ลายพิมพ์ดีเอ็นเอ รูปแบบโครมาโทกราฟี และฤทธิ์ทางชีวภาพของรางจืด

นายพิภพ สุวรรณชัยเกษม

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ปีการศึกษา 2554

DNA FINGERPRINTS, CHROMATOGRAPHIC PATTERNS AND BIOACTIVITIES OF "RANG CHUET"

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmacognosy

Department of Pharmacognosy and Pharmaceutical Botany

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พิภพ สุวรรณชัยเกษม : ลายพิมพ์ดีเอ็นเอ รูปแบบโครมาโทกราฟี และฤทธิ์ทางชีวภาพของรางจืด. (DNA FINGERPRINTS, CHROMATOGRAPHIC PATTERNS AND BIOACTIVITIES OF "RANG CHUET") อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. สุชาดา สุขหร่อง, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร.ชัยโย ชัยชาญทิพยุทธ, 124 หน้า.

Thunbergia laurifolia Lindl. เป็นพืชที่จัดอยู่ในวงศ์ Acanthaceae มีชื่อสามัญว่า "รางจืด" โดยรางจืดเป็นสมุนไพรไทยที่ใช้ในการถอนพิษจากยาฆ่าแมลง แอลกอฮอล์ และโลหะหนัก เป็นต้น อย่างไรก็ตามมีความสับสนในการเรียกสมุนไพร "รางจืด" เนื่องมาจากชื่อและลักษณะภายนอกที่ใกล้เคียงกันของพืชหลายชนิด *T. laurifolia* ซึ่งรู้จักกันโดยทั่วไป มักถูกใช้ทดแทนด้วย *Curcuma* sp., *Rinorea* sp. และ *Crotalaria spectabilis* ซึ่งมีชื่อเรียกเดียวกันว่า "รางจืด" ดังนั้นการตรวจสอบความถูกต้องของสมุนไพรรางจืดจึงเป็นสิ่งจำเป็นเพื่อความถูกต้องและประสิทธิภาพของสมุนไพร ในการศึกษานี้ได้ตรวจสอบองค์ประกอบทางเคมีด้วยวิธีโครมาโทกราฟีแบบผิวบางร่วมกับเทคนิคทางดีเอ็นเอ ได้แก่ อาร์เอพีดีและพีซีอาร์-อาร์เอฟแอลพี จากการทดลองพบว่าสามารถแยก *T. laurifolia* ออกจากสมุนไพรชนิดอื่นที่เกี่ยวข้องได้เป็นอย่างดีด้วยลักษณะเฉพาะทางเคมีของสารในกลุ่มฟลาโวนอยด์และฟีนอลิก และยังพบตำแหน่งนิวคลีโอไทด์ที่แตกต่างกันบนยีน *mat*K ซึ่งสามารถแยกออกจากกันได้ด้วยเทคนิคพีซีอาร์-อาร์เอฟแอลพี โดยการใช้เอนไซม์ตัดจำเพาะ *Dde*I และ *Hae*III พืชในสกุล *Thunbergia* อีกสองชนิด ได้แก่ *T. grandiflora* และ *T. erecta* ยังถูกนำเข้ามาร่วมวิเคราะห์ในลายพิมพ์ดีเอ็นเอชนิดอาร์เอพีดีอีกด้วย จากการใช้ไพรเมอร์เริ่มต้นจำนวน 80 ไพรเมอร์ พบว่ามี 9 ไพรเมอร์ที่ให้ลายพิมพ์ดีเอ็นเอชนิดอาร์เอพีดีที่แม่นยำและชัดเจน ให้แถบดีเอ็นเอชนิดพอลิมอฟิก จำนวน 70 แถบ นอกจากนี้โครมาโทกราฟีแบบสมรรถนะสูงยังถูกนำมาใช้เพื่อตรวจสอบปริมาณของกรดโรสมารินิก ซึ่งเป็นสารที่มีคุณสมบัติในการต้านออกซิเดชันได้ดี และสามารถใช้เป็นสารมาตรฐานในการตรวจสอบสมุนไพร *T. laurifolia* ได้ โดยวิธีที่พัฒนาขึ้นมานี้อาจนำไปใช้ในการตรวจวิเคราะห์คุณภาพของสมุนไพร *T. laurifolia* ในทางการค้า และเนื่องจากสมุนไพรที่เรียก "รางจืด" อาจมาจากพืชต่างชนิดกันและมีฤทธิ์ต้านออกซิเดชันที่ต่างกัน จึงควรตระหนักเมื่อใช้สมุนไพร "รางจืด"

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา เภสัชเวท	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
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KEYWORDS : BIOACTIVITIES / CHROMATOGRAPHIC PATTERNS / DNA FINGERPRINTS / RANG CHUET / *THUNBERGIA LAURIFOLIA*

PIPOB SUWANCHAIKASEM: DNA FINGERPRINTS, CHROMATOGRAPHIC PATTERNS AND BIOACTIVITIES OF "RANG CHUET". ADVISOR: ASSOC. PROF. SUCHADA SUKRONG, Ph.D., CO-ADVISOR: ASSOC. PROF. CHAIYO CHAICHANTIPYUTH, Ph.D., 124 pp.

Thunbergia laurifolia Lindl., a member of the Acanthaceae family, is commonly known in Thai as "Rang Chuet". The "Rang Chuet" herbal drugs have been widely used in Thai traditional medicine for the treatment of poisoning caused by toxic substances such as insecticides, ethyl alcohol, and metals. However, the confusion of the herbal drugs has arisen from the homonymity and close appearances of materials with different botanical origins. *T. laurifolia*, the most known species, has been substituted with other plant materials including *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*. Therefore, authentication of specific "Rang Chuet" herbal drugs is necessary to ensure the authenticity and efficacy. TLC chemical characterization combined with molecular techniques including RAPD and PCR-RFLP offers highly reliable identification procedures. *T. laurifolia* was successfully differentiated from its related species based on the characteristic flavonoids and phenolic compounds. Variation sites in the *mat*K gene of the species can be recognized by the restriction enzymes, *Dde*I and *Hae*III. Two other species of herbaceous *Thunbergia*, *T. grandiflora* and *T. erecta,* were also included in the RAPD analysis. Out of 80 screened RAPD primers, nine primers gave clear and reproducible patterns with a total 70 polymorphic bands. Additionally, an HPLC system was developed for the determination of rosmarinic acid, a potent antioxidant, which represents a suitable biochemical marker for *T. laurifolia*. The established method has potential application at the commercial level for assessing the quality of *T. laurifolia*. Since "Rang Chuet" herbal drugs from different plant origins differ in their antioxidant activities, the use of "Rang Chuet" herbs should be done with care.

Department : Pharmacognosy and Pharmaceutical Botany Student's Signature .....

Field of Study : Pharmacognosy ..... Advisor's Signature .....

Academic Year : 2011 ..... Co-advisor's Signature .....

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CONTENTS

CHAPTER **Page**

LIST OF TABLES

LIST OF FIGURES

FIGURE **Page**

LIST OF ABBREVIATIONS AND SYMBOLS

α	alpha
$[\alpha]_D$	specific rotation measuring at the sodium D line
$AlCl_3$	aluminium chloride
AU	absorption units
β	beta
BHK	baby hamster kidney
bp	basepair
°C	degree Celsius
^{13}C	carbon-13
CE	catechin equivalent
cm	centimeter
δ	chemical shift
d	doublet
dd	doublet of doublets
DEPT	distortionless enhancement of polarization transfer
DMSO-d_6	deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	effective concentration by 50%
e.g.	exempli gratia (for example)
ESI	electrospray ionization
et al.	et alia (and others)
etc.	et cetera (and other things)
EtOH	ethanol
$FeCl_3$	ferric chloride
FRAP	ferric reducing antioxidant power
g	gram

GC	gas chromatography
h	hour
^{1}H	proton
HCl	hydrochloric acid
HMBC	heteronuclear multiple-bond correlation
HMQC	heteronuclear multiple-quantum coherence
HPLC	high performance liquid chromatography
HPTLC	high performance thin-layer chromatography
H_2O	water
Hz	hertz
J	coupling constant
kb	kilobase
kg	kilogram
λ_{max}	maximal absorption wavelength
m	meter
$[M-H]^-$	deprotonated molecular ions in negative ionization mode
MeOH	methanol
MHz	megahertz
µg	microgram
µl	microliter
µM	micromolar
mg	milligram
ml	milliliter
mm	millimeter
mM	millimolar
min	minute
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
m/z	mass-to-charge ratio
Na_2CO_3	sodium carbonate

ng	nanogram
nm	nanometer
NMR	nuclear magnetic resonance
no.	number
OPA	operon-A
OPN	operon-N
PCR	polymerase chain reaction
PDA	photodiode array
pH	negative logarithm of the concentration of hydrogen ions
ppm	part per million
r	correlation coefficient
R_f	retention factor
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rRNA	ribosomal ribonucleic acid
RSD	relative standard deviation
s	second
SCAR	sequence characterized amplified region
SD	standard deviation
sp./spp.	species
TE	trolox equivalent
TLC	thin-layer chromatography
TPTZ	2,4,6-tris(2-pyridyl)-s-triazine
U	unit
UPGMA	unweighted pair group method with arithmetic average
UV	ultraviolet
var.	variety

CHAPTER I

INTRODUCTION

"Rang Chuet" or "รางจืด" in Thai is a local name representing several plants belonging to different families. The most known "Rang Chuet" is *Thunbergia laurifolia* Lindl., the woody climbing plant belonging to the family Acanthaceae. The plant is traditionally used in detoxification and as the first-aid treatment for poisoning with insecticides, ethyl alcohol, arsenic, and strychnine (Thongsaard and Marsden, 2002). Moreover, *T. laurifolia* have been previously reported to exhibit interesting biological activities including antioxidant (Oonsivilai et al., 2008), hepatoprotective (Pramyothin et al., 2005), neuroprotective (Tangpong and Satarug, 2010), and hypoglycemic properties (Aritajat et al., 2004).

However, "Rang Chuet" is a name shared by *Curcuma* sp., *Crotalaria spectabilis,* and *Rinorea* sp. *Curcuma* sp. (Zingiberaceae) is a shrub with underground rhizome. The rhizomes have been widely used for antidote and anti-inflammatory activities in Thai folk medicine (ณรงค์ศักดิ์, 2551). *Crotalaria spectabilis* Roth, a plant in the family Fabaceae, is a short-lived shrub growing in temperate area. The roots have been traditionally used for anti-inflammatory activity while the leaves are used for anti-snake venom properties (สุดารัตน์, 2553). *Rinorea* sp., a shrub in tropical moist range belonging to the family Violaceae, has not been investigated for the activity related to detoxification. However, according to folklore accounts, it has also been used as a detoxifying agent. As above mentioned, all plants named as "Rang Chuet" have been claimed for the treatment of poisoning from drugs to toxic substances (รมย์รวินทร์, 2553). Although all these plants have been regularly used in Thai folk medicine, only *T. laurifolia* has been scientifically examined and proven to have biological activities.

There are two *Thunbergia* species, *T. grandiflora* Roxb. and *T. erecta* (Benth.) T. Anderson, having similar morphology and close relationship with *T. laurifolia*. *T. grandiflora* is a large perennial climber with trumpet-shaped bluish flowers. The leaf has anti-inflammatory effect which is useful for the treatment of urticaria (นิจศิริและธวัชชัย, 2004). *T. erecta*, a small shrub with dense violet flowers, possesses an anti-acaricidal effect (Chungsamarnyart et al., 1992). Both plants have blue trumpet-shaped flowers, resulting in confusion or misidentification of wild-harvested plants. Since the study of *Thunbergia* species has been limited so far, therefore, it is interesting to explore their biological activities and chemical profiles.

Because the name of "Rang Chuet" is used to call several medicinal plants with morphology similar to the aforementioned *Thunbergia* species, "Rang Chuet" herbal products in the form of tea bags and capsules have been confusingly sold in the markets. From the market survey, the scientific name of plant ingredient identified on those products was incongruent with the labeled picture. For this reason, accurate identification is necessary to prove the correct species and also to prevent intentional and inadvertent adulteration of the drugs. Moreover, due to the fact that herbal products may vary in composition and properties, standardization and quality control of the starting herbal material is needed to ensure their safety and efficacy (Liang et al., 2004).

In the current study, we aimed to characterize each "Rang Chuet" species and attempted to differentiate *T. laurifolia* from the others using DNA fingerprinting and chromatographic techniques. DNA molecular method was applied because of its specificity, stability, convenience, and also accuracy (Zhao et al., 2006a). Only a tiny amount of sample is always sufficient for DNA analysis (Novak et al., 2007). The DNA fingerprinting techniques, random amplified polymorphic DNA (RAPD) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), were selected due to their propriety for research purposes and routine uses. Furthermore, chemical profiling techniques, including thin-layer chromatography

(TLC) and high performance liquid chromatography (HPLC), were used to generate chemical profiles of the plants for herbal standardization and differentiation. TLC is the general method for initial screening with a semi-quantitative evaluation for herbal analysis (Liang et al., 2004). The HPLC method is not limited by the volatility or stability of the sample compound; in a consequence it became a popular method for the analysis of herbal medicines (Jiang et al., 2010).

Regarding the use of "Rang Chuet" species, biological assay based on their traditional use is required for clarifying their efficacies. Previously, the antioxidant activities of *T. laurifolia* were proven by Oonsivilai et al. (2008) and some flavonoids and phenolic compounds were suggested to be the active components. Detoxifying effects of *T. laurifolia* were related to antioxidant properties involving hepatoprotective property (Wongchalee et al., 2012), neural protection against lead poison (Tangpong and Satarug, 2010), and antimutagen (Oonsivilai et al., 2007). On the other hand, investigations of antioxidant activities and detoxifying effects in other "Rang Chuet" species have been limited. Moreover, pyrrolizidine alkaloids found in seeds and leaves of *C. spectabilis* can cause hepatotoxicity and pulmonary hypertension in human and animals (Neal et al., 1935; Copple et al., 2003). Therefore, examination of plant extracts for potential activities is considered an important step in evaluating their suitability for application.

T. laurifolia and its related species were characterized by DNA fingerprinting and chromatographic patterns with the aim to reduce the confusion and misidentification of "Rang Chuet" herbal drugs. Biological activity testing was carried out using different antioxidant assays, in order to support the knowledge in health benefits from traditional usages.

CHAPTER II

LITERATURE REVIEW

2.1 Plant Samples

2.1.1 *Thunbergia laurifolia* Lindl.

Thunbergia laurifolia, known as "Rang Chuet" or "รางจืด" in Thai, belongs to Acanthaceae family. The plant was described as a woody vine; stems terete, twining to the left, glabrous, green; leaves opposite, 2-4 times as long as wide, ovate-oblong to oblong-lanceolate, from a cuneate, obtuse, rounded, or subcordate, 3-5 nerved base, acutely acuminate, entire or slightly crenate-dentate, penninerved, glabrous, 7.5-18 cm by 2.5-6 cm; petiole sparsely hispidulous to nearly glabrous, 1-6.5 cm long. Terminal raceme 4-30 cm, dense, sometimes with axillary flowers; pedicels 1.75-3 cm; basal bracteoles of calyx green with violet blotches, persistent till long after the fall of the corolla, 4-5 cm long; calyx outside pubescent and nectariferous only along the outer margin of the top; corolla-tube 3.25-4.75 cm long, inside without a ring of hairs; limb 6-8 cm across, dark blue-violet, rarely white; tube constricted above the conical, much thickened base, above the constriction turbinately widened, with a dorsal longitudinal bulge embrancing stamens and style, yellowish inside; filaments in their lower part very much thickened; 1 cell of posterior anthers ecalcarate, all other cells longitudinally hairy and provided with a subulate spur; pollen grains not echinate; disk yellowish white, including the lower half of the ovary; style thin, apically curved forward; stigmatic lobes broad, flat, anterior one patent, posterior one erect with a pinched upper half; capsule often produced, its basal part 12-16 mm diameter; beak 2.25-3 cm; seeds ventrally smooth, dorsally tuberculate (Figure 2.1A-D) (Backer and Bakhuizen van den Brink, 1965).

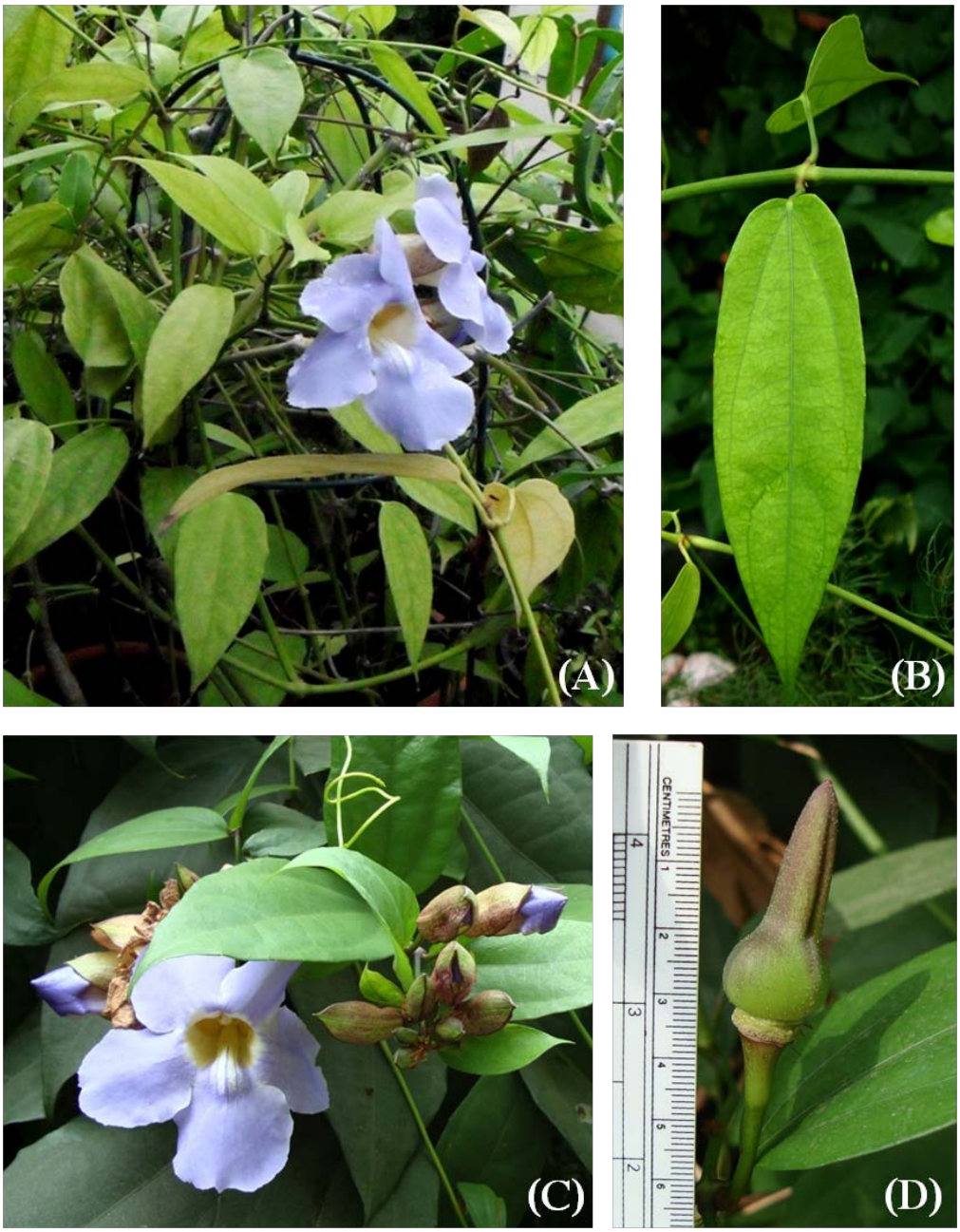


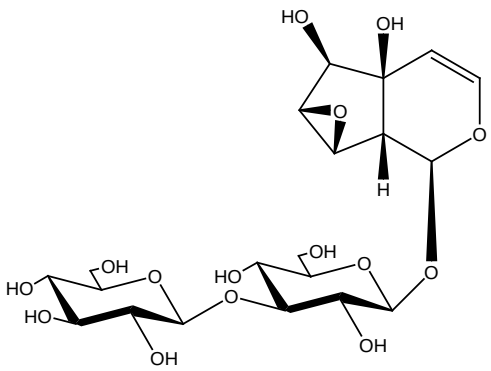
Figure 2.1 *Thunbergia laurifolia* Lindl.

the plant (A); leaves (B); flowering branch (C); and a fruit (D)

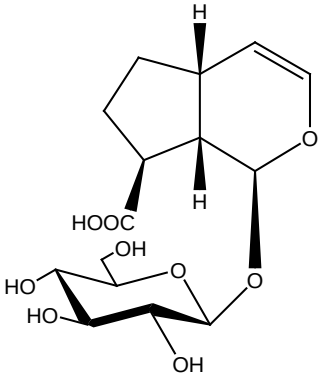
The phytochemistry of *Thunbergia laurifolia* leaves has been studied. Iridoid glycosides, flavonoids, and phenolic acids are main constituents reported from this plant. Two iridoid glycosides of 8-*epi*-grandifloric acid and 3′-*O*-β-glucopyranosyl-stilbericoside have been isolated from the methanolic extract of *T. laurifolia* along with seven glycosides of grandifloric acid, benzyl-β-glucopyranoside, (*E*)-2-hexenyl-β-glucopyranoside, hexanol-β-glucopyranoside, benzyl-β-(2′-*O*-β-glucopyranosyl)-glucopyranoside, 6,8-di-*C*-glucopyranosyl apigenin, apigenin-7-*O*-β-D-glucopyranoside (Kanchanapoom et al., 2002). Leaves and flowers of *T. laurifolia* have also been found to contain phenolic acid of caffeic, protocatechuic, gallic, and chlorogenic as well as the flavonoids, apigenin and delphinidin (Chan et al., 2011). The structures of chemical components reported for *T. laurifolia* are shown in Figure 2.2.

The plant has been traditionally used in Southeast Asia for centuries. In Malaysia, the juice of the leaves is said to be efficacious in case of menorrhagia. It is also applied to the ears for deafness (Bor and Raizada, 1982). In Thailand, the leaves and roots of *T. laurifolia* are used in detoxification and as the first-aid treatment for poisoning with insecticides, ethyl alcohol, arsenic, and strychnine (นันทวันและอรนุช, 2543). It has also been applied for anti-inflammatory and antipyretic effects (วุฒิ, 2540).

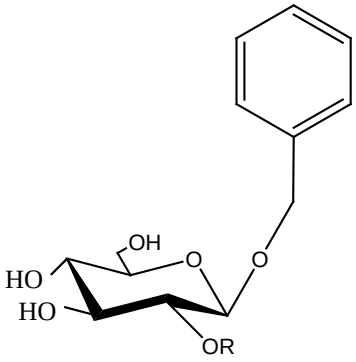
Various physiological and pharmacological activities of *T. laurfolia* were investigated in earlier studies. The aqueous extract of its leaves was found to possess hepatoprotective activity against ethanol-induced liver injury (Pramyothin et al., 2005) and neural protection against lead poisoning in mice (Tangpong and Satarug, 2010). The aqueous extract at appropriate doses increased viability of primary cell cultures of ethanol-treated rat hepatocytes by 2-3 folds and decreased release of alanine transaminase (ALT) and aspatate transaminase (AST). Similarly, the extract reduced neuronal cell death and memory loss caused by lead uptake in mice. The protection of the ethanolic extract of *T. laurifolia* against lead toxicity in Nile tilapia (*Oreochromis niloticus*) by reducing lead levels in the liver and muscle was also reported



3′-*O*-β-glucopyranosyl-stilbericoside

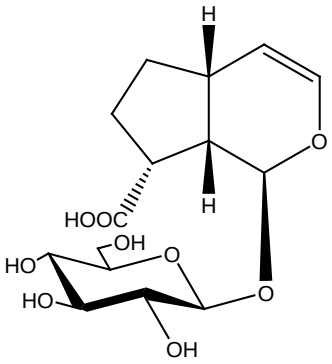


grandifloric acid

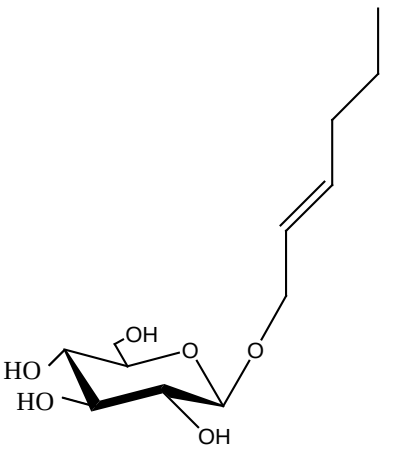


R = H ; benzyl-β-glucopyranoside

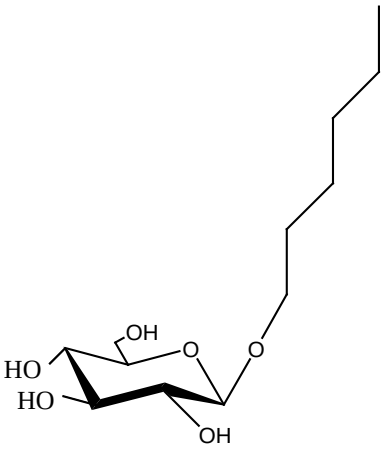
R = β-D-glucose ; benzyl-β-(2′-*O*-β-glucopyranosyl)-glucopyranoside



8-*epi*-grandifloric acid

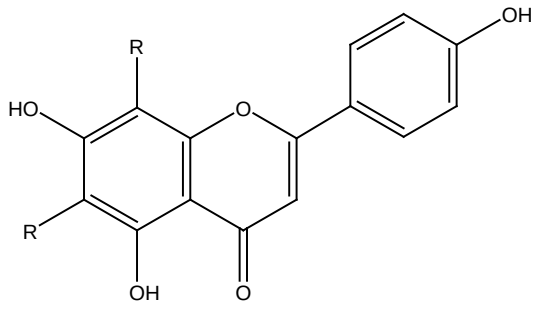


(*E*)-2-hexenyl-β-glucopyranoside

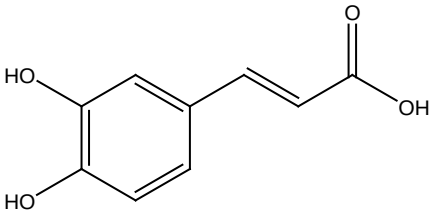


hexanol-β-glucopyranoside

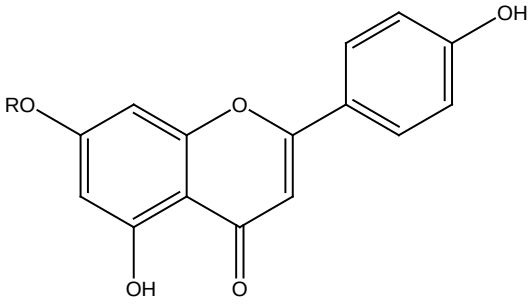
Figure 2.2 Structures of chemical constituents reported for *Thunbergia laurifolia*.



R = glucose ; 6,8-di-*C*-glucopyranosyl apigenin

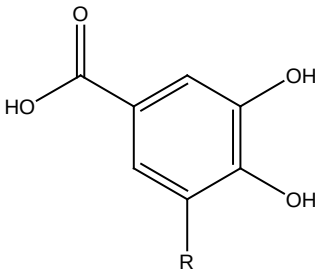


caffeic acid



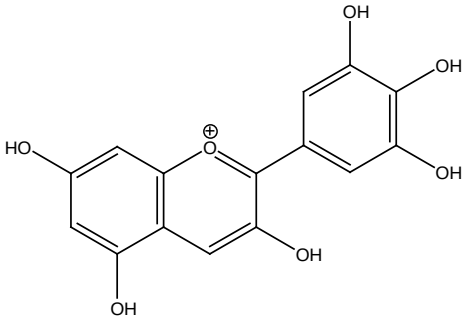
R = H ; apigenin

R = β-D-glucose ; apigenin-7-*O*-β-D-glucopyranoside

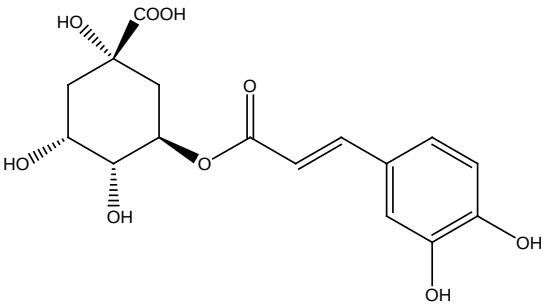


R = H ; protocatechuic acid

R = OH ; gallic acid



delphinidin



chlorogenic acid

Figure 2.2 Structures of chemical constituents reported for *Thunbergia laurifolia* (Continued).

(Palipoch et al., 2011). The leaf extract of the plant was also reported for protective activity against cadmium-induced structural damage in rat kidney (Chattaviriya et al., 2010). *Thunbergia laurifolia* was investigated for detoxifying effects on paraquat-induced toxicity in rats. It was reported that the rats treated with the plant extract had higher survival rates and lower levels of plasma malonaldehyde (Ussanawarong et al., 2000). Moreover, the methanol extract of *T. laurifolia* had potential treatment of drug addiction (Thongsaard et al., 2005). The extract increased significant neuronal activity in the specific brain regions responsible for reward and locomotor behavior and might stimulate dopamine release (Thongsaard and Marsden, 2002).

The aqueous, ethanol, and acetone extracts of *T. laurifolia* were assayed for mutagenic and antimutagenic activity by bacterial reverse mutagenesis. The result indicated that all of three extracts exhibited strong dose-dependent antimutagenic activity (Oonsivilai et al., 2007). Antimutagenic effect was also observed in the aqueous extract of *T. laurifolia* which significantly inhibited the induction of micronuclei in polychromatic erythrocytes induced by *Pueraria mirifica* (Saenphet et al., 2004). Hypoglycemic properties of the aqueous extract of the plant were evaluated in normoglycemic and alloxan-induced diabetic rats. The results showed that a 15-day treatment with the extract (60 mg/ml/day) decreased levels of blood glucose in diabetic rats. The recovery of some β-cells was also found in diabetic rats (Aritajat et al., 2004). The aqueous leaf extract of *T. laurifolia* also possessed anti-inflammatory against carageenin-induced paw edema (Charumanee et al., 1998) and liver fluke-induced inflammation (Wongchalee et al., 2012) in mice. Antioxidant activities of *T. laurifolia* were thoroughly investigated by Chan and Lim (2006), Oonsivilai et al. (2008), and Chan et al. (2011). The aqueous extract of *T. laurifolia* yielded the highest total phenolic contents, free radical scavenging and ferric reducing effects as compared with the ethanol and acetone extracts (Oonsivilai et al., 2008). Total phenolic contents and antioxidant capacities of the commercial tea of *T. laurifolia* showed significantly lower than those of crude material. There is significant difference in antioxidant values between different

batches of the commercial *Thunbergia laurifolia* tea, suggesting that manufacturing procedures have not been standardized (Chan and Lim, 2006; Chan et al., 2011).

Leaves of *T. laurifolia* were tested for antiproliferative activity on SKBR3 human breast cancer cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay. The extract did not show positive antiproliferative activity (Moongkarndi et al., 2004). Moreover, a dried leaf extract of *T. laurifolia* showed very weak cytotoxic activity against BHK and L929 normal cells, and HepG2 and Caco2 cancer cells using MTT assays (Oonsivilai et al., 2008). In addition, a chronic toxicity of the aqueous leaf extract was studied in Wistar rats. The extract at doses ranging from 20 to 2,000 mg/kg/day did not affect their body weight, food consumption, behavior, and general health (Chivapat et al., 2009).

2.1.2 *Thunbergia grandiflora* Roxb.

Thunbergia grandiflora, called as “Soi In Thanin” or “สร้อยอินทนิล” in Thai, is a large woody vine belonging to Acanthaceae family. The plant was botanically described as an extensive much branched liana, young stems strongly angled, glabrous to strongly pilose; flowers on solitary or paired pedicels in upper axils and in long, pendent terminal and axillary racemes or pseudo-racemes, pedicels 3-5 com long, usually 2-several at a node in the racemes; bracteoles broadly oblong, 3-4 cm long, obtuse, sharply mucronate, puberulent and dark glandular-punctate, strongly nerved; calyx reduced to a narrow hispidulous collar with an irregular margin, lobes obsolete; corolla large, tube narrow, contracted in middle, about 8 mm long, throat campanulate, about 4 cm long, 2.5 cm wide at top, limb about 7 cm wide, mauve or white, lobes 2.5-3 cm long, broadly obovate; stamens and pistil included, reaching about half or two thirds the length of throat, anther 7-8 cm long, pilose along margins, spurred at base; stigma lobes equal in length but differently shaped; fruit subglobose, 14 x 13 mm, beak 35 x 6.5 mm, apparently not formed in cultivated clones, said to be self-sterile; leave opposite, the pairs tending to be somewhat unequal, ovate to orbicular cordate, or even sub-reniform, usually somewhat hastately or doubly hastately

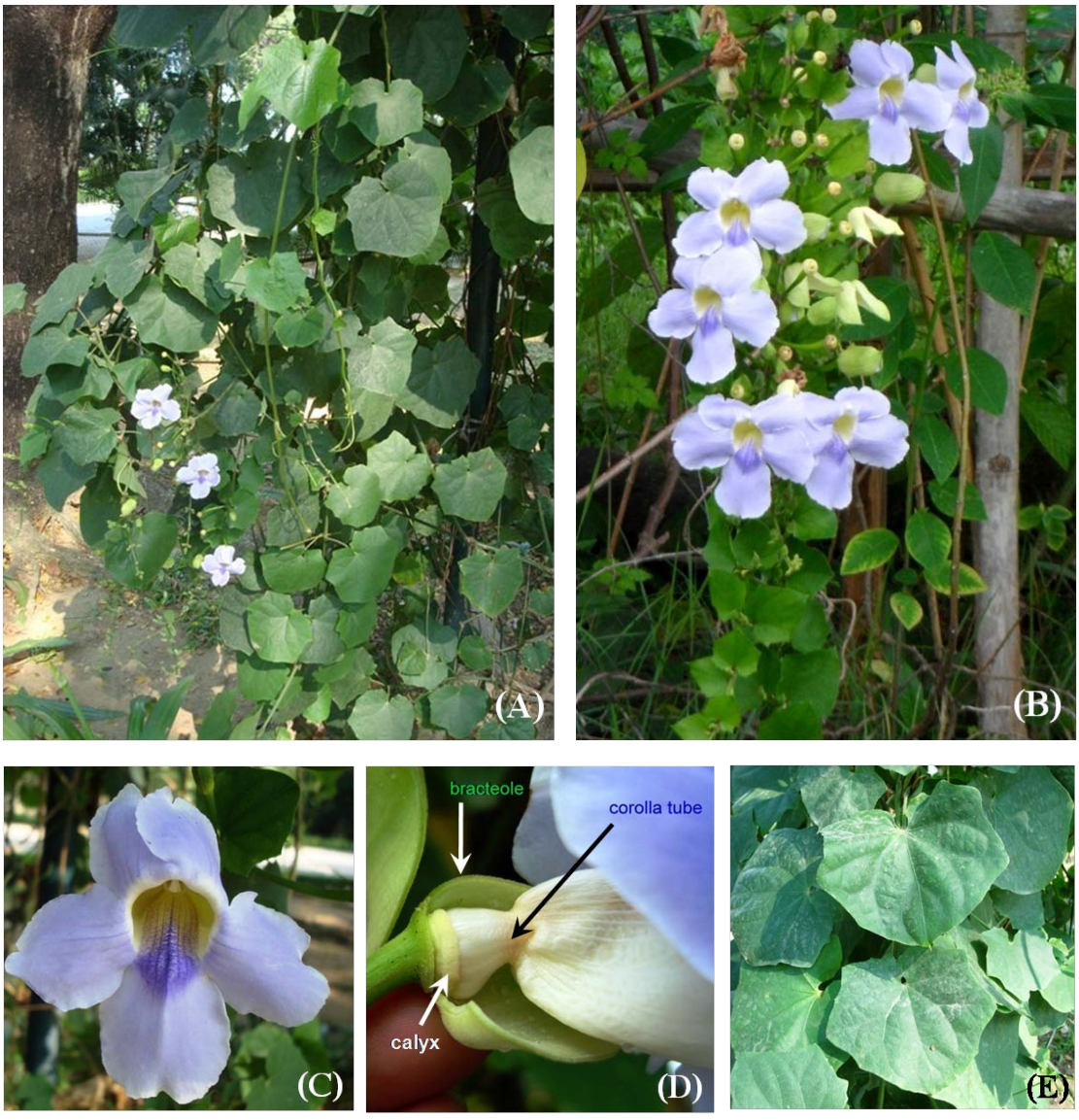


Figure 2.3 *Thunbergia grandiflora* Roxb.

the plant (A); an inflorescence (B); a flower (C); calyx, corolla and bracteole (D); and leaves (E)

lobed, lobes sometimes almost obsolete, to 15 or more cm across, slightly hispidulous to glabrate beneath, scabrous-hispidulous above, apex and lobes acute to slightly acuminate or obtuse, nerves 5, palmate, petiole straight, up to 7 cm, glabrous to pilose, thick and curved at base (Figure 2.3A-E) (Fosberg et al., 1993).

Traditionally, the leaves of *Thunbergia grandiflora* are used for stomach complaints and poultice in Malaya medicine (Bor and Raizada, 1982). In Thai traditional medicine, its leaves have been used for anti-inflammatory activity and treating urticaria (นิจศิริและธวัชชัย, 2547). The methanolic extract of *T. grandiflora* was proved to have moderate analgesic activity. Some chromatographic fractions of the methanolic extract of *T. grandiflora* showed weak activity in terms of both zone of inhibition and spectrum of activity against some gram positive and negative bacteria and fungi. However, all fractions exhibited insignificant activities in antidiarrhoeal test and brine shrimp lethality bioassay (Chowdhury et al., 2012).

Up to date, phytochemistric study of *T. grandiflora* was limited. Two iridoids, isounedoside and grandifloric acid, were isolated along with thunaloside and alatoside in 1996 (Ismail et al., 1996). Stilbericoside was another iridoid found from the leaves of *T. grandiflora* (Jansen and Nielsen, 1989). 5-Hydroxy-4′,6,7-trimethoxy flavone has been recently isolated from the ethanolic extract of the leaves (Chowdhury et al., 2012). In addition, evaluation of phytochemical constituents showed that the flowers of *T. grandiflora* contained alkaloids, phenols, and flavonoids (Jeeva et al., 2011). However, the relation of these phytochemical compounds with biological activities has never been reported.

2.1.3 *Thunbergia erecta* (Benth.) T. Anderson

Thunbergia erecta (Acanthaceae) is called in Thai as “Chong Nang” or “ช้องนาง”. The plant is a shrub 1-2.5 m tall, branched, young internodes strongly angled, glabrous, nodes slightly to prominently woolly; leaves up to 7 x 2.5 cm, usually much smaller, ovate to elliptic or sub-

rhombic, apex acuminate, base obtuse to rounded or subcordate, puberulent on nerves, glabrate, nerves pinnately arranged, 2-4 on each side, network rather obscure except when young, petiole about 5 mm long, slightly margined; flowers on slender pedicels up to 2.5 cm, somewhat dilated distally, axillary on small brachlets; bracteoles broadly oblong, obtuse, 2-2.5 cm long, rather early caducous; calyx a very low collar with about 10 unequal teeth 0.4-1.5 mm long, corolla about 6 cm or less long, tube about 1 cm, slender, throat pale without, yellow within elongate campanulate about 4 cm, slightly dorsiventrally compressed, slightly ventricose, lobes round or broadly ovate, 1.5-2 cm long, spreading deep purple; stamens well included, about half the length of the tube and throat; capsule from a broad base rather gradually narrowed or contracted into a woody beak, 2-2.75 cm long (Figure 2.4A-C) (Fosberg et al., 1993).

Actually, *Thunbergia erecta* is a popular ornamental plant due to its flowering throughout the year. *T. erecta* var. *alba* is a variety with white flowers, but it is by no means as beautiful as the type (Bor and Raizada, 1982). Regarding scientific research, limited studies have been reported on its bioactivity. The screening over 74 Thai plants for of delayed acaricidal effect indicated that 10 samples demonstrated high delayed insecticidal activity on tropical cattle tick (*Boophilus microplus*). The leaf extract of *T. erecta* was one of those exhibiting more than 74% mortality when 5% ethanolic extract was applied (Chungsamarnyart et al., 1992).

Until now, *T. erecta* has never been studied for phytochemical constituents. However, five *Thunbergia* species, *T. laurifolia*, *T. grandiflora*, *T. alata* Boj. ex Sims., *T. fragrans* Roxb., and *T. mysorensis* (Wight) T. Anderson were previously investigated for phytochemical constituents. All of them were found to contain iridoids and iridoid glycosides (Jansen and Nielsen, 1989). The occurrence of iridoid glycosides was utilized for chemotaxonomic characterizing the plants in the family Acanthaceae. The uniqueness and highly derived state of the iridoids demonstrated that the Acanthaceae is an advanced family (Jansen et al., 1988).

Figure 2.4 *Thunbergia erecta* (Benth.) T. Anderson

the plant (A); a flower (B); and a fruit (C)

2.1.4 *Curcuma* sp.

Curcuma sp., known in Thai as "Wan Rang Chuet" or "ว่านรางจืด", is a shrub with underground rhizome belonging to the family Zingiberaceae. The plant is characterized as 0.8-1.2 m tall; petiole 12-30 cm; leaves basal; leaf blade elliptic or elliptic oblong, 35-85 x 13-21 cm, glabrous, base attenuate, oblique, apex caudate. Inflorescences terminal on pseudostems; peduncle 15-22 cm; spike cylindric, 14-20 x 5-8 cm; fertile bracts pale green, ovate, 3.5-4.5 x 2-3.2 cm, apex obtuse; coma bract whitish, ovate or elliptic oblong, apex acute, red and pink. Calyx clavate, apex irregularly 3-toothed; corolla tube pale yellow, funnelform, 2.5-3.2 cm; lobes yellowish, oblong, apex mucronate; lateral staminodes pale yellow, obovate, 2 x 1.1 cm, apex 2-cleft; labellum pale yellow band at center, ovate, 2x 1.6 cm, apex convex, emarginate; rhizomes many branched, white or yellowish inside, aromatic; roots tuberous at tip (Figure 2.5A-E) (author's observation; ณรงค์ศักดิ์, 2551; Wu and Raven, 2000).

Over 80 species are reported in the genus *Curcuma* from Indo-Malayan region (Syamkumar and Sasikumar, 2007) and more than 50 of them are indigenous in Thailand, with only 26 of them being formerly identified (Sirirugsa, 1998). Regarding taxonomic study, there is little taxonomical consensus upon the total number of species that should be recognized. A comprehensive global taxonomic revision of the genus has not yet been attempted. Several problems have hindered a satisfactory systematic treatment of the genus. The original descriptions of many *Curcuma* species are vague and inaccurate, and type specimens are often lacking or fragmentary, which leads to ambiguous assignment of names and usage (Leong-Skornickova et al., 2008). Currently, many DNA-based techniques have been developed to identify and classify the plants in genus *Curcuma*. DNA sequences based on the 18S rRNA, *trn*K gene or *trn*S-*trn*fM region of six *Curcuma* species distributed on Japan and China are different (Cao et al., 2001; Minami et al., 2009). Based on nucleotide dissimilarity on 18S rRNA and *trn*K gene, amplification-refractory mutation system (ARMS) and single-nucleotide polymorphism (SNP)

Figure 2.5 *Curcuma* sp.

the plant (A); an inflorescence (B); flowers (C); roots and rhizomes (D); and rhizomes (E)

were developed to establish more convenient methods for authentication of the *Curcuma* herbal drugs. The methods were successfully applied to discriminate *C. longa* Linn., *C. phaeocaulis* Val., *C. zedoaria* (Berg.) Rosc., and *C. aromatica* Salisb. from each other (Sasaki et al., 2002; Sasaki et al., 2004).

The rhizomes of *Curcuma* sp. called "Rang Chuet" are known as antidote and anti-inflammatory agent in Thai folk medicine. The decoction of its rhizomes is used to treat the abscess and for the treatment of poisoning caused by insecticides and ethyl alcohol (ณรงค์ศักดิ์, 2551). Several researchers have reported different biological actions for the plants in the genus *Curcuma* in various *in vitro* and *in vivo* test models. For instance, different parts of *C. zedoaria* have been found to exhibit antimicrobial, anticancer, antiallergic, and analgesic activities (Lobo et al., 2008). *C. longa* has been reported to exert antioxidant, hepatoprotective, anticarcinogenic, and cardioprotective effects (Cikrikci et al., 2008). Antibacterial, anti-inflammatory, aphrodisiac, and antihypercholesterolemic properties have been recently reviewed for *C. amada* Roxb. (Policegoudra et al., 2011). Most of activities resulted from the presence of curcumin and its derivatives in the plant extract.

Curcuminoids, a chemical group of curcumin and derivatives, is a significant bioactive components reported from several species of *Curcuma*. The naturally occurring ratios of curcuminoids are about 5% bisdemethoxycurcumin, 15% demethoxycurcumin, and 80% curcumin (Ali et al., 2006). Curcumin is an important polyphenolic compound that was isolated from the roots and rhizomes of *C. longa* and related species. A large number of *in vitro* and *in vivo* studies in both animals and human have indicated that curcumin has strong antioxidant, anti-carcinogenic, anti-inflammatory, anti-angiogenic, antispasmodic, antimicrobial, anti-parasitic, and other activities. The mechanisms of some of these actions have been intensively investigated. Curcumin inhibits the promotion/progression stage of carcinogenesis by induction of apoptosis and the arrest of cancer cells in the S, G2/M cell cycle phase (Radhakrishna et al., 2004). The

compound inhibits the activity of growth factor receptors. The anti-inflammatory properties of curcumin are mediated through their effects on cytokines, lipid mediators, eicosanoids, and proteolyitc enzymes (Joe et al., 2004). Curcumin scavenges the superoxide radical, hydrogen peroxide, and nitric oxide, and inhibits lipid peroxidation. These actions may be the basis for many of its pharmacological and therapeutic properties (Ali et al., 2006). Demethoxycurcumin and bisdemethoxycurcumin isolated from *C. zedoaria* were demonstrated to be cytotoxic against human cancer cells (Syu et al., 1998). These two curcumin derivatives were also found to have anti-inflammatory, antioxidant, and antimicrobial properties (Jayaprakasha et al., 2006; Guo et al., 2008; Singh and Jain, 2012).

Besides curcuminoids, terpenoids are other biochemical components mostly abundant in essential oil of *Curcuma* species. The essential oils from rhizomes of *Curcuma* species are important for pharmaceutical therapeutics and industries. Studies on different species of *Curcuma* indicated that sesquiterpenes and sesquiterpene lactones were the major chemical constituents of essential oils. By using gas chromatography-mass spectoscopy (GC-MS), *ar*-curcumene (41.4%) and xanthorrhizol (21.5%) were identified in volatile oils of *C. xanthorrhiza* Roxb. rhizomes (Zwaving and Bos, 1992). 1,8-Cineole (11.2%), α-turmerone (11.1%), and β-caryophyllene (9.8%) were also characterized as the major volatile components in the rhizomes of *C. longa* by using hydrodistillation and GC-MS (Raina et al., 2002). Recently, curzerenone (26.9%), germacrone (12.4%), isocurcumenol (9.7%), β-elemene (6.4%), and curzerene (6.2%) were determined as the major constituents of the essential oil in the rhizomes of *C. sichuanensis* X.X. Chen (Zhou et al., 2007). It was reported that rhizomes of *C. zedoaria* contained curzerenone (22.3%), 1,8-cineole (15.9%), and germacrone (9.0%) (Purkayastha et al., 2006). The rhizome oils were characterized with camphor (28.3%), *ar*-turmerone (12.3%), (Z)-β-ocimene (8.2%), *ar*-curcumene (6.8%) as the major constituents in *C. caesia* Roxb. (Pandey and Chowdhury, 2003). Some of these terpenoids, for instance, xanthorrhizol, germacrone, and isocurcuminol, are natural

bioactive compounds related to anticancer properties (Rukayadi and Hwang, 2007; Zhong et al., 2011; Lakshmi et al., 2011).

2.1.5 *Rinorea* sp.

Rinorea sp., known as “Rang Chuet” or “รางจืด” in Thai, belongs to the family Violaceae. The plant was described as a shrub 1.5 m tall; appendage of connective ovate-triangular, narrowed towards the top, that of the cells of almost equal length. Leaves lanceolate or obovate-lanceolate, acuminate, not or hardly serrate, on either side of midrib with 6-9 lateral nerves, 8.5-18 cm by 2.5-6 cm; petiole 0.25-1.25 cm; stipules subulate-acuminate from a broad base, pubescent, 1.5-3 mm long. Inflorescence sessile, 1.5 cm long peduncle; flower in dense, branched racemiform; filament longer than anther (not computing the appendage); sepals ovate, short-hairy, 1.75-2 mm; petals oblong, short-hairy on the back, 3-3.5 mm; rachises of inflorescence not very thick, not densely scarred; capsule subglobose in shape, 3-valved; seeds broadly ovoid, brown dots (Figure 2.6A-F) (author’s observation; Backer and Bakhuizen van den Brink, 1963). Based on its botanical and morphological characters, *Rinorea* sp. was close to *Rinorea javanica* (Blume) Kuntze (จำลอง, 2552). All of herbarium specimens of *Rinorea* species in the Forest Herbarium, Bangkok remain to be identified, therefore, the plant specimen could not be identified by comparison with voucher samples.

Rinorea sp. called by Thai folk as “Rang Chuet” has been traditionally used for the treatment of poisoning with toxic substance. Until now, there are limited reports regarding the phytochemical components and biological activities of plants in the genus *Rinorea*. To the best of our knowledge, only *R. welwitschii* (Oliv.) Kuntze and *R. anguifera* (Lour.) Kuntze were studied for chemical constituents. Two isoflavones, alpinumisoflavone and di-*O*-methylalpinum isoflavone were obtained from reversed-phase HPLC analysis of the stem-bark of *R. welwitschii* (Stewart et al., 2000). Mauritianin, (+)-syringaresinol, and camptothecin were isolated from *R. anguifera* using bioassay-guided fractionation. The result demonstrated that these compounds

Figure 2.6 *Rinorea* sp.

the plant (A); leaves (B); inflorescences (C, D); a fruit (E); and a granulated capsule (F)

stabilized the topoisomerase I-DNA covalent binary complex, which could be used as topoisomerase I inhibitor (Ma et al., 2005).

With regard to ethnobotanical uses, *Rinorea anguifera* and *R. floribunda* (King) Merr. have been used to treat fever while *R. elliptica* (Oliv.) Kuntze have been used for the treatment of snake venom. *R. kunstleriana* Taub. has the potential to treat syphilis (Duke, 1998). In Gabon, *R. subintegrifolia* (P. Beauv.) Kuntze is used by traditional healers in decoction with lemons to treat malaria symptoms. It is also used as an aroma agent during ancestral cults (Lekana-Douki et al., 2011). By screening for allelopathy in Brazilian Amazon trees, *R. racemosa* (C. Martius) Kuntze possessed allelopathic activity against lettuce seeds (Campbell et al., 1989).

2.1.6 *Crotalaria spectabilis* Roth

Crotalaria spectabilis, belonging to the subfamily Faboideae (Papilionaceae) of the family Fabaceae, has the common name as “Ma Hing Men” or “มะหิ่งเม่น”. The plant is locally known in Thai as “Rang Chuet” or “รางจืด”. The plant is an herb, erect, 0.6-1.5 m tall; braches terete, glabrous; stipules ovate-triangular, approximately 1 cm; racemes terminal, 20-30-flowered; bracts ovate-triangular, 7-10 mm; pedicel 1-1.5 cm; bracteoles inserted at or apical to middle of pedicel, linear, approximately 1 mm. Calyx 2-lipped, 1.2-1.5 cm, glabrous; lobes broadly lanceolate-triangular, longer than tube; corolla pale yellow; standard veined purplish red, suborbicular to oblong, 1-2 cm, base with 2 appendages, apex obtuse to retuse; wings obovate, approximately 2 cm; keel rounded about middle, with a fairly short and slightly incurved twisted beak exserted beyond calyx; legume broadly oblong, 2.5-3 x 1.5-2 cm, 20-30-seeded, shortly stipitate, glabrous; seeds smooth. Leaves simple; petiole 2-8 mm; leaf blade oblanceolate to narrowly elliptic, 7-15 x 2-5 cm, thin, abaxially appressed silky pubescent, adaxially glabrous, base broadly cuneate, apex obtuse and mucronate (Figure 2.7A-C) (Wu et al., 2010).

Figure 2.7 *Crotalaria spectabilis* Roth

the plant (A); an inflorescence (B); and a leaf (C)

A number of reports concerned the toxicity of *Crotalaria spectabilis* in various animal species. The toxic factor was isolated and identified as monocrotaline (Neal et al., 1935). This pyrrolizidine alkaloid mainly found in seeds of *C. spectabilis* produced pulmonary and intrahepatic hemorrhage and liver necrosis in rats (Harris et al., 1942), mice (Neal et al., 1935), cattle (Sanders et al., 1936), and turkeys (Allen, 1963). Veno-occlusive cirrhosis has been attributed to *C. spectabilis* intoxication in cattle (Sanders et al., 1936), swine (McGrath et al., 1975), and humans (Bras and Hill, 1956). The plant was not found to contain other pyrrolizidine alkaloids, such as spectabiline, senecionine, intergerrimine, grantaline-like, trichodesmine-like compounds, which are major components in some *Crotalaria* species (Williams and Molyneux, 1987; Flores et al., 2009). Except for the seeds of *C. spectabilis*, less phytochemical investigation was done on the other parts of the plant. The result of phytochemical screening showed that alkaloids, tannins, unsaturated sterols, and organic acids were presented in all parts of plant including roots, leaves, pods, and stems (Tinker and Lauter, 1956). However, the structures of these compounds have never been identified.

The plant has been used in Thai traditional medicine for the treatment of snake venom (สุดารัตน์, 2553). The root of this plant also possessed anti-inflammatory effect (สุดารัตน์, 2553). In USA, the plant was promoted as a green manure, cover crop, and forage and hay crop (Patterson, 1982), because of the persistence of its dormant seeds. However, seeds and foliage of the plant which are poisonous to livestock, poultry, and wildlife, should be considered during cultivation and harvesting.

2.2 DNA Fingerprinting

DNA-based molecular techniques have been widely used for authentication of plant species of medicinal importance. The molecular technique includes all the aspects of drug

development and discovery, where biotechnology-driven applications play an important role (Joshi et al., 2004). DNA molecular method has proven specific, stable, convenient, and also accurate. The markers created by DNA-based technique are not influenced by age, physiological condition as well as environmental factors (Chan, 2003). Hence, the technique is most useful for the identification of fragile, animal-derived and precious drugs, and it is especially suitable to identify confused herbal medicines with close relationship (Zhao et al., 2006a). Nowadays, various types of DNA-based molecular techniques are utilized to evaluate DNA polymorphism. One of the most frequently used techniques is DNA fingerprinting which does not require conventional sequencing of PCR products and detailed comparison of individual sequence (Peng et al., 2010).

2.2.1 Random amplified polymorphic DNA (RAPD) technique

Unlike traditional PCR analysis, random amplified polymorphic DNA (RAPD) does not require any specific knowledge of the DNA sequence of the target organism. The identical decamer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primer sequence. For example, no fragment is produced if primers annealed too far apart, or 3′ ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site which was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel.

Because of the simplicity and low cost of the RAPD technique, it has found a wide range of applications in many areas of biology. One of the most widely used applications of the RAPD technique is the identification of markers linked to traits of interest without the necessity for mapping the entire genome. The markers are employed for plant species discrimination coupled with methods of identification involving taxonomy, physiology, and histology (Joshi et al., 2004). Various medicinal plants were characterized using RAPD fingerprints to assure the authenticity

and efficacy in herbal medicine. For example, dried fruit samples of *Lycium barbarum* L. were differentiated from its adulteration using RAPD primers (Zhang et al., 2001). Identification of *Desmodium gengeticum* DC. samples in herbal market was also achieved by RAPD profiles (Irshad et al., 2009). RAPD profiling has been utilized for the authentication of *Senna angustifolia* Vahl. as well (Khan et al., 2011). However, the six plants in this study, *Thunbergia laurifolia*, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*, have never been subjected to molecular phylogenetic analysis and botanical identification using RAPD fingerprinting.

The advances in DNA techniques have had a great impact in addressing problems in many aspect of biology. Application of DNA-based approaches to population genetic studies has been limited, probably due to the need for large samples of individuals from each population to provide as accurate estimate of allele and genotype frequencies. The relatively high cost, the requirement for sophisticated equipment and well-trained personnel, and low speed are other limiting factors in population genetic studies. The RAPD technique has received a great deal of attention from population geneticists because of its simplicity and rapidity in revealing DNA-level genetic variation, and therefore has been praised as the DNA equivalent of allozyme electrophoresis. The data obtained from RAPD has been frequently used for phylogenetic studies and generally supported existing taxonomies based on morphology, isozymes, and RFLPs. The most recent studies on the utility of RAPD markers in the phylogeny of tomatoes (Abd El-Hady et al., 2010) and the genus *Lycopersicon* (Kochieva et al., 2002) gave support to classical hypotheses of their phylogenetic relationships.

In conclusion, RAPD technique can be performed in a moderate laboratory for most of its applications. Despite the reproducibility problem, the RAPD method will probably be important as long as other DNA-based techniques remain unavailable in terms of cost, time, and labor.

2.2.2 Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique

PCR-RFLP is a technique wherein genomic DNA is treated with one or more restriction enzymes which cut the DNA whenever certain specific sequence of bases occurs, thus generating a number of fragments of the DNA of varying lengths. In some individuals, random changes in the DNA will cause one or more sites to be lost or may otherwise cause variation between individuals in these fragment lengths. If the DNA is placed on a gel, and an electric field applied, the differing sized fragments will move at varying distances across the gel. The DNA can then be rendered visible by a variety of methods, yielding a pattern of bands, sometimes described as similar to a supermarket barcode (Lewontin, 1994). It is relatively easy to determine that two samples are different, if one has a band that the other lacks, but it is far more difficult to determine, on the basis of identical banding patterns, that two samples must have come from the same individual (Panneerchelvam and Norazmi, 2003).

PCR-RFLP has been commonly applied for species identification of plants, animals, bacteria, etc. and used in paternity cases or criminal cases to determine the source of a DNA sample. It has been successfully used for identification of several medicinal plants and crude drugs. Recently, chloroplast *trn*K intron sequences of six *Eleutherococcus* species were determined and compared in order to establish a convenient and sensitive method for authentication. Based on genetic polymorphism, the restriction site of enzyme *Ase*I was specifically found in *E. senticosus* (Rupr. & Maxim.) Maxim. sequence. Thus, the species can be easily differentiated from the others using PCR-RFLP technique with *Ase*I enzyme (Zhu et al., 2011). Besides, molecular authentication of *Atractylodes*-derived crude drugs was done with the help of PCR-RFLP and direct sequencing of chloroplast *trn*K. Based on polymorphism in the restriction site for *Hindf*I in *trn*K fragment, it was possible to discriminate between the *Atractylodes* rhizome and *Atractylodes lancea* (Thunb.) DC. rhizome (Mizukami et al., 2000).

Moreover, PCR-RFLP can be used in the detection of mutations that is useful for determination of the disease status of an individual. The method has also been used to measure recombination rates which can lead to a genetic map with the distance between RFLP loci. The fact that PCR requires only minute amounts of DNA suggests that herbarium collections will become more valuable as sources of material for DNA study. Automated sequencing is likely to replace manual methods for most large-scale comparative sequencing projects. However, at lower taxonomic levels, where restriction site studies can more rapidly and economically access large portions of the chloroplast genome, restriction site studies will continue to provide an important source of phylogenetic information (Olmstead and Palmer, 1994). Until now, restriction site variation of PCR-amplified chloroplast DNA (PCR-RFLP) has been applied for phylogenetic reconstruction in plants at various taxomonic levels. For example, PCR-RFLP was able to provide unambiguous identification and disposition of true species of Indian *Citrus* including *C. indica* Tanaka, *C. latipes* (Swingle) Tanaka, *C. macroptera* Mont. var. *anammensis*, *C. jambhiri* Lush., *C. karna* Raf., *C. megaloxycarpa* Lush., *C. limettoides* Tan., and *C. pseudolimon* Tan.. The result has been useful in understanding the pattern of molecular differentiation and interpreting the problem origin and systematic position of *Citrus* genotypes (Jena et al., 2009).

Based on this study, the sequence of chloroplast *mat*K gene was used to provide genetic information for identifying the botanical origin of “Rang Chuet” herbal drugs by PCR-RFLP method. However, the *mat*K nucleotide sequences of the six plants are not available in the international database. Three species of herbaceous Thunbergia, *T. laurifolia*, *T. grandiflora*, and *T. erecta* have only the sequencing information on three chloroplast DNA regions, *rps16*, *rpl16*, and *trnT-trnL* (Borg and McDade, 2008). The sequences were utilized for molecular phylogenetic study along with the plants in subfamily Thunbergioideae. The result showed that *T. grandiflora* was placed as sister to *T. laurifolia* while *T. erecta* was distant (Figure 2.8). Similarly, limited DNA sequencing data and molecular research is available for *Crotalaria spectabilis*. The plant

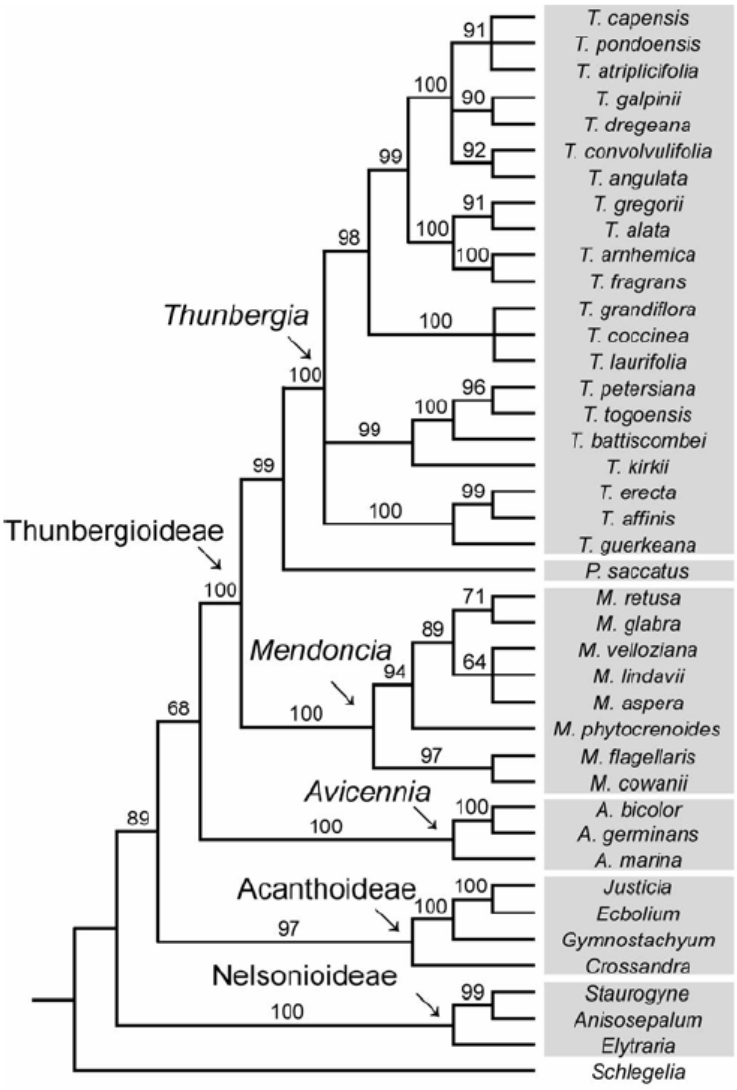


Figure 2.8 Phylogenetic trees of Thunbergioideae species based on analyses of the combined data set. Strict consensus of eight shortest trees resulting from maximum parsimony analysis (source: Borg and McDade, 2008).

was known for partial sequence of ITS region which used for molecular-based systematic of Indian *Crotalaria* taxa. For *Curcuma* sp. and *Rinorea* sp., DNA molecular analysis might be beneficial for assisting the identification of the plants.

2.3 Chromatographic Profiling

Chromatographic fingerprint analysis by which multiple compounds in single herbal drugs and finished products can be identified represents a rational approach for the quality assessment of herbal medicines. It utilizes chromatographic techniques, gas chromatography (GC), high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), etc. to construct specific patterns of recognition for multiple compounds in herbal drugs. The entire pattern of compounds can then be evaluated to determine not only the absence or presence of desired markers or actives but the complete set of ratios of all detectable analytes (Xie et al., 2006). Thus, chromatographic fingerprint analysis of herbal drugs represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality, and ensuring the consistency and stability of herbal drugs and their related products.

2.3.1 Thin-layer chromatography (TLC) method

TLC is widely used in laboratories throughout the world for plant analysis and quality control. The method has the advantages of many-fold possibilities of detection in analyzing herbal medicines (Sherma, 2000). It is rather simple and can be employed for multiple sample analysis. For each plate, more than 30 spots of samples can be applied simultaneously in one time. Although GC, HPLC, and TLC are highly complementary, TLC has advantages in many analyses, including simplicity of operation, the availability of many sensitive and selective reagents for detection and confirmation without interference of the mobile phase, the ability to

repeat detection and quantification at any time with changed parameters, and cost effectiveness (Sherma, 2000).

By application, TLC was applied for detection and determination of various compounds from medicinal plants. For example, two alkaloids, lupanine and 3β-hydroxylupanine were determined in agricultural products by silica gel TLC with cyclohexane-diethylamine (7:3), visualization with Dragendorff reagent (Touche et al., 1997). Flavonol glycosides from the seed coat of a new Manteca-type dry bean (*Phaseolus vulgaris* L.) were separated on silica gel with chloroform-methanol (4:1 and 1:1) and chloroform-methanol-water (8:2:1) and detected by spraying with 20% sulfuric acid, heating, and viewing under UV light (Beninger et al., 1998). Flavonoids from honey bush tea (formononetin, naringenin, hesperitin, medicagol, mangiferin, and luteolin) were analyzed by analytical TLC on silica gel using double development with each of two mobile phases, hexane-benzene-acetone-methanol (40:40:15:5) and chloroform-benzene-acetone (50:45:5), and detection with sulfuric acid-formaldehyde (40:1) (Ferreira et al., 1998). Antimicrobial saponins of *Yucca schidigera* Roezl ex Ortgies were identified by TLC on silica gel with 2-octanol-3-methylbuthanol-acetone-methanol-water (3:5:2:1:1) and visualization by spraying with 10% sulfuric acid and heating at 120°C for 20 min (Killeen et al., 1998). However, TLC characterization has never been used for characterizing chemical profiles of the plants used in this study including *Thunbergia laurifolia*, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*.

In summary, the advantages of using TLC to analyze the chemical components of herbal medicines are its simplicity, versatility, high velocity, specific sensitivity, and simple sample preparation. The method is also used in various pharmacopoeias to provide first characteristic fingerprints of herbs (Liang et al., 2004). Thus, TLC is a convenient method of determining the quality and possible adulteration of herbal products.

2.3.2 High-performance liquid chromatography (HPLC) method

HPLC is a popular method for the analysis of herbal medicines (Xie et al., 2006). The method is basically a highly improved form of column chromatography. The development in the HPLC system is to use stationary phases with an internal diameter of 4.6 nm and small particle size which leads to a great increase in column efficiencies and high pressure. The column of normal phase HPLC is filled with tiny silica particles, but silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface in reversed phase HPLC. HPLC also allows the users to use a very much smaller particle size for the column packing material which gives a much greater surface area for interactions between the stationary phase and the molecules flowing past it. This allows a much better separation of the components of the mixture.

HPLC method is widely applied because it can be used to analyze almost all the compounds in the herbal medicines. The method is easy to learn and use and is not limited by the volatility or stability of the sample compound (Liang et al., 2004). Thus, over past decades, HPLC has received the most extensive application in the analysis of herbal medicines. Generally, one or two markers or pharmacologically active components in herbs and or herbal mixtures were currently employed for evaluating the quality and authenticity of herbal medicines. By application, HPLC equipped with photo diode array (PDA) detector was used to analyze benzophenones and biflavonoids in eight *Garcinia* species. These phenolic compounds contributed to antioxidant activity reported for *Garcinia mangostana* Linn. As a result, *G. intermedia* (Pittier) Hammel demonstrated the highest antioxidant activity and the highest total phenolic contents among the eight *Garcinia* species. It was concluded that *Garcinia intermedia* might be a new source of antioxidants and the described HPLC method is useful to determination of the contents of compounds in the plant and its preparations (Acuna et al., 2012). Additionally, the HLPC with fluorescence detection was applied to determine the amount of five anthraquinones in the plants known “Rhubarb”. The result indicated that the highest amount of

anthraquinones was found in *Rheum palmatum* L. collected from Shaanxi, China whilst *Polygoni multiflori* has the least amount of anthraquinones. This method for simultaneous determination of five anthraquinones was sensitive, reproducible, fully validated and suitable for use as a tool for routine quality assurance and standardization of raw material and commercial preparations containing rhubarb (He et al., 2009).

HPLC was used by Thongsaard et al. (2005) to compare different batches the methanolic extracts of *Thunbergia laurifolia* using gradient 0-100% acetonitrile from 0-20 min as the mobile phase. However, it is noticed that no biochemical maker or pharmacologically active components in plant was used in their chromatographic analysis (Figure 2.9). Moreover, the HPLC method has also been applied to characterize the phytochemical constituents in acetonic, ethanolic, and aqueous extracts of *T. laurifolia* (Oonsivilai et al., 2007). Lutein and major chlorophyll derivatives including chlorophyll a, chlorophyll b, pheophytin a, and pheophorbide a, were detected in the acetonic and ethanolic extracts but were not found in the aqueous extract (Figure 2.10). However, these lipophilic compounds are generally found in the leaves of plants. The chemical fingerprint of these compounds might be not applicable for assessing the quantitative herbal compositions of herbal products. It seems necessary to determine the other phytochemical constituents of *T. laurifolia* in order to ensure the reliability and repeatability of pharmacological and clinical research and to enhance product quality control.

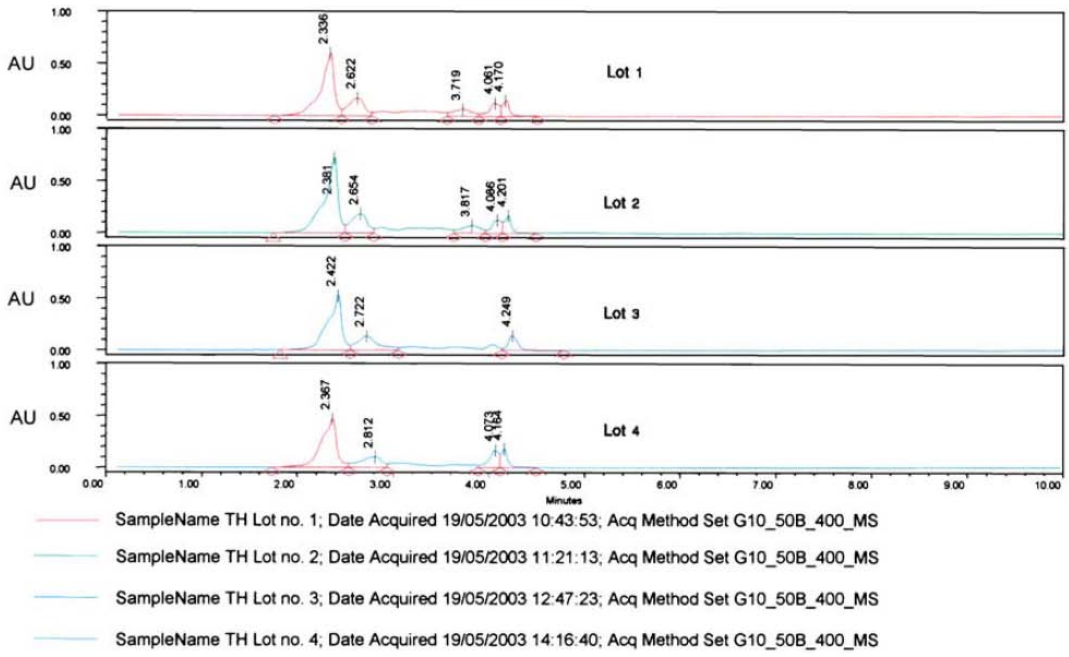


Figure 2.9 HPLC chromatograms of four 80% methanol extracts of *Thunbergia laurifolia* (1 mg/ml) obtained using UV detection at an absorbance of 210 nm; Water Symmetry C18 (4.6x250 mm) column; 25 µl injection volume; flow rate 0.8 ml/min; and gradient 0-100% acetonitrile from 0 to 20 min (source: Thongsaard et al., 2005).

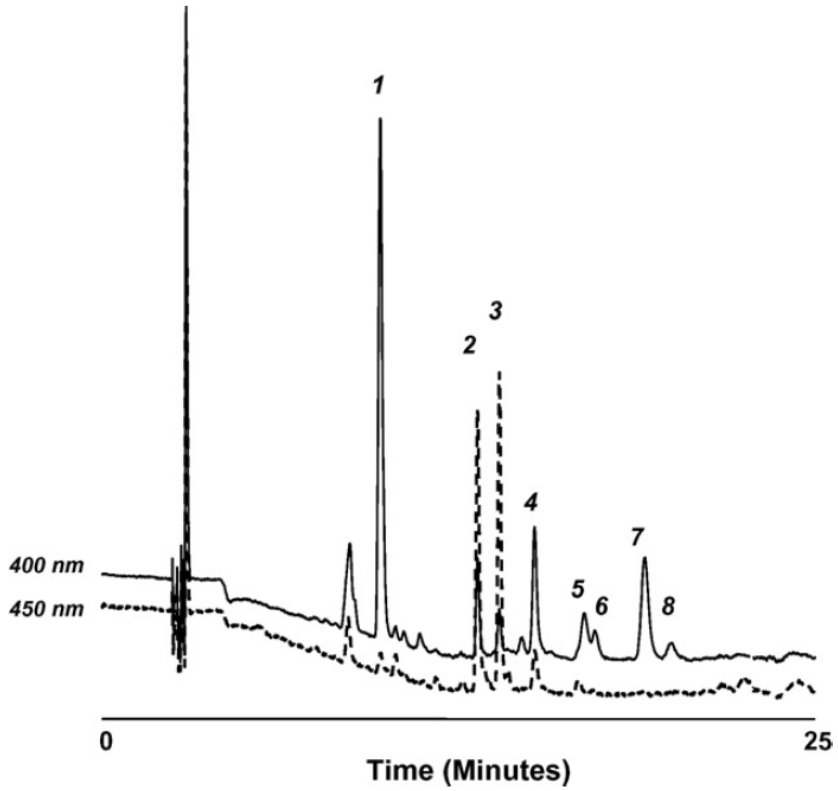


Figure 2.10 HPLC separation of lutein and chlorophyll derivatives from *Thunbergia laurifolia* acetonic extract. Peak identifications: (1) pheophorbide *a*; (2) lutein; (3) chlorophyll *b*; (4) chlorophyll *a*; (5) pheophytin *b*; (6) pheophytin *b'*; (7) pheophytin *a*; (8) pheophytin *a'*. Online electronic absorption spectra were collected from 250 to 600 nm. Responses at 400 and 450 nm are shown (source: Oonsivilai et al., 2007).

2.4 Antioxidant Assays and Determination of Total Phenolics

The adverse effects of oxidative stress on human health have become a serious issue. Under stress, our bodies produce more reactive oxygen species (e.g., superoxide anion, hydroxyl radical, and hydrogen peroxide) than enzymatic antioxidants (e.g., superoxide dismutase, glutathione peroxidase, and catalase) and non-enzymatic antioxidants (e.g., ascorbic acid, α-tocopherol, glutathione, carotenoids, and flavonoids). This imbalance leads to cell damage and health problems (Krishnaiah et al., 2011). Numerous experimental data indicate that free radical mechanisms contribute to ethanol-induced liver injury (Kono et al., 2000, Gramenzi et al., 2006). Increased generation of oxygen- and ethanol-derived free radicals has been observed at the microsomal level, especially through the intervention of the ethanol-inducible cytochrome P450 isoform (CYP2E1). Furthermore, an ethanol-linked enhancement in free radical generation can occur at the level of the cytosolic molybdo-flavoenzymes xanthine oxidase and aldehyde oxidase, as well as through the mitochondrial respiratory chain. The preferential localization of CYP2E1 and xanthine oxidase within perivenous hepatocytes is likely playing a role in the higher incidence of ethanol toxicity on these hepatocytes as compared to the periportal ones (Nordmann et al., 1992). It has been proposed that the vulnerable fatty liver is injured by reactive oxygen species generated from microsomal, mitochondrial, and other hepatocellular pro-oxidant pathways when the antioxidant defenses are critically lowered. Antioxidant enzymes reduce the levels of lipid peroxides as well as hydrogen peroxide and are important in preventing lipid peroxidation and maintaining the structure and function of biologic membranes (Koruk et al., 2004). Free radical mechanisms also appear to be implicated in the injuries of many toxin-induced on various extrahepatic tissues. Organophosphate insecticides are known to stimulate enzymatic and non-enzymatic antioxidant system and lipid peroxidation in the erythrocytes and serum of rats (Lukaszewicz-Hassian and Moniuszko-Jakoniuk, 2002). Redox-active metals, e.g., iron, copper, and chromium, undergo redox cycling whereas redox-inactive metals, e.g., lead,

cadmium, mercury, and others deplete cells' major antioxidants, particularly thiol-containing antioxidants and enzymes. Either redox-active or redox-inactive metals may cause an increase in protection of reactive oxygen species such as hydroxy radical, superoxide radical or hydrogen peroxide. Enhanced generation of reactive oxygen species can overwhelm cells' intrinsic antioxidant defenses, and result in a condition of oxidative stress. Cells under oxidative stress display various dysfunctions due to lesions caused by reactive oxygen species to lipids, protein, and DNA (Ercal et al., 2001).

Natural plant antioxidants can serve as a type of preventive medicine and therefore solve the imbalance of reactive oxygen species in the cells body (Krishnaiah et al., 2011). Antioxidants including phenolic compounds (e.g., flavonoids, phenolic acid, and tannins) have diverse biological effects, such as anti-inflammatory, anti-carcinogenic, and anti-atherosclerotic effects, as a result of their antioxidant activity. Among the six plants studied here, only *Thunbergia laurifolia* has been found to exhibit significant antioxidant properties. Flavonoids and phenolic compounds in the extract might contribute to important biological and pharmacological functions of *T. laurifolia* (Oonsivilai et al., 2008). However, antioxidant properties of the other plants including *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis* remain to be investigated. As proved by many previous studies, a lot of medicinal plants have been reported to possess the antioxidant potential. For instance, ethyl acetate extract of *Acacia auriculiformis* A.Cunn. ex Benth. showed scavenging activity against DPPH radical and high ferric reducing power. Stem bark of the plant contained tannins, triterpenoid saponins, and phenolic compounds which are probably responsible for antioxidant effect (Singh et al., 2007). The methanolic extract of the bark of *Ficus microcarpa* L.f. exhibited strong antioxidant activity when assayed by the DPPH-scavenging method, ABTS-scavenging method, superoxide radical scavenging assay, and the β-carotene linoleic acid system. Triterpenoids involving lupenyl acetate, friedelin, glutinol, epifriedelinol, and β-amylin, and the phenolic compounds have been reported to be present in *F. microcarpa* (Ao et al., 2008). Moreover, the cultured cells of

Carthamus tinctorius L. were found to contain Kinobeon A. This alkene compound has been demonstrated as being a remarkably strong antioxidant by quenching various reactive oxygen species and inhibiting lipid peroxidation (Kanehira et al., 2003; Kambayashi et al., 2005).

The antioxidant properties could be evaluated using a number of methods. For instance, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP), total radical trapping antioxidant potential (TRAP) assay, oxygen radical absorbance capacity assay, nitro blue tetrazolium reduction (NBT) assay, 2,2′-azinobis(3-ethylbenztriazoline-6-sulphonic acid) (ABTS) radical scavenging method, and trolox equivalent antioxidant capacity (TEAC) method. Moreover, the antioxidant extracts were evaluated in terms of total phenols, total flavonoids, total flavonols, phenolic acids, catechins, lignans, and tannins. The phenolic concentration was determined using the Folin-Ciocalteu method. Although, many methods are available to determine antioxidant activity, it is important to employ a consistent and rapid method. While each method has its own merits and drawbacks, it has been found that DPPH and FRAP assays were the most common methods regularly used by several studies (Krishnaiah et al., 2011).

2.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging assay was first described by Blois (1958) and was later modified slightly by numerous researchers. It is one of the most extensively used antioxidant assays for plant samples. DPPH is a stable free radical that reacts with compounds that can donate a hydrogen atom. This method is based on the scavenging of DPPH through the addition of a radical species or an antioxidant that decolorizes the DPPH solution. The antioxidant activity is then measured by the decrease in absorption at 515 nm.

2.4.2 Ferric reducing antioxidant power (FRAP) assay

FRAP assay is a technique to determine the total antioxidant power interpreted as the reducing capacity. The ferric reducing ability assay was first given by Benzie and Strain (1996). The method is based on the ability of the antioxidants to reduce Fe^{3+} to Fe^{2+}. The Fe^{2+} is measured spectrophotometrically via determination of its colored complex with 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), which has a high absorbance at 595 nm.

2.4.3 Determination of total phenolic contents

Total phenolic contents in the plant extract are usually determined by the Folin-Ciocalteu reagent method. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxide (Javanmardi et al., 2003).

CHAPTER III

RAPD FINGERPRINTING OF "RANG CHUET"

The counterfeits of *Thunbergia laurifolia* have been commonly found in Thai herbal markets and have arisen based on their similarity in appearance and shared common names. Therefore, the comprehensive identification method is required for differentiation of the herbal materials. Random amplified polymorphic DNA (RAPD) technique is considered as a useful tool to distinguish between different botanical species due to its low cost and good reliability of RAPD markers and also because it is a relatively simple procedure and previous sequence information is not required. Herein, the RAPD method was used to facilitate the rapid detection of medicinal materials of *T. laurifolia*, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*. In addition, the classification tree based on the DNA banding patterns of the plants studied is also discussed.

3.1 Materials and Methods

3.1.1 Plant materials

Thunbergia laurifolia, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis* were collected from different locations in Thailand (Table 3.1). The plants were identified by the Associate Professor Thatree Phadungcharoen (Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand), and then confirmed by comparison with related herbarium specimens at the Forest Herbarium (Department of National Parks, Wildlife and Plant Conservation, Thailand). Voucher specimens were deposited in the Museum of Natural Medicines (Faculty of

Table 3.1 Details of the plant samples used in RAPD analysis.

Plant name	**Code**	**Family**	**Location (Province)**	**Voucher No.**
Thunbergia laurifolia	TL	Acanthaceae	Bangkok	SS-0809101
Lindl.			Nakhon Si Thammarat	SS-1009102
			Buri Ram	SS-1009103
			Prachin Buri	SS-1009104
			Nakhon Pathom	SS-0510105
			Nonthaburi	SS-0510106
			Uttaradit	SS-1010107
			Chiang Mai	SS-1110108
T. grandiflora Roxb.	TG	Acanthaceae	Bangkok	SS-1109201
			Prachuap Khiri Khan	SS-1209202
			Bangkok	SS-0510203
			Bangkok	SS-0810204
			Nakhon Pathom	SS-1210205
T. erecta (Benth.) T.	TE	Acanthaceae	Bangkok	SS-0809301
Anderson			Nakhon Pathom	SS-0510302
			Ubon Ratchathani	SS-1010303
			Chiang Mai	SS-1110304
Curcuma sp.	Cur	Zingiberaceae	Chachoengsao	SS-0909401
			Ratchaburi	SS-0710402
			Prachin Buri	SS-0710403
Rinorea sp.	Rin	Violaceae	Chachoengsao	SS-0909501
			Bangkok	SS-1209502
Crotalaria spectabilis Roth	CS	Fabaceae	Nakhon Pathom	SS-0809601
			Bangkok	SS-1209602

Pharmaceutical Sciences, Chulalongkorn University, Thailand). Young leaves were collected from each plant and stored at -80°C until the DNA isolation.

3.1.2 DNA isolation and RAPD-PCR amplification

A total of 100 mg of leaves from each sample was frozen using liquid nitrogen and ground with a mortar and pestle to obtain a fine powder. The isolation of the total DNA from the powder was performed using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The quality of the genomic DNA was estimated after electrophoresis on an agarose gel and staining with ethidium bromide.

Eighty RAPD primers (OPA 01-20, OPD 01-20, OPN 01-20, and OPO 01-20) obtained from Operon Technologies (California, USA) were tested in the initial screening against 6 selected species to choose the suitable RAPD primers. The DNA templates were added to 25 µl of the PCR reaction mixture consisting of 1X amplification buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 U of *Taq* DNA polymerase (Fermentas, Canada), and 0.4 µM random decamer primers (Operon Technologies, USA). The amplification was performed using a DNA thermal cycler (Bio-Rad, USA) with an initial denaturation of 2 min at 95°C, followed by 40 cycles of 45 s at 95°C, 1 min at 35°C, 2 min at 72°C, and a final elongation of 5 min at 72°C. The amplification products were separated on 1% agarose gels and stained with ethidium bromide. The RAPD fragments were photographed using a UV transilluminator and analyzed with a gel documentation system (Bio-Rad, USA).

3.1.3 RAPD data analysis

The RAPD bands were scored as 0 or 1 for the absence or presence of bands, respectively. Only clear and reproducible bands were scored as 1. The molecular weights of the bands were estimated based on DNA markers (1 kb Plus, Invitrogen, USA). The similarity index was calculated from the data that was generated using Nei and Li's similarity index coefficient

(Nei and Li, 1979). The dendrogram was constructed based on the similarity matrix data using the unweighted pair group method with arithmetic averages (UPGMA) clustering and FreeTree software (Pavlicek et al., 1999). To evaluate the strength of the resulting branches, bootstrap probabilities were calculated using 1,000 bootstrap resampling data with the above software.

3.2 Results and Discussion

The confusion surrounding the identification of herbs in traditional medicine has been reported in many studies (Zhong et al., 2009; Bauer and Franz, 2010). Some of the various reasons that have been reported for such confusion have included herbs from multiple sources, region-specific herbs, similarities in appearance, the complexity of processed products and confusing nomenclature (Zhao et al., 2006a). Specifically, the confusion regarding crude *Thunbergia laurifolia* preparations in herbal markets has been attributed to confusing nomenclature and the similarities to other plants in terms of appearance. However, curative effects of the substitutes or adulterants have not been reported. Moreover, the mistaken use of *Crotalaria spectabilis* in place of *T. laurifolia* can increase the risk of hepatotoxicity. Therefore, the correct identification and quality assurance of the starting material is an essential prerequisite to ensure the reproducible curative effects of herbal medicines.

RAPD analysis has been widely used to differentiate between many medicinal plants and their close relatives or adulterants, including *Derris* spp. (Sukrong et al., 2005), *Desmodium* spp. (Irshad et al., 2009), *Encephalatos* spp. (Prakash and van Staden, 2008), and *Phyllanthus* spp. (Manissorn et al., 2010). The advantages of this technique include its speed, simplicity and the requirement for only small amounts of DNA (Mahmood et al., 2010). Indeed, RAPD analysis has been successfully used for taxonomic and systematic classification and phylogenetic or genetic diversity studies in plants (Neog et al., 2010). Although RAPD markers have few disadvantages,

some of the problems regarding the reproducibility of RAPD data and scoring errors have been addressed (Skroch and Nienhuis, 1995). As the standardization of DNA isolation techniques and PCR reaction conditions are key in limiting errors, the use of intense staining, clearly resolvable bands and unvaried DNA samples have been shown to provide consistent results (Aruna et al., 1993).

In the current RAPD study, all six plants, *Thunbergia laurifolia*, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*, were differentiated from each other based on unique band patterns that were obtained after RAPD amplification. RAPD was performed in triplicate to test the reproducibility. Because each species which was collected from different localities showed the same pattern of RAPD profiles, an individual representative sample of each species was selected. Nine RAPD primers, OPA-03, OPA-04, OPA-07, OPA-10, OPN-02, OPN-10, OPN-12, OPN-16, and OPN-18, produced clear and reproducible polymorphic fragments in all six of the species, and all of the primers detected significant polymorphisms in the genomic DNA analysis. The sequences of nine RAPD primers chosen are listed in Table 3.2. A total of 164 amplified bands with 70 polymorphic bands were scored from the population (Figure 3.1A-I). Polymorphic fragments were used to distinguish and to construct the dendrogram among the six species. The results demonstrated that different primers generated different fragment numbers and lengths. The size of the amplification products ranged from 0.4 kb to 4.9 kb (Table 3.2). The largest number of RAPD bands (25 bands) was detected using the OPA-04 primer (Figure 3.1B), whereas the smallest number of bands (8 bands) was generated with OPA-17 (Figure 3.1C).

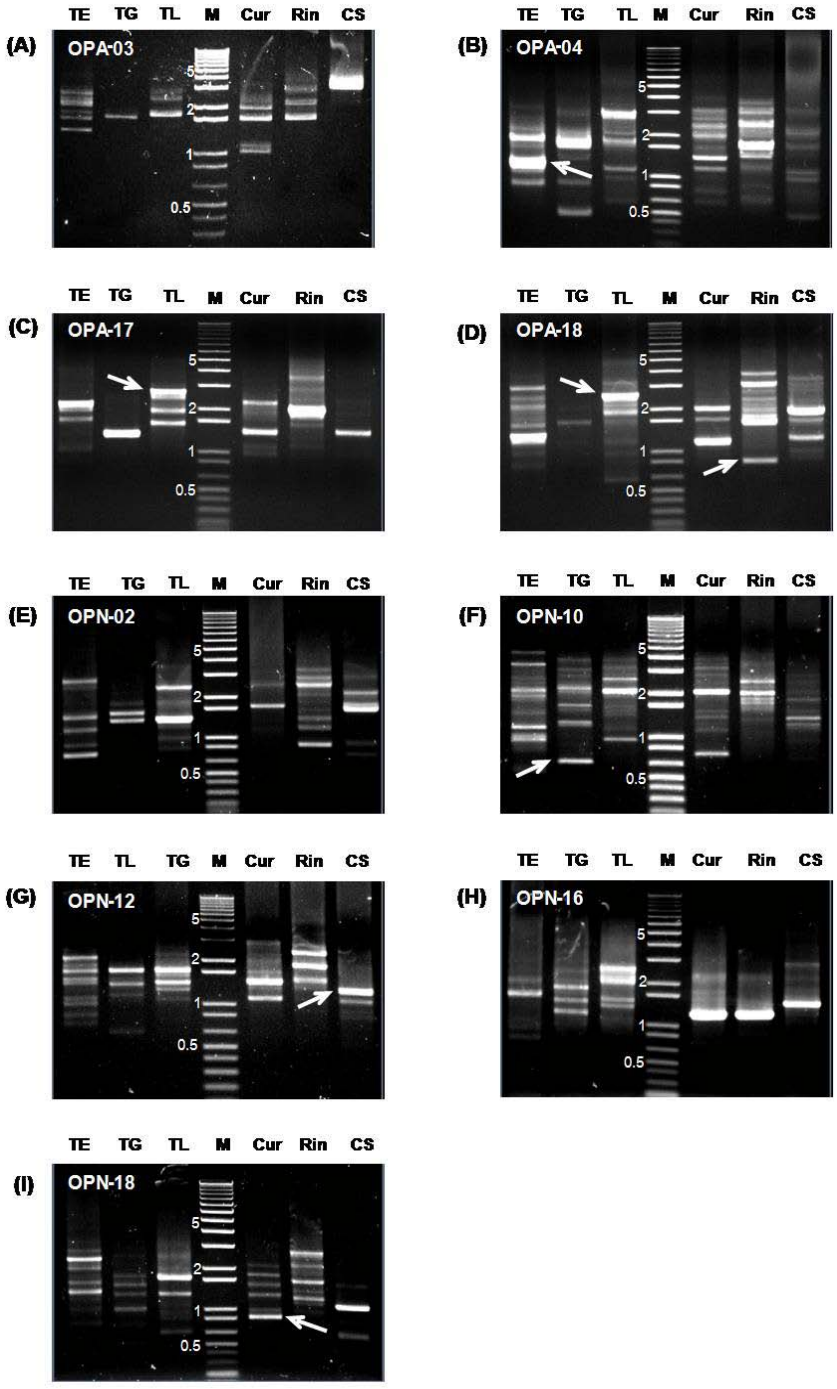


Figure 3.1 RAPD fingerprint of the six plant species obtained with the OPA-03 (A), OPA-04 (B), OPA-17 (C), OPA-18 (D), OPN-02 (E), OPN-10 (F), OPN-12 (G), OPN-16 (H) and OPN-18 (I) primers. TE: *Thunbergia erecta*, TG: *T. grandiflora*, TL: *T. laurifolia*, M: 1 kb Plus DNA marker (sizes shown in kb), Cur: *Curcuma* sp., Rin: *Rinorea* sp., CS: *Crotalaria spectabilis*. The unique fragments of each species are indicated with arrows.

Table 3.2 The sequence of the oligonucleotide primers used for the RAPD analysis and the banding patterns obtained from the six plant species.

Primer	Primer sequence (5′ to 3′)	No. of bands	Size of bands	No. of polymorphic bands[a]	No. of unique bands[b]
OPA-03	AGTCAGCCAC	19	1040 - 3830	4	15
OPA-04	AATCGGGCTG	25	420 - 3760	10	15
OPA-17	GACCGCTTGT	8	1000 - 3770	4	4
OPA-18	AGGTGACCGT	19	510 - 4050	7	12
OPN-02	ACCAGGGGCA	16	590 - 3450	8	8
OPN-10	ACAACTGGGG	22	490 - 4890	16	6
OPN-12	CACAGACACC	20	580 - 3370	6	14
OPN-16	AAGCGACCTG	13	670 - 2660	5	8
OPN-18	GGTGAGGTCA	22	530 - 2700	10	12

[a] polymorphic band: more than one band present at a given locus, but not entire population

[b] unique band: only one band individually occur at a given locus

Unique RAPD bands were recognized in all of the investigated species. For *Thunbergia laurifolia*, the OPA-17 (Figure 3.1C) and OPA-18 (Figure 3.1D) primers consistently amplified an intense band at 2.8 and 2.6 kb, respectively. However, these bands were absent in the other species. Our results indicate that these fragments can be used as markers to distinguish between authentic *T. laurifolia* and its related species. Similarly, RAPD fragments of approximately 560 bp using OPN-10 (Figure 3.1F) and 1.2 kb using OPA-04 (Figure 3.1B) were only detected in *T. grandiflora* and *T. erecta*, respectively. Furthermore, a 710 bp fragment that was generated by the OPN-18 primer (Figure 3.1I) was a specific fragment in the *Curcuma* sp., OPA-18 (Figure 3.1D) produced a specific band of 730 bp in the *Rinorea* sp., and OPN-12 (Figure 3.1G) generated a unique band of 1.3 kb in *Crotalaria spectabilis*. Using OPN-12 (Figure 3.1G), the intense fragments were approximately 1750 bp in *T. laurifolia*, *T. grandiflora*, and *T. erecta*. Matching bands of similar size were not observed in the other species and therefore these monomorphic bands were useful to characterize the species in the genus *Thunbergia*. However, patterns from other primers indicated the presence of genetic variability, which was also beneficial for identification of the different *Thunbergia* species.

The pair-wise comparisons of the RAPD profiles which were based on both the shared and unique amplification products were used to generate a similarity index. Among the six species, the range of genetic similarity was from 0.1235 to 0.3137 (Table 3.3). The highest genetic similarity value was detected between *T. erecta* and *T. laurifolia* (0.3137), whereas the lowest similarity value was found between *Crotalaria spectabilis* and *T. laurifolia* (0.1235).

Table 3.3 Similarity matrix of the six plant species generated using Nei and Li's similarity coefficient.

Species		**TE**	**TG**	**TL**	**Cur**	**Rin**	**CS**
Thunbergia erecta	**TE**	1.0000					
T. grandiflora	**TG**	0.2222	1.0000				
T. laurifolia	**TL**	0.3137	0.3095	1.0000			
***Curcuma* sp.**	**Cur**	0.2245	0.2000	0.1522	1.0000		
***Rinorea* sp.**	**Rin**	0.2056	0.2247	0.2574	0.2474	1.0000	
Crotalaria spectabilis	**CS**	0.2069	0.2029	0.1235	0.1558	0.2791	1.0000

A dendrogram was constructed according to the UPGMA cluster analysis using Nei and Li's similarity coefficient. Based on the dendrogram, the six species were categorized into three major groups (Figure 3.2), which strongly correlated with the parents. The first group was comprised of *Curcuma* sp., which was the only monocotyledon plant in this study and was clearly separated from the other species based on molecular phylogeny. In the second group, *Thunbergia erecta* and *T. laurifolia* were clustered together, with a similarity value of 0.3137 and were comparatively separated from *T. grandiflora*. Our results contrast with those of a previous study, which has reported the comparison of the *trnT-trnL*, *rpl-16* and *rps-16* sequences in Thunbergioideae species and had identified a close relationship between *T. laurifolia* and *T. grandiflora* (Borg and McDade, 2008). However, the similarity value that we calculated from the RAPD data between *T. laurifolia* and *T. erecta* (0.3137) was similar to the value of *T. laurifolia* and *T. grandiflora* (0.3095). This result indicates the close genetic relationship of *Thunbergia* species compared with other species. The third group consisted of the *Rinorea* sp. and *C. spectabilis*. Although *Rinorea* sp. and *Crotalaria spectabilis* are classified in different botanical families, the plants were genetically placed together in the third group. The arrangement of these six plants in this dendrogram is relatively correlated with the plant classification and floral taxonomy following botanical principles.

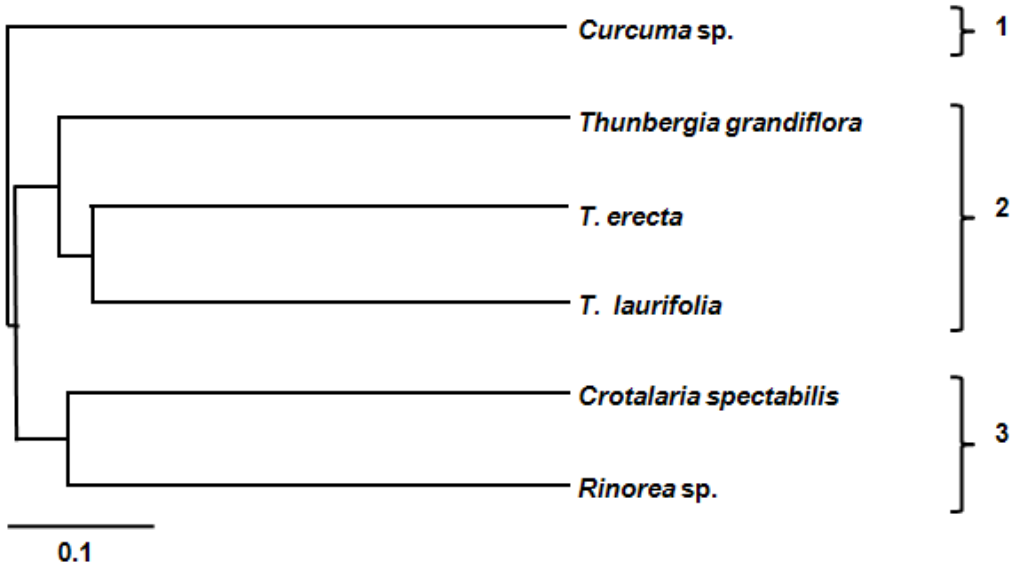


Figure 3.2 Dendrogram produced by UPGMA cluster analysis of RAPD data showing the genetic relationship among the six plant species. The similarity scale is indicated at the bottom left corner.

3.3 Conclusions

The confusion of “Rang Chuet” herbal drugs has arisen based on confused nomenclature and similarity in appearance of plant material. RAPD techniques have now become a popular means for the identification and authentication of herbal medicines from plants and animals. Our RAPD study demonstrated that *Thunbergia laurifolia* was successfully distinguished from its related species based on their molecular signatures. The results affirm RAPD analysis as a technique that is able to examine the phylogenetic relationship of different plant species. Additionally, sequence characterized amplified regions (SCARs) can be further developed to identify specific and sensitive DNA markers.

CHAPTER IV

TLC AND PCR-RFLP FINGERPRINTS AND ANTIOXIDANT ACTIVITIES OF "RANG CHUET"

Commercial preparations of "Rang Chuet" in tea, capsule, and powder forms in herbal markets are claimed to have antidote, antipyretic, and anti-inflammatory effects. However, the confusion of "Rang Chuet" herbal drugs has arisen because of the similarity in the vernacular name of several plant materials. Therefore, two tasks must be accomplished to address these problems and ensure the safety and efficacy of the "Rang Chuet" herbal drugs: (1) the biological potencies of the herbal drugs must be clarified based on their medicinal uses; and (2) the herbal drugs derived from different plants must be distinguished from each other.

4.1 Materials and Methods

4.1.1 Chemicals and equipment

The 2,2-diphenyl-1-dipicrylhydrazyl (DPPH) radical, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu reagent, ascorbic acid, and catechin were purchased from Sigma-Aldrich (Missouri, USA). A UV-160A spectrophotometer (Shimadzu, Japan) was used for spectrophotometric measurements. A Victor3 microplate reader (Perkin Elmer, USA) was used for the DPPH assay.

The DNeasy Plant Mini Kit and Dream *Taq* polymerase were obtained from Qiagen (Hilden, Germany) and Fermentas (Ontario, Canada), respectively. The restriction enzymes *Dde*I and *Hae*III were purchased from New England Biolabs (Massachusetts, USA). PCR was

performed in a C1000 thermal cycler (Bio-Rad, USA) and documented with a Gel Doc XR^{+} system (Bio-Rad, USA).

4.1.2 Plant materials and preparation of plant extracts

Details of the plants recognized as "Rang Chuet" are shown in Table 4.1. Dried leaves of *Thunbergia laurifolia* and *Crotalaria spectabilis* and dried rhizomes of *Curcuma* sp. were collected from various locations and identified by Associate Professor Thatree Phadungcharoen at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand. Voucher specimens of the plant materials were preserved in the Museum of Natural Medicines at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Plant extracts were prepared as follows. Plants were ground into a powder with an electric blender. The fine powder (20 g) of each species was independently extracted with 200 ml of either ethanol or distilled water. The ethanolic extracts were macerated at room temperature for 72 h, whereas the aqueous extracts were boiled on a hotplate for 30 min (Thongsaard and Marsden, 2002). Subsequently, each extract was filtered with Whatman No. 1 filter paper. The filtrates obtained from the ethanolic and aqueous extracts were individually dried by evaporation at 50°C or lyophilization, respectively. The residues were redissolved with the extracted solvents in the concentration 2 mg/ml and 20 mg/ml for the application of antioxidant assays and TLC characterization, respectively.

4.1.3 DPPH radical scavenging activity

Radical scavenging activity was evaluated with a standard spectrophotometric assay employing the DPPH radical in a 96-well microplate with modifications (Kintzios et al., 2010). The plant extracts were diluted into 5 concentrations (0.2 to 1.5 mg/ml). A 20-µl aliquot of the samples and 180 µl of a 0.1 mM methanolic DPPH solution were added to each well. The plate

Table 4.1 Details of the three plant samples known as “Rang Chuet”.

Plant	**Code**	**Family**	**Location (Province)**	**Voucher specimen**	**Accession number**
Thunbergia laurifolia Lindl.	TL	Acanthaceae	Bangkok	SS-0809101	AB649970
			Nakhon Si Thammarat	SS-1009102	
			Buri Ram	SS-1009103	
			Prachin Buri	SS-1009104	
			Nakhon Pathom	SS-0510105	
			Nonthaburi	SS-0510106	
			Uttaradit	SS-1010107	
			Chiang Mai	SS-1110108	
Curcuma sp.	CUR	Zingiberaceae	Chachoengsao	SS-0909401	AB649974
			Ratchaburi	SS-0710402	
			Prachin Buri	SS-0710403	
Crotalaria spectabilis Roth	CS	Fabaceae	Nakhon Pathom	SS-0809601	AB649973
			Bangkok	SS-1209602	

was covered with aluminum foil and incubated at room temperature for 30 min. The absorbance was measured at 510 nm against a solvent blank to estimate the radical scavenging capacity of each antioxidant sample. The free radical scavenging activities of the plant extracts were compared with ascorbic acid, which served as a positive control (Arabshahi-Delouee and Urooj, 2007). The scavenging capacity was reported as the effective concentration at which 50% of the DPPH radicals were scavenged (EC_{50} value).

4.1.4 Ferric-reducing antioxidant power

Ferric-reducing antioxidant power (FRAP) was measured according to the method developed by Benzie and Strain (1996). The FRAP working solution was prepared from 300 mM acetate buffer, pH 3.6 (3.1 g $CH_3COONa.3H_2O$ and 16 ml of CH_3COOH adjusted to 1000 ml with distilled water), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1. The plant extract solutions (2 mg/ml, 20 µl) was allowed to react with 600 µl of the FRAP working solution for 4 min at room temperature. The absorbance of the colored product was then measured at 595 nm. A Trolox standard solution was used to prepare the calibration curves. The values are expressed as µM Trolox equivalents (TE) per g dry weight.

4.1.5 Total phenolic content

The total phenolic content (TPC) was analyzed according to the method developed by Amarowicz et al. (2010), with modifications. Five concentrations of catechin were prepared for use as standards. Each reaction mixture contained 20 µl of a standard catechin solution or 20 µl of a sample solution (2 mg/ml), 770 µl distilled water, and 60 µl Folin-Ciocalteu reagent. After 1 min but before 8 min, 200 µl of a saturated Na_2CO_3 solution was added. The reagent blank was prepared by replacing the sample solutions with 20 µl of the extracted solvent. After 2 h of reaction at ambient temperature, the absorbance of each reaction mixture was measured at 760 nm. The value of the total phenolic contents was expressed as catechin equivalents (CE) per g of

dry weight. The values were determined from the linear equation based on the calibration curve of catechin.

4.1.6 TLC characterization

The plant extracts (0.2 mg) were applied to a silica gel TLC plate as 8-mm-wide bands. For the ethanolic extract, the plates were developed in chloroform-methanol-formic acid (7:3:0.5). For the aqueous extract, the bands were eluted with a solvent solution composed of ethyl acetate-formic acid-acetic acid-water (10:0.8:0.8:2.8). The chromatograms were evaluated under UV light at 254 and 365 nm to detect the target compounds. To detect flavonoids, the TLC plate was also sprayed with a 1% $AlCl_3$ solution and monitored under UV light at 365 nm (Gage et al., 1951). In the aqueous extracts, phenolic compounds were detected with 20% Na_2CO_3 solution and Folin-Ciocalteu reagent (Keith et al., 1958) because no flavonoids were detected in these extracts. The TLC bioautography assay of radical scavenging activity with the DPPH radical was also employed. The chromatogram was sprayed with a 0.5 mM methanolic solution of DPPH to detect antioxidant compounds.

4.1.7 DNA isolation and the PCR-RFLP method

A total of 100 mg of the plant samples was frozen and ground to obtain a fine powder. The total DNA was isolated with the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol.

A pair of amplification primers, the matK-465F primer (5′-ACT AAT ACC CTA TCC TGT CCA T-3′) and the matK-1483R primer (5′-CCA AAT ACC AAA TCC GWC CTC TA-3′), was designed to amplify an approximately 1 kb fragment of the *mat*K gene from *Thunbergia laurifolia*, *Curcuma* sp., and *Crotalaria spectabilis*. The 50 µl reaction mixture was composed of 1X amplification buffer, 2.5 mM $MgCl_2$, dNTPs (0.2 mM each), the two primers (0.5 µM each), 1.25 U Dream *Taq* Polymerase, and 20 ng of total DNA as the template. The PCR amplifications

were performed with a DNA thermal cycler with the cycling conditions of a hot start at 95°C for 2 min; 40 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 2 min; and a final extension at 72°C for 5 min. A 5-µl sample of the resulting PCR product was subjected to electrophoresis on a 1.0% agarose gel, and the remaining sample was sequenced with the primers listed above. The determined nucleotide sequences were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database (Table 4.1).

RFLP patterns were analyzed with the CLC Sequence Viewer software (Joo et al., 2010). According to the preliminary computerized analysis, *Dde*I and *Hae*III were selected as suitable candidate enzymes for the identification of the three species. A 10-µl aliquot of the PCR product was digested with *Dde*I and *Hae*III for 5 h at 37°C in a total volume of 20 µl according to the manufacturer's protocol. The restriction products were examined by gel electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining with a gel documentation system.

4.2 Results and Discussion

Confusing herb nomenclature is clearly a significant problem in the herbal markets of many countries (Zhao et al., 2006a). In Belgium, *Aristolochia fangchi* has been mistakenly used as "Fangji" instead of *Stephania tetrandra* S. Moore in sliming drugs (Vanherweghem et al., 1993). In Hong Kong markets, *Aristolochia mollissima* Hance and *Solanum lyratum* Thunb. share the same common Chinese name "Baimaoteng" (Zhao et al., 2006b). When one herb is used in place of another, the curative effects are not as expected. Similarly, the confusion caused from using the same name ("Rang Chuet") for the three different herbs, *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* sp., in herbal drugs and herbal preparations is currently a problem in Thai herbal markets. Each of these three species is claimed to have detoxifying effects; however, there is insufficient information to prove whether they are, in fact, equally

effective as herbal drugs. The identification of the plant materials is also necessary to assure the safe and effective use of the "Rang Chuet" herbal drugs.

4.2.1 Antioxidant effects and phenolic content of plant extracts

Reactive oxygen species are known mediators of many toxin-induced organ injuries. Most of the available data concern the liver, gastric mucosa, the central nervous system, the heart, and the testes (Nordmann et al., 1992). Antioxidants are known to protect cells of the body organ from damage and/or death caused by free radical. Antioxidant properties of different plant extracts are also known to relate with several detoxifying properties including hepatoprotective (Sohn et al., 2003), gastroprotective (Jaiswal et al., 2011), cytoprotective (Yoo et al., 2008), and immunomodulatory (Awah et al., 2010).

In the present study, two assays, the DPPH radical scavenging assay and the FRAP assay, which are based on different reaction mechanisms, were employed to evaluate the antioxidant capacity of *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* sp. In the DPPH analysis, the scavenging activities of the three species against the DPPH radical were concentration-dependent (Figure 4.1). As shown in Table 4.2, the scavenging capacity of the ethanolic extract of *C. spectabilis* (EC_{50} value = 26.56 ± 1.41 μg/ml) was notably higher than that of *T. laurifolia* (EC_{50} value = 119.97 ± 12.95 μg/ml) or *Curcuma* sp. (EC_{50} value > 150.00 μg/ml). The ethanolic extracts of *C. spectabilis* also showed the highest ability reducing Fe^{3+} to Fe^{2+} (203.53 ± 38.20 μM TE/g). This is the first report of the radical scavenging activity and reducing power of an ethanolic extract of *C. spectabilis*, which represents a good source of a potential natural antioxidant. Of the aqueous extracts, *T. laurifolia* possessed both the highest DPPH scavenging activity (EC_{50} value = 86.04 ± 3.94 μg/ml) and the highest ferric-reducing power (148.41 ± 8.46 μM TE/g). In addition, the aqueous extract of *T. laurifolia* exhibited greater antioxidant properties than its ethanolic counterpart, in agreement with an earlier report (Oonsivilai et al., 2008). Those

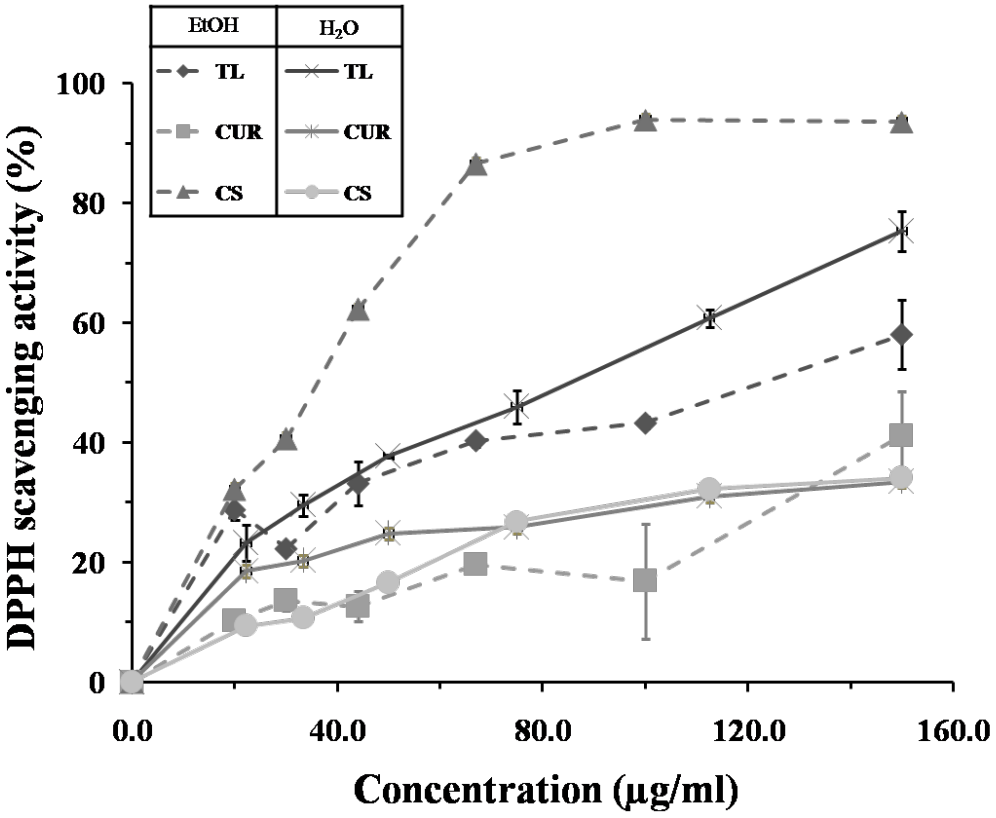


Figure 4.1 DPPH radical scavenging activities of the ethanolic and aqueous extracts of *Thunbergia laurifolia* (TL), *Crotalaria spectabilis* (CS), and *Curcuma* sp. (CUR).

Table 4.2 Antioxidant properties and total phenolic contents of the ethanolic and aqueous extracts of *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* sp. The values are expressed as the mean of three replicates ± SD.

Plant	**Extract**	**EC_{50} (µg/ml) of DPPH test**	**FRAP (µM TE/g dry weight)**	**TPC (mg CE/g dry weight)**
T. laurifolia	Ethanol	119.97 ± 12.95	155.05^{a} ± 7.59	26.54^{a} ± 0.09
	Aqueous	86.04 ± 3.94	148.41^{x} ± 8.46	35.84^{x} ± 0.09
Curcuma sp.	Ethanol	n.d.*	65.80^{b} ± 13.59	17.92^{b} ± 0.09
	Aqueous	n.d.*	34.62^{y} ± 4.26	12.28^{y} ± 0.09
C. spectabilis	Ethanol	26.56 ± 1.41	203.53^{a} ± 38.20	40.75^{c} ± 1.10
	Aqueous	n.d.*	67.05^{z} ± 12.18	14.89^{z} ± 0.00
Ascorbic acid	-	12.73 ± 1.18	-	-

* not detectable because the maximum concentration used in this experiment was not efficient to scavenge 50% of the DPPH radicals. The significance levels were individually calculated for each ethanolic or aqueous extracts. Different letters (a, b, c or x, y, z) within the same column indicate a significant difference at $p < 0.05$ by Tukey's test.

results support the traditional application of *Thunbergia laurifolia* as an herbal tea in a decoction with boiling water (วุฒิ, 2540).

Phenolic compounds have been recognized as natural antioxidants in various plants (Krishnaiah et al., 2011), including *T. laurifolia* (Oonsivilai et al., 2008). In this study, the highest total phenolic content was found in the ethanolic extract of *Crotalaria spectabilis* (40.75 ± 1.10 mg CE/g), which possessed the most active antioxidant activity, followed by the aqueous and ethanolic portions of *T. laurifolia*, which included 35.84 ± 0.09 and 26.54 ± 0.09 mg CE/g, respectively. The antioxidant activities of the extracts appear to be influenced by the total phenolic levels. Several studies have revealed that the phenolic contents of these plants are associated with their antioxidant activities, most likely due to the redox properties of these compounds, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers (Chang et al., 2001). According to the results, the antioxidant potencies of *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp. differ, implying that the substituted use of medicinal plants known as “Rang Chuet” requires caution. However, more specific pharmacological activities related to traditional indications should be further investigated to better verify the biological functions and medicinal uses of these plants.

4.2.2 Chemical characterization by TLC

The characteristic chemical pattern indicated by TLC profiling is useful for the primary identification of plant materials. The TLC mobile phases for the crude extracts of *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp. were optimized empirically. The chemical profiles of the ethanolic extracts of the three species are shown in Figures 4.2A and 4.2B. Flavonoids, which turn yellow under UV light at 365 nm after being sprayed with an $AlCl_3$ solution, were found in the ethanolic extracts of *T. laurifolia* and *C. spectabilis* at R_f 0.05 and 0.17, respectively (Figure 4.2A). However, no flavonoid compounds were detected in the ethanolic extract of *Curcuma* sp. When

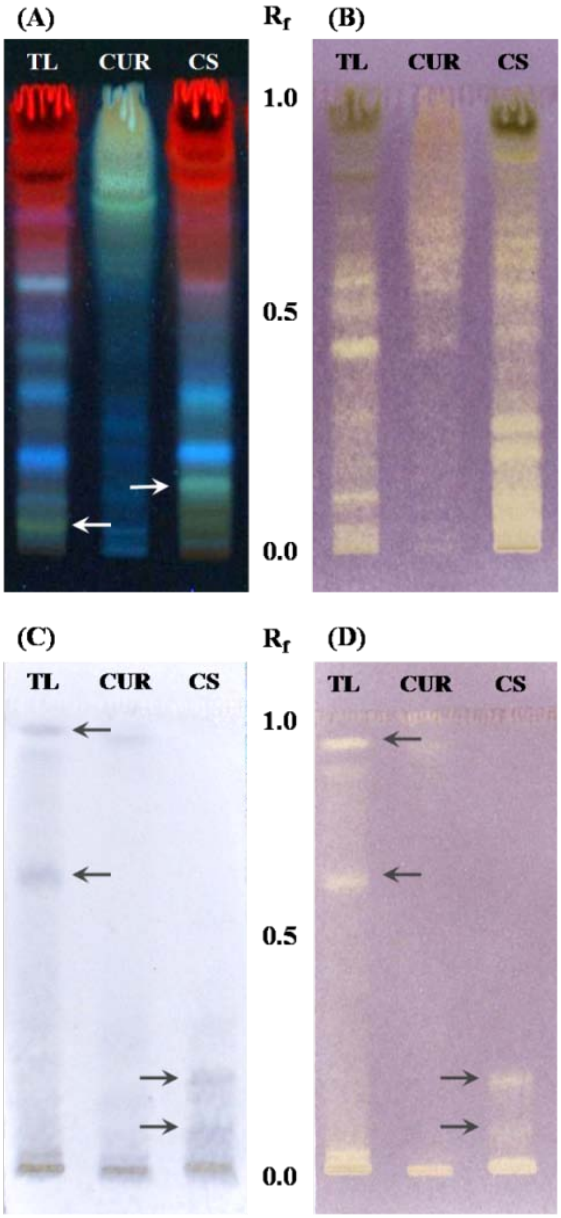


Figure 4.2 TLC chromatograms of the ethanolic and aqueous extracts of *Thunbergia laurifolia* (TL), *Curcuma* sp. (CUR), and *Crotalaria spectabilis* (CS). The plates of the ethanolic extracts were developed with chloroform-methanol-formic acid (7:3:0.5) and then viewed under UV light at 365 nm after being sprayed with 1% $AlCl_3$ (A) or under visible light after being sprayed with a 0.5 mM DPPH solution (B). The plates of the aqueous extracts were developed with ethyl acetate-formic acid-acetic acid-water (10:0.8:0.8:2.8) and then viewed under visible light after being sprayed with the Folin-Ciocalteu reagent (C) or under visible light after being sprayed with 0.5 mM DPPH solution (D). The characteristic flavonoid and phenolic bands are indicated with white and black arrows in the chromatograms, respectively.

the DPPH solution was applied to the ethanolic extracts of *Thunbergia laurifolia* and *Crotalaria spectabilis*, numerous bands were detected on the TLC plate (Figure 4.2B). These results indicate that the ethanolic extracts of *T. laurifolia* and *C. spectabilis* are rich in the natural antioxidant compounds detected in the free radical scavenging assay. These results also agree well with the spectrophotometric analysis of antioxidant activity, which indicated the high antioxidant capacities of the ethanolic extracts of *C. spectabilis* and *T. laurifolia*.

Because flavonoids were not detected in the aqueous extracts of *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp., the components of the aqueous extracts were characterized based on the phenolic compounds. The bands that were visible on the developed TLC plate after the plate was sprayed with 20% Na_2CO_3 and the Folin-Ciocalteu reagent were identified as phenolic compounds. As shown in Figure 4.2C, the compounds were observed in the aqueous extracts of *T. laurifolia* and *C. spectabilis*. The characteristic bands of phenolic compounds were observed at R_f 0.62 and 0.95 for *T. laurifolia*, whereas those of *C. spectabilis* were detected at different positions (R_f 0.05 and 0.20). By contrast, phenolic compounds were not observed in the extract of *Curcuma* sp. The DPPH solution was also applied to the TLC plates on which the aqueous extracts were separated to detect radical scavengers. The DPPH-scavenger-active bands of *T. laurifolia* and *C. spectabilis* coincided with the phenolic compounds detected on the TLC plates (Figure 4.2D). These results suggest that these phenolic compounds may contribute to the antioxidant properties of the plants. On the basis of the chemical characteristics of these medicinal plants, TLC analysis should be one of methods used to rapidly characterize “Rang Chuet” herbal drugs. The three medicinal plants, *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp., can be distinguished based on their flavonoid and phenolic constituents and their DPPH-scavenging profiles, which exhibited characteristic marks upon the development of the TLC plates of the ethanolic and aqueous extracts.

4.2.3 Development of the PCR-RFLP method for species differentiation

Although there are a variety of identification methods available, no single method is sufficient to identify herbal drugs. Applying various methods in concert is necessary to conclusively confirm an identification or authentication (Zhao et al., 2006a). A DNA-based technique is a supplementary method for this task. Herein, a convenient PCR-RFLP method based on the *mat*K gene that would enable rapid and accurate identification was developed to differentiate between *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* sp. As indicated in many previous studies, the sequence of the chloroplast *mat*K gene provides useful information to assist in the taxonomic classification and identification of the botanical origin of herbal drugs (Boonsom et al., 2012; Gao et al., 2011). The primer set matK-465F and matK-1483R was designed to amplify a short fragment of the *mat*K gene because the DNA extracted from the crude drug sample was generally degraded, thus making the amplification of a long PCR fragment difficult (Zhu et al., 2011). The PCR product of each species exhibited a single band in the electrophoresis profile, corresponding to a fragment size of approximately 1 kb.

Based on the alignment of the products, polymorphic nucleotides were observed at various sites of the *mat*K products of the three species, permitting the differentiation of *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp. with the restriction enzymes *Dde*I and *Hae*III, which can recognize and cleave the sequences C^TXAG and GG^CC, respectively. The locations of the restriction sites recognized by *Dde*I and *Hae*III differed among the three sequences of *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp. (Figure 4.3), yielding different fragment sizes that can be observed by gel electrophoresis (Figure 4.4). *Dde*I cleaved the 1046-bp fragment of *T. laurifolia* that was amplified by matK-465F and matK-1483R into 934- and 112-bp fragments. The *Curcuma* sp. sequence includes one restriction site for *Dde*I at position 687, and fragments of 627 bp and 407 bp were observed after digestion. For *C. spectabilis*, the 1037-bp PCR product was cleaved into 230-bp and 807-bp fragments by *Dde*I. There is one restriction site specific to

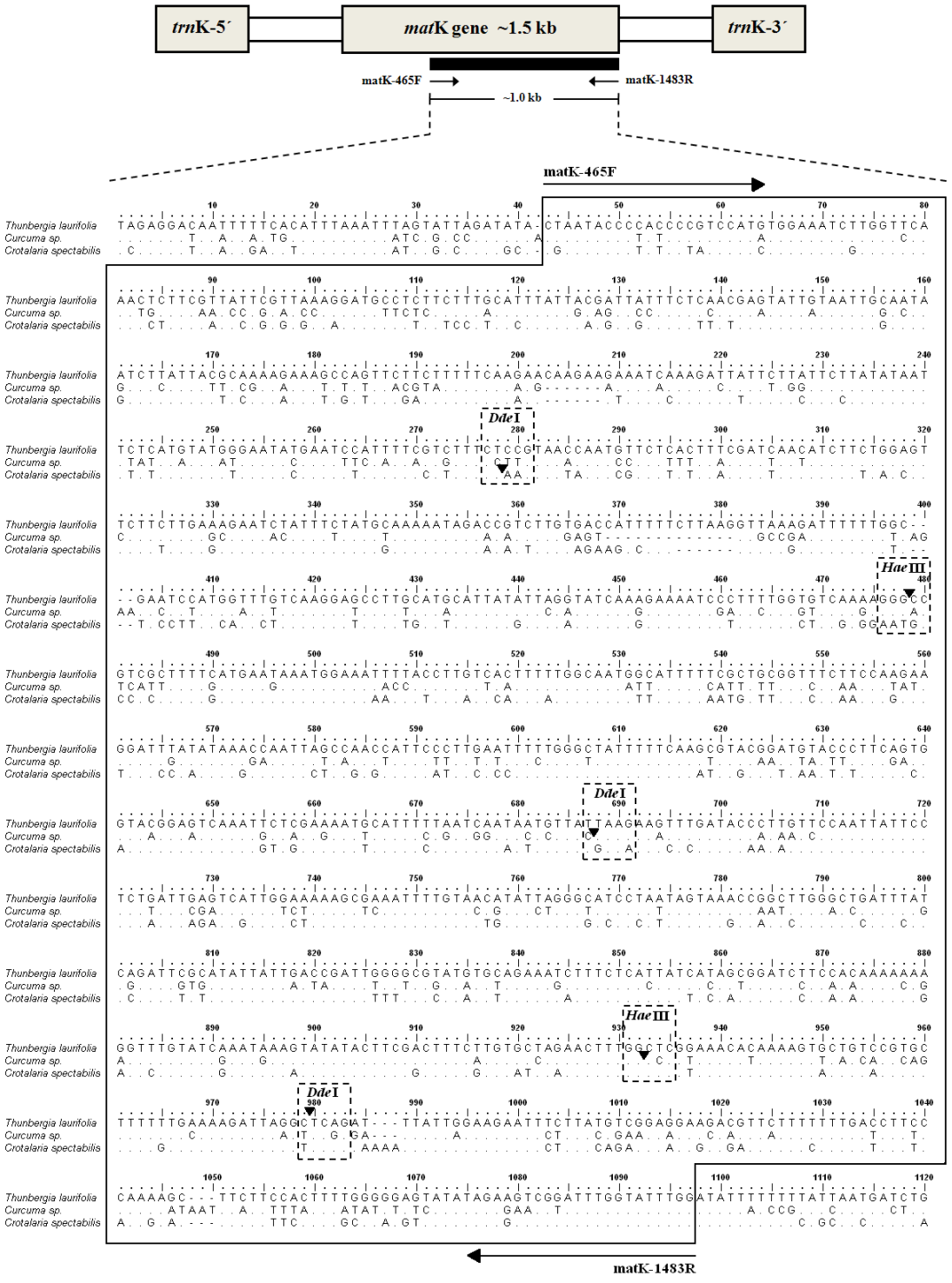


Figure 4.3 Structure of the *mat*K gene and sequence alignment of the partial *mat*K gene from *Thunbergia laurifolia*, *Crotalaria spectabilis*, and *Curcuma* sp. The chloroplast *mat*K gene is embedded in the intron of the *trn*K gene. The first position of the alignment corresponds to nucleotide position 404, 425, and 407 of the *mat*K genes of *T. laurifolia*, *Curcuma* sp., and *C. spectabilis*, respectively. The arrows indicate the locations of the amplified primers matK-465F and matK-1483R. Dots denote nucleotides identical to those of the *T. laurifolia* sequence. The gaps that were introduced to maintain alignment are indicated by dashes. The restriction sites for *Dde*I (C^TXAG) and *Hae*III (GG^CC) in the sequences are indicated by a dotted squared box.

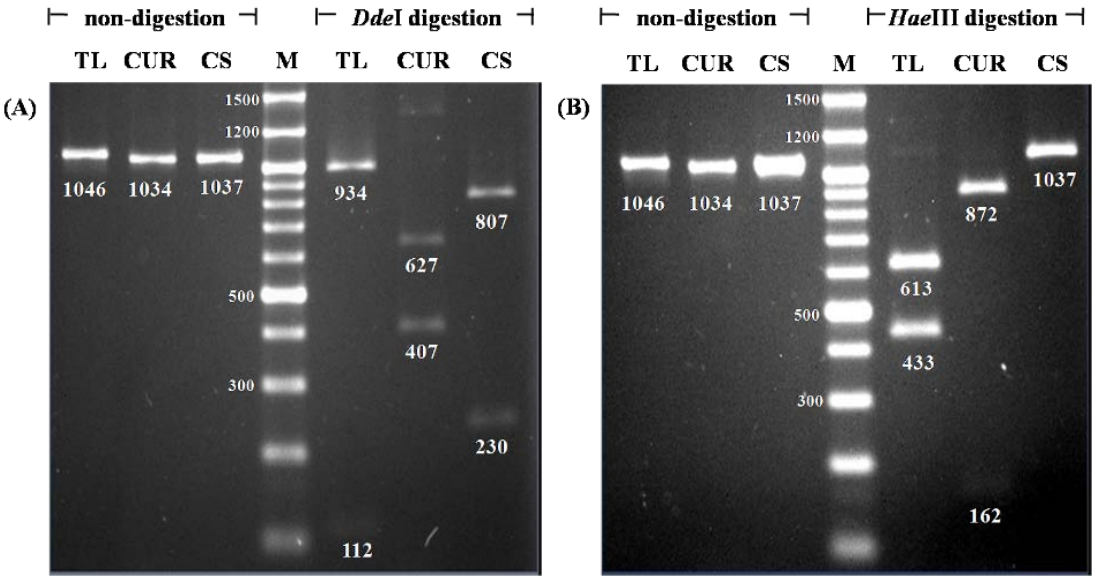


Figure 4.4 PCR-RFLP patterns of the *mat*K products of *Thunbergia laurifolia* (TL), *Crotalaria spectabilis* (CS), and *Curcuma* sp. (CUR) digested with *Dde*I (A) and *Hae*III (B). Gel electrophoresis was performed on a 1.5% agarose gel, which was stained with ethidium bromide. The DNA markers (VC 100 bp plus, Vivantis, USA) are indicated as bp units in lane M.

*Hae*III in the sequence of *Thunbergia laurifolia*, resulting in the cleavage of the PCR product into two fragments of 433 bp and 613 bp in length. *Hae*III can digest the 1034-bp *mat*K fragment of *Curcuma* sp. into 872-bp and 162-bp fragments, whereas there is no *Hae*III restriction site in the *C. spectabilis* sequence. The different fragment sizes observed via gel electrophoresis after digestion with *Dde*I and *Hae*III represent a good diagnostic tool to differentiate between *T. laurifolia*, *Crotalaria spectabilis*, and *Curcuma* sp.

4.3 Conclusions

Confusion regarding "Rang Chuet" herbal drugs has arisen in Thai herbal markets because of similar nomenclature. *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp. are all known by the same local name. However, these plants exhibit different antioxidant effects, as demonstrated by DPPH and FRAP assays. High antioxidant capacities were observed in *T. laurifolia* and *C. spectabilis* that may be influenced by the presence of phenolic components in the extracts. Because herbal drugs are often processed into powders during preparation, species identification based on histological characteristics may not be applicable. Chemical profiling and DNA analysis were used in tandem to identify the plant materials. The TLC results indicated that these different species can be identified based on the flavonoid and phenolic constituents of the ethanolic and aqueous extracts, respectively. Development of the TLC plates with the DPPH radical permitted the detection of radical-scavenging compounds in *T. laurifolia* and *C. spectabilis*. The PCR-RFLP technique has potential for identifying species present in "Rang Chuet" herbal drugs. After digestion with specific restriction enzymes, *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp. were easily distinguished based on the different sizes of the digested fragments. The use of both TLC and PCR-RFLP analyses permits the identification of species via the comparison of unknown samples with standard patterns.

CHAPTER V

ISOLATION OF ROSMARINIC ACID FROM *THUNBERGIA LAURIFOLIA* AND ITS USE AS A BIOACTIVE PRINCIPLE FOR HERBAL STANDARDIZATION

Currently, herbal teas, powders, and capsule preparations of *Thunbergia laurifolia* are commonly available in the herbal and nutraceutical markets. Despite its common use, no commercially available preparations with determined content or standardized products exist, likely due to a lack of information about the active phytochemicals in *T. laurifolia*. In the present study, guided isolation through TLC bioautography using the DPPH radical as a detection reagent led to the isolation of a potent antioxidant compound from the leaves of *T. laurifolia*. This method provides rapid detection and localization of the active compounds in the complicated plant extract. Moreover, an HPLC method with UV detection was developed to determine the bioactive compounds in *T. laurifolia*. The analysis of the active components in herbs and herbal mixtures is essential for evaluating the quality and authenticity of herbal medicines. The developed HPLC method was also used to test *T. laurifolia* leaf extracts collected from different locations.

5.1 Materials and Methods

5.1.1 Sample collection and preparation

The *T. laurifolia* leaves used for the isolation of the active components were collected from the Nakhon Pathom province of Thailand. An additional five samples were collected from different locations in Thailand. All plant samples were identified by Associate Professor Thatree Phadungcharoen from the Department of Pharmacognosy and Pharmaceutical Botany at the

Faculty of Pharmaceutical Sciences at Chulalongkorn University in Thailand. The voucher specimens were kept in the Museum of Natural Medicines at the Faculty of Pharmaceutical Sciences at Chulalongkorn University.

The plant samples were ground into powders using an electric blender and extracted with ethanol. The ethanolic extracts were macerated at room temperature for 72 h. Subsequently, each extract was filtered though Whatman No. 1 filter paper and evaporated to dryness at 50°C.

5.1.2 Antioxidant-guided isolation of rosmarinic acid

The dried *Thunbergia laurifolia* leaves (900 g) collected from the Nakhon Pathom province were macerated with 95% ethanol at room temperature for 72 h. The extract was filtered and evaporated to dryness at 50°C in a rotary evaporator. The extraction of the residue was repeated using the same conditions, and the two filtrates were combined. The ethanolic extract (64.75 g) was applied to a column of ion-exchange resin and eluted with a gradient mixture of water and acetone. All of the obtained fractions were examined for DPPH-scavenging properties via TLC bioautography. The DPPH-scavenging active fraction was identified as a yellowish spot on the purplish background of the TLC plate and was further separated on a silica gel column, eluted with a chloroform-methanol-formic acid (8.5:1.5:0.5) mixture. The fraction containing the targeted compound was then submitted to sephadex LH-20 with methanol, resulting in four fractions. The DPPH-scavenging active fraction was purified on a silica gel column with a gradient system ramping from dichloromethane-methanol (7:3) to 100% methanol to obtain the pure, yellow antioxidant compound (235.3 mg).

As a result, the isolated rosmarinic acid was identified using spectral analysis and comparison to previous data (Mehrabani et al., 2005). The structure of rosmarinic acid is presented in Figure 5.1. The NMR spectra were measured on a JEOL Lambda 400 MHz or 600 MHz spectrometer (JEOL Ltd., Japan). The sample was prepared in DMSO-d_6. In addition, the

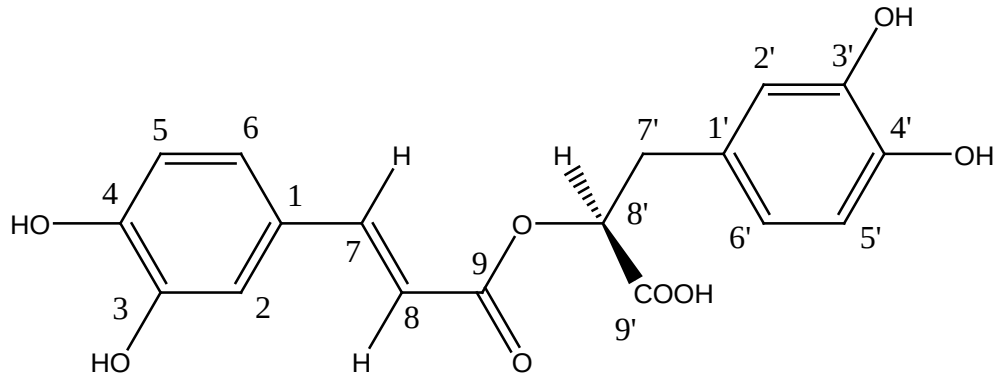


Figure 5.1 Chemical structure of *trans*-rosmarinic acid isolated from the leaves of *Thunbergia laurifolia*.

structure assignment was confirmed by mass spectroscopy using an AB-SCIEX QTRAP 5500 spectrometer (AB-SCIEX, USA).

5.1.3 HPLC analysis

The plant extracts and the isolated rosmarinic acid were analyzed using an HPLC system equipped with a LC-20AD HPLC, a CTO-20A column oven, and a SPD-M20A diode array detector (Shimadzu, Japan). The separation was performed on a 4.6 x 250 mm Cosmosil® $5C_{18}$-AR-II column (Nacalai Tesque, Japan) at ambient room temperature. The mobile phase was isocratic water-methanol-acetic acid (65:35:0.1). The flow rate was 1.0 ml/min, and the injection volume was 20 µl. The signal was monitored at 320 nm in accordance with the maximum absorption of the rosmarinic acid measured with a UV spectrophotometer. The standard solutions of rosmarinic acid were prepared to yield concentrations in the range of 10 to 1000 µg/ml. The chromatographic peaks of the plant extracts were identified by comparing their retention times and UV spectra against a reference sample of rosmarinic acid.

5.1.4 Method validation

The suitability of the HPLC system was evaluated by determining the intra- and inter-day precision of the replicates. The accuracy was evaluated through recovery studies by adding known amounts of standard rosmarinic acid to the extract. The recovery experiments were performed with three concentrations of the standard. To measure the linearity, a calibration line was made and correlation coefficient was calculated based on the obtained calibration line. Both the calibration line and the residuals were graphically inspected and evaluated.

5.1.5 DPPH-scavenging assay

The radical scavenging activity was evaluated using the DPPH radical and a standard spectrophotometric assay in a 96-well microplate with slight modifications (Kintzios et al., 2010).

The plant extracts were diluted into 5 concentrations (0.5 to 2.0 mg/ml). A 20 µl sample of the extract and 180 µl of a 0.1 mM methanolic DPPH solution were added to each well. The plate was covered with aluminum foil and left at room temperature for 30 min. Each well's absorbance was measured at 510 nm against a solvent blank to estimate the scavenging capacity of each sample. The free-radical scavenging activities of the ethanolic extracts from each *Thunbergia laurifolia* sample and the isolated rosmarinic acid were evaluated. Trolox and quercetin were used as positive controls. The scavenging capacity was calculated as the effective concentration at which the DPPH radicals were scavenged by 50% (EC_{50}).

5.2 Results and Discussion

The isolation of an antioxidant compound from the 95% ethanolic extract from the dried leaves of *T. laurifolia* was achieved with DPPH autography-directed separation. The isolated compound was identified as rosmarinic acid and represented at least 0.36% of the dry weight. This compound was obtained as a yellow powder with UV (MeOH) maxima (λ_{max}) at 290 and 326 nm. A detailed analysis of the ^{1}H and ^{13}C NMR spectra of the isolated rosmarinic acid agreed well with previous reports (Mehrabani et al., 2005) (Table 5.1). The signals of olefinic protons at δ 7.46 and 6.24 (each 1H, d, 15.6 Hz) specified the structure as *trans*-rosmarinic acid with the assignment of *R*-configuration at C-8′ position based on the optical rotation, $[\alpha]_D$ +22.9° (c = 0.2, MeOH). Its identification was confirmed by a negative ESI-MS analysis (m/z 359, $[M-H]^-$). This work is the first report of the isolation of rosmarinic acid from *T. laurifolia*. This successful bioassay-guided fractionation is in accordance with earlier studies that effectively applied this method to the rapid separation of active compounds from several medicinal plants. For example, vasorelaxant compounds were isolated from *Ziziphora clinopodioides* Lam. (Senejoux et al., 2012), anthelmintic compounds were separated from *Semen pharbitidis* (Hao et al., 2012), and anxiolytic compounds were isolated from *Melissa officinalis* L. (Award et al., 2009).

Table 5.1 ^{1}H- and ^{13}C-NMR data of the isolated rosmarinic acid in DMSO-d_6.

Position	Isolated rosmarinic acid		Rosmarinic acid (Mehrabani et al., 2005)	
	δ ^{13}C-NMR (ppm)	δ ^{1}H-NMR (ppm)	δ ^{13}C-NMR (ppm)	δ ^{1}H-NMR (ppm)
1	125.36	-	126.20	-
2	114.94	7.05 (d, *J* = 1.8 Hz)	115.74	7.06 (s)
5	115.77	6.76 (d, *J* = 8.0 Hz)	116.64	6.77 (d, *J* = 8.0 Hz)
6	121.60	7.00 (dd, *J* = 8.0, 1.8 Hz)	122.45	7.01 (d, *J* = 8.0 Hz)
7	148.63	7.46 (d, *J* = 15.6 Hz)	146.73	7.47 (d, *J* = 16.0 Hz)
8	113.26	6.24 (d, *J* = 15.6 Hz)	114.18	6.24 (d, *J* = 16.0 Hz)
9	165.93	-	166.81	-
1′	127.29	-	128.28	-
2′	116.70	6.67 (d, *J* = 1.8 Hz)	117.55	6.69 (s)
5′	115.40	6.63 (d, *J* = 8.2 Hz)	116.25	6.64 (d, *J* = 7.6 Hz)
6′	120.05	6.52 (dd, *J* = 8.2, 1.8 Hz)	120.90	6.53 (d, *J* = 7.6 Hz)
7′	36.10	2.98 (dd, *J* = 14.4, 4.2 Hz)	37.01	2.99 (dd, *J* = 14.0, 4.0 Hz)
		2.90 (dd, *J* = 14.4, 8.4 Hz)		2.91 (dd, *J* = 14.0, 8.6 Hz)
8′	72.81	5.02 (dd, *J* = 8.4, 4.2 Hz)	73.84	5.03 (d, *J* = 8.6, 4.0 Hz)
3-OH	145.60	8.73	144.85	8.81
4-OH	145.94	9.63	145.79	9.68
3′-OH	144.02	8.72	146.47	8.75
4′-OH	144.94	9.15	149.49	9.20
9′-OH	170.84	12.87	171.84	12.90

The free-radical scavenging capacities of the isolated rosmarinic acid and two positive controls, trolox and quercetin, were measured using a DPPH assay. The rosmarinic acid exhibited a higher antioxidant potential (2.71 ± 0.08 μg/ml) that was higher than that of trolox (3.51 ± 0.04 μg/ml) but lower than that of quercetin (0.62 ± 0.03 μg/ml). A good correlation (r = 0.9611) was observed between the rosmarinic acid concentration and its free-radical scavenging activity. This result is in agreement with previous reports that showed that rosmarinic acid has a strong capacity for scavenging free radicals (Tepe et al., 2007; Tepe, 2008). The presence of two *ortho*-dihydroxyphenyl groups and multiple conjugated double bonds in the structure of rosmarinic acid contributes to its ability to scavenge free radicals (Nakamura et al., 1998). Rosmarinic acid has been reported to have other biological activities, including anti-inflammatory (Osakabe et al., 2004), antimutagenic (Furtado et al., 2008), hepatoprotective (Lima et al., 2006), anticholinesterase (Dastmalchi et al., 2009), and antiviral (Mazzanti et al., 2008) properties. The presence of rosmarinic acid in *Thunbergia laurifolia* may be related to the anti-inflammatory (Wongchalee et al., 2012), antimutagenic (Oonsivilai et al., 2007), and hepatoprotective (Pramyothin et al., 2005) properties previously reported for this plant and may provide key chemical evidence for determining the contribution of rosmarinic acid to these activities.

Not only is rosmarinic acid an active compound in the leaves of *T. laurifolia*, but it is also the major constituent. The compound can be used as a chemical marker for the quality control of raw materials or any preparations containing *T. laurifolia* as an active ingredient. In this study, an HPLC elution procedure was developed and used to determine the amount of rosmarinic acid in a sample. The mobile phase consisted of water-methanol-acetic acid (65:35:0.1) and provided good resolution for the target compound and satisfied the analysis. A typical chromatogram of the isolated rosmarinic acid and its absorption spectrum measured by the HPLC photodiode array detector are shown in Figure 5.2A. The rosmarinic acid peak had a retention time of approximately 33 min and acceptable symmetry. The chromatogram of the rosmarinic acid standard showed another small peak at approximately 30 min. This impurity peak

was also found in the rosmarinic acid standard reported by Yuan et al. (1998). Rosmarinic acid was identified in the crude extracts of *Thunbergia laurifolia* by comparing its retention time and absorption spectrum with those from the standard rosmarinic acid sample under the same chromatographic conditions. Good separation between rosmarinic acid and the other constituents in the crude ethanolic extracts of *T. laurifolia* was observed (Figure 5.2B). The established HPLC method was subsequently validated to ensure its suitability for the analysis of rosmarinic acid.

The results for the evaluation of the calibration model are shown in Table 5.2. The rosmarinic acid exhibited good linearity in the range of 25 to 200 µg/ml for the calibration curve generated from the HPLC data. The calibration line was described by the following equation, y = 36921.74x – 136340.40, and the correlation coefficient (*r*) was at least 0.99. The HPLC determination of rosmarinic acid also yielded precise and accurate results. The precision was evaluated by analyzing a reference solution of rosmarinic acid six times within the same day and once daily for three consecutive days. The intra- and inter-day variation studies indicated that the relative standard deviations were less than 1.76% and 2.68%, respectively (Table 5.3). The standard deviation data indicated that the precision results were acceptable. The recovery experiment was evaluated by adding known quantities of rosmarinic acid to the *T. laurifolia* extracts. The results were calculated by comparing the actual and theoretical amounts of detected rosmarinic acid. The amount of rosmarinic acid recovered from the extract at three different concentrations, 50, 100, and 200 mg/g, were 109.87% (± 3.94 RSD), 109.10% (± 5.90 RSD), and 102.02% (± 3.05 RSD), respectively (Table 5.4), which indicates that the method has good accuracy. Mean recovery should be appropriate to the concentration tested encompassing a tolerance interval, 20% below the lowest expected concentration and 20% above the highest expected concentration (Walfish, 2006). The developed method was also more accurate and precise than the others studies of HPLC method validation in plant extracts (Obmann et al., 2012, Ferreira and Gonzalez, 2009).

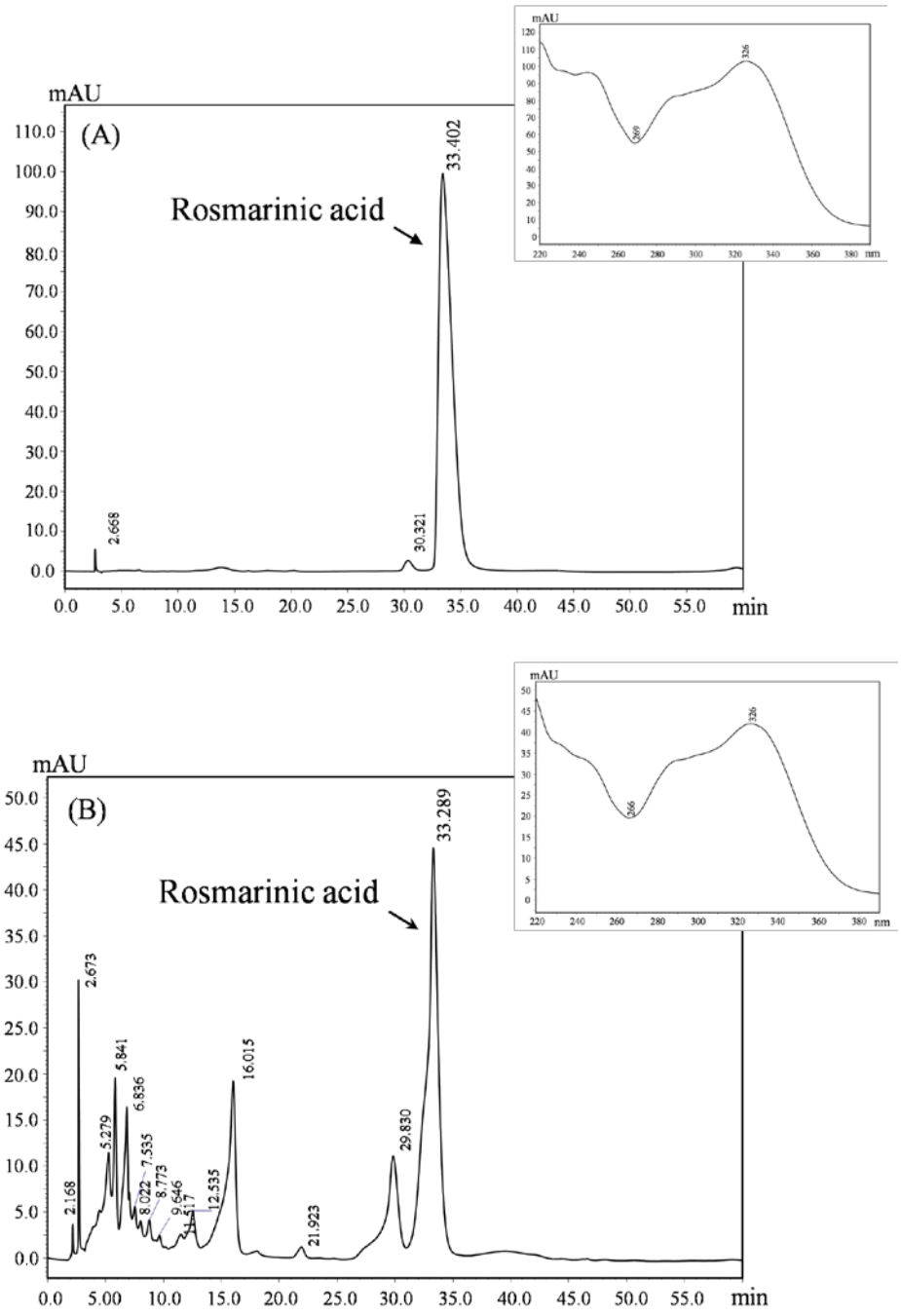


Figure 5.2 HPLC chromatogram of the isolated rosmarinic acid (A) and the ethanolic extract of *Thunbergia laurifolia* (B). A Cosmosil® $5C_{18}$-AR-II (250 mm x 4.6 mm) column was used with an isocratic water-methanol-acetic acid (65:35:0.1) at a flow rate of 1.0 ml/min and a detection wavelength of 320 nm. The inset shows the online UV spectrum of the arrowed peak (rosmarinic acid).

Table 5.2 Linear regression data of rosmarinic acid analyzed by HPLC.

Compound	**Linear range (µg/ml)**	**Slope ± SD**	**Intercept ± SD**	***r***
Rosmarinic acid	25.00-200.00	36921.74 ± 875.18	-136340.40 ± 74324.98	0.9996

Table 5.3 Intra- and inter-day precision values of rosmarinic acid analyzed by HPLC.

	Day 1	**Day 2**	**Day 3**	**Inter-day**
Number of replicates	6	6	6	18
Area	8,215,010	8,294,414	8,638,269	8,382,564
RSD (%)	0.81	1.57	1.76	2.68

Table 5.4 Recovery data of rosmarinic acid from the extracts of *Thunbergia laurifolia* analyzed by HPLC.

Amount added (mg/g)	**Amount detected (mg/g)**	**Recovery (%)**	**RSD (%)**
50.00	54.93 ± 2.16	109.87	3.94
100.00	109.10 ± 6.44	109.10	5.90
200.00	204.04 ± 6.22	102.02	3.05

The HPLC method developed as described above was applied to the determination of the rosmarinic acid concentration in *Thunbergia laurifolia* extracts obtained from various locations. HPLC methods have been successfully used to determine the rosmarinic acid content in several plants, such as *Rosmarinus officinalis* L. (Wang et al., 2004), *Melissa officinalis* (Sanchez-Medina et al., 2007), and *Salvia officinalis* L. (Bandoniene et al., 2005). As shown in Table 5.5, the rosmarinic acid content in *Thunbergia laurifolia* varied from 1.56 to 53.32 mg/g of dry weight. This high variability in the rosmarinic acid level is similar to those reported for *Salvia officinalis*, one of the plants with an abundance of rosmarinic acid, which varied from 6 to 47 mg/g of dry weight (Lamien-Meda et al., 2010). The highest concentration of rosmarinic acid (53.32 mg/g dry weight) in *T. laurifolia* was observed in sample no. 4, whereas sample no. 2 (1.56 ± 0.14 mg/g dry weight) and sample no. 6 (3.01 ± 0.22 mg/g dry weight) contained the lowest concentrations in comparison to the others. The high degree of variability could be due to environmental factors such as altitude, growing conditions or the age of the plants during sample collection (Lamien-Meda et al., 2010).

Our results also indicated a high degree of variability in the DPPH-scavenging activity for *T. laurifolia* collected from different locations. As shown in Table 5.5, sample no. 1 (32.84 ± 0.72 µg/ml) and sample no. 4 (30.62 ± 0.36 µg/ml) produced the highest DPPH-scavenging effects. The lowest activity was reported for sample no. 2, which had the highest EC_{50} value of 114.51 ± 1.21 µg/ml. The variability in DPPH-scavenging capacity among the six *T. laurifolia* samples might be influenced by the quantity of phenolic compounds in the extract (Oonsivilai et al., 2008). Previously, *T. laurifolia* has been reported to contain the caffeic, chlorogenic, gallic, and protocatechuic acids (Chan et al., 2011), which contribute to its antioxidant activity. However, a good correlation (0.8399) between the free-radical scavenging activity against DPPH and the amount of rosmarinic acid found in each *T. laurifolia* sample was observed in this study (Figure 5.3). This correlation is in agreement with several studies that showed that the rosmarinic acid

Table 5.5 Details of the *Thunbergia laurifolia* plants used for the experiments and their rosmarinic acid contents and DPPH-scavenging capacities.

Sample	**Sample no.**	**Location collected (province)**	**Voucher specimen**	**Rosmarinic acid (mg/g dry weight)**	**EC_{50} DPPH (μg/ml)**
T. laurifolia	1	Nakhon Pathom	SS-0510105	38.82 ± 2.54^{a}	32.84 ± 0.72^{a}
	2	Nonthaburi	SS-0510106	1.56 ± 0.14^{b}	114.51 ± 1.21^{b}
	3	Nakhon Sawan	SS-0401109	14.86 ± 0.83^{c}	67.96 ± 1.89^{c}
	4	Prachin Buri	SS-1009104	53.32 ± 2.18^{d}	30.62 ± 0.36^{a}
	5	Phetchabun	SS-0611110	8.68 ± 0.76^{e}	86.24 ± 2.24^{d}
	6	Samut Prakan	SS-0911111	3.01 ± 0.22^{b}	78.05 ± 0.34^{e}
Rosmarinic acid (isolated)	-	-	-	-	2.71 ± 0.08
Trolox	-	-	-	-	3.51 ± 0.04
Quercetin	-	-	-	-	0.62 ± 0.03

Values are expressed as the mean of three replicates ± SD. The different letters (a, b, c, d, and e) within the same columns indicate a significant difference at $p < 0.05$ by Tukey's test.

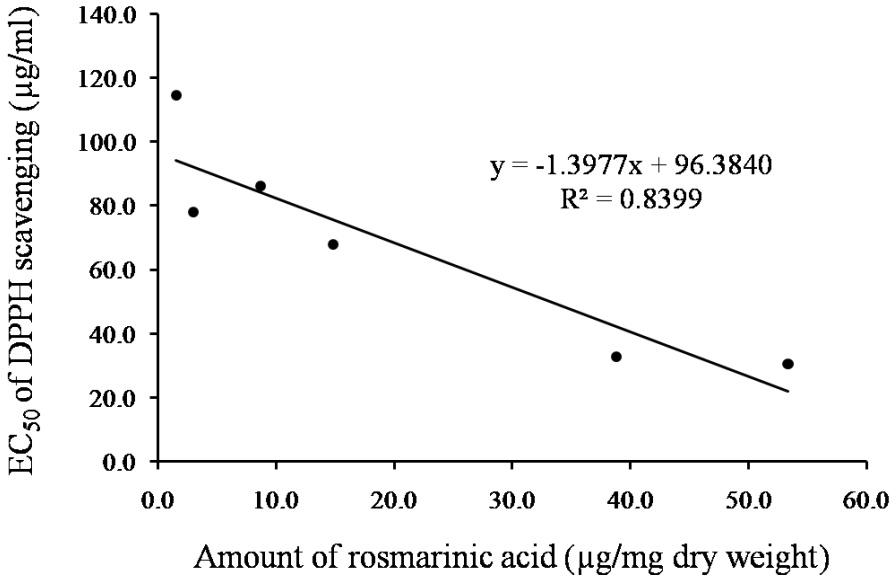


Figure 5.3 A graph depicting the correlation between the rosmarinic acid content and the EC_{50} value of DPPH-radical scavenging activity found for each *Thunbergia laurifolia* samples.

concentration accounted for the antioxidant potential (Chizzola et al., 2008; Lamien-Meda et al., 2010). Hence, rosmarinic acid is most likely responsible for the majority of *Thunbergia laurifolia*'s antioxidant activity.

5.3 Conclusions

Currently, there are no standardized production methods or accepted measures for the manufacture of *T. laurifolia* as an herbal drug; therefore, there is no guarantee of potency or efficacy. Searching for the bioactive components of *T. laurifolia* is the first step in designing a quality control procedure. As a result, a potent antioxidant compound, rosmarinic acid, was isolated from *T. laurifolia*. Because rosmarinic acid demonstrated a significant free-radical scavenging effect, it can be used as the reference compound for the quality assurance of *T. laurifolia*. In the present study, an HPLC method was established and applied to determine rosmarinic acid levels in *T. laurifolia*. The method is simple, sensitive, and suitable for the standardization of raw materials and commercial preparations containing *T. laurifolia*. The present study also showed that the rosmarinic acid content in *T. laurifolia* leaves correlated well with its DPPH-scavenging capacity, indicating that it contributes significantly to the overall antioxidant activity. The bioactivity of this compound might provide a chemical basis for some of the health benefits seen with the leaves of *T. laurifolia* in folk medicine and warrant further studies to assess its potential as an effective natural remedy.

CHAPTER VI

CONCLUSION

Authentication is fundamental for the quality assessment and reliability of herbal medicines. Herbal authentication mainly addresses quality-related issues using routine botanical and organoleptic parameters and chemical characterization with chromatographic and spectroscopic techniques. DNA-based techniques have now become a popular means for the identification of herbal medicines. In the present study, exploratory on the use of molecular markers were undertaken. RAPD technique was successfully used to identify the six plant species, *Thunbergia laurifolia*, *T. grandiflora*, *T. erecta*, *Curcuma* sp., *Rinorea* sp., and *Crotalaria spectabilis*. Relationship among the investigated species is well presented in the RAPD clustered dendrogram. RAPD represents a useful tool for differentiating *T. laurifolia* and its related species and assisting the phylogenetic classification of the plants.

Proper integration of molecular techniques and analytical tools will lead to the development of a comprehensive system of botanical characterization. In the present study, the TLC and PCR-RFLP fingerprinting were jointly applied to differentiate the three “Rang Chuet” species, *T. laurifolia*, *C. spectabilis*, and *Curcuma* sp., which are commonly found in herbal markets. Combination of TLC and PCR-RFLP fingerprinting might be helpfully applied at the commercial level for quality control of the “Rang Chuet” herbal drugs.

Quality control currently plays a very important role in the application and development of herbal drugs. Several modern techniques and methods of characterization are used in the quality control of herbal medicines. Chromatographic fingerprint analysis represents a comprehensive qualitative approach for the purpose of species authentication, evaluation of quality, and ensuring the consistency and stability of herbal drugs and their related products. The best known as “Rang Chuet” species, *T. laurifolia*, has been used in Thai traditional medicine for

a long time. However, limited information is available regarding the characterization of the chemical components in *Thunbergia laurifolia*. Herein, the HPLC quantification of rosmarinic acid was accomplished. The rosmarinic acid was isolated from *T. laurifolia* for the first time using guided isolation through TLC bioautography using the DPPH radical as a detection reagent. It is a major and potent antioxidant compound contained in the leaves of *T. laurifolia*. The developed HPLC method was applied to determine the amount of rosmarinic acid in *T. laurifolia* collected from different locations and also validated in terms of linearity, precision, and accuracy. The established HPLC method for the determination of rosmarinic acid, a bioactive principle, is a useful approach for the herbal standardization of the raw material and herbal preparations containing *T. laurifolia*.

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APPENDICES

APPENDIX A

RAPD fingerprinting of “Rang Chuet”

Table A1 RAPD scoring data of amplification products among the six plant species. The RAPD products were scored as 0 or 1 for the absence or presence of bands, respectively.

Bands no.	Plant species[a]					
	TE	**TG**	**TL**	**CUR**	**RIN**	**CS**
OPA-03						
1	0	0	0	0	0	1
2	0	0	0	1	0	0
3	0	0	0	0	1	0
4	1	0	1	0	0	0
5	0	0	1	0	0	0
6	0	0	0	1	0	0
7	0	0	0	0	1	0
8	0	0	0	1	0	0
9	1	0	0	0	1	1
10	0	1	0	0	1	0
11	1	0	0	0	0	0
12	0	0	1	0	0	0
13	1	1	0	1	0	0
14	0	0	1	0	0	0
15	1	0	0	0	0	0
16	0	0	0	0	1	0
17	0	0	0	1	0	0
18	0	0	0	1	0	0
19	0	0	0	0	0	1
OPA-04						
1	0	0	0	1	0	0
2	0	0	1	0	0	0
3	0	0	0	0	1	0
4	1	0	0	0	0	0
5	0	1	0	0	0	0
6	0	0	0	1	0	0
7	0	0	1	0	1	1
8	0	0	0	1	0	0
9	0	1	0	0	1	0
10	1	0	0	1	0	0
11	0	0	1	0	0	0
12	0	0	0	0	1	1
13	1	0	0	1	0	0
14	0	1	1	0	0	0
15	1	0	0	0	0	0
16	0	0	0	0	1	1
17	0	1	0	0	0	0
18	0	0	0	1	1	0
19	1	0	0	0	0	0
20	0	0	1	1	1	0
21	0	0	0	0	0	1
22	1	1	1	0	0	1
23	0	0	0	1	0	0
24	0	1	0	0	0	0
25	0	0	0	0	0	1

Bands no.	Plant species[a]					
	TE	**TG**	**TL**	**CUR**	**RIN**	**CS**
OPA-17						
1	0	0	0	0	1	0
2	0	0	1	0	0	0
3	0	0	1	0	0	0
4	1	0	0	1	0	0
5	1	0	1	0	1	0
6	1	0	0	0	0	0
7	0	0	1	0	1	0
8	0	1	0	1	0	1
OPA-18						
1	0	0	1	0	0	0
2	0	0	0	0	1	0
3	0	0	0	0	0	1
4	0	0	1	0	1	0
5	1	0	0	0	0	0
6	0	0	0	0	1	0
7	0	0	1	0	0	0
8	1	0	0	0	1	0
9	1	0	0	1	0	0
10	0	0	0	0	1	1
11	0	0	1	0	0	0
12	0	0	0	0	1	0
13	0	0	0	1	0	1
14	0	1	0	0	1	0
15	1	0	0	0	0	0
16	1	0	0	0	0	1
17	0	0	0	1	0	0
18	0	0	0	0	1	0
19	0	0	1	0	0	0
OPN-02						
1	0	0	0	0	1	0
2	1	0	0	0	0	0
3	0	0	0	1	1	0
4	1	0	1	0	0	0
5	0	0	0	0	1	1
6	0	1	1	0	0	0
7	0	0	0	1	0	0
8	0	0	0	0	1	1
9	0	1	0	0	0	0
10	1	1	1	0	1	0
11	0	0	1	0	1	0
12	1	0	0	0	0	0
13	0	0	1	0	0	0
14	1	0	1	0	1	1
15	0	0	0	0	0	1
16	1	0	0	0	0	0
OPN-10						
1	1	0	0	0	0	0
2	0	0	0	1	0	0

Bands no.	Plant species[a]					
	TE	TG	TL	CUR	RIN	CS
3	0	1	1	0	0	0
4	0	0	0	0	1	1
5	1	0	0	0	0	0
6	0	1	1	1	1	0
7	1	1	0	0	0	1
8	1	0	1	1	0	0
9	0	0	0	0	1	0
10	1	0	0	0	0	0
11	0	1	0	0	1	0
12	0	0	1	1	1	0
13	1	1	1	0	1	1
14	0	1	0	1	1	1
15	1	0	1	0	0	0
16	0	0	0	1	0	1
17	0	0	0	1	1	0
18	1	1	1	0	0	0
19	0	0	0	0	0	1
20	1	0	1	1	0	1
21	1	1	0	0	0	0
22	0	1	0	1	1	0
OPN-12						
1	0	0	0	1	0	0
2	0	0	0	0	1	0
3	0	0	0	0	1	0
4	0	0	1	0	0	0
5	1	0	0	0	0	0
6	0	0	0	1	0	0
7	1	0	0	0	1	0
8	1	1	1	0	0	0
9	0	0	0	1	0	0
10	0	0	0	0	0	1
11	1	0	0	1	1	0
12	0	0	1	0	0	0
13	0	1	0	0	0	0
14	0	0	1	0	1	0
15	0	0	0	0	0	1
16	0	1	0	0	0	0
17	1	0	1	1	0	0
18	1	0	0	0	0	1
19	1	0	0	0	0	0
20	0	1	0	0	0	0
OPN-16						
1	0	0	0	0	0	1
2	0	0	1	0	0	0
3	0	1	0	0	0	0
4	0	0	0	1	1	0
5	1	0	1	0	0	0
6	0	1	0	0	0	0
7	1	0	0	1	0	0
8	0	1	1	0	0	0
9	0	0	0	0	0	1

Bands no.	Plant species[a]					
	TE	**TG**	**TL**	**CUR**	**RIN**	**CS**
10	0	0	1	0	0	0
11	0	1	0	0	0	0
12	0	0	0	1	1	0
13	1	0	0	0	0	0
OPN-18						
1	1	0	0	0	0	0
2	1	0	0	0	0	0
3	0	0	0	0	1	0
4	0	1	1	0	0	0
5	1	0	0	0	0	0
6	0	0	0	1	0	0
7	0	0	0	0	1	0
8	1	0	1	0	0	0
9	0	0	0	1	0	0
10	1	1	1	0	0	0
11	1	0	0	0	1	0
12	0	1	0	1	0	0
13	0	0	0	0	0	1
14	1	0	0	0	1	0
15	0	1	1	1	0	0
16	0	0	0	0	1	0
17	0	0	1	0	1	0
18	1	1	0	1	1	1
19	0	0	0	1	0	0
20	1	0	0	0	0	0
21	0	0	1	0	0	0
22	0	1	0	0	0	1

[a] TE: *Thunbergia erecta*, TG: *Thunbergia grandiflora*, TL: *Thunbergia laurifolia*, CUR: *Curcuma* sp., RIN: *Rinorea* sp., CS: *Crotalaria spectabilis.*

APPENDIX B

TLC and PCR-RFLP fingerprints and antioxidant activities of “Rang Chuet”

Figure B1 Raw data of DPPH-radical scavenging activities of the three "Rang Chuet" species**.**

Extract	**Concentration**	**DPPH-scavenging activity (% ± SD)**		
	(µg/ml)	***T. laurifolia***	***Curcuma* sp.**	***C. spectabilis***
Ethanol	150.00	57.96 ± 5.76	41.19 ± 7.40	93.54 ± 0.09
	100.00	43.29 ± 0.96	16.89 ± 9.59	93.86 ± 0.15
	67.00	40.25 ± 0.98	19.62 ± 1.29	86.50 ± 0.25
	44.00	33.19 ± 3.62	12.67 ± 2.44	62.20 ± 2.62
	30.00	22.30 ± 0.93	13.58 ± 1.62	40.61 ± 4.10
	20.00	28.80 ± 1.79	10.26 ± 1.41	32.21 ± 2.15
Aqueous	150.00	75.30 ± 3.33	33.47 ± 2.85	34.06 ± 9.25
	112.50	60.76 ± 1.45	31.02 ± 2.58	32.19 ± 1.21
	75.00	45.94 ± 2.79	25.86 ± 0.19	26.89 ± 0.10
	50.00	37.74 ± 0.21	24.79 ± 1.25	16.58 ± 0.97
	33.33	29.54 ± 1.81	20.26 ± 2.30	10.70 ± 1.47
	22.22	23.28 ± 3.04	18.53 ± 1.14	9.37 ± 4.85

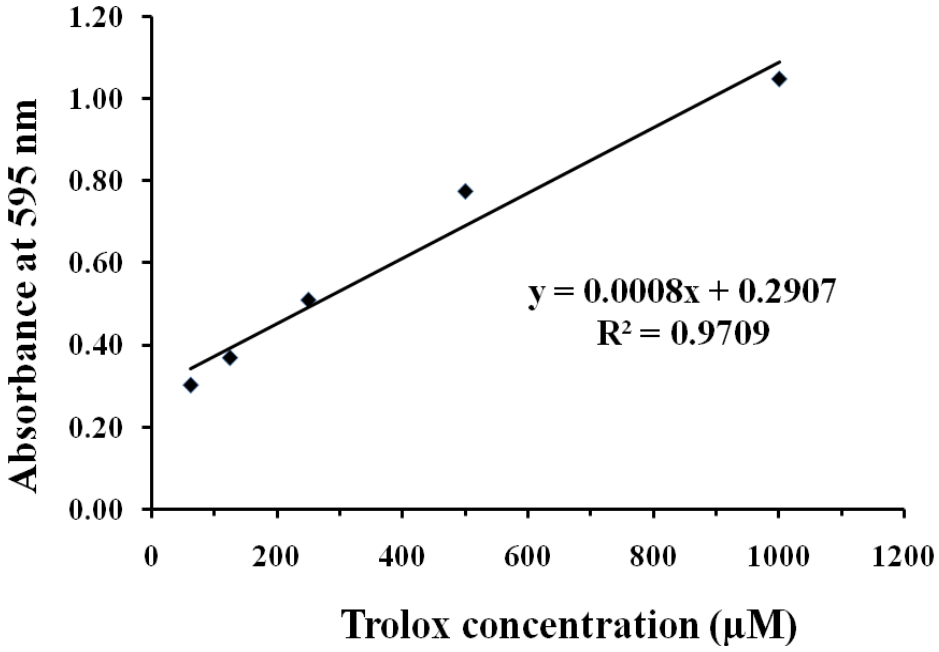


Figure B1 Standard curve of trolox used for calculating of trolox equivalent in FRAP assay.

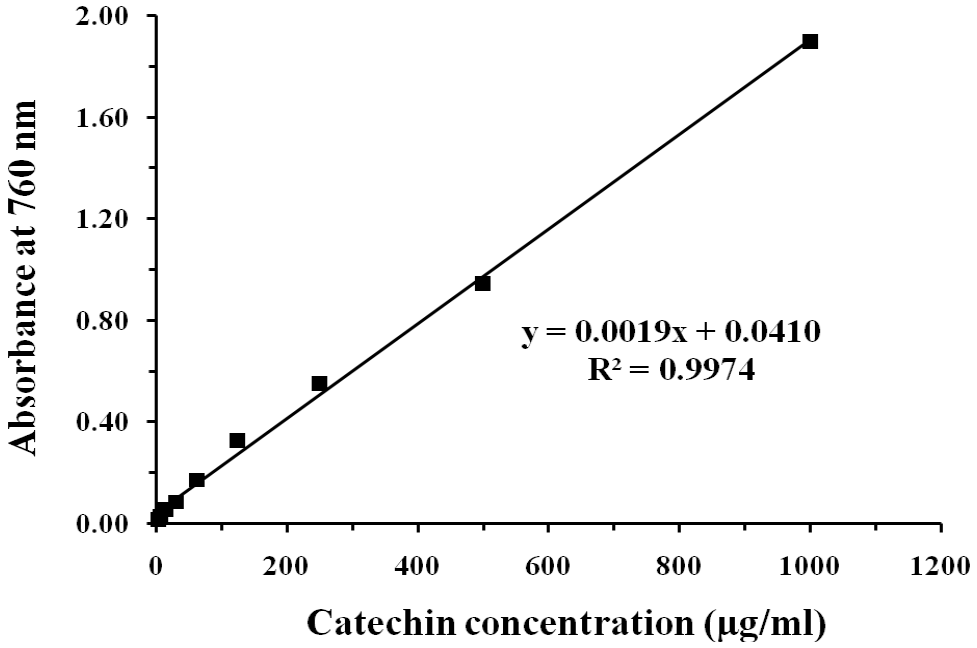


Figure B2 Standard curve of catechin used for calculating of catechin equivalent in the determination of total phenolic contents.

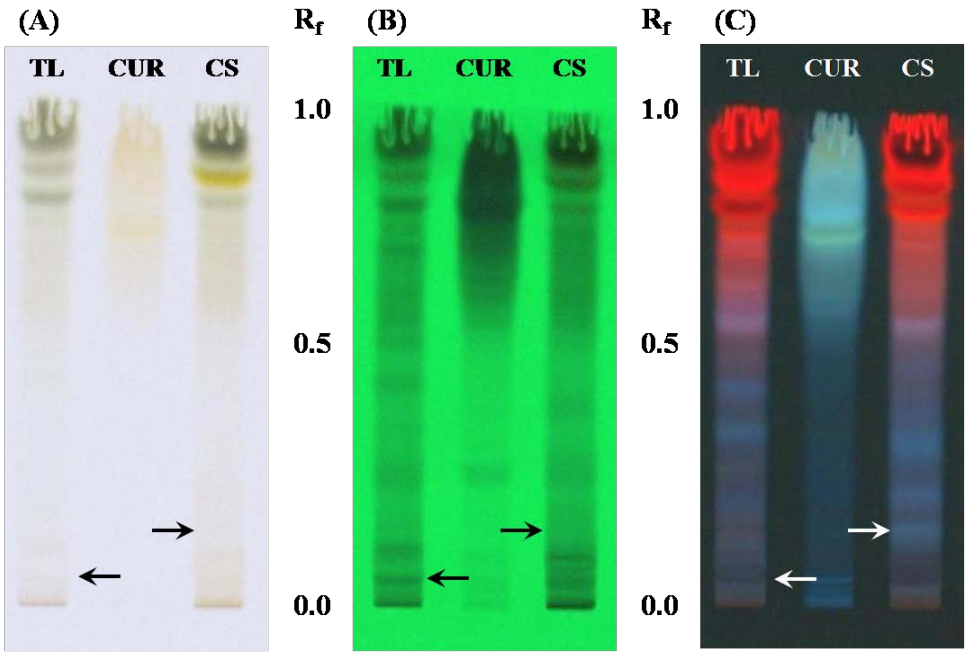


Figure B3 TLC chromatograms of the ethanolic extracts of *Thunbergia laurifolia* (TL), *Curcuma* sp. (CUR), and *Crotalaria spectabilis* (CS). The plates were developed with chloroform-methanol-formic acid (7:3:0.5) and then viewed under visible light (A), under UV light at 254 nm (B), and under UV light at 365 nm (C). The arrows indicate the position of characteristic flavonoid bands detected by $AlCl_3$ spraying solution.

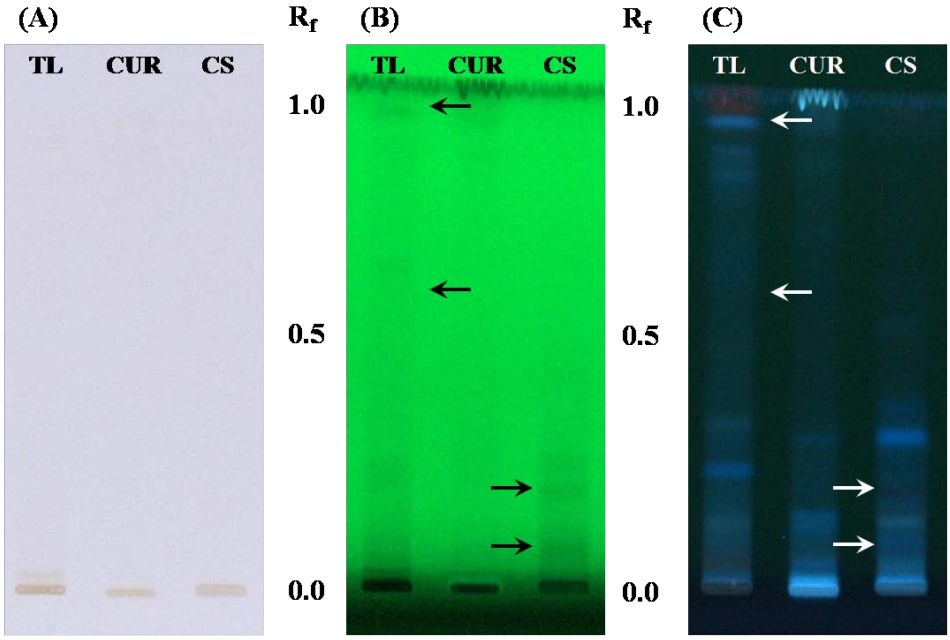


Figure B4 TLC chromatograms of the aqueous extracts of *Thunbergia laurifolia* (TL), *Curcuma* sp. (CUR), and *Crotalaria spectabilis* (CS). The plates were developed with ethyl acetate-formic acid-acetic acid-water (10:0.8:0.8:2.8) and then viewed under visible light (A), under UV light at 254 nm (B), and under UV light at 365 nm (C). The arrows indicate the position of characteristic phenolic bands detected by 20% Na_2CO_3 and Folin-Ciocalteu spraying solution.

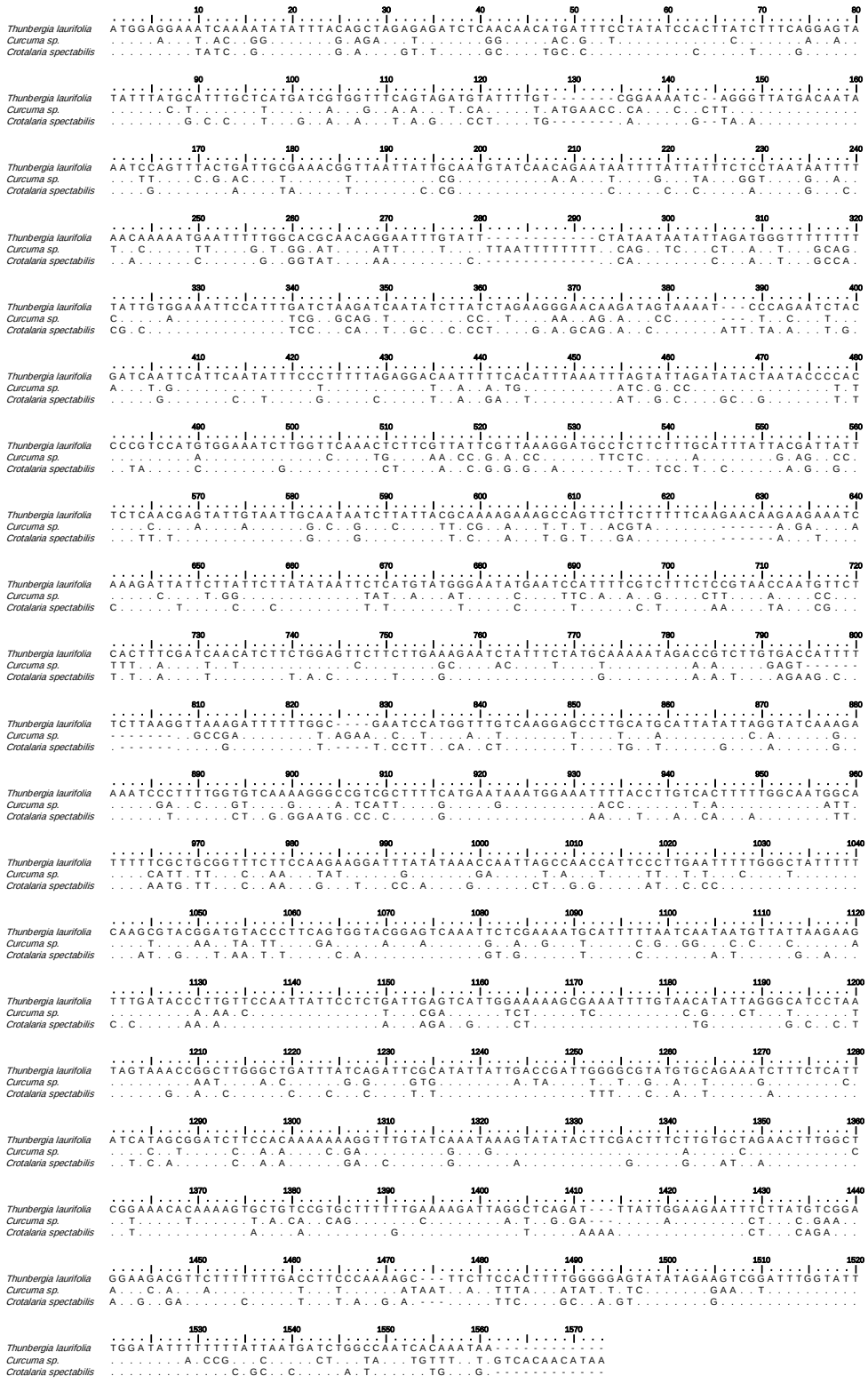
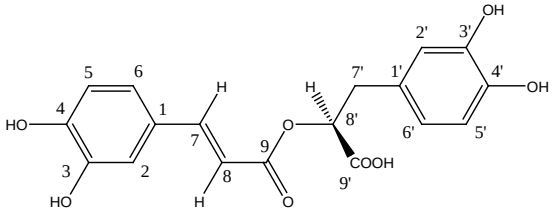


Figure B5 Sequence alignment of full length *mat*K gene among *Thunbergia laurifolia*, *Curcuma* sp., and *Crotalaria spectabilis*. Dots denote a nucleotide identical to those of the *T. laurifolia* sequence. The gaps that were introduced to maintain alignment are indicated by dashes.

APPENDIX C

Isolation of rosmarinic acid from *Thunbergia laurifolia* and its use as a bioactive principle for herbal standardization



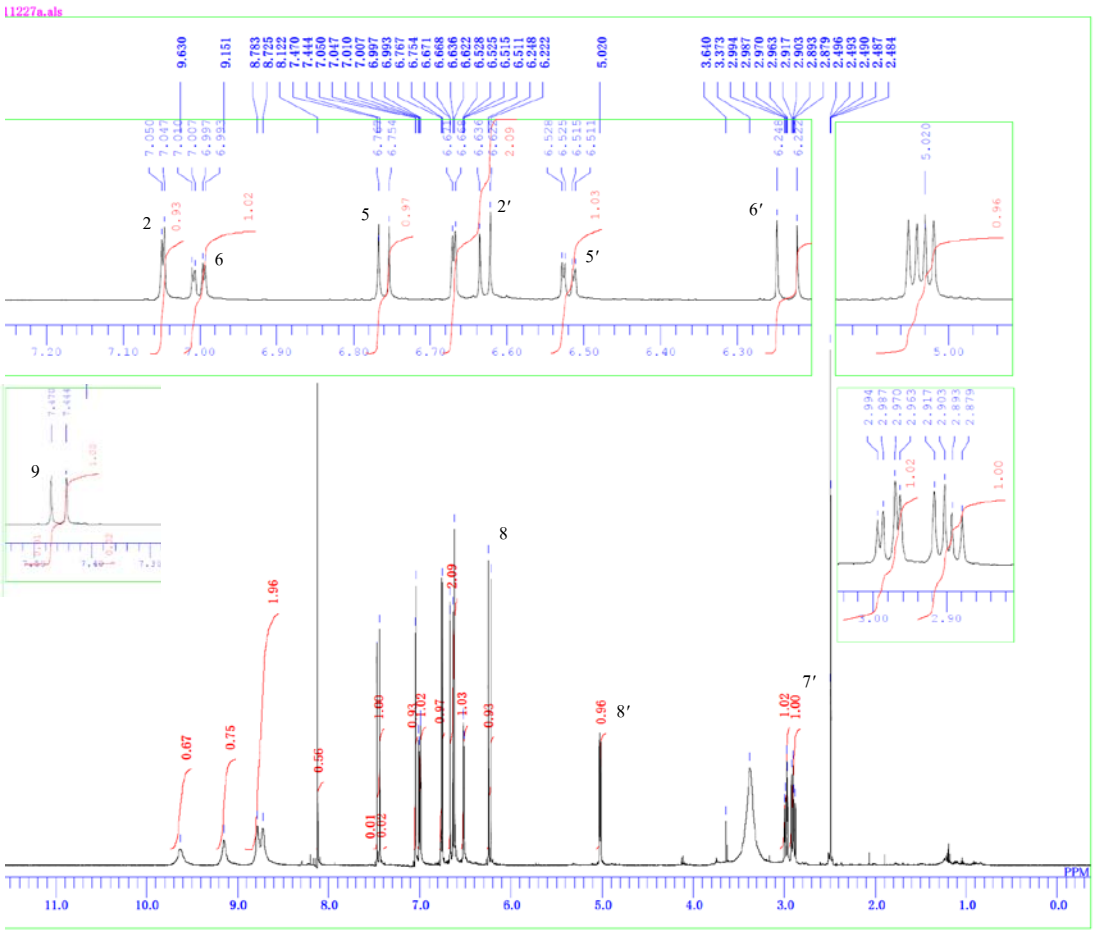


Figure C1 ^{1}H-NMR (600 MHz) spectrum of the isolated rosmarinic acid.

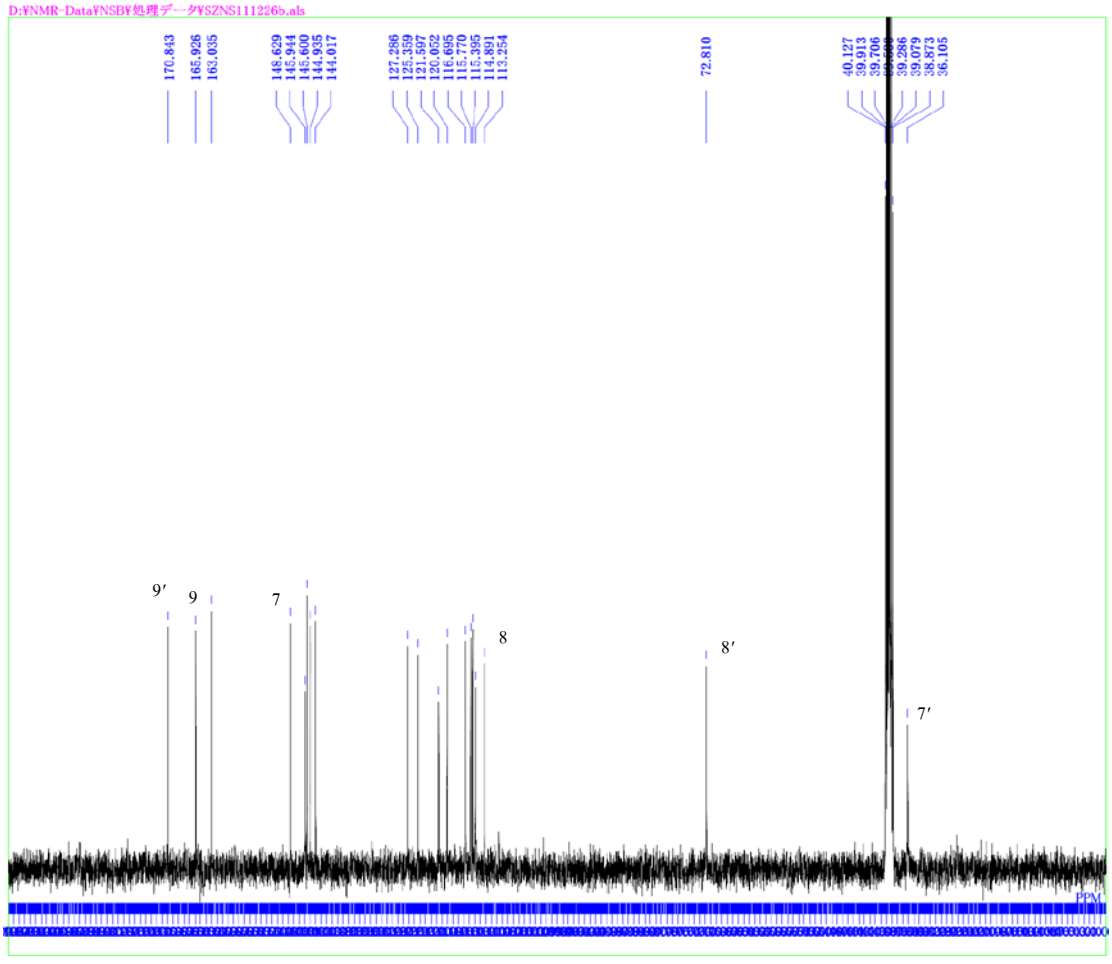


Figure C2 ^{13}C-NMR (100 MHz) spectrum of the isolated rosmarinic acid.

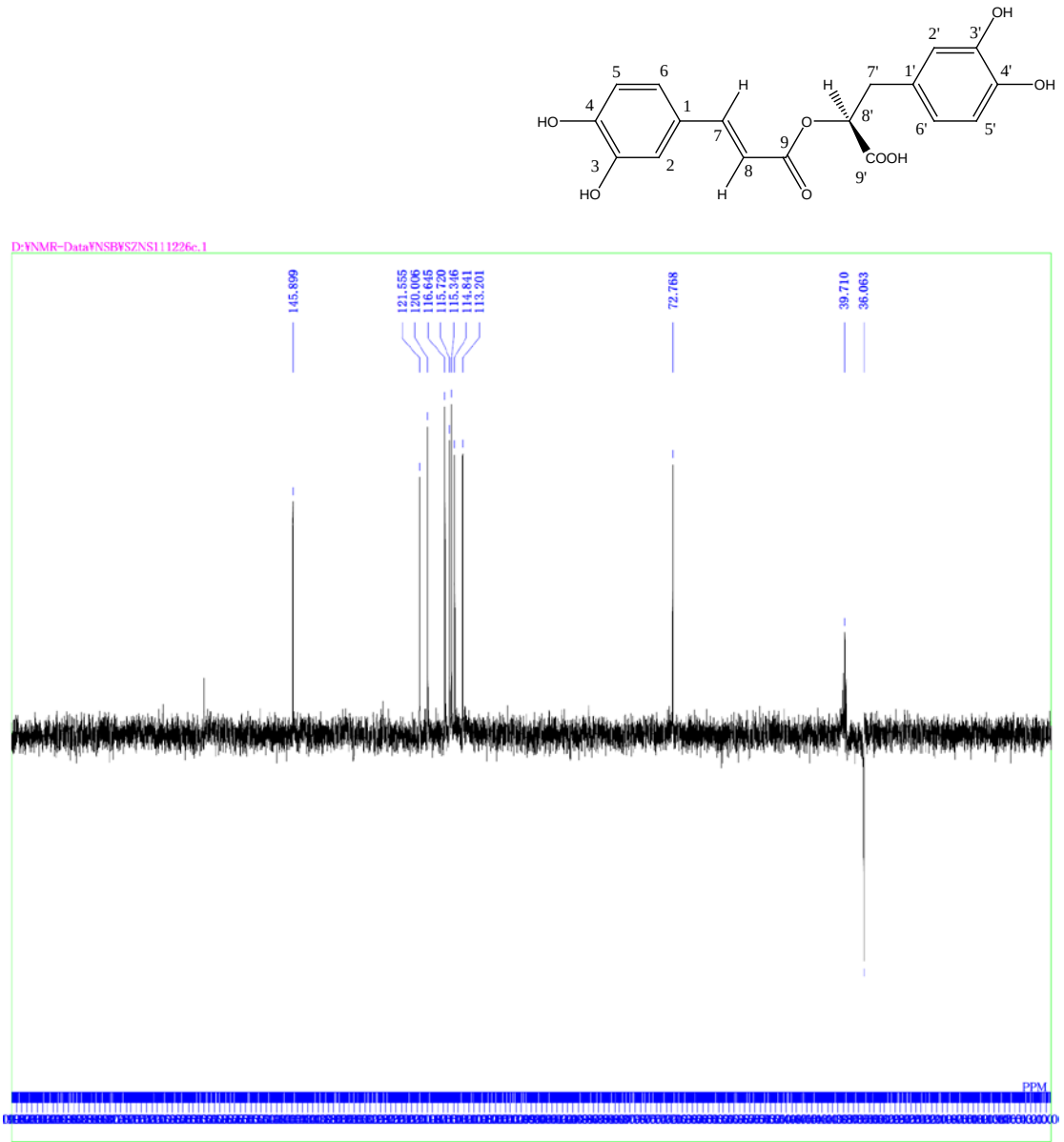


Figure C3 DEPT135 (100 MHz) spectrum of the isolated rosmarinic acid.

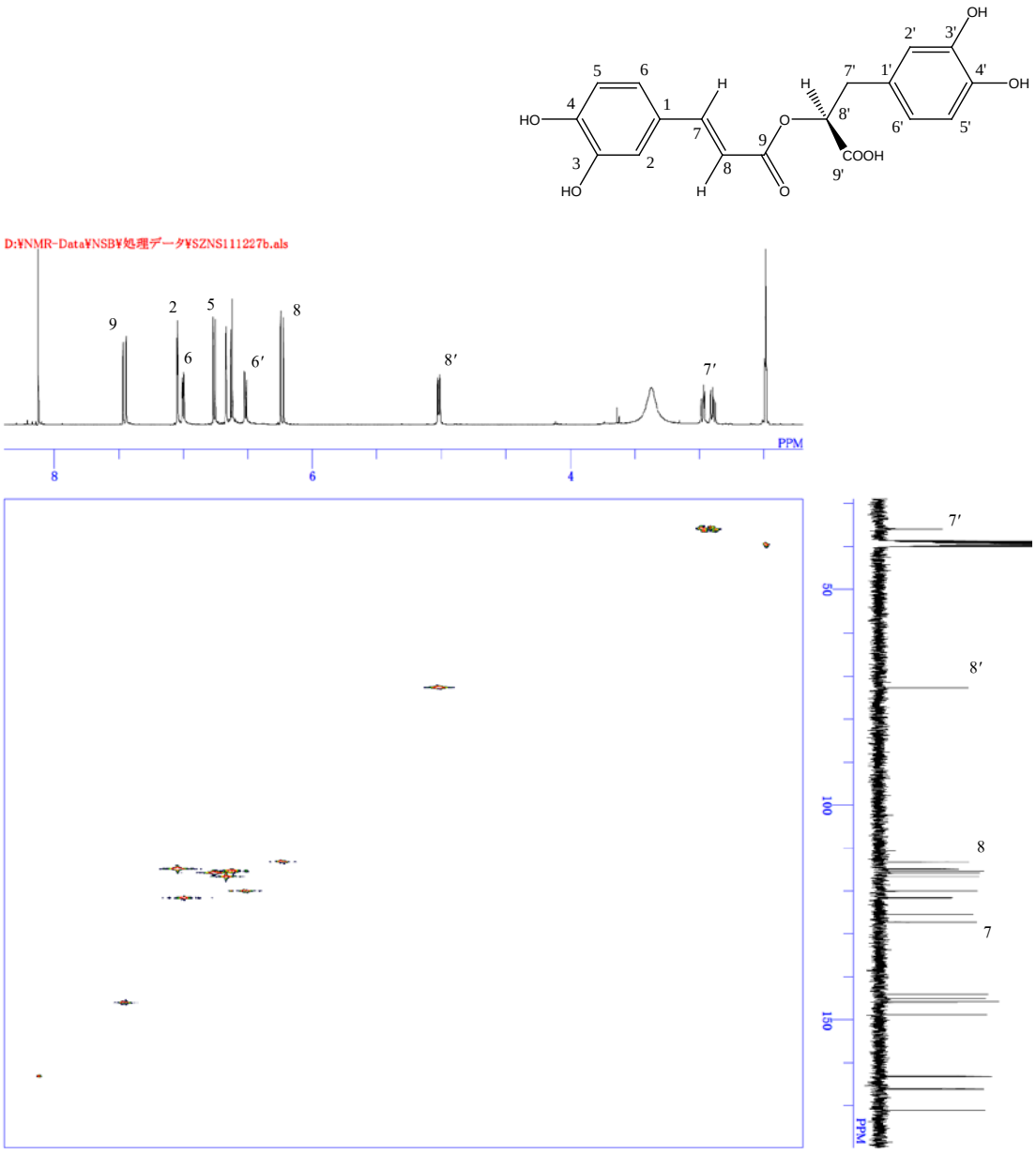


Figure C4 HMQC (600 MHz) spectrum of the isolated rosmarinic acid.

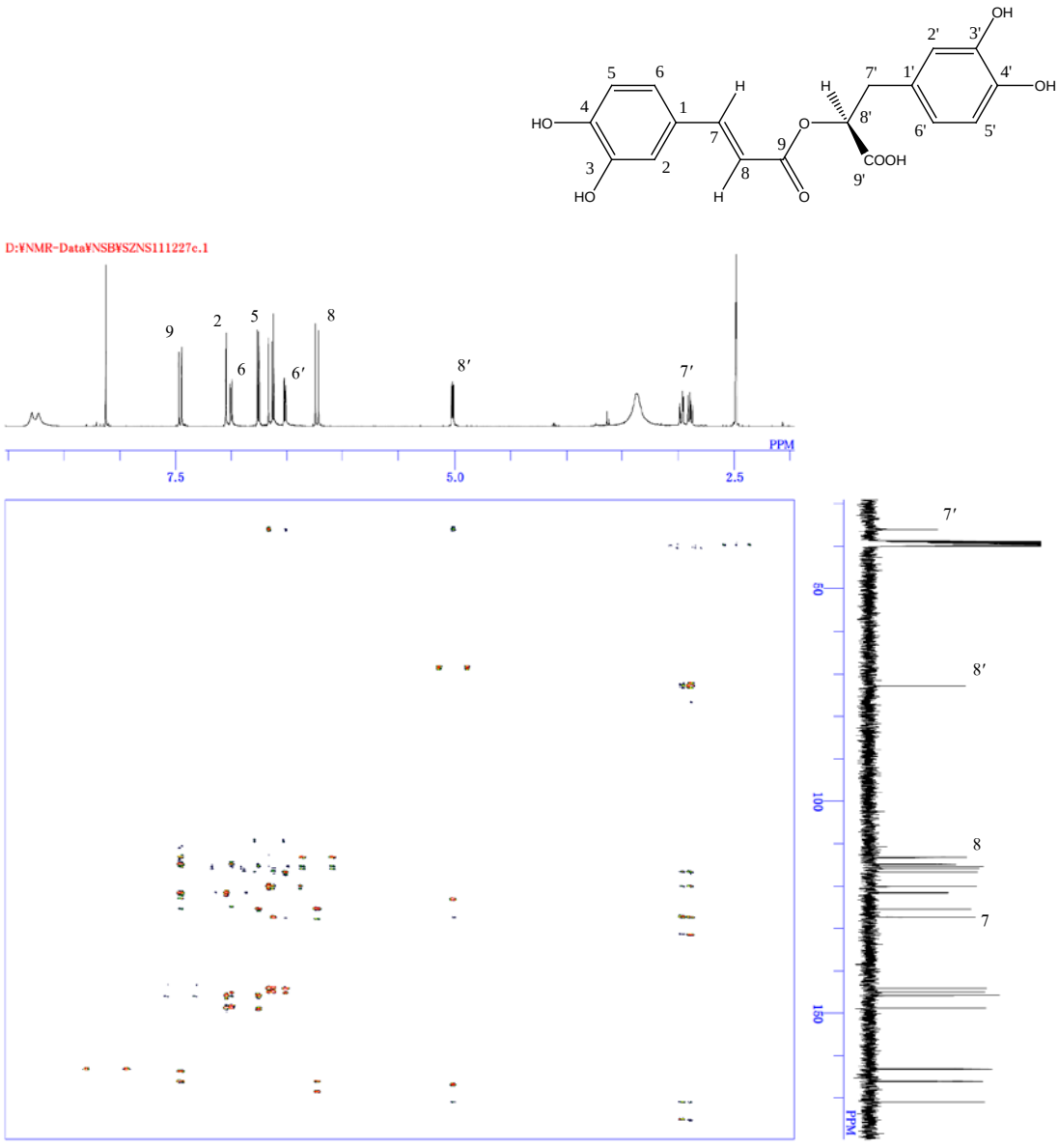
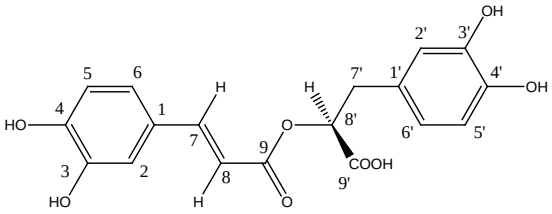


Figure C5 HMBC (600 MHz) spectrum of the isolated rosmarinic acid.



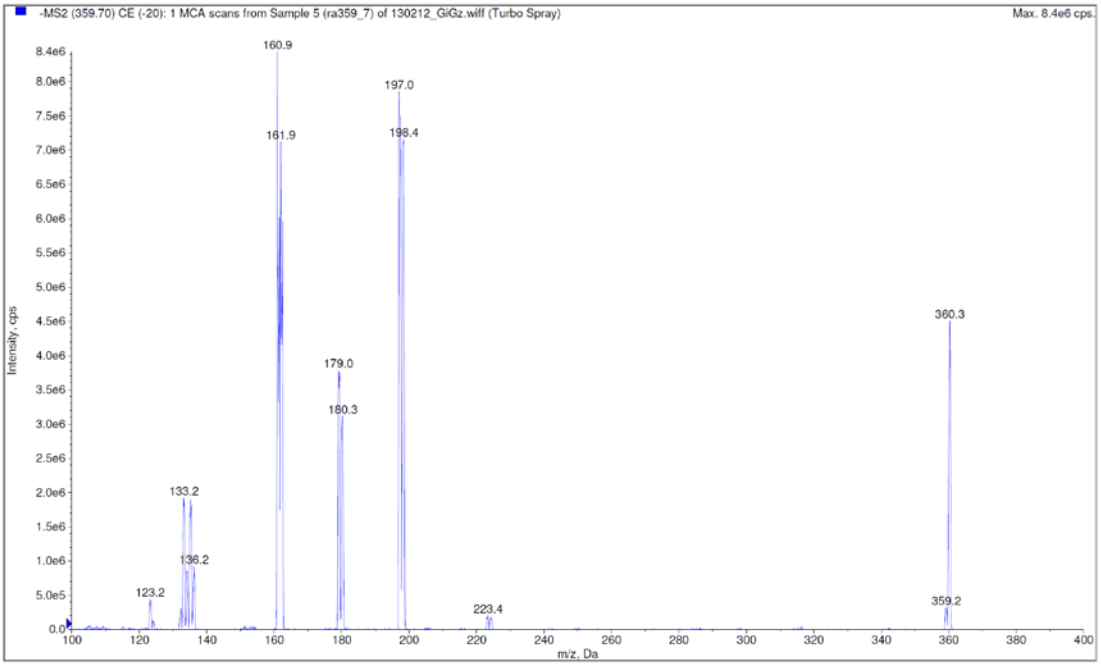


Figure C6 Mass spectrum of the isolated rosmarinic acid.

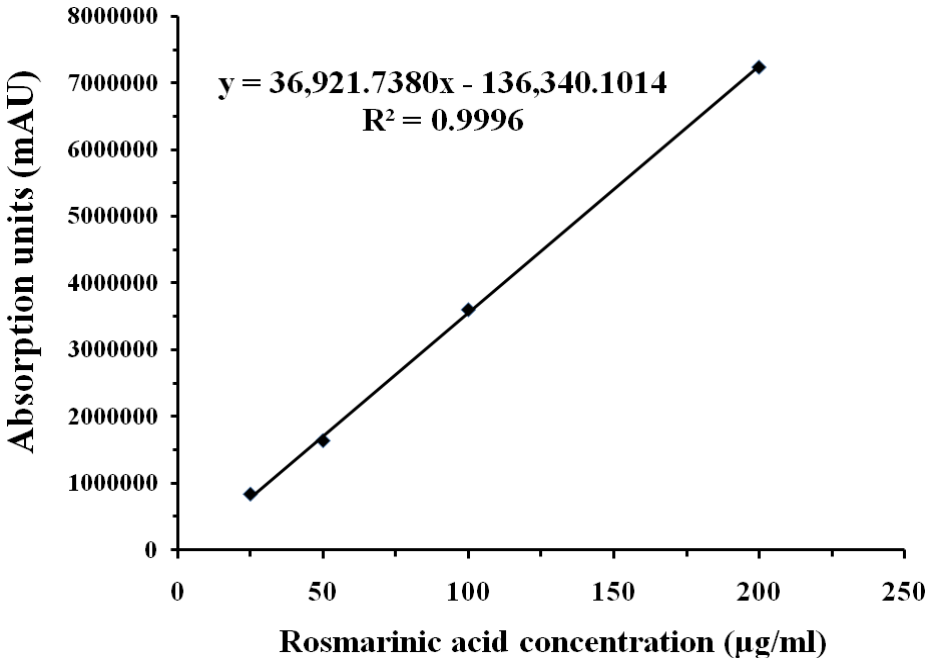


Figure C7 Calibration curves of HPLC generated by the response in absorption units for the isolated rosmarinic acid. The compound was injected (20 µl) in amounts ranging from 0.5-4 µg on column (25-200 µg/ml).

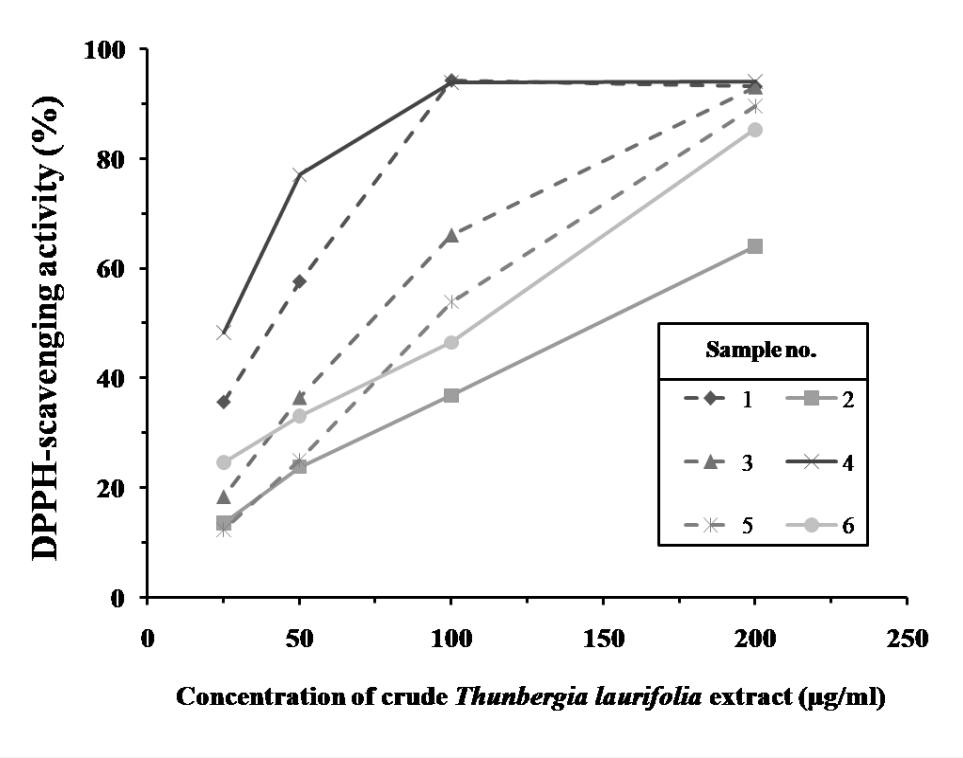


Figure C8 DPPH-scavenging activity of six *Thunbergia laurifolia* samples collected from different locations (1-6).

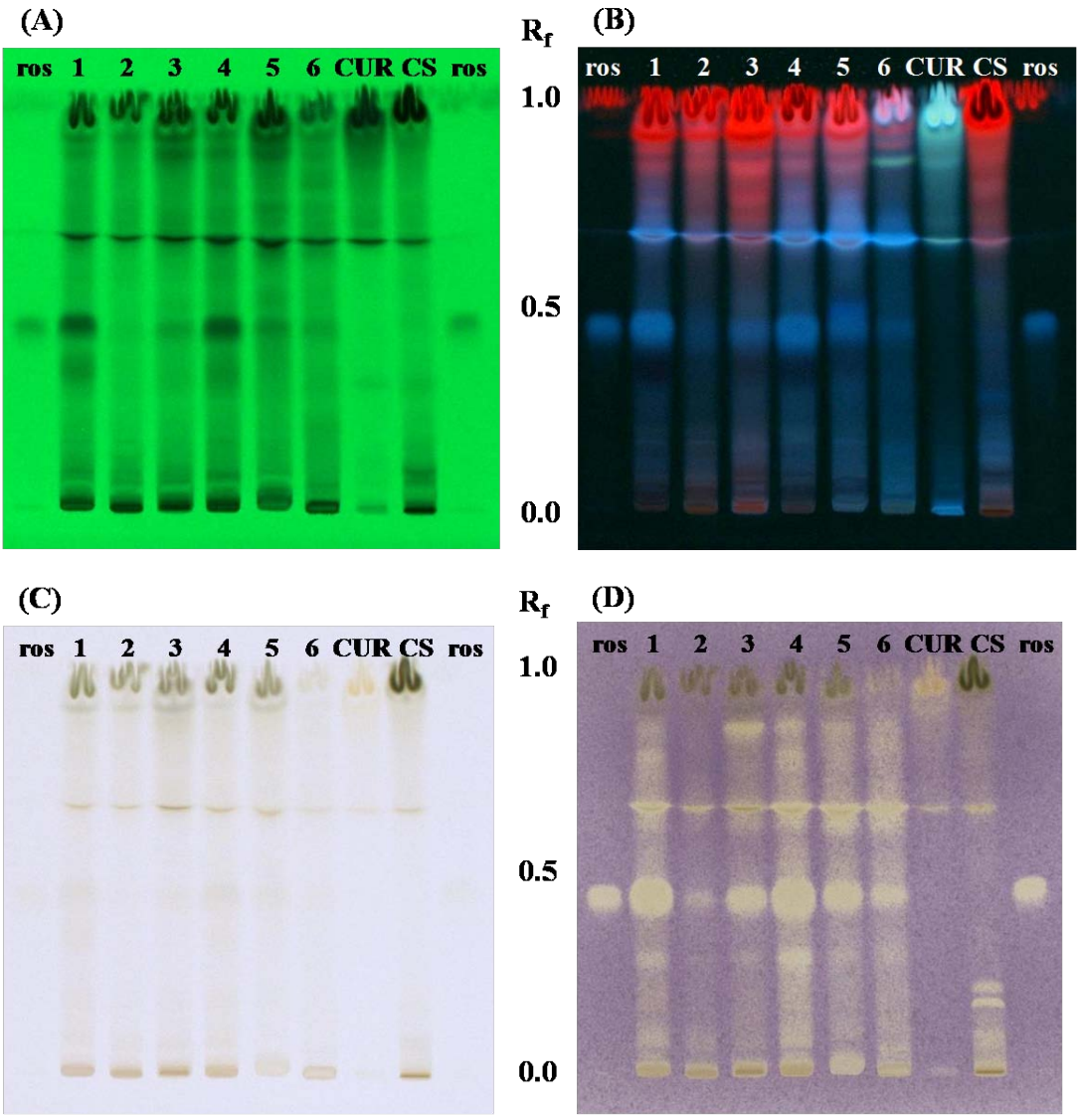


Figure C9 TLC chromatograms of the ethanolic extracts of *Thunbergia laurifolia* collected from different locations (1-6), *Curcuma* sp. (CUR), and *Crotalaria spectabilis* (CS) compared with the isolated rosmarinic acid (ros). The plates were developed with chloroform-methanol-formic acid (7:3:0.5) and then viewed under UV light at 254 nm (A), under UV light at 365 nm (B), under visible light (C), and under visible light after being sprayed with 0.5 mM DPPH solution (D).

BIOGRAPHY

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ORAL PRESENTATIONS

Suwanchaikasem, P., Chaichantipyuth, C., Phadungcharoen, T., and Sukrong, S. Identification of crude drug "Rang Chuet" by combination of pharmacognostic characteristics with DNA fingerprints. TRF-Master Research Congress V, March 30th – April 1st, 2011. Chonburi, Thailand.

Suwanchaikasem, P., Phadungcharoen, T., Chaichantipyuth, C., and Sukrong, S. Identification of *Thunbergia laurifolia* by thin-layer chromatography and random amplified polymorphic DNA. The 5th RSPG Club Conference and RSPG Symposium 2011, November 3rd – 5th, 2011. Nakhon Ratchasima, Thailand.

PUBLICATION

Suwanchaikasem, P., Chaichantipyuth, C., Amnuoypol, S., Sukrong, S. 2012. Random amplified polymorphic DNA analysis of *Thunbergia laurifolia* Lindl. and its related species. Journal of Medicinal Plants Research 6(15): 2955-2961.