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SAFETY EFFICACY AND QUALITY ASSESMENTS OF BEN CHA LO KA WI CHIAN REMEDY

Mr. Chatubhong Singharachai

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By	Mr. Chatubhong Singharachai
Field of Study	Research for Health Development
Thesis Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.
Thesis Co-advisor	Chanida Palanuvej, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Assistant Professor Pongchai Harnyutthanakorn, Ph.D.)

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..... Thesis Advisor
(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

Chanida Palanuvej
..... Thesis Co-advisor
(Chanida Palanuvej, Ph.D.)

Kulwara Meksawan
..... Examiner
(Assistant Professor Kulwara Meksawan, Ph.D.)

Wandee Gritsanapan
..... External Examiner
(Associate Professor Wandee Gritsanapan, Ph.D.)

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ยาตำรับเบญจโลกวิเชียร เป็นยาแผนโบราณที่มีการใช้มาอย่างยาวนานโดยแพทย์แผนโบราณซึ่งใช้เป็นยาถอดไข้ อีกทั้งยังได้ถูกบรรจุไว้ในบัญชียาจากสมุนไพร พ.ศ. 2549 สมุนไพรในตำรับนี้ประกอบไปด้วยผงยาจากรากชิงชี่ รากไม้เท้ายายม่อม รากคนทา รากมะเดื่ออุทุมพร และรากย่านาง ในอัตราส่วนที่เท่ากัน จากการทบทวนวรรณกรรมโดยละเอียดและมีข้อมูลบางส่วนพบว่า ยาตำรับนี้ได้ถูกปนเปื้อน และปนปลอมจากส่วนเหนือดินของพืชชนิดนั้นๆ ดังนั้นคุณภาพของสมุนไพรแต่ละชนิดที่ใช้ในตำรับนี้จึงควรได้รับการประเมิน โดยการจัดทำเป็นข้อกำหนดทางเภสัชเวท และการใช้สถิติวิเคราะห์พหุตัวแปร โดยวิธีโครมาโทกราฟีชนิดของเหลวประสิทธิภาพสูงแบบสามมิติ การศึกษาความปลอดภัยของยาตำรับเบญจโลกวิเชียรและสมุนไพรแต่ละชนิด โดยการทดสอบความเป็นพิษต่อเซลล์ ด้วยวิธีทดสอบการตายของไรทะเล ทดสอบการก่อกลายพันธุ์ด้วยการทดสอบเอมส์ และการก่อให้เกิดความเสียหายต่อดีเอ็นเอด้วยวิธีโคเมต ตามลำดับ การศึกษาประสิทธิภาพของตำรับยาและสมุนไพรแต่ละชนิดโดยศึกษาฤทธิ์ลดไข้และฤทธิ์ระงับปวดในสัตว์ทดลอง ศึกษาฤทธิ์ยับยั้งการก่อกลายพันธุ์โดยใช้วิธีการทดสอบเอมส์ ศึกษาฤทธิ์ในการจับกับอนุมูลอิสระโดยวิธีดีพีพีเอช ศึกษาฤทธิ์ต่อการแบ่งตัวของเซลล์ โดยวิธี เอ็มทีที และศึกษาการเกิดไนตริกออกไซด์โดยวิธี griess reagent พืชตัวอย่างได้รับการเก็บมาจากแหล่งต่างๆ รวมทั้งสิ้น 14 แหล่งทั่วประเทศไทย โดยใช้ลักษณะทางสัณฐานวิทยาและจุลกายวิภาคศาสตร์ของเซลล์และเนื้อเยื่อ รวมถึงกระสวนโครมาโตแกรม เป็นหลักในการจำแนกความแตกต่างของรากสมุนไพรทั้งห้าชนิด จากลักษณะทางจุลกายวิภาคศาสตร์ของเซลล์และเนื้อเยื่อทำให้สามารถกำหนดรูปวิธานซึ่งเป็นลักษณะความแตกต่างของรากสมุนไพรแต่ละชนิด เพื่อใช้ในการพิสูจน์เอกลักษณ์ของผงยาที่ได้จากรากสมุนไพรแต่ละชนิด ซึ่งข้อมูลดังกล่าวจะเป็นประโยชน์ต่อการแก้ปัญหาการปนปลอม และปนเปื้อนของวัตถุดิบที่ได้จากร้านขายยาสมุนไพรได้ จากการศึกษามาตรฐานโดยวิธีโครมาโทกราฟีชนิดของเหลวประสิทธิภาพสูง แบบสามมิติ พบว่ามี 12 พีคหลักในยาตำรับเบญจโลกวิเชียร ซึ่งตำรับยาทุกชุดแสดงลักษณะคล้ายคลึงกันอย่างมาก ตั้งแต่ชุดที่ 2 ถึง ชุดที่ 12 ยกเว้นในชุดที่ 1 การทดสอบการตายของไรทะเล แสดงให้เห็นว่า สารตัวอย่างโดยส่วนใหญ่ไม่มีความเป็นพิษต่อไรทะเล ยกเว้นสารสกัดเอทานอลจากรากย่านาง ซึ่งมีค่า LC_{50}= 44 มคก/มล อีกทั้งยังไม่มีฤทธิ์ก่อกลายพันธุ์โดยตรง แม้กระนั้นสารสกัดโดยส่วนใหญ่ยังคงมีฤทธิ์ก่อกลายพันธุ์ทางอ้อมหลังจากการเกิดปฏิกิริยาในโครเซซูน แต่อย่างไรก็ตาม สารสกัดจากตำรับเบญจโลกวิเชียร และสารสกัดจากรากสมุนไพรแต่ละชนิด แสดงฤทธิ์ยับยั้งการก่อกลายพันธุ์ต่อผลิตภัณฑ์ที่เกิดจากปฏิกิริยาของอมิโนพัยรีนทำปฏิกิริยากับไนไตรท และมีเพียงสารสกัดด้วยน้ำและเอทานอลจากรากชิงชี่ และสารสกัดด้วยน้ำจากรากย่านางที่แสดงฤทธิ์ที่ทำให้เกิดความเสียหายต่อดีเอ็นเอในระดับสูงเทียบเท่ากับไฮโดรเจนเปอร์ออกไซด์ ซึ่งถูกใช้เป็นตัวควบคุมบวก ยาตำรับเบญจโลกวิเชียรทุกขนาดมีผลทำให้การเพิ่มขึ้นของอุณหภูมิที่วัดได้ทางทวารหนักของหนูทดลองซึ่งถูกกระตุ้นด้วยไลโปโพลีแซคคาไลด์ ลดลงอย่างมีนัยสำคัญทางสถิติโดยค่า $p<0.05$ โดยมีประสิทธิภาพเทียบเคียงกับแอสไพริน และยาตำรับเบญจโลกวิเชียรยังแสดงค่านัยสำคัญทางสถิติต่อการทดสอบฤทธิ์ระงับปวดด้วยวิธีการใช้แผ่นความร้อนอีกด้วย สารสกัดตัวอย่างส่วนมากแสดงฤทธิ์ดีในการจับกับอนุมูลอิสระโดยเฉพาะอย่างยิ่งสารสกัดด้วยเอทานอลของตัวอย่างที่ทดสอบ ในกรณีของการทดสอบฤทธิ์ต่อการแบ่งตัวของเซลล์พบว่าสารสกัดตัวอย่างเกือบทั้งหมดมีค่าความเข้มข้นที่ทำให้เกิดเซลล์ตายร้อยละ 50 (LD_{50}) มากกว่า 2,000 มคก/มล ในขณะที่ สารสกัดจากตำรับเบญจโลกวิเชียร แสดงค่า LD_{50} มากกว่า 20,000 มคก/มล ความสามารถในการจับกับอนุมูลอิสระในการทดสอบไนตริกออกไซด์แสดงให้เห็นว่า สารสกัดส่วนใหญ่ที่ได้จากรากสมุนไพรแต่ละชนิดให้ค่าการดูดกลืนแสง (OD) สูงกว่าวิตามินซี ในขณะที่ตำรับยาเบญจโลกวิเชียร ให้ค่าการดูดกลืนแสง ต่ำกว่าวิตามินซี ดังนั้นการศึกษานี้จึงเป็นหลักฐานที่สนับสนุนข้อมูลทางด้านความปลอดภัย ประสิทธิภาพ และคุณภาพของตำรับยาเบญจโลกวิเชียรและสมุนไพรทั้งห้าชนิดซึ่งเป็นส่วนประกอบของตำรับนี้ อย่างไรก็ตาม ผู้บริโภคควรพิจารณาถึงผลข้างเคียงจากการใช้ยาตำรับนี้ร่วมกับไนไตรทซึ่งเจือปนอยู่ในอาหาร นอกจากนี้ ผลจากการศึกษาครั้งนี้ไม่เพียงแต่อธิบายความปลอดภัย ประสิทธิภาพ และคุณภาพของสมุนไพรแต่ละชนิดได้แล้ว ยังทำให้เกิดความเข้าใจผลลัพธ์จากการรวมเป็นตำรับอีกด้วย สุดท้ายนี้จึงสรุปได้ว่าการศึกษานี้ช่วยทำให้เกิดความเข้าใจด้านความปลอดภัย ประสิทธิภาพ และคุณภาพของสมุนไพรแต่ชนิดและตำรับยาเบญจโลกวิเชียรอีกทั้งยังใช้เป็นหลักฐานทางวิทยาศาสตร์เพื่อสนับสนุนยาตำรับเบญจโลกวิเชียรซึ่งเป็นตำรับยาไทยที่เป็นที่รู้จักอย่างแพร่หลายต่อไป

สาขาวิชา วิจัยเพื่อการพัฒนาสุขภาพ ลายมือชื่อนิสิต จตุพงศ์ สิงหราไชย.

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม

4989653520 : MAJOR RESEARCH FOR HEALTH DEVELOPMENT
KEYWORDS : BEN CHA LO KA WI CHIAN REMEDY/ STANDARDIZATION/ 3D-HPLC/ MULTIVARIATE ANALYSIS/ CYTOTOXIC ACTIVITY/ MUTAGENICITY/ ANTI-MUTAGENICITY/ DNA DAMAGE/ ANTIPYRETIC ACTIVITY/ ANALGENIC ACITIVITY/ FREE RADICAL SCAVENGING ACTIVITY/ CELL PROLIFERATION/ NITRIC OXIDE

CHATUBHONG SINGHARACHAI: SAFETY EFFICACY AND QUALITY ASSESSMENTS OF BEN CHA LO KA WI CHIAN REMEDY. ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., CO-ADVISOR: CHANIDA PALANUVEJ, Ph.D., 210 pp.

Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW remedy) is a Thai traditional medicine that has long been used as an antipyretic drug by traditional practitioners and has been notified in the List of Medicine Products of the National List of Essential Drugs A.D. 2006. It is used as mixed powders of the roots of *Capparis micracantha* DC., *Clerodendrum petasites* S. Moore, *Harrisonia perforata* (Blanco) Merr., *Ficus racemosa* L. and *Tiliacora triandra* (Colebr.) Diels, in equal part by weight. From an exhaustive review and few reported data, the remedy has been contaminated and adulterated with upper ground of the plant used. Therefore, the quality of each root species, the pharmacognostic evaluation and multivariate analysis by 3D-HPLC were measured. The safety studies, cytotoxic acitivity, mutagenic testing and DNA damage using Brine shrimp method, Ames test and comet assay were investigated respectively, The efficacy study, antipyretic and analgesic activity by animal model, anti-mutagenic activity by Ames test, free radical scavenging activity by DPPH assay, cell proliferation by MTT assay and nitric oxide by Griess reagent assay were determined. Fourteen samples were collected from wild or non-cultivated places throughout Thailand. The main distinguishable features of five root species were obtained from the morphological and histological characters as well as TLC chromatogram. The histological results allowed an establishment of dichotomous key for the identification of each crude powdered species which is beneficial in resolving the adulteration and contamination of crude drugs in traditional medicine market. Three-dimensional of HPLC was showed clear twelve high major peaks in BLW remedy. All batches remedies were revealed a close relationship between batch 2 to 12 excepted batch 1. The Brine shrimp method demonstrated that most of samples are non-toxic except for the ethanol extract of *T. triandra* (LC_{50} 44 µg/ml). Along with a no-direct mutagenic activity, however most of the extracts exhibited indirect mutagenic activity when combined with nitrosation. Nevertheless, the remedy extracts and the components herb extracts strongly inhibited mutagenicity when nitrite-treated 1-aminopyrene was used as a mutagen. Only water and ethanol extract of *C. micracantha* and water extract of *T. triandra* were exhibited higher damage in DNA as same as the positive control, H_2O_2. All doses of BLW remedy significantly (p<0.05) attenuated the increased rectal temperature produced by lipopolysaccharide (LPS) and were found to be as potent as acetylsalicylic acid (ASA). BLW remedy (400 mg/kg) also produced a significant analgesic response in the hot-plate test. Most of samples also showed good scavenging activity particularly in the ethanol extract samples. In case of cell proliferation, the entire samples were demonstrated LD_{50} more than 2,000 µg/ml, whilst BLW remedy exhibited the LD_{50} more than 20,000 µg/ml. The scavenging activities on nitric oxide demonstrated that most of samples that prepared from each root species were demonstrated the optical density higher than vitamin C, while BLW remedy was exhibited lower optical density than vitamin C. Consequently, the present study provided further evidence to support the safety, efficacy and the quality of Thai traditional medicine: Ben-Cha-Lo-Ka-Wi-Chian remedy and its component herbs. Nevertheless, consumers should be advised on the adverse effects of using the remedy with nitrite containing foods. Moreover, the results of the current study could be described that not only each species which need to carry out for the safety, efficacy and quality, the combination of remedy were need to understand the consequences of such combined used also. Finally, this study helps clarifying the safety, efficacy and quality of each plant species and Ben-Cha-Lo-Ka-Wi-Chian remedy as well as providing additional scientific support for this well-known Thai traditional medicine.

Field of Study: Research for Health Development Student's Signature Chatubhong Singharachai

Academic Year: 2010 Advisor's Signature Nijsiri Ruangrungsi

Co-advisor's Signature Chanida Palanuvej

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LIST OF ABBREVIATIONS

BLW remedy	=	Ben Cha Lo Ka Wi Chian remedy
cm	=	centimeter
m	=	meter
ED_{50}	=	median effective dose
IC_{50}	=	Concentration at 50% inhibition
LD_{50}	=	Medium Lethality dose
LC_{50}	=	Medium lethal concentration
μg	=	microgram
ml	=	milliliter
mg	=	milligram
g	=	gram
kg	=	kilogram
g/kg	=	gram per kilogram
i.p.	=	intraperitoneal
i.m.	=	intramuscular
/	=	per
%	=	percent
%MPE	=	percent of the maximum possible effect
°C	=	degree celsius
ASA	=	acetylsalicylic acid
AUC	=	area under the curve (area of analgesia)
BW	=	body weight
LPS	=	lipopolysaccharide
Min	=	minute
MO	=	morphine sulphate

NAL	=	naloxone
n	=	sample size
GACP	=	good agricultural and collection practices
WHO	=	World Health Organization
TLC	=	thin layer chromatography
AHP	=	American Herbal Pharmacopoeia
3D-HPLC	=	three-dimensional of high performance liquid chromatography
HPLC	=	high performance liquid chromatography
OD	=	optical density
mAU	=	milliabsorbance units
PCA	=	principle component analysis
HCA	=	Hierarchical clustering analysis
r	=	correlation coefficient
his^+	=	histidine independence or histidine prototrophy
his^-	=	histidine dependent
$NaNO_2$	=	sodium nitrite
$NaNO_3$	=	sodium nitrate
DNA	=	deoxyribonucleic acid
SCGE	=	single cell gel electrophoresis
TNFα	=	tumor necrosis factor-alpha
IL	=	interleukin
PG	=	postaglandin
IASP	=	The International Association for the Study of Pain
CNS	=	central nervous system
BHT	=	butylated hyroxytoluene

BHA	=	butylated hydroxyanisole
TBHQ	=	*tert*-Buthylhydroquinone
DPPH	=	1,1-diphenyl-2-picryhydrazyl
MTT	=	4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ROS	=	reactive oxygen species
DMSO	=	dimethylsulfoxide
1-AP	=	1-aminopyrine
MI	=	mutagenic index
M	=	mole per liter
IWGT	=	the international workshop in genotoxicity tests
LMP	=	low melting point agarose
PBS	=	phosphate buffered saline
EDTA	=	ethylenediaminetetraacetic acid
CU-ACUC	=	the Animal Care and Use Committee
ELISA	=	Enzyme-linked Immuno sorbent Assay
NED	=	*N*-1-napthylethylenediamine dihydrochloride
CM	=	*Capparis micracantha* DC.
CP	=	*Clerodendrum petasite* S. Moore
HP	=	*Harrisonia perforata* (Blanco) Merr.
FR	=	*Ficus racemosa* L.
TT	=	*Tiliacora triandra* (Colebr.) Diels

CHAPTER I

INTRODUCTION

Background and Significance of the study

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies [1]. Additionally, herbal medicines are prepared from a variety of plant materials – leaves, stems, roots, bark and so on. They usually contain many biologically active ingredients and are used primarily for treating mild or chronic ailments. Herbs can be prepared at home in many ways, using either fresh or dried ingredients. Herbal teas and infusions can be steeped to varying strengths. Roots, bark or other plants can be boiled into strong solutions called decoctions. Honey or sugar can be added to infusions and decoctions to make syrups. Herbal remedies can also be purchased in the form of pills, capsules or powders, or in more concentrated liquid forms called extracts and tinctures. They can be applied topically in creams or ointments, soaked into cloths and used as compresses, or applied directly to the skin as poultices [2, 3].

In the last decade, there has been a global upsurge in the use of traditional medicine in both developed and developing countries. Today, therefore, certain forms of traditional medicines play an increasingly important role in health care and health sector reform globally [4, 5]. According to The World Health Organization (WHO), 80% of the world's population primarily those of developing countries rely on plant-derived medicines for their healthcare [1, 6]. Most population in the developing countries still relies mainly on indigenous traditional medicine for satisfying their primary health care needs. Traditional medicine has not, however, been incorporated in most national health systems, and the potential of services provided by traditional practitioners is far from being fully utilized. During the last decade, in many developed countries, there has also been a growing interest in herbal medicine, acupuncture and alternative systems of medicine. Consequently, an increase in international trade in herbal medicines and other types of traditional medicines has occurred. Proper use of these different types of medicine has therefore become a concern [7, 8].

One of the main reasons for the increasing use of traditional medicine is growing trend for patients to take a more proactive approach to their own health and to seek out different forms of self-care. There is a worldwide "green" revolution, which is reflected in the belief that herbal remedies are safe and less damaging to the human body than synthetic drugs, under the assumption that "natural means safe" [3, 9]. Moreover, underlying this upsurge of interest in plants is the fact that many important drugs in use today were derived from plants or from starting molecules of plant origin. Digoxin/ digitoxin, the vinca alkaloids, reserpine and tubocurarine are some important examples. Plants have also yielded molecules, which are extremely valuable tools in the characterization of enzymes and the classification of receptor systems where physostigmine, morphine, muscarine, atropine, nicotine and tubocurarine are

important examples. Some scientists thus expect that the plant kingdom hold the key to the understanding of complex human biochemistry/pathology and the cure of man's perplexing diseases. The initial optimism, engendered by the idea that a sophisticated understanding of receptor systems and of the biochemistry of disease would pave the way to predictable drug development, has not been realized. Therefore, laboratories around the world are engaged in the screening of plants for biological activity with therapeutic potential [4, 6, 9-11].

One positive aspect of the use of medicinal plants is their low cost compared to the high price of new synthetic drugs, which have become totally inaccessible to the vast majority of people. Another consideration in favor of the use of medicinal plants, when they are the only recourse available is that they have comparatively few side effects. Synthetic drugs, in general, have very potent pharmacodynamic effects; but as they are active, many also have strong and possibly dangerous and harmful side effects of pharmaceuticals. On the contrary, medicinal plants, with a few exceptions, do not have great therapeutic potency, but neither do they have intense or serious side effects. Therefore, their direct administration in folk medicine offers little risk. Thus, there exists a wide field for research in the phytochemistry of those hundreds of plants that are used in folk medicine in each country, research confirming the presence of pharmacodynamic chemicals such as alkaloids, glucosides to a lesser degree, and essential oils and other substances, indispensable knowledge that justifies the practices of naturalist and folk medicine. The natural products (botanicals) have played the major role in drug discovery [6, 11].

The variety and sheer number of plants with therapeutic properties is quite astonishing. It is estimated that around 70,000 plant species from lichens to towering trees have been used for treating various ailments. Today western herbal medicine still makes use of at least a thousand indigenous European plants, as well as many thousand species of other varieties native to America, Africa and Australia. In Ayurveda (India Traditional Medicine) about 2,000 plants are considered to have medicinal values, while in Chinese Pharmacopoeia 5,700 various traditional medicines of plant origin have been reported. About 500 herbs are employed within the conventional medicine, although whole plants are rarely used. From time immemorial the herbs have played a major role by providing us lead compounds for the isolation and synthesis of so many conventional drugs [4, 6, 11].

Thailand, herbal medicine has long been practiced in Thai history. Herbs which are the integral parts of traditional systems of Thai medicine often combine as herbal mixtures. Either in the Royal or Folk Thai traditional medicine, most herbal recipes contain dozens of herb ingredients. According to Thai medicine, all aliments involve multiple symptoms which point toward multiple imbalances of the four elements or Dhaatu (Earth, Water, Air and Fire). Herbal medicines are traditionally classified by the primary taste of the herb into ten tastes (Astringent, Oily (Nutty), Salty, Sweet, Bitter, Toxic (Nauseating), Sour, Hot (spicy), Bland and Aromatic (Cool)). Combinations of herbs with same or different tastes are the arts of traditional and holistic healing. Herbal combinations have beneficial effects for maximum

potency (synergistic efficacy), minimum certain side effects (antagonistic toxicity) and palatableness [12, 13].

Phikud is a set of herbals with equal quantity and has been used as an ingredient in Thai traditional preparations of medicines. Herb components in same Phikud must have the taste that do not interfere with each other and also have equivalent quality or medical property [14]. This is the traditional wisdom to organize group of herbs for the purpose of synergistic effect and serving healer's conveniently use. Phikud Ben-Cha-Lo-Ka-Wi-Chian is one of the Thai traditional remedies that have been used as an antipyretic drug. First revealed of these remedy has been in the national traditional medical textbook named "Paad Sard Song Kro" which printed by Phra ya Pis-Sa-Nu-Pra-Saad-Vej [15]. It is the first ranke in The Thai traditional medicines notified in the List of Herbal Medicine Product of the National List of Essential Drugs A.D.2006 [16]. The remedy composes of five roots in an equal part by weights. The components of Ben-Cha-Lo-Ka-Wi-Chian Remedy are shown in table 1.

Table 1 Components of Ben Cha Lo Ka Wi Chian Remedy

Scientific name [17]	**Thai name** [17]	**Family** [17]	**Plant Part**
Capparis micracantha DC.	ชิงชี่	Capparidaceae	Root
Clerodendrum petasites S. Moore	ไม้เท้ายายม่อม	Verbenaceae	Root
Harrisonia perforata (Blanco) Merr.	คนทา	Simaroubaceae	Root
Ficus racemosa L.	มะเดื่ออุทุมพร	Moraceae	Root
Tiliacora triandra (Colebr.) Diels	ย่านาง	Menispermaceae	Root

Currently, Herbal medicines are of great importance to the health of individuals and communities, but their quality assurance needs to be developed [4]. Additionally, adverse events arising from consumption of herbal medicines may be due to the term "adulteration" or debasement, which may be deliberate or accidental. Usually in crude drug, that included substitution of the original crude drugs partially of fully with other substances which are either free from or inferior in therapeutic and chemical properties. Inferiority is a natural substance condition (e.g. where a crop is taken whose natural constituent is below the minimum standard for that particular drug) which can be avoided by more careful selection of the plant material [18]. Spoilage is substandard condition produced by microbial of other pest infestation, which makes a product unfit for consumption, which can be avoided by careful attention to the drying, and storage conditions. Deterioration is an impairment of the quality or value of an article due to destruction of abstraction of valuable constituents by bad treatment or aging or to the deliberate extraction of the constituents and the sale of the residue as the original drugs. Admixture is the addition of one article to another through accident, ignorance or carelessness e.g. inclusion of soil on an underground organ or the co-collection of two similar species. Sophistication is the deliberate addition of spurious of inferior material with intent to defraud; such materials are carefully produce and may appear at first sight to be

genuine e.g. powder ginger may be diluted with starch with addition of little coloring material to give the correct shade of yellow colour. Substitution is the addition of an entirely different article in place of that which is required e.g. supply of cheap cotton seed oil in place of olive oil [19,20].

Additionally, Ben Cha Lo Ka Wi Chian Remedy is one of the several Thai traditional remedy that has been provided in the market. It is could be adulterated with upper ground parts of plants (not only roots) or substances with low quality. Moreover, the general lack of knowledge about Ben Cha Lo Ka Wi Chian remedy such as standardization of each plant, biological activities and efficacy of the remedy. Hence, the safety and efficacy, as well as the quality control, of Ben Cha Lo Ka Wi Chian remedy have become important concerns for both health authorities and the public.

Objective of the study

1. To develop standardization parameters of each species in Ben Cha Lo Ka Wi Chian remedy.
2. To evaluate some toxicity of Ben Cha Lo Ka Wi Chian remedy.
3. To evaluate some biological activities of Ben Cha Lo Ka Wi Chian remedy and its components.

Expected Benefits

1. This research provides pharmacognostic specification of each species in Ben Cha Lo Ka Wi Chian remedy which needed for drug standardization and drug quality improvement.
2. This research provides scientific evidences in efficacy and safety of Ben Cha Lo Ka Wi Chian which needed for the step of clinical trials.
3. This research protocol can be applied to other traditional medicine formularies.

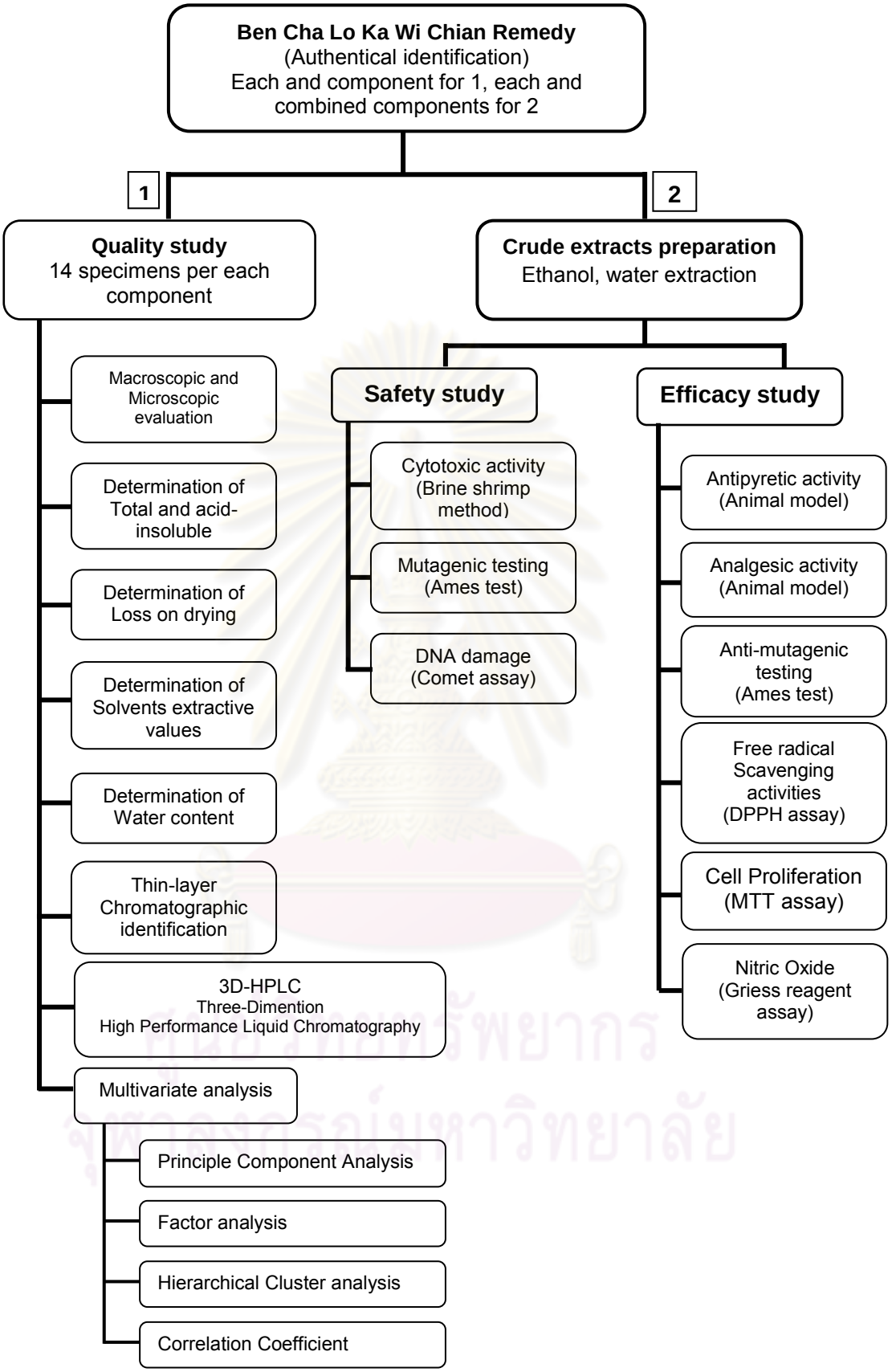


Figure 1 Scopes of the study

CHAPTER II

LITERATURE REVIEWS

Ben-Cha-Lo-Ka-Wi-Chian Remedy

Ben-Cha-Lo-Ka-Wi-Chian is one of the Phikud in Thai traditional medicine. Phikud is a set of herbs with equal quantity and has been used as an ingredient in Thai traditional preparations of medicines. Herb components in same Phikud must have the taste that do not to interfere with each other and also have equivalent quality or medical property. Earliest, it was revealed in Paad Sard Song Kaor scripture.

In the present time, Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW remedy) has been notified in The list of Herbal Medicine Products of the National List of Essential Drugs A.D. 2006. The Thai Government also supports to be used in Primary Health Care as a general antipyretic drug in the form of powder or tablet.

BLW remedy is composed of five root species, including *Capparis micracantha* DC., *Clerodendrum petasites* S. Moore*., Harrisonia perforata* (Blanco) Merr*., Ficus racemosa* L. and *Tiliacora triandra* (Colebr.) Diels, in an equal part by weight.

Recently, Koncue *et al.* [21] had examined the antipyretic effect of each root powder and the remedy utilizing yeast-induced fever model in rats. From the study, BLW remedy was found to have antipyretic effect at doses 100, 200 or 400 mg/kg and BLW remedy 200 mg/kg seemed to have the highest antipyretic efficacy. Each root also seemed to be active as an antipyretic drug, except the root of *C. petasites*. This is the only scientific evidence that support its use as an antipyretic drug. Thus BLW remedy has inadequate scientific evidence to support the above claimed.

***Capparis micracantha* DC.**

C. micracantha is a half-erect shrub or small tree with drooping branches, 1-6 m tall, leaves oval to oblong-lanceolate: 9.5 -20 cm x 3 -11 cm, flowers 2-6 in row, pedicel about 1 cm long, white with yellow base, later turning dark red [22].

A book of Thai Herbs mentioned that the flowers of *C. micracantha* can inhibit cancer [30]. The hexane extracts of the leaves and flowers of *C. micracantha* showed inhibiting lung cancer and anti-tuberculosis. Moreover, the hexane and dichloromethane extracts of the flowers have been shown anti-tuberculosis [23]. As well, The root of *C. micracantha* using Heinz body model has been shown antioxidation [24].

***Clerodendrum petasites* S. Moore**

C. petasites is vines, shrubs or small trees, usually unarmed, glabrous or pubescent. Leaves are opposite of whorled, simple, sometimes lobed, Flowers are zygomorphic, bisexual, usually large, showy, mostly white.

In Thai folklore medicine, its leaves and roots are traditionally used for the treatment of fever, inflammation and skin diseases as well as asthma [25]. In India, a mixture of the fruits of *C. petasites* is used to produce sterility [26], whereas Chinese use it (part not specified) for the treatment of fever and malaria [27].

There were several phytochemical, biological and pharmacological studies of *C. petasites.* [28-30]. One pronounced effect of *C. petasites*is antibacterial activity of two flavonoid compounds, which isolated from the stem and roots of the plant [30]. In addition, Chatluang found that the ethanol extract from this plant possessed the bronchodilator effect [31]. The active principle responsible for this effect was isolated and identified as the flavonoid hispidulin. The ethanolic extract of *C. petasites* caused relaxation of tracheal smooth muscle which was contracted by exposure to histamine [32]. Moreover, it was found that *C. petasites* extract possessed moderate inhibitory activity on acute phase of inflammation in a dose-related manner as seen in ethyl phenylpropiolate-induced ear edema (ED_{50} = 2.34 mg/ear) as well as carrageenin-induced hind paw edema (ED_{30} = 420.41 mg/kg) in rats. *C. petasites* extract possessed an excellent antipyretic effect when tested in yeast-induced hyperthermic rats [33].

***Harrisonia perforata* (Blanco) Merr.**

H. perforata is a scandent to erect prickly shrub up to 4 - 6 m tall, leaves imparipinnate up to 20 cm long, with 1-15 pairs of leaflets. Flowers are 4-5 merous, red outside, pale red to white inside. Fruits are a berry [22].

H. perforata has been used for diarrhea and dysentery [34, 35]. The extracts of the leaves and the branches showed *in vitro* antimalarial activity against *Plasmodium falciparum* [36, 37]. Chemical investigations have shown the presence of several chromones, peucenin-7-methyl ether, *O*-methylalloptaeroxylin (perforine A) [38], perforatin B, perforatic acid, perforatic acid methyl ester, perforatin, perforatin C—G [39, 40], two limonoids, perforatinolone [41] and tetranortriterpenes in the obacunol series [42], that have been isolated from the roots [43, 44], leaves [39, 41], branches [45] and wood [46] of *H. perforata* [46]. Quassinoids were also identified from the root bark of this plant [38, 39].

***Ficus racemosa* L.**

F. racemosa is evergreen or sometime deciduous, woody epiphytic climbers or stranglers, shrubs or small to large trees up to 40-50 m tall or banyans. Bark surface is smooth, often pale grey, sometime whitish or brown. The inner bark is yellowish [22].

The leaves, bark and fruits of *F. racemosa* are employed in native medicine to treat several diseases [47]. It has been reported in the indigenous system of medicine such as in Sri Lanka, which has been used as treatment of skeletal fracture [48], hypoglycemic [49] and antidiarrheal activity [50]. Experimental studies have demonstrated its anti inflammatory, hepatoprotective and hypoglycemic effect [61-63]. It has been reported to have many

medicinal properties [54]. The roots are used as a medicine against hydrophobia. Its fruits are effective against leprosy, diseases of the blood, fatigue, bleeding nose and cough. Its bark is helpful against asthma and its leaves are used against bronchitis. It is used as carminative, astringent, vermifuge and an anti-dysentery drug [55]. The bark is used for treatment of dysentery [56-59], antiseptic, antipyretic and vermicidal, and a decoction of the bark is used in treating various skin diseases and ulcers. It is used as a plaster in inflammatory swellings and boils. It is reported to be effective in the treatment of piles, dysentry, asthma, gonorrhea, gleet, menorrhagia, leucorrhea, hemoptysis, and urinary diseases [55, 60, 61]. Its stem bark has shown anti-diarrhoeal, antidiuretic, antitussive, anti-pyretic and hypoglycemic activities [53, 62-65]. The root of *F. racemes* is useful in dysentery, diarrhea and diabetes [56, 66]. The plant is used locally to relieve inflammation of skin wounds, lymphadenitis, sprains and fibrositis. It is also used in the treatment of mumps, smallpox, heamaturia and inflammatory conditions [67].

The chemical composition and medicinal uses of *F. racemosa* extract have been reported widely. *F. racemosa* extract has been found to possess significant anti-inflammatory activity on the tested experimental models. It has hepatoprotective, immunostimulant, antibacterial, antiedemic, antihistaminic, antipyretic and analgesic activities [67, 68]. The extract of fruit is used in diabetes and leucoderma. The alcoholic extract of the stem bark of the plant possessed antiprotozoal activity against *Entamoeba histolytica* [55]. Moreover, the extract of *F. racemosa* bark with IC_{50} of 100 µg/ml showed more potent inhibitory effect of COX-1 [79]. Also, another studies were carried out to evaluate the anti-pyretic effect of a methanol extract of stem bark of *F. racemosa* on normal body temperature and yeast-induced pyrexia in albino rats. The methanolic extract of stem bark of *F. racemosa* possessed significant antipyretic effect in yeast-provoked elevation of body temperature in rats, and its effect was comparable to that of paracetamol (standard drug) [53]. The chemomodulatory effect of *F. racemosa* against ferric nitrilotriaceatate (Fe-NTA) induced renal carcinogenesis and oxidative damage response in rats were reported recently [35, 70].

This plant has been reported to contain tannins, kaempferol, rutin, arabinose, bergapten, psoralenes, flavonoids, ficusin, coumarin and phenolic glycosides [71]. All these compounds act as strong antioxidant and anti-inflammatory agents [55].

***Tiliacora triandra* (Colebr.) Diels**

T. trianda has been widely distributed throughout Thailand and common in deciduous and dry evergreen forests [72]. It is used particularly in many cuisines of the northeast of Thailand and Lao PDR especially in bamboo shoot soup. It is climbing plant with deep green leaves and yellowish flowers, stem usually slender, leaves simple, alternate, green to dark-green ovate about 5-10 cm long and 3-4 cm wide, fruits yellowish obovate druplets [22, 73].

Chemical analysis revealed that *T. triandra* leaves contain high levels of beta-carotene and minerals, such as calcium and iron. A number of alkaloids, especially bisbenzylisoquinoline

alkaloids have been identified in *T. triandra* [73, 74]. Three know alkaloids, tiliacorinine, tiliacorine, and nortiliacorinine A, together with a new alkaloid, tiliacorinine 2'-*N*-oxide, have been isolated from the roots of *T. triandra* [75]. Also, Tilitriandrin is a new bisbenzylisoquinoline alkaloid from *T. triandra* [76]. Moreover, alkaloids magnoflorine, nortiliacorine A, and tiliacorinin-2'- N-oxide, two new bisbenzylisoquinoline alkaloids, noryanangine and norisoyanangine were isolated from the aerial parts of *T. triandra* Diels. [77]. The major constituent monosaccharide of *T. triandra* gum was xylose, together with substantial amounts of rhamnose, arabinose, glucose and galactose [78].

The roots of *T. triandra* were found to have antimalarial activity against *Plasmodium falciparum in vitro* [29]. However, the water extract from *T. triandra* does not produce acute or subchronic toxicities in female and male rats [30].

Standardization Parameters

Quality Control of Plant material

Comparing with the conventional preparations, herbal products represent a number of unique problems when quality aspects are considered. These are because of the nature of the herbal ingredients present therein, which are complex mixtures of different secondary metabolites that can vary considerably depending on environmental and genetic factors. Furthermore, the constituents responsible for the claimed therapeutic effects are frequently unknown or only partly explained and this precludes, the level of control which can routinely be achieved with synthetic drugs so much so with conventional pharmaceutical preparations. These complex positions of quality aspects of herbal drugs are further complicated by the use of combinations of herbal ingredients as are being used in traditional practice. It is not uncommon to have as many as five (sometimes even more) different herbal ingredients in one product.

Standards of any drug relate to the uniformity in quality, which are numerical quantities by which the quality of commodities may be assessed. The information upon which standards may be based is obtained by a study of the genuine drug, the methods used for adulteration and the means adopted for the detection of adulterants. While proposing the standards for crude drugs, several aspects are to be considered as pharmacognostical standards.

In the meantime, The World Health Organization (WHO) has conducted a recent global survey on the regulatory control of herbal medicines and has reported finding from 141 countries [50]. This work provides a valuable update to the earlier WHO reviews and illustrates the wide differences in the approach to regulation between these countries [51, 52]. The recent survey confirms that during the past four years many countries have established, or initiated, the process of establishing national policy and regulations regarding herbal medicines. The most important challenges faced by countries were those related to regulatory status, assessment of safety and efficacy, quality control and safety monitoring. In response

to requests from Member States, WHO has resolved to provide technical support for the development of methodology monitor or ensure product safety, efficacy and quality, preparation of guidelines, and promotion of information exchange. WHO guidelines have recently been developed in a number of important areas including consumer information, pharmacovigilance, good agricultural and collection practices (GACP) [53-55] and quality control methods for medicinal plant materials: World Health Organization Geneva [81].

The World Health Assembly – in resolutions WHA31.33 (1978), WHA40.33 (1987) and WH42.43 (1989) – has emphasized the need to ensure the quality of medicinal plant products by using modern control techniques and applying suitable standards. The Quality Control Method for Medicinal Plant Materials: WHO manual describes a series of tests for assessing the quality of medicinal plant materials. The tests are designed primarily for use in national drug quality control laboratories in developing countries, and complement those described in the international pharmacopoeia, which provides quality specifications only for the few plant materials that are included in the WHO Model List of Essential Drugs. This manual does not constitute a herbal pharmacopoeia, but a collection of test procedures to support the development of national standards based on local market conditions, with due regard to existing national legislation and national and regional norms. The test methods described are the best methods currently available [82].

The Quality Control Methods for Medicinal Plant Materials are consisting of several experiments namely,

Parameters standardization

The evaluation of these parameters gives a clear idea about the specific characteristic of crude drugs under examination, beside it's macro-morphological or cyto morphological characters, microscopical natures in both it's entire and it's powder form. While these diagnostic features enable the analyst to know the nature and characteristics of the crude drugs, further evaluation of different parameters indicate their acceptability by criteria other than the morphological characteristics. The procedures normally adopted to get the qualitative information about the purity and standard of a crude drug include the determination of various parameters as described in this sections.

Determination of ash

Ashing involves an oxidation of the components of the product. A high ash value is indicative of contamination, substitution, adulteration or carelessness in preparing the crude drug for marketing. Total ash is designed to measure the total amount of material produced after complete incineration of the ground drug at as low temperature as possible (about $450^{O}C$) to remove all the carbons. At higher temperature, the alkali chloride may be volatile and may be lost by this process. The total ash usually consists of carbonates, phosphate, silicates and silica which include both physiological ash-which is derived from the plant tissue itself and

non-physiological ahs which is the residue of the adhering material to the plant surface e.g. sand and soil.

Determination of acid-insoluble ash

Ash insoluble in hydrochloric acid is the residue obtained after extracting the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

Determination of loss on drying

An excess of water in medicinal plant materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water.

Determination of solvent extractive values

This method determines the amount of active constituents in a given amount of medicinal plant material when extracted with solvents. It is employed for materials for which as yet no suitable chemical or biological assay exists. As mentioned in different official books [83, 84] the determination of water soluble and alcohol soluble extractives, is used as a means of evaluating crude drugs which are not readily estimated by other means.

The extraction of any crude drug with a particular solvent yields as solution containing different phyto-constituents. The composition of these phyto-constituents in that particular solvent depends upon the nature of the drug and solvent used. The use of a single solvent can be the means of providing preliminary information on the quality of a particular drug sample; for example, in a drug where the extraction procedure for the constituents commences with as the solvent, any subsequent aqueous extraction on the re-dried residue will give a very low yield of soluble matter.

Determination of water content

Moisture is an inevitable component of crude drugs, which must be eliminated as far as practicable. The preparation of crude drug from the harvested drug plants involves cleaning or garbling to remove soil or other extraneous material followed by drying which play a very important role in the quality as well as purity of the material. Insufficient drying favors spoilage by molds and bacteria and makes possible the enzymatic destruction of active principles. The moisture requirements for the active growth of some of the common molds and bacteria that may be found in or on drugs are relatively low. Therefore, the drying process should reduce the moisture content of the drug below this critical, or threshold level.

Thin-layer Chromatographic identification

Thin-layer chromatography is particularly valuable for the qualitative determination of small amounts of impurities. The principles of thin-layer chromatography and application of the technique in pharmaceutical analysis are described in Quality control methods for medicinal

plant materials [82]. As it is effective and easy to perform, and the equipment required is inexpensive, the technique is frequently used for evaluating medicinal plant materials and their preparations. Thin-layer chromatography is used for the rapid separation of compounds by means of a uniform layer of dry, finely powdered material applied to a glass, plastic, or metal sheet or plate, glass plates being most commonly employed. The most common stationary phase is silica gel. The separation achieved may be based upon adsorption, partition, or a combination of both effects, depending on the particular type of adsorbant, it preparation, and its use with different solvents.

Out of the numerous practical applications of pharmacognosy, the great importance for the pharmaceutical industry is in the evaluation of the crude drugs. This involves the determination of identity, purity and quality. Purity depends upon the absence of foreign matter whether organic or inorganic, while quality refers essentially to the concentration of the active constituents in the drugs that make it valuable to medicine. By virtue of these constituents or components, the product is used and it's economic and commercial value is estimated. Based on the concentration and nature of the constituents though, a crude drug may confirm to all the official standards of purity and be good quality.

Thin layer chromatography: TLC

TLC is the common method of choice for herbal analysis before instrumental chromatography methods like GC and HPLC were established. Even nowadays, TLC is still frequently used for the analysis of herbal medicines since various pharmacopoeias such as American Herbal Pharmacopoeia (AHP), WHO still use TLC to provide first characteristic fingerprints of herbs. Rather, TLC is used as an easier method of initial screening with a semi-quantitative evaluation together with other chromatographic techniques. Also there is relatively less change in the simple TLC separation of herbal medicines than with instrumental chromatography [85, 86]

TLC has the advantages of many-fold possibilities of detection in analyzing herbal medicines. In addition, TLC is rather simple and can be employed for multiple sample analysis. For each plate, more than 30 spots of samples can be studied simultaneously in one time. Thus, the use of TLC to analyze the herbal medicines is still popular [87] and possible to get useful qualitative and quantitative information from the developed TLC plate.

In summary, the advantages of using TLC to construct the fingerprints of herbal medicines are its simplicity, versatility, high velocity, specific sensitivity and simple sample preparation. Thus, TLC is a convenient method of determining the quality and possible adulteration of herbal products.

Three-Dimension of High Performance Liquid Chromatography (3D-HPLC)

The most remarkable advances in chromatography have occurred in the domain of HPLC, despite the fact that the technique itself has only been in existence for about 40 years. The year 1967 was a landmark in the introduction of HPLC, with papers from Horvath, Huber and Scott, but the first automatic liquid chromatograph with gradient elution was an amino acid analyser described by Moore and co-workers in 1958 [88]. Until the advent of HPLC, most phytochemical separations were performed by open-column, paper or thin-layer chromatography. Open-column chromatography was time consuming and tedious, often requiring a large amount of sample. With paper chromatography and TLC, very small samples could be analysed and the resolution and reproducibility improved. However, quantitation was still inadequate and resolution of similar compounds was difficult. Gas chromatography provided excellent resolution but restriction to volatile samples (less than 20% of organic compounds can be separated by gas chromatography) and derivatization was often necessary. A technique was needed which could separate water-soluble, thermallylabile, non-volatile compounds with speed, precision and high resolution. HPLC fulfilled these criteria and is now one of the most powerful tools in analytical chemistry, with the ability to separate, identify and quantitate the compounds present in any sample that can be dissolved in a liquid. The viscosity of liquids is higher than that of gases by a factor of 100 – hence the need for pressure in the columns and the original name ‘‘high-pressure liquid chromatography’’. But ‘‘pressure’’ was replaced by ‘‘performance’’ as particles got smaller and columns became shorter. The wide variety of stationary and mobile phases should give a large potential for finding suitable separation conditions. However, at first, only relatively large particles were available. The introduction of small porous silica particles with a diameter of approximately 10 μM radically changed the situation, as did the production of chemically-bonded phases, notably the reversed-phase (RP) octadecyl (RP-18) and octyl (RP-8) materials. Standardization of silica surfaces by defined hydroxylation, application of very pure silica, improvement in bonding and endapping procedures now gives very versatile reversed-phase systems, with high selectivity, high separation power, robustness, high stability and efficiency. Reviews on equipment and instrumentation are published periodically in journals such as Analytical Chemistry – for example, by La Course [89]. High-performance liquid chromatography has become by far the most widely used chromatographic technique. In fact, the liquid chromatography, mass spectrometry and thermal analysis segments account for USD 4.4 billion of the global USD 20 billion analytical instrumentation market.

Three-dimensional of HPLC, the Optical density (mAU) and absorbance of the eluant from the HPLC columns were collected three-dimensionally: the retention time along the x-axis, the absorbance or Optical density (mAU) along the y-axis, and the wavelength along the z-axis. That is, the photometric data were collected in optional time intervals between optional wavelengths on a computer, which was connected to the spectrophotometers with a communication interface. After the analysis, the filed data could be presented in three-dimensional (3D) form by a computer program.

Analytical HPLC

High performance liquid chromatography (HPLC) has been the biggest revolution in analytical chemistry over the past 40 years [90]. The implications are enormous, with applications of HPLC being found in hundreds of areas, not least of which is phytochemistry. HPLC is also used routinely in phytochemistry to ‘‘pilot’’ the preparative isolation of natural products (optimization of the experimental conditions, checking of the different fractions throughout the separation) and to control the final purity of the isolated compounds. For chemotaxonomic purposes, the botanical relationships between different species can be shown by chromatographic comparison of their chemical composition. Chromatograms, which are used as fingerprints, are compared with authentic samples and known substances to permit identification of drugs and/or search for adulteration. HPLC is thus the best suited technique for an efficient separation of the crude extracts, as shown by Sakakibara *et al.* [91] who claim to have found a method capable of quantifying every polyphenol in vegetables, fruits and teas.

Multivariate analysis [92]

The subject of multivariate analysis deals with the statistical analysis of the data collected on more than one (response) variable. These variables may be correlated with each other, and their statistical dependence is often taken into account when analyzing such data. In fact, this consideration of statistical dependence makes multivariate analysis somewhat different in approach and considerably more complex than the corresponding univariate analysis, when there is only one response variable under consideration.

Response variables under consideration are often described as random variables and since their dependence is one of the things to be accounted for in the analyses, these response variables are often described by their joint probability distribution. This consideration makes the modeling issue relatively manageable and provides a convenient framework for scientific analysis of the data. Multivariate normal distribution is one of the most frequently made distributional assumptions for the analysis of multivariate data. However, if possible, any such consideration should ideally be dictated by the particular context. Also, in many cases, such as when the data are collected on nominal or ordinal scales, multivariate normality may not be an appropriate or even viable assumption. In the real world, most data collection schemes or designed experiments will result in multivariate data.

In this study, multivariate analysis is based on the statistical principle of multivariate statistics which involves observation and analysis of more than one statistical variable at a time.

Factor analysis

Factor analysis is used to uncover the latent structure (dimensions) of a set of variables. It reduces attribute space from a larger number of variables to a smaller number of factors. Factor analysis originated a century ago with Charles Spearman's attempts to show that a wide variety of mental tests could be explained by a single underlying intelligence factor.

Factor analysis is a statistical method used to describe variability among observed variables in terms of a potentially lower number of unobserved variables called factors. In other words, it is possible, for example, that variations in three or four observed variables mainly reflect the variations in a single unobserved variable, or in a reduced number of unobserved variables. Factor analysis searches for such joint variations in response to unobserved latent variables. The observed variables are modeled as linear combinations of the potential factors, plus "error" terms. The information gained about the interdependencies between observed variables can be used later to reduce the set of variables in a dataset. Factor analysis originated in psychometrics, and is used in behavioral sciences, social sciences, marketing, product management, operations research, and other applied sciences that deal with large quantities of data.

Factor analysis is related to principal component analysis (PCA), but the two are not identical. Because PCA performs a variance-maximizing rotation of the variable space, it takes into account all variability in the variables. In contrast, factor analysis estimates how much of the variability is due to common factors ("communality"). The two methods become essentially equivalent if the error terms in the factor analysis model (the variability not explained by common factors, see below) can be assumed to all have the same variance.

Principle component analysis (PCA)

Principle component analysis [93] is a sophisticated technique widely used for reducing the dimensions of multivariate problems. As a non-parametric method of classification, it makes no assumptions about the underlying statistical data distribution [94-96]. It reduces the dimensionality of the original data set by explaining the correlation amongst a large number of variables in terms of a smaller number of underlying factors (Principal Components or PCs) without losing much information [97,98]. PCA always results in scores plots that provide a visual determination of the similarity among the fingerprints. When a new fingerprint is measured with unexpected features that significantly differ from those of major good fingerprints, it would be excluded from the model and diagnosed different. The principal component line depends upon the scaling of the data, and therefore a transformation procedure of the raw data is important for the improvement of PCA analysis [99-103].

Hierarchical Cluster analysis

Hierarchical cluster analysis is a multivariate analysis technique that is used to sort samples into groups. This technique comprises an unsupervised classification procedure that involves measuring either the distance or the similarity between the objects to be clustered. It is unsupervised because it does not require previous information in the system under study and, therefore, is an ideal technique when no previous information is at the scientist’s disposal. The similarity or dissimilarity between samples (objects) is usually represented in a dendrogramfor ease of interpretation. Each object is similar to the others within a group but different from those in other groups with respect to a predetermined selection criterion [94].

Correlation

Correlation is a statistical technique that can show whether and how strongly pairs of variables are related or describes the degree of relationship between two variables.

Correlation Coefficient

The main result of a correlation is called the correlation coefficient (r). It ranges from -1.0 to +1.0. The closer r is to +1 or -1, the more closely the two variables are related. If r is close to 0, it means there is no relationship between the variables. If r is positive, it means that as one variable gets larger the other gets larger. If r is negative it means that as one gets larger, the other gets smaller (often called an "inverse" correlation). While correlation coefficients are normally reported as r = (a value between -1 and +1), squaring them makes then easier to understand. The square of the coefficient (or r square) is equal to the percent of the variation in one variable that is related to the variation in the other. After squaring r, ignore the decimal point. An r of .5 means 25% of the variation is related (.5 squared =.25). An r value of .7 means 49% of the variance is related (.7 squared = .49).

Biological Activities Evaluation: Safety and Efficacy Evaluation

Safety Evaluation

Cytotoxic activity using Brine shrimp method

Brine shrimp (*Artemia salina*) has been used as “bench top bioassay” for the discovery bioactive natural products and it is an excellent choice for elementary toxicity investigations of consumer products [104-105]. The brine shrimp lethality assay was proposed by Michale *et al.* [106] and later developed by Vanhaeche *et al*. [107] and Sleet and Brencel [108]. It is based on the ability to kill laboratory-cultured *Artemia* nauplii brine shrimp.

Brine shrimp *(Artemia salina)*[109-112]

Brine shrimp is a species of aquatic crustaceans of the genus *Artemia*. First discovered in Lymington, England, in 1755, *A. salina* is found worldwide in inland saltwater lakes, but not in oceans. *A. salina* is a well known genus as one variety (sometimes identified as a new species, *Artemia salina* x nyos, a cultivated subspecies of *A. slina*) is sold as a novelty gift, most often under the marketing name Sea-Monkeys.

Life cycle of the *Artemia salina* [111-113]

Brine shrimp eggs are metabolically inactive and can remain in total stasis for several years while in dry oxygen-free conditions, even at temperatures below freezing. This characteristic is called *cryptobiosis* meaning "hidden life" (also called *diapause*). The eggs of brine shrimp, *Artemia salina*, are readily available at low cost in pet shops as a food for tropical fish, and they remain viable for years in the dry state. Upon being placed in water, the eggs hatch within 48 hours, providing large numbers of larvae (nauplii). The nauplii are less than 0.5 mm in length when they first hatch. Brine shrimp has a biological life cycle of one year, during which it grow to a mature length of around one cm on average. This short life span, along

other characteristics such as its ability to remain dormant for long periods, has made them invaluable in scientific research, including space experiments.

Brine shrimp has been previously utilized in various bioassay systems. It has been analysis of pesticide residues[106, 114 -116], mycotoxins [116-120], stream pollutants [121], anesthetics [122] dinoflagellate toxins [123], morphine-like compounds [124], toxicity of oil dispersants [125], cocarcinogenicity of phorbol asters [126], and toxicants in marine environments [107]. Most workers have made use of the hatched nauplii, although inhibition of hatching of the eggs has also been studied (encased embryos that are metabolically inactive) [127]. In bioactivity for screening natural products, brine shrimp has been used as cytotoxicity screening of various medicinal plant such as South American Solanceae [128], Indian medicinal plants [129], Kenya medicinal plants [130], Brazilian Medicinal Plants [131],and Thai medicinal plants in the family Meliaceae [132]. Therefore, the method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the tube

The advantages of *A. salina* as the first choice for toxicity studies can be summarized as follows [107].

- The cysts are commercially and readily available so that the tests can be carried out worldwide with the same original material and without any problems of provisioning. moreover, the quantity of cysts required per test is very small so that the price of the biological material is negligible.
- The necessity of year-round maintenance of stock cultures, with all the biological and technical difficulties and the considerable economic repercussions, is completely eliminated.
- Large numbers of test organisms of exactly the same age and physiological condition can be easily obtained to start the tests.

Mutagenic activity using Ames test

The Ames test is used world-wide as an initial screen to determine the mutagenic potential of new chemicals and drugs. The test is also used for submission of data to regulatory agencies for registration or acceptance of many chemicals, including drugs and biocides. International guidelines have been developed for use by corporations and testing laboratories to ensure uniformity of testing procedures.

The Ames *Salmonella*/microsome mutagenicity assay (Salmonella test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test employs several histidine dependent Salmonella strains, each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that

cause DNA damage *via* different mechanisms. When the Salmonella tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (*his*$^+$) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner.

Nitrite as converter for Direct-Acting Mutagens

Nitrate and nitrite occur in the diet from numerous different sources [133]. Vegetables are major sources of nitrate; nitrates alone are not toxic, which is converted to nitrite when such foods are stored at room temperature [134]. The salts of nitrate and nitrite were used as a food additive for preserveation due to antimicrobial properties. Particularly inhibition of the grown of *Clostridium botulinum* and their ability give a well color and taste [135-136].

Sodium nitrite, with chemical formula $NaNO_2$, is used as a color fixative and preservative in meats and fish. When pure, it is a white to slight yellowish crystalline powder. It is very soluble in water and is hygroscopic. It is also slowly oxidized by oxygen in the air to sodium nitrate, $NaNO_3$. As a food additive, it serves a dual purpose in the food industry since it both alters the color of preserved fish and meats and also prevents growth of *Clostridium botulinum*, the bacterium which causes botulism. In the European Union it may be used only as a mixture with salt containing at most 0.6% sodium nitrite. While this chemical will prevent the growth of bacteria, it can be toxic in high amounts for animals, including humans. Sodium nitrite's LD_{50} in rats is 180 mg/kg and its human LD_{50} is 71 mg/kg.

A principal concern about sodium nitrite is the formation of carcinogenic nitrosamines in meats containing sodium nitrite when meat is charred or overcooked. Such carcinogenic nitrosamines can be formed from the reaction of nitrite with secondary amines under acidic conditions (such as occurs in the human stomach) as well as during the curing process used to preserve meats.

Mechanism of action

Carcinogenic nitrosamines are formed when amines that occur naturally in food react with sodium nitrite found in cured meat products.

$$R_2NH \text{ (amines)} + NaNO_2 \text{ (sodium nitrite)} \rightarrow R_2N\text{-}N{=}O \text{ (nitrosamine)}$$

In the presence of acid (such as in the stomach) or heat (such as via cooking), nitrosamines are converted to diazonium ions.

$$R_2N\text{-}N{=}O \text{ (nitrosamine)} + \text{(acid or heat)} \rightarrow R\text{-}N_2^+ \text{ (diazonium ion)}$$

Certain nitrosamines such as N-nitrosodimethylamine and N-nitrosopyrrolidine form carbocations that react with biological nucleophiles (such as DNA or an enzyme) in the cell.

$R\text{-}N_2^+$ (diazonium ion) → R^+ (carbocation) + N_2 (leaving group) + :Nu (biological nucleophiles) → R-Nu

If this nucleophilic substitution reaction occurs at a crucial site in a biomolecule, it can disrupt normal cell functioning leading to cancer or cell death.

1-Aminopyrene-Nitrite Mutagenicity Model for Antimutagenicity Study

1-Aminopyrene is a derivative of 1-nitropyrene found in human gastrointestinal tract. Anaerobic bacteria metabolize 1-nitropyrene to 1-aminopyrene. 1-Nitropyrene is generally a product of incomplete combustion and is the predominant nitro-PAHs emitted in diesel engine exhaust, exhaust of kerosene heaters, petroleum gas burners and food products as a result of pyrolysis of fat in meat during barbecuing [137–141].

1-Aminopyrene was known to be non-mutagenic when it was tasted without metabolic activation [140]. Kato *et.al*., [142] demonstrated that 1-aminopyrene treated with nitrite at pH 3.0 and 37°C showed mutagenicity to *Salmonella typhimurium* strains TA98 and TA100 without metabolic activation. The results agreed with the work of Kangsadalampai, Butryee and Manonophol [143] which stated that nitrite-treated 1-aminopyrene exhibited stronger mutagenicity than the authentic aminopyrene towards *Salmonella typhimurium* strains TA98 (frame-shift mutation) and TA100 (base-pair substitution mutation), in the absence of metabolic activation.

DNA damage using Comet assay

In the last two decades, the search for new methodologies which are able to assess DNA damage has been developed. Rydberg and Johanson [144] were the first to directly quantitate DNA damage in individual cells by lysing and embedding them in agarose on slides under mild alkali conditions to allow the partial unwinding of DNA. After neutralization, cells were stained with acridine orange and the extent of DNA damage quantitated by measuring the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence using a photometer. To improve the sensitivity for detecting DNA damage in isolated cells, Ostling and Johanson [145] developed a microgel electrophoresis technique, commonly known as the Comet assay. In this technique cells embedded in agarose gel were placed on a microscope slide, the cells lysed by detergents and high salt treatment and the liberated DNA electrophoresed under neutral conditions (pH of 9.5); the DNA then stained with a fluorescent dye (ethidium bromide), resembled a comet with head and tail. However this technique permits the detection of double-stranded DNA breaks only and the presence of RNA can lead to potential artifacts [146–148].

Two versions of the Comet assay are currently in use, one introduced by Singh *et al.* [149], who used alkaline electrophoresis (pH.13) to analyze DNA damage after treatment with X-rays or HO, which is capable of detecting DNA single-strand breaks and alkali labile sites in individual's cells. This version is known as the ''single cell gel electrophoresis (SCGE)

technique'', although for historical reasons, many investigators refer to this method as the''Comet assay''. Subsequently, Olive and co-workers developed versions of the neutral technique of Ostling and Johanson, which involved lysis in alkali treatment followed by electrophoresis at either neutral [150] or mild alkaline (pH 12.3) conditions [151] to detect single strand breaks.

The Singh and Olive methods are identical in principle and similar in practice, but the Singh method appears to be at least one- or two-orders of magnitude more sensitive [152,153].

In the Singh version of the assay, a single cell suspension of the mammalian cell culture or tissue under study is embedded in low-melting-point agarose in an agar gel sandwich on a microscope slide, lysed by detergents and high salt concentration at pH 10 and then electrophoresed for a short time under alkaline conditions. Lysis removes the cell contents except for the nuclear material. DNA remains highly supercoiled in the presence of a small amount of non-histone protein but when placed in alkali, it starts to unwind from sites of strand breakage. Cells with increased DNA damage display increased migration of the DNA from the nucleus towards the anode under an electrical current, giving the appearance of a ''comet tail''.

About the sensitivity of the (SCGE) Comet assay, McKelvey-Martin *et al.* [154] and Collins *et al.* [155] reported that the assay resolves break frequencies up to a few hundred per cell, definitely well beyond the range of fragment size for which conventional electrophoresis is suitable.

Depending on pH conditions for lysis and electro phoresis, the sensitivity of the technique can change. Employing neutral conditions for both variables, allows to detect DNA double strand breaks; but the pH 12.3 detects single strand breaks and delay DNA repair sites, while at pH 13 the sensitivity allows to evaluate alkali labile sites, single strand breaks and delay repair sites of DNA.

Efficacy evaluation

Antipyretic activity

Fever

Fever is a non–specific clinical manifestation associated with various pathophysiological conditions mediated by endogenously produced prostaglandin and cytokines such as tumor necrosis factor (TNFα), interleukin-1 (IL – 1) and interleukin –6 (IL – 6). These cytokines IL-1, IL – 6 and TNFα induce increase in body temperature *via* direct and indirect actions on the brain and are believed to act as endogenous pyrogens. They act at the level of organum vasculosum of the lamino terminalis of the central nervous system inducing the synthesis of prostaglandins which are central mediators in the coordinated response leading to fever.

Pathology of fever

Many of meditors underlying pyrexia have been described in recent years. The critical "endogenous pyrogens" involved in producing a highly regulated inflammatory response to tissue injury and infections are polypeptide cytokines. Pyrogenic cytokines, such as interleukin-β (IL-1β), tumor necrosis factor (TNF-α), and interleukin-6 (IL-6) are those that act directly on the hypothalamus to effect a fever response. Exogenous pyrogens, such as microbial surface components, evoke pyrexia most commonly through the stimulation of pyrogenic cytokines. The gram-negative bacterial outer membrane lipopolysaccharide (endotoxin), however, is capable of functioning at the level of the hypothalamus, in much the same way as IL-1β. These signals trigger the release of other mediators most notably prostaglandin E_2 (PGE_2), in the region of the POAH. PGE_2 is believed to be the proximal mediator of the febrile response. Preoptic neurons bearing E-prostanoid receptors alter their intrinsic firing rate in response to PGE_2, evoking an elevation in the thermoregulatory set point. There are four known cellular receptors for PGE_2: EP_1 through EP_4. The particular receptor subtype involved in pyrogenesis is unknown. Although mice lacking the neuronal PGE_2 receptor subtype EP_3 demonstrate an impaired febrile response to both exogenous (endotoxin) and endogenous pyrogens, studies in rats appear to implicate the EP_4 receptor. The intracellular events triggering pyrexia after PGE_2-EP receptor coupling among species are unclear. Fever is tightly regulated by immune response. Inflammatory stimuli triggering the generation of propyretic messages provoke the release of endogenous antipyretic substances, Substances such as arginine vasopressin (AVP), amelanocyte stimulalting hormone, and glucocorticoids act both centrally and peripherally to limit pyrexia. The cytokine interlukin-10 (IL-10) has numerous anti-imflammatory properities, including fever suppression, In addition, a class of liquid compounds known as epoxyeicosanoids generated by certain cytochrome P-450 enzymes plays an important role in limiting the fever and inflammation. Analogous to a biochemical feedback pathway, fever itself appears capable of countering the release of pyrofenic cytokines. For example, febrile temperatures augment early TNF release in endotoxin-challenged mice, yet limit its prolonged (and perhaps detrimental) expression after either lipopolysaccharide injection or bacterial infection [156].

Pain

The International Association for the Study of Pain (IASP) has been defined pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage or both" as defined by cause by noxious stimuli including thermal, chemical or mechanical. Pain is both a sensation (conscious awareness of a noxious stimulus) and an emotional experience (intense feeling of displeasure resulting in a pattern of reactive behavior).

Pain can be classified in several different ways. Anatomic (somatic or visceral pain) and temporal (acute or chronic pain) classification schemes have some clinical utility, but do not suggest appropriate analgesic therapy. Mechanistically, pain can also be classified as either

inflammatory or neuropathic. As the names suggest, inflammatory pain is associated with tissue trauma and inflammation, while neuropathic pain is associated with nerve injury. Both types of pain can occur as a result of surgical trauma, but inflammatory pain is by far the most common type of pain and its physiology is better understood [157].

Physiologic pathways

Specialized receptors provide information to the central nervous system (CNS) about the state of the environment in the vicinity of the organism. Each receptor is specialized to detect a particular type of stimulus (e.g. touch, temperature, pain, etc.). Those receptors in the skin and other tissues that sense pain are free nerve endings, while those for temperature detection can be free nerve endings, bulbs of Krouse or Ruffinigs corpuscles. Receptors are distributed with varying densities in different tissues. Pain receptors may be stimulated by mechanical damage, extremes of temperature, or by irritating chemical substances. While certain pain receptors are responsive to only one of the above stimuli, most can be stimulated by two or more. When the pain receptors in peripheral tissues (such as skin) are stimulated, the nociceptive (pain) impulses are transmitted to the CNS by two distinct types of neurons the A-delta and C nerve fibers. The A-delta fibers are large-diameter, fast conducting myelinated fibers, which transmit first pain-sharp, prickling and injurious. The C fibers are small-diameter, slower conducting unmyelinated fibers that are responsible for second pain-dull, aching and visceral type.

The primary afferent sensory neurons from the periphery then enter the spinal cord and synapse with neurons in the dorsal horn. The second-order neurons, arising from the dorsal horn, have long axons that decussate in the anterior commissure and travel cephalad in the contralateral anterolateral pathway (also known as spinothalamic tract). Some of the long axons that synapsed with type C neurons do not decussate, but pass cranially in the ipsilateral anterolateral spinal pathway. The anterolateral spinal pathway fibers terminate in the thalamus, from which neuronal relays are sent to other CNS centers and the sensory cortex. These higher centers are responsible for the perception of pain and the emotional components that accompany it.

Free radical scavenging activity

Oxidation is one of the most important processes, which produce free radicals in food, chemicals, and even in living systems. Free radicals have an important role in processes of food spoilage, chemical materials degradation, and also contribute to more than one hundred disorders in humans [158-163].

Antioxidants are defined as substances that even at low concentration significantly delay or prevent oxidation of easy oxidizable substrates [164]. The applications of antioxidants are industrially widespread in order to prevent polymers oxidative degradation, autooxidation of

fats, synthetic and natural pigments discoloration, etc. There is an increased interest of using antioxidants for medical purposes in the recent years [165-167].

The importance of oxidation in the body and in food stuffs has been widely recognized. Oxidative metabolism is essential for the survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species that cause oxidative changes. There is increasing evidence for the involvement of such species in a variety of normal *in vivo* regulatory systems [168] When an excess of free radicals is formed, they can overwhelm protective enzymes such as superoxide dismutase, catalase and peroxidase and cause destructive and lethal cellular effects (e.g., apoptosis) by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration. Furthermore, reactive oxygen species seem to influence cell signaling pathways in ways that are only now being unraveled [169, 170] Oxidation can also affect foods, where it is one of the major causes of chemical spoilage [171], resulting in rancidity and/or deterioration of the nutritional quality, colour, flavor, texture and safety of foods [172]. It is estimated that half of the world's fruit and vegetable crops are lost [173] due to postharvest deteriorative reactions. Defense mechanisms against the effects of excessive oxidations are provided by the action of various antioxidants and the need to measure antioxidant activity is well documented.

Antioxidant

An antioxidant may be defined [174] as 'any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate'. For convenience, antioxidants have been traditionally divided into two classes, primary or chainbreaking antioxidants and secondary or preventative antioxidants [175]. Secondary or preventative antioxidants are compounds that retard the rate of oxidation. This may be achieved in a number of ways including removal of substrate or singlet oxygen quenching [176]. Primary antioxidants, AH, when present in trace amounts, may either delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals [175]:

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$$

The antioxidant free radical may further interfere with chain propagation reactions by forming peroxy antioxidant compounds:

$$A^{\bullet} + LOO^{\bullet} \rightarrow LOOA$$

$$A^{\bullet} + LO^{\bullet} \rightarrow LOA$$

The activation energy of the above reactions [172] increases with increasing A–H and L–H bond dissociation energy. Therefore, the efficiency of the antioxidant increases with decreasing A–H bond strength. Chain-breaking antioxidants may occur naturally or they may be produced synthetically as in the case of Butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA), *tert*-Butylhydroquinone (TBHQ) and the gallates. The synthetic antioxidants are widely used in the food industry [177] and are included in the human diet [178]. The use of naturally occurring antioxidants [179] has been promoted because of concerns regarding the safety of synthetic antioxidants, [180, 181] with natural alternatives (*e.g.*, plant biophenols) possessing antioxidant activity similar to or even higher than that of synthetic antioxidants [165, 182].

Several methods are used for the estimation of efficiency of synthetic/natural antioxidants, like the ferric reducing antioxidant power (FRAP) assay [183], *β*- carotene/linoleic acid assay [184, 185], Rancimat method [186, 187], inhibition of low-density lipoprotein (LDL) oxidation [16], DPPH assay [185, 186] etc. This method diversity is due to the complexity of the analyzed substrates, often a mixtures of dozens of compounds with different functional groups, polarity, and chemical behavior.

In this paper the attention is focused on the DPPH assay, which is one of the best-known, frequently employed, and accurate methods. DPPH (1,1-diphenyl-2-picrylhydrazyl) is a stable free radical because of its spare electron delocalization over the whole molecule. The delocalization causes a deep violet color with λmax around 520 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable nonradical form of DPPH is obtained with simultaneous change of the violet color to pale yellow [188].

Cell Proliferation (MTT Assay)

The MTT assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue insoluble MTT formazan product by mitochondrial succinic dehydrogenase [189]. Since its development, this assay has been modified by various investigators [190-192] and has been used primarily with tumour cells and, to a lesser extent, with fibroblast cell lines, to evaluate the cytotoxicities of chemotherapeutic agents [190, 193]. The MTT assay has also been adapted for detecting lymphotoxins [194] and for measuring cell activation [195] and radiation effects [196].

The MTT assay is a semi-automated assay based on cleavage of the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] into a colored product formazan by the mitochondrial enzyme succinate dehydrogenase [197]. Since this conversion can only be accomplished by viable cells, the amount of formazan produced is a direct measure of viable cells. The original protocol of Mosmann [197] was established in adherent cells where complete removal of medium ensured absence of phenol red, an indicator in culture media known to cause high background absorbances [198]. In case of non adherent cells such as

Leishmania parasites, the incomplete removal of culture medium results in a high background absorbance.

Fibroblast cell [199]

Dense connective tissue, in particular, is used to form ligaments and tendons. Ligaments are rope-like tissue bundles that attach bones to each other and are found at the joints. Tendons attach bones to the surrounding skeletal muscle tissue. Dense connective tissue also makes up the dermis. The tissue matrix is made almost entirely of collagen, which is the most abundant protein in mammals. Interspersed between the collagen fibers of dense connective tissue, are fibroblasts, which produce a collagen subunit, tropocollagen, which is used to construct larger collagenous aggregates.

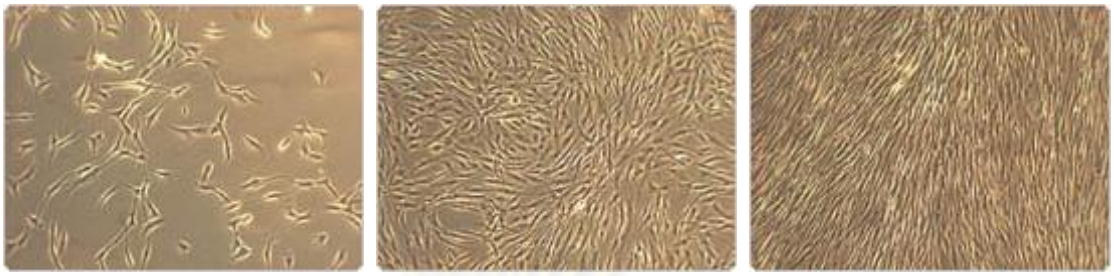
Fibroblast, therefore, is the principal active cells of connective tissue. Fibroblasts are large, flat, elongated (spindle-shaped) cells possessing processes extending out from the ends of the cell body. The cell nucleus is flat and oval. Fibroblasts produce tropocollagen, which is the forerunner of collagen, and ground substance, an amorphous gel-like matrix that fills the spaces between cells and fibres in connective tissue. Fibroblasts appear to play an important role in wound healing, and this activity is thought to be regulated by cells known as fibrocytes residing in the tissue stroma. Following tissue injury, fibroblasts migrate to the site of damage, where they deposit new collagen and facilitate the healing process. Additionally, fibroblast cells are large and flat, with elongated processes protruding from the body of each cell, creating the spindle-like appearance of the cell. the nucleus in the body of the cell, is oval.

Connective Tissue

Connective tissue is one of four main types of tissue in the body. The others are epithelial, muscle, and nervous tissue. Connective tissue is a fibrous tissue made largely of collagen, the most abundant protein in mammals. There are many kinds of connective tissue. These include, loose, dense, elastic, reticular, and adipose connective tissue. In addition, there are embryonic connective tissues, as well as specialized connective tissues, which include bone, cartilage, and blood.

Origin:

Fibroblasts are derived from primitive mesenchyme, like all connective tissue cells. Their ability to express filament protein vimentin alludes to their mesodermal origin.



Day 3 **Day 5** **Day 7**

Figure 2 Characteristic of fibroblast cell in different time**.**

According to the generally view as mentions before, MTT is cleaved in intact mitochondria of living cells to formazan by the “succinate-tetrazolium reductase” system of the respiratory chain activity [200]. Under this assumption, the formazan produced by cellular suspensions directly correlates with the number of metabolically active living cells, and the colorimetric assay for MTT is used as an assay for cell proliferation. Indirectly, cell death can just as well be quantified by the MTT assay, by determining the percentage of viable cells [201]. Therefore in this study, fibroblast cell was selected as a target through the experiments.

Nitric oxide (Griess reagent assay)

Free radical oxidative stress, usually resulting from deficient natural anti-oxidant defenses [202], has been implicated in the pathogenesis of a wide variety of clinical disorders, such as the degenerative diseases [203], aging [204] and the progressive decline in the immune functions [205]. Nitric oxide (NO) is one of the reactive oxygen species (ROS), and plays an important role in diverse physiological processes, including vasodilatation, neurotransmission and immune responses [206]. The pathological roles of NO have been implicated in a wide range of inflammatory diseases, such as sepsis, arthritis, multiple sclerosis and systemic lupus erythematosus [207]. Therefore, the supernatant from fibroblast cell after treated with differences concentration of samples as represented the inhibited of NO production were collected and investigated using Griess reagent assay.

Due to nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues [208, 209]. Due to its involvement in these diverse systems, interest in measuring NO in biological tissues and fluids remains strong.

One means to investigate nitric oxide formation is to measure nitrite (NO_2^-), which is one of two primary, stable and nonvolatile breakdown products of NO. This assay relies on a diazotization reaction that was originally described by Griess in 1879 [210]. Through the years, many modifications to the original reaction have been described.

The Griess Reagent System is based on the chemical reaction shown in Figure 3, which uses sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO_2^- in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium. The nitrite sensitivity depends on the matrix.

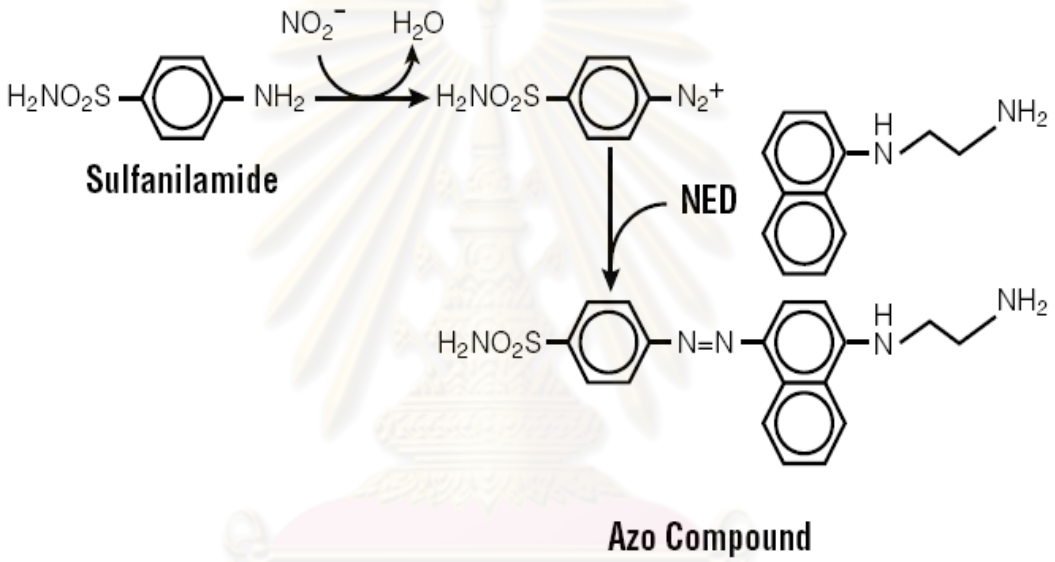


Figure 3 Chemical reactions involved in the measurement of NO_2^- using the Griess Reagent System.

CHAPTER III

MATERIALS AND METHODS

Plant materials

Five root species were collected from 14 different places throughout Thailand as described follows. All set of crude drugs were authenticated by Ruangrungsi N. and identified by comparison with the herbarium at Department of National Parks, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok. Voucher specimens are deposited at College of Public Health Sciences, Chulalongkorn University, Bangkok, Thailand. Each authentic sample species were air dried and ground to coarse powders.

***Capparis micracantha* DC. Root (CAPPARIDACEAE)**

Collected place: Chiang mai, Lopburi (Lumnarai), NongKhai (Sea-ka), Lampang, Petchabun, Rayong, Nakhornnayok, Nongkhai (Sriwilai), Lopburi (Muang), Uthai thani, Nan, Yasothon, Kanchanaburi, Kalasil.

***Clerodendrum petasites* S. Moore Root (VERBENCEAE)**

Collected place: Chiang mai, Lopburi (Lumnarai), NongKhai (Sea-ka), Lumpang, Petchabun, Rayong, Nakhornnayok, NongKhai (Sriwilai), Lopburi (Muang), Authaitanee, Nan, Yasothon, Kalasil, Phuket.

***Harrisonia perforata* (Blanco) Merr. Root (SIMAROUBACEAE)**

Collected place: Chiang mai, Lobburi (Lumnarai), NongKhai (Sea-ka), Lumpang, Petchabun, Rayong, Nakhornnayok, NongKhai (Sriwilai), Lopburi (Muang), Uthai thani, Nan, Yasothon, Kalasil, Kanchanaburi.

***Ficus racemosa* L. Root (MORACEAE)**

Collected place: Chiang mai, Lopburi (Lumnarai), NongKhai (Sea-ka), Lumpang, Petchabun, Rayong, NongKhai (Sriwilai), Lopburi (Muang), Uthai thani, Nan, Yasothon, Kalasil, Kanchanaburi, Songkla.

***Tiliacora triandra* (Colebr.) Diels Root (MENISPERMACEAE)**

Collected place: Chiang mai, Lopburi (Lumnarai), NongKhai (Sea-ka), Lumpang, Petchabun, Rayong, Nakhornnayok, NongKhai (Sriwilai), Lopburi (Muang), Uthai thani, Nan, Yasothon, Kalasil, Lopburi (Tha voung).

Plant extraction

After the authenticated five root species were sliced into small pieces and shade dried, all dried crude materials were pulverized to a course powdered by Universal Cutting Mill.

Ethanol (EtOH) extracts

Each coarsely powdered of five roots species was macerated extract in ethanol for 24 h, filtered through filter paper no.1 with Buchner funnel. The ethanol extracts were evaporated to

dryness by using Buchi rotary evaporator under vacuum (*in vacuo)* and the recovered ethanol was again poured into the macerated powdered, filtered and concentrated. The entire protocol was repeated until exhausted. The pooled dried residue was weighed and stored at -20^{o} C.

Water extracts

The marc of each root species coarsely powdered after successively extracted with ethanol was dried in a laboratory dryer until dryness, then macerated gently with boil distilled water for 1 h and allow to standing for 24 h at room temperature, filtered through filter paper no.1 with Buchner funnel. The whole process was repeated until exhausted. The pooled water extract was lyophilized to dryness, weighed and stored at -20^{o} C.

BLW Remedy preparation

The remedy extract was prepared by mixing each extract in according to their yields as to make a mixture of powder of component herbs in equal proportions by weight.

Parameters Standardization [11, 211-214]

Macroscopic and Microscopic examinations

The organoleptic characteristics so much so the macroscopic identify of medicinal plant materials is based on the shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface and other visual inspection. Visual inspection provides the simplest and quickest means by which to establish identity, purity and, possibly, quality. Also, these features are useful in judging the material in its entirety. No preliminary treatment is necessary for evaluating the sample in this manner excepting the softening and stretching of the wrinkled etc.

Microscopical techniques provide detail information about the crude drugs (broken or powdered materials) by virtue of its two main analytical uses. Firstly, its property to magnify permits the fine structures of minute objects to be visualized and thereby confirm the structural details of the plant drugs under evaluations. Secondly, these techniques can be used in the determination of the optical as well as micro-chemical properties of the crude drug specimen under study. Microscopical observations are based on optical phenomena, which are governed by the optical system of the microscope and the nature of light passing through it. Microscopically inspection of crude drugs from plant origin is essential for the identification of the grounded of powdered materials.

Perform microscopic inspection as described: powder material, sift through a 250 micron sieve then inspect under microscope with a magnification of 4x, 10x, 20x and 40x compared the scale with the 0.01 mm micrometer.

Determination of total ash

Place about 3.0 g of the ground air-dried sample, accurately weighed, in a previously ignited and tared crucible. Spread the sample in an even layer and ignite it by gradually increasing

the heat to 500 – 600 °C until it is white, indicating the absence of carbon. Cool in a desiccator and weigh without delay.

Determination of acid-insoluble ash

To the crucible containing the total ash, add 25.0 ml of hydrochloric acid (70g/l), cover with a watch-glass and boil gently for 5 minutes. Rinse the watch-glass with 5 ml of hot water and add this liquid to the crucible. Collect the insoluble matter on an ashless filter-paper and wash with hot water until the filtrate is neutral. Transfer the filter-paper containing the insoluble matter to the original crucible, dry on a hot plate and ignite to constant weight. Allow the residue to cool in a desiccators and weigh without delay.

Determination of loss on drying

Loss on drying is the loss of mass expressed as per cent w/w. To estimate the loss on drying 3.0 g of the air dried crude drug of the prescribed quantity of the material as specified for that specific substance is accurately weighed in a dried and tared flat weighing bottle. The substance is to be constant mass or for the prescribed time as specified, following the procedure as mentioned below.

Weigh 3.0 g of the ground sample in a tared small beaker and dry with heat (105^{O} C in an oven) to constantly weight.

Determination of solvent extractive values

Determination of ethanol-soluble extractive

Macerate 5.0 g of the ground sample with 100.0 ml of absolute ethanol in a closed conical flask for 6 hours in shaking bath then stand for 18 hours. Filter rapidly to avoid loss of ethanol, evaporate 20.0 ml of the filtrate to dryness in a tared small beaker and dry with heat to constantly weight.

Determination of water-soluble extractive

Macerate 5.0 g of the ground sample with 100.0 ml of distilled water in a closed conical flask for 6 hours in shaking bath then stand for 18 hours. Filter rapidly to avoid loss of water, evaporate 20.0 ml of the filtrate to dryness in a tared small beaker and dry with heat to constantly weight.

Determination of water content

Weight 50.0 g of the ground sample, add 200.0 ml of water-saturated toluene and distill by azeotropic distillation. As soon as water is completely distilled, rinse the inside of the condenser tube with toluene and continue the distillation for 5 more minutes. Allow the receiving tube to cool to room temperature. Allow the water and toluene layers to separate and read off the volume of water.

Thin-layer chromatographic identification

Extract 1 g of the ground sample with 20 ml of methanol, filter and evaporate to dryness. Dissolve the residue in 0.5 ml of methanol then apply 10 µl to the thin-layer plastic plate coat with siliga gel 60 F 254 (0.25 mm thickness, 20 cm x 20 cm). Develop the chromatogram in the chamber with the specified solvent. Remove the plate, allow it to dry in air and observe the produced spots in daylight, under short-wave and long-wave ultraviolet light. Spray the spots with the specified reagents such as anisaldehyde, vanillin-sulfuric acid, Ehrlich's reagent etc.

Three-dimensional (3-D) HPLC analysis

Ten milligrams of each root species and BLW remedy extracts were dissolved in 1 ml of HPLC grade methanol then filtered through a 0.45 µm membrane filter. The filtrate was transfer into the HPLC tube and submitted to the 3D-HPLC analyses.

The 3-D HPLC profile of each root species and BLW remedy extract were obtained by Ito *et al.* [215] using Agilent 1100 3D-HPLC system by Agilent Technologies, Tokyo, Japan equipped with a photodiode-array detector by using a column 4.6 x 250 mm TSK gel ODS-80Ts (Tosoh Corp. Tokyo, Japan) and kept at 40° C. The elution of mobile phase was performed by 10 mM phosphoric acid-acetonitrile linear gradient (95:5-5:95) by 60 min at a flow rate of 0.8 ml/min. The UV spectrum used was monitored by a range of 200-400 nm. All the measurements were done in triplicate.

Assignments of all major peaks were supposed by comparing the UV spectrum patterns of each peak with the data registered in The Dictionary of natural products Program (DNP 19.1) provided by Tayle & Francis Group.

Data extraction and processing for multivariate analysis

The data of area under curve from each chromatorgraphic peaks between 0 – 60 min and the retention time in each sample (n=12, triplicate) were register and extract as raw data for multivariate data analysis. Hierarchical clustering analysis HCA) and principle component analysis (PCA) were performed using Multivariate analysis software at Kitasato Institute for Life Sciences and Infection Control Sciences, Kitasato University, Tokyo, Japan. A cluster method called average linkage between groups was applied and simple correlation was selected as measurement.

Biological Activities Evaluation: Safety and Efficacy Evaluations

Safety Evaluation

Cytotoxic activity (Brine shrimp method) [216-221]

Brine shrimp has been used as “benchtop bioassay” for the discovery bioactive natural products. It is an excellent choice for elementary toxicity investigations of consumer products and based on the ability to kill laboratory-cultured *Artemia* nauplii brine shrimp.

The bioactivity screening of the extracts was investigated by the brine shrimp lethality test by Meyer B.N. *et al*. with some modifications. Briefly, Brine shrimp eggs were hatched in shallow rectangular dish filled with artificial sea water. A plastic divider with several 2 mm holes was clamped in the dish to make two equal compartments. The eggs were sprinkled into the dark compartment, while another compartment was illuminated. After 24 hours the phototropic nauplii were collected by pasteur pipette from light side. Ten brine shrimp (48 hours age) were transferred to each vial, and artificial sea water was added to make 5 ml the vials were maintained under illumination. Apply the different concentrations of Ben Cha Lo Ka Wi Chian remedy and each species extracts in methanol and control (methanol only) into 0.5x2 cm filter paper, air dry place in vial that fill with artificial sea. Five replicated were done for each dose level. The survivors were counted after 6 and 24 hours and the concentrations that kill 50% of the brine shrimps (LC_{50}) or the Medium Lethal Concentrations (LC_{50}) were determined.

Mutagenic and antimutagenic testing [222-225]

The *Salmonella* mutagenicity test, or bacterial reverse mutation assay, is also commonly known as the Ames test. It was developed by Dr. Bruce Ames, the recipient of 2001 LPI Prize for Health Research and his colleagues in UC Berkeley in the 70’s. The principle of the test is to expose histidine-dependent *Salmonella typhimurium* strains (the tester strains, which have artificially induced point mutations; base-pair substitution (T98), frameshift mutation (T100)) to a compound to be examined in a histidine (His) deficient medium. His-independent bacterial colonies may arise from spontaneous reversions (backward mutations) or chemically induced reversions. The mutagenicity of a chemical can be assessed by comparing the control with the treated bacterial culture. Conversely, the antimutagenicity of a compound to a selected positive mutagen can be investigated when the two chemicals are co-administered to the bacteria.

Mutagenic and Anti-mutagenic activity by Ames test

The ethanol extracts of each species and the remedy extract were diluted in dimethylsulfoxide (DMSO) whereas the water extracts were diluted in sterile distilled water at the adequate doses. All aliquots were filtered through 0.45 µm sterile membrane filter discs. Ames tests were performed on *Salmonella typhimurium* strains TA98 for frame-shift mutation and TA100 for base-pair substitution mutation in an acidic condition (pH 3 - 3.5) without metabolic activation. Both direct and in-direct mutagenicity were assayed in the condition without and

with sodium nitrite. The anti-mutagenicity against standard mutagen (nitrite treated 1-aminopyrine) was also investigated. Each assays was investigated in triplicate.

Nitrite treatment

An aliquot of ethanol and water extracts of each species and BLW remedy in responding to 5, 10, 20, 40 mg/ml were performed into a sterile test tube. The volume was adjusted to 200 µl with DMSO or sterile distilled water. Two-hundred fifty µl of 2M sodium nitrite and 550 µl of 0.25 N hydrochloric acid were added respectively for acidify the reaction mixture to pH 3-3.5 [226-227]. The mixtures were incubated at 37°C in shaking water bath for 4 hr then placed for 1 min into the ice bath to stop the reaction and 250 µl of 2 M ammonium sulfamate was added. All tubes were allowed to standing 10 min in the ice bath again.

Ames mutagenicity assay

S. typhimurium (His^-) strains TA98 and TA100 were grown in nutrient broth (NB) liquid medium for 16 h at 37°C in agitation (90 rpm). One-hundred µl of untreated or nitrite treated mixture was transferred into sterile test tubes and mixed with 500 µl of 0.5 M phosphate buffer (pH 7.4), followed by 100 µl of TA98 or TA100 strains suspension. The final volume was 700 µl. The mixtures were incubated at 37°C for 20 min. Next, 2 ml of top agar, which consisted of 0.5 mM L-histidine and 0.5 mM D-biotin at 45°C was added to the mixture and poured onto a minimal glucose agar plate. The plates were incubated at 37 °C in darkness for 48 h and the numbers of his+ revertant colonies were manually counted.

1-Aminopyrine (1-AP) treated with nitrite in acid solution at 0.06 and 0.12 µg/plate on strains TA98 and TA100 was used as positive mutagenic respectively. Dimethyl sulfoxide or sterile distilled water was used as a spontaneous reversion.

The results data were assessed by mean and standard deviation of histidine (His^+) reverstants per plate. The mutagenic index (MI) was also calculated for each concentration. MI is the average number of revertants per plate divided by the average number of the spontaneous revertants per pate. The mutagenic effect of each sample was pronounced if the number of His^+ revertants per plate was higher than twice of spontaneous revertants (MI > 2) with a concentration-response relationship was shown [228].

Anti-mutagenicity with modification by nitrite treated 1-aminopyrene

One-hundred µl of *S. typhimurium* strain suspension was added into the sterile test tube containing 500 µl 0.5 M phosphate buffer (pH 7.4), 0.15 µg of nitrite-treated 1-AP and 5, 10 and 15 mg/ml of each sample solutions. Dimethylsulfoxide (DMSO) or sterile distilled water was added to adjust the final volume to 700 µl. Subsequently, the mixtures followed the protocol as described in *Ames mutagenic assay.* The percent modification was calculated by the following formula:

$$\% \text{ Inhibition} = [(A - B) / (A - C)] \times 100$$

Where A is the number of histidine revertants per plate induced by nitrite treated 1-AP, B is the number of histidine revertants per plate induced by nitrite treated 1-AP in the presence of extract and C is the number of spontaneous histidine revertants per plate. The percentage of inhibition was classified as strong (higher than 60%), moderate (60-41%), weak (40-21%) and negligible (20-0%) [229].

***In vitro* DNA damage: Comet assay** [155, 230-232]

The comet assay (also known as the single cell gel electrophoresis or SCGE) is one of the very widely used assays to microscopically detect DNA damage at the level of a single cell. It is attractive for many reasons. A part from the appeal of the images it produces, it is a quick, simple, sensitive, reliable and fairly inexpensive way of measuring DNA damage. The determination of damage is carried out either through visual scoring of cells (after classification into different categories on the basis of tail length and shape). The assay works upon the principle that strand breakage of the supercoiled duplex DNA leads to the reduction of the size of the large molecule and these strands can be stretched out by electrophoresis. Also, under highly alkaline conditions there is denaturation, unwinding of the duplex DNA and ecpression of alkali labile sites as single strand breaks. Comets form as the broken ends of the negatively charges DNA molecule become free to migrate in the electric field towards the anode. Two principles in the formation of the comet are:

1. DNA migration is function of both size and the number of broken ends of the DNA.
2. Tail length increases with damage initially and then reaches a maximum that is dependent on the electrophoretic conditions, not the size of fragments.

Isolation of lymphocytes: Whole peripheral blood samples were collected by venepuncture from healthy volunteers. Blood samples were aseptically collected in heparinized sterile tubes. Lymphocytes were isolated using Ficoll–histopaque. Blood was diluted 1:1 with PBS and layered onto histopaque with the ratio of blood and Phosphate Buffered Saline (PBS):histopaque maintained at 4:3. The blood was centrifuged at 1340 rpm for 35 min at room temperature. The lymphocyte layer was removed and washed twice in PBS at 1200 rpm for 10min each, and then washed with RPMI-1640 media. The number of lymphocytes was counted using a haemocytometer and the viability of the cells was assayed by the trypan blue exclusion test. Approximately 1 x 10^6 cells were present in 1.0 ml lymphocyte suspension.

The lymphocytes contained in the cryovials were quickly thawed in a water bath at 37 °C and immediately after added to the treatment. The treatments with the crude drugs: Ben Cha Lo Ka Wi Chian remedy and each species extracts (1, 5, 10 µg/ml) were carried out in eppendorfs containing PBS at 37 °C for 1 h. After 1 h treatment, the cells were centrifuged for 5 min at 4000 rpm, washed with PBS and centrifuged again in the same conditions.

The Comet assay was performed as recommended in the international workshop in genotoxicity tests (IWGT) Comet assay guidelines. Briefly, cells were washed in PBS once or twice after treatment and the remaining pellet was embedded in 100 µl of 1 % low melting

point agarose (LMP). The agarose and the pellet were quickly mixed, and 100 µl were pipetted onto agarose (1% normal melting point) pre-coated slides. Afterwards, the slides were placed on a try on ice for 5 min. A third agarose layer, 100 µl of 0.5 % LMP, was added on top of the second layer and left again on ice (5 min) prior to place them in lysing buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, pH 10) overnight. Next day, they were electrophoresed at 25 V and 300 mA (0.75 V/cm) for 30 min after 30 min unwinding at pH > 13. Slides were neutralized with neutralizing buffer (0.4 M Tris, pH 7.5) 3 times for 5 minutes each and stained with ethidium bromide (20 µg/ml). Slides were studied under microscope (Zeiss Axioskop, Germany) attached to a fluorescence microscope (Leica, Germany) with a final magnification of 400x.

Arbitary units

The five categories used for comet classification were those proposed by Collins [155], with modification to take account of characteristic of the silver stain. Several pictures of classes 1-3 were included for better classification during the scoring process. The Excel template provided to participation laboratories allowed the use of internal coded in each laboratory, and was designed in order to register: (a) data separately for each gel of the slide. (b) the number of comet classified as 0 -4, and (C) the total number of cell scored. Arbitrary units with possible values from 0 to 400 were programmed into the Excel sheet to be calculated automatically by multiplying the number o f observed comets (from 0 to 100) by the comet classification (0-4), and then summing the values obtained in each gel.

Efficacy evaluation

Antipyretic activity [233-236]

Antipyretics (literally "against the fire") are drugs that reduce body temperature in situations such as fever. However, they will not affect the normal body temperature if one does not have a fever. Antipyretics cause the hypothalamus to override an interleukin-induced increase in temperature. The body will then work to lower the temperature and the result is a reduction in fever.

Animals

Male albino Wistar rats, 160-180g weight, were housed and maintained at 22± 2^{O} C with a 12 h light-dark cycle and allowed free access to food and water. The animals were fasted overnight before the experiments. All animal studies were performed in compliance to guidelines on the use of animals in research after approval by the Animal Care and Use Committee (CU-ACUC), Faculty of Pharmaceutical sciences, Chulalongkorn University.

Lipopolysaccharide-induced fever

Lipopolysacharides:LPS (50 µg kg^{-1}) induced fever was administered intra-muscularly to the animals an hour before the administration of test drugs. Rectal temperatures were taken at

hourly intervals for 7 h. Only those animals whose rectal temperatures increased 1^{o}C or above from normal rectal temperature were used for the study.

The Ben Cha Lo Ka Wi Chian remedy and each species extracts (25, 50, 100, 200 and 400 mg/kg) were administered. Such animals were allocated to groups of six and are treated orally. Control group received 10 ml/kg Tween 80. Aspirin (300 mg/kg orally) was used as reference drug. The rectal temperatures of the rats were recorded at initial, 1 h intervals starting 1 h drug administration and continuing for 7 h after extracts/drug administration.

Hot-plate analgesic Testing

The male ICR mice weighing 18-25 were used (n=10 per group). Analgesic testing was determined using the hot-plate method. The surface of the hot-plate (Harvard Apparatus) measuring 28x28 cm was set at 55± 0.5 °C and was surrounded by a clear Plexiglas wall cylinder, 20 cm in diameter and 30 cm in height to confine the animal to the heated surface during testing, On the day of testing, animals were randomly assigned to one of eight treatment groups and underwent 3 per drug baseline trials on the hot-plate latency time of less than 45 sec were utilized in these studies. Mice were then administered various treatments and retested. Each mice was placed on the hot-plant from an elevation of 5 cm and the latency to the licking of hind paw or vigorous jumping up from the surface of the metal plate was used as the end point and recorded with a stopwatch. If this behavior was not observed within 45 sec the animal was removed from the hot-plate, given a score of 45 for its paw-lick latency and returned to its cage (the maximum time allowed for an animal to remain on the surface of the plate during testing was 45 sec). The average of the last two trials served as the baseline pre drug paw-lick latency.

Immediately, after the third baseline trial on the hot-plate, the drug administration took place with either intraperitoneal (i.p.) 0.9% sodium chloride solution (NSS; 10 ml/kg), morphine sulphate (MO; 10 mg/kg) or oral administration of 2% tween 80 (10 ml/kg), various dosed of CM, CP, HP, FR, TT and BLW remedy (25, 50,100, 200 and 400 mg/kg). All animals were placed on the hot-plate at 15, 30, 45, 60, 92,120 and 240 min after drug administration. The time-course of hot-plate latency were expressed as the mean percent maximum possible effect (%MPE) according to the following formula:

%MPE = {(post drug latency) – (predrug latency)/ (cut-off time) – (predrug latency)} x 100

* cut-off time for hot-plate test = 45 sec

Thus, ED_{50} were computed and dose-and time response curve was generated. Dose effect curves for hot-plate assays were derived by computing the area under the corresponding 0-240 min time-course- %MPE curves; area was calculated using the trapezoidal rule (Tallarida and Murray, 1987).

Free radical scavenging activity [237-240]

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) assay method is based on the reaction of methanolic solution of colored free radical DPPH by free radical scavenger. DPPH is a stable free radial with red color (absorbed at 517nm). If free radicals have been scavenged, DPPH will generated it's color to yellow. This assay uses this character to show medicinal plants free radical scavenging activity.

The free radical scavenging activity of each extracts and remedy were analyzed by the DPPH assay as described by Brand-William *et al.* [240] with some modifications. Briefly, $6x10^{-5}$ M solution of DPPH in methanol was prepared. A 100 µl of DPPH solution was added to 100 µl of crude drugs of Ben Cha Lo Ka Wi Chian remedy and each species extracts or positive control (Quercetin and Buthylated Hydroxyl Toluene:BHT) prepared in methanol in different concentrations. The assay was carried out in a 96 well microplate at room temperature for 30 minutes. Each concentration is performed in triplicate. The percentage of radical scavenging activity against DPPH was determined from % decreasing of absorbance at 517 nm. The concentration of the extracts led to 50% inhibition (IC_{50}) is determined from the plotted graph of % scavenging activity against the concentration of the extract.

Cell Proliferation: MTT assay *(3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide viability assay)* [241-242]

The MTT cell proliferation and viability assay is a safe, sensitive, *in vitro* assay for the measurement of cell proliferation or, when metabolic events lead to apoptosis or necrosis, a reduction in cell viability. In addition, this is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (e.g. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The absorbance of this colored solution is quantified by measuring the absoprtion at 570 nm in a spectrophotometer.

Incubate monolayer cultures in microtitration plates (96-well) in a range of sample concentration (Ben Cha Lo Ka Wi Chian remedy and each species extracts). Remove the sample, and wash the cell for two times PBS (buffer); then added MTT to each well. Incubate the plates in the dark for 2 h, and then remove the MTT (MTT is reduced by metabolically active cells to insoluble purple formazan dye crystals). Then added DMSO in each well and keep in the dark 20 minute, solubilizing the crystals so the absorbance can be read using a spectrophotometer. Samples are read directly in the wells. The optimal wavelength for absorbance is 570 nm in a plate reader (ELISA:Enzyme-Linked Immuno Sorbent Assay). The data is analyzed by plotting cell number versus absorbance, allowing quantitation of changes

in cell proliferation. The rate of tetrazolium reduction is proportional to the rate of cell proliferation.

Nitric Oxide: Griess reagent assay [243-245]

This assay determines nitric oxide concentration based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction was followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess Reaction is based on the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to *N*-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 – 570 nm.

Allow the Sulfanilamide Solution and NED Solution to equilibrate to room temperature (15-30 min.) Add 50 µl of each experiment sample (Ben Cha Lo Ka Wi Chian remedy and each species extracts) to wells in triplicate. Using a multichannel pipettor, dispense 50 µl of the sulfanilamide Solution to all experimental samples. Incubate 5-10 min at room temperature, protected from light. Dispense 50 µl of NED Solution to all well. Incubate 5-10 min. at room temperature, protected from light. Measure the absorbance within 30 min, in a plate reader, with a wavelength 540 nm.

Statistic analysis [246]

The parameter standardization was carried out as mean± standard deviation (SD). Also, another results and data obtained in this study are evaluated using nonparametric statistical tests, with the Mann-Whitney *U* test between two mean groups: control and test groups. Significant levels were at $P < 0.05$ (95% confident limits).

In part of multivariate analysis, the data were analyzed using the software MULTIVARIATE ANALYSIS at Kitasato University, Tokyo, Japan. Analyses of variances were performed using the factor analysis, hierarchical cluster analysis (HCA) and principle component analysis (PCA). Correlation analysis were achieved for describes the degree of relationship between two variables.

CHAPTER IV

RESULTS

Capparis micracantha DC.

Family: CAPPARIDACEAE

Synonyms: *Capparis bariensis* Pierre ex Gagnep. *Capparis billardieri* DC. *Capparis callosa* Blume, *Capparis donnaiensis* Pierre ex Gagnep., *Capparis forsteniana* Miq., *Capparis hainanensis* Oliv., *Capparis liangii* Merr. & Chun, *Capparis myrioneura* Hallier f., *Capparis odorata* Blanco, *Capparis petelotii* Merr., *Capparis roydsiifolia* Kurz, *Capparis venosa* Merr.

Vernacular names: Thailand: Ching-chee (Central), Kra-dat Khaow (Central), Nuat meaw dang (northern); **Indonesia:** Balung, Kledugn (Javanese), Sanek (Madurese); **Malaysia:** Kaju tuju,; Philippines; Salimbang (Tagalog), Tarabtab (Iloko), Salimono (Bisaya); **Cambodia:** Kanchoen bai dach; **Loas:** Say sou; **Vietnam:** B[uf]ng ch[ef], c[as]p gai nh[or].

Distribution: From Burma, Indo-China, Thailand and Peninsular Malaysia, to Indonesia and Philippine.

Observation: A half-erect shrub or small tree with dropping branches, 1-6 m tall, rarely a vine 2-4 m tall, young braches zigzag, glabrous; leaves oval to oblong-lanceolate, 9.5-20 cm x 3-11 cm, base rounded, apex variable, rarely acuminate, coriaceous, shining, petiole 0.7-1.5 cm long, thorns patent, straight or slightly curved, 2-7 mm long, on flowering branches often absent; flowers 2-6 in a row, pedical about 1 cm long; sepals ovate, 5.5-13 mm long, petals oblong or elliptical, 10-26 mm long, thin, white with yellow base, later turning dark red, stamens 20-45, filaments 2.5-3 cm long, white, gynophores 15-35 mm long , ovary and gynophores sometimes abortive; berry globular or ellipsoid, 2-6 cm in diameter, with 4 longitudinal sutures, yellow, orange or red and strongly smelling when ripe; seeds numerous, in whitish, slimy, sweet pulp. *Capparis micracantha* is found in brush wood, hedges and open forest, also along the seashore and in sandy locations, mostly below 500 m attitude.

Uses: Root: carminative; treatment of chronic infected skin diseases. Stem: crush with small amount of water and topically apply to relieve sprains and swelling . Leaf: used for muscular cramps; boil with water, drink or bathe to relieve fever with chronic vesicular skin diseases; smoke to treat bronchitis. Root or leaf; antiasthmatic; treatment of chest pain, fever with vesicular skin diseases, such as meales.

Macroscopic

Figure 4 A, C, D, G and H. Flowers of *C. micracantha*, B and F Leaves, E and I Fruits

Figure 5 Crude drug of *Capparis micracantha* DC. Root

The fragment of dried root crude drug is harden, grayish yellow, coarse surface and quite big sized.

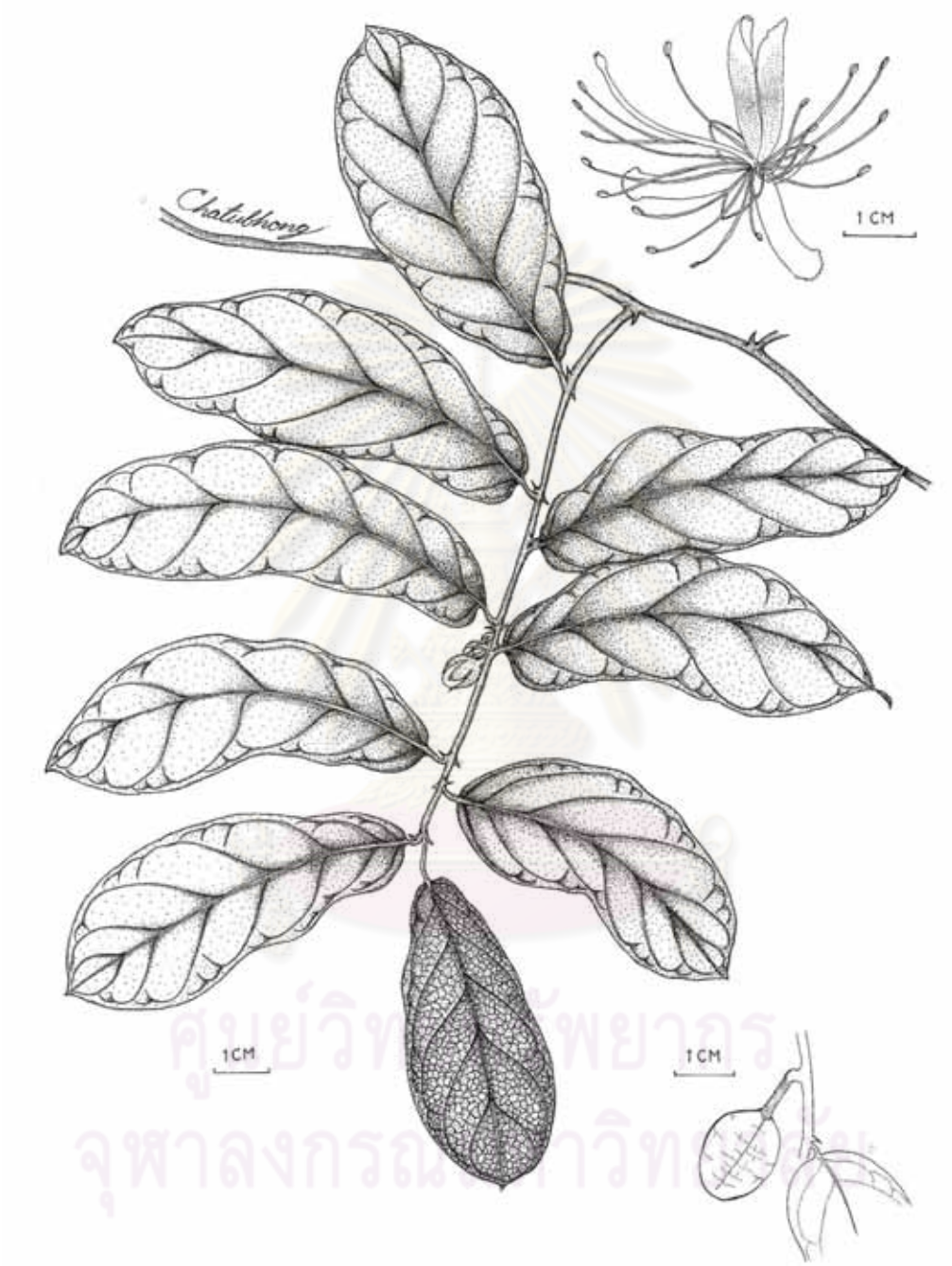


Figure 6 Whole plant of *Capparis micracantha* DC.

Whole plant is half erect shrub, oblong of leaves, armed with very short conical straight thorns and white flower with petals oblong, long filament and style.

Microscopic : Anatomical Character

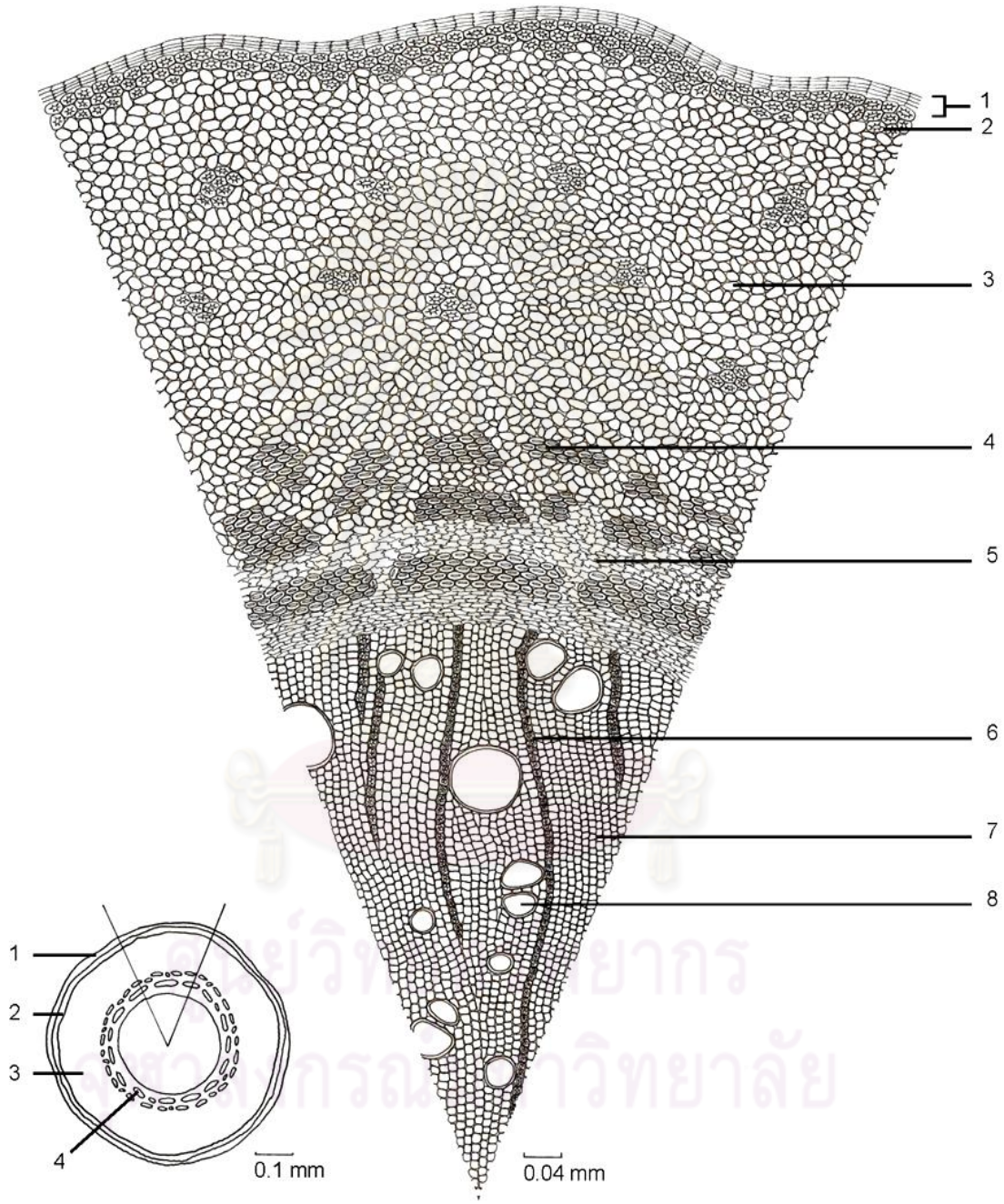


Figure 7 Transverse section of *Capparis micracantha* DC. root:

1.Periderm 2. Sclereid 3. Parenchyma of cortex 4. Fiber 5. Phloem tissue

6. Xylem ray with starch granules 7. Xylem parenchyma 8. Xylem vessle

Histological character

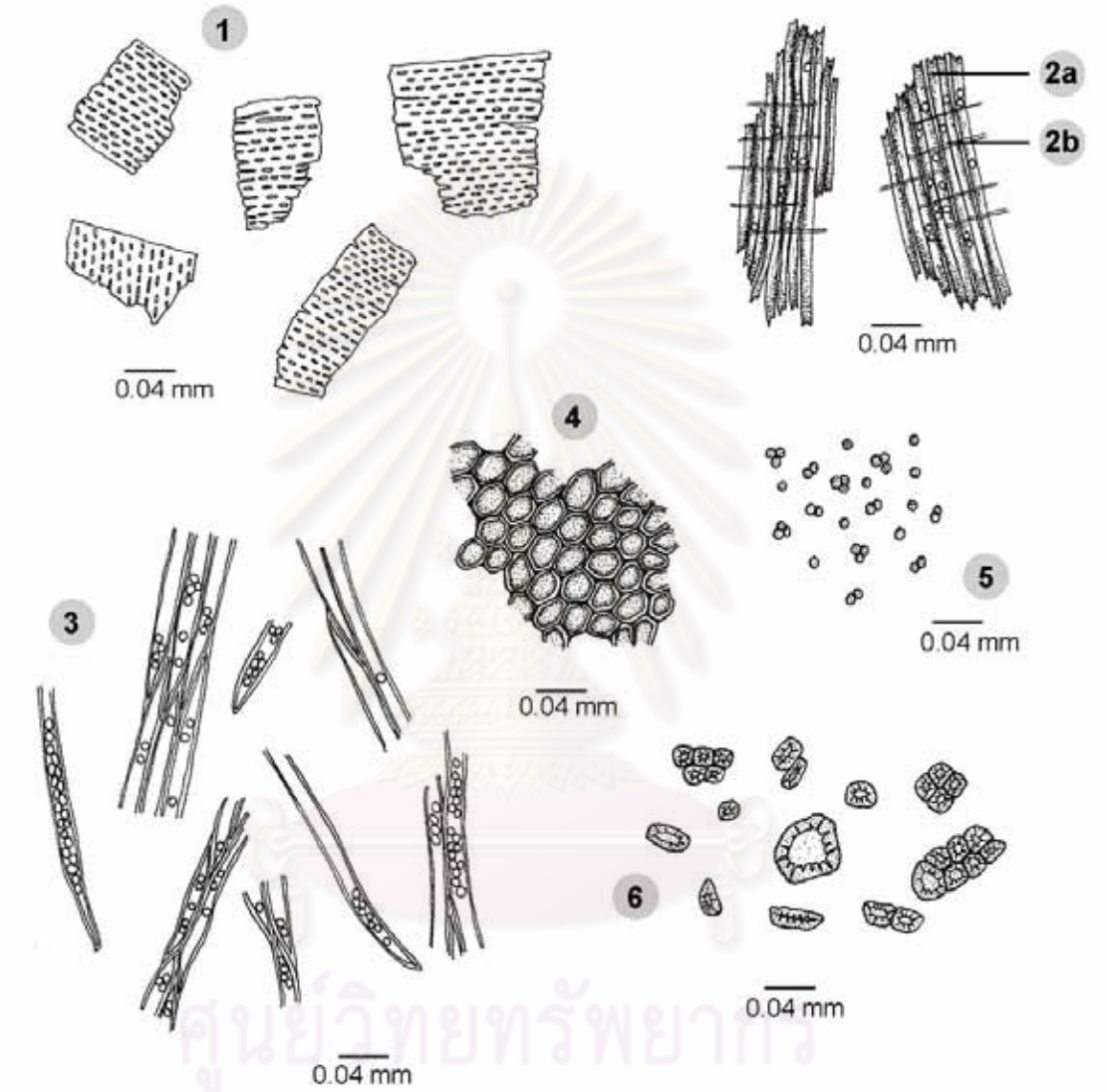


Figure 8 Powdered *Capparis micracantha* DC. Root:

1.Fragment of pitted vessels 2. Part of xylem in radial longitudinal section showing wood fiber containing starch granules(2a) and medullary ray(2b) 3. Fragment of fibers containing starch granules 4. Parenchyma in sectional view 5. Starch granules 6. Sclereids

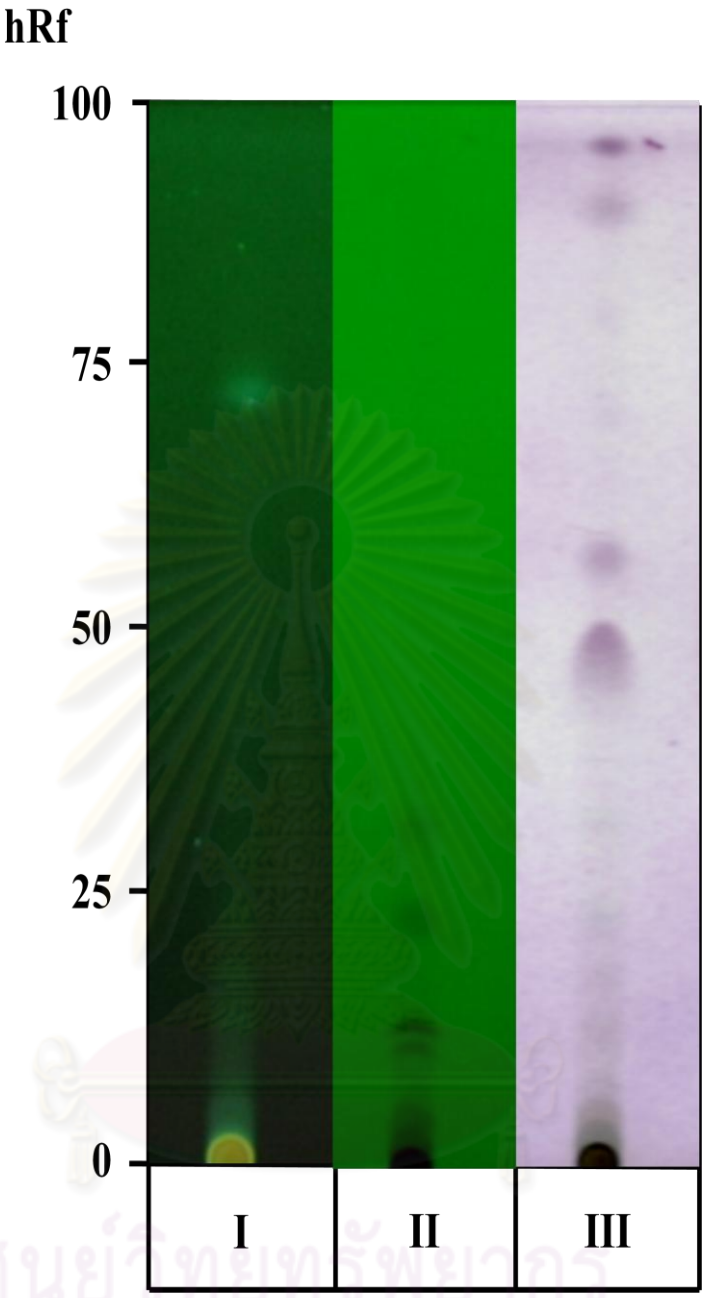


Figure 9 Thin-layer chromatogram of
methanolic extract of the root of *Capparis micracantha* DC.

Solvent system	**Toluene : Ethyl acetate 75:25**
Detection	I = detection under UV light 254 nm
	II = detection under UV light 366 nm
	III = detection with Anidaldehyde*,**

*Anisaldehyde regent
Preparation: Anisaldehyde (0.5 ml), Glacial acetic acid (10 ml), methanol (85 ml), Sulfuric acid (5 ml)
**Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

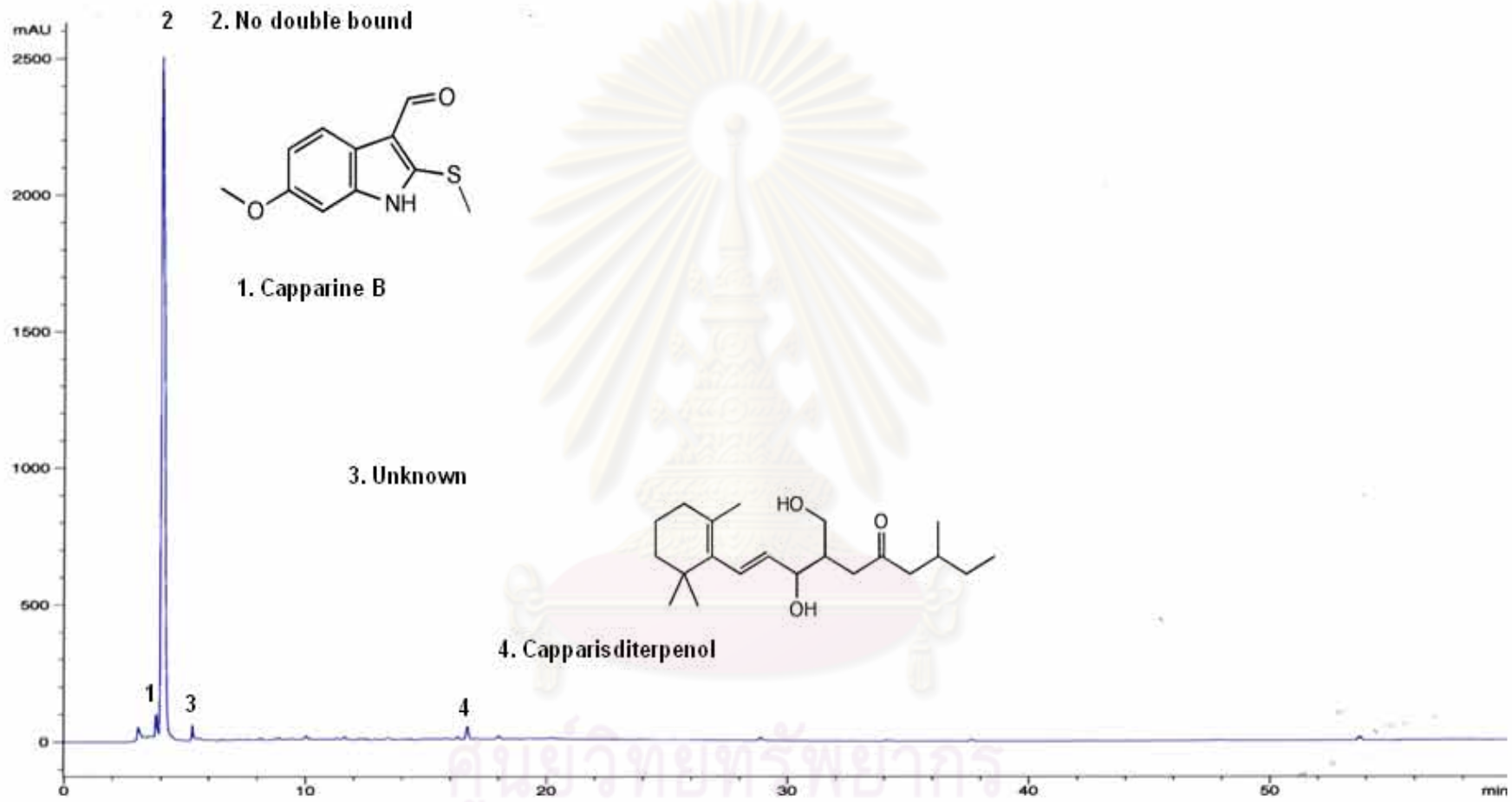
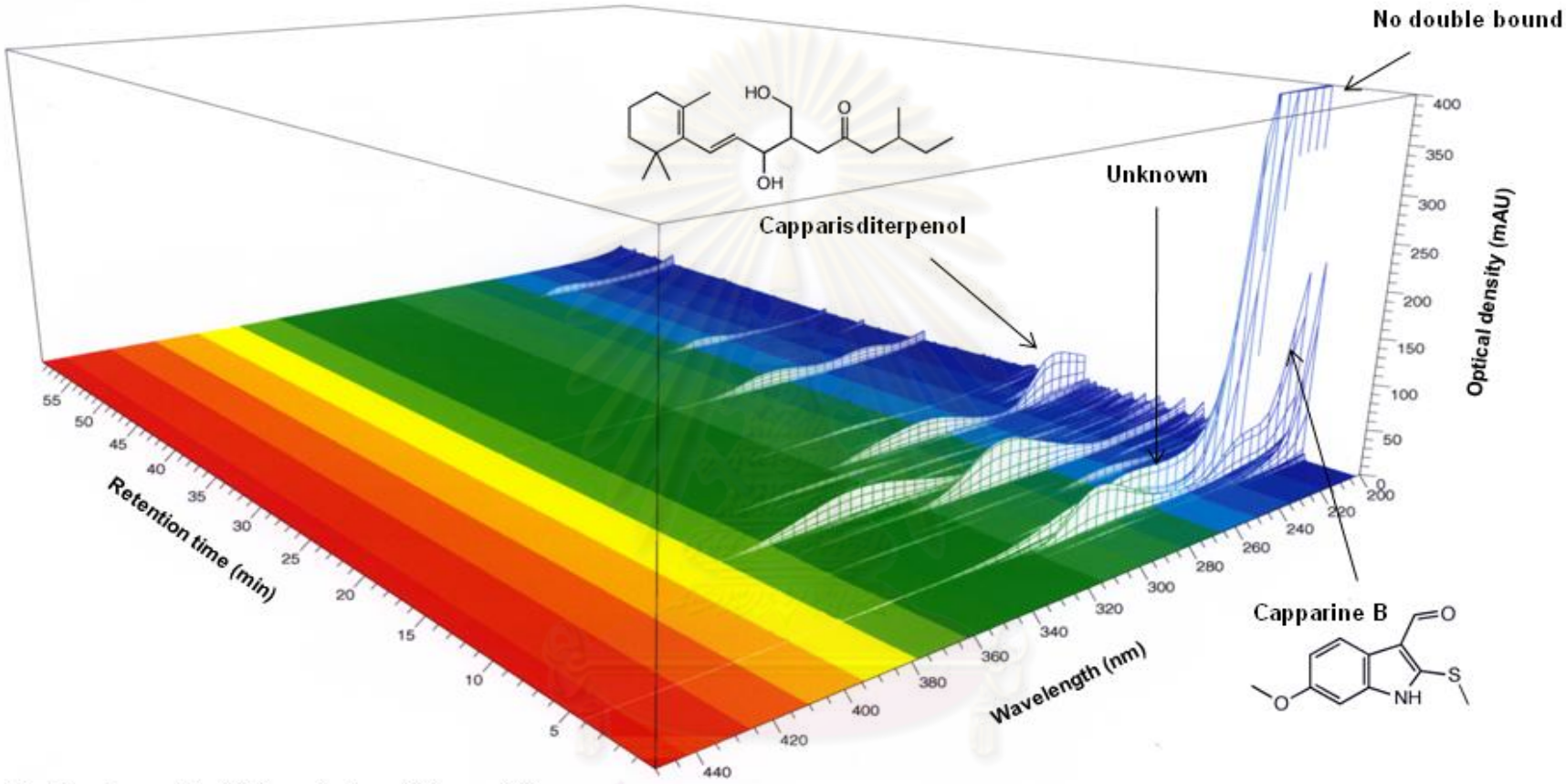


Figure 10 The HPLC Chromatogram of *Capparis micracantha* DC.



Mobile phase: 10 mM Phosphoric acid-Acetonitrile
Linear gradient: (95:5→ 5:95, 60 min)
Flow rate: 0.8 mL/min
Infusion volume: 5 mL
Temperature: 40°C

Figure 11 The 3D HPLC profile of *Capparis micracantha* DC.

Table 2 Specification of *Capparis micracantha* DC. Root

Content (% by weight)	Mean ± SD	Min – Max	n
Acid - insoluble ashes	2.09 ± 0.59	0.36 - 5.65	14
Total ash	4.91 ± 0.34	2.43 - 11.47	14
Loss on drying	6.76 ± 0.13	5.44 - 8.90	14
Ethanol-soluble extractive	0.52 ± 0.03	0.04 - 1.41	14
Water-soluble extractive	2.16 ± 0.46	1.21 - 4.82	14
water content	8.39 ± 0.15	7.00 - 9.80	14

N = 14, each sample was done in triplicate

***Clerodendrum petasites* S Moore.**

Family: VERBENACEAE

Synonym: *Clerodendrum petasites* (Lour.) A. Meeuse, *Clerodendrum robinsonii* Dop, *Clerodendrum subpandurifolium* Kuntze, *Volkameria petasites* Lour.

Vernacular names: Thailand: Tao-yai-mom, Mai-tao-ru-sri.

Distribution: *C. petasites* is widely grown in India, Malaysia and Thailand.

Observation: Vines, shrubs or small trees, 1-2 meter high, usually unarmed, glabrous or pubescent. Leaves is 15-20 cm long and 1.5-2.5 wide, opposite or whorled, simple, sometimes lobed, entire or dentate; petiolate or not; stipules absent. Inflorescence a terminal or axillary cyme, sometimes arranged in panicles or corymbs. Flowers long tubes with white color, zygomorphic, bisexual, usually large, showy, mostly white, blue, violet or red; calyx campanulate or tubular, truncate or 5-dentate to 5-partite, often accrescent; corolla salverform, tube cylindrical, straight or curved, limb 5-lobed, spreading or reflexed, stamens 4, long-exserted, didynamous, inserted in corolla tube; ovary imperfectly 4-locular, style exserted. Fruit a drupe(or berry) with a large kernel, obovoid or globose, 4-lobed or 4-sulcate, usually separating in 4 pyrenes. Seed exalbuminous. Seedling with epigeal germination; cotyledons emergent, green, fleshy.

Uses: Root is used to trat fever decrease body temperatures, ant-allergic, anti-inflammatory, anticonvulsant.

Macroscopic

Figure 12 A, B, C and F Folwers of *C. petasite*, D and E Leaves, G Root

Figure 13 Crude drug of *Clerodendrum petasites* S. Moore Root

The fragment of crude drug is small, harden, the color of surface cutting is soft white, smooth, glisten and normally empty of pitted.

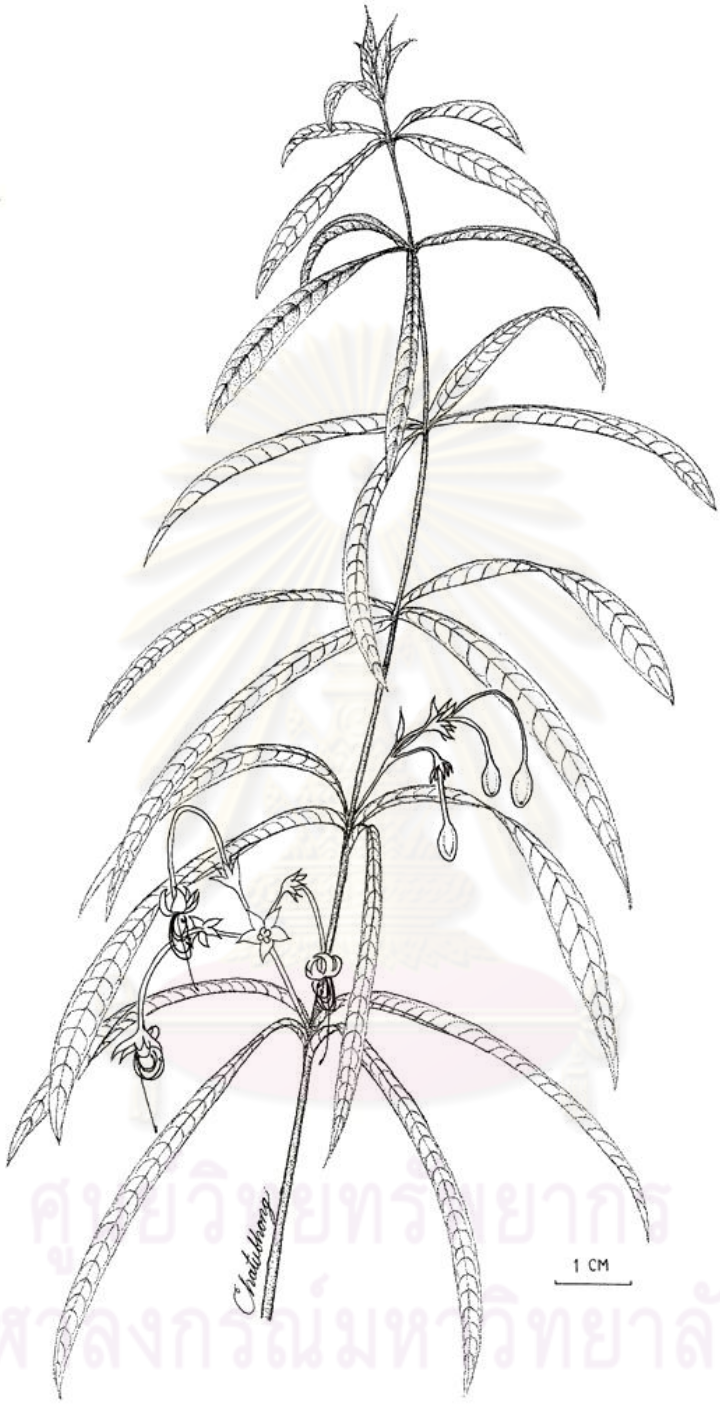


Figure 14 Whole plant of *Clerodendrum petasites* S Moore.

Whole plant is erect shrubs or small trees, generally unarmed, opposite leaves, inflorescence axillary cyme, large flowers and showy, mostly white or sometime red, calyx campanulate, truncate and 5-dentate. The root is conical small size and hardens, less branch root and root hairs.

Microscopic: Anatomical Character

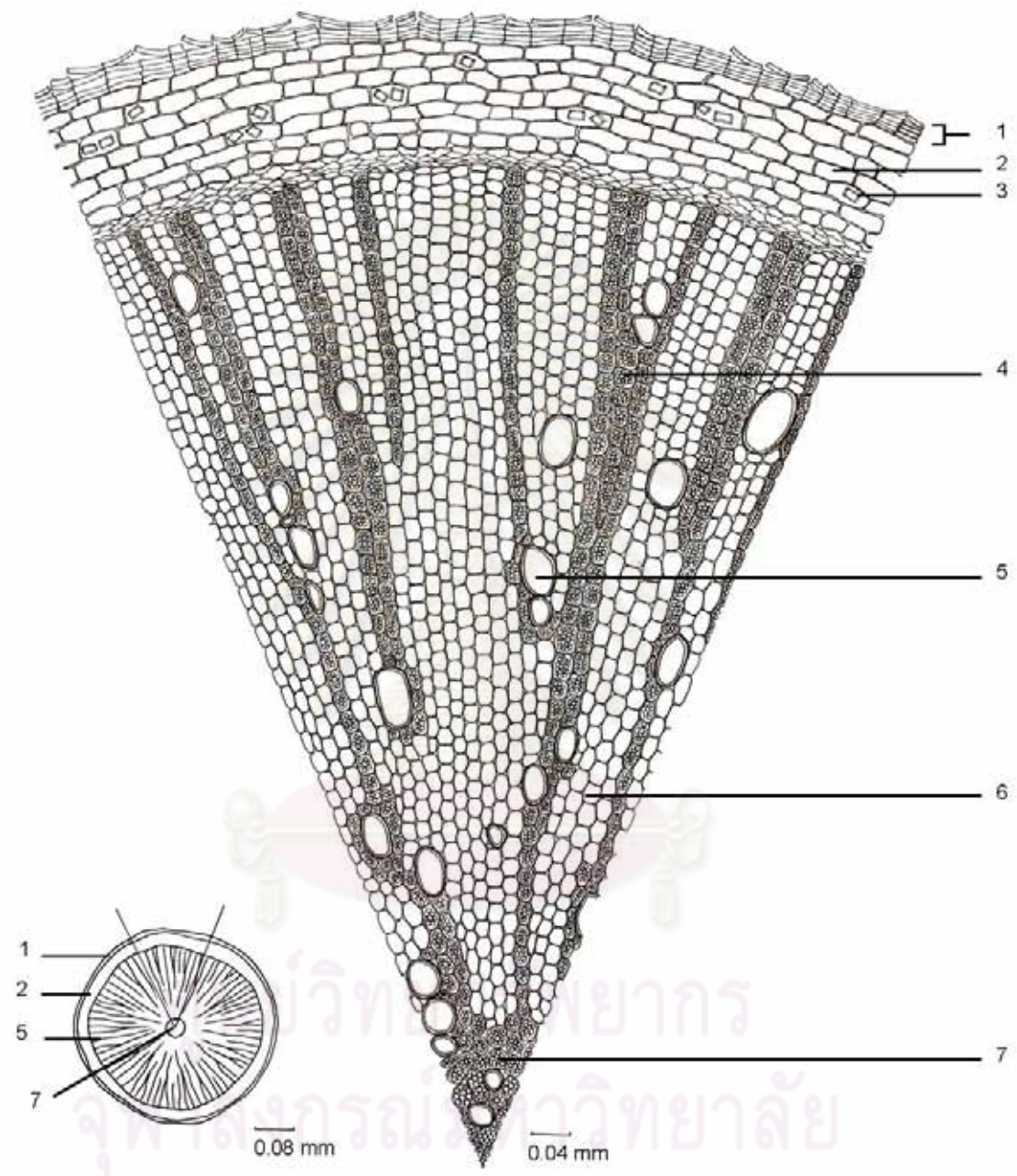


Figure 15 Transverse section of *Clerodendrum petasites* S. Moore. root:
1.Periderm 2.Parenchyma of cortex 3. Prism crystal in reserved parenchyma 4. Xylem ray with starch granules 5. Xylem vessel 6. Xylem parenchyma 7. Parenchyma of pith containing with starch granules

Histological Character

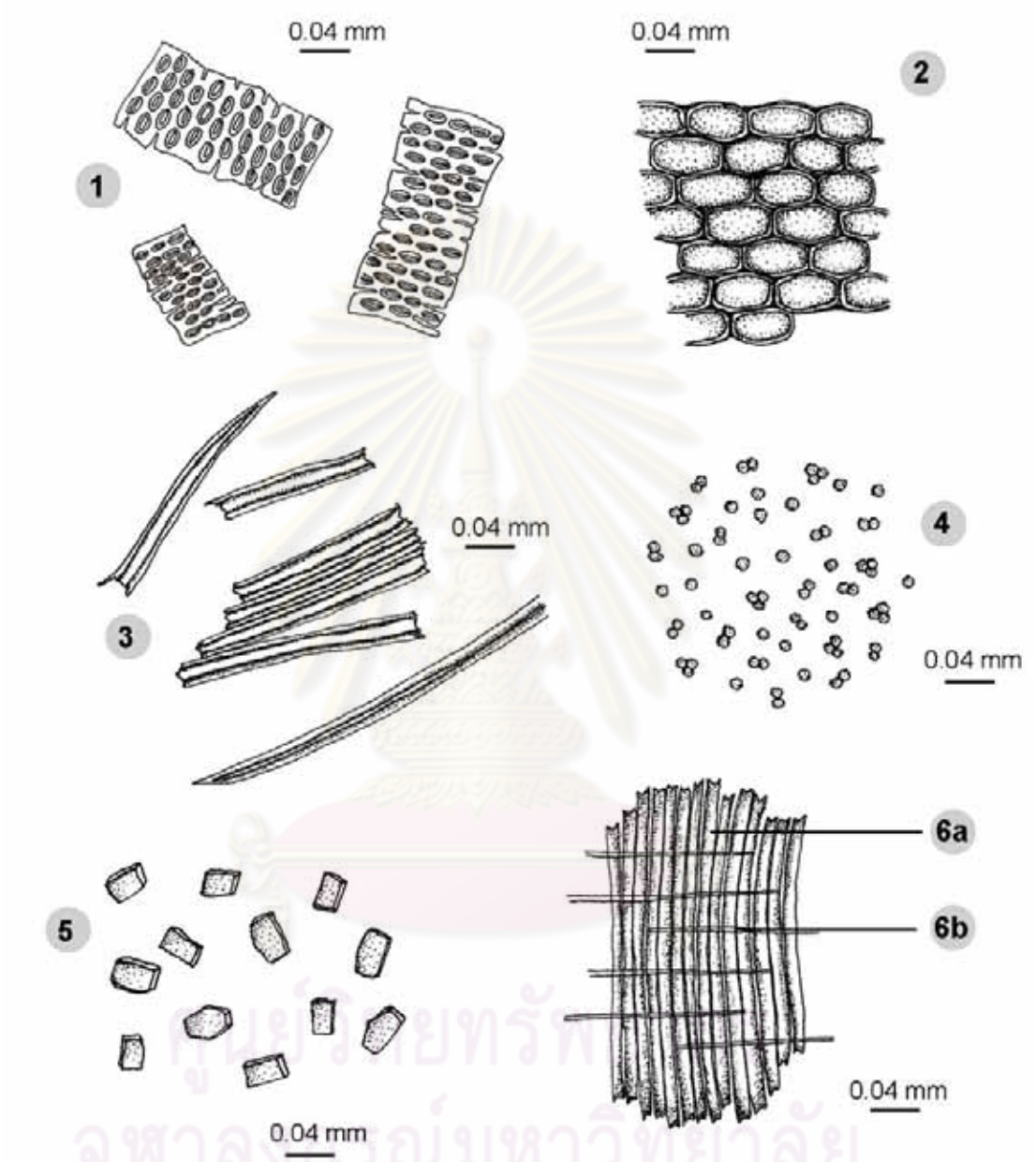


Figure 16 Powdered of *Clerodendrum patasites* S. Moore. Root:

1. Fragment of bordered pitted vessels 2. Parenchyma in sectional view 3. Fragment of fibers 4. Starch granules 5. Prism crystals of calcium oxalate 6. Part of xylem in radial longitudinal section showing wood fiber(6a) and medullary ray(6b)

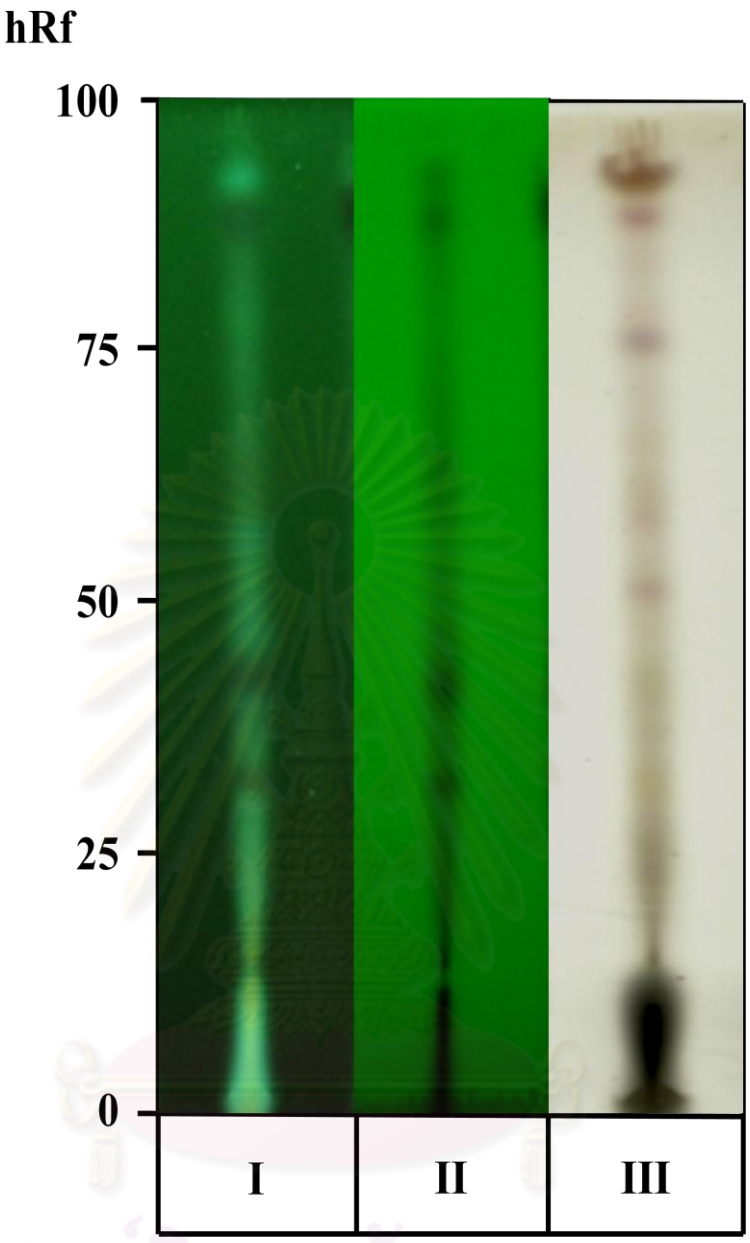


Figure 17 Thin-layer chromatogram of
methanolic extract of the root of *Clerodendrum petasites* S. Moore

Solvent system **Butanol : Acetic acid : Water 4 : 1 : 5**
Detection I = detection under UV light 254 nm
II = detection under UV light 366 nm
III = detection with Anisaldehyde*,**

*Anisaldehyde regent
Preparation: Anisaldehyde (0.5 ml), Glacial acetic acid (10 ml), methanol (85 ml), Sulfuric acid (5 ml)
**Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.
.

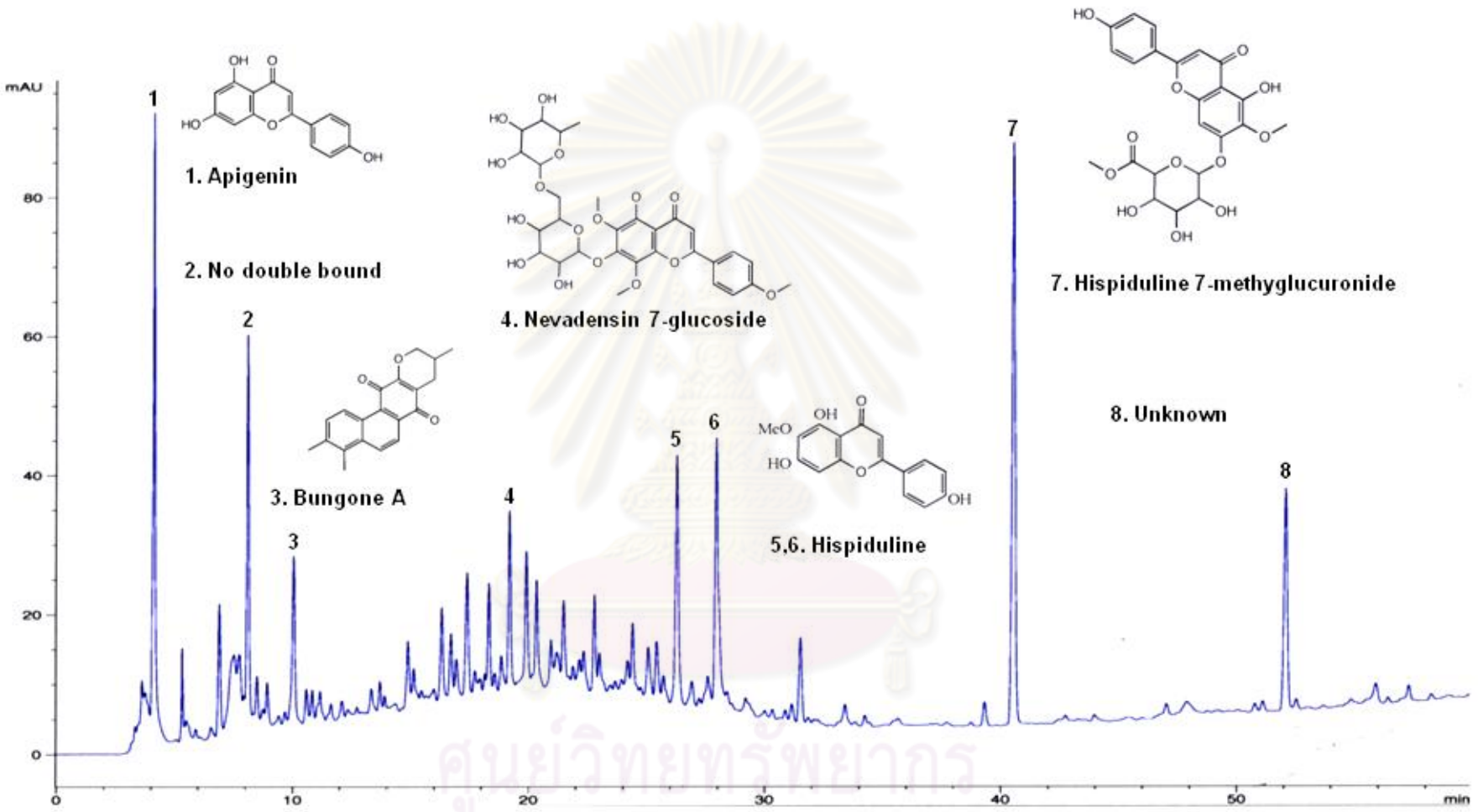


Figure 18 The HPLC chromatogram of *Clerodendrum peatasites* S. Moore.

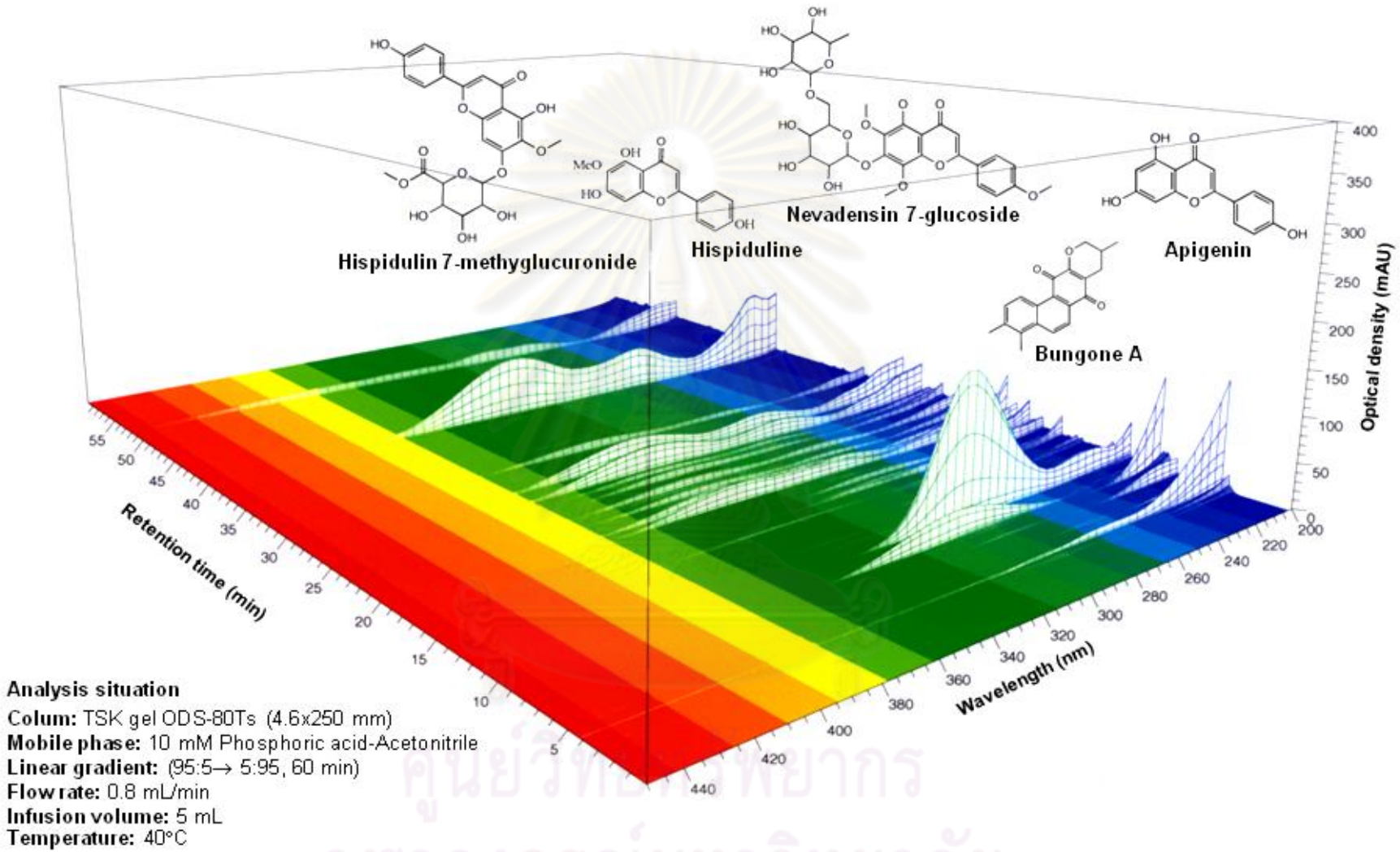


Figure 19 The 3D-HPLC profile of *Clerodendrum petasites* S. Moore.

Table 3 Specification of *Clerodendrum petasites* S. Moore

Content (% by weight)	Mean ± SD	Min – Max	n
Acid - insoluble ashes	0.98 ± 0.29	0.35 - 3.67	14
Total ash	4.33 ± 0.50	1.93 - 8.49	14
Loss on drying	6.09 ± 0.18	3.09 - 9.02	14
Ethanol-soluble extractive	0.65 ± 0.06	0.22 - 1.01	14
Water-soluble extractive	1.59 ± 0.18	0.90 - 2.73	14
water content	8.08 ± 0.28	5.20 - 10.60	14

N = 14, each sample was done in triplicate

***Harrisonia perforata* (Blanco) Merr.**

Family: SIMARUBACEAE

Synonyms: *Harrisonia paucijuga* Oliv., *Harrisonia bennettii* Benn., *Anisifolium pubescens* (Wall.) Kuntze, *Feroniella puberula* Yu.Tanaka, *Feroniella pubescens* (Wall. ex Hook.f.) Yu.Tanaka, *Feroniella pubescens* (Wall. ex Hook. f.) Tanaka, *Harrisonia citrinaecarpa* Elmer, *Lasiolepis multijuga* Benn., *Lasiolepis paucijuga* Benn. & R. Br., *Limonia pubescens* Wall. ex Hook.f., *Paliurus dubius* Blanco, *Paliurus perforatus* Blanco

Vernacular names: Thailand: Khon-tha (Central), Naam chee (Northern). Indonesia: Sesepang (Lampung), Garut (Sundanese), Ri kengkeng (Javanese). Malaysia: Kait-kait (Murut, Sabah). Philippines: Asimau, Mamiki (Tagalog), Muntani (Bisaya). Laos: Dok kin ta. Vietnam: S[aa]n, da da, h[ar]l s[ow]n.

Distribution: Harrisonia perforata is found in the drier part from Burma (Myanmar) eastward through Thailand to Indo-China and the Philippines, southward to Peninsular Malyasia (Paris), South Sumatra, Borneo (Sabah), Sulawesi, Java and the Lesser Sunda Islands.

Observations: A scandent to erect prickly shrub up to 4-6 m tall, leaves imparipinnate with unpaired terminal leaflet up to 20 cm long, with 1-15 pairs of leaflets supported by 5-30 mm long stalk; stipulate thorns slightly recurved backward to downward, accrescented to 7 mm; leaflets rhomboid to ovate-laceolete, 10-20 mm x 5-15 mm, subentire to lobed, rachis narrowly winged; inflorescence 8-20 flowered, flowers 4-5 merous, pedicellate, calys small, lobes triangular, petals lanceolate, 6-9 mm x 2-4 mm, red outside, pale red to white inside, stamens 8-10, anthers 1.5-4.5 mm long, filaments 7-10 mm long, at base with ligule which is densely woolly at the margin, disk cup-shaped, ovary slightly lobed, styles 5-8 mm long, pubescent; fruit a berry, 4-9 mm x 11-15 mm, exocarp coriaceous, at least 1 mm thick, endocarp hard, without suture.

Uses: In Indonesia, young shoots are considered a remedy against diarrhea. In the Philippines, a decoction of the root bark is recommended in the treatment of diarrhea and dysentery as well as against cholera. In Indo-China, ashes of the roasted leaves mixed with oil or simply crushed leaves are applied to relieve itch. In Thailand, the dried root is considered antipyretic and anti-inflammatory; it is used in wound healing and in the treatment of diarrhea. The stems are also employed in the treatment of diarrhea.

Macroscopic

Figure 20 A Fruits of *Harrisonia perforata* (Blanco) Merr., B and D Flowers, D Whole plant

Figure 21 Crude drug of *Harrisonia perforata* (Blanco) Merr. Root

The fragment of crude drug is harden and plain surface. The outermost is grayish in color and leathery texture.

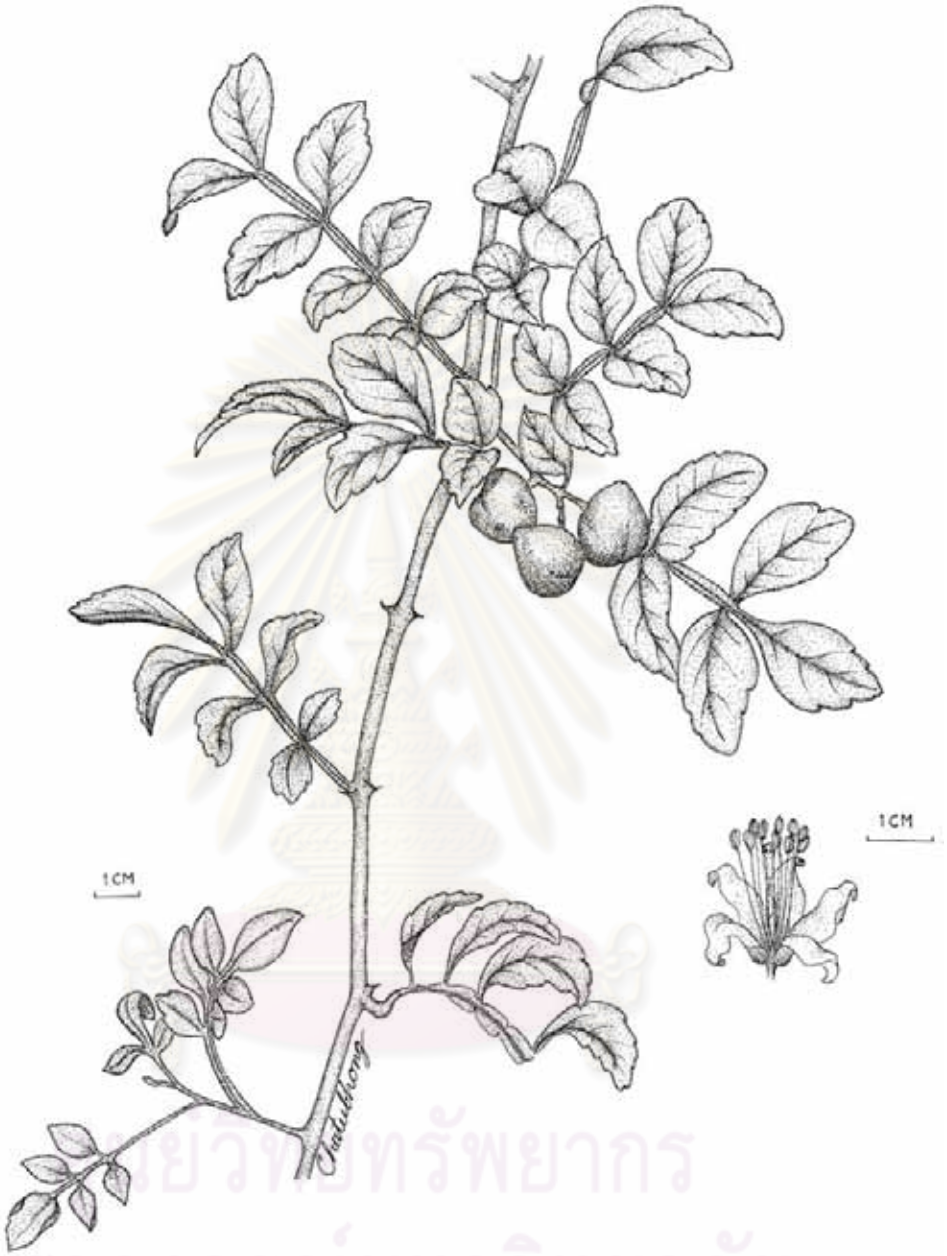


Figure 22 Whole plant of *Harrisonia perforata* (Blanco) Merr.

Whole plant is a climbing to erect prickly shrub. The branches are armed with short, sharp spines, the leaves pinnate with unpaired terminal leaflet and the leaflets are ovate-lance-shaped. Flowers are a pedicel with 4-5 merous, triangular lobes,petals are lance-shaped which are red outside and pale red to white inside. The fruit is globular, exocarp of leathery texture and freshly.

Microscopic: Anatomical Chracter

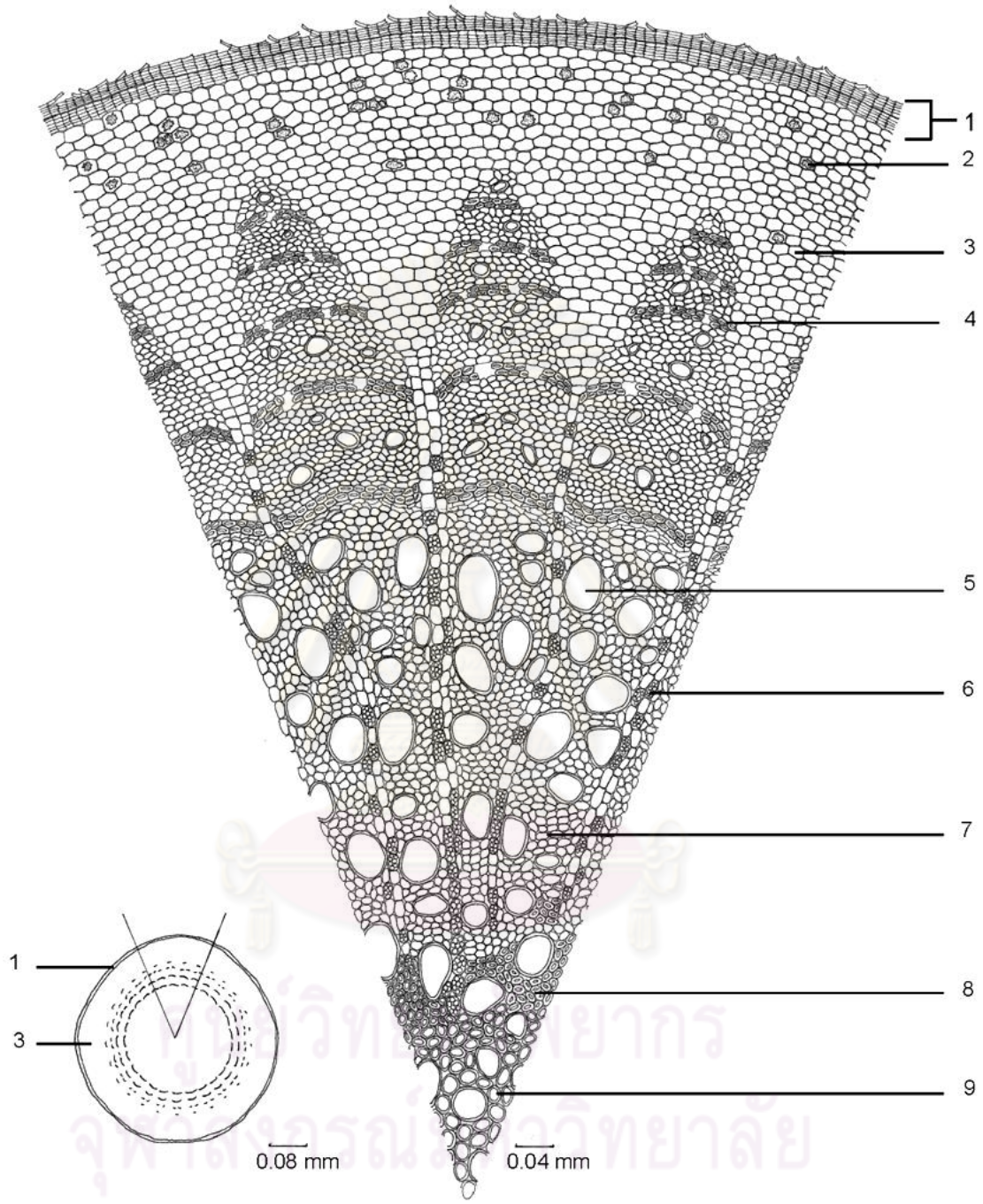


Figure 23 Transverse section of *Harrisonia perforata* (Blanco) Merr. root:

1.Periderm 2. Parenchyma containing rosette aggregate crystal 3. Parenchyma of cortex 4. Fiber 5. Xylem vessel 6. Xylem ray with starch granules 7. Xylem parenchyma 8. Xylem fiber 9. Parenchyma of pith

Histological character

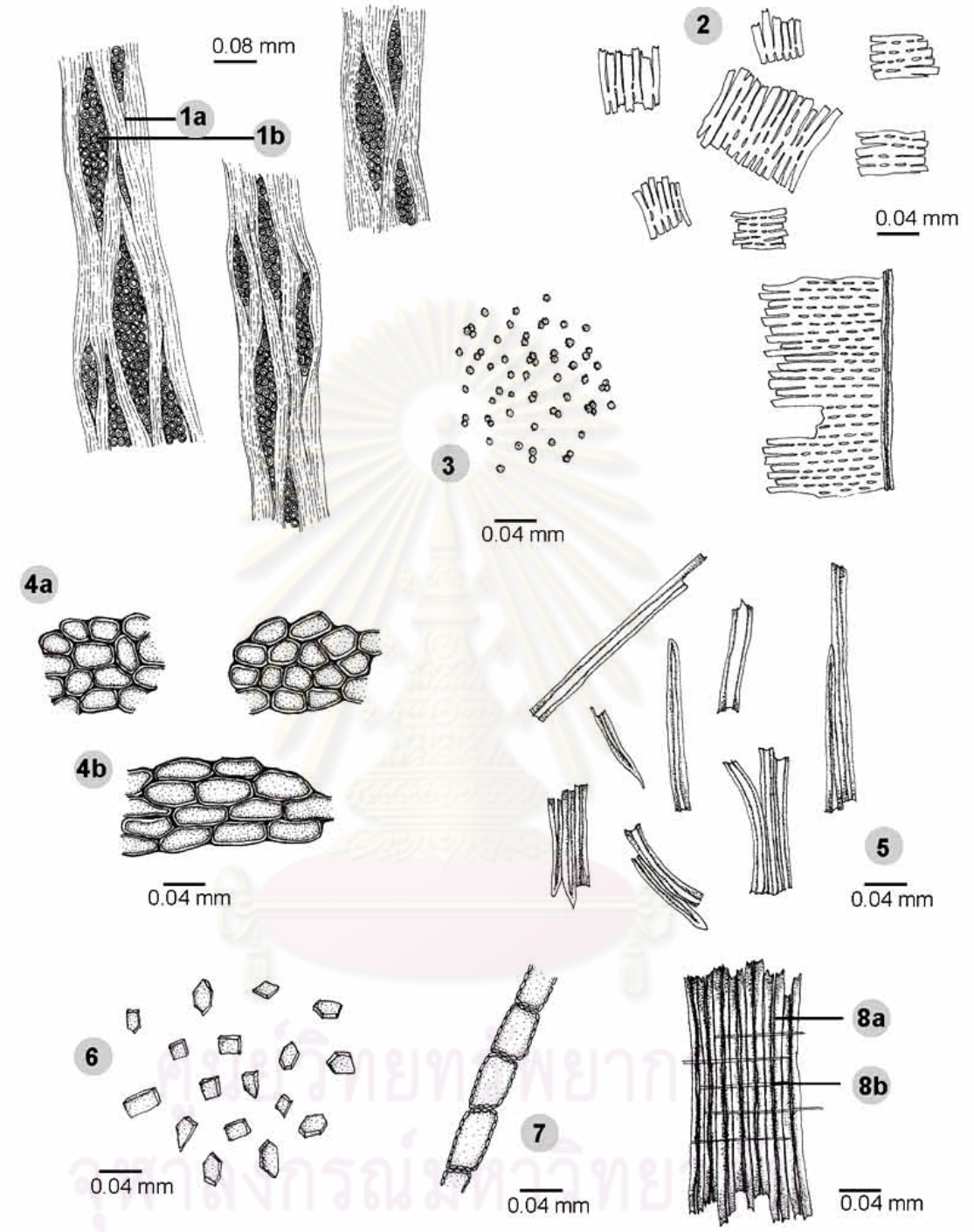


Figure 24 Powdered of *Harrisonia perforata* (Blanco) Merr. root:

1. Part of xylem in tangential longitudinal section showing wood fiber(1a) and medullary ray(1b) 2. Fragment of pitted vessels 3. Starch granules 4. Parenchyma in sectional view(4a) and longitudinal view(4b) 5. Fragment of fiber 6. Prism crystals of calcium oxalate 7. Part of xylem parenchyma 8. Part of xylem in radial longitudinal section showing wood fiber(8a) and medullary ray(8b)

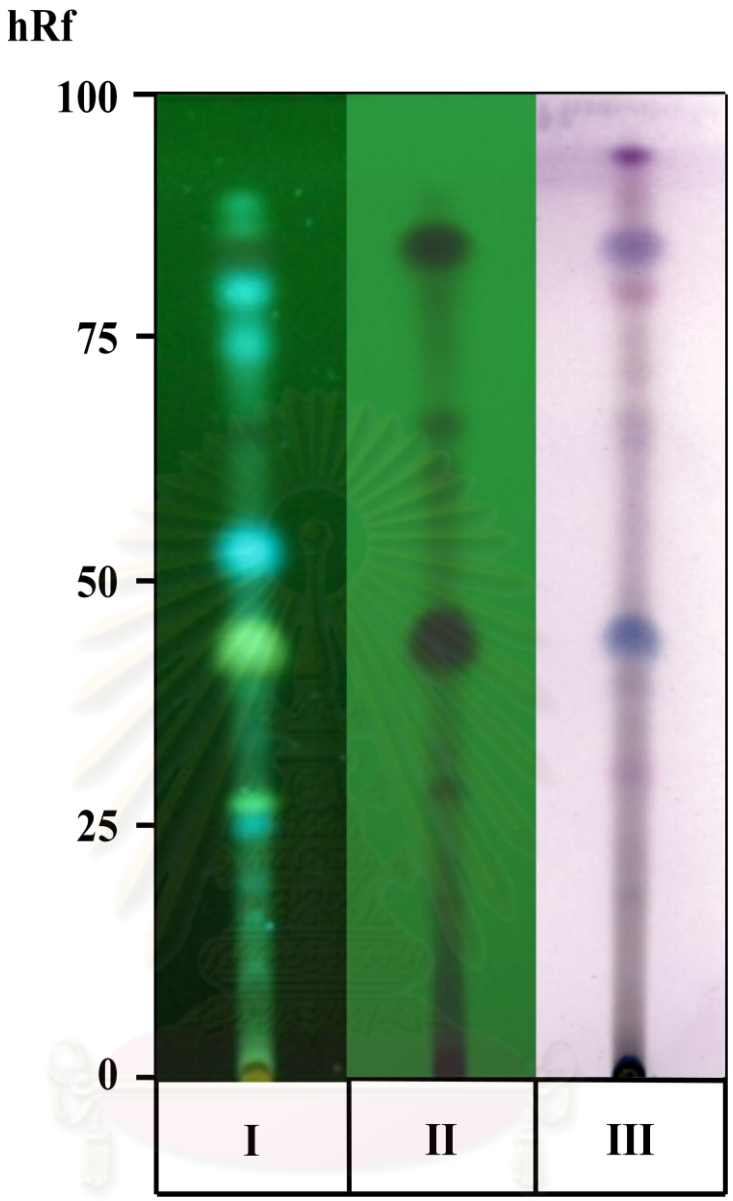


Figure 25 Thin-layer chromatogram of
methanolic extract of the root of *Harrisonia perforat*a (Blanco) Merr.

Solvent system **Dichloromethane : Methanol 95 : 5**
Detection I = detection under UV light 254 nm
II = detection under UV light 366 nm
III = detection with Anisaldehyde*,**

*Anisaldehyde regent
Preparation: Anisaldehyde (0.5 ml), Glacial acetic acid (10 ml), methanol (85 ml), Sulfuric acid (5 ml)
**Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

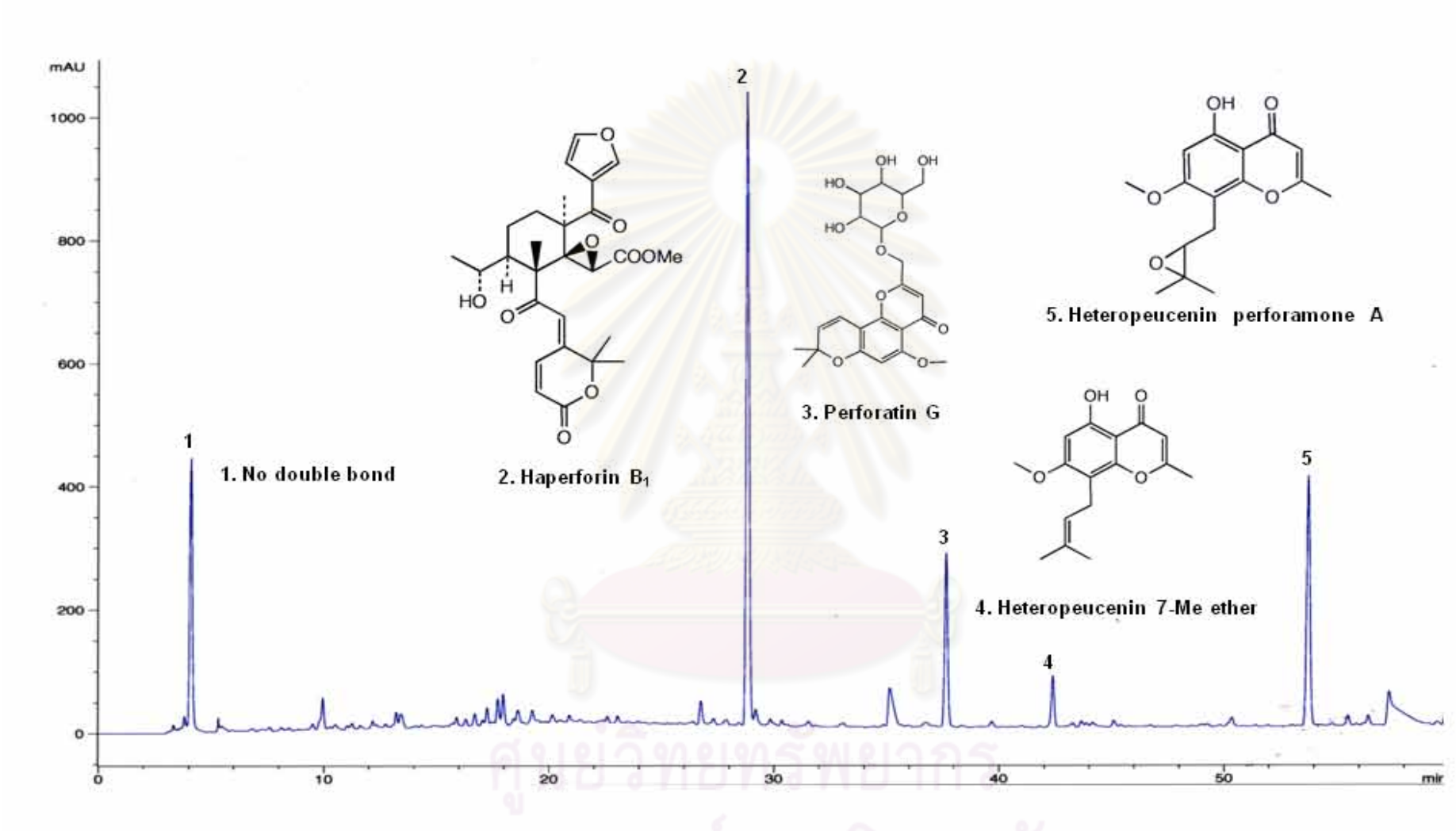


Figure 26 The HPLC chromatogram of *Harrisonia perforata* (Blanco) Merr.

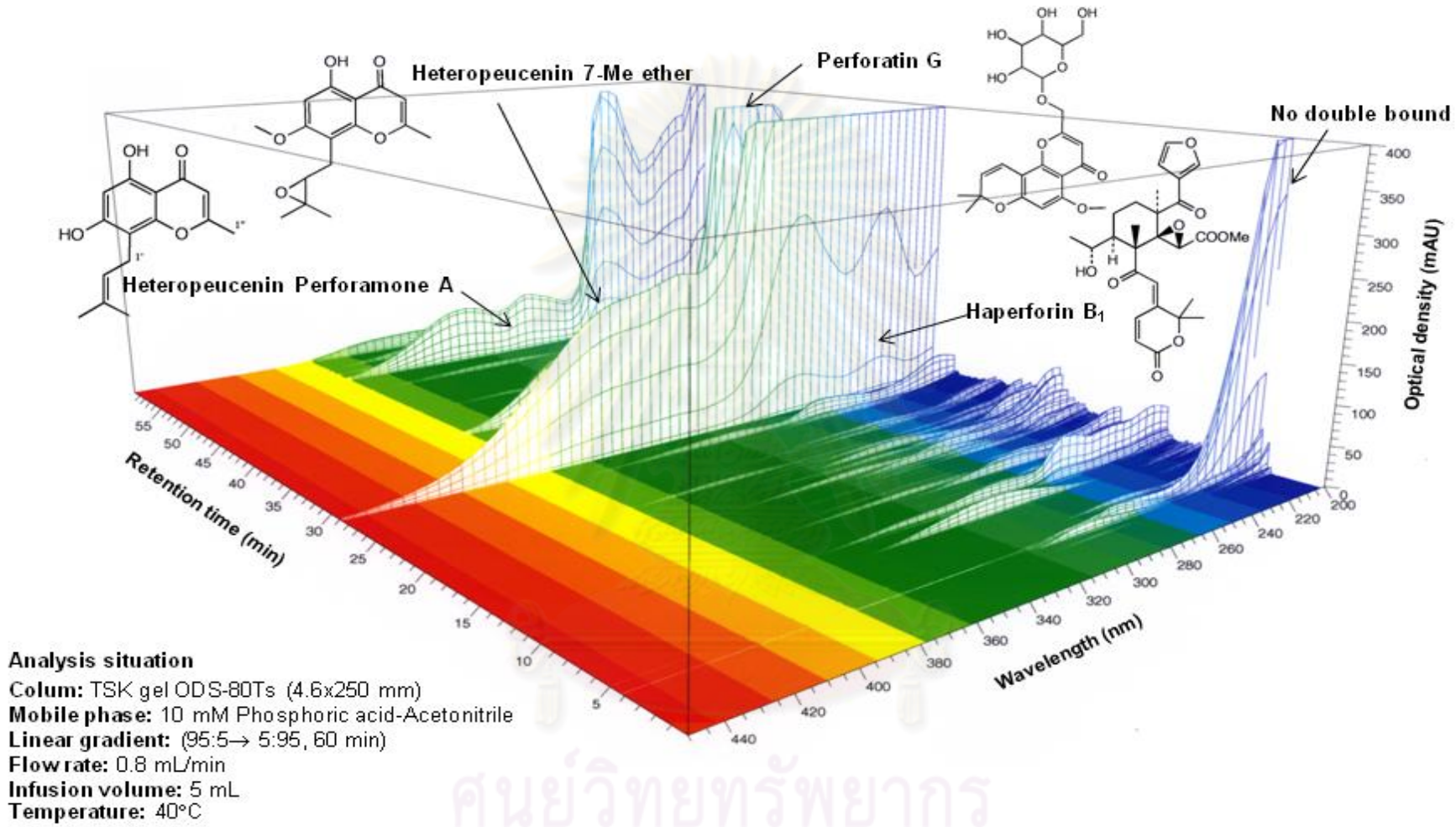


Figure 27 The 3D-HPLC profile of *Harrisonia perforata* (Blanco) Merr.

67

Table 4 Specification of *Harisonia perforata* (Blanco) Merr. Root

Content (% by weight)	Mean ± SD	Min – Max	n
Acid - insoluble ashes	0.67 ± 0.17	0.20 - 1.36	14
Total ash	3.62 ± 0.32	1.36 - 6.56	14
Loss on drying	6.36 ± 0.70	5.03 - 8.00	14
Ethanol-soluble extractive	0.83 ± 0.06	0.22 - 1.36	14
Water-soluble extractive	1.17 ± 0.21	0.56 - 4.06	14
water content	8.34 ± 0.40	5.50 - 10.60	14

N = 14, each sample was done in triplicate

***Ficus recemosa* L.**

Family: MORACEAE

Synonyms: *Ficus glomerata* Roxb., *Covellia glomerata* (Roxb.) Miq., *Ficus vesca* F.Muell. ex Miq.

Vernacular names: Thailand: Duea-kling (Central, northern), Duca nam (southern). English: Cluster fig, Red river fig. Indonesia: Elo (Javanese), Loa (Sundanese), Arah (Madurese). Singapore: Atteeka. Burma; Atti, Umbar. Combodia: Lovie. Laos: Dua kiengz. Vietnam: Sung.

Distribution: North-eastern Africa, India to Indo-China, Malaysia to northern and western Australia. Not in the Philippines. In India also cultivated.

Observations: *Monoecious tree,* up to 18 m tall, 25 cm diameter, buttressed, occasionally deciduous, the crown often irregular and shabby; Bark pinkish-brown, smooth then coarsely flaky; latex copious, cream buff, inner bark yellowish. Young shoots and figs finely white hairy, soon glabrous; young leaves pale green. Twigs 1.5-3 mm thick, slender, reddish brown. Stipules -12 mm long, -30 mm long on opening shoots, often persistent on young twigs. Lamina 6-19 x 3.5 – 8.5 cm, elliptic varying subovate, shortly oblong or somewhat lanceolate, tapered to a subacute or subacuminate apex, base broadly to narrowly cuneate, rearely subcordate, subcoriaceous, smooth, entire (dentate in saplings); lateral veins 4-8 pairs, -7 intercostals slightly raised below; basal veins 1 pair, short, often with a slight axillary gland; petiole 15-70 mm long, becoming brown scurfy. Figs in large clusters on the main branches and trunk, on branching leafless twigs -25 cm long, 2.5 cm thick at the base, ripening rose-red; peduncle 3-12 mm long, stout; basal bracts 1-2 mm long, ovate-triangular, obtuse; pedicel 0-5 mm long; body 20-30 mm wide (35-50 mm, living), subglobose to pyriform, often lenticellate-verrucose, orifice plane or slightly sunken, closed by 5-6 apical bracts; internal bristles non; sclerotic cells none in the thick, soft wall. Perianth with 3 (-4) dentate-lacerate lobes joined below, red, glabrous. Male flowers ositolar in 2-3 rings, sessile, much compressed; stamens (1-) 2, rarely 3, often with an abortive ovary. Gall-flowers long-stalked; ovary dark red; style short. Female flowers sessile or very shortly stalked among the gallflowers; ovary sessile or substipitate, red-spotted; style 2-3 mm long, glabrous, simple. Seed 1 mm long, lenticular, smooth not or scarcely keeled. Lamina with cystoliths only on the lower side.

Uses: The figs, which are rather insipid but sweet, are edible. They are used in various preserves and side-dishes. Leaves eaten as vegetable and are said to be used against diarrhoea. They are also used as animal fodder and they provide valuable mulch. In India the tree is also cultivated as host plant for lac insects, shade tree for coffee and a rootstock for Ficus carica L. The latex is used in production of water-resistant paper and as plasticizer for Hevea rubber.

Macroscopic

Figure 28 A and F Fruits of *Ficus racemosa* L., B and E Leaves, C and D Bark with Fruits

Figure 29 Crude drug of root of *Ficus racemosa* L. Root

The fragment of crude drug is dark red-brown, coarse surface. The outermost is thin and leathery texture.

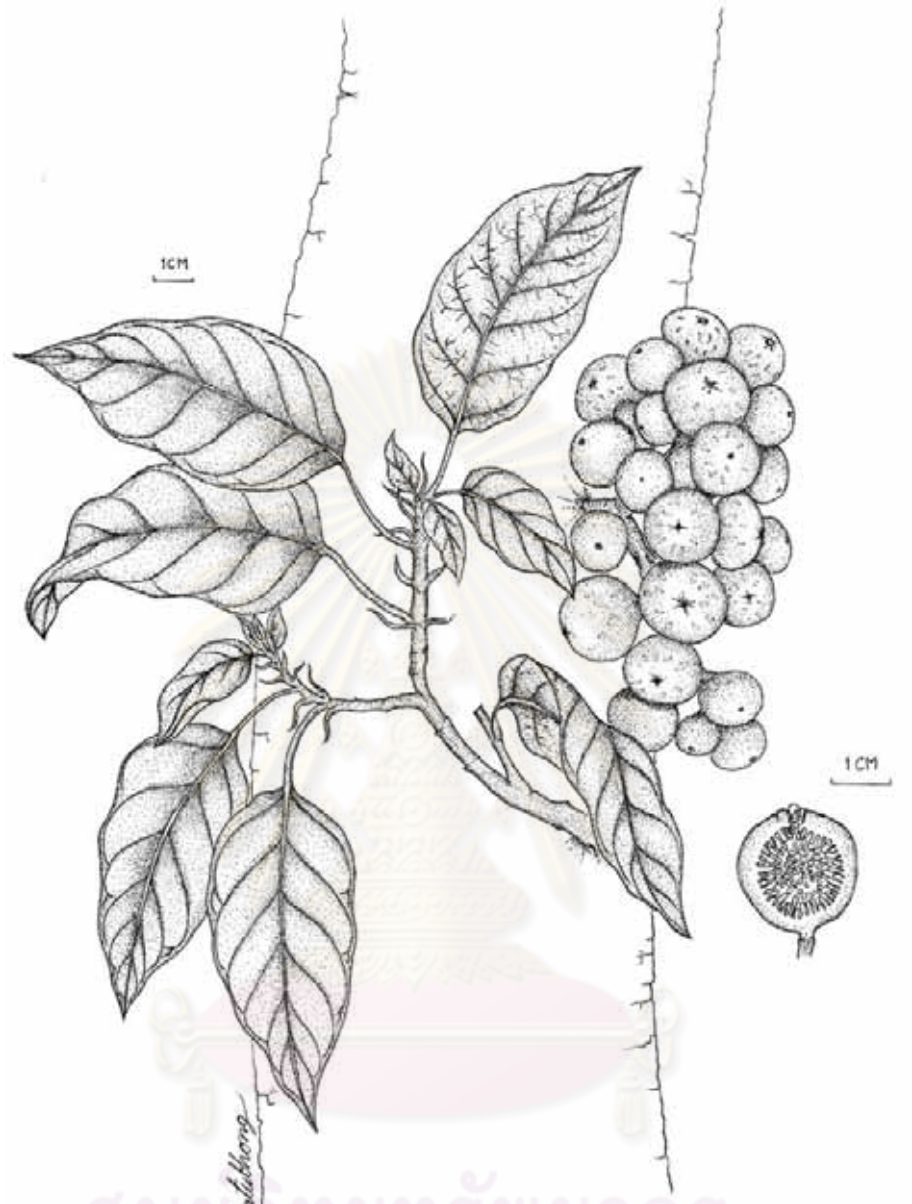


Figure 30 Whole plant of *Ficus racemosa* L.

Whole plant is tree or banyans, evergreen or occasionally deciduous, the crown often irregular and shabby, woody epiphytic climbers or stranglers, creepers. The trees whose branches send down aerial roots that thicken (pillar roots). Leaves arranged spirally or alternate, simple to palmately lobed. Fruits are in large clusters on the main branches and trunk, ripening rose-ripe, subglobose to pyriform and soft.

Microscopic: Anatomical Character

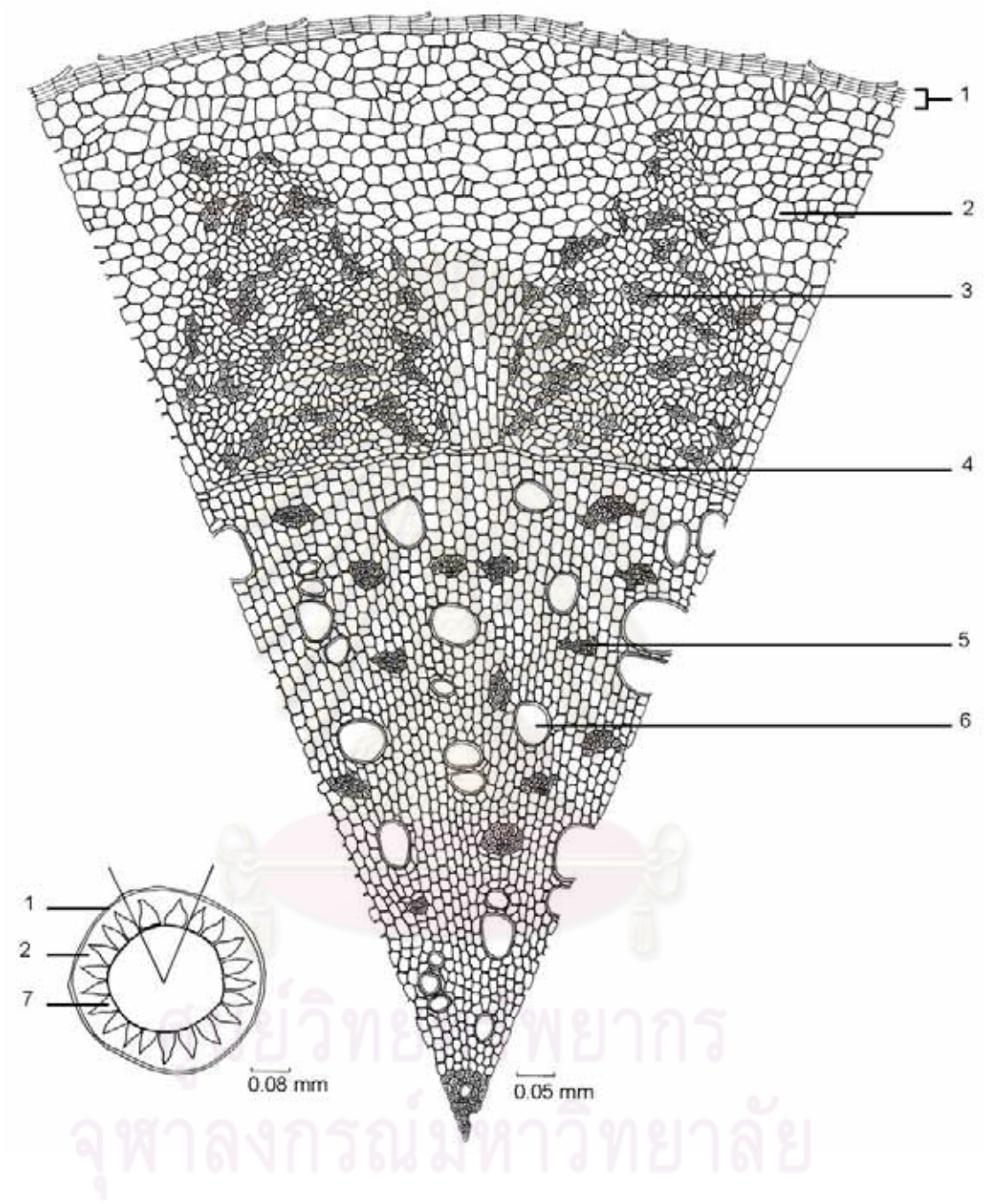


Figure 31 Transverse section of *Ficus racemosa* L. root:

1. Periderm 2. Parenchyma of cortex 3. Phloem fiber 4. Vascular cambium 5. Xylem fiber 6. Xylem vessle 7. Phloem tissue

Histological Character

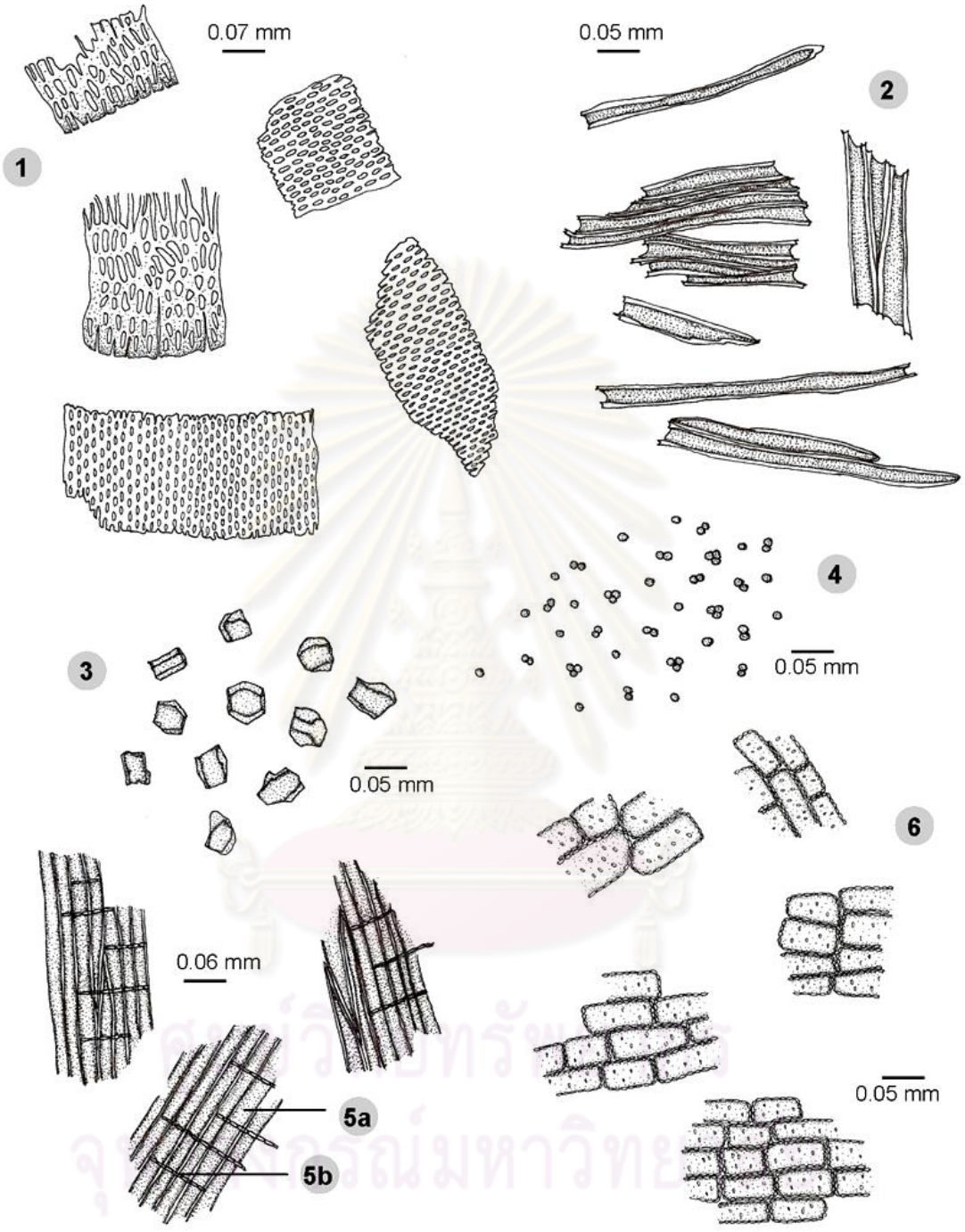


Figure 32 Powdered of *Ficus racemosa* L. root:

1. Fragment of pitted vessels 2. Fragment of fibers 3. Prism crystals of calcium oxalate 4. Starch granules 5. Part of xylem in radial longitudinal section showing wood fiber(5a) and medullary ray(5b) 6. Xylem parenchyma in longitudinal view

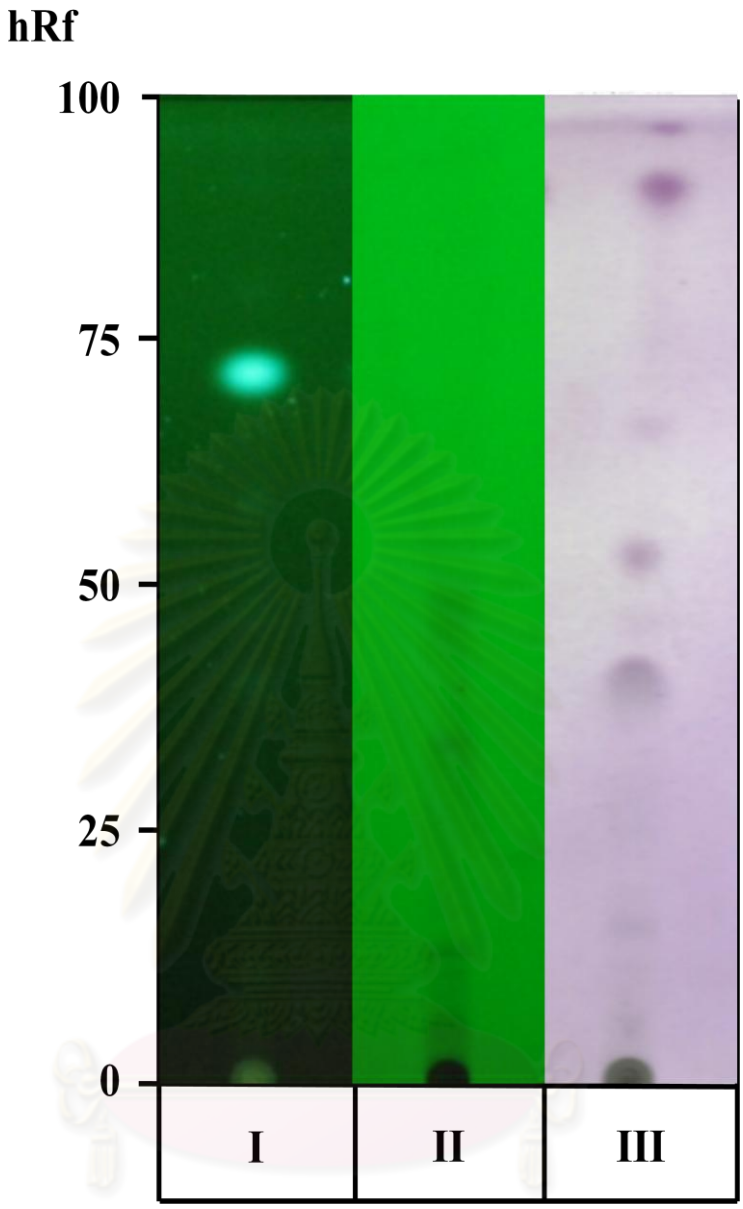


Figure 33 Thin-layer chromatogram of methanolic extract of the root of *Ficus racemosa* L.

Solvent system **Toluene : Ethyl acetate 75:25**

Detection

I = detection under UV light 254 nm

II = detection under UV light 366 nm

III = detection with vanillin-sulfuric acid*,**

*Anisaldehyde regent

Preparation: Anisaldehyde (0.5 ml), Glacial acetic acid (10 ml), methanol (85 ml), Sulfuric acid (5 ml)

**Spot color Development

Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

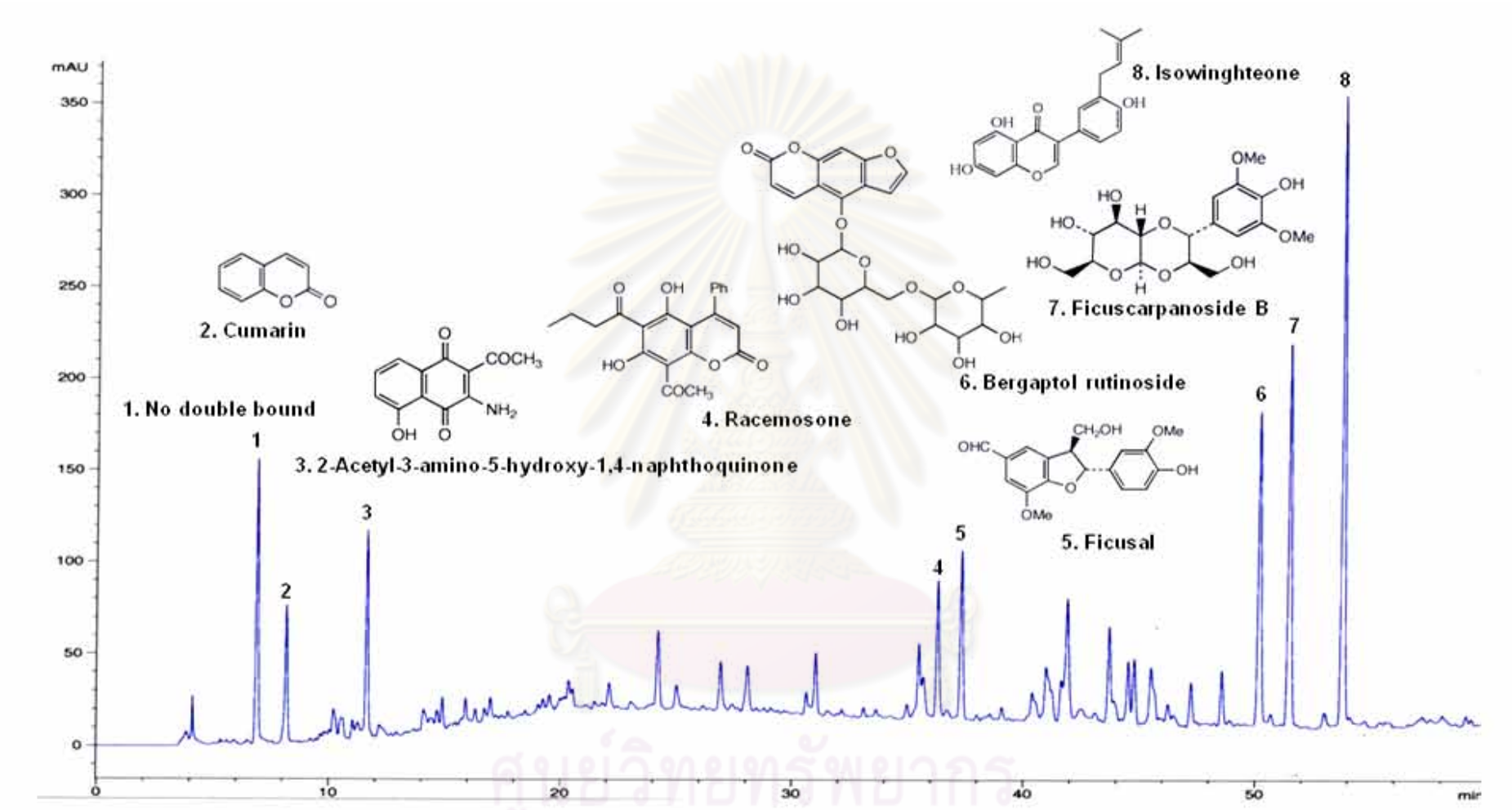


Figure 34 The HPLC chromatogram of Ficus racemosa L.

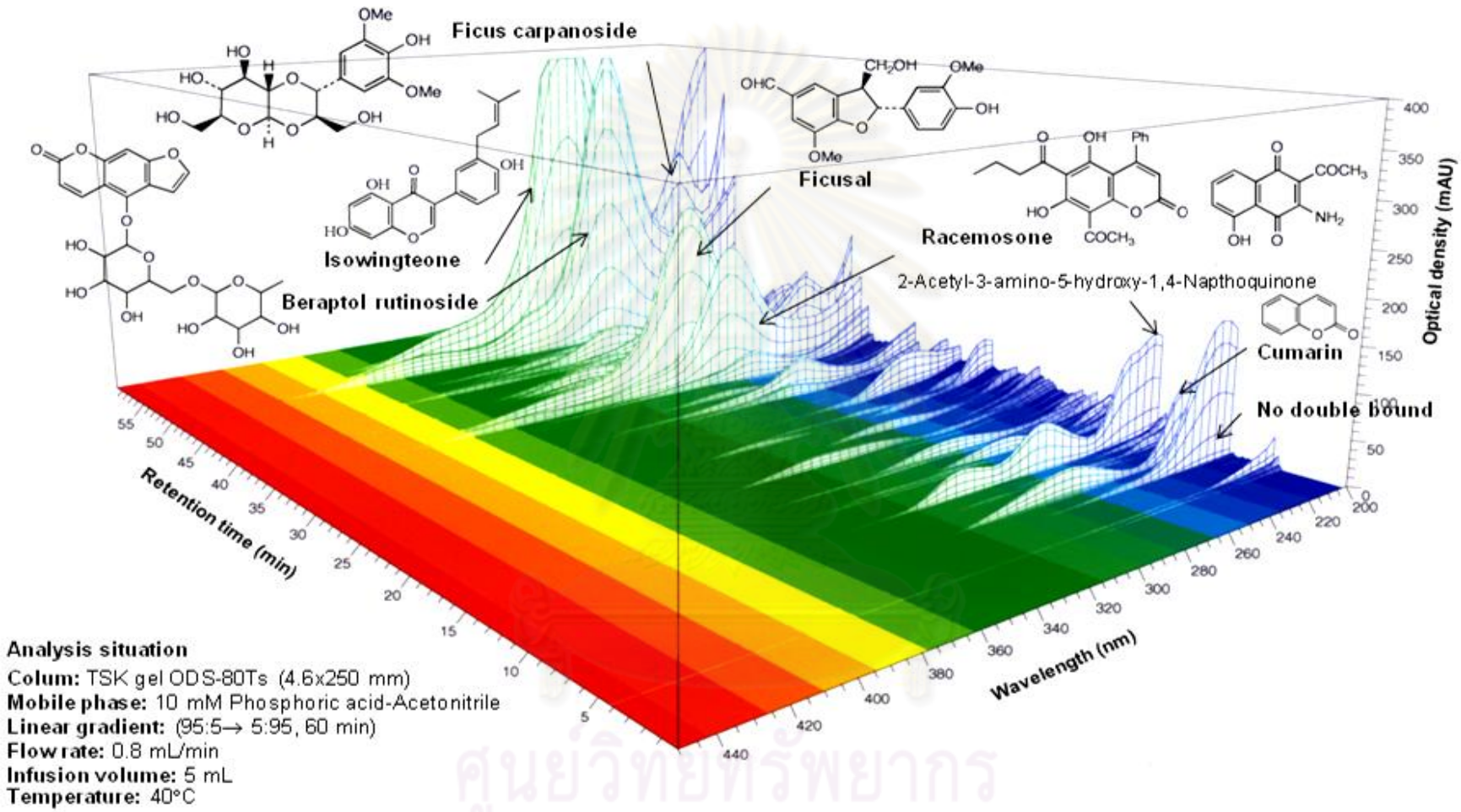


Figure 35 The 3D-HPLC profile of *Ficus racemosa* L.

Table 5 Specification of *Ficus racemosa* L. Root

Content (% by weight)	Mean ± SD	Min – Max	n
Acid - insoluble ashes	1.08 ± 0.24	0.28 - 2.72	14
Total ash	5.94 ± 0.34	3.07 - 8.37	14
Loss on drying	6.28 ± 0.10	4.88 - 7.49	14
Ethanol-soluble extractive	0.56 ± 0.40	0.07 - 1.03	14
Water-soluble extractive	1.22 ±0.24	0.12 - 2.25	14
water content	8.48 ± 0.64	5.30 - 11.20	14

N = 14, each sample was done in triplicate

Tiliacora triandra (Colebr.) Diels

Family: MENISPERMACEAE

Synonyms: *Cocculus triandrus* Colebr., *Limacia triandra* (Colebr.) Hook.f. & Thomson.

Vernacular names: Thailand; Choi nang (northern), Thao wan khiew (Central), Yat nang (southern). Malaysia: Akar kunyitkunyit, Berkunyit, Akar kusin. Vietnam: Xanh tam.

Origin and geographic distribution: *T. triandra* occurs in India (Assam), southern Burma, Inco-china, Thailand and Penisular Malaysia.

Observations: A dioecious liana with puberulous to glaborous and striate stems. Leaves alternate, simple and entire,elliptical, lanceolate or sometimes subovate, 6.5-11-17 cm x 2-4 cm, base cuneate to rounded, apex usually acuminate, with 3-5 basal veins and 2-6 pairs of lateral veins; petiole 0.5-2 cm long; stipules absent. Inflorescence an axillary or cauliflorous pseudo-receme, up to 2-8(-17) cm long, composed of 1-few-flowered peduncled cymes. Flowers unisexual, yellowish; sepals 6-12 , the outermost smellest, innermost up to 2 mm long; male flowers with 3 or 6 petals 1 mm long and 3 stamens; demale flowers with 6 petals c. 1 mm long and 8-9 carpels inserted on a gynophores. Fruit consisting of several drupes borne on a brached carpophores; drupes obovoid, 7-10 mm x 6-7 mm, red, glabrous, endocarp transversely and irregularly ridged. In Indo-China T. Triandra can be found flowering and fruiting throughout the year, but in Thailand from December-July only. As in other Menispermaceae, the pollinators are probably small insects, which are undoubtedly attracted by the scent of the flowers. Tiliacora consists of 19 species in Africa, 2 in tropical Asia and 1 in Australia.

Uses: In Thailand aerial parts of *T. triandra* are widely used as an antipyretic. In Cambodia the leafy shoots enter into a prescription for the treatment of dysentery. They are also used as a flavoring in cooking in Thailand. In Indo-china the flexible stems are used for rough cordage, thatching and basketry. *T. acuminata* (Lamk) Hook.f. & Thomson, an Indian-Burmese species, appreciated for its ornamental foliage and fragrant flowers and mentioned as a remedy for snakebites, is cultivated in the botanical garden in Bogor, Indonesia.

Macroscopic

Figure 26 A and B Fruits of *Tiliacora triandra* (Colebr.) Diels, C and F Leaves, D and E Flowers

Figure 37 Crude drug of *Tiliacora triandra* (Colebr.) Diels Root

The fragment of crude drug is grayish-yellow, soft, leathery texture of epidermis and clear brown patches radiating from pitted.

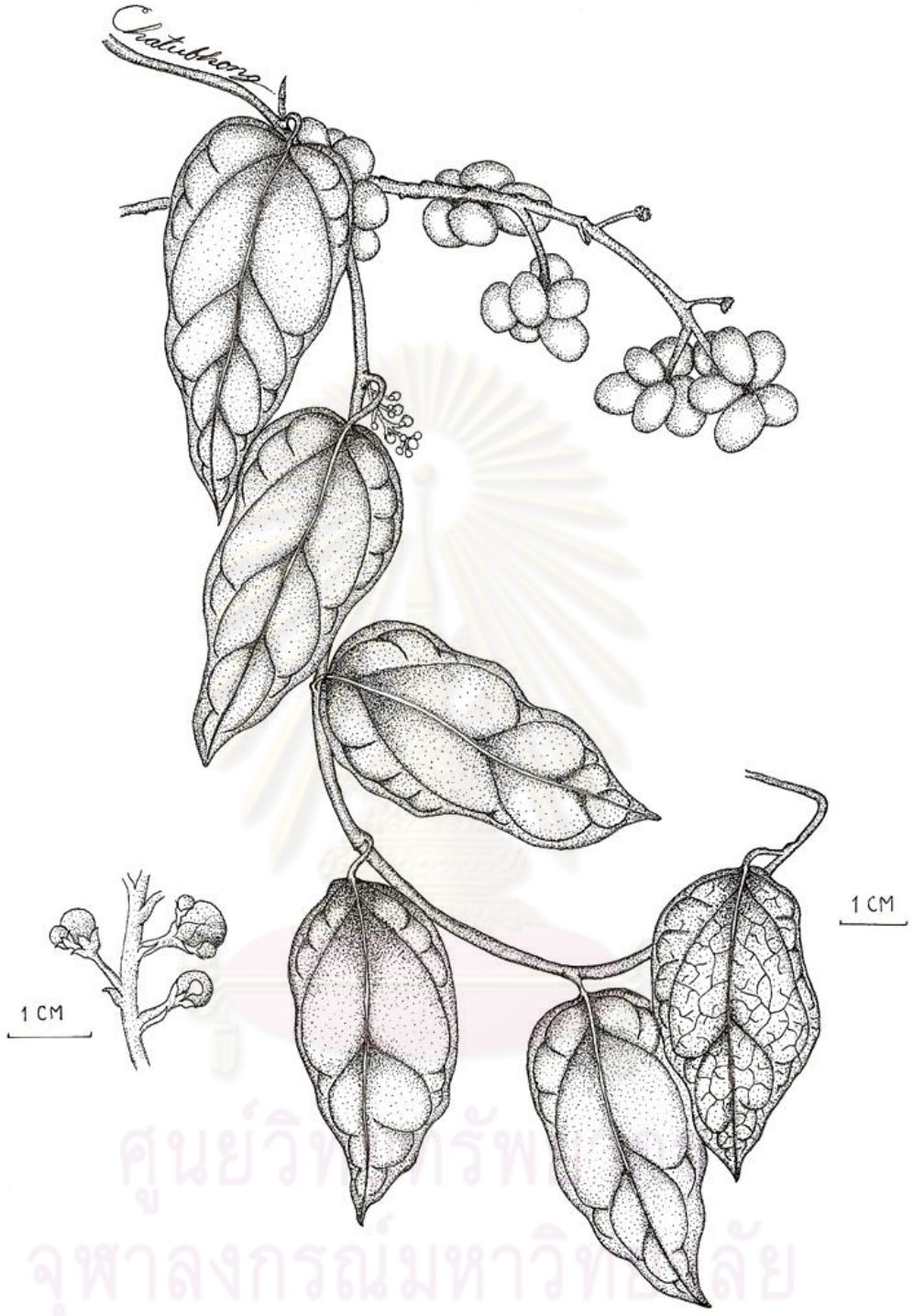


Figure 38 Whole plant of *Tiliacora tirandra* (Colebr.) Diels

Whole plant is climbing shrub, dioecious. The leaves are ovate, alternate, glabrous and dark-green. Flower is small and yellowish. The fruits are yellowish obovate druplets.

Microscopic: Anatomical Character

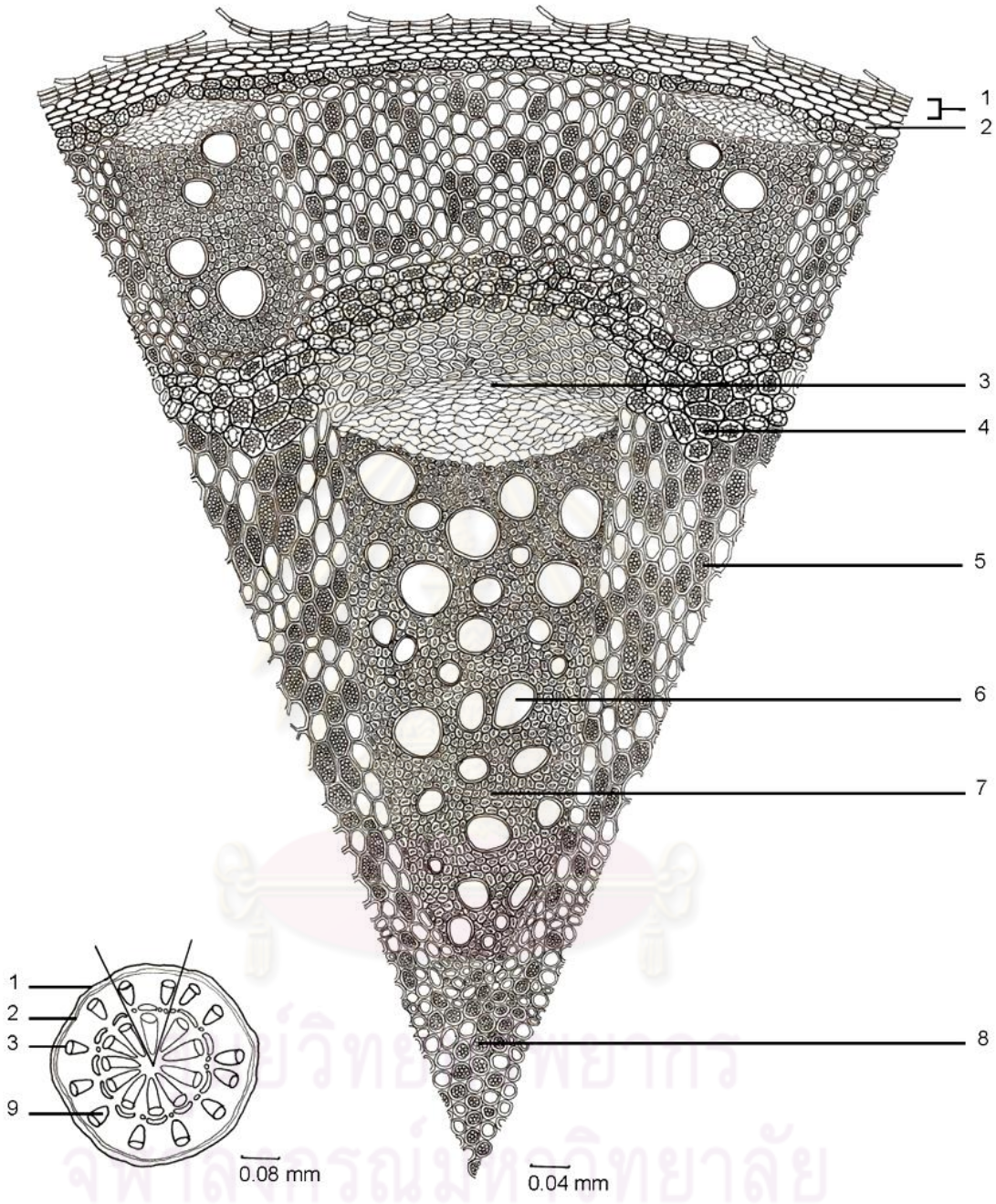


Figure 39 Transverse section of *Tiliacora triandra* (Colebr.) Diels root:
1.Periderm 2.Parenchyma of cortex 3. Phloem tissue 4. Sclereid containing with starch granules 5. Starch granules in reserved parenchyma 6. Xylem vessel 7. Xylem fiber 8. Parenchyma of pith containing with starch granules 9. Xylem tissue

Histologicla Character

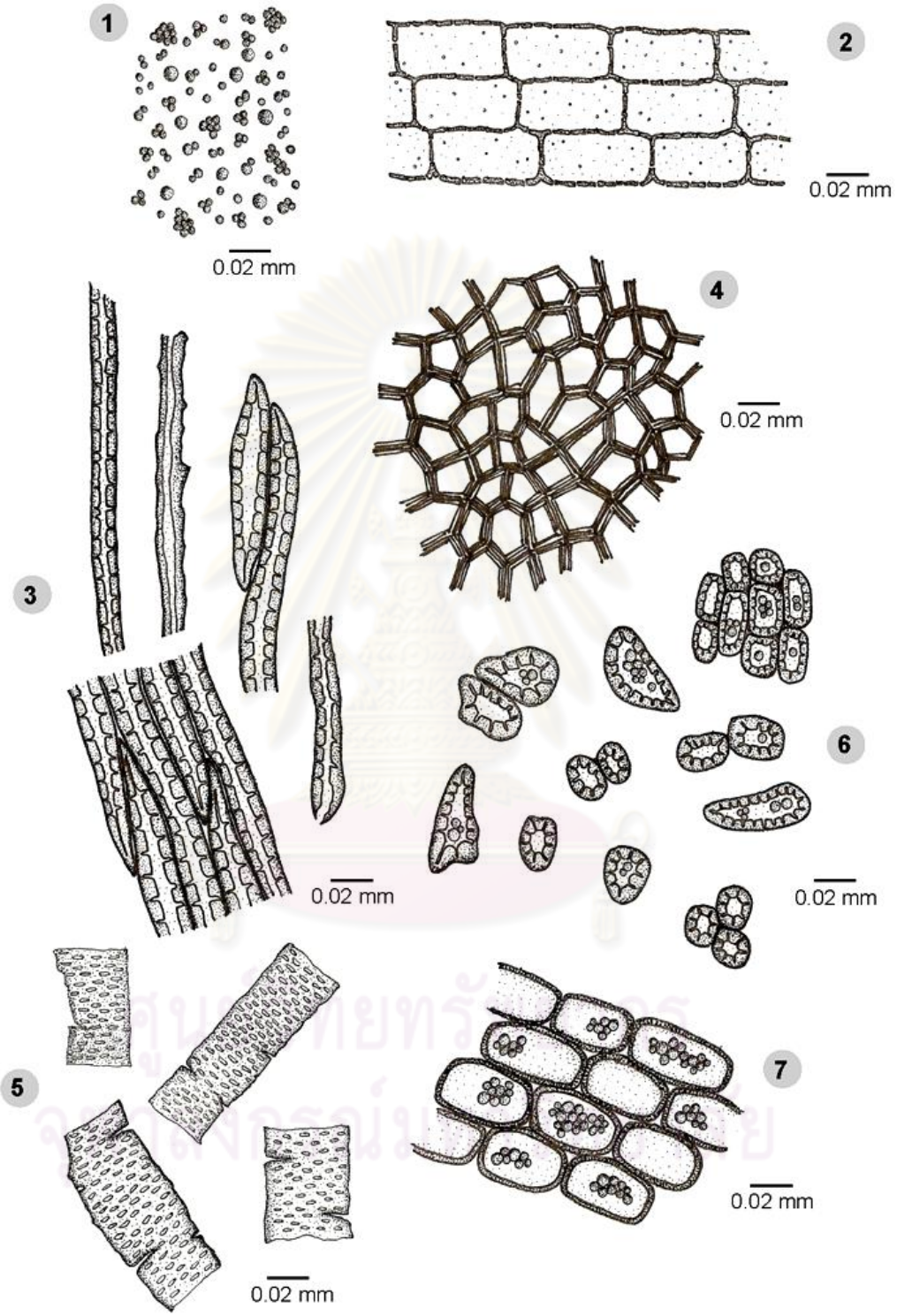


Figure 40 Powdered of *Tiliacora triandra* (Colebr.) Diels root:

1. Starch granules 2.Part of xylem parenchyma 3. Fragment of fibers 4.Cork in surface view 5. Fragment of pitted vessels 6. Sclereids 7. Parenchyma in sectional view containing starch granules

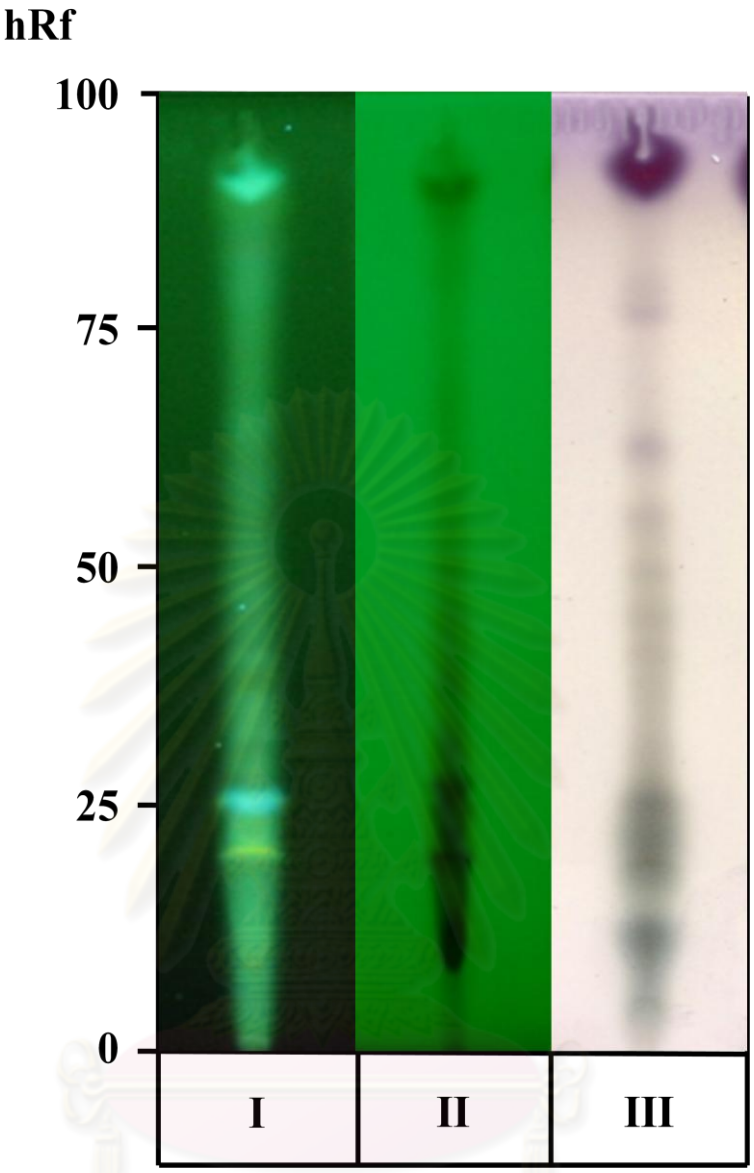


Figure 41 Thin-layer chromatogram of
methanolic extract of the root of *Tiliacora triandra* (Colebr.) Diels

Solvent system **Butanol : Acetic acid : Water 4 : 1 : 5**
Detection
I = detection under UV light 254 nm
II = detection under UV light 366 nm
III = detection with Anisaldehyde*,**

*Anisaldehyde regent
Preparation: Anisaldehyde (0.5 ml), Glacial acetic acid (10 ml), methanol (85 ml), Sulfuric acid (5 ml)
**Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

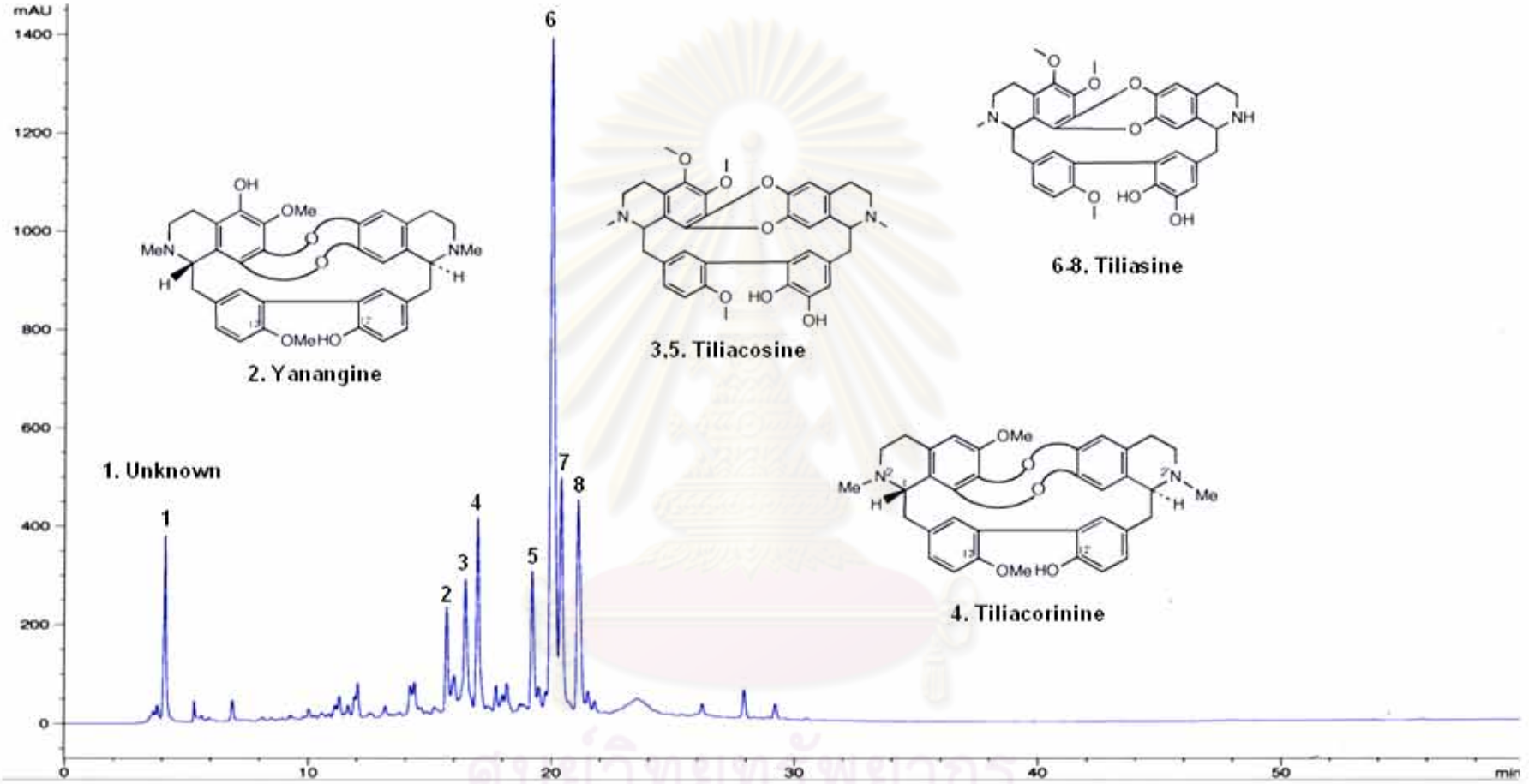


Figure 42 The HPLC chromatogram of *Tilliacora triandra* Diels.

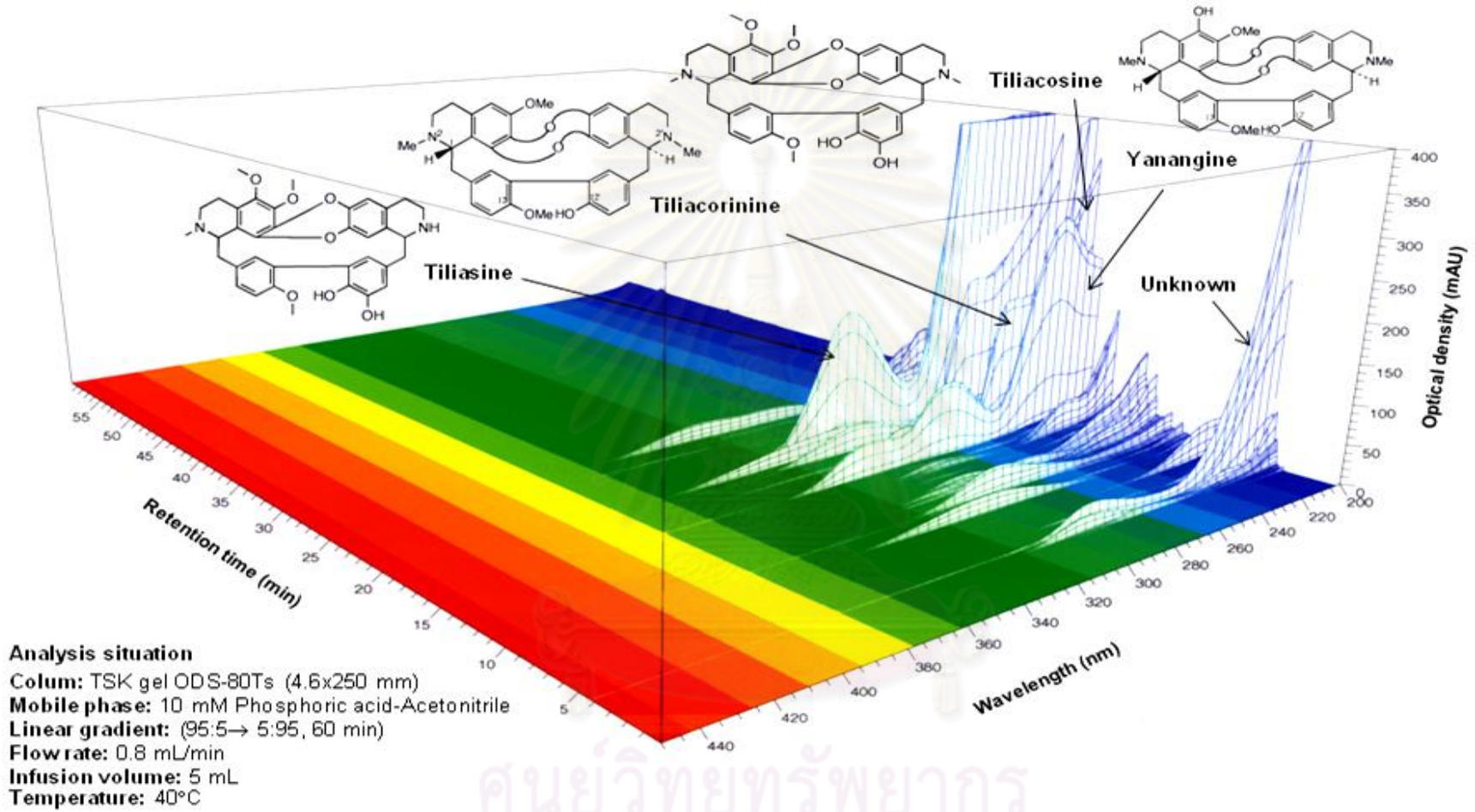


Figure 43 The 3D-HPLC profile of Tiliacora triandra (Colebr.) Diels

Table 6 Specification of *Tiliacora triandra* Diels. Root

Content (% by weight)	Mean ± SD	Min – Max	n
Acid - insoluble ashes	1.15 ± 0.18	0.51 - 2.29	14
Total ash	4.39 ± 0.43	2.97 - 7.30	14
Loss on drying	6.80 ± 0.13	2.67 - 11.43	14
Ethanol-soluble extractive	1.22 ± 0.13	0.50 - 1.74	14
Water-soluble extractive	2.17 ± 0.25	1.47 - 3.23	14
water content	8.14 ± 0.66	4.80 - 11.40	14

N = 14, each sample was done in triplicate

A: Prism crystal of calcium oxalate present

B: Sclereids present

BB: Sclereids absent

C: Part of bordered pitted vessel present **: *Clerodendrum petasites* S. Moore**

CC: Part of bordered pitted vessel absent

D: Part of xylem in tangential longitudinal section present **: *Harrisonia perforata* (Blanco) Merr.**

DD: Part of xylem in tangential longitudinal section absent **: *Ficus racemasa* L.**

AA: Prism crystal of calcium oxalate absent

B: Sclereids present

C: Sclereids containing starch granules present **: *Tiliacora trianda* (Colebr.) Diels**

CC: Sclereids containing starch granules absent **: *Capparis micracantha* DC.**

BB: Sclereids absent

Figure 44 Key Identification for each roots powder of five species in Ben-Cha-Lo-Ka-Wi-Chian remedy

Regarding to the presence or absence of some histological characters of each root species was explored and the dichotomous key was firstly established. This key identification can be used for detection of the contamination or adulteration of raw materials without high technology instrument (Figure 44). The examination of the physicochemical parameters in this study can help to evaluate the quality of the crude drugs and confirm raw materials standardization.

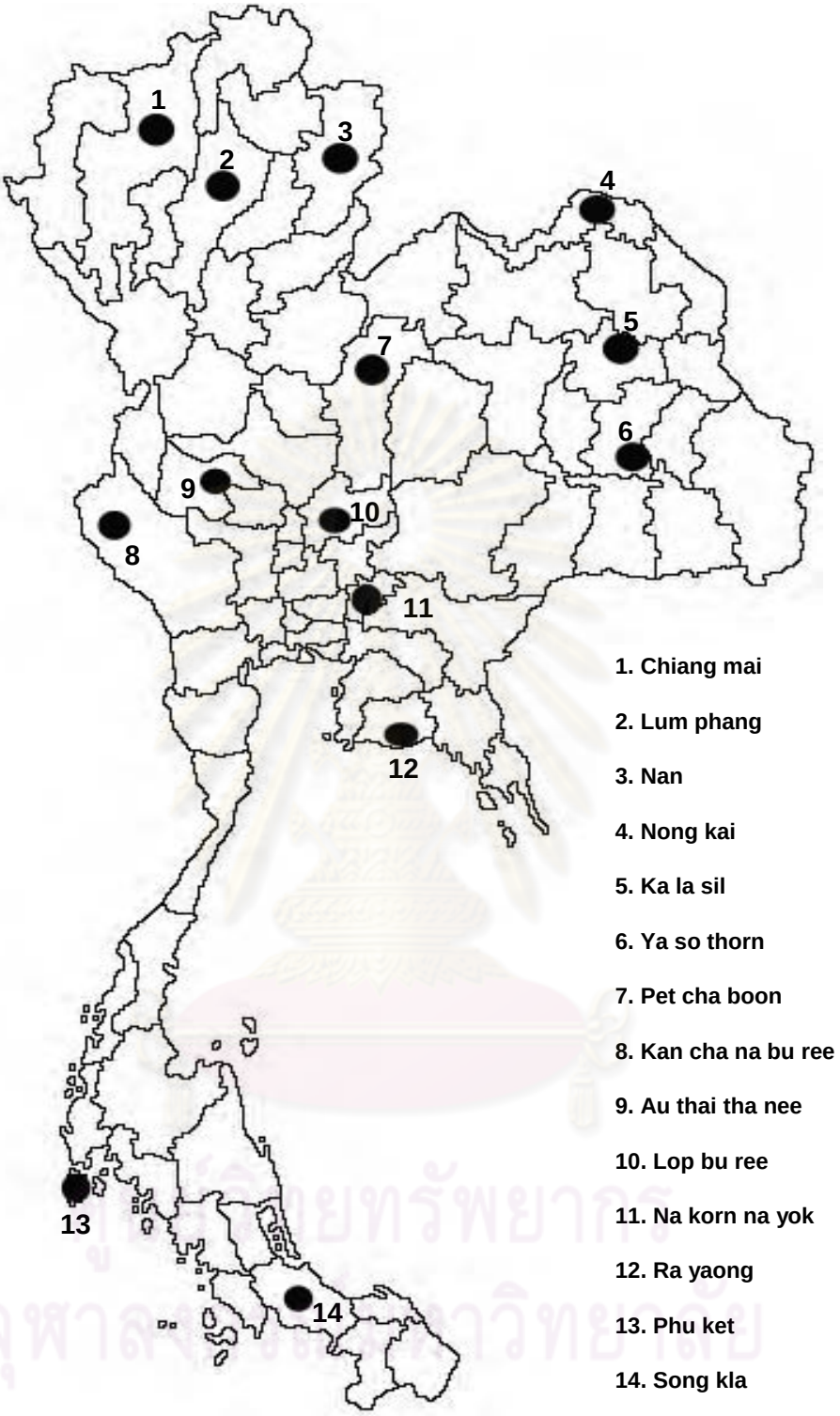


Figure 45 The resources of each species plant in Ben-Cha-Lo-Ka-Wi-Chian Remedy

Five root plant species were collected from several places throughout Thailand as reveals in above figure.

Plant extraction

Course powdered of five root species were extracted using maceration technique in ethanol and distilled water respectively. All crude extract from the all specimens yielded range from 1.6586 % to11.8171 % as shown in Table 7.

Table 7 Crude extracts of five root species in Ben Cha Lo Ka Wi Chian remedy.

Plant	Yield of ethanol	Yield of water	Total yield
C. micracantha	5.1660	3.8182	8.9842
C. petasite	8.3499	3.4672	11.8171
H. perforata	4.5109	1.6586	6.1699
F. racemosa	4.0135	2.4821	6.4965
T. triandra	8.2818	3.2479	11.5298

Table 8 A summary resources of Ben Cha Lo Ka Wi Chian Remedy Batches.

No	sample	sample code	Collected location				
			C. micracantha	***C.petasite***	***H.perforata***	***F.racemosa***	***T.triandra***
1	BLW1	A1-A3	Lampang	Nakhonnayok	Uthai thani	Songkhla	Nan
2	BLW2	B1-B3	Rayong	Rayong	Rayong	Rayong	Rayong
3	BLW3	C1-C3	Uthai thani	Lumphang	Nongkhai(Se-ka)	Yasothon	Nakhonnayok
4	BLW4	D1-D3	Lumnarai	Phuket	Nan	Lampang	Kalasil
5	BLW5	E1-E3	Kanchanaburi	Nongkhai(Se-ka)	Lampang	Authaitanee	Muang Lopburi
6	BLW6	F1-F3	Nongkhai(Se-Ka)	Rayong	Nakhonnayok	Kanchanaburi	Nakhonnayok
7	BLW7	G1-G3	Nongkhai(sri wi lai)	Kalasil	Yasothon	Lampang	Nan
8	BLW8	H1-H3	Nongkhai(Sri wi lai)	Yasothon	Uthai thani	Kanchanaburi	Lampang
9	BLW9	I1-I3	Lampang	Phuket	Nan	Nongkhai(Sri wi lai)	Nan
10	BLW10	J1-J3	Yasothon	Yasothon	Yasothon	Yasothon	Yasothon
11	BLW11	K1-K3	Uthai thani	Uthai thani	Uthai thani	Uthai thani	Uthai thani
12	BLW12	L1-L3	Lampang	Lampang	Lampang	Lampang	Lampang

All batches of BLW remedy that included BLW1 to BLW12 were prepared from several places as mention in above.

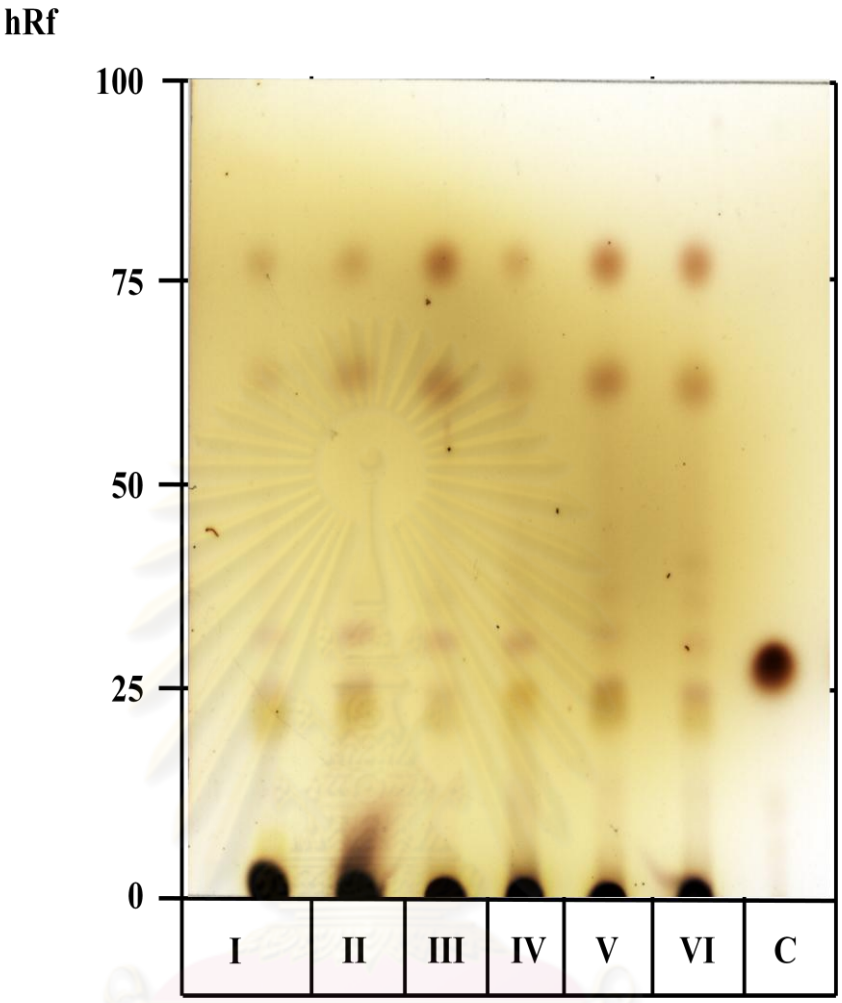


Figure 46 Thin-layer chromatogram of Ben Cha Lo Ka Wi Chian Remedy

Solvent system	**n-Hexane:Ethyl-Acetate (4:1)**
	Sample 10 ml/ml: volume 5 μl
Detection	detection with 10% sulfuric acid*
Standard sample (C)	Dihydrofernesol 1 mg/ml: volume 0.25 μl
Abbreviation	I: BLW1, II: BLW2, III: BLW3, IV: BLW4, V: BLW5, VI: BLW6
	C: Standard sample

*Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

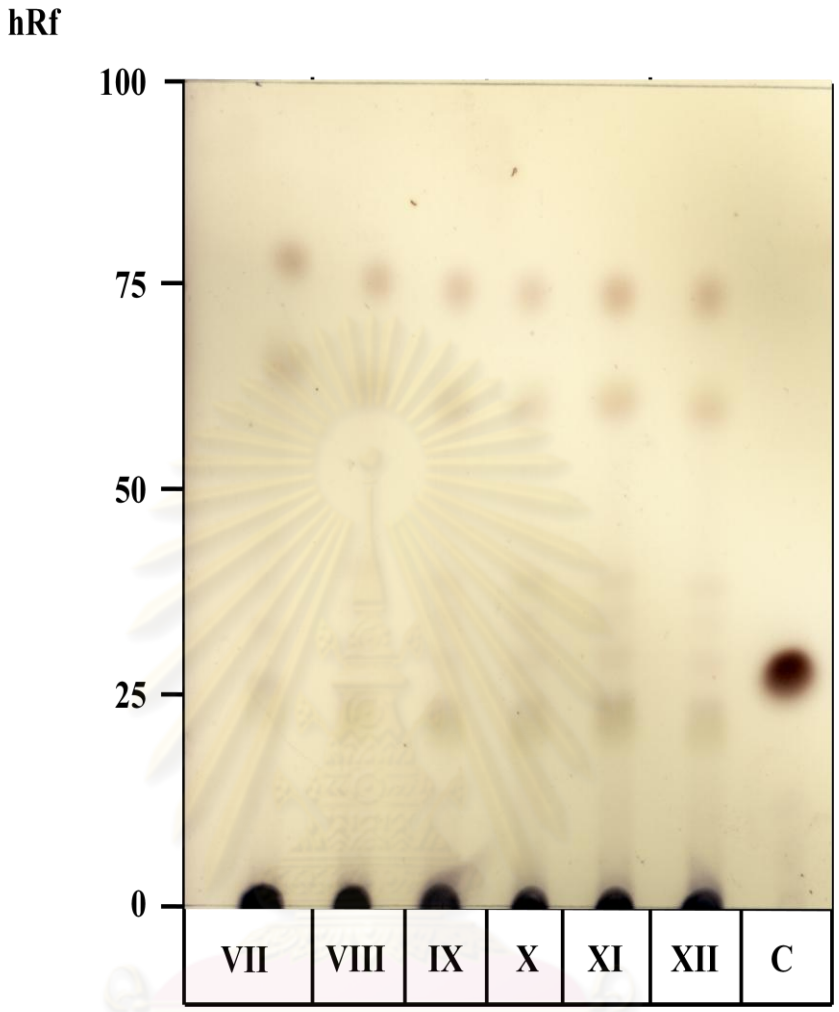


Figure 47 Thin-layer chromatogram of Ben Cha Lo Ka Wi Chian Remedy

Solvent system	**n-Hexane:Ethyl-Acetate (4:1)**
	Sample 10 ml/ml: volume 5 μl
Detection	detection with 10% sulfuric acid*
Standard sample (C)	Dihydrofernesol 1 mg/ml: volume 0.25 μl
Abbreviation	VII: BLW7, VIII: BLW8, IX: BLW9, X: BLW10, XI: BLW11, XII: BLW12
	C: Standard sample

*Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

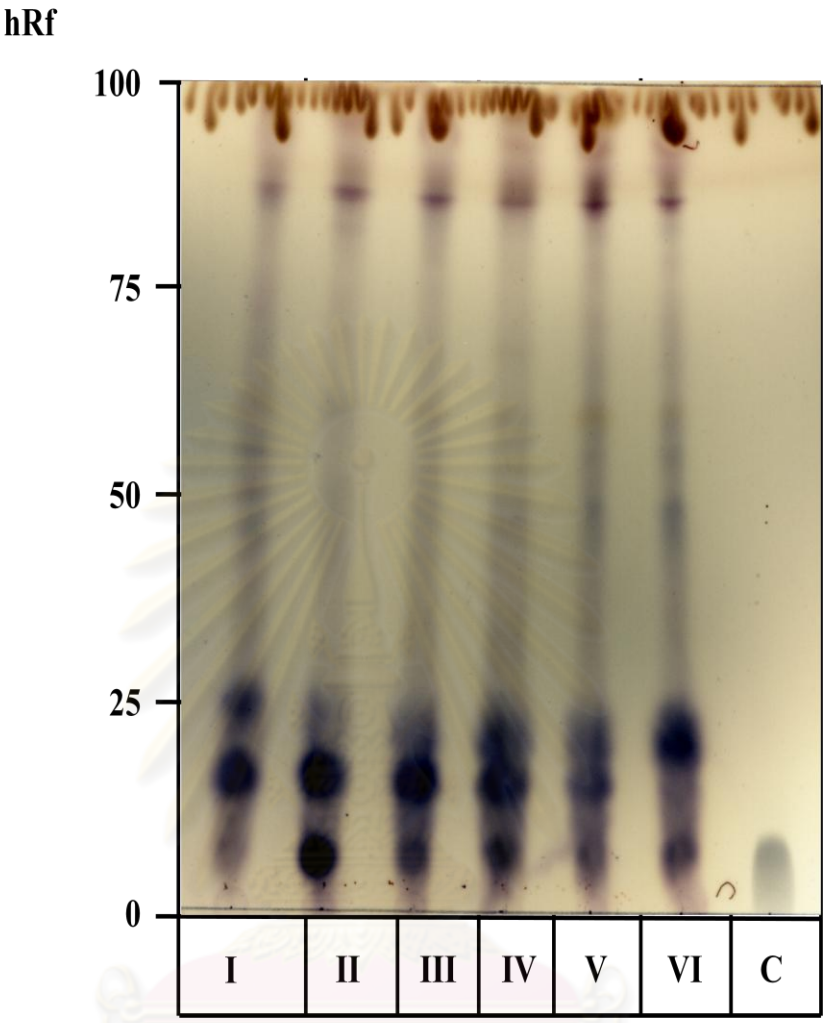


Figure 48 Thin-layer chromatogram of Ben Cha Lo Ka Wi Chian Remedy

Solvent system	**Chlorofrom: Methanol: Acetic acid: Water (15: 9: 1: 2)**
	Sample 10 ml/ml: volume 5 μl
Detection	detection with 10% sulfuric acid*
Standard sample (C)	Tannic acide1mg/ml :volume 0.25 μl
Abbreviation	I: BLW1, II: BLW2, III: BLW3, IV: BLW4, V: BLW5, VI: BLW6
	C: Standard sample

*Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

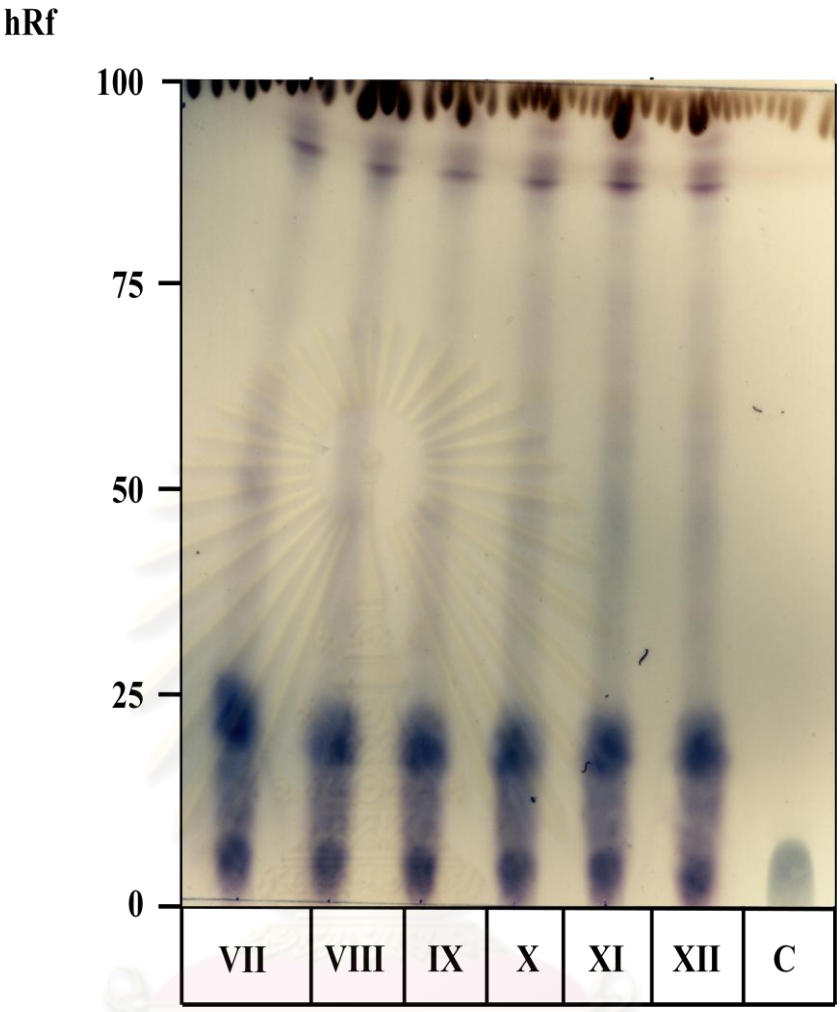


Figure 49 Thin-layer chromatogram of Ben Cha Lo Ka Wi Chian Remedy

Solvent system	**Chlorofrom: Methanol: Acetic acid: Water (15: 9: 1: 2)**
	Sample 10 ml/ml: volume 5 μl
Detection	detection with 10% sulfuric acid*
Standard sample (C)	Tannic acid 1mg/ml :volume 0.25 μl
Abbreviation	VII: BLW7, VIII: BLW8, IX: BLW9, X: BLW10, XI: BLW11, XII: BLW12
	C: Standard sample

*Spot color Development
Heat the plate at 120 ^{o} C for 10 minutes after sprayed.

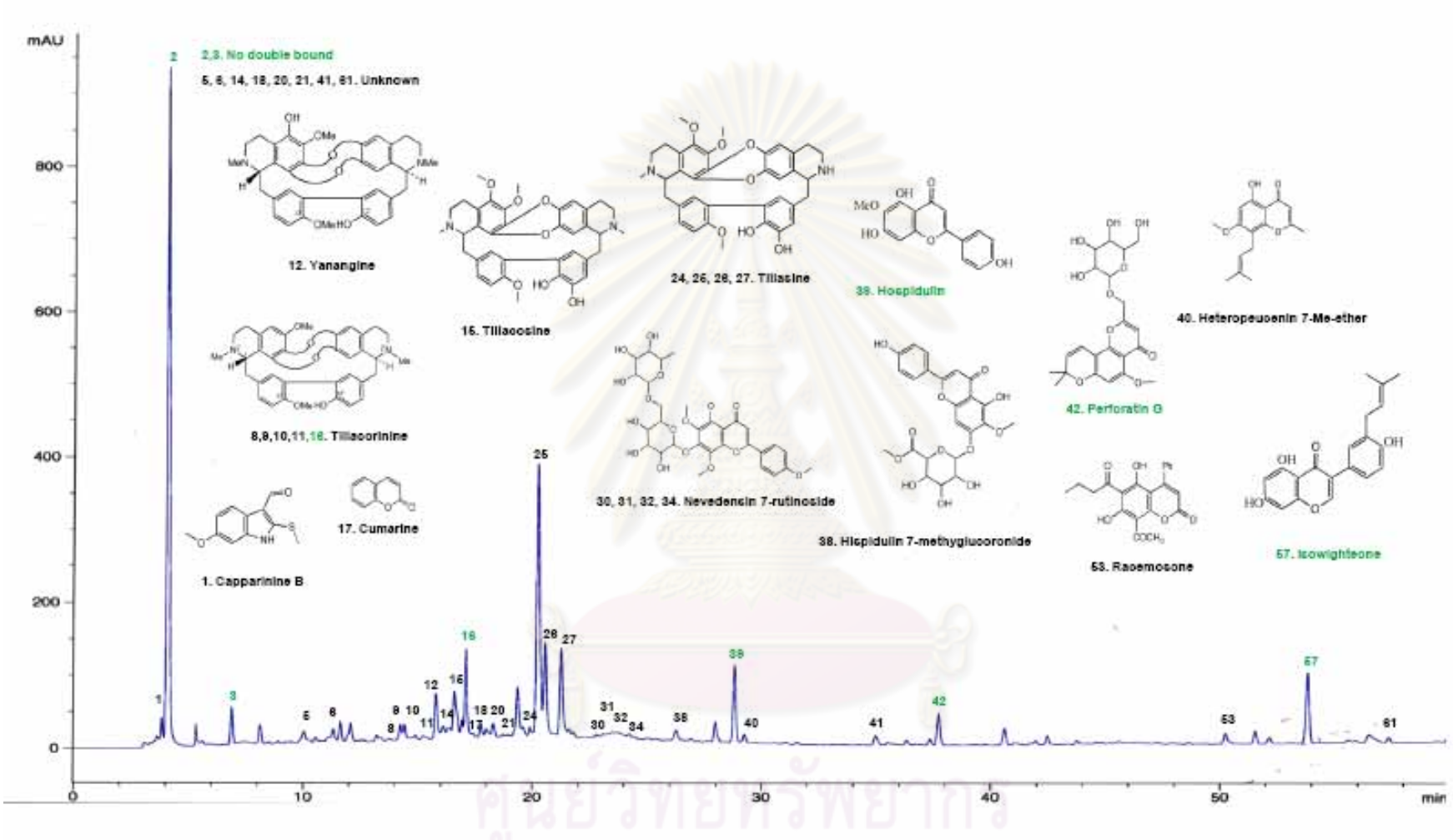


Figure 50 The HPLC chromatogram of Ben Cha Lo Ka Wi Chian Remedy

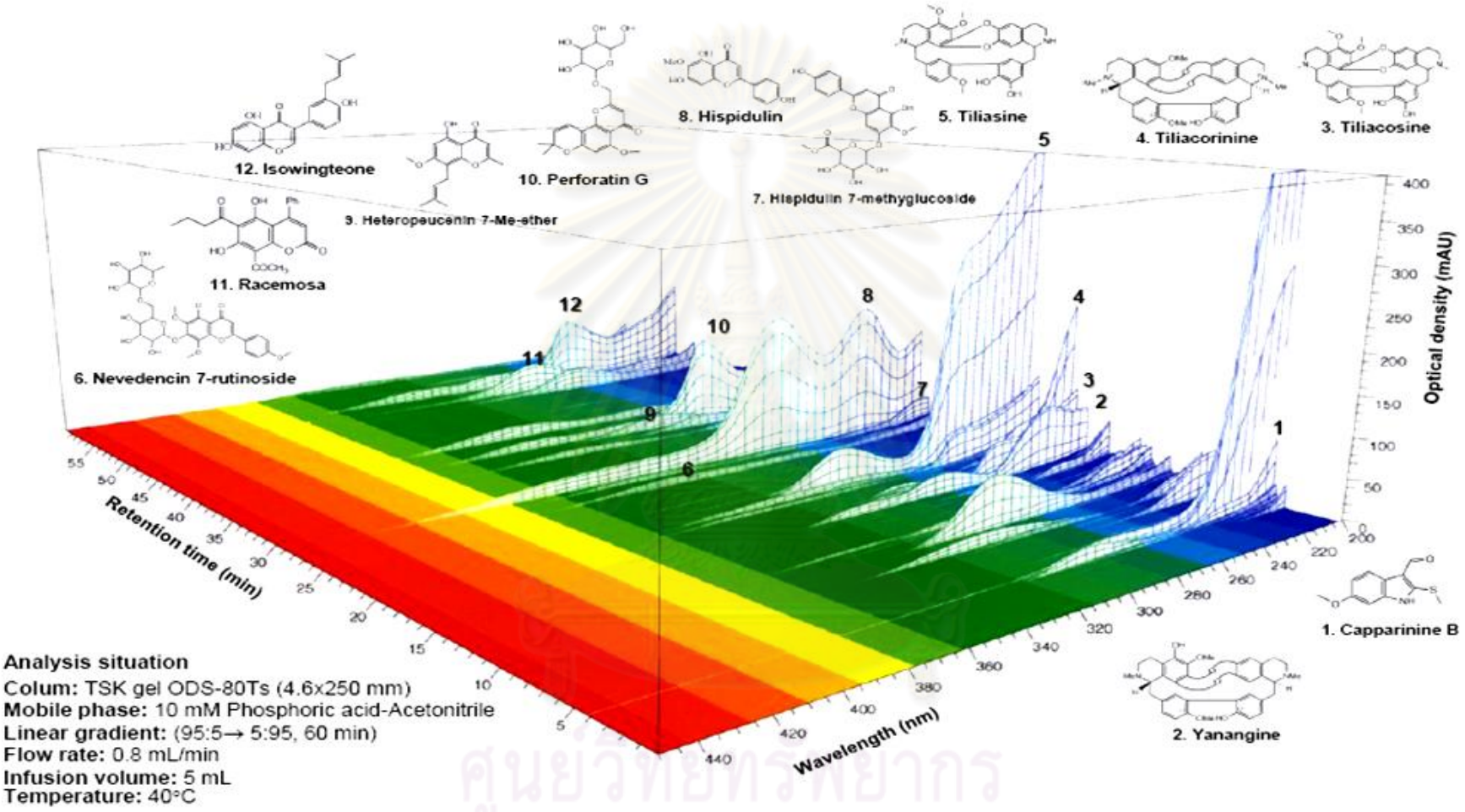


Figure 51 The 3D-HPLC profile of Ben Cha Lo Ka Wi Chian Remedy

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Multivariate Analysis

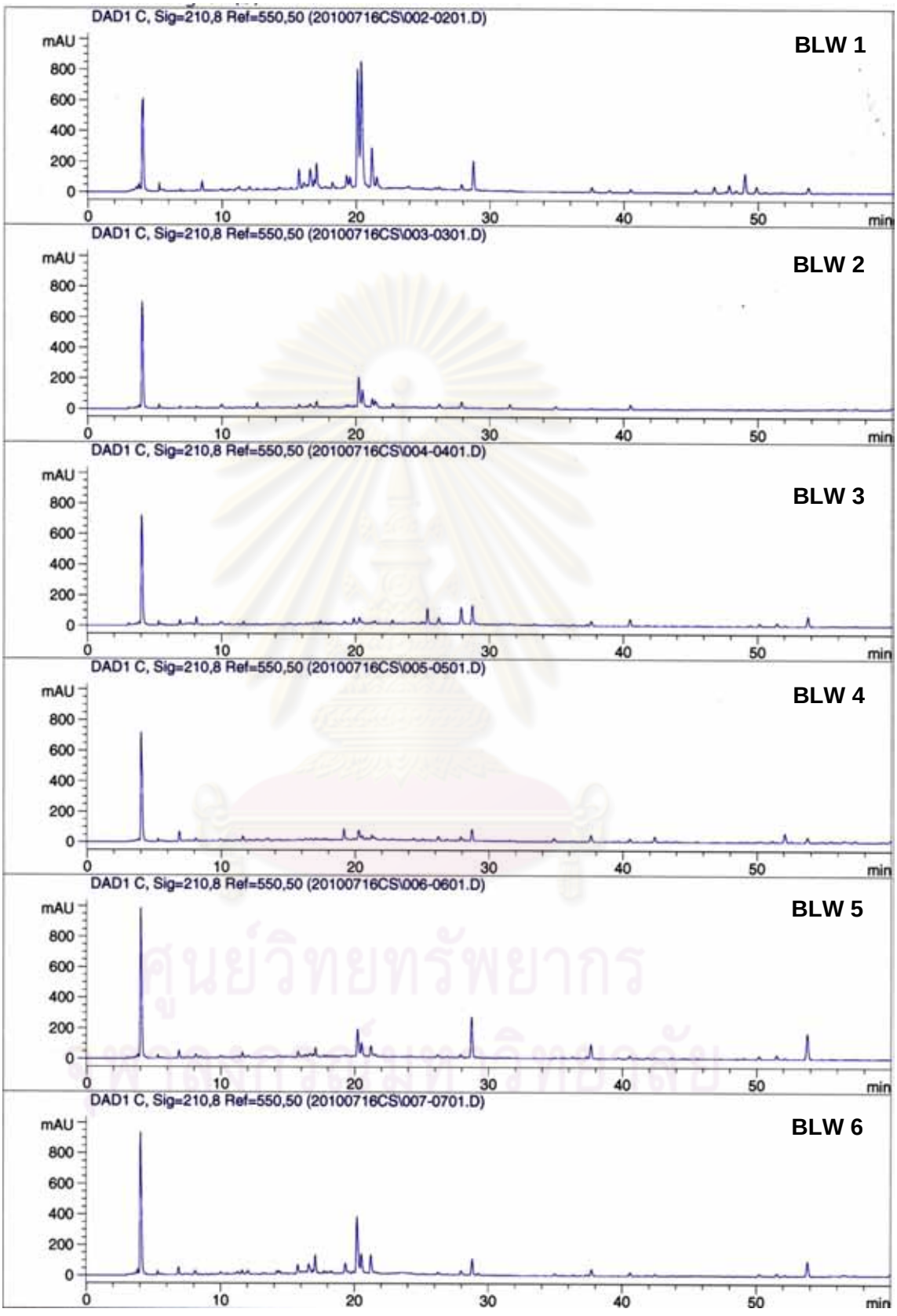

Figure 52 Representative 2D HPLC chromatogram from each BLW batches 1-12 that composed from several crude extracts as mention above.

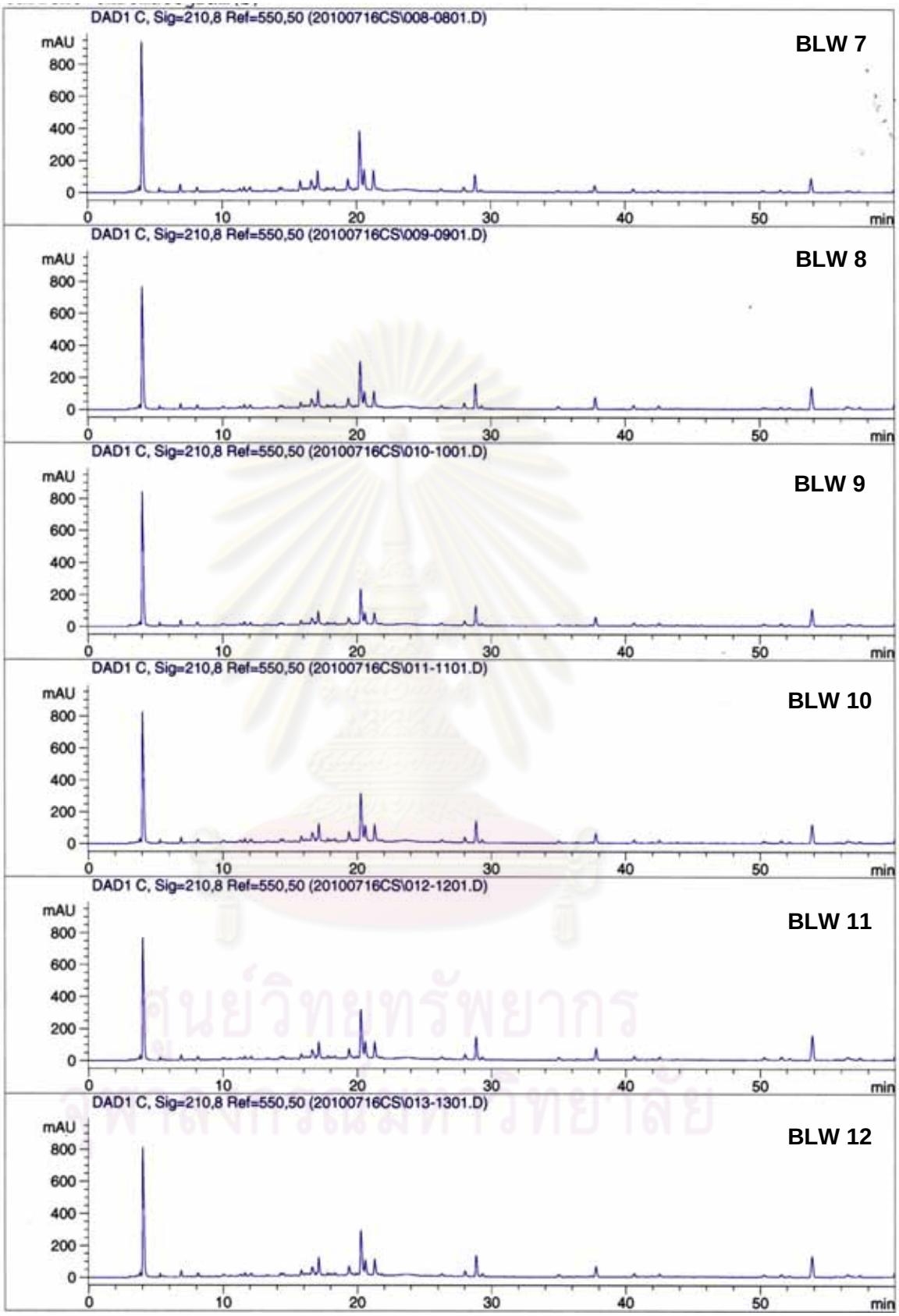

Figure 52 Representative 2D HPLC chromatogram from each BLW batches 1-12 that composed from several crude extracts as mention above. (Cont.)

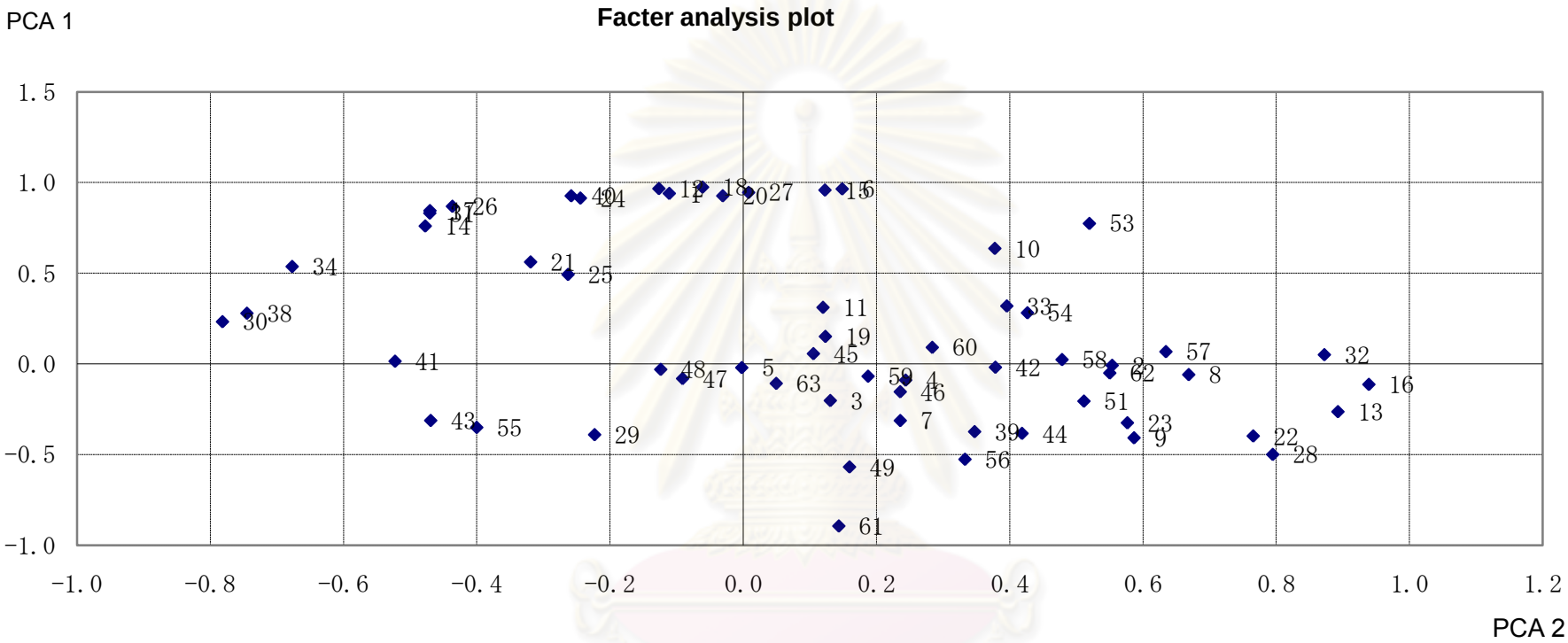

Figure 53 The factor analysis plot base on the first comparison among the area under curve of BLW1 until BLW 12

The plot based on the first principle component analysis, Factor analysis, was conducted. The result was demonstrated that all of factor plots were disseminated through fact sheet as shown in above figure. It was not separated or grouping into groups. Thus the first factor analysis was not enough to distinguish all samples of BLW 1 – BLW 12.

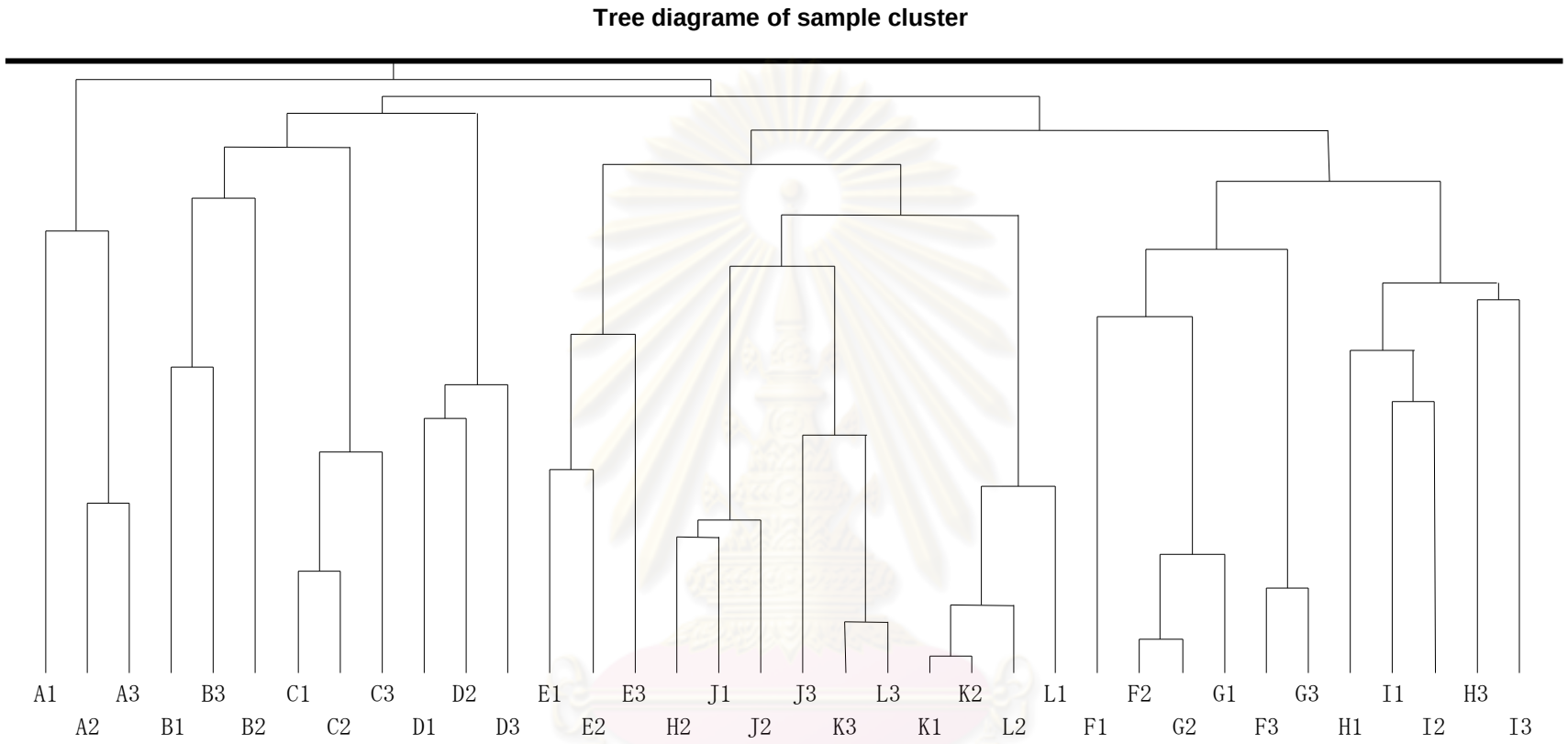

Figure 54 Hierarchical cluster analysis (HCA) dendrogram plot of fingerprint-based data (33x33 matrix)

Figure 54 Illustrates the HCA result. It is apparent that most of sample batches were more similar. Only BLW 1(A1-A3) was reveals clearly fell into separate clusters. While BLW 2 (B1-B3), BLW 3 (C1-C3) and BLW 4 (D1-D3) were group into one cluster. Due to a close relationship, BLW 5 (E1-E3), BLW 6(F1-F3), BLW 7(G1-G3), BLW 8 (H1-H3), BLW 9 (I1-I3), BLW 10 (J1-J3), BLW 11 (K1-K3) and BLW 12 (L1-L3) were demonstrated into the big one clusters.

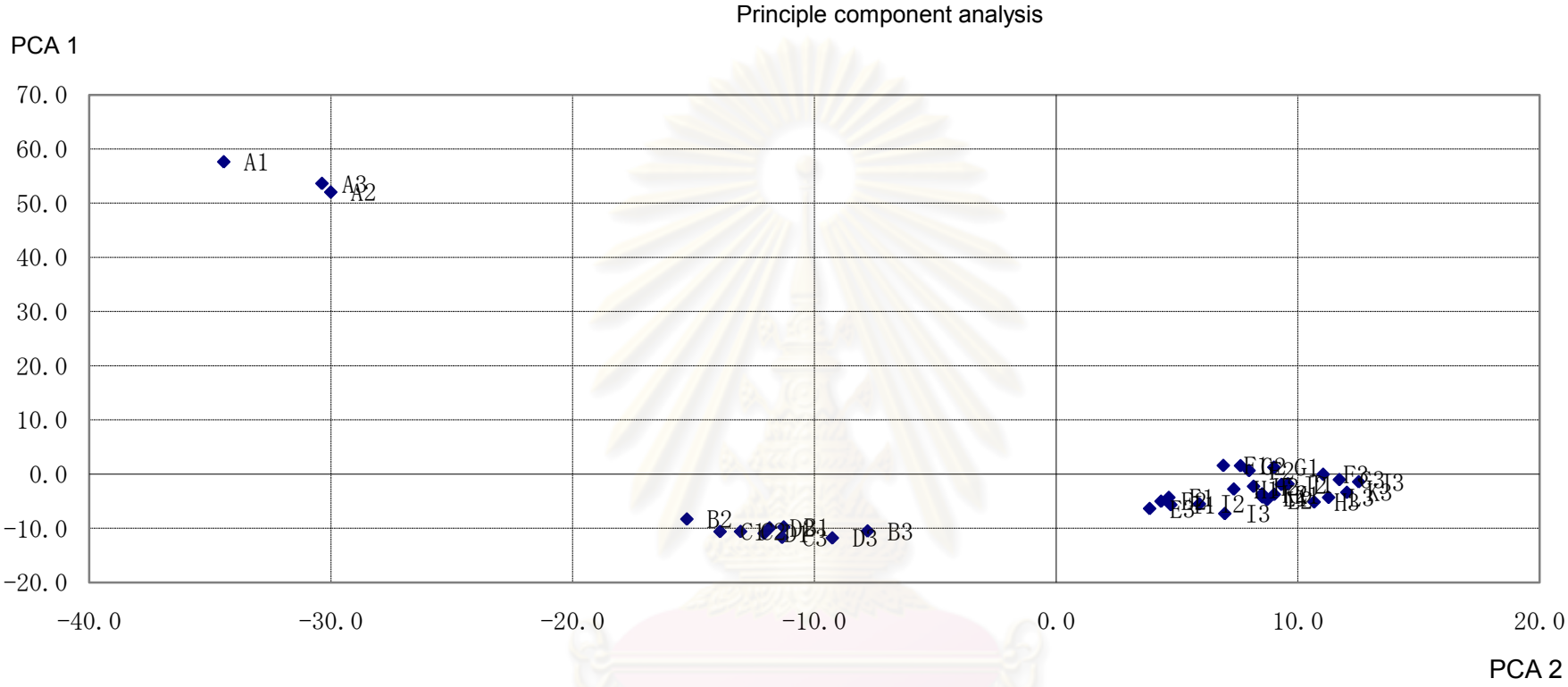

Figure 55 Principle component analysis (PCA) plot based on the reduce data set form

PCA was conducted in order to find some characteristic constituents as ideal constituents markers. The plot base on the first factor analysis as present in Figure 55 in clear identification, PCA can divide all samples into tree groups. Only BLW 1(A1-A3) was shown clearly separated from other samples.

Table 9 revealed the Correlation analysis from cluster analysis. The significant Batches were demonstrated as double star [**] whilst not significant (its have no correlation) data were demonstrated as one star [*] or blank [ ]. All samples were significant that means most of them are similar or same standard of BLW remedy except only BLW 1 (A1-A3) was not significant, means its have no correlation or not the same standard as same as all samples.

	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3	E1	E2	E3	F1	F2	F3
A1	-	[**]	[**]	[**]	[*]	[*]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[**]	[**]	[**]
A2	[**]	-	[**]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
A3	[**]	[**]	-	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
B1	[**]	[ ]	[ ]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
B2	[*]	[ ]	[ ]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
B3	[*]	[ ]	[ ]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
C1	[ ]	[ ]	[ ]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
C2	[ ]	[ ]	[ ]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
C3	[ ]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
D1	[ ]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
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K2	[**]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
K3	[**]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
L1	[*]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
L2	[*]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
L3	[*]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]

Table 9 Correlation analysis from cluster analysis. (Cont.)

	G1	G2	G3	H1	H2	H3	I1	I2	I3	J1	J2	J3	K1	K2	K3	L1	L2	L3
A1	[ ]	[**]	[**]	[*]	[*]	[*]	[ ]	[ ]	[ ]	[**]	[**]	[**]	[**]	[**]	[**]	[*]	[*]	[*]
A2	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
A3	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
B1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
B2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
B3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
C1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
C2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
C3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
D1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
D2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
D3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
E1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
E2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
E3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
F1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
F2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
F3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
G1	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
G2	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
G3	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
H1	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
H2	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
H3	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
I1	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
I2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
I3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
J1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]
J2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]	[**]
J3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]	[**]
K1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]	[**]
K2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]	[**]
K3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]	[**]
L1	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]	[**]
L2	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-	[**]
L3	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	[**]	-

Safety study

Cytotoxic activity using Brine Shrimp method

Results were expressed as the concentration of the extracts necessary to cause 50% of lethality (LC_{50}) to the brine shrimp. The ethanolic extract of *T. triandra*, *H. perforata* and *C. micracantha* exhibited brine shrimp lethality with LC_{50} of 44, 600 and more than 1,700 µg/ml. respectively. The water extracts of *T. trianda* and *H. perforata* also showed toxicity to the brine shrimp (LC_{50} 200 and 560 µg/ml respectively). Both ethanolic and water extracts of *C. petasites* and *F. racemosa* showed LC_{50} more than 10,000 µg/ml. Finally, Ben-Cha-Lo-Ka-Wi-Chian Remedy extract demonstrated LC_{50} of 265 µg/ml. (Table 10) According to Meyer *et al.* (1982), who classified crude extracts into toxic (LC_{50} value < 1,000 µg/ml) and non-toxic (LC_{50} value > 1000 µg/ml), Ben-Cha-Lo-Ka-Wi-Chian remedy, *H. perforata* and *T. triandra* had potential to be toxic to brine shrimp.

Table 10 Brine shrimp lethality (LC_{50}) of the ethanol, water extracts of five roots species and the remedy

Species	Ethanol extract LC_{50} (µg/ml)	Water extract LC_{50} (µg/ml)
Harrisonia perforata (Blanco) Merr	600	560
Tiliacora triandra (Colebr.) Diels	44	200
Ficus racemosa L.	>10,000	>10,000
Clerodendrum petasites S. Moore	>10,000	>10,000
Capparis micracantha DC.	>1,700	>10,000
Ben-Cha-Lo-Ka-Wi-Chian remedy	265	

Mutagenic activity using Ames test

Ben-Cha-Lo-Ka-Wi-Chian remedy and its component extracts at all doses (5, 10, 20 and 40 mg/plate) were not directly mutagenic (MI < 2) towards *S. typhimurium* TA98 (**Figure 56A**) and TA100 (**Figure 56B**). Most of water extracts except *T. triandra* illustrated higher histidine (His^+) reverstants per plate than ethanol extracts.

Figure 56A (TA98)

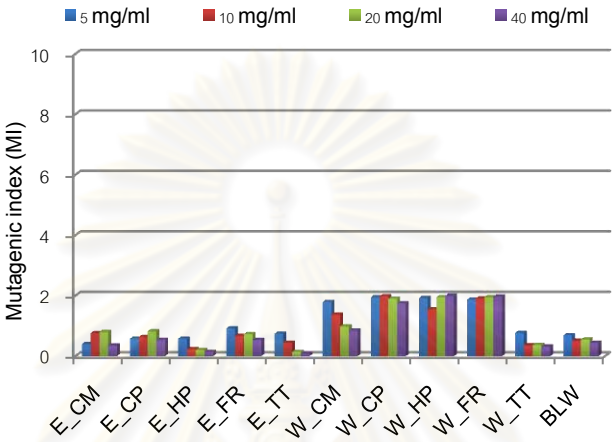

Figure 56B (TA100)

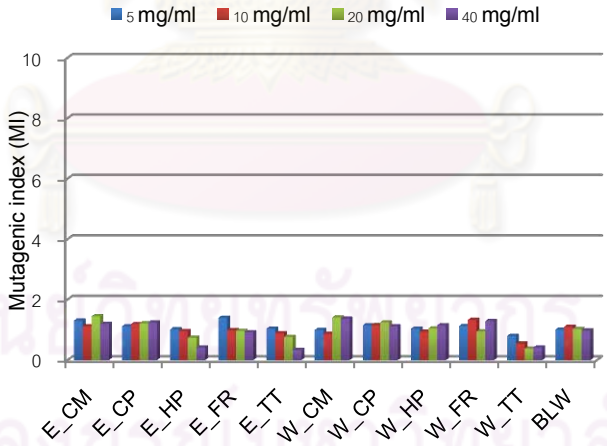

Figure 56 The mutagenic index (MI) of mutagnenic without nitrite effect induced by each plant species and BLW remedy on *S.typhimurium* strains TA98 (56A) TA100 (56B). Abbreviations including E: ethanol extract, W: water extract, CM: *C. micracantha*, CP: *C. petasites*, HP: *H. perforata*, FR: *F. racemosa*, TT: *T. triandra* and BLW: Ben-Cha-Lo-Ka-Wi-Chian Remedy.

On the contrary, most of the extracts including BLW remedy extract was shown indirect mutagenictiy induced by nitrosation (sodium nitrite treated in acid solution) as shown in **Figure 57** (TA 98 in **Figure 57A** and TA100 in **Figure 57B**). Most of ethanol extracts demonstrated the mutagenic index higher than the water extracts including the water extract of *T. triandra* and BLW remedy extract, whereas, ethanol and water extracts of *C. petasites* were shown highest mutagenic index in both strains.

Figure 57A (TA98)

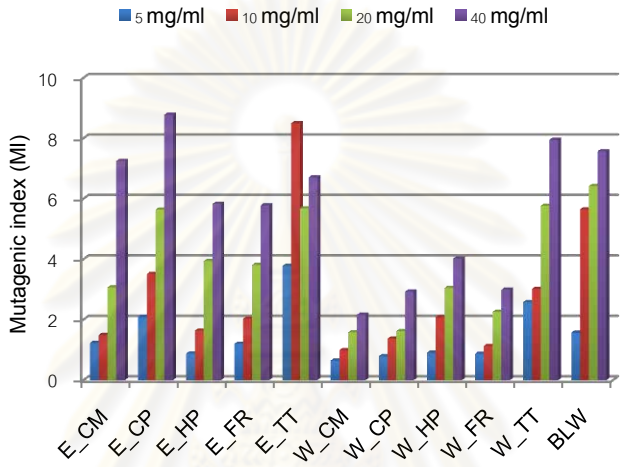

Figure 57B (TA100)

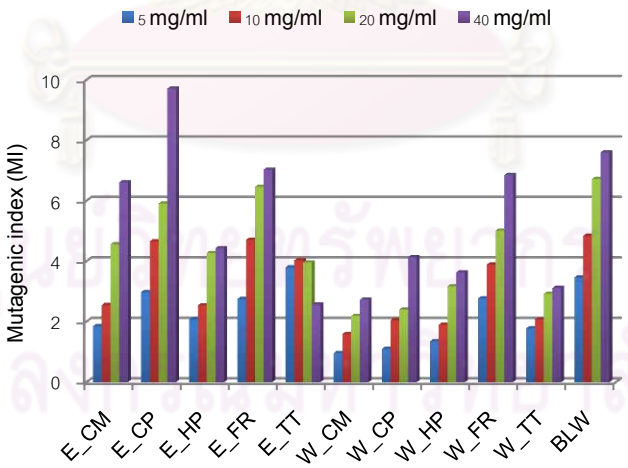

Figure 57 The mutagenic index (MI) of mutagnenic with nitrite effect induced by each plant species and BLW remedy on *S.typhimurium* strains TA98 (57A) TA100 (57B). Abbreviations including E: ethanol extract, W: water extract, CM: *C. micracantha*, CP: *C. petasites*, HP: *H. perforata*, FR: *F. racemosa*, TT: *T. triandra* and BLW: Ben-Cha-Lo-Ka-Wi-Chian Remedy.

Anti-mutgenic activity using Ames test

For anti-mutagenicity, most of the remedy extracts and the components extracts exhibited strongly active inhibition (more than 60% inhibition) against nitrite treated 1-aminopyrine induced mutagenicity in both TA98 and TA100. Ethanol extracts *of H. perforata* and *T. triandra* were particularly presented the higher percentage of inhibition (> 100% of inhibition), whereas, the water extracts of *F. racemosa* and *C. petasites* were demonstrated moderately active inhibitor (40 – 60 % inhibition) (**Table 11**).

Table 11 Percentage of the mutagenicity inhibition on the Ben-Cha-Lo-Ka-Wi-Chian remedy and its components

Sample	Solvent extract	Percentage of inhibition					
		5 mg/ml		10 mg/ml		15 mg/ml	
		TA 98	TA100	TA98	TA100	TA98	TA100
BLW remedy		89.35	75.20	94.19	95.07	96.56	102.18
C.micracantha	Ethanol	79.04	85.83	93.45	83.29	93.09	86.25
	water	72.44	98.52	85.87	103.07	89.81	104.97
C. petasites	Ethanol	71.82	83.85	85.13	99.48	87.01	106.07
	Water	39.40	41.71	50.18	56.36	57.17	57.30
H. perforata	Ethanol	101.48	120.20	102.15	118.36	102.28	124.25
	Water	82.16	99.37	88.71	106.77	96.00	108.31
F. racemosa	Ethanol	97.39	93.74	99.03	110.31	100.75	106.54
	Water	35.13	70.06	61.18	39.22	58.01	52.59
T. triandra	Ethanol	98.98	112.85	101.35	123.12	101.53	121.61
	WAter	88.42	101.93	97.21	120.24	98.69	119.02

DNA damage using Comet assay

The number of cell to be scored per gel was 100. The five categories used for this comet classification were those proposed by Collins [155] as 0-4 that shown in figure 58. All doses were done in triplicates.

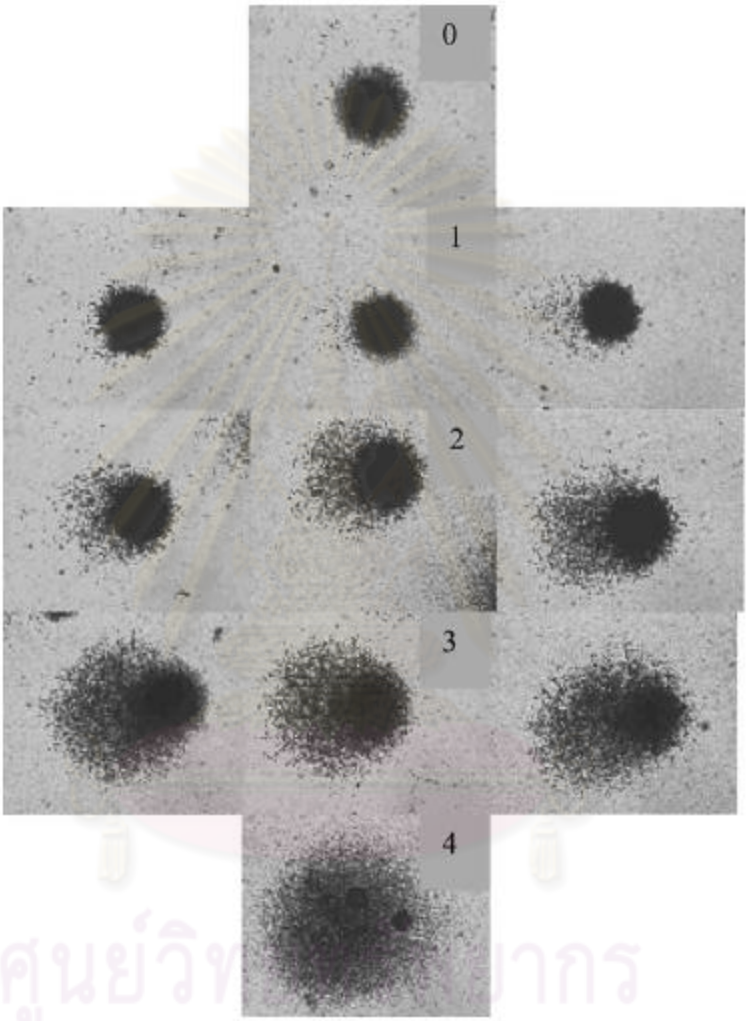

Figure 58 Images of human lymphocytes with various degrees of DNA damages. Class 0 represents undamaged cells and class 4 the most heavily damaged cells.

Figure 59 illustrated the DNA damage in lymphocytes treated with different concentrations of ethanol extract of each plant species and BLW remedy extract. The results of ethanol extracts indicated that ethanol extract of *C. micracantha* and *C. petasites* showed high damaged in normal human lymphocytes as compared with all of samples. While, ethanol extract of *H. perforata*, *F. racemosa, T. triandra* and BLW remedy exhibited no difference from each others. H_2O_2, a positive control, demonstrated highest damage to DNA in lymphocytes.

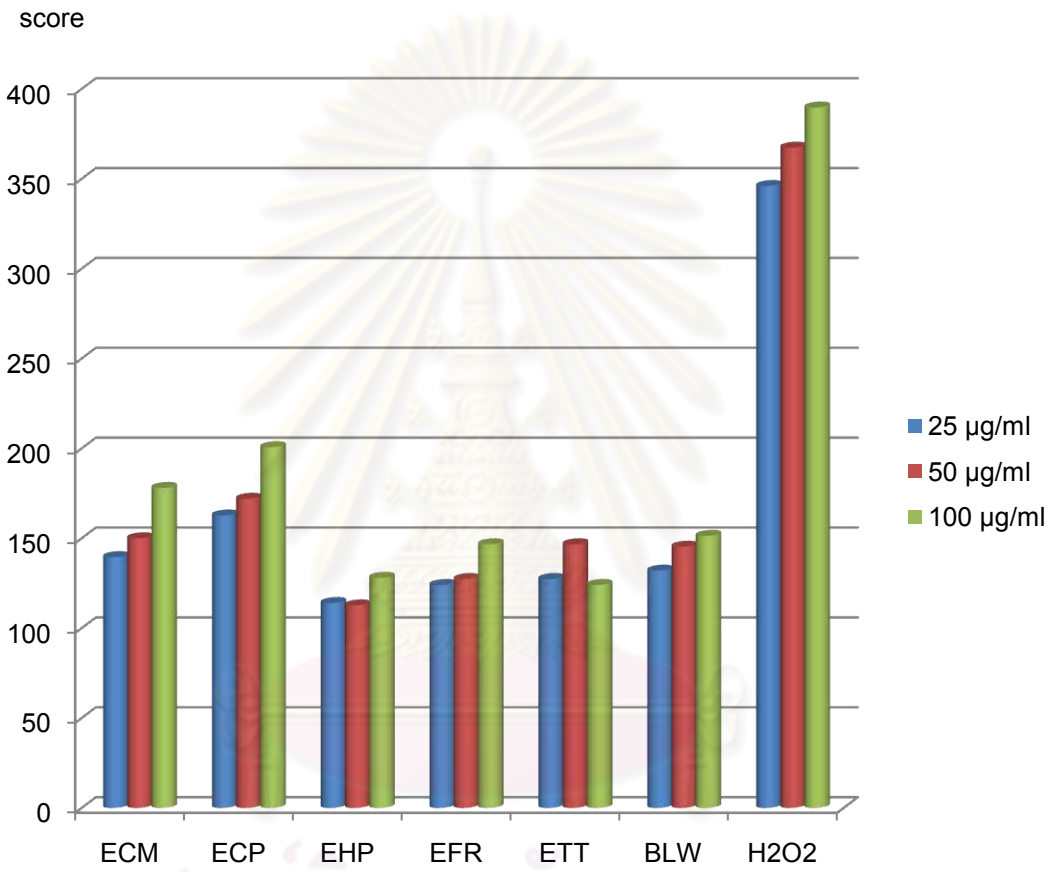

Figure 59 The total summing values (out of 400) of the number of comet classification, which obtained from each ethanol extract samples (ECM; ethanol extract of *C. micracantha*, ECP; ethanol extract of *C. petasites,* EHP; ethanol extract of *H. perforata*, EFR; ethanol extract of *F. racemosa* and ETT; ethanol extract of *T. triandra*) and BLW remedy. Doses of all samples were 25, 50 and 100 µg/ml. H_2O_2 was used as a positive control.

Figure 60 Illustrated the DNA damage in lymphocytes treated with different concentrations of water extract of each plant species and BLW remedy extract. The results of water extracts indicated that water extract of *C. micracantha* and *T. triandra* showed high damaged in normal human lymphocytes as compared with all of samples and revealed as same as the positive control H_2O_2. Water extract of *C. petasites, H. perforata* and *T. triandra* exhibited no difference from each others. BLW remedy revealed the lowest DNA damage. H_2O_2, a positive control, demonstrated the highest damage to DNA in lymphocytes.

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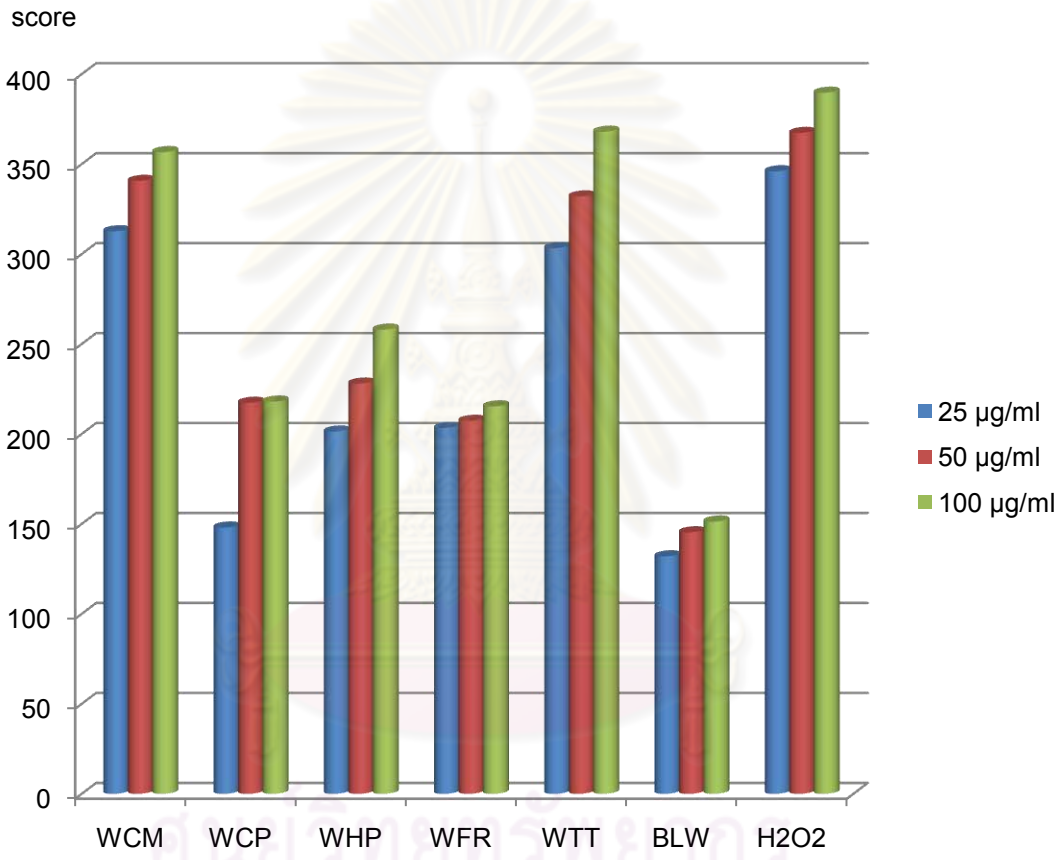
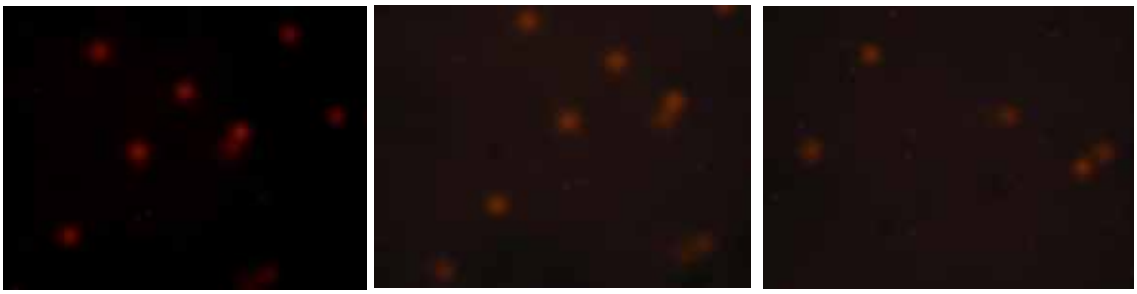


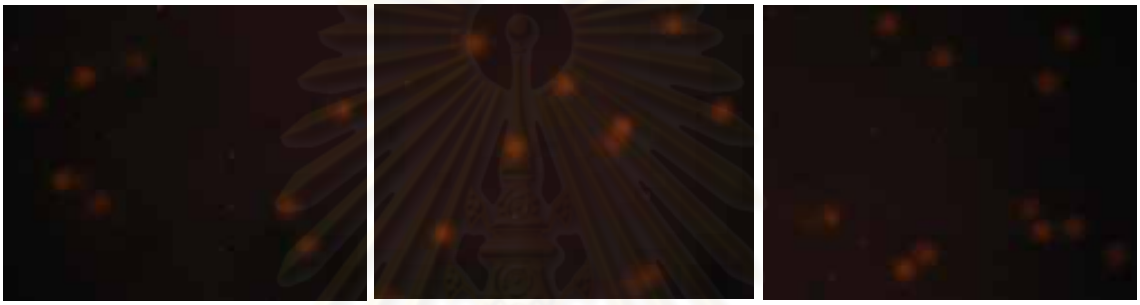
Figure 60 The total summing values (out of 400) of the number of comet classification, which obtained from each water extract samples (WCM; ethanol extract of *C. micracantha*, WCP; ethanol extract of *C. petasites,* WHP; ethanol extract of *H. perforata*, WFR; ethanol extract of *F. racemosa* and WTT; ethanol extract of *T. triandra*) and BLW remedy. Doses of all samples were 25, 50 and 100 μg/ml. H_2O_2 was used as a positive control

Ethanol extract of *C. micracantha* root



25 μg/ml **50 μg/ml** **100 μg/ml**

Water extract of *C. micracantha* root



25 μg/ml **50 μg/ml** **100 μg/ml**

Ethanol extract of *C. petasites* root

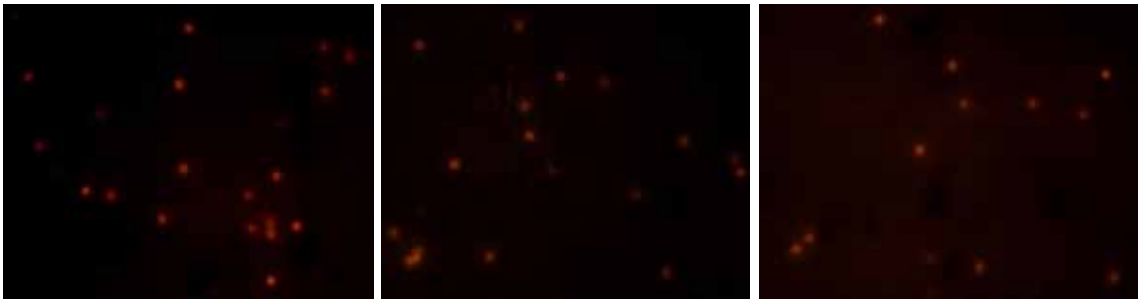
25 μg/ml **50 μg/ml** **100 μg/ml**

Water extract of *C. petasites* root

25 μg/ml **50 μg/ml** **100 μg/ml**

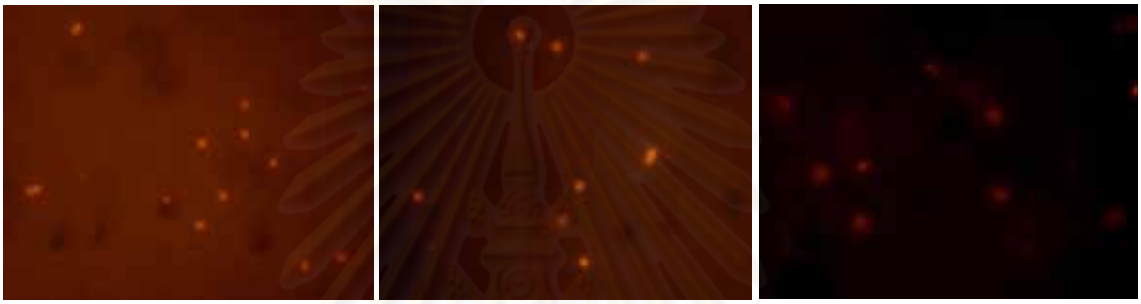
Figure 61 The DNA damage in lymphocytes treated with different concentration

Ethanol extract of *H. perforata* root



25 μg/ml **50 μg/ml** **100 μg/ml**

Water extract of *H. perforata* root



25 μg/ml **50 μg/ml** **100 μg/ml**

Ethanol extract of *F. racemosa* root

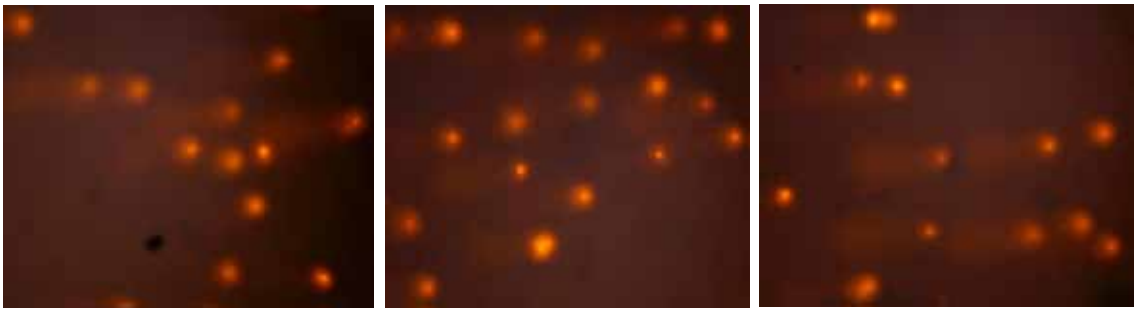
25 μg/ml **50 μg/ml** **100 μg/ml**

Water extract of *F. racemosa* root

25 μg/ml **50 μg/ml** **100 μg/ml**

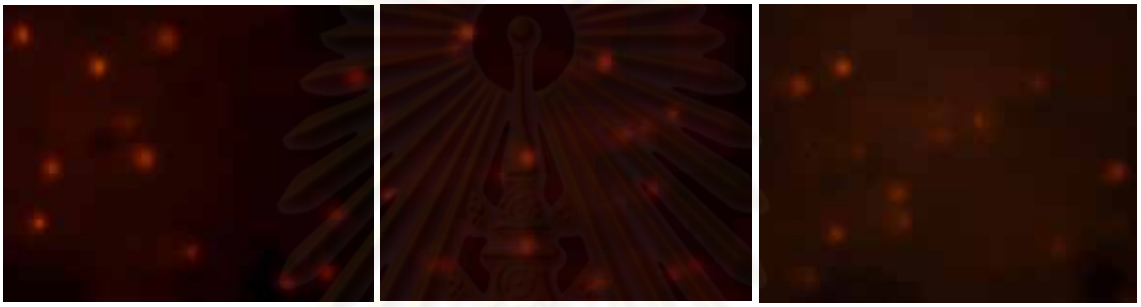
Figure 62 The DNA damage in lymphocytes treated with different concentration

Ethanol extract of *T. triandra* root



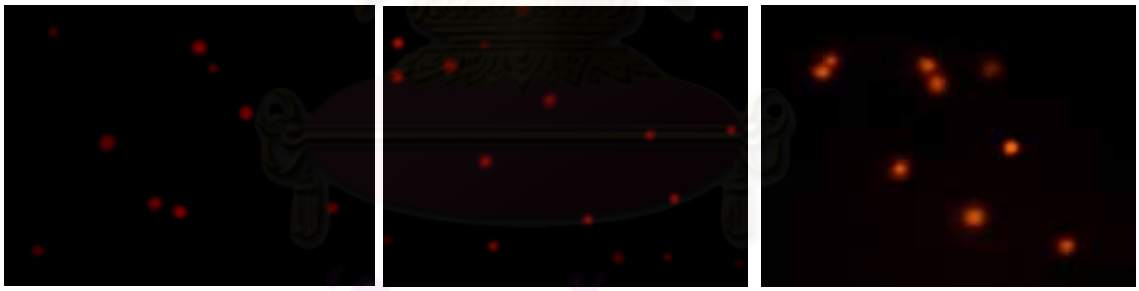
25 μg/ml **50 μg/ml** **100 μg/ml**

Water extract of *T. triandra* root



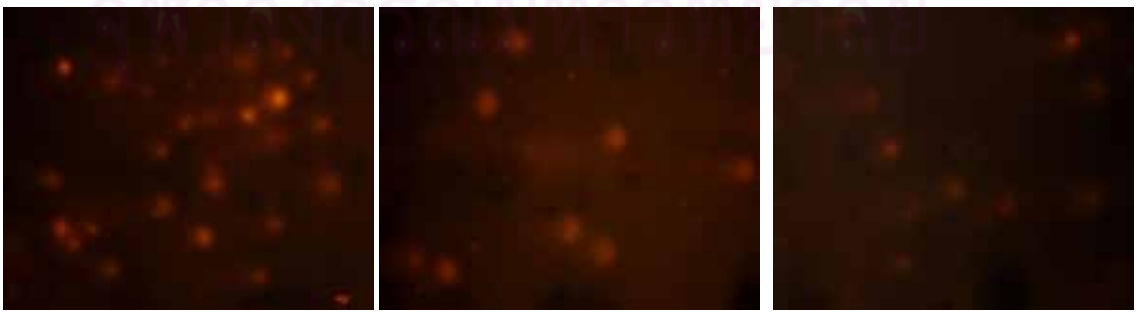
25 μg/ml **50 μg/ml** **100 μg/ml**

BLW remedy



25 μg/ml **50 μg/ml** **100 μg/ml**

H_2O_2



25 μM **50 μM** **100 μM**

Figure 63 The DNA damage in lymphocytes treated with different concentration

Efficacy study

Antipyretic activity test: *Lipopolysaccharide-induced fever*

Lipopolysaccharide injected intramuscularly significantly (p<0.001) produced a time-dependent increase in rectal temperature in vehicle pretreated rats starting from 1 hr, and this effect was maintained for 7 hr after LPS injection. The maximum increase in rectal temperature was reached at 3 hr (1.76°C) giving a maximum observed mean rectal temperature of 38.19±0.09°C after which there was a decrease. During the same period, the maximum mean rectal temperature of normothermic rats was 36.85±0.05°C. Thus, LPS significantly (p<0.001) increased the rectal temperature.

ASA 300 mg/kg significantly (p<0.05) attenuated the increase in rectal temperature produced by LPS at 2 hr and the antipyretic effect was maintained over the 7 hr period. The maximum mean rectal temperature in the presence of ASA was 37.21±0.13°C. All doses of the root extract of Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW; 25, 50, 100, 200 and 400 mg/kg) also significantly attenuated the increase in rectal temperature produced by LPS (p<0.05) with a maximum reduction at 7 hr. The antipyretic effect of increasing doses of BLW was noted at 4, 4, 2, 2 and 3 hr respectively, and the effect was maintained for the full 7 hr after LPS injection. The maximum mean rectal temperature produced by LPS in the presence of 25, 50, 100, 200 and 400 mg/kg of BLW were 37.53±0.19°C, 37.82±0.19°C, 37.25±0.19°C, 37.64±0.13°C and 37.49±0.09°C, respectively. BLW 100 and 200 mg/kg were found to be as potent as ASA (Table 12).

Table 12 Effect of the root extract of Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW; 25- 400 mg/kg) on lipopolysaccharide-induced fever in rats. N=6 for all groups

Treatments	Rectal Temperature (°C) before and after LPS injection								
	-1 hr	0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr
Normothermic rats[a]	36.83 ± 0.09	36.91 ± 0.26	37.11 ± 0.12	36.88 ± 0.05	36.85 ± 0.05	36.86 ± 0.05	36.89 ± 0.15	36.30 ± 0.24	36.44 ± 0.15
Control LPS[b]	36.79 ± 0.14	36.44 ± 0.24	37.77 ± 0.11#	38.12 ± 0.11#	38.19 ± 0.09#	38.06 ± 0.11#	37.95 ± 0.10#	37.75 ± 0.09#	37.56 ± 0.12#
ASA 300 mg/kg	36.74 ± 0.14	36.99 ± 0.11	37.21 ± 0.13	37.01 ± 0.13*	36.85 ± 0.13*	36.90 ± 0.18*	36.97 ± 0.14*	36.74 ± 0.16*	36.73 ± 0.17*
BLW 25 mg/kg	36.54 ± 0.22	36.94 ± 0.14	37.53 ± 0.19	37.30 ± 0.30	36.88 ± 0.25	36.76 ± 0.18*	36.58 ± 0.13*	36.34 ± 0.18*	35.97 ± 0.23*
BLW 50 mg/kg	36.51 ± 0.20	37.06 ± 0.15	37.82 ± 0.19	37.40 ± 0.24	37.19 ± 0.23	36.85 ± 0.11*	36.67 ± 0.11*	36.58 ± 0.11*	36.46 ± 0.07*
BLW 100 mg/kg	36.76 ± 0.15	36.93 ± 0.11	37.25 ± 0.19	37.00 ± 0.22*	36.79 ± 0.13*	36.68 ± 0.04*	36.41 ± 0.06*	36.29 ± 0.15*	36.05 ± 0.16*
BLW 200 mg/kg	36.45 ± 0.34	37.21 ± 0.14	37.64 ± 0.13	37.22 ± 0.17*	36.90 ± 0.13*	36.59 ± 0.06*	36.54 ± 0.06*	36.42 ± 0.15*	36.33 ± 0.10*
BLW 400 mg/kg	36.86 ± 0.05	37.16 ± 0.17	37.49 ± 0.09	36.61 ± 0.54	36.69 ± 0.10*	36.49 ± 0.21*	36.32 ± 0.09*	36.32 ± 0.10*	36.09 ± 0.26*

Each value represents mean± S.E.M. [a]Normothermic rats received 0.9% NSS. [b]Control LPS received 2% Tween 80 solution * p<0.005 significantly different compared to normothermic rat values for the corresponding hour

# p<0.001 significantly different compared to normothermic rat values for the corresponding hour

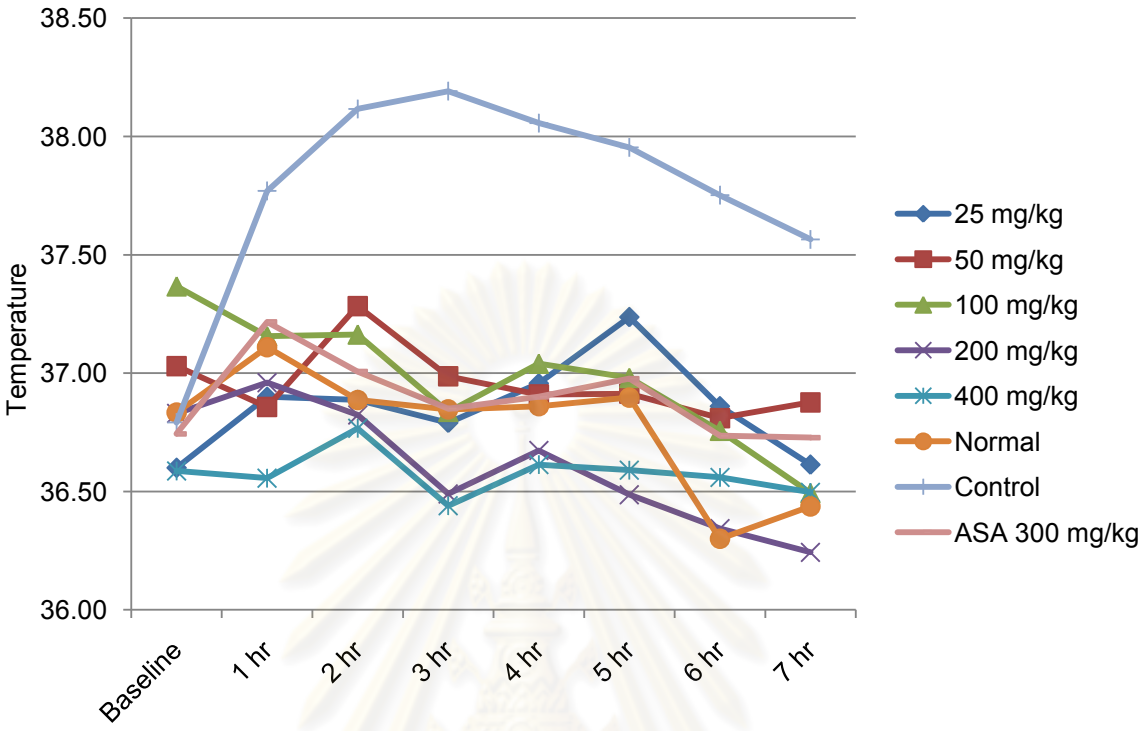


Figure 64 The effect of *C. micracantha* root extract on lipopolysaccharide-induced fever in rats.

Figure 64 Reatal temperature after oral administration of 2% Tween 80, Acetylsalicylic acid (ASA; 300 mg/kg) and various doses of *C. micracantha* root extract (CM; 25 – 400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysacharide (LPS; 50 μg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all group.

C. micracantha at doses 25, 100 and 200 mg/kg significantly ($p< 0.05$) reduced LPS induced increase in rectal temperature over a period of 2-7 hr with a maximum reduction at 7 hr. *C. micracantha* at doses of 50 and 400 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 1-7 hr with a maximum reduction at 6 and 3 hr, repectively. The mean rectal temperature produced by LPS in the presence of 25, 50, 100, 200 and 400 mg/kg of *C. micracantha* were reduced to 36.61 ± 0.26 °C, 36.81 ± 0.23 °C, 36.49 ± 0.14 °C, 36.24 ± 0.22 °C and 36.44 ± 0.36 °C respectively. *C. micracantha* showed antipyretic effect with all dose tested, especially at the dose of 100 mg/kg.

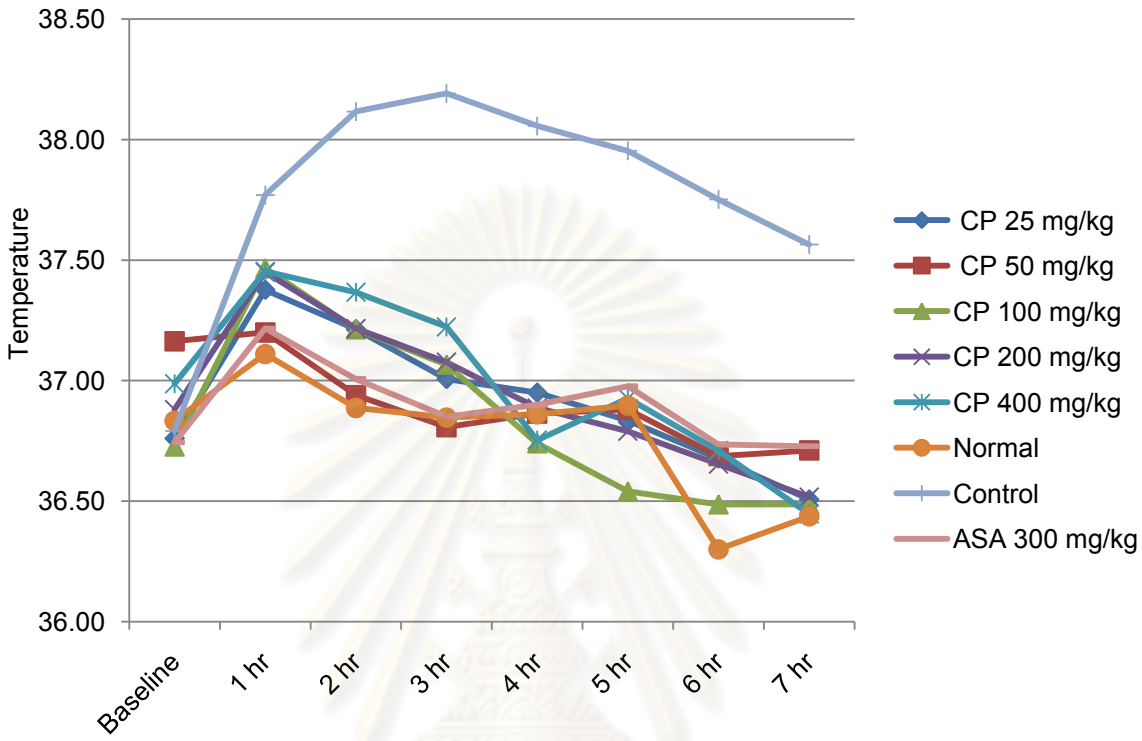


Figure 65 The effect of *C. petasite*s root extract on lipopolysaccharide-induced fever in rats.

Figure 65 Rectal temperature after oral administration of 2% Tween 80, Acetylsalicylic acid (ASA; 300 mg/kg) and various doses of *C. petasite*s root extract (CP; 25 – 400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysacharide (LPS; 50 μg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all group.

C. petasites at doses 25, 50 and 100 mg/kg significantly ($p< 0.05$) reduced the increased rectal temperature produced by LPS over a period of 2-7 hr with a maximum reduction at 7 hr. *C. petasites* at doses of 200 and 400 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 1-7 hr with a maximum reduction at 7 hr. The maximum reduction of mean rectal temperature produced by LPS in the presence of 25, 50, 100, 200 and 400 mg/kg of *C. petasites* were 36.51 ± 0.23 °C, 36.69 ± 0.05 °C, 36.49 ± 0.08 °C, 36.52 ± 0.09 °C and 36.45 ± 0.20 °C respectively. *C. petasites* showed antipyretic effect with all dose tested, especially at the dose of 100 mg/kg.

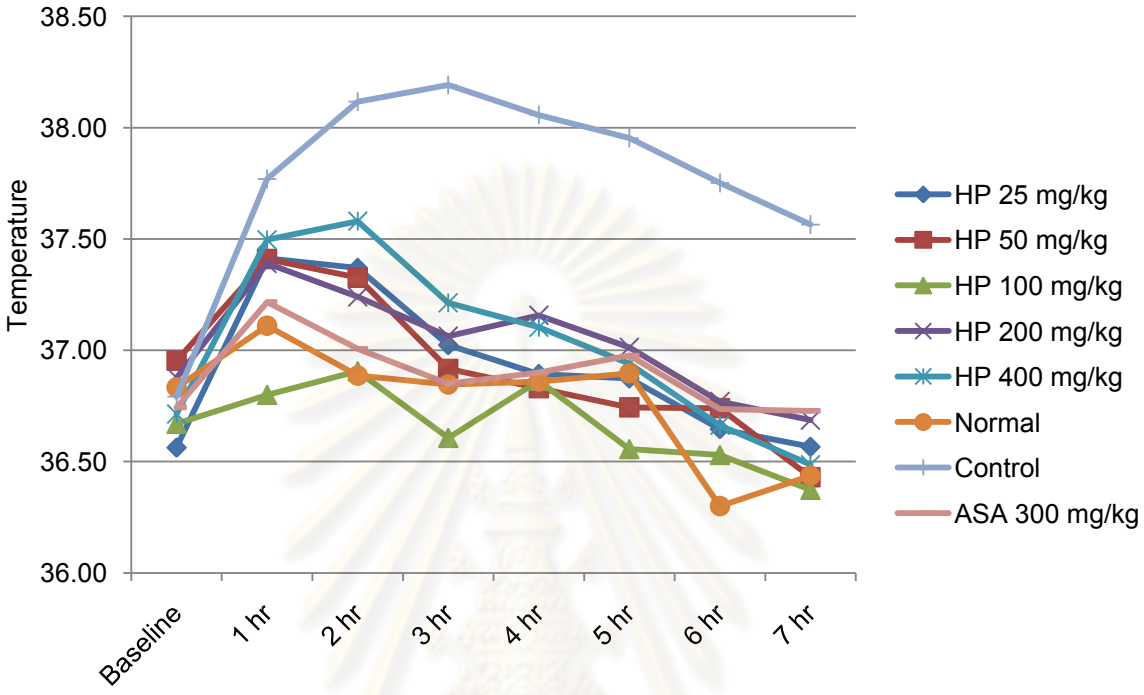


Figure 66 The effect of *H. perforata* root extract on lipopolysaccharide-induced fever in rats.

Figure 66 Rectal temperature after oral administration of 2% Tween 80, Acetylsalicylic acid (ASA; 300 mg/kg) and various doses of *H. perforata* root extract (HP; 25 – 400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysacharide (LPS; 50 μg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all group.

H. perforata at doses 25 mg/kg significantly ($p< 0.05$) reduced the increased rectal temperature produced by LPS over a period of 2-7 hr with a maximum reduction at 7 hr. *H. perforata* at doses of 50, 100 and 400 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 3-7 hr with a maximum reduction at 7 hr. *H. perforata* at doses of 200 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 5-7 hr with a maximum reduction at 7 hr. The maximum reduction of mean rectal temperature produced by LPS in the presence of 25, 50, 100, 200 and 400 mg/kg of *H. perforata* were 36.57 ± 0.09 °C, 36.43 ± 0.07 °C, 36.37 ± 0.12 °C, 36.69 ± 0.12 °C and 36.49 ± 0.07 °C respectively. *H. perforata* showed antipyretic effect with all dose tested, especially at the dose of 100 mg/kg.

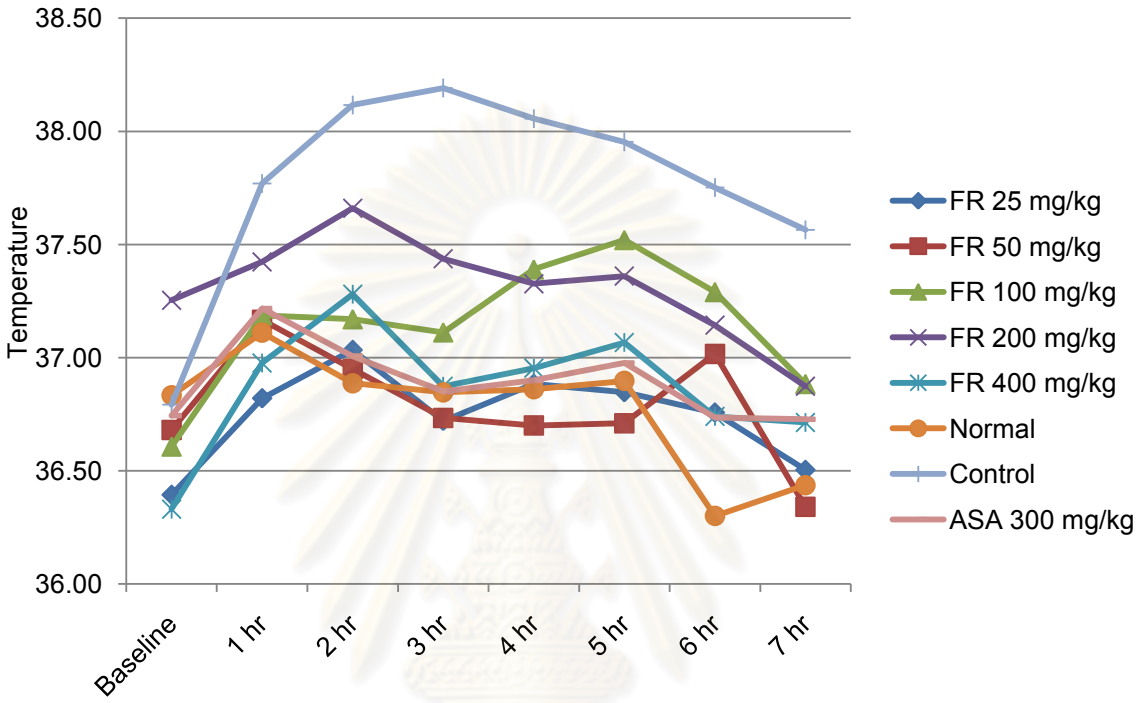


Figure 67 The effect of *F. racemosa* root extract on lipopolysaccharide-induced fever in rats.

Figure 67 Rectal temperature after oral administration of 2% Tween 80, Acetylsalicylic acid (ASA; 300 mg/kg) and various doses of *F. racemosa* root extract (FR; 25 – 400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysacharide (LPS; 50 μg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all group.

F. racemosa at doses 25 and 400 mg/kg significantly ($p< 0.05$) reduced the increased rectal temperature produced by LPS over a period of 1-7 hr with a maximum reduction at 7 hr. *F. racemosa* at doses of 50 and 100 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 2-7 hr with a maximum reduction at 7 hr. *F. racemosa* at doses of 200 mg/kg failed to reduced the increased rectal temperature produced by LPS over the entire period. The maximum reduction of mean rectal temperature produced by LPS in the presence of 25, 50, 100, 200 and 400 mg/kg of *F. racemosa* were 36.50 ± 0.19 °C, 36.34 ± 0.24 °C, 36.88 ± 0.26 °C, 36.87 ± 0.21 °C and 36.87 ± 0.34 °C respectively. *F. racemosa* showed antipyretic effect with all dose tested, especially at the dose of 50 mg/kg.

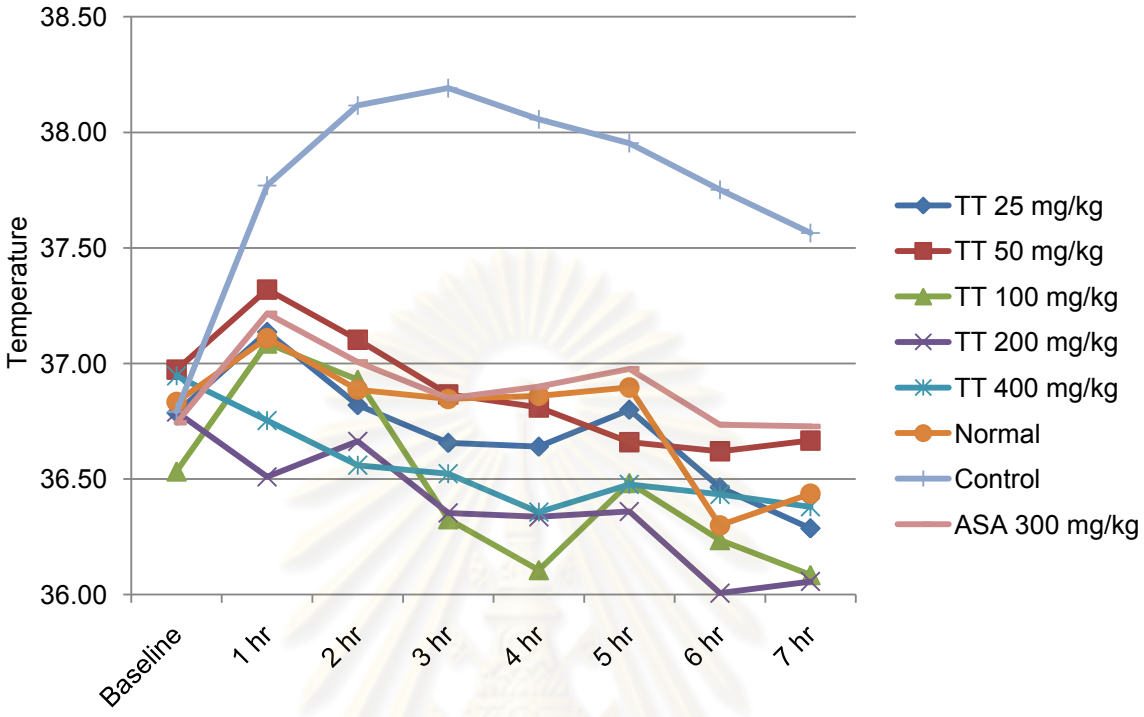


Figure 68 The effect of *T. triandra* root extract on lipopolysaccharide-induced fever in rats.

Figure 68 Rectal temperature after oral administration of 2% Tween 80, Acetylsalicylic acid (ASA; 300 mg/kg) and various doses of *T. triandra* root extract (TT; 25 – 400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysacharide (LPS; 50 μg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all group.

T. triandra at doses 25, 50 and 100 mg/kg significantly ($p< 0.05$) reduced the increased rectal temperature produced by LPS over a period of 2-7 hr with a maximum reduction at 7, 6, 7 hr respectively. *T. triandra* at doses of 200 and 400 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 1-7 hr with a maximum reduction at 6, 4 hr respecitvely. The maximum reduction of mean rectal temperature produced by LPS in the presence of 25, 50, 100, 200 and 400 mg/kg of *T. triandra* were 36.29 ± 0.38 °C, 36.67 ± 0.25 °C, 36.08 ± 0.26 °C, 36.06 ± 0.23 °C and 36.36 ± 0.28 °C respectively. *T. triandra* showed antipyretic effect with all dose tested, especially at the dose of 100 mg/kg.

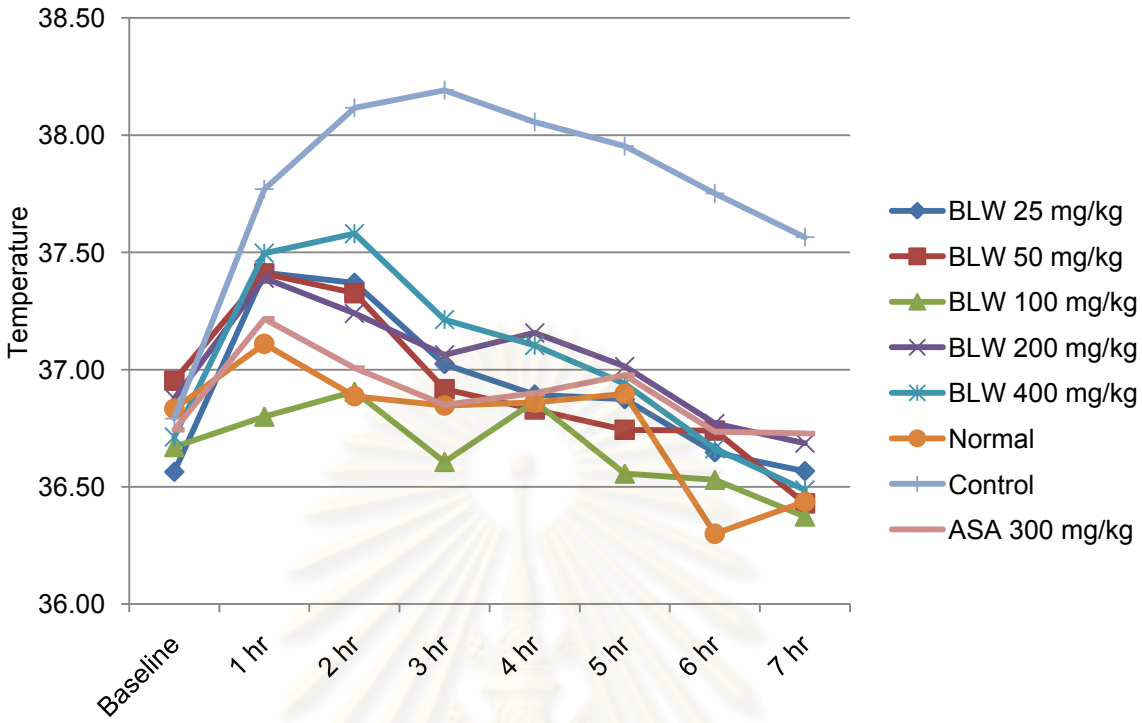


Figure 69 The effect of Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW) extract on lipopolysaccharide-induced fever in rats.

Figure 69 Rectal temperature after oral administration of 2% Tween 80, Acetylsalicylic acid (ASA; 300 mg/kg) and various doses of BLW remedy extract (BLW; 25 – 400 mg/kg) to febrile rats. Fever was induced by intramuscular injection of lipopolysacharide (LPS; 50 μg/ml) at 0 hr. All drugs were administered 1 hr before LPS. N=6 for all group.

BLW remedy at doses 25 and 50 mg/kg significantly ($p< 0.05$) reduced the increased rectal temperature produced by LPS over a period of 4-7 hr with a maximum reduction at 7 hr. BLW remedy at doses of 100 and 200 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 2-7 hr with a maximum reduction at 7 hr. BLW remedy at doses of 400 mg/kg significantly ($p<0.05$) reduced the increased rectal temperature produced by LPS over a period of 3-7 hr with a maximum reduction at 7 hr. The maximum reduction of mean rectal temperature produced by LPS in the presence of 25, 50, 100, 200 and 400 mg/kg of *BLW remedy* were 35.97 ± 0.23 °C, 36.46 ± 0.07 °C, 36.05 ± 0.16 °C, 36.66 ± 0.10 °C and 35.42 ± 0.64 °C respectively. BLW remedy showed antipyretic effect with all dose tested, especially at the dose of 400 mg/kg.

Antinociceptive activity test: Mouse Hot-plate

Latency in mouse hot-plate test

Initial studies utilizing the hot-plate test in mice to examine the efficacy of five root species and BLW remedy extract in producing analgesia. Mice were administered orally 2% Tween 80 or various doses of five root species and BLW remedy extract (25, 50, 100, 200 and 400 mg/kg).

Morphine 10 mg/kg significantly (p<0.01) increased the hot-plate latency producing an area of analgesia of 16,992.68 ± 1,940.94 %MPE-min compared with that of normal saline solution (NSS) (-6,908.17±2,505.75 %MPE-min; Figure 70). BLW 400 mg/kg significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group.

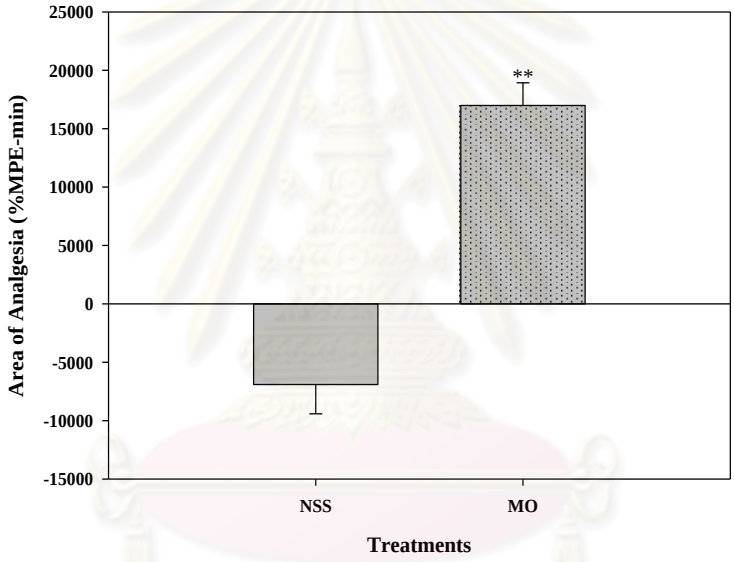


Figure 70 Mouse Hot-Plate Test. Area of analgesia (%MPE-min) from 0-240 minutes after intraperitoneal administration of 0.9% normal saline solution (NSS) and morphine sulphate (MO; 10 mg/kg). ***p*<0.01 significantly different compared to control animals.

All five root species extracts at dose of 25 mg/kg demonstrated similar analgesic effect when compared to BLW 24 mg/kg. *C. micracantha* and *F. racemosa* at dose of 50 mg/kg revealed significant (p<0.05) analgesic efficacy when compared to BLW 50 mg/kg. *C. micracantha*, *T. triandra* and *F. racemosa* at the dose of 100 mg/kg exhibited significant (p<0.05) analgesic efficacy when compared to BLW 100 mg/kg. *C. micracantha*, *H. perforata*, *T. triandra* and *F. racemosa* at the dose the 200 mg/kg showed significant (p<0.05) analgesic efficacy when compared to BLW 200 mg/kg. *C. micracantha* and *F. racemosa* at the dose of 400 mg/kg showed significant (p<0.05) analgesic efficacy when compared to BLW 400 mg/kg.

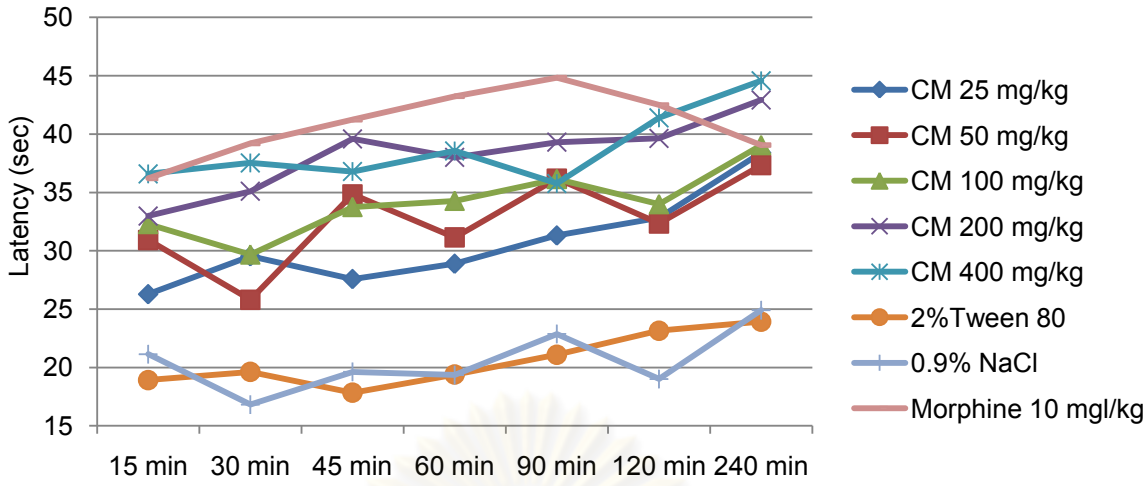


Figure 71 Latency (sec) in mouse hot-plate test from 0-240 min after administration of various doses of *C. micracantha* root extract (CM; 25-400 mg/kg).

All doses of *C. micracantha* tested significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group. The analgesic peak effects of all doses of C. micracantha (25-404 mg/kg) were reached within 240 min after oral administration.

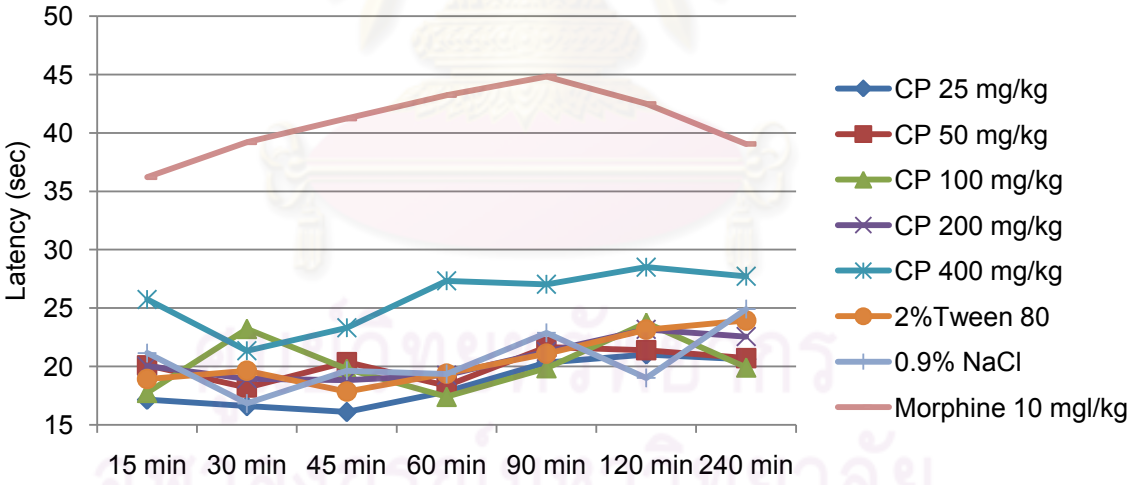


Figure 72 Latency (sec) in mouse hot-plate test from 0-240 min after administration of various doses of *C. petasites* root extract (CP; 25-400 mg/kg).

C. petasites at dose 400 mg/kg was significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group. The analgesic peak effects of *C. petasites* (25, 50, 100, 200 and 400 mg/kg) were reached within 120, 90, 120, 120 and 120 min respectively after oral administration.

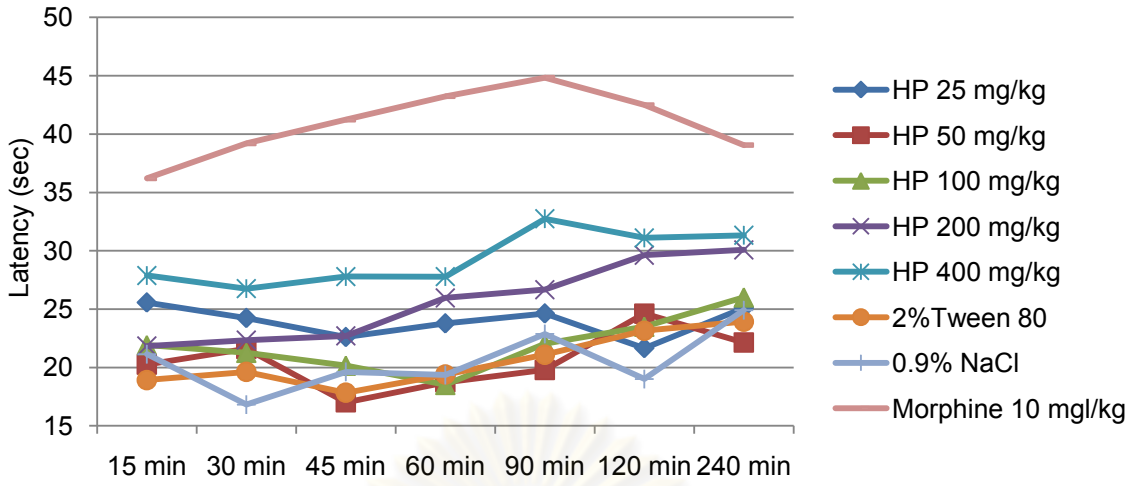


Figure 73 Latency (sec) in mouse hot-plate test from 0-240 min after administration of various doses of *H. perforata* root extract (HP; 25-400 mg/kg).

H. perforata at the doses of 200 and 400 mg/kg were significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group. The analgesic peak effects of *H. perforata* (25, 50, 100, 200 and 400 mg/kg) were reached within 15, 120, 240 120 and 90 min respectively after oral administration.

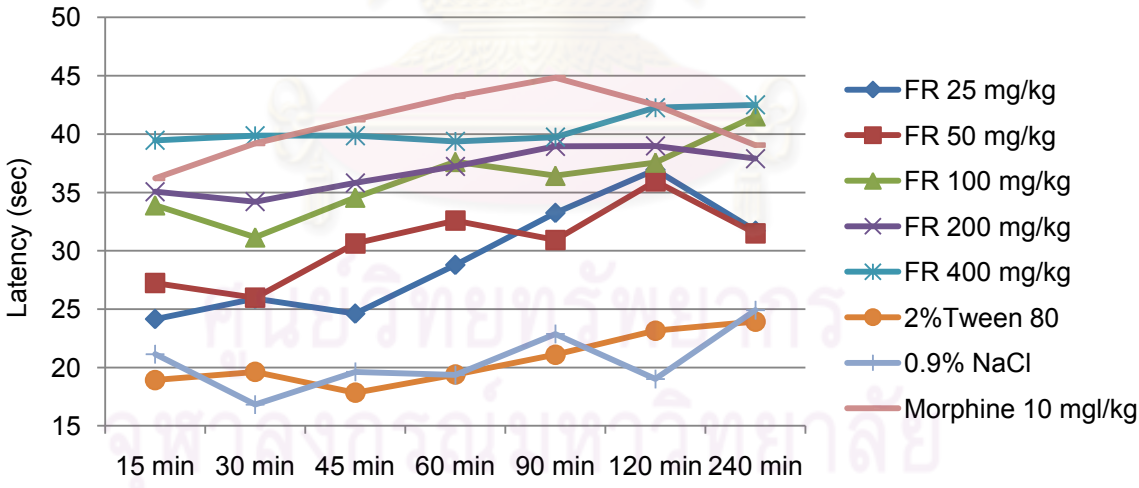


Figure 74 Latency (sec) in mouse hot-plate test from 0-240 min after administration of various doses of *F. racemosa* root extract (FR; 25-400 mg/kg).

F. racema at the doses of 100, 200 and 400 mg/kg were significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group. The analgesic peak effects of *F. racemosa* (25, 50, 100, 200 and 400 mg/kg) were reached within 120, 120, 240 120 and 240 min respectively after oral administration.

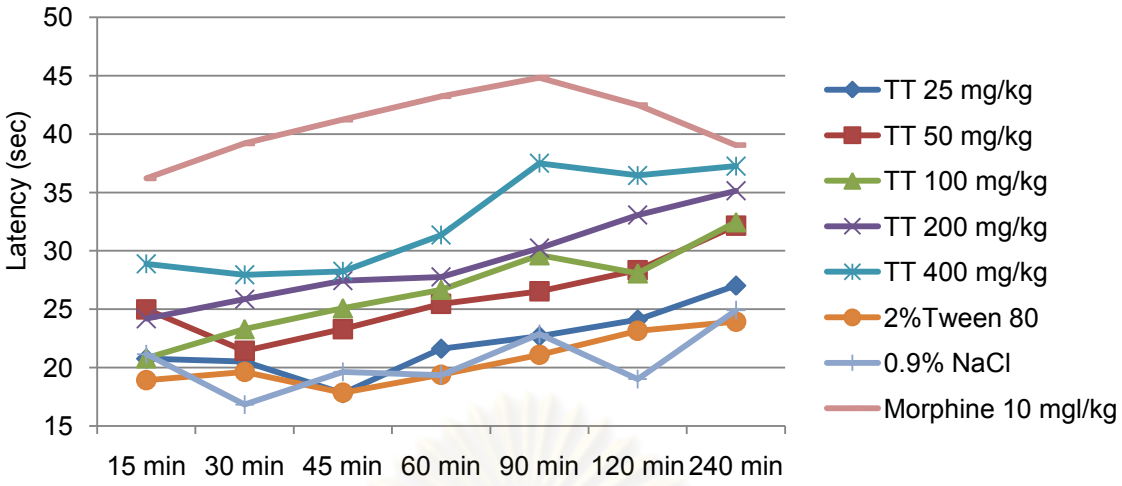


Figure 75 Latency (sec) in mouse hot-plate test from 0-240 min after administration of various doses of *T. triandra* root extract (TT; 25-400 mg/kg).

T. triandra at the doses of 100, 200 and 400 mg/kg were significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group. The analgesic peak effects of *T. triandra* (25, 50, 100, 200 and 400 mg/kg) were reached within 240, 240, 240 120 and 90 min respectively after oral administration.

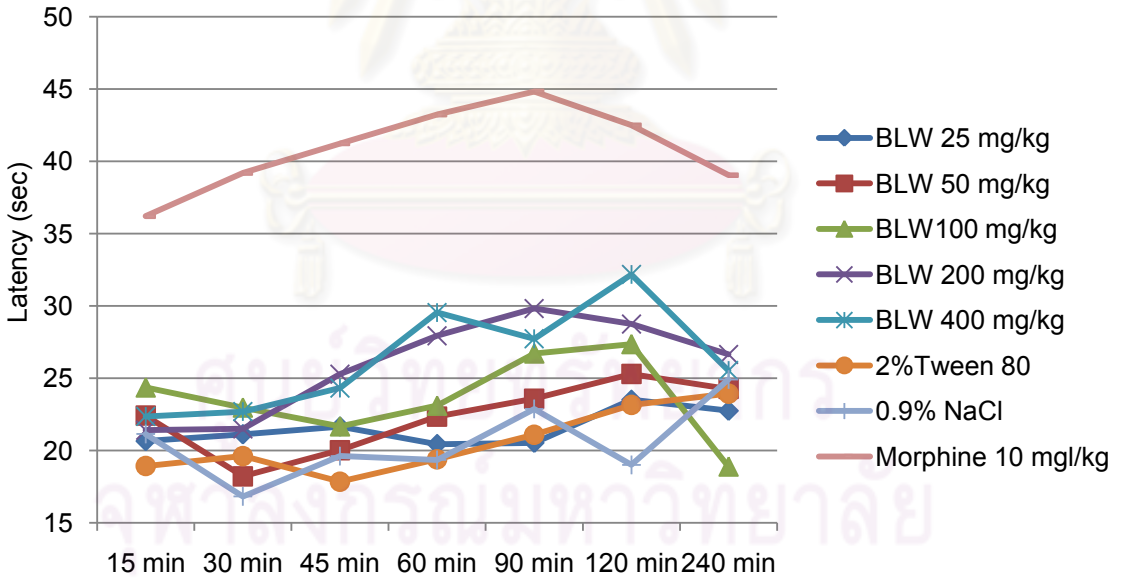


Figure 76 Latency (sec) in mouse hot-plate test from 0-240 min after administration of various doses of *BLW remedy* extracts (TT; 25-400 mg/kg).

BLW remedy at the doses of 400 mg/kg were significantly (p<0.05) increased the hot-plate latency when compared to the vehicle group. The analgesic peak effects of BLW remedy (25, 50, 100, 200 and 400 mg/kg) were reached within 120, 120, 120, 90 and 90 min respectively after oral administration.

Mouse Hot-plate Test; Area of analgenia (% MPE-min)

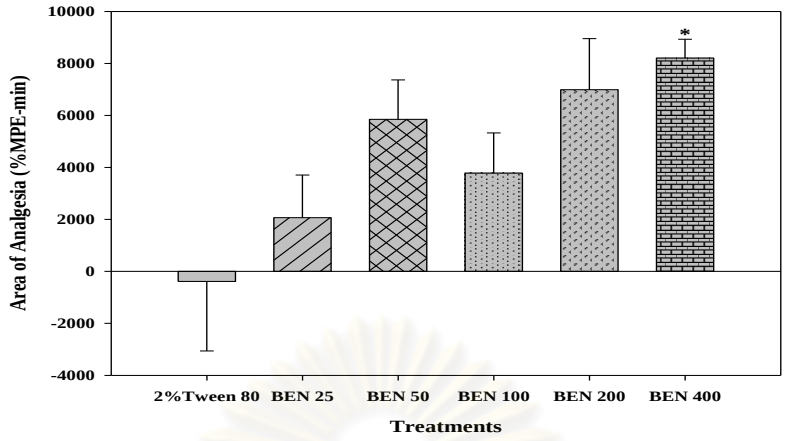


Figure 77 Mouse Hot-Plate Test. Area of analgesia (%MPE-min) from 0-240 minutes after oral administration of 2% Tween 80 and various doses of the root extract of Ben-Cha-Lo-Ka-Wi-Chian remedy (BLW; 25- 400 mg/kg). N=10 for all groups. Values represent the mean±S.E.M. *p<0.05 significantly different compared to control animals.

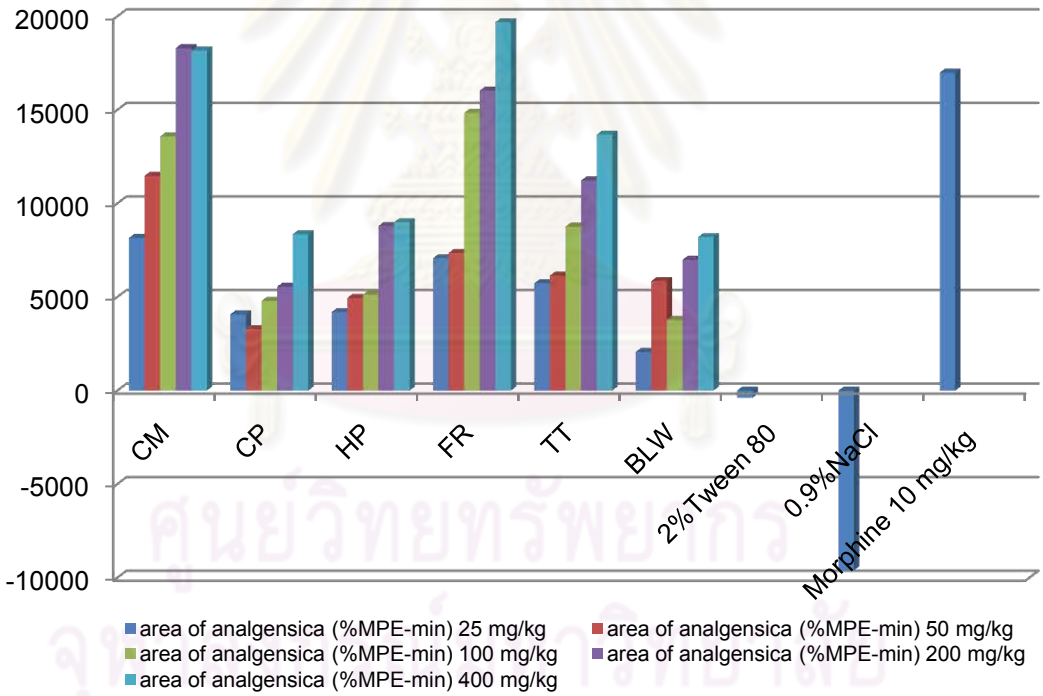


Figure 78 Percentage of the Mean Percent Maximum Possible effect (%MPE-Time) in mouse hot-plate test from 0-240 after oral administration of five root species and BLW remedy extract (doses; 25 – 400 mg/kg*). C. micracantha* and *F. racemosa* at doses 200 and 400 mg/kg (CM; 18303.52±1134.09, 18175.59±1460.05 and FR 16030.41±1552.24, 19682.32±708.32 of %MPE) were presented the highest values, whilst *C. petasites*, *H. perforata* and BLW remedy were demonstrated similar % MPE as 8354.51±1628.98, 8991.86±2563.81 and 8205.52±724.12 respectively.

Scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals

The ethanol and water extracts of five roots and the remedy extract were assessed for their antioxidation potential by the DPPH assay. The ethanol extracts of *H. perforata* and *T. triandra* showed the scavenging activity with IC_{50} of 71.46 and 83.64 µg/ml respectively, whilst BLW remedy extract showed the IC_{50} of 83.53 µg/ml. Only ethanol extract of *C. micracantha* exhibited weak free radical scavenger (IC_{50} > 1,000 µg/ml). Most of the water extracts showed weak radical scavenging activities including *C. micracantha, C. petasites* and *T. triandra* (IC_{50} > 1,000 µg/ml), excepted *F. racemosa* (IC_{50} 93.15µg/ml) (**Table 13**).

Table 13 Mean inhibition concentration (IC_{50}) of the ethanol, water extract of five roots species and the remedy

Species	Ethanol extract Mean IC_{50} (µg/ml)	Water extract Mean IC_{50} (µg/ml)
Capparis micracantha DC.	>1,000	>1,000
Clerodendrum petasites S. Moore	249.10	>1,000
Harrisonia perforata (Blanco) Merr	71.46	404.64
Ficus racemosa L.	111.87	93.15
Tiliacora triandra (Colebr.) Diels	83.64	>1,000
Ben-Cha-Lo-Ka-Wi-Chian remedy	83.53	
Quercetin	0.45	
Buthylated Hydroxyl toluene(BHT)	3.47	

Cell Proliferation using MTT assay

In the present result, the median lethal dose, LD_{50} (abbreviation for "Lethal Dose, 50%"), of each extract and BLW remedy were the dose required to kill half Fibroblast cells.

Table 14 The lethal dose (LD_{50}) of BLW remedy and each root species in BLW remedy.

Species	Ethanol extract LD_{50} (μg/ml)	Water extract LD_{50} (μg/ml)
Capparis micracantha DC.	>2,000	>2,000
Clerodendrum petasites S. Moore	>2,000	>2,000
Harrisonia perforata (Blanco) Merr	>2,000	>2,000
Ficus racemosa L.	>2,000	>2,000
Tiliacora triandra (Colebr.) Diels	>2,000	>2,000
Ben-Cha-Lo-Ka-Wi-Chian remedy	>20,000	
Vit C	200	

The LD_{50} of five root species and BLW remedy were tabulated as mention manner. Most of sample exhibited the LD_{50} effects on proliferation of fibroblast cell as measured with the MTT assay after the incubation times with each extracts and BLW remedy of 24 h. The concentrations of each extract and BLW remedy were platted against the absorbance of reduced MTT formazan which – according to the basic principle of the MTT assay – is expected to be proportional to the number of living cells. The results of Table 14 suggested that, all of the sample including ethanol and water extracts of each root species component in BLW remedy were revealed the LD_{50} values more than 2,000 μg/ml while LD_{50} value of BLW remedy extract was more than 20,000 μg/ml. Thus, If the sample were good, it should have less effect to Fibroblast cells that mean should have LD_{50} higher than vitamin c that used as a positive control.

Nitric Oxide using Griess reagent assay

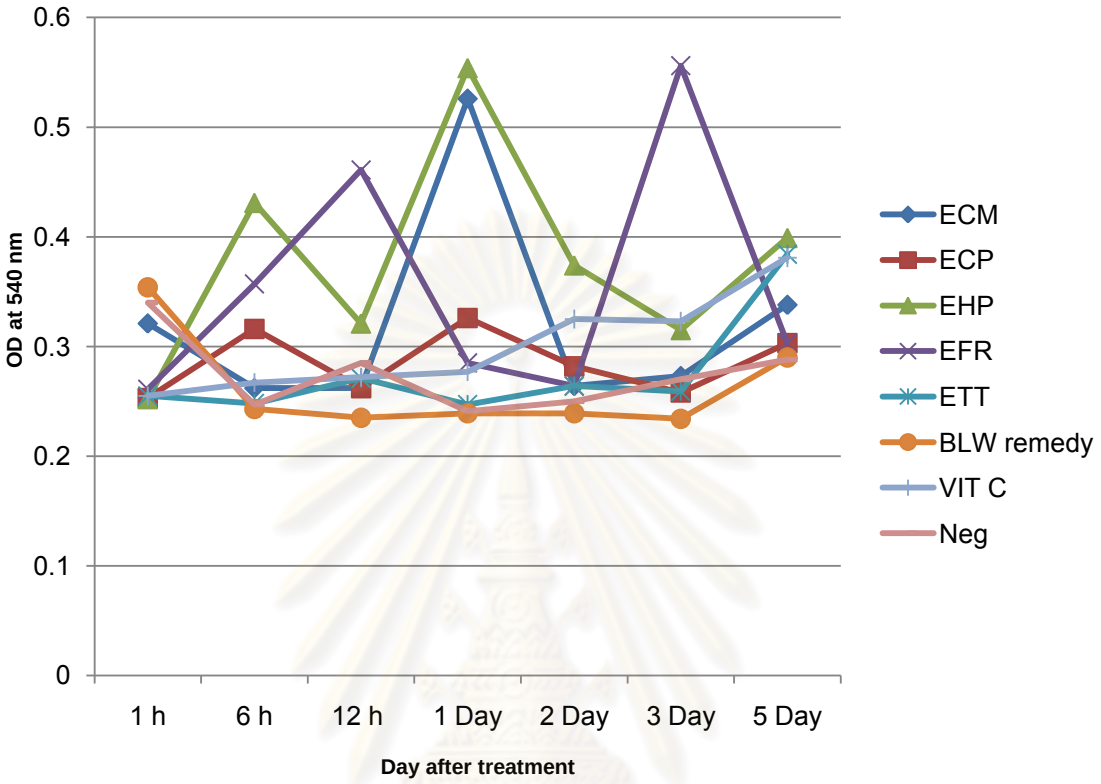


Figure 79 Effect of ethanol extracts induced by Griess reagent assay

Figure 79 The level of Optical Density (OD) that can be represented the level of Nitrite using Griess reagent assay at 540 nm after treated with ethanol extract of five root species (ECM; ethanol extract of *C. micracantha*, ECP; ethanol extract of *C. petasites*, EHP; ethanol extract of *H. perforata*, EFR; ethanol extract of *F. racemosa* and ETT; Ethanol extract of *T. triandra*) and BLW remedy extract from 1 h. – 5 day at dose 2,000 μg/ml. Vitamin C was used as positive control and DMEM in 10% FCS was used as Negative control.

Form the results in Figure 79, ethanol extract of *H. perforata* and *F. racemosa* demonstrated highest value of optical density at first day for *H. perforata* and third day for *F. racemosa*. Most of samples revealed higher optical density than vitamin C but only BLW remedy showed optical density lower than vitamin C. (For more understanding, if Optical Density (OD) trend to be increase that mean the level of Nitric oxide also increases, so the free radical scavenging are enhances as well).

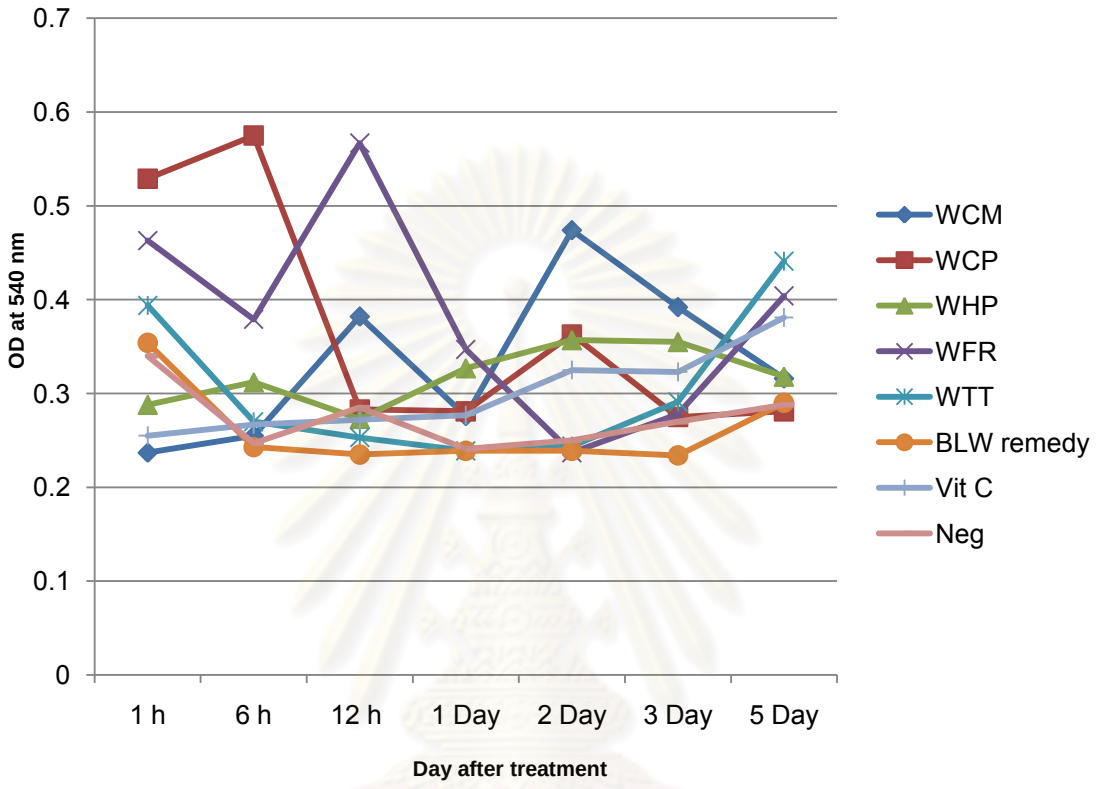


Figure 80 Effect of water extracts induced by Griess reagent assay

Figure 80 The level of Optical Density (OD) that can be represented the level of Nitrite using Griess reagent assay at 540 nm after treated with water extract of five root species (WCM; water extract of *C. micracantha*, WCP; water extract of *C. petasites*, WHP; water extract of *H. perforata*, WFR; water extract of *F. racemosa* and WTT; water extract of *T. triandra*) and BLW remedy extract from 1 h – 5 day by dose 2,000 μg/ml. Vitamin C was used as positive control and DMEM in 10% FCS was used as Negative control.

Twelve hours after treated with different water extract samples, *C. micracantha* and *F. racemosa* exhibited higher OD while *C. micracantha, C. petasites* and *H. perforata* at the second day demonstrated higher OD than all of sample. On the other hand, BLW remedy displayed lower OD, as illustrated in figure 80.

CHAPTER V

DISCUSSION AND CONCLUSION

As a consequence of increasingly interest to herbal medicine, the concern about the safety, efficacy and quality of all medicinal plants uses are also increasing. Some of Thai traditional remedies, although have been in practice, they are possible for adverse effects. Therefore, information on the safety, efficacy and quality of BLW remedy and its ingredients are important for public health issue. This study attempted to study the safety, efficacy and quality assessment of BLW remedy and each component herb. Firstly, it was undertaken using WHO guideline as quality control methods for medicinal plant materials for assessment of the quality of five root species that used as BLW herbs ingredients. Including this, the chemical fingerprint of the remedy was undertaken by using 3D-HPLC then performed the multivariate analysis for further standardization of BLW remedy according to chemical characteristics. Secondly, the safety of each root species extract and BLW remedy were performed for the cytotoxicity, mutagenicity and DNA damage activities using Brine shrimp method, Ames test and Comet assay respectively. Thirdly, the efficacy studies included antipyretic and analgesic activities in animal model, anti-mutagenic activity by Ames test, free radical scavenging activity by DPPH assay, cell proliferation by MTT assay and nitric oxide by griess reagent assay.

Although each species of BLW remedy could easily be distinguished on the basis of whole plant morphology as revealed in Figure 6,14,22,30 and 38, it became very difficult when the raw materials were in dried crude drugs and/or in powdered form. Especially in the form of the root powder that might be contaminated or adulterated in the traditional markets. In this study, the results obtained in morphological characters of five species were a good tool to authenticate the crude drugs. According to the study of Mecalfe [247], it was stated that only the methods of comparative histology can be used for identification of fragmentary or partly decomposed condition of vegetable material. Narayanan *et al*. [248] also mentioned that the correct identification of roots could often be achieved only by microscopical investigation. Consequently, the presence or absence of some histological characters of each root species was also explored and the dichotomous key was firstly established. This key identification could be used for detection of the contamination or adulteration of raw materials without high technology instrument (Figure 44). The examination of the physicochemical parameters in this study can help to evaluate the quality of the crude drugs and confirm raw materials standardization.

In 3D-HPLC results, the chromatogram from several places in each species, which were used as fingerprints, were compared and demonstrated no difference in the same species but for a variation species, there was distinction. According to Marston [249], who described for chemotaxonomic purposes, the botanical relationship between different species can be shown by chromatographic comparison of their chemical composition and Sakakitbara *et. al.*

[250] also explained that the HPLC was the best technique for an efficient separation of the crude extracts. Therefore, 3D-HPLC profiles were served well as fingerprint to differentiate all five species of BLW remedy. Based on the principle that profile-based classification could investigate variations within and among species by comparison of fingerprints.

Moreover, the fingerprint similarity - based taxonomy, which relies on the ratio of selected constituents, can improve the misclassifications caused by large qualitative differences. All batch samples were analyzed using the comparisons of the fingerprints by multivariate analysis. The chromatographic region from 0 to 60 min was selected for further studies. Sixty-three peaks within the studied region (0 - 60 min) were selected as characteristic peaks since they had relatively large areas. By comparisons of the retentions time, the area under curve of all selected peaks were extracted as raw data and used for multivariate analysis later.

The multivariate analysis, hierarchical clustering analysis (HCA), principle component analysis (PCA) and factor analysis were used to investigate the similarity of fingerprints. For the factor analysis results, most of factor plots were disseminated through fact sheet. Hierarchical clustering analysis illustrated clearly fell into three separate clusters. Principle component analysis also grouped into three clusters due to their similar area under curve or the quantitative of compounds in each peaks. The results indicated a close relationship between batch 2 to batch 12, except batch 1 that had no relationship with another batches. In this study, therefore, multivariate analysis was shown to be able to discriminate between various preparations including differentiation between various batches from different places throughout Thailand.

In conclusion, this study showed the effectiveness of the use of HPLC spectra combined with multivariate analysis of BLW remedy preparations from several places throughout Thailand. Most of batch samples were classified as same group which represented to the same standardization. There was also batch established different chemical characteristics which might be duet to the uncertainties constituents from different types of ecological environment. However, the results of this study revealed the value of the multivariate analysis spectral data as a sensitive method for characterization of very complex traditional medicines preparations, which took all detectable constituents into account in the description of sample composition.

Cytotoxic assay by *A. salina* lethality assay has been used as bench top bioassay for the discovery of bioactive natural products. It is an excellent choice for elementary toxicity investigations of consumer products based on the ability to kill laboratory-cultured *Artemia nauplii* (brine shrimp larva) [217, 218, 251]. Good correlation between the *in vitro* and the *in vivo* test ($r = 0.85$ and $p<0.05$) using LC_{50} of *A. salina* and mice model reported by Perra *et al.* [252] showed that the *A. salina* was a useful method to predict oral acute toxicity in plant extracts. From the results, BLW remedy extract showed toxicity to *A. salina*, and the toxicity was assumed to be due to ingredients in *T. triandra* and *H. perforata*. In contrary with Paowin *et al*., [253], who has reported the different results that the water extract of leaves of *T.*

triandra showed no acute or subchronic toxicities in female and male rats. This discrepancy might be due to the different parts used of this plant in the present study.

Mutagenic and anti-mutagenic were undertaken by using *Salmonella typhimurium* as target, TA98 and TA100 strains were used for detected frame-shift and base-paired substitution mutagenicity respectively. The studies of Kato *et al.* [142] demonstrated that 1-aminopyrine treated with nitrite at pH 3.0 and 37°C showed mutagenicity to *S. typhimurium* strain TA98 and TA100 without metabolic enzyme in the system. In concordance with Kangsadalampai *et al.* [21, 22] who found that 1-Nitropyrene was a potent direct mutagen toward *S. typhimurium* strains TA 98 and TA100 with similar condition that occurring in the stomach digestion. International Agency for Research on Cancer (IARC) [31] had revealed that the mutagenicity of 1-aminopyrine needed to be activated by nitroreductase. From the literature of Thai traditional remedy, only Kangsadalampai *et al.* [226] had undertaken the investigation of mutagenicity modification of Thai folklore medicine by nitrite in Ames mutagenicity test. The results of present study showed that all of the extract samples were non mutagenic directly but most of them were mutagenic indirectly under the nitrosation condition (nitrite treated in acidic condition). On the other hand, for anti-mutagenic property, most of ethanol extract and BLW remedy exhibited strongly active inhibition of mutagenicity whereas the water extracts were shown moderately active inhibition. These results were in accordance with the studies of Botting *et al.* [255] and Wongwattanasathien *et al.* [256] that the extracts which was derived from low polar solvents caused high inhibition than the crude extracts which derived from high polar solvents.

Comet assay provides a rapid, visual method for assessing DNA breakage quantitatively in single cell. DNA damage was evaluated in peripheral blood lymphocytes treated with different concentrations of each root species and BLW remedy. Water extract of *C. micrantha* and *T. triandra* were exhibited high damage in DNA as same as the positive control, H_2O_2. Additionally, the ethanol extract of *C. micracantha* also exhibited higher DNA damage when compared with the other ethanol extracts samples. The results revealed that most of water extracts samples demonstrated DNA damage higher than ethanol extract samples. Also in each species extract, it caused DNA damage higher than the combination of each species as the remedy.

From the finding data of safety studies, it was interesting to note that most data of water extracts showed non-safety results. While most of ethanol extracts of each root species showed safety results and the BLW remedy also showed the safety results in all bioactivities in the safety studies part.

These studies have demonstrated the antipyretic and antinociceptive effects of five root species and BLW remedy extracts in two animal models. Antipyretic activity was assessed utilizing the LPS-induced fever model. The antinociceptive effect was assessed utilizing thermal by hot-plate models. Antipyretics such as ASA and other nonsteroidal anti-

inflammatory drugs (NSAIDs) reduce fever by depressing inflammatory messages at both peripheral sites of tissue inflammation and within CNS thermoregulation sites. They suppress peripheral producing of pyrogenic cytokines including TNF-α and IL-1β while lower the thermoregulatory set point by blocking central COX production of PGE_2 [257].

LPS is the most potent stimulus known for TNF-α production and release and also increases circulating levels of another pyrogen, IL-1. This exogenous pyrogen has been shown to produce fever in laboratory animals such as guinea pigs and rabbits by stimulating the production of endogenous TNF-α [258, 259]. For characterization of the antipyretic activity of BLW remedy, the LPS-induced fever model in rats was employed in this study.

Orally administered ASA, the positive control, significantly attenuated fever in LPS treated rats at all times tested. This has been due to inhibition of COX and therefore interference with the cascade of the synthesis of PGs which induces fever. The oral administration was chosen in order to imitate the normal consumption of 'Ben-Cha-Lo-Ka-Wi-Chian remedy', the Thai traditional antipyretic herbal medicine. All doses of BLW remedy (25-400 mg/kg) displayed antipyretic activity in the LPS-induced fever model of rats over the period of 2-7 hr after LPS injection. Additional studies are needed to determine whether the antipyretic effect of BLW remedy are due to suppression of TNF-α, inhibition of TNF-α or PG synthesis. The antipyretic effect of BLW remedy (100 and 200 mg/kg) occurred within 2 hr after LPS injection and was sustained for up to 7 hr, similar to that seen with ASA treatment. The antipyretic efficacy of all doses of BLW used was comparable to that of ASA. These results are consistent with the previous study of Konsue *et al.* in 2008 [260] who investigated the antipyretic effect of dried root powder of Ben-Cha-Lo-Ka-Wi-Chian herbal drugs.

In order to investigate the antinociceptive properties of BLW remedy, thermal pain by hot-plate models was performed in mice. The standard hot-plate test, a central analgesic activity testing model, measures two behavioral components including paw licking and jumping which are both considered to be supraspinally integrated responses [261]. This model usually employed morphine (MO) as a reference drug. MO demonstrated potent analgesic effects in this model indicating the sensitivity of this test. The significant analgesic action of BLW (400 mg/kg) was observed during the 240 min test. The results obtained from hot-plate tests indicated that BLW has analgesic activity at supraspinal level.

The scavenging activity was tested by the ability to scavenge DPPH radicals. DPPH (2, 2-diphenyl-1-picrylhydazyl) assay is based on the reaction of metanolic solution of colored free radical DPPH by free radical scavenger. DPPH is a stable free radical with red-purple color (absorbed at 517 nm). If free radical has been scavenged, DPPH will fade its color to yellow. Therefore, from this character, the medicinal plant free radical scavenging activity was carried out. The resultant level of reduced DPPH was measured spectrophotometerically [262]. This result showed that BLW remedy had potential to scavenge free radicals. This due to three plant roots ingredients namely *H. perforata*, *T. triandra* and *F. racemosa.* The stem bark of *F.*

racemosa was previously reported of free radical scavenging activity [263]. This study revealed the same antioxidant activity by *F. racemosa* root as well. In addition, the root of *C. micracantha* has been shown as antioxidant using Heinz body model [264].

The dose dependent effect of each root species and BLW remedy on proliferation of fibroblast cell was measured with the MTT assay after incubation for 24 h and LD_{50} were determined. The results of this assay suggested that most of ethanol and water extracts showed LD_{50} more than 2,000 μg/ml while BLW exhibited the LD_{50} more than 20,000 μg/ml. It was shown that each root species affected fibroblast cell weaker than vitamin C at dose of 200 μg/ml. Moreover the BLW remedy showed less damage to fibroblast cell also. As general from several studies, vitamin C (ascorbic acid) has revealed the effect as a potential for treatment brought enhanced quality and prolongation of cell life. On the other hand, recently emerging evidence indicated that vitamin C (1-10 mM) resulted in effective cell death *in vitro* and inhibition of tumor growth *in vivo* [265-267]. Additionally, the mechanism and physiologic relevance remained to be fully elucidated that vitamin C could lead to H_2O_2 production. It has been known that H_2O_2 involves in the redox control of several physiological processes including cell proliferation and appotosis [268-269]. Regarding to Takemura *et.al.* in 2010 [270], who firstly reported that high dose of vitamin C induced cell death of all mesothelioma cell lines.

Fibroblast cell tested in this study were passage 15, which represented aging cell. The supernatant from cell proliferation assay aforementioned above were further tested for nitric oxide as one indicator of cellular oxidative stress affecting to apoptosis or cell death.

The supernatant of BLW remedy treated fibroblast cells showed lowest optical density relevant to least nitric oxide concentration than the supernatant of each root species and also vitamin C treated cells. *H. perforata*, *F. racemosa* and *C. micracantha* demonstrated higher nitric oxide level than other species. This might be due to either less nitric oxide scavenging activity or more nitric oxide induction [268-269]

On the basis of studies, the selection of plant materials for this study was based on ethobotanical data on the traditional use of the plants in treatment of antipyretic as mentioned manner. The benefit of such subsequent results will be served as scientific evidences in efficacy and safety for BLW remedy use in primary health care. The importance of conservation of the traditional uses among these medicinal plants is unquestionable. This knowledge represents additional information in order to promote a good standard of plant materials for used as a remedy. Even though, some tested species showed limit in safety and efficacy *via* the bioactivity testing, it is important to note that these species have not been employed as single crude drug use in the traditional context.

From such findings as conclusion in the table 15, when each species was investigated the safety and efficacy of BLW ingredient, it was revealed diversity results. While all of ingredient

remedy prepared in form of the remedy as a combination of the remedy, the good results were revealed. It may possibly be affected from the remedy medicine that contains many active ingredients, several combination effects are involved in their efficacy. These combination effects might be classified as pharmacological effects and pharmaceutical effects. The former affect synergic and antagonistic actions, new pharmacological activity, reduction of adverse reaction, and so on. Concurrence with the principle of Kampo medicine in Japan [271], that the efficacy cannot be explained by the pharmacological activity of any one of the active ingredients. Several active ingredients may affect the multiple systems of the whole body by several combined effects. Additionally Kampo medicines are generally administered orally, “inactive” compounds may be activated by endogenous factors such as gastric sections, intestinal enzymes, and bacteria.

Table 15 Conclusion of Safety and Efficacy of each root species and Ben-Cha-Lo-Ka-Wi-Chian remedy extract.

	Experiment	**Sample**										
		C. micracantha		*C. petasites*		*H. perforata*		*F. racemosa*		*T. triandra*		BLW Remdy
		E	W	E	W	E	W	E	W	E	W	
Safety evaluation by	Brine shrimp assay	+++	++	+++	+++	+	+	+++	+++	T	T	++
	Mutagenic assay *(Ames test) without nitrite*	++	++	++	+	++	+	++	+	++	++	++
	(Ames test) with nitrite	T	+	T	+	+	+	T	T	T	T	T
	Comet assay	+++	T	++	++	+++	+	+++	+	+	T	+++
Efficacy evaluation by	Antipyretic activity *(Animal model)*	++		+++		+++		++		+++		+++
	Analgesic activity *(Animal model)*	+++		+++		+++		++		++		+++
	Anti-mutagenic testing *(Ames test)*	+++	+++	+++	+	+++	+++	+++	+	+++	+++	+++
	Free radical Scavenging Activities	+	+	++	+	++	++	++	+	++	+	++

+++: Good, ++: Moderate, +: Mild, T: Toxic, E: Ethanol extract, W: water extract

Although BLW remedy has a long clinical experience to support their efficacies and safety, the efficacy of BLW remedy requires the clarification of the mechanism of action and active ingredients in nonclinical studies and objective clinical evaluations as an evidence-based medicine. Because BLW remedy contain many active ingredients due to the several component herbs, clarification of such combination effects and standardization are also very important in order to understand the mechanism of action and supply quality-controlled materials. Likewise the results more indicate that the often very elaborate traditional knowledge can serve as guideline to provide leads for further testing of potentially interesting plants that can serve for further studies that would allow the clinical validation of the traditional uses and the application of the species in modern treatment forms.

Overall, regarding to the results of the current study, it could be described that not only each species that need to carry out for the safety, efficacy and quality, but the combination of remedy needed to understand the consequences of such combined used for safety, efficacy and quality also. Moreover the finding from the present study provided further evidences to support the safe consumption of Thai traditional medicines: Ben-Cha-Lo-Ka-Wi-Chian remedy and its components. The consumers should beware of using the remedy with nitrite containing food for prospect continuing used as well. Additionally, it is interesting to note that an antipyretic effect revealed the same potential of standard aspirin due to the synergistic effect of its components. Finally, this study helps clarifying the safety, efficacy and quality of each plant species and Ben-Cha-Lo-Ka-Wi-Chian remedy as well as provides additional scientific support for this well-known Thai traditional medicine.

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APPENDICES

APPENDIX A

Data of Pharmacognostic characters (% by weight) of five root species in Ben Cha Lo Ka Wi Chian Remedy

Table 16 Pharmacognostic characters (% by weight) of *Capparis micracantha* DC. root

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
1	Chiang mai	1	8.40	5.77	3.72	1.83	0.52	1.74
		2	8.40	5.73	3.47	1.99	0.55	1.72
		3	8.80	5.66	3.54	1.83	0.54	2.14
2	Lopburi (Lumnarai)	1	8.50	8.81	4.93	1.66	0.58	2.10
		2	8.00	8.90	4.94	1.79	0.55	2.16
		3	8.00	8.81	4.79	1.68	0.57	2.58
3	Nong khai (Sriwilai)	1	9.50	7.56	2.54	0.50	0.46	2.32
		2	9.60	7.63	2.43	0.39	0.59	2.32
		3	9.10	7.67	2.44	0.36	0.51	3.09
4	Lampang	1	8.80	6.56	3.65	0.74	0.73	2.09
		2	9.00	7.19	3.84	1.05	0.75	2.67
		3	9.00	7.21	3.81	0.95	0.80	2.62
5	Petchabun	1	8.60	6.16	11.35	5.60	1.39	1.21
		2	8.50	6.23	11.47	5.65	1.41	2.03
		3	8.80	6.28	10.46	5.21	1.33	2.02
6	Rayong	1	7.80	5.87	3.97	2.14	0.54	2.26
		2	7.60	5.89	3.62	2.03	0.55	2.57
		3	7.60	5.84	3.73	1.88	0.55	2.32
7	Nakornnayok	1	7.00	5.49	7.81	0.71	0.09	1.96
		2	7.10	5.51	7.76	4.28	0.05	1.99
		3	7.00	5.44	7.37	3.74	0.04	2.05
8	Nong khai (Sea ka)	1	9.60	7.92	6.38	4.57	0.10	1.53
		2	9.70	7.89	4.72	2.94	0.10	1.61
		3	9.80	7.85	4.44	2.76	0.11	1.64
9	Lopburi (Muang)	1	9.00	8.00	4.65	1.45	0.52	1.98
		2	9.00	8.04	4.82	1.64	0.52	2.37

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
		3	9.10	7.89	4.71	1.50	0.54	2.27
10	Uthai thani	1	7.40	6.22	5.48	3.13	0.73	2.31
		2	7.50	6.24	5.44	2.94	0.71	2.59
		3	7.50	6.09	5.23	2.94	0.78	2.38
11	Nan	1	8.60	6.88	3.96	1.41	0.64	2.19
		2	8.70	6.81	4.18	1.48	0.77	2.35
		3	8.60	6.87	4.12	1.38	0.68	2.25
12	Yasothon	1	8.20	6.50	3.82	0.99	0.07	1.42
		2	8.40	6.41	4.11	1.18	0.08	1.36
		3	8.30	6.34	3.93	0.96	0.09	1.37
13	Kanchanaburi	1	8.40	6.39	4.80	2.26	0.10	1.91
		2	8.20	6.44	4.43	2.09	0.11	1.98
		3	8.20	6.82	4.44	2.04	0.10	1.82
14	Kalasil	1	7.70	6.04	3.69	1.22	0.66	4.82
		2	7.60	6.05	3.75	1.36	0.64	2.47
		3	7.60	6.02	3.62	1.33	0.67	2.13
Grand mean± Pooled SD			**8.39±0.15**	**6.76±0.13**	**4.91±0.34**	**2.09±0.59**	**0.52±0.03**	**2.16±0.46**
Min - Max			**7.00-9.80**	**5.44-8.90**	**2.43-11.47**	**0.36-5.65**	**0.04-1.41**	**1.21-4.82**

Table 17 Pharmacognostic characters (% by weight) of *Clerodendrum petasites* S. Moore root

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
1	Chiang mai	1	7.60	5.11	2.81	0.58	0.63	1.38
		2	7.50	4.94	1.93	0.44	0.75	1.41
		3	7.50	4.14	2.33	0.43	0.74	1.28
2	Petchabun	1	9.60	8.28	4.42	1.20	0.60	1.30
		2	9.50	8.18	4.25	1.18	0.66	1.38
		3	9.60	8.36	3.50	0.93	0.64	1.31
3	Nan	1	6.50	3.62	4.61	0.83	0.67	1.42
		2	6.50	3.39	4.97	0.84	0.68	1.93
		3	6.60	3.50	4.27	0.53	0.60	1.69
4	Nongkhai (Sriwilai)	1	10.60	8.47	4.24	1.26	1.01	1.70
		2	10.60	8.65	4.29	1.32	1.01	1.72
		3	10.60	9.02	4.39	1.27	1.00	1.92
5	Lumpang	1	7.00	3.32	6.13	1.04	0.49	0.97
		2	5.20	3.19	5.90	1.01	0.57	1.10
		3	5.40	3.09	6.50	0.89	0.60	1.20
6	Nakhornnayok	1	7.60	6.11	3.26	0.61	0.36	1.48
		2	7.70	6.01	3.05	0.64	0.30	1.14
		3	7.60	6.04	3.22	0.79	0.34	1.30
7	Lopburi (Muang)	1	9.80	7.97	4.33	0.71	0.80	1.43
		2	9.40	7.79	4.14	0.65	0.93	1.43
		3	9.40	7.93	4.45	0.82	0.98	1.51
8	Nongkhai (Seaka)	1	8.00	5.77	6.35	2.32	0.35	2.45
		2	8.00	5.82	4.84	1.04	0.36	2.48
		3	8.00	5.90	5.46	1.51	0.40	2.73
9	Lopburi (Lumnarai)	1	9.80	7.78	6.54	0.96	0.60	1.62
		2	9.60	7.64	8.40	1.36	0.75	1.19

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
		3	9.60	8.02	8.49	1.29	0.84	1.38
10	Phuket	1	7.70	5.76	7.13	3.67	0.26	0.92
		2	7.80	5.77	5.32	2.13	0.29	0.90
		3	7.80	5.60	6.20	2.95	0.30	1.19
11	Yasothon	1	7.90	5.83	3.63	0.55	0.26	1.31
		2	8.00	5.91	3.57	0.43	0.22	1.24
		3	8.00	5.90	3.60	0.70	0.23	1.38
12	Rayong	1	6.80	5.23	3.01	0.69	0.89	2.50
		2	7.00	5.12	2.38	0.50	0.92	2.30
		3	7.00	5.25	2.99	0.58	0.96	2.35
13	Uthai thani	1	8.80	7.13	2.87	0.35	0.71	1.34
		2	8.80	7.25	2.98	0.37	0.89	1.39
		3	8.80	7.01	3.10	0.54	0.76	1.93
14	Kalasil	1	6.70	5.37	2.77	0.45	0.90	1.75
		2	6.70	5.29	2.82	0.42	0.94	2.35
		3	6.80	5.34	2.53	0.35	0.99	2.18
Grand mean± Pooled SD			**8.08±0.28**	**6.09±0.18**	**4.33±0.55**	**0.98±0.29**	**0.65±0.06**	**1.59±0.18**
Min - Max			**5.20-10.60**	**3.09-9.02**	**1.93-8.49**	**0.35-3.67**	**0.22-1.21**	**0.90-2.73**

Table 18 Pharmacognostic characters (% by weight) of *Harisonia perforata* (Blanco) Merr. root

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
1	Nakhornnayok	1	7.30	5.80	3.47	0.51	0.37	0.90
		2	7.40	5.99	3.73	0.54	0.28	1.03
		3	7.30	5.89	3.54	0.54	0.39	1.04
2	Kanchanaburi	1	8.40	6.43	3.01	0.83	0.55	0.88
		2	8.30	6.37	3.48	1.00	0.63	0.93
		3	8.40	6.35	2.66	0.70	0.56	0.80
3	Yasothon	1	9.10	7.00	1.82	0.23	0.34	0.79
		2	9.20	7.05	1.71	0.21	0.30	0.60
		3	9.20	7.02	1.36	0.20	0.22	0.68
4	Nongkhai (Seaka)	1	8.40	6.55	2.75	0.58	0.67	0.79
		2	8.40	6.63	2.69	0.43	0.87	0.92
		3	8.40	6.52	2.72	0.55	0.87	0.97
5	Nan	1	8.00	6.71	4.06	0.91	0.92	0.74
		2	8.00	6.70	4.23	0.85	0.91	0.97
		3	7.90	6.71	3.88	0.71	1.07	1.14
6	Lopburi (Muang)	1	7.60	6.23	6.56	1.36	1.26	1.08
		2	7.60	6.09	6.20	0.97	1.21	1.32
		3	7.70	6.06	6.43	1.27	1.32	1.27
7	Lopburi (Lumnarai)	1	10.30	7.48	6.13	0.71	1.32	0.69
		2	10.20	7.49	5.59	0.94	1.25	1.14
		3	10.00	7.70	5.85	1.22	1.32	1.28
8	Lumpang	1	5.70	5.13	3.33	0.47	0.67	0.99
		2	6.50	5.27	3.83	0.63	0.74	1.01
		3	6.60	5.13	2.09	0.50	0.64	1.08
9	Petchabun	1	10.20	5.22	3.98	0.65	0.94	0.88
		2	10.10	5.21	4.29	0.83	0.91	0.70

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No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
		3	10.10	5.27	4.45	0.77	0.88	0.79
10	Uthai thani	1	7.10	5.90	3.13	0.55	1.00	0.98
		2	7.10	6.06	3.88	0.62	0.97	0.95
		3	7.00	5.93	3.37	0.45	0.93	0.83
11	Nongkhai (Sriwilai)	1	10.00	6.71	1.98	0.29	0.66	0.56
		2	8.60	6.63	1.83	1.10	0.70	0.79
		3	8.60	6.58	1.83	0.31	0.64	0.68
12	Rayong	1	8.60	6.83	4.51	0.64	0.50	4.06
		2	8.60	6.73	4.21	0.59	0.50	2.89
		3	10.60	6.71	4.28	0.71	0.54	3.52
13	Chiang mai	1	10.40	7.84	3.82	0.57	1.08	1.80
		2	10.40	7.89	3.83	0.99	1.20	1.63
		3	10.40	8.00	3.85	0.78	1.06	1.70
14	Kalasil	1	5.50	5.13	2.84	0.46	1.17	0.84
		2	5.60	5.07	2.54	0.45	1.24	1.39
		3	5.50	5.03	2.45	0.50	1.36	1.25
Grand mean± Pooled SD			**8.34±0.40**	**6.36±0.07**	**3.62-0.32**	**0.67±0.17**	**0.83±0.06**	**1.17±0.21**
Min - Max			**5.50-10.60**	**5.03-8.00**	**1.36-6.56**	**0.20-1.36**	**0.2-1.36**	**0.59-4.06**

Table 19 Pharmacognostic characters (% by weight) of *Ficus racemosa* L. root

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
1	Kanchanaburi	1	8.00	6.86	5.63	0.98	0.34	0.92
		2	8.10	6.64	5.83	1.25	0.52	0.75
		3	8.10	7.03	5.74	0.97	0.35	0.73
2	Nongkhai (Seaka)	1	10.10	7.32	6.99	1.66	0.45	0.92
		2	10.20	7.41	7.68	2.72	0.42	1.00
		3	10.30	7.49	8.37	2.36	0.47	0.93
3	Yasothon	1	8.30	6.19	3.41	0.48	0.10	0.70
		2	8.40	6.15	3.07	0.28	0.07	0.58
		3	8.40	6.29	3.15	0.35	0.07	0.76
4	Songkla	1	8.40	6.39	5.32	0.64	0.59	1.62
		2	8.50	6.31	5.10	0.64	0.55	1.49
		3	8.60	6.39	5.25	0.67	0.53	1.60
5	Nongkhai (Sriwilai)	1	9.00	6.69	6.15	0.70	0.92	1.44
		2	8.90	6.53	7.64	1.40	1.03	1.16
		3	9.10	6.47	7.43	1.14	0.86	1.35
6	Uthai thani	1	8.50	6.01	6.98	1.18	0.49	1.16
		2	8.40	5.99	7.01	1.03	0.49	0.97
		3	8.50	5.96	6.87	1.19	0.45	1.19
7	Petchabun	1	11.20	6.54	5.14	0.71	0.60	1.08
		2	11.00	6.94	5.47	1.00	0.49	1.32
		3	10.90	6.93	5.71	1.06	0.51	1.65
8	Chiang mai	1	7.20	5.75	4.51	0.64	0.82	1.08
		2	7.40	5.74	4.50	0.74	0.79	1.18
		3	7.40	5.76	4.51	0.77	0.90	1.18
9	Lumpang	1	5.30	4.93	6.11	0.98	0.43	1.27
		2	5.40	4.88	6.03	1.17	0.45	0.12

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
		3	5.50	4.88	6.00	1.01	0.45	1.32
10	Lopburi (Lumnarai)	1	9.10	6.52	7.41	1.87	0.34	1.15
		2	9.10	6.60	8.21	2.02	0.32	1.40
		3	8.90	6.55	7.37	1.30	0.34	1.25
11	Nan	1	5.40	6.88	5.53	1.18	0.63	1.31
		2	9.50	6.87	5.51	1.09	0.62	1.40
		3	9.40	6.99	5.42	0.91	0.63	1.65
12	Rayong	1	7.70	5.71	6.19	0.89	0.72	1.68
		2	7.70	5.56	6.62	0.88	0.76	2.25
		3	7.80	5.56	6.36	0.99	0.75	2.20
13	Lopburi (Muang)	1	8.00	6.22	7.42	1.87	0.39	1.12
		2	8.50	6.30	7.21	1.37	0.39	1.35
		3	8.50	6.41	7.00	1.27	0.37	1.51
14	Kalasil	1	9.20	5.65	4.66	0.69	0.99	1.02
		2	9.20	5.68	4.42	0.62	0.99	1.41
		3	9.10	5.75	4.62	0.69	0.99	1.20
Grand mean± Pooled SD			**8.48±0.64**	**6.28±0.01**	**5.94±0.34**	**1.08±0.24**	**0.56±0.04**	**1.22±0.42**
Min - Max			**5.30-11.20**	**4.88-7.49**	**3.07-8.37**	**0.28-2.72**	**0.07-1.03**	**0.12-2.25**

Table 20 Pharmacognostic characters (% by weight) of *Tiliacora triandra* (Colebr.) Diels root.

No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
1	Petchabun	1	11.20	11.40	3.80	0.96	1.19	1.94
		2	11.40	11.04	2.97	0.73	1.20	1.89
		3	11.20	11.43	5.62	1.75	1.17	2.11
2	Lopburi (Muang)	1	8.30	7.12	5.88	1.53	1.58	1.99
		2	8.40	6.97	5.34	1.52	1.62	2.36
		3	8.50	7.27	5.37	1.38	0.83	2.32
3	Lopburi (Lumnarai)	1	9.80	9.61	4.83	1.29	1.62	1.51
		2	5.70	9.75	3.87	1.09	1.74	2.11
		3	10.00	9.43	4.95	0.93	1.71	1.90
4	Nan	1	8.20	7.10	4.36	0.98	1.40	1.77
		2	8.20	6.61	4.39	1.17	1.62	2.22
		3	8.30	6.60	4.53	1.15	1.55	2.19
5	Rayong	1	8.00	6.23	3.59	0.77	1.34	2.05
		2	8.00	6.29	3.96	0.90	1.32	2.35
		3	8.00	6.23	3.45	0.73	1.36	2.41
6	Nongkhai (Sriwilai)	1	8.60	7.27	5.23	1.49	1.50	2.46
		2	8.10	7.29	4.86	1.40	1.58	2.25
		3	8.48	7.23	4.76	1.39	1.62	1.94
7	Lumpang	1	4.80	2.72	3.26	0.71	1.34	2.16
		2	5.00	2.76	3.33	0.72	1.46	2.86
		3	5.10	2.67	3.23	0.53	1.40	2.33
8	Uthai thani	1	7.50	5.10	4.00	0.74	1.57	1.74
		2	7.40	5.18	3.93	0.77	1.55	2.28
		3	7.40	4.98	4.24	0.84	1.54	2.59
9	Nongkhai (Sea ka)	1	9.70	8.27	4.19	1.57	0.52	2.09
		2	9.80	8.26	4.28	1.45	0.53	2.36

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No	Sources	Lot.	Moisture content	Loss on drying	Total ash content	Acid insoluble ash content	Extractive value	
							EtOH	Water
		3	9.70	8.32	4.39	1.51	0.50	2.33
10	Lopburi (Tha voung	1	7.90	6.60	4.17	0.91	0.66	1.99
		2	7.80	6.61	4.11	0.91	0.63	1.82
		3	8.00	6.61	4.35	0.92	0.62	2.08
11	Yasothon	1	7.10	5.36	6.80	1.97	0.57	1.50
		2	7.00	5.30	7.18	1.81	0.63	1.47
		3	7.20	5.66	7.30	2.29	0.63	1.67
12	Nakhornnayok	1	7.30	6.08	4.94	1.51	0.60	2.18
		2	7.00	6.14	4.73	1.65	0.60	2.08
		3	7.40	6.10	5.10	1.88	0.58	2.18
13	Chiang mai	1	8.60	6.49	3.20	0.97	1.53	1.92
		2	8.80	6.52	3.27	0.87	1.53	2.44
		3	8.90	6.52	3.25	0.89	1.54	2.29
14	Kalasil	1	8.10	6.12	3.03	0.51	1.60	2.68
		2	8.00	6.18	3.09	0.63	1.59	3.17
		3	8.10	6.06	3.16	0.69	1.66	3.23
Grand mean± Pooled SD			**8.14±0.66**	**6.80±0.13**	**4.39±0.43**	**1.15±0.18**	**1.12±0.13**	**2.17±0.25**
Min - Max			**4.80-11.40**	**2.67-11.43**	**2.97-7.30**	**0.51-2.29**	**0.50-1.74**	**1.47-3.23**

APPENDIX B

Data from the comparisons between retention time and area under peak from each peaks (Batch 1- Batch 12)for multivariate analysis.

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	1	2	3	4	5	6
BLW1-1	480.34906	5704.63818	191.25595	285.24170	417.27335	680.29730
BLW1-2	476.66873	5798.77980	194.16803	280.75616	414.80618	789.29291
BLW1-3	495.59808	5951.19873	205.25792	287.87988	428.31769	674.05176
BLW2-1	215.18517	5871.20801	170.43932	222.76312	525.62097	142.22246
BLW2-2	189.65518	5854.75781	119.17624	155.66499	414.49936	0.00000
BLW2-3	235.30714	6247.14063	181.12112	225.10988	442.75058	130.54567
BLW3-1	188.94850	6047.12012	328.92200	496.51480	428.20477	0.00000
BLW3-2	202.20763	6241.52881	339.63309	516.25787	445.46292	0.00000
BLW3-3	216.04082	5991.74805	324.52420	489.33414	469.95172	0.00000
BLW4-1	196.18742	5806.53955	549.62701	260.97400	366.34027	0.00000
BLW4-2	203.74844	5879.52539	561.90723	268.63428	375.63174	0.00000
BLW4-3	222.82135	6008.71045	571.60370	263.17236	373.87491	0.00000
BLW5-1	235.64508	8052.37170	462.94049	402.52115	333.63739	274.75659
BLW5-2	244.03136	7945.23193	458.73361	400.67587	336.61386	276.91968
BLW5-3	250.27872	8002.60986	458.79343	410.04871	330.63792	214.06871
BLW6-1	271.23972	7442.60645	453.96625	391.23071	439.73679	306.36096
BLW6-2	285.10153	7559.89648	464.51422	398.32068	437.87454	311.38107
BLW6-3	315.84808	7767.21289	598.87423	413.02933	408.62006	296.98746
BLW7-1	283.56134	7531.76367	435.97992	401.82458	451.91159	318.33679
BLW7-2	291.66351	7634.10791	439.70807	400.65717	422.06268	317.01395
BLW7-3	323.11868	7818.84619	579.23719	415.53259	311.55579	301.73895
BLW8-1	227.34666	6144.60010	309.91824	343.54230	413.39243	242.19423
BLW8-2	238.20630	6306.61670	319.11200	352.83771	420.76862	253.11067
BLW8-3	256.23846	6372.22119	329.11960	359.02563	370.42075	241.43063
BLW9-1	217.39920	6579.93994	330.52585	323.42484	433.42938	210.78516
BLW9-2	231.54388	6759.37402	342.30194	336.47351	449.37598	222.59308
BLW9-3	238.51291	6560.79443	332.06503	321.01096	310.05524	213.97035
BLW10-1	238.65775	6658.76904	341.09561	356.92349	509.23459	256.45557
BLW10-2	242.30725	6538.84863	338.61823	351.53543	503.42001	260.74316
BLW10-3	273.71921	6977.67285	364.42114	377.65256	384.26953	256.65067
BLW11-1	225.41554	6269.95947	305.08600	321.55414	414.44965	239.71109
BLW11-2	227.09122	6182.39258	303.35974	316.12903	480.09821	242.62187
BLW11-3	256.06436	6493.99170	321.72043	337.88434	366.14200	244.02135
BLW12-1	226.33678	6366.80127	344.75629	335.67038	478.10284	244.34663
BLW12-2	239.76593	6563.05322	356.67752	346.64532	495.11618	255.56367
BLW12-3	253.32730	6438.94287	353.69919	340.47058	346.89590	243.90282

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	7	8	9	10	11	12
BLW1-1	183.83476	0.00000	0.00000	304.67349	375.30344	1924.05531
BLW1-2	203.65451	0.00000	0.00000	368.06631	387.89917	1923.86776
BLW1-3	188.43198	0.00000	0.00000	469.48361	457.53659	1745.08500
BLW2-1	233.44823	219.45839	148.59346	157.13731	360.36989	372.12592
BLW2-2	172.62694	0.00000	102.78662	111.17088	166.46219	308.44443
BLW2-3	258.38602	195.13893	198.52087	203.60446	322.80273	341.82074
BLW3-1	304.48145	0.00000	206.15642	155.94804	412.29108	265.94531
BLW3-2	320.93408	0.00000	214.90929	167.47200	387.25107	280.66406
BLW3-3	303.42313	0.00000	217.22563	152.61269	331.08185	177.87332
BLW4-1	435.53229	0.00000	379.53348	285.98001	385.72309	405.83398
BLW4-2	444.15115	0.00000	378.19306	301.53845	477.87862	424.62302
BLW4-3	513.07581	0.00000	372.80835	303.49869	411.47339	321.19904
BLW5-1	461.47672	410.50304	318.22345	291.54483	383.11861	571.87817
BLW5-2	461.61719	423.61415	323.41101	293.93881	382.73424	573.56812
BLW5-3	409.57550	417.77932	269.88083	359.93430	320.84833	515.97577
BLW6-1	398.23581	265.15959	370.86072	333.48944	409.85291	764.62543
BLW6-2	381.90131	282.19518	432.20975	334.35226	425.93311	870.68793
BLW6-3	442.08502	216.93079	390.44812	398.44476	470.90669	747.90125
BLW7-1	401.01807	280.02155	383.39517	426.57098	431.00470	794.17480
BLW7-2	374.98938	289.11779	388.12979	338.31439	434.40277	889.10529
BLW7-3	376.24826	227.01476	400.46884	392.44492	481.79919	761.32843
BLW8-1	360.17178	248.55593	366.78128	291.34186	398.63474	531.57916
BLW8-2	358.67685	256.43837	371.95261	298.78726	410.10093	632.65991
BLW8-3	381.35486	153.90445	331.27167	359.73575	342.32724	602.02559
BLW9-1	347.64185	220.35504	320.34177	243.84634	348.88968	512.68578
BLW9-2	347.17853	233.71746	325.83276	253.13611	359.54852	530.67487
BLW9-3	244.39560	130.72269	272.13504	294.66843	294.12274	390.03015
BLW10-1	343.30716	266.75663	402.13541	308.06573	418.52884	554.31873
BLW10-2	373.93286	275.63930	383.24948	305.72174	412.65710	638.68048
BLW10-3	407.15842	162.47906	354.32684	395.76563	491.47568	525.55365
BLW11-1	345.73660	251.67156	370.76294	283.39252	400.00833	594.97046
BLW11-2	337.99109	264.63062	349.19614	285.78232	394.80576	591.22522
BLW11-3	359.42899	146.60843	323.73563	361.92551	358.98825	477.77814
BLW12-1	325.85425	248.85665	379.18140	287.65256	397.60712	523.55334
BLW12-2	337.93903	265.22818	383.82471	301.91025	406.23279	631.22693
BLW12-3	373.31235	143.86720	323.33273	356.40732	352.09802	478.46719

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	13	14	15	16	17	18
BLW1-1	0.00000	1436.93387	2083.37134	0.00000	2897.40276	754.96029
BLW1-2	0.00000	1413.40036	1926.65881	0.00000	2895.26620	738.85028
BLW1-3	0.00000	1381.89148	2251.60821	0.00000	3100.13883	634.92211
BLW2-1	254.40462	257.13831	111.39204	538.93256	331.70514	188.89577
BLW2-2	192.31223	633.98869	0.00000	466.94052	251.97296	142.93317
BLW2-3	284.36984	666.94757	147.72043	644.18097	330.28784	189.67365
BLW3-1	105.02019	297.22839	277.63541	447.17958	398.45551	218.29729
BLW3-2	102.74120	322.50961	287.24530	471.17882	420.31592	231.71719
BLW3-3	220.12482	327.12949	241.34409	385.56839	457.28461	214.60776
BLW4-1	120.78842	404.32657	538.04465	403.12628	333.11633	307.44333
BLW4-2	113.21208	429.40112	534.70541	409.94885	337.99860	322.37375
BLW4-3	318.27927	352.18976	477.53270	444.57736	469.89648	325.88101
BLW5-1	246.59541	379.01199	810.50403	717.72687	200.58168	301.06345
BLW5-2	237.28455	388.58157	812.48941	722.53564	283.22238	309.63620
BLW5-3	306.58212	352.16580	609.08114	900.07635	260.22626	306.74536
BLW6-1	396.92035	384.64966	993.15094	1546.09692	224.55139	369.43710
BLW6-2	380.39984	404.76602	1033.94214	1565.22607	329.42627	382.29938
BLW6-3	476.48793	365.93872	841.40356	1870.67429	363.82114	394.56598
BLW7-1	417.25006	401.52510	1025.91943	1598.43579	235.25911	385.78705
BLW7-2	393.10272	421.29325	1055.99194	1595.16900	339.86957	388.58105
BLW7-3	483.47095	384.93920	847.38354	1914.44434	354.90131	394.00299
BLW8-1	366.67914	222.12386	1045.18007	1346.23888	222.02840	353.40027
BLW8-2	358.17801	247.86490	1073.52378	1380.42891	323.72748	363.97681
BLW8-3	454.33090	349.98849	1027.74676	1364.28330	339.87653	372.86230
BLW9-1	284.62678	192.19588	834.82679	1091.11050	191.25858	295.78516
BLW9-2	281.17566	208.07263	867.01609	1122.25779	277.58914	304.92694
BLW9-3	348.49081	283.30881	786.40980	1074.75183	263.29971	298.78513
BLW10-1	393.13956	220.95532	1123.05172	1464.60083	222.41852	374.98764
BLW10-2	369.37173	230.21725	1113.39865	1442.01992	322.56497	369.79343
BLW10-3	491.11081	367.34021	766.89001	1876.48752	341.78946	403.92343
BLW11-1	363.31189	218.68022	1083.44444	1356.66336	215.40262	356.70917
BLW11-2	345.24344	226.57957	1076.48248	1338.46731	309.39835	352.39807
BLW11-3	453.52765	346.45435	1060.04178	1396.69604	316.46518	377.66040
BLW12-1	355.15100	211.64821	1030.36670	1384.48629	209.08957	346.93976
BLW12-2	352.70044	225.47795	1080.35993	1427.48190	316.37338	359.53314
BLW12-3	431.38095	338.45093	982.76101	1396.38977	301.76593	357.12073

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	19	20	21	22	23	24
BLW1-1	426.43787	1102.18677	354.02124	0.00000	0.00000	2170.81720
BLW1-2	435.85056	1009.20099	383.23730	0.00000	0.00000	2078.49350
BLW1-3	0.00000	1199.08142	588.01149	0.00000	0.00000	1987.15692
BLW2-1	228.52718	354.37888	234.22641	195.57787	106.62641	509.41278
BLW2-2	172.84735	277.54849	163.58376	145.12059	209.88858	288.75052
BLW2-3	194.57150	395.18622	138.02943	251.97113	438.50159	361.17737
BLW3-1	189.28174	348.52297	270.00711	143.84343	534.85309	0.00000
BLW3-2	198.13065	367.73886	290.83063	149.12053	571.25250	0.00000
BLW3-3	194.20297	363.74283	257.47270	148.69633	523.92609	0.00000
BLW4-1	379.11856	232.77005	366.31213	187.98265	1055.77319	0.00000
BLW4-2	389.04962	241.50800	478.12389	185.83978	1088.91357	0.00000
BLW4-3	411.71351	232.64720	388.04184	184.48218	1068.36358	0.00000
BLW5-1	351.12253	305.97485	372.90610	344.44003	517.79123	349.62543
BLW5-2	343.15146	313.82553	387.19901	342.24799	516.33902	362.99701
BLW5-3	278.89474	352.37647	227.44591	124.05292	803.18500	330.69510
BLW6-1	274.65656	569.80540	290.86699	304.66843	1057.21484	369.26242
BLW6-2	394.31302	473.15521	318.81635	277.59689	1075.77417	373.64883
BLW6-3	323.65756	570.71860	321.68582	299.89346	995.65515	358.61154
BLW7-1	286.59161	465.06314	302.88328	316.20300	1096.18250	378.42664
BLW7-2	400.49048	481.66931	319.38646	283.41711	1093.49597	383.53629
BLW7-3	336.11679	575.56731	311.52374	319.51196	1000.83826	365.56229
BLW8-1	273.04898	514.33033	292.65581	284.90939	996.55744	314.18350
BLW8-2	378.28992	432.35742	310.40788	277.62878	1004.60968	329.03284
BLW8-3	317.59531	511.05446	306.18805	303.68546	1222.91565	259.45334
BLW9-1	237.54173	341.15945	254.29004	230.67863	814.44298	256.78543
BLW9-2	324.60590	362.53366	269.80911	224.98640	824.90082	271.82605
BLW9-3	260.10892	414.28576	252.67783	234.12978	953.69664	211.09135
BLW10-1	279.64426	542.99235	310.72215	294.12857	1055.85999	329.14734
BLW10-2	376.14865	440.00504	316.81243	272.27542	1017.23871	339.04779
BLW10-3	333.42447	542.48398	335.03103	314.84790	979.63971	341.45184
BLW11-1	273.20261	519.42206	299.10027	281.05820	1023.59357	326.98715
BLW11-2	366.73489	420.33618	303.32467	265.62994	994.49884	329.00186
BLW11-3	323.35220	511.01297	317.51230	292.37949	939.10620	324.85422
BLW12-1	256.65045	516.81054	284.48245	274.83261	972.48663	306.40561
BLW12-2	368.65741	436.15833	302.23795	266.37448	987.76196	324.79498
BLW12-3	306.41602	494.91248	293.17744	281.94458	1171.72043	256.38828

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	25	26	27	28	29	30
BLW1-1	**111086000**	**126498000**	3745.96899	0.00000	0.00000	541.29651
BLW1-2	8796.20410	**104737000**	3334.68311	0.00000	0.00000	507.31039
BLW1-3	9630.48438	**113908000**	3794.23981	0.00000	0.00000	555.71582
BLW2-1	2791.87915	1755.31689	885.85284	209.13687	131.51494	454.60480
BLW2-2	2035.64880	1312.46228	705.60742	242.94905	0.00000	375.91605
BLW2-3	2188.10522	1615.55432	0.00000	191.83885	143.96938	468.33578
BLW3-1	741.07129	349.84424	393.04611	241.52419	254.56906	456.41779
BLW3-2	765.44208	396.06815	405.88339	257.35645	258.59848	480.50101
BLW3-3	427.52692	644.93842	395.86874	243.99097	259.70459	482.57687
BLW4-1	995.43890	546.59619	568.39001	186.30304	738.72626	352.00653
BLW4-2	1110.38171	557.57751	586.26373	195.65373	766.53949	353.56689
BLW4-3	806.19678	891.84497	738.45960	200.48351	776.73895	370.99695
BLW5-1	2092.92700	1216.79626	1154.29517	183.78831	646.11414	258.06989
BLW5-2	2077.00562	1239.38477	1138.03418	203.62633	664.52020	259.28760
BLW5-3	2011.53284	1380.93872	1028.28589	245.55164	663.06400	263.12869
BLW6-1	3947.08301	1599.28687	1612.10352	316.98999	256.58737	287.47870
BLW6-2	4069.02490	1695.34167	1622.40369	316.69412	265.80768	285.80896
BLW6-3	4085.51416	1872.19812	1716.56506	353.94113	283.22003	273.32492
BLW7-1	0.00000	1610.61072	1645.87830	330.19955	264.14856	297.49719
BLW7-2	4045.37231	1712.04578	1640.11401	317.93790	266.08698	289.01053
BLW7-3	3992.11914	1860.45154	1725.14746	353.44724	283.25931	272.84592
BLW8-1	3037.71704	1249.71716	1377.06555	324.23813	239.39247	267.56906
BLW8-2	3089.46802	1365.71924	1400.00012	330.34680	244.20599	270.47107
BLW8-3	3076.52100	1484.75903	1309.46570	353.71378	261.05081	241.54823
BLW9-1	2383.68579	968.44708	1099.51636	271.29050	217.71797	229.44310
BLW9-2	2457.21997	1237.88757	1122.11987	279.54037	221.62813	235.59862
BLW9-3	2247.38867	1122.88867	988.22742	285.71341	219.64987	198.77879
BLW10-1	3314.08643	1585.60596	1490.31189	331.98157	254.16617	268.96976
BLW10-2	3232.88013	1607.36511	1446.63562	328.45483	247.41365	265.78598
BLW10-3	3432.34131	1622.72876	1455.56030	369.14209	275.62048	243.67087
BLW11-1	3310.96484	1372.66541	1436.20740	317.87650	248.69887	258.59644
BLW11-2	3265.18799	1644.69910	1403.96985	314.15427	248.29370	255.42671
BLW11-3	3370.08008	1619.67322	1379.67383	345.24557	259.77835	228.37424
BLW12-1	2921.23193	1215.37427	1358.19910	312.65372	239.17339	254.38928
BLW12-2	3004.60352	1542.20410	0.00000	324.18115	248.53999	262.08118
BLW12-3	2899.26318	1427.49805	1267.99963	328.82043	243.22543	219.86063

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	31	32	33	34	38	39
BLW1-1	699.35596	0.00000	377.28815	906.81798	997.74969	0.00000
BLW1-2	702.40320	0.00000	339.62976	827.68030	860.64905	0.00000
BLW1-3	739.31384	0.00000	469.58264	861.18207	1011.54303	0.00000
BLW2-1	195.44554	0.00000	280.00677	144.14449	738.38409	439.70604
BLW2-2	146.74306	0.00000	213.13266	0.00000	584.29047	333.91895
BLW2-3	174.30498	0.00000	259.34741	132.04143	721.41089	409.01694
BLW3-1	190.71593	0.00000	177.04652	596.07886	873.05383	1413.36047
BLW3-2	206.96384	0.00000	190.84937	625.36365	905.01379	1453.14502
BLW3-3	168.95839	0.00000	238.87253	515.48901	892.79211	1623.06116
BLW4-1	425.88721	0.00000	153.82246	559.75850	744.12415	1034.86536
BLW4-2	429.98705	0.00000	152.22826	671.17621	772.78442	1058.81079
BLW4-3	436.94147	0.00000	313.24149	449.94390	785.31366	1128.34851
BLW5-1	260.58017	238.60739	248.12126	557.60295	636.14490	2672.73340
BLW5-2	268.29318	254.21721	251.92775	565.01241	651.36084	2674.20605
BLW5-3	250.10445	274.65042	209.62444	551.86083	670.50012	2665.24365
BLW6-1	268.74442	537.30063	583.03503	272.84900	652.21968	1127.10681
BLW6-2	268.84897	592.43170	559.89441	297.48389	657.21369	1171.47229
BLW6-3	288.82324	948.52856	223.58333	299.28455	686.64970	1163.70886
BLW7-1	278.98584	394.63641	595.40680	285.56827	522.73138	1134.30212
BLW7-2	269.60049	424.54004	568.35498	298.41730	532.86029	1157.97913
BLW7-3	295.91217	952.67108	224.06287	294.09991	563.95142	1136.03516
BLW8-1	265.35480	394.77237	550.67621	253.02663	521.52185	1538.21545
BLW8-2	268.48914	595.47943	546.56763	270.71948	544.37543	1692.12122
BLW8-3	288.01395	1136.18750	0.00000	272.32031	583.79712	1594.36414
BLW9-1	225.81905	345.05792	454.96667	234.00258	454.82605	1239.66699
BLW9-2	230.12923	506.15115	461.81427	248.27989	473.23822	1286.15442
BLW9-3	236.64583	914.90857	0.00000	241.43964	489.57858	1236.05896
BLW10-1	269.41489	614.23779	564.51910	274.62604	534.47974	1416.58374
BLW10-2	265.50702	615.60455	550.48560	279.31415	532.05725	1409.01904
BLW10-3	300.45969	768.46155	465.54233	302.50827	702.75690	1505.98328
BLW11-1	263.82849	586.31134	521.75195	274.00427	515.80481	1484.60510
BLW11-2	259.73932	580.18372	514.84894	282.04153	516.29163	1481.36755
BLW11-3	285.27533	710.66949	420.35635	298.86240	657.85919	1538.18481
BLW12-1	255.03508	578.03577	528.43365	258.63141	505.53452	1342.31714
BLW12-2	263.75803	610.12305	535.53540	281.03549	533.87988	1401.53052
BLW12-3	270.83838	690.56781	419.24881	278.04019	638.36072	1371.30408

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	40	41	42	43	44	45
BLW1-1	2711.07516	412.60091	470.99380	502.55011	135.32820	224.20357
BLW1-2	2656.32590	417.21280	479.91599	520.05408	143.52818	219.29145
BLW1-3	2544.57016	303.23717	481.39026	525.16040	134.38177	237.67868
BLW2-1	208.06924	545.06714	307.16025	557.70209	233.91269	0.00000
BLW2-2	142.75955	313.73715	187.06909	420.45166	118.42435	0.00000
BLW2-3	363.15634	228.43893	328.62119	560.32739	236.41068	0.00000
BLW3-1	0.00000	338.97501	550.27374	704.55627	258.47015	231.41046
BLW3-2	0.00000	277.76538	563.79376	736.40930	271.64578	237.59015
BLW3-3	0.00000	190.77409	552.84937	713.56258	267.48193	235.82040
BLW4-1	0.00000	574.84021	708.07788	566.77396	293.05624	394.54435
BLW4-2	0.00000	596.25751	728.26617	593.21051	305.50049	416.41484
BLW4-3	0.00000	373.74658	742.98236	607.92731	314.90201	417.41666
BLW5-1	287.68570	293.15228	1228.52209	532.78925	365.50639	432.72142
BLW5-2	432.55713	303.31512	1227.67822	556.86865	403.92036	469.93082
BLW5-3	369.19867	210.96724	1234.36523	554.63220	365.19044	449.39972
BLW6-1	268.86639	303.85516	596.24231	370.68210	230.85350	196.31776
BLW6-2	298.82214	311.75903	617.55444	469.86154	259.53937	217.94267
BLW6-3	430.09442	151.16537	633.11298	471.84174	257.44217	220.40352
BLW7-1	284.99899	314.40460	622.44873	409.02405	276.74573	226.50288
BLW7-2	294.96109	295.85056	619.92670	499.63943	281.29950	229.28881
BLW7-3	421.98062	152.81346	635.42908	508.88977	288.81995	237.82674
BLW8-1	326.29318	364.88348	918.84894	421.81592	258.81970	216.72102
BLW8-2	352.85406	379.90558	949.01593	451.53223	287.79071	238.24393
BLW8-3	417.61026	189.67307	972.84607	543.59674	296.12448	238.05479
BLW9-1	285.28870	317.91721	764.39185	501.46084	317.07162	368.57046
BLW9-2	309.57840	328.57520	789.20465	541.92285	347.43536	406.26211
BLW9-3	345.35449	188.63097	776.79462	510.78760	335.88654	379.04721
BLW10-1	336.96301	338.78687	826.37103	414.72775	300.37833	350.11128
BLW10-2	347.74640	337.08517	820.42175	421.68622	320.76868	368.41306
BLW10-3	429.43777	196.31653	877.80219	535.41919	340.08963	378.13508
BLW11-1	331.64691	395.40891	963.01648	573.11578	338.50464	394.84765
BLW11-2	346.76465	390.79221	955.36707	576.24358	344.17413	405.85801
BLW11-3	413.06705	193.70560	1004.41779	592.36548	380.55966	418.87671
BLW12-1	322.34396	354.79599	870.05042	562.57827	344.68076	401.05864
BLW12-2	350.45782	366.37891	895.25488	576.95598	351.93494	411.93861
BLW12-3	397.05502	195.00102	898.52972	573.40289	381.30698	419.30221

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	46	47	48	49	51	53
BLW1-1	221.96252	212.65285	220.55472	150.37463	197.34549	671.49835
BLW1-2	179.34242	151.81769	238.87192	103.53192	207.63261	707.58063
BLW1-3	177.45659	222.35161	235.69859	191.33640	219.52760	720.85950
BLW2-1	201.31250	162.11260	216.81555	266.47116	406.74660	0.00000
BLW2-2	119.81766	0.00000	0.00000	145.71185	176.40332	0.00000
BLW2-3	203.11249	164.27591	0.00000	254.42443	427.07388	0.00000
BLW3-1	205.93333	205.76157	297.58122	272.76111	289.34152	0.00000
BLW3-2	211.67258	220.48602	301.36264	286.92801	318.43087	0.00000
BLW3-3	204.21915	216.32726	371.94978	297.05414	374.28638	0.00000
BLW4-1	521.54419	345.27695	363.41461	334.21667	214.78577	0.00000
BLW4-2	531.82520	363.73361	379.32141	345.71075	361.59485	0.00000
BLW4-3	534.72888	367.39792	385.79447	334.35303	124.59232	0.00000
BLW5-1	345.21387	379.83460	224.46913	302.95813	240.81926	469.84076
BLW5-2	347.92368	410.65444	252.70374	329.99792	105.08463	511.78848
BLW5-3	291.85846	392.23917	246.83133	332.08749	105.18276	495.33383
BLW6-1	333.31845	114.57375	164.84825	245.46260	289.75595	382.86505
BLW6-2	287.83438	109.64833	182.37001	282.98495	338.36036	423.90854
BLW6-3	269.97354	190.15115	181.29303	288.85223	385.17374	421.89371
BLW7-1	378.03500	116.88708	278.73734	254.62115	330.70264	427.21262
BLW7-2	305.13519	118.61325	283.25754	249.47958	339.25797	432.68680
BLW7-3	293.64612	123.71783	192.47501	279.99039	408.10501	446.27957
BLW8-1	440.62704	268.60211	267.61124	241.70142	326.79575	436.48103
BLW8-2	398.40524	294.67310	288.35449	256.42792	358.48865	454.80826
BLW8-3	381.92108	272.32712	191.86623	275.93961	418.81317	468.20627
BLW9-1	436.08704	307.22528	294.60852	263.42703	333.60901	471.66423
BLW9-2	494.75412	323.50180	320.93472	280.23914	359.68628	485.07253
BLW9-3	334.94702	238.45624	301.45572	283.27988	417.57175	484.75069
BLW10-1	446.36438	294.56686	294.84979	265.78036	325.12671	466.50785
BLW10-2	479.93494	302.46057	309.77673	279.37988	356.22198	472.47699
BLW10-3	370.63837	266.11682	320.34308	305.10168	434.11722	506.08132
BLW11-1	499.43356	325.20853	331.88492	286.51114	342.48734	541.29834
BLW11-2	513.89117	331.46939	340.51682	297.96518	374.20541	548.97420
BLW11-3	404.75934	285.45523	251.39020	320.63940	452.25128	580.05434
BLW12-1	496.85495	335.35562	326.55743	293.46326	343.41376	502.88022
BLW12-2	518.25415	341.73386	337.22620	301.87411	359.05490	508.55498
BLW12-3	392.56479	286.45950	343.47482	327.07965	436.49738	537.27718

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	54	55	56	57	58	59
BLW1-1	340.61569	210.13805	0.00000	715.13489	169.43153	239.31291
BLW1-2	361.29521	223.86240	163.50757	743.82971	173.66121	238.99045
BLW1-3	369.30823	232.13544	237.66107	756.60919	177.98126	275.91736
BLW2-1	138.09650	373.78351	290.47131	337.54688	272.72617	260.08325
BLW2-2	0.00000	181.97603	166.99751	210.01035	178.70013	200.86974
BLW2-3	145.97437	394.37164	292.91394	361.14209	255.50580	306.24609
BLW3-1	375.04166	319.24268	207.04320	914.65259	0.00000	278.98770
BLW3-2	426.72672	301.91961	219.73512	948.51288	0.00000	309.15262
BLW3-3	427.81873	316.85098	232.26718	926.88171	0.00000	352.24353
BLW4-1	189.56866	938.35405	264.28958	667.00128	329.35583	225.97749
BLW4-2	196.41632	965.61237	275.20270	681.99890	327.89969	258.08615
BLW4-3	206.58009	985.05652	281.50616	713.56042	326.90436	324.01700
BLW5-1	501.82172	334.01718	239.07549	2123.62744	237.51646	248.45300
BLW5-2	482.74689	426.83517	270.52878	2153.65493	262.34467	353.80380
BLW5-3	474.37961	408.47888	251.75012	2140.02295	235.43500	354.29889
BLW6-1	340.52957	321.27441	222.33061	1196.41528	240.92949	205.13269
BLW6-2	367.27194	354.99649	245.84171	1245.41870	307.60281	272.11169
BLW6-3	373.50882	357.66852	237.42577	1368.13904	255.52284	340.86890
BLW7-1	369.40002	336.38489	249.86603	1170.93811	272.74332	178.03880
BLW7-2	373.50989	342.81223	244.98564	1168.63867	267.23044	258.52209
BLW7-3	385.90125	375.53610	255.96210	1231.56177	261.55420	329.04578
BLW8-1	323.54337	353.88547	253.43805	1824.54344	308.24377	442.06516
BLW8-2	354.25290	374.70172	265.51886	1874.43402	298.04047	224.65294
BLW8-3	361.32581	388.64456	267.92734	1846.64323	310.16672	311.98175
BLW9-1	376.48297	324.45319	240.86981	1391.05005	322.23383	128.06160
BLW9-2	405.39297	349.09995	253.89531	1438.75403	295.17328	241.87471
BLW9-3	392.03021	351.57364	247.49190	1331.33179	305.18823	299.30661
BLW10-1	388.13580	333.99710	253.60260	1670.35424	319.21866	434.70129
BLW10-2	407.28088	345.55365	259.79303	1664.07067	294.84164	256.21185
BLW10-3	417.26218	281.68402	266.35291	1689.70700	312.21872	340.69974
BLW11-1	457.46136	383.08682	266.14261	2072.42883	336.33661	547.18317
BLW11-2	469.86713	386.29245	273.24576	1972.78223	329.41595	217.39195
BLW11-3	484.66348	422.98569	286.25043	2072.16967	351.19919	341.12372
BLW12-1	433.15851	341.43011	254.60843	1668.98572	332.02606	524.72961
BLW12-2	434.92065	358.88409	250.84767	1807.95220	318.83688	239.22249
BLW12-3	453.24866	380.84326	269.67450	1736.12130	365.01334	339.81339

Table 21 Raw data, that from the comparisons between retention time and area under peak from each peaks for multivariate analysis.(cont.)

	60	61	62	63
BLW1-1	425.90878	0.00000	156.92006	196.75336
BLW1-2	326.70914	0.00000	160.83342	202.76497
BLW1-3	385.98746	0.00000	180.03456	192.96254
BLW2-1	315.84225	814.42419	478.51219	287.29376
BLW2-2	191.75084	603.29701	151.34839	146.38747
BLW2-3	396.41901	735.00055	423.55701	113.57089
BLW3-1	148.44946	696.49625	290.60304	162.48267
BLW3-2	136.70459	737.53054	305.94732	167.36296
BLW3-3	171.30782	594.53490	435.61454	107.37234
BLW4-1	724.27667	601.58862	0.00000	567.23892
BLW4-2	741.81091	634.73877	0.00000	593.07745
BLW4-3	518.10910	685.02972	0.00000	780.35583
BLW5-1	514.38112	512.53833	344.91978	217.05165
BLW5-2	476.73420	616.93274	396.12711	262.59988
BLW5-3	196.35986	641.02143	372.42780	181.65645
BLW6-1	733.59094	390.92538	314.67462	247.57130
BLW6-2	765.24786	541.19362	351.53610	266.05563
BLW6-3	416.55940	591.19746	941.98676	0.00000
BLW7-1	777.18115	456.35370	348.39145	267.75946
BLW7-2	708.57532	452.28818	338.54089	280.64166
BLW7-3	229.09160	726.50061	742.47797	226.21892
BLW8-1	610.56970	470.74127	267.00500	631.38129
BLW8-2	900.88385	506.15826	286.72464	668.75854
BLW8-3	268.44724	769.98693	1071.86377	271.75320
BLW9-1	810.31030	457.61435	264.15524	590.51773
BLW9-2	769.10419	492.28372	284.41479	625.98196
BLW9-3	239.23726	696.11035	1096.80450	240.30843
BLW10-1	568.69354	465.73944	268.07346	618.45358
BLW10-2	817.84357	481.81946	281.55634	644.32181
BLW10-3	426.55923	507.74915	1050.27368	263.14420
BLW11-1	601.08359	522.99591	290.90558	664.58692
BLW11-2	968.78137	554.25519	310.61270	543.53911
BLW11-3	517.18076	581.46820	1310.35278	287.80109
BLW12-1	560.66544	494.15494	275.76367	620.50152
BLW12-2	860.94775	515.01910	280.33838	629.85150
BLW12-3	480.98821	529.74927	1230.95605	269.55069

APPENDIX C

Figure of HPLC chromatogram and 3D-HPLC profile of

BLW 1- BLW12

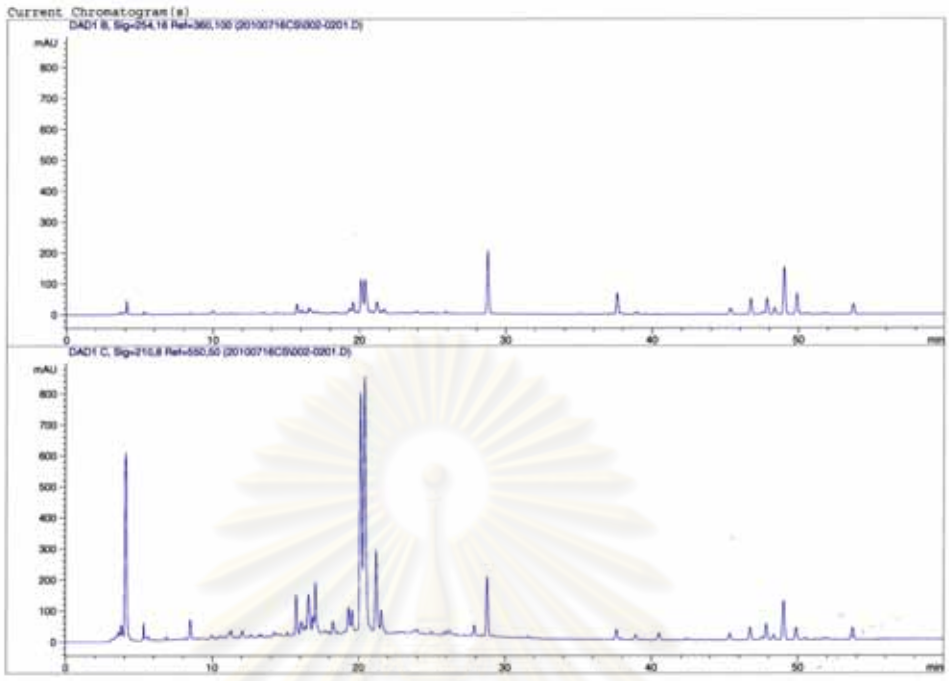

Figure 81 HPLC Chromatogram of BLW 1

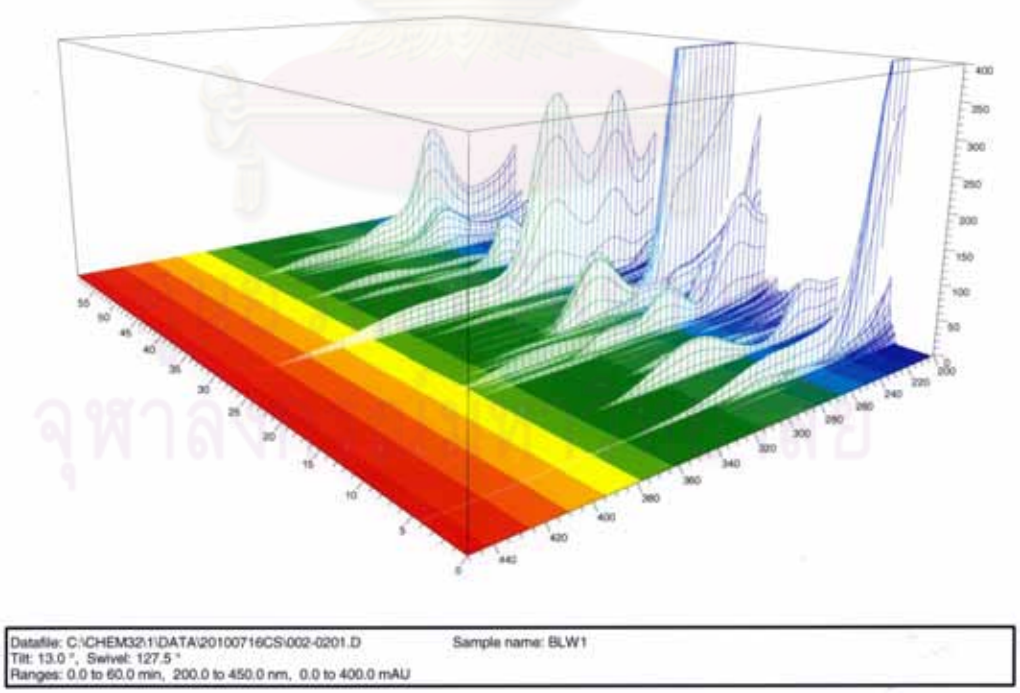

Figure 82 3D-HPLC Profile of BLW 1

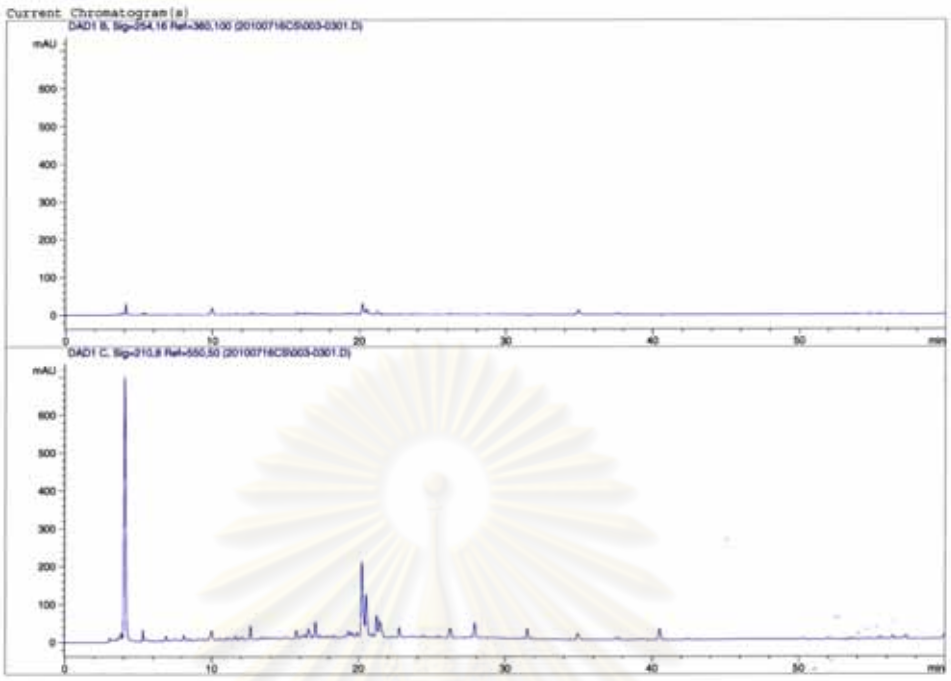

Figure 83 HPLC Chromatogram of BLW 2

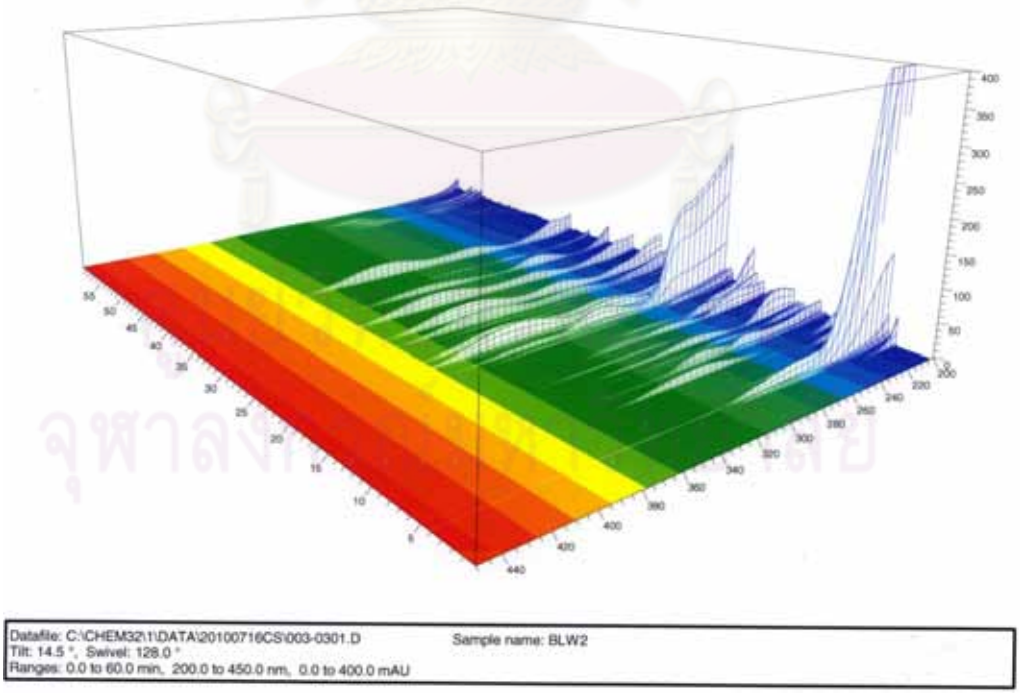

Figure 84 3D-HPLC Profile of BLW 2

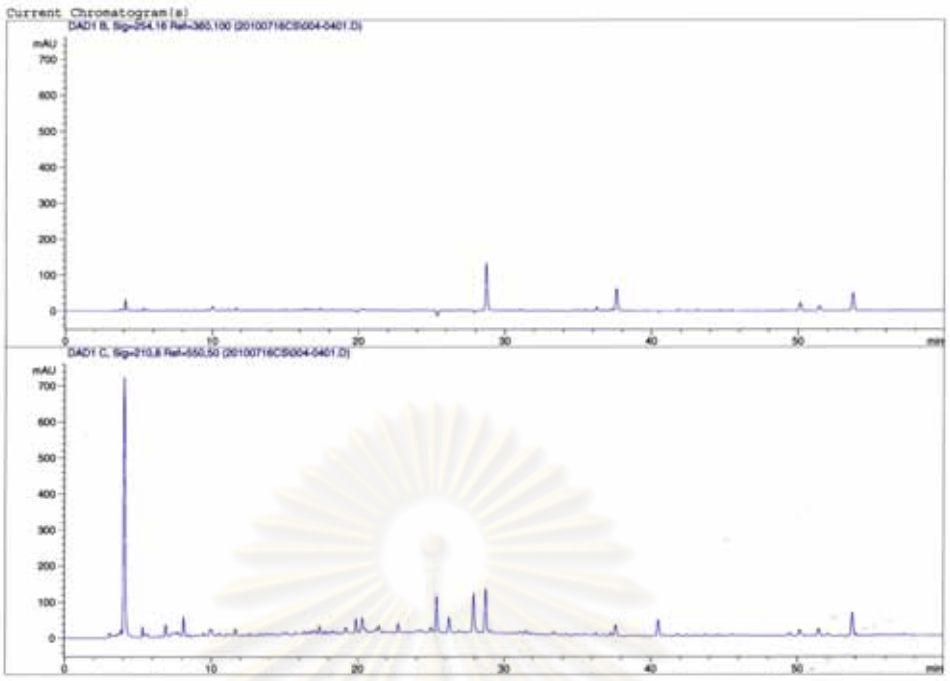

Figure 85 HPLC Chromatogram of BLW 3

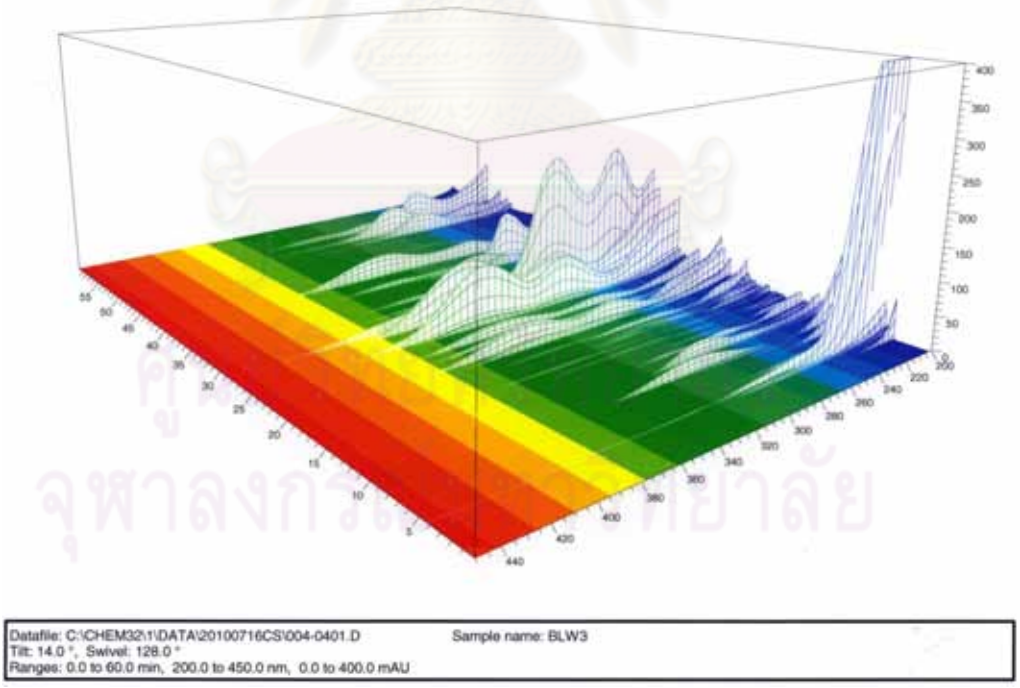

Figure 86 3D-HPLC Profile of BLW 3

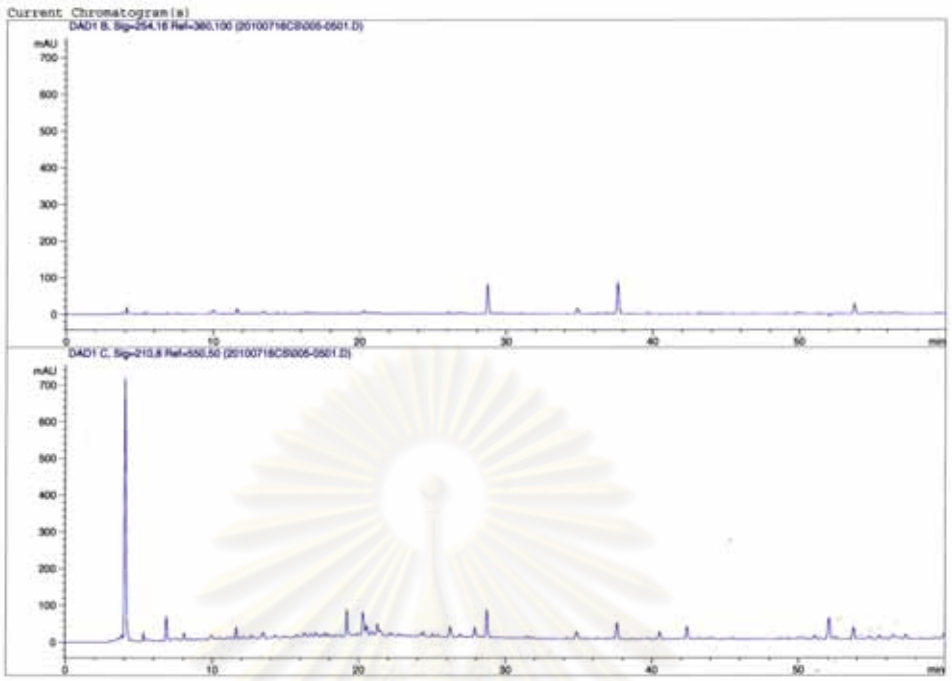

Figure 87 HPLC Chromatogram of BLW 4

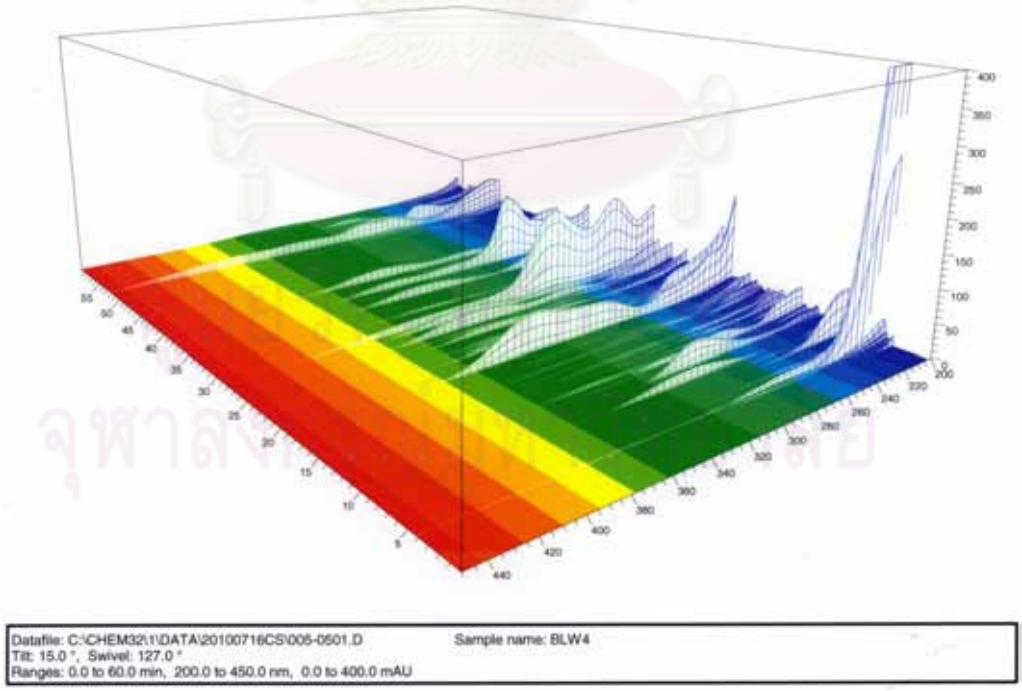

Figure 88 3D-HPLC Profile of BLW 4

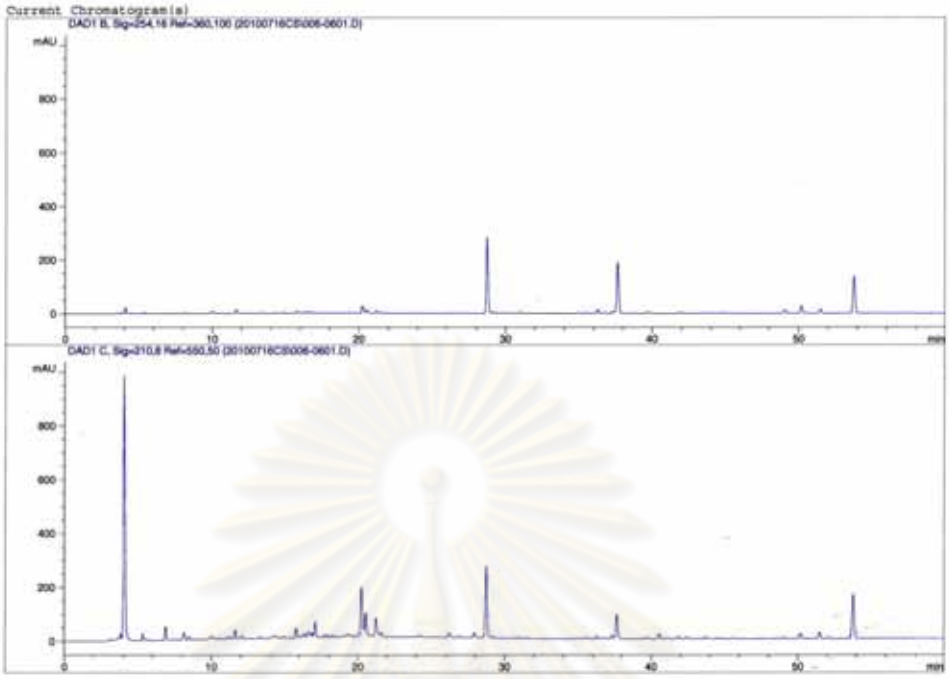

Figure 89 HPLC Chromatogram of BLW 5

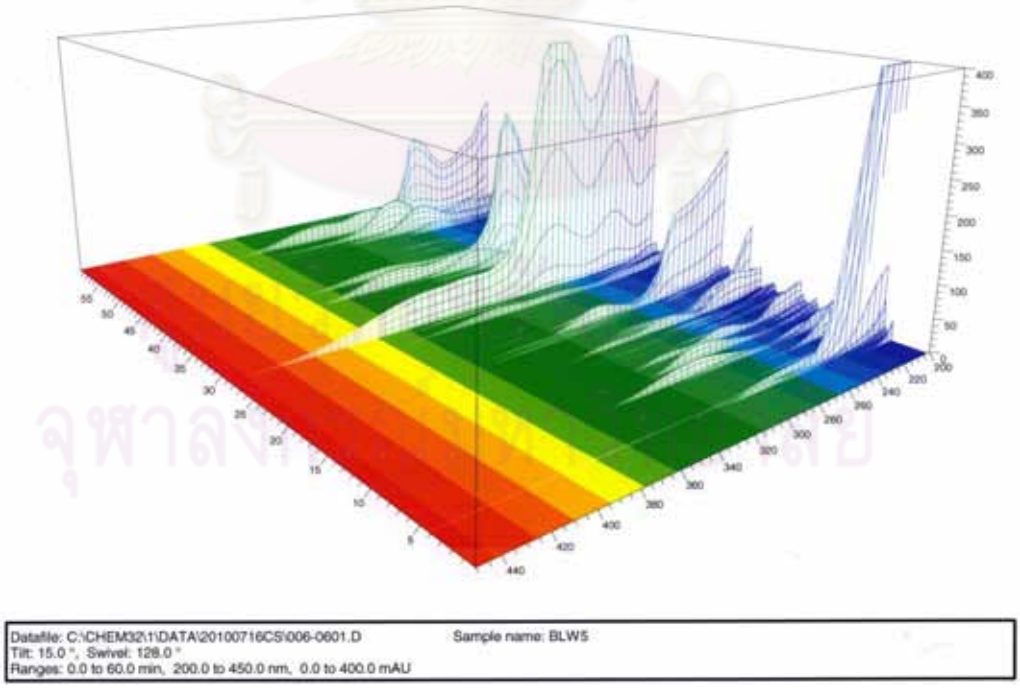

Figure 90 3D-HPLC Profile of BLW 5

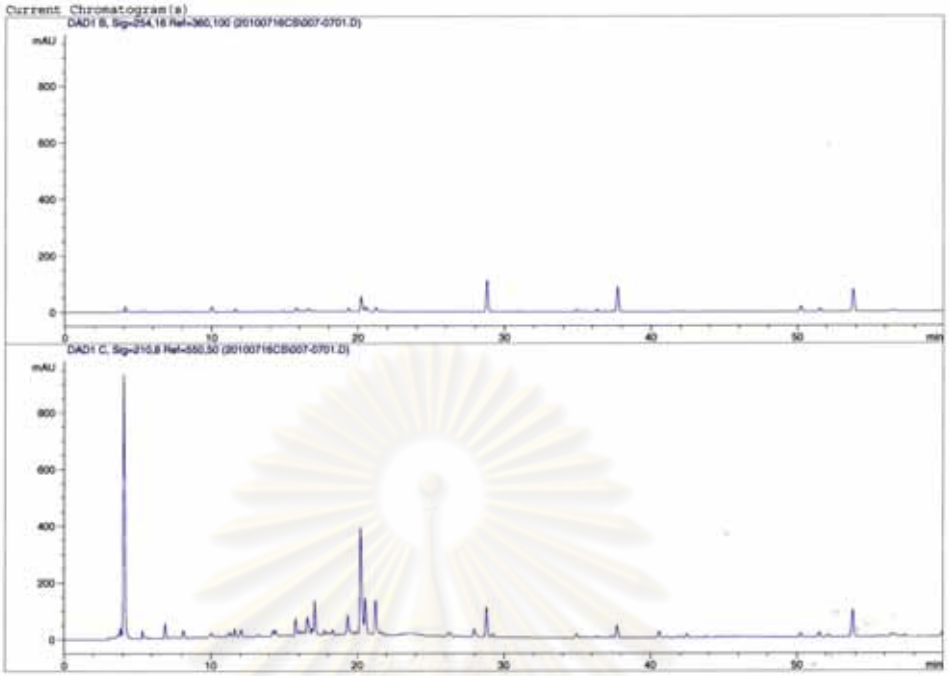

Figure 91 HPLC Chromatogram of BLW 6

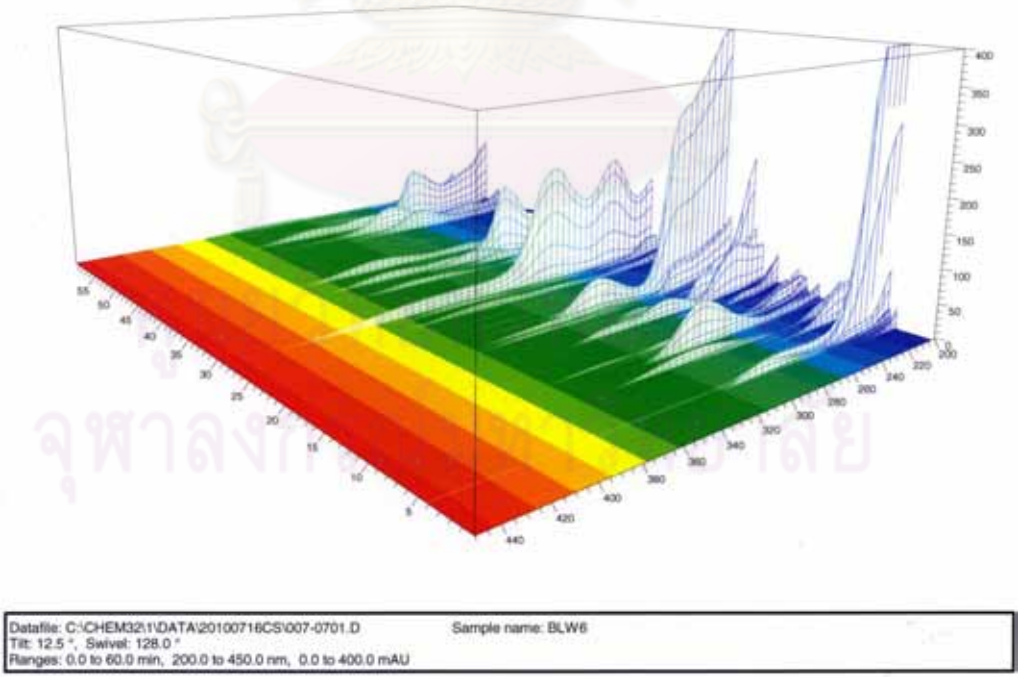

Figure 92 3D-HPLC Profile of BLW 6

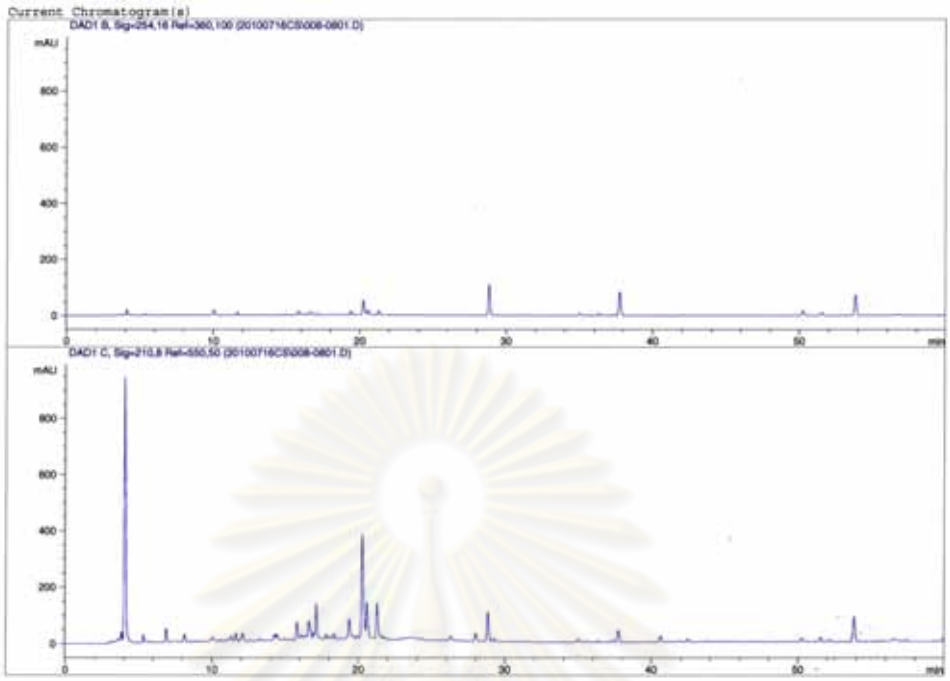

Figure 93 HPLC Chromatogram of BLW 7

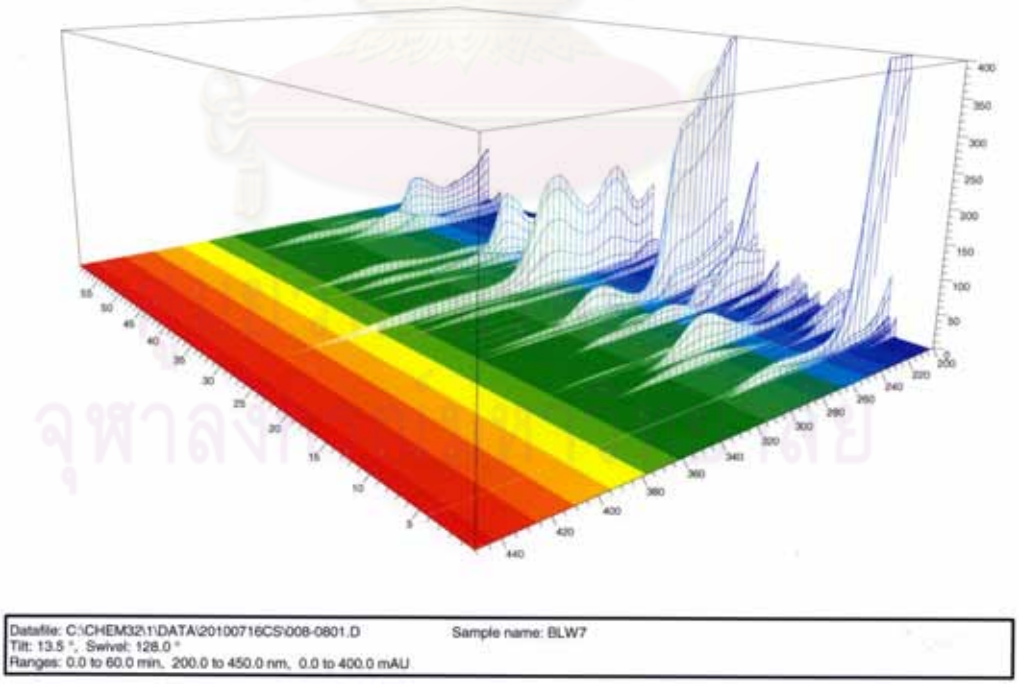

Figure 94 3D-HPLC Profile of BLW 7

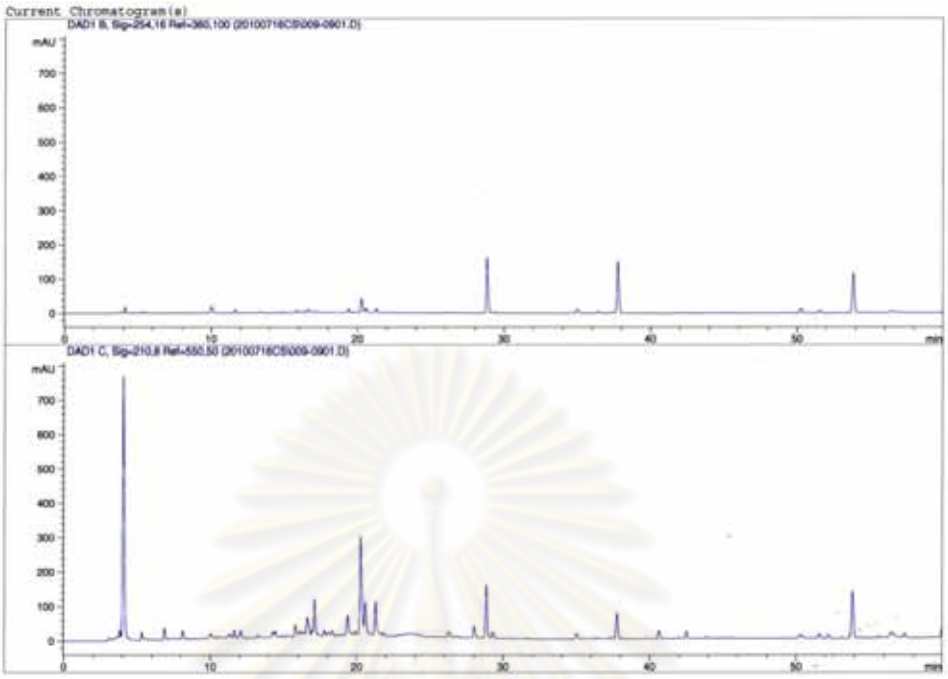

Figure 95 HPLC Chromatogram of BLW 8

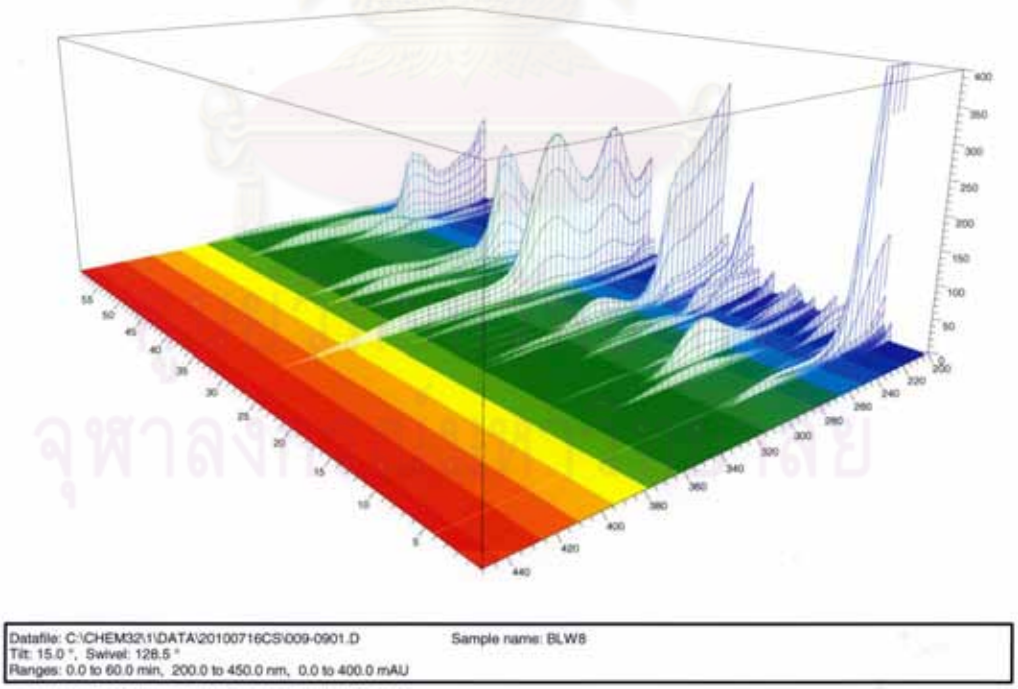

Figure 96 3D-HPLC Profile of BLW 8

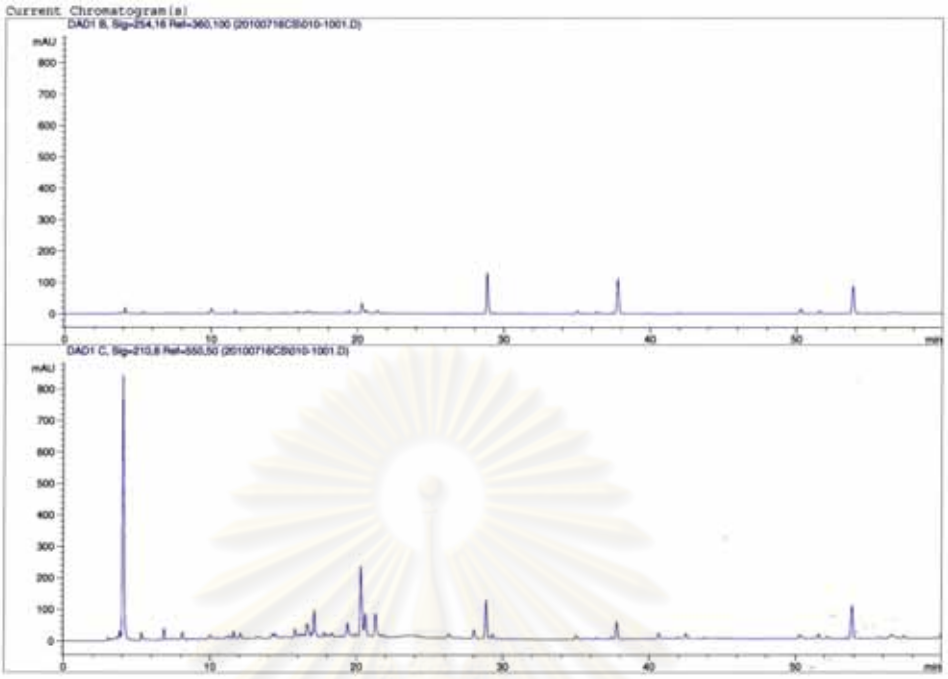

Figure 97 HPLC Chromatogram of BLW 9

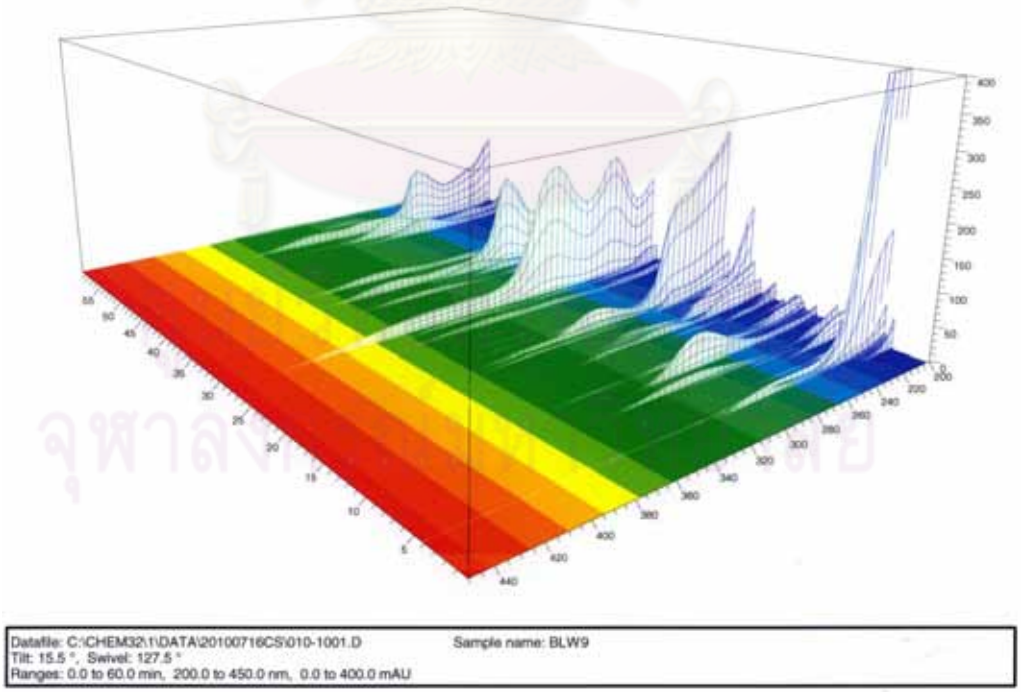

Figure 98 3D-HPLC Profile of BLW 9

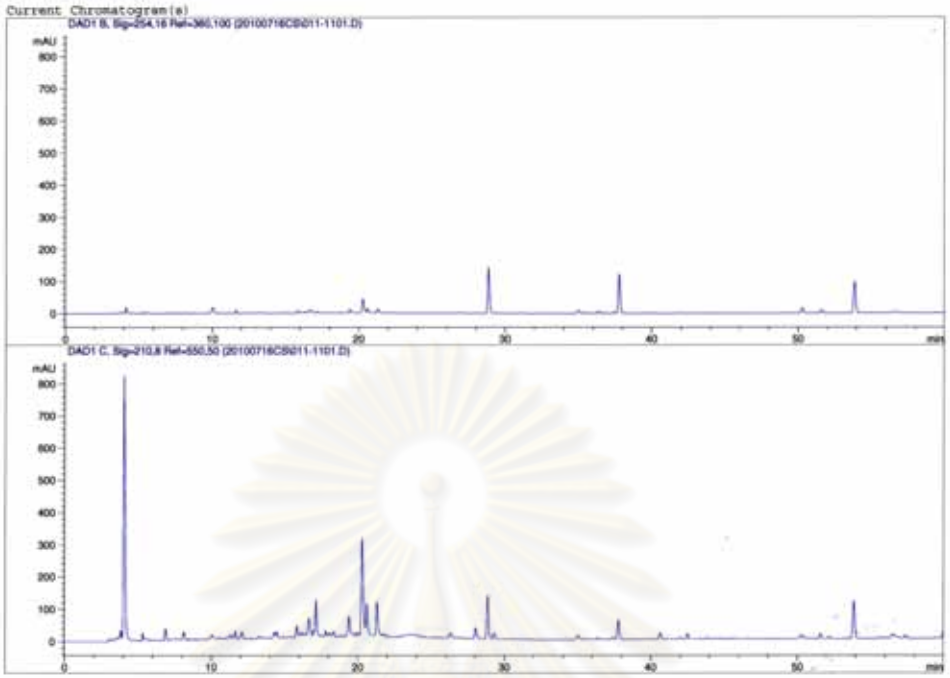

Figure 99 HPLC Chromatogram of BLW 10

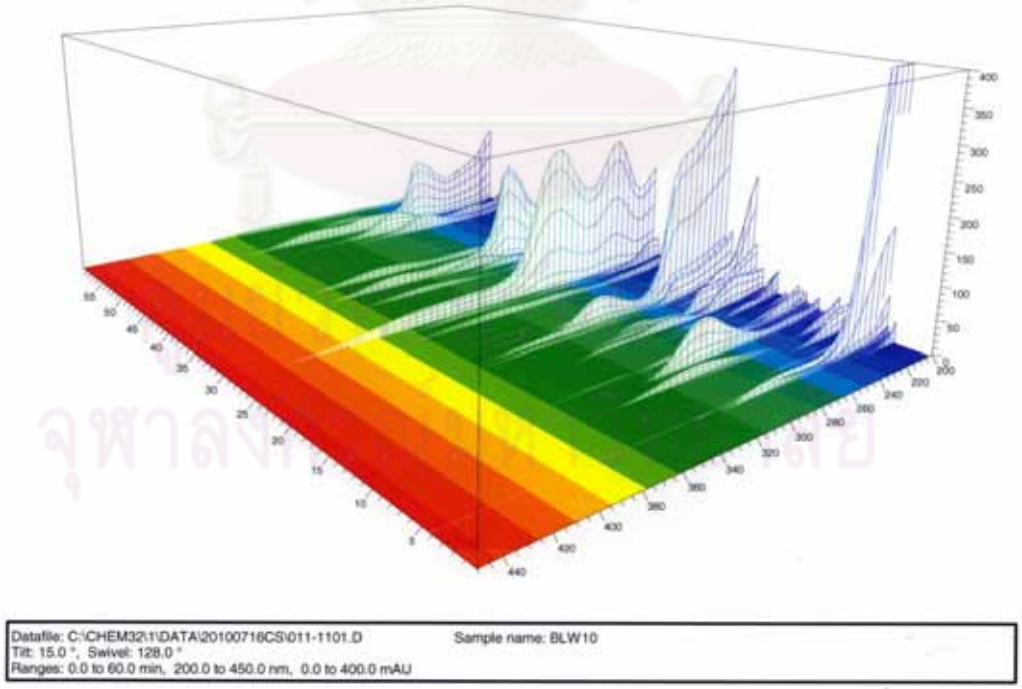

Figure 100 3D-HPLC Profile of BLW 10

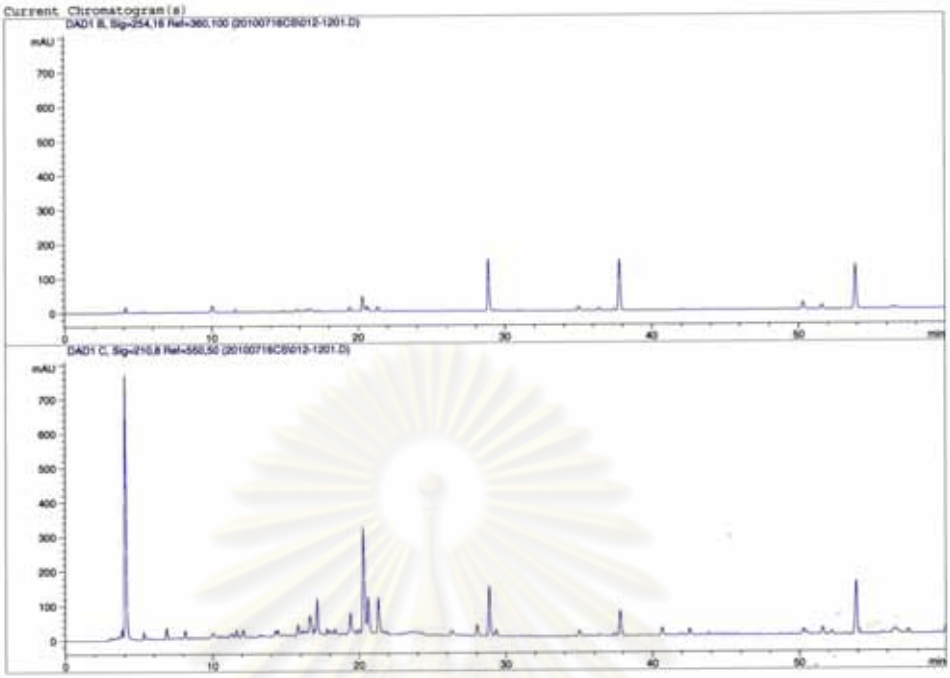

Figure 101 HPLC Chromatogram of BLW 11

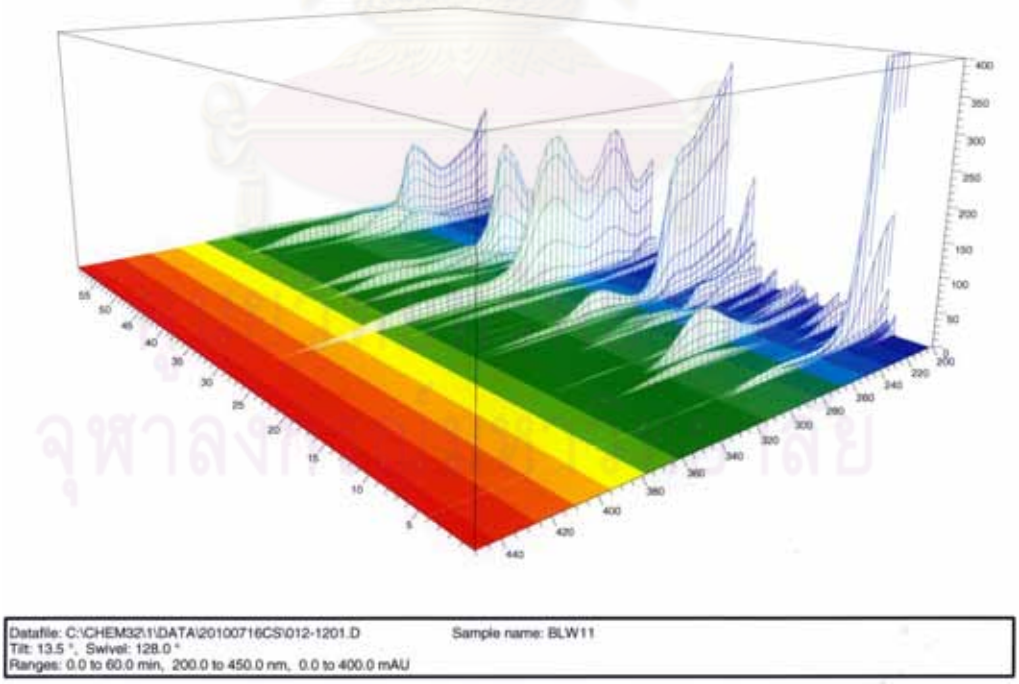

Figure 102 3D-HPLC Profile of BLW 11

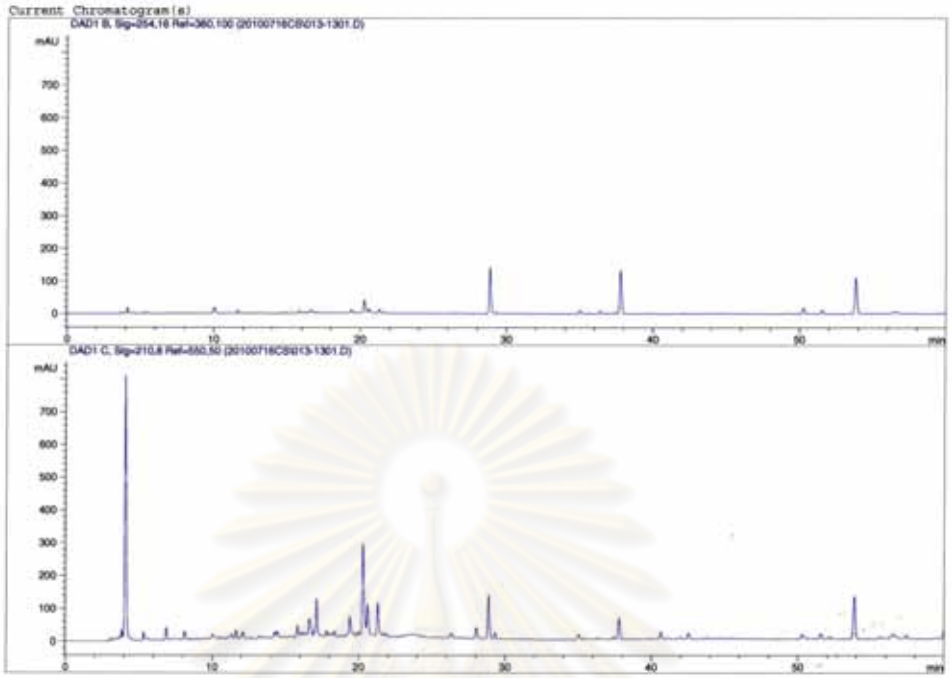

Figure 103 HPLC Chromatogram of BLW 12

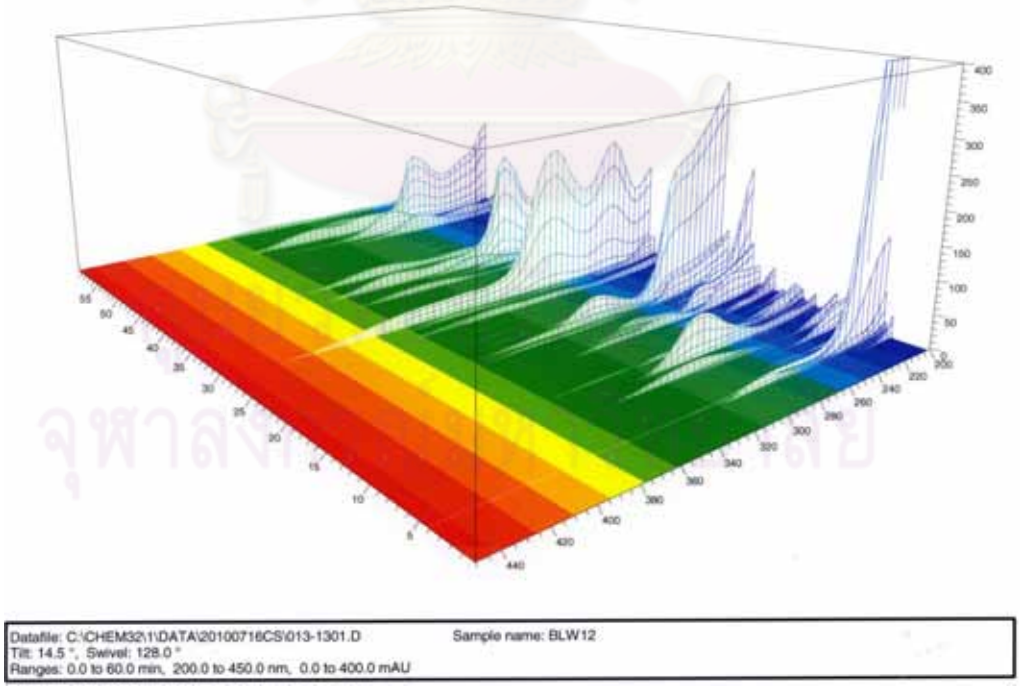

Figure 104 3D-HPLC Profile of BLW 12

APPENDIX D

Data Antipyretic and Antinoceceptive activity by
Ben Cha Lo Ka Wi Chian Remedy and root of five plant species

ที่ ศธ 0512.14 ศตว 021/ 2552

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 09-33-007	Approval No. 09-33-007
Protocol Title Antipyretic, antinociceptive and anti-inflammatory effects of bencha-loga-wichien herbal drug in animal models	
Principal Investigator Pasarapa Towiwat, Ph.D.	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval January 30, 2009	**Date of Expiration** January 30, 2010
Applicant Faculty/Institution Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan BKK-THAILAND. 10330	
Signature of Chairperson	**Signature of Authorized Official**
Name and Title WITHAYA JANTHASOOT Chairman	**Name and Title** RUNGPETCH SAKULBUMRUNGSIL, Ph.D. Associate Dean (Research and Academic Service)
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

Figure 105 Certificate of Project Approval

Table 22 Effect of NSS (10 ml/kg), 2% Tween 80 (10 ml/kg) and ASA 300 mg/kg, on lipolysaccharide-induced fever.

GROUP	BASELINE	HOUR 1	HOUR 2	HOUR 3	HOUR 4	HOUR 5	HOUR 6	HOUR 7
2% Tween80+NSS	36.98	37.32	36.82	36.94	36.76	37.02	36.48	36.32
	36.96	36.72	36.84	36.76	36.98	37.00	36.60	36.18
	36.96	37.20	37.08	36.96	36.82	36.38	35.26	36.70
	36.66	37.42	36.98	36.94	36.90	37.46	36.96	37.04
	36.44	36.78	36.74	36.62	36.98	36.62	36.02	36.32
	37.00	37.22	36.86	36.86	36.72	36.90	36.48	36.06
Mean	36.83	37.11	36.89	36.85	36.86	36.90	36.30	36.44
SD	0.23	0.29	0.12	0.13	0.11	0.37	0.59	0.37
2% Tween80+LPS	36.98	38.34	38.18	38.50	38.40	37.98	37.94	37.76
	37.12	37.68	38.00	38.42	38.62	38.10	37.82	37.40
	37.02	38.06	38.02	38.24	38.44	38.42	38.12	37.96
	36.96	37.90	38.94	38.68	37.38	38.16	37.56	37.42
	37.10	38.00	38.40	38.32	38.28	38.32	38.04	37.82
	37.12	38.02	38.00	38.46	38.08	37.72	37.62	37.92
	36.50	37.74	37.92	38.02	38.14	38.00	37.98	37.82
	36.94	37.94	38.40	38.10	38.00	37.76	37.62	37.56
	36.82	37.12	38.38	38.10	38.30	38.38	38.18	38.00
	35.34	37.30	37.56	37.64	37.94	37.86	37.32	36.86
	36.68	37.90	37.90	37.98	37.60	37.30	37.28	36.70
	36.92	37.24	37.70	37.84	37.50	37.44	37.54	37.56
Mean	36.79	37.77	38.12	38.19	38.06	37.95	37.75	37.57
SD	0.49	0.37	0.37	0.30	0.39	0.36	0.30	0.42
ASA 300 mg/kg	36.64	36.70	36.58	36.74	36.90	37.08	36.62	36.54
	36.98	36.86	36.12	36.74	37.14	36.92	36.80	36.48
	36.42	37.56	36.94	37.56	37.58	37.64	37.18	37.42
	36.14	38.08	37.46	37.38	37.88	38.16	37.50	36.92
	35.68	36.38	36.80	36.52	36.14	36.50	35.90	36.86
	36.98	36.90	36.66	36.94	36.20	36.94	36.66	37.68
	36.82	37.50	37.68	37.06	37.52	37.84	37.28	36.62
	37.00	37.34	37.72	37.18	38.08	37.32	37.40	37.70
	38.20	38.16	37.58	37.66	37.70	37.16	37.38	37.66
	36.88	36.26	36.18	35.66	36.28	36.78	36.20	36.38
	37.52	37.18	37.30	37.30	37.18	37.16	37.20	37.22
	36.82	36.80	36.40	36.00	35.20	36.06	35.66	35.46
	36.76	37.72	37.24	36.48	36.08	36.30	35.66	35.54
	36.60	37.50	37.22	36.90	37.24	36.98	37.50	36.80
	36.98	37.02	36.76	36.54	36.60	36.10	36.20	36.24
	36.44	37.64	37.72	37.76	37.50	37.42	37.50	37.64
	36.82	37.62	37.50	36.36	36.44	36.72	36.48	35.84
	35.70	36.68	36.26	36.52	36.54	36.50	36.12	36.10
Mean	36.74	37.22	37.01	36.85	36.90	36.98	36.74	36.73
SD	0.58	0.55	0.56	0.57	0.76	0.57	0.66	0.73

Table 23 Effect of *C. micracantha* root extract (CM; 25-400 mg/kg) on lipolysaccharide-induced fever in rat

GROUP	BASE LINE	HOUR 1	HOUR 2	HOUR 3	HOUR 4	HOUR 5	HOUR 6	HOUR 7
MCM 25 mg/kg	35.38	36.88	37.18	37.18	38.02	37.70	37.14	36.68
	36.66	35.80	35.88	35.96	36.24	36.88	35.98	35.58
	36.88	37.42	37.18	37.06	37.34	37.50	37.16	37.10
	37.62	37.02	37.08	36.64	36.42	36.98	36.64	36.18
	37.86	36.88	36.96	36.86	36.62	37.02	36.84	36.86
	35.20	37.40	37.04	37.04	37.10	37.34	37.40	37.28
Mean	36.60	36.90	36.89*	36.79*	36.96*	37.24*	36.86*	36.61*
SD	1.11	0.59	0.50	0.45	0.67	0.33	0.51	0.63
MCM 50 mg/kg	36.70	38.00	37.52	37.18	37.48	37.18	37.28	37.42
	36.80	35.58	38.22	38.02	37.84	38.30	37.68	37.48
	36.88	37.08	36.48	36.44	36.34	36.14	36.44	37.08
	37.14	36.60	36.74	36.66	36.70	36.76	36.52	36.26
	37.30	36.66	37.24	36.40	36.42	36.24	36.16	36.34
	37.36	37.22	37.50	37.22	36.68	36.86	36.78	36.68
Mean	37.03	36.86*	37.28*	36.99*	36.91*	36.91*	36.81*	36.88*
SD	0.28	0.80	0.62	0.62	0.61	0.78	0.57	0.53
MCM 100 mg/kg	37.40	36.46	37.16	36.24	37.84	36.84	35.88	35.80
	37.62	37.60	36.90	36.80	36.94	37.58	37.64	36.64
	36.42	37.22	36.92	37.00	36.88	36.82	36.90	36.60
	37.92	37.50	37.24	36.68	36.96	36.98	36.96	36.58
	37.46	36.48	37.04	36.56	36.18	36.70	36.50	36.82
	37.38	37.68	37.72	37.74	37.44	36.96	36.66	36.52
Mean	37.37	37.16	37.16*	36.84*	37.04*	36.98*	36.76*	36.49*
SD	0.51	0.55	0.30	0.51	0.56	0.31	0.58	0.35
MCM 200 mg/kg	36.84	36.72	36.80	36.28	36.64	35.82	36.34	35.90
	36.62	36.16	36.66	36.14	36.14	36.56	35.72	35.62
	37.20	37.32	36.64	36.74	37.64	37.58	36.86	37.16
	36.02	37.32	36.78	36.62	36.26	36.26	36.30	36.10
	37.24	37.60	37.60	36.80	36.86	36.62	36.70	36.56
	37.06	36.64	36.46	36.36	36.50	36.08	36.14	36.12
Mean	36.83	36.96	36.82*	36.49*	36.67*	36.49*	36.34*	36.24*
SD	0.46	0.54	0.40	0.27	0.54	0.61	0.41	0.54
MCM 400 mg/kg	36.78	37.96	37.88	37.76	38.16	38.12	37.38	36.72
	36.32	35.88	35.92	35.82	36.24	35.70	36.64	36.30
	35.80	36.46	36.08	35.34	35.72	35.74	35.92	36.60
	36.46	36.20	36.92	36.06	36.28	36.36	36.36	36.28
	36.96	36.90	36.94	36.70	36.56	36.90	36.60	36.62
	37.20	35.94	36.86	36.96	36.72	36.72	36.46	36.46
Mean	36.59	36.56*	36.77*	36.44*	36.61*	36.59*	36.56*	36.50*
SD	0.50	0.78	0.71	0.87	0.83	0.90	0.48	0.18

$p<0.05$ singnificantly different compared to control values for the corresponding hour.

Table 24 Effect of *C. petasites* root extract (CP; 25-400 mg/kg) on lipolysaccharide-induced fever in rat

GROUP	BASELINE	HOUR 1	HOUR 2	HOUR 3	HOUR 4	HOUR 5	HOUR 6	HOUR 7
CP 25 mg/kg	36.84	37.20	37.38	36.98	36.68	36.24	36.08	35.80
	36.20	37.76	37.76	37.52	37.48	37.62	37.34	37.26
	36.24	37.20	36.96	36.30	36.56	36.64	36.68	36.52
	37.44	37.46	37.32	37.14	37.40	37.10	36.76	36.88
	36.94	37.46	36.78	37.02	36.86	36.66	36.26	35.94
	36.90	37.18	37.08	37.08	36.72	36.74	36.94	36.64
Mean	36.76	37.38	37.21*	37.01*	36.95*	36.83*	36.68*	36.51*
SD	0.47	0.23	0.35	0.40	0.39	0.47	0.46	0.56
CP 50 mg/kg	36.82	37.38	36.98	37.00	37.06	36.88	36.88	36.92
	36.52	37.12	36.96	36.88	36.84	36.82	36.56	36.56
	36.32	36.90	36.40	36.64	37.08	37.12	36.82	36.90
	36.68	37.14	36.74	36.26	36.68	36.82	36.50	36.84
	36.92	37.66	37.80	37.46	36.92	36.86	36.74	36.52
	39.72	37.00	36.76	36.60	36.60	36.78	36.62	36.52
Mean	37.16	37.20	36.94*	36.81*	36.86*	36.88*	36.69*	36.71*
SD	1.27	0.28	0.47	0.41	0.20	0.12	0.15	0.20
CP 100 mg/kg	36.62	37.38	36.98	37.02	36.96	36.66	36.82	36.36
	36.82	37.44	37.34	37.16	37.24	36.98	36.92	36.80
	36.80	37.26	37.38	36.82	35.92	35.26	35.16	36.56
	36.74	37.68	37.22	36.88	37.00	37.20	36.98	36.48
	36.56	37.88	37.50	37.76	36.76	36.40	36.34	36.20
	36.82	37.16	36.86	36.76	36.56	36.74	36.70	36.52
Mean	36.73	37.47	37.21*	37.07*	36.74*	36.54*	36.49*	36.49*
SD	0.11	0.27	0.25	0.37	0.46	0.68	0.69	0.20
CP 200 mg/kg	37.34	37.58	36.94	37.24	37.18	36.96	36.92	36.80
	37.10	37.42	37.84	37.64	36.82	36.78	36.52	36.72
	36.78	37.70	37.30	37.18	37.18	36.90	36.68	36.48
	36.46	36.74	36.50	36.58	36.70	36.90	36.64	36.28
	36.62	37.54	37.06	36.84	36.70	36.70	36.74	36.54
	36.98	37.72	37.66	36.98	36.74	36.50	36.42	36.28
Mean	36.88	37.45*	37.22*	37.08*	36.89*	36.79*	36.65*	36.52*
SD	0.32	0.36	0.49	0.37	0.23	0.17	0.17	0.22
CP 400 mg/kg	36.98	37.32	37.08	37.04	36.70	36.76	36.44	36.44
	37.46	37.96	38.12	37.92	37.86	37.80	37.76	37.34
	36.76	36.98	36.48	36.42	36.32	36.50	36.30	35.82
	36.86	37.76	37.82	37.32	35.84	36.92	36.50	36.26
	36.90	36.90	36.90	37.14	36.60	36.60	36.54	36.40
	36.96	37.80	37.80	37.50	37.20	36.98	36.72	36.42
Mean	36.99	37.45*	37.37*	37.22*	36.75*	36.93*	36.71*	36.45*
SD	0.24	0.45	0.64	0.50	0.70	0.47	0.53	0.50

$p<0.05$ singnificantly different compared to control values for the corresponding hour.

Table 25 Effect of *H. perforata* root extract (HP; 25-400 mg/kg) on lipolysaccharide-induced fever in rat

GROUP	BASE LINE	HOUR 1	HOUR 2	HOUR 3	HOUR 4	HOUR 5	HOUR 6	HOUR 7
HP 25 mg/kg	35.08	37.08	37.16	36.52	36.04	36.50	36.66	36.66
	37.28	37.32	37.24	37.24	37.50	37.30	37.02	36.88
	36.98	37.32	37.08	37.04	36.92	36.94	36.50	36.42
	36.96	37.48	37.90	37.54	37.18	36.94	36.50	36.54
	35.66	37.44	37.40	36.92	37.10	37.02	36.86	36.68
	37.42	37.84	37.44	36.88	36.62	36.54	36.34	36.22
Mean	36.56	37.41	37.37*	37.02*	36.89*	36.87*	36.65*	36.57*
SD	0.96	0.25	0.29	0.35	0.51	0.30	0.25	0.23
HP 50 mg/kg	36.34	37.86	37.18	36.64	36.44	36.96	36.86	36.52
	37.20	36.78	36.86	37.04	36.94	36.76	37.16	36.66
	36.84	38.04	38.08	37.10	36.98	36.78	36.58	36.36
	36.96	37.70	37.88	37.22	37.04	36.88	36.60	36.50
	37.70	37.02	37.00	36.78	37.08	36.80	36.26	36.20
	36.68	37.06	36.96	36.72	36.50	36.28	36.98	36.34
Mean	36.95	37.41	37.33	36.92*	36.83*	36.74*	36.74*	36.43*
SD	0.47	0.52	0.52	0.23	0.28	0.24	0.32	0.16
HP 100 mg/kg	36.32	36.46	36.34	36.26	36.74	36.12	36.44	36.80
	37.08	38.24	37.94	37.54	37.72	37.00	36.88	36.64
	36.62	36.88	37.10	36.90	36.78	36.52	36.34	36.12
	36.96	36.80	37.46	36.76	36.82	36.58	36.56	36.32
	35.52	35.60	35.68	35.44	35.96	36.12	36.38	36.10
	37.52	36.82	36.92	36.74	37.16	37.00	36.58	36.26
Mean	36.67	36.80	36.91	36.61*	36.86*	36.56*	36.53*	36.37*
SD	0.70	0.85	0.80	0.70	0.58	0.39	0.20	0.29
HP 200 mg/kg	36.68	37.16	36.60	36.38	36.90	37.06	36.98	36.70
	37.20	37.74	37.44	37.10	37.50	37.38	37.00	36.88
	36.26	37.84	38.04	38.08	37.96	37.52	37.20	37.00
	36.90	37.08	36.90	36.70	36.42	36.32	36.12	36.88
	36.92	36.62	36.94	36.88	37.12	36.94	36.74	36.46
	37.30	37.90	37.52	37.24	37.04	36.86	36.58	36.20
Mean	36.88	37.39	37.24	37.06	37.16	37.01*	36.77*	36.69*
SD	0.38	0.52	0.52	0.58	0.53	0.42	0.39	0.30
HP 400 mg/kg	36.00	37.72	37.72	37.32	37.04	36.96	36.56	36.46
	37.12	38.12	38.12	37.90	37.66	37.50	37.28	36.86
	37.00	36.94	37.40	37.48	37.18	36.96	36.60	36.42
	37.60	37.94	38.12	37.02	36.96	36.84	36.62	36.14
	35.52	36.84	36.80	36.68	37.14	37.02	36.74	36.62
	37.04	37.42	37.32	36.88	36.64	36.36	36.18	36.42
Mean	36.71	37.50	37.58	37.21*	37.10*	36.94*	36.66*	36.49*
SD	0.78	0.53	0.51	0.44	0.33	0.37	0.36	0.24

$p<0.05$ singnificantly different compared to control values for the corresponding hour.

Table 26 Effect of *F. racemosa* root extract (FR; 25-400 mg/kg) on lipolysaccharide-induced fever in rat

GROUP	BASELINE	HOUR1	HOUR2	HOUR3	HOUR4	HOUR5	HOUR6	HOUR7
FR 25 mg/kg	36.14	36.78	36.54	36.52	36.78	36.76	36.98	36.42
	36.86	37.62	37.78	37.56	38.22	37.92	37.80	37.36
	36.54	36.48	36.96	36.48	36.62	36.80	36.96	36.52
	35.74	36.60	36.90	36.66	35.90	36.04	36.38	36.34
	36.68	37.56	37.72	36.92	37.92	37.40	36.50	36.44
	36.40	35.88	36.30	36.18	35.86	36.16	35.92	35.94
Mean	36.39	36.82*	37.03*	36.72*	36.88*	36.85*	36.76	36.50*
SD	0.40	0.67	0.61	0.48	1.00	0.72	0.65	0.47
FR 50 mg/kg	35.68	37.08	36.88	37.46	37.36	36.94	39.86	36.80
	36.96	37.64	37.36	37.50	37.14	37.48	37.10	37.00
	36.50	37.06	37.66	37.02	36.64	36.62	36.76	36.42
	36.40	37.10	37.08	36.64	36.92	37.44	36.74	36.18
	37.34	36.56	36.64	35.94	36.18	35.60	35.30	35.26
	37.20	37.56	36.08	35.84	35.96	36.18	36.34	36.38
Mean	36.68	37.17	36.95*	36.73*	36.70*	36.71*	37.02	36.34*
SD	0.62	0.39	0.56	0.73	0.55	0.73	1.53	0.61
FR 100 mg/kg	36.12	36.90	37.06	36.95	36.96	37.26	37.50	37.38
	37.12	36.82	36.90	36.82	37.90	37.74	37.96	37.60
	36.58	37.14	36.86	37.28	37.16	37.48	37.52	36.86
	37.18	37.88	37.74	37.14	37.62	37.84	36.84	36.24
	36.66	37.56	38.16	37.84	38.22	37.88	37.52	37.20
	35.98	36.82	36.30	36.64	36.48	36.92	36.40	36.02
Mean	36.61	37.19	37.17*	37.11*	37.39	37.52	37.29	36.88
SD	0.50	0.44	0.67	0.42	0.64	0.38	0.56	0.64
FR 200 mg/kg	36.88	37.42	37.46	36.96	36.82	37.14	36.94	36.84
	37.06	36.94	37.50	37.66	36.64	36.40	36.42	36.10
	36.76	36.78	38.26	37.68	37.98	37.90	37.36	37.50
	36.92	37.76	37.68	37.50	37.46	37.46	37.60	36.54
	37.78	38.24	37.96	37.72	37.84	37.94	37.58	37.34
	38.12	37.40	37.10	37.10	37.22	37.32	36.96	36.92
Mean	37.25	37.42	37.66	37.44	37.33	37.36	37.14	36.87
SD	0.56	0.53	0.41	0.33	0.54	0.57	0.46	0.51
FR 400 mg/kg	36.56	36.90	37.34	37.58	37.14	36.98	36.94	36.64
	36.92	37.50	38.04	37.96	37.84	37.70	37.38	37.44
	35.80	36.62	36.62	36.44	36.42	37.00	36.56	36.76
	36.62	37.56	37.56	36.76	36.90	37.26	36.58	36.34
	36.16	37.24	38.00	36.90	37.08	36.86	36.46	36.40
	35.92	36.04	36.12	35.60	36.34	36.60	36.52	36.70
Mean	36.33	36.98*	37.28*	36.87*	36.95*	37.07*	36.74*	36.71
SD	0.44	0.58	0.77	0.84	0.55	0.38	0.36	0.39

$p<0.05$ singnificantly different compared to control values for the corresponding hour.

Table 27 Effect of *T. triandra* root extract (TT; 25-400 mg/kg) on lipolysaccharide-induced fever in rat

GROUP	BASELINE	HOUR 1	HOUR 2	HOUR 3	HOUR 4	HOUR 5	HOUR 6	HOUR 7
TT 25 mg/kg	36.82	37.60	37.28	36.78	36.52	37.04	36.42	36.46
	37.32	37.26	37.20	37.94	37.60	37.48	36.96	36.64
	36.08	36.80	36.34	35.96	35.86	35.58	35.10	34.78
	36.86	36.32	35.98	35.06	35.78	35.94	35.78	36.10
	36.44	37.22	37.12	37.52	37.68	37.90	37.70	37.64
	37.20	37.62	37.00	36.68	36.40	36.86	36.82	36.10
Mean	36.79	37.14	36.82*	36.66*	36.64*	36.80*	36.46*	36.29*
SD	0.47	0.50	0.53	1.04	0.83	0.89	0.92	0.93
TT 50 mg/kg	37.36	37.96	37.48	37.18	36.64	36.58	36.60	36.62
	37.30	36.80	36.38	35.90	36.54	36.14	36.16	36.00
	37.10	37.56	37.06	37.36	37.18	36.56	36.56	36.58
	36.84	37.40	37.42	37.22	37.16	37.52	37.56	37.64
	36.78	36.78	36.78	36.44	36.46	36.74	36.84	37.06
	36.46	37.42	37.50	37.10	36.88	36.42	36.00	36.10
Mean	36.97	37.32	37.10*	36.87*	36.81*	36.66*	36.62*	36.67*
SD	0.34	0.46	0.45	0.57	0.31	0.47	0.55	0.61
TT 100 mg/kg	36.16	37.32	36.58	36.18	35.78	35.66	35.02	35.06
	36.72	37.16	36.94	36.38	35.94	36.30	36.00	35.83
	37.18	37.04	37.54	36.56	36.76	37.06	36.56	35.86
	36.50	37.10	36.90	36.56	36.30	36.64	36.40	36.84
	36.24	37.00	36.30	36.18	35.62	36.68	36.56	36.36
	36.40	36.90	37.32	36.10	36.24	36.56	36.88	36.56
Mean	36.53	37.09	36.93*	36.33*	36.11*	36.48*	36.24*	36.09*
SD	0.37	0.14	0.46	0.20	0.41	0.47	0.66	0.64
TT 200 mg/kg	36.16	36.14	36.14	36.04	35.78	35.88	35.16	35.44
	37.32	37.80	37.84	37.14	37.44	37.38	37.10	36.98
	37.02	37.34	37.66	36.90	36.42	36.56	36.10	36.00
	36.64	35.54	35.68	36.08	36.22	36.18	36.12	35.86
	36.76	35.38	35.40	35.22	35.30	35.86	35.74	35.68
	36.84	36.86	37.26	36.74	36.86	36.30	35.82	36.38
Mean	36.79	36.51*	36.66*	36.35*	36.34*	36.36*	36.01*	36.06*
SD	0.39	0.98	1.06	0.71	0.76	0.57	0.64	0.55
TT 400 mg/kg	37.28	38.10	37.72	36.94	36.50	37.12	36.54	36.16
	37.46	36.22	36.24	35.46	35.44	36.28	35.68	35.84
	36.82	36.90	36.98	36.78	36.64	35.42	36.28	36.60
	36.86	36.22	35.02	36.62	36.74	37.08	37.14	37.10
	36.96	36.64	37.16	37.28	37.20	37.42	37.42	37.50
	36.30	36.44	36.24	36.06	35.62	35.54	35.54	35.08
Mean	36.95	36.75*	36.56*	36.52*	36.36*	36.48*	36.43*	36.38*
SD	0.40	0.71	0.94	0.66	0.68	0.86	0.76	0.88

$p<0.05$ singnificantly different compared to control values for the corresponding hour.

Table 28 Effect BLW root extract (BLW; 25-400 mg/kg) on lipolysaccharide-induced fever in rat

GROUP	BASELINE	HOUR 1	HOUR 2	HOUR 3	HOUR 4	HOUR 5	HOUR 6	HOUR 7
BLW 25 mg/kg	36.14	36.74	36.30	35.98	36.20	36.64	36.28	36.20
	35.60	37.60	38.00	37.76	37.70	37.20	37.04	36.72
	36.90	37.90	37.96	36.84	36.62	36.38	35.78	35.20
	36.78	38.02	37.86	37.28	36.80	36.46	36.02	35.44
	36.96	37.60	36.96	36.74	36.68	36.44	36.48	36.16
	36.86	37.32	36.70	36.68	36.58	36.36	36.44	36.12
Mean	36.54	37.53	37.30	36.88	36.76*	36.58*	36.34*	35.97*
SD	0.55	0.46	0.74	0.60	0.50	0.32	0.43	0.56
BLW 50 mg/kg	35.66	38.08	37.96	37.94	37.66	37.14	36.88	36.72
	36.24	38.04	36.74	36.60	36.50	36.80	36.42	36.32
	36.88	38.34	38.08	37.66	36.94	36.54	36.28	36.34
	36.84	37.90	37.74	37.40	36.86	36.50	36.36	36.38
	36.94	37.02	36.88	36.64	36.34	36.40	36.76	36.60
	36.52	37.52	36.98	36.92	36.80	36.64	36.80	36.42
Mean	36.51	37.82	37.40	37.19	36.85*	36.67*	36.58*	36.46*
SD	0.50	0.47	0.60	0.56	0.46	0.27	0.26	0.16
BLW 100 mg/kg	37.28	37.26	37.12	36.90	36.74	36.42	36.22	36.16
	37.04	37.40	36.70	36.32	36.70	36.42	36.40	36.36
	36.88	36.40	36.20	36.56	36.52	36.16	35.78	35.34
	36.46	37.86	37.80	37.28	36.78	36.58	36.00	35.84
	36.48	37.24	37.14	36.88	36.76	36.56	36.66	36.36
	36.42	37.36	37.06	36.78	36.60	36.34	36.68	36.24
Mean	36.76	37.25	37.00*	36.79*	36.68*	36.41*	36.29*	36.05*
SD	0.36	0.48	0.53	0.33	0.10	0.15	0.36	0.40
BLW 200 mg/kg	35.02	37.32	36.98	36.68	36.46	36.72	36.54	36.42
	37.10	37.78	37.16	36.84	36.38	36.66	36.62	36.22
	36.96	37.80	37.56	37.06	36.68	36.46	35.86	36.00
	36.82	38.10	37.88	37.48	36.76	36.32	36.08	36.14
	36.96	37.34	36.84	36.70	36.60	36.48	36.72	36.52
	35.84	37.48	36.92	36.64	36.64	36.58	36.70	36.68
Mean	36.45	37.64	37.22*	36.90*	36.59*	36.54*	36.42*	36.33*
SD	0.84	0.31	0.41	0.32	0.14	0.15	0.36	0.25
BLW 400 mg/kg	36.88	37.40	36.62	36.24	36.90	36.04	36.06	35.82
	36.72	37.44	37.54	36.64	36.84	36.42	36.34	36.00
	36.76	37.88	37.64	36.82	36.44	36.22	36.04	35.76
	37.06	37.42	34.04	36.74	35.52	36.32	36.28	32.24
	36.80	37.60	36.72	36.76	36.48	36.26	36.54	36.36
	36.92	37.22	37.12	36.98	36.74	36.68	36.66	36.34
Mean	36.86	37.49	36.61	36.70*	36.49*	36.32*	36.32*	35.42*
SD	0.12	0.22	1.33	0.25	0.51	0.22	0.25	1.58

$p<0.05$ singnificantly different compared to control values for the corresponding hour.

Table 29 Latency (sec) in mouse hot-plate test from 0-240 min after oral administration of various doses of each root species components in BLW remedy (25-400 mg/kg). N = 10 for all groups and data were expressed as mean ± S.E.M.

sample/time	15 min	30 min	45 min	60 min	90 min	120 min	240 min
CM 25 mg/kg	26.28±1.95	29.53±1.19	27.58±2.18	28.89±1.22	31.31±2.41	32.82±1.76	38.40±1.69
CM 50 mg/kg	30.93±2.68	25.80±2.46	34.83±2.14	31.14±2.32	36.18±2.01	32.34±2.09	37.34±2.07
CM 100 mg/kg	32.29±2.76	29.68±2.57	33.75±1.36	34.28±1.98	36.15±2.13	33.99±2.68	39.05±1.93
CM 200 mg/kg	32.98±3.16	35.10±2.63	39.59±1.91	38.00±2.20	39.30±1.37	39.64±2.01	42.93±1.28
CM 400 mg/kg	36.59±1.89	37.53±1.64	36.79±1.63	38.56±1.80	35.77±4.14	41.40±1.06	44.57±0.43
CP 25 mg/kg	17.14±1.23	16.59±1.29	16.09±0.59	17.83±1.07	20.32±1.35	21.02±0.55	20.61±1.37
CP 50 mg/kg	20.09±1.28	18.19±1.34	20.37±1.58	18.34±1.49	21.66±1.61	21.39±0.78	20.71±1.66
CP 100 mg/kg	17.74±0.89	23.21±2.99	19.79±1.45	17.40±1.21	19.85±0.99	23.74±1.58	19.91±0.67
CP 200 mg/kg	20.02±1.44	18.94±1.35	18.82±1.06	19.25±0.76	21.27±1.26	23.17±0.93	22.55±1.49
CP 400 mg/kg	25.74±2.66	21.34±1.51	23.3±1.24	27.33±2.06	27.03±2.74	28.50±2.27	27.72±2.48
HP 25 mg/kg	25.56±3.68	24.23±3.03	22.60±2.69	23.77±2.23	24.62±2.68	21.65±1.25	25.10±1.91
HP 50 mg/kg	20.25±1.69	21.60±1.69	17.01±0.76	18.73±1.12	19.79±1.70	24.61±1.97	22.13±1.24
HP 100 mg/kg	21.92±2.15	21.26±2.33	20.16±2.47	18.51±1.76	22.03±2.89	23.50±2.46	26.01±2.68
HP 200 mg/kg	21.84±2.12	22.34±1.91	22.71±2.30	25.96±2.45	26.67±2.57	29.62±2.59	30.08±2.75
HP 400 mg/kg	27.88±2.36	26.75±2.21	27.79±3.59	27.78±2.65	32.74±2.77	31.11±3.01	31.33±2.61

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Table 30 Latency (sec) in mouse hot-plate test from 0-240 min after oral administration of various doses of each root species components in BLW remedy (25-400 mg/kg). N = 10 for all groups and data were expressed as mean ± S.E.M. (cont.)

sample/time	15 min	30 min	45 min	60 min	90 min	120 min	240 min
FR 25 mg/kg	24.15±2.19	25.89±1.94	24.61±2.03	28.79±2.25	33.25±2.18	36.94±2.14	31.72±2.26
FR 50 mg/kg	27.23±2.22	25.98±2.65	30.63±2.85	32.58±2.20	30.93±2.63	35.96±1.75	31.50±0.98
FR 100 mg/kg	33.90±3.14	31.15±2.14	34.56±2.94	37.62±2.08	36.43±2.34	37.56±2.18	41.53±2.12
FR 200 mg/kg	35.06±3.23	34.21±3.05	35.82±2.41	37.24±2.34	38.95±2.24	38.98±2.12	37.90±2.58
FR 400 mg/kg	39.47±1.85	39.87±2.02	39.86±1.47	39.37±1.94	39.74±2.21	42.27±1.58	42.51±1.44
TT 25 mg/kg	20.76±1.66	20.52±0.84	17.78±0.74	21.61±1.47	22.68±1.52	24.11±1.34	27.02±2.52
TT 50 mg/kg	24.99±2.04	21.43±1.69	23.29±2.10	25.45±1.55	26.53±1.14	28.35±1.49	32.16±2.24
TT 100 mg/kg	20.80±1.48	23.30±2.31	25.09±2.01	26.67±2.19	29.63±2.15	28.08±1.96	32.46±2.77
TT 200 mg/kg	24.18±2.02	25.85±1.93	27.44±1.31	27.76±2.55	30.23±1.63	33.07±1.80	35.15±1.90
TT 400 mg/kg	28.88±1.87	27.94±1.85	28.24±2.08	31.35±2.18	37.50±1.82	36.46±2.36	37.26±1395
BLW 25 mg/kg	20.65±1.53	21.12±1.59	21.65±1.51	20.44±1.69	20.53±0.51	23.51±1.38	22.75±2.09
BLW 50 mg/kg	22.41±6.57	18.21±4.93	20.02±4.19	22.35±5.98	23.58±2.85	25.29±8.07	24.26±11.99
BLW100 mg/kg	24.36±2.31	22.97±2.63	21.67±2.12	23.10±2.32	26.71±2.75	27.36±1.89	18.89±2.85
BLW 200 mg/kg	21.41±2.67	21.50±2.21	25.28±3.72	27.94±2.65	29.82±2.41	28.75±2.58	26.65±2.53
BLW 400 mg/kg	22.36±2.63	22.69±2.15	24.32±1.74	29.55±1.57	27.73±1.66	32.18±1.52	25.53±1.41

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Table 31 % MPE – Time in mouse hot-plate test from 0-240 min after oral administration of various doses of each root species components in BLW remedy (25-400 mg/kg). N = 10 for all groups and data were expressed as mean ± S.E.M.

sample/time	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
CM 25 mg/kg	1.8±17.66	20.30±6.41	3.03±17.67	11.14±12.24	29.89±10.54	32.58±12.42	58.63±12.14	8159.37±2193.70
CM 50 mg/kg	38.13±11.62	15.89±12.68	52.75±12.71	40.64±9.96	62.57±8.39	42.79±12.62	64.55±11.99	11475.66±2360.57
CM 100 mg/kg	50.15±10.08	34.32±12.50	51.83±7.25	55.59±7.96	56.67±8.34	53.14±10.44	76.72±11.99	13584.04±1380.13
CM 200 mg/kg	53.44±11.88	60.60±10.47	67.65±7.43	71.27±7.90	74.69±5.73	82.37±8.54	88.92±5.12	18303.52±1134.09
CM 400 mg/kg	61.47±8.56	68.01±6.86	74.98±6.62	76.39±7.50	82.46±29.77	92.57±5.55	100.00±1.65	18175.59±1460.05
CP 25 mg/kg	5.78±3.76	4.44±3.64	2.02±4.29	7.99±4.20	17.11±3.12	18.61±2.12	17.89±3.30	4074.49±436.22
CP 50 mg/kg	12.07±5.38	5.52±5.52	13.76±5.54	5.54±6.93	17.45±6.81	16.59±4.17	15.31±5.09	3280.93±912.09
CP 100 mg/kg	9.65±3.13	30.32±9.03	15.60±6.44	7.68±6.19	16.99±3.29	30.62±3.68	16.26±4.01	4788.47±472.01
CP 200 mg/kg	18.88±4.26	15.57±3.76	14.68±4.11	15.87±3.05	25.62±4.15	33.91±3.26	27.25±5.17	5550.33±586.79
CP 400 mg/kg	34.38±7.53	16.00±5.24	20.93±5.66	33.24±8.79	46.98±10.30	51.14±9.23	44.85±9.53	8354.51±1628.98
HP 25 mg/kg	28.84±12.94	15.31±13.76	11.23±11.62	14.89±12.21	19.95±9.82	5.99±6.65	20.87±6.49	4193.84±1163.55
HP 50 mg/kg	16.58±4.75	19.99±6.32	4.32±4.56	9.82±5.96	14.25±5.98	31.69±6.66	21.82±5.29	4948.31±684.19
HP 100 mg/kg	18.06±7.13	15.83±7.41	11.82±8.81	5.31±6.89	16.30±10.61	23.57±9.07	34.47±8.82	5129.97±1309.66
HP 200 mg/kg	16.91±7.23	19.95±5.73	19.01±8.29	43.99±8.58	44.14±8.53	59.29±9.55	57.16±9.59	8793.58±1710.23
HP 400 mg/kg	29.15±10.49	25.72±9.39	49.80±15.11	45.11±12.83	66.65±13.20	62.75±13.77	60.16±11.36	899.86±2563.81

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Table 32 % MPE – Time in mouse hot-plate test from 0-240 min after oral administration of various doses of each root species components in BLW remedy (25-400 mg/kg). N = 10 for all groups and data were expressed as mean ± S.E.M. (cont.)

sample/time	15 min	30 min	45 min	60 min	90 min	120 min	240 min	Area of analgesia
FR 25 mg/kg	12.31±15.82	0.14±11.16	13.45±14.44	2.60±16.39	50.83±11.01	61.11±14.29	29.85±12.84	7074.25±2285.75
FR 50 mg/kg	15.27±20.05	6.58±11.54	38.92±14.69	40.00±9.07	26.53±12.66	59.52±9.40	8.48±17.40	7353.23±1976.46
FR 100 mg/kg	49.19±14.29	29.88±10.57	48.02±15.23	64.74±9.29	54.78±13.42	62.39±11.67	85.89±8.54	14839.99±1223.01
FR 200 mg/kg	82.77±12.65	16.79±11.65	61.88±10.72	83.19±9.14	68.24±10.54	89.08±8.79	70.66±12.26	16030.41±1552.24
FR 400 mg/kg	91.53±6.98	93.76±7.56	88.79±5.77	66.17±10.24	76.15±8.78	94.89±6.33	93.24±5.24	19682.32±708.32
TT 25 mg/kg	11.86±6.58	11.17±3.95	1.08±4.96	14.99±6.09	18.45±5.84	23.79±5.14	32.87±9.83	5736.97±1219.76
TT 50 mg/kg	11.43±13.06	_5.14±15.15	2.28±8.67	18.25±8.67	22.07±6.88	28.53±8.40	46.72±11.06	6144.08±1733.65
TT 100 mg/kg	8.98±4.45	17.46±10.22	25.64±6.27	32.36±7.34	39.86±6.99	37.74±5.90	53.79±9.25	8762.85±1174.41
TT 200 mg/kg	21.86±8.76	27.95±7.69	25.87±5.81	23.12±10.04	41.03±6.09	51.60±7.26	49.81±8.02	11227.57±1306.75
TT 400 mg/kg	33.80±9.02	29.49±9.48	33.74±10.37	51.44±7.91	66.62±8.27	59.74±9.47	59.47±8.86	13679.46±1432.75
BLW 25 mg/kg	2.69±3.49	1.47±9.88	4.67±7.78	0.04±7.80	0.72±6.70	11.47±7.60	6.13±11.70	2066.04±1640.06
BLW 50 mg/kg	22.32±7.03	7.09±6.72	14.05±5.50	23.43±4.86	26.13±4.33	31.11±9.37	25.91±14.57	5850.36±1516.25
BLW100 mg/kg	17.94±10.36	16.12±8.62	8.86±8.46	12.65±11.51	18.53±14.80	30.71±8.58	0.13±8.67	3779.60±1548.00
BLW 200 mg/kg	7.86±10.21	7.70±7.93	10.34±15.86	30.93±10.69	38.98±9.54	26.42±11.56	34.17±9.68	6989.44±1963.43
BLW 400 mg/kg	17.48±7.83	12.14±10.35	27.72±4.25	34.80±6.41	34.80±6.41	54.97±5.21	26.75±4.82	8205.52±724.12

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BIOGRAPHY

Name : Mr. Chatubhong Singharachai

Born : November 21, 1975, Nan, Thailand

Education : Bachelor of Public Health (Hons.), Faculty of Medicine, Chiangmai University, Chiang mai, Thailand, 2002

Poster Presentations:

1. Singharachai C, Palanuvej C, Vipanngeun N, Ruangrungsi N. "Microscopic Characters of Five Roots Species in Ben-Cha-Lo-Ka-Wi-Chian Remedy" 5th Annual Conference in Thai Traditional medicine & Alternative medicine,September 3-5, 2008, Ministry of Public Health, Thailand.
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Honor:

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