คุณสมบัติเชิงหน้าที่และโภชนเภสัชของสารสกัดรางจืด (*Thunbergia laurifolia* Lindl.)

นางรัชฎาพร อุ่นศิวิไลย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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FUNCTIONAL AND NUTRACEUTICAL PROPERTIES OF RANG CHUET (*Thunbergia laurifolia* Lindl.) EXTRACTS

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

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FUNCTIONAL AND NUTRACEUTICAL PROPERTIES OF RANG CHUET (*Thunbergia laurifolia* Lindl.) EXTRACTS

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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รัชฎาพร อุ่นศิวิไลย์ : คุณสมบัติเชิงหน้าที่และโภชนเภสัชของสารสกัดรางจืด (*Thunbergia laurifolia* Lindl.) (FUNCTIONAL AND NUTRACEUTICAL PROPERTIES OF RANG CHUET (*Thunbergia laurifolia* Lindl.) EXTRACTS) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. สุเวทย์ นิงสานนท์, 105 หน้า.

รางจืดเป็นพืชพื้นบ้านของประเทศไทยที่ถูกนำมาใช้สำหรับป้องกันและแก้พิษจากอาหารและสิ่งแวดล้อม เพื่อศึกษาคุณสมบัติเชิงหน้าที่และโภชนเภสัชของสารสกัดรางจืด จึงนำใบรางจืดไปสกัดด้วยน้ำ เอทานอล และอะซีโตน จากการตรวจหาส่วนประกอบและตรวจสอบคุณสมบัติเชิงหน้าที่และโภชนเภสัชของสารสกัดรางจืด พบว่ารางจืดมีองค์ประกอบหลักคือฟินอลิก คาโรทีนอยด์และคลอโรฟิลล์ โดยพบปริมาณสารฟินอลิกสูงสุด (24.3 ไมโครกรัมของกรดกาลิค) ในสารสกัดน้ำ รองลงมาเป็นสารสกัดเอทานอล (5.65 ไมโครกรัมของกรดกาลิคและน้อยที่สุดในสารสกัด อะซีโตน(1.42 ไมโครกรัมของกรดกาลิค) จากการตรวจหาส่วนประกอบหลักโดยวิธี HPLC พบว่ากรดคาเฟอิก และ อะพิจินิน(apeginin) เป็นส่วนประกอบหลักในสารสกัดน้ำ และสารประกอบคลอโรฟิลล์เป็นสารประกอบหลักในสารสกัดเอทานอลและสารสกัดอะซีโตน

ในการทดสอบคุณสมบัติในด้านการแก้พิษของสารสกัดรางจืดโดยวัดค่าการเพิ่มการออกตามฤทธิ์ของเอนไซม์ NAD(P)H: quinine oxidoreductase (NQO1)ในเซลล์ตับชนิด Hepa 1c1c7 พบว่า สารสกัดอะซีโตนมีฤทธิ์ในการเพิ่มปฏิกิริยาของเอนไซม์ NQO1 สูงสุด 2.8 เท่าเมื่อเทียบกับตัวควบคุม รองลงมาคือสารสกัดเอทานอลและสารสกัดน้ำซึ่งมีฤทธิ์ในการเพิ่มปฏิกิริยาของเอนไซม์ 1.35 และ 1.56 เท่าตามลำดับ

การทดสอบฤทธิ์การเป็นสารก่อกลายพันธุ์และการต่อต้านฤทธิ์ของสารก่อกลายพันธุ์ พบว่าสารสกัดรางจืดทุกชนิดมีฤทธิ์ต้านการก่อกลายพันธุ์ของ 2 Amino-anthracene สูงสุดที่ สายพันธุ์ TA 98 และ TA100.

สารสกัดน้ำแสดงฤทธิ์ต้านอนุมูลอิสระโดยการตรวจสอบด้วยวิธี DPPH assay ที่ค่า EC_{50} สูงสุดที่ 0.13 มิลลิกรัมกรดกาลิคต่อมิลลิลิตร ขณะที่สารสกัดเอทานอลและอะซีโตนแสดงค่า EC_{50} ที่ 0.26 และ 0.61 มิลลิกรัมกรดกาลิคต่อมิลลิลิตร ตามลำดับ นอกจากนี้การแสดงฤทธิ์ต้านอนุมูลอิสระโดยการตรวจสอบด้วยวิธี FRAP assay สูงสุดที่ 0.93 มิลลิโมลต่อกรัมในสารสกัดน้ำ รองลงมาเป็นสารสกัดเอทานอลและอะซีโตนที่ค่า 0.18 และ 0.04 มิลลิโมลต่อกรัม ตามลำดับ

ส่วนการทดสอบความเป็นพิษของสารสกัดรางจืดในเซลล์ไลน์ L929, BHK(21)C13, Hep G2, และ Caco-2 พบว่ามีค่าความเป็นพิษต่ำกับทุกเซลล์ไลน์ที่ระดับความเข้มข้นของสารสกัดมากกว่า 100 ไมโครกรัมต่อมิลลิลิตร

ในการศึกษาอายุการเก็บของใบรางจืดแห้งและสารสกัดในระยะเวลา 6 เดือน ณ อุณหภูมิแตกต่างกันที่ -25, 25, 30, และ 50^{o}C พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญของระดับฟีนอลิกในช่วงอายุการเก็บที่ทดลองทั้งในใบแห้งและสารสกัด ส่วนระดับคลอโรฟิลล์มีระดับลดลงซึ่งแปรผันกลับกับอุณหภูมิของการเก็บที่สูงขึ้นและระยะเวลาการเก็บรักษา ส่วนระดับของลูทีนจะลดลงในช่วงระยะเวลาการเก็บรักษา ณ ทุกอุณหภูมิที่ทำการทดลอง

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

RATCHADAPORN OONSIVILAI : FUNCTIONAL AND NUTRACEUTICAL PROPERTIES OF RANG CHUET (*Thunbergia laurifolia* Lindl.) EXTRACTS. THESIS ADVISOR : ASST. PROF. SUWAYD NINGSANOND, Ph.D. 105 PP.

FUNCTIONAL AND NUTRACEUTICAL PROPERTIES OF RANG CHUET (*Thunbergia laurifolia* Lindl.) EXTRACTS

The *Thunbergia laurifolia* Lindl or Rang Chuet (RC) is widely described in traditional medicine for protection against dietary and environmental toxicants with little substantiation. To better assess the potential of RC as a medicinal plant, extract were prepared by infusion with water, ethanol, and acetone. Extracts were subsequently assayed for major phytochemical constituents including phenolics, carotenoids, and chlorophylls. Total phenolic content was 24.33, 5.65, and 1.42 ug gallic acid equivalent (GAE) per mL water, ethanol and acetone extract respectively. HPLC analysis identified caffeic acid and apigenin as primary constituents of water extract. Acetone and ethanol extracts contained primarily chlorophyll *a* and *b*, pheophorbide *a*, pheophytin *a*, and lutein.

The detoxification potential of RC was determined from the ability of the standardized extracts to increase NAD(P)H:quinone oxidoreductase (NQO1) in Hepa 1c1c7 cells. Treating hepatoma cells with RC extracts resulted in a dose dependent increase in NQO1 specific activity for all extracts. Acetone extract (92 µg GAE/mL)

increased NQO1 activity 2.8 folds, compared with the control while ethanol (120 μg GAE/mL) and water (1000 μg GAE/mL) extracts increased 1.35 and 1.56 folds respectively.

RC extracts were subsequently assayed for mutagen and antimutagenic activity using bacterial reverse mutagenesis assay. All three RC extracts exhibited strong dose-dependent antimutagenic activity inhibiting 2-aminoanthracene induced matagenesis up to 87% in *Samonella typhimurium* TA 98 and TA 100. Rang Chuet water extract possessed the highest antioxidant activities using free radical scavenging at the EC_{50} values of 0.13 mg GAE/mL whereas ethanol and acetone extract showed EC_{50} at 0.26 and 0.61 mg GAE/mL respectively. In addition, the water extract also showed the highest total antioxidant activity using FRAP assay at 0.93 mmol/g following with ethanol and acetone extracts (0.18 and 0.04 mmol/g).

Cytotoxicity of RC crude extracts were investigated in L929, BHK(21)C13, HepG2, and Caco-2 cell lines. The toxicity was indicated at high concentrations over 100 μg/mL for all extracts, which would be the index for further recommended concentration.

Storage changes of polyphenols, chlorophyll derivatives, and carotenoids in RC leaves and extracts over 6 months were studied at different storage temperatures of -25, 25, 30 and 50°C. There were no significant variations of total phenolics occurred during storage. Chlorophyll contents decreased with increased storage temperature and time in ethanol extract. Carotenoids identified as luteins in RC

ethanol extract decreased in both leaves and the extract during storage at all temperatures.

School of Food Technology

Academic Year 2006

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Ratchadaporn Oonsivilai

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continuesd)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figures **Page**

LIST OF FIGURES (Continued)

Figures **Page**

LIST OF FIGURES (Continued)

Figures

Page

LIST OF ABBREVIATIONS

2-AA	2-Aminoanthracene
^{o}C	Degree Celsius
DMSO	Dimethyl Sulfoxide
Fig	Figure
g	Gram
GAE	Gallic acid equivalent
h	Hour
HCl	Hydrochloric acid
Kg	Kilogram
M	Molar
mM	Millimolar
µM	Micromolar
mg	Milligram
µg	Microgram
min	minute
mL	Milliliter
µL	Microliter
mmol	Millimole
MTT	3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyltetrazolium bromide
NQO1	NAD(P)H: Quinine oxido reductase 1
UV	Ultraviolet
rpm	round per minute

CHAPTER I

INTRODUCTION

1.1 Introduction

Throughout the ages, humans have relied on nature for their basic needs including production of food-stuffs, shelters, clothing, means of transportation, fertilizers, flavours and fragrances, and, not the least, medicines. Plants have formed the basis of sophisticated traditional medicine systems existing for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical evidence from use over hundreds and thousands of years. Interest in natural sources of potential chemotherapeutic agents continues. Natural products and their derivatives represent more than 50% of all the drugs in clinical use in the world. Higher plants contribute no less than 25% of this total. During the last 40 years, at least a dozen potent drugs have been derived from flowering plants. Approximately half (125,000) of the world's flowering plant species grow in the tropical forests. Tropical rain forests continue to support a vast reservoir of potential drug species. They continue to provide natural product chemists with invaluable compounds of starting points for the development of new drugs. The potential for finding more compounds is enormous as at date only about 1% of tropical species have been studied for their pharmaceutical potential (Gurib-Fakim, 2005). This proportion is even lower for species confined to the tropical rain forests. To date, approximately 50 drugs have been derived from tropical plants, and the the potentialexistence of undiscovered pharmaceuticals for modern medicine has often been

cited as one of the most important reasons for protecting tropical forests (Gurib-Fakim, 2005).

Food components are normally grouped in five great families: carbohydrates, lipids, proteins, and vitamins, with each having a particular biological function in nutrition. Besides these basic nutrients, many other food constituents are present that may exert beneficial effects to the consumer. These are often termed bioactive food constituents. Many compounds have been grouped into this category including natural pigments, polyphenols, and flavor components. Specific examples include chlorophylls, carotenoids, flavonoids, coumarins and many others (Table1). While not essential for correct nutrition and are therefore not nutrients, and they are not considered substances having direct therapeutic activity, although some do at concentrations much higher than those found in normal diet. However when foods containing these compounds are a regular part of the diet, the incidence and prevalence of certain diseases are changed (Hurst, 2002).

Of all food groups, plant derived materials are the most abundant in the bioactive food components. Plants have always played a significant role in maintaining the health and improving the quality of human life. Many Western drugs owe their origin to plant extracts. For example, reserpine, which is widely used for treatment of high blood pressure, was originally extracted from the plant *Rauwolfia serpentina* while digitalis, used as a heart stimulant, was derived from the foxglove plant (*Digitalis purpurea*) (Hurst, 2002).

Self prescribed herbal preparations are commonly consumed today for a whole host of common aliments or conditions, such as anxiety, arthritis, colds, coughs, constipution, declining mental acuity, depression, fever, headaches, infections, insomnia, intestinal disorders, premenstrual syndrome, stress, ulcers, and weakness. Culinary herbs to flavor foods have also been grown and used since antiquity. While these herbs are

generally consumed in small amounts, it is interesting to note that they contain similar health-promoting phytochemicals as do fruits and vegetables as shown in Table 1.1 (Watson, 2002)

Table 1.1 Phytochemicals found and their botanical sources.

Phytochemical	Herbal source of phytochemical
Carotenoids	Green leafy herbs, paprika, rose hips
Coumarins	Alfalfa, aniseed, chamomile, fenugreek, parsley, red clover
Curcuminoids	Ginger, turmeric
Flavonoids	Chamomile, ginkgo, hawthorn, licorice root, milk thistle, nettles, passionflower, rosemary, thyme.
Lignans	Flaxseed, milk thistle, schizandra.
Phthalides	Anise, caraway, cilantro, cumin, dill, fennel, parsley.
Phytosterols	Nettles, pumpkin seeds, saw palmetto.
Phenolic acids	Echinacea, rosemary, sage, St.John's wort, parsley.
Polyacetylenes	Anise, caraway, cilantro, cumin, dill, fennel, parsley.
Saponins	Alfalfa, black cohosh, ginseng, horse-chestnut, licorice root, snakeroot.
Sulfides	Chives, garlic, leeks, onions.
Terpenens	Basil, buchu, ginkgo, juniper, lemon grass, oregano, peppermint, rosemary, sage, thyme.

Source: Watson (2002)

In Thailand, traditional medicine is commonly practiced with the use of herbal remedies. Rang Chuet (*Thunbergia laurifolia* Lindl) is a local Thai plant in the family of *Acanthaceae* found in the North. It has twinning shrub, with simple, opposite, oblong or

ovate leaves, 4-7 cm \in width, 8-14 cm in length (Figure 1.1). It has influorescence in terminal raceme; flower bluish purple; bracts green with reddish brown blotches, and fruit capsule. It can be divided into three types; white flower, yellow flower, and purple flower. The purple flower cultivar has been reported to have several distinct pharmacological properties particularly from extracts derived from stems, roots and leaves. For example, aqueous extracts of fresh leaves, dried leaves, dried root and bark are used as antidote for insecticide, ethyl alcohol, arsenic and strychnine poisoning; the dried root is also used as an anti-inflammatory agent and antipyretic (Thongsaard and Marsden., 2002). In addition, it has been reported that Rang Chuet leaves affect body temperature of rats by centrally act at the thermo regulating center and/or cause vasodilatation and thereby increase heat dissipation (Chamreondararassame, 2003).

Figure 1.1 *Thunbergia Laurifolia* Lindl (Rang Chuet)

Source: Faculty of Pharmacy, Mahidol University (1987)

The pharmacological properties of Rang Chuet has also been reported as the antidote to insecticide, treatment for drug addiction, toxicity as well as having reported antimicrobial and antioxidant activity (Tejasen and Thongthapp, 1979; Rengyutthakan, 1980; Thongsaard and Marsden, 2002; Khunkitti, Taweechaisupapong, Aromdee, and Pesee, 2003; Srida, Hankete, Khunkitto, Aromdee, and Pese, 2002). Recently, it has been

reported that extracts of *Thunbergia laurifolia* Lindl leaves have a protective effect on ethanol-induced hepatotoxicity using hepatic lipid peroxidation, boold dehydrogenase activity as indicator (Chanawirat, Toshulkao, Temcharoen, and Glinsukon, 2000). Treatment of alcoholism is also claimed using its aqueous extract. Alcohol and hexane extract from *Thunbergia laurifolia* also posses anti-inflammatory activity against carageenin-induced paw edema in mice (Charumanee *et al*., 1998). While results for rang chuet are promising, efforts to elucidate mechanism by which this plant protects against dietary and environmental toxicants is not well described. It is not known whether rang chuet biological activity is consistent with natural detoxification processes through induction of phase II xenobiotic enzymes or by other other mechanisms. Furthermore, while often consumed as water extracts or leaf, solvent extracts are becoming more prevalent in the Thai market. As solvent extraction may generate a different bioactive component profile, the potential toxicity as well as classic antioxidant, and mutagen-antimutagen activity of these extracts need to be investigated relative to the parent plant. However, there is little information available in the literature on the chemical composition and functional properties of this plant extract.

Therefore, the purpose of this research is to investigate the chemical composition and functional properties of Rang Chuet extract, especially its detoxification mechanism in order to understand its useful functionality including active components. In addition, the functional properties such as antioxidant, mutagen-antimutagen activity of this plant would be investigated. Furthermore, the stability of plant extract components would be studied for potential application in functional foods and nutraceuticals.

1.2 Research objectives

(1) To determine chemical and functional properties of Rang Chuet extracts prepared with several commercial solvents.

(2) To screen solvent extracts for toxic effect using on appropriate in vitro and cell based assays.

(3) To investigate the antioxidant and antimutagenic activity of Rang Chuet extracts .

1.3 Research hypothesis

Rang chuet leaves contain phytochemical that enable non-toxic themselves and have biological activity toward detoxification system.

1.4 Scope and limitation of the study

Rang Chuet's leaf extract will be analyzed for antioxidant and antimutagenic activities and its chemical and functional properties will be determined. As well, the extracts will be tested for cytotoxicity to liver and intestinal cell lines.

1.5 Expected results

1. To understand the chemical and functional properties of the Rang Chuet extract.
2. To produce active components and/or Rang Chuet preparations to may be used as food ingredient and neutraceutical product.

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CHAPTER II

LITERATURE REVIEW

Thailand is located in the Sub-Continent of Southeast Asia, or tropical zone, where the climate is suitable for agricultural and forestry, thus a lot of medicinal plants are found all over the country. Native doctors have, for centuries, used these plants to combat diseases in the form of herbal drugs, traditional drugs, or old-style preparations. Much of knowledge of traditional medicine had been transferred from one generation to the next through experience, but without any scientific proof as in modern medicine (Mokkhadmit, Ngarmwathana, Sawasdimongkol, and Permphiphat, 1971).

2.1 *Thunbergia Laurifolia* Lindl

Thunbergia laurifolia Lindl (*Acanthaceae*, Thai name: Rang-Chuet), a vine distributed in Southeast Asia and found to be a native in the North of Thailand, is in the family of *Acanthaceae* shown in Fig. 2.1.

As Rang Chuet has been used as traditional medicine, researchers have studied the compounds in Rang Chuet leave extracts (Jitpuwngam, 1979). Kanchanapoom, Kasai, and Yamasaki (2002) reported two iridoid glucosides, 8-*epi*-grandiforic and 3'-*O*- β-glucopyranosyl-stibericoside isolated from the aerial part of *Thunbergia laurifolia* Lindl along with seven known glucosides compounds. Purnima and Gupta (1978) reported that the flowers of *Thunbergia laurifolia* yielded Delphinidin 3:5-di-o-β-D-glucopyranoside, apigenin and apigenin-7-o-β-D-glucopyranoside. The plant was also reported to contain flavonoids such as apigenin, casmosiin, delphinidin-3-5-di-*O*-β-D-glucoside and

chorogenic acid (Thongsaard and Marsden., 2002). Furthermore, Jitpuwngam (1979) studied the compounds in Rang Chuet using its leaves' extract. The results showed that it composed of eight steroids and β-carotene.

The pharmacological properties of Rang Chuet extracts have been reported as the antidote to insecticide, treatment for drug addiction and toxicity induced from enivonenmental and dietary compounds. Modes of action of rang chuet are believed to include antioxidant and antimicrobial activity (Tejasen and Thongthapp, 1979; Rengyutthakan, 1980; Thongsaard and Marsden, 2002; Khunkitti, Taweechaisupapong, Aromdee, and Pesee, 2003; Srida, Hankete, Khunkitto, Aromdee, and Pese, 2002).

Fig. 2.1 *Thunbergia Laurifolia* Lindl (Rang Chuet)

Source: Faculty of Pharmacy, Mahidol University, 1987

2.2. Pharmacological studies of Rang Chuet

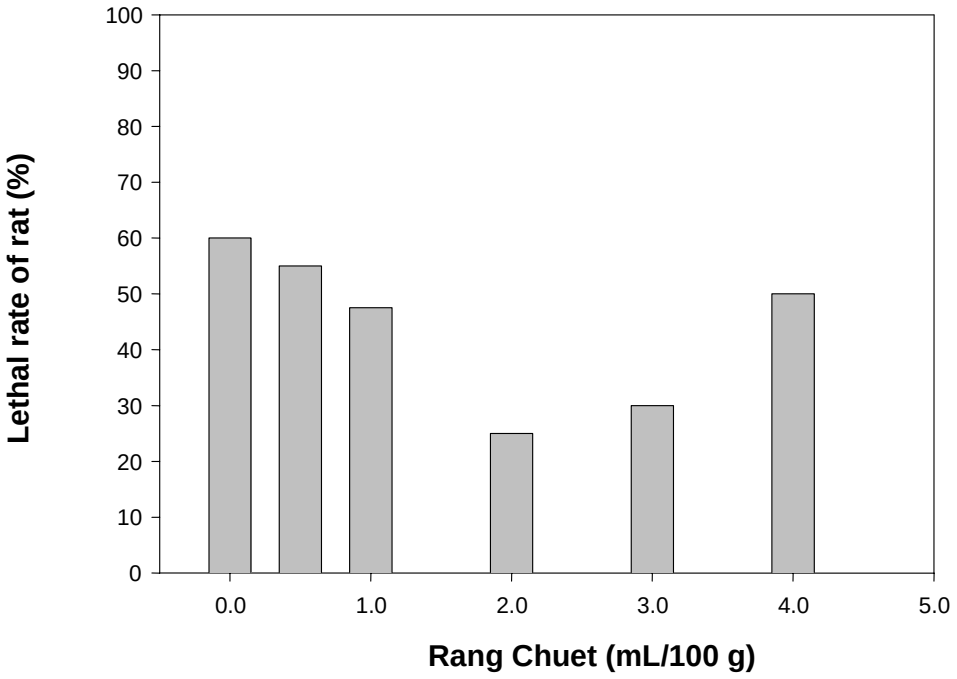

Fig. 2.2 Lethal rate of rat after received Rang Chuet extracts solution via stomach tube

Source: Tajasen and Thongthap, 1979

The lethal rate of folidol in a rat model was significantly decreased as concentration (ml extract/ml diluent) of rang chuet extract increased ($p < 0.001$) (Fig. 2.2). The most effective dose for antidote to Folidol was 20 μL /kg. There was no significant difference ($p < 0.001$) between group four and group five although the concentration of extract was increased, the lethal rate did not change significantly (Tejasen and Thongthapp, 1979).

2.2.1. Route of Administration the extract

Tejasen and Thongthapp (1979) studied the route of choice for introducing the extract of Rang Chuet to study antidote to Folidol and the results were concluded in Table 2.1, showing that the route for introducing Rang Chuet was oral and the optimum dose was 2.0 μL per 100 g body weight.

Table 2.1. Comparison of route of choice for introduce Rang Chuet extract.

Group	Amount of rat	Folidol (μL/kg)	Route	Rang Chuet (μL/100 g)	Route	Lethal rate (%)*
1	60	20.0	Subcu-taneous	-	-	63.33±3.33
2	40	20.0	Subcu-taneous	2.0	Oral	25.00±8.66**
3	10	20.0	Subcu-taneous	0.5	Abdomen cavity	50.00
4	10	20.0	Subcu-taneous	0.5	Subcu-taneous	50.00
5	10	20.0	Subcu-taneous	0.5	Subcu-taneous same area as Folidol	50.00

* Lethal rate percentage ± standard deviation

** Significant at $p < 0.001$ when compared with group one.

Source: Tajasen and Thongthap, 1979

2.2.2. Comparison between Rang Chuet and modern medicial treatment of Folidol exposure.

Tejasen and Thongthapp (1979) compared the effect of Rang Chuet extracts to pharmacological medicines often used in treatment of folidol exposure. The results indicated that Rang Chuet extract could significantly ($P<0.001$) reduce lethal rate from

56.67% to 16.67%. When Rang Chuet was used together with atropine, the lethality of folidol exposure significantly ($p < 0.001$) decreased from 56.67% to 5.00 % in a manner similar to the effect of atropine together with 2-PAM, the standard modern medical treatment for Folidol exposure. On the other hand, using Rang Chuet with atropine and 2-PAM did not change the lethal rate. While the exact mechanism by which Rang Chuet may act as an antidote of insecticides and similar environmental toxicant, it has been postulated to be biochemical antagonism instead of pharmacological antagonism that requires further investigation.

2.2.3. Effects of Rang Chuet on cardiovascular system

The phamacological effect and mechanism of actions of Rang Chuet extract concentration 15% w/v) on the cardiovascular system were studied in 91 pentobarbital anesthetized rats by Ruengyutthakan in 1980. It was found that intravenous injection of the aqueous extract produced hypotension, with a fall in blood pressure reaching a maximum extent (46.42 ± 3.75 %) at the dose of 120 mg/kg body weight. The mechanism of action was determined through comparative use of several traditional blocking drugs including: atropine (cholinergic blocking drug), propranolol (beta-adrenoceptor blocking drug) and selective antihistamines (mepyramine and cimetidine which were H_1 and H_2 receptor antagonists, respectively). Atropine attenuated the hypotensive response without complete antagonism whereas propanolol, mepyramine and cimetidine could not alter the hypotensive action of Rang Chuet. It was also observed that the amount of K^+ presence in the extract had no significant effect on the blood pressure. Furthermore normal saline solution had no significant effect on the blood pressure (Ruengyutthakan, 1980).

2.2.4. Effects of Rang Chuet and its mode of actions on smooth muscles

The direct stimulating effect of Rang Chuet on non-vascular smooth muscle might be acted through cholinergic receptors to produce hypotension. Aqueous extract of Rang Chuet (concentration 15% w/v) caused and increased and followed by a decrease in tension in isolated human umbilical arteries. Transient relaxation followed by a prolonged contraction with high intensity of both tonus and contractile force of the smooth muscle of isolated rat small intestine (48 preparations) were also observed. The response of the intestinal smooth muscle appeared to be dose related. The stimulation on the smooth muscle was also established in the isolated guinea pig tracheal chain and isolated rat uterus.

2.2.5. Rang Chuet used in the treatment of drug addiction

Thongsaard and Marsden (2002) studied *Thunbergia laurifolia Linn* (Rang Chuet) as herbal medicine used in the treatment of drug addiction. They investigated the effects of Rang Chuet on dopaminergic neurotransmission in comparison with amphetamine. The effect of crude water extract (0.1 g/ml) of Rang Chuet on K^+ (20mM) stimulated dopamine release from rat strial slices were compared with amphetamine (10^{-4}M) using high-performance liquid chromatography with electrochemical detection to measure endogenous dopamine. Amphetamine and Rang Chuet significantly increased K^+ stimulated dopamine release ($P< 0.001$) from rat strial slices when compared with K^+ stimulated alone. Rang Chuet potentiated the effect of amphetamine on K^+ stimulated dopamine release ($P< 0.001$) when compared with amphetamine alone. The results indicate that Rang Chuet may stimulate dopamine release in the similar manner to amphetamine. It remains to be determined whether the effect of these extracts on dopamine function is important in their therapeutic use in the treatment of drug addiction or in response to insult by other environment or dietary toxicants.

Recently, it has been reported that extracts of *Thunbergia laurifolia* Lindl leaves have a protective effect on ethanol-induced hepatotoxicity by modulating hepatic lipid peroxidation, blood ethanol concentration as well as hepatic alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) as the indicators (Chanawirat, Toshulkao, Temcharoen, and Glinsukon, 2000). Also *Thunbergia laurifolia* Lindl extract had hepatoprotective activity in rats treated with ethanol both in *in vitro* and *in vivo* studies, treatment of alcoholism was also claimed using its aqueous extract. Alcohol and hexane extract from *Thunbergia laurifolia* Lindl also posses anti-inflammatory activity against carageenin-induced paw edema in mice (Charumanee *et al*., 1998).

2.3. Separation methods for the analysis and isolation of plant constituents

The large portion of the population globally still relies on local traditional medicinal plants and other materials (*materia medica*) for everyday health care needs. It is also a fact that one quarter of all medical prescriptions are based on substances derived from plants or plant-derived synthetic analogs, and according to the WHO, 80% of the world's population—primarily those of developing countries—rely on plant-derived medicines for their healthcare (Nyiredy, 2004).

People who use traditional remedies may not understand the scientific rationale behind their medicines, but they know from personal experience that some medicinal plants can be highly effective if used at therapeutic doses. Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health. A single plant may, for example, contain bitter substances that stimulate digestion, anti-inflammatory compounds that reduce swellings and pain, phenolic compounds that can act as an antioxidant and venotonics, anti-bacterial and anti-

fungal tannins that act as natural antibiotics, diuretic substances that enhance the elimination of waste products and toxins, and alkaloids that enhance mood and give a sense of well-being. While modern approaches are intent on development of a patentable single compound or a 'magic bullet' to treat specific conditions, traditional medicine often aims to restore balance by using chemically complex plants, or by mixing together several different plants in order to maximize a synergistic effect or to improve the likelihood of an interaction with a relevant molecular target (Nyiredy, 2004).

2.3.1. Classification of separation methods

Separation of the constituents of dried, powdered plants can be classified into three main categories: extraction, purification, and chromatography. In general, the first two belong to sample preparation, while the various chromatographic methods ensure qualitative and quantitative analysis as well as isolation (Nyiredy, 2004).

In order to obtain the plant constituents, the first separation method is the solid–liquid extraction of the dried and powdered plant materials which contain many chemically different classes of compounds. A schematic view of plant tissue structure with damaged and undamaged cells is shown in Fig. 2.3 indicating which extraction process is decisive for the different types of cells. Using various solvents (A, B, C, D, …) with increasing polarity, different extracts (Extracts A, B, C, D, …) can be obtained. By applying an appropriate purification method, a certain extract (e.g., Extract C) can be divided into fractions (e.g., Fraction I, Fraction II), as the different shapes indicate it in the Fig.. Using chromatographic methods, the fractions can be separated into their constituents; in ideal cases baseline separations can be achieved (Nyiredy, 2004).

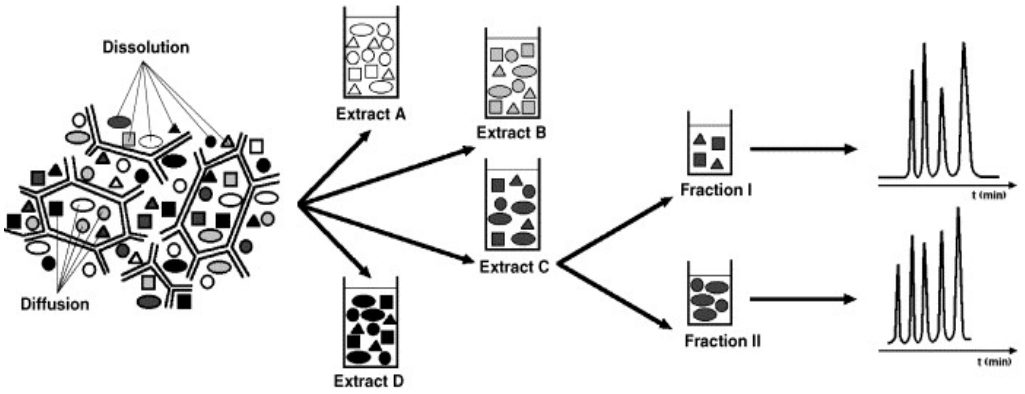

Fig. 2.3 Separation methods for the analysis and isolation of plant constituents

Source: Nyiredy, 2004

For research purposes, extraction methods can be classified according to whether the aim of the extraction is analytical or preparative. In the first case, exhaustive methods have to be used, or such methods through the repetition of which we obtain quantitative results. For the preparative isolation of plant constituents, exhaustive extraction is not essential but is obviously advantageous.

2.3.2. Methods used in natural product chemistry

Spectroscopic methods coupled with good extraction techniques like chromatography, have contributed to the phenomenal success of natural product chemistry over the past 50 years. A sound isolation strategy has helped in the isolation and characterization of many bioactive molecules. Nowadays, bioassay-guided fractionation of medicinal plants is a feature of routine in the attempt to isolate bioactive components from natural sources. These techniques are not restricted only to plant sources but they are also applied to microbial and even fungal sources of metabolites (Gurib-Fakim, 2006).

Numerous extraction techniques are available and typically produces vary distinct finished extracts. They are as the follows:

2.3.2.1. ***Cold extraction***: whereby the plant material is extracted with solvent of differing polarity at room temperature. It allows maximum extraction of most components (Gurib-Fakim, 2006).

2.3.2.2. ***Hot percolation***: the plant is heated in a solvent usually under reflux. This extraction method allows extraction of a large number of metabolites, from the most insoluble material like waxes to lipophilic natural products (Gurib-Fakim, 2006).

2.3.2.3. ***Supercritical fluid extraction***: In this extraction technique, gases, most commonly CO_2 is placed under pressure and at a temperature above its critical point while in a supercritical state where it shares properties of both a gas and a liquid. The polarity of carbon dioxide can be enhanced by adding a modifying agent such as carbon tetrachloride (Gurib-Fakim, 2006).

2.3.2.4. ***Soxhlet extraction***: Perhaps the most widely and commonly used extraction technique for the extraction of natural products. Solvents of different polarities may be applied. Although some components may be destroyed in the process, it is still the best method for natural product chemistry (Gurib-Fakim, 2006).

Once the extraction is complete, solvent is typically removed under vacuum or heat. Aqueous extracts are generally freeze-dried and stored frozen as this low temperature reduces the degradation of bioactive natural products. Extraction protocols may sometimes be modified depending on the type of molecules being extracted, e.g. sometimes acids may be added to extract alkaloids as their salts (Gurib-Fakim, 2006).

2.3.3. Isolation methods

Once the extract has been obtained, the activity within can be determined by bioassay methods using both the crude extract or fractionated extracts. Fractionation has an additional advantage of obtaining specific biologically active materials faster. One of the simplest separation methods is partitioning which is a widely used method as an initial

purification step. A combination of solvents—miscible and immiscible ones is used for separating phytochemicals making up the extract. This method relies on the ability of the components to be either soluble in water or in the organic phase (Gurib – Fakim, 2006).

2.3.3.1. Chromatography

Reverse high performance chromatographic techniques have been instrumental in the separation and characterization of natural products. These methods are very popular and widely used for the analysis and isolation of bioactive natural products. The analytical capabilities are further enhanced depending on the detector being used. UV detectors such as a photodiode array (PDA), which enables the acquisition of electronic absorption spectra of eluting peaks between has the advantage of detecting even compounds with UV and visible light absorption and further characterization by spectral on-line data. Coupling with electric library searching of compounds along with the 'fingerprinting' of biologically active extracts, HPLC-PDA has become a standard tool for initial analysis of a majority of natural products in the food pharmaceutical industry (Gurib- Fakim, 2006).

2.4. Assays used in evaluating the activity of extracts

2.4.1 Bioassay

2.4.1.1. *Requirements for screening medicinal plant materials*

Bioassays are a very critical step in assessing the biological actions of plant extracts and their ethnomedical uses. In the initial stage, *in vitro* testing have priority over *in vivo* studies involving laboratory animal models. This decision is usually based on scientific, economic and ethical grounds. *In vivo* studies may be preferable at later stages of the research project but still depends on the amount and the nature of evidence or bioactivity already collected by means of *in vitro* studies and the quest for additional information under life conditions. Bioactive components that are candidates for therapeutic application

will still have to undergo extensive clinical and toxicological screening programmes before they can be registered as medicines (Gurib – Fakim, 2006).

2.4.1.2. *Common pharmacological screening methods*

There are many types of pharmacological screens. They are specific for bacteria, fungi, protozoa, intestinal worms, viruses etc. The efficacy of compounds against health problems such as cancer and inflammation is often probed while the effect on physiological and anatomical systems such as reproduction, digestion *etc.* can be judged (Gurib-Fakim, 2006).

The Ames *Samonella*/microsome mutagenicity assay is a short term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutation. The Ames test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs (Mortelmans and Zeiger, 2000). Antioxidants reduce damage cause by free radicals which is a rodents from Phase I detoxification pathway. If lacking of antioxidants and toxin exposure is high, toxic chemicals become from more dangerous to potentially carcinogenic substances (www.brassica.com). Hasimoto *et al* (2002) reported that some dietary anticarcinogens may inhibit carcinogen activation by tipping the balance between carcinogen-activating phase I enzymes and phase II detoxifying enzymes. Food factors which are able to inhibit phase I enzymes and/or induce or activate phase II enzymes, including NAD(P)H: (quinone-acceptor) oxidoreductase (quinone reductase (NQO1) are likely to play a protective role against xenobiotic-mediated cellular damage. Thus antimuatgenic, antioxidant activity, and induction of NQO1 had been chosen for bioactivity assay toward to detoxification properties of Rang Chuet extracts.

2.4.2. Bacterial reverse mutation (Ames) assay

The bacterial reverse mutation assay evaluates a substance's genotoxicity by comparing its ability to induce reverse mutations at selected loci in several bacterial strains. The assay, commonly referred to as the Ames test, was originally developed by Dr. Bruce Ames and others in the early 1970's. It is sensitive to a wide range of mutagenic and carcinogenic chemicals (McCann and Ames, 1976; McCann, Choi, Yamasaki, and Ames, 1975; Maron and Ames, 1983). This simple, quick and inexpensive genotoxicity assay is one of several genotoxicity assays required for product safety testing of a variety of products including drugs, medical devices, food additives, industrial chemicals and pesticides. This assay measures genetic damage at the single base level in DNA by using five or more tester strains of bacteria. *Salmonella typhimurium* and *Escherichia coli* strains used in the assay each have a unique mutation that has turned off histidine biosynthesis in Salmonella or tryptophan biosynthesis in *E. coli* (Ames, McCann, and Yamasaki, 1975; Green and Muriel, 1976). Because of these original mutations, the bacteria require exogenous histidine or tryptophan to survive and will starve to death if grown without these essential nutrients (auxotrophy). The key to the assay is the bacteria can undergo a reverse mutation turning the essential gene back on, permitting the cell to grow in the absence of either histidine or tryptophan (prototrophy). Each bacterial strain was created by a specific type of mutation - either a base-pair substitution or frame-shift mutation. Because of reversion, compensating mutation must occur by the same mutagenic mechanism, mechanistic toxicology information is also available from Ames assay results based on the pattern of which strain(s) reverted.

The standard Ames assay designs include preliminary toxicology tests or combined toxicity and mutation tests followed by a definitive mutation assay. In both toxicity and mutation tests, tester strains are combined with S9 mix or buffer, test or control article, a trace of histidine or tryptophan and molten agar. The bacteria use the trace histidine or tryptophan to undergo several cell divisions, but will stop growing once

they have run out, leaving a characteristic "background lawn" that decreases in density with increasing toxicity (Fig.2.4).

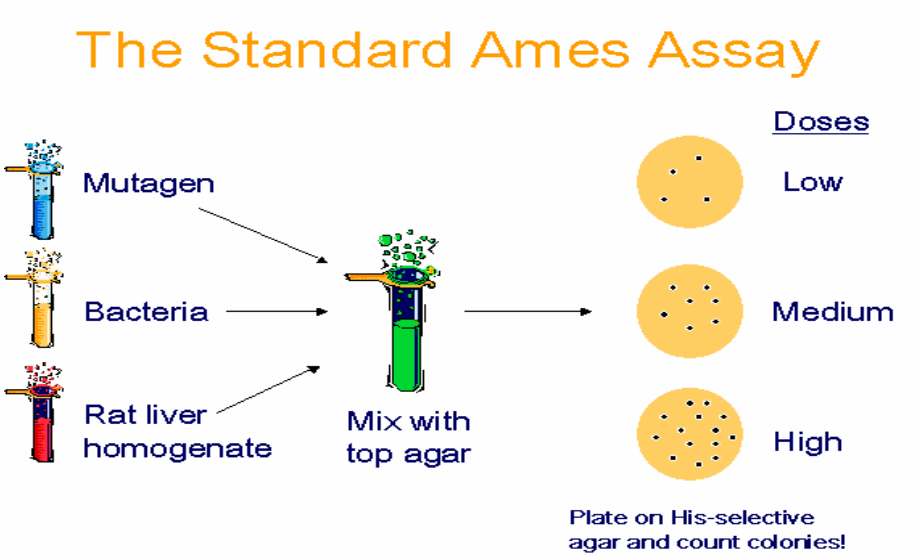

Fig. 2.4 Standard Ames Assay

Source: www.BioReliance.com

After 48 hours, only those cells that have undergone a reverse mutation turning the essential gene back on have survived, producing mutant colonies. The background lawn density is scored followed by counting the number of revertant colonies. Mutation results are reported as revertants per plate. Fig. 2.5(A) shows a vehicle control with strain TA 100 and (B) shows a positive control plate with TA 100. Each strain has a characteristic spontaneous background rate and positive control response pattern. Assay evaluation criteria for a positive response look for a dose-related increase of reverting colonies in test article treated cells over concurrent vehicle controls.

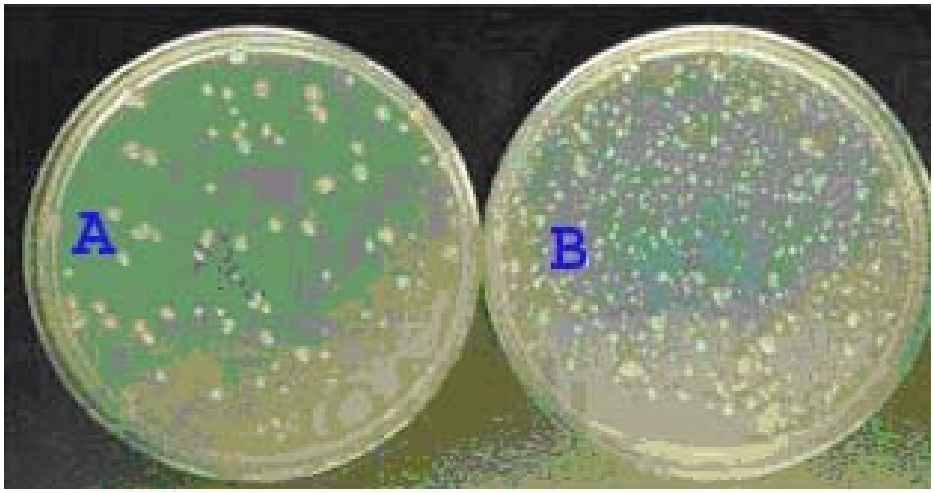

Fig. 2.5 Ames Bacterial Reverse Mutation Assay strain TA 100;

A: Vehicle Control , B: Positive Control.

Source: www.BioReliance.com

The principle of this bacterial reverse mutation test is that it detects chemicals that induce mutations which revert mutations present in the tester strains and restore the functional capability of the bacteria to synthesize an essential amino acid. The revertant bacteria are detected by their ability to grow in the absence of the amino acid required by the parent tester strain. The specificity of the tester strains can provide some useful information on the types of mutations that are induced by genotoxic agents. A very large data base of results for a wide variety of chemical structures is available for bacterial reverse mutation tests and well-established procedures have been developed for testing chemicals with different physicochemical properties, including volatile compounds (www.fda.gov).

2.4.3. Antioxidant Activity of extracts

2.4.3.1 *Chemistry of oxidation*

Thermodynamic equilibrium strongly favors the net oxidation of reduced, carbon-based biomolecules. The kinetic stability of all biological molecules in an oxygen-rich atmosphere results from the unique spin state of the unpaired electrons in ground-state molecular (triplet) oxygen in the atmosphere. This property renders atmospheric oxygen relatively inert to reduced, carbon-based biomolecules. Hence, reactions between oxygen and protein, lipids, polynucleotide, and carbohydrates proceed at vanishingly slow rates unless they are catalyzed. However, once a free radical chain reaction is limited. The free radicals generated rapidly propagate and interact directly with various targets and also yield hydroperoxides. The hyperoxides are rapidly attacked by reduced metals, leading to a host of decomposition products. Some of these products cause further damage, and some, formed through self-propagating reactions, are themselves free radicals; thus, oxidation is reinitiated (German and Dillard, 1998).

Initiators of oxidation eliminate the reactive impediments imposed by the spin restrictions of ground-state oxygen by converting stable organic molecules, RH, to free radical-containing molecules, $R^{\cdot}$. Oxygen reacts readily with such species to form the peroxy radical, $ROO^{\cdot}$. Initiators of lipid oxidation are relatively ubiquitous, primarily single-electron oxidants, and include trace metals, hydroperoxide cleavage products, and light. A risk of biological systems that use polyunsaturated fatty acids (PUFAs) is that these molecules are oxidized by the $ROO^{\cdot}$ species to yield another free radical, $R^{\cdot}$, and a lipid hydroperoxide, ROOH. This effectively sets up a self-propagting free radical chain reaction, $R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \longrightarrow ROOH + R^{\cdot}$, that can lead to the complete consumption of PUFA in a free radical chain reaction. The ability of the peroxy radical to act as an initiating, single-electron oxidant drives the destructive and self-perpetuating reaction of PUFA oxidation (German and Dillard, 1998).

2.4.3.2 ***Antioxidant control of oxidation by radicals scavenging***

The chemistry of free radical oxidations is multistage and complex. Oxidation is not a single catastrophic event. There is no single initiating oxidant that generates all free radicals; there are a great many sources of single-electron oxidants. Similarly, there is no single reactive product of oxidation; there are classes of products, many of which are both selectively and broadly damaging. Free radicals and their products react with virtually all biological molecules, and there is no single defense against all targets of oxidative damage. Thus, organisms have evolved a spectrum of mechanisms to prevent or respond to oxidative stresses and free radicals and their products at one or more of the many steps of oxidation. The potential health effects of phytochemicals as antioxidant protectors must be considered in the context of the overall response of living organisms to oxidation (German and Dillard, 1998).

2.4.3.3 ***Antioxidants***

Antioxidant is a board classification for molecules that may act prior to, or during, a free radical chain reaction at initiation, propagation, termination, decomposition, or subsequent reaction of oxidation products at sensitive targets. Antioxygenic compounds can participate in several of the protective strategies described for higher animals. Differences in point of activity are not trivial and influence the efficacy of a given compound to act as a net antioxidant or protectant. Different molecular behavior can also affect the impact of oxidation, and its inhibition, on biological function and damage. The alkyl radicals, $R^{\cdot}$, is too reactive in an oxygen-rich environment for many competing species to successfully re-reduce $R^{\cdot}$ to RH before oxygen adds to form the peroxy radical, $ROO^{\cdot}$. Antioxidant activity is not limited to prevention of hydroperoxide formation. Hydroperoxides are not damaging to foods or biological molecules, but their presence is

an indication that oxidation has occurred. Although hydroperoxides are not directly damaging, their decomposition by reduced metals generates the reactive hydroxyl radical, $HO^{\cdot}$, or the alkoxyl radical, $RO^{\cdot}$. These strongly electrophilic oxidants react with and oxidize virtually all biological macromolecules. The alkoxy radical typically fragments the parent lipid molecule and liberates electrophilic aldehydes, hydrocarbons, ketonesand alcohols. Both the highly reactive hydroxyl and alkoxy radicals and the electrophilic aldehydes liberated with their reduction react readily with polypeptides (proteins) and polynucleotides (DNA). Thus, additional antioxidant actions include preventing hydroperoxide decomposition, reducing alkoxyl radicals, or scavenging the electrophilic aldehydes. The efficacy of different antioxidants varies during this phase of the oxidation process. Even tocopherol isomers differ with respect to their ability to prevent decomposition of hydroperoxides. Plant phenolics vary in their ability to interrupt a free radical chain reaction with differences detectable among different lipid systems, oxidation initiators and other antioxygenic components (German and Dillard, 1998).

2.4.3.4 *Phytochemicals as antioxidants*

There is a growing information on usefulness of naturally occurring antioxidants. It has been proposed that diets high in natural antioxidants have been associated with reduced risk of specific chronic disease for specific populations. This is particulary true for antioxidant polyphenolics in fruits and vegetables drawing considerable attention on the actions of these products and compounds in human health (German and Dillard, 1988). This interest has produced epidemiological studies, hypotheses for mechanisms of action, and testing of oxidant/antioxidant effects in the progression of several diseases that can be classified by the aberration in oxidation balance that is believed to cause them. The breadth of associations between consumption of phenolic phytochemicals and improved human health emphasizes the need for further scientific investigation. The research group

of German (German and Dillard, 1998) has focused on candidate molecules and their absorption and mechanisms of action. The research community is now developing testable hypotheses to further assess the mechanisms of these associations (German and Dillard, 1998).

A neutraceutical is any substance that is a food, or part of a food, and provides medical or health benefits, including the prevention or treatment of disease. Nutraceuticals may be isolated nutrients, dietary supplements, specific diets, designer foods, herbal products, processed foods, or processed beverages. Recently, the ability of phenolic substances including flavonoids and phenolic acids to act as antioxidants has been extensively investigated (Siddhuraju and Becker, 2006). Phenolic compounds such as flavonoids, phenolic acid, and tannins are considered to be a major contributor to the antioxidant activity in Chinese medicinal plants. These antioxidants also possess diverse biological activities, such as anti-inflammatory, anti-carcinogenic and anti- atherosclerotic activities *in vitro*. These activities may be related to their antioxidant activity (Wong, Li, Cheng, and Chen, 2006) Antioxidant compounds present in plant extracts may therefore be multi-functional and their activity and mechanism would largely depend on the composition and conditions of the test system. Many authors had stressed the need to perform more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant action (Wong, Li, Cheng, and Chen, 2006).

Many herbs and spices, usually used to flavors dishes, are an excellent source of phenolic compounds which have been reported to show good antioxidant activity. Therefore, they may serve as natural food preservatives (Hinneburg, Damien Dorman, and Hiltunen, 2006)

2.4.3.5 *DPPH scarvenging*

DPPH· is a stable nitrogen-centre free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron-donation. Substances which are able to perform this reaction can consider as antioxidants and therefore radical scavengers (Hinneburg, Damien Dorman, and Hiltunen, 2006). A methanol solution of the sample extracts at various concentrations was added to DPPH solution. The decrease in absorbance at 515 nm was determined continuously at every minute with spectrophotometer until the reaction reached a plateau. Srida, Hankete, Khunkitti, Aromdee, and Pese (2002) reported the antioxidant activity of Rang Chuet ethanol extract at the EC_{50} varied from 20 – 99 $ug.ml^{-1}$. Also, there was some difference in the fingerprint which correlated to the antioxidant activity. However, this finding is still inconclusive, fractionation for the spot and test for the activity have to be confirmed (Srida, Hankete, Khunkitti, Aromdee, and Pese , 2002).

2.4.3.6 *Total antioxidant power of extracts by FRAP assay*

FRAP assay is a simple assay that gives fast, reproducible results. The FRAP is versatile and can be readily applied to both aqueous and alcohol extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric (III) iron to ferrous (II) iron. The standard curve was constructed using iron (II) sulfate solution (100-2000 μM), and the results were expressed as μmol Fe(II)/g dry weight of plant materials (Wong, Li, Cheng, and Chen, 2006).

2.4.4 Direct measurement of NAD(P)H: Quinone reductase (NQO1) from cells cultured in microtiter wells

Quinone reductase is a widely distributed, primarily cytosolic, discoumarol-inhibitable flavoprotein that catalyse the reduction of a wide variety of quinones and quinoneimines. Quinone reductase protects cell against the toxicity of quinones and their metabolic precursors by promoting the obligatory two-electron reduction of quinones to hydroquinones which are then susceptible to glucuronidation. In addition, quinine

reductase is induced coordinately with other electrophile-processing Phase II enzymes (glutathione S-transferase and UDP-glucuronosyltransferase) by a variety of compounds that protect rodents from the toxic, mutagenic, and neoplastic effects of carcinogens. There is a large body of evidence which suggests that the induction of Phase II enzymes is the predominant mechanism by which these heterogeneous compounds are chemoprotective and it is clear that the monitoring of Phase II enzyme induction is a convenient method for screening for anticarcinogenic activity (Prochaska and Santamaria, 1987

2.5 Cytotoxicity of extracts

Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are a chemical substance or an immune cell. Cytotoxicity can be measured by the MTT assay.

2.5.1 MTT assay

A tetrazolium salt [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyl tetrazolium bromide] has been used for a quantitative colorimetric assay of mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The MTT assay is based on the reduction of soluble yellow tetrazolium salt to a blue insoluble formazan product by mitochondrial succinate dehydrogenase activity of viable cells. After MTT addition (0.5 mg /ml), the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was removed from the wells, and the formazan product was dissolved in dimethyl sulfoxide. The result can be read on a multiwell scanning spectrophotometer (ELISA reader) with high degree of

precision. Main advantages of the colorimetric assay are rapidity, precision, and no radioisotope (Mosmann, 1983).

The term EC_{50} represents the concentration of a compound where 50% of its effect is observed. It is commonly used as a measure of drug potency. It is also related to IC_{50} which is a measure of a compound's inhibition (50% inhibition). According to the FDA, IC_{50} represents the concentration of a drug that is required for 50% inhibition *in vitro* whereas EC_{50} represents the plasma concentration required for obtaining 50% of a maximum effect *in vivo* (www.fda.gov).

Concentration measures typically follow an S-shaped curve, increasing rapidly over a relatively small change in concentration. The point at which the effectiveness slows with increasing concentration is the IC_{50}. This can be determined mathematically by derivation of the best-fit line. However, it is more easily observed from a graph and estimated rather than through complex calculus equations (www.fda.gov).

IC_{50} values are dependent on conditions under which they are measured. For example for enzymes, IC_{50} value increases as enzyme concentration increases. Furthermore depending on the type of inhibition other factors may influence IC_{50} value; for ATP dependent enzymes IC_{50} value has an interdependency with concentration of ATP, especially so if inhibition is competitive. It's commonly used as a measure of drug effectiveness (www.fda.gov).

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CHAPTER III

PHYTOCHEMICAL PROFILE AND DETOXIFICATION PROPERTIES OF THUNBERGIA LAURIFOLIA LINDL (RANG CHUET) EXTRACTS

3.1 Abstract

Thunbergia laurifolia Lindl or Rang Chuet (RC) is widely described in traditional medicine for protection against dietary and environmental toxicants with little substantiation. To better access the potential of RC as a medicinal plant, extract were prepared by infusion with water, ethanol, and acetone. Extracts were subsequently assayed for major phytochemical constituents including phenolics, carotenoids, and chlorophylls. Total phenolic content was determined to be 24.33, 5.65, and 1.42 gallic acid equivalent (GAE) per gram dry leaf water, ethanol and acetone extract respectively. HPLC analysis recognized caffeic acid and apigenin as primary constituents of water extracts. Acetone and ethanol extracts contained primarily chlorophyll *a* and *b*, pheophorbide *a*, pheophytin *a*, and lutein. The detoxification potential of RC was determined by measuring the standardized extracts to increase NAD(P)H:quinone oxidoreductase in Hepa 1c1c7 cells. Treatment with hepatoma cells with RC extracts resulted in a dose dependent increase in NQO1 specific activity for all extracts. Acetone extract (92 µg GAE/mL) increased NQO1 activity 2.8 folds, compared to control while ethanol (120 µg GAE/mL) and water (1000 µg GAE/mL) extracts increased 1.35 and 1.56 folds respectively.The RC extracts were subsequently assayed for mutagen and antimutagenic activity by bacterial reversemutagenesis assay. All three RC extracts exhibited strong dose-dependent

antimutagenic activity inhibiting 2-aminoanthracene induced matagenesis up to 87% in *Samonella typhimurium* TA 98 and TA 100. These results demonstrated that RC preparations have biological activities consistent with protection from toxicants and that both phenolic and pigment constituents likely contribute to these activities.

Key words : Phenolic acids; NQO1; Antimutagenicity; Flavonoids; RC; HPLC

3.2 Introduction

Thunbergia laurifolia Lindl (*Acanthaceae*, Thai name: Rang-Chuet), is a vine widely distributed in Southeast Asia belonging to the larger family of *Acanthaceae*. Rang Chuet is a shrub with small oblong or ovate leaves and bluish-purple flowers (Faculty of Pharmacy, 1987). It can be divided into three types designated by flower color; white, yellow, or purple. Purple varieties are believed to possess compounds that deliver beneficial pharmacological activities particularly from materials of stems, roots and leaves (Faculty of Pharmacy, 1987). Various parts of this Rang Chuet cultivar including fresh leaves, dried leaves, dried root and bark are utilized in preparation of aqueous extracts for traditional Thai medicine. These extracts are reported to have "detoxification" benefits in treatment of acute overexposure to ethyl alcohol, insecticides arsenic and strychnine (Tejasen and Thongthapp, 1979; Ruengyutthakan, 1980; Thongsaard and Marsden, 2002; Khunkitti, Taweechaisupapong, Aromdee, and Pesee, 2003; Srida, Hankete, Khunkitto, Aromdee, and Pese, 2002; Chanawirat, Toshulkao, Temcharoen, and Glinsukon, 2000). Dried Rang Chuet extracts are also widely used as anti-inflammatory and antipyretic agents (Charumanee *et al*., 1998; Thongsaard and Marsden, 2002).

While applications of Rang Chuet increase, the characterization of physiologically active components has been limited. Kanchanapoom, Kasai, and Yamasaki (2002) reported two iridoid glucosides, 8-*epi*-grandiforic and 3'-*O*- β-glucopyranosyl-

stibericoside isolated from aerial parts of Rang Chuet along with seven known compounds; benzyl β-glucopyranoside, benzyl β- 2'-*O*- β-glucopyranosyl, glucopyranoside, grandifloric acid, *E*-2-hexenyly β-glucopyeanosdie, hexanol β-glucopyranoside, 6-*C*-glucopyranosylapigenin, and 6,8-di-*C*-glucopyranosylapigenin. Both flowers and leaf materials of Rang Chuet have been found to contain other bioactive phenolic constituents including delphinidin 3:5-di-o-β-D-glucopyranoside, apigenin, apigenin-7-o-β-D-glucopyranoside and chlorogenic acid (Purnima and Gupta, 1978; Thongsaard and Marsden., 2002).

Although these findings are promising, quantities of bioactive lipophilic compounds generally associated with leaf materials, chlorophyll and carotenoids, have not been assayed. While carotenoids are well characterized as antioxidants and potential cancer preventative compounds (Bertram and Vine, 2005), chlorophyll derivatives have demonstrated bioactivity consistent with detoxification benefits attributed to Rang Chuet. Specifically, chlorophyll derivatives including chlorophyll *a* and pheophorbide *a* have demonstrated the ability to modulate xenobiotic detoxification pathways including induction of NAD(P)H:quinine oxido reductase 1 (NQO1) (Fahey, Stephenson, Dinkova-Kostova, Egner, and Kensler, 2005). Furthermore, natural chlorophylls and chlorophyllins (more water soluble derivatives) have demonstrated appreciable antimutagenic activity against complex environmental and dietary mutagens (Ferruzzi, Nguyen, Sander, Rock, and Schwartz, 2001).

Considering the broad use of Rang Chuet leaf materials in preparation of extracts and herbal products for Thai medicine, it is critical to better understand the qualitative and quantitative profiles of key bioactive phytochemical constituents. Furthermore, understanding of these phytochemical rich extracts modulating key markers of detoxification activity, induction of NQO1 and antimutagenic activity is critical to define the role of these traditional medicines that may play in disease prevention and health

promotion. The specific objectives of this work are to quantify total phenolics, carotenoids and chlorophyll derivatives present in Rang Chuet leaf extracts and to assay specific preparations for their ability to modulate *in vitro* measures of protection against dietary/environmental toxicants.

3.3 Materials and Methods

3.3.1 Plant materials

The medicinal plant *Thunbergia laurifolia* Lindl. (*Acanthaceae*) was collected from December 2005 to February 2006 from local area in Nakhon Ratchasima Province, Thailand. Leaves were air dried at 60°C for 6 h, after which they were ground in a blender (National, MX-T2GN, Taiwan) to fine powder and stored in a vacuum package at 4 °C until use.

3.3.2 Chemicals and standards

Solvents including acetone, acetonitrile, ethanol, ethyl acetate and methanol (Mallinckrodt-Baker, Phillipsburg, NJ) were of certified HPLC and ACS grades. A 1.0 M ammonium acetate buffer solution (Fluka; Ronkonkoma, NY, USA) was prepared with double distilled (dd) water and adjusted to pH 4.6 with glacial acetic acid. The following standards were obtained: chlorophyll *a*, chlorophyll *b*, lutein, phenolic acids, and apigenin. (Sigma Aldrich, St. Louis, MO).

Pheophytin *a* and *b* standards were synthesized from chlorophyll *a* and *b* as described by Ferruzzi, Nguyen, Sander, Rock, and Schwartz (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. Complete conversion to pheophytins was followed by HPLC analysis as described below. Pheophytins were extracted with 5 mL of petroleum ether, dried under a stream of nitrogen and kept at -80 ^{o}C until use. Prior to HPLC calibration, each standard was dissolved in appropriate

solvent and filtered through a 0.45 μm PTFE membrane. 2-Aminoanthracene(2-AA), the test mutagen for the bacterial reverse mutagenicity assay, was purchased from Moltox (Boone, N.C., U.S.A.).

3.3.3 Preparation of Rang Chuet extracts

Approximately 100 mg leaf powder was extracted with three 12 mL portions of boiling water, ethanol, or acetone in a shaking water bath at 25^{o}C for 15 min. Centrifugation at 3000 g (Thermo IEC, Waltham, MA) was applied for 3 min between extraction. The filtrates obtained from vacuum filtration were combined and the volume was adjusted to 50 mL. Aliquots of 2 mL were transferred to culture test tubes (VWR, Wilmington, NC) and dried under vacuum (Rapid Vap® Vacuum Evaporation Systems, Labconco Corporation, Kansas City, MO). Samples were stored frozen at -20^{o}C until use.

3.3.4 Instrumentation and chromatography

Chlorophyll and carotenoid analyses were achieved as described by Ferruzzi, Nguyen, Sander, Rock, and Schwartz (2001) with modification. Briefly, a Hewlett-Packard 1090A system equipped with a diode array detector was utilized. Separation was achieved using a Grace-Vydac 201TP54 reversed-phase (4.6 x 250mm) polymeric C18 column with a guard column containing the same stationary phase (Grace Vydac, Apple Valley, MN). A gradient elution profile was based on a binary mobile phase system consisting of methanol:water:ammonium acetate (73:23:2 v/v/v) in reservoir A and ethyl acetate in reservoir B. A flow rate of 1.0 mL/min was applied with initial setting at 100% A with a linear gradient to 50:50 A/B over 10 min. The gradient was held for 10 min followed by a 5 min linear gradient back to 100% A and equilibrated at the initial condition for 5 min for a total run time of 30 min. Detection and tentative identification of all chlorophylls and carotenoid were accomplished using in-line diode array data between

250 and 600 nm. Quantification of chlorophylls and carotenoid was accomplished using multilevel response curves constructed with authentic chlorophyll and carotenoid standards.

Analysis of phenolic compounds were achieved as described by Ferruzzi and Green (2005) with modification. The analysis was performed using a Waters model 2695 HPLC system equipped with a model 2996 photodiode array detector. A Waters NovaPak C18 (3.8 mm i.d.× 150 mm) reversed phase (RP) column (Milford, MA) with a guard column also packed with Waters NovaPak C18. Phenolic compounds were separated by gradient elution with a flow rate of 1.0 mL/min at 35^{o}C using a binary mobile phase of water/acetic acid (98:2, v/v) in reservoir A and acetonitrile in reservoir B. Initial solvent proportion of 99:1 A/B with a linear gradient to 70:30 A/B over 20 min was used, followed by a 5 min linear gradient back to 99:1 A/B and 5 min equilibration at the initial condition for a total chromatographic run time of 30 min. Detection and tentative identification of major Rang Chuet phenolic compounds was accomplished using in-line PDA data between 200 and 500 nm. Calibrations plots for quantification were constructed from injection of authentic standards of caffeic acids and apigenin.

3.3.5 Total phenolics

Total soluble phenolic constituents of the extracts (water, ethanol, and acetone) were determined employing the method of Waterhouse (2002), involving Folin-Ciocalteu reagent and gallic acid as the standard. Twenty µl of freshly prepared RC extract was added into a 1.5 mL cuvette, after that 1.58 mL water was added followed with 100 µl Folin-Ciocalteu reagent before thoroughly mixed and incubated for 5 min. 300 µl of the $NaCO_3$ (2% w/v) solution was added and the mixture was allowed to stand at room temperature for 2 h. Absorbance was measured at 765 nm and expressed as gallic acid equivalents.

3.3.6 Antimutagenic assay

The microscreen method of Diehl, Wilaby, and Snyder (2000) was employing with modifications as described by Ferruzzi, Bohm, Courtney, and Schwartz (2002). *Salmonella typhimurium* TA98 and TA100 (Moltox; Boone, N.C., USA) were utilized as the test strain. Six-well plates pre-poured with 5 mL of Vogel-Bonner minimal glucose agar per well and top agar (2%) were purchased from Moltox (Boone, N.C., U.S.A.). Bacterial stock cultures were inoculated into 10 mL of Oxoid#2 nutrient broth and incubated at 37 ^{o}C for 6 h until optical density at 600 nm of 0.8 was achieved. Mammalian microsomal activation system (S-9 mix) was prepared by diluting Aroclor 1254-induced rat liver microsomes (Moltox; Boone, N.C., U.S.A.) into 1.0 molL^{-1} glucose-6-phosphate-NADP solution at 4% (v/v). 2-Aminoanthracene (AA) stock solution (100 μg/mL) was prepared in sterile dimethyl sulfoxide (DMSO) 99.8%. Individual Rang Chuet extract was dissolved in DMSO, sterilized by filtration and diluted to concentrations between 10 to 400 μg GAE/mL for incorporation into mutagenicity assay.

The assays were conducted in two different incorporations of extracts, during and after metabolic activation. Microscreen assays were plated by incorporation of 25 μL of 2 AA (0.625 μg), 100 μL of S-9 mix, 25 μL of bacterial culture, and 25 μL of test extracts (incorporated during metabolic activation) into a 2 mL vial. Samples were pre-incubated for 20 min at 37 ^{o}C, then combined with 500 μL of molten top agar, mixed and poured onto the surface of wells. Upon hardening of the top agar, plates were incubated for 48 h at 37 ^{o}C after which his^{+} revertant colonies were counted. Spontaneous mutations were determined by plating controls containing DMSO in place of 2-AA and Rang Chuet extracts. Antimutagenic activity of each extract was determined from the ability of each extract in reducing the number of his^{+} revertants caused by 2-AA corrected for spontaneous background revertants.

3.3.7 Quinone reductase (NQO1) assay

The NQO1 assay was conducted as described by Bomser, Madhavi, Singletary, and Smith (1996). Hepa 1c1c7 cells (ATCC CRL 2026) were grown and maintained as described by following. Cells were seeded at a density of 10,000 cells/well. RC extracts were dissolved in ethanol and diluted in basal media to concentrations between 0.07 and 150 μg/mL. The NQO1-inducing activity of all fractions was normalized based on the total phenolics. The concentration of the fractions, expressed as μg GAE/mL required to significantly inducing NQO1-specific activity relative to controls, was used as an indicator for induction potency.

Cells were initially seeded in 96 well dishes, and treated for 48 h with control (basal media) and test media containing dried RC extract dissolved in ethanol and diluted to concentration with basal media. At appropriate times, cellular NQO1 activity was measured as previously described by Prochaska and Santamaria (1988). After the plates were exposed to inducers for 48 hrs, the media from one set of plates were decanted and the cells were lysed by agitating the plates on an orbital shaker in the presence of 50 μL/well of 0.8% digitonin and 2 mM EDTA, pH 7.8 for 10 min. Before the plates were assayed for NQO1 activity, a cocktail containing 25 mM Tris·Cl (pH 7.4), 0.67 mg/mL bovine serum albumin, 0.01% Tween-20, 5 μM FAD, 30 μM $NADP^+$, 1 mM glucose 6-phosphate, 2 U/mL Bakers yeast glucose-6-phosphate dehydrogenase, and 0.3 mg/mL MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide was prepared for all plates to be assayed (25 mL/plates). Shortly before each plate was scanned, 25 μL of 50 mM menadione dissolved in acetronitrile was mixed with 25 mL of assay cocktail. After 200 μL/well of the resulting solution was added to the plates, the plates were placed in an UVmax microtiter plate scanner (Molecular Devices, Menlo Park, CA USA), and the rate of formation of the formazan dye was quantitated at 610 nm for two min. The dicoumarol-inhibitable rate of MTT reduction was measured by rescanning the plates after the addition

of 50 µL/well of 0.3 mM dicoumarol dissolved in 0.5% DMSO and 5 mM potassium phosphate (pH 7.4).

3.3.8 Statistical analyses

Descriptive statistics including mean and standard error of mean (SEM) were calculated for phytochemical constituents of Rang Chuet extracts (n=3). NQO1 induction data were expressed as fold-induction of NQO1 specific activity (n=8) in treated versus control cells receiving no RC treatment. The reduction of total his^+ revertants relative to 2AA controls, corrected for spontaneous mutations, is defined as % Inhibition. Descriptive statistics including mean and standard error of mean (SEM) were calculated for each extract fold induction of NQO1 and % inhibition. Group differences were determined by analysis of variance with Tukey-Kramer post-hoc test ($\alpha < 0.05$).

3.4 Results and Discussion

3.4.1 Chlorophylls and carotenoid profile of RC extracts

Resolution of seven chlorophyll derivatives and lutein, one major carotenoid, was achieved from RC acetone and ethanol extracts by a RP HPLC (Fig. 3.1). Chlorophyll and carotenoid pigments were not detected in hot water extracts of RC. Lutein and major chlorophyll derivatives including, chlorophyll *a*, chlorophyll *b*, pheophytin *a* and *b* were identified based on UV-vis spectra and co-chromatography with authentic standards. Due to the lack of an authentic standard for pheophorbide *a*, this derivative was tentatively identified by comparison of online spectra and previous separation of water soluble chlorophyll derivatives by this method (Canjura and Schwartz, 1991).

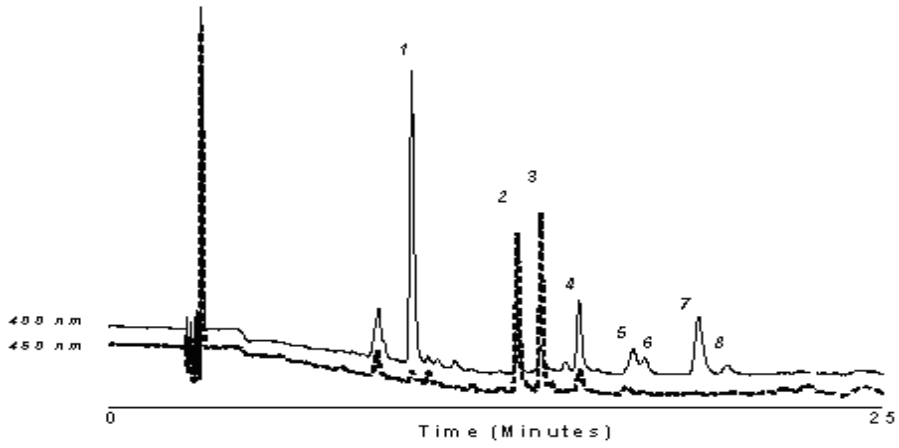


Fig. 3.1 HPLC separation of RC lutein and chlorophyll derivatives in acetone extract detected at 400 and 450 nm: (1) Pheophorbide *a*; (2) Lutein; (3) Chlorophyll *b*; (4) Chlorophyll *a*; (5) Pheophytin *b*; (6) Pheophytin *b′*; (7) Pheophytin *a*; and (8) Pheophytin *a′*

Total chlorophyll contents were determined to be 5.5 and 6.0 μg/mL for ethanol and acetone extracts, respectively (Table 3.1). Water, ethanol, and acetone extracts gave yield percentage at 0.3, 0.2, and 0.4 weight by weight respectively. Of the chlorophyll derivatives found in RC extracts, pheophytin *a* (1.5 and 1.6 μg/mL) and pheophorbide *a* (1.1 and 2.0 μg/mL) were most abundant. Chlorophyll *a*, chlorophyll *b*, and pheophytin *a* were found to be 0.5, 1.3, 1.5 and 0.7, 1.4, 1.6 μg/mL for ethanol and acetone extract, respectively. Only pheophytin *a′* was identified as the pheophytin epimer in ethanol and acetone extracts at 1.1 and 1.0 μg/mL respectively. Lutein content of ethanol and acetone RC extracts was determined to be 0.02 μg/mL and 0.04 μg/mL for ethanol and acetone extracts, respectively.

Table 3.1 Yield and phytochemical profile of RC extracts.

	Extraction Solvent		
	Water	**Ethanol**	**Acetone**
%Yield (wt/wt x100)	32.6	23.2	36.6
Phytochemical Contents			
Lutein (μg/mL)	ND	0.02±0.0	0.04±0.0
Chlorophyll *a* (μg/mL)	ND	0.5±0.0	0.6±0.1
Chlorophyll *b* (μg/mL)	ND	1.3±0.0	1.4±0.1
Pheophytin *a* (μg/mL)	ND	1.5±0.0	1.6±0.1
Pheophytin *a′* (μg/mL)	ND	1.1±0.1	1.0±0.0
Pheophorbide *a* (μg/mL)	ND	1.1±0.0	2.0±0.2
TOTAL Chlorophylls (μg/mL)	ND	5.5±0.1	6.0±2.7

These results indicate that for ethanol and acetone RC extracts chlorophyll derivatives are present at significant levels. Furthermore, the presence of significant amounts of chlorophyll degradation products (pheophytins and pheophorbides) is likely a result of the post harvest handling of leaves prior to extract preparation. Hot air drying and milling of the RC leaves during raw material preparation, similar to the method generally used by local people, would expose leaf materials to conditions favorable to degradation of chlorophylls.

3.4.2 Phenolics content and phenolics profile of RC extracts

All extracts contained appreciable levels of phenolic compounds as assessed by the Folin-Cicalteau method. Water extracts of RC had the highest total phenolics with 2433.9 mg GAE/100 g followed by ethanol and acetone RC at 565.1 and 142.1 mg GAE /100g, respectively.

HPLC separation of the phenolic RC extracts was recognized to contain several phenolic acids and flavonoid including caffeic acid, gallic acid, protocatechuic acid and apigenin and an apigenin glucoside. The separation profile from water extract was illustrated in Fig. 3.2. Caffeic acid and apigenin including apigenin glucoside were identified by co-chromatography with authentic standards and quantified to be 142.1, 38.8,

55.4 mg/100 g and 2413.7, 140.5, 120.9 mg/100 g in water, ethanol, and acetone extracts, respectively. In addition, gallic acid in ethanol extract was not detectable and protochuic acid in water and acetone extract was 0.2, and 0.1 mg/100 g, respectively.

Both flowers and leaf materials of Rang Chuet have been found to contain other bioactive phenolic constituents including delphinidin 3:5-di-o-β-D-glucopyranoside, apigenin and apigenin-7-o-β-D-glucopyranoside, and chlorogenic acid (Purnima and Gupta, 1978; Kanchanapoom, Kasai, and Yamasaki, 2002). This phytochemical profile confirms the presence of apigenin and apigenin glucosides in the RC leaves. Moreover, findings in this study further can identify other phenolic acids in the leaves i.e. caffeic acid, gallic acid and protochuic acid. Differences in phenolic profiles are due to variation of plant cultivars and environmental conditions of growth.

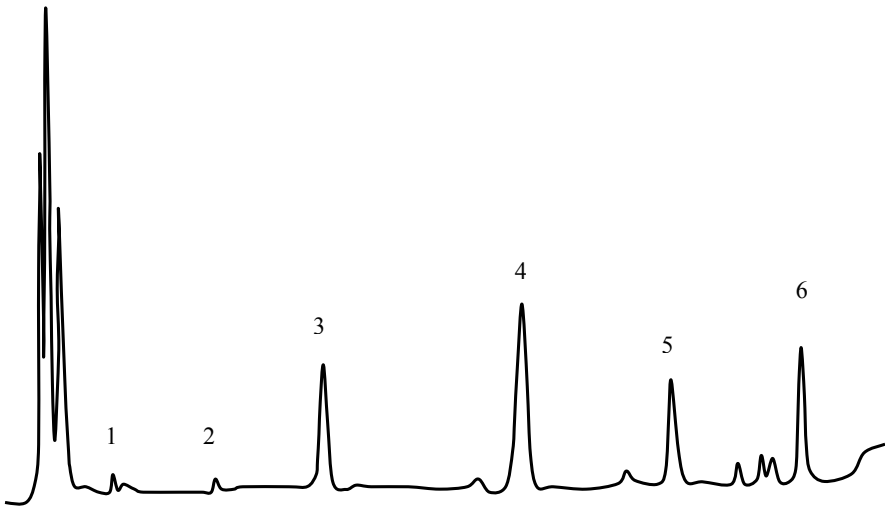


Fig. 3.2 HPLC separation of RC phenolic derivatives in water extract detected at 240 nm: (1)protocatechuic acid; (2) gallic acid (3) Caffeic acid; (4) unknown phenolic; (5) Apigenin-7-glucoside; and (6) Apigenin

Table 3.2. Polyphenol profile and total phenolics of RC extracts.

Phytochemical contents	Extraction Solvent		
	Water	**Ethanol**	**Acetone**
[1,2]Apigenin (mg/100g)	2413.7±27.9	140.5±19.4	120.9±14.2
[1]Caffeic Acid (mg/100 g)	142.1±4.7	38.8±2.1	55.4±3.8
Gallic acid (mg/100 g)	0.0006±0.0	ND	ND
Protocatechuic acid (mg/100 g)	ND	0.05±0.004	0.15±0.001
[3]TOTAL Phenolics (mgGAE/100g)	2433.9±57.7	565.1±7.9	142.1±0.8

Note: [1]Determined by LC as described in Materials and Methods.

[2]Indicates apigenin plus apigenin-7-glucoside

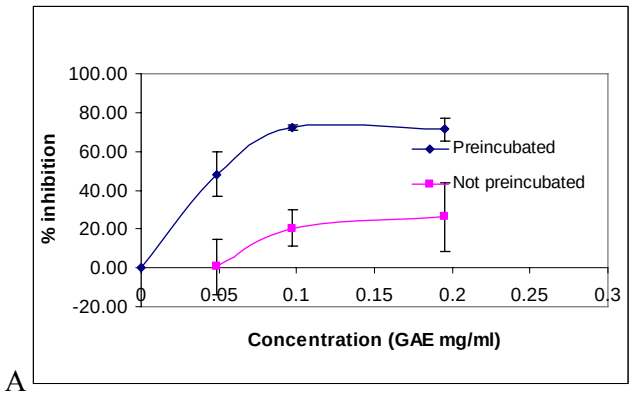
[3]Determined by Folic-Cicalteau Method as described by (Waterhouse, 2002)

3.4.3 Antimutagenic activity of RC extracts

Rang Chuet extracts were screened to inhibit the mutagenicity of 2-AA in a *Samonella typhimurium* TA 98 and TA100 microscreen assay. Extracts were dissolved in sterile DMSO at concentrations between 0.01-0.40 mg GAE/mL. RC extracts were either mixed with 2AA and s-9 fraction (pre-incubated), or added just prior to combination (not pre-incubated) with top agar and plated on 5 mL of Vogel-Bonner minimal glucose agar in 6 well plates. Plates were incubated for 48 h at 37 ^{o}C after which his^{+} revertant colonies were counted. Assays were conducted with 0.625 ug/well of 2-amino-anthracene providing 285 ± 4.3 his^{+} revertants per well corrected for spontaneous background revertants.

All Rang Chuet extracts demonstrated pronounced dose-dependent inhibition of the mutagenic activity of 2-AA (Fig. 3.3 and Fig. 3.4). Acetone extracts of Rang Chuet were found to inhibit a maximum of 81.1 ± 12.2 % of 2-AA induced mutagenicity at its highest experimental concentration of 0.17 mg GAE/mL (0.004 mg GAE per well) in Fig. 3.3 and 47.2 ± 12.2% in Fig. 3.4 while ethanol and water extracts were found to inhibit 86.6 ± 9.8

% and 71.2 ± 5.3 % in Fig. 3.3, 57.7 ± 11.5 and 60.7 ± 10.8 in Fig. 3.4 at concentrations of 0.15 and 0.2 mg GAE/mL (0.004 and 0.005mg GAE per well), respectively. Interestingly, RC water extract was found to be cytotoxic to *Samonella typhimurium* TA 98 and TA100 observed as thinning of background lawn accompanied by a decrease in the total number of colonies but an increase in size of reverting colonies (data not shown). Several phytochemical constituents identified in this study are known to be antimutagenic agents. Chlorophyll derivatives abundant in both ethanol and acetone extracts have previously demonstrated powerful antimutagenic activity in bacterial mutagenicity assays (Ferruzzi, Bohm, Courtney, and Schwartz, 2002). Furthermore, phenolic constituents including caffeic acid have been reported to have antimutagenic activity *in vivo* (Moridani, Scobie, and Brien, 2002).



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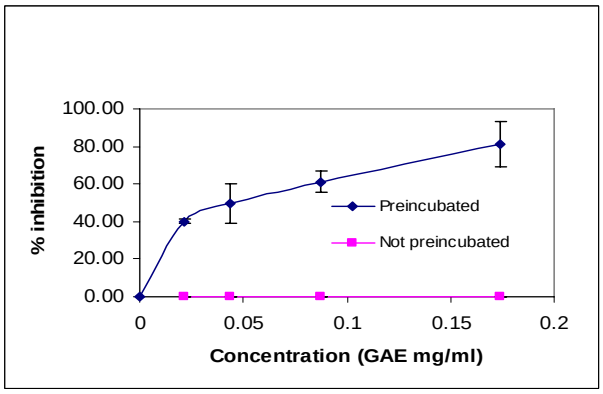

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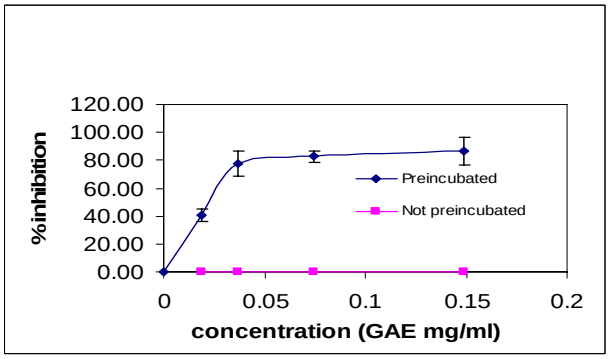

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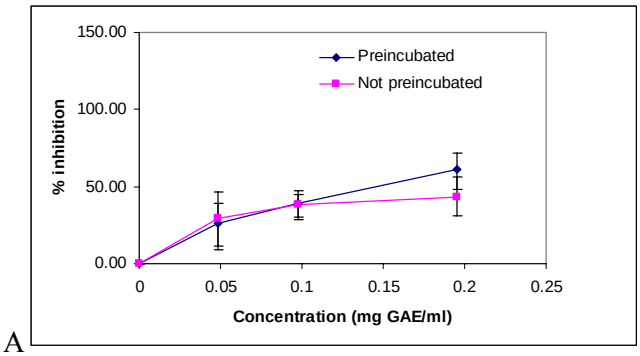
Fig. 3.3 Inhibition of 2-Amino-anthracence induced mutagenesis in *Samonella typhimurium* TA 98 by water extract (panel A); acetone extract (panel B); ethanol extract (panel C).



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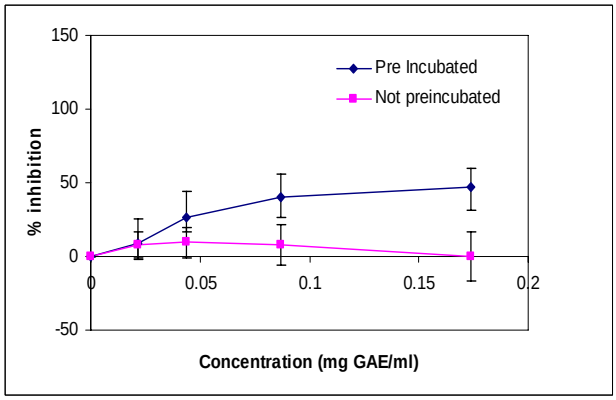

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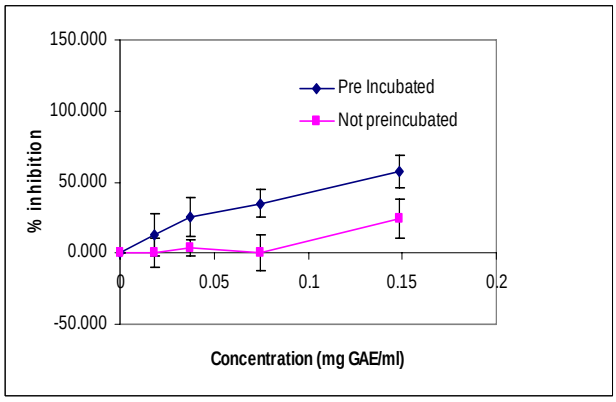

C

Fig. 3.4 Inhibition of 2-Amino-anthracence induced mutagenesis in *Samonella typhimurium* TA 100 by water extract (panel A); acetone extract (panel B); ethanol extract (panel C).

Addition of RC extracts after metabolic activation of 2 AA (non pre-incubated experiment) did not appear to modulate mutagenic activity of 2AA (Fig.3.3). For RC ethanol and acetone extracts, no change in mutagenic activity of 2AA was observed in the treatment without pre-incubation. For water extract, decreasing in mutagenic activity was noted and attributed to partial killing of *Samonella typhimurium* TA 98 and TA100. 2AA is an indirect mutagen requiring metabolic activation by Phase 1 cytochrome P450 enzymes presenting in the microsomal S-9 fraction. Observed differences in antimutagenic activity of RC extracts incubated together with 2AA and S-9 during metabolic activation and RC extracts added after cells incubated with 2AA and S-9 raise the possibility that RC extracts may inhibit transformation of 2AA and/or specific components presenting in RC extracts which require metabolic activation by Phase 1 enzymes in order to provide protective effects.

Ethanol extract showed antimutagenicity with *Samonella typhimurium* TA 98 and TA 100 (Fig. 3.5). Moreover, it should be noted that water extract showed partial killing effect (Fig. 3.6) from which colonies were quite big with clear background lawn. This might be due to cytotoxicity of water extract (Maron and Ames, 1983).

Potent carcinogens were used to induce cancer, and compounds being able to inhibit this chemically induced cancer were called ‘anticarcinogens’. Plant phenols may interfere with all stages of the cancer process, potentially resulting in a reduction of cancer risk (Hollman, 2001).

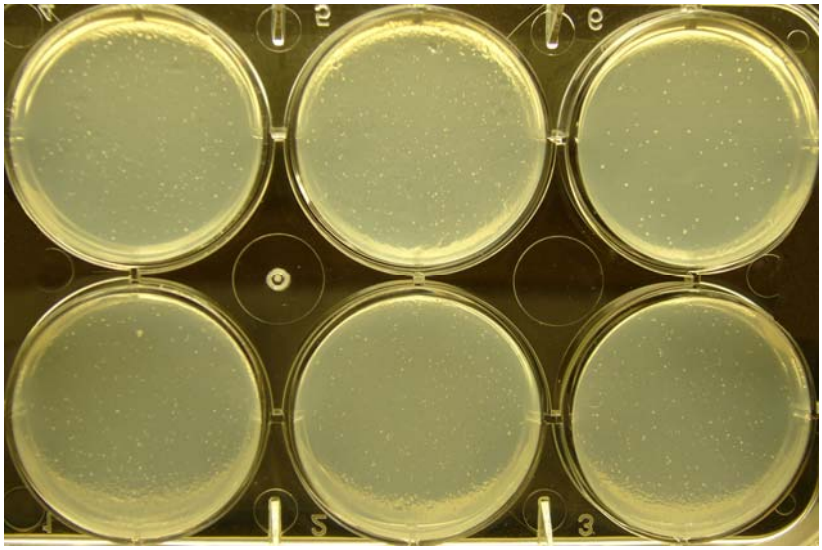

Fig. 3.5 Antimutagenicity of ethanol extract at concentration from 0.02 to 0.15 mg GAE/mL.

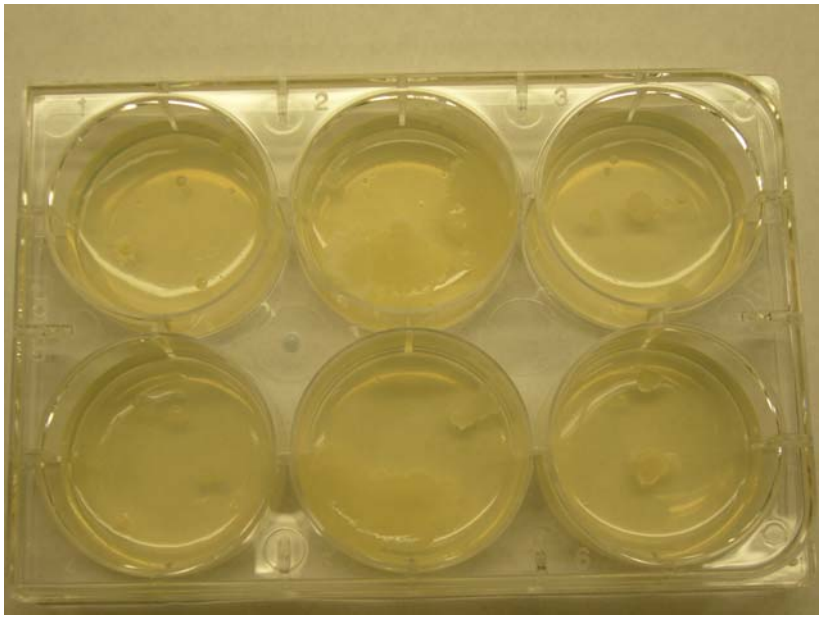

Fig. 3.6 Antimutagenicity of water extract at concentration from 0.02 to 0.20 mg GAE/mL.

Kanchanapoom, Kasai, and Yamasaki (2002) found that the constituents in this plant were iridoid glucosides. In these experiments, the main constituents identified were chlorophylls, chlorophyll derivatives, lutein, phenolic acids (gallic acid and caffeic acid), and some flavonoids (from spectroscopic data) (Mabry, Markham, and Thomas, 1970). Caffeic acid has been reported to exhibit anti-inflammatory, anticarcinogenic, and antimutagenic activities (Moridani, Scobie, and Brien, 2002). Flavonoids were reported to have antimutagenic effects by Edenharder, Petersdorff, and Rauscher(1993) with 21 flavones and 16 flavonols possessing the highest antimutagenic potencies.

These results indicate that for ethanol and acetone RC extracts chlorophyll derivatives are present at significant levels. Furthermore, the presence of significant amounts of chlorophyll degradation products (pheophytins and pheophorbides) is likely a result of the post harvest handling of leaves prior to extract preparation (Martile and Hortensteiner, 1999). Hot air drying and milling of the RC leaves during raw material preparation, similar to the method generally used by local people, would expose leaf materials to conditions favorable to degradation of chlorophylls. Chlorophylls have been recognized as chemopreventive properties more recently. Also chlorophylls exhibit antimutagenic activity in short-term genotoxicity assays, and protect against various intermediate biomarkers of cancer *in vivo*. The anticarcinogenic activity of sodium-copper chlorophyllin (CHL), a clinically-used water-soluble salt of chlorophylls, has been studied in several species. *In vivo* mechanism studies have indicated that inhibition is most effective when CHL is administered simultaneously with the carcinogen because of molecular complex formation between CHL and the carcinogen (Dashwood, 1997).

3.4.4 RC induction of NAD(P)H:quinine oxido reductase 1 (NQO1)

The ability of RC extracts to modulate NQO1 specific activity was investigated in cell culture. Dried extracts were solubilized in ethanol, diluted in basal media and applied

to 80-90% confluent cultures of Hepa 1c1c7 cells. NQO1 was assessed after 48 h of treatment with Rang Chuet extracts. Results are expressed as concentration of RC extracts required to increase inductive capacity, compared with vehicle controls.

Treatments of hepatoma cells with RC extracts resulted in increasing NQO1 specific activity for all extracts (Fig. 3.7). Acetone extract showed the highest induction potency followed by ethanol and water extracts, respectively. Acetone extract at its highest concentration (140 μg GAE/mL) increased NQO1 activity by 2.8 fold compared with controls, while ethanol (120 μg GAE/mL) and water (1000 μg GAE/mL) extracts increased NQO1 activity by a maximum of 1.4 and 1.6 fold, respectively. As with antimutagenicity assays, Rang Chuet water extracts exhibited cytotoxic activity above 1000 μg GAE/mL.

Compounds that increase specific NQO1 activity may also act as inducers of glutathione-*s*-transferase and thus may be an indirect assessment of more general phase II enzyme activity consistent with xenobiotic detoxification pathways (Hashimoto, Kawamata, Usui, Tanaka, and Uda, 2002). Chlorophyll rich acetone extract appear to be superior to both water and ethanol extracts with regards to increasing specific activity of NQO1 in hepa1c1c7. More recently, Fahey *et al* (2005) demonstrated that chlorophyll derivatives can be potent inducers of NQO1 in murine hepatoma cells. The chlorophyll content of acetone and ethanol extracts would explain, in part, the observed activity of these extracts. Although water extract showed minimal increase in specific activity, cytotoxicity of the water extract was apparent in the reduced cell densities determined by crystal violet staining (data not shown). This would in fact limit observable induction of NQO1 in water extract and point to potential negative effects of this extract form.

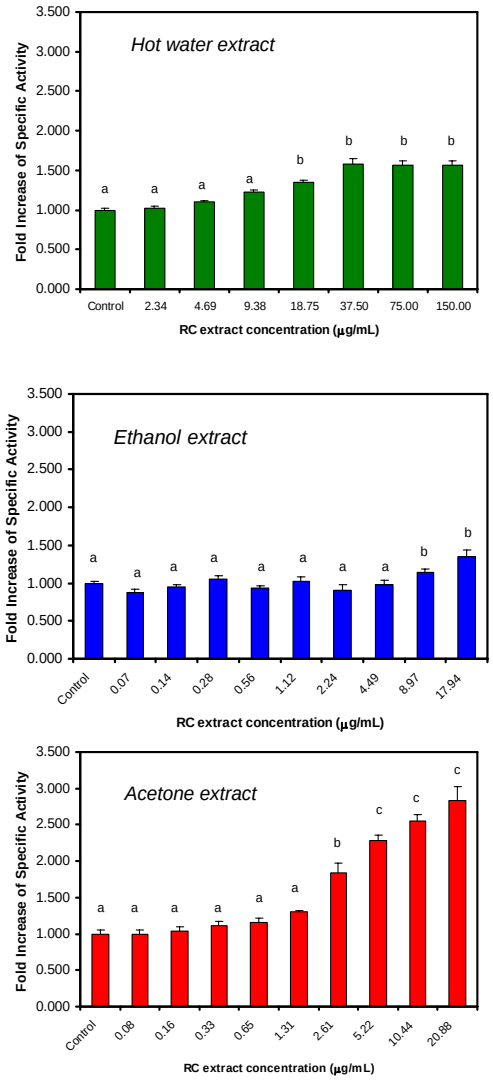

Fig. 3.7 Increases in specific activity of NQO1 in Hepa1c1c7 cells treated with RC extracts.

3.5 Conclusion

Rang Chuet extracts produced from common solvents (hot water, acetone and ethanol) were analyzed for major phytochemicals. Acetone and ethanol extracts of Rang Chuet were found to be good sources of chlorophyll derivatives particularly in form of water soluble pheophorbide *a*. Rang Chuet water extract has highest content of total phenolic acids. Chlorophyll rich acetone and ethanol extracts demonstrated significant antimutagenic activity by inhibiting the 2-AA induced mutagenicity in a *Samonella typhimurium* model. Furthermore, Rang Chuet extracts demonstrated the ability to induced phase II xenobiotic detoxification enzyme quinine reductase (NQO1) activity in cultured Hepa 1c1c7 cells. These results combined with those of Pramyothin, Chierdchupunsare, Runsipipat, and Chaichantipyuth (2005) reporting hepatoprotective activity of Rang Chuet aqueous extract and Fahey *et al* (2005) demonstrated the ability of chlorophyll derivatives to induce phase II enzyme (NAD(PH: quinine oxidoreductase 1 (NQ01), support the notion that chlorophyll rich extracts of Rang Chuet may provide protective activity. While only preliminary and not completely extendable to in vivo conditions, these results demonstrate that Rang Chuet preparations have biological activities consistent with protection against dietary and environmental toxicants and that both phenolic acids and natural chlorophyll constituents likely to contribute to these activities.

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CHAPTER IV

ANTIOXIDANT ACTIVITY OF RANG CHUET EXTRACTS

4.1 Abstract

Antioxidant activities and total phenolic content of Rang Chuet extracts were evaluated using free radical scavenging, ferric reducing antioxidant power assay (FRAP), and the Folin-Ciocalteu method. Rang Chuet was extracted with water, ethanol, and acetone. The extraction efficiency among these methods were compared and found that water extraction of phenolic compounds was the most efficient (2433.9 mg GAE/100g) compared to ethanol and acetone extraction which had phenolic contents 565 and 142.1 mg GAE /100g, respectively. Rang Chuet water extract possessed the highest antioxidant activities using free radical scavenging at the EC_{50} values of 0.13 mg GAE/mL while as ethanol and acetone extract showed EC_{50} at 0.26 and 0.61 mg GAE/mL, respectively. The EC_{50} value of water extract less than ascorbic acid (0.10 mg GAE/mL). Relating, the water extract also showed the highest total antioxidant activity using FRAP assay at 0.93 mmol/g following by ethanol and acetone extract (0.18, 0.04 mmol/g) while BHT, ascorbic acid, and trolox at 1.42, 119.5, 7.2 mmol/g respectively.

Free radical reactions occur in the human body and food systems (Wong, Li, Cheng, and Chen, 2006). Free radicals, in the form of reactive oxygen and nitrogen species, are an integral part of normal physiology. An over-production of these reactive species can occur, due to oxidative stress brought about by the imbalance of the bodily

antioxidant defense system and free radical formation. The reactive species can react with micromolecules, causing cellular injury and death. This is believed to contribute to the etiology of several chronic disease states including cancers as well as cardio- and cerebro-vascular systems (Wong, Li, Cheng, and Chen, 2006). Dietary antioxidants are believed to play a protective role by augmenting cellular defenses and preventing oxidative damage to cellular components (Wong, Li, Cheng, and Chen, 2006).

Reactive oxygen species (ROS), such as superoxide radical ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and peroxyl radicals ($ROO^{\cdot}$), are produced as a part of normal metabolic processes. The oxidative damages caused by ROS on lipids, proteins and nucleic acids may trigger various chronic diseases, such as coronary heart diseases, atherosclerosis, cancer and aging. The health-promoting effect of antioxidants from plants is thought to arise from their protective effects by counteracting specific ROS forms. In view of these potential health benefits, many intensive research projects have been conducted on natural antioxidants derived from plants (Wong, Li, Cheng, and Chen, 2006).

Besides playing an important role in physiological systems, antioxidants have been used in the food industry to prolong the shelf life of food, especially those rich in polyunsaturated fats which are readily oxidized by molecular oxygen causing quality deterioration, nutritional losses, off-flavors development and discolouration (Wong, Leong, and William, 2006). Synthetic antioxidants have been used industrially to control lipid oxidation in foods. However, their uses have been questioned due to their potential health risks and toxicity (Wong, Leong, and William, 2006). The search for antioxidants from natural sources has received much attention and efforts have been put into identify compounds that can act as suitable antioxidants to replace synthetic ones. In addition, these naturally-occurring antioxidants can be formulated as supplements and nutraceuticals to aid in prevention of oxidative damage occurring in the body. Antioxidant compounds in plant extracts are therefore multi-functional and their activity

and mechanism would largely depend on the composition and conditions of the test system (Wong, Leong, and William, 2006).

In this study, the free radical scavenging activities of the Rang Chuet plant extracts were followed via their reaction with the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical and their ferric ions reducing antioxidant activity potential (FRAP) assay. Results will provide a better understanding of the antioxidant properties of this plant and allow further evaluation of the role this medicinal plant may play in promotion of health and elucidate potential applications and development opportunities for value-added foods and neutraceuticals.

4.3 Materials and Methods

4.3.1 Plant materials

The Rang Chuet, *Thunbergia laurifolia* Lidnl. (*Acanthaceae*) used in this experiment was collected in December 2005 - Febuary 2006 from local area in Nakhon Ratchasima Province, Thailand. Leaves were air dried at 60^{o}C for 6 h, after which they were ground in a blender (National, MX-T2GN, Taiwan) to fine powder and stored in vacuum packages at 4^{o}C until use. Leaf powder like this is a typical raw material used for manufacture of extracts and/or herbal tea products from Rang Chuet.

4.3.2 Chemicals

All chemicals of analytical grade including: 1,1-diphenyl-2-picryhydrazyl free radical (DPPH), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), Ferric, chloride-6-hydrate, Ferrous, sulfate 7-hydrate, acetate buffer pH 4.6, Gallic acid, Folin-Ciocalteu phenol reagent, and anhydrous sodium carbonate (Na_2CO_3) were obtained from Sigma-Aldrich Co. (St. Louis, USA). Solvents including acetone, ethanol, hydrochloric acid, and methanol were purchased from Mallinckrodt-Baker (Phillipsburg, NJ).

4.3.3 Extraction

Leaf powder of about 100 mg was extracted with three 12 mL portions of a boiling water (100^{o}C), ethanol, and acetone in a shaking water bath at 25^{o}C for 15 min. Centrifugation at 3000 g (Thermo IEC, Waltham, MA) was applied for 3 min between extraction three times. The filtrates obtained after vacuum filtration were combined and the volumes were adjusted to 50 mL with the same solvent. Aliquots of 2 mL were transferred to culture test tubes (VWR, Wilmington, NC) and dried in a vacuum dryer (Rapid Vap® Vacuum Evaporation Systems, Labconco corporation, Kansas city, MO). Samples were stored at -20^{o}C until use.

4.3.4. Assaying methods

4.3.4.1 Determination of total phenolics

Total soluble phenolic constituents of the extracts (water, ethanol, and acetone) were determined with Folin-Ciocalteu reagent and gallic acid as standard (Waterhouse, 2002). Extracts were dissolved in same solvent (water dissolved in 150 µl, ethanol and acetone dissolved in 75 µl) then extracts were mixed thoroughly. 20 µl of the crude extract solution, standard or blank was added into a 1.5 mL plastic cuvette, after that 1.58 mL water was added followed by 100 µl Folin-Ciocalteu reagent. Then the mixture was inversely and thoroughly mixed and incubated for 5 min. After that, 300 µl of the $NaCO_3$ (2% w/v) solution was added and mixed before the mixture was allowed to stand at room temperature for 2 h. Absorbance was measured at 765 nm. Gallic acid solution (0-1000 µg/0.1 mL) was used as a standard.

4.3.4.2 DPPH free radical scavenging assay

The DPPH free radical scavenging activity of Rang Chuet extracts (acetone,

ethanol, and water), BHT, and Ascorbic acid were determined using DU 800 Spectrophotometer (Beckman Coulter, CA) according to the method described by Ferruzzi, BohM, Courtney, and Schwartz (2002) in terms of hydrogen donating or radical scavenging ability. Briefly, 0.1 mM solution of DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol was measured at 515 nm and did not change throughout the period of assay. An aliquot (100 µL) of an extract diluted at the concentration range of 0.01-0.15 mg GAE/mL was mixed with 1.9 mL of methanolic DPPH solution. The change in absorbance at 515 nm was measured at 15 min. The percent of scavenging was calculated as the ratio of the absorption of the sample relative to the control DPPH solution, without the extracts. The BHT and Ascorbic acid in MeOH solution were used as positive controls. The EC_{50} of the extracts was also calculated at the 15 min scavenging value using nonlinear regression of Sigma Plot 9.1 (Systat software Inc, Illinois). Inhibition of free radical DPPH (%) was calculated according to the formula:

$$\text{Inhibition \%} = (A_{blank}\text{-}A_{sample}/A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the tested compound.

Exact concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted of %inhibition against extract concentration. Tests were carried out in triplicate. The synthetic antioxidant BHT and ascorbic acid were included in experiments as positive controls (Schlesier, Harwat, Bohm, and Bitsch, 2002).

4.3.4.3 Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the procedure of Wong, Li, Cheng,

and Chen, 2006. Briefly, the FRAP reagent was prepared from acetate buffer (pH 3.6), 10 mmol TPTZ solution in 40 mmol HCl and 20 mmol ferrous chloride solution in the proportion of 10:1:1 (v/v), respectively. The FRAP reagent was fresh and daily prepared and was warmed to 37^{o}C in a water bath prior to use. The extract of 50 µl was added to 1.5 mL of the FRAP reagent. The absorbance of the reaction mixture was then recorded at 593 nm after 4 min. The standard curve was constructed using ferric sulfate solution (100-2000 µM), and the results were expressed as µmol ferric/g dry weight of plant materials. All measurements were taken in triplicate and the mean values were calculated.

4.3.5 Statistical analysis

Descriptive statistics including mean and standard error of mean (SEM) were calculated for each extract antioxidant activity (n=3).

4.4 Results and Discussion

4.4.1 Total phenolic contents

The phenolic contents of the aqueous, ethanol, and acetone extracts of Rang Chuet were tested using the Folin-Ciocalteu reagent. The yield and total phenolics in Rang Chuet extracts are presented in Table 4.1. The phenolic contents of water extract was found to have highest phenolic contents (2433.9 mg GAE/100g) followed by ethanol (565 mg GAE/100g), and acetone (142.1 mg GAE/100g). Comparing the efficiency of extraction with boiling water, ethanol, and acetone, the method using boiling water showed the highest efficiency in the extraction of phenolic compounds. Therefore, Rang Chuet water extract would be expected to have the highest antioxidant activity. The acetone extracts gave the highest yield of extract at 36.6% (g/g leaf powderx100) but this extract showed the lowest phenolic contents.

Extraction method is also critical to the recovery of antioxidant phytochemicals. The nature of both plant materials and the bioactive components should be considered in order to achieve good extraction efficiency. Lipophilicity or hydrophilicity affects the solubility of a phytochemical in the extracting solvent, and conversely, polarity of a solvent also has an impact on the extraction efficiency. Many different extractions methods exist for antioxidant phytochemicals, but most of them are based on solvent extraction using water, organic solvent or liquefied gas, or combinations of them under different temperature and pressure, although other methods such as physical press, filtration, stream distillation and solid absorption have been used (Tsao and Deng, 2004).

Polar antioxidants such as phenolic acids and glycosides of many flavonoids are generally extracted using water, alcohol or a mixture of water and alcohols. Our results (Table 4.1) are consistent with these previous notions. For antioxidants such as aglycones of some flavonoids, non-aqueous solvents are used (Tsao and Deng, 2004). The efficiency of the boiling water in extracting compounds producing antioxidant activity is higher than that of methanol extract (Wong, Li, Cheng, and Chen, 2006).

Table 4.1. Yield and total phenolics of Rang Chuet using various extraction solvents.

Solvent	Yields (g/g *100)	Total phenolic contents (mg GAE/100g)
Water	32.6	2433.9 ± 57.7
Ethanol	23.3	565.0 ± 7.9
Acetone	36.6	142.1 ± 10.6

4.4.2. DPPH free radicals scavenging activity

DPPH is a stable nitrogen-centered free radical. The color changes from violet to yellow upon reduction by either the process of hydrogen or electron-donation. Substances which are able to perform this reduction can be considered as antioxidants and therefore radical scavengers (Hinneburg, Damien Dorman, and Hiltunen, 2006). The hydrogen atoms or electrons donation ability of the corresponding extract will be measured from the bleaching of violet colored MeOH solution of DPPH.

The studied extracts exhibited the scavenging activity of various strengths and were dose dependent in all species. In addition, positive controls with BHT and ascorbic acid were tested for their DPPH radical scavenging. The calculated EC_{50} for 15 min incubation time are reported in Table 4.2.

Table 4.2 DPPH scavenging activity of Rang Chuet extracts**.**

Rang Chuet Extracts	EC_{50}
Water	0.129 ± 0.01 (mgGAE/mL)
Ethanol	0.261 ± 0.04 (mgGAE/mL)
Acetone	0.607 ± 0.06 (mgGAE/mL)
BHT	0.278 ± 0.04 (mg/mL)
Ascorbic acid	0.103 ± 0.02 (mg/mL)

DPPH radical scavenging activity of water, ethanol, and acetone crude extracts of Rang Chuet revealed antioxidant potency considering the fact that the EC_{50} values. A lower value of EC_{50} indicates a higher antioxidant activity. Water extract has the highest scavenging activity among all extracts. Moreover, scavenging activity of water extract and ascorbic acid are closely comparable. There appears to be highly correlation between

scavenging activity and phenolics content of Rang Chuet extracts.

The different in antioxidant activities among Rang Chuet extracts due to multiple factors including concentration of the extracts and qualitative profile of extracts (Table 3.1 and 3.2, Chapter 3). Water extract had primarily components as apigenin and phenolic acids as caffeic acid and gallic acid as well as acetone and ethanol had main components as chlorophyll derivatives and lutein.

4.4.3 Antioxidant activity of Rang Chuet extracts by FRAP assay

The extracts of Rang Chuet were tested for antioxidant activity using the FRAP assay, which is simple, fast, and reproducible (Wong, Li, Cheng, and Chen, 2006). The FRAP is versatile and can be readily applied to both aqueous and alcohol extracts of different plants. In this assay, the antioxidant activity is determined on the basis of the ability to reduce ferric to ferrous. The results are expressed as mol ferrous equivalents per gram of sample.

Table 4.3 shows the antioxidant activity of Rang Chuet extracts including positive controls of BHT, ascorbic acid and Trolox. For the Rang Chuet extracts, the antioxidant activity ranged from 0.044 to 0.928 mmol Fe(II)/g. Water extract showed the highest antioxidant activity (0.928 mmol Fe(II)/g), followed by the ethanol extract (0.079 mmol Fe(II)/g) and the acetone extract (0.044 mmol Fe(II)/g). Wong, Li, Cheng, and Chen (2006) classified categories of medicinal plants based on their antioxidant activities: extremely high (> 500 µmol Fe(II)/g), high (100 – 500 µmol Fe(II)/g), medium (10 – 100 µmol Fe(II)/g), and low (<10 µmol Fe(II)/g). Under this classification, the water extract exhibited extremely high antioxidant activity, ethanol and acetone extracts showed medium antioxidant activity. In this study, Rang Chuet was extracted with boiling water similar to traditional Thai method used for herbs. The other samples were extracted with a more modern method, using ethanol and acetone.

Table 4.3 Antioxidant activity of water, ethanol, and acetone Rang Chuet extracts using FRAB assay.

Rang Chuet Extracts	Total antioxidant activity (mmol/g)
Water	0.928 ± 0.050
Ethanol	0.079 ± 0.002
Acetone	0.044 ± 0.005
BHT	1.421 ± 0.593

Furthermore, the leaves of Rang Chuet are available at a low cost and thus provide an economic source of potential natural antioxidants for use as food supplements or food additives. The results combined with those of chapter 3 showed that Rang Chuet leaves contain phenolic acid as gallic acid, caffeic acid, protocatechuic acid, other than that it contains flavonoid as apigenin and apigenin glucosides (Table 3.2, Chapter 3) Caffeic acid had been reported to have antioxidant activity (Son and Lewis, 2002) and this phenolic acid was found in all extracts especially found the highest in water extract (142.1 mg/100g) thus related to the highest antioxidant activities by both radical scavenging and FRAP assay. Interestingly, it was found that gallic acid is component in water extract which related to the highest antioxidant activity. Wong, Li, Cheng, and Chen (2006) reported that gallic acid isolated from Chinese medicinal plant has shown strong DPPH radical scavenging activities. However when compared to standards in FRAP assay, BHT (1.421 mmol Fe(II)/g), ascorbic acid (119.5 mmol Fe(II)/g), and trolox (7.2 mmol Fe(II)/g), all RC extracts showed relatively modest total antioxidant activity.

Antioxidant activity can be explained as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species (oxidant) is reduced at the expense of the oxidation of another antioxidant. The FRAP assay measures the antioxidant effect of any substance in the reaction medium as reducing ability. In this

assay, the antioxidant activity is determined on the basis of the ability of reduce ferric to ferrous. In the DPPH assay antioxidants reduce the free radical 2,2-diphenyl-1-picrylhydrazyl, which has an absorption max at 515 nm (Wong, Li, Cheng, and Chen, 2006).

Method use to measure antioxidant activity could be classified into two groups depending on oxidizing agent. DPPH assay use radical while as FRAP assay works employing metal ions for oxidation. In addition, depending on the reaction procedure, DPPH assay analyses the ability to reduce the radical cat ion and FRAP assay analyses the ability to reduce the ferric ion. When DPPH encounters proton radical scavengers, its purple color fades rapidly. This assay determines the scavenging of stable radical species of DPPH by antioxidants. Furthermore, test acting by radical reduction use preformed radicals and determine the decrease in absorbance while frap assay measures the increased absorbance of the formed ferrous ions (Schesier, Harwat, Bohm, and Bitsch, 2002).

Water extract showed the highest antioxidant activity both by DPPH and FRAP assay. It could be explained that water extract had the ability to reduce both radicals and ferric ions and also better reduce radicals. Moreover, all extracts showed antioxidant activity differently depend on components in each extract. The water extract showed the highest antioxidant activity might be from the main constituent is phenolic acids and flavonoids and flavonoids glucosides. Researcher reported that flavonoids and phenolic acids is the source of antioxidant activity in plants (Cook and Samman, 1996. The ethanol extract and acetone extract which showed less antioxidant activity composed of chlorophylls, chlorophyll derivatives and luteins. It could be explained that chlorophylls and chlorophylls derivative exhibited low antioxidant activity, also luteins that identified in ethanol and acetone extract are very low amount thus did not showed antioxidant activity.

Several hundreds of different polyphenols have been identified in foods. The two main types of polyphenols are flavonoids and phenolic. As antioxidants, polyphenols may protect cell constituents against oxidative damage and therefore, limit the risk of various degenerative diseases associated to oxidative stress. The phenolic groups in polyphenols can accept an electron to form relative stable phenoxyl radicals, thereby disrupting chain oxidation reactions in cellular components (Scalbert, Manach, Morand, and Remesy, 2005).

Flavonoids are potent antioxidants, free radical scarvengers, and metal chelators and inhibit lipid peroxidation. The structural requirement for the antioxidant and free radical scavenging functions of flavonoids include hydroxyl carbon in position three, a double bonds between position two and three, a carbonyl group in carbon position four, and polyhydroxylation of the A and B aromatic rings (Cook and Samman, 1996). The radical scavenging activities of flavonoids were highly controlled by the number and configuration of phenolic hydroxyl groups in the molecules and also influenced by glycosylation and configuration of other substituents. The flavonoids without any hydroxyl group (e.g., *trans*-chalcone, flavone, flavanone, and isoflavone) had no radical scavenging capacity (Cai, Sumb, Xingc, Luod, and Corkea, 2006).

The results from the antioxidant assays show that all extracts can act as radical scavengers to a certain extent. Water extract showed the highest activity in the iron reduction assay and DPPH assay. Yet, the EC_{50} values for these extracts were still lower than those of the tested reference antioxidant, ascorbic acid and BHT. From these results, it could be suggestion that RC extract could be natural sources for antioxidant in food but the amount required to produce antioxidant activity similar to standard use antioxidants could be large.

4.5 Conclusion

In conclusion, the antioxidant activity and total phenolic contents of Rang Chuet extracts were evaluated. The Rang Chuet extracts, in general, showed high antioxidant activities and phenolic content particular in water extract followed by ethanol and acetone extract respectively. Relatively, the antioxidant activity by DPPH and FRAP assay both highest in water extract at 0.129 mg GAE/mL and 0.928 mmol Fe (II)/g respectively. In addition, the antioxidant activity values of water extract was less than positive control(BHT) one fold by DPPH assay and 1.6 fold in FRAP assay. Moreover, there are relative between antioxidant activities and total phenolic contents in all extracts. The high total phenolic contents showed high antioxidant activity in all extracts. It could be conclude that polyphenols compounds contribute to antioxidant activity in RC extracts.

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CHAPTER V

CYTOTOXICITY OF THUNBERGIA LAURIFOLIA LINDL EXTRACTS

5.1 Abstract

Thunbergia aurifolia Lindl or Rang Chuet (RC) is widely described in Thai traditional medicine for protection against dietary and environmental toxicants with little substantiation. To better access the potential of RC as a medicinal plant, extracts were prepared by infusion with water, ethanol, and acetone. Extracts were subsequently investigated for their cytotoxicity. Cytotoxicity of Rang Chuet crude extracts were investigated in L929, BHK(21)C13, HepG2, and Caco-2 cell lines. The IC_{50} was affected differently in each cell line. The IC_{50} value of all RC extracts showed between 145 to more than 200 μg/mL in L929 cell lines and between 118 to more than 200 μg/mL for BHK(21)C13 cell lines. In addition, IC_{50} in HepG2 cell lines showed between 140 to more than 200 μg/mL and 117 and 147 μg/mL in Caco2 cell lines. The toxicity was indicated at high concentrations over 100 μg/mL for all extracts, which would be the index for further recommended concentration.

5.2 Introduction

5.2.1. *Thunbergia laulifolia* Lindl

Thunbergia laulifolia Lindl, commonly known as Rang Chuet, belongs to the

botanical family of *Acanthaceae*. It has been used in Thailand as a natural remedy for centuries. Rang Chuet is commonly consumed as herbal tea. Various parts of Rang Chuet could be used, for examples, aqueous extracts of fresh leaves, dried leaves, dried root and bark are used as antidote for insecticide, ethyl alcohol, arsenic, and strychnine poisoning; the dried root is also used as anti-inflammatory and antipyretic agents (Thongsaard and Marsden., 2002). As well, it has been reported that Rang Chuet leaves change body temperature of rats by centrally act at the thermo regulating center and/or cause vasodilatation and thereby increase heat dissipation (Chamreondararassame, 2003).

As Rang Chuet has been used as traditional medicine, researchers are interested in studying the compounds in Rang Chuet leaves extracts. Kanchanapoom, Kasai, Yamasaki (2002) reported two iridoid glucosides, 8-*epi*-grandiforic and 3'-*O*- β-glucopyranosyl-stibericoside isolated from the aerial parts of *Thunbergia laurifolia* Lindl along with seven known glucosides compounds. The flowers of *Thunbergia laurifolia* was also reported to contain Delphinidin 3:5-di-o-β-D-glucopyranoside, apigenin and apigenin-7-o-β-D-glucopyranoside (Purnima and Gupta, 1978). In addition, the plant was reported to contain flavonoids such as apigenin, casmosiin, delphinidin-3-5-di-*O*-β-D-glucoside and chorogenic acid (Thongsaard and Marsden., 2002)

The pharmacological properties of Rang Chuet water crude extracts has been reported as the antidote to insecticide, treatment for drug addiction, reducing toxicity of insecticide (Folidol), and antimicrobial activity, as well as antioxidant activity (Tejasen and Thongthapp, 1979; Rengyutthakan, 1980; Thongsaard and Marsden, 2002; Khunkitti, Taweechaisupapong, Aromdee, and Pese, 2003; Srida, Hankete, Khunkitti, Aromdee, and Pese, 2002). Recently, it has been reported that extracts of *Thunbergia laurifolia* Lindl leaves have a protective effect on ethanol-induced hepatotoxicity using hepatic lipid peroxidation, blood ethanol concentration as well as hepatic alcohol dehydrogenase

(ADH) and aldehyde dehydrogenase (ALDH) as indicators (Chanawirat, Toshulkao, Temcharoen, and Glinsukon, 2000). Treatment of alcoholism is also claimed using its aqueous extract. Alcohol and hexane extracts from *Thunbergia laurifolia* also posses anti-inflammatory activity against carageenin-induced paw edema in mice (Charumanee *et al*., 1998)

In this study, cytotoxicity of Rang Chuet crude extracts was investigated in L929, BHK(21)C13, HepG2, and Caco2 cell lines. Results would provide a better understanding of the cytotoxicity of this plant and IC_{50} value of extracts which would suggest recommended uses.

5.2.2 Cytotoxicity

The cytotoxicity of plant extracts can be evaluated with MTT assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells with a detergent results in solubilization and liberation of the crystals. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay. The results `can be read on a multiwell scanning spectrophotometer (ELISA reader).

5.3. Materials and Methods

5.3.1 Plant materials

Rang Chuet, *Thunbergia laurifolia* Lidnl. (*Acanthaceae*) was collected in December 2005- Febuary 2006 from local area in Nakhon Ratchasima province, Thailand.

Leaves were air dried at 60^{o}C for 6 h, after which they were ground in a blender (National, MX-T2GN, Taiwan) to fine powder and stored in vacuum packages at 4^{o}C until use.

5.3.2. Cell culture

The target cells were L929 (mouse connective tissue ECACC Cat. No. 85011425), BHK(21)C13 (baby hamster Syrian kidney ECACC Cat. No. 85011433), HepG2 (human liver hepatocarcinoma ATCC Cat. No. HB-8065) and Caco2 (a human colon adenocarcinoma ATCC Cat. No. HTB-37, ATCC, USA). The L929 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 2mM L-glutamine. The BHK(21)C13 cells were grown in Glasgow Modified Eagle's Medium (GMEM) supplemented with 5% fetal bovine serum, 1.5 g/L tryptose phosphate broth and 2mM L-glutamine. The HepG2 and Caco2 were grown in Minimun Essential Medium (MEM) supplemented with 10% fetal bovine serum, 0.1mM MEM non-essential amino acid, 1.0 mM Sodium pyruvate and 2 mM L-glutamine. All cells were incubated at 37^{o}C in a fully humidified incubator with 5% CO_2 : 95% air atmosphere.

5.3.3 Chemicals

All chemicals used were of at least analytical grade. Solvents including acetone, ethanol, methanol, and petroleum ether were purchased from Italma (Bangkok, Thailand). Cell culture, Media, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], DMSO, Sorensen's Glycine buffer, were purchased from American Type Culture Collection; ATCC (Manassas, VA).

5.3.4 Extraction

5.3.4.1. Solvent extraction

The extraction method of Kanchanapoom *et al*. (2002) was modified. Rang Chuet

powder of 5g was homogenized with 200 mL of solvents which were water, ethanol, and petroleum ether. The homogenate was placed in a shaker at 55^{o}C for 72 h. and then filtered using Whatman No.1 paper. The filtrate was concentrated using a vacuum evaporator (BUCHI Rotavapor R-114, Switzerland) and kept at 4^{o}C until use.

5.3.4.2. Supercritical fluid extraction

Rang Chuet powder was sieved through 425 micron screen before extraction in a supercritical fluid extractor SFE (SFX200, ISCO, Fortune scientific Co.Ltd, Thailand) using supercritical fluid CO_2. The extraction, modified method of Yamini, Asghari-Khiavi, and Bahramifar (2002), was performed with 3.0 g of the sieved leaves powder filling in extraction vessels under 250 atm pressure and 50^{o} C for 5 min static followed by 15 min dynamic extraction. A duraflow manual variable restrictor was used in SFE system with the flow rate of approximately 0.3-0.4 mL /min to collect the extracted analytes in 3 mL of petroleum ether.

5.3.5. Cytotoxicity assay

L929, BHK(21)C13, HepG2 and Caco2 were seeded in a 96-well plate with 500 cells/well for L929 and 2000 cells/well for the others, and incubated for 48 h. All the *Thunbergia laurifolia* Lindl dried crude extracts were dissolved in ethanol to make stock solution then diluted to various extracts concentrations in media from 1.56 ug/mL to 200 ug/mL. The extracts were added to the wells and incubated for 24 h. The test samples were removed from the cell cultures and the cells were re-incubated for a further 24 h in fresh medium and then tested with MTT.

Briefly, 50 µl of MTT in PBS at 5 mg/mL was added to the medium in each well and the cells were incubated for 4 h. Medium and MTT were then aspirated from the

wells, and formazan solubilized with 200 μL of DMSO and 25 μl of Sorensen's Glycine buffer, pH10.5. The optical density was read with a plate reader at a wavelength of 570 nm. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each extract sample. Two controls were set up, one with medium as reagent control and the second with the ethanol 1% as solvent control.

A dose-response curve was derived from 8 concentrations in the test range of 200-1.56 μg/mL using 4 wells per concentration to determine the mean of each point. Within each experiment 2-dose response curves were obtained. Results of toxic compounds were expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compared with controls.

5.4 Results and discussion

Over the last few decades several in vitro assays using mammalian cell cultures have been developed thus avoiding the excessive use of laboratory animals which is expensive, time consuming, and often involves ethical problems. Cell culture systems can be more sensitive and more reproducible than tests involving intact animals (Cetin and Bullerman, 2005).

The cytotoxicity of *Thunbergia laurifolia* Lindl extract was evaluated in BHK, L929, Hep G2, and Caco-2 cell line with the MTT assay (Mossman, 1983). When cells were treated for 24 h with concentration range from 1.56 to 200 μg/mL of extract, the relative cell survival progressively decreased in a dose-dependent manner. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaved the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced was directly proportional to the number of viable cells (Mossmann, 1983). The IC_{50} value for *Thunbergia laurifolia* Lindl is shown in Table 1. In addition,

there was no difference between the two controls of medium and Ethanol 1% with no toxicity to the tested cell lines.

Table 5.1. IC_{50} values for *Thunbergia laurifolia* lindl extracts (water, petroleum ether, ethanol, and SFE).

Sample	IC_{50} (ug/mL)			
	L929	BHK(21)C13	HepG2	Caco2
Vehicle control	677±11	689±13	5299±72	2993±27
Water crude extract	182±8	>200	>200	147±15
Petroleum ether crude extract	>200	103±1	140±4	117±9
Ethanol crude extract	>200	134±2	>200	ND
SFE	145±4	118±4	154±1	120

The results showed that the IC_{50}'s was affected differently in each cell line. The petroleum ether extract showed the highest cytotoxic toward BHK(21)C13 cell line at concentration of 103±1 μg/mL. The water extract showed the lowest cytotoxic toward BHK(21)C13 and HepG2 cell lines at concentration over 200 μg/mL. Also petroleum ether and ethanol extracts showed low cytotoxic toward L929 at concentration over 200 μg/mL. However, the toxicity for all extracts was indicated at high concentration of 200 ug/mL, which would be the index for further recommended concentration being applied.

All RC extracts exhibited extremely high value of IC_{50} (>100 µg/mL) against all cell lines tested indicating low cytotoxic to the cells (Okonogi, Duangrata, Anuchpreedab, Tachakittirungroda, and Chowwanapoonpohna, 2006). When compared among tested cell lines, BHK(21)C13 cells was found to be sensitive to the cytotoxic effect of RC extracts including petroleum ether, ethanol, and SFE extracts followed by Caco-2 and HepG2 after 24-h exposed whereas L929 cells showed low response to RC extracts toxicity. The highest cytotoxicity of BHK(21)C13 cells, baby hamster Syrian kidney, toward RC extracts (Petroleum ether, ethanol, and SFE) could lead to understanding that these RC extracts affect kidney cells when concentration at specifically value was applied. In petroleum ether extract, cytotoxicity may cause from solvent itself and components in extracts which are from non-polar extraction. The use of large amount of organic solvents poses health and safety risks (Tsao and Deng, 2004).

The water extracts showed low cytotoxic (> 200 µg/mL) toward BHK(21)C13 and HepG2 cell lines which lead to suggestion use this kind of RC extract due to low toxicity to kidney cells and human liver cell lines. The water extraction is the non-toxic method for extraction and also the components be extracted could be polyphenol parts including flavonoids and phenolic acids which are generally extracted using water (Tsao and Deng, 2004). In opposite, the water extract showed high cytotoxic at IC_{50} of 147 µg/mL toward Caco-2 cells which lead to information that water extract even though low toxic toward kidney and liver cells but moderately toxic toward intestinal cells. For Caco-2 cells, the cytotoxicity of RC extracts at IC_{50} of 117, 120, and 147 for SFE, water, and Petrolum ether extracts respectively. SFE extracts is high cytotoxicity toward human intestinal cells and this lead to suggestion for carefully use at specifically concentration of this extract. When compared between cell lines tested, L929 cells is the lowest cytotoxic from all RC extracts which might due to type of cell lines (connective tissue) and if compared between types of RC extracts, the water extract is the lowest cytotoxic for all cell lines tested.

The L929 and BHK(21)C13 cells line were selected based on they are the basic cell lines use for generally cytotoxicity test. For HepG2 and Caco-2 cell lines, they are selected for cytotoxicity tested with all RC extracts due to they are human liver and intestinal cell lines which should give information about toxicity relating to how this plant affect liver and intestinal cells of human after consume this plant as herbal tea. Furthermore, Pramyothin, Chirdchupunsare, Runsipipat, Chaichantipyuth (2005) reported that RC extracts had protective activity in rats treated with ethanol both *in vitro* and *in vivo* thus human liver cells should be selected to study cytotoxicity from RC extracts.

The Caco-2 cell line is derived from a human colon adenoma and has been used routinely in drug absorption screening, because the Caco-2 monolayer displays several features of the small intestinal epithelial barrier. The cytotoxicity against the Caco-2 cell line could provide preliminary information for studying of toxicity on the normal intestinal cell type and for the selection of appropriate concentrations required in a future permeability study of active components (Okonogi, Duangrata, Anuchpreedab, Tachakittirungroda, and Chowwanapoonpohna, 2006).

Okonogi, Duangrata, Anuchpreedab, Tachakittirungroda, and Chowwanapoonpohna (2006) studied antioxidant capacities and cytotoxicities of certain fruit peels in Caco-2 cells and peripheral blood mononuclear cells and reported that the peels of rambutan may be considered potential useful as a source of natural antioxidants for food or drug product because of its high antioxidant activity and non-toxic property to normal cells (IC_{50} > 100 ug/mL).

5.5. Conclusion

In the MTT assay, the *Thunbergia laurifolia* Lindl crude extracts were indicated toxicity to L929, BHK (21)C13, HepG2, and Caco2 over the tested concentration ranges

of 200 to 1.56 ug/mL. The water extract showed the lowest cytotoxicity toward all cells line tested at IC_{50} of 182, > 200, > 200, and 147 μg/mL for L929, BHK(21C13, HepG2, and Caco-2 cell lines respectively. In addition, the petroleum ether extract is the highest toxic toward BHK(21)C13, HepG2, and Caco-2 cell lines at IC_{50} of 103, 140, and 117 μg/mL respectively but low toxic toward L929 cells at IC_{50} of > 200 μg/mL. Moreover, the ethanol extract showed low cytotoxic toward L929 and HepG2 cells at IC_{50} of > 200 μg/mL but high toxic toward BHK(21)C13 at IC_{50} of 134 μg/mL. Finally, the SFE extract showed moderate to high toxic toward HepG2, L929, Caco-2, and BHK(21)C13 cells at IC_{50} of 154, 145, 120, and 118 μg/mL respectively. The IC_{50} was summarized low cytotoxicity at concentration over 100 ug/mL for all crude extracts.

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CHAPTER VI

STORAGE STUDIES OF THUNBERGIA LAURIFOLIA LINDL EXTRACTS

6.1 Abstract

Storage changes of polyphenols, chlorophyll derivatives, and carotenoidss in RC leaves and ethanol extract were studied at different storage temperatures of -25, 25, 30 and 50°C over 6 months. There were relative stability of total phenolics occurred during storage both in leaves and extracts. Chlorophyll contents decreased with increases storage temperature and time in ethanol extract. Carotenoids identified as luteins in RC decreased both in leaves and ethanol extract during storage time and at all temperatures.

6.2 Introduction

Chlorophyll is the most widely distributed plant pigment, and the importance of chlorophylls *a* and *b* in food Industry derives from their part in the green color of vegetables. However, they are highly susceptible to degradation during processing and storage. The conversion of chlorophylls to pheophytins and other derivatives results in a change from bright green to dull olive-green or olive-yellow, which ultimately is perceived by the consumer as a loss of quality. A challenge to food processors has been to prevent or to minimize this degradation in attempts to produce higher quality vegetable products.

The properties of chlorophyll pigment in green tissues may depend on the nature of its association with lipoproteins of chloroplast. Disruption of the chlorophyll structure can

not take place until the membrane array of the chloroplast has been disorganized. Chlorophyll degradation could occur during processing and storage, depending on temperature, pH, time, enzyme, oxygen, and light. Although the most common mechanism of chlorophyll degradation seems to be acid-catalyzed transformation into pheophytin, oxidation through the action of lipoxygenase has also been observed (King, Liu, and Jing Liu, 2001)

Polyphenols are the most abundant antioxidants in our diets. The main classes of polyphenols are phenolic acids (mainly caffeic acid) and flavonoids (the most abundant in diets are flavonols, catechins plus proanthocyanins, anthocyanins and their oxidation product), which account for one-and two-thirds, respectively. Polyphenols are reducing agents, and together with other dietary reducing agents, such as vitamin C, vitamin E and carotenoids, referred to as antioxidants, protect the body's tissues against oxidative stress and associated pathologies such as cancers, coronary heart disease and inflammation. The biological properties, bioavailability, antioxidant activity, specific interactions with cell receptors and enzymes are related to the chemical structure of polyphenols. It is, therefore, essential to know the nature of the main polyphenols ingested, their dietary origin, the amounts consumed in different diets, their bioavailability and the factors controlling their bioavailability (Tapiero, Tew, Nguyen, Mathe, 2002).

The objective of this study was to investigate the retention of chlorophyll, lutein, and total phenolic contents in dried RC leaves and RC extracts under various storage temperatures for stability of their phytochemicals.

6.3 Materials and Methods

6.3.1 Plant materials

The plant *Thunbergia laurifolia* Lindl. (*Acanthaceae*) collected in December 2005-Febuary 2006 from local area in Nakhon Ratchasima Province, Thailand was used in this

study. Leaves were air dried at 60°C for 6 h, after which they were ground in a blender (National, MX-T2GN, Taiwan) to fine powder and stored in vacuum packages at 4°C until use.

6.3.2 Chemicals and standards

Solvents including acetone, acetonitrile, ethanol, ethyl acetate and methanol (Mallinckrodt-Baker, Phillipsburg, NJ) were of certified HPLC and ACS grade. A 1.0 *M* ammonium acetate buffer solution (Fluka; Ronkonkoma, NY, USA) was prepared with double distilled (dd) water and adjusted to pH 4.6 with glacial acetic acid. The following standards were obtained: chlorophyll *a*, chlorophyll *b*, lutein (Sigma Aldrich, St. Louis, MO), caffeic acid, and apigenin.

Each standard was dissolved in solvent and filtered through a 0.45 μm PTFE membrane prior to HPLC injection. Pheophytin *a* and pheophytin *b* standards were prepared according to the method described by Ferruzzi, Nguyen, Sander, Rock, and Schwartz (2001). One 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. After that 5 ml of petroleum ether was added then washed out by dd water. The petroleum ether part was collected and 2 ml aliquot was added into test tubes and dried by nitrogen. The pheophytin *a* and *b* were kept at -20 °C until use.

6.3.3 Extraction

Approximately 100 mg leaves powder was extracted with three 12 ml portions of boiling water (100°C), ethanol, or acetone in a shaking water bath at 25°C for 15 min. Centrifugation at 3000 g (Thermo IEC, Waltham, MA) was applied for 3 min between extractions three times. The filtrates obtained from vacuum filtration were combined and the volumes were adjusted to 50 ml. Aliquots of 2 ml were added in test tubes dried in a

vacuum dryer (Rapid Vap® Vacuum Evaporation Systems, Labconco corporation, Kansas city, MO). Samples were stored at -20^{o}C until use.

6.3.4 Chlorophyll and carotenoids analyses

Chlorophyll and carotenoids analyses were achieved as described by Ferruzzi *et al*. (2001) with modification. Briefly, a Hewlett-Packard 1090A system equipped with a diode array detector was utilized. Separation was achieved using a Grace-Vydac 201TP54 reversed-phase (4.6 x 250mm) polymeric C18 column with guard column containing the same stationary phase (Grace Vydac, Apple Valley, MN). A gradient elution profile was used based on a binary mobile phase system consisting of methanol:water:Ammonium acetate (73:23:2 v/v/v) in reservoir A and Ethyl acetate in reservoir B. A flow rate of 1.0 mL/min was applied with initial setting at 100% A with a linear gradient to 50:50 A/B over 10 min. The gradient was held for 10 min followed by a 5 min linear gradient back to 100% A and equilibration at the initial condition for 5 min for a total run time of 30 min. Detection and tentative identification of all chlorophylls and carotenoids were accomplished using in-line diode array data between 250 and 600 nm. Quantification of chlorophylls and carotenoids were accomplished using multilevel response curves constructed with authentic chlorophyll and carotenoids standards.

6.3.5 Phenolic compounds analyses

Total soluble phenolic constituents of water and ethanol extracts and dried leaves were determined, employing Folin-Ciocalteu reagent and gallic acid as a standard (Waterhouse, 2002). Dried leaves were extracted with hot water or acetone described previously before test. The crude extract solution of 20 µl of, standard or blank was added into a 1.5 ml plastic cuvette, after that 1.58 ml water was added, followed by 100 µl Folin-Ciocalteu reagent before the mixture was mixed thoroughly by inverting and incubated for

5 min. $NaCO_3$ (2% w/v) solution of 300 µl was added and mixed and the mixture was allowed to stand at room temperature for 2 h. Absorbance was measured at 765 nm. The gallic acid solution (0-1000 µg/0.1 mL) was used to construct a standard curve.

Analysis of individual phenolic compounds was performed using a Waters model 2695 HPLC system equipped with a model 2996 photodiode array detector. A Waters NovaPak C18 (3.8 mm i.d.× 150 mm) reversed phase (RP) column (Milford, MA) with a guard column also packed with Waters NovaPak C18 was employed. Phenolic compounds were separated by gradient elution with a flow rate of 1.0 mL/min at 35^{o}C using a binary mobile phase of water/acetic acid (98:2, v/v) in reservoir A and acetonitrile in reservoir B. Initial solvent proportion of 99:1 A/B with a linear gradient to 70:30 A/B over 20 min was used, followed by a 5 min linear gradient back to 99:1 A/B and 5 min equilibration at the initial condition for the total chromatographic run time of 30 min. Detection and tentative identification of major Rang Chuet phenolic compounds were accomplished using in-line PDA data between 200 and 500 nm. Calibration plots for quantation were constructed from injection of authentic standards of caffeic acids and apigenin.

6.3.6 Stability of Rang Chuet phytochemicals

Stability studies were performed for RC extracts and dried leave powder at various at 25, 25, 30, and 50 ^{o}C. Samples of rang chuet extracts and dried leaves powder were aliquot in glass tubes, flushed with nitrogen and wrapped in foil to avoid light induced degradation. At predetermined intervals samples were pulled for analysis (0.5, 1, 3, 6 months) and analyzed for chlorophyll/carotenoid contents by HPLC and total phenolic by spectrophotometry as previously described (Chapter 3).

6.3.7 Statistical data analysis

Descriptive statistics including mean and standard error of mean (SEM) were

calculated for total phenolic contents and chlorophyll/carotenoids contents (n=3).

6. 4. Results and Discussion

6.4.1 Total phenolic contents

Total phenolic contents of water extract was found to be the highest with 2433.9 mg GAE/100g followed by ethanol with 565 mg GAE/100g, and acetone with 142.1 mg GAE/100g at the beginning of storage.

The change in total phenolic content in water extract, ethanol extract and dried leaves are reported in Fig. 6.1 to Fig. 6.4. RC water extract was found to be relatively stable for 3 months storage at all temperature while total phenolic contents in ethanol extract, acetone extract, and dried leaves were stable for 6 months storage at all temperature.

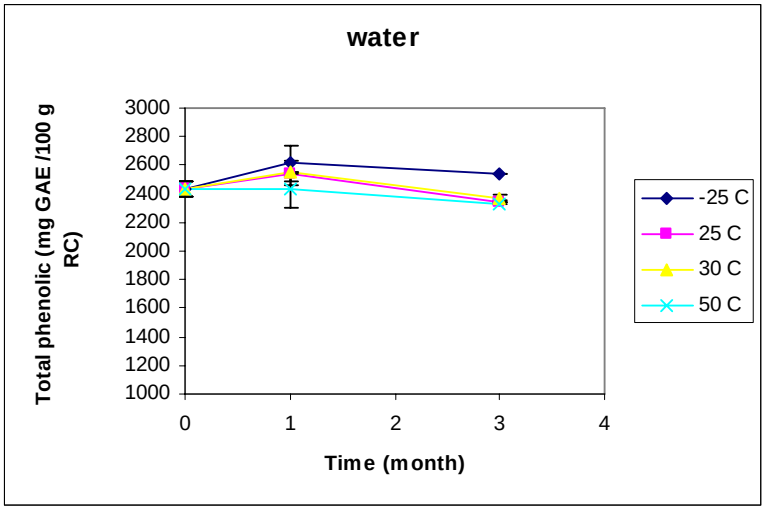

Fig. 6.1 Changes of total phenols in RC water extract during storage at -25, 25, 30, and 50^{o}C.

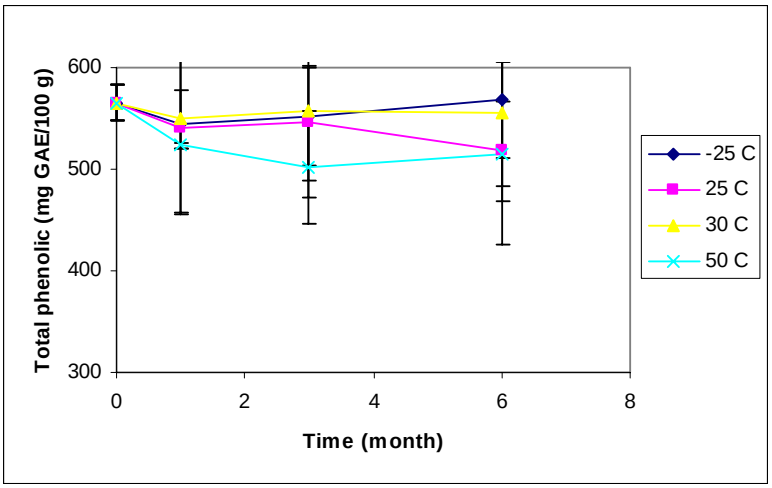

Fig. 6.2 Changes of total phenols in RC ethanol extract during storage at -25, 25, 30, and 50°C.

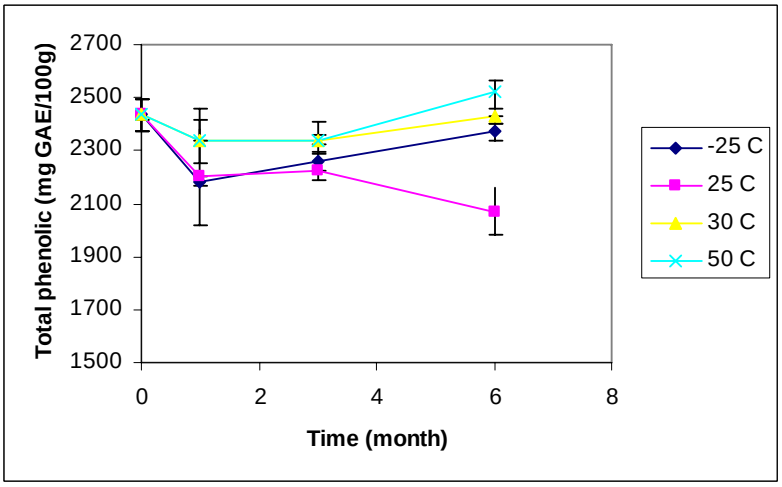

Fig. 6.3 Changes of total phenols in RC dried leaves (extracted with water) during storage at -25, 25, 30, and 50°C.

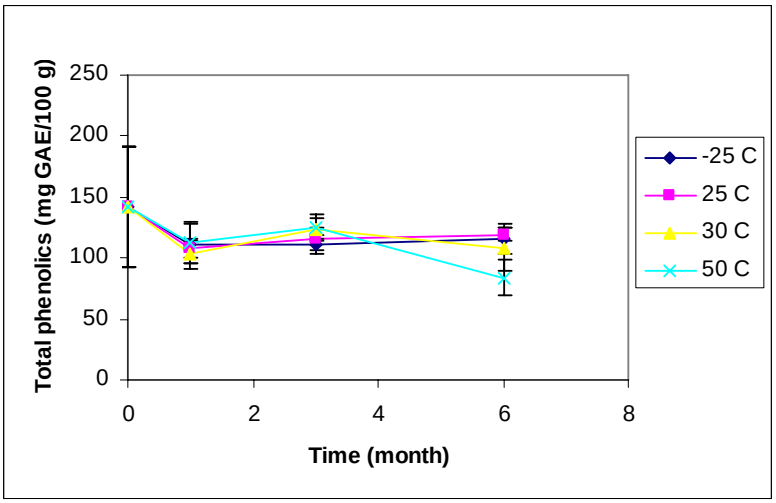

Fig. 6.4 Changes of total phenols in RC dried leaves (extracted with acetone) during storage at -25, 25, 30, and 50°C.

6.4.2 Chlorophylls and carotenoids

Changes in chlorophylls and carotenoids profiles of RC dried leaves are reported in Fig. 6.5 to Fig. 6.8. During the 6 month storage of RC leaves and the extracts in the dark, the chlorophyll *a* and *b* decreased in dried leaves for 3 months. A concurrent increase in pheophytin and pheophorbide was observed indicating that transformation of native chlorophyll to magnesium free endproducts occurred. Higher loss in chlorophyll composition was observed during the first two weeks in dried leaves for all temperatures. The abundant pheophytins and pheophorbide were found after storage for 2 weeks for all temperatures. Chlorophyll *a* and chlorophyll *b* decreased rapidly in 2 weeks and all degraded in 3 months for all temperatures except at 50°C which chlorophyll *a* all degraded in 2 weeks. Pheophytin *a* was stable for 3 months at 30 °C and 50 °C.

Total chlorophyll decreased more rapidly as temperature increased. Furthermore, total chlorophyll was more stable in dried leaves than in ethanol extract as shown in Fig. 6.9 and Fig. 6.10.

For ethanol extract storage, chlorophyll *a* decreased gradually at temperature -25^{o}C, and all degraded within 2 weeks at temperature 25, 30 and 50 ^{o}C. Chlrophyll *b* was stable for 6 month at temperature -25 ^{o}C (Fig. 6.11), degraded all in 3 month at temperature 25 and 30 ^{o}C (Fig. 6.12 and Fig. 6.13), and degraded all in 2 weeks at temperature 50 ^{o}C (Fig. 6.14). Pheophytin *a* and pheophorbide *a* were stable at temperature -25, 25, and 30 ^{o}C (Fig. 6.11, 6.12, 6.13) for 6 months but pheophytin *a* degraded all after 3 months, also pheophorbide *a* degraded all after 1 month at temperature 50 ^{o}C (Fig. 6.14).

Structurally, chlorophyll is a substituted tetrapyrole with a centrally bound magnesium atom. The porphyrin macrocycle is further esterified to a diterpene alcohol, phytol, to form chlorophyll (Sheer, 1991). The sensitivity of natural chlorophylls to extremes in pH and temperature allows for the formation of several distinct derivatives through processing of vegetable tissue and human digestion (Simpson, 1985). It is well known that heat or solvent treatments of these pigments cause slight modifications of the molecules giving in total eight structurally similar derivatives: Chlorophyll *a*, *a'*, *b*, and *b'*, and the corresponding Pheophytins (Watanabe *et al*., 1984). From this study, Rang Chuet commercial preparation should be in dried leaves form due to stability of their phytochemicals and total phenolic contents. Furthermore, the storage temperature should be at -25^{o}C.

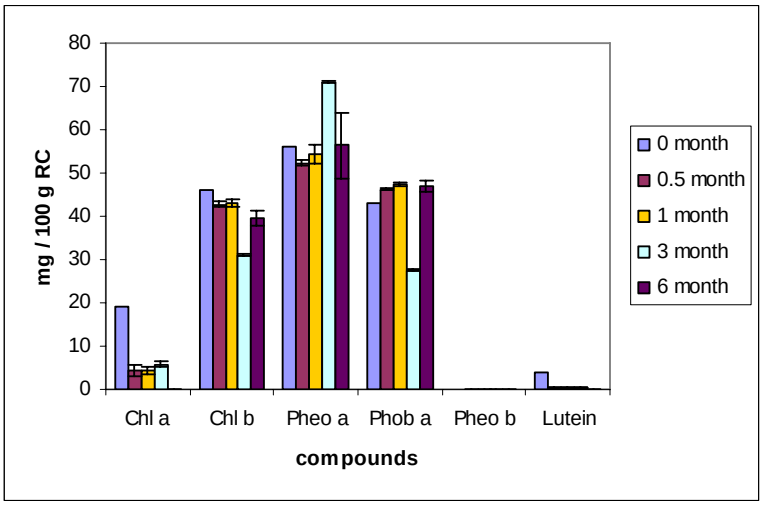

Fig. 6.5 Changes of chlorophylls, chlorophylls derivatives, and lutein in RC leaves during storage at -25^{o}C

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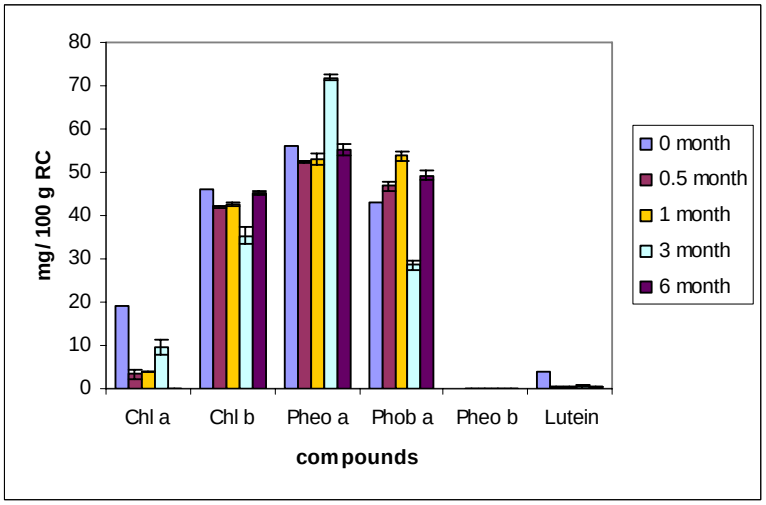

Fig. 6.6 Changes of chlorophylls, chlorophylls derivatives, and lutein in RC leave during storage at 25^{o}C.

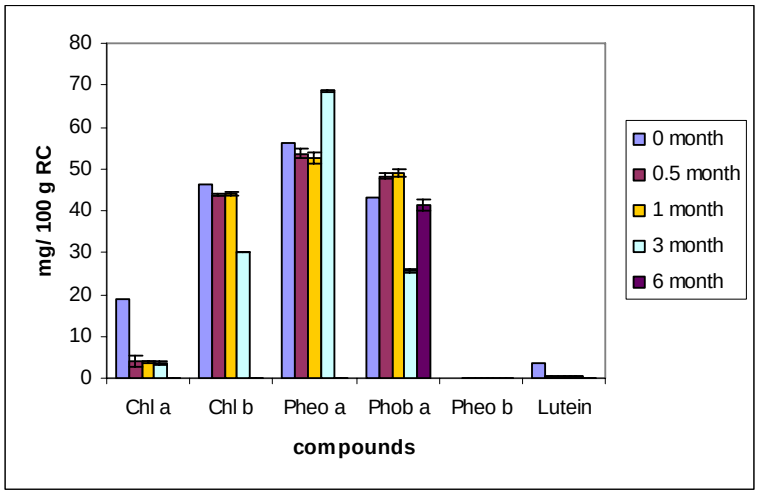

Fig. 6.7 Changes of chlorophylls, chlorophylls derivatives, and lutein in RC leaves during storage at 30^{o}C.

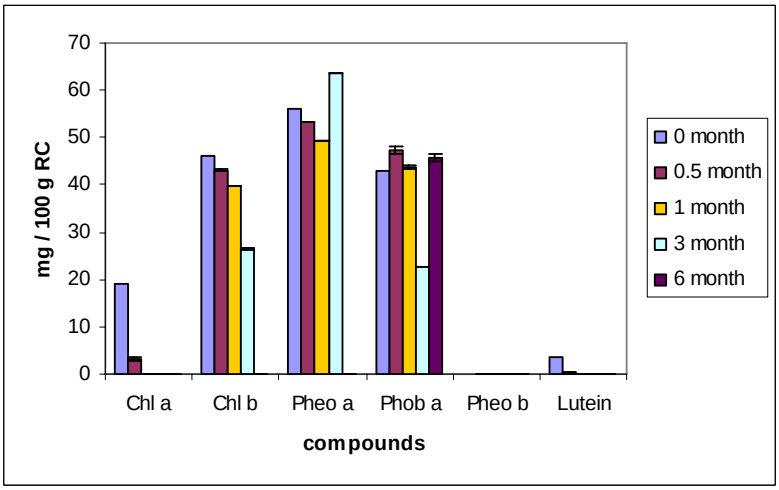

Fig. 6.8 Changes of chlorophylls, chlorophylls derivatives, and lutein in RC leaves during storage at 50^{o}C.

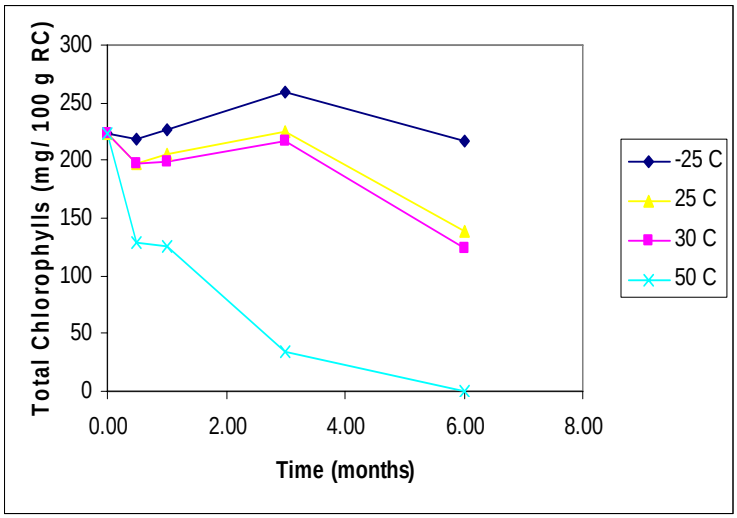

Fig. 6.9 Changes of total chlorophylls in RC ethanol extract during storage at -25, 25, 30, and 50°C.

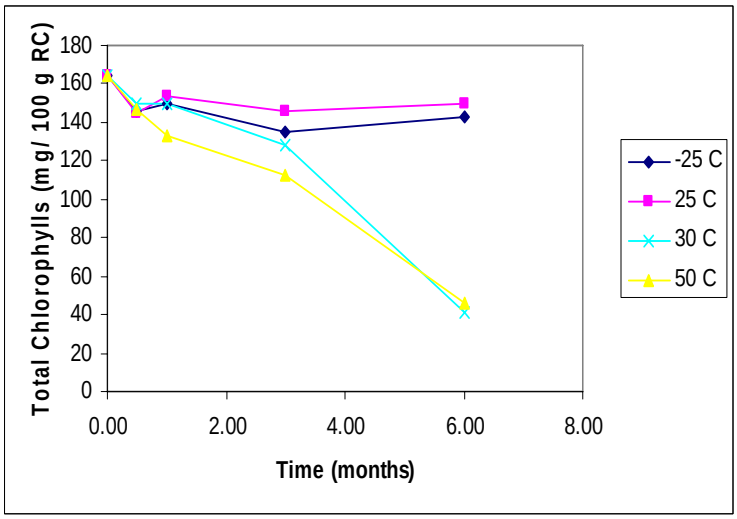

Fig. 6.10 Changes of total chlorophylls in RC leaves during storage at -25, 25, 30, and 50°C.

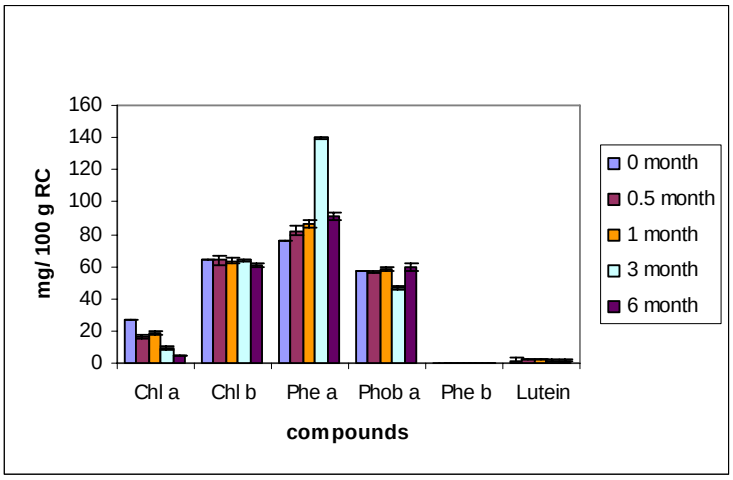

Fig. 6.11 Changes of chlorophylls derivatives and lutein in RC ethanol extract during storage at -25 ^{o}C.

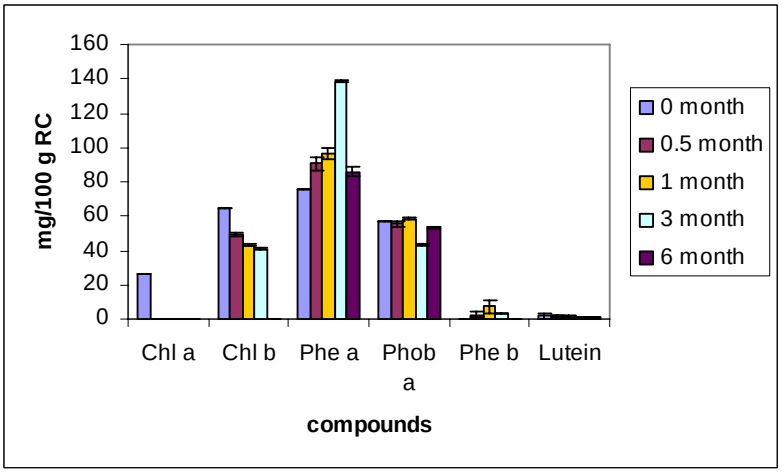

Fig. 6.12 Changes of chlorophylls derivatives and lutein in RC ethanol extract during storage at 25 ^{o}C.

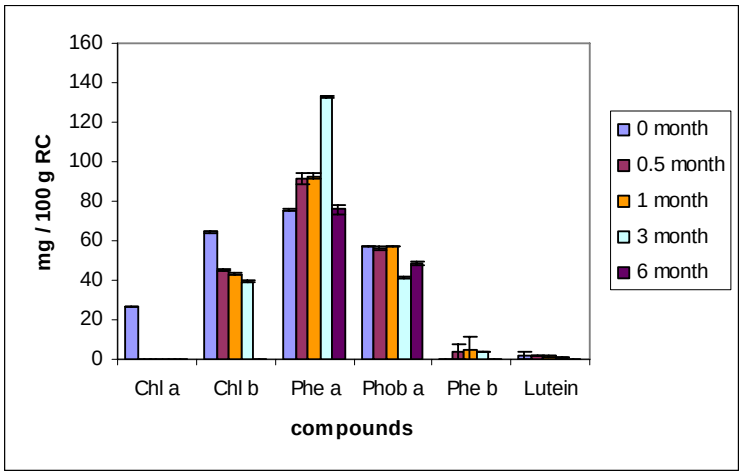

Fig. 6.13 Changes of chlorophylls derivatives and lutein in RC ethanol extract during storage at 30^{o}C.

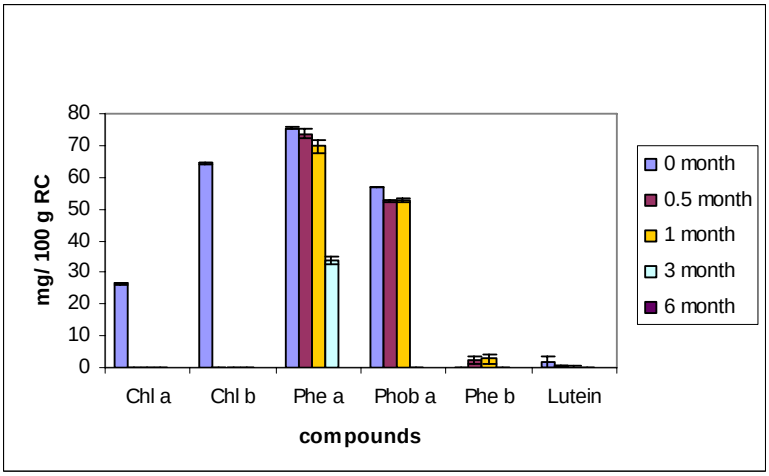

Fig. 6.14 Changes of chlorophylls derivatives and lutein in RC ethanol extract during storage at 50^{o}C.

6.5. Conclusion

Storage studies of RC in dried leaves and extracted form showed that dried leaves storage provides longer thermal storage stability than that of extracted form all temperatures, -25, 25, 30, and 50^{o}C. An increase in pheophytin *a* and pheophorbide *a* has been found in RC extract and leaves composition as a result of the degradation of chlorophyll *a* during storage. Chlorophyll derivatives as pheophytin *a* and pheophorbide *a* in leaves are stable over 6 months. Total chlorophylls decreased more rapidly as temperature increased because chlorophylls degradation had been shown to follow a first-order model and described by the Arrhenius equation (Ryan-Stoneham and Tong, 200)

Total phenolic contents was stable in water extract over 3 months and total phenolics in ethanol extract has changed in range of around 50 mg GAE/100g at all temperatures storage. In addition, total phenolics in dried leaves storage and then extracted with water and acetone when test for total phenolics not changed over six months storage.

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CHAPTER VII

SUMMARY AND RECOMMENDATIONS

Thunbergia laurifolia Lindl or Rang Chuet (RC) was extracted with water, ethanol, and acetone at 25^{o}C. Drying provided crude ethanol and acetone extracts and freeze drying provided a crude water extract. It was found that main chemical compositions of RC ethanol and acetone extracts contained chlorophylls derivatives, especially abundant in pheophytin *a* and pheophorbide *a*, and carotenoids.

Rng Chuet extracts were analyzed for major chlorophylls and carotenoids profiles as well as major phenolic profiles. RC water extract showed the highest total phenolic contents and also rich in apigenin and apigenin glucosides including some phenolic acid such as caffeic acid. In addition, both RC acetone and ethanol extracts composed of major constituents as chlorophylls and chlorophyll derivatives particularly abundant in form of pheophorbide *a*.

Rang Chuet extracts (acetone, ethanol, and water) at concentrations of 0.03 to 1 μg GAE/μl induced the phase II xenobiotic detoxification enzyme, quinone reductase (NQO1), and activity on Hepa 1c1c7 cells up to 2.8 fold at the highest concentration of Rang Chuet. In addition, acetone extract showed the highest antimutagenic activity 81.11% at concentration of 0.17 mg GAE/ml. Ethanol extract showed the highest antimutagenic activity 86.56% at concentration of 0.15 mg GAE/ml. Thus, chlorophyll constituents could play the major role in bioactivity as NQO1 induction due to the highest activity in acetone extract which is rich in chlorophylls and chlorophyll derivatives. Therefore the presence of chlorophylls derivative (pheophorbide *a*) in high concentration in the extract may account for its protective against dietary and environmental toxicants.

In fact, RC extracts might have a synergistic effect on antimutagenic activity due to the highest activity found in ethanol extract rich in chlorophyll derivatives, phenolic acid (caffeic acid), and flavonoids (apigenin and apigenin glucoside). Furthermore, both NQO1 induction and antimutagenic activity of Rang Cuet extracts supported traditional use this plant for protection against dietary and environmental toxicants for local people in Thailand.

Rang Chuet extracts were subsequently investigated for their cytotoxicity in L929, BHK(21)C13, HepG2, and Caco2 cell lines. The IC_{50} was affected differently in each cell line. The toxicity was indicated at high concentration over 100 μg/ml for all extracts, and this level would be an index for further recommended concentration to be used. The IC_{50} was summarized low cytotoxicity at extract concentration over 100 ug/mL for all crude extracts.

Antioxidant activities and total phenolic contents of Rang Chuet extracts were evaluated using free radical scavenging, ferric reducing antioxidant power assay, and the Folin-Ciocalteu method. Comparing the efficiency of extraction methods, water extraction is very efficient for phenolic compounds, providing the highest antioxidant activity among all the extraction methods used Thus this water extract is a modest natural antioxidants.

In storage studies, total phenolic contents were found to be relatively stable for 6 months both in dried leaves and ethanol extract. Chlorophyll contents decreased with increases storage temperature and time in ethanol extract. Carotenoids identified as luteins in RC decreased both in leaves and ethanol extract during storage time and at all temperatures.

BIOGRAPHY

Ratchadaporn Oonsivilai was born and brought up in Nakhonpanom province, Thailand. She attended Khon Kaen University, Thailand, and received her Bachelor's degree in Science (nursing) (1987). She worked at Srinakarin hospital, Faculty of medicine, Khon Kaen University for 10 years. In 2000, she received a Master's degree in Food Science from Dalhousie University, DalTech, Halifax, Nova Scotia, Canada and worked at Suranaree University of Technology as a lecturer in the School of Food Technology. From 2002-2006 she obtained a scholarship from the Commision for Higher Education, Ministry of Education under Consortium Sanwich Ph.D Program, to pursue her Ph.D. study at the School of Food Technology, Suranaree University of Technology, Thailand and the Department of Food Science, Purdue University, USA.