oleate and 13-O-acetylphorbol-4-deoxy-4β -phorbol-20-linoleate showed significant inhibition of liver cancer cell growth, with IC_{50} values of 1.9 µg/mL and 0.71µg/mL for SNU387, and 13.5 µg/mL and 18.2 µg/mL for SNU398, respectively.

Table 2.1 IC_{50} values for liver cancer cell inhibition

Compounds	**Inhibitory concentration for liver cancer cell growth (IC_{50})**	
	SNU387	**SNU398**
1	59.5 ± 2.1	9.5 ± 1.5
2	30.2 ± 1.4	30.2 ± 3.7
3	1.9 ± 0.2	13.5 ± 1.1
4	0.71 ± 0.08	18.2 ± 1.7
Paclitaxel	0.35 ± 0.05	0.35 ± 0.5

2.4.1.2 Leukemia

Phorbol derivatives isolated from *C. tiglium* seeds were tested against leukemia cell lines K562, U937, MOLT-4, and HL60. The compound 20-deoxy-20-oxophorbol-12-tiglate-13-(2-methyl) butyrate effectively inhibited all four leukemia cell types, with IC_{50} values of 2.2, 1.0, 2.6, and 1.2 μM, respectively[4, 18]

2.4.1.3 Lung cancer

C. tiglium seeds were dried at 180°C for 90 minutes, washed with ethyl ether to remove oils, and extracted with methanol at 50°C for 5 hours. The methanol extract was evaporated under reduced pressure, yielding a yellow crude extract. Chemical analysis revealed the presence of isoguanosine, 12-*O*-acetylphorbol-13-tigliate, and 13-*O*-acetylphorbol-20-linoleate.

The extracts were tested against the A549 human lung cancer cell line using the MTT assay. Isoguanosine showed significant inhibitory effects, with an IC_{50} value of 97.6 μg/mL, and a 95% cell growth inhibition rate. Other compounds, i. e. 12-*O*-acetylphorbol-13-tigliate and 13-*O*-acetylphorbol-20-linoleate, did not show inhibitory effects[5].

Additional analysis revealed that *C. tiglium* seed extracts induced apoptosis via the Bax/Bcl-2 pathways[5]. Other studies identified compounds such as B10G5, which activate PKC-mediated RAF/MEK/ERK signaling pathways to inhibit non-small cell lung cancer (NSCLC) cells. The compound showed IC_{50} values of 0.11 μM for H1975 and <20 μM for A549 cells[25].

2.4.1.4 Prostate cancer

Ethanol extracts of *C. tiglium* leaves and branches containing 12-*O*-tiglylphorbol-13-isobutyrate showed strong inhibition of DU145 prostate cancer cells, with an IC_{50} value of 1.1 μM. Other compounds, such as 12-*O*-acetylphorbol-13-isobutyrate and 12-*O*-benzoylphorbol-13-(2-methyl) butyrate, exhibited IC_{50} values ranging from 4.3 to 11.0 μM[4].

2.4.1.5 Breast cancer

Among the compounds isolated from ethanol extracts from the leaves and branches of the *Jatropha* sp. 12-*O*-acetylphorbol-13-isobutyrate showed the most effective inhibition of the growth of MCF-7 (breast cancer cells), with an IC_{50} value of 13.0 μM. Other compounds such as 12-*O*-benzoylphorbol-13-(2-methyl)butyrate, 12-*O*-tiglyl-7-oxo-5-ene-phorbol-13-(2-methylbutyrate), and 13-*O*-(2-methyl)butyryl-4-deoxy-4α-phorbol exhibited IC_{50} values of 20.0, 20.0, and 24.0 μM, respectively[4].

2.4.1.6 Gastric cancer

Among the compounds isolated from ethanol extracts of the leaves and branches, of *Jatropha* sp.,13-*O*-(2-methyl)butyryl-4-deoxy-4α-phorbol was found to be the most effective inhibitor of the growth of SGC-7901 gastric cancer cells, with an IC_{50} value of 5.4 μM. Other compounds, including 12-*O*-benzoylphorbol-13-(2-methyl)butyrate, 12-*O*-acetylphorbol-13-isobutyrate, 12-*O*-tiglyl-7-oxo-5-ene-phorbol-13-(2-methylbutyrate), and 12-*O*-tiglylphorbol-13-isobutyrate, showed IC_{50} values of 10.0, 13.0, 23.0, and 43.0 μM, respectively[4].

Based on the results of these studies, it was found that the IC_{50} values for inhibition of prostate cancer, breast cancer, lung cancer, and gastric cancer cells were all below 50 μM.

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2.4.1.7 Colorectal cancer

The 80% ethanol, petroleum ether, and aqueous extracts of *Jatropha* sp. fruits were tested for their growth inhibition of human colon carcinoma cells at concentrations of 5, 10, 20, 40, 80, and 160 µg/mL using the sulforhodamine B (SRB) assay. The ethanol and aqueous extracts were found to significantly inhibit human colon carcinoma cells, with IC_{50} values of 36.3 and 33.9 µg/mL, respectively[18].

The phorbol derivatives were shown to inhibit various types of cancer cells, such as 3-*O*-Acetylphorbol-4-deoxy-4β-phorbol-20-oleate, 13-*O*-acetylphorbol-4-deoxy-4β-phorbol-20-linoleate, 20-deoxy-20-oxophorbol-12-tiglate13-(2-methyl) butyrate, 12-*O*-Acetylphorbol-13-tigliate, among others. The study by Isakov et al. also indicated that phorbol compounds activate protein kinase C (PKC), a family of serine/threonine kinases consisting of 10 isozymes. Some PKC isozymes, such as PKCδ, are associated with tumor suppression, while others like PKCα, PKCβ, and PKCγ are involved in tumor growth[26]. Research focusing on PKC-specific cancer therapies, including substances such as bryostatins, staurosporine alkaloids, curcumin, and resveratrol, as well as phorbol-based compounds such as phorbol 12-myristate 13-acetate (PMA) combined with bryostatin 1 was also carried out[27]. Some of these compounds, like ingenol mebutate and bryostatin 1, were already tested in clinical trials for cancer treatment, often in combination with paclitaxel[28].

2.4.2 Laxative activity

The methanol extract of *Jatropha* fruits was partitioned with petroleum ether, ethyl acetate, n-butanol, and water. These extracts were administered to fasting rats (12 hours) at doses of 10-200 mg/kg using the charcoal method. After 30 minutes, the animals were euthanized, and their intestines were examined. All *Jatropha* fruit extracts

significantly increased intestinal motility compared to the control group, with the highest motility observed in the ethyl acetate extract (100%), followed by petroleum ether (83.23%), methanol (77.62%), and water (75.06%)[12]. Additionally, when the extracts were administered at doses of 100-500 mg/kg, the amount of feces increased proportionally, with the 500 mg/kg dose resulting in the highest fecal output (3.2 g), which was significantly higher than the control group ($P<0.05$)[29].

The hexane and ethanol extracts of *Jatropha* seed revealed that they enhanced intestinal transit. Doses of 0.03, 0.06, and 0.09 mL/30 g body weight resulted in 61.89%, 72.52%, and 65.08% of intestinal transit, respectively, compared to 48.36% and 50.60% in the water and oleum ricini (OR) groups. These results suggest that *Jatropha* extracts can significantly enhance intestinal motility, with an effective dosage (ED_{50}) of 0.027 mL and a lethal dose (LD_{50}) of 0.071 mL[30].

Safety and effectiveness tests showed that *Jatropha* seed extracts induced laxative effects with a significant reduction in intestinal transit time in rats, ranging from 198.6 minutes at low doses to 94.6 minutes at higher doses[20].

2.4.3 Antifungal, antibacterial, and anti-HIV-1 activities

A study on the antifungal property of various parts of the *S. lonica* plant (including the bark, leaves, and fruit) was conducted using 95% ethanol extract (10:1 ratio) by two methods: cold soaking for 48 hours and reflux for 2 hours. After evaporating the solvent at 40°C, the extracts were tested against three fungi: *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Epidermophyton floccosum* using the disc diffusion method. The fungi tested are known to cause tinea infections. Results showed that extracts from both the bark and fruit, obtained via reflux, were effective in inhibiting fungal growth. The minimum inhibitory concentrations of the

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bark extract were 0.16, 0.31, and 0.16 mg/mL for *T. mentagrophytes*, *T. rubrum*, and *E. floccosum*, respectively. The fruit extract showed MIC values of 0.31, 1.25, and 0.63 mg/mL, respectively[31]. Additionally, the crude extract was extracted by ethyl acetate,and the ethyl acetate extract showed improved antifungal activity, particularly against *E. floccosum*, with MIC values of 0.04 and 0.16 mg/mL for the bark and fruit, respectively. Oleic acid was identified as the most effective compound in the extracts when compared with other componentes[32]. In a similar study using 80% aqueous ethanol extract and assayed by the disc diffusion method, the extracts showed inhibition zones of 16-25 mm for *Mucor mucedo* and 6-15 mm for *Aspergillus niger* after 48 hours[33].

Leaf and fruit extracts of *S. lonica* were assayed for antifungal activity against (*Candida albicans*, *T. rubrum*, and *Microsporum canis*, using the agar well diffusion method, and the results showed significant antifungal activity. Water extracts of the leaves showed MIC values of 0.5 mg/mL against all the three fungi. Ethanol and methanol extracts of the leaves and fruit also displayed significant inhibition, with MIC values of 0.0625-0.125 mg/mL, depending on the fungal strain. The acetone extract of the leaves and fruit showed lower MIC values of 0.25 mg/mL. The ethanol and methanol extracts of both the leaves and fruit were particularly effective in killing *C. albicans*, with the minimum fungicidal concentration (MFC) of 0.125 mg/mL[34].

Regarding antibacterial activity, extracts of the fruit were tested against *Pasteurella multocida* and *Bacillus subtilis* using the disc diffusion method with 80% ethanol. The inhibition zone ranged from 16 to 25 mm after 48 hours[33]. Further assays of leaf and fruit extracts against *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* using the agar well diffusion method revealed

significant antibacterial activity. Water extracts showed MIC values ranging from 125 to 500 µg/mL, while ethanol and methanol extracts exhibited better activity, with lower MIC values of 31.25 to 125 µg/mL. Ethanol and methanol extracts were the most effective against *E. coli*, with a Minimum Bactericidal Concentration (MBC) of 62.5 µg/mL[35].

Additionally, phorbol esters such as 12-*O*-acetylphorbol-13-decanoate and 12-*O*-decadienoylphorbol-13-(2-methylbutyrate) were found to inhibit HIV-1 with IC_{50} value of 0.02 µg/mL[22].

2.4.4 Anti-inflammatory activity

The anti-inflammatory effect of fruit extracts was studied using a hot extraction method with methanol[36] for 3 hours, followed by drying in an evaporator. The extract further partitioned with petroleum ether (CP), ethyl acetate (CE), n-butanol, and water to give the petroleum ether , ethyl acetate, n-butanol and aqueous extracts. The writhing test was performed by administration of these extracts to the rats with inflammation induced by acetic acid. The results showed that petroleum ether and ethyl acetate extracts had the highest inhibition of inflammation at 20 mg/kg with inhibition rates of 38% and 36.7%, respectively. Aspirin, a standard reference at 100 mg/kg showed an inhibition rate of 80%[12], as shown in Table 2.2.

Table 2.2 Acetic acid-induced Writhing test

Substance	Dosage (mg/kg)	Number of Writhes (30 min)	Inhibition (%)
CM (p.o.)	200	21.7	11.4
CE (p.o.)	20	15.5	36.7
CP (p.o.)	20	15.2	38.0
Aspirin (p.o.)	100	8.1	80.0

2.4.5 Vaccine response enhancement effect

An extract was obtained from *C. tiglium* (600 grams) using 90% ethanol by a fermentation process for 3 hours, then evaporated to yield a crude extract (12 g). The crude extract was dissolved in water and a buffer solution, followed by partitioning with n-hexane (3 × 500 mL), $CHCl_3$ (3 × 500 mL), EtOAc (3 × 500 mL), and n-BuOH (3 × 500 mL). The crude extract was further partitioned with mixtures of n-hexane/acetone with the ratios ranging from 20:1 to 1:3, with 200 mL fractions producing 10 different fractions. Fraction 8 was then further fractionated into 9 subfractions using mixtures of MeOH/H_2O (from 1:2 to 10:1). Subfraction 2, containing 30.2 mg of PMA, was selected for testing on Human Embryonic Kidney 293 (HEK293) T cells to assess vaccine response using NF-κB reporter assay and cytokine assay. The results revealed that cells treated with the protein FlaB showed lower absorbance compared to those treated with PMA. When FlaB was combined with PMA, there was a significant synergistic effect, indicated by a statistically significant increase in absorbance ($P<0.05$). This suggests that the extract from *C. tiglium* enhances and stimulates vaccine responses via Toll-like receptors (TLRs). Moreover, the extract

activates immune functions such as IgG, IgE, and interleukin-12 (IL-12) production, which are involved in T-cell development in spleen[37].

2.4.6 Immune-stimulating effect

PMA is reported to act as both a tumor promoter and an immune activator. PMA, with a structure similar to diacylglycerol, activates PKC, which plays a role in T-cell activation. Additionally, PMA has been shown to stimulate immune responses by inducing IL-2 production through non-specific receptor binding[38].

2.4.7 Antioxidant activity

The antioxidant properties of extracts from *C. tiglium* fruits using three solvents: petroleum ether, 80% ethanol, and water at concentrations of 0.2-1 mg/mL, were evaluated. The total polyphenolic content was highest in the water extract, yielding 699.21 mg of gallic acid/100 g, followed by ethanol (617.87 mg/100 g) and petroleum ether (388.24 mg/100 g) extracts. The antioxidant activity was determined using DPPH (2,2-diphenyl-1-picryl-hydrazyl and ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) assays. The water extract exhibited the highest antioxidant activity with the antioxidant values of 8.81 µg/mL and 41.523 mg/mL, respectively, followed by the ethanol extract, with the antioxidant values of 3.30 µg/mL and 21.205 mg/mL, and the petroleum ether extract had the lowest values at 0.37 µg/mL and 0.838 mg/mL, respectively. The percentage of antioxidant activity (%) of the water extract was 87% (IC_{50} = 33.9 µg/mL), while the ethanol and petroleum ether extracts showed activities of 53% and 22.2% (IC_{50} = 36.3 µg/mL and 50 µg/mL, respectively)[18].

2.5 Toxicity of croton oil

A study on the tumor-promoting properties of diterpene compounds in croton oil, including phorbol-diacetate, phorbol-dibenzoate, phorbol-didecanotate, and tetradecanoyl-phorbol-acetate, was conducted using 6-10 week-old rats. The rats' back fur was shaved 1-2 days before being induced with tumors using 7,12-dimethylbenzanthracene (DMBA) at 51.2 µg in 0.2 mL of acetone for 2 weeks. The results showed that tetradecanoyl-phorbol-acetate at a concentration of 1.83 µM /week induced the highest number of papillomas in the rats, followed by phorbol-diacetate and phorbol-didecanotate. Phorbol-dibenzoate, however, at a concentration of 0.017 µM did not induce tumor and required a dose 10 times greater than the others to cause tumors in the rats[39].

Additionally, plants in the Euphorbiaceae family often contain ester compounds of saturated fatty acids attached to the C-12 and C-13 positions of phorbol and the C-3 position of ingenol. These can form diterpene esters that exhibit tumor-promoting properties[40]. In a study using 1,2-dimethyl-9,10-benzanthracene (DMBA) at 0.1 µM as an initiator and 0.5% PMA as a tumor promoter, croton oil (5.5 µg) induced tumors in rats after 5 weeks. After 25 weeks, 80% of the rats developed tumors[7]. Similarly, Van Duuren and Sivak (1968) found that PMA and phorbol-12,13-didecanoate promoted tumor formation with phorbol esters at concentrations as low as 50 µg/mL capable of releasing acid hydrolases that trigger tumor development[41].

In an experiment on croton oil, the ethyl acetate extract of croton seeds was tested on male and female rats aged 7 weeks with varying doses: 125, 250, 500, 1000, and 2,000 mg/kg body weight. The rats were observed for 0, 1, 7, and 14 days after administration, with hematological tests, serum biochemistry, gross findings, organ

weights, and histopathological assessments. The hematological results showed normal levels for white blood cells, red blood cells, hemoglobin, hematocrit, platelets, MCV, MCH, MCHC, reticulocytes, and white blood cell subtypes. Serum biochemistry, including BUN, TC, TP, albumin, and other parameters, also remained normal. Organ weights, except for testis weight (which decreased) and heart weight (which increased at 500 mg/kg), showed no significant changes. Histopathological examinations revealed normal cell structures in all organs. Therefore, the study concluded that the 2000 mg/kg dose of croton oil extract did not cause acute toxicity in rats[42].

In India, a study on the acute toxicity of croton oil was carried out before and after processing via Shodhana. The seeds were boiled in cow milk for 3 hours, washed with hot water, ground into powder, and soaked in citrus juice (*Citrus medica* L.) for three cycles. Then, rats were administered with the extract as a single dose of 2,000 mg/mL, with observations for 14 days. The results showed that rats treated with unprocessed croton oil exhibited central nervous system abnormalities, including hypoactivity, passivity, ataxia, ptosis, hypothermia, and abdominal pain within the first hour. Additionally, gastrointestinal bleeding, gastric ulcers, and severe diarrhea were observed. In contrast, rats treated with processed croton oil showed no significant abnormalities except for diarrhea, and behavioral changes, such as hyperactivity, were observed during the study[43].

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2.6 Laxatives and Cathartics

Based on the mechanism of action, laxatives are classified into six major groups[44, 45].

2.6.1 Bulk-forming laxatives

These substances are hydrophilic colloids derived from fruits, vegetables, and seeds. Some of these compounds are digested during the digestive process, while others remain undigested and absorb water, forming a viscous substance or gel. This results in the expansion of the stool volume, stimulating the intestinal walls to expand, which in turn encourages peristalsis and facilitates the passage of waste. Bulk-forming laxatives are considered the first choice due to their natural mechanism of action, as they do not interfere with nutrient absorption and are safe for long-term use. Examples include bran, psyllium seeds, mucilage, basil seeds, and synthetic water-insoluble resins such as polycarbophil.

2.6.2 Osmotic laxatives

This group primarily includes substances that are poorly absorbed or not absorbed in the digestive tract. they exert their effects by drawing water into the gastrointestinal tract via osmotic pressure. this category can be divided into five subgroups:

2.6.2.1 Saline Cathartics

Examples include magnesium sulfate,magnesium citrate, magnesium hydroxide, and sodium phosphate salts.

2.6.2.2 Glycerine

Glycerine also draws water through osmotic pressure and stimulates bowel contractions due to irritation of the intestinal mucosa.

2.6.2.3 Sorbitol

Sorbitol, such as glycerine, draws water through osmotic pressure and induces bowel contractions by irritating the intestinal lining.

2.6.2.4 Lactulose

Lactulose is a disaccharide that is not absorbed or is resistant to digestion by enzymes in the small intestine. This sugar acts as an osmotic agent in the large intestine, where bacteria break it down into lactic and acetic acids. These acids are not absorbed and increase osmotic pressure, thereby drawing more water and facilitating bowel movements.

2.6.2.5 Lactitol

Like lactulose, lactitol is a disaccharide with similar osmotic properties.

2.6.3 Stool softeners

These laxatives are anionic surfactants that work by reducing surface tension, allowing water and fats to penetrate the stool and soften the stool. These laxatives also stimulate the secretion of water and electrolytes which enhance a bowel motility. Examples of these laxatives include dioctyl sulfosuccinate and sodium, potassium, and calcium salts of cocuset. However, some of these agents can be absorbed and may cause liver toxicity.

2.6.4 Stimulant laxatives

This group includes agents that stimulate intestinal motility. They can be divided into four subgroups:

2.6.4.1 Anthraquinones

These compounds are often metabolized by bacteria into absorbable forms and converted into anthrols, which stimulate peristalsis by inhibiting Na^{+}-K^{+}

ATPase. Examples include emodin from *Aloe vera*, anthracene glycosides such as cascara sagrada bark (*Frangula pushiana*), and casanthranol from senna bark.

2.6.4.2 Diphenylmethane derivatives

After ingestion, these compounds can inhibit Na^{+}-K^{+} ATPase, increase the production of prostaglandin E, and enhance adenylate cyclase activity, leading to an increase in cyclic AMP. This also irritates the intestinal lining, stimulating fluid secretion into the lumen. Examples of these compounds include bisacodyl, phenolphthalein, and sennosides.

2.6.4.3 Castor oil

Castor oil is hydrolyzed by lipase, in the small intestine, into glycerol and ricinoleic acid, which inhibits Na^{+}-K^{+} ATPase and increases cyclic AMP. It also irritates the intestinal mucosa.

2.6.4.4 Dehydrocholic acid

Dehydrocholic acid stimulates intestinal motility and inhibits water and electrolyte reabsorption. This acid also reduces surface tension. It is excreted via bile and is suitable for use in patients with gall bladder problems and with constipation.

2.6.5 Other laxatives

In addition to the above-mentioned laxatives, other agents can also be used for treatment of constipation. These are:

2.6.5.1 Rectal preparations

These include water, saline, sodium phosphate solutions, and glycerine. When administered rectally, these substances generate pressure against the rectal wall, triggering bowel contractions. However, they are not suitable for long-term use.

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2.6.5.2 Prokinetic agents

Cisapride is a prokinetic active substance belonging to the pharmacotherapeutic group of propulsives. This drug increaes motility in the upper gastrointestinal tract, and is used in chronic constipation cases or when laxative use is prolonged. This drug has been either withdrawn from the market or had its indications limited in many countries due to incidence of serious cardiac side-effects.

2.6.5.3 Opioid antagonists

Opioid-related constipation is one of the most frequent side effects of chronic pain treatment. Enteral administration of naloxone blocks opioid action at intestinal receptor level, thus inhibits intestinal motility. By blocking these receptors, naloxone stimulates bowel movement. However, naloxone has low systemic bioavailability due to maked heptatic first-pass metabolism.

2.6.5.4 *Hovenia dulcis* extract

Vanillic acid is a constituent of the hot-water extract of branches of *Hovenia dulcis* that is resonsible for improving the intestinal transit and the frequency and weight of stools in rat model. Therefore, the extract can stimulate intestinal activity and is classified as a stimulant laxative[37].

2.7 Detoxification of herbal medicine according to Thai pharmaceutical principles

In Thai traditional medicine, it is mentioned that certain herbs, animals, and minerals, need to be processed before use because some of them are toxic or too potent. The processing of herbs is crucial not only to reduce their toxicity but also to enhance their effectiveness. There are three commonly used methods of preparing medicinal herbs: satu (treatment with heat), prasa (cleaning and detoxifying), and khi-thit

(neutralizing the potency). These methods all aim to reduce toxicity or decrease the strength of medicinal properties, with slight differences in their objectives and procedures.

Satu (treatment with heat). This method is used for herbs with strong effects, such as YADUM and gamboge (Thai: rong thong), which are potent purgatives, or for external herbs that may easily be contaminated with foreign substances or pathogens, such as alum, talc, and *Ferula asafoetida* (Thai: mahahingkhu). The herb is often wrapped or placed in a container and then directly heated over a fire, which is similar to the khi-thit method. This method is typically applied to toxic components like arsenic and mercury.

Prasa (cleaning and detoxifying): This method reduces toxicity, cleanses the herb, and ensures the herb is standardized with other ingredients in a formulation. Unlike satu, the herb is not directly heated. Instead, hot water is poured over the herb repeatedly until it becomes softened, such as in the case of prasa ylang-saladai (a process for certain herbs).

2.7.1 Detailed descriptions of various herbs and methods of processing:

2.7.1.1 Satu (treatment with heat):

1) Satu *Aloe vera* (L.) Burm.f. resin (Black Medicine): Black medicine is a potent purgative. The process involves placing it in a clay pot or heat-resistant container, adding a little water, and heating it until the medicine becomes brittle. This heat causes some of the volatile components to evaporate, reducing its potency.

2) Satu *Garcinia hanburyi* Hook F. resin (Rong Thong) is another strong purgative. It is ground finely, wrapped in seven layers of lotus or ginger

leaves, and roasted over a fire until it becomes crispy, making it ready for use in medicine.

3) Satu *Ferula assafoetida* L. (Mahahing) used in combination with alcohol, is used to treat infant stomach upset. It is prepared by boiling fresh red basil leaves in water, adding mahahingkhu, and straining it to cleanse the herb to reduce its potency.

4) Satu Potassium Alum (Alum): Alum is used both internally and externally for various purposes like menstrual disorders, blood purification, and oral health. The process involves grinding alum into a fine powder, placing it in a clay pot, and heating it until it becomes crisp and white.

5) Satu Talc: Talc is calcium carbonate found in nature and commonly used for healing. It is processed by placing it in a clay pot, covering it, and heating it until it is fully processed, after which it is ready for medicinal use.

6) Satu Sodium borate (Nam Prasarn Thong) is a naturally occurring sodium borate salt. It has medicinal properties for treatment of mucous-related diseases like cough and nasal congestion. The process involves heating borax until it melts and becomes white and crystalline, at which point it can be used in medicine.

7) Satu Sodium chloride: Salt, whether obtained from the sea or underground, is used to nourish the body’s four elements and for treatment of digestive system. The process involves roasting sea salt or rock salt at high temperatures until moisture and water evaporated, making it purer and suitable for medicinal use.

8) Satu Iron: Iron filings are used in traditional medicine for improving blood circulation and treatment of liver and spleen disorders. The process

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involves grinding iron and adding lime juice, then heating it several times until it becomes brittle and ready for use in formulations.

9) Satu Shells: Shells are rich in calcium carbonate. When processed, they convert to calcium oxide and carbon dioxide, which are used in medicine as a remedy for stomach acid symptoms, digestive issues, and to strengthen bones. The process involves heating the shells until they become soft, then grinding them into a fine powder for use.

10) Satu Serpentes head: (a type of venomous snake) head is traditionally used for treatment of convulsions in children and reducing body heat. The method involves placing the snakehead in a clay pot, sealing it, covering it with a mixture of talc and water, and then heating it overnight before use.

11) Satu Vulture Head and Other Animal Parts: Parts from animals without venom, such as vulture heads, animal skin, and bones, are processed by heating them in a clay pot until they become crisp. These parts can then be used to make medicines.

2.7.1.2 Preparation (Prasa)

Preparation refers to the process of reducing the toxicity of medicinal substances, purifying them, and ensuring they contain the appropriate dosage for various medicinal purposes. This term is often used to describe medicines that have been purified or treated to become safe and effective for use. For example, "Prasa Nammom" refers to a medicinal preparation that purifies breast milk, making it safe for the infant. Some sources also describe this preparation as a process for increasing the effectiveness or potency of the substance.

In Thai traditional medicine, one of the methods of preparation of herbs is called "Prasa Yang Salad Dai" (Preparation of the *Euphorbia antiquorum* L.). The process involves placing the herb in a bowl and pouring boiling water over it. The mixture is stirred thoroughly and then allowed to cool. After cooling, the water is discarded, and boiling water is added again. This process is repeated seven times until the herb reaches its desired state, after which it can be used for making medicinal formulas.

2.7.2 Detoxification of the medicinal potency

Detoxification of a potency refers to a process similar to "Satu" (a method for preparing substances), but it specifically involves, reducing the harmful effects of substances with high toxicity to make them safe for use.

2.7.2.1 Detoxification of Arsenic

In traditional medicine, arsenic is known for its bitter taste and is believed to have various medicinal properties, including treatment of skin diseases, ulcers, blood problems, sexually transmitted diseases, and healing wounds. To detoxify arsenic, it is finely ground and placed in a ceramic or clay pot. Then, lemon or kaffir lime juice is squeezed into the pot, and the mixture is heated until it dries. This process is repeated seven to eight times until arsenic becomes brittle, after which it can be used in medicine. The container used for this process should be destroyed and buried properly.

2.7.2.2 Detoxification of Mercury

Mercury is known for its bitter taste and is used for treatment of various skin diseases, lymphatic disorders, cancer, ulcers, joint problems, and other conditions. The process of detoxifying mercury involves placing copper, brass, or silver into

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mercury until the mercury is fully absorbed by the metal (this means the mercury infiltrates the metal completely). The metal can then be used to prepare medicinal formulas, especially in the form of decoctions.

2.7.2.3 Detoxification of *C. tiglium* seed

The *C. tiglium* seed, or "Salod," is known for its potent purgative effects. It is believed to be able to expel phlegm, in Thai traditional medicine, the seed is regarded as dangerous due to its strong medicinal properties. There are several methods for detoxifying *C. tiglium* seeds, including:

1) The first method: The *C. tiglium* seed is cut in half ground finely and fried in coconut oil until it becomes charred. Some sources recommend roasting the seeds until they become charred instead.

2) The second method: The *C. tiglium* seed is peeled and boiled with various herbs over several days. Each day, the fruit is boiled with a different herb, such as betel leaves, cloves, chili leaves, tamarind leaves as well as salt water, rice, or cow dung, for a period of seven days.

3) The third method: The *C. tiglium* seeds are mixed with cooked rice and roasted until they are charred.

4) The fourth method: The *C. tiglium* seed is boiled in water until it becomes soft.

5) The fifth method: The *C. tiglium* seed is soaked with fermented fish sauce overnight, then inserted into kaffir lime fruits and heated it over a straw fire until it is warm and then ground into a mixture. Some sources suggest inserting the slod seed into kaffir lime or lime fruits and roasting it until the seeds are charred.

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6) The sixth method: The *C. tiglium* seed is peeled and soaked in salt water for two nights, then inserted into kaffir lime fruits and roasted until charred. The resulting mixture can then be combined with other medicinal ingredients.

7) The seventh method: The *C. tiglium* seed is processed by peeling it, removing the seed, soaking it in salt water, and then drying it under the sun. Afterward, it is roasted with fish sauce and crushed to extract oil.

8) The eight method: The *C. tiglium* seed t is boiled with *Tamarindus indica* leaves and *Acacia concinna* leaves (1 handful each), along with salt (1 handful), and then dried under the sun.

9) The ninth method: The *C. tiglium* seed is peeled and the seeds removed, soaked in fermented fish sauce overnight, then roasted until it becomes golden brown. A cloth wrap is used to extract the oil from the mixture.

10) The tenth method: The *C. tiglium* seed is placed in a coconut shell, heated over a straw fire for one night, then removed and squeezed to extract the oil. Afterward, the mixture is roasted until charred.

11) The eleventh method: The *C. tiglium* seed is peeled, boiled in various liquids (such as dung, coconut water, and sugar cane juice), then dried under the sun.

12) The twelfth method: The *C. tiglium* seed is peeled, seeds removed, and the fruit is wrapped in cooked rice, boiled, and dried under the sun. This process is repeated several times with different herbs such as tamarind leaves, Sophi leaves, and salt[46].

2.7.2.4 Killing the effect of Civet Musk

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Civet Musk is a fragrant substance obtained from the secretion or oil of a civet, which is rubbed onto sticks or the bars of its cage. In traditional herbal medicine, Civet Musk has a cold, pungent aroma and is used as a mental stimulant and a tonic, as well as to relieve blood toxicity and to nourish bile. It is also used in the production of perfumes. The method of neutralizing its effects involves mixing the Civet Musk with finely chopped onion or kaffir lime peel. The mixture is then placed on a betel leaf or silver spoon and exposed to candlelight until the Civet Musk melts. Once the civet musk has been sufficiently heated and its fragrance has developed, the Civet Musk-infused liquid is filtered and ready for use in preparation of medicine.

From the above information, it is clear that the methods for neutralizing the effects of these substances are often related to temperature. Increasing temperature reduces the bonding strength between particles or causes the particles to aggregate less. When these substances are used in medicine, it is likely to improve the body's ability to detoxify. Heat also causes certain temperature-sensitive chemicals to degrade. These herbs are often combined with other ingredients in herbal formulations and are rarely used alone due to their potential danger to the user.

2.8 Cancer

2.8.1 Causes of cancer

Cancer is a leading cause of death globally, with increasing mortality rates. The development of cancer is associated with two main mechanisms: the intrinsic pathway, which is triggered by cancer-causing genes (oncogenes) such as gene mutations or chromosomal rearrangements that lead to inflammation, and the extrinsic pathway, which involves infection causing inflammation and increasing the risk of cancer[47, 48].

Cancer cells arise from the detoxified of normal cells into cancerous ones. These cancerous cells possess properties such as maintaining growth signals, resisting cell death, proliferating, stimulating angiogenesis, invading and spreading to other organs, and inducing inflammation[49].

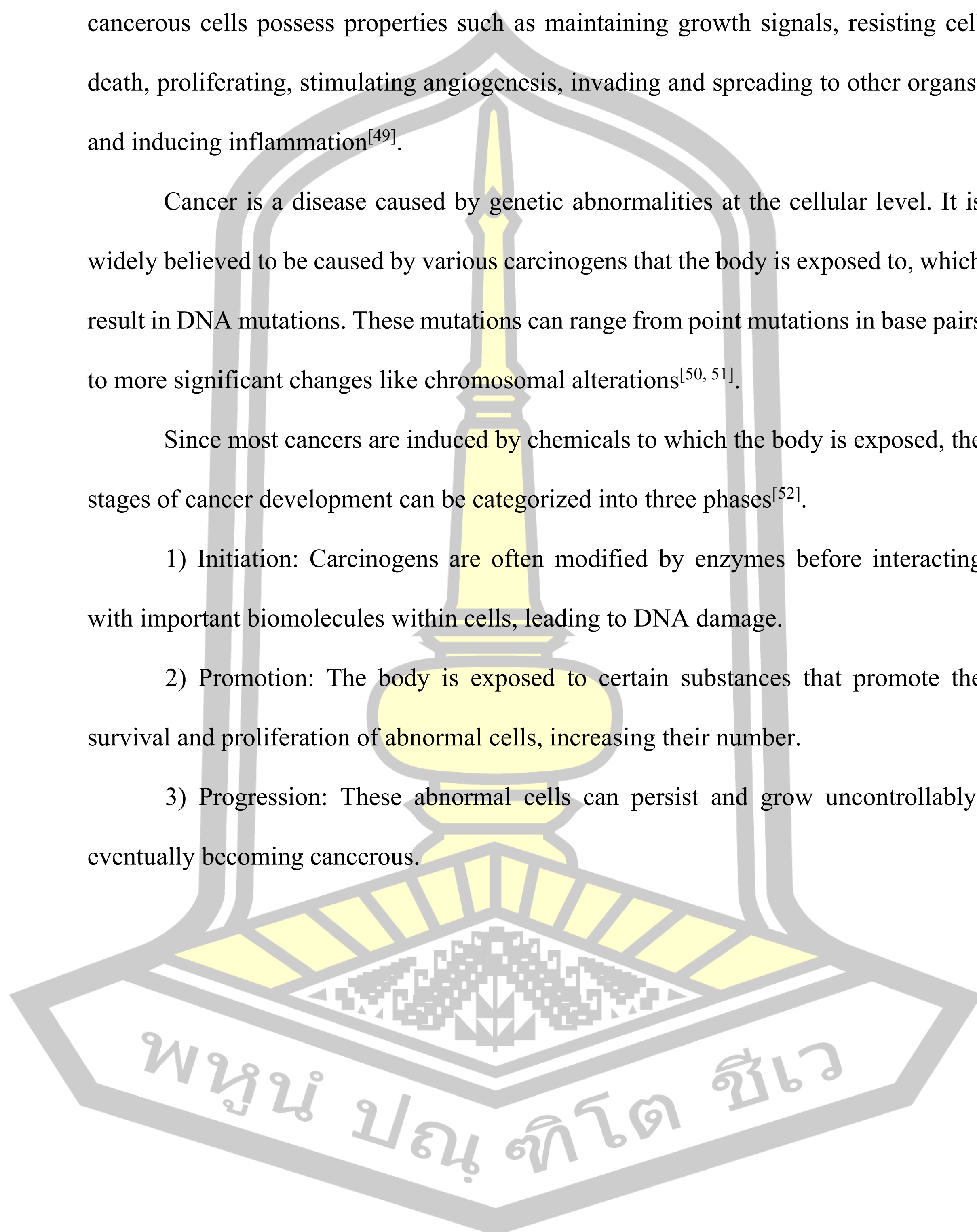
Cancer is a disease caused by genetic abnormalities at the cellular level. It is widely believed to be caused by various carcinogens that the body is exposed to, which result in DNA mutations. These mutations can range from point mutations in base pairs to more significant changes like chromosomal alterations[50, 51].

Since most cancers are induced by chemicals to which the body is exposed, the stages of cancer development can be categorized into three phases[52].

1) Initiation: Carcinogens are often modified by enzymes before interacting with important biomolecules within cells, leading to DNA damage.

2) Promotion: The body is exposed to certain substances that promote the survival and proliferation of abnormal cells, increasing their number.

3) Progression: These abnormal cells can persist and grow uncontrollably, eventually becoming cancerous.



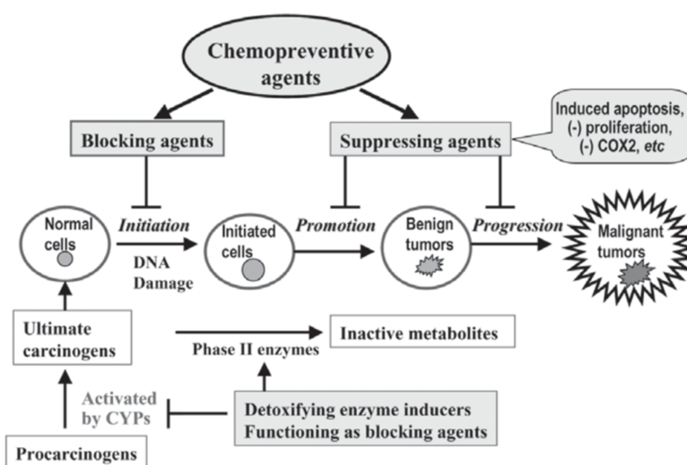


Figure 2.3 The development of cells in various stages before cancer formation[53]

2.8.2 Cancer treatment approaches[54, 55]

Current chemotherapy treatments for cancer are divided into two types:

2.8.2.1 Blocking agents

These drugs work by inhibiting the formation of carcinogenic agentes of the compounds that are not carcinogenic in their original form or are in the procarcinogenic stage. These chemicals block specific enzymes involved in the conversion process, especially those in the (cytochrome P450) CYP enzyme group, and induce increased destruction of carcinogenic substances by promoting conjugation with phase II drug-metabolizing enzymes. This helps to accelerate the excretion of these compounds and enhances the destruction of free radicals or electrophilic compounds formed from procarcinogens.

2.8.2.2 Suppressing agents

These drugs act by inhibiting the promotion and progression stages of cancer development. They prevent the proliferation of cancer cells and block the mitogenic effects of carcinogens, leading to the inhibition of the cell cycle in cancer cells. This

promotes apoptosis in cancer cells, potentially returning them to a normal state or slowing the progression of the disease.

2.9 *In vitro* assays for anticancer and pre-cancer effects

There are several methods to test the pharmacological effects of extracts and compounds for anticancer and pre-cancer properties:

2.9.1 Cell viability assay

This assay can be performed in various ways, each using different biological characteristics of cells, including membrane integrity, membrane function, the release of products after cell damage or death, cellular metabolism, enzyme activity, and cloning survival. Examples of cell viability assay methods include:

2.9.1.1 Trypan blue dye exclusion assay

This assay is based on the fact that living cells do not take up the dye, as their membrane functions to exclude it, On the other hand, dead cells absorb the dye due to membrane damage[56].

2.9.1.2 MTT assay

This method is based on the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) by the enzyme succinate dehydrogenase in metabolically active cells to purple-blue formazan, which absorb light at 560 nm The absorbance is directly proportional to the amount of formazan produced, which correlates with the number of viable cells[56].

2.9.1.3 XTT assay

The XTT (2,3-bis-2-Methoxy-4-nitro-5-sulfophenyl-2*H*-tetrazolium-5-carboxyanilide inner Salt) assay is similar to the MTT assay, but XTT is used instead

of MTT. XTT is reduced to an orange-yellow formazan product, by succinate dehydrogenase in metabolically active cells,which absorbs the light at 450 nm. The absorbance is used to measure cell viability[56].

2.9.1.4 Sulforhodamine B (SRB) assay

This method measures cell toxicity by fixing cells with 40% trichloroacetic acid (TCA) and staining with SRB, a dye that binds to basic amino acids in proteins of surviving cells. The intensity of SRB staining correlates with the number of surviving cells, and the results are analyzed using ultraviolet light absorbance at 392 nm. The IC_{50} value, which indicates cytotoxic activity, is derived from a dose-response curve[57].

2.9.2 Detection of specific protein expression

Specific markers can be used to detect the expression of particular proteins. These markers bind to receptors on the cell surface and allow for the differentiation of cell types. A common method for protein analysis is Western Blot, also known as immunoblot, which tracks proteins in samples like tissue homogenates or protein extracts. This technique is similar to Southern blot hybridization but uses protein instead of DNA. The steps for Western blotting are as follows:

2.9.2.1 Sample sreparation

Samples for Western blotting can be derived from cell tissue or extracted protein. Protease inhibitors, such as lysis buffer, are added to prevent protein degradation by cellular proteases.

2.9.2.2 Protein separation by gel electrophoresis

Protein separation is achieved through gel electrophoresis, which sorts proteins based on their isoelectric point (pI) or molecular weight. Sodium dodecyl

sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is typically used, with SDS acting as an ionic detergent that denatures proteins and imparts a negative charge, ensuring separation by size.

2.9.2.3 Electrotransfer of proteins

After protein separation, proteins are transferred from the gel to a membrane (e.g. nitrocellulose or polyvinylidene fluoride (PVDF)) using an electrotransfer method. This can be done in three ways:

1) Wet tank transfer: This method uses a buffer tank, though it which requires a large volume of buffer and longer transfer times.

2) Semi-dry transfer: This is a popular method which uses less buffer and allows multiple membranes to be transferred simultaneously, achieving nearly 100% transfer efficiency.

3) Dry bufferless transfer: In this method, no buffer is used, and transfer takes about 7 minutes, making it quick and efficient.

2.9.2.4 Blocking

After protein transfer, unbound areas on the membrane are blocked to prevent non-specific binding of antibodies using substances like bovine serum albumin (BSA) or non-fat dry milk.

2.9.2.5 Detection

Detection involves probing the membrane with antibodies specific to the protein of interest. These antibodies may be linked to enzymes or fluorescent markers, and the signal is visualized through color change or fluorescence. Detection can be done in two ways:

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1) Two-step detection: This method involves the use of primary and secondary antibodies, with the secondary antibody tagged with an enzyme or other detectable marker.

2) One-step detection: This method uses a single antibody, already tagged with an enzyme, to bind directly to the protein and generate a detectable signal.

2.9.3 Colony-formation cell (CFC) or clonogenic assay:

This assay assesses the proliferative capacity and survival of progenitor cells. Typically, 0.5% w/v crystal violet is used to visualize colonies when viewed under a stereomicroscope[58].

2.9.4 Nucleic acid amplification[59]

Techniques for amplifying nucleic acids from samples are widely used, especially in laboratory diagnostics for viral and bacterial infections, genetic diseases, cancers, and forensic applications. Several methods are used:

2.9.4.1 Conventional PCR method that synthesizes new DNA strands from a single DNA template strand using DNA polymerase. It is commonly used for DNA labeling and sequencing studies. PCR can synthesize two DNA strands simultaneously using a pair of primers. The PCR process consists of three steps that repeat under optimal conditions for each step:

1) Denaturing: The DNA template is separated into single strands at high temperatures (92-95°C).

2) Annealing: The temperature is lowered to allow short primers (composed of 14-13 nucleotide bases) to bind to complementary sequences on the DNA template, typically at 37-60°C.

3) Extension: New DNA strands are synthesized starting from the 5' end of the primers, using DNA polymerase at temperatures around 72-75°C.

The PCR products cannot be seen directly; thus, the amplified DNA must be separated and detected using agarose gel electrophoresis. DNA fragments are separated by size and can be visualized by staining with a special dye that fluoresces under ultraviolet light, showing the bands of DNA on the gel[60].

2.9.4.2 Reverse transcription-PCR (RT-PCR): RT-PCR is a variation of PCR that starts with RNA templates. Initially, RNA (e.g., mRNA) is reverse-transcribed into complementary DNA (cDNA) using reverse transcriptase. The resulting cDNA serves as the template for further amplification by PCR. The primers used for RT-PCR can be random primers, oligo(dT) primers, or gene-specific primers. RT-PCR is widely used to examine gene expression and in viral research, as many viruses have RNA genomes[61].

2.9.4.3 Real-time PCR: Real-time PCR, also known as quantitative PCR (qPCR), is a method for specifically amplifying and monitoring DNA amplification in real-time during each cycle of the PCR process. The increase in DNA quantity is measured by detecting the fluorescence emitted by the system, with the emitted light proportional to the amount of DNA produced. Typically, the data are displayed in an S-shaped curve (sigmoid or exponential), with the Y-axis showing the fluorescence signal and the X-axis representing the number of PCR cycles (Figure 2.4). For accurate results, detection is performed during the exponential phase of the PCR, when the fluorescence signal is above the threshold and amplification follows an exponential pattern[62].

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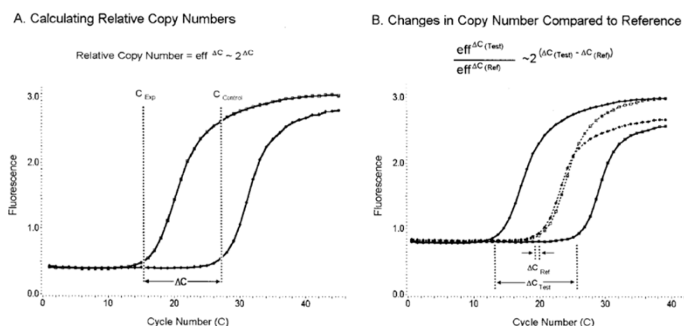


Figure 2.4 DNA amplification using Real-Time PCR technique[63]

2.10 Acute oral toxicity testing

Acute oral toxicity testing (acute oral toxicity: fixed dose) is conducted according to the OECD Guidelines for the Testing of Chemicals (OECD 420). This method evaluates the toxicity of chemicals by considering both the survival and the expression of toxic symptoms in the test animals. The classification of chemical toxicity is based on the Globally Harmonized System for Classification and Labeling of Chemicals (GHS), which categorizes the toxicity into five levels:

1. Category 1: Extremely toxic chemicals, with an LD_{50} value of 5 mg/kg body weight.

2. Category 2: Highly toxic chemicals, with an LD_{50} value of 25-50 mg/kg body weight.

3. Category 3: Moderately toxic chemicals, with an LD_{50} value of 200-300 mg/kg body weight.

4. Category 4: Low toxicity chemicals, with an LD_{50} value of 500-2,000 mg/kg body weight.

5. Category 5: Chemicals with low toxicity or no toxicity, with an LD_{50} value of 2,000 mg/kg body weight or greater, or no observable toxicity.

Test animals (typically rats) are observed for abnormalities within 24 hours after administration of the substance. Observations focus on changes in skin, fur, mucous membranes, respiration, behavioral patterns, tremors, diarrhea, lethargy, and other signs of distress. The animals are monitored daily for 14 days, with regular weight measurements and weekly recording of food and water intake. If any signs of distress or abnormalities in the well-being of the animals are noted, they must be euthanized immediately.

At the end of the experiment, a necropsy is performed to identify any internal organ abnormalities through gross examination, and histopathological examination is conducted on any organs showing abnormalities[34, 64, 65].

2.11 Other related research

Other related research can be divided into three key areas: research on the detoxified of herbs, research on PKC and its role in tumor suppression, and research on PMA. The details are as follows:

Khongkam et al. (2014) explored four different methods for the Thai TDP of croton seeds. Method 1 consists of placing the seeds in a clay pot, then rice husks, salt, and water are added, and boiled until the rice husks expanded. Method 2 is performed by mixing the seeds with leaves of tamarind and senna plant and the mixture is placed in a clay pot. Then water is added and boiled. Method 3 involved soaking the seeds in fermented fish sauce overnight, followed by stuffing them into kaffir lime fruits, place them in a clay pot, and roasting with rice husks. Method 4 involved grinding the seeds into a fine powder and roasting them until the oil was completely evaporated. The study found that the levels of PMA decreased by 44.47%, 7.33%, and 79.76% in methods 1,

3, and 4, respectively. However, method 2 resulted in a 13.91% increase in PMA content. The study also identified a new compound in the phorbol ester group that may have resulted from the hydrolysis of phorbol esters during the detoxified process, which occurs in alkaline conditions. The detoxified methods that involve heat and acid or neutral to slightly alkaline conditions seem to facilitate the hydrolysis, which results in free phorbol that reacts with fatty acids to form new compounds[13].

Pal et al. (2014) the prepare of croton seeds by soaking 250 g of seeds in water overnight, grinding them into poder, wrapping them in cotton cloth, and hanging them over boiling cow milk at 120°C for 3 hours. Afterward, the seeds were washed three times with clean water and dried before being extracted with methanol for 24 hours. HPLC analysis revealed that PMA and crotonic acid were present in the untransformed seeds at 5.2 mg/100g and 0.102 mg/100g, respectively. After detoxified, the PMA content decreased to 1.8 mg/100g, and crotonic acid was not detectable. Further physicochemical analysis showed significant differences in the composition of the seeds before and after detoxified, with increases in total ash value (from 2.71% to 3.11%) and alcohol-soluble extractive value (from 8.26% to 12.35%). Meanwhile, the acid value (from 14.46 to 8.53) and saponification value (from 163.98 to 139.53) decreased. Milk was found to be able to dissolve both polar and non-polar compounds, leading to a reduction in the levels of PMA and crotonic acid in the final extract[66].

Srichayanuruk (2011) studied the antioxidant effects, tyrosinase inhibition, and cytotoxicity of the extract of "Lot Tanong Daeng" (a type of herb) before and after detoxification process. The study involved testing cytotoxicity on mouse fibroblast cells (L929) as a model for inflammatory or allergic skin reactions. The detoxification process was done by soaking the herb in water for varying periods, as it was reported

to have toxic properties due to diterpene compounds. The study found that detoxified extracts of the herb had slightly better inhibition of cell growth compared to non-detoxified extracts, suggesting that longer soaking times might enhance the solubility and extraction of the active compounds from the plant[67].

Vichayawiwattana (2011) examined the effects of heating the extract of *Trigonostemon reidioides* on antioxidant activity, tyrosinase inhibition, and cytotoxicity toward mouse fibroblast cells (L929). The heating process ranged from 80°C to 180°C and was tested over 60 - 150 minutes. The results showed no significant difference in cytotoxicity between detoxified and non-detoxified extracts. However, heating was found to reduce the tyrosinase inhibition activity, likely due to the effects of heat on the structure modification or degradation of the compounds, which reduced their biological activity[68].

Newton (2018) compiled research regarding PKC and its role in tumor suppression. PKC is a family of proteins activated by tumor-promoting phorbol esters, which impact genes by generally causing loss-of-function mutations, promoting unexpected tumor-suppressor functions, and contributing to loss-of-function mutations in cancer. The germ Line mutations of certain PKC isozymes are associated with some diseases, including Alzheimer's disease[69].

Choi et al. (2006) investigated the effects of bryostatin 1 and PMA on the proliferation of HOP-92 non-small cell lung cancer cells (NSCLC). The study used different concentrations (1–1,000 nM) for 24 hours. It was found that bryostatin 1 (at 1 and 10 nM) significantly stimulated cell proliferation, while at 1,000 nM, it inhibited cell proliferation. PMA did not affect cell growth. Western blot and PCR analyses revealed that bryostatin 1 and PMA influenced different PKC isozymes, with bryostatin

1 at 10 nM inducing anti-PKCα expression, while PMA tended to increase PKCδ expression in HOP-92 cells. This suggests that bryostatin 1 and PMA have distinct effects on cellular proliferation in non-small cell lung cancer cells[70].

Kang (2014) gathered data on PKC and its association with cancer. These PKC isozymes are well-known for their involvement in cell proliferation, survival, invasion, migration, apoptosis, angiogenesis, and drug resistance. PKC plays a crucial role in cellular signaling processes and is associated with the development of therapeutic approaches for various diseases, including cardiovascular diseases, immune and inflammatory diseases, neurological disorders, metabolic conditions, and multiple types of cancer[71].

Compiled information on toxic plants, highlighting that plants are important sources of biologically active chemicals. Some plants are used for treating diseases in humans and animals, while others, especially those containing alkaloids, proteins, glycosides, and resins, are toxic. Toxic proteins, such as toxalbumins, are known to cause harm to living organisms. Examples include abrin from *Abrus precatorius* Linn., crotin from *C. tiglium* Linn., ricin from *Ricinus communis* Linn., and crucin from *Jatropha curcas* Linn. Among these, a toxic substance from *C. tiglium* Linn. seeds, known as crotonosinde, is particularly harmful to the circulatory system and can agglutinate red blood cells, causing their precipitation[72].

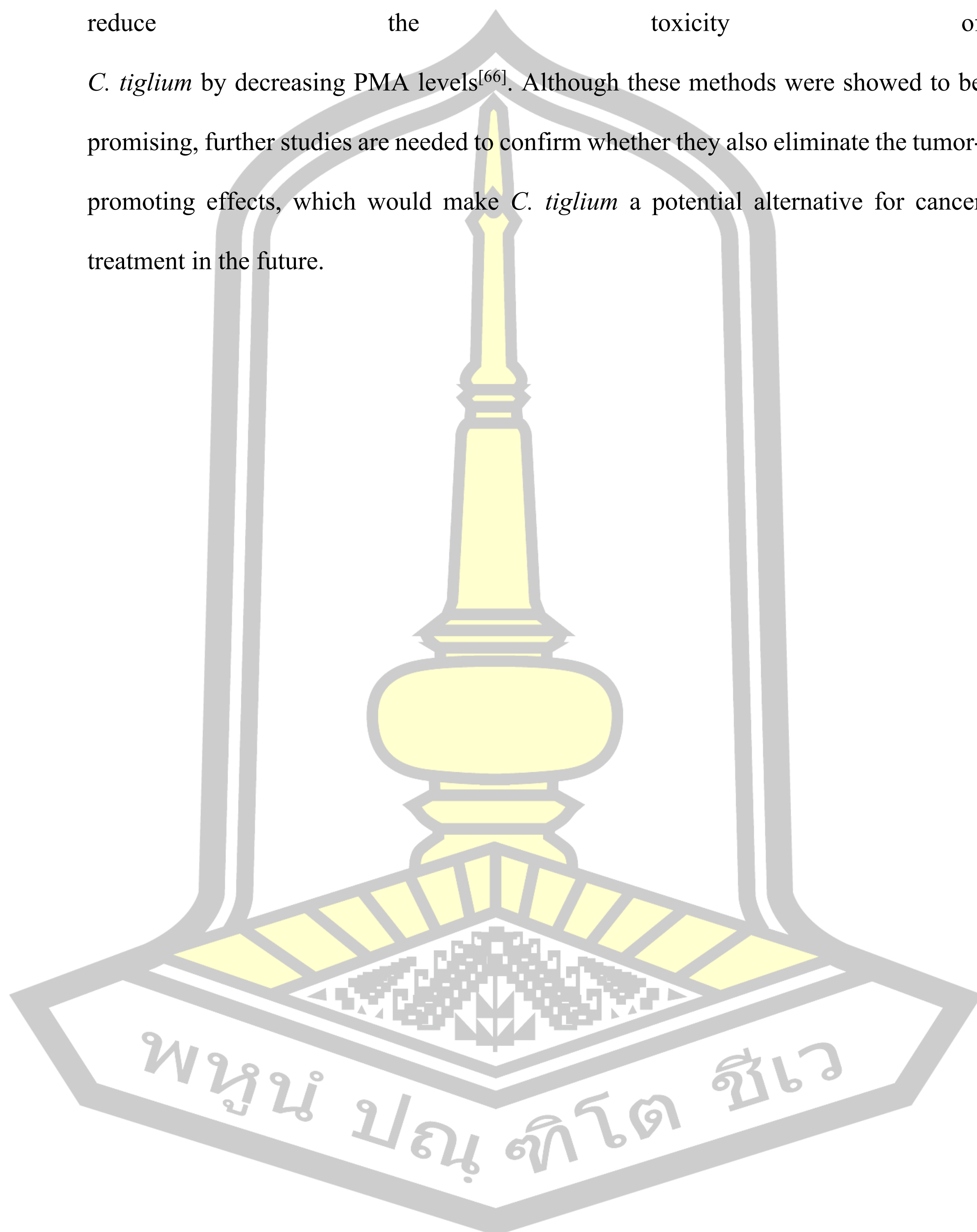
Onoja et al. (2015) studied the purgative effects of extracts from *Operculina turpethum* (150g). The dried leaves were cold-extracted with three solventes, i. e. hexane, chloroform, and 70% metanol, for 48 hours. The extracts were filtered, dried, and tested on rats using the fecal consistency method. The rats were divided into seven groups, including a control group receiving distilled water and various groups receiving

different concentrations of *O. turpethum* extracts. The study found that rats receiving 200 mg/kg doses of chloroform and methanol extracts had significant fecal output, and higher doses increased the percentage of fecal matter excreted. The study also examined intestinal transit and found that extracts from hexane, chloroform, and methanol promoted intestinal movement, suggesting that *O. turpethum* extracts have purgative effects[73].

Liu et al. (2017) studied the toxicity of proteins extracted from *C. tiglium* seeds. Proteins were extracted by grinding and extracting the seeds with petroleum ether, followed by mixing with PBS, and then precipitating proteins with ammonium sulfate. The protein extracts were tested for toxicity in rats. Oral administration at various concentrations showed the values of LD_{50} ranging from 2,752.8 to 3,407.5 mg/kg. Moreover, the intraperitoneal injection resulted in the LD_{50} values of 195.8-272.69 mg/kg. Histopathological examinations revealed organ damage, gastrointestinal bleeding, and intestinal swelling in rats. The study also found that these proteins increased the levels of TNF-α and IL-1β, which are related to cancer development. Therefore, caution should be taken when using croton oil or other preparations that may contain these toxic proteins[74].

From the information above, it is evident that *C. tiglium* seeds are a potent herb with several pharmacological effects, including antifungal, antiviral, antibacterial, anticancer, purgative, anti-inflammatory, and immune response-enhancing properties. Particularly noteworthy is the herb's anticancer activity, as its active compounds can inhibit the growth of various cancer types such as liver, leukemia, lung, and stomach cancer, which are major health concerns globally. However, substances in *C. tiglium*, including PMA and phorbol-12,13 didecanoate, are tumor-promoting agents that can

stimulate abnormal cell division and contribute to cancer development. The methods to reduce the toxicity of *C. tiglium* by decreasing PMA levels[66]. Although these methods were showed to be promising, further studies are needed to confirm whether they also eliminate the tumor-promoting effects, which would make *C. tiglium* a potential alternative for cancer treatment in the future.



CHAPTER 3

Materials and methods

This study is experimental research aimed at investigating the chemical composition, pharmacological effects, and toxicity of *C. tiglium* seed before and after processes through Thai traditional detoxification process. The researcher has divided the research procedure into the following sections:

3.1 Materials, equipment, instruments, and chemicals

3.1.1 Materials/equipments used in the experiment

1) Rotary vacuum evaporator (Buchi, Switzerland)

2) Thin layer chromatography (TLC Silica gel 60 F254 (Merck, Germany))

3) UV-VIS spectrophotometer (Camag, Switzerland)

4) Autoclave (Rexmed, China)

5) Water bath (Memmert, Germany)

6) Vortex mixer (Biosan, Germany)

7) Filter paper (Whatman, China)

8) High-performance liquid chromatography (HPLC) (Agilent, USA)

9) he mass spectrometry (MS) data was obtained from the Bruker Compact QTOF mass spectrometer (Bruker Daltonik, Bremen, Germany).

10) The Nuclear Magnetic Resonance (NMR) spectra were recorded in CDCl3 using a Bruker Ascend™-400 spectrometer (Bruker Biospin AG, Fällanden, Switzerland).

3.1.2 Chemicals used in the experiment

1) Ethanol AR grade (Merck, Germany)

2) Dimethyl sulfoxide (Amresco, USA)

3) n-Hexane AR grade (Merck, Germany)

4) Methanol HPLC grade (Merck, Germany)

5) Ethyl acetate AR grade (Merck, Germany)

6) Dichloromethane AR grade (Merck, Germany)

7) Phorbol-12-myristate-13-acetate (PMA) (Merck, Germany)

3.1.3 Cells used in the experiment

1) HT-29, Caco-2 human colon carcinoma cells (colon cancer cells)

2) KKU213, KKU100 human leukemic cell lines (leukemia cells)

3) A549, H2228 (lung cancer cells)

3.1.4 Cell culture media

1) RPMI-1640 (Gibco, USA)

2) Fetal bovine serum (Gibco, USA)

3.1.5 Animal subjects

The experimental animals used in this study are Wistar rats, aged approximately 6 weeks, weighing 150-200 grams. The study was carried out following the approval from the Ethical Committee of the Institution Animal Care and Use Committee of Khon Kaen University Record No. IACUC-KKU-28/64, Reference No. 660201.2.11/122 (30).

3.2 Research methodology

3.2.1 Chemical composition analysis of *C. tiglium* seeds

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3.2.1.1 Preparation of herbal samples

The preparation of *C. tiglium* seed for chemical composition analysis was done using mature *C. tiglium* seeds collected from Sawi District, Chumphon Province. The samples were authenticated by two Thai traditional medicine doctors and one local herbalist in Chumphon. The seeds were thoroughly cleaned and then air-dried for 48 hours. Once dried, they were stored in amber-colored bottles containing desiccants to maintain freshness.

3.2.1.2 Thai TDP of *C. tiglium* seeds

This experiment used four methods for detoxification processed *C. tiglium* seeds to reduce their toxicity, following traditional Thai medical methods. The methods are as follows[46]:

1) Method 1: 50 grams of *C. tiglium* seeds and placed in a clay pot, with 150 grams of paddy, 100 grams of salt, and 2,000 milliliters of water. The mixture was boiled until the rice husks expanded.

2) Method 2: 50 grams of *C. tiglium* seeds and mixed with 100 grams of *Tamarindus indica* leaves, 100 grams of *Acacia concinna* leaves, and 2,000 milliliters of water in a clay pot, then boiled until it reached a boil.

3) Method 3: 50 grams of *C. tiglium* seeds and soaked overnight in fermented fish sauce, then stuffed into *Citrus hystrix* fruit. This was placed in a clay pot and steamed with paddy fire for 5 hours.

4) Method 4: 50 grams of *C. tiglium* seeds, ground finely, and roasted until the oil was completely evaporated for 40 minutes.

3.2.1.3 Extraction of *C. tiglium* seed compounds

1) To extract and compare PMA and crotonic acid levels in the *C. tiglium* seeds before and after detoxification processes by the four methods, high-performance liquid chromatography (HPLC) was used. The detoxification processes method that most reduced the amounts of PMA and crotonic acid, while maximizing the area under the chromatogram for newly formed compounds after detoxified, was selected. This compound was then extracted using methanol.

The process steps are as follows:

1.1) Remove *C. tiglium*.seeds from both before and after Thai TDP

1.2) Grind the seeds finely using a grinder and pass them through a 16 mesh screen.

1.3) Split the samples into two portions of 25 grams each and place them in Erlenmeyer flasks. Add 150 milliliters of Methanol. Soak the samples 7 day.

1.4) Filter the extracts using Whatman No. 1 filter paper and evaporate the solvents using a Rotary Evaporator until dry.

1.5) Weigh the extracts from each sample and store them in a freezer at -20°C until further use.

2) To purify the extracts for compound separation, the following fermentation process was used with Methanol:

2.1) Take 2000 grams of processed *C. tiglium* seeds.

2.2) Grind the seeds finely and pass them through a 16 mesh screen.

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2.3) Place the seeds in Erlenmeyer flasks and add 150 milliliters of methanol. Leave the mixture to ferment for 7 days, then replace the solvent and repeat triplication.

2.4) Filter each batch of extract using Whatman No. 1 filter paper, then evaporate the solvents using a rotary evaporator until dry.

2.5) Purify the extract using chromatographic techniques.

3.2.1.4 Preparation of PMA and crotonic acid standard compounds

Prepare standard solutions of PMA and crotonic acid in methanol at concentrations of 5, 50, 100, 350, and 500 µg/mL. These solutions are then analyzed by HPLC. The area under the chromatogram and the concentration are used to create a standard calibration curve.

3.2.1.5 Method validation

1) Linearity test

Dilute the PMA and crotonic acid standards with 100% Methanol to prepare five concentrations: 5, 50, 100, 350, and 500 µg/mL. Analyze these samples using HPLC, calculate the area under the chromatograms and correlate them with the concentrations. The correlation coefficient (R^2) is then calculated to determine the linearity.

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2) Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ refer to the minimum concentration of the target compounds that can be detected and quantified in the sample. These limits are determined by analyzing sample blanks spiked with low concentrations of standard

compounds. The standard deviation is calculated, and LOD = 3 × SD and LOQ = 10 × SD[75].

3) Precision test

The precision of the method is tested by repeating the analysis multiple times. Precision is measured by performing intraday (same day) and interday (over three consecutive days) tests, calculating the % relative standard deviation (% RSD) for each repetition:

$$RSD = SD/Mean \times 100$$

4) Accuracy test

The accuracy of the method is assessed by determining the percentage recovery (% Recovery) using spiked samples at three different concentration levels, with at least seven replicates per concentration. The recovery is calculated as follows:

$$\%Recovery = (C1 - C2)/C3 \times 100$$

Where:

C1 = the value from the spiked sample

C2 = the value from the unspiked sample

C3 = the concentration of the spiked standard compound.

3.2.1.6 Comparison of percentage and amount of extract before and after detoxification processes

1) Comparison of the percentage of PMA in the *C. tiglium* seed extract before and after processes using all four methods.

2) Comparison of the percentage of PMA and crotonic acid in the *C. tiglium* seed extract before and after processes using all four methods.

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3.2.1.7 Comparison of high-performance liquid chromatography (HPLC) fingerprint

The *C. tiglium* seed extract, before and after processes (as described in section 3.2.1.3), was analyzed using high-performance liquid chromatography (HPLC). The mobile phase used consisted of 0.05% trifluoroacetic acid: methanol, and the analysis was conducted with a photo diode array (PDA) detector at a wavelength of 254 nm. Subsequently, the HPLC fingerprint and the amount of active compounds in the *C. tiglium* seed extract before and after processes were analyzed.

Following the analysis of HPLC fingerprints, *C. tiglium* seeds processed through Thai TDP exhibiting the chromatograms with the highest presence of newly formed compounds will be selected for further studies. These studies include the isolation of pure compounds, evaluation of purgative activity, acute toxicity testing, anti-cancer activity assessment, and tumor-promoting assay.

3.2.2 Purification of active compounds in *C. tiglium* seed

Studies on the chemical composition of *C. tiglium* seeds after undergoing Thai TDP revealed the formation of new compounds. Therefore, the compounds need to be purified for further structural analysis and to identify the types of compounds present. The purification process is as follows:

3.2.2.1 Separation of compounds by quick column chromatography (Absorption chromatography)

The stationary phase used was Silica Gel, and the process involves the following steps:

1) Column preparation: The column is washed with a solvent. The stationary phase used is Silica Gel 60 (0.063-0.200 mm), and the mobile phase is

dichloromethane: ethyl acetate (DCM: EAC) in a ratio of 8:2 - 0:10, followed by ethyl acetate: methanol (EAC: MET) in a ratio of 9:1 - 6:4.

2) Loading the extract: The *C. tiglium* seed extract, mixed with Silica Gel, is added to the column at a ratio of 30:1 (adsorbent to extract). A layer of sand is placed on top of the extract to prevent disruption of the surface.

3) Solvent flow: The solvent is allowed to flow out of the column until it is 1 cm above the level of the adsorbent. The solvent mixture, dichloromethane: ethyl acetate (DCM: EAC) in a ratio of 8:2 - 0:10, is then added, followed by ethyl acetate: methanol (EAC: MET) in a ratio of 9:1 to 6:4. A total of 17 fractions, each with a volume of 500 mL, are collected, corresponding to different solvent concentrations. Fraction 1 is further separated using Resin-20 as the stationary phase and a mobile phase of water and methanol in varying ratios from 10:0 to 0:10. Furthermore, Fractions 10 and 11 are subjected to partitioning with dichloromethane. The dichloromethane-soluble phase is subsequently dried for further analysis.

4) Thin layer chromatography (TLC) analysis: each fraction is tested using TLC to obtain a fingerprint. Fractions with similar patterns on the TLC plate are combined. These combined fractions are further separated using liquid-liquid extraction.

3.2.3 Pharmacological testing

3.2.3.1 *In vitro* cancer activity testing

1) Cancer cell culturing

Since *C. tiglium* seeds is a herb used in Thai traditional medicine for treating gastrointestinal diseases and immune-related disorders, cancer cells that are relevant to these conditions were selected for the study. These include colon cancer

cells (CaCo-2, Ht-29), leukemia cells (THP-1, K562), cholangiocarcinoma cells (KKU213, KKU100), and lung cancer cells (A549, H2228). The cancer cells were cultured in RPMI-1640 or DMEM media, supplemented with 10% fetal bovine serum, 1% L-glutamine, and 10 units/mL Penicillin G. The cells were seeded in 96-well plates and incubated for 18-24 hours. The cells were then treated with *C. tiglium* seeds extract, prepared using the appropriate extraction method, and diluted to suitable concentrations. After that, the cells were incubated at 37°C in a CO_2 incubator with 5% CO_2.

2) Cytotoxicity testing using MTT assay

Approximately 2 x 10^4 cells were cultured in the appropriate medium for each type of cancer cell (lung, colon, and cholangiocarcinoma cells) at a volume of 200 µL per well in a 96-well culture plate. The cells were incubated at 37°C with 5% CO_2 for 24 hours. After 24 hours, the medium was removed, and the cells were treated with different concentrations of the extract (before and after detoxification processes, as well as new compounds) in 400, 200, 100, 50, 25, and 12.5 µL of cell culture medium. The cells were then incubated for 24, 48, and 72 hours. After incubation, the medium was removed, and MTT reagent was added at a volume of 100 µL per well. The cells were incubated for 4 hours at 37°C with 5% CO_2. After the incubation, the medium was discarded, and DMSO was added (100 µL per well). The color intensity was measured using a microplate reader at a wavelength of 570 nm. The intensity of the color is proportional to the number of viable cells. The optical density was used to calculate the IC_{50} value, which was then analyzed to determine the cell type most effectively inhibited by the *C. tiglium* seeds extract for further testing.

After the cytotoxicity testing, the colon cancer cell line Caco-2 was found to be the most sensitive to the after detoxification processed *C. tiglium* seeds extract. Therefore, the concentrations corresponding to the IC_{50} values were chosen for further tests.

3) Cell death testing

The Caco-2 colon cancer cells were treated with *C. tiglium* seeds extract at concentrations of 50, 10, and 200 µg/mL, and incubated for 72 hours. The cells were then centrifuged and washed twice with cold PBS. The cell pellet was resuspended in 1X Binding Buffer and incubated with FITC Annexin V and PI for 15 minutes at room temperature. After incubation, 1X Binding Buffer was added, and the results were analyzed using a flow cytometer.

4) Measurement of protein levels (p53, p21, Bax, Bcl-2)

The Caco-2 colon cancer cells were treated with *C. tiglium* seeds extract at concentrations of 50, 10, and 200 µg/mL and incubated for 72 hours. Afterward, the cells were centrifuged and lysed using lysis buffer containing protease inhibitors. The samples were incubated on ice for 5 minutes and then centrifuged at 10,000 x g at 4°C for 10 minutes. The protein concentration was measured, and the samples were stored at -80°C until further analysis. The proteins were mixed with LaemmLi Sample Buffer, boiled at 95°C for 5 minutes, and separated using 10% SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to a PVDF membrane and incubated with primary antibodies, followed by secondary HRP-conjugated goat polyclonal antibodies. Chemiluminescent detection was performed using a chemiluminescent kit, and the results were recorded using a Biomolecular Imager.

5) Gene expression analysis by qRT-PCR

The Caco-2 colon cancer cells were treated with *C. tiglium* seeds extract at concentrations of 50, 10, and 200 µg/mL and incubated for 72 hours. RNA was extracted using a commercial RNA extraction kit, and the quantity and purity of the RNA were measured using a Nanodrop ND-1000 spectrophotometer. cDNA was synthesized using a reverse transcription kit. Real-time PCR was then performed to evaluate the expression of genes associated with carcinogenesis biomarkers using the QuantiFast SYBR Green PCR Kit.

P21 Forward: 5'CGATGGAACTTCGACTTTGTCA3'

Reverse: 5'GCACAAGGGTACAAGACAGTG3'

P53 Forward: 5'CCAACAACACCAGCTCCTCT3'

Reverse:5'TCAGGAAGTAACACCATCGTAAG3'

Cyclin B1 Forward: 5'GAA ACATGAGAGCCATCCT3'

Reverse: 5'TTCTGCATGAACCGATCAAT3'

CDK-1 Forward: 5'TGAAACTGCTCGCACTTG3'

Reverse: 5'ATG GTAGATCCCGGCTTATT3'

Bax Forward: 5'GGGCCCACCAGCTCTGA3'

Reverse: 5'CCTGCTCGATCCTGGATGA3'

Bcl-2 Forward: 5'CCAACAACACCAGCTCCTCT3'

Reverse:5'TCAGGAAGTAACACCATCGTAAG3'

Caspase -3 Forward: 5'CATTGAGACAGACAGTGGTGTT3'

Reverse: 5'CACAAAGCGACTGGATGAAC3'

Caspase-9 Forward: 5'GTTTGAGGACCTTCGACCAGCT3'

Reverse: 5'CAACGTACCAGGAGCCACTCTT3'

β-actin Forward 5'CATGTACGTTGCTATCCAGGC3'

Reverse 5'CTCCTTAATGTCACGCACGAT3'

3.2.4.2 Carcinogenesis induction test

1) Cell culture

The cells, which have been tested for toxicity and have the best IC_{50} values, are cultured in cell culture medium consisting of 10% fetal bovine serum and 1% L-glutamine. The cells are plated in 24-well culture plates and incubated for 18-24 hours. Then, they are stimulated with the appropriate extract of *C. tiglium* seeds at suitable dilution concentrations and incubated at 37°C with 5% CO_2.

2) Colony formation inhibition test

Approximately 1,000 cells are plated in the wells of a 6-well culture plate and treated with *C. tiglium* seeds extract at concentrations of 50, 100, and 200 µg/mL. The cells are incubated at 37°C with 5% CO_2 for an appropriate amount of time. After the incubation, the medium is replaced with fresh cell culture medium containing 10% fetal bovine serum, 1% L-glutamine, and 10 units/mL of Penicillin G, and incubated at 37°C with 5% CO_2. Colony formation is monitored in the control wells. The cells are then fixed with 10% paraformaldehyde and stained with 0.05% crystal violet. The number and size of the colonies are counted.

3) Migration and invasion test

The migration and invasion capabilities of the cancer cells are tested to assess their potential to metastasize. For the migration assay, approximately 20,000 cells are plated into 24-well Transwell inserts and incubated for 3 hours. The *C. tiglium* seeds extract is added at concentrations of 50, 100 and 200 µg/mL, and the cells are incubated for another 24 hours. Afterward, the cells are fixed with 3.7%

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paraformaldehyde and stained with 0.05% crystal violet. The results are analyzed under a microscope. The invasion test is performed similarly, but the 24-well Transwell inserts are pre-coated with Corning Matrigel for 2 hours to simulate in vivo tissue characteristics.

3.2.3.3 Laxative effect test in experimental animals[73, 76, 77]

1) Preparation of experimental animals[76, 78]

The animals used in this test are male Wistar rats, approximately 6 weeks old, weighing between 150-200 grams, with a total of 48 rats. They are housed in sterile animal cages in a controlled environment at a temperature of 23 ± 3°C, relative humidity of 60 ± 20%, and a 12-hour light/dark cycle. They are fed standard food and provided with drinking water throughout the experiment. Prior to the experiments, the rats are acclimatized for one week. The dosage for evaluating purgative activity is calculated based on the proportion of *C. tiglium* seeds present in the traditional Thai herbal formulation.

$SD = SEM \sqrt{n}$, where $SEM = 0.77$ and $n = 5$ $SD = 0.77 * \sqrt{5} = 1.72$

d = change in stool volume between experimental and control groups $d = 3.703 - 0.784 = 2.955$

Sample size formula $= 2 * SD^2 * (1.96 + 0.842)^2 / d^2$ $n = 2 * (1.72)^2 * (1.96 + 0.842)^2 / (2.955)^2$ $n = 2 * 2.95 * 7.85 / 8.73$ $n = 46.45 / 8.73$ $n = 5.32$

Adding a 10% margin for potential error: $n = 5.32 / 0.9 = 5.91$

Therefore, the number of rats used for each group is 6.

2) Laxative effect test in experimental animals

The procedure for the laxative effect test is as follows:

2.1) Fasting of animals

The rats are fasted from both food and water for 12 hours before the experiment. Each rat is placed in an individual cage lined with filter paper to collect fecal samples from each animal.

2.2) Group division and treatment

The rats are divided into 8 groups, each consisting of 6 rats. Each group is orally administered different substances as follows:

2.2.1) Group 1 receives the solvent used for preparing the extract solution (vehicle control).

2.2.2) Group 2 receives castor oil (0.3 mL/animal), a standard substance with known laxative effects (positive control).

2.2.3) Groups 3-5 receive *C. tiglium* seed powder before deactivation at doses of 10, 50, and 100 mg/kg, respectively.

2.2.4) Groups 6-8 receive *C. tiglium* seed powder after deactivation at doses of 10, 50, and 100 mg/kg, respectively.

2.3) Fecal monitoring

The number and weight of the feces, both normal and wet, are recorded hourly for 16 continuous hours.

2.4) Fecal drying and water content calculation

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All collected feces are dried using a vacuum freeze-drying machine and reweighed. The water content of the feces is then calculated per gram of feces.

2.5) Data collected from the experiment

The following data are collected:

2.5.1) The number (number of fecal pellets) and weight (fecal weight) of both normal and wet feces during the 16-hour period (results may be presented as 0-8 hours compared to 8-16 hours).

2.5.2) The percentage of wet feces (percentage wet feces).

2.5.3) The amount of water in the feces (fecal water content).

2.6) Isoflurane use for euthanasia and sample collection

The procedure for euthanizing the rats using Isoflurane and collecting samples is as follows:

2.6.1) Anesthesia with isoflurane: the rats are anesthetized with saturated Isoflurane until they lose consciousness. A standard inhalation anesthesia setup is used, with the anesthetic mask placed over the nose of the rat, and the isoflurane concentration is increased above the typical range (greater than 5%, whereas the normal dose is 3-4%). Once the rats are calm, reflex tests are performed (corneal reflex and pedal reflex). If no reflex is observed, blood is drawn from the heart, and once blood is fully drained, the animal is considered to have passed away. In some cases, cervical dislocation may be performed to ensure death in rats weighing less than 250 grams.

2.6.2) Animal weighing: the weight of each animal is recorded either before the post-mortem examination or after euthanasia.

2.6.3) Recording animal history detailed: animal history is recorded for diagnostic purposes, including the type, sex, strain, weight, and age of the animal.

2.6.4) Examination of the rectum and reproductive organs: If abnormalities are observed, such as diarrhea, feces may be found attached to the anus. In male rats, the preputial glands may show signs of swelling or inflammation.

2.6.5) Necropsy: the animal is dissected by opening the abdominal cavity to examine the stomach and intestines. The stomach and intestines are then removed for further examination for abnormalities.

3.2.3.4 Acute toxicity test in experimental animals

The acute toxicity test through oral administration (acute oral toxicity: fixed dose) is conducted according to the OECD guidelines for the testing of chemicals (Figure 3.1-3.2)[65]. This method assesses the toxicity of chemicals based on both the survival of the experimental animals and the manifestation of toxic symptoms. The toxicity of the chemical is classified under the Globally Harmonized System for Classification and Labeling of Chemicals (GHS). The classification includes 5 categories:

Category 1: Extremely toxic chemicals, with an LD_{50} of 5 mg/kg body weight.

Category 2: Highly toxic chemicals, with an LD_{50} between 25-50 mg/kg body weight.

Category 3: Moderately toxic chemicals, with an LD_{50} between 200-300 mg/kg body weight.

Category 4: Low toxicity chemicals, with an LD_{50} between 500-2,000 mg/kg body weight.

Category 5: Chemicals with low toxicity or no toxicity, with an LD50 between 2,000 mg/kg body weight or no toxicity[65].

The procedure for the experiment is as follows:

1) Preparation of experimental animals

The animals used are female Wistar rats, approximately 6 weeks old, weighing 150-200 grams, with a total of 12 rats. They are housed in a sterile animal cage setup within an animal room at a temperature of 22 ± 3°C, relative humidity of 60 ± 20%, with alternating light/dark cycles of 16-18 hours. They are provided with commercial animal feed and chlorine-treated RO water (5-6 ppm chlorine) throughout the study. Prior to the experiment, the rats are allowed to acclimatize for 1 week.

2) Preliminary acute oral toxicity test (sighting study)

Group 1: Receives *C. tiglium* seed extract before deactivation.

Group 2: Receives *C. tiglium* seed extract after deactivation.

Group 3: Receives an extract formulation containing *C. tiglium*.

The purpose of this preliminary test (sighting study) is to determine the starting dose for the main study on toxicity testing. Female Wistar rats are used for the sighting study, with 1 rat per group.

2.1) Preparation of experimental animals

Female Wistar rats, 6 weeks old, weighing 150-200 grams, a total of 36 rats, are housed in sterile cages in an animal room with a

temperature of 22 ± 3°C, relative humidity of 60 ± 20%, alternating light/dark cycles of 16-18 hours, with commercial feed and chlorine-treated RO water (5-6 ppm chlorine). The rats are allowed to acclimatize for 1 week before starting the experiment.

2.2) Administering the test substance

C. tiglium seed powder is administered orally at a dose of 300 mg/kg body weight through gastric lavage.

2.3) Observation and recording of abnormalities

The rats are observed for any abnormalities within 24 hours of administration. Observations include changes in skin, fur, mucous membranes, breathing patterns, behavior, trembling, diarrhea, lethargy, and general condition. The animals are monitored once a day for 14 days.

2.4) Weighting and recording food and water intake

The weight of the rats and their food and water intake are recorded weekly. If any abnormalities affecting the welfare of the animals are observed, the animal must be euthanized immediately.

2.5) Interpretation of results

The following guidelines are used to interpret the results:

If an animal dies, the dose is reduced to 50 mg/kg body weight, and further tests are conducted to determine the starting dose for the main study.

If the animal shows signs of toxicity, the starting dose for the main study is set at 300 mg/kg body weight.

If no toxicity is observed in the animals, the dose is increased to 2,000 mg/kg body weight, and further tests are conducted to determine the starting dose for the main study.

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Note: The dose that causes death in the experimental animals during the sighting study will not be used in the main study.

2.6) Post-mortem examination

After the experiment, the animals are euthanized for necropsy. Internal organs are examined for abnormalities with the naked eye, and histopathological examination is performed on any organs showing abnormalities. The rats are anesthetized using saturated Isoflurane until they lose consciousness. A standard anesthetic apparatus is used, and the Isoflurane concentration is increased above the normal level (greater than 5%, while the normal dose is 3-4%). Once the rats are calm, reflex tests (corneal and pedal reflexes) are performed. If no reflex is observed, blood is drawn from the heart, and once the blood is fully collected, the animal is considered dead. In some cases, cervical dislocation may be performed on rats weighing less than 250 grams to ensure complete death.

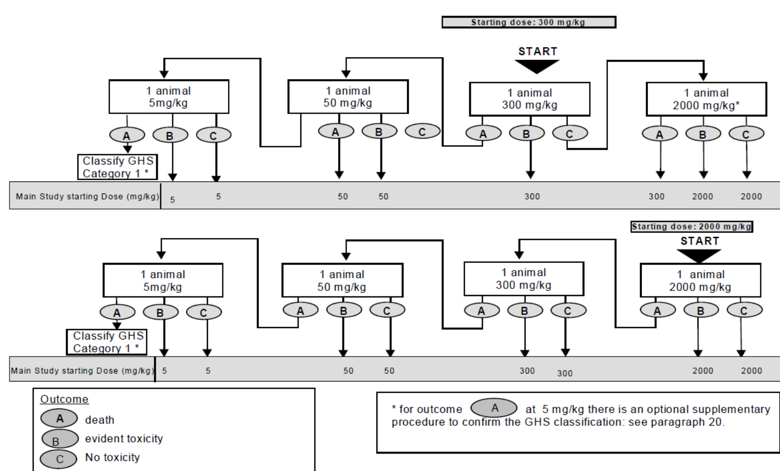

Figure 3.1 Procedure for preliminary acute toxicity test (sighting study)[65]

3) Acute oral toxicity test: fixed dose procedure: main study

The main study for acute toxicity involves administering the test substance orally to the experimental animals once, using the starting dose determined from the sighting study. The test is then continued by adjusting the dose based on the results of the trial.

3.1) Fasting of the rats

The rats are fasted (no food or water) for 16-18 hours before starting the experiment. Each rat is housed in an individual cage with sterile bedding placed underneath.

3.2) Testing on female rats

Six female rats per group are used for the test. The *C. tiglium* seeds extract is administered orally to the rats at the starting dose determined from the sighting study. One rat from the test group should be one that survived from the sighting study.

3.3) Observation and recording of abnormalities

The rats are observed for 24 hours following administration of the test substance, noting any changes in skin, fur, mucous membranes, breathing patterns, behavior, trembling, diarrhea, lethargy, and general condition. Symptoms are monitored once a day for 14 days.

3.4) Weighing and recording of food and water intake

The rats' body weight, as well as the amount of food and water consumed, are recorded weekly. If any abnormality affecting the animal's welfare is observed, the animal must be euthanized immediately.

3.5) Testing procedure example

For example, if the starting dose of the substance is 300 mg/kg body weight, the results are interpreted as follows:

If 2 or more animals in a group die, the dose is reduced to 50 mg/kg body weight and the test continues.

If 1 or more animals show signs of toxicity but no animals die, the substance is classified as GHS Category 4.

If no toxicity is observed in the experimental animals, the dose is increased to 2,000 mg/kg body weight and the test continues.

3.6) Post-test necropsy

After the test, the animals are euthanized, and a necropsy is performed to inspect the internal organs for abnormalities. Any organs showing abnormalities are further examined through histopathological analysis.

3.7) Euthanasia procedure using isoflurane

The rats are euthanized using isoflurane anesthesia until they lose consciousness. A standard anesthetic apparatus is used, and the isoflurane is administered at a concentration higher than normal (greater than 5%, compared to the typical 3-4%). Once the rat is calm, reflex tests (corneal and pedal reflexes) are performed. If no reflex is observed, blood is drawn from the heart until the animal is completely exsanguinated. The animal is then considered dead. In some cases, cervical dislocation may be performed on rats weighing less than 250 grams to ensure complete death.

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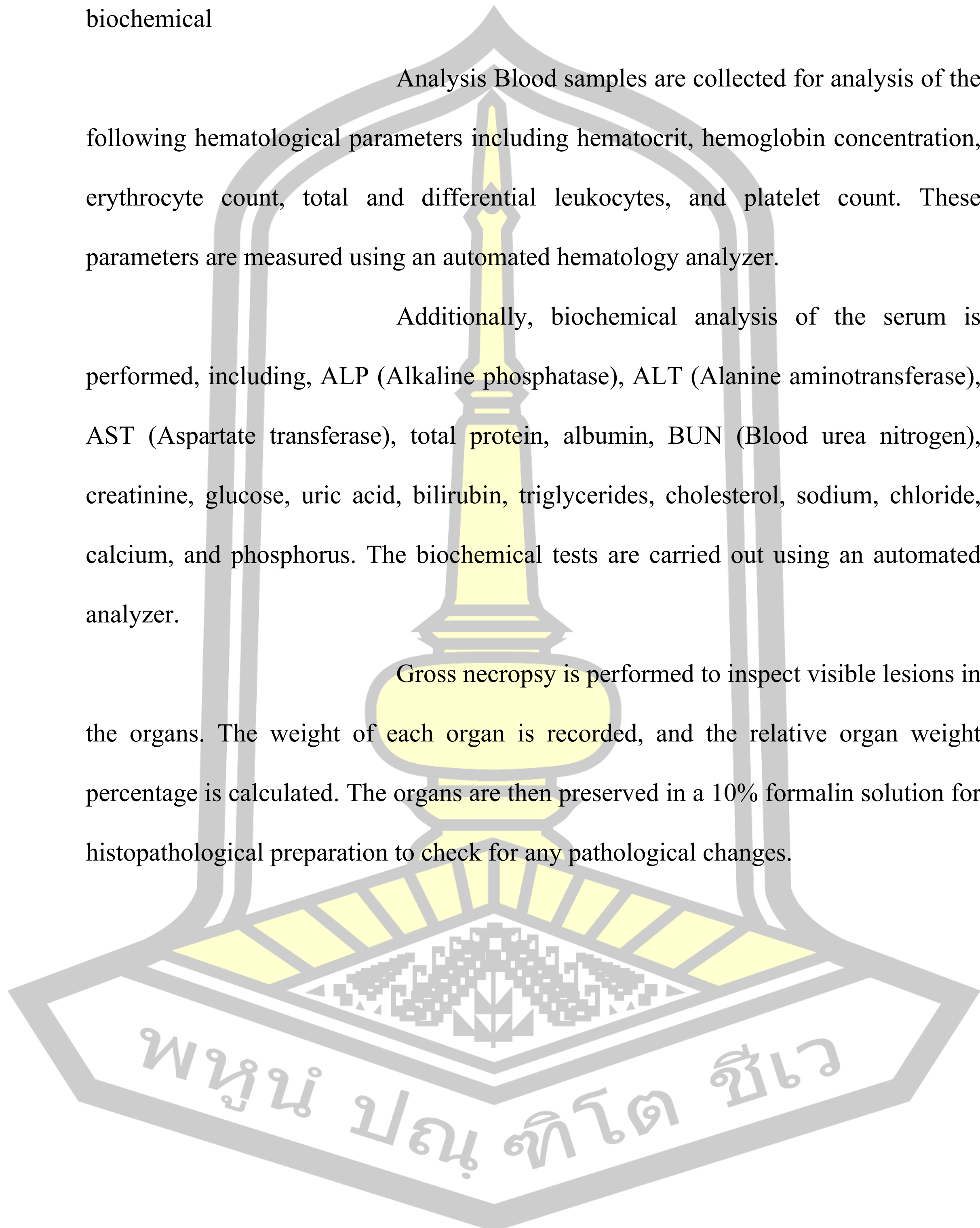
3.8) Blood sample collection for hematological and biochemical

Analysis Blood samples are collected for analysis of the following hematological parameters including hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocytes, and platelet count. These parameters are measured using an automated hematology analyzer.

Additionally, biochemical analysis of the serum is performed, including, ALP (Alkaline phosphatase), ALT (Alanine aminotransferase), AST (Aspartate transferase), total protein, albumin, BUN (Blood urea nitrogen), creatinine, glucose, uric acid, bilirubin, triglycerides, cholesterol, sodium, chloride, calcium, and phosphorus. The biochemical tests are carried out using an automated analyzer.

Gross necropsy is performed to inspect visible lesions in the organs. The weight of each organ is recorded, and the relative organ weight percentage is calculated. The organs are then preserved in a 10% formalin solution for histopathological preparation to check for any pathological changes.



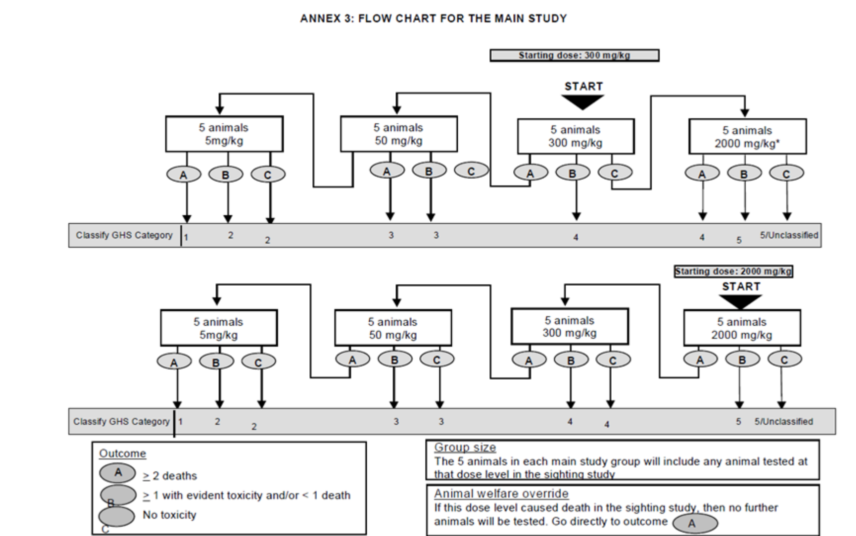


Figure 3.2 Example of acute toxicity test procedure (main study)[65]

3.3 Data collection

For data collection, the researchers carried out the following procedures:

3.3.1 Extraction of substances

The quantity of the extract was recorded before and after undergoing the detoxification processes methods based on traditional Thai pharmacological theories. Afterward, the extract was analyzed preliminarily using thin layer chromatography (TLC), observed under an ultraviolet light source, and the results were documented in a lab notebook.

3.3.2 Measurement and analysis using HPLC

The extracts were tested, and data were recorded before and after the detoxification processes methods based on Thai pharmacological theories, comparing them to standard substances. This was done using HPLC.

3.3.3 Testing for anticancer and pre-cancer effects

The results of tests for inhibition of cell cycle were recorded using Flow cytometry. The tests included assessing cell death, colony formation inhibition, inhibition of cell division, gene expression using qRT-PCR, changes in the cell cycle, and cell morphology testing.

3.3.4 Testing for laxative effects

The number of fecal pellets and fecal weight were recorded, including both normal and wet feces over a 16-hour period. The percentage of wet feces and the fecal water content were also calculated.

3.3.5 Acute toxicity testing

Observations and recordings of abnormalities were made within 24 hours after the administration of the substance, including changes in skin, fur, mucous membranes, breathing patterns, behavior, trembling, diarrhea, lethargy. Body weight, food, and water intake were recorded weekly. At the end of the experiment, a necropsy was performed to detect any internal organ abnormalities under a microscope, and the organs were weighed, including the liver, kidneys, testes, heart, and lungs.

3.4 Statistics used for data analysis

The statistics used for data analysis are as follows:

3.4.1 Descriptive statistics

These include the mean, standard deviation, and percentage values.

3.4.2 Statistics for testing and comparing PMA and crotonic acid levels

For comparing the levels of PMA and crotonic acid in *C. tiglium* seeds before and after processes, the paired sample t-test was used.

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3.4.3 Statistics for testing anticancer and carcinogenic effects

To test the anticancer and carcinogenic effects of *C. tiglium* seed extracts before and after processes, as well as the new substances formed after processes, Two-way ANOVA was applied.

3.4.4 Statistics for testing anticancer and carcinogenic effects of new substances

For testing the anticancer and carcinogenic effects of the new substances formed after processes, One-way ANOVA was applied.

3.4.5 Statistics for testing laxative effects in experimental animals

For testing the laxative effects of *C. tiglium* seed extracts before and after processes, as well as the new substances formed after processes, Two-way ANOVA was used.

3.4.6 Statistics for testing acute toxicity

For testing the acute toxicity of *C. tiglium* seed extracts before and after processes, as well as the new substances formed after processes, Two-way ANOVA was used.

When using ANOVA, normality tests were performed on the data, which should show a normal distribution. Additionally, the homogeneity of variance test was conducted, and results should indicate no significant differences. If these conditions were not met, nonparametric statistics were used as follows:

For paired sample t-test, the Mann-Whitney test.

For One-way ANOVA, the Bonferroni test.

For Two-way ANOVA, the Friedman test.

3.4.7 Post-hoc analysis

When the hypothesis test results showed statistically significant findings, pairwise comparisons of the means were conducted using the Bonferroni test, and the data were analyzed using software programs for statistical computations.

CHAPTER 4

Literature review

The comparison of chemical composition, pharmacological activities, and toxicity of *C. tiglium* Linn. seeds before and after Thai Traditional Detoxification Processes (Thai TDP) yielded the following findings:

4.1 Analysis of chemical constituents in *C. tiglium* seeds

4.2 Comparison of Chromatographic Characteristics

4.3 Isolation of occurring compound from *C. tiglium* seeds after Thai TDP

4.4 Molecular structure analysis of the isolated compounds

4.5 Pharmacological activity tests

4.5.1 Acute toxicity tests in animal models

4.5.2 Laxative activity tests in animal models

4.5.3 *In vitro* anticancer activity tests

4.5.4 Carcinogenesis induction activity tests

4.1 Analysis of chemical constituents in *C. tiglium* Linn. seeds

4.1.1 Comparison of crude extract yields

A comparison of crude extract yields from *C. tiglium* seeds before and after Thai TDP using four different methods was conducted. The methods are described as follows:

4.1.1.1 Method 1: Fifty grams of *C. tiglium* seeds placed in a clay pot with 150 grams of paddy rice, 100 grams of salt, and 2,000 milliliters of water. The mixture was boiled over medium heat until the rice grains were fully expanded

(approximately 4 hours). The seeds were then washed, separated, air-dried, and oven-dried at 45°C for 48 hours. Finally, the seeds were ground into a fine powder for further analysis.

4.1.1.2 Method 2: Fifty grams of *C. tiglium* seeds were separated and mixed with 100 grams of tamarind leaves and 100 grams of *Acacia concinna* leaves. The mixture was placed in a clay pot, filled with 2,000 milliliters of water, and boiled until fully cooked.

4.1.1.3 Method 3: Fifty grams of *C. tiglium* seeds were separated and soaked in fermented fish sauce for one night. The soaked seeds were then stuffed into a kaffir lime, placed in a clay pot, sealed, and roasted in rice husk ash for 5 hours.

4.1.1.4 Method 4: Fifty grams of *C. tiglium* seeds were separated, finely ground, roasted until all oils evaporated (approximately 40 minutes) and each sample dispensed in methanol at a 1:7 ratio. The mixture was subjected to ultrasonic extraction for 30 minutes and filtered through Whatman No. 1 filter paper. The filtrate was evaporated under reduced pressure (250 mbar) at 60°C.

The results showed that the crude extract yield before Thai TDP was higher than after Thai TDP using all four methods, with yields of 12.32%, 12.0%, 8.0%, 14.8%, and 11.52%, respectively. Detailed results are presented in Table 4.1.

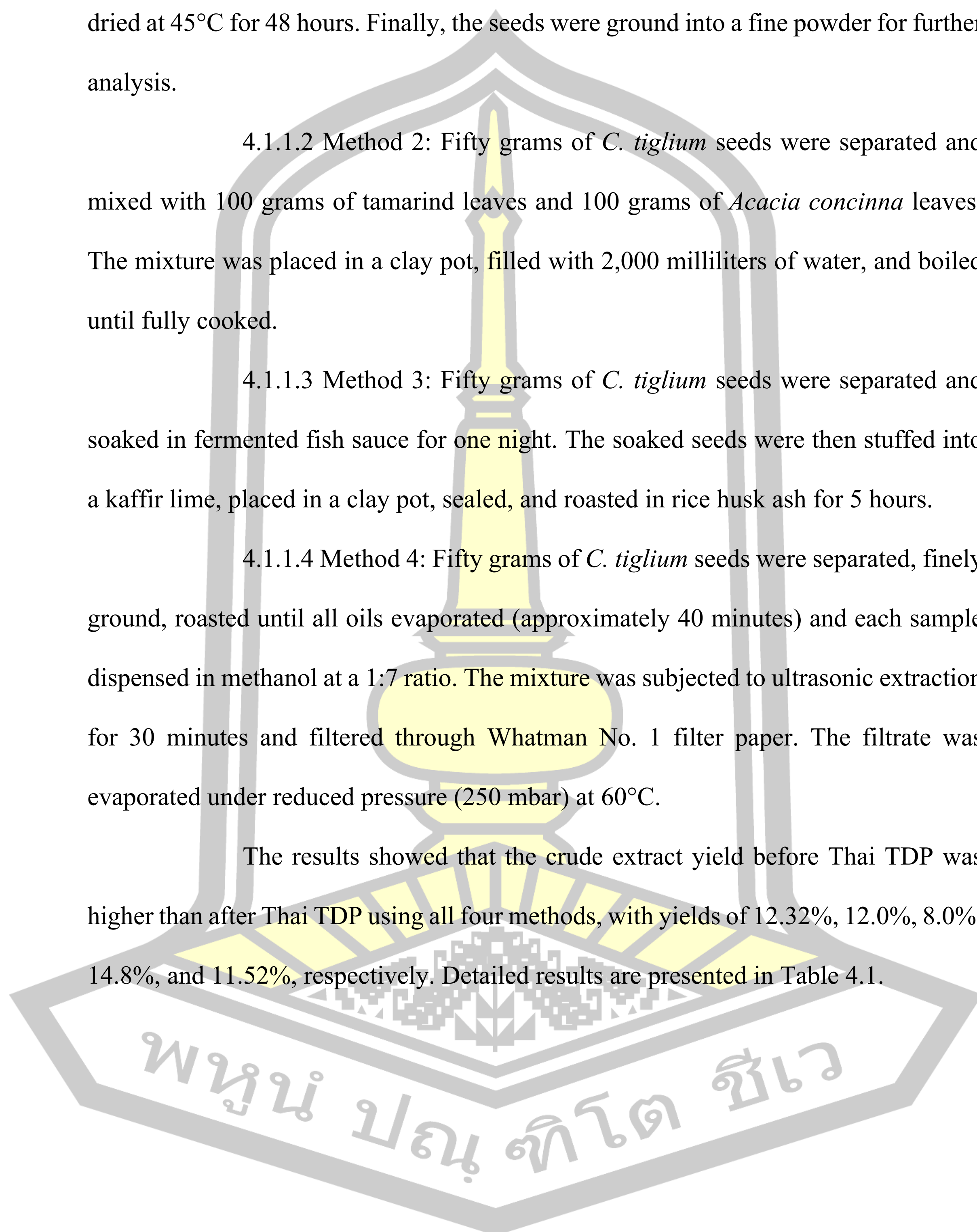

Table 4.1 Crude extract yields of *C. tiglium* seeds before and after Thai TDP

Sample	**Extract Characteristics**	**Methanol extract yield (%)**
Before Thai TDP	Clear, viscous, dark yellow liquid	12.32
After Thai TDP		
Method 1	Clear, viscous, dark yellow liquid	12.00
Method 2	Clear, viscous, dark yellow liquid	8.00
Method 3	Clear, viscous, dark yellow liquid	14.80
Method 4	Clear, viscous, dark brown liquid	11.52

4.1.2 Comparison of high-performance liquid chromatography (HPLC) fingerprints

The comparison of HPLC fingerprints involved analyzing both the number of peaks and the area under the curve (AUC) of each peak. Particulary focus was given to peaks with high quantities or major peaks from methanol-extracted *C. tiglium* seeds. The details are as follows:

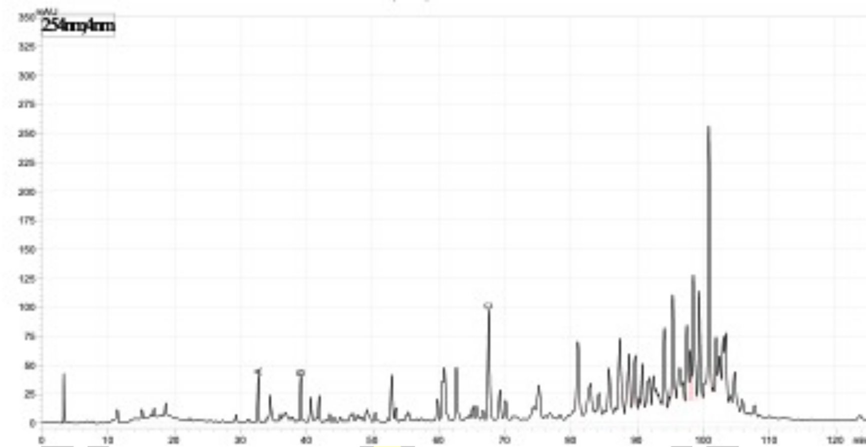

Figure 4.1 Chromatogram of *C. tiglium* seeds before Thai TDP

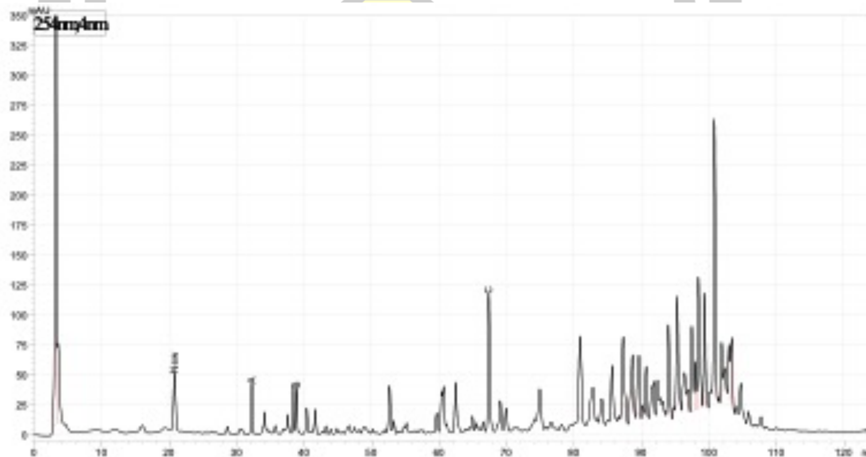

Figure 4.2 Chromatogram of *C. tiglium* seeds after Thai TDP (method 1)

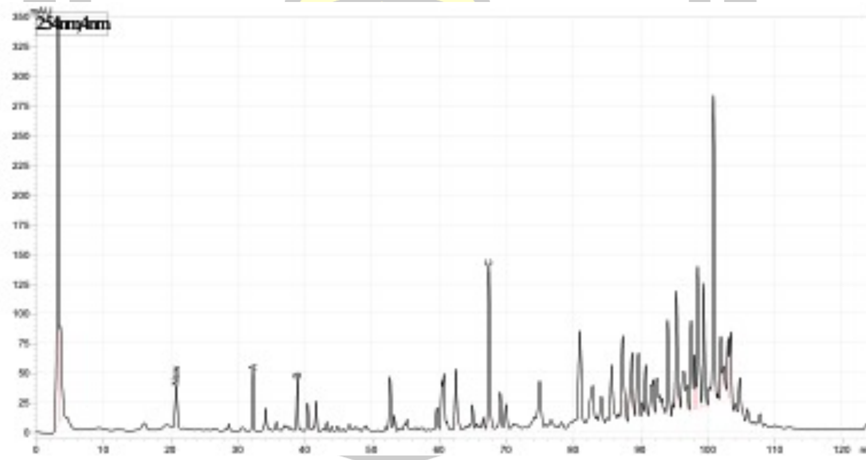

Figure 4.3 Chromatogram of *C. tiglium* seeds after Thai TDP (method 2)

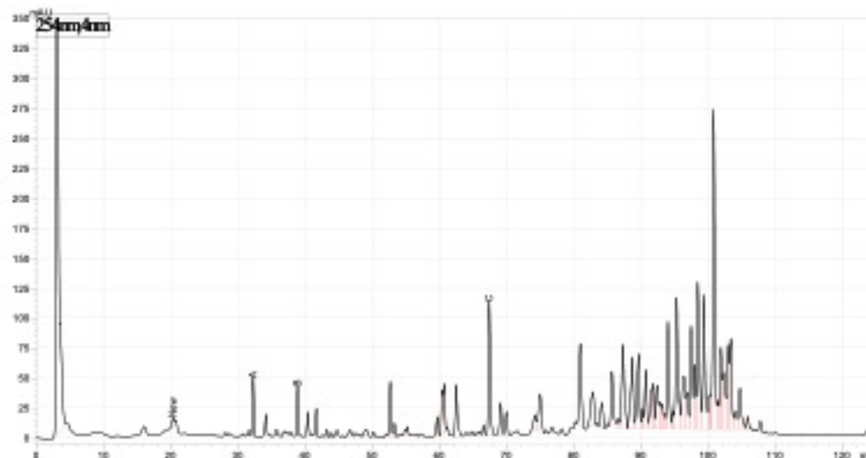

Figure 4.4 Chromatogram of *C. tiglium* seeds after Thai TDP (method 3)

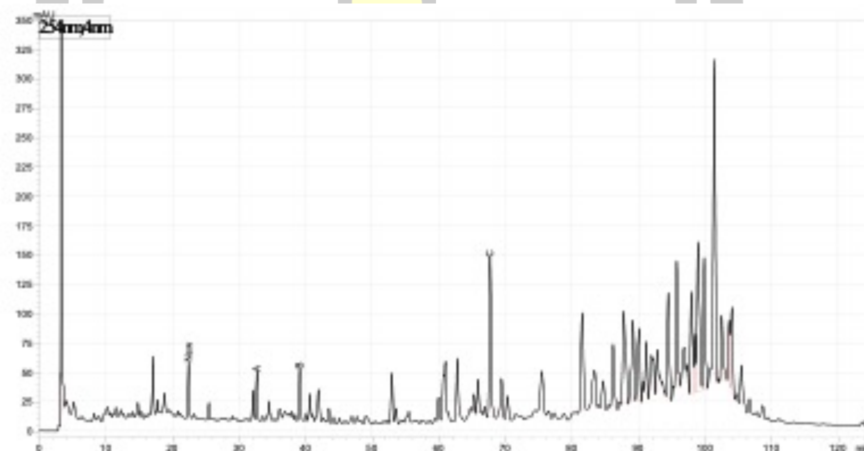

Figure 4.5 Chromatogram of *C. tiglium* seeds after Thai TDP (method 4)

4.1.2.1 Chemical composition analysis of *C. tiglium* seeds before Thai TDP

From the chromatogram shown in Figure 4.1, the analysis revealed three major peaks, namely Peak A, B, and C, with average retention times of 32.65, 39.23, and 67.54, respectively. The average area under the curve (AUC) of the peaks was $67.32 \times 10^4 \pm 1.14 \times 10^4$, $56.66 \times 10^4 \pm 1.12 \times 10^4$, and $26.46 \times 10^4 \pm 1.71 \times 10^4$ mAU, respectively, as shown in Table 4.2.

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4.1.2.2 Chemical composition analysis of *C. tiglium* seeds after Thai TDP (method 1)

The chromatogram shown in Figure 4.2 revealed four major peaks: New, A, B, and C, with average retention times of 20.82, 32.29, 38.98, 53, and 67.40, respectively. The average AUC of the peaks was $87.98 \times 10^4 \pm 5.74 \times 10^4$, $54.179 \times 10^4 \pm 1.86 \times 10^4$, $47.88 \times 10^4 \pm 1.87 \times 10^4$, and $20.13 \times 10^4 \pm 2.72 \times 10^4$ mAU, respectively, as shown in Table 4.2.

4.1.2.3 Chemical composition analysis of *C. tiglium* seeds after Thai TDP (method 2)

The chromatogram shown in Figure 4.3 revealed four major peaks: New, A, B, and C, with average retention times of 20.84, 32.29, 38.89, and 67.40, respectively. The average AUC of the peaks was $62.85 \times 10^4 \pm 9.28 \times 10^3$, $70.21 \times 10^4 \pm 1.46 \times 10^4$, $56.59 \times 10^4 \pm 1.98 \times 10^4$, and $25.12 \times 10^5 \pm 3.45 \times 10^5$ mAU, respectively, as shown in Table 4.2.

4.1.2.4 Chemical composition analysis of *C. tiglium* seeds after Thai TDP (method 3)

The chromatogram shown in Figure 4.4 revealed four major peaks: New, A, B, and C, with average retention times of 20.39, 32.30, 38.92, and 66.61, respectively. The average AUC of the peaks was $87.85 \times 10^3 \pm 1.56 \times 10^3$, $73.26 \times 10^4 \pm 1.69 \times 10^4$, $57.36 \times 10^4 \pm 2.19 \times 10^4$, and $21.68 \times 10^4 \pm 1.05 \times 10^4$ mAU, respectively, as shown in Table 4.2.

4.1.2.5 Chemical composition analysis of *C. tiglium* seeds after Thai TDP (method 4)

The chromatogram shown in Figure 4.5 revealed four major peaks: New, A, B, and C, with average retention times of 22.43, 32.71, 39.14, and 67.73. The average AUC of the peaks was $56.40 \times 10^4 \pm 1.56 \times 10^4$, $61.80 \times 10^4 \pm 1.69 \times 10^4$, $56.73 \times 10^4 \pm 2.19 \times 10^4$, and $16.62 \times 10^5 \pm 4.50 \times 10^5$ mAU, respectively, as shown in Table 4.2.

Table 4.2 Average area under the curve of *C. tiglium* seeds extracts

Peak	Before Thai TDP	Thai TDP method1	Thai TDP method2	Thai TDP method3	Thai TDP method4
New	-	8.79×10^5	6.28×10^5	8.78×10^5	5.64×10^5
A	6.73×10^5	5.41×10^5	7.02×10^5	7.32×10^5	6.18×10^5
B	5.66×10^5	4.78×10^5	5.65×10^5	5.73×10^5	5.67×10^5
C	$2.64 x 10^6$	2.03×10^6	2.51×10^6	2.16×10^6	1.66×10^6

From the comparison of the area under curve (AUC) of *C. tiglium* seeds extracted with methanol before and after detoxified using the four methods, statistical analysis using One-Way ANOVA showed that there were significant differences in the AUC values of the methanol extract before and after treated with Thai TDP at the 95% confidence level ($p<0.05$). When pairwise comparisons were made, the following differences were observed:

Peak New: The AUC values of the detoxified *C. tiglium* seeds were significantly higher than the undetoxified *C. tiglium* seeds. No peak was detected in the undetoxified *C. tiglium* seeds, while the highest AUC was observed in the *C. tiglium*

seeds detoxified by method 1, followed by methods 3, 2, and 4 in descending order (1 > 3 > 2 > 4).

Peak A: The AUC of the detoxified *C. tiglium* seeds varied as follows: the highest AUC was observed in the *C. tiglium* seeds detoxified by method 3, followed by method 2, the undetoxified *C. tiglium* seeds, and method 1 in descending order (3 > 2 > 0 > 1).

Peak B: The *C. tiglium* seeds before detoxified had the highest AUC, followed by the detoxified by method 3, method 2, method 4, and method 1 in descending order (0 > 3 > 2 > 4 > 1).

Peak C: The undetoxified *C. tiglium* seeds had the highest AUC, followed by the seeds detoxified by method 2, method 3, method 1, and method 4 in descending order (0 > 2 > 3 > 1 > 4).

Comparison by peak: In peak new, the AUC of the *C. tiglium* seeds before detoxified was higher than the *C. tiglium* seeds undetoxified for all detoxified methods. In peak A, the after detoxified using methods 2 and 3 showed higher chemical content compared to the undetoxified *C. tiglium* seed, while methods 1 and 4 showed lower chemical content than the detoxified *C. tiglium* seeds. In peak B, the after detoxified using method 3 showed higher chemical content compared to the *C. tiglium* seeds before detoxified, while methods 2 and 4 showed similar levels, and method 1 had lower chemical content than the undetoxified *C. tiglium* seeds. In peak C, the after undetoxified using method 2 had chemical content most similar to the *C. tiglium* seeds undetoxified , while methods 1, 3, and 4 showed lower chemical content than the *C. tiglium* seeds undetoxified.

4.1.3 Comparison amount of PMA and crotonic acid before and after Thai TDP

4.1.3.1 Method of validation

1) Linearity: The peak area of PMA and crotonic acid analyzed by HPLC at a wavelength of 254 nm, with concentrations ranging from 0.125 – 2.0 mg/mL and 0.001 – 0.016 mg/mL is shown in Table 4.3. The equation derived from the analysis is: $y = 8058.4x - 312.32$, with $r^2 = 0.999$ and $y = 53628x + 0.4444$ with $r^2 = 0.9997$ indicating a very high linear relationship. standard curve shown in Figure 4.6

Table 4.3 Peak area and concentration of PMA

PMA (mg/mL)	Peak area (Mean ± SD, n=3)
2.00	15946.47 ± 24.37
1.00	7404.95 ± 28.66
0.5	3780.25 ±3.21
0.25	1768.65 ±43.73
0.125	764.22. ± 20.16
Crotonic acid (mg/mL)	**Peak area (Mean ± SD, n=3)**
0.016	51.28± 4.37
0.008	111.23± 6.37
0.004	210.46± 10.37
0.002	410.92± 11.37
0.001	841.84± 15.37

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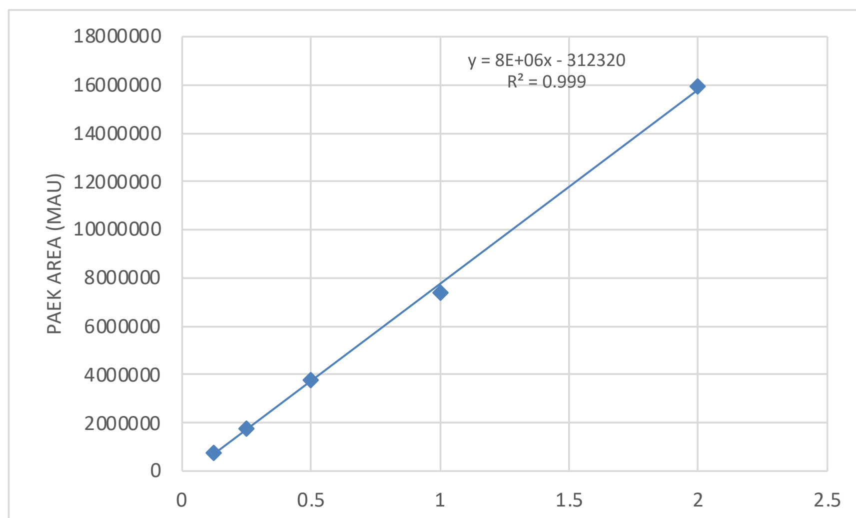
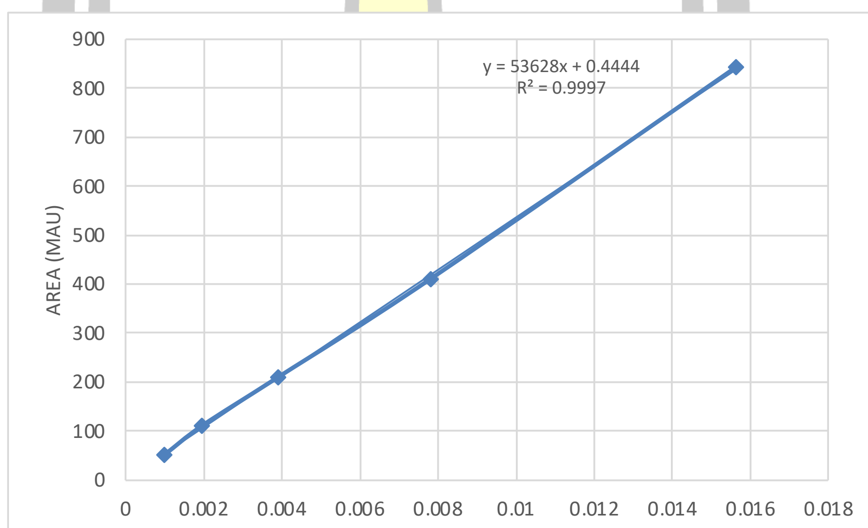


(A)



(B)

Figure 4.6 Standard curve of PMA (A) and crotonic acid (B)

2) Precision: Intraday and interday precision of PMA and crotonic acid from experiments conducted at 3 concentrations, with 10 repetitions per concentration, showed that the % RSD ranged from 0.98 - 5.08 and 1.03 – 4.82, shown in Table 4.4 - 4.5

Table 4.4 Intraday precision and interday precision of PMA

% RSD (n=10)	Concentration (mg/mL)					
	0.125		0.50		2.0	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
1	0.123	0.127	0.467	0.523	1.98	2.04
2	0.121	0.131	0.469	0.531	1.995	2.08
3	0.124	0.129	0.476	0.554	2.18	2.21
4	0.125	0.141	0.479	0.522	1.98	2.11
5	0.122	0.131	0.481	0.531	1.985	1.995
6	0.126	0.1245	0.475	0.524	2.18	2.08
7	0.121	0.126	0.47	0.556	1.988	1.998
8	0.125	0.129	0.468	0.485	1.998	1.99
9	0.123	0.131	0.481	0.522	2.18	2.18
10	0.123	0.129	0.48	0.493	2.108	2.128
Mean	0.1233	0.12985	0.4746	0.5241	2.0574	2.0811
SD	0.0016	0.00437	0.0054	0.0217	0.0900	0.0752
% RSD	1.360	3.880	5.080	4.820	2.870	4.055

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Table 4.5 Intraday precision and interday precision of crotonic acid

% RSD (n=10)	Concentration (mg/mL)					
	0.10		0.80		1.16	
	Intraday	Interday	Intraday	Interday	Intraday	Interday
1	0.1033	0.102	0.821	0.811	1.141	1.119
2	0.1021	0.1011	0.812	0.8	1.132	1.108
3	0.0901	0.101	0.818	0.814	1.159	1.159
4	0.0991	0.101	0.811	0.816	1.12	1.145
5	0.0981	0.1001	0.808	0.902	1.14	1.124
6	0.1011	0.102	0.812	0.81	1.142	1.14
7	0.105	0.101	0.821	0.812	1.156	1.136
8	0.102	0.101	0.832	0.83	1.156	1.146
9	0.104	0.101	0.82	0.812	1.162	1.152
10	0.105	0.1001	0.801	0.802	1.149	1.149
Mean	0.1009	0.1010	0.8156	0.8209	1.1457	1.1378
SD	0.0042	0.0006	0.0081	0.0281	0.0125	0.0152
% RSD	0.980	1.030	1.950	2.613	1.233	1.914

From the interday and intraday precision results, it was found that the majority of % RSD values, indicating that the accuracy was within an acceptable range < 5[79].

3) Accuracy: When analyzing the amount of PMA and crotonic acid at three different concentrations, the % recovery was found to be in the range of 101.09 - 102.08, as shown in Table 4.6, which is within the acceptable range of 95-

105%. This indicates that the analytical method has high accuracy and can be used for quantifying PMA.

4) Limit of detection and quantitation: The standard PMA and crotonic acid solution was prepared at a concentration of 2.0 mg/mL, and after dilution, the limit of detection (LOD) was found to be 0.94 and 0.98 µg/mL, while the limit of quantitation (LOQ) was 3.12, 3.27 µg/mL, as shown in Table 4.6-4.7.

Table 4.6 Accuracy and limit of detection and quantitation of PMA

% RSD	**Concentration (mg/mL)**		
(n=10)	**0.125**	**0.5**	**2.0**
%Recovery (Mean ± SD, n=7)	101.41±7.12	102.08±6.90	101.09±6.43
LOD (µg/mL)		0.94	
LOQ (µg/mL)		3.12	

Table 4.7 Accuracy and limit of detection and quantitation of crotonic acid

% RSD	**Concentration (mg/mL)**		
(n=10)	**0.1**	**0.8**	**1.16**
%Recovery (Mean ± SD, n=7)	104.08±3.12	102.08±5.20	99.79±5.43
LOD (µg/mL)		0.98	
LOQ (µg/mL)		3.27	

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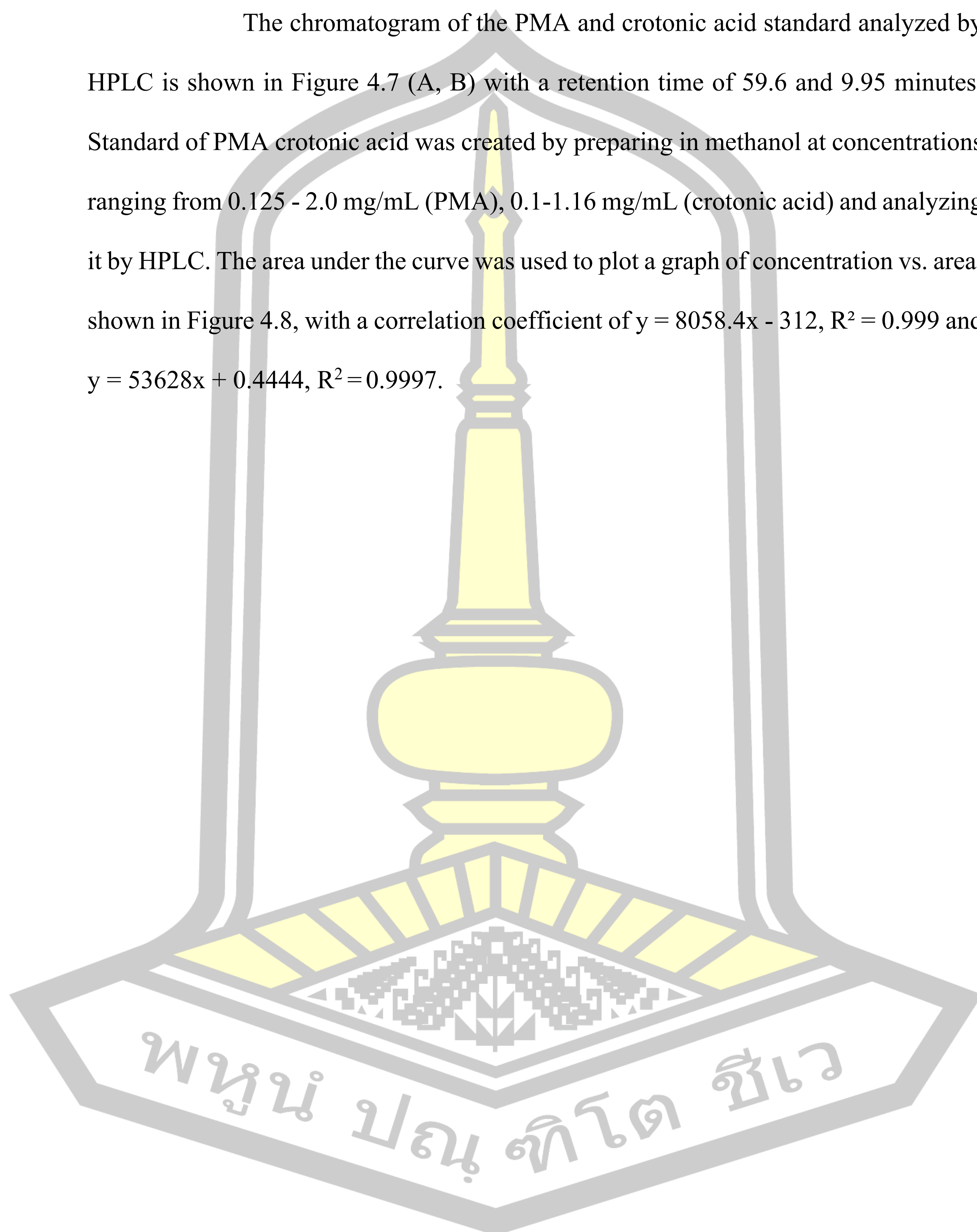
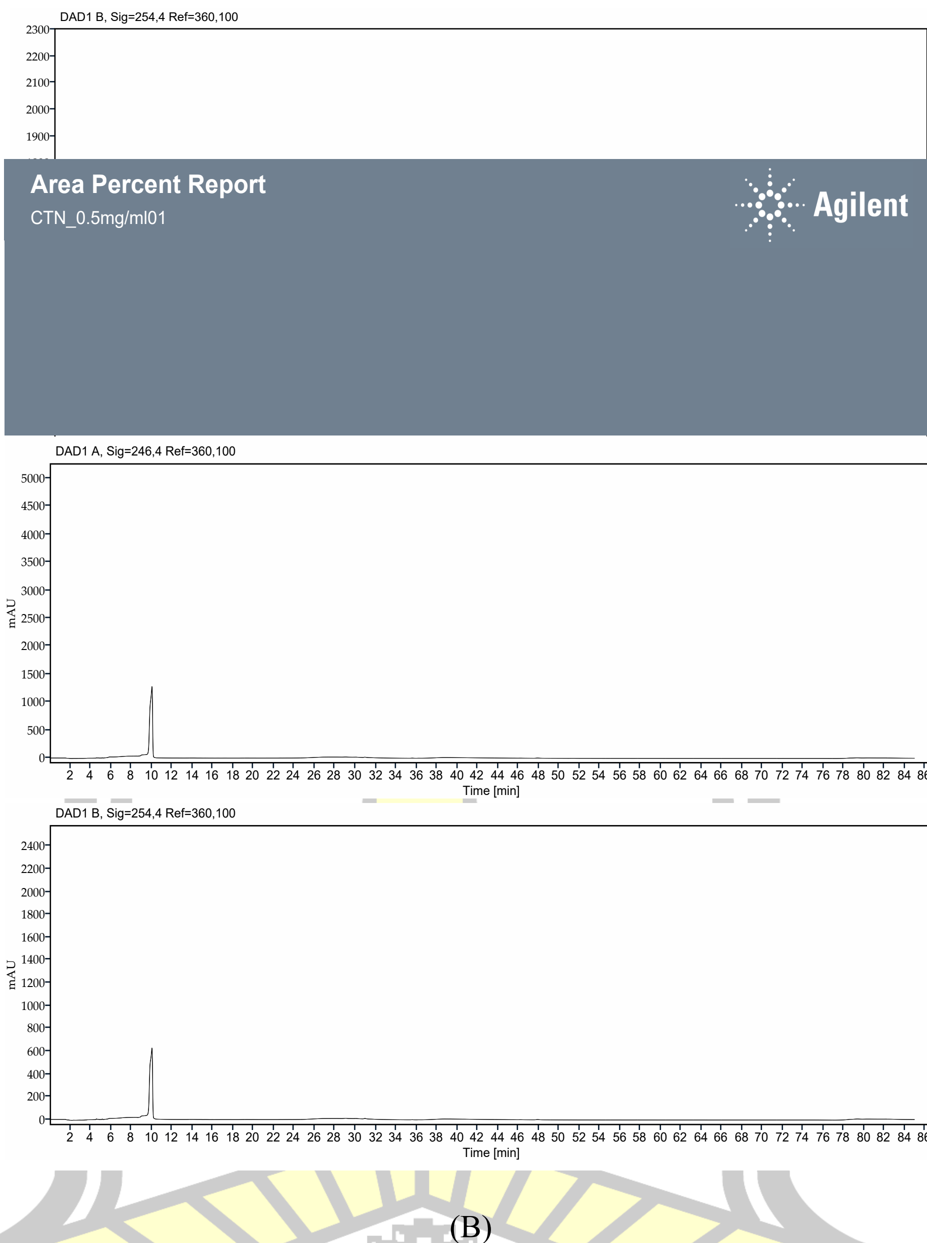
4.1.3.2 Analysis of PMA standard by HPLC

The chromatogram of the PMA and crotonic acid standard analyzed by HPLC is shown in Figure 4.7 (A, B) with a retention time of 59.6 and 9.95 minutes. Standard of PMA crotonic acid was created by preparing in methanol at concentrations ranging from 0.125 - 2.0 mg/mL (PMA), 0.1-1.16 mg/mL (crotonic acid) and analyzing it by HPLC. The area under the curve was used to plot a graph of concentration vs. area, shown in Figure 4.8, with a correlation coefficient of y = 8058.4x - 312, R^2 = 0.999 and y = 53628x + 0.4444, R^2 = 0.9997.





(B)

Figure 4.7 Chromatogram of the reference standards PMA (A) and crotonic acid (B)

4.1.3.3 Comparison of phorbol-12-myristate-13-acetate (PMA) content before and after Thai TDP

From the analysis of PMA content in the *C. tiglium* seeds extract before and after Thai TDP, it was found that the extract undetoxified contained 1.59 mg of PMA per gram of dry *C. tiglium* seeds. After detoxified, the PMA content for each

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method was as follows: 1.25 mg, 1.69 mg, 1.16 mg, and 1.10 mg per gram of dry *C. tiglium* seeds for methods 1-4, respectively. Methods 1, 3, and 4 resulted in a decrease in PMA content, with method 4 showing the largest reduction, a decrease of 31.07%. Meanwhile, method 2 increased the PMA content by 5.86%, as shown in Table 4.8.

Table 4.8 Comparison of average phorbol-12-myristate-13-acetate (PMA) content of *C. tiglium* seeds before and after Thai TDP

Method	Content of PMA		
	PMA content (mg/g)	**Percentage decrease**	**Percentage increase**
Before Thai TDP	1.59	-	-
After Thai TDP 1	1.26	21.46	-
After Thai TDP 2	1.69	-	5.86
After Thai TDP 3	1.16	27.43	-
After Thai TDP 4	1.10	31.07	-

4.2 Comparison of chromatographic characteristics

When analyzed using high-performance liquid chromatography (HPLC), the extracts separated through quick column chromatography with Solvent flow: dichloromethane: ethyl acetate (DCM: EAC) in a ratio of 8:2 - 0:10, is then added, followed by ethyl acetate: methanol (EAC: MET) in a ratio of 9:1 to 6:4. A total of 17 fractions, each with a volume of 500 mL, are collected, corresponding to different solvent concentrations. Fraction 1 is further separated using Resin-20 as the stationary

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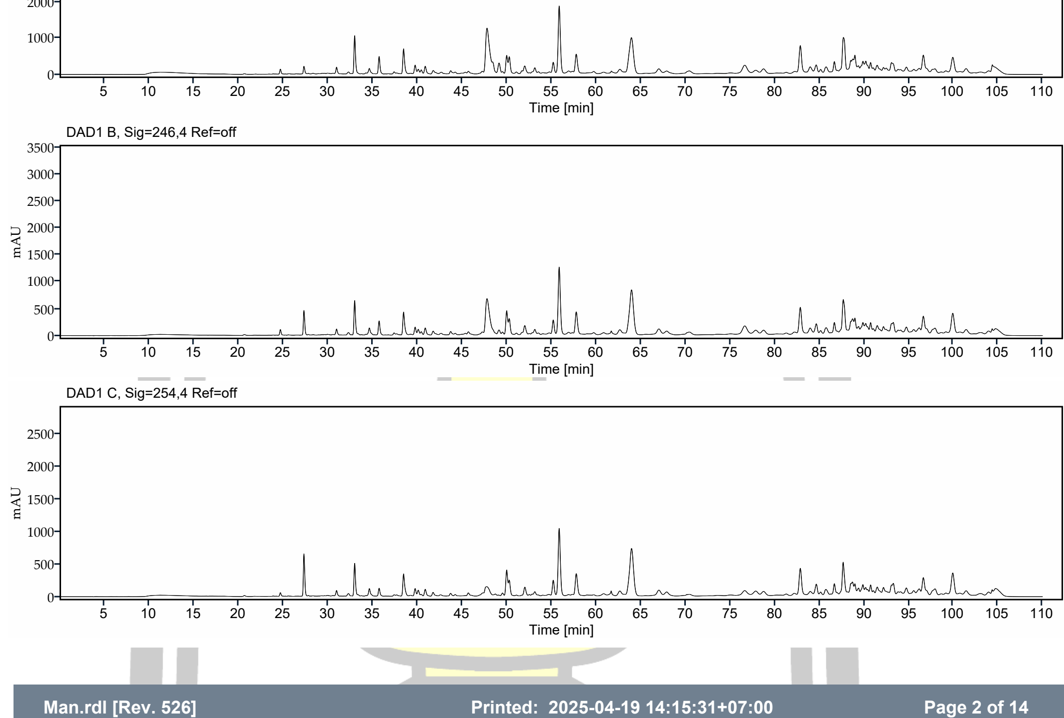

Figure 4.8 Chromatograms of *C. tiglium* seed before (A) and after Thai TDP (B)

The extract was further separated using open column chromatography with resin as the stationary phase. The mobile phase was applied in the following proportions ac :2 (R3), 7:3 (R4), 6:4 (R5), 5:5 (R6), 4:6 (R7), 3:7 (R8), 2:8 (R9), 1:9 (R10), and 0:10 (R11). Subsequently, methanol: ethyl acetate was used with ratios of 9:1 (R11) and 8:2 (R12), respectively. The fractions were then evaporated to dryness using a rotary evaporator. HPLC analysis indicated that fractions R10 and R11 displayed

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chromatographic characteristics matching the "New" compound, with a retention time of 27.50 minutes (Figure 4.9).

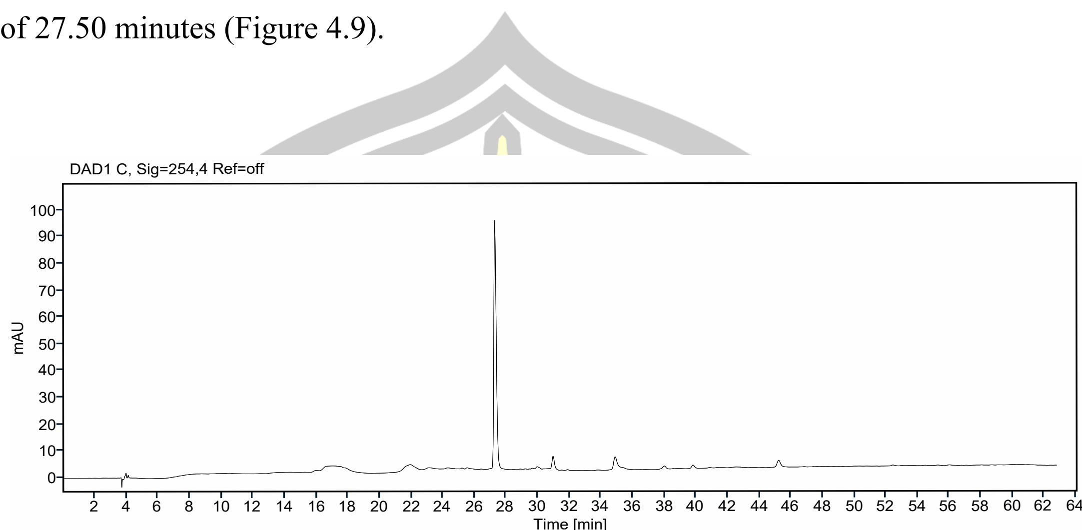

Figure 4.9 Chromatograms of the compound isolated from fraction R10

When analyzed using thin-layer chromatography (TLC), the compounds isolated from fractions R10 and R11 absorbed at a wavelength of 254 nm but did not fluoresce at 366 nm. Upon spraying with anisaldehyde H_2SO_4, the compounds produced a pink coloration with an Rf value of 0.32. Moreover, in the extracts of *C. tiglium* seed after Thai TDP across all four methods, spots with matching Rf values and pink coloration were observed after anisaldehyde H_2SO_4 spraying (Figure 4.10).

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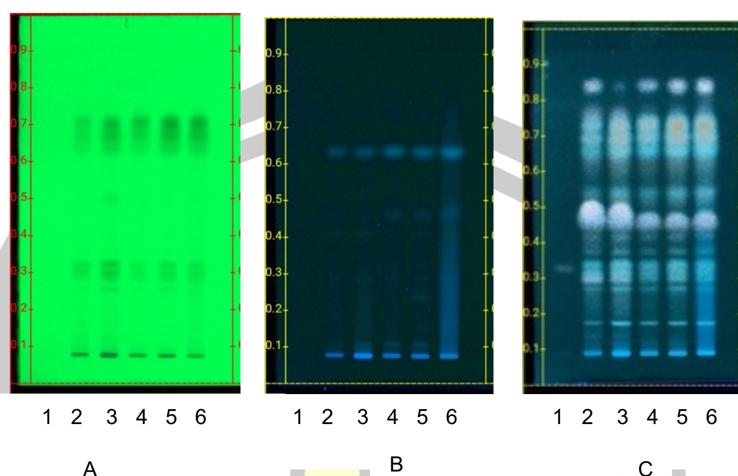


Figure 4.10 TLC chromatogram of: 1. Compounds isolated from R10 and R11 2. Crude extract from *C. tiglium* seed before Thai TDP 3. Crude extract from *C. tiglium* seed after Thai TDP (Method 1) 4. Crude extract from *C. tiglium* seed after Thai TDP (Method 2) 5.Crude extract from *C. tiglium* seed after Thai TDP (Method 3) 6.Crude extract from *C. tiglium* seed after Thai TDP (Method 4), analyzed using the solvent system dichloromethane: ethyl acetate: formic acid (6:4:0.1) and visualized under UV 254 nm (A), UV 366 nm (B), and anisaldehyde H_2SO_4/UV 366 nm (C)

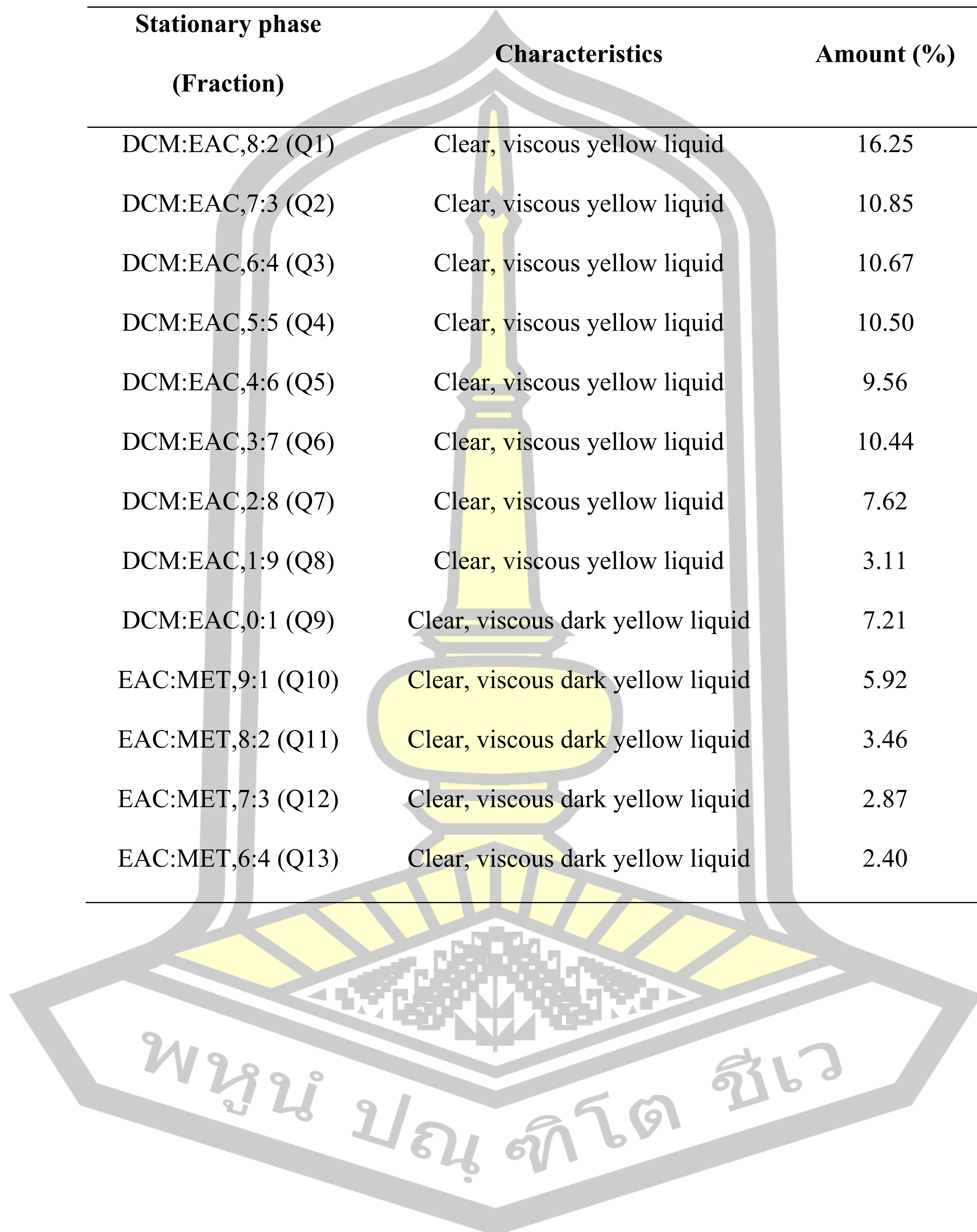
4.3 Isolation of occurring compound from *C. tiglium* seeds after Thai TDP

4.3.1 Extraction using quick column chromatography

10.00 grams sample of methanol extract from *C. tiglium* seeds after Thai TDP was subjected to quick column chromatography. The stationary phase used was Silica gel 60 (0.063-0.200 mm), and the mobile phase consisted of a dichloromethane: ethyl acetate (DCM: EAC) mixture in ratios ranging from 8:2 to 0:10, followed by a mixture of ethyl acetate: methanol (EAC:MET) in ratios from 9:1 to 6:4. Thirteen fractions (Q1-Q13) were collected. After evaporating the solvents using a rotary evaporator, the fractions were concentrated. Fraction 1 contained the highest amount of crude extract, while fraction 13 had the least. The other fractions had varying amounts, as shown in Table 4.9.

Table 4.9 Amount of crude extract obtained from quick column chromatography

Stationary phase (Fraction)	Characteristics	Amount (%)
DCM:EAC,8:2 (Q1)	Clear, viscous yellow liquid	16.25
DCM:EAC,7:3 (Q2)	Clear, viscous yellow liquid	10.85
DCM:EAC,6:4 (Q3)	Clear, viscous yellow liquid	10.67
DCM:EAC,5:5 (Q4)	Clear, viscous yellow liquid	10.50
DCM:EAC,4:6 (Q5)	Clear, viscous yellow liquid	9.56
DCM:EAC,3:7 (Q6)	Clear, viscous yellow liquid	10.44
DCM:EAC,2:8 (Q7)	Clear, viscous yellow liquid	7.62
DCM:EAC,1:9 (Q8)	Clear, viscous yellow liquid	3.11
DCM:EAC,0:1 (Q9)	Clear, viscous dark yellow liquid	7.21
EAC:MET,9:1 (Q10)	Clear, viscous dark yellow liquid	5.92
EAC:MET,8:2 (Q11)	Clear, viscous dark yellow liquid	3.46
EAC:MET,7:3 (Q12)	Clear, viscous dark yellow liquid	2.87
EAC:MET,6:4 (Q13)	Clear, viscous dark yellow liquid	2.40



4.4 Structural elucidation of the compound

4.4.1 ¹H NMR spectroscopic data

When the compound isolated from the column was analyzed for its chemical structure using NMR spectroscopy, it was dissolved in chloroform prior to the analysis. The ^{1}H NMR spectrum revealed that the purified compound isolated from *C. tiglium* seed after Thai TDP is likely a member of the phthalic acid group, based on a comparison of the NMR spectral data as shown in Figure 4.11.

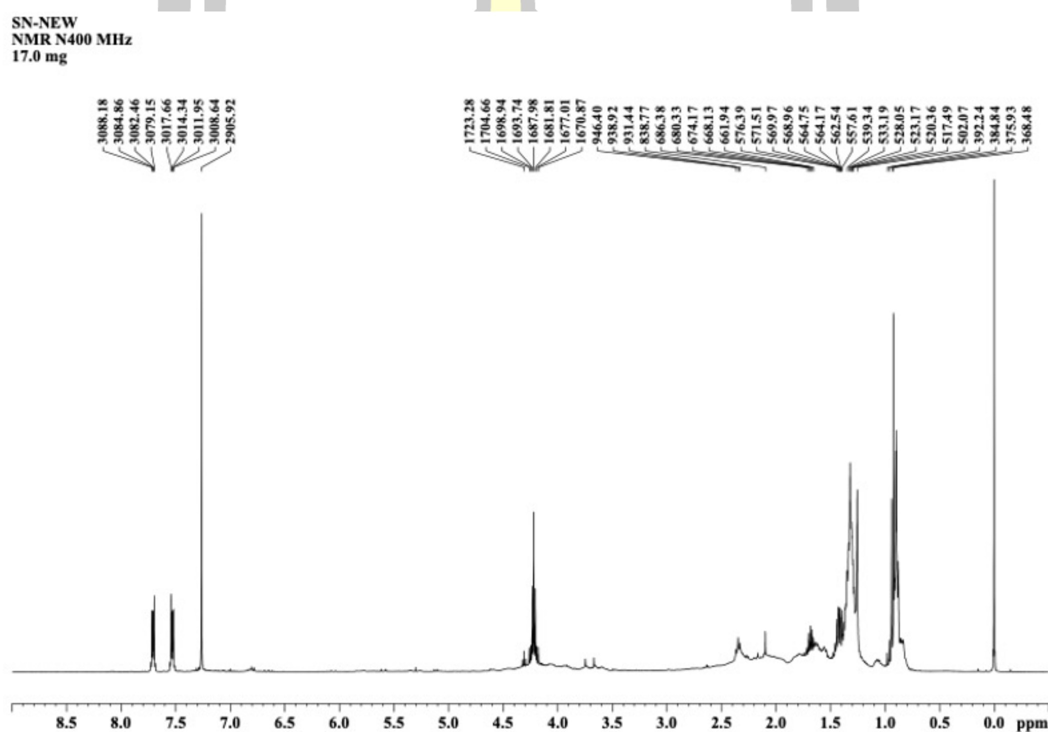


Figure 4.11 ^{1}H NMR spectroscopic data

The ^{1}H NMR spectrum of the isolated compound shows distinct signals consistent with the expected structure of Di-(2-ethylhexyl) phthalate. The signal at δ 0.84–0.92 ppm (triplet) is attributed to terminal methyl protons, while the broad multiple between δ 1.25–1.75 ppm corresponds to multiple methylene (–CH_2–) groups along the alkyl chains. The signals at δ 2.1–2.3 ppm likely represent methylene groups

situated near electronegative groups such as esters or oxygen. Additionally, the signals at δ 3.6–4.2 ppm represent CH_2 groups adjacent to oxygen atoms, indicating ester linkages. Finally, the multiples at δ 7.2–7.7 ppm confirm the presence of aromatic protons from the phthalate moiety. These observations strongly support the presence of a symmetrical diester structure as shown in Figure 4.11.

4.4.2 ^{13}C NMR and DEPT-135 spectroscopic data

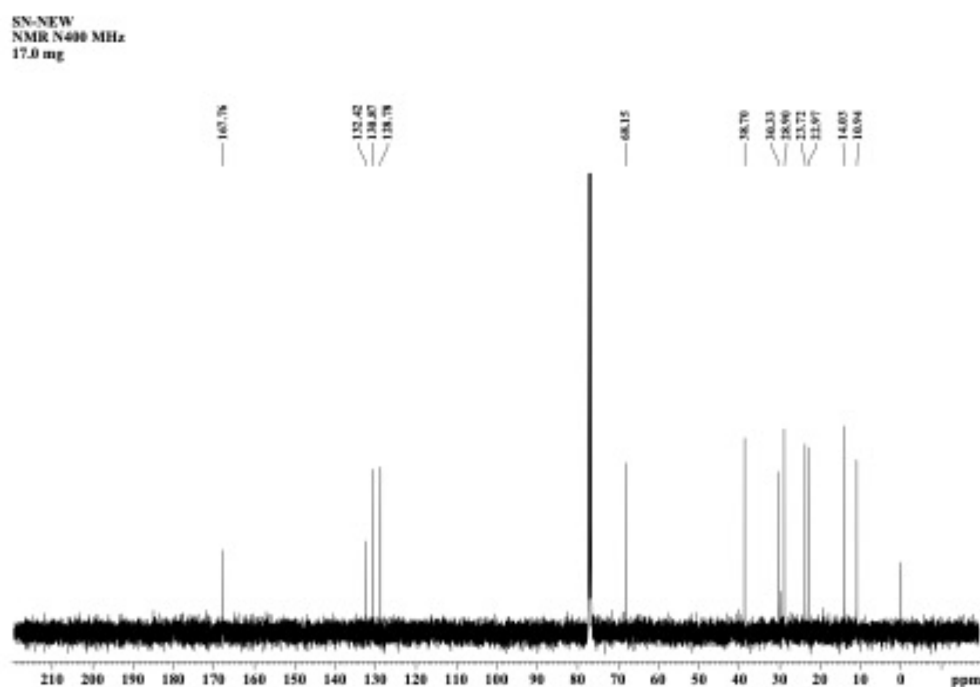


Figure 4.12 ^{13}C NMR spectroscopic data

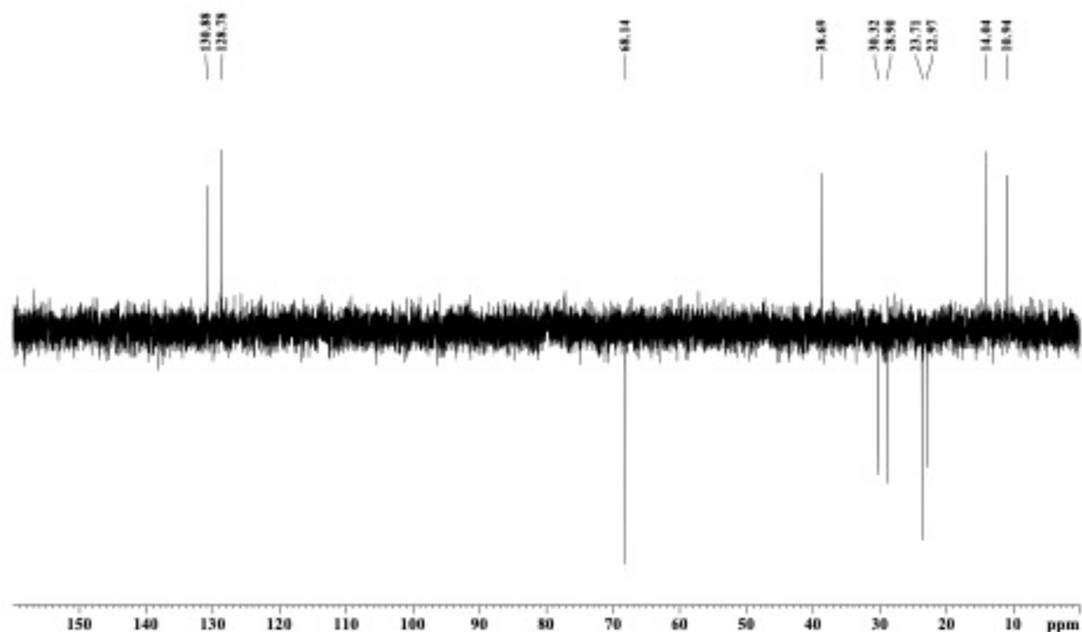


Figure 4.13 DEPT-135 spectroscopic data

The ^{13}C NMR and DEPT-135 spectra further confirm the identity of the isolated compound. Methyl carbons appear at δ 10.94 and 14.03 ppm, while methylene carbons in the alkyl chains appear between δ 22.97 and 30.32 ppm. The carbon bonded to oxygen in the ester moiety shows a chemical shift at δ 68.14 ppm. The aromatic carbons appear as expected at δ 128.78–132.42 ppm, with the ester carbonyl carbon appearing at δ 167.76 ppm as a quaternary carbon (no DEPT signal). The DEPT-135 results help distinguish between CH_3, CH_2, and CH carbons, offering unambiguous assignments consistent with the structure of Di-(2-ethylhexyl) phthalate as shown in Figure 4.12-4.13.

4.4.3 2D NMR spectroscopic analysis

The results of 2D-NMR, COSY, HSQC, HMBC and NOESY, as well as NMR spectral data of previously reported were also used for identification. These data, reinforced by COSY and HMBC interactions, provide strong support for the complete assignment and structural integrity of the Di-(2-ethylhexyl) Phthalate (DEHP) molecule as shown in Figure 4.14 and Table 4.10.

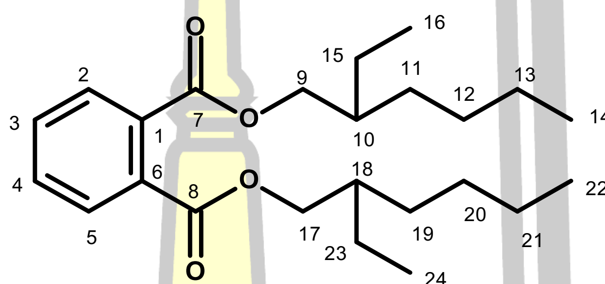


Figure 4.14 (+)-HRESIMS: *m/z* 391.2850 $(M+H)^+$, calculated for $C_{24}H_{39}O_4$ (391.284972)

Table 4.10 ^{1}H and ^{13}C NMR data of di-(2-ethylhexyl) phthalate ($CDCl_3$, 400 and 100 MHz)

Position	δ_C, type	δ_H (*J* in Hz)*	COSY	HMBC
1 (6)	132.4, C	-		
2 (5)	128.8, CH	7.71, dd (5.8, 3.3)	H-3	
3 (4)	130.9, CH	7.53, dd (5.8, 3.3)	H-2	
7 (8)	167.8, CO	-		
9 (17)	68.2, CH_2	4.82, m	H-10	C-11, 15,
10 (18)	38.7, CH	1.70, m	H-9	C-9, 12
11 (19)	30.3, CH_2	1.35, m		C-12
12 (20)	28.9, CH_2	1.32, m		
13 (21)	23.0, CH_2	1.30, m		
14 (22)	14.0, CH_3	0.90, t (6.9)		C-12, 13,
15 (23)	23.7, CH_2	1.42, m		
16 (24)	10.9, CH_3	0.92 t (7.5)		C-C-10, 15

4.4.4 Mass analysis of isolated compound

It was assigned the molecular formula $C_{24}H_{39}O_4$ on the basis of the HRESIMS: m/z 391.2850 (M+H)+, calculated 391.284972. The result of HRESIMS: m/z 391.2850 (M+H)+ was showed in Figure 4.15.

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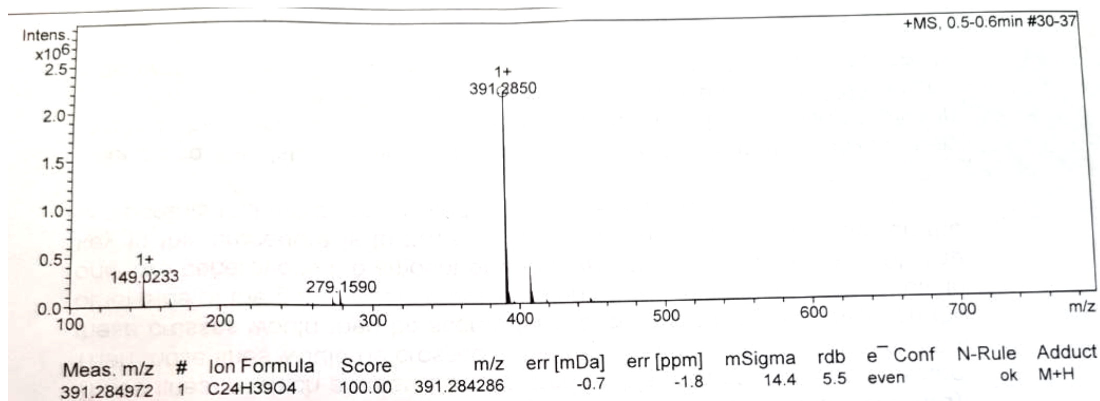

Figure 4.15 Mass analysis of an isolated compound

4.4.5 Chemical structure of Di-(2-ethylhexyl) phthalate

From the data of 1D and 2D NMR spectra and its high-resolution mass spectrum, as well as comparison of its NMR spectral data with those previously reported, it was assigned Di-(2-ethylhexyl) Phthalate showed in Figure 4.16.

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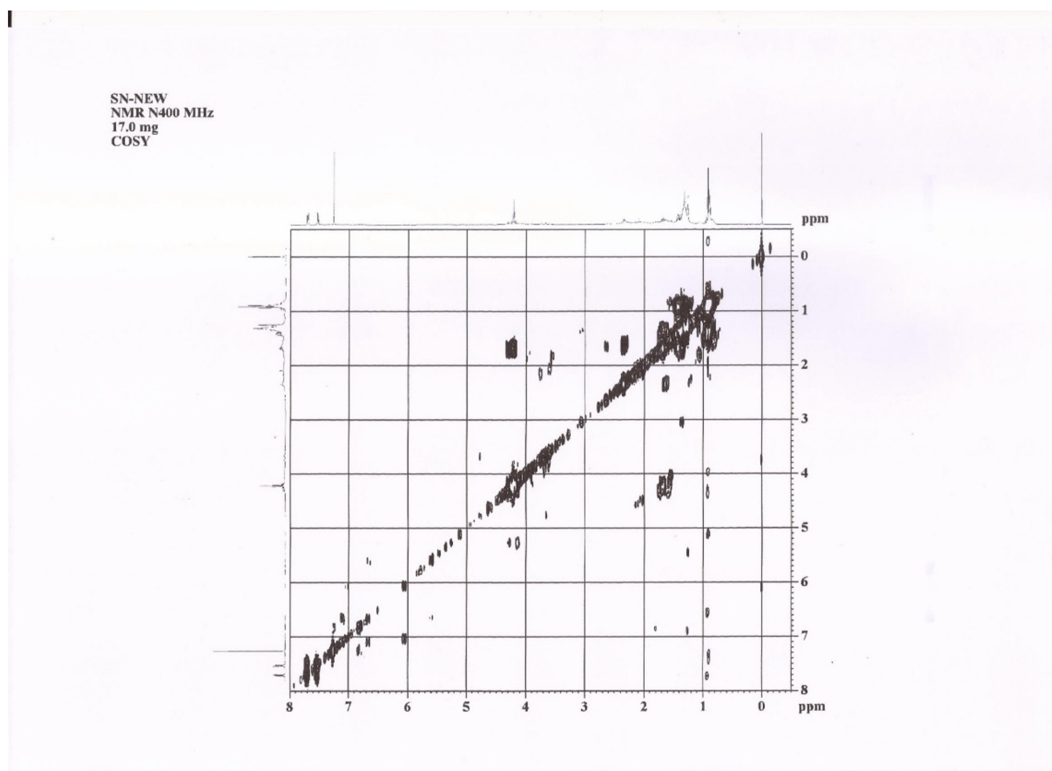
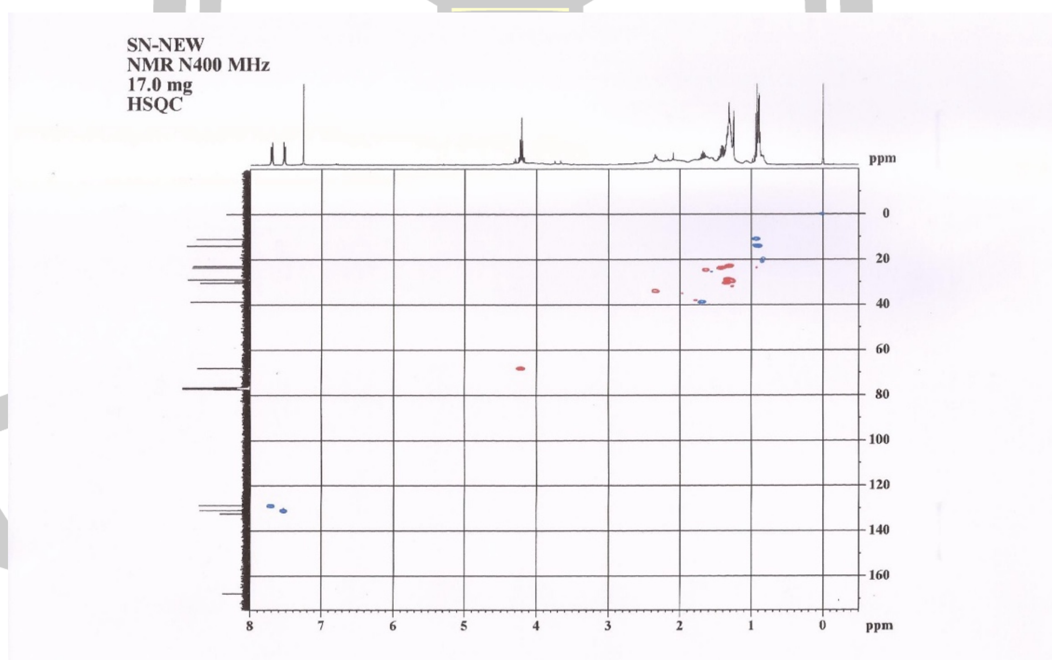


(A)



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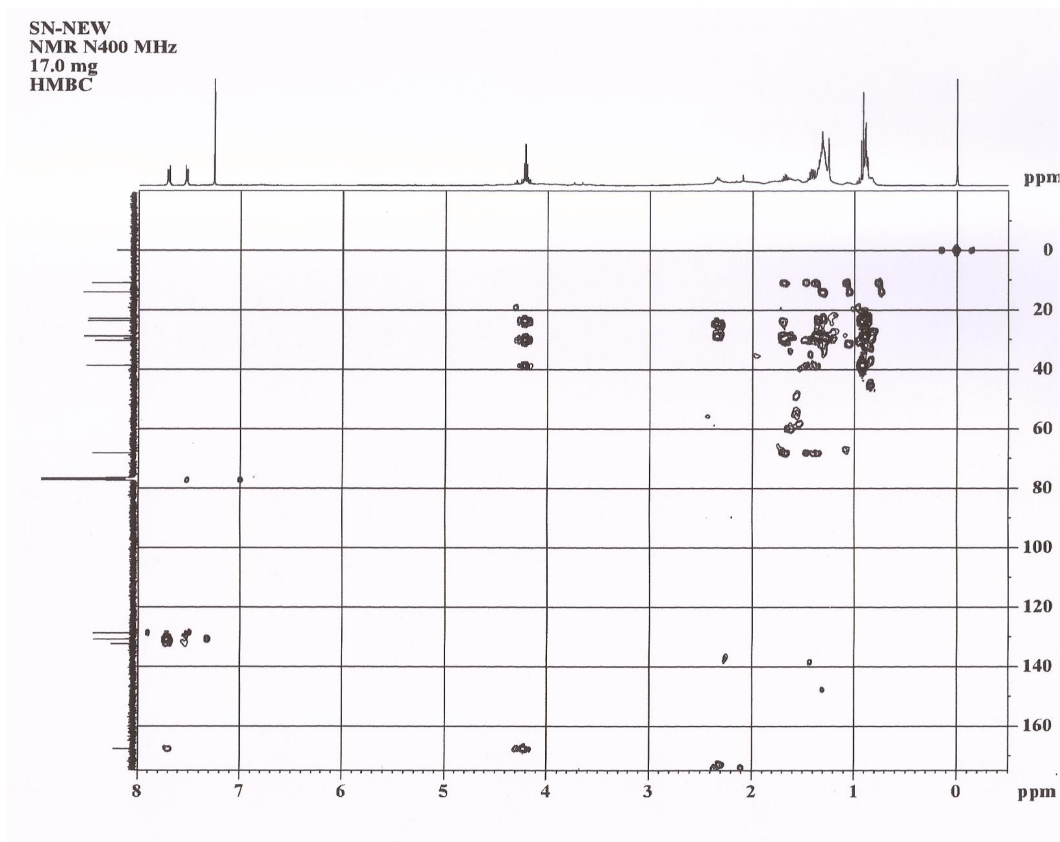

(C)

Figure 4.16 COSY spectrum of Di-(2-ethylhexyl)phthalate ($CDCl_3$, 400 MHz). (A), HSQC spectrum of Di-(2-ethylhexyl)phthalate ($CDCl_3$, 400 MHz). (B), HMBC spectrum of Di-(2-ethylhexyl)phthalate ($CDCl_3$, 400 MHz) (C).

4.5 Pharmacological activity assays

Pharmacological activity testing was divided into four experiments:

- Laxative effect testing in experimental animals.

- Acute toxicity testing in experimental animals.

- Anticancer activity testing in vitro.

- Carcinogenesis induction testing

4.5.1 Laxative effect testing in experimental animals

4.5.1.1 General information

The laxative effect was tested using male Wistar rats aged approximately 6 weeks, weighing 150–200 grams, with 48 rats in total. The rats were housed in metabolic cages in an animal testing room maintained at a temperature of 23 ± 3°C, relative humidity of 60 ± 20%, and a 12-hour light/dark cycle. They were fed standard diets and provided water *ad libitum*. Before the experiment, the rats were acclimated for one week. The rats were divided into eight groups, with six rats per group, and were orally administered different substances as follows:

1) Group 1: distilled water (vehicle control).

2) Group 2: castor oil (0.3 mL/animal) (positive control).

3) Groups 3–5: crude (undetoxify) *C. tiglium* powder at doses of 10, 50, and 100 mg/kg, (Pre-10, Pre-50 and Pre-100), respectively.

4) Groups 6–8: *C. tiglium* seed powder detoxify at doses of 10, 50, and 100 mg/kg, (Post-10, Post-50, Post-100) respectively. The detoxify *C. tiglium* seed powder used in the experiment was prepared according to processing method 1 (Topic 3.2.4.3 in Chapter 3). Briefly, *C. tiglium* seeds were dehulled, and 50 g of seeds were boiled with 150 g of paddy rice, 100 g of salt, and 2,000 mL of water in an earthen pot until the rice expanded completely, taking approximately 3 hours.

The body weight of each rat was measured before and after the test. Food consumption, water intake and general behavior of the rats were observed and recorded daily. Concurrently, wet and dry fecal pellets were also weighed and counted. The results were shown in Figure 4.17 and Table 4.11.

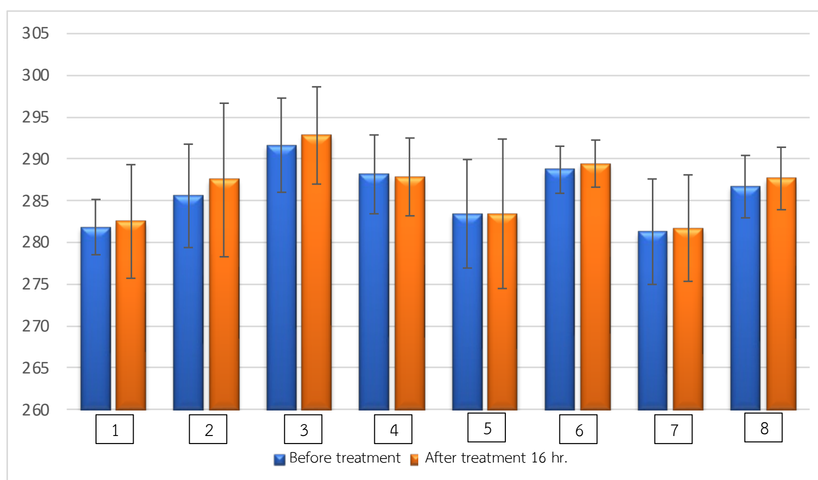

Figure 4.17 Graph comparing the average body weight of rats before and after the 16-hour experimental period (y-axis is body weight (g), x-axis is Group). Rats were divided into 8 groups, Paired sample t-test were used for statistical analysis.
1. Vehicle control. 2. Positive control. 3.*C. tiglium* powder before Thai TDP at 10 mg/kg. 4. *C. tiglium* powder before Thai TDP at 50 mg/kg. 5. *C. tiglium* powder before Thai TDP at 100 mg/kg. 6.*C. tiglium* powder after Thai TDP at 10 mg/kg. 7. *C. tiglium* powder after Thai TDP at 50 mg/kg. 8. *C. tiglium* powder after Thai TDP at 100 mg/kg.

Figure 4.17 illustrates the rats' body weights at baseline (pre-experiment) and 16 hours post-experiment. The initial body weights of the rats ranged from 281.24–291.63 g. At 16-hour after the experiment, most of the rats showed weight gain. The rats that received 10 mg/kg of unprocessed *C. tiglium* powder at 10 mg/kg, gained the maximum weight, increasing by 2.28 ± 0.351 g. However, the rats that were given 100 mg/kg of *C. tiglium* seeds powder before Thai TDP at 100 mg/kg showed the greatest weight loss, reducing by 0.33 ± 0.52 g. Groups 2, 3, 4, and 7 showed statistically significant weight changes ($P<0.05$) according to the One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis. (Table 4.11).

Table 4.11 Food and water consumption of the rats in each group after 16 hours.

Group (each N=6)	**General Data (Mean ±SD)**					
	Weight change after 16 hours (grams)		**Food consumption (grams)**		**Water intake (grams)**	
1) VC	+0.70	±0.65	10.67	±0.12	37.62±	.17
2) pre-10	+2.28**	±0.51	9.00	±0.34	37.84±	.40
3) pre-50	+2.37**	±0.67	9.67	±0.61	42.50±	.36
4) pre-100	-0.33**	±0.52	7.33	±0.67	32.63±	.26
5) post-10	-0.02	±0.65	8.00	±0.89	31.94±	.73
6) post-50	+2.38	±0.81	10.00	±0.15	40.45±	.50
7) post-100	+0.79**	±0.58	11.00	±0.68	31.79±	.27
8) PC	+0.95	±0.99	10.67	±0.42	46.73±	.58

*P<0.05, **P<0.01 compared to control group. The One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis.
Vehicle control group, Group receiving *C. tiglium* powder before Thai TDP at 10 mg/kg (Pre-10), Group receiving *C. tiglium* powder before Thai TDP at 50 mg/kg (Pre-50), Group receiving *C. tiglium* powder before Thai TDP at 100 mg/kg (Pre-100), Group receiving *C. tiglium* powder at after Thai TDP 10 mg/kg (Post-10), Group receiving *C. tiglium* powder after Thai TDP at 50 mg/kg (Post-50), Group receiving *C. tiglium* powder after Thai TDP at 100 mg/kg (Post-100) and Positive control group (PC).

The group with the highest food intake was post-100, consuming 11.00 ± 0.68 g, followed by the vehicle control and positive control groups, which consumed 10.67 ± 1.12 g and 10.67 ±0.42 g, respectively (Table 4.10). The group with the lowest food intake was Pre-100, consuming 7.33 ± 0.67 g. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test showed no significant differences compared to the control group.

For water consumption, the group consuming the highest amount was the positive control group, at 46.73 ± 6.58 g, followed by Pre-50, Post-50, Pre-10, the vehicle control, Pre-100, and Post-10, which consumed 42.50 ± 1.36, 40.45 ± 2.50, 37.84 ± 5.40, 37.62 ± 5.17, 32.63 ± 6.26, and 31.94 ± 4.73 g, respectively (Table 4.11). The group with the lowest water consumption was Post-100, consuming 31.79 ± 1.27 g. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test revealed no significant differences compared to the control group.

4.5.1.2 Fecal quantity

The examination of fecal quantity included counting the number of fecal pellets, measuring the weight of wet feces, determining the weight of dry feces, and calculating the water content in the feces. The results were obtained at 8 and 16 hours after the administration of the substances to each group of rats.

The average number of fecal pellets at 8 hours after administration showed that the group with the highest fecal pellet count was the Pre-100 group, with 8.17±0.31 pellets. The group with the lowest count was the group receiving distilled water, with 4.83±0.40 pellets. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test revealed statistically significant differences between groups, as shown in Table 4.12.

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The average wet fecal weight indicated that the group with the highest wet fecal weight was the group receiving castor oil (positive control), with a weight of 2.99±0.17 grams. The group with the lowest wet fecal weight was the group receiving distilled water, with a weight of 1.65±0.11 grams. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test showed no significant differences between groups, as shown in Table 4.12.

The average dry fecal weight showed that the group with the highest dry fecal weight was the Pre-100 group, with a fecal weight of 1.29±0.12 grams. The group with the lowest dry fecal weight was the Pre-50 group, with a weight of 0.93±0.22 grams. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test showed no significant differences between groups, as shown in Table 4.12.

The group with the highest water content percentage was the positive control group, with the % water content of 56.69±2.41. The group with the lowest water content percentage was the vehicle control group, with the % water content of 38.69±4.18. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test showed no significant differences between groups, as shown in Table 4.12.

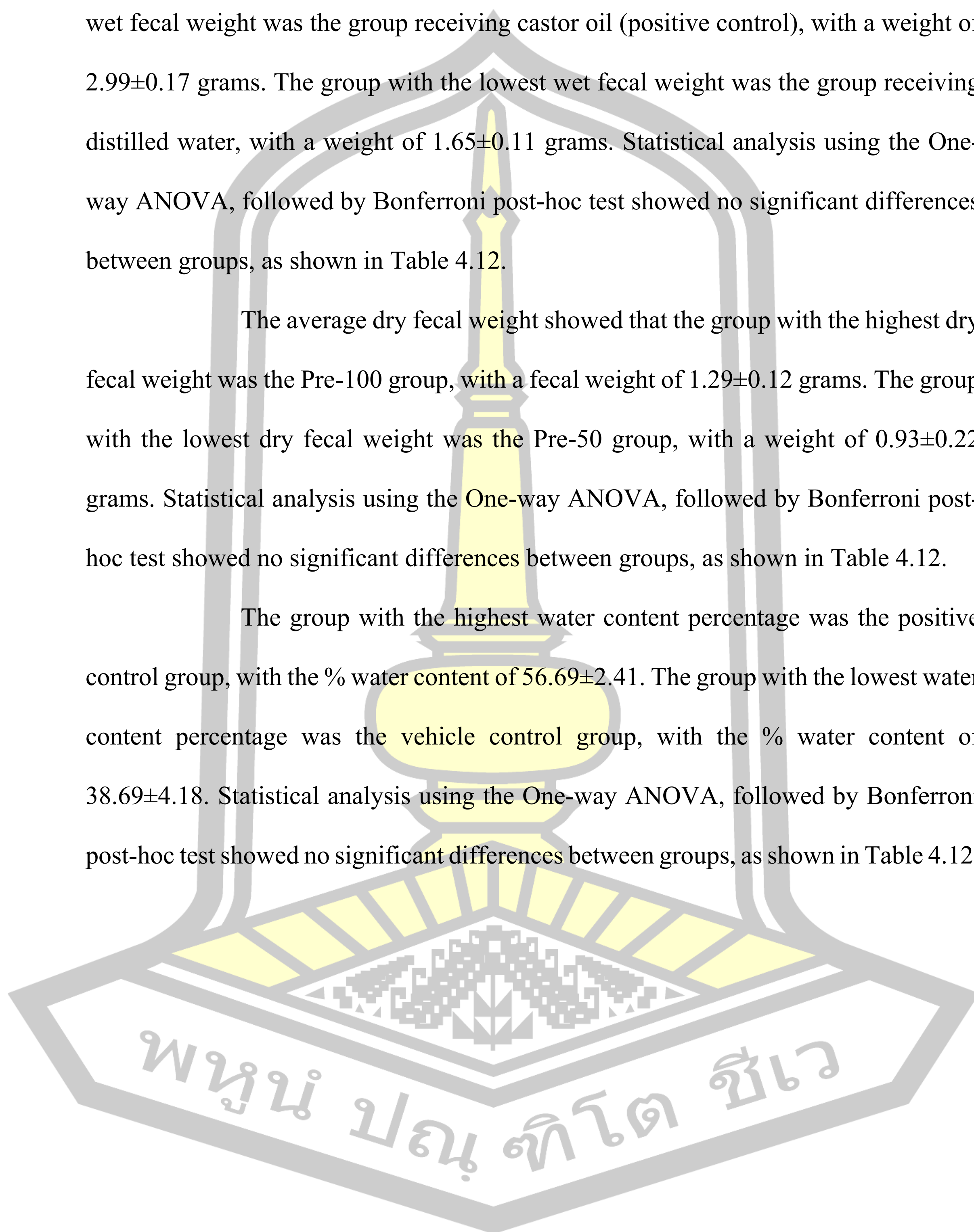

Table 4.12 Number of fecal pellets, fecal weight and water content in feces of the rats at 8-hour after the administration of the test agents

Group (N=6)	Number of fecal pellets (Mean ± SEM) (pellets)		Wet fecal weight (Mean ± SEM) (grams)		Dry fecal weight (Mean ± SEM) (grams)		Water content (Mean ± SEM) (%)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
VC	4.83	0.40	1.65	0.11	0.99	0.05	38.69	4.18
pre-10	5.83	0.79	1.88	0.40	1.01	0.20	44.92	2.14
pre-50	6.33	0.67	2.05	0.53	0.93	0.22	48.37	8.33
pre-100	8.17**	0.31	2.87	0.27	1.29	0.12	55.09	2.34
post-10	5.83	0.70	1.86	0.22	1.08	0.13	41.35	2.59
post-50	6.67	0.67	1.98	0.14	1.09	0.06	44.01	4.39
post-100	7.50	0.56	2.32	0.15	1.21	0.05	47.15	2.69
PC	6.83	0.40	2.99	0.17	1.28	0.05	56.69	2.41

*P<0.05, **P<0.01 compared to the control group. The One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis.
Vehicle control group, Group receiving *C. tiglium* powder before Thai TDP at 10 mg/kg (Pre-10), Group receiving *C. tiglium* powder before Thai TDP at 50 mg/kg (Pre-50), Group receiving *C. tiglium* powder before Thai TDP at 100 mg/kg (Pre-100), Group receiving *C. tiglium* powder at after Thai TDP 10 mg/kg (Post-10), Group receiving *C. tiglium* powder after Thai TDP at 50 mg/kg (Post-50), Group receiving *C. tiglium* powder after Thai TDP at 100 mg/kg (Post-100) and Positive control group (PC).

The mean number of fecal pellets at 16-hour after the administration showed that the group with the highest number of fecal pellets was the positive control group, with the fecal pellets of 19.83 ± 0.87. The group with the lowest number of fecal pellets was the Post-10 group, with the fecal pellets of 15.67 ± 1.02. Statistical analysis

using the One-way ANOVA, followed by Bonferroni post-hoc test revealed no significant differences between groups, as shown in Table 4.13.

The mean wet fecal weight revealed that the group with the highest wet fecal weight was the Pre-10 group, with the fecal weight of 5.31 ± 0.29 grams. The group with the lowest wet fecal weight was the vehicle control group, with the fecal weight of 3.57 ± 0.17 grams. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test showed significant differences between groups, as shown in Table 4.13.

The mean dry fecal weight indicated that the group with the highest dry fecal weight was the Pre-100 group, with the dry fecal weight of 2.40 ± 0.15 grams. The group with the lowest dry fecal weight was the vehicle group, with the dry fecal weight of 2.15 ± 0.06 grams. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test revealed no significant differences between groups, as shown in Table 4.13.

The group with the highest percentage of water content was the Pre-100 group, with the % water content of 54.63 ± 2.39. The group with the lowest percentage of water content was the vehicle control group, with the % water content of 34.81 ± 2.97. Statistical analysis using the One-way ANOVA, followed by Bonferroni post-hoc test indicated significant differences between groups, as shown in Table 4.13.

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Table 4.13 Number of fecal pellets, fecal weight and water content in feces of the rats at 16-hour after the administration of the test agents

Group	Number of fecal pellets (Mean ± SEM) (pellets)		Wet fecal weight (Mean ± SEM) (grams)		Dry fecal weight (Mean ± SEM) (grams)		Water content (Mean ± SEM) (%)	
VC	16.67	0.80	3.57	0.17	2.15	0.06	34.81	2.97
Cpre-10	16.50	1.73	4.05	0.45	2.26	0.26	44.28	2.47
Cpre-50	17.50	2.57	4.57	0.48	2.34	0.19	44.30	2.23
Cpre-100	18.17	1.33	5.31*	0.29	2.40	0.15	54.63**	2.39
Post-10	15.67	1.28	3.62	0.33	2.14	0.29	41.72	3.72
Post-50	16.50	0.67	3.81	0.36	2.24	0.31	42.48	4.58
Post-100	15.67	1.02	4.07	0.29	2.25	0.24	46.00	4.32
PC	19.83	0.87	4.66	0.25	2.33	0.10	49.61	2.75

*P<0.05, **P<0.01 compared to the control group. The One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis.
Vehicle control group, Group receiving *C. tiglium* powder before Thai TDP at 10 mg/kg (Pre-10), Group receiving *C. tiglium* powder before Thai TDP at 50 mg/kg (Pre-50), Group receiving *C. tiglium* powder before Thai TDP at 100 mg/kg (Pre-100), Group receiving *C. tiglium* powder at after Thai TDP 10 mg/kg (Post-10), Group receiving *C. tiglium* powder after Thai TDP at 50 mg/kg (Post-50), Group receiving *C. tiglium* powder after Thai TDP at 100 mg/kg (Post-100) and Positive control group (PC).

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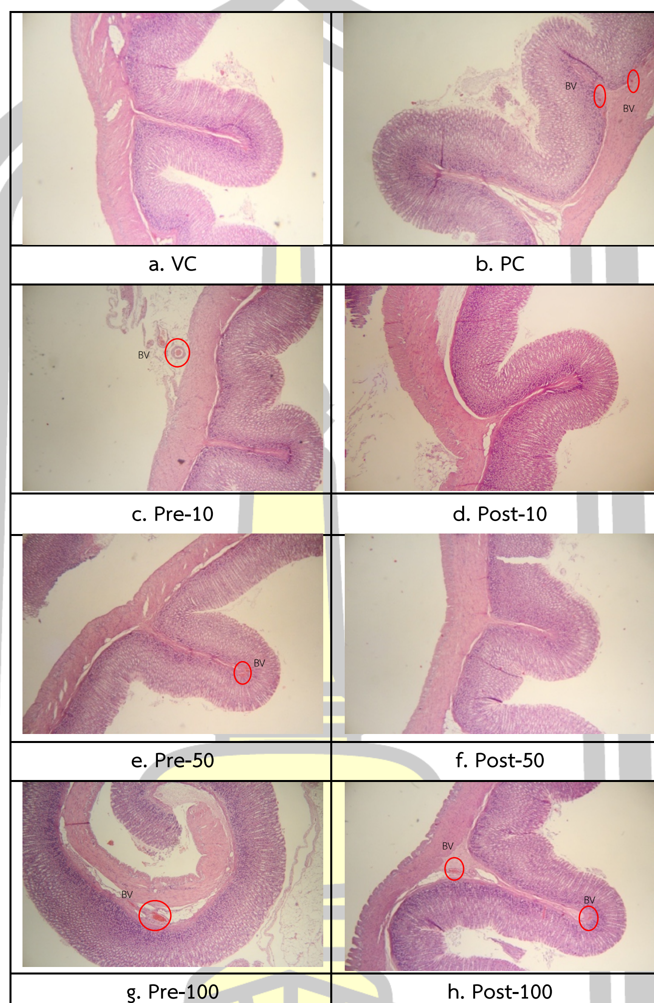
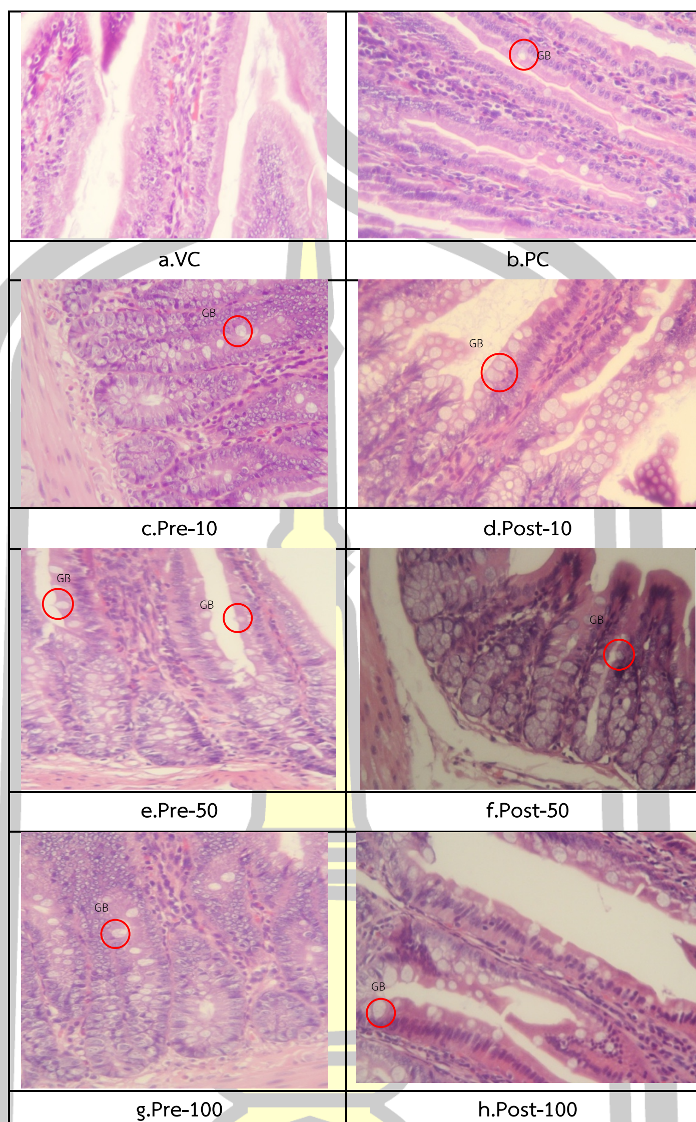
4.5.1.3 Pathological observations of the tissues obtained from stomach, small intestine and colon



*BV, Blood vessels (scale 100X)

Figure 4.18 The effects of *C. tiglium* powder on stomach tissues in Wistar rats stained with hematoxylin and eosin (H&E) at 100x magnification.
a. Vehicle control group, b. Positive control group (PC), c. Group receiving *C. tiglium* powder before Thai TDP at 10 mg/kg (Pre-10), d. Group receiving *C. tiglium* powder before Thai TDP at 50 mg/kg (Pre-50), e. Group receiving *C. tiglium* powder before Thai TDP at 100 mg/kg (Pre-100), f. Group receiving *C. tiglium* powder after Thai TDP at 10 mg/kg (Post-10), g. Group receiving *C. tiglium* powder after Thai TDP at 50 mg/kg (Post-50), h. Group receiving *C. tiglium* powder after Thai TDP at 100 mg/kg (Post-100).

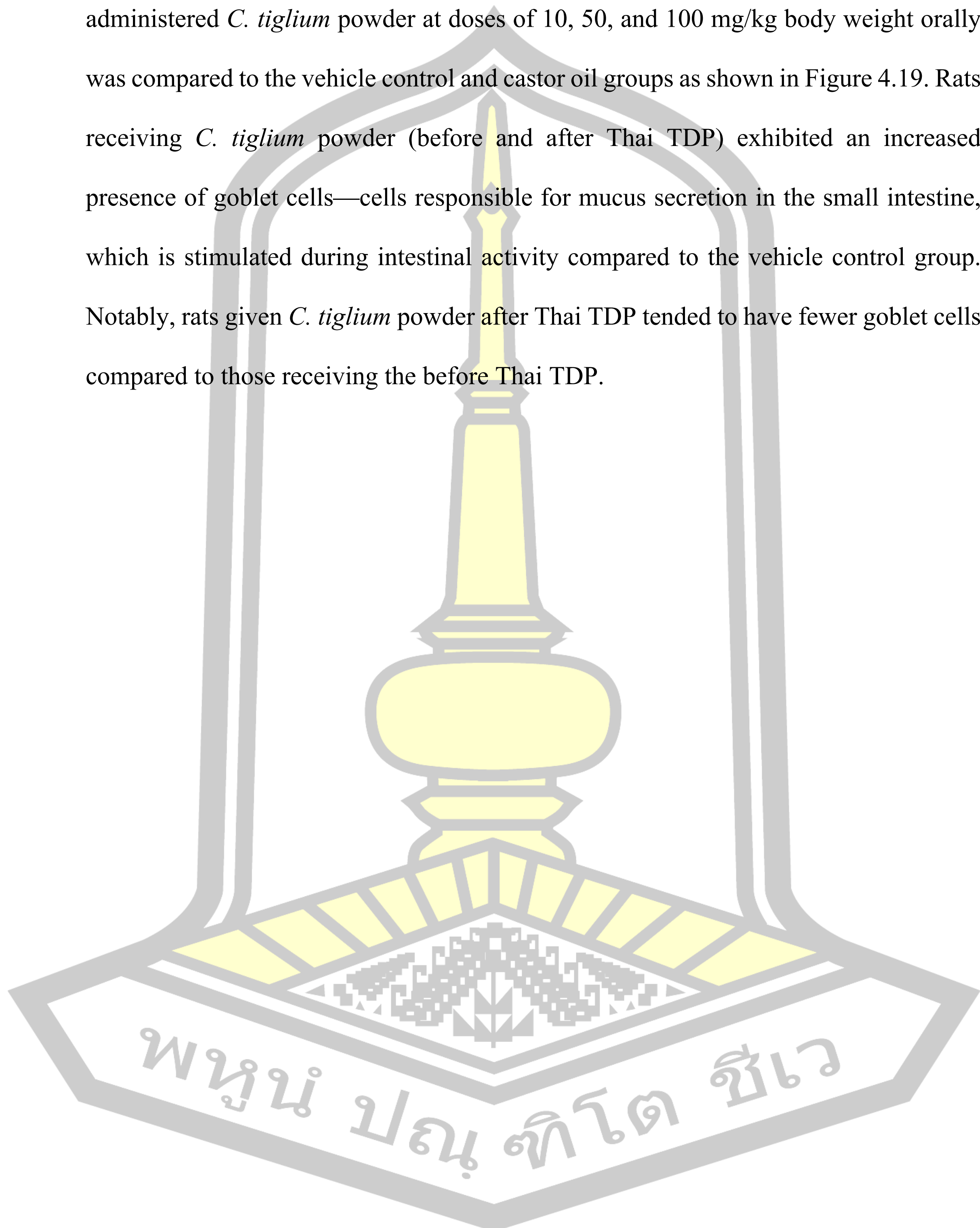
Pathological examinations of stomach tissues after oral administration of *C. tiglium* powder at doses of 10, 50, and 100 mg/kg were compared with the distilled water control and castor oil groups, as shown in Figure 4.18. The presence of blood vessels (BV) containing red blood cells in the submucosa, indicating inflammation in the gastric tissue were observed in Pre-10, Pre-50, Pre-100 as well as Post-100 groups.

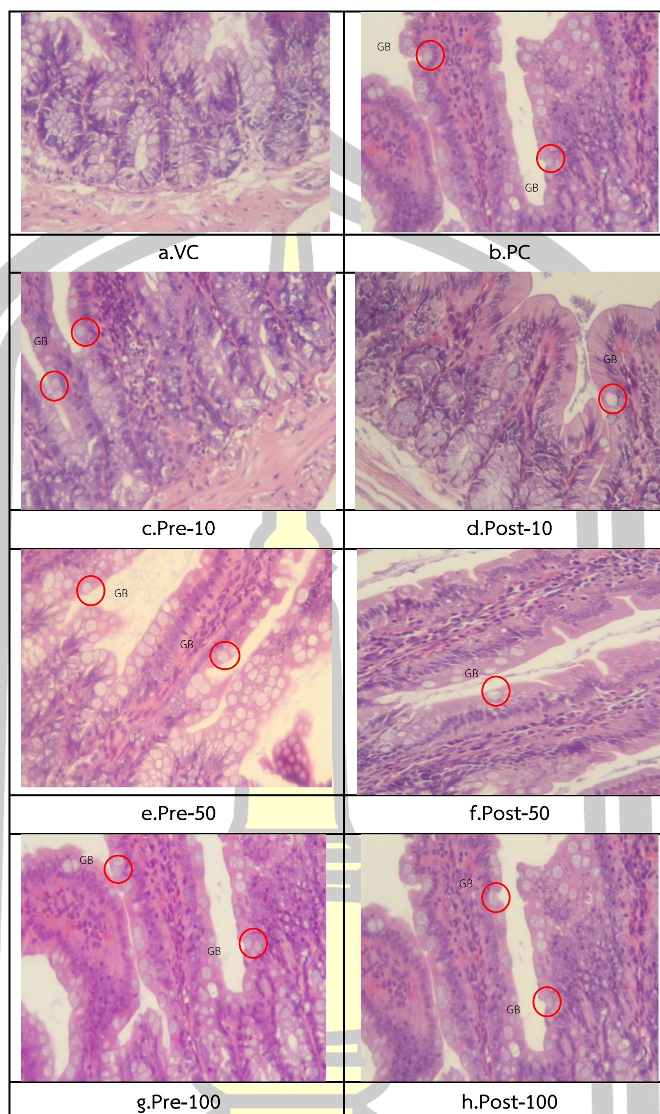


*GB, goblet cell (scale 100X)

Figure 4.19 The effects of *C. tiglium* powder on the small intestine of Wistar rats stained with Hematoxylin and Eosin (H&E) at 100x Magnification:
a. Vehicle control group, b. Positive control group (PC), c. Group receiving *C. tiglium* powder before Thai TDP at 10 mg/kg (Pre-10), d. Group receiving *C. tiglium* powder before Thai TDP at 50 mg/kg (Pre-50), e. Group receiving *C. tiglium* powder before Thai TDP at 100 mg/kg (Pre-100), f. Group receiving *C. tiglium* powder after Thai TDP at 10 mg/kg (Post-10), g. Group receiving *C. tiglium* powder after Thai TDP at 50 mg/kg (Post-50), h. Group receiving *C. tiglium* powder after Thai TDP at 100 mg/kg (Post-100)

The histopathological examination of small intestine tissues in rats administered *C. tiglium* powder at doses of 10, 50, and 100 mg/kg body weight orally was compared to the vehicle control and castor oil groups as shown in Figure 4.19. Rats receiving *C. tiglium* powder (before and after Thai TDP) exhibited an increased presence of goblet cells—cells responsible for mucus secretion in the small intestine, which is stimulated during intestinal activity compared to the vehicle control group. Notably, rats given *C. tiglium* powder after Thai TDP tended to have fewer goblet cells compared to those receiving the before Thai TDP.





*GB, goblet cell (scale 100X)

Figure 4.20 The effects of *C. tiglium* powder on colonic tissues in Wistar rats stained with hematoxylin and eosin (H&E) at 100x magnification.
a. Vehicle control group, b. Positive control group (PC), c. Group receiving *C. tiglium* powder before Thai TDP at 10 mg/kg (Pre-10), d. Group receiving *C. tiglium* powder before Thai TDP at 50 mg/kg (Pre-50), e. Group receiving *C. tiglium* powder before Thai TDP at 100 mg/kg (Pre-100), f. Group receiving *C. tiglium* powder after Thai TDP at 10 mg/kg (Post-10), g. Group receiving *C. tiglium* powder after Thai TDP at 50 mg/kg (Post-50), h. Group receiving *C. tiglium* powder after Thai TDP at 100 mg/kg (Post-100).

Pathological examinations of colonic tissues after oral administration of *C. tiglium* powder at doses of 10, 50, and 100 mg/kg were compared with the distilled water control and castor oil groups (Figure 4.20). The presence of goblet cells (GB), which play a role in mucus secretion in the colon was observed in both unprocessed and processed *C. tiglium* powder groups. The colon stimulation by *C. tiglium* powder increased mucus secretion of the colon when compared to the distilled water control. The group receiving processed powder exhibited a tendency for a lower number of goblet cells compared to the group receiving unprocessed powder.

4.5.2 Acute toxicity testing in experimental animals

The acute oral toxicity test was conducted following the OECD Guidelines for the Testing of Chemicals (OECD 420), which evaluates the toxicity of chemical substances. The study was divided into two experiments:

1. Acute oral toxicity: fixed dose procedure - sighting study.
2. Acute oral toxicity: fixed dose procedure - main study.

4.5.2.1 Acute oral toxicity: fixed dose procedure - sighting study

1) General information:

The acute oral toxicity test was performed in female Wistar rats, aged approximately 10 weeks, weighing between 191-235 grams. The test included 5–7 rats housed in sterile cages within a controlled environment at the temperature of 23 ± 3°C, with relative humidity at 60 ± 20% and a 12-hour light/dark cycle. The rats were provided standard food and water *ad libitum* throughout the study. They underwent a one-week acclimatization period before the experiment.

The test began with a dose of 300 mg/kg of *C. tiglium* seed powder administered to one rat per group. Since no toxicity was observed, a higher dose of 2,000 mg/kg was tested, as detailed below:

Experimental Groups:

1. Vehicle control group (distilled water).
2. *C. tiglium* seed powder before Thai TDP at 300 mg/kg.
3. *C. tiglium* seed powder before Thai TDP at 300 mg/kg.
4. *C. tiglium* seed powder after Thai TDP at 2,000 mg/kg.
5. *C. tiglium* seed powder after Thai TDP at 2,000 mg/kg.

The body weight of the rats was recorded before and after the 14-day test period, and their feeding and drinking behaviors were also observed.

After 14 days, the body weight gain was observed across all groups. The highest weight gain (36.01 g) was seen in the group receiving detoxified powder at 300 mg/kg, followed by the vehicle control group (32.69 g), as shown in Table 4.14.

Food and water consumption:

The group with the highest food consumption (125 g) was the control group. The lowest food consumption (85 g) was observed in the group receiving detoxified powder at 2,000 mg/kg. The group with the highest water consumption (454 mL) was the group receiving detoxified powder at 300 mg/kg, while the lowest (340 mL) was in the group receiving detoxified powder at 2,000 mg/kg.

Survival and Behavioral Observations:

All rats in the 300 mg/kg dose groups survived with no abnormalities in feeding or drinking behaviors. At 2,000 mg/kg, all rats also survived; however, they exhibited lethargy, reduced physical activity, and decreased walking/running during the first two days. The body weight and feeding behaviors in these groups showed lower trends compared to the vehicle control group, as shown in Table 4.14.

Table 4.14 Body weight, food consumption, water intake and mortality of the rats in the sighting study

Group	**Day 0 Body weight (g)**	**Day 14 Body weight (g)**	**Food consumption (g)**	**Water intake (g)**	**Mortality**
pre-300	203.12	234.53	115	448	No
Post-300	194.38	230.39	121	454	No
Pre-2000	208.54	235.58	113	360	No
Post-2000	195.02	222.22	85	340	No
VC	202.11	234.80	125	480	No

P<0.05, ** P<0.01 when compared with the control group. The paired sample t-test analysis was used for statistical analysis.
Group receiving *C. tiglium* powder before Thai TDP at 300 mg/kg (Pre-300), Group receiving *C. tiglium* powder after Thai TDP at 300 mg/kg (Post-300), Group receiving *C. tiglium* powder before Thai TDP at 2000 mg/kg (Pre-2000), Group receiving *C. tiglium* powder after Thai TDP at 2000 mg/kg (Post-2000), control group.

2) Organ size and weight

The test agents at doses of 300 and 2,000 mg/kg were administered to the rats for 14 days. Subsequently, the rats were euthanized under anesthesia using isoflurane, and their organs were examined. The results are presented in Table 4.15. The sizes and weights of other organs showed no significant differences, except for the thymus and spleen. The size of the thymus and spleen in the group receiving *C. tiglium* powder before and after Thai TDP at 300 mg/kg, as well as the group receiving *C. tiglium* powder before Thai TDP at 2,000 mg/kg, were significantly higher compared to the control groups.

Table 4.15 Size and weight of organs in each experimental group of rats from the sighting study

No.	Sample	Brain		Heart		Thymus		Lung		Liver		Stomach		Spleen		L. Adrenal		R. Adrenal		L. Kidney		R. Kidney	
		Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)	Size (cm)	Weight (g)
1	pre-300	2.20	1.77	1.80	0.81	1.80*	0.51	2.40	0.52	5.40	8.71	2.23	1.34	3.40	0.44	0.50	0.04	0.50	0.03	1.50	0.87	1.60	0.86
2	Post-300	2.20	1.78	1.70	0.76	1.80*	0.53	2.00	0.38	5.80	9.14	2.10	1.22	3.40	0.53	0.50	0.04	0.40	0.04	1.60	0.87	1.60	0.84
3	pre-2000	2.40	1.84	1.60	0.77	1.60	0.57	2.00	0.40	4.90	8.15	2.10	1.24	3.50*	0.49	0.40	0.03	0.50	0.03	1.80	0.85	1.70	0.86
4	Post-2000	2.40	1.52	1.60	0.87	1.70	0.55	1.90	0.39	5.10	8.35	2.10	1.24	3.30	0.42	0.50	0.04	0.50	0.03	1.60	0.79	1.60	0.82
5	VC	2.40	1.76	1.70	0.78	1.50	0.54	2.10	0.40	5.80	9.26	2.20	1.33	3.10	0.40	0.40	0.04	0.40	0.03	1.60	0.80	1.70	0.87

*$P<0.05$, ** $P<0.01$ when compared with the control group. The paired sample t-test analysis was used for statistical analysis.
Group receiving *C. tiglium* powder before Thai TDP at 300 mg/kg (Pre-300), Group receiving *C. tiglium* powder after Thai TDP at 300 mg/kg (Post-300), Group receiving *C. tiglium* powder before Thai TDP at 2000 mg/kg (Pre-2000), Group receiving *C. tiglium* powder after Thai TDP at 2000 mg/kg (Post-2000), Control group.

4.5.2.2 Acute oral toxicity testing (fixed dose procedure: main study)

1) General information

The acute oral toxicity test was conducted in 15 female Wistar rats, approximately 10 weeks old, weighing between 215-249 grams. The rats were housed in sterile cages in a laboratory room with a temperature of 23 ± 3°C, relative humidity of 60 ± 20%, and a 12-hour light/dark cycle. They had *ad libitum* access to commercial food and drinking water throughout the study. Prior to the experiment, the rats were allowed to acclimatize for 1 week. The rats were then divided into three groups, each consisting of 5 rats, and were administered the test substance at a dose of 2,000 mg/kg. The details are as follows:

Group 1: *C. tiglium* seed before Thai TDP at a dose of 2,000 mg/kg

Group 2: *C. tiglium* seed after Thai TDP at a dose of 2,000 mg/kg

Group 3: Received distilled water (vehicle control)

The rats were weighed before and after the test, and their eating behavior was observed for 14 days. The results are as follows:

Weight of rats after 7 days: The weights of the rats in all three groups ranged from 231.34 to 233.77 grams. The group that gained the most weight was the distilled water group, with an average increase of 9.27 ± 2.01 grams. The group that received *C. tiglium* seed after Thai TDP an average of 7.01 ± 1.51 grams, while the group that received *C. tiglium* seed before Thai TDP an average of 0.73 ± 2.07 grams. The body weight gain in the *C. tiglium* seed after Thai TDP group was significantly less than the control group ($P<0.05$).

Weight of rats after 14 days: After 14 days, the body weight of the rats ranged from 241.10 to 246.97 grams. The distilled water group gained the

greatest body weight, with an average increase of 20.40 ± 2.39 grams. The *C. tiglium* seed after Thai TDP group gained an average of 14.26 ± 2.12 grams, while the *C. tiglium* seed before Thai TDP group gained an average of 10.78 ± 0.54 grams. Statistical analysis showed a significant difference in the body weight gain of the *C. tiglium* seed before Thai TDP group compared to the control group ($p<0.05$), as shown in Table 4.16.

Food intake during the first 7 days: The group that consumed the most food was the distilled water group, which ate an average of 17.71 ± 0.61 grams per day. The group that consumed the least was the *C. tiglium* seed before group, which ate an average of 14.00 ± 0.53 grams per day. Statistical analysis showed no significant differences in food intake during the first week.

Food intake from day 8-14: The control group that received distilled water consumed an average of 17.88 ± 0.41 grams per day, while the group receiving *C. tiglium* seed after Thai TDP consumed an average of 17.00 ± 1.27 grams per day. There were no significant differences in food intake between the groups during this period, as shown in Table 4.16.

Water intake from day 1-7: The group that drank the most water was the *C. tiglium* seed after Thai TDP group, with an average intake of 34.10 ± 8.14 milliliters per day.

Water intake from day 8-14: The group that drank the most water from Day 8 onward was the *C. tiglium* seed before Thai TDP group, with an average intake of 33.11 ± 3.26 milliliters per day. However, statistical analysis showed no significant differences in water intake between the groups during both the first and second weeks, as shown in Table 4.16.

Toxicity observations: No rats died after receiving the 2,000 mg/kg dose. However, all three groups exhibited lethargy, reduced physical activity, and decreased movement (walking/running) during the first two days. Additionally, the rats in all groups showed a trend of lower weight gain and food intake compared to the control group that received distilled water, as shown in Table 4.16.

Table 4.16 General characteristics and mortality of experimental rats in each group in the main study

Data	**Group**		
	Pre-2,000 (N=5)	**Post-2,000 (N=5)**	**VC (N=5)**
Body weight (g) Day 0	230.31±5.05	232.71±5.17	224.49±4.78
Body weight (g) Day 7	231.04±3.60	239.80±4.35	233.77±6.25
Change in body weight (g) at Day 7	(+) 0.73±2.07**	(+) 7.01±1.51	(+) 9.27±2.01
Body weight (g) Day 14	241.10±5.02	246.97±4.85	244.89±5.42
Change in body weight (g) at Day 14	(+) 10.79±0.54**	(+) 14.26±2.12	(+) 20.40±2.39
Feed intake (g) Day 7	14.00±0.53	17.00±2.42	17.71±0.61
Feed intake (g) Day 14	17.02±0.46	17.00±1.27	17.88±0.41
Water intake (g) Day 7	33.31±5.76	34.10±8.14	29.51±3.21
Water intake (g) Day 14	33.11±3.26	30.31±2.30	28.86±2.21
Mortality	NO	NO	NO

*P<0.05, ** P<0.01 when compared with the control group. The paired sample t-test analysis were used for statistical analysis.

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2) Hematological and biochemical parameters

At 14 days after the treatment, the rats were anesthetized using isoflurane until fully sedated, by using a standard anesthesia machine with a nose cone connected to the rat's nostrils. The isoflurane concentration was set higher than the normal dose, greater than 5%, whereas the usual concentration is 3-4%. Blood samples were then taken from the rat's heart to measure hematological and biochemical blood parameters using an automatic hematology analyzer. The results are as follows:

2.1) Hematological data

White blood cell (WBC) count: The groups that received *C. tiglium* seed before and after Thai TDP had WBC counts ranging from 2.18±0.16 to 2.66±0.34 x10^3/mL, lower than the control group, which had a WBC count of 3.16±0.32 x103/mL.

Red blood cell (RBC) count: The two experimental groups had average RBC counts ranging from 7.04±0.10 to 7.43±0.11 x10^3/mL, which were quite similar to the control group's RBC count of 7.27±0.11 x10^3/mL (Table 4.17).

Hemoglobin concentration (HGB): The experimental groups receiving both forms of *C. tiglium* seed powder showed HGB values ranging from 13.00±0.33 to 13.40±0.13 g/dL, similar to the control group, which had a value of 13.46±0.27 g/dL.

Hematocrit (HCT) was between 37.97±0.67 and 38.78±0.48%.

Platelet (PLT) count: Both experimental groups had higher PLT counts, ranging from 931.20±45.66 to 1,018.10±140.88 x10^3/mL, with the

after Thai TDP group showing the highest increase. The control group had a PLT count of 907.40±41.49 x10^3/mL (Table 4.17).

Mean corpuscular volume (MCV) of both experimental groups was similar, ranging from 52.20±0.54 to 54.62±0.56 fL. The Mean cell hemoglobin (MCH) ranged from 18.04±0.11 to 18.72±0.16 pg. Statistical analysis of MCV and MCH showed a significant decrease compared to the control group ($P<0.05$).

Mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), and mean platelet volume (MPV) values were similar across all groups and did not differ significantly from the control group. MCHC ranged from 34.22±0.37 to 34.56±0.22 g/dL, RDW-SD from 25.90±0.44 to 27.60±1.16%, and MPV from 8.28±0.18 to 8.55±0.03 fL (Table 4.17).

Leukocyte differential counts (Eosinophils %, Lymphocytes %, Monocytes %) in rats that received *C. tiglium* seed powder before Thai TDP were 1.84±0.46%, 86.84±3.11%, and 2.16±0.65%, respectively. Rats receiving after processed *C. tiglium* seed powder had values of 1.20±0.48%, 85.30±4.81%, and 2.32±0.57%, respectively, which were not significantly different from the control group's values of 2.22±0.48%, 84.46±1.70%, and 2.12±0.40%, respectively. Statistical analysis showed no significant differences (Table 4.17).

2.2) Serum biochemistry data

Rats receiving both before and after Thai TDP *C. tiglium* seed powder had blood urea nitrogen levels of 21.20±1.24 and 21.20±1.06 mg/dL, respectively, and uric acid levels of 1.42±0.13 and 1.42±0.11 mg/dL, respectively. Total protein levels were 6.04±0.16 and 5.92±0.09 g/dL, respectively. Albumin levels

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were 4.62±0.19 and 4.42±0.11 g/dL, respectively, and total bilirubin (TB) levels were 0.10±0.00 mg/dL for both experimental groups.

Alkaline phosphatase (ALP) levels in the after Thai TDP *C. tiglium* seed powder group were higher than in the control group, at 81.50±12.55 IU/L. aspartate aminotransferase (AST) levels were higher in both experimental groups, at 77.20±2.35 and 79.80±5.05 IU/L, compared to the control group. Alanine transaminase (ALT) was higher in the before Thai TDP *C. tiglium* seed powder group, with a value of 27.60±2.97 IU/L. However, statistical analysis of biochemical values showed no significant differences between the groups (Table 4.18).

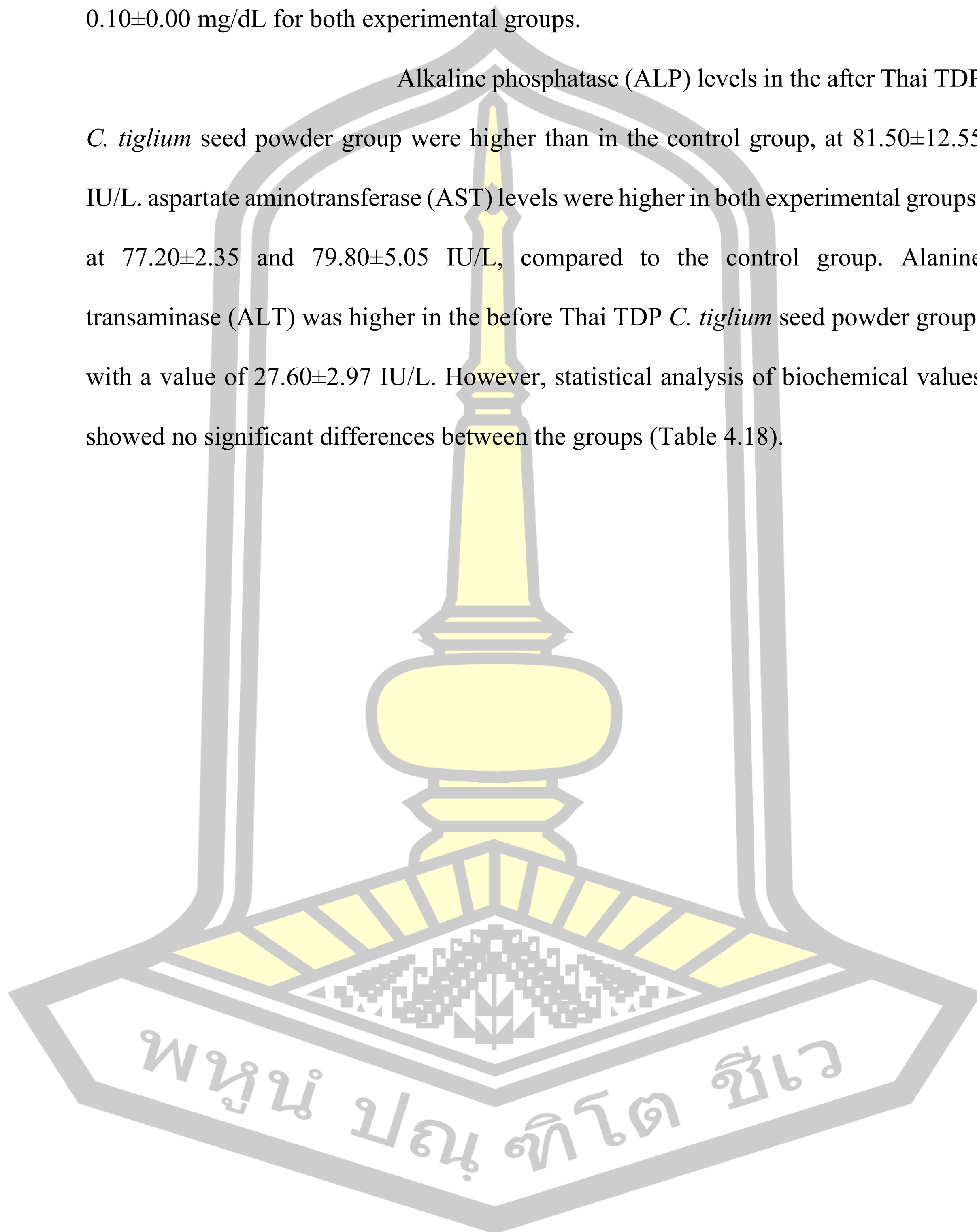

Table 4.17 Hematological data of the rats at 14 days after the administration of the test agents

	pre-2,000 (N=5)	Post-2,000 (N=5)	VC (N=5)
WBC (10^3/mL)	2.66±0.34	2.52±0.21	3.16±0.32
RBC (10^6/mL)	7.43±0.11	7.15±0.15	7.27±0.11
HGB (g/dL)	13.40±0.13	13.00±0.33	13.46±0.27
HCT (%)	38.78±0.48	37.97±0.67	39.16±0.75
PLT (10^3/mL)	976.20±49.92	1,018.10±140.88	907.40±41.49
MCV (fL)	52.20±0.54	53.05±0.75	53.84±0.27
MCH (pg)	18.04±0.11	18.15±0.18	18.50±0.11
MCHC (g/dL)	34.56±0.22	34.22±0.37	34.36±0.09
RDW-SD (%)	25.90±0.44	26.35±0.43	26.68±0.66
MPV (fL)	8.28±0.18	8.55±0.30	7.96±0.12
Neutrophils (%)	9.16±2.06	9.47±0.87	11.20±1.14
Eosinophils (%)	1.84±0.46	1.70±0.57	2.22±0.48
Basophils (%)	*N/A*	0.43±0.42	*N/A*
Lymphocytes (%)	86.84±3.11	85.30±4.81	84.46±1.70
Monocytes (%)	2.16±0.65	2.32±0.57	2.12±0.40

* P<0.05, ** P<0.01 when compared to the control group. The paired sample t-test analysis were used for statistical analysis, N/A means Not Available

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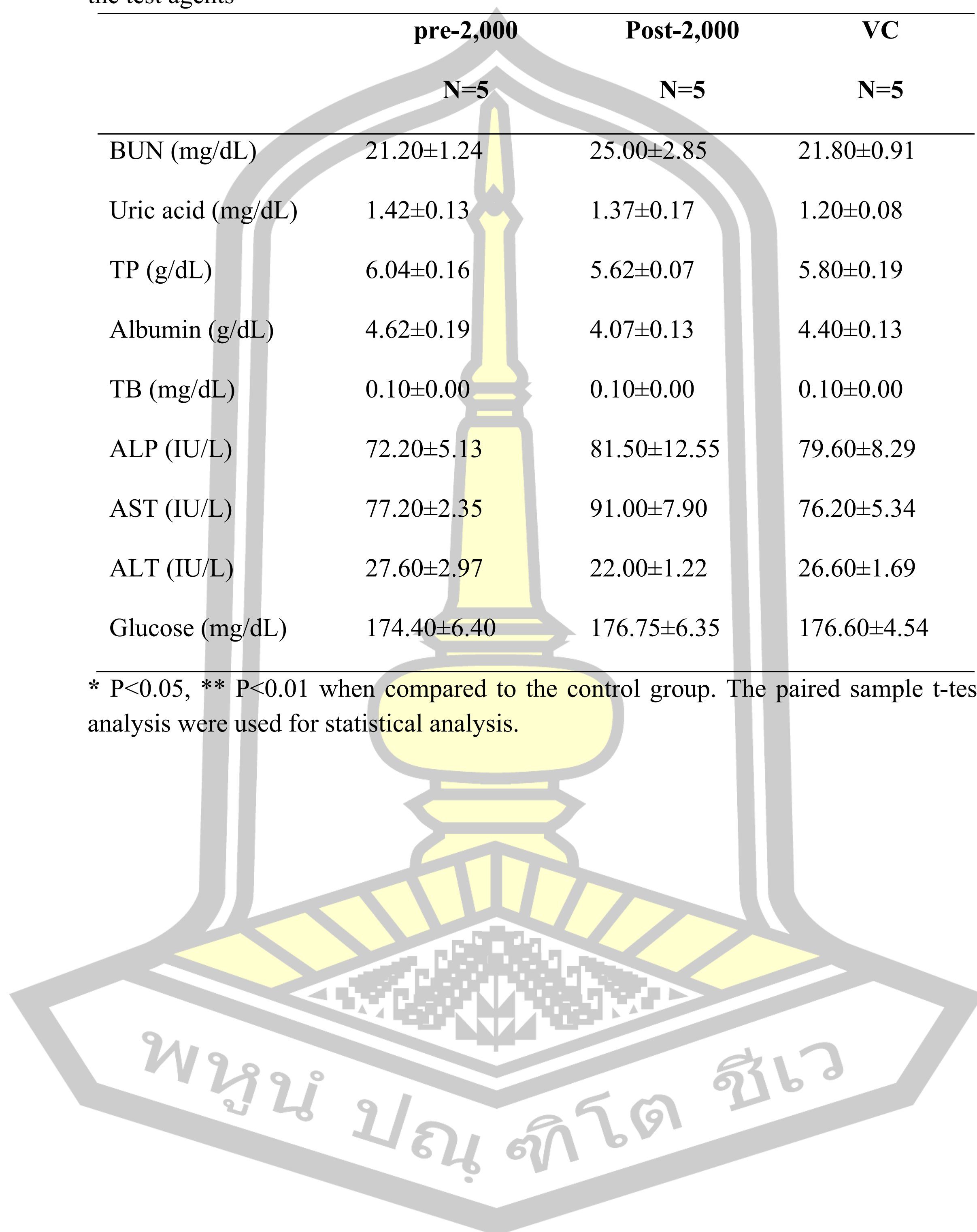
Table 4.18 Serum biochemistry data of the rats at 14 days after the administration of the test agents

	pre-2,000 **N=5**	**Post-2,000** **N=5**	**VC** **N=5**
BUN (mg/dL)	21.20±1.24	25.00±2.85	21.80±0.91
Uric acid (mg/dL)	1.42±0.13	1.37±0.17	1.20±0.08
TP (g/dL)	6.04±0.16	5.62±0.07	5.80±0.19
Albumin (g/dL)	4.62±0.19	4.07±0.13	4.40±0.13
TB (mg/dL)	0.10±0.00	0.10±0.00	0.10±0.00
ALP (IU/L)	72.20±5.13	81.50±12.55	79.60±8.29
AST (IU/L)	77.20±2.35	91.00±7.90	76.20±5.34
ALT (IU/L)	27.60±2.97	22.00±1.22	26.60±1.69
Glucose (mg/dL)	174.40±6.40	176.75±6.35	176.60±4.54

* $P<0.05$, ** $P<0.01$ when compared to the control group. The paired sample t-test analysis were used for statistical analysis.



3.) Histopathology of the organ tissues

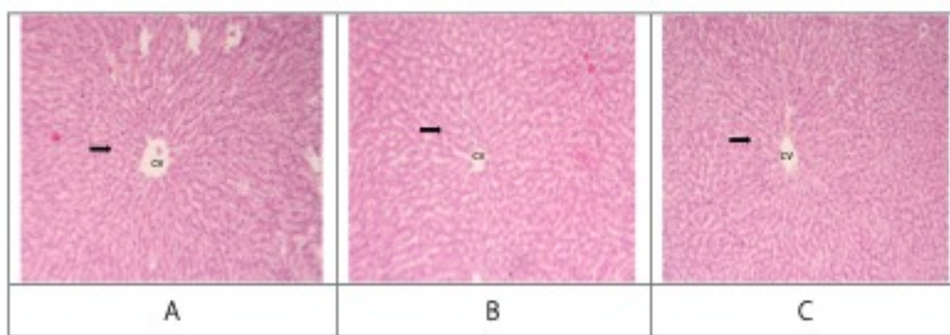


Figure 4.21 Histopathological image of the liver from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg, CV central vein; Arrows: hepatocytes

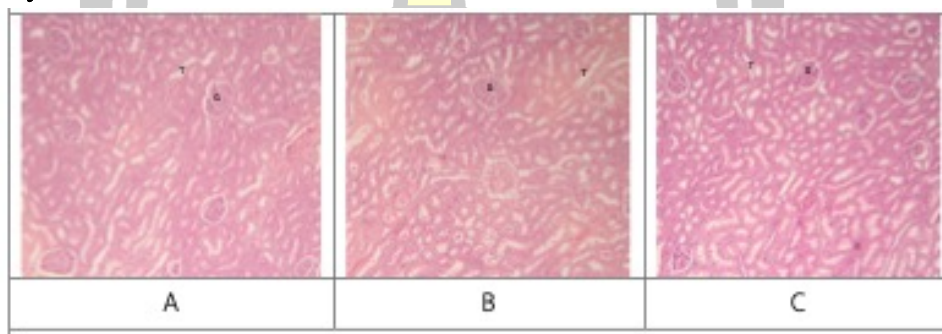


Figure 4.22 Histopathological images of the kidneys from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg, G: glomeruli; T: proximal and distal tubes

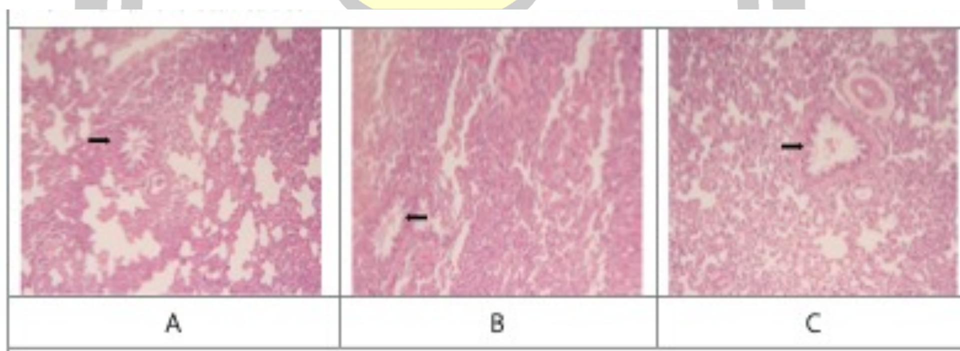


Figure 4.23 Histopathological images of the lungs from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg, ; Arrows: alveoli

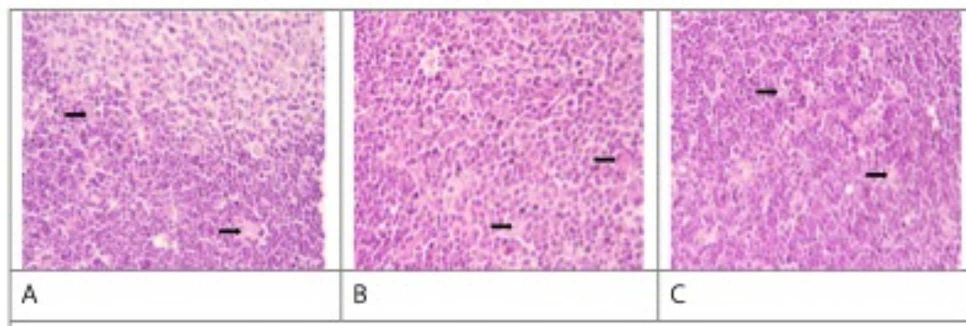


Figure 4.24 Histopathological images of the thymus from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg, ; Arrows: Hassall's corpuscle

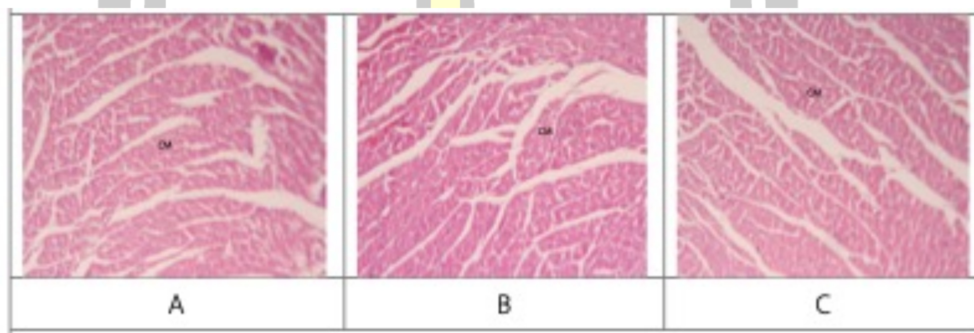


Figure 4.25 Histopathological images of the heart from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg, CM: cardiac muscle

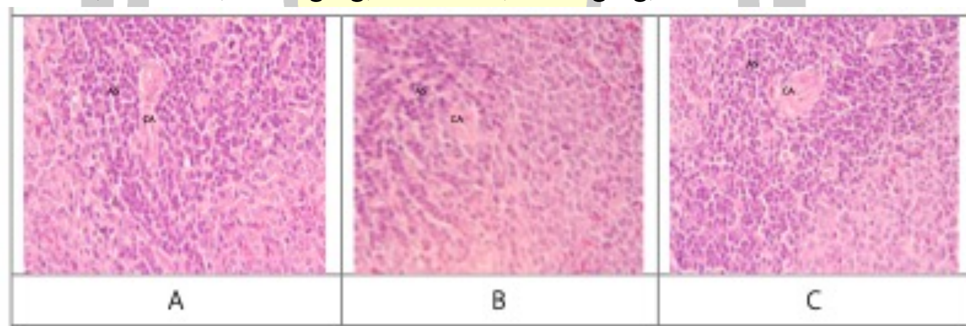


Figure 4.26 Histopathological images of the spleen from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg, ; AS: the periarteriolar sleeves; CA: The central arteries

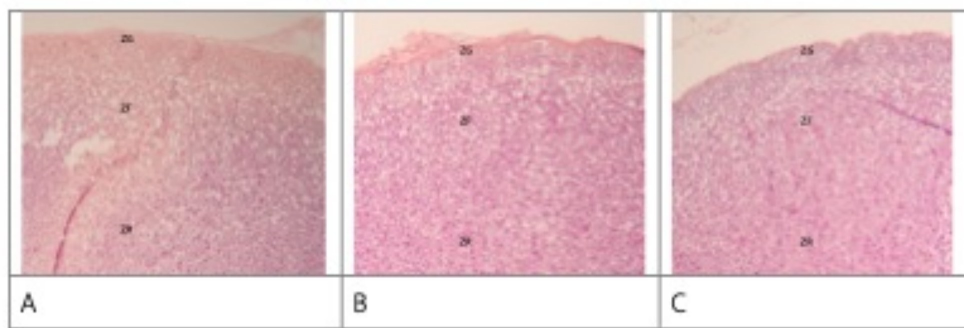


Figure 4.27 Histopathological images of the adrenal glands from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg ; ZG: zona glomerulosa; ZF: zona fasciculata; ZR: zona reticularis

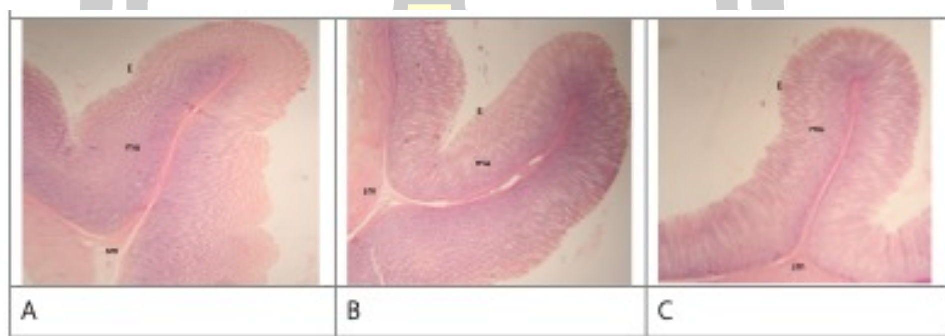


Figure 4.28 Histopathological images of the stomach from the acute oral toxicity study A: controls rats; B: Pre-2,000 mg/kg; C: Post-2,000 mg/kg; E; epithelium, mu; mucosa and sm; Submucosa

The results of the histopathological examination of various organ tissues, including the liver, kidneys, lungs, thymus, heart, spleen, adrenal glands, and stomach, after administering 2,000 mg/kg of *C. tiglium* seed powder orally to the experimental rats, compared with the control group receiving distilled water are shown in Figure 4.21-4.28.

The liver tissue in both the control group and the group receiving *C. tiglium* seed powder did not show any significant differences. No white fatty spots nor changes in the liver tissue were observed, indicating that the high dose of *C. tiglium* seed powder (2,000 mg/kg) did not cause liver toxicity (Figure 4.21).

Similarly, the high dose of *C. tiglium* seed powder (2,000 mg/kg) did not affect the anatomical structure of the kidneys (Figure 4.22). The examination revealed that the capillary appearance in the glomerulus and the sizes of the glomeruli in both the control group and the experimental group were similar, showing no differences.

Further histopathological examinations of other organs, including the lungs, thymus, heart, spleen, adrenal glands, and stomach, revealed no differences compared to the control group, and no pathologies indicating toxicity were found in the experimental animals.

4.5.3 Anticancer activity test *in vitro*

4.5.3.1 Survival rate of cancer cells treated with before and after Thai TDP *C. tiglium* seed extracts using MTT assay

Cancer cell lines, including lung cancer, colorectal cancer, and cholangiocarcinoma, were cultured at a density of approximately 2 x 10^4 cells in 200 µl per well in a 96-well culture plate, and incubated at 37°C with 5% CO_2 for 24 hours. Afterward, the cell culture medium was removed, and various concentrations (400, 200, 100, 50, 25, and 12.5 µg/mL) of before and after Thai TDP *C. tiglium* seed extracts were added. The cells were incubated at 37°C with 5% CO_2 for 24, 48, and 72 hours. The extract that showed the best inhibition of cancer cell growth was selected for further testing, with Gemcitabine as a positive control. The results of the tests were as follows:

The cytotoxicity test of the crude *C. tiglium* seed extract (before processing) showed that the inhibition of cancer cell growth depended on the concentration of the extract and the incubation time. The extract exhibited the strongest inhibition against cholangiocarcinoma cells of the KKU100 type at 72 hours with an

IC_{50} value of 111.1±4.027. The second most effective was the KKU213 cholangiocarcinoma cells with an IC_{50} of 113.4±6.379, while the HT-29 colorectal cancer cells showed the least inhibition with an IC_{50} value of 231.5±5.048, as shown in Table 4.19 and Figure 4.29 showing the percentage of cell growth.

The cytotoxicity test of the crude *C. tiglium* seed extract after Thai TDP also showed that inhibition of cancer cell growth was concentration and time dependent. The extract most effectively inhibited Caco-2 colorectal cancer cells at 72 hours with an IC_{50} value of 103.7±7.194. The second most effective inhibition was observed in KKU100 cholangiocarcinoma cells with an IC_{50} of 111.8±2.429, while H2228 lung cancer cells showed the least inhibition with an IC_{50} value of 173.2±2.760, as shown in Table 4.19 and Figure 4.29 showing the cell survival rate.

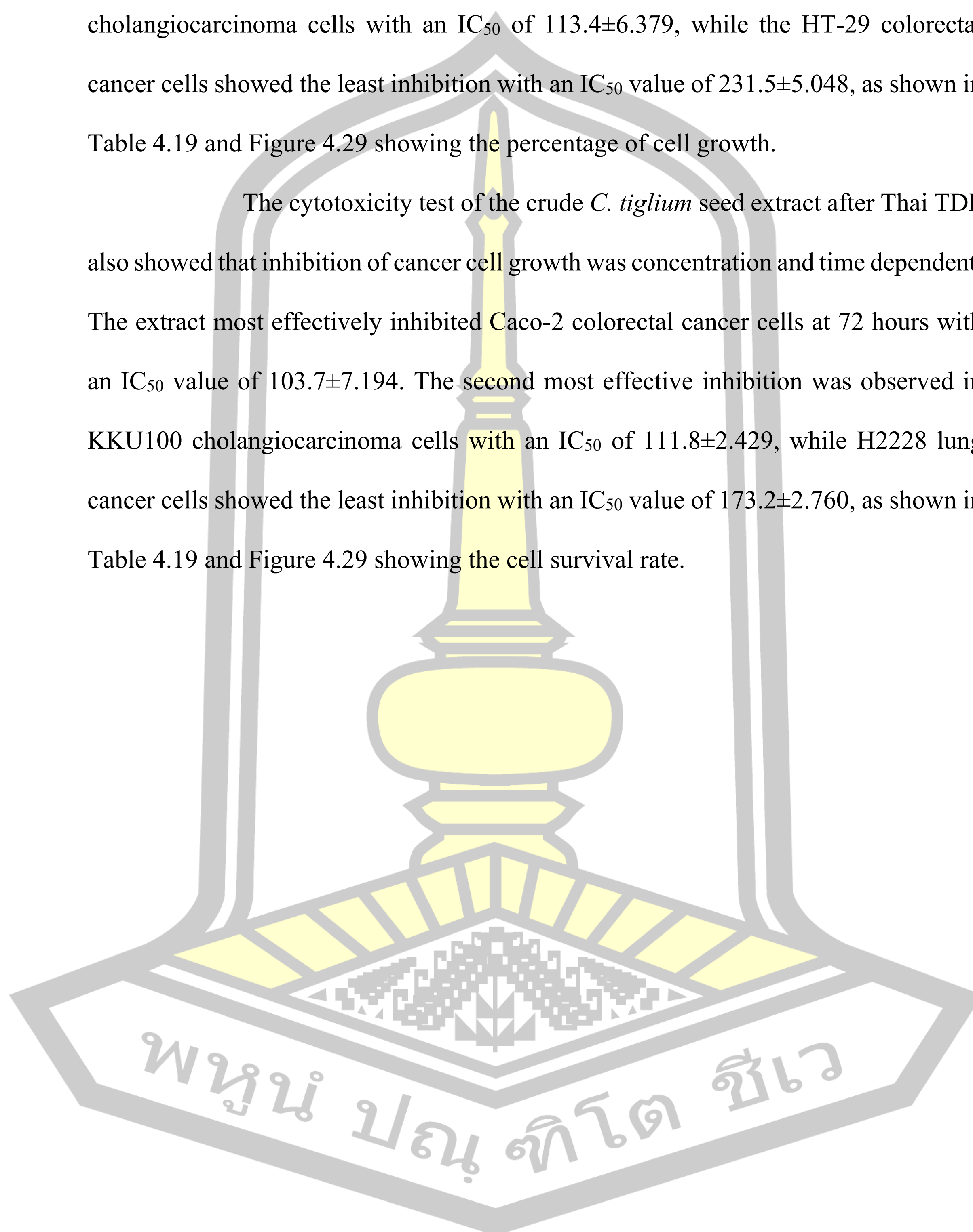
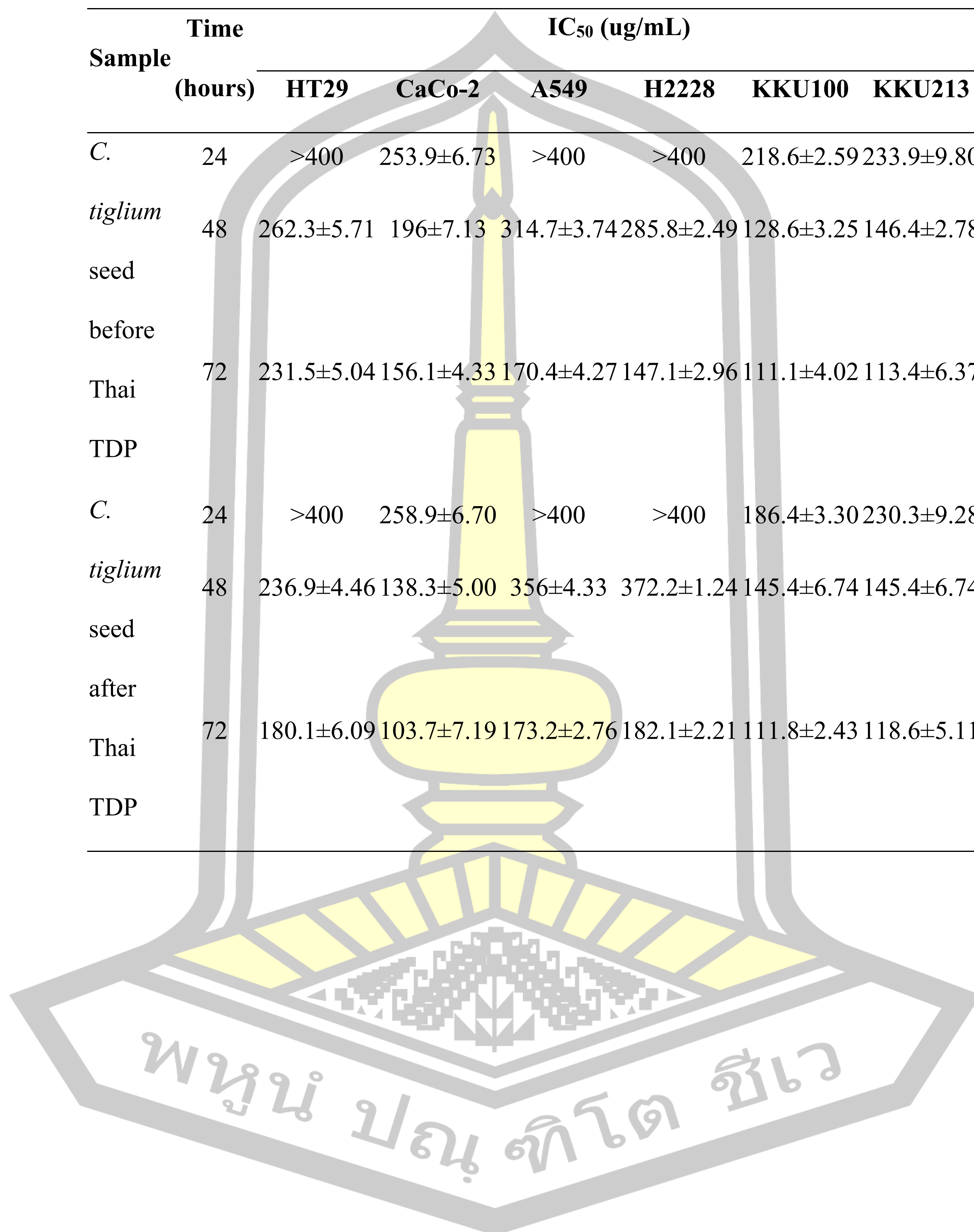


Table 4.19 IC_{50} of crude *C. tiglium* methanol extract against cancer cells

Sample	Time (hours)	IC_{50} (ug/mL)					
		HT29	**CaCo-2**	**A549**	**H2228**	**KKU100**	**KKU213**
C. tiglium seed before Thai TDP	24	>400	253.9±6.73	>400	>400	218.6±2.59	233.9±9.80
	48	262.3±5.71	196±7.13	314.7±3.74	285.8±2.49	128.6±3.25	146.4±2.78
	72	231.5±5.04	156.1±4.33	170.4±4.27	147.1±2.96	111.1±4.02	113.4±6.37
C. tiglium seed after Thai TDP	24	>400	258.9±6.70	>400	>400	186.4±3.30	230.3±9.28
	48	236.9±4.46	138.3±5.00	356±4.33	372.2±1.24	145.4±6.74	145.4±6.74
	72	180.1±6.09	103.7±7.19	173.2±2.76	182.1±2.21	111.8±2.43	118.6±5.11



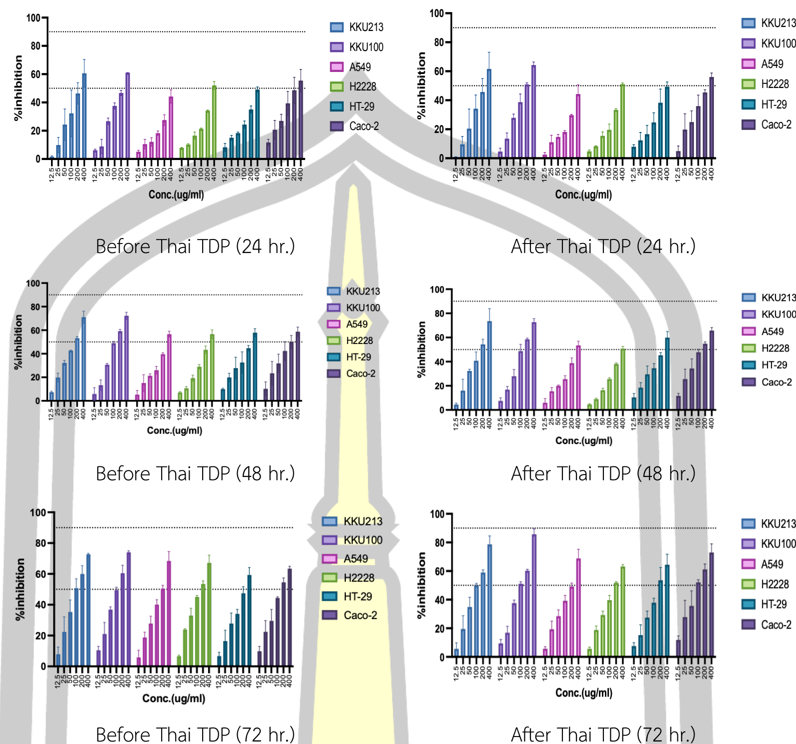
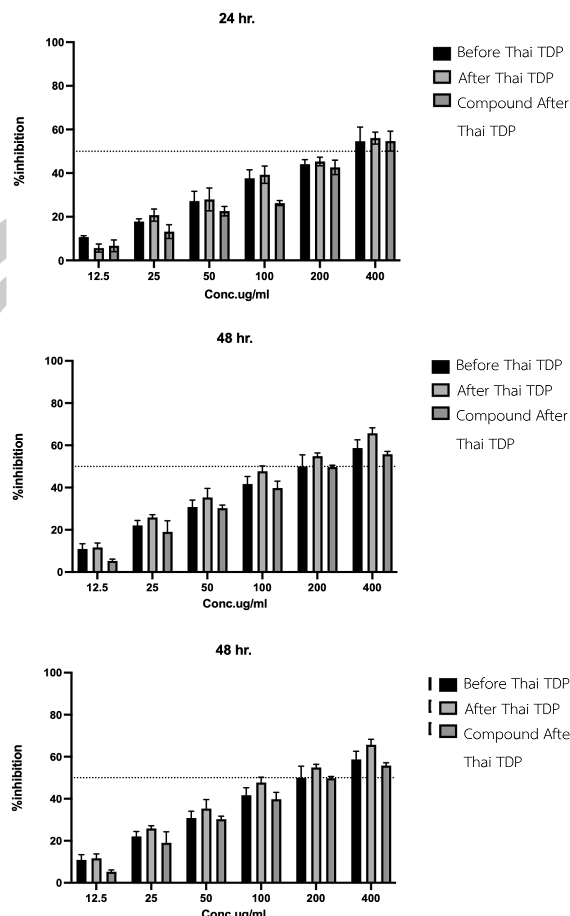


Figure 4.29 The percentage of cancer cell survival after receiving crude *C. tiglium* seed methanol extract from different concentrations at incubation period for 24, 48, and 72 hours

4.5.3.2 Comparison of the inhibition of Caco-2 colorectal cancer cell growth using MTT assay

The inhibition of Caco-2 colorectal cancer cell growth by the after Thai TDP *C. tiglium* seed extract at concentrations ranging from 12.50 to 400 µg/mL was tested. The results showed that the inhibition of cancer cell growth depended on both the concentration of the extract and the incubation time. The percentage of inhibition (% inhibition) was less than 50% at 24 hours with a concentration of 400 µg/mL. At 48 and 72 hours, concentrations of 200 and 100 µg/mL showed inhibition with IC_{50} values of 394.8, 228, and 98.91 µg/mL, respectively. These results were compared with the crude extracts of *C. tiglium* seed before and after processing as shown in Figure 4.30.



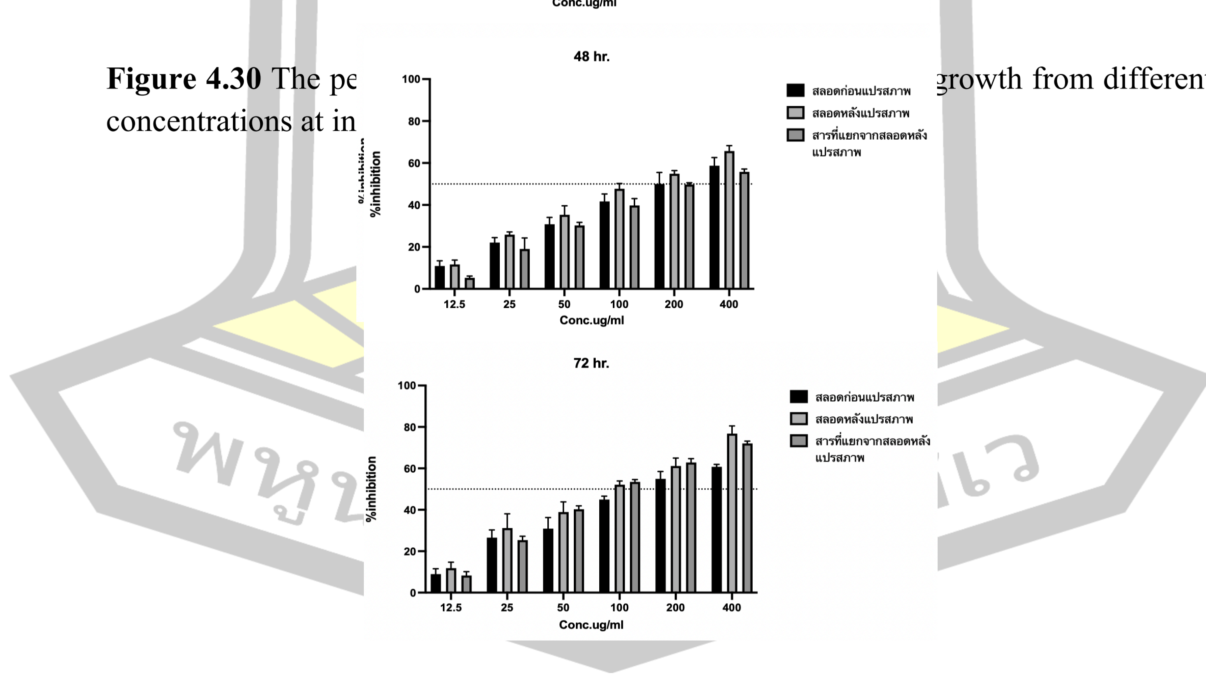


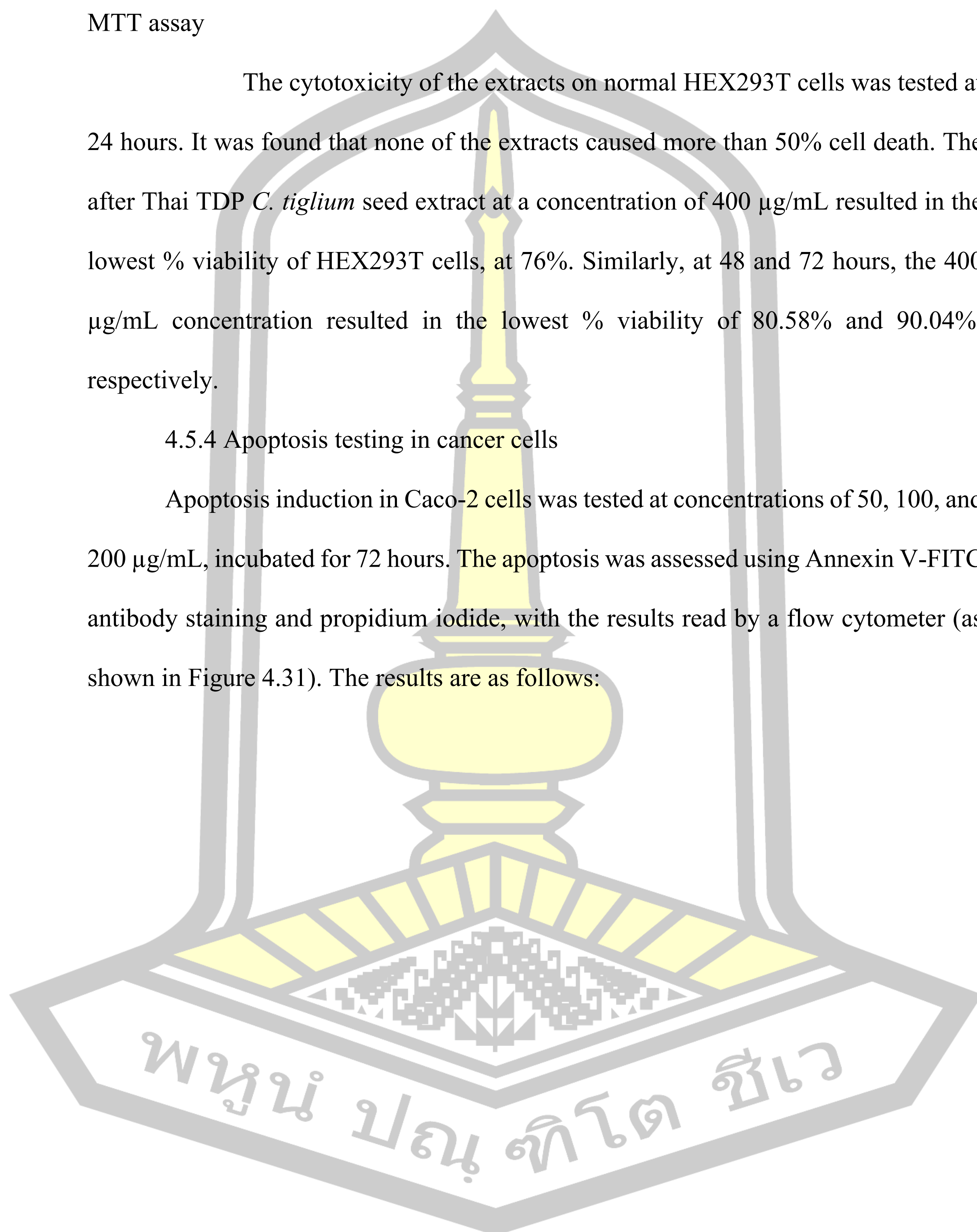
Figure 4.30 The pe growth from different concentrations at in

4.5.3.3 Testing the inhibition of normal HEX293T cell growth using MTT assay

The cytotoxicity of the extracts on normal HEX293T cells was tested at 24 hours. It was found that none of the extracts caused more than 50% cell death. The after Thai TDP *C. tiglium* seed extract at a concentration of 400 µg/mL resulted in the lowest % viability of HEX293T cells, at 76%. Similarly, at 48 and 72 hours, the 400 µg/mL concentration resulted in the lowest % viability of 80.58% and 90.04%, respectively.

4.5.4 Apoptosis testing in cancer cells

Apoptosis induction in Caco-2 cells was tested at concentrations of 50, 100, and 200 µg/mL, incubated for 72 hours. The apoptosis was assessed using Annexin V-FITC antibody staining and propidium iodide, with the results read by a flow cytometer (as shown in Figure 4.31). The results are as follows:



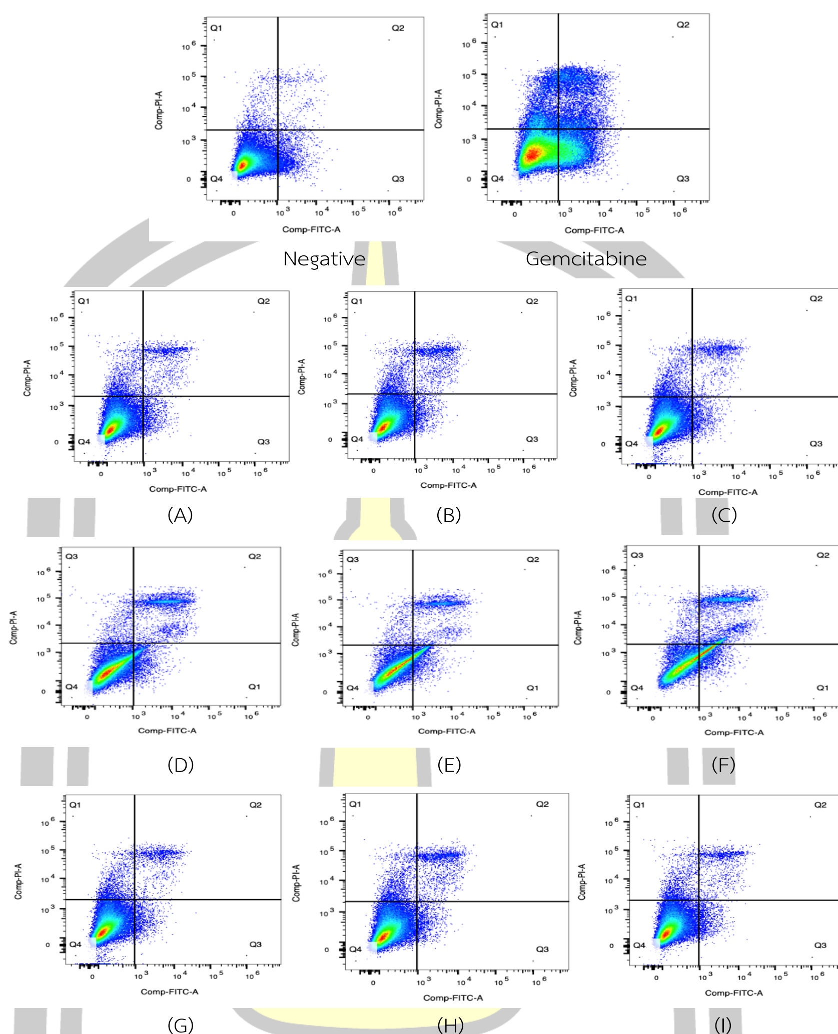


Figure 4.31 Dot plot images showing cells analyzed by flow cytometry. The two axes represent the amount of cell staining with Annexin V and PI. Each dot represents a single cell
(A-C) represents testing with before Thai TDP *C. tiglium* seed extract at concentrations of 50, 100, and 200 μg/mL. (D-F) represents testing with after Thai TDP *C. tiglium* seed extract at concentrations of 50, 100, and 200 μg/mL. (G-I) represents testing with an isolated compound from after Thai TDP *C. tiglium* seed extract at concentrations of 50, 100, and 200 μg/mL.

4.5.4.1 Early apoptosis

Cells treated with both before and after Thai TDP *C. tiglium* seed extracts showed an increase in early apoptosis compared to the control group. The after Thai TDP *C. tiglium* seed extract at a concentration of 200 µg/mL caused the highest apoptosis at 18%, followed by 200 µg/mL of the before Thai TDP extract at 8%, as shown in Figure 4.32.

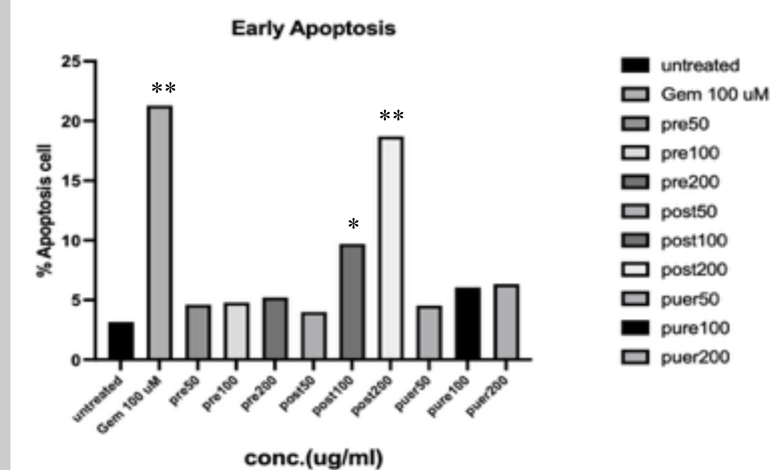


Figure 4.32 Analysis of early apoptosis in colon cancer cells using flow cytometry
* P<0.05, ** P<0.01 when compared to the control group. The paired sample t-test analysis were used for statistical analysis.

4.4.4.2 Late apoptosis

Cells treated with both before and after Thai TDP *C. tiglium* seed extracts exhibited increased late apoptosis compared to the control group. The after Thai TDP *C. tiglium* seed extract at a concentration of 200 µg/mL caused the highest late apoptosis at 10%, followed by the isolated compound from after Thai TDP *C. tiglium* seed extract at 200 µg/mL, which induced 5% apoptosis as shown in Figure 4.33.

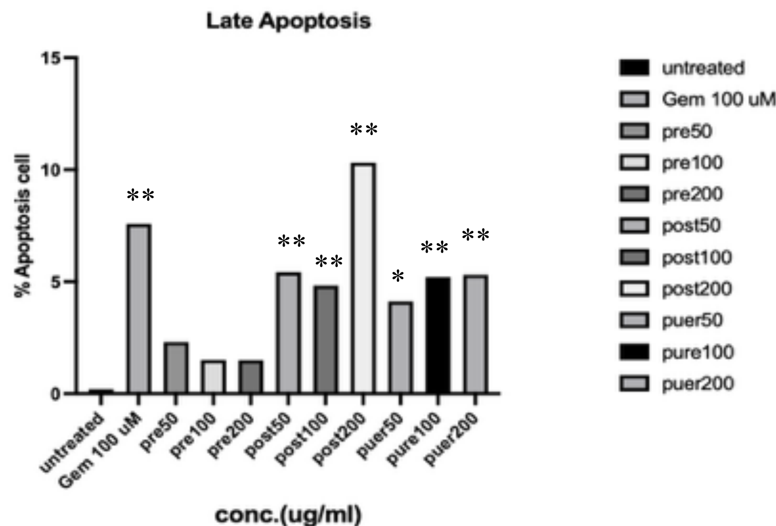


Figure 4.33 Analysis of late apoptosis in colon cancer cells using flow cytometry
* P<0.05, ** P<0.01 when compared to the control group. The paired sample t-test analysis were used for statistical analysis.

4.5.5 Gene expression testing using qRT-PCR

RNA was extracted from Caco-2 cancer cells after 72 hours of exposure to the extract using an RNA extraction kit. RNA quantity and purity were measured using the Nanodrop ND-1000 spectrophotometer, and cDNA was synthesized using a Reverse Transcription Kit. Real-time PCR was then performed to test gene expression as follows:

4.5.5.1 Testing gene expression related to apoptosis pathway

The genes tested include Bax, Caspase 3, and Caspase 9, with Beta-actin used as a reference gene. An increase in the expression of these genes indicates that the cells were triggered to undergo apoptosis. In contrast, a decrease in the expression of the BCL-2 gene suggests apoptosis activation. The results showed that the expression of Bax, Caspase 3, and Caspase 9 increased, with the after Thai TDP *C. tiglium* seed

extract at 200 µg/mL leading to a relative gene expression increase of between 1.5 and 2.5 for all three genes as shown in Figure 4.34.

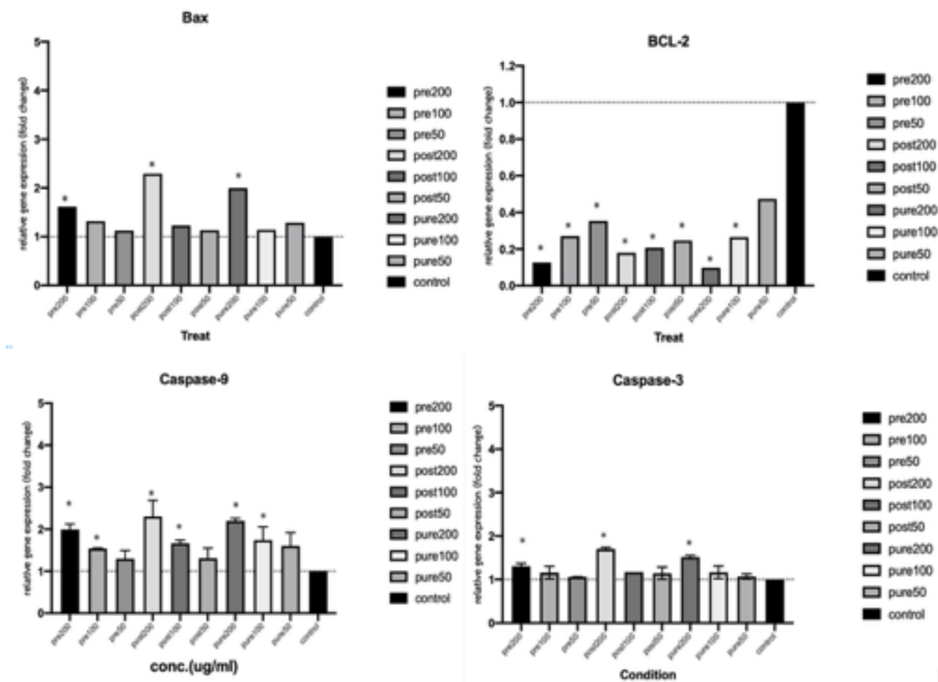


Figure 4.34 Effects of *C. tiglium* seed extract on the gene expression of Bax, Caspase 3, Caspase 9, and BCL-2 in Caco-2 colorectal cancer cells
* $P<0.05$, ** $P<0.01$ when compared to the control group. The paired sample t-test analysis were used for statistical analysis.

4.5.5.2 Testing gene expression related to the cell cycle arrest pathway

The genes tested include P21, P53, Cyclin B, and CDK-1, with Beta-actin used as a reference gene. An increase in the expression of P21 and P53 indicates that the cells are inhibited at a specific point in the cell cycle. On the other hand, a decrease in the expression of Cyclin B and CDK-1 suggests that the cells are being inhibited in the cell cycle. The results showed that the expression of P21 and P53 increased, with the after Thai TDP *C. tiglium* seed extract at 200 µg/mL resulting in a relative gene expression increase of 2.8-7.1 for these two genes. Meanwhile, *C. tiglium*

seed extract caused a slight reduction in the expression of Cyclin B and CDK-1 as shown in Figure 4.35.

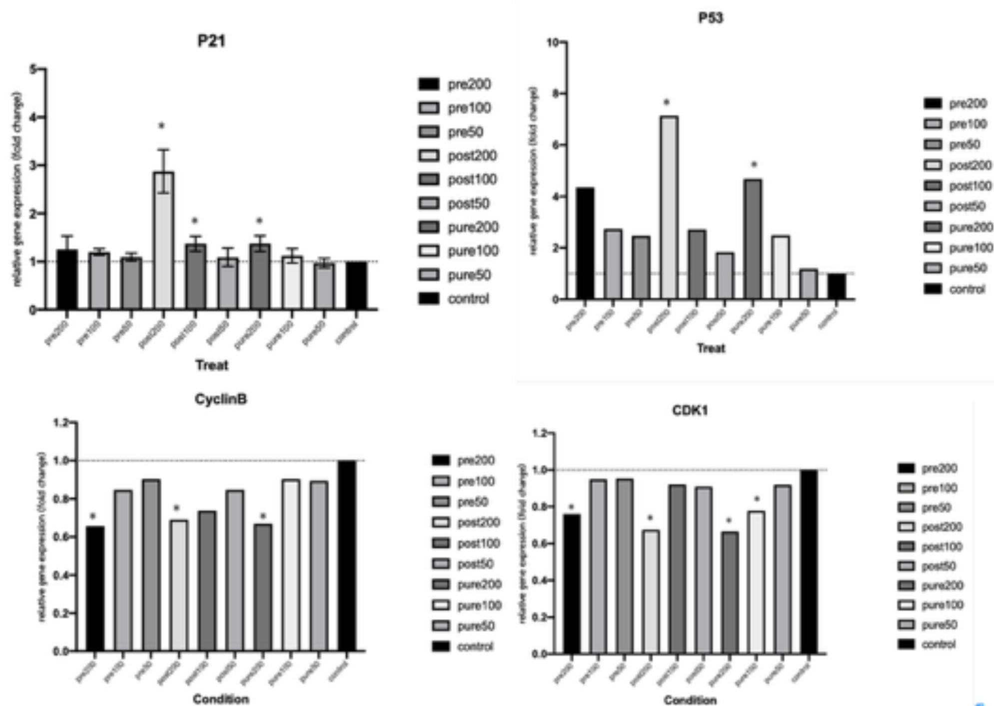
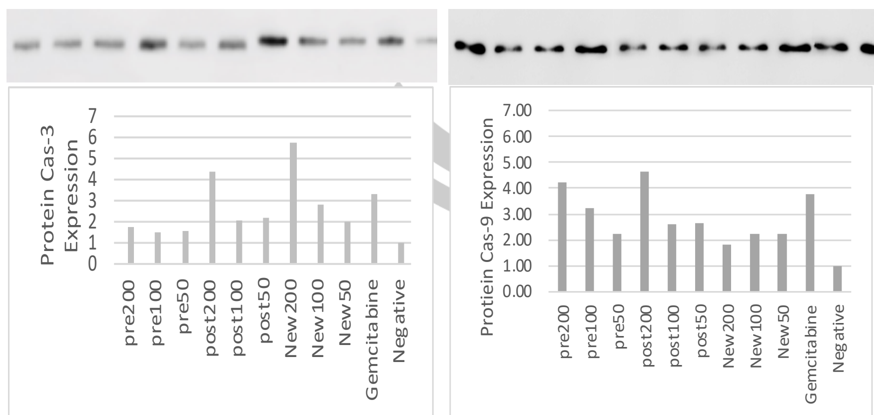


Figure 4.35 Effects of *C. tiglium* seed extract on the gene expression of P21, P53, Cyclin B, and CDK-1 in Caco-2 colorectal cancer cells
* P<0.05, ** P<0.01 when compared to the control group. The paired sample t-test analysis were used for statistical analysis.

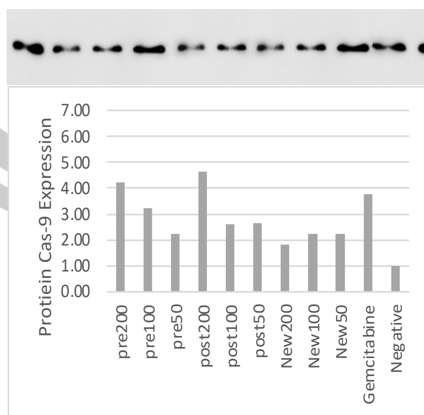
4.5.5 Protein expression testing after treating cells with *C. tiglium* seed extract

Based on the results of gene expression analysis through qRT-PCR, the *C. tiglium* seed extract showed a tendency to affect genes related to the apoptosis pathway. Therefore, further confirmation was made by examining protein expression. Caco-2 cells were treated with *C. tiglium* seed extract for 72 hours, and proteins were extracted using RIPA lysis buffer and a protease inhibitor. The proteins were then subjected to Western blotting using beta-actin as a control protein.

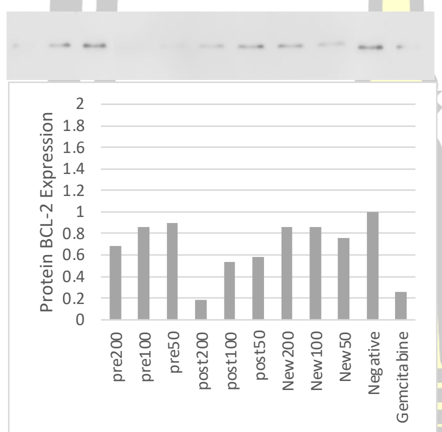
The results for the six proteins tested were consistent with the gene expression tests. Specifically, the protein BCL-2 showed decreased expression in Caco-2 cells treated with *C. tiglium* seed extract, based on the intensity of the bands. In contrast, the proteins Bax, Caspase3, Caspase9, PARP, and Cytochrome c exhibited increased expression as shown in Figure 4.36.



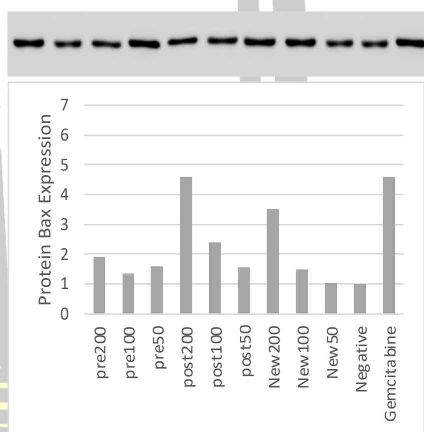
(A)



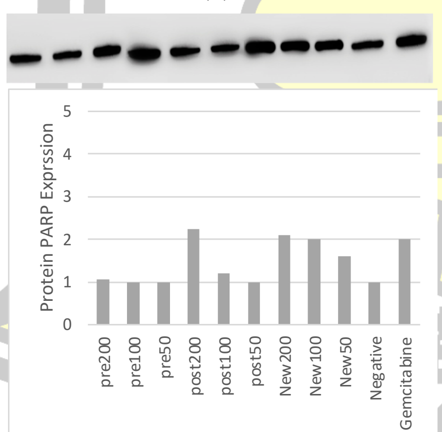
(B)



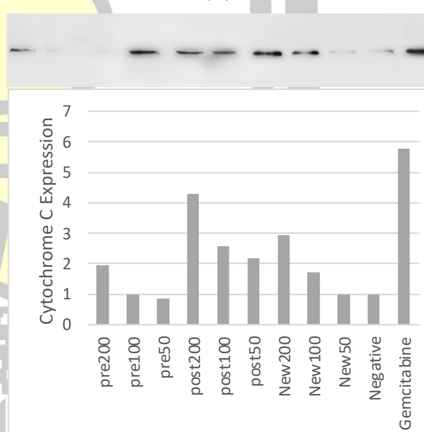
(C)



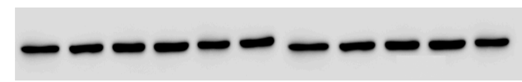
(D)

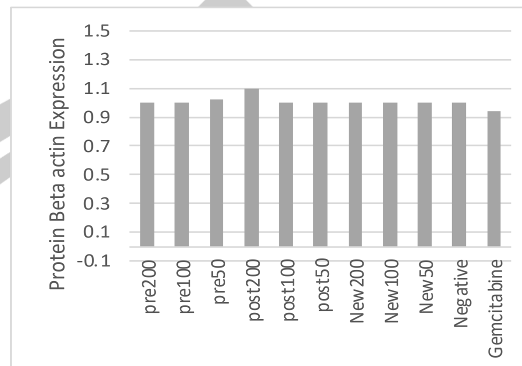


(E)



(F)





(G)

Figure 4.36 Effects of *C. tiglium* seed extract on the protein expression of Caspase3 (A), Caspase9 (B), BCL-2 (C), Bax (D), PARP (E), Cytochrome C (F), and Beta-actin (G) in Caco-2 colorectal cancer cells

4.5.6 Testing for carcinogenic promotion effects *in vitro*

A review of the literature indicates that certain compounds found in *C. tiglium* seed, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA), have been shown to promote increased cancer severity in experimental animals. However, in animals that were not induced to develop cancer and were given TPA, no carcinogenic effects were observed. Based on this, a test was conducted to evaluate the potential carcinogenic promotion of *C. tiglium* seed extract in Caco-2 colorectal cancer cells at concentrations of 50, 100, and 200 µg/mL, with the following results:

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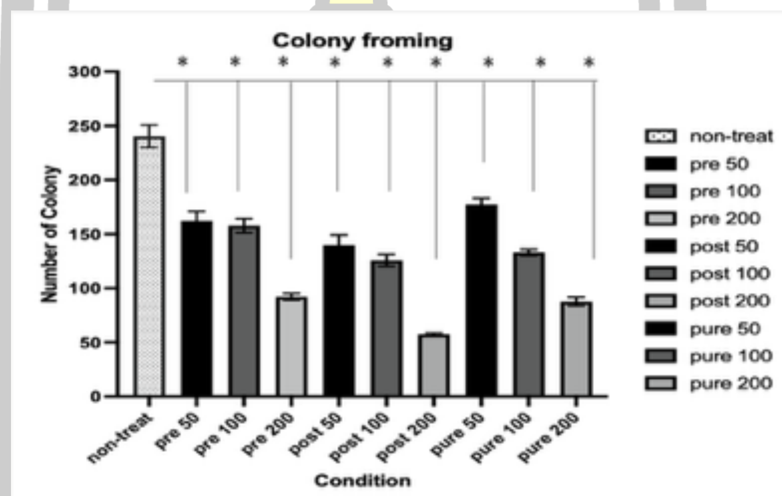
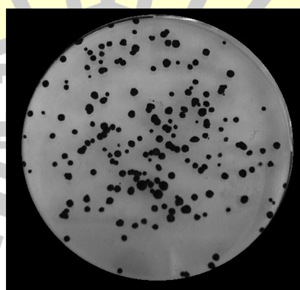
4.5.6.1 Colony formation test

Approximately 200 cells were plated in each well of a 6-well cell culture plate and treated with *C. tiglium* seed extract at varying concentrations. The cells were incubated at 37°C in a 5% CO_2 atmosphere, with media changed every 3 days for 13 days. Afterward, the cells were fixed with 10% paraformaldehyde and stained with 0.05% crystal violet. The results showed that the number of colonies formed by Caco-2 cancer cells treated with *C. tiglium* seed extract was lower compared to the control group as shown in Figure 4.37.



(A)



untreated

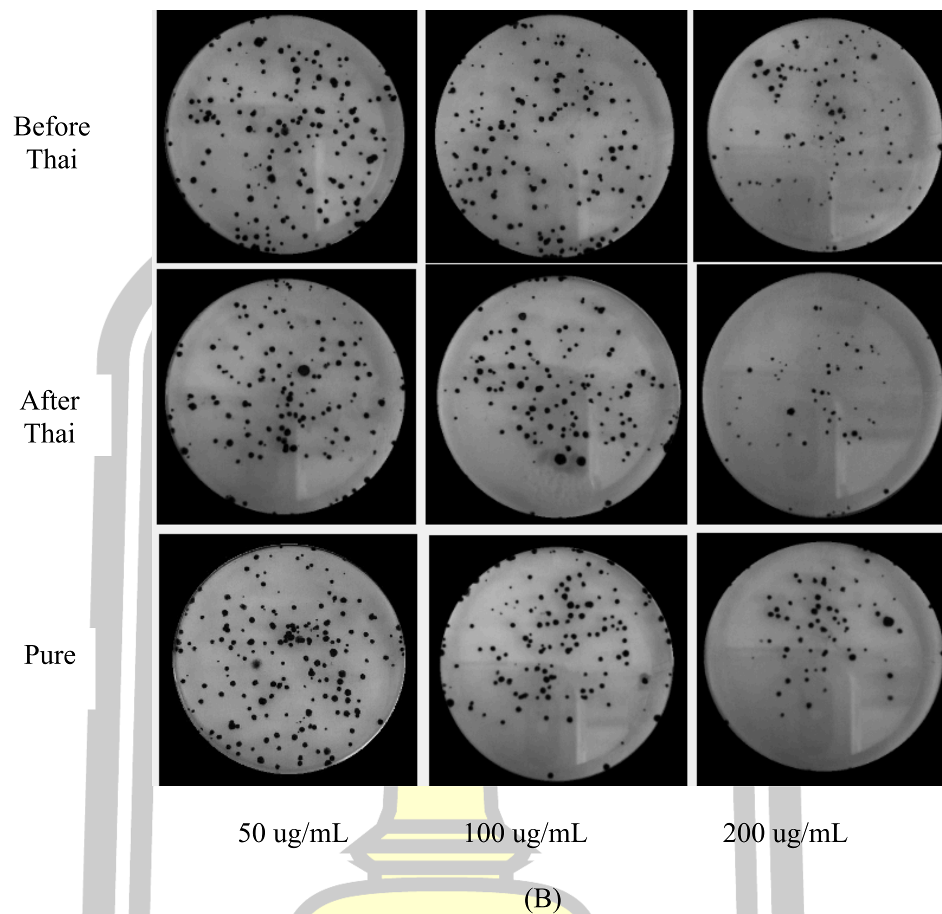
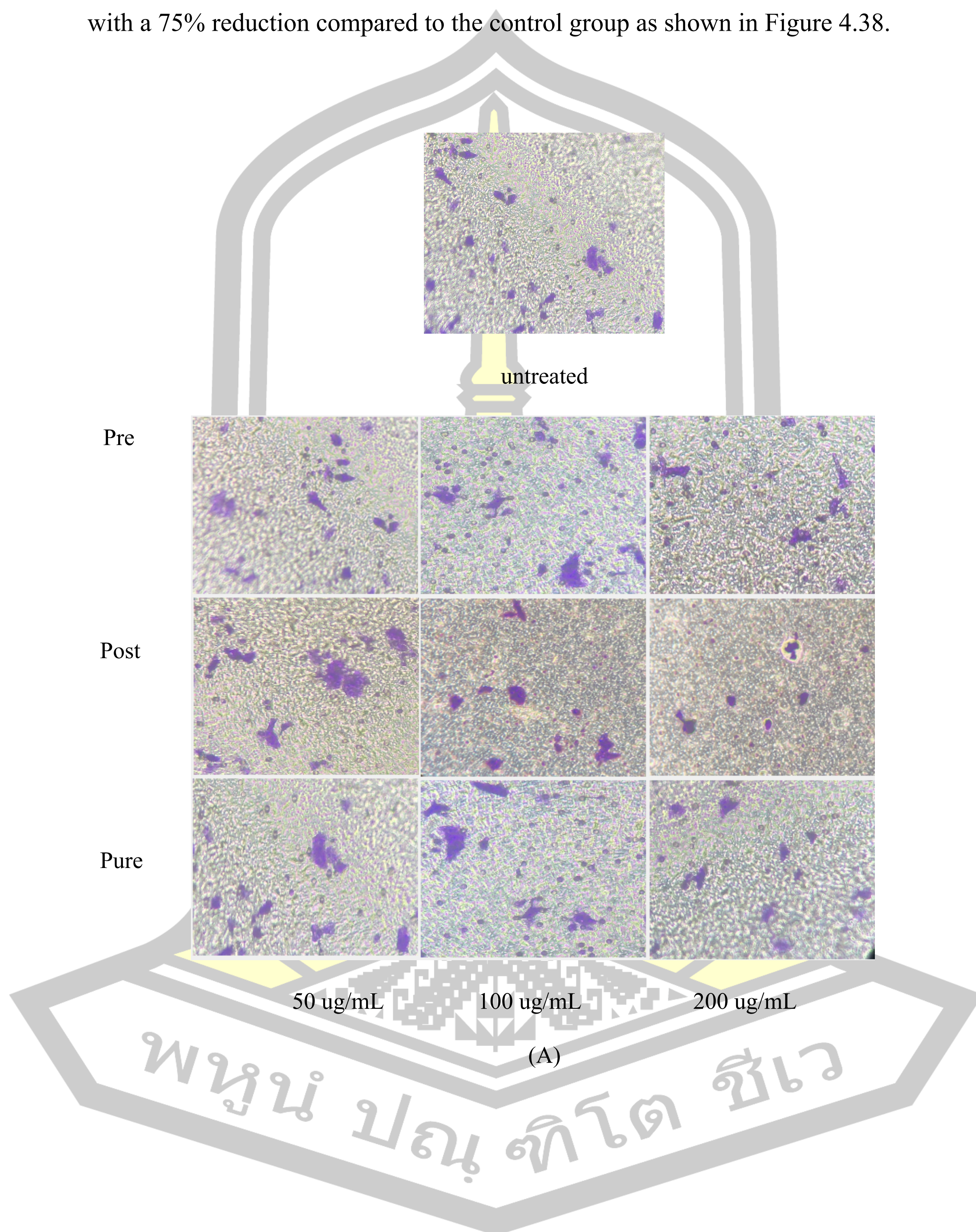


Figure 4.37 Colony formation assay results: (A) Colony count using Image J software, and (B) Photographs showing the appearance of colonies in a 6-well plate
* $P<0.05$, ** $P<0.01$ when compared to the control group. Statistical analysis using the paired sample t-test analysis.

4.5.6.2 Migration assay

After plating approximately 20,000 cells in a 24-transwell plate, cells were incubated for 3 hours, followed by the addition of the *C. tiglium* seed extract at concentrations of 50, 100, and 200 μg/mL, and incubated for an additional 24 hours. The cells were then fixed with 3.7% paraformaldehyde and stained with 0.05% crystal violet. The results showed that the migration of Caco-2 cells treated with *C. tiglium* seed extract decreased when compared to the control group. The post-detoxified C.

tiglium seed extract at a concentration of 200 µg/mL resulted in the least migration, with a 75% reduction compared to the control group as shown in Figure 4.38.



(A)

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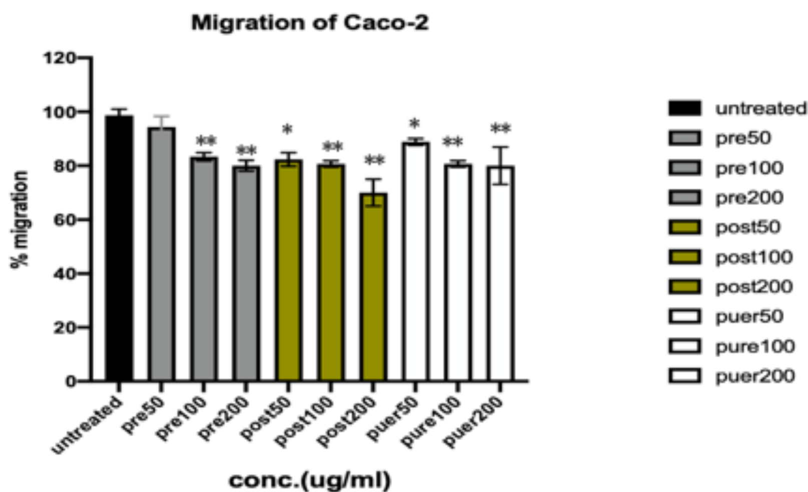
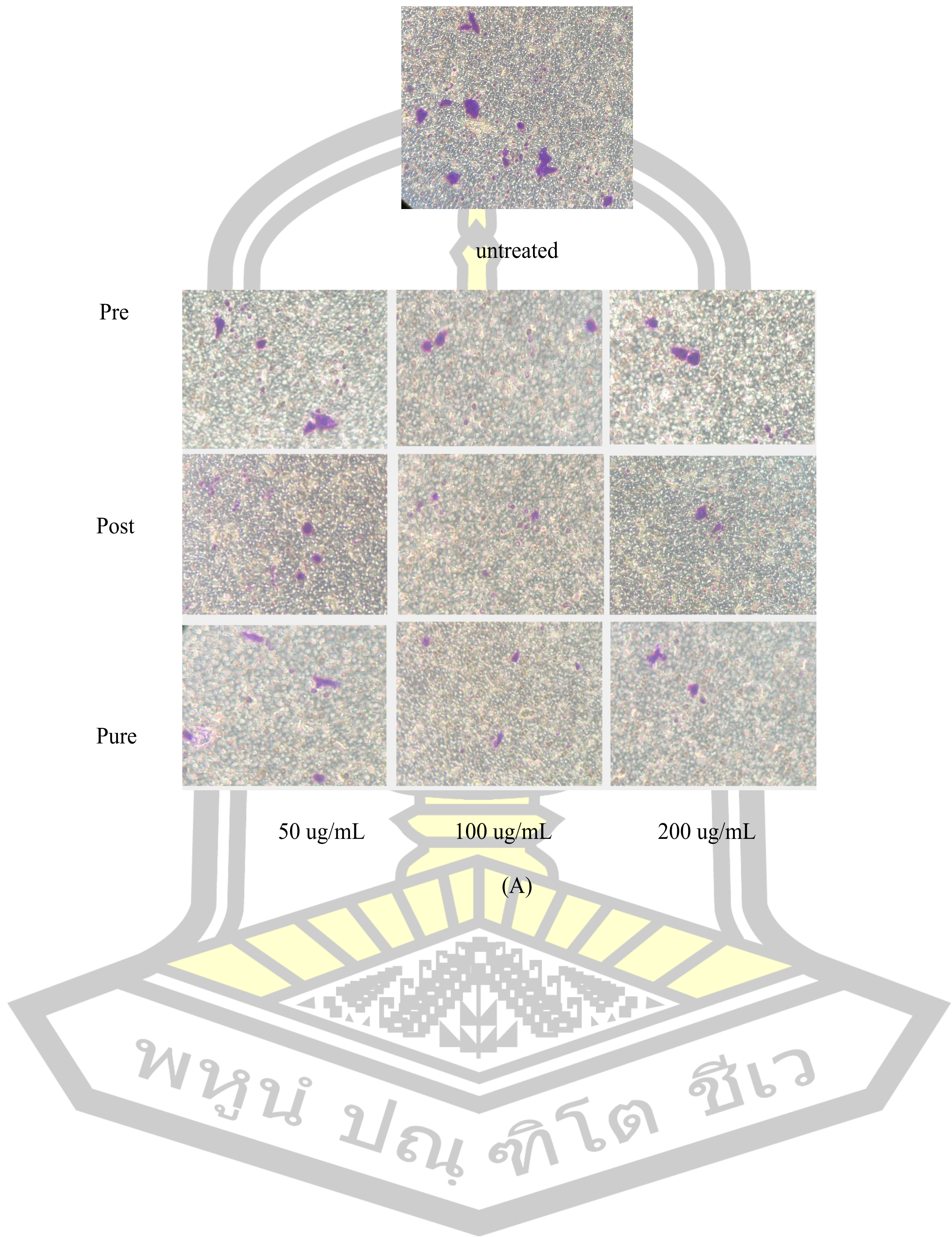


(B)

Figure 4.38 Migration assay results: (A) Migration occurrence image, and (B) Percentage of migration occurrence
* P<0.05, ** P<0.01 when compared to the control group. Statistical analysis using the paired sample t-test analysis.

4.5.6.3 Invasion assay

The 24-transwell plate was coated with Corning Matrigel for 2 hours. After that, approximately 20,000 cells were plated and incubated for 24 hours. The *C. tiglium* seed extract was added at concentrations of 50, 100, and 200 µg/mL. The cells were then fixed with 3.7% paraformaldehyde and stained with 0.05% crystal violet. The results showed that the invasion of Caco-2 cells treated with *C. tiglium* seed extract decreased when compared to the control group. The post-detoxified *C. tiglium* seed extract at a concentration of 200 µg/mL resulted in the least invasion, with a 37% reduction compared to the control group as shown in Figure 4.39.



(A)

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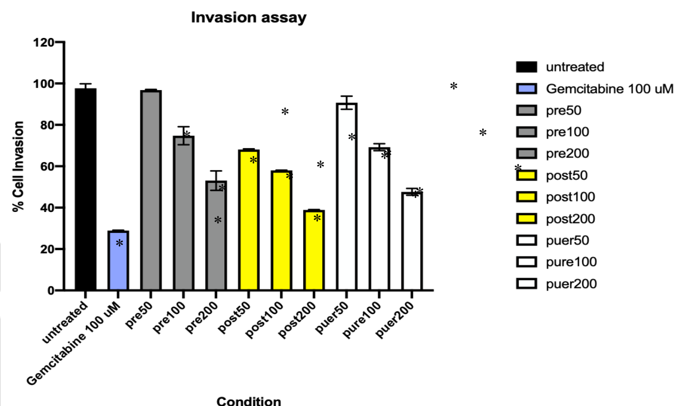

(B)

Figure 4.39 Migration assay results: (A) Image of migration occurrence, and (B) Percentage of migration occurrence
* P<0.05, ** P<0.01 when compared to the control group. Statistical analysis using the paired sample t-test analysis.

CHAPTER 5

Conclusion and discussion

5.1 Conclusion

This study, titled "Comparison of Chemical Constituents, Pharmacological Activities, and Toxicity of *C. tiglium* Linn. Seed Before and After Treatment by Thai Traditional Detoxification Process", addressed various aspects of the effects of Thai Traditional Detoxification Process of *C. tiglium* seed, including comparisons of HPLC and TLC fingerprints and quantification of PMA and crotonic acid of the extracts of *C. tiglium* seed before and after Thai TDP, as well as isolation of compounds that emerged after the detoxification process. The evaluation of purgative effects in experimental animals, toxicity testing, and anticancer cell activity of the extracts of *C. tiglium* seed before and after Thai TDP were also performed. The findings are summarized as follows:

5.1.1 HPLC and TLC fingerprints of *C. tiglium* seed before and after detoxification

HPLC and TLC fingerprinting revealed significant differences in chemical composition of the seed extracts before and after the Thai TDP. Chemical alterations were observed in the *C. tiglium* seed which underwent Thai TDP.

5.1.2 Comparison of the PMA and crotonic acid content

Quantitative analysis of PMA and crotonic acid in *C. tiglium* seed extracts before and after Thai TDP using four methods indicated statistically significant differences ($P < 0.05$). Detoxification methods 1, 3, and 4 reduced PMA content

compared to untreated seeds, with method 4 showing the most substantial reduction (31.07%). In contrast, detoxification method 2 increased PMA content by 5.86%. Additionally, the amount of crotonic acid in the before Thai TDP extract was 0.001 mg/g, while no crotonic acid was detected in the after Thai TDP extract.

5.1.3 Compound isolation using column chromatography

Methanol extracts of detoxified *C. tiglium* seed were fractionated using column chromatography with silica gel 60 (0.063-0.200 mm) as a stationary phase. with mixtures DCM:EtOAc from 8:2 to 0:10 as mobile phases. Fractions 1 and 2 were further purified using open column chromatography with resin as the stationary phase. The elution was performed with H_2O:MeOH (10:0 to 0:10, followed by MeOH: EtOAc (9:1 to 8:2). Chromatographic profiles of fractions R10 and R11 matched well with those of a newly detected compound, with a retention time of 27.50 minutes.

5.1.4 Structural elucidation of isolated compound

When the compound isolated from the column was analyzed for its chemical structure using NMR spectroscopy, it was dissolved in chloroform prior to the analysis. the ^{1}H NMR spectrum revealed that the purified compound isolated from *C. tiglium* seed after Thai TDP processing was identified as di-(2-ethylhexyl) phthalate, by interpretation of its 1D and 2D NMR spectra and its high resolution mass spectrum as well as comparison of its NMR spectral data with those previously reported

5.1.5 Laxative effect testing in animal models

The laxative effect was tested on male Wistar rats aged approximately 6 weeks, 150-200 g of weight, with a total of 48 animals. The test groups were divided as follows: Group 1: Received distilled water (vehicle control), Group 2: Received castor oil (0.3 mL/animal) as a positive control, Groups 3-5: Received *C. tiglium* seed powder before

Thai TDP at doses of 10, 50, and 100 mg/kg, respectively; Groups 6-8: Received *C. tiglium* seed powder which underwent Thai TDP, at doses of 10, 50, and 100 mg/kg, respectively.

Eight hours after administration, there were no significant differences in the average wet stool weight, dry stool weight, or the percentage of water content in dried stool among the groups, except for the number of fecal pellets. The group treated with seed powder of *C. tiglium* without undergoing Thai TDP, at 100 mg/kg, had the highest average pellet count (8.17 pellets), while the control (distilled water) group had the lowest count (4.83 pellets). Statistical analysis using Bonferroni's method showed significant differences between groups.

After 16 h of administration, the highest average pellet count, dry stool weight, wet stool weight, and percentage of water content in dried stool were observed in the group receiving seed powder of *C. tiglium* without undergoing Thai TDP, at 100 mg/kg. Conversely, the control group showed the lowest values, with significant statistical differences.

Histopathological examination of gastric tissues revealed that groups treated with *C. tiglium* seed powder without undergoing Thai TDP, at all doses and powder after undergoing Thai TDP, at 100 mg/kg, exhibited blood vessels and red blood cells in the submucosa, indicating inflammation. This was less pronounced in the control group. Similar phenomena were observed in the large and small intestines, where goblet cells (responsible for mucus secretion) were more prominent in groups treated with *C. tiglium* seed powder. However, the group treated with powder after undergoing Thai TDP showed a lower tendency for goblet cell abundance compared to the group treated with seed powder of *C. tiglium* without undergoing Thai TDP.

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5.1.6 Acute oral toxicity testing (fixed dose procedure)

This study was conducted according to OECD Guidelines for the Testing of Chemicals (OECD 420), which involved two phases, i. e. (i) sighting study-preliminary acute oral toxicity testing, and (ii) main study: comprehensive acute oral toxicity testing.

Sighting study: Female Wistar rats aged approximately 10 weeks, of 191-235 g body weight (5 rats per group), were used. The groups were divided as follows: Group 1: Received distilled water (vehicle control), Group 2: Received *C. tiglium* seed powder, without Thai TDP, at 300 mg/kg, Group 3: Received *C. tiglium* seed powder, after Thai TDP, at 300 mg/kg, Group 4: Received *C. tiglium* seed powder without Thai TDP at 2.0 g/kg, Group 5: Received *C. tiglium* seed powder, after Thai TDP, at 2.0 g/kg.

All groups were found to have weight gain after 14 days of administration. No deaths occurred in the 300 mg/kg groups, and no abnormalities in drinking or feeding behaviors were observed. At the higher dose of 2.0 g/kg, no deaths occurred, but lethargy, reduced activity, and decreased movement were observed within the first two days. Weight and feeding behavior trends were lower compared to the control group.

Main study: The main study involved 15 female Wistar rats (215-249 of body weight), which were divided into three groups (5 rats each) as follows: Group 1: Received *C. tiglium* seed powder without Thai TDP, at 2,000 mg/kg, Group 2: Received *C. tiglium* seed powder, after Thai TDP at 2,000 mg/kg, Group 3: Received distilled water (vehicle control).

At the 2,000 mg/kg dose, no deaths occurred in any group. However, lethargy, reduced activity, and decreased movement were observed during the first two days.

Weight and feeding behaviors were lower in groups received *C. tiglium* seed powder compared to the control group. Four rats in the treated groups died due to complications during oral administration procedures.

Hematological parameters (e.g., hemoglobin concentration, hematocrit, platelet count, mean corpuscular volume) and white blood cell differentials (e.g., eosinophils, lymphocytes, monocytes) were comparable among groups. Neutrophil percentages in treated groups were slightly lower but within normal ranges, with no significant differences.

Biochemical parameters (e.g., blood urea nitrogen, uric acid, total protein, albumin, total bilirubin, alkaline phosphatase, AST, ALT) were not significantly different among groups. Histopathological examination of tissues showed no organ damage in rats administered with both *C. tiglium* seed powder before and after Thai TDP compared to the control group.

5.1.7 *In vitro* anticancer activity testing

In preliminary experiments to evaluate anticancer activity, the effects of the extracts of *C. tiglium* seed with and without Thai TDP were assay on lung cancer cells, colorectal (Caco-2) cancer cells, and cholangiocarcinoma cells by the MTT method. Results indicated that the extract of *C. tiglium* seed, after Thai TDP, exhibited the most potent anticancer activity. Among the tested cancer cell lines, Caco-2 cells showed the lowest IC_{50} value, at 103.7 µg/mL at 72 hours.

Based on this result, Caco-2 cells were selected for further anticancer assay on fractions obtained from the extract of *C. tiglium* seed with Thai TDP, at concentrations of 50, 100, and 200 µg/mL for 72 hours. Additionally, cytotoxicity was assessed on

normal cells (Hex293T), and the results showed that *C. tiglium* extract at concentrations ranging from 12.50 to 400 µg/mL did not exhibit toxicity to normal cells.

Further tests included an evaluation of apoptosis induction in Caco-2 cells. At a concentration of 200 µg/mL, the extract of *C. tiglium* seed, after Thai TDP, induced early apoptosis in 18% of cells and late apoptosis in 10% of cells, the highest percentages observed. These findings were consistent with the expression of apoptosis-related genes and proteins, including Bax, caspase 3, and caspase 9, with beta-actin used as the reference gene and protein.

5.1.8 *In vitro* cancer-promoting activity assay

The colony formation assay was performed by plating Caco-2 cells and stimulating them with the extracts of *C. tiglium* seed before and after Thai TDP, as well as fractions obtained from the extract *C. tiglium* seed after Thai TDP, for 13 days. The results showed that the number of colonies formed by Caco-2 cells treated with *C. tiglium* seed extracts was reduced compared to the control group.

This result aligned with the findings from the migration assay and invasion assay, which showed decreased migration and invasion of Caco-2 cells treated with *C. tiglium* seed extracts compared to the control. These results indicate that the extracts of *C. tiglium* seed before and after Thai TDP, as well as fractions, do not promote cancer aggressiveness.

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5.2 Discussion

This experiment demonstrated the effects of *C. tiglium* seeds, a herb classified as hazardous by the Ministry of Public Health of Thailand. Since 1976, the Ministry has prohibited the use of *C. tiglium* seeds and oil in Thai traditional medicine formulas under the notification of toxic herbs. Previous studies on PMA at dose ranges of 0.016 to 0.0016 μmol have demonstrated significant promoting activity, while a dose of 0.00016 μmol did not exhibit such activity. These findings suggest a threshold for promoting activity between 0.0016 and 0.00016 μmol. Furthermore[7], a study on the effects of croton oil on gastrocarcinogenesis induced by MNNG (83 μg/mL for 3 months) found no tumors in rats treated solely with croton oil and Tween 60[80]. Consequently, the use of *C. tiglium* in Thai traditional medicine has been discontinued until the present day. Despite this restriction, various pharmacological properties of *C. tiglium* have been reported in scientific literature. These include antifungal, antibacterial[81], anti-HIV-1[22], anti-inflammatory activities[12], and anticancer activity against various cancer types[4, 5, 23].

Curiously, *C. tiglium* seeds are still used in many countries[22]. Very interestingly, Thai traditional practitioners have always known about the toxicity and exceedingly strong purgative effect of *C. tiglium* seeds, and therefore, instead of using them directly, the material had to undergo a traditional detoxification process (TDP), which is known in Thai as "Kharith of Salod", before being used in Thai traditional medicine formulas[46]. However, this TDP has never been scientifically proved of its validity or efficiency. Thus, the objective of this study was to evaluate the efficiency of TDP in reducing the purgative activity and toxicity as well as to determine the degree

to which TDP reduced the levels of PMA, the tumor promoter, and crotonic acid, the main irritating chemical, in *C. tiglium* seeds.

It is interesting to mention also that *C. tiglium* seeds are treated before being used in traditional medicine not only in Thailand but also in other countries. In India, the method of treatment consists of wrapping dried *C. tiglium* seeds in a clean white cloth to form a bolus which was soaked in a cow dung solution in a mud pot and boiled, and then washed with water. Then the seeds were treated with cow's urine, followed by lemon juice. Subsequently, the seeds were washed with water, followed by removal of the outer skin and cotyledons and finally fried with ghee. This study was conducted to determine the amount of phorbol and fatty acids, revealing a significant reduction in crotonoside and toxic fatty acids. This method caused a significant reduction of phorbol from 5.18 to 3.86 %. Similarly, the amounts of saturated fatty acids, e. g. arachidic acid, behenic acid, stearic acid and palmitic acid were also decreased whereas all the unsaturated fatty acids such as oleic acid and linoleic acid seemed to be unaltered[82]. Supporting these findings, Pal, Nandi, and Singh (2014) conducted a similar detoxification study using cow's milk and repeated cycles of steaming and washing. HPLC analysis revealed that phorbol esters decreased from 5.2 to 1.8 mg/100 g, while crotonic acid was completely eliminated after Shodhana[66]. Jamadagni et al. (2023) further validated the impact of Shodhana by assessing cytotoxicity and mutagenicity in *C. tiglium* seed extracts. Using MTT assays on Chinese Hamster Ovary (CHO) cells, they found a marked reduction in IC_{50} values, indicating increased potency after Shodhana. LC-MS analysis revealed both the disappearance of mutagenic compounds such as phorbol 13-acetate and the emergence of beneficial metabolites like myristic and palmitic acid. Notably, Ames tests showed no significant mutagenic activity up to

2000 mg/plate, affirming the non-genotoxic nature of the purified extracts [83]. On the contrary, no detoxification process of *C. tiglium* seeds was found before they were used to produce medicine in China[84].

This method not only reduced the amount of crotonic acid and PMA but also produced another compound that appeared in the chromatogram of CA extract, with a retention time of 28 min.This peak was isolated and identified as Di-(2-ethylhexyl) phthalate, which belongs to the phthalic acid ester group. This group of compounds is synthetic and widely used as additives in organic solvents and as plasticizers to enhance flexibility and tensile strength in plastics[85]. These substances are easily detected in the atmosphere, water sources, soil, and sediments. Additionally, reports indicate that this group of compounds can be found in plant roots, essential oils of various plant species, as well as in algae, bacteria, fungi, and some higher plants[86]. Furthermore, these compounds have been found to exhibit various biological activities, such as allelopathic effects, antimicrobial properties, and insecticidal activity[87, 88]. Interestingly, related species such as Croton lachynocarpus have been shown to biosynthesize phthalates, suggesting the potential for natural production under specific conditions[89]. However, contamination during extraction remains a plausible explanation. To differentiate natural biosynthesis from contamination, future studies should employ isotopic labeling, radiocarbon dating, and plastic-free protocols. These methods can clarify whether phthalate esters in medicinal plants are true secondary metabolites or environmental artifacts.

On the other hand, some studies have reported microbial transformation of DEPT in environmental matrices. Notably, *Pseudomonas* sp. DNE-S1 and *Sphingobium yanoikuyae* SHJ have been shown to metabolize DEP through enzymatic

hydrolysis to monoethyl phthalate (MEP) and phthalic acid, or via transesterification to ethyl methyl phthalate , indicating that partial degradation of DEPT may occur biologically under specific conditions[90, 91]. However, no studies to date have provided conclusive evidence that higher plants like Croton tiglium biosynthesize DEP as a secondary metabolite. Thus, the detection of DEPT post-detoxification is more plausibly linked to either 1) laboratory contamination during extraction, or 2) microbial degradation of pre-existing phthalates introduced from prior handling, packaging, or the environment. To differentiate between true natural occurrence and contamination, future studies should implement strict plastic-free protocols and consider radiocarbon (^{14}C) dating or isotopic labeling.

The possibility of natural biosynthesis in certain plant taxa should not be dismissed outright. In fact, evidence has emerged showing that some species of the genus *Croton* are capable of producing phthalates. For instance, Pan et al. (2014) isolated di-n-butyl phthalate and diisobutyl phthalate from the roots of *Croton lachynocarpus* using spectroscopic techniques, strongly indicating their origin as secondary metabolites rather than artifacts of contamination. This finding aligns with broader patterns observed across the Euphorbiaceae family, wherein multiple PAEs have been detected in plant tissues through careful chromatographic separation[89]. Although *Croton tiglium* was not directly studied in Pan et al.'s report, its taxonomic proximity to *Croton lachynocarpus* suggests that it may possess similar metabolic potential under certain physiological or stress-induced conditions such as detoxification. Therefore, the appearance of DEP after the detoxification process could reflect an activated metabolic response rather than laboratory contamination. Nonetheless, because diethyl phthalate was not specifically reported in the cited study,

and given that contamination from solvents and equipment remains a plausible confounder, further research is warranted. Such investigations should include isotopic labeling or ^{14}C analysis to distinguish true biosynthesis from external introduction. These approaches will help elucidate whether phthalate esters in medicinal plants are genuinely natural constituents or merely environmental residues misidentified as endogenous metabolites.

The effectiveness of TDP in reducing the purgative activity of *C. tiglium* seeds was verified by comparing the number of wet feces, dry feces and fecal water content of the Wistar rats receiving *C. tiglium* before and after Thai TDP, water (negative control) and castor oil (positive control). To account for confounding variables, the same conditions were employed for each group: sex, body weight (BW), water and feed intake, and housing conditions. The results showed that water and feed intake in every group was not different before and after the treatment. Almost every group of the rats showed BW gain, except for the group that received *C. tiglium* seed powder before Thai TDP at a dose of 100 mg/kg, which showed a significant weight loss after 16 hours. The results are consistent with the food intake of this group, which consumed significantly less food than the other groups, possibly due to gastrointestinal irritation. The amounts of wet feces, dry feces, and fecal water content after 8 hours of the experiment were comparable across the majority of groups after 8 hours of the experiment, with the exception of the group that received *C. tiglium* seed powder before Thai TDP at a dose of 100 mg/kg and the positive control group, which had significantly higher amount of wet feces than the other groups. After 16 hours of the treatment, the amounts of wet feces and fecal water content in the group that received *C. tiglium* seed powder before Thai TDP at a dose of 100 mg/kg and the positive control group were

also significantly higher than the other groups. These results are consistent with the previous reports which indicated that croton oil can cause a purgative effect via stimulating intestinal contractions[29, 92]. Croton oil is classified as a stimulant laxative, a type of laxative that usually onsets within 6-12 hours, similar to bisacodyl, senna, and sodium picosulfate[93]. Croton oil is known to stimulate nerves that control the muscles in the intestinal wall, helping to induce bowel contractions and facilitate defecation[94]. The rats that received *C. tiglium* seed powder also showed an increase in fecal water content due to the intense stimulation by the croton oil in *C. tiglium* seeds, which have irritant properties on the intestinal mucosa and stimulate excessive intestinal contractions[92, 93]. These effects resulted in an increase in intestinal motility, leading to more frequent contractions of the intestines, which reduce the time for nutrient and water absorption from the feces[95]. Consequently, there is a higher amount of residual water in feces than normal. Furthermore, the intestinal lining may secrete more water and electrolytes into the fecal content as a result of the irritating effects of *C. tiglium* seeds[93]. The water secreted is not reabsorbable, leading to a higher water content in the feces[96].

Pathological examination of the stomach tissue revealed that the rat groups receiving all three doses of *C. tiglium* seed powder before Thai TDP and the group receiving *C. tiglium* seed powder after Thai TDP at 100 mg/kg showed the presence of red blood cells in the submucosa, indicating an inflammation of the stomach tissue. Similarly, in the pathological examination of the small and large intestine tissues, the rat groups receiving *C. tiglium* seed before and after Thai TDP showed the presence of goblet cells, which are responsible for mucus secretion in the intestines. The appearance of goblet cells is consistent with the intestinal stimulating action of *C. tiglium* seeds,

which can cause an increase in mucus secretion. The rat group receiving *C. tiglium* seed powder after Thai TDP tended to have fewer goblet cells than the rat group receiving *C. tiglium* seed powder before Thai TDP. Stomach bleeding is frequently linked to the presence of red blood cells in the stomach tissue[93]. This could be because the chemicals in croton irritate the stomach mucosa, which can result in inflammation and colon ulcers[93]. Several studies have indicated that croton proteins can damage the gastrointestinal lining and stimulate inflammation, which may result in the leakage of blood into the tissues[97, 98]. Goblet cells are commonly found in intestinal tissue and play an important role in the production and secretion of mucus, which helps lubricate and protect the intestinal lining from irritation and injury [93]. The increased number of goblet cells in rats that received *C. tiglium* seed powder before Thai TDP is a response of the intestines to the inflammation caused by the toxic effects of *C. tiglium* seed powder before Thai TDP. The relationship between goblet cell numbers and the inflammatory response is consistent with the previous studies on croton oils that have irritant effects on the gastrointestinal system[99].

The effects of *C. tiglium* seed powder before and after Thai TDP at the doses of 2,000 mg/kg was evaluated in the main study of the acute toxicity test in rats and their effects were compared with those of the control group. The results showed that no rat died in all groups after receiving the samples on day 1 to day 14. The body weight gained by the rats that received *C. tiglium* seed powder before and after Thai TDP was significantly lower than that of the control group, although their food consumption and water intake were similar. The less body weight gained by the rats that received *C. tiglium* seed powder before and after Thai TDP might be due to the purgative and gastric irritation effects of the *C. tiglium* seed powder[100]. The size, weight, color and

lesions of the internal organs (brain, heart, thymus, lung, liver, stomach, spleen, left and right adrenal glands, and left and right kidneys) of the rats that received *C. tiglium* seed powder before and after Thai TDP were not significantly different when compared to the control group. All tested hematological and serum biochemical parameters of the rats that received *C. tiglium* seed powder before and after Thai TDP were also not significantly different from the control group. According to the acute toxicity test, both *C. tiglium* seed powder before and after Thai TDP at doses of up to 2,000 mg/kg BW were not toxic to the rats.

The results of the toxicity study of *C. tiglium* seeds are consistent with those of many previous reports[74, 93]. The toxic effects of *C. tiglium* seed extract have been previously evaluated to determine its single-dose acute toxicity (125 – 2,000 mg/kg), 14-day repeated-dose toxicity (125 – 2,000 mg/kg) and 13-week repeated-dose toxicity (31.25-500 mg/kg) in Sprague-Dawley rats and F344 rats[93]. Hematological, serum biochemical, and histopathological parameters were analyzed to determine its median lethal dose (LD_{50})[93]. The results showed that the acute LD_{50} of *C. tiglium* seed extract in rats was estimated to be greater than 2,000 mg/kg. Moreover, the hematological, serum biochemical, and histopathological parameters were not significantly different between the treatment and the control groups[42]. The sub-chronic toxicity of *C. tiglium* seed extract at a dose of 500 mg/kg was also evaluated in both male and female rats, and the results showed that there were no abnormal changes in mortality and behavioral symptoms[42]. EL-Kamali *et al.*[101] investigated the toxic effects of crushed *C. tiglium* seeds mixed with animal diet at concentrations of 10% or 20% in male albino rats[93]. The results showed that oral administration of *C. tiglium* seeds at doses of 10% and 20% not only had a little effect on some hematological indices relating to red blood

cells and white blood cells but also showed no significant change in aspartate aminotransferase and alkaline phosphatase activity between the control and treated animals. On the other hand, the rat group fed with a diet containing 20% of *C. tiglium* seeds showed a markedly lower level of aspartate aminotransferase activity[101]. Taken together, the results of the current acute toxicity study and the findings of the previous acute and sub-chronic toxicity tests suggested that oral administration of both untreated and treated *C. tiglium* seeds was safe. It is probable that PMA (and other phorbol esters) can be hydrolyzed in the stomach by gastric juice, thus its tumor promotor activity could be lost when *C. tiglium* seeds are administered orally[93].

The carcinogenicity and tumor promotion of *C. tiglium* seeds are main causes for concern. The mutagenicity of *C. tiglium* seeds was studied using the Ames test in *Salmonella typhi* TA 98, 100 and 102, and the result showed that it is non-genotoxic in those strains of *S. typhi*[83]. On the contrary, Kim et al. have found that *C. tiglium* seed extract produced the mutagenic responses in five *S. typhimurium* strains in the Ames assay; however, the frequencies of chromosomal aberrations or micronuclei were not altered, indicating that it exerted mutagenic potential and not clastogenicity[102]. Interestingly, there are many reports insisting that *C. tiglium* seed oil is a tumor promotor and not carcinogenic. Most of the previous studies on the tumor-promoting action of croton seed oil were performed on mouse skin. The promoting action of croton seed oil on skin carcinogenesis in mice by different chemicals, including benzopyrene, dimethylbenzanthracene, and urethan, has been reported[80]. The promoting action of *C. tiglium* seed oil on gastrocarcinogenesis was also investigated. It was found that croton seed oil promoted gastrocarcinogenesis by *N*-methyl-*N′*-nitro-*N*-

nitrosoguanidine, however, no tumors were found in rats given only croton seed oil with Tween 60[103].

The main irritant components of *C. tiglium* seeds are phorbol esters and crotonic acid together with some other organic acid constituents. The amount of crotonic acid in *C. tiglium* seed extracts before Thai TDP is 0.001 mg/g of the seed extract while no crotonic acid was detected after Thai TDP. The result showed that Thai TDP was able to completely eliminate crotonic acid. The amounts of PMA in *C. tiglium* seed extract decreased from 1.59 mg/g of the extract before treatment by Thai TDP to 1.22 mg/g of extract after treatment by Thai TDP. This result indicated that Thai TDP was able to reduce the amount of PMA. The decrease in the amount of crotonic acid and PMA after Thai TDP is likely due to the effect of boiling and salt. The decrease in PMA could be due to the hydrolysis of PMA to phorbol. This method not only reduced the amount of crotonic acid and PMA but also produced another compound that appeared in the chromatogram of *C. tiglium* after Thai TDP, with a retention time of 28 min (Figure 4.8). However, this compound has not yet been identified. Given the condition of the detoxification process, this compound may well be a product of hydrolysis of PMA. Therefore, further study is needed to elucidate the structure of this compound.

C. tiglium seeds are subjected to TDP before being used in traditional medicine not just in Thailand. The treatment of *C. tiglium* seeds is also used in India, however, the method of treatment is completely different. The method used in India consists of wrapping dried *C. tiglium* seeds in a clean white cloth to form a bolus which was soaked in a cow dung solution in a clay pot and boiled and then washed with water. Then the seeds were treated with cow’s urine, followed by lemon juice. Subsequently, the seeds were washed with water and removed from the outer skin and cotyledons and finally

fried with ghee. The study to determine the amount of phorbol and fatty acids revealed that there was a significant reduction of phorbol from 5.18 to 3.86 %. The amounts of saturated fatty acids like arachidic acid, behenic acid, stearic acid and palmitic acid were also decreased whereas all the unsaturated fatty acids like oleic acid and linoleic acid seemed to be unaltered[82]. On the contrary, no detoxification process of *C. tiglium* seeds was found before they were used to produce medicine in China[84].

Most studies consistently indicate that *C. tiglium* seeds and PMA are not carcinogenic but act as tumor promoters[41, 80, 102]. However, cancer caused by PMA requires continuous exposure to carcinogens for a long period of time and with certain concentrations. In addition, numerous studies have reported that PMA and many other phorbol derivatives also exhibited anticancer activity[25, 102, 104, 105]. It is probable that these compounds may display dual actions, i. e. promotor of tumor on the one hand, and antagonist of carcinogenesis on the other hand. However, further studies are needed to investigate chronic toxicity and tumor promoter effect of *C. tiglium* seeds after Thai TDP.

Additionally, tests were conducted to study the toxicity of crude methanol extracts from *C. tiglium* before and after Thai TDP on six types of cancer cells: lung cancer cells H2228 and A549, colon cancer cells HT-29 and Caco-2, and cholangiocarcinoma cells KKU213 and KKU100. Among these, the best results were obtained with the Caco-2 colon cancer cells. After 72 hours, the IC_{50} value was 103.7 µg/mL, consistent with the study by Aboulthana et al. (2019)[18], which tested *C. tiglium* extracts on human colon carcinoma Caco-2 cells using three solvents: 80% ethanol, petroleum ether, and water. Using the MTT assay, significant anticancer activity was

observed with IC_{50} values of 309.70 µg/mL for ethanol extract and 176.90 µg/mL for water extract at 24 hours.

Further investigation on the expression of genes involved in the apoptosis pathway using RT-PCR and protein expression revealed that the extracts induced the expression of apoptosis-related genes, specifically Bax, caspase-3, and caspase-9, using beta-actin as a standard reference. The extract of *C. tiglium* seed after Thai TDP, at 200 µg/mL increased the relative gene expression levels of these genes by 1.5 to 2.5-fold compared to the control group. These findings are in agreement with the results obtained by Li et al. (2016)[5], reported that the inhibition of *C. tiglium* on lung cancer cell growth through apoptosis mechanisms involving the Bax/Bcl-2 pathway. The protein Bcl-2 acts as a bridge between the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway, functioning as a sensor and triggering downstream proteins to initiate apoptosis[106].

This study also examined the effects of crude extracts before and after Thai TDP on colony formation, migration, and invasion of Caco-2 cells at concentrations of 50, 100, and 200 µg/mL to assess the potential tumor-promoting effects. Previous studies have identified diterpene compounds in *C. tiglium*, such as phorbol diacetate, phorbol dibenzoate, phorbol didecanoate, and tetradecanoyl phorbol acetate, which induced tumor formation in mice when combined with 7,12-dimethylbenz[a]anthracene (DMBA)[39]. Tetradecanoyl phorbol acetate, at a concentration of 1.83 µmol/week caused papillomas in mice. Similarly, studies by Van Duuren and Sivak (1968)[41] reported that phorbol esters, such as PMA and phorbol-12,13-didecanoate, promoted tumor formation, with concentrations above 50 µg/mL releasing tumor-promoting compounds like acid hydrolases.

However, the findings in this study showed that crude extracts from *C. tiglium*, both before and after Thai TDP, as well as new compound at concentrations of 50, 100, and 200 µg/mL, did not increase the number of colonies or enhance migration and invasion of Caco-2 cancer cells compared to the control group. These results are consistent with the study by Raick et al. (1972), where 0.1 µM DMBA served as an initiator, and 0.5% croton oil acted as a tumor promoter in mice[7]. While tumor formation increased by week 5 and reached 80% after 25 weeks when both agents were combined, croton oil alone, dissolved in 0.5% acetone, did not induce cancer in the mice. Thus, it can be concluded that crude *C. tiglium* extracts, before and after Thai TDP, and new compound at concentrations of 50, 100, and 200 µg/mL do not promote the severity of Caco-2 colon cancer[93].

In conclusion, the findings of this study provide valuable insights into the toxicological profile and potential applications of *C. tiglium* seeds. While certain processing methods, such as Thai Traditional Detoxification Processes (Thai TDP), can mitigate some of the toxic effects, the inherent risks associated with *C. tiglium* seed usage cannot be entirely eliminated. Therefore, it is recommended that the use of *C. tiglium* in practical applications, particularly in traditional medicine, be strictly regulated. Additionally, chronic toxicity testing should be considered to assess the long-term health effects of its use. Future studies should focus on refining detoxification processes to enhance safety and efficacy, alongside establishing clear dosage guidelines and conducting comprehensive safety evaluations, including chronic toxicity assessments, to ensure that the benefits outweigh the risks in clinical or therapeutic settings.

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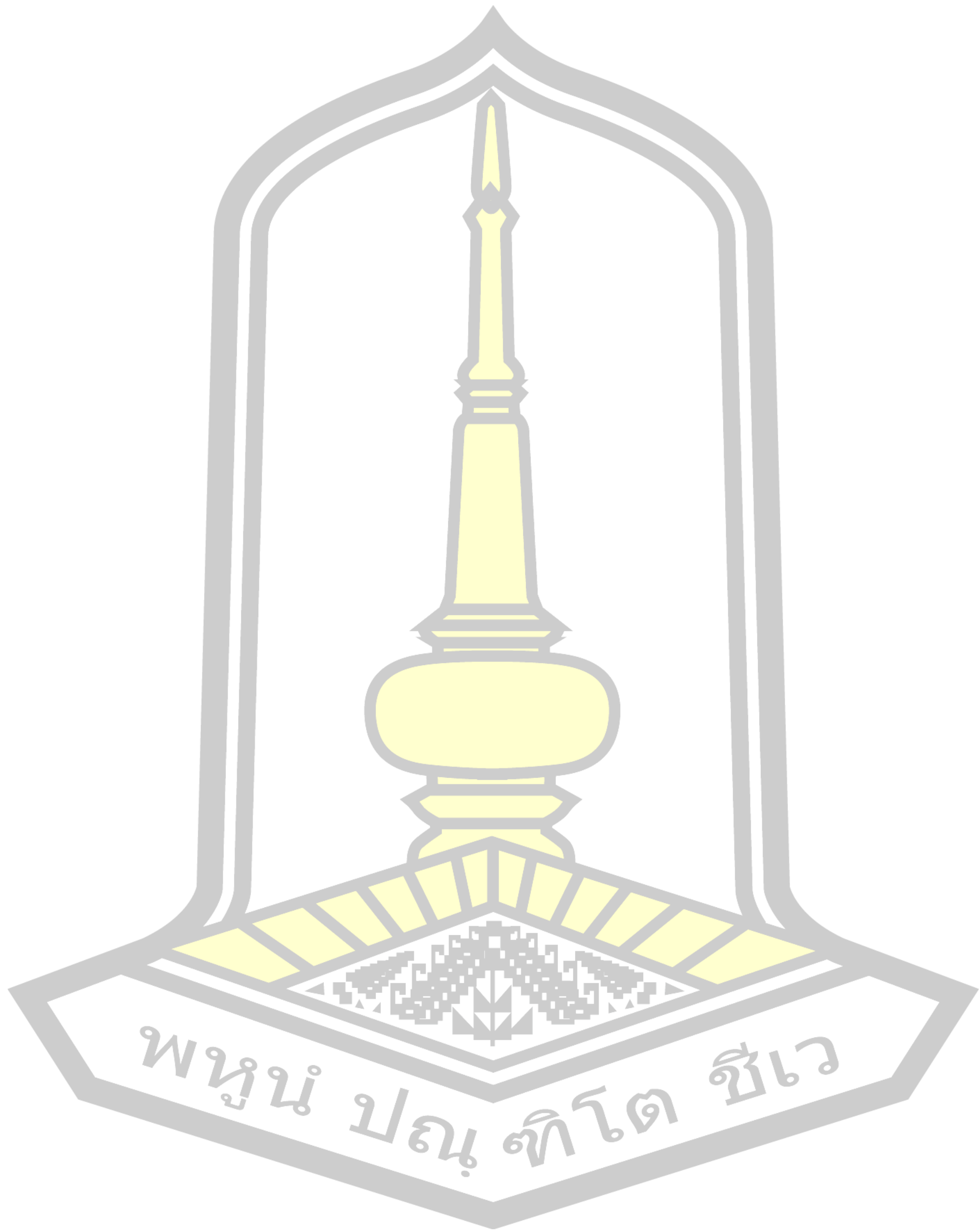
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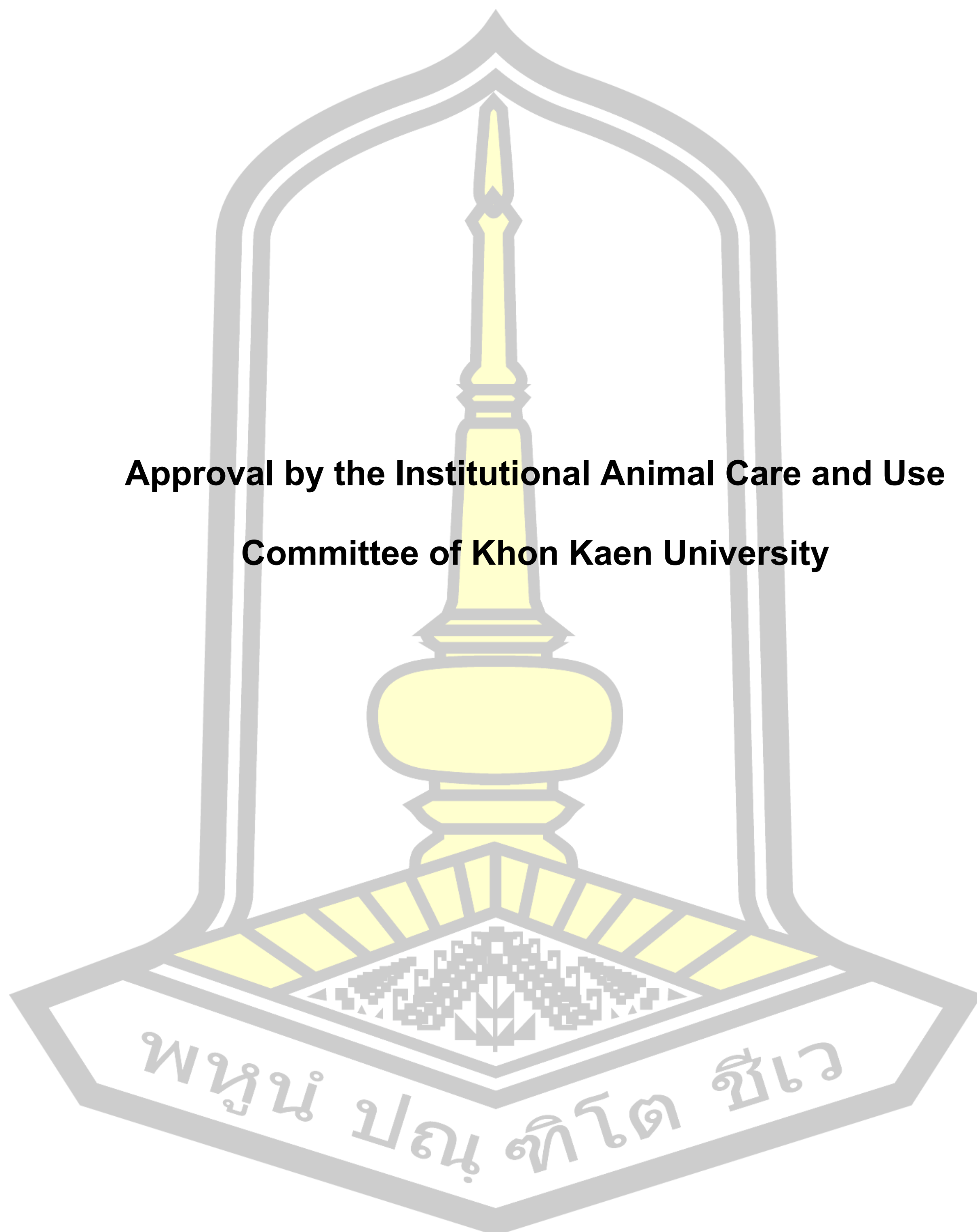
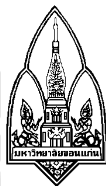
APPENDIX



Approval by the Institutional Animal Care and Use Committee of Khon Kaen University



KHON KAEN UNIVERSITY

This is certified that

The Project Entitled :	Comparison of Chemical Constituents, Pharmacological Activities and Toxicity of *Croton tiglium* L. Seed before and after Treatment by Thai Traditional methods
Researcher :	Assist.Prof.Dr.Somsak Nualkaew
Name of Department :	Faculty of Pharmacy Mahasarakham University

has been reviewed and approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethic of Animal Experimentation of National Research Council of Thailand.

Date of Approval : 18 March 2021

Professor Kanokwan Jarukamjorn, Ph.D.
Chairman,
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Vice President for Research and Graduate Studies,
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Record No. IACUC-KKU-28/64

Reference No. 660201.2.11/122 (30)

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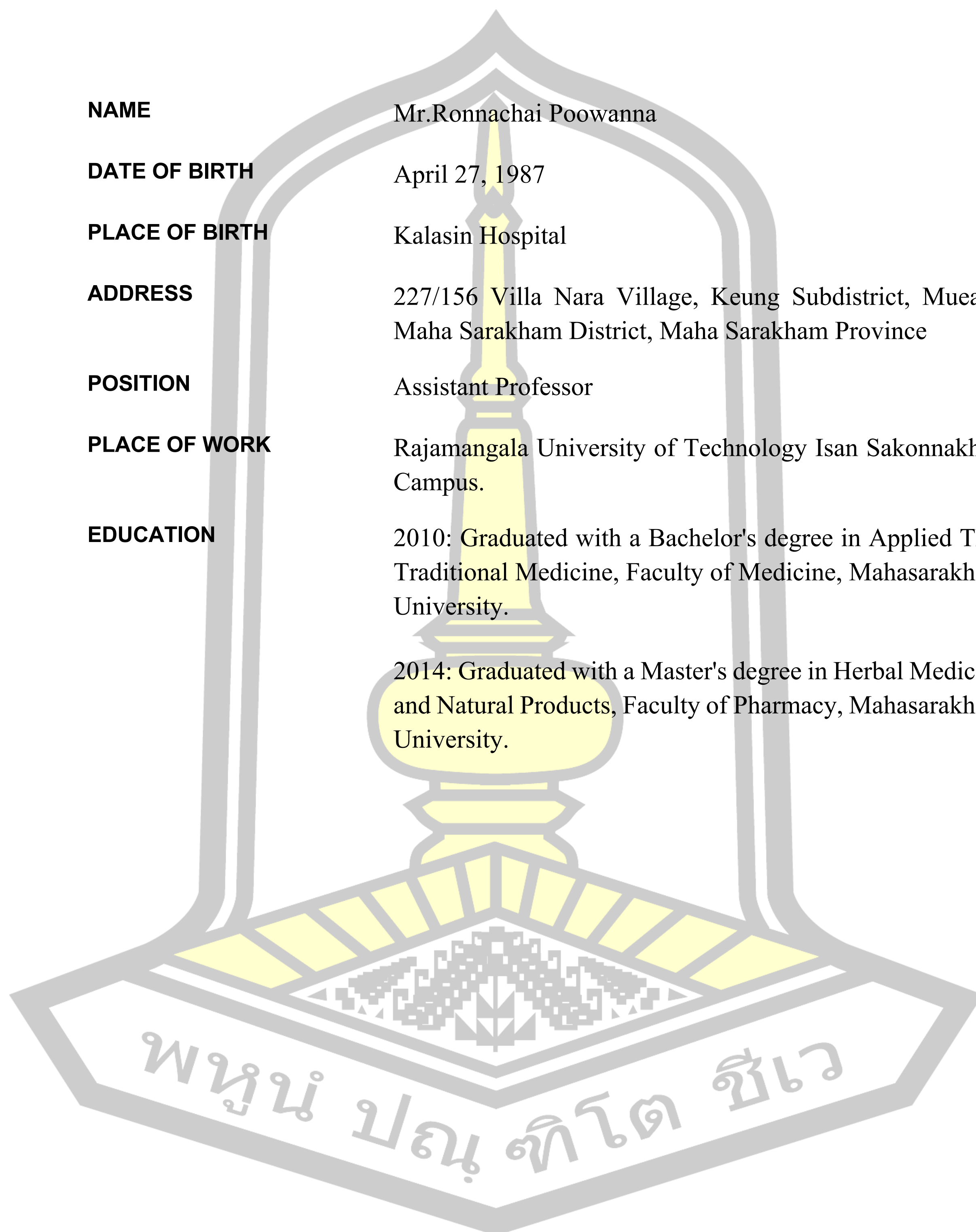
Tel.-66-43-202011

BIOGRAPHY



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