IDENTIFICATION OF BIOACTIVE COMPOUNDS IN *THUNBERGIA LAURIFOLIA* LINDL. (RANG CHUET) EXTRACT AND THEIR DETOXIFICATION IN LIVER CELLS

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การระบุสารออกฤทธิ์ทางชีวภาพในสารสกัดรางจืดและฤทธิ์ของสารสกัดในการกำจัดพิษในเซลล์ตับ

ศรัยลักษณ์ เมือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ศรัยลักษณ์ เมือง : การระบุสารออกฤทธิ์ทางชีวภาพในสารสกัดรางจืดและฤทธิ์ของสารสกัดในการกำจัดพิษในเซลล์ตับ (IDENTIFICATION OF BIOACTIVE COMPOUNDS IN *THUNBERGIA LAURIFOLIA* LINDL. (RANG CHUET) EXTRACT AND THEIR DETOXIFICATION IN LIVER CELLS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. รัชฎาพร อุ่นศิวิไลย์, 72 หน้า.

คำสำคัญ: การระบุกลุ่ม/ *Thunbergia Laurifolia* LINDL. (รางจืด)/ สารออกฤทธิ์ทางชีวภาพ/ ฟีโอไฟติน เอ/ ไฮดรอกซิลฟีโอไฟติน เอ/ HEPG2, AML12

วัตถุประสงค์ของการศึกษาเพื่อระบุสารประกอบออกฤทธิ์ทางชีวภาพในสารสกัดรางจืดแบบหยาบ และตรวจสอบคุณสมบัติการล้างพิษด้วยเซลล์ไลน์ HepG2 และ AML12 วิธีการสกัดรางจืดด้วยคลอโรฟอร์ม โดยใช้เทคนิคสกัดสารด้วยซอกห์เลต จากนั้นวิเคราะห์ปริมาณคลอโรฟิลล์ทั้งหมด (Tchl) แคโรทีนอยด์รวม (Tcar) ปริมาณฟีนอลิกทั้งหมด (TPC) และปริมาณฟลาโวนอยด์ทั้งหมด (TFC) ของสารสกัด นอกจากนี้ใช้โครมาโตกราฟีแบบแบบแผ่นบาง (TLC) ระบุสารประกอบออกฤทธิ์ทางชีวภาพเบื้องต้น และทำการตรวจสอบยืนยันด้วยโครมาโตกราฟีของเหลวประสิทธิภาพสูง (HPLC) นอกจากนี้ส่วนของสารสกัดรางจืดถูกนำไปทดสอบความเป็นพิษต่อเซลล์โดย MTT assay และศึกษากิจกรรมของเอนไซม์ NQO1 โดยส่วนของสารสกัดรางจืดการผ่านการแยกสารที่แสดงกิจกรรมเหนี่ยวนำเอนไซม์ NQO1 ที่สูงที่สุด คือส่วนแยกที่ 3 (F3) ถูกนำไประบุชนิดของสารพฤกษเคมีเบื้องต้นโดยโครมาโตกราฟีแบบชั้นบาง (TLC) จากนั้นจึงใช้เครื่องมือวิเคราะห์ ได้แก่ ลิควิดโครมาโตกราฟีประสิทธิภาพสูง (HPLC) และลิควิดโครมาโตกราฟี-แมสสเปกโตรมิเตอร์ (LC-MS/MS) เพื่อยืนยันชนิดของสารประกอบพฤกษเคมีจากสารสกัดส่วนแยกที่ 3

ผลการศึกษาพบว่าปริมาณคลอโรฟิลล์ทั้งหมด (Tchlo) แคโรทีนอยด์รวม (Tcar) เปอร์เซ็นต์ผลผลิตของสารสกัด, ปริมาณฟีนอลิกทั้งหมด (TPC) และปริมาณฟลาโวนอยด์ทั้งหมด (TFC) ของสารสกัดรางจืดแบบหยาบมีค่า 0.375 ± 0.032 มก./กรัม, 2.682 ± 0.125 มก./กรัม วัตถุดิบ, 15.3 ± 0.1%, 363.776 ± 3.491 มก./กรัม ของสารสกัด และ 112.22 ± 0.367 มก./กรัม ตามลำดับ นอกจากนี้ผลการวิเคราะห์สารพฤกษเคมีของสารสกัดคลอโรฟอร์มของรางจืดด้วย HPLC พบกรดฟีนอลิก (กรดแกลลิก, กรดคาเฟอีน), ฟลาโวนอยด์ (อาพิจินิน), คลอโรฟิลล์ (คลอโรฟิลล์เอ, คลอโรฟิลล์บี, ฟีโอไฟตินเอ, ฟีโอไฟตินบี) และแคโรทีนอยด์คือลูทีน จากผลของการทดสอบความเป็นพิษต่อเซลล์และความมีชีวิตของเซลล์ หลังจากใช้ระดับความเข้มข้นของการแยกส่วนตั้งแต่ 0.03125 – 2 มก./มล. พบว่าเปอร์เซ็นต์ของการมีชีวิตรอดของเซลล์ในเซลล์ไลน์ทั้งสองชนิดสูงกว่า 50 % นอกจากนี้ ค่า IC_{50} ยังสูงกว่า 2 มก./มล. ดังนั้น ส่วนแยกที่ 3 (F3) ถือเป็นส่วนแยกที่มีความสำคัญ ซึ่งมีผลกระตุ้นการทำงานของเอ็นไซม์ NQO1 ทั้งในเซลล์ไลน์ HepG2 และ AML12 ที่ระดับ 3.908 ± 0.079 เท่า และ 1.99 ± 0.047 เท่า ตามลำดับ นอกจากนี้ ในกระบวนการระบุสารพฤกษเคมีใน ส่วนแยกที่ 3

(F3) ประกอบด้วย pheophytin *a* และ hydroxypheophytin *a* ที่มีโครงสร้างทางเคมี $C_{55}H_{74}N_4O_6$ และ $C_{55}H_{74}N_4O_5$.

กล่าวโดยสรุป สารสกัดรางจืด ประกอบด้วยสารพฤกษเคมี ซึ่งสามารถแยกส่วนและระบุชนิดของสารพฤกษเคมีในแต่ละส่วนแยกได้ นอกจากนี้ สารพฤกษเคทีที่ออกฤทธิ์ทางชีวภาพในสารสกัดรางจืด สามารถกระตุ้นการทำงานของเอนไซม์ล้างพิษ NQO1 ในเซลล์ไลน์ทั้งสองชนิด ดังนั้น การค้นพบชนิดของสารพฤกษเคมีในสารสกัดรางจืดที่ออกฤทธิ์ด้านการล้างพิษ สามารถนำไปประยุกต์

สาขาวิชาเทคโนโลยีอาหาร
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ลายมือชื่ออาจารย์ที่ปรึกษา _____
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SREYLAK MOEURNG : IDENTIFICATION OF BIOACTIVE COMPOUNDS IN *THUNBERGIA LAURIFOLIA* LINDL. (RANG CHUET) EXTRACT AND THEIR DETOXIFICATION IN LIVER CELLS. THESIS ADVISOR : ASST. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 72 PP.

Keyword: Identification/ *Thunbergia Laurifolia LINDL*. (RANG CHUET)/ Bioactive Compound/ Pheophytin-a/ Hydroxypheophytin –a/ HEPG2/ AML12 Cells Line.

The objective of the study was to identify a group of bioactive compounds in *Thunbergia laurifolia* Lindl. (Rang Chuet: RC) crude extract that provided the highest detoxification properties in HepG2 and AML12 cell lines. The soxhlet method was performed to extract phytochemical from RC leave powder then bioactive compounds of RC crude extract were separated by flash column chromatography (TLC). The percentage of yield extract, total chlorophylls (Tchl), total carotenoids (Tcar), total phenolic content (TPC), and total flavonoid content (TFC) were determined. Moreover, Thin-layer chromatography (TLC) was used as a primary identification of bioactive compounds following verification with High- performance liquid chromatography (HPLC). Furthermore, RC fractions (0.031 – 5 mg/mL) were applied to HepG2 and AML12 cell lines, and then cell viability and cytotoxicity were investigated by tetrazolium salt (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay. Next, NADPH: quinone oxidoreductase 1 (NQO1) activity was investigated in all fractions. Bioactive compounds in a fraction was further identified by TLC and verified by HPLC, and liquid chromatography-mass spectrometry (LC-MS/MS).

The results showed that the percentage of yield extract, Tchl, Tcar, TPC, and TFC were 15.3 ± 0.1 %, 2.682 ± 0.125 mg/g raw materials (RM), 0.375 ± 0.032 mg/g RM, 363.776 ± 3.491 mg/g of extract, and 112.22 ± 0.367 mg/g of extract, respectively. RC chloroform extract consisted of phenolic acids (gallic acid, caffeic acid), flavonoids

(apigenin), chlorophyll (chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b*), and lutein. As a result of cytotoxicity and cell viability, the percentages of cell viabilities in both cell lines were higher than 50 % and IC_{50} values were higher than 2 mg/mL. Consequently, fraction 3 (F3) was considered as a significant fraction, which induced NQO1 enzyme activity in HepG2 at 3.908 ± 0.079 fold and AML12 1.99 ± 0.047 folds, were compared to control. In the process of compound identification, F3 consisted of pheophytin *a* and hydroxypheophytin *a* with the chemical structures $C_{55}H_{74}N_4O_6$ and $C_{55}H_{74}N_4O_5$.

In conclusion, RC consists of phytochemical constituents which could be purified and identified. Bioactive compounds in RC could induce detoxification enzyme activity NQO1 in cell lines. Thus, phytochemicals in RC extract could be applied for functional food development.

School of Food Technology

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LIST OF ABBREVIATIONS

TL	=	Thunbergia laurifolia Lindl.
RC	=	Rang Chuet
F	=	Fraction
%	=	Percentage
℃	=	Degree Celsius
HPLC	=	High performance liquid chromatography
LC-MS	=	Liquid chromatography mass spectrometry
NMR	=	Nuclear Magnetic Resonance
TLC	=	Thin-layer chromatography
TPC	=	Total phenolic contents
TFC	=	Total flavonoid contents
Tchl	=	Total Chlorophylls
Tcar	=	Total Carotenoids
SFE	=	Supercritical fluid extraction
MAE	=	Microwave-assisted extraction
SEC	=	Size exclusions column chromatography
IEC	=	Ion exchange chromatography
Chl a	=	Chlorophyll a
Chl b	=	Chlorophyll b

LIST OF ABBREVIATIONS (Continued)

Pheo a	=	Pheophytin a
Pheo *b*	=	Pheophytin *b*
H	=	Hour
Rpm	=	Revolution per minute
RM	=	Raw material
m/z	=	Mass to charge ratio
Fractions	=	F
IC_{50}	=	Half maximal Inhibitory Concentration
ABTS	=	(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DPPH	=	2,2-diphenyl-1-picrylhydrazyl
FRAP	=	Ferric ion reducing antioxidant power
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
AML12	=	Alphas-mouse liver
HepG2	=	Hepatoma G2
DMEM F12	=	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMEM	=	Dulbecco's Modified Eagle Medium

LIST OF ABBREVIATIONS (Continued)

PBS	=	Phosphate buffered saline
DEX	=	Dexamethasone and
NQO1 (QR1)	=	NAD(P)H: Quinone oxidoreductase 1
DMSO	=	Dimethyl sulfoxide
FAD	=	Flavin adenine dinucleotide
NQO1 (QR 2)	=	NAD(P)H: Quinone oxidoreductase 2
Keap1	=	Kelch-like ECH-associated protein 1
Nrf2	=	Nuclear factor erythroid 2-related factor 2
ARE	=	Antioxidant response element
GST	=	Glutathione S-transferase
(m, μ) g	=	(milli, micro) Gram
(m, μ) l	=	(milli, micro) Liter
nm	=	Nanometers
SD	=	Significant different
ND	=	Non- detected

CHAPTER 1

INTRODUCTION

1.1 Significance study

Humans have been suffering from toxic chemicals found in food, drugs, and the environment. Continuous exposure to toxicants is a significant source of liver acutes, which is associated with various diseases and multiorgan failure (Mathew et al., 2018). The liver is the largest organ in the human body with hundreds of biochemical reactions including, metabolism, manufacturing, storage of energy, digestion food, and detoxification. The kidney, large intestine, lymphatic system, sweat glands, and liver are involved in detoxification, however, the liver is the main part of detoxifying (Gagliano, Grizzi, & Annoni, 2007). The detoxification enzyme systems are extremely complicated, and several catabolisms are involved. Basically, in phase I reaction, biotransformation of lipid-soluble (toxicants) is polar, however, many of the products are still active metabolites (Johansson, Weidolf, & Jurva, 2007). These mediate substances particularly generate oxidative stress (David Josephy, Peter Guengerich, & Miners, 2005). The reaction of the conjugation pathway in the phase II detoxification system is the main function in the conversion of active metabolites to become completely water-soluble (Kumar & Surapaneni, 2001). There are several substances such as glucuronic acid, sulfate, glycine, glutamine, taurine, ornithine, and glutathione found in Phase II detoxification system (Knights, Sykes, & Miners, 2007).

R. Smith and Williams (1970) reported the mechanism of non-water-soluble are transformed into a hydrophobic compound at hydroxyl reactive site. Phase I detoxification system mainly consists of the cytochrome P450 enzymes family while

the phase II detoxification system is composed of subordinated reactions such as oxidoreduction, hydrolysis, NAD(P)H: quinone oxidoreductase (NQO1), and glutathione S-transferase (GST) as well (Donkotjan, Benjanirut, & Angkanaporn, 2020). A family of CYP450 enzymes is containing a heme group and the conjugation pathway could neutralize chemicals into harmless forms, which be able to remove from the body.

A healthy diet might be a compelling agent in the body, which supports and impels enzyme activities. Plant-based diet might either provide a health benefit in disease prevention or treatment of chronic diseases, chronic inflammation, and cancer. According to World Health Organization found that people used medicinal plants as primary health care; there was about 70% to 80% for treatment of diseases (Matic et al., 2018). Several studies revealed herbal plants are an abundant source of phytochemical substances, that support the liver detoxification pathway, antioxidant, anti-inflammation, and other biological activities (L. Smith & Culvenor, 1981; Wattenberg, 1992). Among these phytochemicals, there might have some active compounds are generated into phase II enzymes while others are related to antioxidation reaction.

Phytochemicals in the plant could act as antioxidants which are generally contains of phenolic structure including, flavonoids, phenolic acids, catechins, carotenoids, lycopene, and diterpene (Moharram & Youssef, 2014). These phenolic structures are water-soluble and probably convey ferric reducing ability of plasma (FRAP) and DPPH free radical scavenging activity. *Thunbergia laurifolia* Lindl. hot water extract induced a very high level of FRAPP at 15.72 and 156.29 mmol Fe^{2+}/g sample (Wonkchalee et al., 2012). As well as Oonsilivaiet al., 2007 reporrted the *Thunbergia laurifolia* Lindl. water extract showed FRAP 0.928 ± 0.050 (mmol/g), while ethanol and acetone extract was 0.079 ± 0.002 and 0.044 ± 0.005 (mmol/g). Furthermore, another antioxidant assay revealed that *Thunbergia laurifolia* Lindl.

water extract showed DPPH 0.129 ± 0.01 (mgGAE/mL) which was higher than ethanol and acetone extracts at 0.261 ± 0.04 and 0.607 ± 0.06 (mgGAE/mL)

Thailand is one of the developing countries in South Asia that has been a facade to air pollution and a huge amount of chemical fertilizer in agricultural products. As a result, unbalanced of toxicants and antioxidant agents may contribute to oxidative stress and a souce of chronic diseasesas well (Arora, Sairam, & Srivastava, 2002). In attendance of free radicals in cellular could induce aging, deficiency of cell operation, and leading diseases (Aritajat, Wutteerapol, & Saenphet, 2004). Consequently, researchers are enthusiastic about plants' facilities in detoxification; the primary goal of Thai tradition is to promote health benefits and balance body circumstances according to Salguero, C. P. (2010). In Thailand, people use their trandition herb in both ready-to used and herbal drugs to maintain their health care (Chan et al., 2012). *Thunbergia laurifolia* Lindl. has known as a Thai traditional herb, which Thai people have used for centuries; they believed that it could antinode alcohol and pesticide (Chivapat, Chavalittumrong, Attawish, Bansiddhi, & Padungpat, 2009). *Thunbergia laurifolia* Linld. contains a large group of phytochemicals such as phenolic acids, chlorophylls, flavonoids, carotenoids, glycosides, and steroids. Phenolic compounds are regularly found in *Thunbergia laurifolia* Lindl. extracts; these active compounds were reported in inducing antioxidation activity (Pattananandecha et al., 2021). Furthermore, flavonoids are also acting as ferric-reducing antioxidant power agents (Wonkchalee et al., 2012). In an in-vitro study, *Thunbergia laurifolia* Lindl. was used for detox, its crude extracts could protect human cell lines from the toxicity of Cd (Junsi et al ., 2020), and decrease the human breast adenocarcinoma (MCF-7) cells cytotoxicity at IC_{50} 843 µg/mL (Jetawattana, Boonsirichai, Charoen, & Martin, 2015). Moreover, *T. laurifolia* Lindl. could induce the expression of CYP450 enzymes in hepatocellular carcinoma (HepG2) cells (Rocejanasaroj, Tencomnao, & Sangkitikomol,

2014); Oonsivilai et al., 2007 found that *Thunbergia laurifolia* Lindl. crude extracts induce the expression of NQO1 enzymes in Hepa 1c1c7.

Thunbergia laurifolia Lindl. leaves crude extracts are involved in several biological functions, however, the distinction of bioactive compounds is still in pursuit. Chromatography technique such as high-performance liquid chromatography (HPLC), Mass spectrometry (MS), and Nuclear magnetic resonance spectroscopy (NMR) has been applied for separating and identifying bioactive compounds in the *Thunbergia laurifoli* Lindl. extract (Kanchanapoom, Kasai, & Yamasaki, 2002). Chromatography methods consist of solid stationary phases like silica gel, which could preferentially adsorb polar substances and separate them in different retention times. The principle of silica gel is very useful in laboratories; essential substances could be isolated, identify, and determined theirs structural (Nakanishi, 1997). Initially, compound identification could be screened through Thin-layer chromatography (TLC) and proved by analytical instruments.

Thus, this study attempted to isolate the group of bioactive compounds in *Thunbergia laurifolia* Lindl. crude extract using silica gel column chromatography then identifying compounds by Thin-layer chromatography and analytical instruments (HPLC, and LC-MS/MS). This research also observed detoxification properties with hepatocyte alpha mouse liver 12 (AML12) and hepatoma G2 (HepG2) cell lines.

1.2 Research objectives and hypotheses

The objectives of this study were to an identified group of bioactive compounds in *Thunbergia laurifolia* Lindl. extract and observed theirs detoxification properties in the liver cells line. Therefore, this research work focused on:

1. Extraction of phytochemicals from *Thunbergia laurifolia* Lindl. leaf using an organic solvent, chloroform.

2. Separation group of compounds from *Thunbergia laurifolia* Lindl. crude extract by silica gel flash column chromatography.
3. To discover phytochemicals in fractions using thin-layer chromatography and High-performance liquid chromatography.
4. Evaluating the cytotoxicity and detoxification properties of fractions with HepG2 and AML12.
5. Identifying bioactive compounds in the fractions that induced the maximum NQO1 enzyme activity by the analytical instrument (HPLC and LC-MS/MS).

1.3 Scope of the study

Thunbergia laurifolia Lindl. leaves were collected in Nakhon Ratchasima province, Thailand. Chloroform was used as solvent extracts in soxhlet methods at 50 °C for 5 hours. The crude extracts were fractionated on silica gel flash column chromatography with solvent systems at a ratio of Hexanes and ethyl acetate. Cytotoxicity was investigated at various concentrations of each fraction (0.03125 -5 mg/mL) with HepG2 and AM12 cell lines. The detoxification was determined by the NAD(P): quinone oxidoreductase NQO1 kit, which measures the quantity of the NQO1 enzyme activity. The fraction that exhibited the highest NQO1 activity, was selected to identify bioactive compounds by LC-MS/MS.

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CHAPTER 2

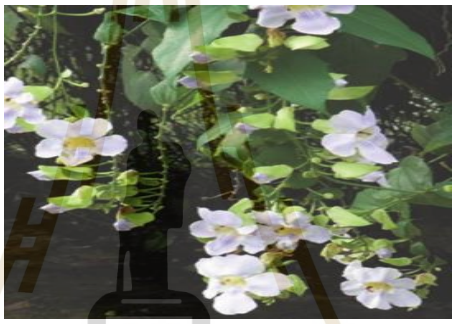
LITERATURE REVIEWS

2.1 *Thunbergia laurifolia* Lindl.

2.1.1 Physical characteristics of *Thunbergia laurifolia* Lindl .

Thunbergia laurifolia Lindl. is a claiming plant which is belonging to the genus *Thunbergia*, *Laurifolia* species, and family of Acanthaceae (Junsi et al., 2017). The plant is edible and distributed in southeast Asia including Malaysia and Thailand (Phyu & Tangpong, 2013). It has trumpet vine flowers with 5-7 rounded and pale purplish-blue petals, along with the leaves size are being up to 20 cm in length and 16 cm in width (E. W. C. Chan et al., 2012a). For years agoes, *Thunbergia laurifolia* Lindl. has found in a Thai' s traditional folk, and used against toxic substances because of their effectiveness in curing fever, antipyretic, mild poisons, hangovers, and other toxification. *Thunbergia laurifolia* Lindl. is called Rang Chuet or RC (da Silva et al.), other familiar names are Yaw Kaew, Kob Sha Nang, Gum Lung, Chang Puak, or Krua Nan Nae (Aritajat, Wutteerapol, & Saenphet, 2004). Thai people consume RC in form of tea, fresh dishes, juice, and capsules (E. W. C. Chan et al., 2012b). According to scientific studies, RC contains serval groups of bioactive compounds namely, phenolic acids, flavonoids, chlorophylls, steroids, glucosides, and cardiac glycosides; these phytochemicals are reported in health promotion due to they are antioxidant agents. RC is also composed of protein (1.983 ± 1.90 %), fat (1.83 ± 0.12 %), ash (19.93 ± 1.90 %), fiber (11.16 ± 0.44 %), and carbohydrate (53.10 ± 2.23 %) (M. Junsi et al., 2017); microelements also reported for instance iron and selenium (Marasri et al., 2020).

RC constituents are probably involved in the degradation of cadmium toxicity in rat liver, inducing the expression of phase II reduction enzyme NQO1, and enchanting CYP450 enzymes (Morkmek et al., 2010; Oonsivilai et al.,2007). Oonsivilai et al., 2007 revealed the effectiveness of RC acetone extract that outperformed water and ethanol by increasing NQO1 enzyme activity. In the study of RC methanolic crude extracts, there were reported any side effects on the hepatic bile duct in hamsters (Wonkchalee et al., 2012). Aritajat et al. (2004) said RC may secretly contain an insulin-like substance or regenerating β-cells (insulin-secreting cells) that



are associated with decreasing blood glucose. Figure 2.1 shows the RC leaves, the young leaf (right) is brighter than the developing (middle), and mature (left) leaves. Figure 2.2 shows the RC purple flower. RC has different colors of flowers, white flower, yellow flower, and purple flower, which was frequently mentioned as an important source of phytochemicals with serval pharmacological properties (Aritajat et al., 2004; Oonsivilai et al., 2007).

Figure 2.1 Rang Chuet leaves, the middle leaf is in the developing stages.

Figure 2.2 The Thunbergia laurifolia purple flowers. Source: (E. W. Chan, Eng, Tan, & Wong, 2011).

2.1.2 Phytochemicals constituent of *Thunbergia laurifolia* Lindl.

2.1.2.1 Phenolic acids and flavonoids

Phenolic acids and flavonoids are organic compounds consisting of a hydroxy-functional group linked to one or more aromatic rings; the aliphatic structure with an -OH group induces the compounds to become weak acids (Pandey and Rizvi 2009; Lattanzio 2013; Kumar, 2019). Generally, phenolic acid are found in many vegetables, fruits, and higher plants; they also acts as plant cellular defenders. The functional group provides an antioxidant potential which could induce therapeutic properties. Phenolic acid constituents include coumarins, ferulic acid, caffeic acid, *p*-coumaric acid, lignin, and sinapic acid, play a major role in reducing oxidative stress (Lanfer-Marquez, Barros, & Sinnecker). Tsao and Deng (2004) showed that carboxylic acid group in phenolic acid could attributes higher antioxidation activity compared to vitamins C. The value of antioxidant substances in plants or food products might

determine their merit potency in biological prevention from oxidation stress (Amorati & Valgimigli, 2018; Munteanu & Apetrei, 2021). Total phenolic contents could be estimated by the Folin–Ciocalteu colorimetric or gallic acid 9 4requivalence method (GAE). The reagent Folin–Ciocalteu consists of molybdate and tungstic that could form a complex of phosphomolybdic/ phosphotungstic acid with phenolic compound and induce visible purple color, which can be detected at wavelength

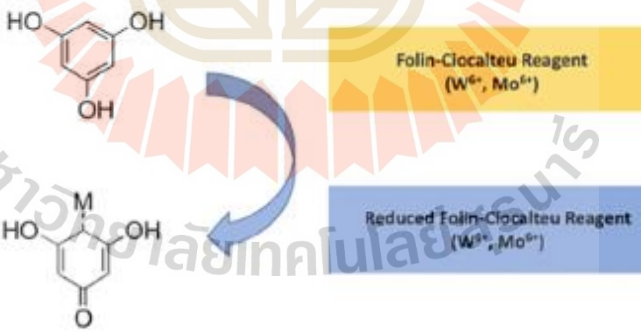
756 nm (Figure 2.3) (Ainsworth & Gillespie, 2007; Magalhães, Segundo, Reis, Lima, & Rangel, 2006).

Figure 2.3 Folin-ciocalteu reagent reaction with phenols.

Source: (Ford, Theodoridou, Sheldrake, & Walsh, 2019).

Flavonoids is a main group that contributes to phenolic compounds; flavones and flavonols are an assembly of phenolics (Pietta, 2000). Flavonoid constituents are extremely important in many biological activities such as antioxidants, antimicrobial, antiinflammation, and against cardiovascular (Sulaiman & Balachandran, 2012). RC existing phenolic acid and flavonoid i.e. gallic acid, caffeic acid, rosmarinic acid, apigenin, and quercetin (Kanchanapoom et al., 2002; Oonsivilai et al., 2007). Galic acid (GA) is strongly antioxidation and inti-inflammation and it could protects cells or tissue from oxidative stress including ABTS, DPPH, and FRAP assay (Gao, Hu, Hu, & Yang, 2019).

2.1.2.2 Carotenoids and chlorophylls



Plant leaves are a source of pigments that is responding to photosynthesis; pigments are chlorophylls, carotenoids, and betalains. Pigments not only provid an attractive signal but also protect plant cellular from being damaged by UV light (Choo, 2018). Pigments are regularly applied as food colorings instead of artificial colors; these natural coloring also exhibits antioxidative capacities (Dikshit & Tallapragada, 2018). In addition, they have been used in pharmaceutical and cosmetic products because of their health promotion (Pangestuti & Kim, 2011).

Carotenoids is yellow pigment, which exhibits distinguishing colors like reddish, yellow, as well as orange. Naturally, carotenoids are distributed by eight isoprenoids with C_{40} backbone, which is a major provitamin A with a great role in visual function (Mitra et al., 2021). Typically, carotene and xanthophyll are regularly found in carrots, potatoes, tomatoes, spinach, mangos, and papaya. Lutein is different from carotene by containing oxygen atoms in the form of a hydroxyl group, whereas, chemical structure of Beta-carotene is a form of hydrocarbons without oxygen in Figure 2.2 (Bohn, 2016). Moreover, carotenoid (lycopene) could be leading to detoxification capacity by inducing phase I and phase II metabolize enzymes (Stahl & Sies, 2005).

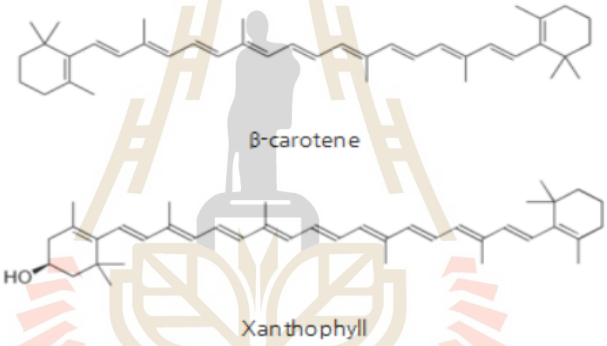


Figure 2.4 Chemical structures of beta-carotene and xanthophyll.

Source: (Zarekarizi, Hoffmann, & Burritt, 2019).

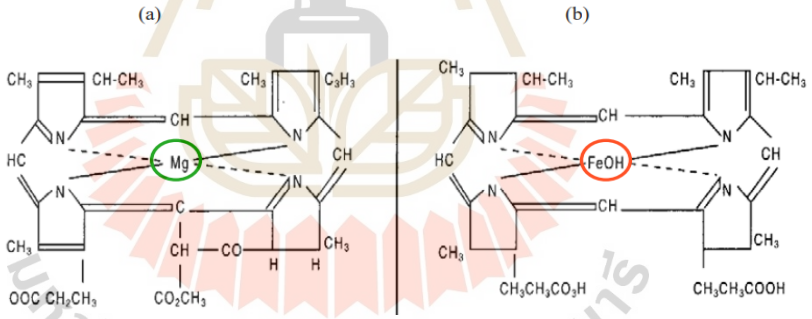
Chlorophyll is necessary in photosynthesis mechanism, which solar light is required. Carbon dioxide and water are converted to chemical energy glucose and oxygen through photosynthesis (Alinia-Ahandani et al. 2019). Chlorophylls are are natural chlorophylls that have used as an alternative form of medicine. Chlorophylls are rapidly self-degradation, they are sensitive to acidification, heat, oxygen, and light (Matile, Hörtensteiner, & Thomas, 1999). The structure compouond is similar to the heme group with a magnesium atom in the center of the porphyrin ring (Figure 2.3**)** (Zepka, Jacob-Lopes, & Roca, 2019). Natural chlorophylls are the most predominant form in green plants; 100 chlorophyll structures are relevant. Chlorophyll *a*, *b*, *c*, *d*,

and *e* are natural chlorophylls while the other compounds are metal-free particularly chlorophylls derivatives and metallochlorophyll derivatives (Mishra, Bacheti, & Husen, 2011). Scientists repotedt that bioactivity function of chlorophylls and their derivatives in particular antioxidation (Lanfer-Marquez et al., 2005), inhibition of cytochrome P450 enzymes (Guengerich, 2008), induction of phase II enzymes, and inducing of glutathione S-transferase (Kensler, 1997).

Figure 2.5 Molecular structure of (a) chlorophyll and (b) red blood cell.

Source: (Mishra, Bacheti, and Husen 2011).

Natural chlorophylls and it derivatives may donate an electron to a quinone that is evidence of reducing free radicals. However, natural chlorophylls could againstoxidative stress, Lanfer-Marquez et al. (2005) reported that natural



chlorophyll were lower antioxidant activity than pheophytin. Pheophytins are metal-free substances, which coulld act through many mechanisms including antioxidant activity, a modifier of genotoxic effects, inhibition of cytochrome P450 enzymes, cell arrest, apoptosis, induction of phase II enzymes even increased level of glutathione S-transferase (Percival 1997; Ikemoto et al. 2004; Tachino et al. 1994).

2.1.3 Biological properties of *Thunbergia laurifolia* Lindl

2.1.3.1 Detoxification study

NAD(P)H: quinone oxidoreductase 1 (NQO1) is considered as a cytosol enzyme involved in detoxification enzyme (Strassburg, Strassburg, Manns, & Tukey,

2002). NQO1 provides an obligation electron to quinones then conjugated with glucuronic acid/ glutathione then toxicants become water-soluble. Regarding to toxic classification, NQO1 is the xenobiotics-metabolizing which could be expressed at high levels in cancer cells compared to normal cells (Hamajima et al., 2002; Oh & Park, 2015).

RC crude extracts were applied to Hepa 1c1c7 cells and investigated the NQO1 enzyme activities. As a result, water, ethanol, and acetone extraction could induce detoxifying enzymes. RC acetone extract showed the highest NQO1 enzyme activity at 2.8 fold compared with the control group. Then ethanol (120 µg GAE/mL) and water (1000 µg GAE/mL) extract could induce NQO1 enzyme activity at levels of 1.4 and 1.6 folds (Oonsivilai et al., 2007).

Aritajats et al., (2004) studied the effectiveness of RC crude extract on diabetic rats. These rats were treated with RC crude extract at a dose of 60 mg/mL/day for 15 days and then histology of the pancreas was investigated. As a result, RC crude extract could decrease the level of blood glucose from 1,284.14 ± 629.12 (mg/100 mL) to 494.47 ± 343.25 (mg/100 mL). Moreover, the β-cells of the pancreas were found to partial recovery, and insulin levels were increased. In conclusion, bioactive compounds in RC might act as cell regenerative agents or probably contain insulin-like substances that are involved in hypoglycemia.

Junsi, Siripongvutikorn, Yupanqui, and Usawakesmanee (2017) found that RC water crude extract could protect RAW264.7 cells from carbamate insecticide methomyl toxicity (Gunderson et al.). MT (100-600 µg/mL) were exposed and measured IC_{50} by MTT assay. The outcome of this study, the pre-treatment group showed a positive result, which could protect macrophage cells from MT.

Tangpong and Satarug (2010) showed that RC water crude extracts are suitable for the protection of mice from Pb poisoning. Several studies mentioned that Pb can decrease acetylcholinesterase AChE activities coupled with increasing the

amount of acetylcholine, which is contributed to neuron dysfunctions, inducing ROS, and DNA damage (Ab Latif Wani & Usmani, 2015). RC crude extract 100 and 200 mg/Kg body weight were treated to exposed mice at level 1 g/L of $(2CH_3COO)_2$ Pb. $3H_2O$. This study also observed the physiology, antioxidant capacity, lipid peroxidation (Malondialdehyde, MDA), and the level of lead in blood and urine in mice. In a group of mice that received RC concentration are last of latency than untreated group; catalysis activity of caspase-3 enzymes lower than untreated and vitamin E supplement. Moreover, MDA also decreased in RC serving mice however the level of Pb was not decreased.

RC water crude extract (0.1 mg/mL 250 ml per day) and purification fraction (0.02 mg/mL 250ml per day) were subjected to Wistar rats, which were exposed with Cd (1 mg/Kg body weight). As result, the group of Wistar rats without RC treatment were dead, while treatment groups have been got along with the experiment. They found that levels of Cd in blood and urine had not reduced but rats' liver and kidneys were less damaging than untreated group. The research mentioned that bioactive compounds obtained from RC water crude extract and its' purification could protect cells and organs. Thus, active compounds in RC might role as a qualitative agent for health prevention (Ruangyuttikkarn et al., 2013).

2.1.3.2 Antioxidant and anti-inflammation activity

Unpair electrons in the body might be leading to an oxidative reaction, which is increased along with lifestyles like dietary, cigarette smoking, alcohol consumption, environmental exposures, and stress. The free radical is a reduction molecule that may attach to the unsaturated lipids and cellular then become a complex or mediation radical. These biological reactions may cause aging and various diseases such as ischemic heart damage, cancer, arthritis, Parkinson's, and Alzheimer's disease (Abdollahi, Moridani, Aruoma, & Mostafalou, 2014).

RC water extract is an abundant source of phenolic compounds, which showed high antioxidation properties including DPPH radical scavenging and free radical scavenging (FRAPP). Oonsivilai et al, (2007) found that DPPH IC_{50} values of water extract (0.129 ± 0.01 mg GAE / mL) are lower than ethanol minutes (0.261 ± 0.04 (mg GAE / mL) and acetone (0.607 ± 0.06 (mg GAE /mL) extract. Along with the antioxidant assay, water extract showed the highest FRAP at values (0.928 mmol Fe(II)/g), ethanol extract (0.079 mmol Fe(II)/g), and acetone extract (0.044 mmol Fe(II)/g).

2.1.3.3 Antimicrobial activities

RC crude extract could antimicrobial activities, antiviral, and antifungals. Aquarium and ethanol extracts were reported against gram-positive bacteria such as Escherichia coli O157: H7, Propionibacterium acnes, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, and methicillin resistant S. aureus.

2.2 Solvent and Extraction methods

Medicinal plant preparation is the initial stage of a research study; it is a critical part that could affect the result. Typically, plants should be prepared and held properly with a manipulated extraction method, an appropriate solvent, and also drying methods (Ingle et al., 2017). The menstruum used for plant extraction should rely on the target compounds, plant part, and further applications. Normally, polar solvents are intended for extraction polar bioactive compounds, whereas non-polar solvents attract non-polar substances (Sasidharan, Chen, Saravanan, Sundram, & Latha, 2011). Non-polar solvents may provide lower extraction yield and antioxidation properties than polar solvents (Nawaz, Shad, Rehman, Andaleeb, & Ullah, 2020). Solvent classification is depending on their polarity. Water, ethanol, and methanol are widely used in the extraction of polar compounds, while hexane,

dichloromethane, ethyl acetate, and chloroform were selected for lower-polar phytochemicals (Pandey & Tripathi, 2014). Chloroform was recommended for low polarity compounds, which are terpenoids, flavonoids, fats, and oils (Cowan, 1999). According to the chemical properties of chloroform, it is bulky chloride group that has low dielectric constant level at 5 while water has 78 of electric constant (Nath & Dubey, 1980). Thus, the chloroform might be non-polar solvent. However, chloroform compose of Carbon, Hydrogen, and Chloride could produce electronegativity not greater than 1, and they also induce dipole moment forces leading chloroform to be considered as a polar substance (Moseley, 1978). It could use as a solvent to dissolve other substances; Baul et al. (2017) found that P. fascicularis chloroform extract contains various bioactive compounds group such as alkaloids, steroids, terpenoids, flavonoids, saponis, proteins and tannins.

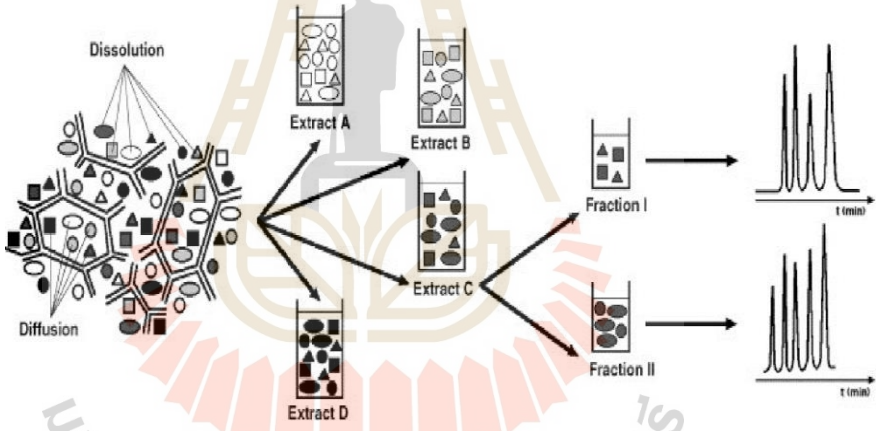
Furthermore, extraction methods are also the main factor of the study. Scientists should be considered, compounds' stability, characteristics of solvent, cost, duration of extraction, final amount of extract, and product design (Abubakar & Haque, 2020). Several extraction methods are mentioned such as maceration, infusion, digestion, decoction, percolation, soxhlet, microwave-assisted, and ultrasound-assisted extraction.

1. Soxhlet method: the continuous extraction method is used for plant extract with an organic solvent. It is appropriated in large-scale extraction. The plant powder in cellulose extraction is placed in thimble and connected with solvent in a round flask. The processing is certainly heating at about 50 ℃ and specific during times. The method could produce a large amount of extract, and economically (Hossain, Al-Hdhrami, Weli, Al-Riyami, & Al-Sabahi, 2014).

2. Maceration: thee method is a term of digestion mode that is appropriated for the thermolabile plant. Plant leaves powder place in container and shock in a solvent for at least 3 days. The method may be used a large amount of solvent and is time-consuming but it is a very convenient method (Singh, 2008).
3. Infusion: The plant material in extract solvent is left at room temperature. This method is able for shaking. Hot water infusion extract is an inexpensive method with sustainable savings (Ozel, Gogus, & Lewis, 2003).
4. Supercritical Fluid Extraction (SFE): the technique has been commonly used for extraction because it could provide good quality and high yield products. Products are non-toxic, lack reactivity, and are environmental. Carbon dioxide (CO_2) contributes to gas-like, liquid-like characteristics, and high diffusion coefficients with low viscosities under the pressure of 73.8 bar at 31.1 °C. The system penetrates solvent into porous plant material inconsequent in mass transfers (Mohammad Azmin et al., 2016).
5. Microwave-assisted extraction (MAE): it is less time-consuming method, which electromagnetic waves at a frequency range from 0.3-300 GHz apply for the extraction of phytochemicals. Solvent is the main factor in this method because it is associated with the absorbance of microwave energy. The advantages of MAE is appropriate for industrial scale, however, it might not suitable for temperature-sensitive compounds (Chemat, Abert-Vian, & Zill-e-Huma, 2009; Sandri, Zacaria, Fracaro, Delamare, & Echeverrigaray, 2007).

2.3 Compound separation techniques

The purification technique is the process used for separating a component from a mixture (Abubakar & Haque, 2020) . The chromatographic technique is generally used in medicinal compounds purification; the method is based on stationary and mobile phase systems (Revathy, Elumalai, & Antony, 2011; Sasidharan et al., 2011). The compound separation is depending on differences in adsorption of compounds to the adsorbent, through the column at different rates, and separated into fractions (Altemimi, Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017). The plant leaves extract contains various types of phytochemicals which are different in molecular weight and polarities. The characteristics of compounds allow them to be separated into several fractions A, B, C, and D (Figure 4) . High-performance



chromatography (HPLC), thin-layer chromatography (TLC), and flash chromatography apply for separation and identification purposes (Sasidharan et al., 2010).

Figure 2.6 The technique of separation method. A mixture of plant constituents could be isolated into extraction A, B, C, and D. The extract of solvent C obtains 2 fragments, I and II. Source: (Nyiredy 2004).

2.3.1 Thin-layer chromatography

Thin-layer chromatography apply as primary identification of bioactive compounds because it is one of the fastest, least expensive, simplest, and easiest techniques. (Cheng, Huang, & Shiea, 2011). A compound matrix drop on a TLC plate

and put in mobile phase, constituent appear as spots by moving on the surface of stationary phase (Coskun 2016). The output of high-affinity compounds is slower than others, their character and nature are suitable for separation technique (Sherma and Fried 2003). The reaction between the stationary phase, mobile phase, and compounds is a basic of compound separation at different distances, the distance (Rf) of the spot sample is over the distance of the solvent.

2.3.2 Flask column chromatography

Flask column chromatography composes stationary phase and system mobile phase. Silica gel powder is a polar stationary phase, which provides an attractive OH group on the surface. Bioactive compounds are interacted and stuck on the silica gel, then compound are eluted by the mobile phase (AMAROWICZ, SHAHIDI, & WICZKOWSKI, 2003).

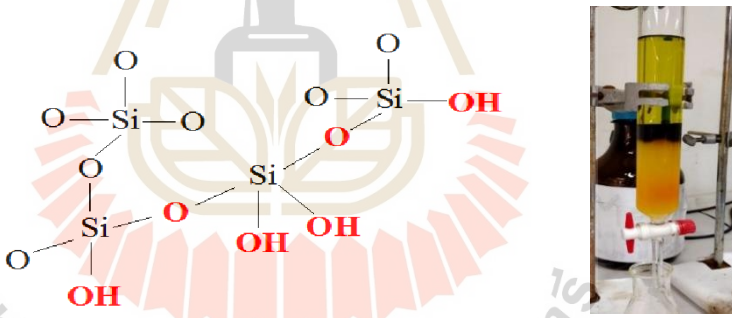


Figure 2.7 The structure of silica gel with silanol groups interacts with the hydroxyl group of polar compounds. Source: (Hamza, Sherif, and Abdalla 2017).

2.3.3 Size exclusions column chromatography (SEC)

Size exclusions column chromatography is composed of resin which is spherical beads with various porous sized. Basically, compounds separation is based on diffusion of molecules into the bead' s pores, incapable molecules would be

eluted first. Whereas compounds with smaller sizes than pores are trapped in resin beads; compounds do not bind or interact with filtration gel mean whiles mobile phases also do not directly affect resolution (Sun, Chance, Graessley, & Lohse, 2004). SEC could use for purifying compounds by their molecules size, geometry, and molecular weight. The technique is suitable for separation protein, amino acids, enzymes, antibodies, DNA, and RNA (Stranska et al., 2018).

2.3.4 Ion exchange chromatography (IEC)

Ion exchange chromatography is also a liquid chromatography technique that consists of mobile phase and stationary phase. The stationary phase is composed of organic matrix substances that are ionizable to produce anion or cation ion exchangers. The technique is depending on the reaction between positive or negative charge of analytes and a fixed ion stationary phase. A cation ion compound would be attached to an anion fixed, molecules show at defferent level of interaction because of their charge property (Jungbauer & Hahn, 2009) . The application of IEC is generally used for separation biomolecules such as peptides, proteins, nucleic acids. Furthermore, IEC also could purify biopolymers compounds (Acikara, 2013).

2.4 Cytotoxicity and MTT assay

Cytotoxicity is the method used for measuring bioactive compounds or chemical concentration being toxic to the living cell. The high concentration, incased of treatment or exposed, cells acutes are alerted. The cell cytotoxicity could determine by a tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT assay. MTT assay is a standard calorimetric laboratory assay involving cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. MTT reagent turns to purple formazan crystals because of viable cells (living cells). Living cells contain NAD(P)H-dependent oxidoreductase enzymes which

it involves in the reduction of MTT to formazan (Maehara et al. 1987). The formazan crystals are soluble in Dimethyl sulfoxide (DMSO) solution and absorb at wavelengths 450-600 nm (Berridge and Tan 1993). The result of cell viability:

$$\% \text{ Cell viability} = (OD_{sample}/OD_{control}) * 100$$

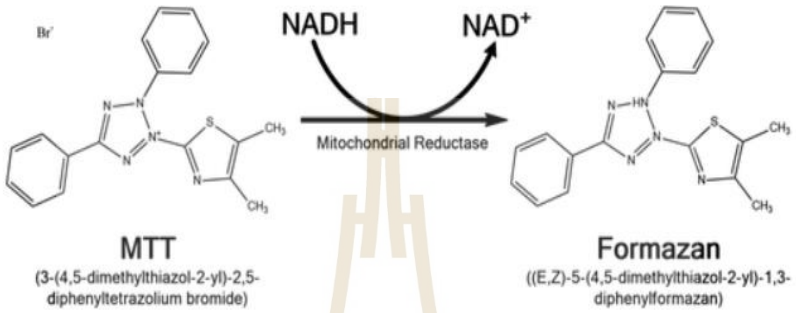


Figure 2.8 The reaction of MTT reagent with cofactor NADH in mitochondrial, a visible formazan could be measured by colorimetric assay.

2.5 NAD(P)H: quinone oxidoreductase 1

Quinone oxidoreductase or QR assay is the biological study used in the exhibition of active enzymes NAD(P)H: NQO1 (quinone oxidoreductase) involved in detoxification (Burke, 2021). NQO1 (QR1) is cytosolic quinone reductase, once among of phase II enzymes found in intracellular detoxification (Fahey, Zhang, and Talalay 1997; Lateef et al. 2015). NQO1 enzyme is encoded by the NQO1 gene, which is mapping to chromosomal location 16q22. 1 (Cleton-Jansen et al., 2008), the transcription of the QR gene that is responded to synthesis enzymes NAD(P)H: NQO1. QR1 protein is consisting of a 53 long residues C-terminal domain, which is absent in QR2. The NQO1 and NQO2 are sharing similar properties on Flavin adenine dinucleotide (FDA) binding site (Figure 2.6), but are different in the hydride donor binding site (Ferruzzi & Blakeslee, 2007). The basic mechanism of QR involves a

hydride transfer from NADH or NADPH to free toxicants or radicals. (Srijiwangsa and Na-Bangchang 2017).

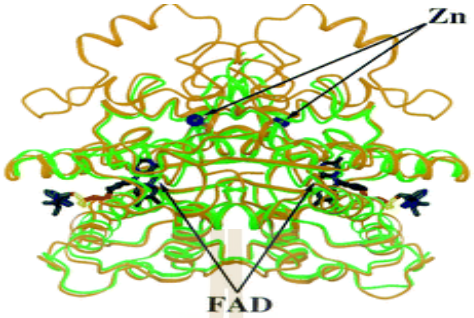


Figure 2.9 The structure of QR 1 (orange) and QR2 (green) enzymes.

Source: (Fischer et al., 2011).

Medicinal plants or herbal are consisting of phytochemicals related to inducing NQO1 activities, which are highly reactive to quinone (Fahey, Dinkova-Kostova, Stephenson, & Talalay, 2004). The enzyme was hypothesized to have a crucial role in the protection against oxidative stress and was shown to be multifunctional, and antioxidant especially acts as a stabilizer for the tumor suppressor protein p53 (Ross et al., 2000). Bioactive compounds induce direct and indirect antioxidants (Tumer, Rojas-Silva, Poulev, Raskin, & Waterman, 2015), however, salvianolic acid B, lithospermic acid, and rosmarinic acid were reported that they are insufficient potency in inducting the NQO1 enzyme in h1c1c7 (Zhang, Song, Xu, & Ma, 2011).

Pigments in plants provide indirect antioxidant, which is phase II enzymes through Keap1/ Nrf2/ ARE pathway, these metabolized enzymes could enlarge the antioxidant capacity with long-lived protection. Chlorophylls and their derivative substances have been reported that they are effective in decreasing cancer. Balder et al. (2006) found that chlorophyll dietary intake patients frequently decreased colon cancer. Similarly, chlorophylls are potentially blocking carcinoma cells (De Vogel, Jonker-Termont, Van Lieshout, Katan, & van der Meer, 2005).

2.6 Analytical instrument

2.6.1 High-performance liquid chromatography

High-Performance Liquid Chromatography (HPLC) is an analytical instrument, that separates chemical compounds. Its application in analytical chemistry, pharmaceutical and drug science, clinical sciences, and food technology. In the operation of HPLC, sample mixture to be separated and analyzed in microliters volume injection, components interact with the stationary phase and pass through the column at different velocities. The peak release coupling to intensity and retention time (RT) (Sahu et al. 2018).

2.6.2 Liquid chromatography-mass Spectrophotometer

Liquid chromatography-mass spectroscopy (LC-MS) is a combination of LC and MS that it could detects chemicals or compounds based on their mass spectrum. LC or HPLC is function in compound separation while MS provides spectral information of fraction ions. This analytical instrument could be applied for identification biological molecules, organic and inorganic compounds. The instrumentt is sensitive and accurate, which could detect analytes in microgram or even nanogram. LC-MS operates by converting analytes to ions or fraction ions by electrospray ionization (ESI) that can be detected by mass to charge ratio (m/z). Typically, LC_MS contains interface that could transfer analyts from LC into MS (Zheng, McErlane, & Ong, 1998). The separated compound from LC column could form a droplet, which rapidly evaporated by N_2 gas and transformed into an ionized analyst using ESI. Then, the ion entires mass analyzers, and the separation is based on their mass-to-charge (Pitt, 2009).

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CHAPTER 3

COMPOUNDS SEPARATION AND IDENTIFICATION IN *THUNBERGIA LAURIFULAI* LINDL. CRUDE EXTRACT.

3.1 Abstract

Thai traditional medicinal plants, the *Thunbergia laurifolia* Lindl. (Rang Chuet) or RC consists of various bioactive compounds involved in biological activities, which could promote health benefits. This research aims to purify and identify a group of bioactive compounds in RC crude extract by using silica gel column chromatography and High-performance liquid chromatography (HPLC). RC leaves powder was extracted in chloroform utilizing the Soxhlet method. RC crude extracts were separated by silica gel flash column chromatography and using mixture of hexan and ethyl acetate as a system mobile phase. We determined the yield extract, total chlorophylls, total carotenoids, total phenolic content (TPC), and total flavonoid content (TFC). Furthermore, Thin-layer chromatography was applied as a primary screening of bioactive compounds following verification on HPLC. The results showed that the total carotenoids, total chlorophylls, yield percentage of extract, TPC, and TFC were 0.375 ± 0.032 mg/g of raw materials (RM), 2.682 ± 0.125 mg/g of RM, 15.3 ± 0.1 % , 363.776 ± 3.491 mg/g of extract, and 112.22 ± 0.367 mg/g of extract, respectively. RC chloroform extract consisted of phenolic acids (gallic acid, caffeic acid), flavonoids (apigenin), chlorophyll (chlorophyll *a*, chlorophyll *b*, pheophytin *a*, pheophytin *b*), and lutein. Moreover, there were 7 fractions collected from RC crude extract and their phytochemicals (TPC, TFC, and Tchl) were also evaluation. The result found that TPC of fractions F1 and F2 were higher than other fractions while

they showed at very low amount of Tchl. In contrast, fraction F3 contained the highest quality of Tchl at 10.70 ± 0.418 mg/g extract. In conclusion, this research could separate and identify phytochemicals in RC chloroform crude extract. Therefore, RC could be applied in further functional food development.

Keywords: *Thunbergia laurifolia* Lindl., primary identification Thin-layer chromatography, purification, High-performance liquid chromatography.

3.2 Introduction

Thunbergia laurifolia Lindl. (Acanthaceae, Thai name: Rang Chuet) is a traditional medicine that has been used in Thai folk for centuries (M. Junsi et al., 2017). Rang Chuet crude extract is a natural source of bioactive compounds including a group of phenolic acids (caffeic acid, rosmarinic acid), flavonoids (apigenin), lutein, and chlorophylls (chlorophyll *a*. chlorophyll *b*, pheophorbide *a*, pheophytin *a)* (Oonsivilai et al., 2007; Kanchanapoom, et al., 2002; M.Junsi et al., 2017). The plant also contains protein (1.983 ± 1.90 %), fat (1.83 ± 0.12 %), ash (19.93 ± 1.90 %), fiber (11.16 ± 0.44 %), and carbohydrate (53.10 ± 2.23 %) (Junsi et al. 2020). Phytochemicals in RC were reported as antioxidant agents such as gallic acid potent in reducing free radical scavenging activities (Rajan & Muraleedharan, 2017). Suwanchaikasem, Chaichantipyuth, and Sukrong (2014) successfully separated rosmarinic acid from RC crude extract by column chromatography. Numerous of bioactive compounds were found in RC crude extract, studied their abiological activity. However, the bioactive compounds purification of RC crude extract have not been widely study.

The main idea of this work is to separate bioactive compounds in RC crude extract by applying silica gel column chromatography, then compounds were identified by thin-layer chromatography and varying on HPLC. The research also

quantifies the total phenolics, total flavonoids, carotenoids, and chlorophylls in Rang Chuet crude extracts and derived fractions.

3.3 Chemicals and standards

The phenolic profiling was studied and eight chemical standards including, garlic acid, caffeic acid, 3,4, dihyroxybenzoesaure (protocatechuic), p-coumaric acid, rosmarinic acid, 4-hydroxycinnamic (ferulic acid), apigenin, and sinapic acid (Sigma-Aldrich Co) were used. As well, lutein, pheophytin *a*, pheophytin *b*, chlorophyll *a*, and chlorophyll *b* were used in chlorophylls profiling investigation. Thin-layer chromatography (TLC) $60F_{254}$ and silica gel powder 60 µm were from Sigma-Aldrich, Germany. Hexane, chloroform, acetic acid, methanol (HPLC grade), and ethyl acetate (HPLC grade) were from Carlo Erba, France.

Pheophytin *a* and *b* standards were extracted from chlorophyll *a* and *b* as described by Ferruzzi et al. (2001). Briefly, 1 mg of chlorophyll *a* or chlorophyll *b* was dissolved in 10 ml of acetone, then 400 µl of 0.1 N HCl was added into 5 ml of chlorophyll *a* or *b* solution. The extract solution was kept at room temperature and notified color change from green to olive-brown. The solvent was evaporated by N_2 gas, and the residue was dissolved in acetone followed by filtering through a 0.22 µm PTFE membrane, then kept at -80 ℃ until used.

3.4 Raw material and extraction

RC leaves were collected from Nakhon Ratchasima province, Thailand. Fresh leaves were collected from December of 2020 to January of 2021. Leaves were washed with tap water, rinsed, and dried under microwave vacuum (March Cool, USA) at 24000 W to reach a moisture content of 8 – 9 % . Sample leaves were ground (Multi-purpose blender Philips, 7000 W), and sieved by mesh size 2 - 4 nm, then were kept in freezer at -20 ℃.

The plant extraction method is followed by Pandey and Singh (2014). Rang Chuet leaf powder 5 grams was placed in a cellulose thimble (Whatman, GE Healthcare, UK), then 200 ml of chloroform was used as solvent extracted utilizing the soxhlet method. The extraction was performed at 50 ℃ for 5 hours and evaporated, after that the crude extract was collected and stored in a freezer at temperature - 20 ℃ for further experiments.

3.5 Phytochemical profiling

3.5.1 Total phenolic contents

The total phenolic content of the extracts was determined by the method of Deng et al., (2014). One hundred microliters of Folin-Ciocalteu reagents were added into RC crude extract and its derivatives fraction 20 µl (0.5 mg/mL) followed by 80 µl of the $NaCO_3$ (7.5 % w/v) mixed and incubated at darkroom temperature for 30 minutes. The absorbance was measured at wavelength 765 nm using microplate reader (Spectro star Nano Microplate reader, BMG LABTECH) and results were expressed as mg gallic acid equivalents (GAE) per gram extracts. Gallic acid concentration (0, 50, 100, 250, 500, and 750 µg/mL) was used for the calibration of a standard curve. At least 3 replications were repeated for mean values calculated at $p < 0.05$.

3.5.2 Total flavonoid contents

Total flavonoid contents was described by Dewanto et al., (2002); quercetin was used as a chemical standard. The plant extract of 0.25 µl was added to 100 µl distilled water followed by 10µl of 5 % $NaNO_2$. The mixture was incubated for 5 min and added 10 µl of 10 % $AlCl_3$ before measuring by a microplate reader at wavelength 510 nm. Standard curve constructed in various concentrations at (0, 50, 100, 250, 500, and 750 µg/mL) . Results were expressed as QAE quercetin.

3.5.3 Total chlorophylls and Carotenoids

RC leave powder weight 0.2 gram was extracted in 15 ml of 80 % acetone and measured at 663, 647, and 470 nm by UV-Vis spectrophotometer (Thermo scientific, Genesys 10S). The quantities of total chlorophylls and carotenoids were determined by the following equation which was described by Lichtenthaler (1987).

chla (µg/mL) = 12.25*A663 - 2.79*A647

chlb (µg/mL) = 21.50*A647 - 5.1*A663

Tchl (µg/mL) = chla + chlb

Tcar (µg/mL) = (1000*A470 - 1.8*chla - 85.02*chlb)/ 198

3.5.4 High-performance liquid chromatography

3.5.4.1 Phenolic profiling

The experiment was described by Oonsilivai (2007). The crude extracts were prepared at a concentration of 20 mg/mL and filtrated through a 0.22 µm nylon membrane. RC crude extract and chemical standard, 20 µl injected into Agilent ZORBAX Eclipse Plus C18 (4.6 mm × 250 mm) column with a constant flow rate of 1 mL/min at 35 °C. This experiment was performed by using Agilent 1260_1 Series HPLC system equipped with a diode array detector. The mobile phases consist of (A) 2 % acetic acid in the water, (B) DI water, and acetonitrile (C). The detection wavelength was performed at 270 nm and 320 nm.

3.5.4.2 Chlorophylls profiling

In the study of chlorophyll profiling, the method of Oonsivilai (2007) was applied. The chloroform crude extract, (25 mg/mL), and standard solution range 50–500 µg/mL were filtrated through 0.22 µm nylon membrane and injected into C18 HPLC Column (L 250 x ID 4.6 mm), GRACE Vydac. The mobile phase system consists of methanol: water: ammonium acetate (73:25:2, v/v) in reservoir A and ethyl acetate in reservoir B with a flow rate of 1.0 mL/min. The injection volume is 25 µl for a total

run time of 30 min and perform at a wavelength between 250 and 600 nm. Calibration curves were prepared for measuring the quantities of the compound.

3.6 Compounds separation and TLC screening

3.6.1 Thin-layer chromatography

The bioactive compounds in RC crude extract were separated by thin-layer chromatography before applying them into silica gel column. The experiment was carried out for screening a proper solvent system. Then, the RC crude extract gently dropped on 1 cm in width and 5 cm length of TLC plate. The plate was placed in solvent composed of Hexan: ethyl acetate at ratio (70:30, v/v), after that spots separation was investigated. The distance of each spot was calculated by:

R_f = (distance of spot / distance of mobile phase).

3.6.2 Flash column chromatography

The column chromatography was prepared by the following; first flash column size (7 x 50 cm) was washed with pure hexane and dried. Little pieces of cotton were used as a barrier to hold the stationary phase. Second, silica powder was homogeneously dissolved in hexane and packed in flash column chromatography. Third, 0.5 g of RC crude extract passed through silica gel column and then all fractions were collected while applying mobile phase system which was hexane, and ethyl acetate at ratios 80:20, followed by 70:30. All oft fractions (F) were evaporated by rotary evaporator (IKA RV8, Germany) and these fractions were primarily identified by a TLC plate.

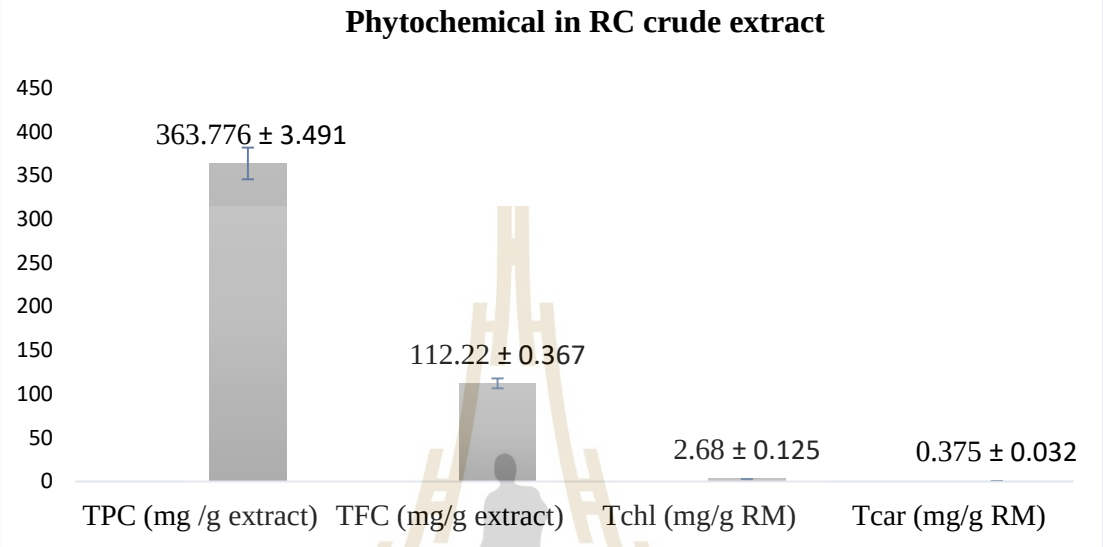
3.7 Statistics analysis

Data for the analysis was prepared in at least triplicate and reported as mean and standard deviation errors. The group of fractions was compared by using a one-way ANOVA at a significance level of $P < 0.05$.

3.8 Results and Discussion

Total phenolic contents refer to the quantities of polyphenols contents in the RC plant extract. Phenolic compounds are usually found in plants and vegetables; these active compounds may induce antioxidation activity (Pattananandecha et al., 2021). Furthermore, flavonoids showed as ferric-reducing antioxidant power agents (Wonkchalee et al., 2012). In the present study, RC chloroform extracts were found TPC and TFC 363.776 ± 3.491 mg/g GAE and 112.22 ± 0.367 mg/g extract as quercetin equivalents in Figure 3.1. Numbers of TPC and TFC are normally detected in RC extracts, but chloroform was outperformed by containing several groups of bioactive compounds; the solvent could extract TPC, TFC, TClh, and Tcar. Oonsivilai, et al., (2007) revealed that TPC in RC water extract was at 24.33 ± 0.57 mg / 100g of RM; the result was supported by researched data 123.68 ± 2.94 mg/g extract and TFC 62.83 ± 2.85 mg/g extract (Junsi et al., 2017). The quantities of phenolic and flavonoid content might be extremely important to the biological study, especially in terms of antioxidation and anti-inflammation. However, Nawaz, Shad, Rehman, Andaleeb, and Ullah (2020) reported that the TPC and TFC of polar solvents extracts are significantly lower than non-polar solvents despite the yield extracts being more effective. In this study, RC chloroform extract released yield extract at 15.3 ± 0.1 % , whereas other solvents such as water, ethanol, and acetone found at 32.6, 23.2, and 36.6 % of yield extraction (Oonsivilai et al., 2007). In addition, Oonsivilai et al., (2007) also reported Tchl by 6.0 ± 2.7 mg/mL of acetone RC extraction while this research detected the total chlorophylls at 2.68 ± 0.125 mg/

g RM, and total carotenoids 0.375 ± 0.032 mg/g RM. However, the extraction yield and phytochemicals are also related to raw material preparation, especially for plant leaves drying method. Phahom, Phoungchandang, and Kerr (2017) revealed that RC



microwave drying shows TPC at 3080 ± 202 (mg GAE/100 g extract) followed by RC oven-drying showed at 2433.9±57.7 (mg GAE/100 g extract) reported by Oonsivilai et al., (2007). Other drying methods, freeze-drying provided TPC at 488 ± 44 (mg GAE/100 g extract) (Eng SY et al., 2011). The microwave-drying method provides the most efficient than other methods because microwave drying is a rapid hydration technique that could remain green color to plant leaves. The short drying time may not disrupt phytochemicals in plant materials and it also improved product quality (Haghi & Amanifard, 2008).

Figure 3.1 Total phenolic, total flavonoid contents and chlorophylls, total carotenoids in chloroform Rang Chuet crude extract. The results were reported in means ± standard deviation (SD).

Table 3.1 Phytochemical profiling of RC crude extract.

Phenolic profile (mg/g extract)		Chlorophyll profile (mg/g extract)	
Gallic acid	1.448 ±0.015[a]	Chlorophyll *a*	1.943±0.037[a]
Caffeic acid	1.13±0.012[a]	Chlorophyll *b*	4.661±0.005[b]
Apigenin	2.167±0.014[b]	Pheophytin *a*	1.571±0.041[c]
Sinapic acid	ND	Pheophytin *b*	1.665±0.024[c]
Ferulic acid	ND	Lutein	1.222± 0.045[d]

Data are presented in means ± SD, the different letters in the same column responding to significant differences ($p < 0.05$).

The phytochemical profiling of crude extract are shown in Table 3. 1. Chloroform RC extract consisted of chlorophyll *a* (1.943 ± 0.037), chlorophyll *b* (4.661 ± 0.005), pheophytin *a* (1.571 ± 0.041), pheophytin *b* (1.665 ± 0.024), and lutein (1.222 ± 0.045). The results matched well with previous findings (Oonsivilai et al., 2007), in which they found chlorophylls and their derivatives in ethanol and acetone RC extracts. Moreover, RC chloroform extracts also contained phenolic acids and flavonoid including, gallic acid and caffeic acid, and apigenin at levels of 1.448 ± 0.015, 1.13 ± 0.012, and 2.167 ± 0.014 (mg/g extract), respectively.

RC crude extract was separated by using flash column chromatography and solvent system with Hexane: ethyl acetate (80:20 and 70:30, v/v). Seven fractions were collected, evaporated, and primarily detected by a TLC plate.

Table 3.2 The retardation factor (Rf) values of separated compounds on the TLC plate of RC crude extract.

Spots	1	2	3	4	5	6	7
Rf.	0.875	0.8	0.75	0.55	0.475	0.325	0.225
Rf. STDS	Carotene	Pheo-*b*	Pheo-*a*	Chl -*b*	Chl-*a*	Unknown	Lutein

		0.82	0.76	0.6	0.48		0.23

The spots of RC crude extract were compared with the standard solution in Table 3.2. The results showed that the first spot (F1) was a yellow color compound, which was identified as a group carotene. F2 has one spot of pheophytin-*b* and F3 contained pheophytin-*a* on the TLC plate. Moreover, Fractions 4, 5, and 6 consisted of chlorophyll-*b* , chlorophyll-*a*, and an unknown derivative compound while F7 was lutein. The results are well consistent with Oonsivilai (2007) , who studied phytochemical profiling in RC extracts.

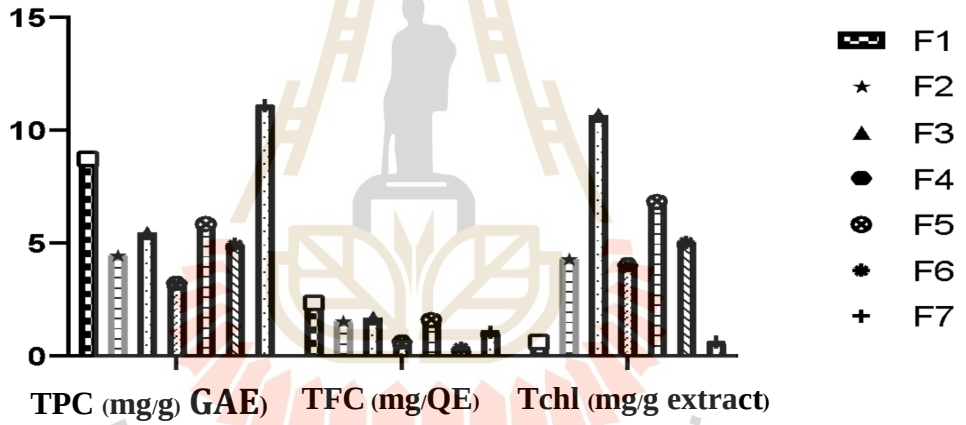


Figure 3.2 Total phenolic, total flavonoids, and total chlorophylls in each fraction of RC extract.

Figure 3.2 shows the quantities of TPC, TFC, and Tchl of each fraction. The outcome showed that TPC of F1 and F7 were at 8.749 ± 0.60 and 11.095 ± 0.02 mg/g GAE extract while both fractions consisted of Tchl at 0.635 ±.0.0255 and 0.62 ± 0.0288 mg/ g extract. Lutein contains hydroxyl group that could induce a redox reaction in possessive of an antioxidation whereas carotene is an isoprenoids with hydrocarbon structure by containing only hydrogen and carbon atoms. β-carotene and other carotenes presumably reduce free radical oxidative (Diplock, 1995) .

Fraction 3 showed the highest amount of Tchl at 10.70 ± 0.418 mg/ g extract followed by F5, F6, F4, and F2. Most of all phytochemicals in each fraction is significantly different at $p < 0.05$ in Table 3.3. In this research, all fractions contained low level of total flavonoids and may consist of polar compounds, which could stick on the surface of silica gel.

Table 3.3 The polyphenol content, and total chlorophyll in each fraction.

Fraction	TPC (mg/ g GAE extract)	TFC (mg/g QE extract)	Total chloro (mg/ g extract)
F1	8.749±0.608[a]	2.361±0.133[a]	0.635±0255[a]
F2	4.474±0.094[b]	1.548±0.249[b]	4.29±0.171[b]
F3	5.463±0.31[c]	1.71±0.095[c]	10.70±0.418[c]
F4	3.23±0.197[d]	0.631±0.133[d]	4.039±0.095[b]
F5	5.858±0.718[e]	1.613±0.305[e]	6.86±0.455[d]
F6	4.937±0.01[f]	0.361±0.064[f]	5.043±0025[e]
F7	11.095±0.02[g]	1.069±0.205[g]	0.62±0.288[a]

Note: Data are presented in means ± SD, the different subscriptions in the same column responding to significant differences ($p < 0.05$).

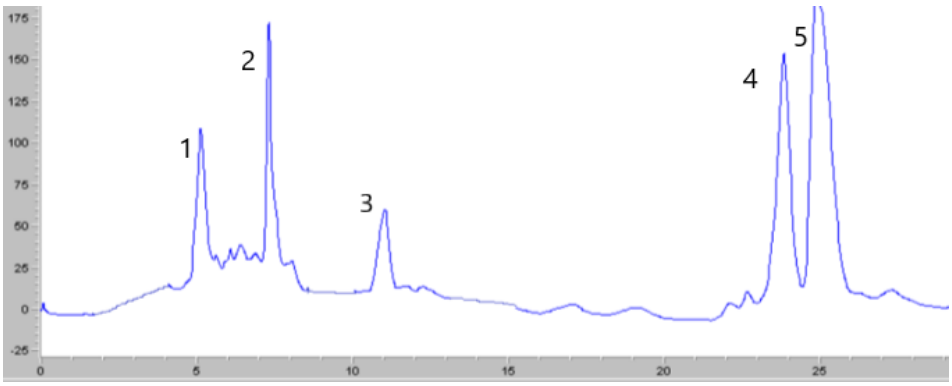


Figure 3.3 HPLC chromatograms of phenolic profiling in RC crude extract; (1) gallic acid, (2) Unknown, (3) caffeic acid, (4) apigenin, (5) Unknown.

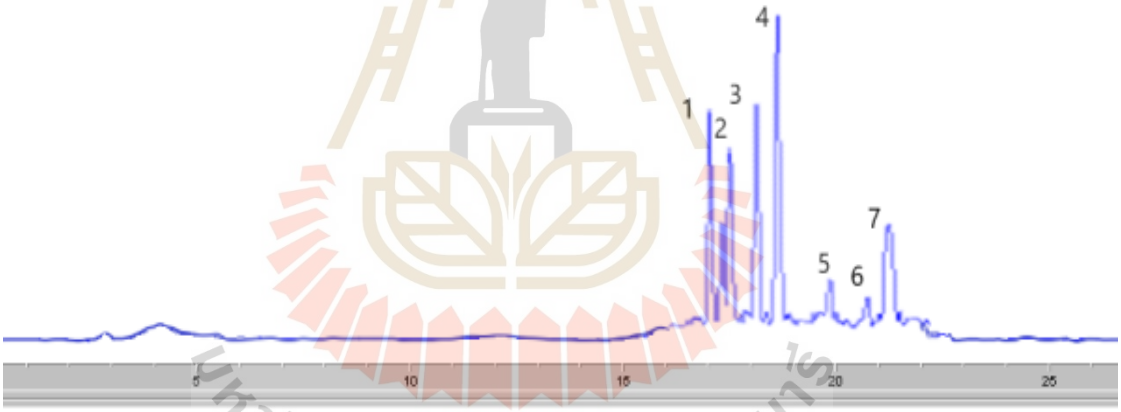


Figure 3.4 HPLC chromatograms of chlorophylls and its derivatives in RC crude extract. (1) lutein, (2) Unknown, (3) Chlorophyll *b*, (4) Chlorophyll *a*, (5) pheophytin-b, (6) Unknown, and (7) pheophytin-*a.*

According to the HPLC chromatogram, chloroform RC crude extract contains five phenolic compounds (Figure 3.3). The first peak was recorded as gallic acid, third peak was caffeic acid, and the fourth peak was apigenin. RC chloroform extract also consisted of other two unknown compounds in the second and fifth peaks. The

second peak might be a phenolic compound that has a mass molecule ranging from 170 -180 Da, meanwhile, peak number 5 might be a group of flavonoids (Catechins) that consists of higher mass molecules than apigenin. The elution peak is based on principles of bioactive compounds' interaction with stationary phase and mobile phase (Bushra, 2018) . C18 column chromatography is very high degree of hydrophobicity, which would elute carbons C-7 before C-9, then C-15 (Mendes, Cartaxana, & Brotas, 2007). Thus, the retention time of compounds at C-15 will be shown their peaks lately. Mass molecular of apigenin ($C_{15}H_{14}O_7$) is 270 Da while catechin is 290 Da with $C_{15}H_{14}O_6$. Even though both compounds consist of the same carbon number but apigenin existed more hydroxy group that provides more polarity than catechine. More over, catechin was also found in RC extract (Junsi et al., 2017).

Figure 3.4 shows chlorophylls profiling of RC crude extract. The outcome of chlorophylls profiling found that seven compounds were detected. The first release was recorded as lutein (1), Chlorophyll *b* (3), Chlorophyll *a* (4), pheophytin-*b* (5), and pheophytin-*a* (7). The unknown compounds were also detected at peak numbers 2 and 6 which they might be derivatives of chlorophylls.

3.9 Conclusion

RC leaf crude extract consists of serval groups of phytochemicals such as phenolic acids, flavonoids, chlorophylls, and carotenoids. Chloroform is properly used as an extraction solvent for bioactive compounds. The RC extract contains bioactive active compounds that are suitable for separation on silica gel column chromatography. The bioactive compound in RC and its derived fractions are able detected on Thin-layer chromatography. Moreover, the analytical instrument HPLC could apply for compound identification in RC crude extract. RC extract consists of

bioactive compounds such as gallic acid, caffeic acid, apigenin, chlorophyll-*a*, chlorophyll-*b,* pheophytin-*a*, pheophytin-*b,* and it also contains other derivatives substances.

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CHAPTER 4

DETOXIFICATION PROPERTIES OF *THUNBERGIA LAURIFOLAI* LINDL. FRACTIONS IN HEPG2 AND AML12 CELL LINES

4.1 Abstract

The objectives of the study was to investigated the detoxification properties of an isolated fractions of *Thunbergia laurifolai* Lindl. (Rang Cheut or RC) extract. NAD(P)H: quinone oxidoreductase (NQO1) assay was utilized to examine the NQO1 enzyme activity in these assessment fractions. In addition, a fraction with the greatest inducing of NQO1 enzyme activity was identified by thin-layer chromatography (TLC). Other analytical instruments such as high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS/MS) were used for further verification. Consequently, fraction 3 (F3) was considered as a significant fraction, which could induce the highest NQO1 enzyme activity in both HepG2 and AML12 at levels 3.908 ± 0.079 fold and 1.99 ± 0.047 folds. In the process of compound identification, HPLC resulting was pheophytin *a*. Furthermore, F3 not only contained pheophytin *a* but also hydroxypheophytin-*a* by verifying on LC-MS/MS. These bioactive compounds showed mass-to-charge ratios (m/z) at 871.59^{+} $[M + H]^{+}$ and 887.59^{+} $[M + H]^{+}$ which were pheophytin-*a* and 13^{2}-hydroxypheophytin-*a*. The chemical molecules of 13^{2}-hydroxy-pheophytin-*a* and pheophytin-*a* is $C_{55}H_{74}N_4O_6$ and $C_{55}H_{74}N_4O_5$. Additionally, F3 showed the highest quantity of total chlorophylls (Tchl) at 10.7 ± 0.418 mg/g extract. In conclusion, the F3 of RC extract shows the

highest inducing of NQO1 enzyme activity; bioactive compounds were also identified. This new finding could benefit for functional food ingredients development.

Keywords: *Thunbergia laurifolia* Lindl., Rang Chuet (RC), detoxification, NQO1 enzyme, pheophytin *a,* hydroxypheophytin *a,* Liquid chromatography-mass spectrometry.

4.2 Introduction

The liver is a main organ in the human body with multifunctions, especially in detoxification through metabolism in phases I and II reactions (Liska, 1998). The reaction could convert toxicants into water-soluble, however, the overdose of toxic compounds in the body could be leading to liver dysfunctions (Badr, Saadat, & Saadat, 2016). Recently, many studies have shown that herbal plants are abundant in phytochemicals either involved in hepaprotective or detoxicity (Al-Snafi, 2015; Smith & Culvenor, 1981). Rang Chuet or RC is a Thai traditional herb, which roles in several pharmacological properties. According to scientific data from analytical instruments (HPLC and NMR), RC composes of bioactive compounds such as phenolic acids and flavonoids i.e. gallic acid, caffeic acid, rosmarinic acid, and quercetin (Kanchanapoon et., al 2002; Oonsilivai et., al 2007). In addition, a group of pigments was identified by reverse-phase HPLC, active constituents including chlorophylls, chlorophylls derivation, and carotenoids (Oonsivilai, Cheng, Bomser, Ferruzzi, & Ningsanond, 2007). Recently, Junsi, Siripongvutikorn, Yupanqui, and Usawakesmanee (2017) operated LC-MS to identify polyphenols including, catechin, caffeic acid, rosmarinic acid, rutin, isoquercetin, apigenin, and quercetin.

Toxicity data revealed that RC roles as an antidote to poison heavy metals, insecticides, and toxic drugs. In the *Vivo* studies, the RC leaf extract could lower Pb in Nile Tilapia fish (Palipoch et al., 2011). Ruangyuttikarn et al. (2013) revealed that the

RC water extract reduce organ damage by Cd (the liver and kidney), however, the level of Cd in blood and urine were unfaltering. Likewise, Rana et al. (2020), the RC water crude extract partially recovers damaged kidney organs by Pd toxicity. Another study also reported that the RC water extract treatment (100, 200 mg/Kg BW) could reduce the expression of tumor necrosis factor (TNF)-α, inducible nitric oxide (iNOS), and cyclooxygenase-2 (COX-2) (Rana et al., 2020).

Both hepatocytes and hepatoma cell lines have been used in in-vitro studies. Either hepatic cell line (Huh7s) or HepG2 could produce CYP3A4 expression, which is a family of CYP450 enzymes in phase I detoxification reaction (Bulutoglu et al., 2020). HepG2 cell line exhibits CYP3A4 at very low levels but the cell line could induce NADPH (NQO1) enzyme 0.237-0.255 arbitrary units at low-density 10^4cell/well (Del córdoba-pedregosa et al., 2006). Alpha-mouse liver 12 (AML12) is a normal cells line, that was isolated from a 3-month -old mouse. Dai et al. (2021) claimed that Wogonin could activate Nuclear factor erythroid (Nrf) signaling in AML12. Nuclear erythroid 2-related factor 2 (Nrf2) functions as regulator of antioxidative machinery and also induced NAD(P)H: quinone oxidoreductase (NQO-1), glutathione S-transferase (GST) (Skelly et al., 1999).

Detoxification is the process of eliminating endogenous and xenobiotics from the body through a biotransformation reaction. Phase II enzymes are UDP-glucuronosyltransferases, sulfotransferases, N-acetyltransferases, glutathione S-transferases, and methyltransferases; these enzymes role as the electron donator to intermediated substances (Jancova, Anzenbacher, & Anzenbacherova, 2010). NQO1 enzyme is one of the detoxification enzyme. NQO1 not only function in

detoxification but also acts as a chaperone protein, which could prevent non-specific aggregation by binding to unfolded or non-native protein (Scott, Barnes, Whitehead, Stratford, & Nolan, 2011). Figure 4.1 shows the transformation of quinone to hydroquinone. The mechanism of NQO1 catalysis consists of two steps, hydride transferring from the NAD(P)H to Flavin adenine dinucleotide (FAD) cofactor and released NAD(P)+. The second step is hydride transfer from reduced cofactor to quinone substrate (K. Zhang et al., 2018).

Figure 4.1 Roles of NAD(P)H in quinone transformation.

Source: (Srijiwangsa & Na-Bangchang, 2017).

Several research studies found that RC crude extract had the potential for detoxification, however, the bioactive compounds that show detoxification properties have not been identified. Thus, this research focused on detoxification study and identification of bioactive compounds in RC extract. Furthermore, a fraction that induced the highest level of specific enzyme activity NQO1 in the liver cells (AML12 and HepG2) was selected for bioactive compound identification, using HPLC and LC-MS/MS.

4.3 Chemicals

DMEM/F12 - Dulbecco's Modified Eagle Medium, dexamethasone (Gibco™, 10mM in DMSO), Insulin-Transferrin-Selenium (Gibco™ Insulin-Transferrin-Selenium), NQO1 activity assay kit abcam184867 were obtained from Abcam. Phosphate buffered saline (PBS) composites of Sodium chloride, Potassium Chloride, Sodium

Phosphate Dibasic, and Potassium Phosphate Monobasic. Dulbecco' s Modified Eagle' s Medium (DMEM), and fetal bovine serum (FBS) are supplied by Gibco (Carlsbad, California). Hepatoma HepG2 liver cells received from Asso.Prof.Dr. Parinya Noisa, School of Biotechnology; Hepatocyte AML12 was a gift from Asst. Prof. Dr. Annyanee Kamkaew, School of Chemistry, Suranaree University, Thailand.

4.4 Cell Culture

Human hepatocellular carcinoma (HepG2) and Alpha mouse liver 12 (AML12) cell lines were used in this research. HepG2 cell was cultured in a completed medium containing Dulbecco's Modified Eagle's Medium (DMEM) fortified with 10 % of fetal bovine serum (FBS). HepG2 cells were performed in a T-75 ml flask; the flask was incubated at the temperature of 37 °C and 5 % CO_2. These cells line was refreshed with a new medium every day.

AML12 is a normal liver cells line was grown in DMEM/ F12 - Dulbecco's Modified Eagle Medium, which consisted of 0.5 % Insulin-Transferrin-Selenium (Gibco™ Insulin-Transferrin-Selenium), 0.1 % of dexamethasone (Gibco™, 10 mM in DMSO), and 10 % FBS. The cells was incubated and performed under the same condition as the HepG2 cells line. In *in-vitro* studies, the insulin could stimulate cell growth and proliferation. The insulin interacts with other hormones such as platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) (Stout, Bierman, & Ross, 1975). Dexamethasone (DEX) is a cellular regulator which could increase cell proliferation(McCulloch & Tenenbaum, 1986). DEX is also a synthetic glucocorticoid that activates protein kinase B/AKT and p38 (Guendisch et al., 2012).

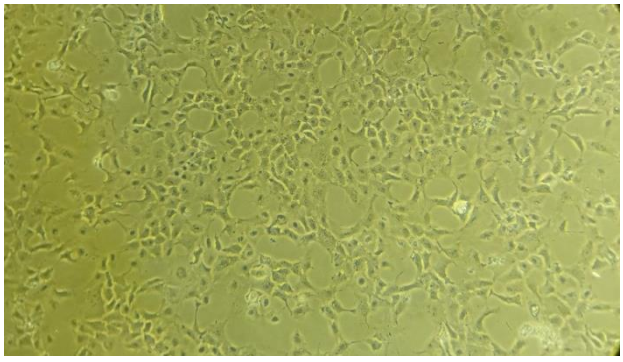


Figure 4.2 Alpha mouse liver 12 (AML12) cultured on DMEM/ F12 medium, captured microscopic (IKA, German).

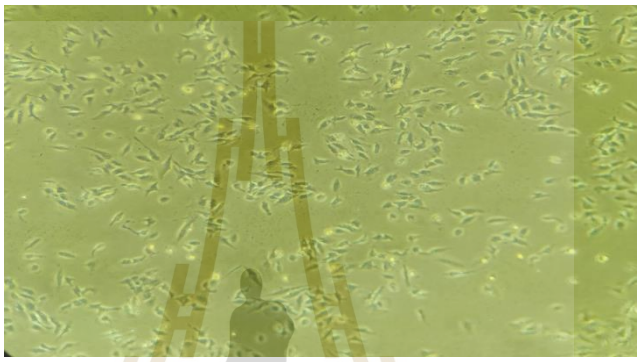


Figure 4.3 Human hepatocellular carcinoma (HepG2) cultured on DMEM medium, captured microscopic (IKA, German).

4.5 Cytotoxicity of HepG2 and AML12 cell lines

Cytotoxicity is the biological assays which cell viability or cell proliferation could be measured by colorimetric assays. In addition, the method could determine the toxic concentration of compounds that cause cell death (Aslantürk, 2018). Briefly, HepG2 and AML12 cells were seeded at the density of 10^4 cells/ well on 96 microplates and incubated for 24 hours before compound treatments. The concentration of crude extract and fractions were applied from 5, 2.5, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.0312 (mg/ mL) then cells were incubated for further 24hours. MTT assays were used to assess cytotoxicity and cell viability. In the experiment of MTT assay, the 100µl (0.5 mg/ mL) of MTT reagent (3-(4,5-dimethylthiazol-2-yl) -2–5-diphenyltetrazolium bromide) was added to cells and incubated for 3 hours. Then

MTT reagent was removed and washed with Phosphate buffered saline (PBS); Dimethyl sulfoxide (DMSO) 100 µl was added to each well. The OD was measured by colorimeter spectroscopy (Spectro star Nano Microplate reader (BMG LABTECH) at wavelength 590 nm. The percentage of cell viability was calculated using the following equation (Adewusia, Foucheb, & Steenkamp, 2013), whereases IC_{50} was determined.

Cell Viability (%) = (Absorbance of cell treatments x 100) / Absorbance of control cells

$IC_{50} = (50 - b) / a$

The dose-response was constructed for finding y = ax + b

4.6 NAD(P)H dehydrogenase quinone 1 (NOQ1)

NQO1 enzyme activity was investigated by NQO1 activity assay kit (AB184867, Abcam). After cells were treated with RC extract for 24 hours, the medium was removed and cells was washed with PBS before the process of cell lysate. The 100 µl of extraction buffer (1 x) was added. Cells was transferred to clean tubes and incubated on ice for 15 min then centrifuged (18000 g, 20 min, 4 °C) and supernatant was collected. Two folds dilution of supernatant (50 µl) was loaded in pairs for activity buffer and inhibitor reaction. The plate was immediately recorded at a wavelength 440 nm. Each fraction was measured in 3 replicates and expressed the results as folds control.

4.7 Compound identification by LC-MS

From our previous study, the results of fractionation, compound identification (TLC, total phenolic contents (TPC), total flavonoids (TFC), and total

chlorophylls (Tchl) were determined. The result of TLC found that there were 7 fractions (F) obtained. F1 and F7 were yellow spots that is a group of Carotenoids, which F1 may be of β-carotene and F7 was lutein. F2 showed one spot of pheophytin-*b* and F3 contained pheophytin-*a* on the TLC plate. Moreover, F4, 5, and 6 consisted of a group of chlorophylls. The results were well consistent with Oonsivilai et a., (2007) who studied phytochemical profiling of RC extracts.

Our research was intended to identify the bioactive compound in fraction 3, which showed the highest NQO1 activity, thus the fraction was selected for further verification by HPLC and LC-MS/MS. Fraction 3 was dissolved in methanol absolutes, and filtered, then kept at -80 °C until further identification. The 10 µl of sample was directly injected into LC-MS/ QTOP (Thermo scientific, ultimate 3000) mass spectrometer. The process is carried out in an isocratic mode by 5 % (A): 95 % (B). Whereas solvent A consisted of water and 0.1% formic acid, solvent B is a mixture of acetonitrile and 0.1% formic acid. The condition of Mass spectrometry was held at the scan range m/z 50 -1700 and the source of voltages was 4.5kV. The operation temperature was set at 180 °C in positive ionization mode.

4.8 Statistics

Sample was prepared and analyzed in triplicate. The results were calculated and reported as mean and standard deviation errors. The fractions were compared by using one-way ANOVA test at a significance level of $P < 0.05$.

4.9 Results and discussion

4.9.1 Cell viability and cytotoxicity of HepG2 and AML12

Cell viability was determined using MTT assay. The reaction is based on the activities of cellular oxidoreductase enzymes and tetrazolium dyes; as a result, the purple color was inducted (Stockert et al., 2012). RC crude extract and fractions at concentrations of (5 - 0.031 mg/mL) were accessed to HeG2 and AML12 cells. Then, the cell viability and cytotoxic were evaluated. Healthy cells in a population remained higher than 50 % at concentrations higher than 2 mg/mL in both cell lines (Figures 4.4 and 4.5). Despite our experiment being studied on fractions, the result was in line with Marasri Junsi et al., 2020, which showed that the percentage cell viability of HepG2 was higher than 80% at concentrations of 0.01-2 mg/mL of RC crude extract. The results were agreed with Rocejanasaroj, Tencomnao, and Sangkitikomol (2014), who found that cell viability of HepG2 cells line was higher than 50 % at a treatment concentration 3 mg/mL of RC crude extract. Another research data also reported that consuming RC extract 9-12 mg/day showed no side effects (Preechasuk et al., 2020). According to these results, bioactive compounds in RC might function in cells protection.

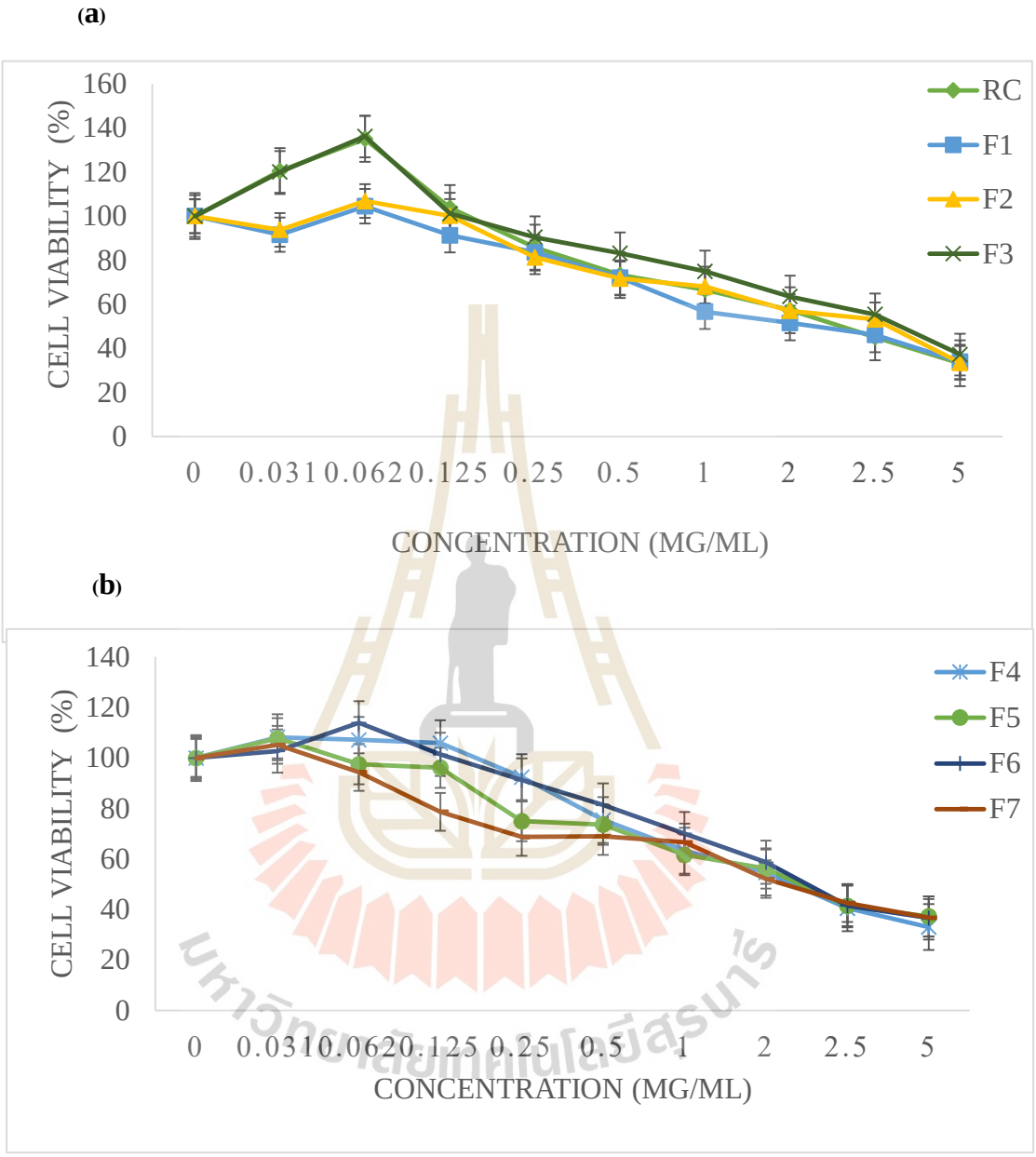


Figure 4.4 Percentage of cell viability in AML12 after incubation with RC and derived fraction, (a) showed cell viability of RC, F1, F2, and F3 while (b) exhibits of F4, F5, F6, and F7. The cell viability was individually calculated by mean and standard deviation, n = 3.

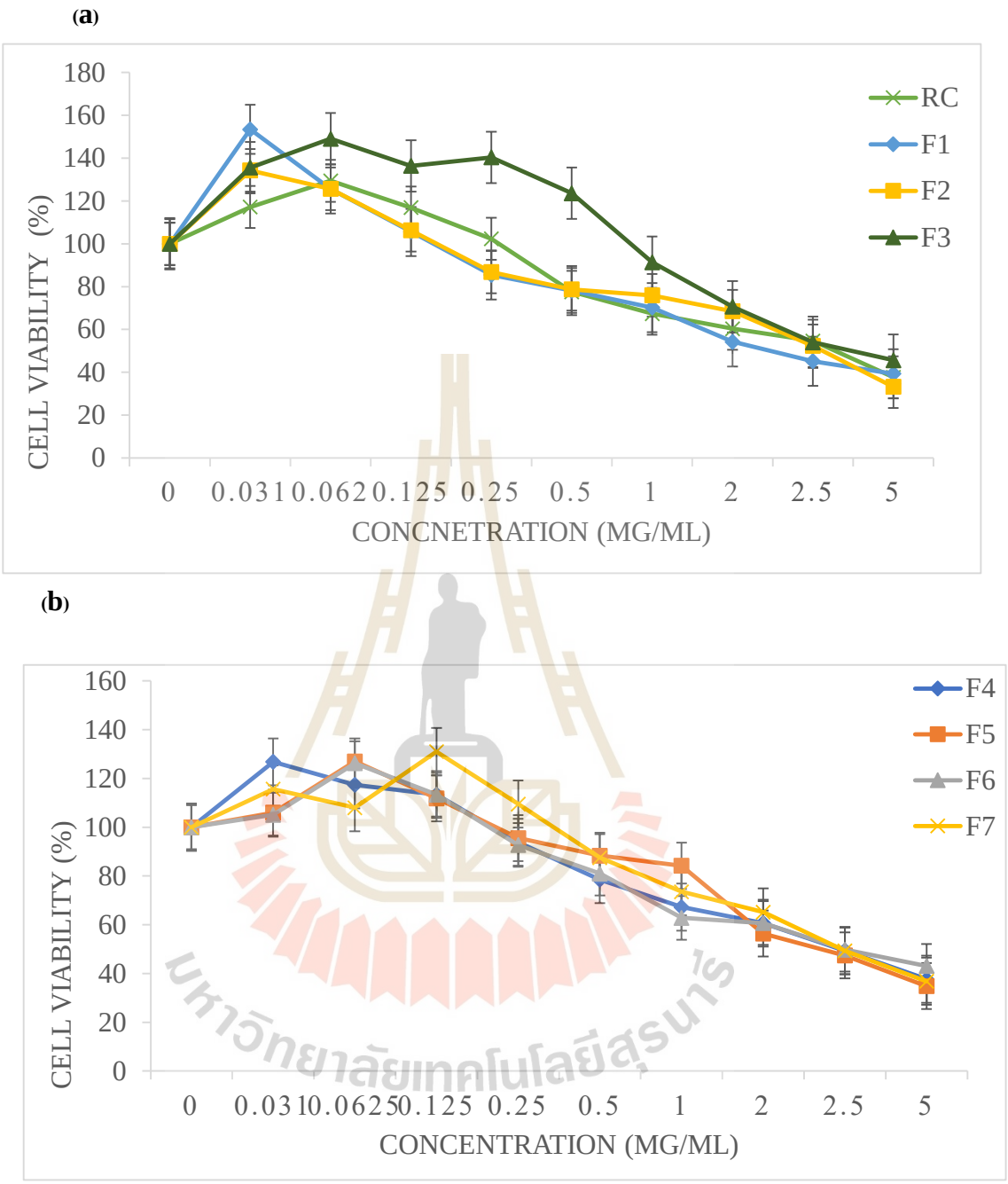


Figure 4.5 Cell viability of HepG2 cell line was measured by MTT assay. The cell viability represents the percentage compared with 100% of control. Sample concentration was subjected overnight with concentration (5 – 0.031 mg/mL). The results were reported in mean ± SD, n = 3. (a)

showed the cell viability of RC crude extract, F1, F2, and F3 also (b) revealed the cell viability of F4, F5, F6, and F7.

Cell viability in both cell lines was higher than 100 % of control at low concentration RC crude extract and its derived fractions, then cell viabilities were decreased at higher concentrations. Bioactive compounds in RC extract might induce cell proliferation, and the results of this research become information for future research studies.

Table 4.1 Cytotoxicity of RC crude extract and fraction in HepG2 and AML12 cell lines.

Active compounds/cell line	IC_{50} AML12	IC_{50} HepG2
RC crude extract	2.042 ± 0.067^{a}	2.676 ± 0.095^{a}
F1	2.056 ± 0.142^{a}	2.377 ± 0.11^{b}
F2	2.556 ± 0.045^{b}	2.818 ± 0.109^{c}
F3	3.139 ± 0.096^{c}	3.583 ± 0.13^{d}
F4	2.25 ± 0.078^{d}	2.546 ± 0.078^{e}
F5	2.228 ± 0.070^{d}	2.583 ± 0.12^{e}
F6	2.288 ± 0.079^{d}	$2.836 \pm 0.14c^{c}$
F7	2.07 ± 0.12^{a}	2.67 ± 0.13^{a}

IC_{50} value calculated in means ± SD in the same row with the different subscripts are significantly different at $P < 0.05$. Data were analyzed by a one-way ANOVA test. Cells were incubated for 24 hours after treatment concentration 5 – 0.031 mg/mL.

Additionally, IC_{50} values determination was carried out. In principle, cytotoxicity has been used for indicating drugs' therapeutic properties and safety

evaluation. The IC_{50} value in RC crude extract and its derives fractions were higher than 2 mg/mL (Table 4.1). From the cytotoxicity test of all fractions in AML12, the IC_{50} values of fractions F1 and F7 were non-significant different from RC crude extract. Whilst other fractions are significantly different from RC crude extract at $p < 0.05$. Moreover, the IC_{50} values of cytotoxicity in HepG2 cells line were also higher than 2 mg/mL. Interestingly, Fraction 3 showed maximum IC_{50} values in both HepG2 and AML12 which were at 3.583 ± 0.13 and 3.139 ± 0.096, respectively.

According to IC_{50} values, these results indicated that bioactive compounds in RC are non-toxic to cells (da Silva et al., 2018). The similarities of IC_{50} values in AML12 and HepG2 cells might be useful in cells application. Although HepG2 is a cancer cell line, it is very convenient for studying drug evaluation and toxicity due to inexpensive and high proliferation (Knasmüller et al., 2004). Sefried, Häring, Weigert, and Eckstein (2018) found that insulin expression in HepG2 and AML12 cells are comparable.

4.9.2 Specific enzymes activities of NQO1

NQO1 enzyme is encoded by the NQO1 gene at chromosomal location 16q22.1; the enzyme could neutralizes electrophiles and oxidant (Cleton-Jansen et al., 2008). NQO1 has been known as a cellular defender in mammalian cells, especially function in stabilizing tumor suppressor protein, p53 (Oh & Park, 2015). Moreover, NQO1 enzyme is very important in phase II detoxification because it can reduce toxicity of substrates in both endogenous and environmental quinones (Ross et al., 2000). The ping-pong reaction between NOQ1 enzyme, cofactor NADH, and quinone could cover toxicants to become hydroquinone which is a harmless substance (Srijiwangsa and Na-Bangchang 2017).

The results of detoxification study dedicated that RC crude extract and its fractions could induce NAD(P)H: quinone oxidoreductase enzyme activitty. Table 4.2 shows the NQO1 enzyme activity after being applied with RC extract for 24 hours in

both liver cell lines. The results found that RC crude extract induced the specific NQO1 activity in AML12 was at about 1.387 ± 0.073 fold compare to control. F3 showed the highest quantity of the enzyme at 1.99 ± 0.047 fold. Likewise, F4, F2, F5, and F6 showed activities enzyme at levels of 1.66 ± 0.45, 1.472 ± 0.064, 1.420 ± 0.299, and 1.392 ± 0.041 folds, respectively. Fraction 1 showed the lowest enzyme activity at 1.174 ± 0.263 fold whereas F7 induced enzyme activity at level 1.445 ± 0. 122 fold. The specific enzyme NQO1 activities were mostly non-significant differences from the specific enzyme activity of controls, whilst, fractions F3 and 4 were significantly different at $p < 0.05$. Also HepG2 cells line, the finding showed that F3 extremely induced NQO1 enzyme activity at 3.908 ± 0.124 fold followed by F4, F5, F6, F2, F7, and F1 at levels 2.67 ± 0.187, 2.562 ± 0.083, 2.332 ± 0.15, 2.033 ± 0.079, 2.281 ± 0.034, 1.701 ± 0.074-folds, respectively. NQO1 enzyme activities in all fractions are significantly different from control in HepG2 cells line. The potency of bioactive compounds in RC extract may induce NQO1 gen to express NQO1 enzyme.

Moreover, the specific enzyme activity of NQO1 in AML12 cells was significantly different from the HepG2 cells in each treatment (Figure 4.6). Despite, NQO1 activity was being constitutively expressed in a variety of tissues throughout the body, it is overexpressed in many tumors and cancer cells (Srijiwangsa & Na-Bangchang, 2017). The significant difference of enzyme activity might be related to metabolism of cells. Basically, proliferation or enzyme production of normal cells occurs after receiving signal from neighboring cells. They consist of tumor suppressors genes p53, which control cell proliferation (Weinberg, 1991). In contrast, cancer has been known as uncontrollable in proliferation, these results are due to tumors ignoring cell signaling. The abnormal characteristic may induce overproduction of proteins, which could be abnormal or function differently (Cairns, Harris, McCracken, & Mak, 2011). Fang, Hai-Ping, and Guang-Ji (2012) found that HepG2 cells could express a high amount of NQO1 activity at 1782 ± 72 (nmoL/mg of extracts) at the

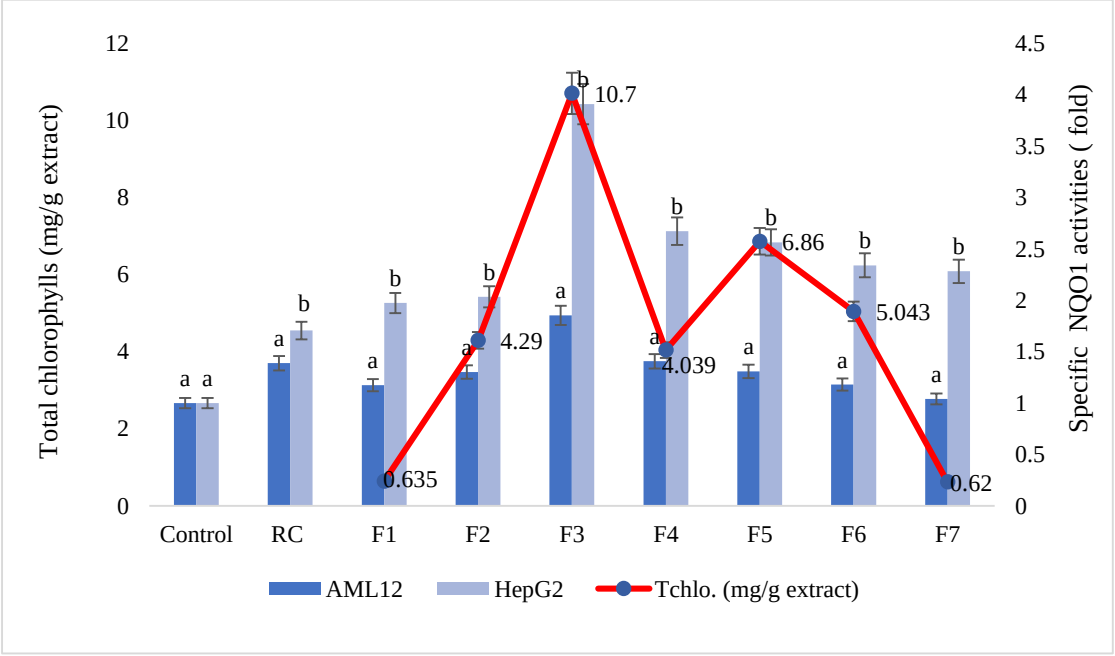
same condition of tanshinone IIA treatment compared to the Caco2 cell line which showed NQO1 activity at values 10 ± 1.0 (nmoL/mg).

Table 4.2 NQO1 enzyme activity in HepG2 and AML12 cells lines.

Fraction	NQO1 AML12	NQO1 HepG2
Control	1^{a}	1^{a}
RC	$1.387 \pm 0.073^{a*}$	$1.705 \pm 0.142^{b**}$
F1	$1.174 \pm 0.263^{a*}$	$1.973 \pm 0.0742^{c**}$
F2	$1.472 \pm 0.064^{a*}$	$2.033 \pm 0.079^{c**}$
F3	$1.99 \pm 0.047^{b*}$	$3.908 \pm 0.124^{d**}$
F4	$1.66 \pm 0.45^{b*}$	$2.67 \pm 0.187^{e**}$
F5	$1.420 \pm 0.299^{a*}$	$2.562 \pm 0.083^{e**}$
F6	$1.392 \pm 0.041^{a*}$	$2.339 \pm 0.15^{f**}$
F7	$1.445 \pm 0.122^{a*}$	$2.281 \pm 0.034^{f**}$

Mean in columns with different letters are significantly different ($P < 0.05$). The different star * in the same row shows significantly different.

Figure 4.6 shows that F1 and F7 had lower enzyme activities than other fractions, which might be contained difference bioactive compounds. Based on the



result of NQO1 and Tchl, the bioactivities might comply with the quantities of Tchl. F3 exerted the highest expression of the NQO1 enzyme coupling with 10.7 ± 0.418 (mg/g extract) of Tchl. Chlorophylls and their derivatives compound could promote detoxification enzymes because of the chemical characteristics of each compound. The double bond may increase their ability or metabolites reaction with thiol group (R-SR), which is found in Cysteine amino acid and functions in enzymes expression (Fahey et al., 2005).

Figure 4.6 NQO1 enzyme activity in both cell lines and quantities of total chlorophylls in each fraction. Noted: significantly different was compared by groups of treatment at p-value < 0.05 with n =3.

As a result of the primary identification of fractions on the TLC plate, the results found that the F1 and F7 fractions consisted of the carotenoids group. The F2 fraction showed one spot of pheophytin-*b* and the F3 fraction contained pheophytin-*a* while the F4, 5, and 6 fractions reported that they were chlorophyll-*a*, chlorophyll *b*, and unknown fraction. Our finding showed pheophytin *a* was detected in F3 fraction that exhibited maximal detoxification enzymes. Similar to other researchers, Fahey et al. (2005), chlorophylls could induce NQO1 enzymes in murine hepatoma cells. Also, Oonsivilai et al. (2007) demonstrated that RC could induce specific NQO1 activities in the hepatoma hepa1c1c7 cell line. Moreover, acetone extract provided a high enzyme activity of 2.8 fold compared to water and ethanol extract by increasing 1.4 and 1.6 fold, respectively. The RC acetone extract also detected a high amount of chlorophylls and its derivatives. Whilst RC water extract showed the lowest NQO1 activities along with contained high values of phenolic acids and flavonoids compounds. Thus, the chlorophylls and its derivative might

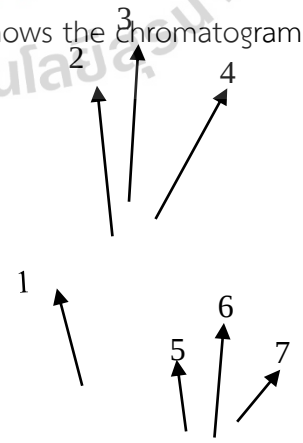
potent to induce the expression of NAD(P)H: quinone oxidoreductase (NQO1). Chlorophylls and chlorophyllin are associated with accumulation of transcription factor Nrf2 and activated PI3K/Akt signaling pathway then could induce HO-1 and NQO1 (Y. Zhang et al., 2008). Chlorophylls and their derivative substances have been reported that they are effective in decreasing cancer. Balder et al. (2006) found that chlorophyll dietary intake patients frequently decreased colon cancer. Similarly, chlorophylls are potentially blocking carcinoma cells (De Vogel at al., 2005).

Although several studies revealed effective of chlorophylls in antioxidant activity and detoxification, there was also mentioned that chlorophylls were lower antioxidant activity than pheophytin (Solymosi & Mysliwa-Kurdziel, 2017). Furthermore, pheophytins are metal-free substances, which could act through many mechanisms including antioxidant activity, a modifier of genotoxic effects, inhibition of cytochrome P450 enzymes, cell arrest, apoptosis, induction of phase II enzymes even increased level of glutathione S-transferase (Fahey et al., 2005).

4.9.3 Compounds identification

The pheophytin-*a* was found in F3 fraction on the primary identification of TLC plate and this fraction was also verified by HPLC and liquid chromatography-Mass spectrometry (LC-MS/MS). Figure 4.5 shows the chromatogram of RC chloroform crude extract (a) and fraction 3 (b).

(a)



(b)

Figure 4.7 HPLC chromatograph represented the compounds in RC crude extract and F3 fraction, and the results showed peaks: (1) lutein, (2) Unknown, (3) Chlorophyll *b*, (4) Chlorophyll *a*, (5) pheophytin-b, (6) Unknown, and (7) pheophytin-*a*. (b) showed a peak of pheophytin-*a*.

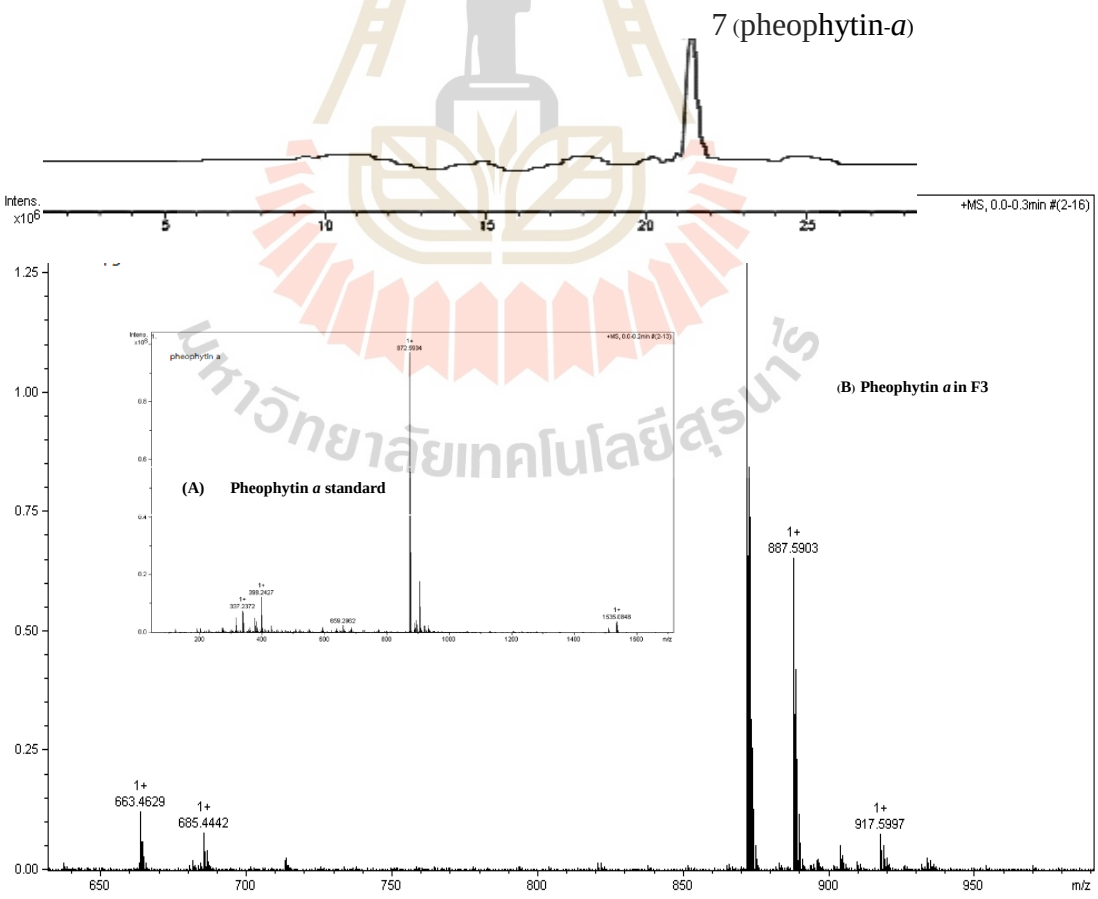


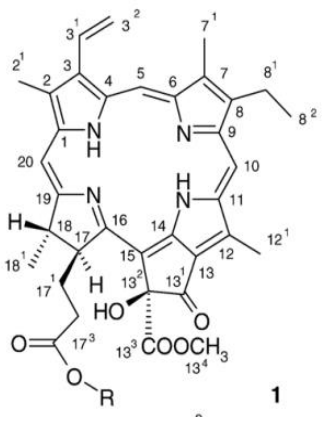
Figure 4.8 LC-MS spectra of (A) pheophytin *a* standard and fraction 3 (B).

Table 4.3 MS spectral data of chlorophylls and their derivative compounds.

Compound	[M+H] (On-line)	[M+H] (reported)
Chlorophyll a	893	-
Chlorophyll b	907	-
Hydroxypheophytin b	901	-
Pheophytin b	885	-
Pheophytin a	871.5	871.59
Hydroxypheophytin a	887.4	887.59

Source: (Jerz, Arrey, Wray, Du, & Winterhalter, 2007).

As the result of LC-MS/MS, Fraction F3 showed at m/z 871.59^{+} $[M + H]^{+}$, corresponding to pheophytin-*a* that was matched with standard pheophytin *a*. There also contained another peak at m/z 887.59^{+} $[M + H]^{+}$, which was hydroxypheophytin *a* (Figure 4.6). Jerz et al., (2007) reported that m/z 887.5697 value for [M + H] + was monoisotopic of $C_{55}H_{74}N_4O_6$ that is a 13^2-hydroxy-pheophytin *a*.



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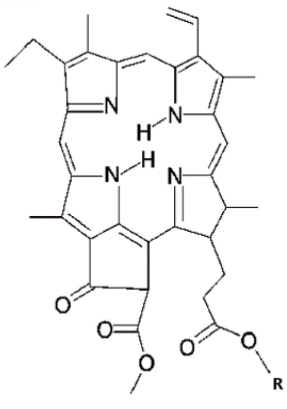

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Figure 4.9 The structure of (1) 13^2-hydroxy-pheophytin *a* [$C_{55}H_{74}N_4O_6$] and (2) pheophytin *a* [$C_{55}H_{74}N_4O_5$]. Source: (Jerz et al., 2007).

According to the result, fraction F3 contained pheophytin-*a* and its hydroxylation compound showed a great capacity for detoxification activity. The fraction F3 consists large quantity of Tch and also found some numbers of TPC and TFC, thus the degree of biological activity of this fraction may be accompanied by other synthetics compounds in the fraction.

4.10 Conclusion

According to this experiment, RC leaves extract are rich in bioactive compounds that shoed detoxification properties in AML12 and HepG2 cell lines. IC_{50} values of RC extract is shown at concentration higher than 2 mg/mL which could be evaluated plant qualifications. The pheophytin-*a* and hydroxypheophytin-*a* in RC extract are remarked as the essential substance in inducing of detoxification activity. These bioactive compounds in RC may provide health benefits, thus it could be developed as functional food ingredients.

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CHAPTER 5

CONCLUSIONS

Thai' s traditional herb *Thunbergia laurifo*lia Lindl. (RC) exhibits pharmacotherapeutic properties in detoxification in the liver cell lines. As result, the RC chloroform extract consisted of serval groups of phytochemicals such as phenolic acids, flavonoids, carotenoids, and chlorophylls and its derivatives. Column chromatography utilized silica gel as a stationary phase, which was very convenient for purifying bioactive compounds from plant extract. The results showed that fractions 1 and 7 contained the higher level of total phenolic contents while in fraction 3 showed the greatest amounts of total chlorophylls. From the cytotoxicity test by MTT assays, the percentages of cell viabilities in HepG2 and AML12 were higher than 50 % at the concentration of 0.03125 – 2 mg/ml, including IC_{50} value were higher than 2 mg/ml. RC crude extract and its fractions could induce NQO1 enzyme activities, however, fraction 3 exhibited the highest induction of NQO1 enzyme activity in both HepG2 and AML12 at 3.908 ± 0.079 fold and 1.99 ± 0.047 folds compared to control, respectively. Pheophytin *a* and Hydroxypheophytin *a* were found in F3, which showed the m/z 871.59^{+} $[M + H]^{+}$ and 887.59^{+} $[M + H]^{+}$, chemical structure of 13^{2}-hydroxy-pheophytin *a* and pheophytin *a* is $C_{55}H_{74}N_4O_6$ and $C_{55}H_{74}N_4O_5$. RC leaves consist of pheophytin *a* that is related to substantially induce the phase II detoxification enzymes H: quinone oxidoreductase 1 (NQO1) in liver cells line. Moreover, in the fraction F3 might contains other bioactive compounds that can be synergetic. This research demonstrated that bioactive compounds in RC crude extract could induce the expression of phase II enzyme NQO1, which not only

functions in detoxification of exogenous but also antioxidation of endogenous. The capacity of Rang Chuet isolation fractions are worth for future studies on which toxic substances would be exposed and observing their potential in detoxication.

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APPENDIX

Calculation of Total chlorophylls and Carotenoids

Chlorophyll and carotenoid contents were determined according to Lichtenthaler (1987). RC leave powder 0.2 g was mixed with 15 mL 80% acetone and transferred 1ml of extract for reading at the absorption 663.2, 646.8, and 470 nm, against acetone 80% blank. The concentration of chlorophyll (chl) and carotenoid (car) was calculated using the following formulas:

Equation of Chlorophyll a (Output equation = μg/mL extract)

chla (μg/mL) = 12.25*A663.2 - 2.79*A646.8

Equation of Chlorophyll b (Output equation = μg/mL extract)

chlb (μg/mL) = 21.50*A646.8 - 5.1*A663.2

Equation of Carotenoids (Carotenes and Xanthophylls)

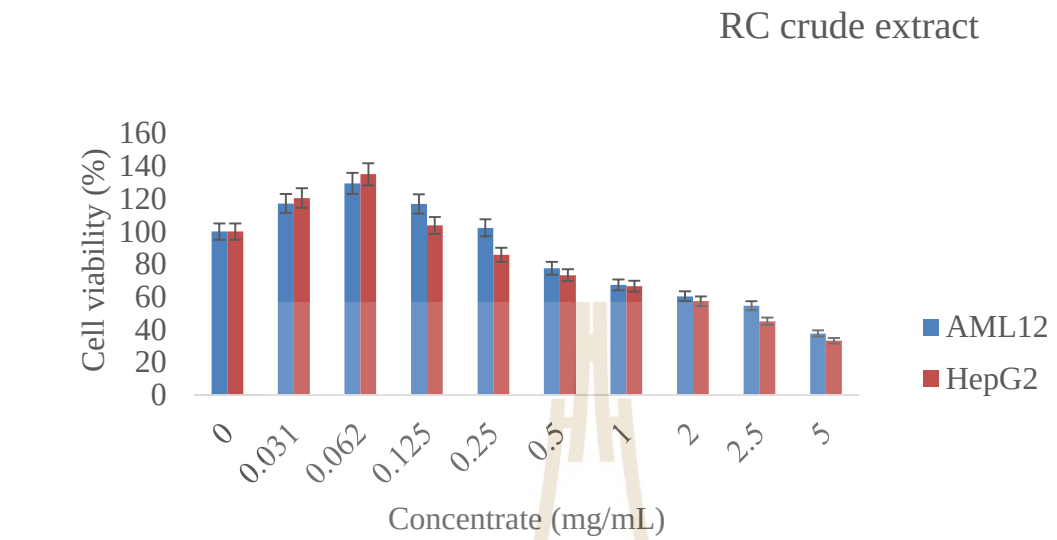
(Output equation = μg/mL extract)

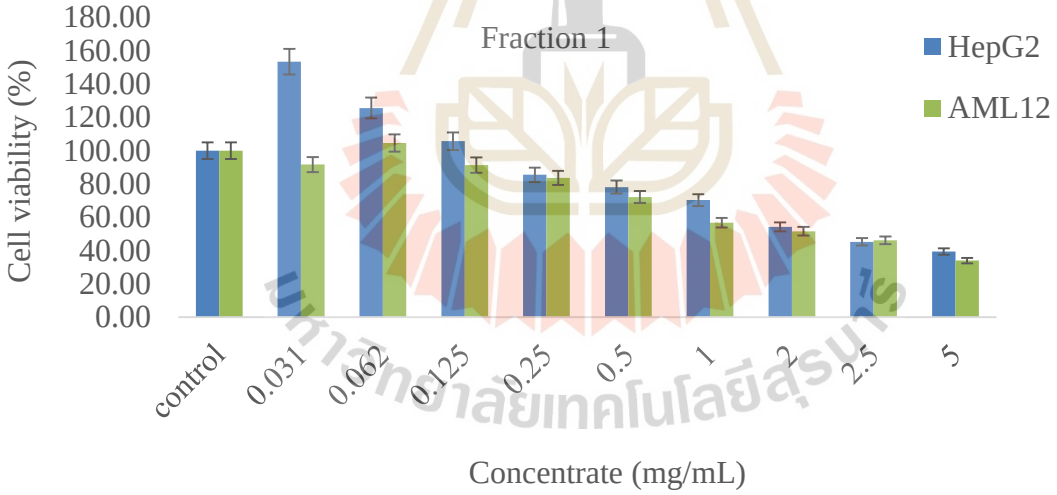
car (μg/mL) = (1000*A470 - 1.8*chla - 85.02*chlb)/ 198

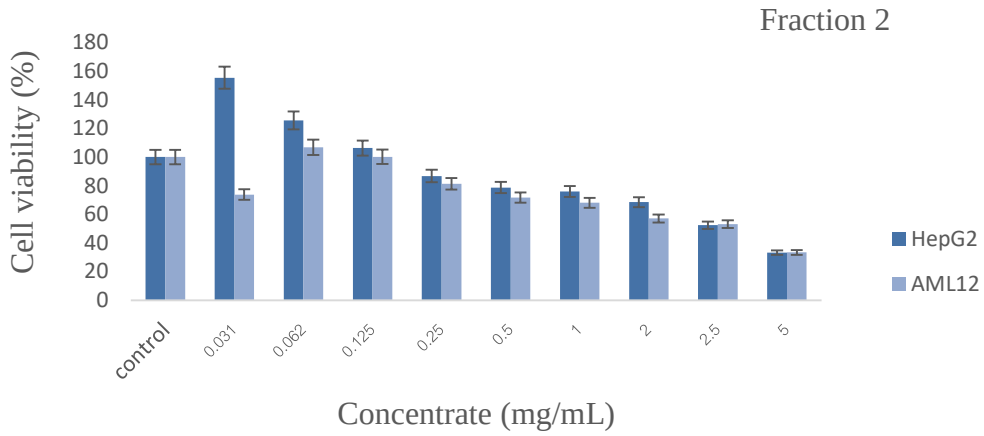
chla = output of chla equation, chlb = output of chlb equation

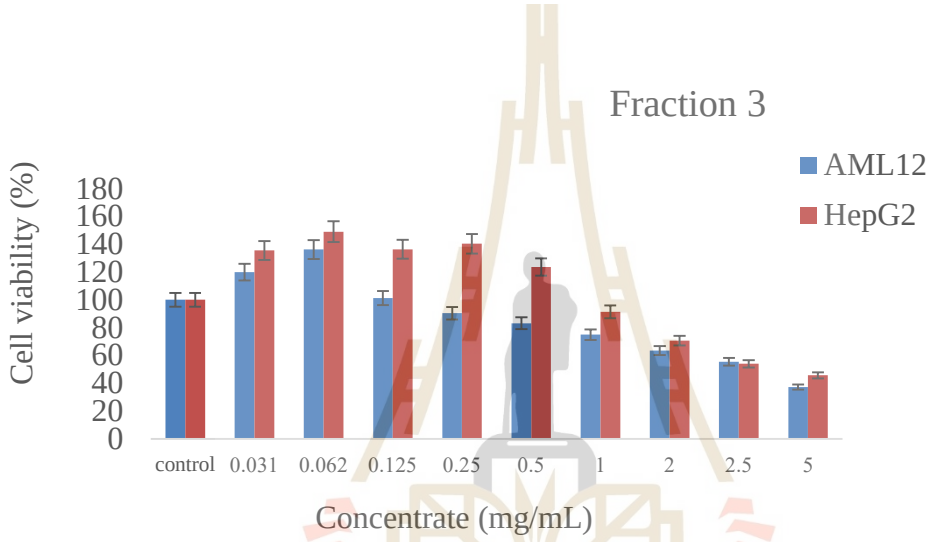
Cell culture

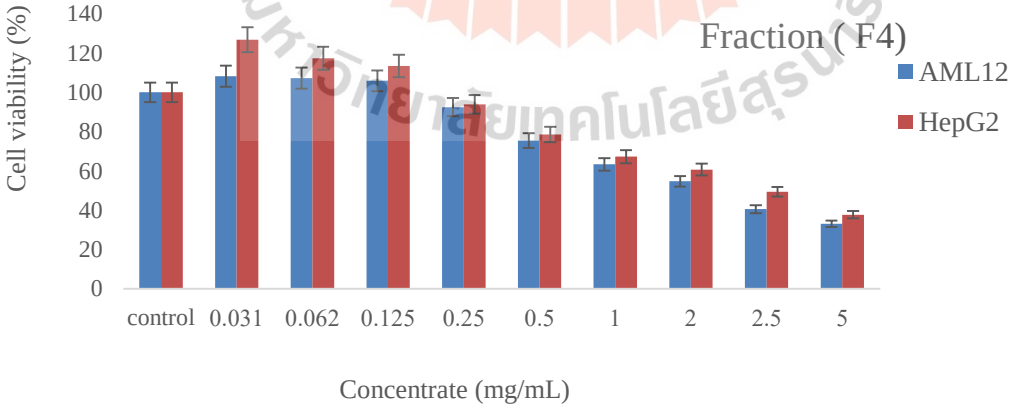
% Cell viability of RC crude extract and fractions in AML12 and HepG2

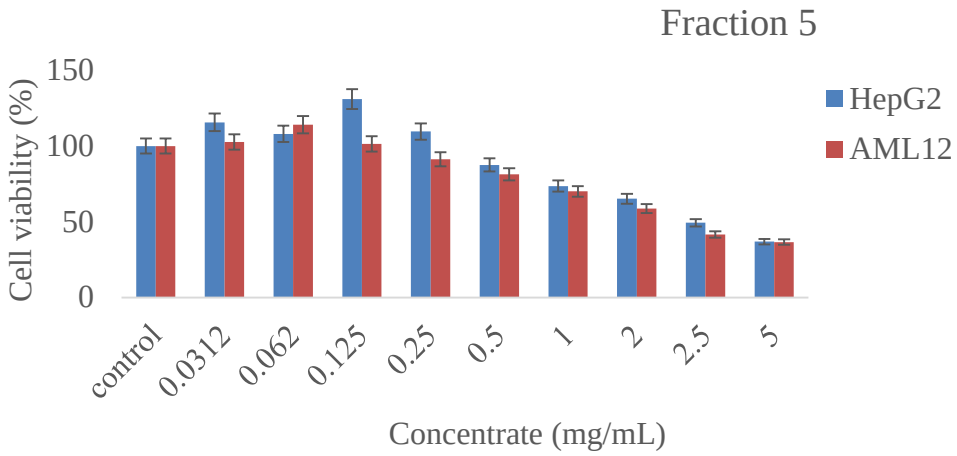


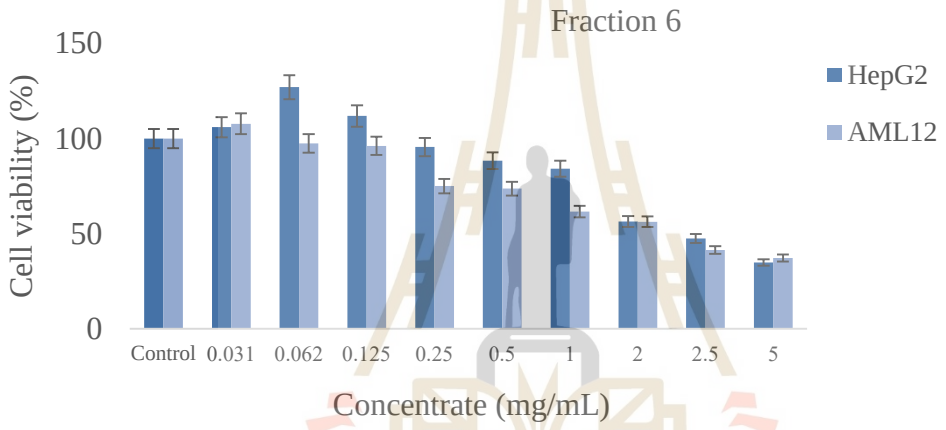



Fraction 2
Cell viability (%)
180
160
140
120
100
80
60
40
20
0
control
0.031
0.062
0.125
0.25
0.5
1
2
2.5
5
HepG2
AML12
Concentrate (mg/mL)


Fraction 3
Cell viability (%)
180
160
140
120
100
80
60
40
20
0
control
0.031
0.062
0.125
0.25
0.5
1
2
2.5
5
AML12
HepG2
Concentrate (mg/mL)


Fraction (F4)
Cell viability (%)
140
120
100
80
60
40
20
0
control
0.031
0.062
0.125
0.25
0.5
1
2
2.5
5
AML12
HepG2
Concentrate (mg/mL)


Fraction 5
Cell viability (%)
150
100
50
0
control
0.0312
0.062
0.125
0.25
0.5
1
2
2.5
5
Concentrate (mg/mL)
HepG2
AML12


Fraction 6
Cell viability (%)
150
100
50
0
Control
0.031
0.062
0.125
0.25
0.5
1
2
2.5
5
Concentrate (mg/mL)
HepG2
AML12

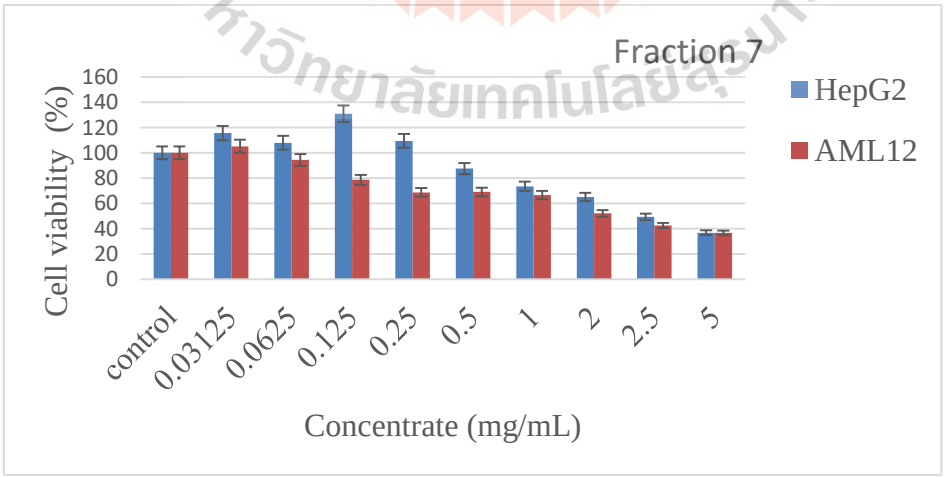

Fraction 7
Cell viability (%)
160
140
120
100
80
60
40
20
0
control
0.03125
0.0625
0.125
0.25
0.5
1
2
2.5
5
Concentrate (mg/mL)
HepG2
AML12

Figure 4B: IC50 value of each fraction in both cell lines.

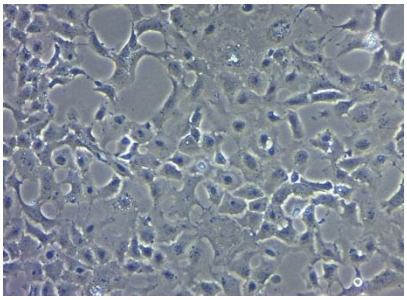
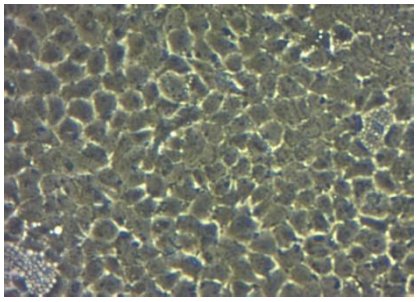
(a)   (b)

Figure 4B AML12 cells line. (a) at low cells density, (b) at high cells density.

VITAE

Ms. Sreylak Moeurng was born in Kampong Spue province, Cambodia. In 2018, she received a bachelor's degree at department of Bioengineering, Faculty of Engineering at Royal University of Phnom Penh, Cambodia. The topic of her project was "Prevalence and concentration of Escherichia coli and Salmonella species in fresh vegetables collected from different types of markets in Phnom Penh". In 2020, she enrolled Master's degree at School of Food Technology, Suranaree University of Technology, Thailand. Her research title was " Group of bioactive compounds identification in *thunbergia luarifolia* lindl. (rang chuet) extract with detoxification in hepg2 and aml12 cell lines". The results from some parts of this research had an oral presentation and proceeding at The international conference on 6th of Food Applied Bioscience, February 14-15, 2022, Chiang Mai University, Bangkok, Thailand. Sreylak Moeurng, Anyanee Kamkeaw, Ratchadaporn Oosilivia., (2022). Group of Bioactive Compounds Identification in *Thunbergia Laurifolia* Linn. (Rang Chuet) Crude Extract.