QUALITY SAFETY AND EFFICACY EVALUATION OF CHA TU KA LA THAD AND TREE PHON THAD REMEDIES

Miss Pravaree Phuneerub

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Public Health Sciences
College of Public Health Sciences
Chulalongkorn University
Academic Year 2014

การประเมินคุณภาพ ความปลอดภัย และประสิทธิผลของตำรับจตุกาลธาตุและตำรับตรีผลธาตุ

นางสาวปราวรี ภูนีรับ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์สาธารณสุข
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	QUALITY SAFETY AND EFFICACY EVALUATION OF CHA TU KA LA THAD AND TREE PHON THAD REMEDIES
By	Miss Pravaree Phuneerub
Field of Study	Public Health Sciences
Thesis Advisor	Assistant Professor Chanida Palanuvej, Ph.D.
Thesis Co-Advisor	Associate Professor Nijsiri Ruangrungsi, Ph.D.
	Assistant Professor Wacharee Limpanasithikul, Ph.D.

Accepted by the Faculty of College of Public Health Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the College of Public Health Sciences

(Associate Professor Sathirakorn Pongpanich, Ph.D.)

THESIS COMMITTEE

.....Chairman

(Professor Surasak Taneepanichskul, Ph.D.)

.....Thesis Advisor

(Assistant Professor Chanida Palanuvej, Ph.D.)

.....Thesis Co-Advisor

(Associate Professor Nijsiri Ruangrungsi, Ph.D.)

.....Thesis Co-Advisor

(Assistant Professor Wacharee Limpanasithikul, Ph.D.)

.....Examiner

(Assistant Professor Naowarat Kanchanakhan, Ph.D.)

.....Examiner

(Assistant Professor Kanchana Rungsihirunrat, Ph.D.)

.....External Examiner

(Assistant Professor Thanapat Songsak, Ph.D.)

ปราวรี ภูนีรับ : การประเมินคุณภาพ ความปลอดภัย และประสิทธิผลของตำรับจตุกาลธาตุและตำรับตรีผลธาตุ (QUALITY SAFETY AND EFFICACY EVALUATION OF CHA TU KA LA THAD AND TREE PHON THAD REMEDIES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: นิจศิริ เรืองรังษี, วัชรี ลิมปนสิทธิกุล, 323 หน้า.

ตำรับยาจตุกาลธาตุและตรีผลธาตุ เป็นยาแผนโบราณที่บรรจุอยู่ในตำราแพทย์ศาสตร์สงเคราะห์ ตำรับจตุกาลธาตุ ประกอบด้วยพืชสี่ชนิด ได้แก่ รากเจตมูลเพลิงแดง เหง้าว่านน้ำ รากแคแตร และรากพนมสวรรค์ในอัตราส่วนที่เท่ากันโดยน้ำหนัก ส่วนตำรับตรีผลธาตุ ประกอบด้วยพืชสามชนิด ได้แก่ เหง้ากะทือ เหง้าไพล และรากตะไคร้หอมในอัตราส่วนที่เท่ากันโดยน้ำหนัก ทั้งสองตำรับนี้มีการใช้เป็นยาแก้ไข้ และแก้อักเสบมาเป็นเวลานาน การศึกษาครั้งนี้ได้ประเมินมาตรฐานเครื่องยาสมุนไพร ศึกษาความเป็นพิษ และประสิทธิผลของสารสกัดเอทานอลและสารสกัดน้ำของสมุนไพรทั้งเจ็ดชนิด สารสกัดตำรับจตุกาลธาตุ และสารสกัดตำรับตรีผลธาตุ ด้วยวิธีการทดสอบในห้องปฏิบัติการและในสัตว์ทดลอง ค่ามาตรฐานของสมุนไพรทั้งเจ็ดชนิดในตำรับจตุกาลธาตุและตรีผลธาตุถูกจัดทำขึ้นโดยประเมินลักษณะทางมหทรรศน์และจุลทรรศน์ของเครื่องยาสมุนไพร ลักษณะทางเคมี-ฟิสิกส์ และเอกลักษณ์ทางเคมี การประเมินฤทธิ์การก่อกลายพันธุ์และฤทธิ์ต้านการก่อกลายพันธุ์ของทุกสารสกัดด้วยวิธีการทดสอบเอมส์ พบว่าสารสกัดส่วนใหญ่ไม่มีฤทธิ์การก่อกลายพันธุ์ต่อเชื้อ *Salmonella typhimurium* สายพันธุ์ TA98 และ TA100 นอกจากสารสกัดเอทานอลของเหง้าว่านน้ำมีฤทธิ์ก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* ทั้งสองสายพันธุ์ และสารสกัดน้ำของรากเจตมูลเพลิงแดงและตะไคร้หอมมีฤทธิ์ก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* สายพันธุ์ TA98 อย่างไรก็ตามพบว่าสารสกัดส่วนใหญ่มีฤทธิ์ก่อกลายพันธุ์ต่อเชื้อ *S. typhimurium* ทั้งสองสายพันธุ์หลังถูกกระตุ้นด้วยไนไตรท นอกจากนี้ยังพบว่าสารสกัดส่วนใหญ่มีฤทธิ์ต้านการก่อกลายพันธุ์ในปฏิกิริยาของอะมิโนไพรีนและไนไตรทภายใต้สภาวะกรดในวิธีทดสอบเอมส์ การศึกษาพบว่าสารสกัดเอทานอลของเหง้าว่านน้ำมีความเป็นพิษสูงสุดต่อเซลล์ไรทะเล โดยมีค่า LC_{50} = 129.88 มคก/มล การศึกษายังพบว่าสารสกัดน้ำของเหง้าว่านน้ำและรากเจตมูลเพลิงแดงมีฤทธิ์ทำให้เกิดความเสียหายต่อดีเอ็นเอที่สกัดได้จากลิมโฟไซค์ของมนุษย์ในการทดสอบโคเมท โดยมีผลรวมของคะแนนอยู่ที่ 233.67 และ 158.33 ตามลำดับ ขณะที่สารสกัดตำรับจตุกาลธาตุและตรีผลธาตุนั้นไม่มีฤทธิ์ทำให้เกิดความเสียหายต่อดีเอ็นเอในการทดสอบโคเมท การศึกษาพบว่าสารสกัดเอทานอลของเหง้าไพล เหง้าว่านน้ำ สารสกัดน้ำของรากเจตมูลเพลิงแดง สารสกัดตำรับจตุกาลธาตุและสารสกัดตำรับตรีผลธาตุมีฤทธิ์ต้านอนุมูลอิสระด้วยวิธีการทดสอบในห้องปฏิบัติการหลายวิธี นอกจากนี้ยังพบว่าสารสกัดตำรับจตุกาลธาตุและสารสกัดตำรับตรีผลธาตุทุกขนาดมีประสิทธิผลในการออกฤทธิ์ต้านการอักเสบ

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5479052553 : MAJOR PUBLIC HEALTH SCIENCES
KEYWORDS: CHA TU KA LA THAD / TREE PHON THAD / PHARMACOGNOSTIC SPECIFICATION / GAS CHROMATOGRAPHY-MASS SPECTROMETER (GC-MS) / MUTAGENICITY / ANTIMUTAGENICITY / GENOTOXICITY / CYTOTOXICITY / ANTI-INFLAMMATORY / ANTIOXIDANT ACTIVITY

PRAVAREE PHUNEERUB: QUALITY SAFETY AND EFFICACY EVALUATION OF CHA TU KA LA THAD AND TREE PHON THAD REMEDIES. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., 323 pp.

Cha Tu Ka La Thad (CKT) and Tree Phon Thad (TPT) remedies are the remedies of traditional Thai medicine recorded in Tamra Paetsat Songkhrau. CKT remedy is composed of four species (i.e., *Plumbago indica*, *Acorus calamus*, *Clerodendrum paniculatum* and *Dolichandrone serrulata*) and each in contains equal part by weight. TPT remedy consists of three species (i.e., *Zingiber zerumbet*, *Zingiber montanum* and *Cymbopogon nardus*) and each contains in equal part by weight. Both remedies have been used as antipyretic and anti-inflammatory indications for a long time. This study was carried out to provide pharmacognostic specification of each species in CKT and TPT remedies. In addition, the ethanol and fractionated water extracts of both remedies were investigated for the safety and efficacy *in vitro* and *in vivo*. The macroscopic and microscopic, physico-chemical parameters, and chemical fingerprints were performed to estabish the standard parameters of each species in CKT and TPT remedies. The mutagenic and antimutagenic activities of all extracts were studied using Ames test with pre-incubation method. Most extracts without nitrite treatment exhibited non-mutagenicity toward *Salmonella typhimurium* TA98 and TA100, except the ethanol extract of *A. calamus* which exhibited mutagenicity to both strains and the fractionated water extracts of *P. indica* and *C. nardus* showed slightly mutagenicity toward *S. typhimurium* TA98. However, most of the extracts were mutagenic on both strains of *S. typhimurium* after being treated with sodium nitrite. Additionally, most of the extracts exhibited strong antimutagenic potential against the nitrite-treated 1- aminopyrene under acidic condition in the Ames test. Among CKT and TPT remedies and their ingredient extracts, results demonstrated the ethanol extract of *A. calamus* indicated the highest toxicity against brine shrimp with LC_{50} of 129.88 µg/ml. The fractionated water extracts of *A. calamus* and *P. indica* showed the highest DNA damage in human lymphocytes using comet test with total score of 233.67 and 158.33 respectively, while CKT and TPT remedies presented non-genotoxicity in comet test. These studies demonstrated the ethanol extracts of *Z. montanum, A. calamus* and the fractionated water extracts of *P. indica,* CKT and TPT remedies showed the highest antioxidant capacity in various *in vitro* models. Furthermore, all doses of CKT and TPT remedy extracts demonstrated anti-inflammatory activities.

Field of Study: Public Health Sciences
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

The author wishes to express her heartily gratitude and appreciation to her thesis advisor, Assistant Professor Dr. Chanida Palanuvej, for her continuous guidance, suggestion, and support throughout the course of this study.

The author is sincerely grateful to her thesis co-advisor, Associate Professor Dr. Nijsiri Ruangrungsi and Assistant Professor Dr. Wacharee Limpanasithikul, for his kindness and valuable suggestion to complete the study.

Gratitude is grateful to thesis committee members, Professor Dr. Surasak Taneepanichskul, Assistant Professor Dr. Thanapat Songsak, Assistant Professor Dr. Naowarat Kanchanakhan and Assistant Professor Dr. Kanchana Rungsihirunrat for their important and constructive suggestion in finalizing this thesis.

The author is thankful to The Strategic Scholarships Fellowship Frontier Research Network from The Commission on Higher Education, Ministry of Education, Thailand, Research Fund; the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) and grateful to the Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 Years Chulalongkorn University Fund. The author is Mae Fah Luang University for The scholarships of Ph.D. programme.

Appreciation is also extended to College of Public Health Sciences, Chulalongkorn University and all staff members for their assistances and instrumental supports.

The author's final thank goes to her family and Mr. Pantakan Jantarachote for their love, understanding, encourage, and moral support thoughout her study.

CONTENTS

Page

Page

Page

Page

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg	=	Microgram
µl	=	Microlitre
AC	=	*Acorus calamus*
BHT	=	Buthylated hydroxytolen
$^{\circ}C$	=	Degree Celsius
CKT	=	Cha Tu Ka La Thad remedy
cm	=	Centimeter
CN	=	*Cymbopogon nardus*
CP	=	*Clerodendrum paniculatum*
DC	=	*Dolichandrone serrulata*
DPPH	=	2, 2-diphenyl-1 picryl hydrazyl
EDTA	=	Ethylenediaminetetraacetic acid
Fe^{2+}	=	Iron (II)
Fe^{3+}	=	Iron (III)
$FeSO_4$	=	Iron (II) sulfate
FRAP	=	Ferric reducing antioxidant power
g	=	Gram
GC/MS	=	Gas chromatography/Mass spectrometry

h	=	Hour
HCl	=	Hydrochloric acid
hr	=	Hour
i.p.	=	intraperitoneal
IC_{50}	=	Half maximal inhibitory concentration
IND	=	Indomethacin
kg	=	Kilogram
l	=	Litre
LC_{50}	=	Lethal concentration 50%
mg	=	Milligram
mg/kg	=	Milligram per kilogram
min	=	Minute
ml	=	Microlitre
mm	=	Millimetre
mM	=	Millimolar
nm	=	Nanometre
NO	=	Nitric oxide
NSS	=	Normal salinesolution
pH	=	Potential of hydrogen ion

PI	=	*Plumbago indica*
r^2	=	Correlation coefficients
RNS	=	Reactive nitrogen species
ROS	=	Reactive oxygen species
rpm	=	Revolutions per minute
β	=	Beta
TLC	=	Thin layer chromatograghy
TNF-α	=	Tumor necrosis factor –alpha
TPT	=	Tree Phon Thad remedy
UV	=	Ultraviolet
WHO	=	World Health Organization
ZM	=	*Zingiber montanum*
ZZ	=	*Zingiber zerumbet*
α	=	Alpha

CHAPTER 1
INTRODUCTION

Background and significance of the study

The use of herbal medicines for the prevention and treatment of various health ailments has been in practice from time immemorial and become more popular over the past decades [1]. Due to fear of side effects from Western medicine, more and more people every year turn to herbal medicine because it is considered to be generally effective and safe.

In definition, herbal medicine is “a plant derived material or preparation with therapeutic or other human health benefits which contains either raw or processed ingredients from one or more plants that in some traditions, materials of inorganic or animal origin may also be present” [2].

In Thailand, there are numerous medicinal plants that have been clinically used for a long time because they are easily available, inexpensive, effective and have less harmful side effects than synthetic drugs. Preparation of these plants for treatment involves preparing a tea, tincture or filtrate [3]. Moreover, consuming local medicinal plants can reduce the import of synthetic drugs from foreign countries as well [1].

For herbal medicines, the purpose of a study is not only to screen out bioactive compounds from herbal extracts for new drug development, but also to

standardize and control the quality of raw herbal materials and their products to ensure the safety and efficacy; and more importantly, to reveal their preventative and therapeutic mechanisms [4]. Quality control for an herbal material usually includes not only the quantitative analysis of the main compounds from herbs, but also other analyses related to hygiene or safety examination such as heavy metal, pesticides, and microorganisms.

Pharmacological research plays an important role in the modernization of traditional herbal medicines, because the experimental method is the most fundamental method of modern science. Information that cannot be obtained from human beings can be obtained through animal experiments. Therefore, the results of pharmacological studies in the research of traditional herbal medicine will lay a foundation of clinical study [5].

The safety of herbal medicines is of particular importance in that the majorities of these products are self-prescribed and used to treat minor and often chronic conditions. The extensive traditional use of plants as medicines has enabled those medicines with acute and obvious signs of toxicity to be well recognized and their use avoided. However, the principle that traditional use of a plant for many hundreds of years establishes its safety does not necessarily hold true. The more subtle and chronic forms of toxicity, such as carcinogenicity, mutagenicity, and hepatotoxicity, may well have been disregarded by previous generations and it is

these types of toxicities that are of most concern when assessing the safety of herbal remedies.

Currently, the major pharmaceutical companies have demonstrated renewed interest in investigating higher plants as sources for new lead structures and also for the development of standardized phytotherapeutic agents with proved efficacy, safety and quality.

Phikud is a set of herbals with equal quantity and has been used as an ingredient in Thai traditional preparations of medicine. Herb components in same Phikud must have the taste that do not interfere with each other and also have equivalent quality or medical property. This is the traditional wisdom to organize group of herbs for purpose of synergistic effect and serving healer's conveniently use. Phikud Cha Tu Ka La thad and Phikud Tree Phon Thad remedies are the Thai traditional remedies that have been used as an antipyretic and anti-inflammatory drug that has been provided in the market. The revealed of these remedies have been in the Thai traditional medical text book called "Paad sard song khor". Phikud Cha Tu Ka La thad remedy composes of the four species and Phikud Tree Phon Thad remedy composes of the three species in an equal part by weights. The component of Phikud Cha tu ka la thad and Phikud Tree Phon Thad remedies are shown in table 1 and table 2.

Table 1 Component of Cha Tu Ka La Thad Remedy

Scientific name	Thai name	Family	Plant Part
Plumbago indica L.	เจตมูลเพลิงแดง	Plumbaginacea	Root
Acorus calamus L.	ว่านน้ำ	Araceae	Rhizome
Clerodendrum paniculatum L.	พนมสวรรค์	Verbernaceae	Root
Dolichandrone serrulata (DC.)	แคแตร	Bignoniaceae	Root

Table 2 Component of Tree Phon thad Remedy

Scientific name	Thai name	Family	Plant Part
Zingiber zerumbet (L.) Smith.	กระทือ	Zingiberaceae	Rhizome
Zingiber montanum (Koenig)-Link ex Dietr	ไพล	Zingiberaceae	Rhizome
Cymbopogon nardus Rendle	ตะไคร้หอม	Gramineae	Root

Most herbal medicines still need to be studied scientifically, the genaral lack of knowledge about Cha Tu Ka La Thad and Tree Phon Thad remedies such as standardization of each plant material, biological activities and efficacy of both remedies have become important concerns for both health authorities and the public.

Objectives

1. To establish the parameters of quality each plant material in Cha Tu Ka La Thad and Tree Phon Thad remedies.

2. To assess *in vitro* safety potential of each plant material in Cha Tu Ka La Thad and Tree Phon Thad remedies for their cytotoxic, mutagenic and genotoxic activities.

3. To perform the evaluation of the efficacy of each plant material in Cha Tu Ka La Thad and Tree Phon Thad remedies for their antioxidation, antimutagenic and anti-inflammation

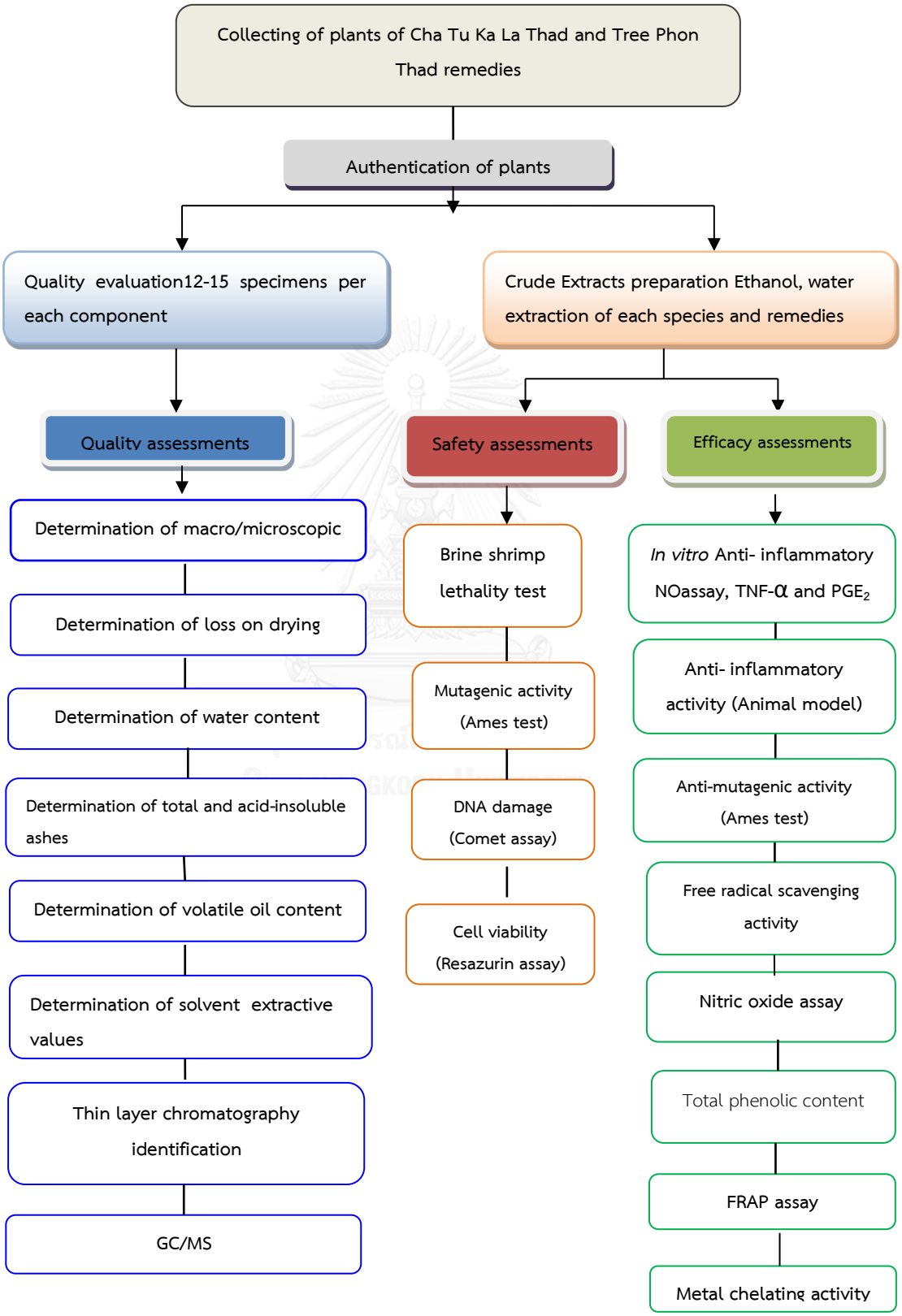
Expected benefit and application

1. This research provides the pharmacognostic specification of each species in Cha Tu Ka La Thad and Tree Phon Thad remedies.

2. This research provides the scientific evidences in efficacy and safety of Cha Tu Ka La Thad and Tree Phon Thad remedies

3. This research protocol can be a model to study other traditional medicine formularies.

Conceptual framework



CHAPTER 2
REVIEW OF RELATED LITERATURES

***Plumbago indica* Linn.**

Herb, perennial, 0.5-2 m tall, evergreen. Stems pliable, often lianous, simple or branched from base. Petiole base without auricles; leaf blade narrowly ovate to elliptic-ovate, papery, base rounded to obtuse, apex acute. Inflorescences spicate-racemose, flowered; peduncle 1-3 cm, not glandular; rachis, not glandular; bracts ovate, 2-3 X 1.5-2 mm, apex acuminate; bractlets obovate-elliptical to ovate, 2-2.5 X 1.5-2 mm, apex acute. Calyx 7.5-9.5 mm, glandular almost throughout, tube ca. 2 mm in diam. at middle. Corolla purple to dark red, tube 2-2.5 cm, limb ca. 2 cm in diam.; lobes obovate, ca. 12 X 7 mm, apex rounded and mucronate. Anthers blue, 1.5-2 mm. Ovary ellipsoid-ovoid, indistinctly angular. Heterostylous; style basally pilose; short-styled form with style arms only partly exserted, stigmatic glands without enlarged apex; long-styled form with style arms completely exserted from corolla throat, stigmatic glands capitate [6].

Botanical classification [6].

Scientific name	:	*Plumbago indica* L.
Domain	:	Eukaryota
Kingdom	:	Plantae
Subkingdom	:	Tracheobionta

Phylum	**:**	Tracheophyta
Division	**:**	Magnoliophyta
Class	**:**	Magnoliopsida
Subclass	**:**	Caryophyllidae
Order	***:***	Plumbaginales
Family	**:**	Plumbaginaceae
Genus	**:**	*Plumbago*
Species	**:**	*indica*

Synonyms [7] [8].

Bengali	Rakt-chitrak
Burmese	Chuvondacovaillie, Kanchopni, Kenkhyokeni
Chinese	Zi hua dan
English	Radix Plumbago, Fire plant, Scarlett Leadwort, Chitrakmool, Rosy flowered leadwort
Gujarati	Kalochitrak
Hindi	Lal chitrak
Kannada	Chitramulika
Konkani	Tambdi chitrak
Malayalam	Chethikoduveli, Chivappu-koduveli, Koduveli, Chuvana-koduveli

Manipuri	Mukaklei, Telhidak
Oriya	Ogni
Tamil	Akkini
Thai	Khui-wu, Chetra mun phloeng daeng, Tang-chu-wo, Pit pio daeng, Fai tai din, Ubakucha
Vietnamese	Xich hoa xa, Duoi cong hoa dor

Figure 1 *Plumbago indica* Linn.

Phytochemistry

Constituent:

Plumbago indica L. demonstrated the presence of alkaloids, steroids, saponins and reducing sugars. tannins, steroid glycosides, flavonoids and alkaloids. Plumbagin is a therapeutically important natural naphthoquinone occurs mainly in the roots of *P. indica* [9]. It suggested a simple method for isolation of plumbagin by addition of water to an an acetone extract through precipitation [10].

Plumbagin

Chemical Name :5-hydroxy-2-methyl-naphthalene-1,4-Dione,5-hydroxy-2-methyl-1,4-naphthoquinone

Molecular Formula :$C_{11}H_8O_3$

Molecular Weight :188.18 g mol−1

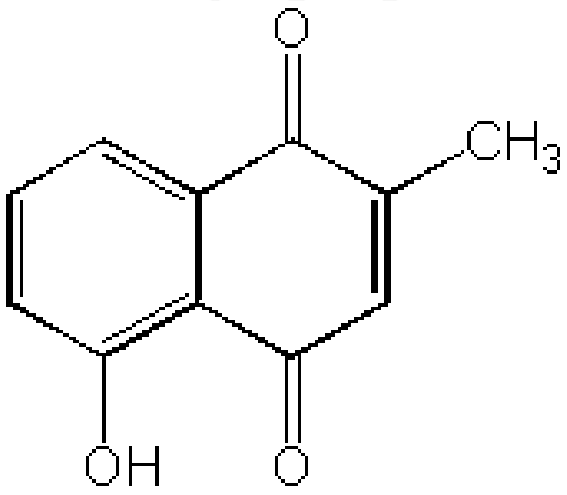


Figure 2 Structure of Plumbagin

Ethnomedicinal / traditional use

In traditionally, *P. indica* Linn. has several medicinal uses. It is used in inflammatory disorder, skin diseases, stomachache, acidity, constipation, abdominal pain and as abortifacient [11-13].

Inflammation diseases:

Plumbago indica is commonly used plants in folk medicine for the treatment of inflammation. A liniment made from bruised root mixed with a little bland oil is used as a rubefacient in rheumatism, paralytic affections, in enlarged glands, buboes etc. This herb cures certain cases of leucoderma. It is also useful for other skin diseases and for scorpion-sting. In Myanmar, it used for leprosy and syphilis [14].

Gastrointestinal diseases:

The juice of root of *P. indica* is considered a powerful sudorific, vesicant, sialagogue, alternative digestive and promotes appetite [15].

Gynaecological and obstetrical diseases:

In India, root is used as abortifacient and antifertility medicine; root is used as irritant, introduced into the vagina and applied directly to the neck of the uterus [15].

Pharmacological activities

Plumbago indica plant is anti-bacterial, anti-fungal, anti-cancer, anti-inflammatory, antianalgesic, anti-diabetic, anti-fertility, and antioxidant.

Antibacterial activity:

Studying antimicrobial of *Plumbago indica* L. showed the most active plants against *Staphylococcus aureus* with MIC and MBC of 0.726; 1.453 mg/ml and 0.782; 0.782 mg/ml, respectively. At Rf = 2.3 of TLC finger print of *P. indica* L. was equivalent to morin with antibacterial property against *S. aureus* [16].

Antifungal activity:

Plumbagin expressed the inhibition of *S. aureus* and very high inhibitory activity against *C. albicans* [17].

Antianthelmic activity:

The study screened for anthelmic and antitumour activity of ethnomedicinal plants from Thailand showed plumbagin, a pure compound from *Plumbago indica*, had the strongest activity against *Caenorhabditis elegans* [18].

Anticancer activity:

The study on experimental mouse tumors indicated Plumbago extract may have only a weak antitumor effect, but may be a good potential for use with radiation to enhance the tumor killing effect [19].

Antidiabetic activity:

Plumbago indica indicated the presence of reducing sugars, steroids, alkaloids, flavonoids and gums by standard procedure [20].

Antifertility activity:

The effective acetone extract was further studied on estrogenic functionality in rats. The acetone extract showed significant estrogenic and antiestrogenic activity ($p < 0.05$) ($p < 0.001$). The acetone extract of stem of *Plumbago rosea* showed the antifertility activity in female Wistar rats [21].

Antioxidant activity:

This study showed that the antioxidant activity observed with the *P.indica* extracts agree well with the amount of phenolic constituents detected in the extract. The present data suggested that PLBM can effectively scavenge ROS, including hydroxyl radical as well as other free radicals, under *in vitro* conditions, and can be a potential source of natural antioxidant that could be of great importance for the treatment of radical-related diseases [22].

Lipid metabolism activity:

Plumbagin was reported to reduce serum cholesterol and LDL-cholesterol by 53% - 86% and 61% - 91 % respectively; lower cholesterol/ phospholipid ration by 45.8%; elevates decreased HDL-cholesterol significantly in rabbits. It was also observed that plumbagin could prevent accumulation of cholesterol and triglycerides in liver and aorta and regressed atheroma plaques of thoracic and abdominal aorta. The treated rabbits excreted more faucal cholesterol and phospholipids [23].

Analgesic activity:

Methanol Extract of *Plumbago indica* (L.) showed significant inhibition in pain response induced by thermal and mechanical stimuli in dose dependent manner [24].

Anti-inflammatory activity:

The aqueous extracts of *P. indica* had significantly ($P<0.001$) inhibited the Carrageenan induced paw volume at the dose of 300 mg /kg body weight when compared to the control group and was comparable to the standard drug Indomethacin [25].

Toxicity:

The ethanolic root extract of *P. rosea* root was investigated acute toxicity and subacute toxicity in animal models. The result showed 24 h LD50 was 239.88 mg for intraperitoneal and 1148.15 mg/kg b.wt. for oral routes. However, at above 1250 mg/kg effected to severe diarrhea in mice by oral administration [26].

***Acorus calamus* Linn.**

Perennial herb up to 80 cm tall. Rootstock stout, 1-1.5 cm broad, creeping, with long fibrous roots from the lower surface. Stem erect, glabrous, grooved at one side, and ribbed at the opposite. Leaves ensiform or linear, 55-100 x 8-1.5 cm. Spathe leaf-like, up to 46 cm long, not enclosing the spadix. Spadix 5-6.5 cm long, cylindrical, obtuse, 1-1.4 cm broad. Tepals c. 2 mm long, oblong-obovate, slightly curved, margin membranous, surface with embedded raphides. Filaments 2 mm long, flat, anthers less than 1 mm long, ± orbicular. Ovary 3 mm long, obconical; seeds obconical, 2 mm long [27].

Botanical classification [27].

Scientific name	:	*Acorus calamus* Linn.
Domain	:	Eukaryota
Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Phylum	:	Tracheophyta
Division	:	Magnoliophyta
Class	:	Liliopsida
Subclass	:	Arecida
Order	:	Acorales
Family	:	Acoraceae

Genus : *Acorus*

Species : *Calamus*

Synonyms [27, 28].

Arabic	Vaj, Vash, Oudul Vaj
Ayurvedic	Vacha
English	Sweet Flag, Beewort, Bitter Pepper Root, Calamus Root, Flag Root, Gladdon, Myrtle Flag, Myrtle Grass, Myrtle Root, Myrtle Sedge, Pine Root, Rat Root, Sea Sedge, Sweet Cane, Sweet Cinnamon, Sweet Grass, Sweet Myrtle, Sweet Root, Sweet Rush and Sweet Sedge
French	Acore Calame, Acore Odorant, Acore Vrai
German	Kalmus
Gujarati	Gandhilovaj, Godavaj
Hindi	Bach, Ghorbach, Safed bach
Italy	Plant of Venus
Kannada	Baje, Vasa
Kashmir	Vachi,
Malayalam	Vayambu
Marathi	Vekhand
Nepali	Bojho

Persian	Agar, Agarturki
Sanskit	Bhadra, Bhutanashini, Vacha
Spanish	Calamo Aromatico, Calamis
Swedish	Kalmus, Kalmusrot
Tamil	Vashambu, pullai-valathi
Thai	Wan Nam, Hang khao pha, Som chuen, Wan nam lek.
Urdu	Bach
Unani	Vaj turki

Figure 3 *Acorus calamus* Linn.

Phytochemistry

Constituent:

Photochemical studies have reported the presence of glycosides, flavonoids, saponins, tannins, polyphenolic compounds, mucilage, volatile oil and bitter principle. The plant has been reported for the presence of glucoside, alkaloid and essential oil containing calamen, clamenol, calameon, asarone and sesquiterpenes. It also contains a bitter glycoside named acorine along with eugenol, pinene and camphene. The plant has been extensively investigated and a number of chemical constituents from the rhizomes, leave and roots of the plant have previously reported which includes beta- asarone, alpha- asarone, elemicine, cisisoelemicine, cis and trans isoeugenol and their methyl ethers, camphene, P-cymene, alpha-selinene, bgurjunene, beta-cadinene, camphor, terpinen-4-ol, aterpineol and a calacorene, acorone, acrenone, acoragermacrone, 2-deca -4,7 dienol, shyobunones, linalool and preisocalamendiol are also present [29, 30].

beta- Asarone

Chemical Name :cis-1-Propenyl-2,4,5-trimethoxybenzene

Molecular Formula : $C_{12}H_{16}O_3$

Molecular Weight :208.254 g mol−1

:

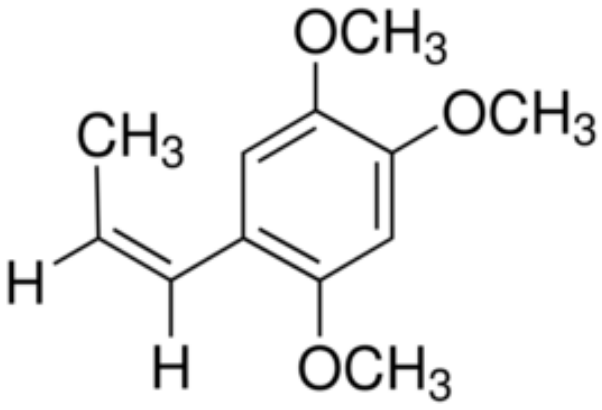


Figure 4 Structure of beta- asarone

<u>alpha- Asarone</u>

Chemical Name :trans-1,2,4-Trimethoxy-5-(1-propenyl)benzene, trans-1-Propenyl-2,4,5-trimethoxybenzene, alpha-Asarone

Molecular Formula :$C_{12}H_{16}O_3$

Molecular Weight :208.254 g mol−1

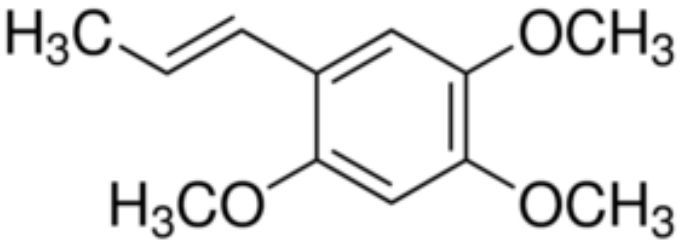


Figure 4 Structure of alpha- asarone

Ethnomedicinal / traditional use

The rhizomes of sweet flag (*Acorus calamus*) are a very long used for numerous traditional medicinal uses in Chinese and Indian herbal traditions. The leaves, stems, and roots are used in various Siddha and Ayurvedic medicines [31]. In Ayurveda traditional medicine used *A. calamus* for psychoneurosis, insomnia, hysteria, epilepsy, memory loss, Cough, fever, bronchitis, depression, inflammation, tumors, and general debility. The rhizome was used as remedy for rheumatism in Teheran. In Chinese medicine, used to aid digestion and regulate gastrointestinal fermentation, acidity, relieve swelling and constipation. Traditional medicine in Greek-Arab used to treat gastritis, anorexia, epilepsy, rheumatoid arthritis. Early Europeans, considered it a strong aphrodisiac; while in North America and New Guinea, once used to induce abortion [32].

Pharmacological activities

Antibacterial activity:

The ethanolic extracts of *A. calamus* was active against all the investigated bacterial strains while aqueous extract was totally inactive against the studied gram negative bacterial strains (*E. coli, P. mirabilis* and *P. aeruginosa*) and showed moderate antibacterial activity against gram positive bacteria *B. subtilis* and *S. aureus* at high concentration (200 ml) [33].

Antifungal activity:

The rhizome extract of *A. calamus* exhibited highest antifungal activity inhibiting the mycelial growth completely (100%) against all the 6 test pathogens. *P. betel* exhibited more than 50% inhibition against most of the test fungi. The ethanolic extract of several higher plants could be used as alternative source of antifungal agents for protection of plants or crops against fungal infection [34].

Synergistic Anthelmintic Activity:

The ethanolic extract of *A. Calamus* and *V. Negundo* showed anthelmintic activity against earthworms. Also the synergistic anthelmintic activity of *A. Calamus* and *V. Negundo* is significant than the individual activity of both the plants. For this study marketed drug was used as a standard reference drug [35].

Anticancer activity:

Methanolic extract of *A. calamus* rhizome (25, 50, 100 µg/plate) showed no sign of mutagenicity to *Salmonella tryprimurium* tester strains. Nevertheless, the extract of *A. calamus* (100 µg/plate) assessed percentage inhibition of mutagenicity against NaN3 and MMS at 60.92% and 83.60%, respectively. This study indicated that the methanolic extract of *A. calamus* rhizome possessed anti-tumor in MDA-MB-435S (human breast carcinoma) and Hep36 (humam liver carcinoma) cells [36, 37].

Antidiabetic activity:

A. calamus extract (200 mg/kg) was administered orally to diabetic rats for 21 days to determine the anti-hyperglycemic activity by estimating various biochemical parameters. Results showed significant restoration of the levels of blood glucose level [38].

Antidepressant Activity:

In a clinical study in fifty cases of depression at OPD of S.S. Hospital BHU, Varanasi, *Acorus calamus* (500 mg in a dose of 2 tablets three times a day after meal with water) given for six weeks showed reduction in the degree of severity of depression and better rehabilitation. There was also a significant improvement in assessment based on rating of symptoms on Hamilton depression rating scale. The rate of improvement before and after treatment was significant ($P < 0.001$) [39].

Antioxidant activity:

The aqueous extract showed strong dose dependent reducing activity. The results showed that *Acorus calamus* exhibits free radical scavenging, reducing power and metal chelating property [40].

Antihypertensive Effect:

Ethyl acetate extract of *A. calamus* rhizomes (EAAC) treated rats that underwent hypertension, demonstrated significant ($P < 0.01$) lower systolic blood pressure and diastolic blood pressure when compared with 2K1C rats indicated blood pressure lowering activity. In conclusions, EAAC treatment attenuated renal

artery occlusion induced hypertension via nitric oxide generation and decreases the plasma rennin activity [41].

Analgesic activity:

In the previous study, the LC50 and LC90 value of *A. calamus* extract were 50 and 300 µg/ml and LD50 values was 865 mg/kg body weight (oral) in acute toxicity study. Moreover, root of *A. calamus* showed significant analgesic activity by acetic-induced writhing test [42].

Anti-inflammatory activity:

The anti-inflammatory activities of *A. calamus* extracts (ACL) were investigated using RT-PCR, ELISA assay, immunoblotting, and immunofluorescence staining. Result shows that the HaCaT cells induced the pro-inflammatory cytokines, interleukin-8 (IL-8) and/or interleukin-6 (IL-6) expressions after treatment with polyI: C or PGN. ACL inhibited the expression of IL-8 and IL-6 RNA and protein levels, and attenuated the activation of nuclear factor kappa-light-chain-enhancer of activated B cells and IRF3 (Interferon regulatory factor 3) after poly I: C treatment. ACL also inhibited expression of IL-8 and activation of NF-kB following PGN induction [43].

Toxicity:

The ethanolic extract of *A. calamus* did not appear to have toxicity on acute and chronic administration in Wistar rat [44].

Other:

In 2012, there was study about anticonvulsant activity of *A. calamus* rhizome. The result revealed that A. *calamus* clearly confirmed the anticonvulsant activity [45].The study revealed the presence of unique combination of airways relaxant constituents in crude extract of *A. calamus*, provide a pharmacological basis for traditional use of *A. calamus* in disorders of airways [46].

***Dolichandrone serrulata* (DC)Seem.**

It is a deciduous tree to 25 m with narrow cylindrical crown and slender branches. Bark is pale brown, smooth or slightly flaking. Leaf is to 43 cm, once-pinnate, 3-5 pairs of leaflets, 5-14 × 3-6 cm, elliptic with tapering tip and strongly asymmetric base, usually with scattered teeth. Flower is 12-21 cm, pure white, opening at night, in short unbranched clusters of 3-7 flowers at end of twigs, 2-3 cm. Fruit is up to 85 × 1.8 cm, pointed, spirally twisted, seeds 2.2-2.8 × 0.5-0.8 cm, rectangular, thin with transparent wing.

Botanical classification [47].

Scientific name	:	*Dolichandrone serrulata* (DC)Seem.
Domain	:	Eukaryota
Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	*:*	Lamiales
Family	:	Bignoniaceae
Genus	:	*Dolichandrone*
Species	:	*serrulata*

Synonyms [47].

Thai Khae haen hae, Khae ket thawa, Kaepa, Khae khao, Khae ket thawa, Khae tui, Khae nae, Khae fa, Khae foi, Khae yui ho, Khae haen hae, Khae sai, Khae na, Khae pa

Vietnamese Quao rang

Other: *Bignonia serratula* Wall. ex DC.*, Bignonia serrulata* Wall. ex DC., *Spathodea serrulata* (Wall. ex DC.) DC.*,Stereospermum serrulatum* DC.

Figure 5 *Dolichandrone serrulata* (DC) Seem.

Phytochemistry

Constituent:

The methanolic extract of *D. serrulata* found triglycoside which was a new phenolic. Dolichandroside was isolated from the branches of *D. serrulata* together with decaffeoyl-verbascoside, verbascoside, isoverbascoside, markhamioside A, 2”-0-apiosylverbascoside, luteoside B and ixoside. The structure elucidations were based on analyses of spectroscopic data [48].

Ethnomedicinal / traditional use

The flower of this plant has a bitter taste and has been used as a vegetable. The bark is used in Thai traditional medicine as an antipyretic and anti-inflammatory agent [49].

Pharmacological activities

Antimutagenicity activity:

The ethanolic and aqueous extracts showed non mutagenicity and exhibited the antimutagenic effect against mutagen toward *Salmonella typhimurium* TA98 and TA100 in the Ames test. Moreover, they did not induce DNA damage in cultured lymphocyte cells by the comet assay [50].

Analgesic activity:

D. serrulata displayed both central and peripheral analgesic activities by hot plate, formalin and writhing tests in animal models and the highest dose (200-400

mg/kg) significantly reduced paw edema at 3 hour as second phase of carrageenan-induced paw edema in rats [51].

Antioxidant activity:

The result revealed that the flower extract from this plant exhibited low antioxidant capacities in three different antioxidant assay such as DPPH free radical scavenging activity, trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP) and total phenolic content [52, 53].

Anti-inflammatory activity:

Cycloheylethanoid isolated from flower of *D. serrulata* demonstrated moderate anti-inflammatory activity in ethylphenylpropiolate-induced ear edema in rats [54].

***Clerodendrum paniculatum* Linn.**

Shrubs ca. 1 m tall. Branchlets 4-angled, subglabrous to pubescent, nodes villous. Leaves palmately lobed; petiole 3-11 cm, yellow-brown pubescent; leaf blade broadly ovate to subrounded, 5-17 X 7.5-19 cm, abaxially sparsely pubescent and sandy glandular, adaxially sparsely pubescent to subglabrous, base cordate, margin entire or minutely denticulate, apex acute. Inflorescences conical to rounded thyrses, 15-26 X 16-22 cm; peduncle long; bracts ovate-lanceolate to ovate; bractlets linear. Calyx ca. 7 mm, deeply 5-lobed, dotted; lobes usually lanceolate, ca. 5 mm. Corolla red to orange, tube 1-1.5 cm, dotted, outside pubescent, inside subglabrous; lobes oblong to ovate, spreading. Stamens and style 4 X as long as corolla tube. Drupes globose, 5-9 mm in diam [55].

Botanical classification [55].

Scientific name	:	*Clerodendrum paniculatum* Linn.
Domain	:	Eukaryota
Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Phylum	:	Tracheophyta
Division	:	Magnoliophyta
Class	:	Equisetopsida
Subclass	:	Asteridae

Order : Lamiales

Family : Verbenaceae

Genus : *Clerodendrum*

Species : *paniculatum*

Figure 6 *Clerodendrum paniculatum* Linn.

Synonyms [56, 57].

Chinese Bao da lung chuan hua

English Pagoda Flower, Red pagoda plant

Indonesian Bunga pagoda

Malayalam Pangil-Pangil, Panggil-Panggil, Cheema perigalam, Krishnakireetam, Hanuman kireetam

Sanskit Krishnakireeta

Thai Norm Sawan, Pa nom Sawan, Chatt faa

Phytochemistry

Constituents:

Phytochemical screening of various extracts of leaves of *Clerodendrum paniculatum*. Linn carried out and indicates the presence of carbohydrates, glycosides and tannins and phenolic compounds [58].

Ethnomedicinal / traditional uses

It is used traditionally in India, China and Japan, in the treatment of rheumatism, neuralgia, ulcer, inflammation, and for healing wounds [59]. Crused leaves are used in the treatment of dysentery. Roots contain an antidote for certain snake bites. A paste of the leaves applied to infected burns. The whole plants of Red pagoda plant is used for vata, pitta, inflammation, wounds and skin diseases [60].

Pharmacological activities

Antimicrobial activity:

Previous study revealed that the effective antimicrobial activity against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans* of methanolic and chloroform extracts of the leaves of *C. paniculatum*. Furthermore, the petroleum ether, chloroform, ethyl acetate, methanol extracts of leaves of *C. paniculatum* showed LC50 value with 400, 1600, 1700, and 1700 µg/ml, respectively in brine shrimp assay (BSA) test [61]. Alcohol extract had good antibacterial activity against *Salmonella newport* and aqueous extract had good antibacterial activity

against *E. coli* compared to standard streptomycin [62]. The extract of *C. paniculatum* exhibited potent activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Klebsiella pneumoniae*. The study reveals that *Clerodendron paniculatum* possess better antibacterial activity than *Clerodendron infortunatum* Linn. [63].

Anticancer activity:

The alcoholic extract and their fractions of *C. paniculatum* showed moderate anticancer potential by in vitro methods like Trypan blue dye exclusion, MTT and SRB assays [64]. The alcoholic extract of roots of *Clerodendron paniculatum* showed significant antioxidant activity, its anticancer potential was not significant [65].

Antioxidant activity:

The methanolic extract of leaf and root of *C. paniculatum* presented flavanoids, tannins, cardio glycosides, saponins, and terpenoids. The plant showed good antioxidant by enzymatic and non- enzymatic methods [66].

Anti-inflammatory activity:

The leaf extracts of *C. paniculatum* showed the presence study indicate that petroleum ether and chloroform extracts possess significant ($P<0.001$) anti-inflammatory potential which provide scientific basis for the traditional claims of *Clerodendrum paniculatum* Linn leaves as an antiinflammatory drug [67].

***Zingiber zerumbet* (L.) Smith.**

It is a herbaceous plant, so from autumn until spring it goes dormant above ground as the leafy stems shrivel and die away, leaving the pale brown, creeping stems (rhizomes) at ground level. In the spring the plant springs up anew. The 10-12 blade-shaped leaves 15–20 cm long grow in an alternate arrangement on thin, upright stem to 1.2 m tall. The flower heads are reddish-green 3–10 cm long with overlapping scales, enclosing small yellowish-white flowers that poke out a few at a time. As the flower heads mature, they gradually fill with an aromatic, slimy liquid and turn a brighter red color.

Botanical classification [68].

Scientific name	:	*Zingber zerumber* (L.) Smith.
Domain	:	Eukaryota
Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Phylum	:	Tracheophyta
Division	:	Magnoliophyta
Class	:	Liliopsida
Subclass	:	Zingiberidae
Order	:	Zingiberales
Family	:	Zingiberaceae

Genus : *Zingiber*

Species : *zerumbet*

Synonyms [69].

Arabic	Zurunbad, Zurunbahburmese
Chinese	Hong Qui Jiang
English	Shampoo Ginger, Bitter Ginger, 'Awapuhi, 'Awapuhi kuahiwi, Pinecone Ginger, Wild Ginger
French	Gingembre Blanc, Gingembre D'oceanie, Gingembre Fou, Gingembre Tauvage, Zerumbet
Bangladesh	Jangli Adha
Brazil	Jenjibre-Amargo
Cambodia	Khnhei Phtu, Prateal Vong Prenh Atit
Dominican Republic	Jengibre Amargo
Germany	Wilder Ingwer
India	Ghatian, Yaiimu
Indonesia	Lampojang, Lampuyang, Lempuyang
Italy	Zenzero Bianco, Zenzero Salvatico
Japan	Niga Shouga
Laos	Hvanz Phai Chai Hluang
Malaysia	Lampoyang

Palau	Terriabek
Philippines	Barik, Lampuyang (Llonggo), Langkawas
Polynesia	Moeruru, Rea
Portugal	Gengibre Amargo
Puerto Rico	Jengibre Amargo
Samoa	Avapui
Spanish	Jengibre
Thailand	Haeo Dam Hiao Dam, Kathue, Kathue-Pa, Kawaen

Figure 7 *Zingber zerumber* (L.) Smith.

Phytochemistry

Constituent:

All report showed the presence of approximately 86% sesquiterpenoids with zerumbone being the major component in the essential oil of *Zingber zerumbet* root. In contrast, the oils from leaves and flowers contained high amount of (E)-nerolidol, beta-caryophyllene, and linalool, respectively, with the former differed from the others by their high levels of alpha- and beta-pinene. In addition to these, leaves and flowers also contain zingiberene. Interestingly, in most of the studies that described the presence of zerumbone, the compound has been reported as the predominant compound in rhizome of *Zingber zerumbet*. Recent study revealed the presence of a sesquiterpene, zederone, in ethanol extract of rhizome of *Zingber zerumbet.* Phytochemical screening to the aqueous extract of rhizome of *Zingber zerumbet* was reported to contain phenolic, saponins, and terpenoids [70].

Zerumbone [70]

Chemical Name : (2E,6E,10E)-2,6,9,9-tetramethylcycloundeca-2,6,10-trien-1-one

Molecular Formula : $C_{15}H_{22}O$

Molecular Weight :218.33 g mol−1

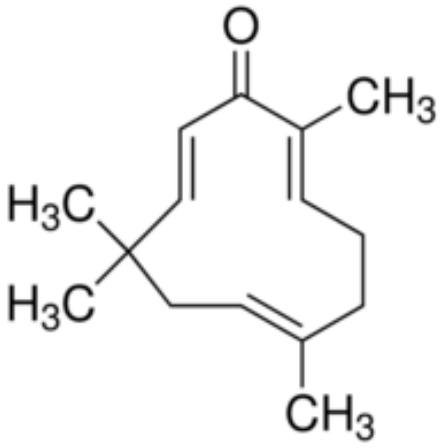


Figure 8 Structure of Zerumbone

Ethnomedicinal / traditional use

The rhizome of *Zingiber Zerumbet* traditional usages as botanical medicine include the treatment of inflammation, fever, toothache, indigestion, constipation, diarrhea, severe sprains, and to relieve pain, as well as antispasmodic, antirheumatic, and diuretic agents. The Malays used the fresh rhizomes as a cure for edema, stomach ache, sores, and loss of appetite while the juice of the boiled rhizomes is used to treat worm infestation in children. In Thailand, the fresh rhizomes are also used as antiflatulent agent. The Chinese macerated the rhizomes in alcohol and use it as a tonic, depurative, or stimulant. Taiwanese used the plant as an anti-inflammatory adjuvant for stomach ache, sprain, and fever. In India, the rhizome powder is mixed with ripe *Morinda citrifolia* for the treatment of severe pain, the cooked and softened rhizome is used to treat toothache, cough, asthma, worms,

leprosy, and other skin diseases, and the ground and strained rhizome is mixed with water and drank to treat stomach ache. The Hawaiians apply the compressed the rhizome of *Z. zerumbet* to sore spots, bruises, and cuts and also used it to treat headaches, toothache, ringworm/other skin disease, achy joints/sprains, stomach-ache. In addition, they also used ashes from burnt *Z. zerumbet* leaves, which are combined with a mixture of ashes of *Schizostachyum glaucifolium*, nut sap of *Aleurites moluccana*, and tuber sap of *Z. zerumbet*, as a remedy for cuts and bruised skin while the rhizome of *Z. zerumbet* was mashed with salt and rubbed on the head to treat headaches [71].

Pharmacological activities

Various studies have revealed the different pharmacological potentials of *Z. zerumbet* in a range of in vitro and in vivo test models.

Hepatoprotective Activity:

Previous study indicated the ethyl acetate extract of *Z. zerumbet* have the effective in preventing paracetamol-induced hepatotoxicity and this hepatoprotective effects was probably mediated through the reduction in oxidative stress [72].

Anticancer activity:

In vitro studies of zerumbone and cisplatin toward HeLa Cervical Cancer Cells showed that both compounds were able to induce cell death towards the

cancer cells. So, zerumbone was a new chemo-natural drug for treatment of cervical cancer [73].

Antioxidant activity:

The ethyl acetate extract of *Z. zerumbet* rhizome has a protective role against PCM-induced nephrotoxicity and the process is probably mediated through its antioxidant properties in rats [74].

Antipyretic activity:

The water and ethanol extracts of *Zingiber zerumbet* possessed significant anti-pyretic activities in Brewer's yeast-induced pyrexia in rats. In acetic-induced writhing test showed the writing movement in mice was decreased by the ethanol extract of rhizome of *Z. zerumbet* [75].

Analgesic activity:

Zerumbone was isolated from *Z. zerumbet* showed significant peripheral and central antinocciceptive effect in laboratory animals at dose investigated (10, 50 and 100 mg/kg) [76]. Essential oil from *Zingiber zerumbet* produced significant central and peripheral antinociceptive effects in laboratory animals that are probably mediated both by inhibition in the production of inflammatory mediators and activation of an opioidergic mechanism [77].

Anti-inflammatory activity:

At a dose of 5, 10, 50 and 100 i.p.mg/kg of zerumbone showed significant dose-dependent inhibition of paw edema induced by carrageenan. Besides, it

produced significantly suppressed granulomatous tissue formation in cotton pellet-induced granuloma test [78].

Toxicity:

The ethanol extracts of *Z. zerumbet* and *C. zedoaria* were strongly cytotoxicity with LC_{50} value of 1.24 and 33.593 µg/mL, respectively) after 24 h in brine shrimp nauplii assay [79]. The actie subfractions of *Z. zerumbet* showed a strong anti-proliferative effect with EC_{50} of 2.81 µg/mL on human breast carcinoma (*MCF-7*) but less cytotoxic to normal cells [80].

***Zingiber montanum* (Koenig) Link ex Dietr.**

A tuft rhizomatous herb, leafy stems 1-2 m high. Rhizome greenish-yellow to pale orange. Leaves simple, alternate, oblong-laceolate, 20-30 cm long and 2-5 cm wide, apex acuminate, lower surface often pubescent. Inflorescence ovoid to ellipsoid; peduncle 10-20 cm long, borne from the rhizome. Bract greenish brown purple. Flower pale yellow. Fruit spherical, dry when mature.

Botanical classification [81]

Scientific name	:	*Zingiber montanum (Koenig) Link ex Dietr.,* *Zingiber cassumunar Roxb.*
Domain	:	Eukaryota
Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Phylum	:	Tracheophyta
Division	:	Magnoliophyta
Class	:	Liliopsida
Subclass	:	Zingiberidae
Order	:	Zingiberales
Family	:	Zingiberaceae

Genus : *Zingiber*

Species : *cassumunar*

Synonyms [82]

English	Cassumunar ginger, Bengal root, Bengal ginger, bitter ginger
French	Gingembre marron
German	Blockzitwer, Gelber Zitwer
Hindi	Bun-ada
Indonesia	Banglai, Bengle, Mungle (Acheh), Bungle (Batak), Kunik Bolai, Panglai, Pandhiyang, Panglai, Pandhiyang, Bale, Panini, Unin makei
India	Jangliadrak, Karallamu, Kadushunti, Peju, Peyu, Aardikaa, Shringaberikaa, Adarakhi, Bana-adarakhi
Malaysia	Bong,lai, Bangle, Bolai, Kunyit terus putih, Lampoyang kuning, Lia Vetong, Tepus Merah
Puerto Rico	Jengibre amargo
Sanskit	Vanardraka
Spanish	Jengibre amargo
Telagu	Car-puspoo
Thailand	Phlai, Puloie, Wan-fai

Figure 9 *Zingiber montanum* (Koenig) Link ex Dietr.

Phytochemistry

Constituent:

In Indonesia, the main constituents the essential oil of *Z. cassumunar* was sabinene and terpinen-4-ol that examined by GC and GC-MS [83]. The essential oils of rhizome and leaves of *Z. cassumunar* were investigated compound oils by GC and GC/MS. The composition of the rhizome and leaf oils is no similar. Terpinen-4-ol (50.5%),(E)-l-(3,4-dimethoxyphenyl)buta-1,3-diene(19.1%),(E)-1-(3,4 dimethoxyphenyl) but-1-ene (6.0%) were main constituents of the rhizome oil and 4-furanodien-6-one (27.3%), curzerenone (25.7%) and beta-sesquiphellandrene (5.7%) were main constituents of the leaf oil [84]. Moreover, It Also present is curcuminoids (cassumunin A and B, cassumunarin A, B and C) with potent anti-oxidant activity [85].

Sabinene [86]

Chemical Name :4-methylidene-1-propan-2-ylbicyclo

Molecular Formula :$C_{10}H_{16}$

Molecular Weight :136.23404 g mol−1

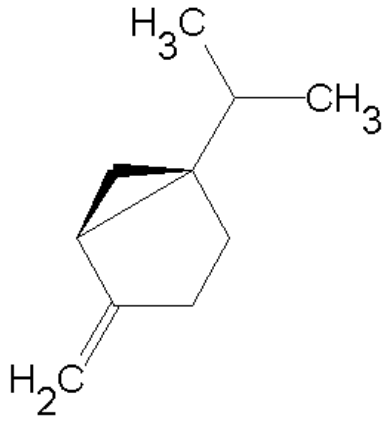


Figure 10 Structure of sabinene

Terpinen-4-ol [87]

Chemical Name :(R)-p-Menth-1-en-4-ol, (R)-1-Isopropyl-4-methyl-3-cyclohexen-ol

Molecular Formula : $C_{10}H_{18}O$

Molecular Weight : 154.25 g mol−1

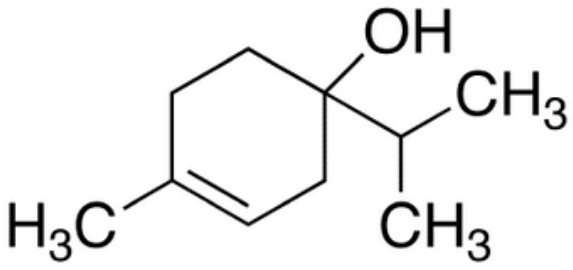


Figure 11 Structure of terpinen-4-ol

Cassumunin A [88]

Chemical Name : (1E,6E)-1-[3-[(E)-4-(3,4-dimethoxyphenyl)but-3-en-2-yl]-4-hydroxy-5-methoxyphenyl]-7-(4-hydroxy methoxyphenyl)hepta-1,6-diene-3,5-dione

Molecular Formula :$C_{33}H_{34}O8$

Molecular Weight :558.61826 g mol−1

Cassumunin B [89]

Chemical Name : (1E,6E)-1-(4-hydroxy-3-methoxyphenyl)-7-[4-hydroxy-3-methoxy-5-[(E)-4-(2,4,5-trimethoxyphenyl)but-3-en-2-yl]phenyl]hepta-1,6-diene-3,5-dione

Molecular Formula : $C_{34}H_{36}O9$

Molecular Weight : 588.64424 g mol−1

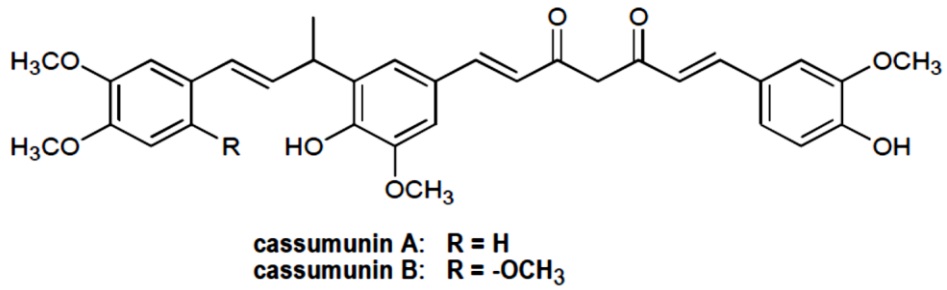


Figure 12 Structure of cassumunin A and B

Ethnomedicinal / traditional use

Zingiber cassumunar Roxb., commonly known as plai, is widely used in folklore remedies as a single plant or as component of herbal recipes in Thailand and many Asian countries for the treatments of conditions, such as: inflammation, sprains and strains, rheumatism, muscular pain, wounds, and asthma, cough and respiratory problems, and as a mosquito repellant, a carminative, a mild laxative and an antidysenteric agent [90, 91]. In Thai traditional medicine, the rhizomes are consumed to relieve asthma, and muscle and joint pain. In Indonesia, the rhizomes are chewed together with clove and the residue is rubbed over the abdomen to relieve colics in children. For treat hepatitis or jaundice, the Indonesian use grated rhizome juice mixed with water and honey by given twice a day. In Malaysia a decoction of the rhizome with pepper given orally is used instead to treat

stomachache. Plain decoction of the rhizome or an infusion of it is given for constipation, diarrhea, flatulence and as a vermifuge. *Zingiber cassumunar* is one of the components of a compound medicine given to women immediatedely after delivery. For purpose of cleansing the uterus the rhizome is pounded together with the rhizome of *Acorus calamus*, the juice is squeeze and mixed with vinegar. *Zingiber cassumunar* is also used to treat all forms of inflammatory conditions. It is used to treat infective inflammatory processes like non-healing ulcers, abscesses and gonorrhea. For non-healing ulcers the pounded rhizome is applied over the ulcer and secured with a bandage. Haematoma is treated by immersing the pounded rhizome in arrack and this is compressed over the lesion.

Pharmacological activity

Antimicrobail Activity:

The essential oil of *Z. cassumunar* (Plai oil) possessed a potent antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria, dermatophytes and yeasts [92]. *Z. cassumunar* had very low or weak activity against the tested microorganisms: *Bacillus cereus* (*B. cereus*), *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*); and two fungi: *Candida albicans* (*C. albicans*) and *Cyptococcus neoformans* (*C. neoformans*), using disc-diffusion and broth microdilution methods [93].

Antitumor activity:

The results of previous study indicated that the rhizome extracts of *Zingiber cassumunar* were able to anti-tumour promoter activity using the short-term assay of inhibition of 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced Epstein-Barr virus early antigen (EBV-EA) in Raji cells [94].

Antioxidant activity:

The essential oil components among the original sources of rhizomes were found no significant differences. The antioxidant activities of the rhizome extract from *Z. montanum* collected from various regions of Thailand obtained from the north showed the highest activity (80.88%), followed by those from the east (76.47%), the south (72.51%), the northeast (67.38%), the west (66.66%) and the central region (57.63%) [95].

Anti-inflammatory activity:

Not only the methanol extract (p.o.) of the rhizomes of *Z. cassumunar* assessed anti-inflammatory activity activity on carrageenin-induced edema in rats but it showed and anti-analgesic on acetic acid-induced vascular permeability and writhing symptoms in mice. These results suggest that the (E)-1-(3,4-dimethoxyphenyl)but-1-ene was one compound which acted to the anti-inflammatory action and analgesic action of *Z. cassumunar* . DMPBD was one compound which was isolated from *Z. casssumunar* in this study. *In vitro* and in vivo,

it showed a potent anti-inflammatory activity through the inhibition of CO and LO pathways [96, 97].

***Cymbopogon nardus* Rendle**

Perennial from a stout rootstock. Culms tufted, robust, up to 2.5 m tall, 1–2 cm in diam. Leaf sheaths reddish purple at base, smooth, glabrous; leaf blades dark green or dark brown when dry, drooping for 1/3 of their length, 30–100 × 1–2 cm, glabrous, abaxial surface scabrid, adaxial surface smooth, base narrow, apex long acuminate; ligule 2–3 mm. Spathate panicle large, narrow, congested, interrupted, 60–90 cm; spatheoles reddish brown, 1.2–2.5 cm; racemes 1–1.5 cm; rachis internodes and pedicels ciliate on margins; pedicel of homogamous pair not swollen. Sessile spikelet oblong-lanceolate, 3–4.5 × 1–1.2 mm; lower glume flat or slightly concave, reddish brown or purplish upward, sharply 2-keeled, keels narrowly winged, obscurely 0–3-veined between keels; upper lemma linear, entire or slightly 2-lobed, mucronate or very shortly awned. Pedicelled spikelet 3.5–7 mm [98].

Botanical classification [99]

Scientific name : *Cymbopogon nardus* Rendle, *Cymbopogon afronardus* Stapf, *Cymbopogon validus* (Stapf) Burtt Davy, *Andropogon nardus* L., *Cymbopogon afronardus* Stapf.

Domain : Eukaryota

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Phylum	:	Tracheophyta
Division	:	Magnoliophyta
Order	:	Poales
Family	:	Graminaceae
Genus	:	*Cymbopogon*
Species	:	*nardus*

Figure 13 *Cymbopogon nardus* Rendle

Synonyms [98]

Ayurvedic	Pangiri
English	Citronella grass
Kenya	Blue Citronella Grass
India	Naid Grass
Thailand	Takrai-hom
Uganda	Tussocky Guinea grass

Phytochemistry

Constituent:

The main constituent of essential oils from *C. nardus* leaves was citronellal (35.5%), geraniol (27.9%) and citronellol (10.7%) [100].

Citronellal [101]

Chemical Name : 3,7-dimethyloct-6-en-1-al

Molecular Formula : $C_{10}H_{18}O$

Molecular Weight : 154.25 g mol−1

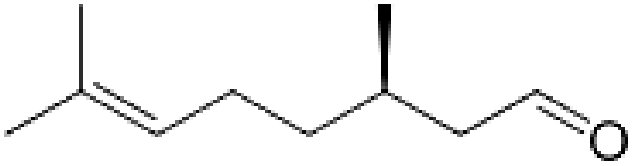


Figure 14 Structure of citronellal

Ethnomedicinal / traditional use

Citronella grass (Cymbopogon nardus) is relatively new to clinical aromatherapy as it has been primarily developed for the fragrance industry. It has little traditional use information. *Cymbopogon nardus* are used for the production of citronella oil, which is used in soaps, as an insect repellent especially mosquitoes in insect sprays and candles, and in aromatherapy, which is famous in Bintan Island, Indonesia and the Philippines. Scientifically known as *Cymbopogon nardus*, this aromatic herb is called as Pangiri in Ayurveda and have been used in this ancient healing system in the treatment of toothaches, redness, irritation and inflammation of the skin, infectious diseases, digestive problems, headache, rheumatism, fatigue and as an after childbirth wash. It was used in traditional medicine for treating menstrual problems, increased perspiration, mental fatigue, depression and as a safe repellent for pets [102].

Pharmacological activity

Antimicrobail Activity:

The in vitro cytotoxicity bioassays on human epidermic cell line HaCaT revealed that the toxicity of the essential oil from *C. nardus* (IC_{50}: 450 µL.mL^{-1}) [99]. A total of 22 chemical compounds were detected in *C. nardus* essential oil with 6-octenal, 3, 7-dimethyl- or citronellal representing the major compounds (29.6%). The MIC values of the citronella oil ranged from 0.244 µg/ml to 0.977 µg/ml when tested against the bacterial isolates. [101]. Lemongrass and citronella oil (*Cymbopogon nardus* L.) revealed the highest antibacterial effect as they possessed the MIC value of 0.125% v/v for all of 5 strains bacterial tested of *Propionibacterium acnes (P. acnes*) [103].

Antifungi activity:

The major constituents of the oils of aerial parts of *Cymbopogon nardus* were geraniol, trans-citral, cis-citral, geranyl acetate, citronellal and citronellol. The essential oils from *Cymbopogon nardus* completely inhibited all 9 tested fungal strains at a dose of 250 mg/m [104].

Standardization parameter

Determination of Macroscopic and microscopic [105]

Plant materials are categorized according to sensory macroscopic and microscopic characteristics. The macroscopic identity of medicinal plant materials is based on the shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface. Microscopic identification is a method using a microscope to identify the structural features, cells, and ergastic substances of herbal samples with application of the knowledge of plant morphology and anatomy so as to authenticate plant species. The procedure of identification contains three main steps: selection of typical materials, preparation of slides or powder, and observation of features. The drawings will be made using microscope and drawing attachment.

Determination of loss on drying [105]

The test for loss on drying determines both water and volatile matter. For materials, which contain little balance combines the drying, process and weight recording; it is suitable where large numbers of samples are handled and where a continuous record of loss in weight with time is required. Loss on drying is the loss of mass expressed as per cent w/w.

Determination of ash values [105]

The earthy matter or inorganic salt of carbonate, silicate and oxalate were measured by two different methods such as the total ash and acid-insoluble ash. This value varies within fairly wide limits and is therefore and important parameter for the purpose of evaluation of crude drugs. In certain drugs, the percentage variation of the weight of ash from sample to sample is very small and any marked difference indicates a change in quality.

The total ash method is designed to measure the total amount of material remaining after ignition of the ground drug at as low temperature as possible (about 500 °C) to remove all the carbons. This includes both "physiological ash", which is derived from the plant tissue itself, and "non-physiological" ash, which is the residue of the extraneous matter adhering to the plant surface.

Acid-insoluble ash is the residue obtained after boiling 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 water content [105]

The azeotropic method (toluene distillation method) gives a direct measurement of the water present in the material being examined. When the sample is distilled together with an immiscible solvent, such as toluene R or xylene

R, the water present in the sample is absorbed by the solvent. The water and the solvent are distilled together and separated in the receiving tube on cooling. The volume of water distilled over can be read and the percentage initially present in the sample can be calculated as a percentage using the formula:

$$\text{Percent of water content (\%)} = (N/W) \times 100$$

Where W = the weight in g of the material examined and N = the number of ml of water obtained.

Determination of volatile oil content [105]

Volatile oils are characterized by their odor, oil-like appearance and ability to volatilize at room temperature. Chemically, they are usually composed of mixtures of, for example, sesquiterpenes, monoterpenes, and their oxygenated derivatives. Aromatic compounds predominate in certain volatile oils.

Determination of extractive value [105]

This method determines the amount of active constituents in a given amount of plant material when extracted with solvents. Then, calculate the content of extractable matter in mg per g of air-dried material. For ethanol-soluble extractable matter, use the concentration of solvent specified in the test procedure for the plant material concerned; for water-soluble extractable matter, use water as the solvent.

TLC identification [106]

Thin layer chromatography is particularly valuable for the qualitative determination of small amounts of impurities. Separation by TLC is effected by the application of the mixture or extract as a spot or thin line on to a sorbent that has been applied to a backing plate. Samples could be directly compared and identified by the fluorescence or color reaction of the developed spots. Silica gel 60 F_{254} precoated TLC plates (Merck, Germany) 0.063–0.200 mm will be used and developed over a 10 cm path. The plate is then placed into a tank with sufficient suitable solvent to just wet the lower edge of the plate sorbent but not enough to wet the part of the plate where the spots were applied. The solvent front then migrates up the plate through the sorbent by capillary action, a process known as development. Remove the plate, mark the position of the solvent front and allow the solvent to evaporate at room temperature. The spots will be visualized under UV light at 254 nm and 365 nm. Then spray with detecting reagent and heat at 110 °C for 10 min. The information provided by a finished chromatogram includes the "migrating behavior" of the separated substances. It is given in the form of the R_f value.

After development, the plate is dried in an oven or fume hood to evaporate the solvent. Compounds are detected on thin layers by their natural color, natural fluorescence under UB light, quenching of fluorescence on a phosphor containing

layer, or as colored, UV absorbing, or fluorescent zones after reaction with an appropriate reagent.

GC/MS qualitative analysis

GC/MS is an instrument that uses to separate mixtures into individual components, identifying and then providing quantitative and qualitative information on the amounts and chemical structure of each compound. Gas liquid chromatography is a popular, powerful, reasonably inexpensive, and easy-to-use analytical tool. Mixtures to be analyzed are injected into an inert gas stream and swept into a tube packed with a solid support coated with a resolving liquid phase. Absorptive interaction between the components in the gas stream and the coating leads to a differential separation of the components of the mixture, which are then swept in order through a detector flow cell. Gas chromatography suffers from a few weaknesses such as its requirement for volatile compounds, but its major problem is the lack of definitive proof of the nature of the detected compounds as they are separated.

The mass spectrometer takes injected material, ionizes it in a high vacuum, propels and focuses these ions and their fragmentation products through a magnetic mass analyzer, and then collects and measures the amounts of each selected ion in a detector. A mass spectrometer is an excellent tool for clearly identifying the structure of a single compound, but is less useful when presented with a mixture.

The combination of the two components into a single GC/MS system forms an instrument capable of separating mixtures into their individual components, identifying, and then providing quantitative and qualitative information on the amounts and chemical structure of each compound. It still possesses the weaknesses of both components. It requires volatile components, and because of this requirement, has some molecular weight limits. The mass spectrometer must be tuned and calibrated before meaningful data can be obtained. The data produced has time, intensity, and spectral components and requires a computer with a large storage system for processing and identifying components. A major drawback of the system is that it is very expensive compared to other analytical systems. With continual improvement, hopefully the cost will be lowered because this system and/or the liquid chromatograph/mass spectrometry system belong on every laboratory bench top used for organic or biochemical synthesis and analysis. Determination of the molecular structure of a compound from its molecular weight and fragmentation spectra is a job for a highly trained specialist. It is beyond the scope and intent of this book to train you in the interpretation of compound structure [107].

Cytotoxic activity (Brine Shrimp lethality assay)

The brine shrimp lethality assay is developed might be used as a simple tool to guide screening and fractionation of physiologically active plant extracts, where

one of the simplest biological responses to monitor is lethality, since there is only one criterion: either dead or alive. This general bioassay detects a broad range of biological activities and a diversity of chemical structures. One basic premise here is that toxicology is simply pharmacology at a higher dose, thus if we find toxic compounds, a lower, non-toxic, dose might elicit a useful, pharmacological, perturbation on a physiologic system [108].

Mutagenic and antimutagenic testing (The Ames Test)

The Ames mutagenicity assay was developed in the early 1970s by Dr. Bruce Ames and has been used for many years as a screen for chemicals that may have mutagenic potential as determined by the frequency of mutations produced in strains of bacteria. The assay is based on a reverse mutation occurring that permits the respective bacterium to survive and grow in the absence of these essential amino acids [109].

The *Salmonella typhimurium*/microsome assay (Ames test) is a widely accepted short-term bacterial assay for identifying substances that can produce genetic damage that leads to gene mutations. The test uses a number of *Salmonella* strains with preexisting mutations that leave the bacteria unable to synthesize the required amino acid, histidine, and therefore unable to grow and form colonies in its absence. The Ames *Salmonella*/microsome mutagenicity assay evolved over the years from the initial screening of a number of histidine mutants which led to the

selection of mutants that were highly sensitive to reversion by a variety of chemical mutagens [110, 111].

DNA damage: Comet assay

The comet assay, which is also referred to as the single cell gel electrophoresis assay (SCG or SCGE assay), is a rapid and quantitative technique by which visual evidence of DNA damage in eukaryotic cells may be measured. It is based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis [112].

Comet assay has been found highly effective in biomonitoring of natural compounds. When damaged, DNA strands are broken into fragments and then migrated towards the anode during electrophoresis. The extent of DNA damage is then quantified using normalized tail length. This normalized tail length of the comet indicated the amount of damage as longer tails indicated that the strand breaks are frequent and that the DNA is fragmented to several smaller molecules. One hundred comets on each slide are scored according to the length and relative intensity of fluorescence in the tail is compared to the head and expressed as tail to head ratio [113].

Once a suspension of cells is obtained, the basic steps of the assay include (1) preparation of microscope slides layered with cells in agarose; (2) lysis of cells to liberate DNA; (3) exposure to alkali (pH 13) to obtain single-stranded DNA and to

express ALS as SSB; (4) electrophoresis under alkaline (pH 13) conditions; (5) neutralization of alkali; (6) DNA staining and comet visualization; and (7) comet scoring [114].

Resazurin cell viability assay

Resazurin dye (7-hydroxy-3H-phenoxazin-3-one-10-oxide) has been broadly used as an indicator of cell viability in several types of proliferation and cytotoxicity assays. The reduction of resazurin correlates with the number of live organisms, such as bacterial, fungi and mammalian cells. Mitochondrial enzymes, as carriers of diaphorase activities, like NADPH dehydrogenase, are probably responsible for the transference of electrons from NADPH + H+ to resazurin, which is reduced to resorufin. Cellular viability quantification based on resazurin has advantages, including rapidity, reliability, sensitivity, safety and cost. In addition, it keeps cells intact, which permits other parallel analyses, such as mRNA, cytogenetic, apoptosis, and immunophenotyping. The level of reduction can be quantified by spectrophotometers using appropriate filters, since resazurin exhibits an absorption peak at 600 nm and resorufin at 570 nm wavelengths [115].

Inflammation

Inflammation is the most common biological reaction to a variety of stimuli and local injury. It generally occurs in response to tissue injury and is associated with the release of different mediators like bradykinin, nitric oxide (NO), vasoactive amines, interleukin 1(IL-1), tumor necrosis factor alpha (TNF-alpha) and eicosanoids (prostaglandins, thromboxones, leukotrienes, lipoxins). Inflammation protects the body against infection and injury but it can produce deleterious consequences to the host. The inflammatory response can lead different diseases, such as rheumatoid arthritis, inflammatory bowel disease, psoriasis [116].

***In vitro* anti-inflammatory activity**

An *in vitro* test is a study that takes place outside a living body and offers data of a different quality compared with tests by using live animals.

Determination of nitric oxide production by griess assay

Griess reaction Assay has been used extensively in analysis of numerous biological samples including plasma (serum), urine, CSF, saliva and cell culture media. In this method, nitrite is first treated with a diazotizing reagent, e.g., sulfanilamide (SA), in acidic media to form a transient diazonium salt. This intermediate is then allowed to react with a coupling reagent, N-naphthyl-ethylenediamine (NED), to form a stable azo compound. The overall reaction is described in the scheme below. The intense purple color of the product allows

nitrite assay with high sensitivity and can be used to measure nitrite concentration as low as 0.5 μM level. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample. Because of the two-step nature of the Griess Reaction, variations exist among published Griess Reaction assays. For example, SA and NED can be pre-mixed in an acid medium before reacting with nitrite. Yet in a different version, after reacting nitrite with SA in acidic medium, NED is added 10minutes later. The most popular version seems to be the sequential method in which nitrite is mixed with SA first, followed immediately by addition of NED. This method seems to give highest yield of the chromophore, and therefore most sensitive way to perform Griess Reaction assay [117].

Enzyme Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a common laboratory technique which is used to measure the concentration of an analyte (usually antibodies or antigens) in solution. The basic ELISA, or enzyme immunoassay (EIA), is distinguished from other antibody-based assays because separation of specific and non-specific interactions occurs via serial binding to a solid surface, usually a polystyrene multiwell plate, and because quantitative results can be achieved. The steps of the ELISA result in a colored end product which correlates to the amount of analyte present in the original sample [118].

Tumor Necrosis Factor alpha (TNF - alpha)

Tumor necrosis factor-alpha (TNF-alpha) is a central regulator of inflammation, and TNF-alpha antagonists may be effective in treating inflammatory disorders in which TNF-alpha plays an important pathogenetic role.

A monoclonal antibody specific for TNF alpha has been coated onto the wells of the microtiter strips provided. Samples, including standards of known TNF alpha concentrations, control specimens or unknowns are pipetted into these wells. During the first incubation, the standards or samples and a biotinylated monoclonal antibody specific for TNF alpha are simultaneously incubated. After washing, the enzyme Streptavidin-HRP, that binds the biotinylated antibody is added, incubated and washed. A TMB substrate solution is added which acts on the bound enzyme to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of TNF alpha present in the samples [119].

Interleukin-1 alpha

Interleukin-1 (IL-1) is a key mediator of the host response to various infectious, inflammatory and immunologic challenges. Two distinct polypeptides, IL-1 alpha and IL-1beta, mediate the biological activities and bind to the same cell surface receptors.

The Human Interleukin 1 Alpha (IL-1alpha) ELISA Assay Kit (enzyme-linked immunoassay kit) is intended for the quantitative determination of human Interleukin

1 Alpha (IL-1alpha) concentrations in cell culture supernates, serum, and plasma. An antibody specific for IL-1alpha has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu IL-1alpha content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a biotinylated second antibody. During the first incubation, the Hu IL-1alpha antigen binds simultaneously to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site. After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu IL-1alpha present in the original specimen [120, 121].

Prostaglandin E2

Prostaglandin E2 (PGE2) is one of the prostaglandins, a group of hormone-like substances that participate in a wide range of body functions such as the contraction and relaxation of smooth muscle, the dilation and constriction of blood vessels, control of blood pressure, and modulation of inflammation. PGE2 is produced by several cell types including macrophages, fibroblasts and some malignant cells and exerts its actions through 4 receptors EP1, EP2, EP3 and EP4. All these receptors are

rhodopsin-type receptors with seven transmembrane-spanning domains. However each receptor is coupled to different G proteins and uses different second messenger signaling pathways. Accounting on those receptors coupled to different G proteins, PGE2 has been shown to have both pro and anti-inflammatory actions. It induces vasodilatation by activating cAMP-coupled EP2 receptors on vascular smooth muscle and increases vascular permeability. But as inflammation progresses PGE2 promotes bronchodilatation. PGE2 also inhibits T helper cells type 1 (Th1) cytokines productions such as IL-12, IFNγ, IL-2 and seems to favor Th 2 type of immune response. Although it has also been described that PGE2 decreases IL-4 and IL-5 production as well as IgE production. PGE2 is involved in several pathologies such as periodontal disease and promotes tumor-cellssurvival.

The Mouse PGE2 ELISA Kit is an Vitro enzyme-linked immunosorbent assay for the quantitative measurement of Mouse PGE2 in serum, plasma, tissue,cell culture supernatants and urine. The Mouse PGE2 polyclonal antibodies are precoated onto 96-well plate. Standard and samples are pipetted into the wells and Mouse PGE2 present in a sample is bound to the wells by the immobilized antibody. The biotinylated detection antibodies are added to the wells and then followed by washing with PBS or TBS buffer. After washing away unbound biotinylated antibody, Avidin-Biotin -Peroxidase Complex is pipetted to the wells. The wells are washed again, a TMB substrate solution is added to the wells and the color changes after

adding acidic stop solution. The indensity is proportional to the amount of Mouse PGE2 bound and measured at 450nm±10nm [122, 123].

***In vivo* test anti-inflammatory activity**

Carrageenan induced rat paw edema test

The processes associated with the inflammatory response are complex but important aspects which have been exploited for screening for anti-inflammatory compounds are the various functions of neutrophils, the metabolic products of arachidonic acid and the role played by reactive oxygen species. Inhibition of carrageenan-induced inflammation has been shown to be highly predictive of antiinflammatory drug activity in human inflammatory disease. The subcutaneous injection of carrageenan causes extravasation of plasma with an increased formation of arachidonic acid metabolites via the cyclooxygenase and lipoxygenase enzyme pathways [124, 125].

Prostaglandin E_2-induced mouse paw edema

PGE2, a major cyclooxygenase product, exhibits a broad range of biological actions in diverse tissues through its binding to specific receptors on plasma membrane. These receptors belong to the family of G protein-coupled receptors, and they can be divided into four subtypes (EP1-4), each of which is encoded by distinct genes. Whereas the EP1 receptor induces calcium mobilization by phospholipase C activation via Gq protein, EP2 and EP4 receptors are known to

activate adenylyl cyclase via stimulatory G protein. On the other hand, the EP3 receptor reduces cAMP levels as it is coupled to inhibitory G proteins. In addition, the EP3 receptors are the only receptors to display multiple splice variants identified in several species. PGE2 is generally considered as a key proinflammatory mediator, and its role has been extensively studied in several inflammatory events.Thus, high levels of PGE2 have been found in inflammatory exudates, and the injection of PGE2 directly into tissue has been shown to induce a number of classical sign of inflammation [126].

Antioxidant activity

Free radicals are electrically charged molecules, i.e., they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves. Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. And until subsequent free radicals are deactivated, thousands of free radical reactions can occur within seconds of the initial reaction. Antioxidants are capable of stabilizing, or deactivating, free radicals before they attack cells. Antioxidants are absolutely critical for maintaining optimal cellular and systemic health and well-being [127].

Total phenolic content by Folin-Ciocalteu method

Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins. These compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators, contributors to plant pigmentation, antioxidants, and protective agents against UV light, among others. Colorimetric reactions are widely used in the UV/VIS spectrophotometric method, which is easy to perform, rapid and applicable in routine laboratory use, and low-cost . However, it is important that colorimetric assay need to use a reference substance, then this method measures the total concentration of phenolic hydroxyl groups in the plant extract. Polyphenols in plant extracts react with specific redox reagents (Folin-Ciocalteu reagent) to form a blue complex that can be quantified by visible-light spectrophotometry. The Folin-Ciocalteu reaction forms a blue chromophore constituted by a phosphotungstic-phosphomolybdenumcomplex, where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds [128].

DPPH free radical-scavenging

DPPH is very stable free radical. Unlike in vitro generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme

inhibition. A freshly prepared DPPH solution exhibits a deep purple colour with an absorption maximum at 517 nm. This purple colour generally fades when antioxidant molecules quench DPPH free radicals (i.e. by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them into a colourless-/bleached product (i.e. -2,2diphenyl–1hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517nm [129].

Ferric Reducing Antioxidant Power (Frap) Assay

The ferric reducing antioxidant power(FRAP) method is based on the reduction of a ferroin analog, the Fe3+ complex of tripyridyltriazine Fe(TPTZ)3+, tothe intensely blue coloured Fe2+ complex Fe(TPTZ)2+ byantioxidants in acidic medium. Results are obtained as absorbance increases at 593 nm and can be expressed as micromolar Fe2+ equivalents or relative to an antioxidant standard [130].

Nitric oxide : Griess reagent assay

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions. NO is known to be a ubiquitous free-radical moiety, which is distributed in tissues or organ systems

and is supposed to have a vital role in neuromodulation or as a neurotransmitter in the CNS.

Nitric oxide generated from sodium nitroprusside and measured by the Griess reaction. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitric ions that can be estimated by use of Griess reagent. Scavenger of nitric oxide competes with oxygen leading to reduced production of nitric oxide. Sodium nitroprusside (5 mM) in phosphate–buffered saline (PBS) was mixed with 3.0ml of different concentrations (10-100 µg/ml) of the drugs dissolved in the suitable solvent systems and incubated at 250C for 150 min. The samples from the above were reacted with Griess reagent (1% sulphanilamide, 2%H3PO4 and 0.1% napthylethylenediamine dihydrochloride). The absorbency of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 540 nm [131, 132].

Metal chelating assay

Transition metal ions, especially iron can stimulate lipid per oxidation by Fenton reaction (H2O2 + Fe2+ ---> Fe3+ OH- +OH-) and can also accelerate lipid per oxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction. Metal ion chelating capacity is significant

since it reduces the concentration of the transition metal that catalyzes lipid per oxidation [133].

CHAPTER 3

RESEARCH METHODOLOGY

Chemicals

1. Cirtic acid monohydrate (BDH Prolabo chemical, England)
2. Magnesium sulfate (MgSO47H20) (Ajax Finechem Pty Ltd, Australia)
3. Sodium ammonium phosphate Tetrahydrate (Fluka Chemika, Switzerland)
4. Potassium phosphate dibasic(androus) (Ajex Finechem Pty Ltd, Australia)
5. Bacto agar, 40%glucose (Merck, Damstadt, Germany)
6. Oxiod nutrient broth No.2 (Himedia Laboraties. Pvt. Ltd., India)
7. Sodium chloride (NaCl) (Mallinckrodt Labaratory Chemicals, U.S.A.)
8. L-histidine HCL (Fluka Chemika, Switzerland)
9. Biotin (Sigma Chemical, St Louis, USA)
10. Sodium dihydrogen phosphate (NaH2PO4) (Sigma Chemical, St Louis, USA)
11. Disodium hydrogen phosphate dehydrate (BDH Prolabo chemical, England)
12. Potassium chloride (KCl) (Ajex Finechem Pty Ltd, Australia)
13. Sodium nitrite (Ajex Finechem Pty Ltd, Australia)
14. Ammonium sulfamate (Fluka Chemika, Switzerland)
15. Conc. Hydrochloric acid (Mallinckrodt Labaratory Chemicals, U.S.A.)
16. Acetonitrile (J.T. Baker, Phillipsburg, USA.)
17. 1-Aminopyren (Aldrich, St, Louis, USA.)

18. Dimethysulfoxide (DMSO) (Merck, Damstadt, Germany)
19. Crytal violet indicator (Fluka Chemika, Switzerland)
20. 2% Tween 80 (Sigma Chemical, Co., USA)
21. Acetylsalicylic acid (aspirin) (Sigma Chemical, Co., USA)
22. Lipopolysacharide (LPS) from *E. coli* (Sigma Chemical, Co., USA)
23. Indomethacin (IND; Sigma Chemical, Co., USA)
24. Serotonin (Sigma Chemical, Co., USA)
25. Histamine (Sigma Chemical, Co., USA)
26. Prostaglandin (Sigma Chemical, Co., USA)
27. Cyproheptadine (Sigma Chemical, Co., USA)
28. Chlorpheniramine (Sigma Chemical, Co., USA)
29. 0.9% Normal saline solution (General hospital products Public company, Thailand)
30. Gramma-carrageenan (Sigma Chemical, Co., USA)
31. Methanol HPLC grade (Merck, Damstadt, Germany)
32. Phosphoric acid (Merck, Damstadt, Germany)
33. Ficoll-Histopaque 1077 (Sigma-Aldrich, St Louis, USA)
34. Ethylenediaminetetra-acetic acid disodium salt (Ajex Finechem Pty Ltd, Australia)
35. Low melting point agarose (Sigma-Aldrich, St Louis, USA)
36. Triton X-100 (Sigma-Aldrich, St Louis, USA)

37. 2, 2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, St Louis, USA)
38. 3, 5-Di-*tert*-4-butylhydroxytoluene (BHT) (Sigma-Aldrich, St Louis, USA)
39. Quercetin (Sigma-Aldrich, St Louis, USA)
40. *N*-1-napthylethylenediamine dihydrochloride (Sigma-Aldrich, St Louis, USA)
41. Sodium nitroprusside (Sigma-Aldrich, St Louis, USA)
42. Sulfanilamide (Sigma-Aldrich, St Louis, USA)
43. Folin-Ciocalteu reagent (Sigma Chemical, Co., USA)
44. Ferric chloride (Ajex Finechem Pty Ltd, New Zealand)
45. Ferrozine (Sigma-Aldrich,USA)
46. Potassium ferricyanide (Sigma-Aldrich,USA)
47. Ascorbic acid (Merck, Damstadt, Germany)

Equipments and instumentations

1. Rotary evaporation (Buchi R210, Switzerland)
2. Filter paper Whatman No.1 and No.4
3. Lyophilizer (Labconco, Missouri, USA)
4. Gas chromatography (GC) / mass spectrometry (Thermo Finnigan model Trace GC Ultra equipped with Finnigan DSQ MS detector, USA)
5. Spectrophotometer (Shimadzu-W 1800, Shimadzu corp., Japan)
6. Spectrophotometer (T60, PG Instrument Ltd., United Kingdom)
7. Autoclave

8. Hot air oven (WTB binder No. 4940006, Germany)
9. Incubator (Memmert, Germany)
10. Ultrasonic sonicator (Analytical Lab Science Co., Ltd., Thailand)
11. Water bath
12. Microplate reader (ASYS UVM340, Biochrom Ltd., United kingdom)
13. Microplate with 96 wells (Costar, USA)
14. Microscope (Zeiss Axioskop, Germany)
15. Vortex mixer (Scientific Industries, USA)
16. Plethysmometer (Ugo Basile, Italy)
17. Stomach tube No. 18 (Poper & Sons, USA)
18. Needle No. 26,30 (Nipro, Japan)
19. Syring 1 ml (Nipro, Japan)
20. Syring 50 µl (Hamilton, USA)
21. Digital thermometer (YSI PrecisionTM model 4000A, USA)

Sample collection

Plants were collected from several places in Thailand. All set of crude drugs were authenticated by Ruangrungsi N. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Cha Tu Ka La Thad and Tree Phon Thad remedies extraction

Plants species were shade-dried and ground to coarse powders. The powders of plants were continuous macerated with ethanol and water respectively. The ethanol extracts were evaporated under vacuum and the water extracts were lyophilized to dryness. The remedy extract will be prepared by mixing each extract in the quantity equivalent to the formula. The extracts yield were weighed, recorded and stored at -20 ℃ to decrease the possibility of degradation of active compound.

Standardization parameter

The standardization parameters were examined by standard methods of World Health Organization (WHO) [105].

Determination of Macroscopic and microscopic

The characters of each species were carried out by macroscopic and microscopic examinations. The physical properties such as shape, size, color, odor, texture and other visual inspection of crude drug were observed during the macroscopic evaluation. The transverse section and powder were studied under microscopic with 10X, 20X, and 40X objective lens magnifications and 10X eyepiece lens. The results were displayed by hand drawing in proportional scale related to the original size.

Determination of loss on drying

Three grams of the air dried crude drug for that specific substance was accurately weighed in a dried and tarred flat weighing bottle. The substance was to be dried by heating at 100-105 ℃ to constant mass.

Determination of total ash value

Three grams of the ground sample was placed in a previously ignited and tared crucible. The sample was spread in an even layer and ignited by gradually increasing the heat to 500 ℃ at 5 hours until white that ash was obtained. Then, the sample was cooled in a desiccator and weighed.

Determination of acid-insoluble ash

The crucible containing the total ash was added 25 ml of 2N HCl was added into the remaining ash and gently boiled. It was filtrated and burned at 500 ℃ for 5 h then measured the amount of silica presented.

Determination of water content

Fifty grams of sample in 200 ml water saturated toluene was subjected to an azeotropic distillation. As soon as the water was completely distilled, the inside of condencer tube was rinsed with toluene and the distillation was continued for 1 more hours.The volume of water distilled over can be read and the percentage initially present in the sample can be calculated as a percentage using the formula:

$$\text{Percent of water content (\%)} = (N/W) \times 100$$

Where W = the weight in g of the material examined and N = the number of ml of water obtained.

Determination of volatile oil content

Weigh 100.0 grams of ground crude drug, add with 600.0 ml of water and distill by Clevenger apparatus. When volatile oil was completely distilled, allow the receiving tube to cool room temperature. When volatile oil and water layers were separated, read off the volume of volatile oil.

Determination of extractive value

The ground sample 5.0 g was macerated with 70 ml 95% ethanol or water in a closed conical flask in shaking bath for 6 hours and allowed to stand for 18 hours. The extract was filtrated and adjusted to 100 ml by washing the residue. The filtrate (20 ml) was evaporated to dryness on a water bath. Then, it was dried at 105 □ C for 6 hours, cooled in a desiccator and weighed without delay.

TLC identification

Ten milligrams of the sample extract was mixed with 1 ml of ethanol. Five microliters of the solution was applied on to a thin-layer plate coated with siliga gel 60 $F_{254.}$ The TLC plate was then placed in a chamber with mobile phase. After development, the plate was removed and allowed to dry at room temperature and examined under ultraviolet light with 254 nm and 365 nm. Then, the plate was sprayed with sulfuric acid reagent and heated in an oven at 105 ℃ for 5 minutes.

GC/MS analysis

The volatile oil analyses were performed on a Finnigan Trace GC ultra with Finnigan Trace DSQ mass spectrometry and ZB-5 capillary column (30 m x 0.25 mm, 0.25 µm film thicknesses). The oven temperature was 60°C for 1 min. then ramped to 240°C with the rate of 3°C/min. Auto injector (Finnigan AI 3000) was set at 180°C and 1 µl solution with split ratio of 100:1. Helium was used as carrier gas. The constituents of the volatile oils were identified by matching their mass spectra and retention indices with Adams Essential Oil Mass Spectral library and NIST02 Mass Spectral library.

Safety assessment

Cytotoxic activity (Brine Shrimp Bioassay)

Brine shrimp lethality bioassay will be carried out according to the procedure described by Meyer *et al*. [134]. Brine shrimp eggs were hatched in artificial sea water. After 48 hours of incubation, ten brine shrimps were transferred to each sample vial using a Pasteur pipette and artificial sea water was added to make 5 ml. Filter papers impregnated with extracts at the concentration of 1000, 100 and 10 µg/ml in methanol were air dried before placed in vials containing the brine shrimps. Control was prepared as mentioned above using only methanol instead. Five replicates were prepared for each concentration. The vials were maintained under illumination. Twenty-four hours later, the number of survivors was counted, recorded and concentration which caused 50% of brine shrimp lethality (LC_{50} value) was

obtained from a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts.

Mutagenic and antimutagenic testing (The Ames test)

Preparation of bacterial suspension

S. typhimurium strain for frame-shift mutation, TA98 (*hisD3052, bio, uvrB-bio, rfa,* and *pKM101*) and strain for base-pair substitution mutation, TA100 (*hisG46, bio, uvrB-bio, rfa,* and *pKM101)* were kindly provided by the Biochemistry and Chemical Carcinogenesis Section, Research Division, National Cancer Institute, Bangkok, Thailand. All tester strains were grown in an Oxoid nutrient broth No.2 and incubated overnight in a shaking water bath at 37 °C. The culture were reisolated by streaking the bacteria on a minimal glucose agar plates enriched with ampicillin, L-histidine HCl and biotin, then incubated at 37 °C for 48 h. After incubation, picked a well isolated colony with a sterile loop, then overnight cultured in an Oxoid nutrient broth No.2 at 37 °C in a shaking water bath. The mutant strains were confirmed for the genotypes of histidine/biotin dependence, *rfa* marker, *uvrB* deletion gene mutations and presence of plasmid *pMK101*.

Mutagenic testing

Aliquots (0, 25, 50, 100 and 200 mg/ml) of both extracts of each species and remedies were dissolved in DMSO or water to add to the tube at 200 µl for mutagenic screening using the Ames test. They were acidified to pH 3.0–3.5 by

adding 200 µl of each solution to the tube containing 550 µl of 0.2 N hydrochloric acid and adjusted to 1000 µl by 250 µl of DMSO (without nitrite treatment) or 250 µl of 2 M sodium nitrite (with nitrite treatment) then incubated under shaking at 37°C for 4 h. One hundred microliters of the acidified extracts were neutralized with 500 µl of 0.5 M phosphate buffer (pH 7.4) before adding 100 µl of bacterial suspension and incubated at 37°C in shaking water bath for 20 min. Two milliliters of top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin at 45°C were added, mixed well, and poured over the surface of a minimal glucose agar plate. The plates were incubated at 37°C for 48 h and the numbers of his + revertant colonies were counted. DMSO was used as a negative control to determine the spontaneous reversion activity. All tests were performed in triplicate. The mutagenic index (MI) was calculated from the number of revertant colonies of the sample treatment divided by the number of spontaneous revertant colonies. Positive mutagenic effect was considered when the number of induced revertant colonies increases in a dose-response relationship manner, at least two doses were higher than spontaneous revertant (MI ≥1) and at least one dose gave rise to twice over the spontaneous revertant (MI >2) [135-137].

Antimutagenic testing

The antimutagenic effect of extracts against 1-aminopyrene treated with sodium nitrite was determined by the preincubation method of Ames test similar to

the mutagenic testing. Ten microliters (tested on TA98) or 20 µl (tested on TA100) of 0.075 mg/ml 1-aminopyrene was transferred into the sterile test tube. Then, 740 µl or 730 µl of 0.2 N hydrochloric acid and 250 µl of 2M sodium nitrite were added to obtain the total volume at 1 ml. The mixtures were shaken at 37 C for 4 h. Stopped reaction for 1 min in an ice bath, then added 250 µl of 2 M ammonium sulfamate and allowed the test tube to stand in an ice bath for 10 min before Ames test. Mixed 25 µl of nitrite treated 1-aminopyrine with 500 µl of 0.5 M phosphate buffer (pH 7.4), added 100 µl of each tester strains that overnight cultured. An aliquot (0, 25, 50 and 75 µl) of both extracts of *C. nardus* roots (200 mg/ml in DMSO or water) were added and the final volume was adjusted to 700 µl with DMSO or water. The mixture was incubated at 37 °C in shaking water bath for 20 min, after that, added to 2 ml top agar containing histidine–biotin. The mixture was poured over the surface of a minimal agar plate and incubated for 48 h at 37 °C. After incubation, revertant (mutant) colonies were counted. All tests were performed in triplicate. The percent inhibition was calculated by the following formula [138].

$$\% \text{ modification} = \left(\frac{A - B}{A - C} \right) \times 100$$

Where A is the number of histidine revertants colonies per plate induced by nitrite treated 1-Aminopyrene, B A is the number of histidine revertants colonies per

plate by nitrite treated 1-Aminopyrene in the present of each extract and is the number of spontaneous revertants colonies per plate.

DNA damage: Comet assay

The comet assay with crude extracts was performed by the modified method of Singh *et al*. The human lymphocyte cells were obtained from the study of Manohan *et al*. The human lymphocyte cells were treated with the extracts, incubated at 37 °C for 1 hour. Then the samples were centrifuged at 3000 rpm at 4 °C for 5 min, discarded RPMI-1640 and added 10 µl of phosphate buffer saline pH 7.4 in to adherent cells in test tubes. The slides were pre-coated with 1 % Normal melting agarose which was the first layer. After incubated 1 hour, an 85 µl of treated samples were layered into the pre-coated slide, placed the cover slips over the second layer and kept on ice to solidify agarose for 10 min. After agarose gel has solidified, the cover slips were removed. The third layer was applied by 0.5 % of low melting point agarose (LMP) and allowed to solidify on ice for 10 min. Then the freshly lysis solution was prepared by mixed 2.5 M NaCl, 100 mM EDTA, 10 mM Tris (PH 10) with 10 % of DMSO and 1 % of Triton X-100 being added just before use. The slide were incubated with lysis solution at 4 °C for 1 hour. After lysis process, the slides were drained and placed in horizontal gel electrophoresis tank and immersed by electrophoresis solution incubated at 25 min. The electrophoresis was conducted under alkali condition at 0.7 v/cm for 25 min. After electrophoresis, the slides were

placed horizontally and neutralization buffer containing 0.4 M Tris uffer, pH 7.5 was added, and allowed to sit for 5 min wuth three times. Each slide was stained with 75 µl of 20 µg/ml Ethidium bromide for 5 min. Hydrogen peroxide treated cell was used as a positive control wheras phosphate buffer saline was used as a negative control. The migrated DNA (comet) images are observed under fluorescence microscope with a final magnification of 400x. [139]. The degrees of damage will be classified, based on the length of migration or the perceived relative proportion of the DNA in tail, into 5 classes from 0 (no tail) to 4 (almost all DNA in tail), and then summing the values obtained in each gel [140].

Efficacy assessment

***In vitro* anti - inflammatory activity for Cha Tu Ka La Thad remedy**

An *in vitro* test is a study that takes place outside a living body and offers data of a different quality compared with tests by using live animals.

Cells

Murine macrophages J774A.1 were obtained from ATCC. The cells were subcultured three times weekly and maintained in the completed Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated at 37 °C in 5%CO2/ 95% air [141].

Cell viability

The viability of the cells was performed to evaluate the cytotoxicity of extracts using the resazurin assay [142] .The 1×10^5 cell/well of J774A.1 murine macrophage cells were treated with extracts at the concentrations 0, 6.25, 12.5, 25, 50 and 100 µg/ml in -96well plates. % 0.2DMSO and 20 µM dexamethasone were used as the negative and positive controls respectively. The cells were stimulated with 0.1 µg/ml LPS for 24 h at 37 °C. Supernatants were removed from the treated cells to investigate in NO scavenging inhibition assay. The complete DMEM medium containing 50 µg/ml of resazurin 70 µl was added in each wells and incubated for 2 h at 37 °C. The amount of resorufin, the product from resazurin production in viable cells was determined by measuring the absorbance of each well was read at 570 and 600 nm by microplate reader.

Determination of nitric oxide production

From the resazurin assay, the supernatants were investigated the amount of NO released by using Griess reagent [143]. Aliquots of 100 µl of supernatants were mixed with 20 µl of 1% sulfanilamide in 5% phosphoric acid and incubated 10 min at room temperature. Then added 20 µl of 0.1% napthyl-ethylenediamin dihydrochloride and incubated 10 min at room temperature. After that, the reaction mixture was detected with a microplate reader at 540 nm. The amount of nitrite was calculated from a sodium nitrite standard curve.

TNF-alpha inhibition assay

J774A. 1murine macrophage cells were seeded at a density of 1× 105 cell/well and incubated overnight. The macrophage were pretreated with all ethanol extracts at different concentrations (0, 12.5, 25, 50and 100µg/ml) and incubated for 1 h. After, LPS (0.1 µg/ml) was added followed by overnight incubation at 37 °C in %5CO%95 /2 air. The supernatants were collected and used to evaluate the levels of TNF-alpha as according to the method described by the kit manufacturer (ELISA Development kit, Peprotech, Rocky Hill, USA). Dexamethasone (20 µM) was used as reference standard.

PGE2 inhibition assay

J774A. 1murine macrophage cells were seeded at a density of 1× 105 cell/well and incubated overnight. The macrophage were pretreated with extracts at different concentrations (0, 12.5, 25, 50and 100µg/ml) and incubated for 1h. After, LPS (0.1µg/ml) was added followed by overnight incubation at 37°C in %5CO %95 /2 air. The supernatants were collected and used to evaluate the levels of PGE 2as according to the method described by the kit manufacturer (PGE 2ELISA Kit, Thermo scientific, Pierce Biotechnology, USA). Dexamethasone (20µM) was used as reference standard.

In vivo anti - inflammatory activity for Tree Phon Thad

Experimental animal

Experiments were conducted using adult male ICR mice weighing 18-25g purchased from National Laboratory Animal Centre, Salaya, Nakhon Pathom, Thailand. They were housed at 25±2 ℃ under a 12-h light/12-h dark cycle, relative humidity (50-60%) and with access to food and water *ad libitum*. Animals were starved 2-3 h prior to administration of test compounds. At the end of each experiment, animals were sacrificed with carbon dioxide asphyxiation. This animal protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Carrageenan-induced paw edema in mice

Paw edema is one of the most commonly employed techniques for screening of anti-inflammatory drugs. This method is based upon the ability of such agents to inhibit the edema produced in the hind paw of rodent after injection of a phlogistic agent. Carrageenan induced rat paw edema test will be used to assess the anti-inflammatory activity according to the method described by Winter et al. in 1962 [144]. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil®, sulfated polysaccharides like with 2% Tween 80 (10 mL/kg), IND (10 mg/kg), various doses of the root extract of Tree-Phon-Thad remedy (TPT; 75, 150 and 300 mg/kg) or three herbal root extracts of Tree-Phon-Thad remedy (ZZ, ZM, CN; 25, 50, 100, 200 and 400 mg/kg) orally. One

hour later, 1% carrageenan solution (50 µL) were injected subcutaneously into the plantar surface of the left hind paw of each mouse. The mouse's paw was marked with black ink at the level of the lateral malleolus and immersed in dipping solution up to this mark. The volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls at 1, 2, 3, 4, 5 and 6 h using plethysmometer (Ugo Basile7150, Italy). The percentage of inhibition of edema was analyzed using the following fomular:

$$\% \text{ Inhibition of edema} = \left[\frac{(V_c - V_t)}{V_c}\right] \times 100$$

Vc = edema volume in control group; Vt = edema volume in tested group [145, 146].

Analysis of the mechanism of anti-inflammatory action of three herbal roots extracts of Tree-Phon-Thad remedy

The mechanism of anti-inflammatory action of three herbal root extracts of Tree-Phon-Thad remedy (ZZ, ZM and CN) were investigated using PGE_2-induced acute inflammation model in mice. Male ICR mice (18-25 g) were used. Group of 8 animals were used for controls and treated mice. On the day of testing, animals were treated orally with 2% Tween 80 (10 mL/kg), IND (10 mg/kg), ZZ (25 mg/kg), ZM (400 mg/kg) or CN (200 mg/kg). One hour later, animals were challenged by subcutaneous injection of 0.01% PGE_2 solution (50 µL) into the plantar surface of the left hind paw.

The volume of the injected paw is measured before and after injection of PGE_2 and the paw volume of the treated animals is compared to the controls at 0.5, 1, 1.5, 2, 3 and 4 h using plethysmometer (Ugo Basile7150, Italy). The percentage of inhibition of edema was analyzed using the formula as described above [147].

Acute toxicity

Animals employed in the study were observed for 72 h and morbidity or mortality was recorded, if happens, for each group at the end of observation period.

Determination of antioxidant activity

Total phenolic contant

The total phenolic content was determined by the spectrophotometric method. In brief, 25 µl of extracts (0.5- mg/ml) was mixed with 125 µl of 10% Folin-Ciocalteu's phenol reagent in with a 96 well microplate reader. After 5 min, 100 µl of a 7.5% Na_2CO_3 solution was added to the mixture then incubation at room temperature at 60 min, after which the absorbance was read at 756 nm [148].

DPPH free radical-scavenging

The antioxidant activity of plant extracts will be assessed by ability to scavenge DPPH free radical as described by Brand-William *et al.* [149]. Various concentrations of samples dissolved in methanolic are added to DPPH radical methanolic solution (120 µM). After 30 minutes of incubation at room temperature in the dark, the absorbance is measured at 517 nm with a 96 well microplate reader.

BHT and Quercetin are used as positive controls. Three replicates are made for each test sample. The scavenging activity will be evaluated from the decrease in absorbance value at 517 nm and calculated using the following formula: DPPH radical inhibition (%) = [(absorbance of control-absorbance of sample)/absorbance of control]×100. The activity is expressed as IC_{50} values which indicate the concentration of sample required to scavenge 50% of DPPH free radical.

Ferric Reducing Antioxidant Power (Frap) Assay

The FRAP assay was carried out by the method of Benzie and Strain (1996) with minor modification. The FRAP solution was freshly prepared by mixing: 300 mM of Acetate buffer at P.H. 3.6, 10 mM of TPTZ in 40 mM HCL and 20 mM $FeCl_3$ solution at (10:1:1) parts per volume respectively. To 25 µl of extracts, quercitin and BHT were added 175 µl of FRAP solution in 96 well plate, and then were incubated in room temperature for 30 min. The absorbance was recorded at 593 nm by micro plate reader. The change in absorbance from the initial blank reading was compared to that of a standard that was run simultaneously. The extracts and positive controls were expressed as mM of ferrous iron (Fe)ll()/mg dry mass of sample [150].

Nitric oxide : Griess reagent assay

Sodium nitroprusside (5 mM) in phosphate–buffered saline (PBS) was mixed with 3.0 ml of different concentrations (10-100 µg/ml) of the drugs dissolved in the suitable solvent systems and incubated at 25 ℃ for 150 min. The samples from the

above were reacted with Griess reagent (1% sulphanilamide, 2%H3PO4 and 0.1% napthylethylenediamine dihydrochloride). The absorbency of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was read at 540 nm [151-153].

Metal chelating assay

The assay was carried out according to with modifications. In the 96 well microplate, 15 µl of iron (II) chloride ($FeCl_2$) in ultrapure water (2 mM) was added into 110 µl of ethanol and water extracts (1-10 mg/ml). The reaction started by addition of 75 µl of aqueous Ferrozine (5 mM). The mixture was left for 10 minutes, before the absorbance of reaction was measured at 562 nm. Control was ethanol reacted with FeCl2 and Ferrozine. The Fe^{2+} chelating activity of test compound was calculated as: Chelating activity (%) = (Absorbance control – Absorbance sample)/Absorbance control × 100. EDTA was used as positive control (0.01-0.1 mg/ml) [154].

DATA ANALYSIS

The parameter standardization will be carried out as mean ± standard deviation (SD). The results of anti-inflammatory activities were expressed as mean ± standard deviation (SD) of triplicate in each experiment. After that, one-way analysis of variance (ANOVA) was performed to see the differences in mean values between

groups, followed by a Dunnett's test for multiple comparisons. Statistical significance will be assessed as $p < 0.01$ and $p < 0.05$.

Limitation

Quality assessments are performed according to Thai herbal pharmacognostic specifications. Moreover, biological activities testing in this study are performed in only pre-clinical stages prior to further clinical trial evaluation.

Ethical consideration

For ethical consideration, the proposal be submitted for approval from Animal Care and Use Committee (CU-ACUC), Faculty of Pharmaceutical Sciences, Chulalongkorn University for some biological activities testing.

CHAPTER IV
RESULTS

Pharmacognostic specifications

Cha Tu Ka La Thad remedy

Plumbago indica Linn.

Common Name	CHET-TA-MUN-PHLOENG-DAENG
Other Name	PIT-PIU-DAENG
English Name	Rosy-flowered leadwort, Officinal leadwort
Scientific Name	*Plumbago indica* Linn.
Synonym	*P. rosea* Linn.
Family	PLUMBAGINACEAE
Distribution	Tropical Africa, tropical Asia and the Pacific region. Common throughout South-East Asia.
Used Part	Root
Ethnomedical Use	Emmenagogue, stomachic and carminative activities, treatment of haemorrhoids, as an abortifacient and as a means to purify the blood and stimulate digestion.

Description of plant

A shrub up to 1.5 m tall, branched from the base, stems drooping, sometimes rooting; leaves oblong, 5-15 cm x 2-8 cm, petiole not auriculate; inflorescence a rather sparsely flowered spike, not corymbose, rachis glabrous, 10-30 cm long; flowers with calyx about 1 cm long, covered in glands, red, corolla tube 2.5-4 cm long, lobes 2-3 cm in diameter, distinctly mucronate, red; fruit unknown.

Macroscopic

Whole plant

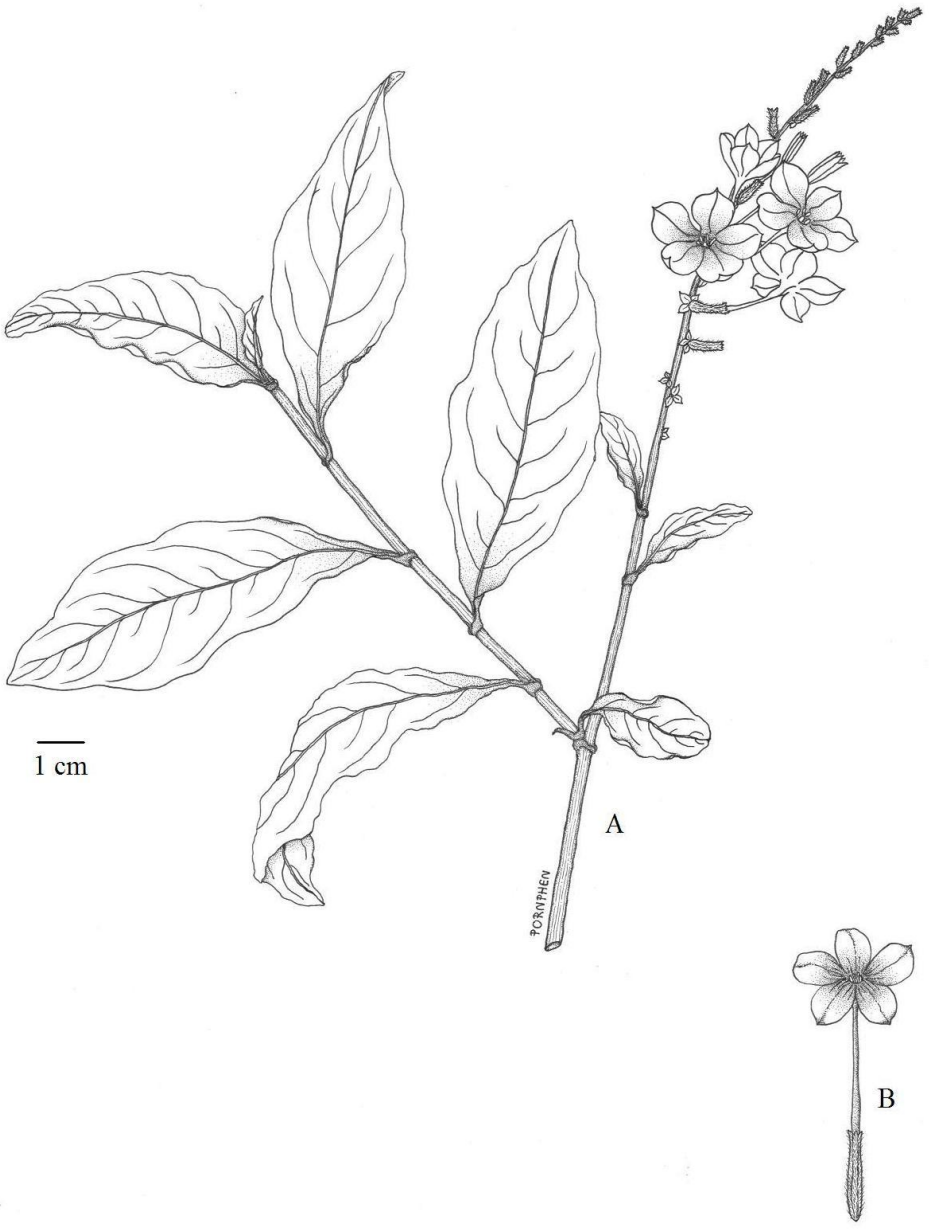


Figure 15 The whole plant of *Plumbago indica* Linn. A. the flowering branch B. flower

Crude drug

Figure 16 Dried roots of *Plumbago indica* Linn.

Microscopic

Anatomical character

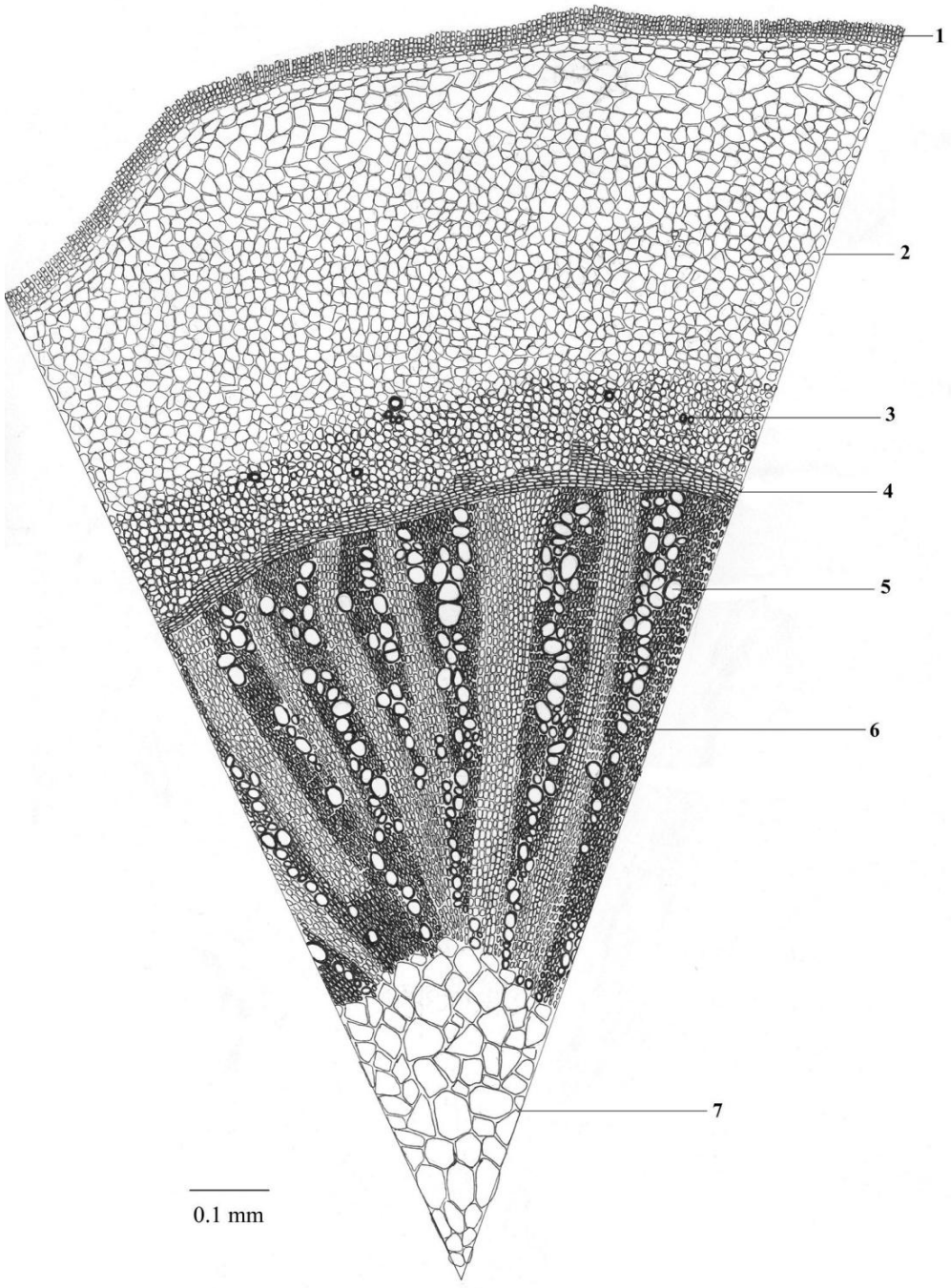


Figure 17 Transverse section of *Plumbago indica* Linn. root ; 1. Periderm 2. Cortex 3. containing brownish resin 4. Vascular cambium 5. Second xylem 6. Rays 7. Pith

Microscopic

Histological character

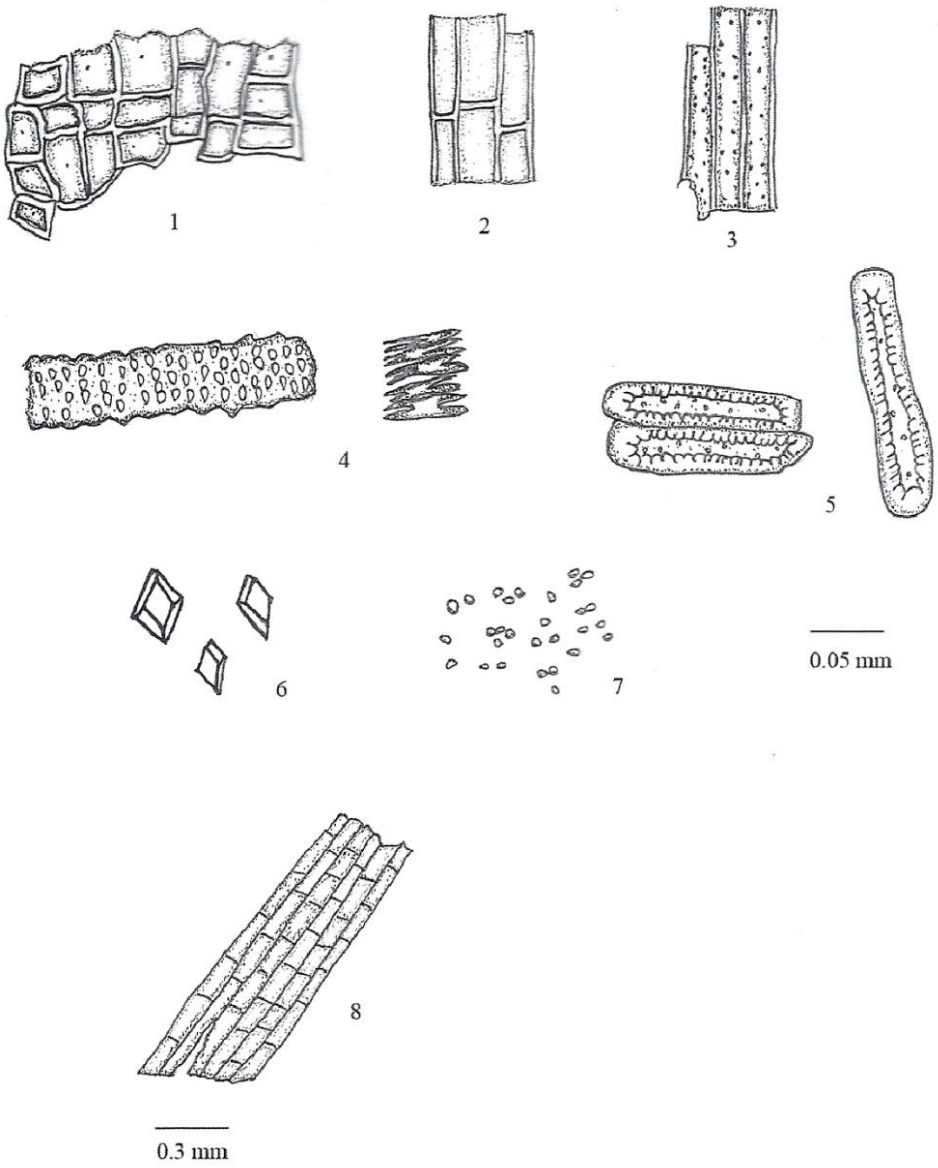


Figure 18 Powder of *Plumbago indica* Linn. root; 1. epidermal cells containing brownish resin 2. Parenchyma in longitudinal view 3. Part of xylem parenchyma 4. Reticulate vessel 5. Sclerenchymatous sclerids 6. Prism crytals of calcium oxalate 7. Starch grains 8. Fragment of fiber

Identification

TLC fingerprint

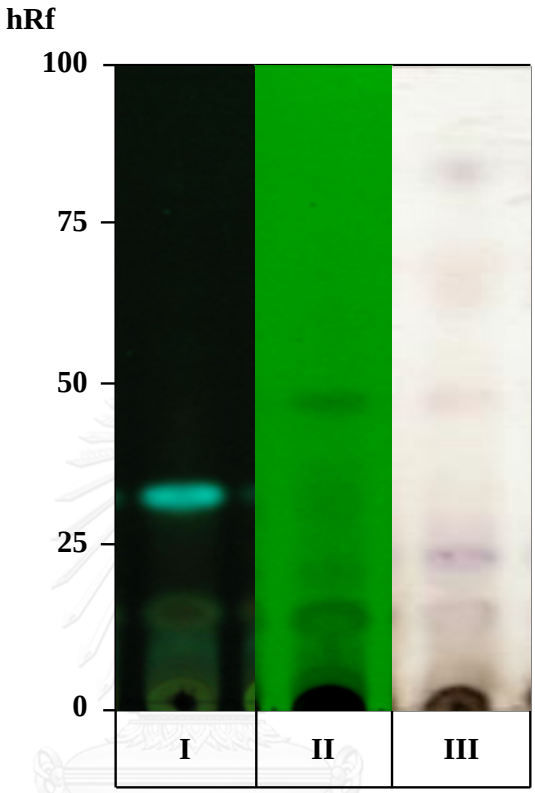


Figure 19 Thin-layer chromatogram of the ethanol extract from *Plumbago indica* Linn. Root

Solvent system Hexane : Ethyl acetate 8 : 2

Detection

I	=	detection under UV light 365 nm
II	=	detection under UV light 254 nm
III	=	detection with 10% sulfuric acid*

*10% sulfuric acid reagent

Preparation: conc. sulphuric acid 10 ml in methanol 90 ml

Spot color development

Heat the plate at 105 $^{\circ}$ C for 5 minutes after sprayed

Table 3 Physico-chemical specification (% by weight) of *Plumbago indica* Linn. root

Content (% by weight)	Mean	SD	Range (Mean ± 3SD)
Acid-insoluble ash	3.227	0.514	1.685 – 4.769
Total ash	9.331	0.434	8.028 – 10.634
Ethanol-soluble extractive	6.990	1.098	3.697 – 10.283
Water-soluble extractive	18.372	2.649	10.425 – 26.320
Loss on drying	8.873	0.144	8.443 – 9.304
Volatile oil	0	0	0
Water	10.498	0.211	9.865 – 11.130

The parameters were shown as grand mean ± pooled SD of 15 sources. Each source was performed in triplicate.

***Acorus calamus* Linn.**

Common Name	WAAN-NAM
Other Name	TA-KRAI-NAM, KHA-CHIANG-CHEE
English Name	Calamus, Sweet flag, Sweet root
Scientific Name	*Acorus calamus* Linn.
Synonym	*A. terrestris* Spreng., *A. asiaticus* Nakai
Family	ACORACEAE
Distribution	China, Afghanistan, Bangladesh, Bhutan, India, Indonesia, Japan, Korea, Malaysia (Sarawak), Mongolia, Nepal, Pakistan, Russia (Far East, Siberia), Sri Lanka, Thailand, Vietnam; SW Asia, Europe (except S), North America
Used Part	Rhizome
Ethnomedical Use	Carminative, analgesic, anthelmintic, treatment of diarrhoea and dysentery

Description of Plant

Perennial herb up to 80 cm tall. Rootstock stout, 1-1.5 cm broad, creeping, with long fibrous roots from the lower surface. Stem erect, glabrous, grooved at one side, and ribbed at the opposite. Leaves ensiform or linear, 55-100 x 8-1.5 cm. Spathe leaf-like, up to 46 cm long, not enclosing the spadix. Spadix 5-6.5 cm long, cylindrical, obtuse, 1-1.4 cm broad. Tepals c. 2 mm long, oblong-obovate, slightly curved, margin membranous, surface with embedded raphides. Filaments 2 mm long, flat, anthers less than 1 mm long, ± orbicular. Ovary 3 mm long, obconical; seeds obconical, 2 mm long [ref eflora 27].

Macroscopic

Whole plant

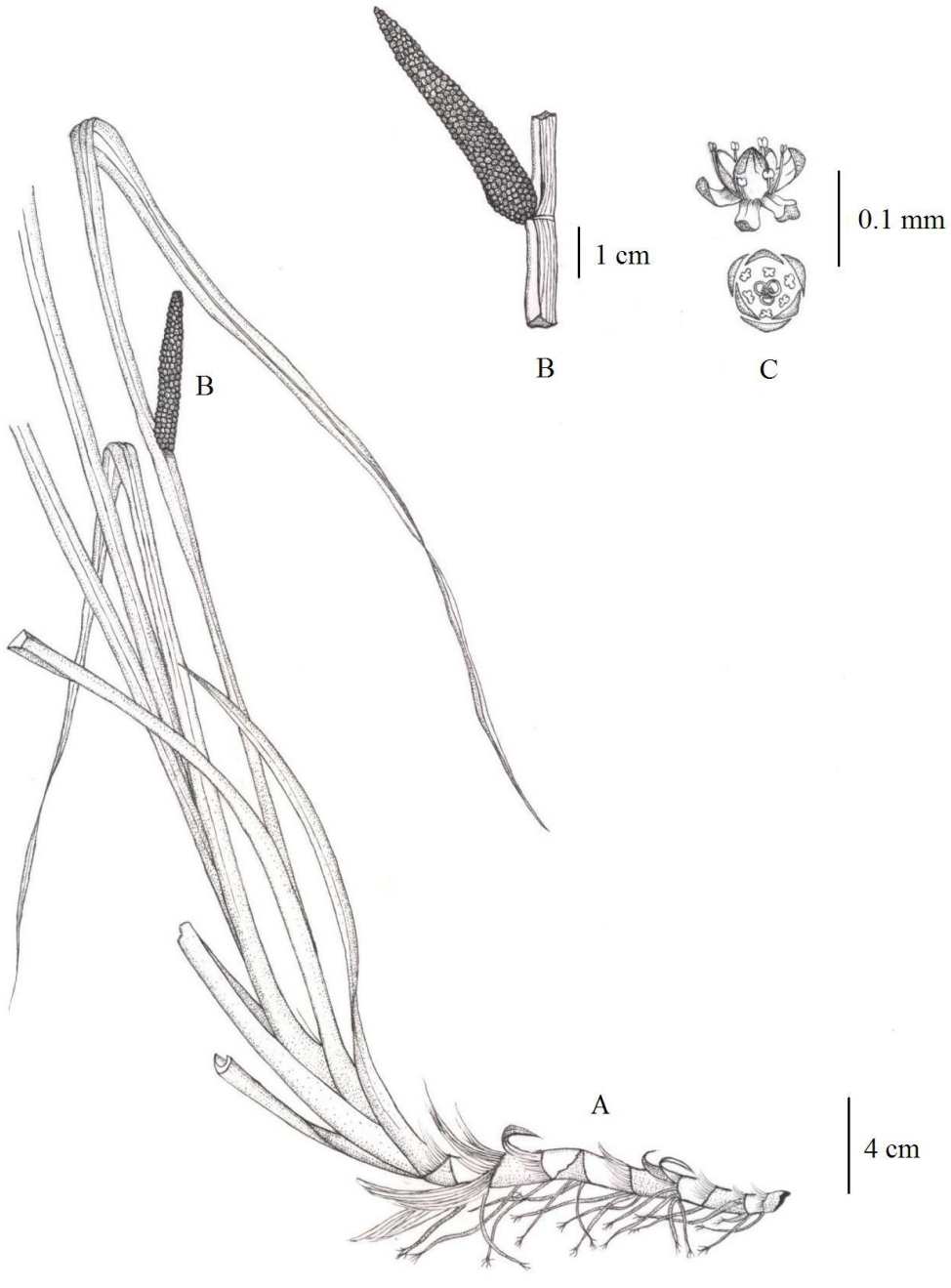


Figure 20 The whole plant of *Acorus calamus* Linn. A. Rhizome B. Inflorescence C. Single flower

Crude drug

Figure 21 Dried rhizomes of *Acorus calamus* Linn.

Microscopic

Anatomical character

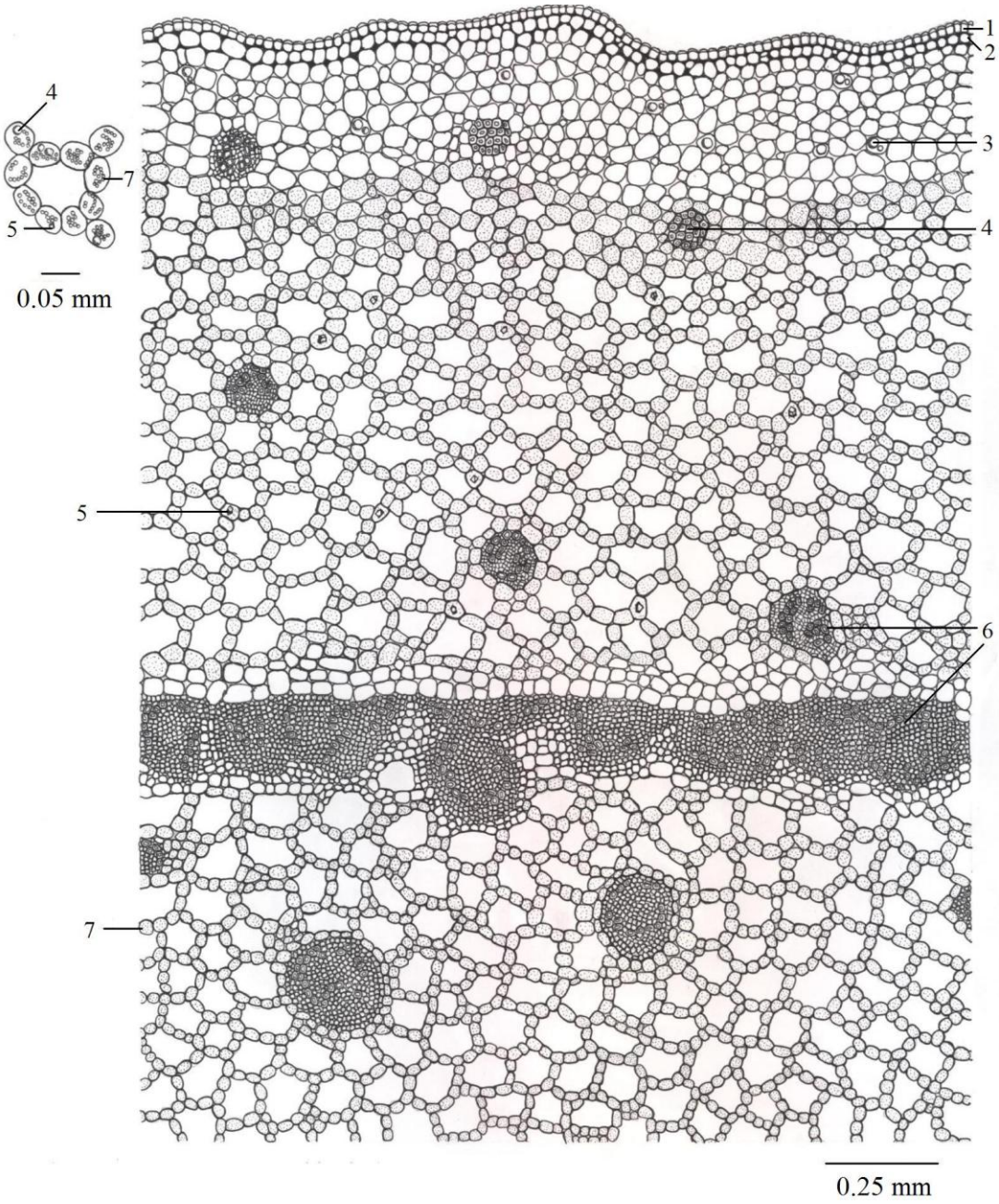


Figure 22 Transverse section of *Acorus calamus* Linn. rhizome ; 1. Epidermis 2. Collenchyma 3. Oil cell 4. Cortical fibers 5. Crystal of calcium oxalate 6. Vascular bundle 7. Starch granules

Microscopic

Histological character

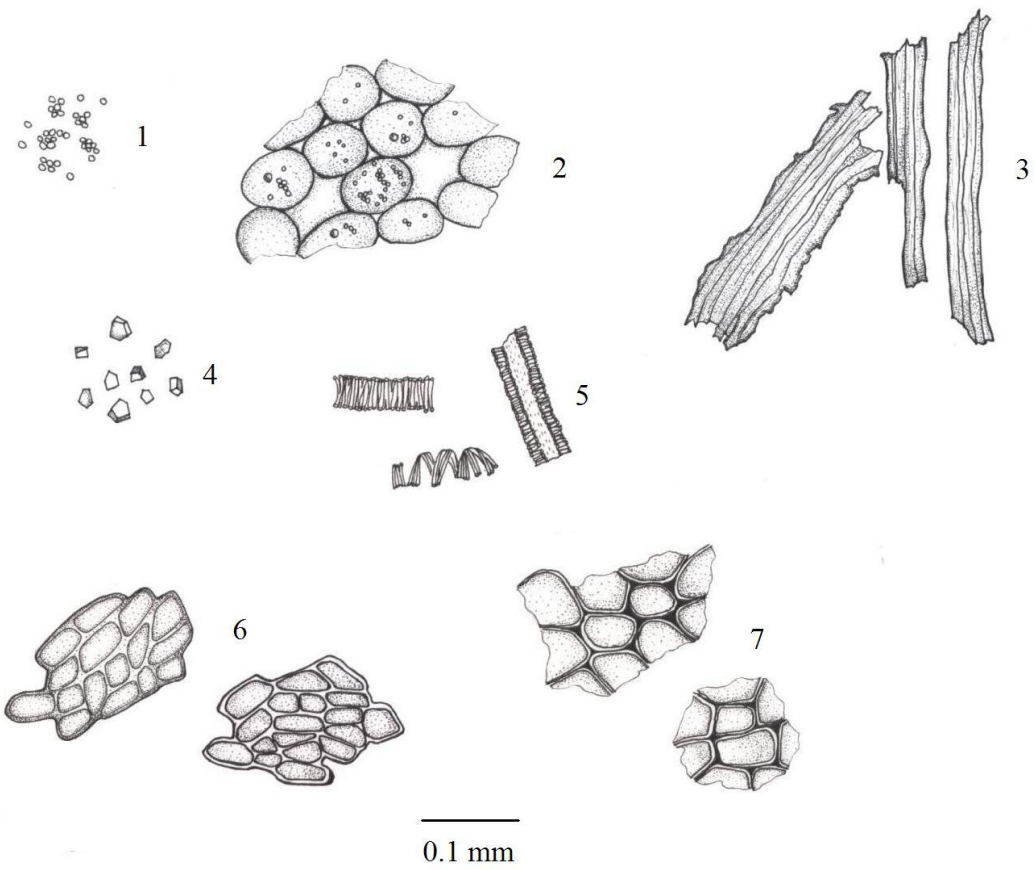


Figure 23 Powder of *Acorus calamus* Linn. rhizome; 1. Starch granules 2. Parenchyma cells in sectional view with starch granules and oil cells 3. Fiber in longitudinal view 4. Calcium oxalate prism 5. Fragment of vessels 6. Epidermis of rhizome in surface view 7. Collenchyma of rhizome in sectional view

Identification

TLC fingerprint

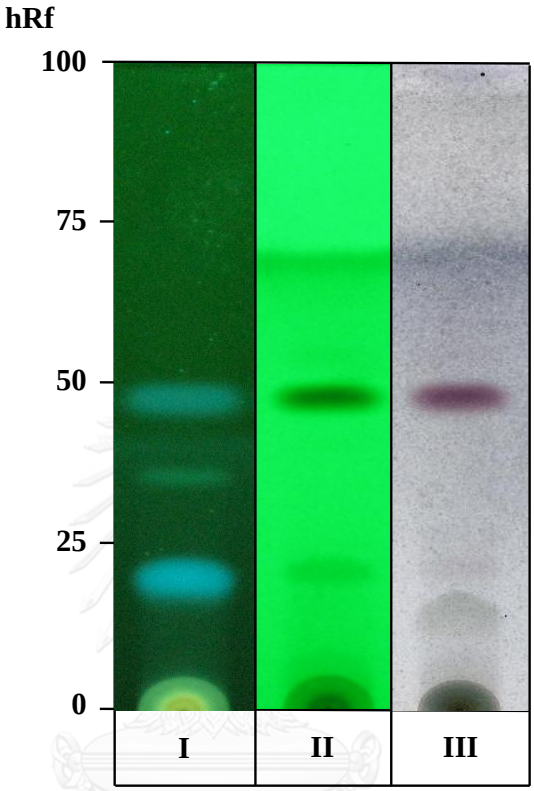


Figure 24 Thin-layer chromatogram of the ethanol extract from *Acorus calamus* Linn. rhizome

Solvent system Toluene : Ethyl acetate 9 : 1

Detection

I	=	detection under UV light 365 nm
II	=	detection under UV light 254 nm
III	=	detection with anisaldehyde staining reagent*

*Anisaldehyde staining reagent

Preparation: Anisaldehyde 1 ml, Methanol 17 ml, Sulphuric acid 1 ml, Acetic acid 2 ml

Spot color development

Heat the plate at 105 $^{\circ}$ C for 5 minutes after sprayed

Table 4 Physico-chemical specification (% by weight) of *Acorus calamus* Linn. Rhizome

Content (% by weight)	Mean	SD	Range (Mean ± 3SD)
Acid-insoluble ash	0.832	0.068	0.627 - 1.036
Total ash	4.493	0.153	4.035 - 4.950
Ethanol-soluble extractive	7.320	0.291	6.449 - 8.192
Water-soluble extractive	9.534	0.453	8.176 - 10.892
Loss on drying	12.229	0.335	11.224 - 13.235
Volatile oil	1.369	0.107	1.046 - 1.691
Water	13.149	0.460	11.769 - 14.530

The parameters were shown as grand mean ± pooled SD of 15 sources. Each source was performed in triplicate.

***Dolichandrone serrulata* (DC.) Seem.**

Common Name	KAE-TRAE
Other Name	KAE-KHAO
Scientific Name	*Dolichandrone serrulata* (DC.) Seem.
Synonym	*Stereospermum serrulatum* DC.
Family	BIGNONIACEAE
Distribution	Africa, Asia, Australia
Used Part	Root
Ethnomedical Use	Expectorant, antiflatulent, blood tonic, antifever and antiinflammatory agent

Description of Plant

Deciduous tree to 25 m with narrow cylindrical crown and slender branches. Bark pale brown, smooth or slightly flaking. Leaf 43 cm, once-pinnate, 3-5 pairs of leaflets, 5-14 × 3-6 cm, elliptic with tapering tip and strongly asymmetric base, usually with scattered teeth. Flower 12-21 cm, pure white, opening at night, in short unbranched clusters of 3-7 flowers at end of twigs, 2-3 cm. Fruit up to 85×1.8 cm, pointed, spirally twisted. Seed 2.2-2.8 × 0.5-0.8 cm, rectangular, thin with transparent wing.

Macroscopic

Whole plant

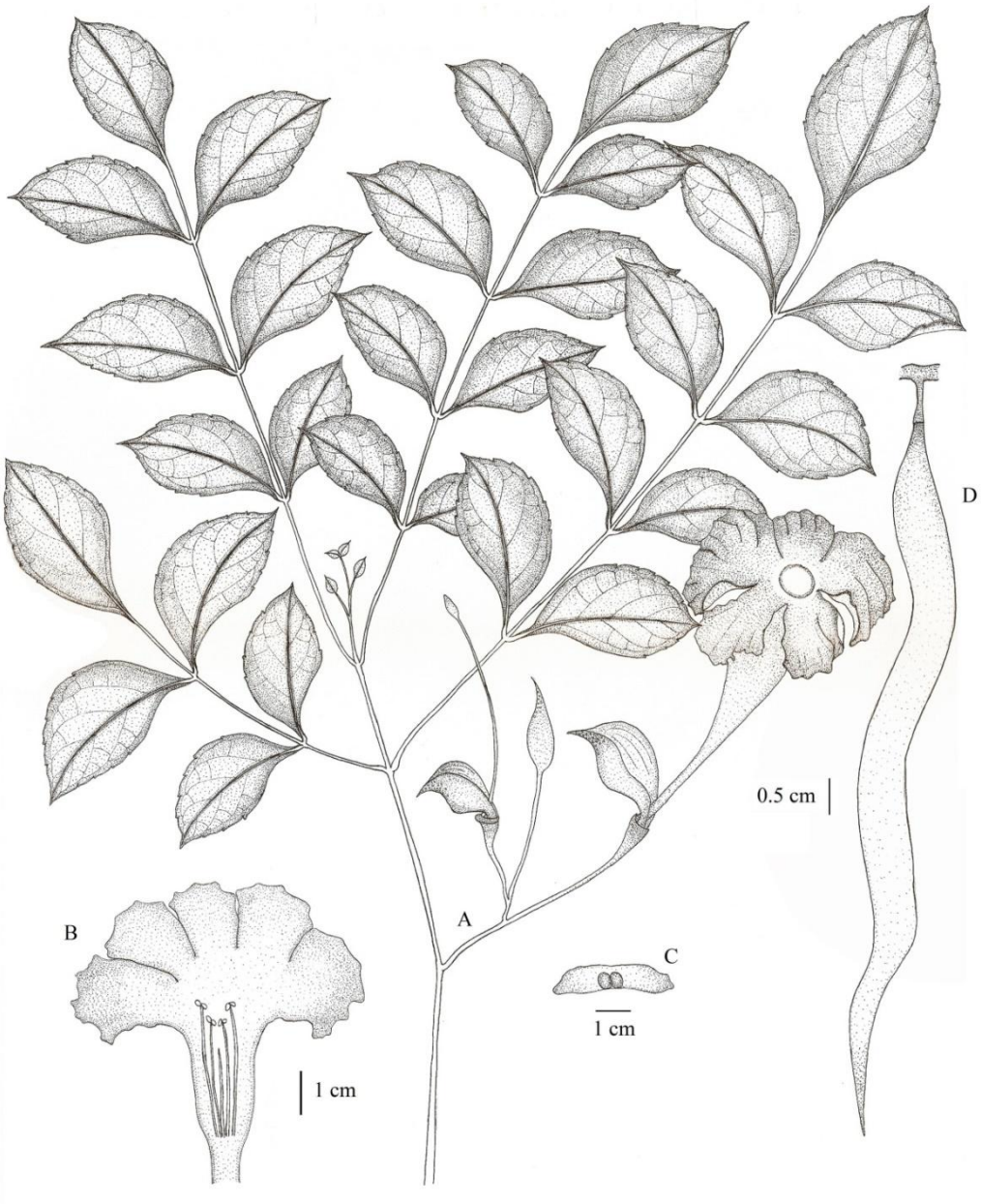


Figure 25 The whole plant of *Dolichandrone serrulata* (DC.) Seem. A. Flowering branch B. Petal and stamen C. Seed D. Pod

Crude drug

Figure 26 Dried roots of *Dolichandrone serrulata* (DC.) Seem.

Microscopic

Anatomical character

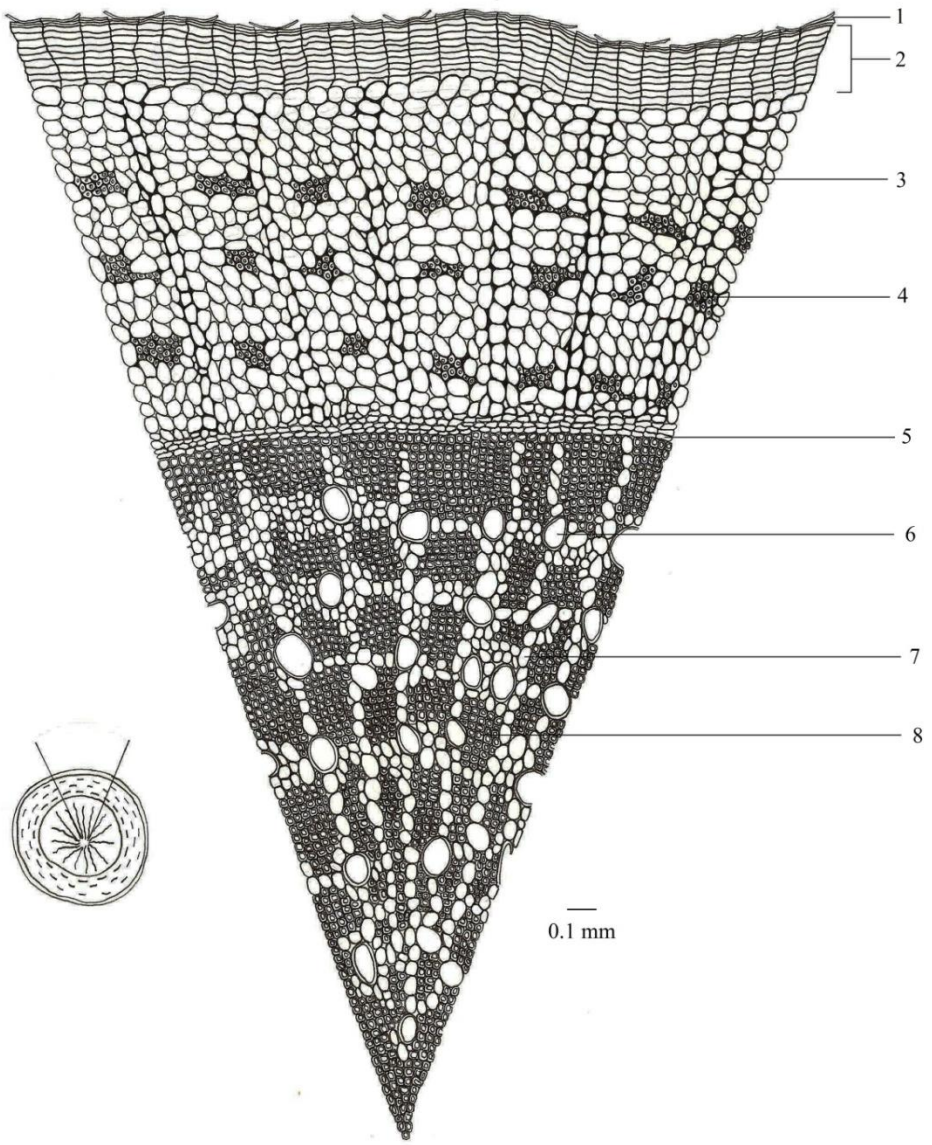


Figure 27 Transverse section of *Dolichandrone serrulata* (DC.) Seem root.; 1. Epidermis 2. Periderm 3. Cortical parenchyma 4. Group of cortical fiber 5. Endodermis 6. Xylem vessel 7. Xylem ray 8. Xylem fiber

Microscopic

Histological character

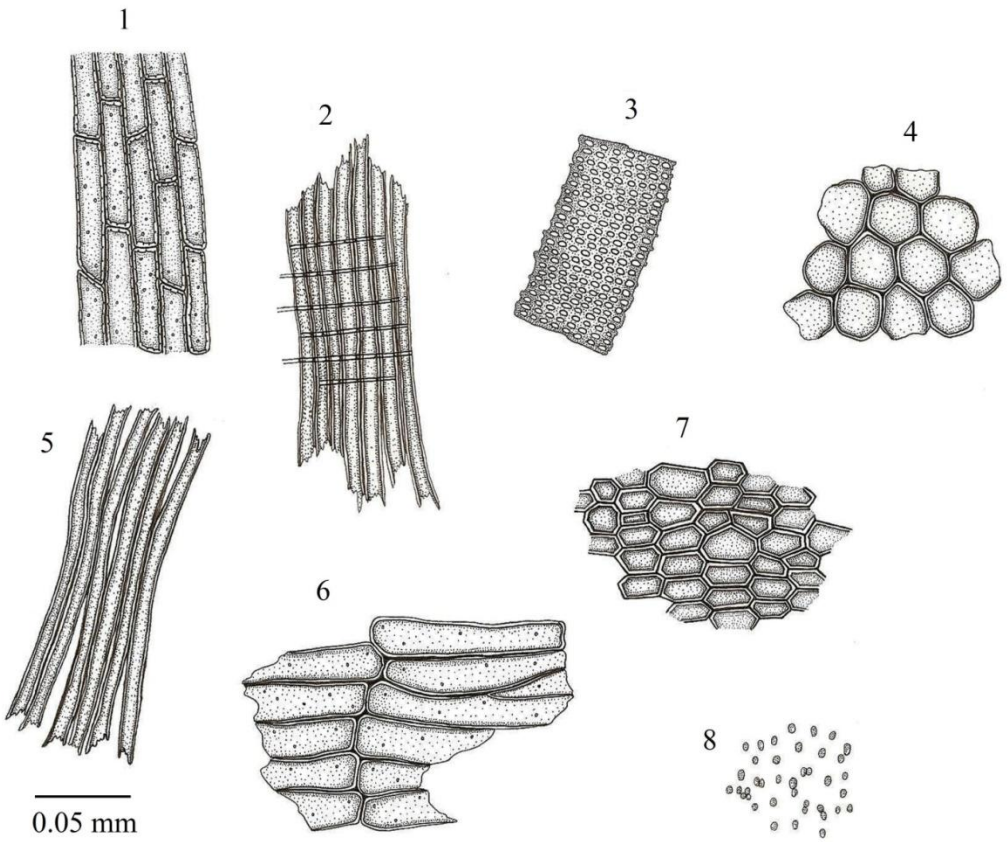


Figure 28 Powder of *Dolichandrone serrulata* (DC.) Seem. root; Powder of the root of Dolichandrone serrulata (DC.) Seem. 1. Xylem parenchyma in longitudinal view 2. Xylem in radial longitudinal view 3. Fragment of pitted vessel 4. Parenchyma in transverse view 5. Fragment of fiber 6. Parenchyma in longitudinal view 7. Epidermis in surface view 8. Starch grain

Identification

TLC fingerprint

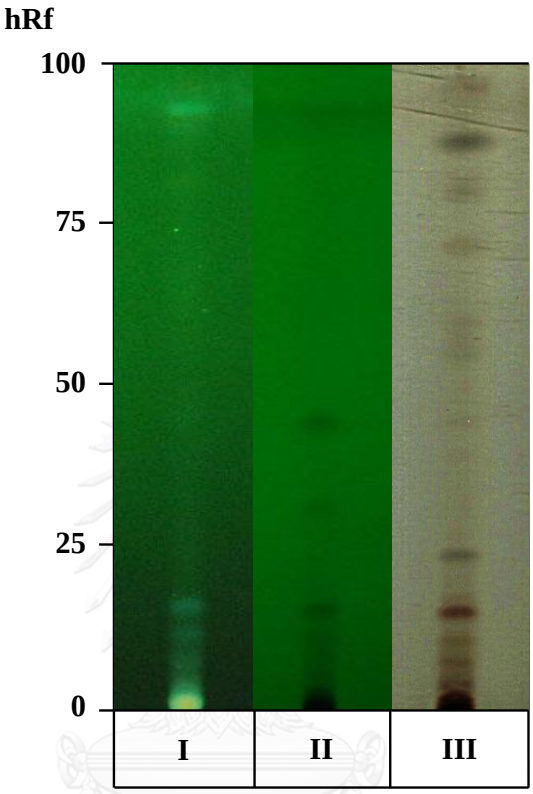


Figure 29 Thin-layer chromatogram of the ethanol extract from *Dolichandrone serrulata* (DC.) Seem. root

Solvent system Chloroform : Methanol 9 : 1

Detection I = detection under UV light 365 nm

II = detection under UV light 254 nm

III = detection with 10% sulfuric acid reagent*

*10% sulfuric acid reagent

Preparation: conc. sulfuric acid 10 ml. in methanol 90 ml.

Spot color Development

Heat the plate at 105 $^{\circ}$ C for 5 minutes after sprayed

Table 5 Physico-chemical specification (% by weight) of *Dolichandrone serrulata* (DC.) Seem. Root

Content (% by weight)	Mean	SD	Range (Mean ± 3SD)
Acid-insoluble ash	0.746	0.210	0.117 – 1.376
Total ash	3.051	0.057	2.880 – 3.221
Ethanol-soluble extractive	4.524	0.152	4.069 – 4.978
Water-soluble extractive	10.167	0.489	8.699 – 11.634
Loss on drying	7.843	0.071	7.631 – 8.055
Volatile oil	0	0	0
Water	12.462	0.985	9.506 – 15.418

The parameters were shown as grand mean ± pooled SD of 15 sources. Each source was performed in triplicate.

***Clerodendrum paniculatum* Linn.**

Common Name	PA-NUM-SA-WAN
English Name	Red pagoda plant
Scientific Name	*Clerodendrum paniculatum* Linn.
Synonym	*C. pyramidale* Andrews, *Volkameria angu-lata* Loureiro.
Family	VERBENACEAE
Distribution	Asia-tropical
Used Part	Root
Ethnomedical Use	Treatment of inflammation, ulcers, wounds and skin diseases

Description of Plant

Shrubs ca. 1 m tall. Branchlets 4-angled, subglabrous to pubescent, nodes villous. Leaves palmately lobed; petiole 3-11 cm, yellow-brown pubescent; leaf blade broadly ovate to subrounded, 5-17 x 7.5-19 cm, abaxially sparsely pubescent and sandy glandular, adaxially sparsely pubescent to subglabrous, base cordate, margin entire or minutely denticulate, apex acute. Inflorescences conical to rounded thyrses, 15-26 x 16-22 cm; peduncle long; bracts ovate-lanceolate to ovate; bractlets linear. Calyx ca. 7 mm, deeply 5-lobed, dotted; lobes usually lanceolate, ca. 5 mm. Corolla red to orange, tube 1-1.5 cm, dotted, outside pubescent, inside subglabrous; lobes oblong to ovate, spreading. Stamens and style 4 x as long as corolla tube. Drupes globose, 5-9 mm in diam.

Macroscopic

Whole plant

Figure 30 The whole plant of *Clerodendrum paniculatum* Linn.

Crude drug

Figure 31 Dried roots of *Clerodendrum paniculatum* Linn.

Microscopic

Anatomical character

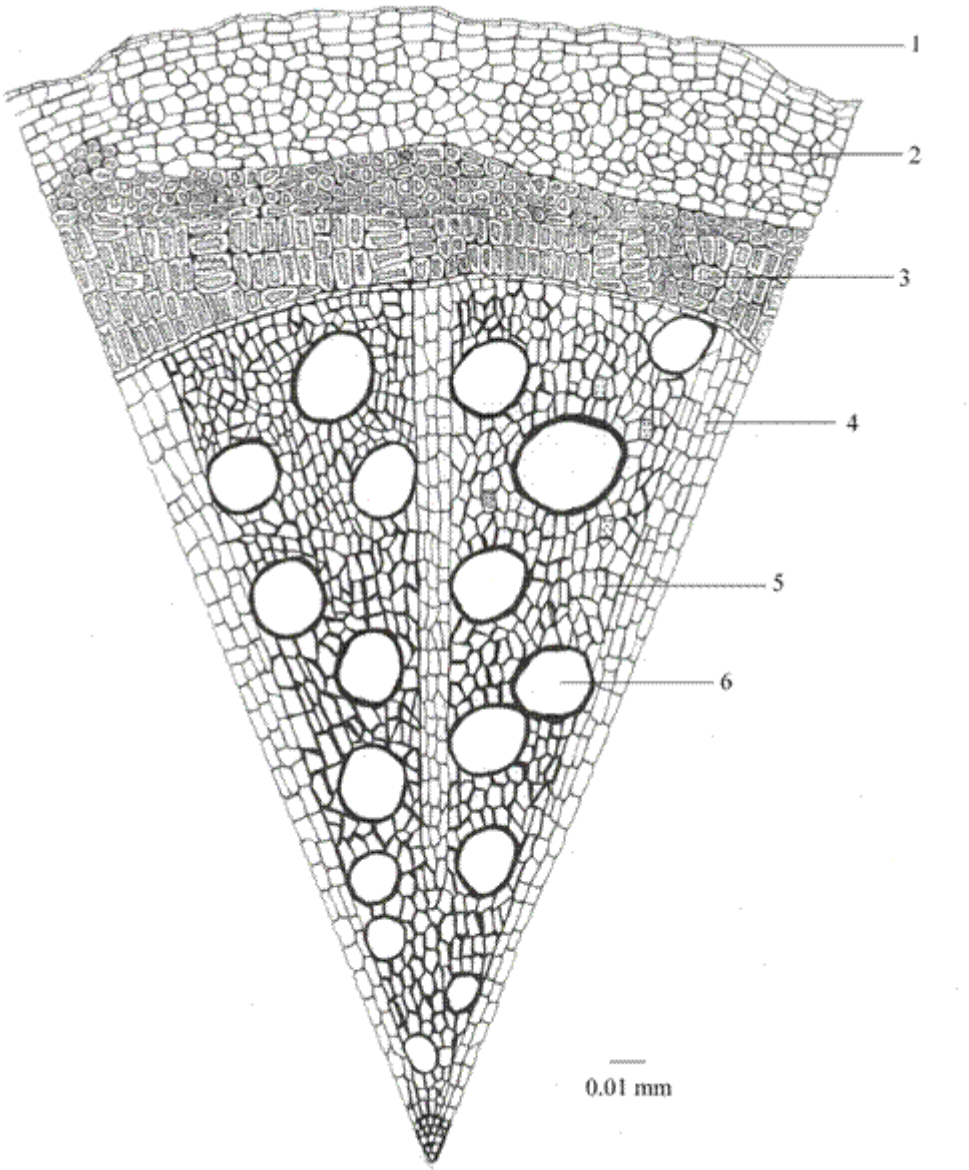


Figure 32 Transverse section of *Clerodendrum paniculatum* Linn. root ; 1. Epidermis 2. Parenchyma of cortex 3. Sclereid cell 4. Xylem ray 5. Xylem parenchyma 6. Xylem vessel

Microscopic

Histological character

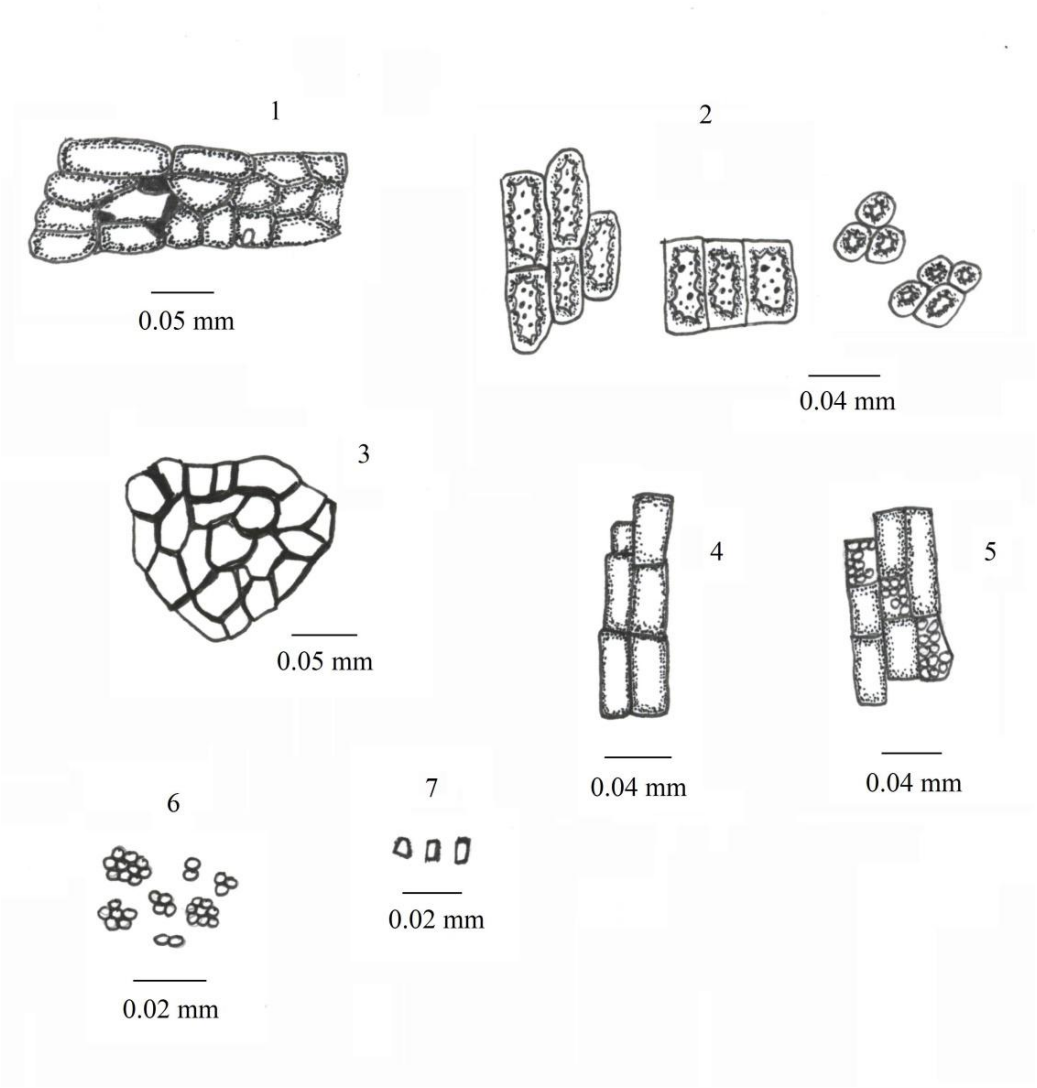


Figure 33 Powder of *Clerodendrum paniculatum* Linn. root; 1. Parenchyma 2. Sclereids 3. Xylem parenchyma 4. Xylem fiber 5. Xylem fiber containing starch 6. Starch granules 7. Crystals

Identification

TLC fingerprint

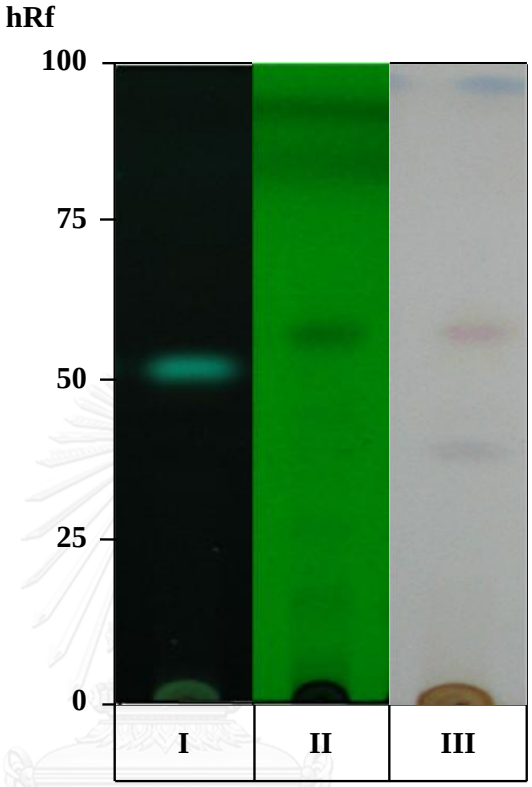


Figure 34 Thin-layer chromatogram of the ethanol extract from *Clerodendrum paniculatum* Linn. root

Solvent system			Hexane : Diethyl ether 4 : 6
Detection	I	=	detection under UV light 365 nm
	II	=	detection under UV light 254 nm
	III	=	detection with anisaldehyde staining reagent*

*Anisaldehyde staining reagent

Preparation: Anisaldehyde 1 ml, Methanol 17 ml, Sulphuric acid 1 ml, Acetic acid 2 ml

Spot color Development

Heat the plate at 105 $^{\circ}$ C for 5 minutes after sprayed

Table 6 Physico-chemical specification (% by weight) of *Clerodendrum paniculatum* Linn. root

Content (% by weight)	**Mean**	**SD**	**Range (Mean ± 3SD)**
Acid-insoluble ash	1.667	0.434	0.366 – 2.968
Total ash	5.560	0.499	4.063 – 7.056
Ethanol-soluble extractive	2.689	0.171	2.175 – 3.203
Water-soluble extractive	8.247	0.912	5.511 – 10.983
Loss on drying	6.375	0.127	5.993 – 6.757
Volatile oil	0	0	0
Water	8.408	0.342	7.382 – 9.435

The parameters were shown as grand mean ± pooled SD of 12 sources. Each source was performed in triplicate.

Tree Phon Thad remedy

***Zingiber zerumbet* (L.) Smith.**

Common Name	KRA-THUE
English Name	Bitter ginger
Scientific Name	*Zingiber zerumbet (L.)* Smith.
Family	ZINGIBERACEAE
Distribution	Tropical and subtropical areas
Used Part	Rhizome
Ethnomedical Use	Treatment of swelling, loss of appetite, diabetes, inflammation, chest pain, rheumatic pains, bronchitis, dyspepsia and sore throat

Description of Plant

Rhizomes yellowish inside, tuberous. Pseudostems 0.6 to 2 m. Leaves sessile or shortly petiolate; ligule entire, 1.5 to 2 cm; leaf blade lanceolate or oblong-lanceolate, 15 to 40 cm long and 3 to 8 cm wide, glabrescent or abaxially somewhat pilose, base narrowed, apex acuminate. Inflorescences arising from rhizomes, conical or ovoid-oblong, 6 to 15 cm long and 3.5 to 5 cm wide, apex obtuse; peduncle 10 to 30 cm, scale like sheaths 5 to 7 cm long; bracts closely imbricate green when young, red when old, slightly hairy, slimy adaxially, margin membranous; bracteoles ca 1.5 cm. Calyx 1.2 to 2 cm, membranous, split on 1 side, apex 3-toothed. Corolla tube 2 to 3 cm long, slender; lobes white or pale yellow, lanceolate, central one 1.5 to 2.5 cm. Labellum pale yellow 1.5 to 2.5 cm in diameter; central lobe suborbicular or subobovate, 1.5 to 2 cm and ca 1.5 cm, apex emarginated; lateral lobes narrower, ca 1 cm, free nearly to base. Stamen ca 1 cm; connective appendage beaklike ca 8 mm. Ovary ca 4 mm, glabrous. Capsule ellipsoid, 0.8 to 1.2 cm. Seeds black.

Macroscopic

Whole plant

Figure 35 The whole plant and inflorescence of *Zingiber zerumbet (L.)* Smith.

Crude drug

Figure 36 Dried rhizomes of *Zingiber zerumbet (L.)* Smith.

Microscopic

Anatomical character

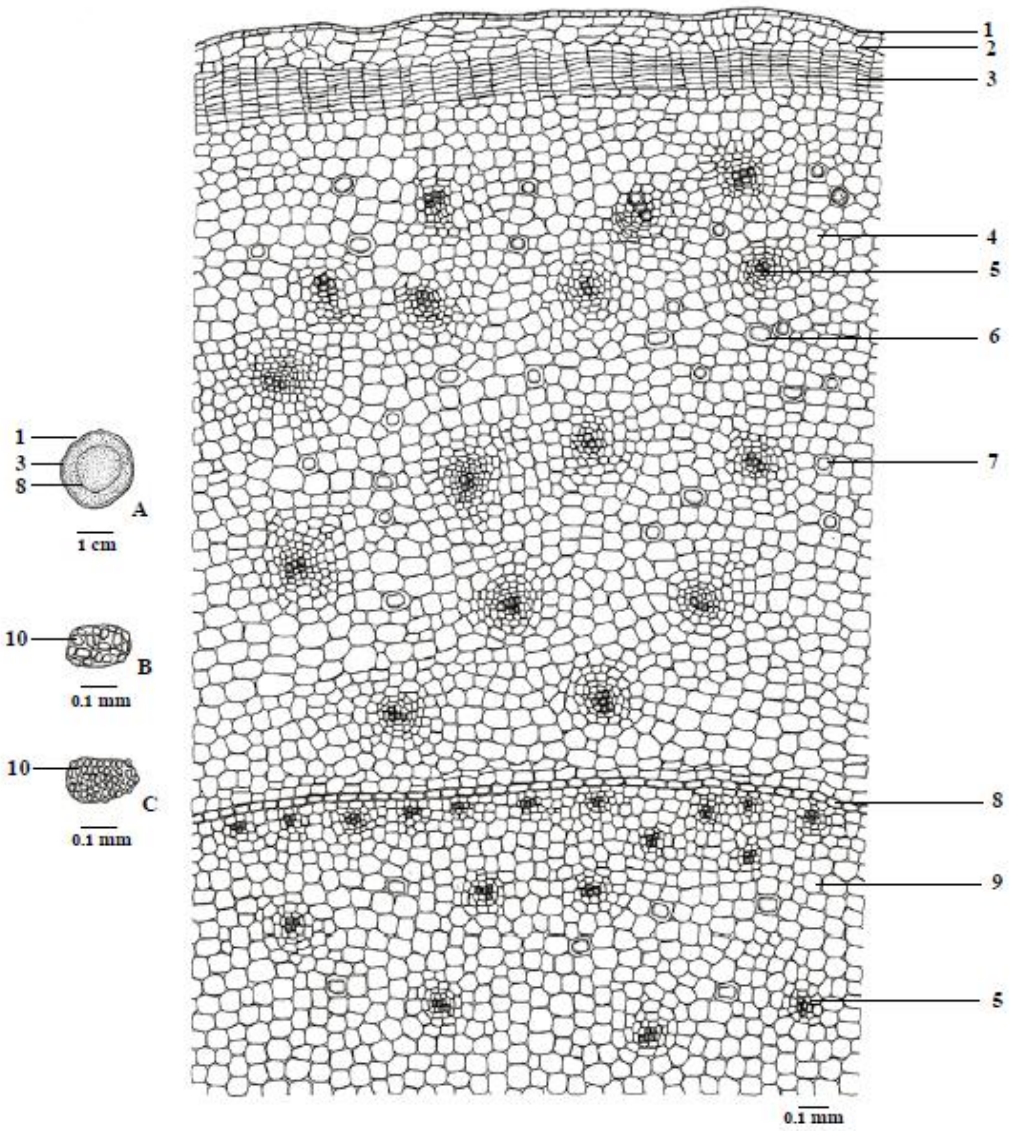


Figure 37 Transverse section of *Zingiber zerumbet (L.)* Smith. rhizome

A. diagram of transverse section B. cortical parenchyma C. stele parenchyma

1. epidermis
2. hypodermis
3. cork cell
4. cortical parenchyma containing starch grain
5. vascular bundle
6. mass of oleoresin
7. oil droplet
8. endodermis
9. stele parenchyma containing starch grain
10. starch grain

Microscopic

Histological character

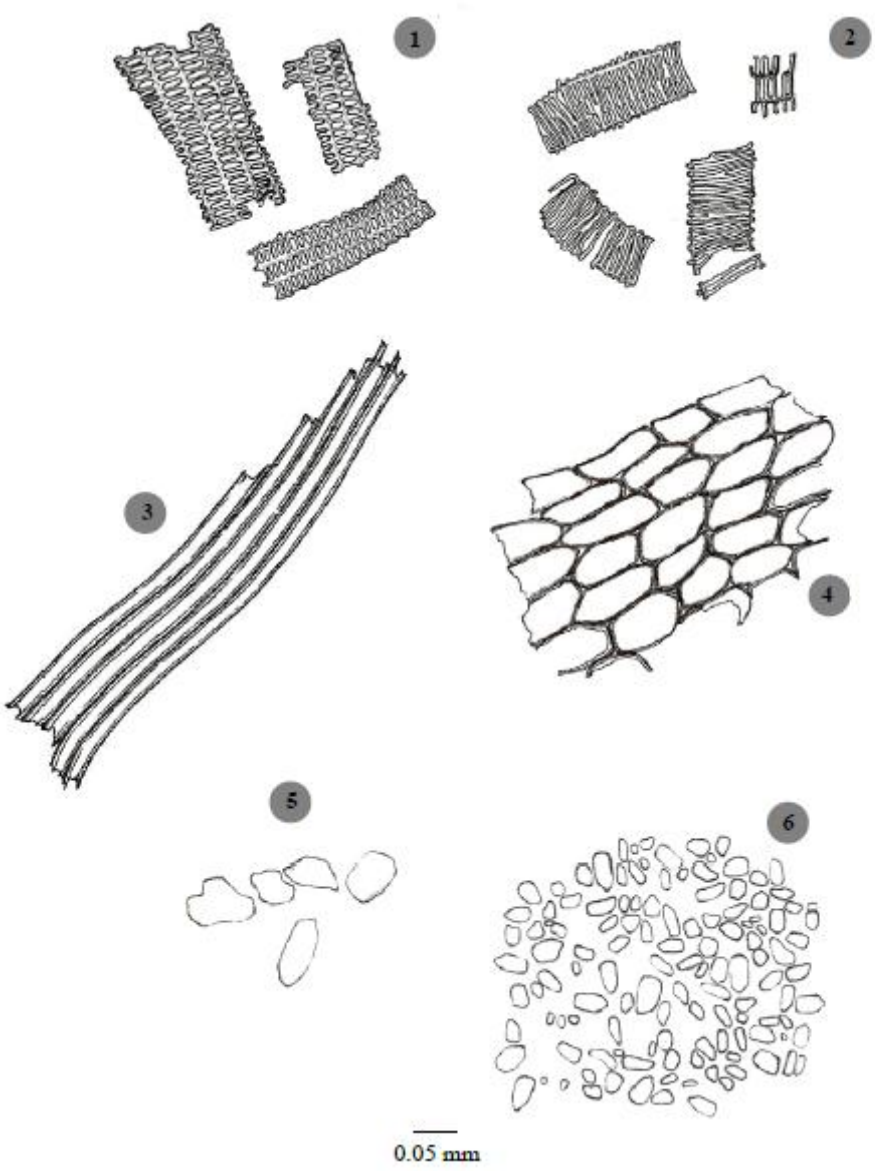


Figure 38 Powder of *Zingiber zerumbet (L.)* Smith. rhizome; 1. scalariform vessel 2. reticulate vessel 3. fragment of fibers 4. parenchyma in transverse view 5. masses of oleoresin 6. starch grains

Identification

TLC fingerprint

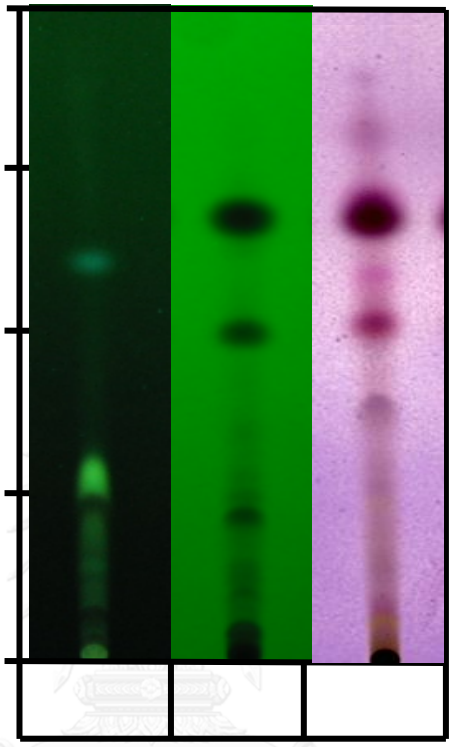


Figure 39 Thin-layer chromatogram of the ethanol extract from *Zingiber zerumbet (L.)* Smith. rhizome

Solvent system			Toluene : Ethyl acetate 75 : 25
Detection	I	=	detection under UV light 365 nm
	II	=	detection under UV light 254 nm
	III	=	detection with anisaldehyde staining reagent*

*Anisaldehyde staining reagent: Anisaldehyde 1 ml, Methanol 17 ml, Sulphuric acid 1 ml, Acetic acid 2 ml

Spot color Development

Heat the plate at 105 $^{\circ}$ C for 5 minutes after sprayed

Table 7 Physico-chemical specification (% by weight) of *Zingiber zerumbet (L.)* Smith. Rhizome

Content (% by weight)	Mean	SD	Range (Mean ± 3SD)
Acid-insoluble ash	3.574	0.230	2.884 - 4.264
Total ash	8.227	0.203	7.617 - 8.837
Ethanol-soluble extractive	3.067	0.276	2.239 - 3.894
Water-soluble extractive	12.378	0.481	10.933 - 13.822
Loss on drying	9.952	0.135	9.546 - 10.357
Volatile oil	1.156	0.055	0.990 - 1.322
Water	9.621	0.414	8.379 - 10.863

The parameters were shown as grand mean ± pooled SD of 15 sources. Each source was performed in triplicate.

***Cymbopogon nardus* Rendel**

Common Name	TA-KRAI-HOM
English Name	Citronella grass
Scientific Name	*Cymbopogon nardus* Rendle
Family	GRAMINEAE
Distribution	Tropical Asia, Africa
Used Part	Root
Ethnomedical Use	Treatment of toothaches, redness, irritation, inflammation of the skin, infectious diseases, digestive problems, headache, rheumatism, fatigue, diuretic and sudorific

Description of Plant

Densely caespitose perennial; culms up to 240 cm high. Leaves often reddish at maturity; ligule (2.5)3.5–10 mm long, membranous, erose; laminas 20–55 cm × 3.5–9 mm, broadest at about the middle, scabrid on the margins, tapering at the apex into a thread-like point. Inflorescence 8–47 cm long, elongated and interrupted, reddish. Racemes 9–18 mm long; internodes and pedicels pilose with silvery-white hairs. Sessile spikelet 3.8–6.5 mm long, glabrous; inferior glume with a shallow V-shaped median groove, occasionally flat, winged in the upper half, notched at the apex; superior lemma linear, bifid in the upper third; awn delicate, 6.5–11 mm long, with a well developed column; anthers 1.2–3 mm long, straw-coloured to brown-red. Pedicelled spikelet male, 2.7–6.2 mm long, awnless.

Macroscopic

Whole plant

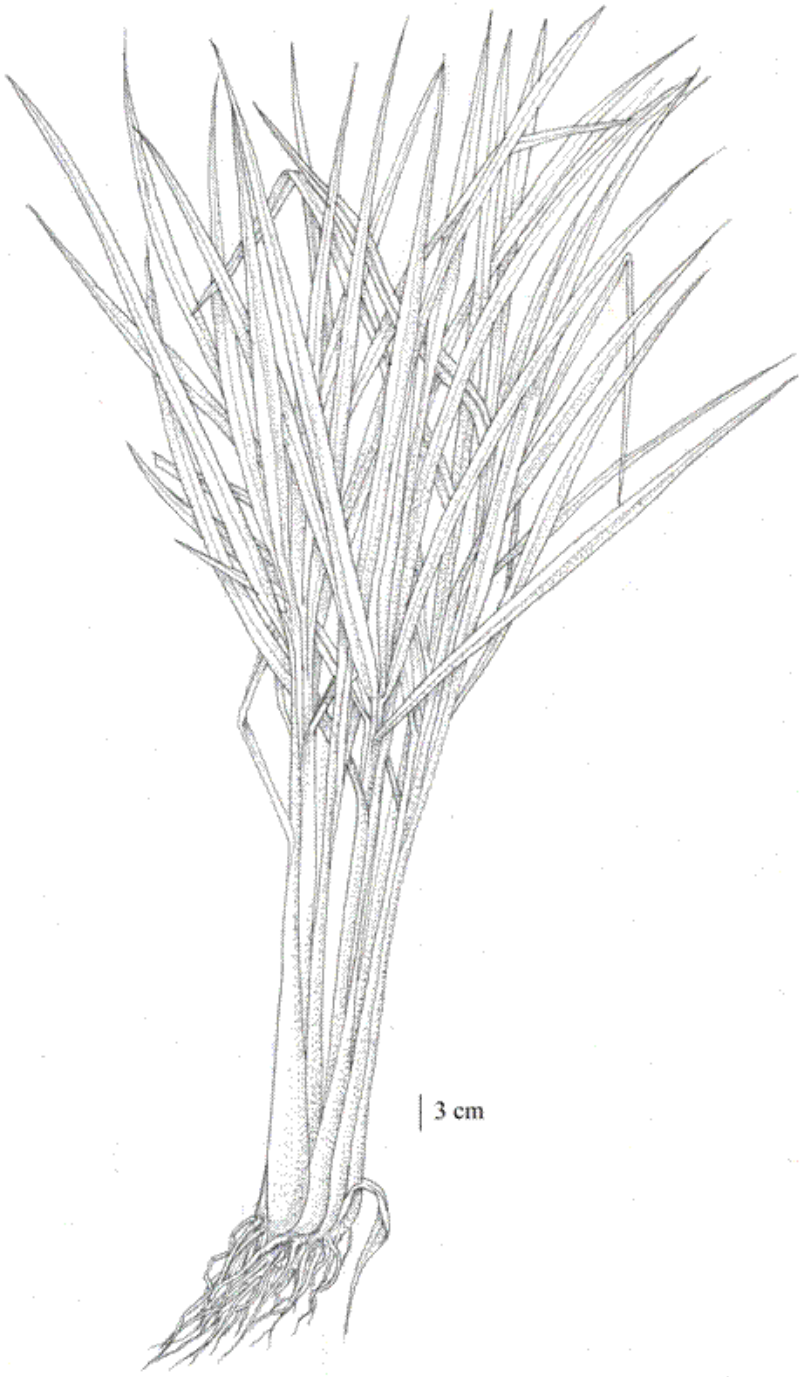


Figure 40 The whole plant of *Cymbopogon nardus* Rendle

Crude drug

Figure 41 Dried roots of *Cymbopogon nardus* Rendle

Microscopic

Anatomical character

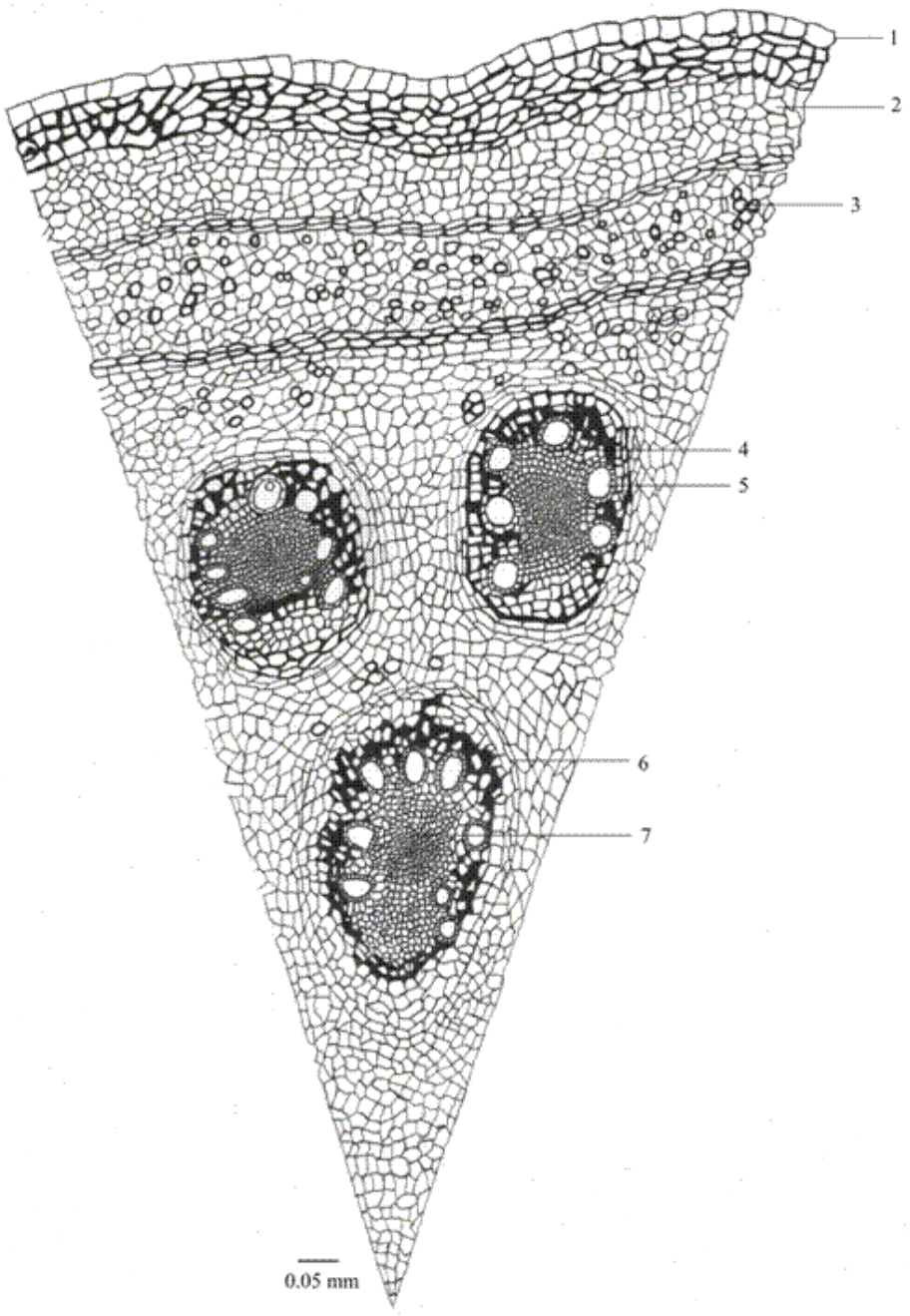


Figure 42 Transverse section of *Cymbopogon nardus* Rendle root 1. Epidermis 2. Parenchyma 3. Oil gland 4. Phloem 5. Xylem vessel 6. Endodermis 7. Pith

Microscopic

Histological character

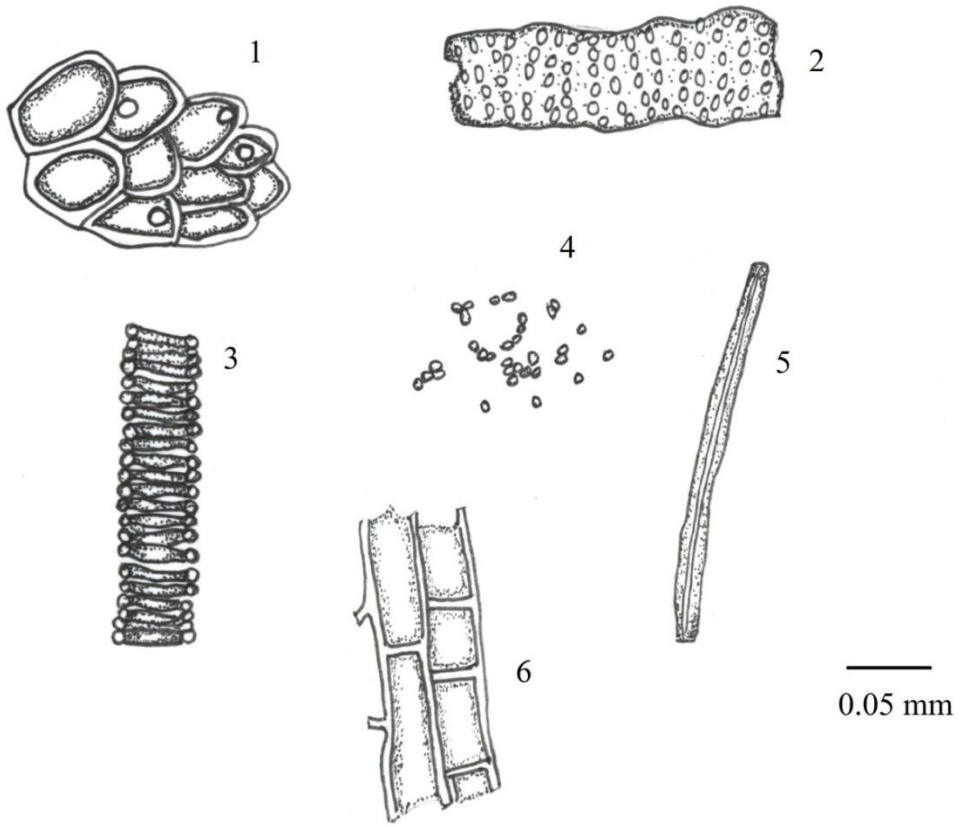


Figure 43 Powder of *Cymbopogon nardus* Rendle root 1. Parenchyma containing oleoresin 2. Reticulate vessel 3. Annular vessel 4. Starch granules 5. Fragment of fiber 6. Parenchyma in longitudinal view

Identification

TLC fingerprint

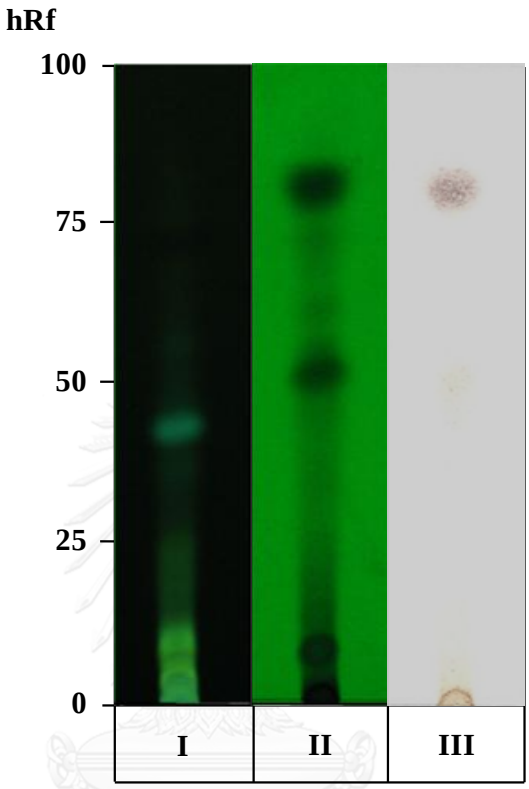


Figure 44 Thin-layer chromatogram of the ethanol extract from *Cymbopogon nardus* Rendle root

Solvent system			Hexane : Diethyl ether 4 : 6
Detection	I	=	detection under UV light 365 nm
	II	=	detection under UV light 254 nm
	III	=	detection with anisaldehyde staining reagent*

*Anisaldehyde staining reagent : Anisaldehyde 1 ml, Methanol 17 ml, Sulphuric acid 1 ml, Acetic acid 2 ml

Spot color Development

Heat the plate at 105 $^{\circ}$ C for 5 minutes after sprayed

Table 8 Physico-chemical specification (% by weight) of *Cymbopogon nardus* Rendle root

Content (% by weight)	Mean	SD	Range (Mean ± 3SD)
Acid-insoluble ash	3.968	0.641	2.046 – 5.889
Total ash	5.815	0.646	3.876 – 7.753
Ethanol-soluble extractive	4.886	0.848	2.342 – 7.431
Water-soluble extractive	8.118	0.539	6.503 – 9.734
Loss on drying	8.286	0.221	7.624 – 8.947
Volatile oil	2.960	0.380	1.820 – 4.100
Water	8.057	0.451	6.704 – 9.410

The parameters were shown as grand mean ± pooled SD of 12 sources. Each source was performed in triplicate.

***Zingiber montanum* (Koenig) Link ex Dietr.**

Common Name	PLAI
Other Name	WAAN-PHI, PU-LOEI
English Name	Cassumunar Ginger, Bengal Root
Scientific Name	*Zingiber montanum* (Koenig) Link ex Dietr.
Synonym	*Z. cassumunar* Roxb.
Family	ZINGIBERACEAE
Distribution	From the Himalayas to Ceylon, Malay Peninsula and tropical Asia
Used Part	Rhizome
Ethnomedical Use	Antiasthmatic, emmenagogue, antidysentery, astringent and laxative

Description of Plant

Rootstock perennial, bright yellow inside. Leafy stem 1.2-1.8 m. Leaves oblong-lanceolate pubescent beneath, 30-45 by 5-8 cm. Spike oblong, 10-15 cm, 3.5-5 cm dia; peduncle 7.5-30 cm; bracts ovate reddish or greenish-red, 2.5-4 cm and nearly as broad. Corolla-tube as long as the bract; segments whitish, 2.5 cm, upper broader and more concave. Lip with an orbicular unspotted midlobe 2 cm, long and broad, stamen yellowish-white, shorter than the lip. Capsule small, globose.

Macroscopic

Whole plant

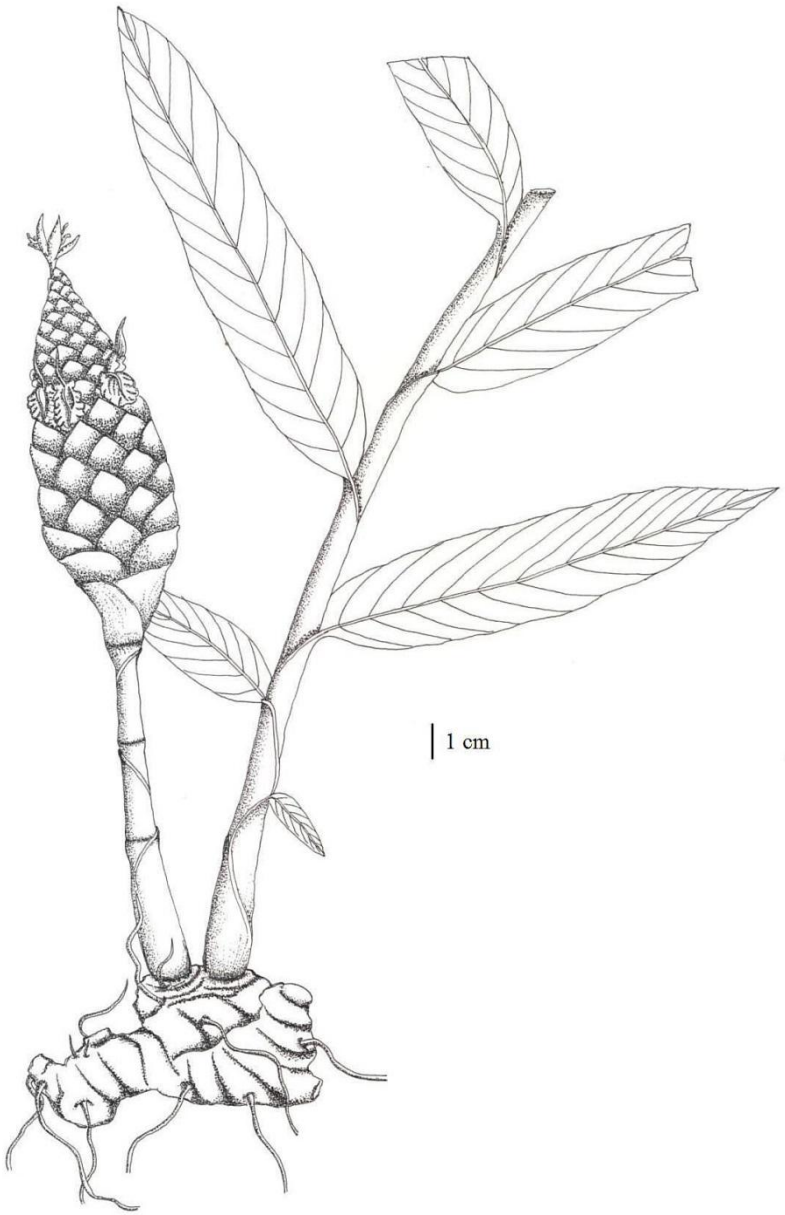


Figure 45 The whole plant of *Zingiber montanum* (Koenig) Link ex Dietr.

Crude druge

Figure 46 Dried rhizomes of *Zingiber montanum* (Koenig) Link ex Dietr.

Microscopic

Anatomical character

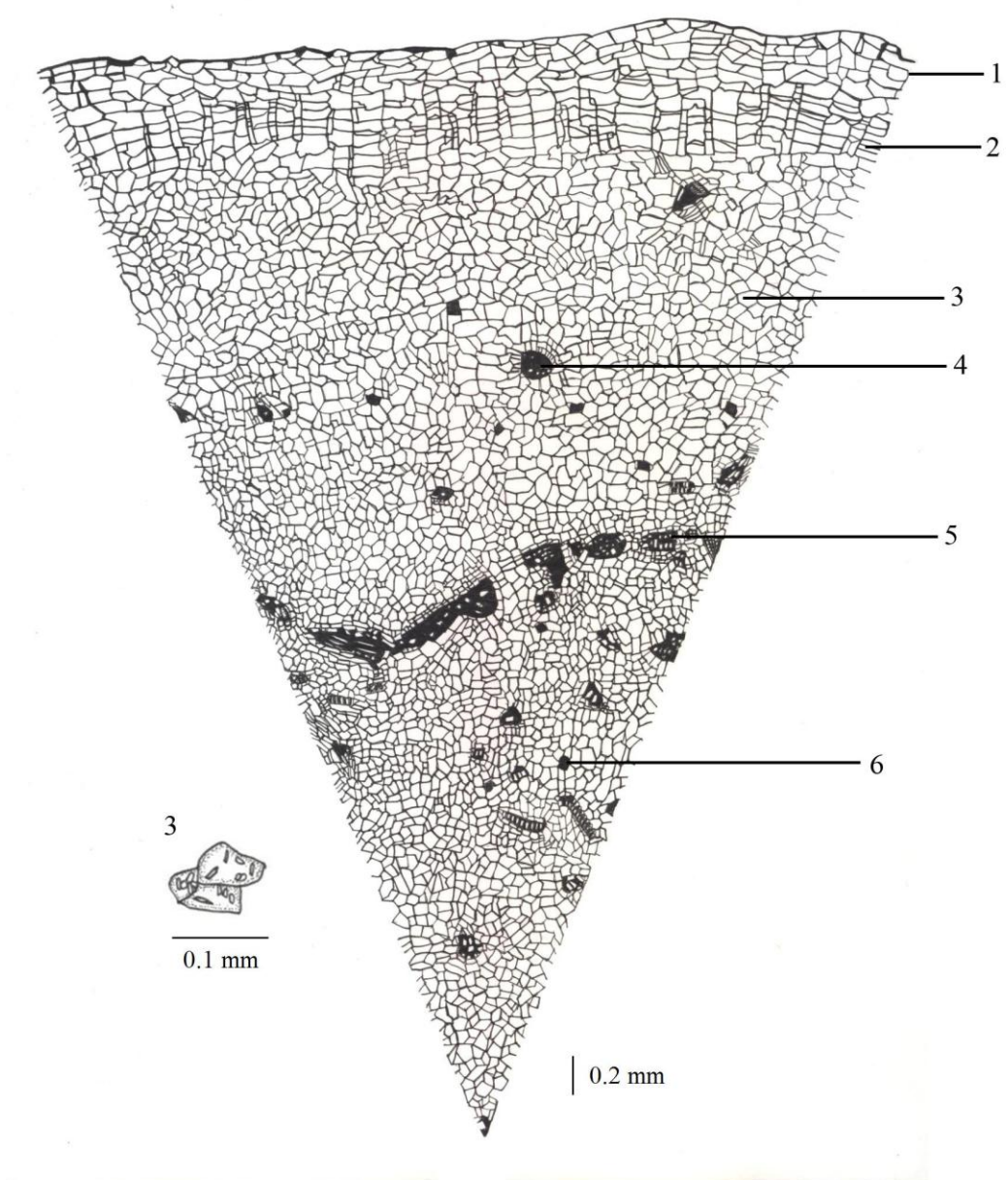


Figure 47 Transverse section of *Zingiber montanum* (Koenig) Link ex Dietr. rhizome; 1. Cork 2. Cork cambium 3. Parenchyma containing starch granules 4. Vascular bundle 5. Pseudoendodermis 6.Parenchyma containing oleoresin

Microscopic

Histological character

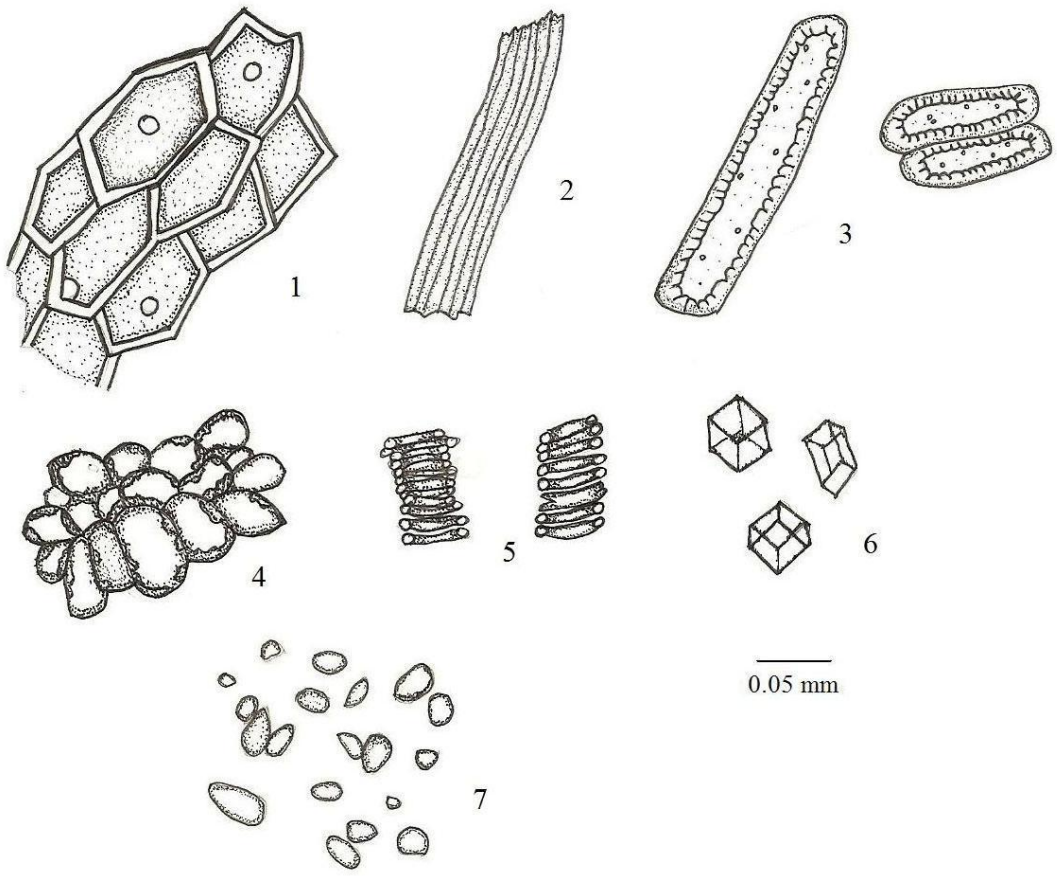


Figure 48 Powder of *Zingiber montanum* (Koenig) Link ex Dietr. rhizome 1. Cork in surface view 2. Fragment of fiber 3. Sclereids 4. Parenchyma 5. Annular vessel 6. Prism crystals of calcium oxalate 7. Starch granules

Identification

TLC fingerprint

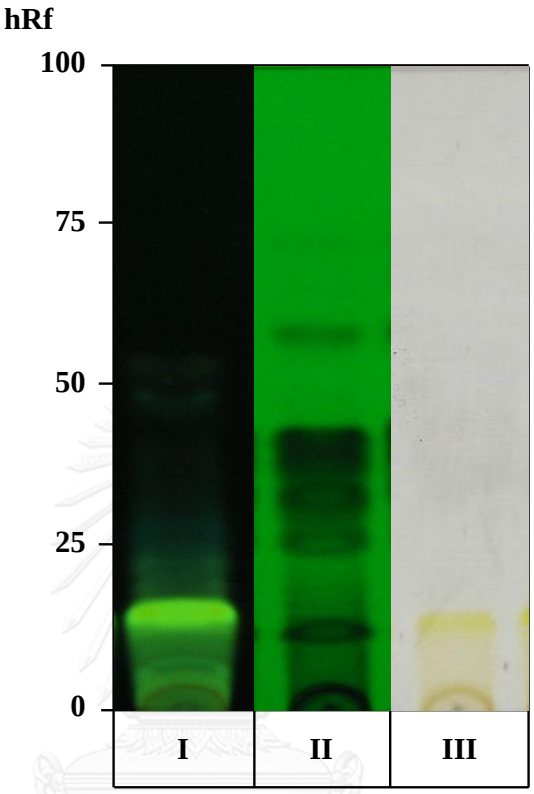


Figure 49 Thin-layer chromatogram of the ethanol extract from *Zingiber montanum* (Koenig) Link ex Dietr. rhizome

Solvent system Chloroform

Detection I = detection under UV light 365 nm

II = detection under UV light 254 nm

III = detection with 10% sulfuric acid reagent*

*10% sulfuric acid reagent

Preparation: conc. sulphuric acid 10 ml. in methanol 90 ml.

Spot color Development

Heat the plate at 105 $^{\circ}$ C for 5 minutes after sprayed

Table 9 Physico-chemical specification (% by weight) of *Zingiber montanum* (Koenig) Link ex Dietr. rhizome

Content (% by weight)	Mean	SD	Range (Mean ± 3SD)
Acid-insoluble ash	2.013	0.139	1.596 - 2.429
Total ash	6.889	0.402	5.683 - 8.094
Ethanol-soluble extractive	7.432	1.048	4.288 - 10.576
Water-soluble extractive	10.400	1.118	7.045 - 13.754
Loss on drying	10.236	1.035	7.130 - 13.342
Volatile oil	3.255	0.311	2.322 - 4.188
Water	9.666	0.479	8.228 - 11.103

The parameters were shown as grand mean ± pooled SD of 15 sources. Each source was performed in triplicate.

GC/MS analysis

There were four plant species (*Zingiber montanum, Cymbopogon nardus, Zingiber zerumbet* and *A. calamus*) whose the volatile oils were analysed by GC/MS.

***Zingiber montanum* dried rhizome volatile oil**

The major chemical constituents of the volatile oil of *Zingiber montanum* from 15 different sources in Thailand were revealed as Terpinene-4-ol (36.48±11.02 %) followed by Sabinene (32.14±8.54 %). Figure 50 and Table 10 showed the GC chromatogram and chemical constituents of the volatile oil of *Zingiber montanum* rhizome.

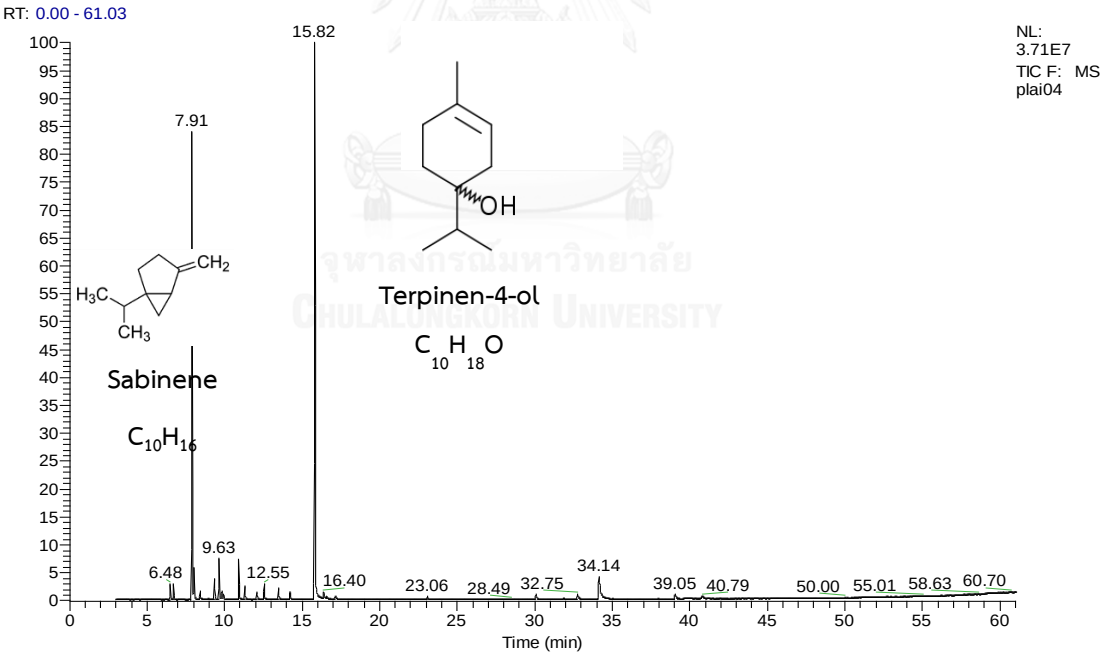


Figure 50 GC chromatogram of the volatile oil of *Zingiber montanum* dried rhizome

Table 10 The chemical constituents of the volatile oil of *Zingiber montanum* rhizome

RT	Compound name	Area% [a]	KI[b]
6.48	Thujene (alpha)	0.95±0.29	930
6.69	Pinene (alpha)	1.13±0.37	939
7.89	Sabinene	32.14±8.54	975
8.01	Pinene (beta)	2.62±0.71	979
8.40	Myrcene (beta)	0.72±0.25	990
9.34	Terpinene (alpha)	1.80±0.73	1017
9.63	Cymol (p)	2.81±1.16	1024
9.81	Phellandrene (Beta)	0.76±0.21	1026
10.91	Terpinene (Gramma)	4.06±1.87	1054
11.30	Sabinene hydrate (cis)	1.02±0.37	1070
12.54	Terpinolene	1.04±0.47	1088
13.48	Sabinene hydrate acetate (trans)	0.77±0.20	1098
14.22	Menth-2-en-1-ol	0.55±0.12	1140
15.82	Terpinene-4-ol	36.48±11.02	1177
16.40	Terpineol (alpha)	0.59±0.1	1188
23.08	Terpineol acetate (alpha)	0.27±0.1	1299
32.77	2-Allyl-1,4-dimethoxy-3-methyl-benzene	1.23±0.84	1474
34.18	Triquinacene, 1,4-bis(methoxy)-	9.17±14.34	-
39.07	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester	1.09±0.65	1454
40.79	Triquinacene, 1,4,7-tris(methoxy)-	0.8±0.22	-

[a] The parameters were shown as mean ± SD. Samples were from 15 different sources throughout Thailand.

[b] Kovat's index [155].

***Cybopogon nardus* dried root volatile oil**

The major chemical constituents of the volatile oil of *Cybopogon nardus* from 12 different sources in Thailand were revealed as elemol (22.87±3.11%) followed by and alpha-eudesmol (16.09±2.61%). Figure 51 and Table 11 showed the GC chromatogram and chemical constituents of the volatile oil of *Cybopogon nardus* root.

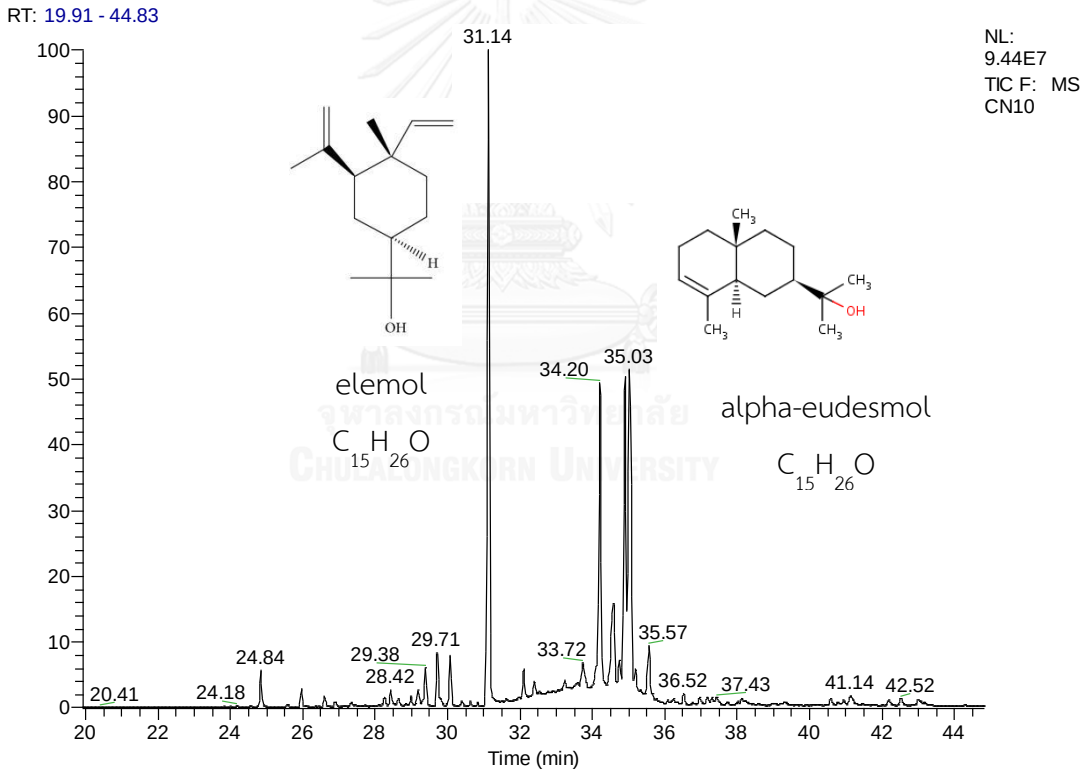


Figure 51 GC chromatogram of the volatile oil of *Cybopogon nardus* dried root

Table 11 The chemical constituents of the volatile oil of *Cybopogon nardus* root.

RT	Compound name	Area% [a]	KI [b]
24.84	Elemene (beta)	2.13±0.95	1390
28.43	Germacrene D	1.82±0.60	1485
28.63	Selinene (beta)	0.9±0.32	1490
28.98	Selinene (alpha)	0.68±0.23	1498
29.19	Muurolene (alpha)	0.80±0.35	1500
29.38	Cuparene	2.16±0.96	1504
29.71	Cadinene (gamma)	2.52±1.03	1513
30.06	Cadinene (delta)	2.44±1.02	1523
31.12	Elemol	22.87±3.11	1549
32.11	Cadinene (alpha)	1.69±0.72	-
32.40	Caryophyllene oxide	1.89±0.81	1583
33.23	Eudesmol (5-epi-7-epi-alpha)	2.00±0.88	1607
33.58	Cubenol	1.91±0.81	1645
33.75	Unidentified	6.75±1.69	-
34.20	Eudesmol (gamma)	11.72±2.23	1630
34.57	Cadinol (epi-gamma)	6.99±1.72	1640
34.74	Torreyol	1.73±0.82	1646
34.91	Eudesmol (Beta)	11.33±2.19	1650
35.05	Eudesmol (alpha)	16.09±2.61	1652
35.20	Eudesm-7(11)-en-4-ol	4.20±1.06	1700

[a] The parameters were shown as mean ± SD. Samples were from 15 different sources throughout Thailand.

[b] Kovat's index [155].

***Zingiber zerumbet* dried rhizome volatile oil**

The major chemical constituents of the volatile oil of *Zingiber zerumbet* dried rhizomes from 15 different sources in Thailand were revealed as zerumbone (51.67 ± 10.23 %) followed by alpha-caryophyllene (14.13 ± 8.05%) and humulene epoxide II (10.85 ± 4.47%). Figure 52 and Table 12 showed the GC chromatogram and chemical constituents of the volatile oil of *Zingiber zerumbet* dried rhizome.

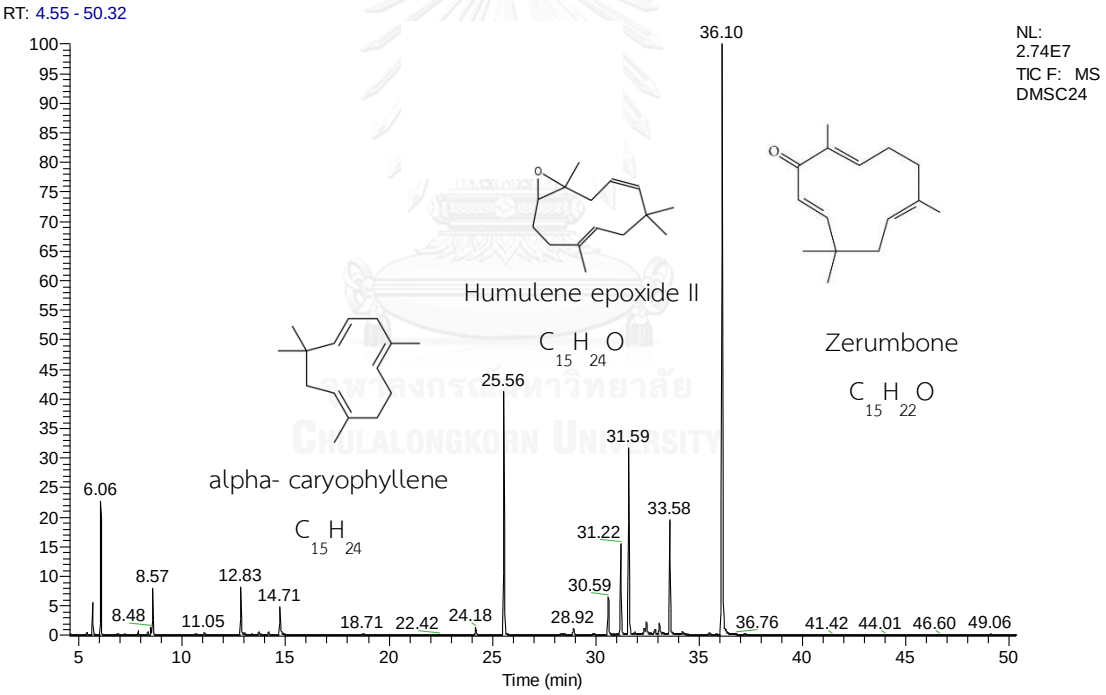


Figure 52 GC chromatogram of the volatile oil of *Zingiber zerumbet* dried rhizome.

Table 12 The chemical constituents of the volatile oil of *Zingiber zerumbet* dried rhizome.

RT	Compound name	Area% [a]	KI[b]
5.67	Pinene (alpha)	1.09 ± 1.16	932
6.06	Camphene	3.39 ± 1.92	954
7.86	3-Carene	0.30 ± 0.14	1011
8.48	Limonene	0.48 ± 0.50	1029
8.57	Eucalyptol	1.70 ± 0.73	-
12.83	Camphor	1.92 ± 0.71	1146
13.70	Borneol	1.01 ± 1.91	1169
14.18	Cyclopenta[c]pyran-1,3-dione, 4,4a,5,6-tetrahydro-4,7-dimethyl-	1.69 ± 1.35	-
14.71	Terpinol (alpha)	1.30 ± 0.41	1186
24.18	Isocaryophyllene	0.63 ± 0.34	1408
25.56	Caryophyllene (alpha)	14.13 ± 8.05	1454
30.59	Caryophyllene oxide	2.11 ± 0.58	1583
31.22	Unidentified	4.64 ± 1.41	-
31.59	Humulene epoxide II	10.85 ± 4.47	1608
33.58	Unidentified	2.18 ± 1.55	-
36.10	Zerumbone	51.67 ± 10.23	1733

[a] The parameters were shown as mean ± SD. Samples were from 15 different sources throughout Thailand.

[b] Kovat's index [155].

Acorus calamus dried rhizomes volatile oil

The major chemical constituents of the volatile oil of *Acorus calamus* rhizome from 15 different sources in Thailand were revealed as beta-asarone (71.84 ± 5.12 %) followed by and alpha-asarone (16.36 ± 1.32 %). Figure 53 and Table 13 showed the GC chromatogram and chemical constituents of the volatile oil of *Acorus calamus* rhizome.

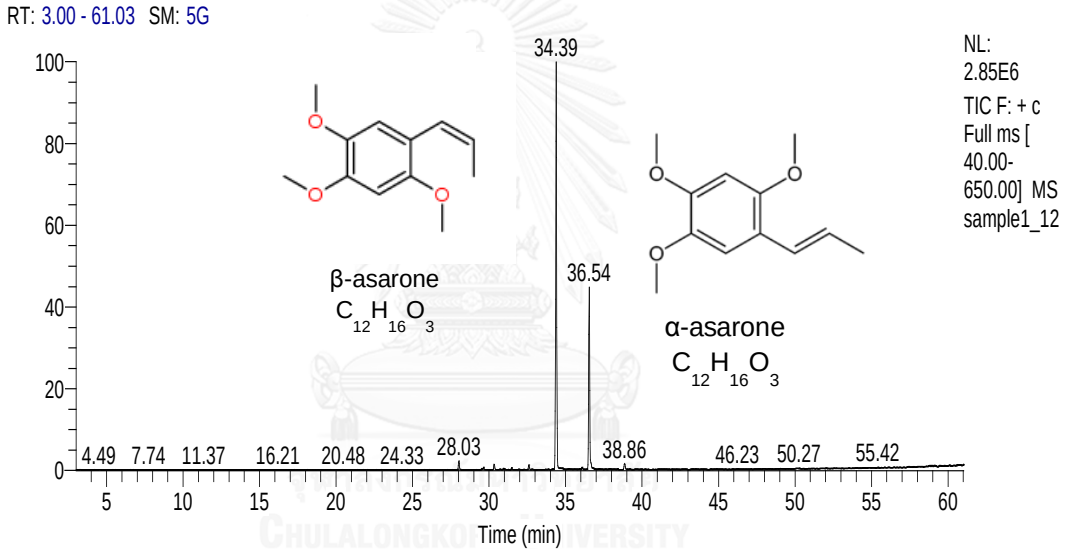


Figure 53 GC chromatogram of the volatile oil of *Acorus calamus* dried rhizome

Table 13 The chemical constituents of the volatile oil of *Acorus calamus* dried rhizome

RT	Compound name	Area% [a]	KI[b]
28.05	Methyl isoeugenol (Z)	4.25 ± 1.20	1453
29.54	Shyobunone	0.29 ± 0.07	-
29.64	Methyl isoeugenol (E)	0.99 ± 0.80	1492
30.35	Shyobunone	1.74 ± 0.08	-
30.72	Cadinene (delta)	0.11 ± 0.12	1523
30.97	Unidentified	0.35 ± 0.10	-
31.48	Calacorene (beta)	0.47 ± 0.02	1565
31.92	Elemicin	0.12 ± 0.01	1557
32.62	Isoelemicin (Z)	1.60 ± 0.8	1570
32.81	Spatulenol	0.11 ± 0.10	1578
33.74	Rosifoliol	0.07 ± 0.06	1600
34.51	Guaiol	0.16 ± 0.11	1600
34.55	Asarone (beta)	71.84 ± 5.12	1617
35.25	tau-Muurolol	0.11 ± 0.09	1642
35.71	Cadinol (alpha)	0.13 ± 0.05	1654
35.96	Unidentified	0.11 ± 0.10	-
36.10	Khusinol acetate	0.47 ± 0.31	1823
36.62	Asaron (alpha)	16.36 ± 1.32	1676
37.90	Unidentified	0.17 ± 0.02	-
38.88	Unidentified	0.55 ± 0.01	-

[a] The parameters were shown as mean ± SD. Samples were from 15 different sources throughout Thailand.

[b] Kovat's index [155].

Plant extraction

The powders of seven species were extracted using macreration technique in 95% ethanol and water respectively. Table 14 showed the percentage yields of crude extracts of each species in Cha Tu Ka La Thad and Tree Phon Thad remedies.

Table 14 Crude extracts of seven species in Cha Tu Ka La Thad (CKT) and Tree Phon Thad (TPT) remedies.

Plant name	Yield of ethanol extract (%W/W)	Yield of fractionated water (%W/W)	Total extract yield (%W/W)
Cha Tu Ka La Thad			
Plumbago indica	22.52	17.65	40.17
Acorus calamus	7.43	6.33	13.76
Clerodendrum paniculatum	7.25	7.51	14.76
Dolichandrone serrulata	4.40	1.37	5.77
Tree Phon Thad			
Zingiber zerumbet	8.15	4.01	12.16
Zingiber montanum	15.26	5.20	20.46
Cymbopogon nardus	17.52	6.09	23.61

Safety studies

Cytotoxic activity using brine shrimp lethality assay

Cha Tu Ka La Thad remedy

The results of the brine shrimp lethality assay were expressed as the concentration of the extracts necessary to cause 50% of lethality)LC_{50}(. The extracts of Cha Tu Ka La Thad remedy and its ingredients showed LC_{50} more than 1,000 µg/ml except the ethanol extract of *A. calamus* which gave LC_{50} values of 129.88 µg/ml and the fractionated water extract of *C. paniculatum* which gave LC_{50} values of 868.92 µg/ml. Finally, CKT remedy extract demonstrated LC_{50} of 577.83 µg/ml)Table 15(. According to Meyer *et al.*)1982(, who classified crude extracts into toxic)LC_{50} values <1,000 µg/ml(and non-toxic)LC_{50} values >1,000 µg/ml(, *A. calamus*, *C. paniculatum* and CKT remedy had potential to be toxic to brine shrimp.

Table 15 Brine shrimp lethality (LC_{50}) of the extracts of Cha Tu Ka La Thad remedy and its ingredients

Species	Ethanol extract LC_{50} (µg/ml)	Fractionated water extract LC_{50} (µg/ml)
Plumbago indica	>1,000	>1,000
Acorus calamus	129.88	>1,000
Clerodendrum paniculatum	>1,000	868.92
Dolichandrone serrulata	>1,000	>1,000
Cha-Tu-Ka-La-Thad Remedy	577.83	

Tree Phon Thad

The LC_{50} values of the brine shrimp obtained for the extracts of Tree Phon Thad remedy and its ingredients were given in Table 16. It was found that only the ethanol extract of *Z. zerumbet* showed LC_{50} of 531.23 µg/ml while TPT remedy showed LC_{50} of 576.66 µg/ml. According to Meyer *et al.*)1982(, who classified crude extracts into toxic)LC_{50} values <1,000 µg/ml(and non-toxic)LC_{50} values >1,000 µg/ml(, *Z. zerumbet* and TPT remedy had potential to be toxic to brine shrimp.

Table 16 Brine shrimp lethality (LC_{50}) of the extracts of Tree Phon Thad remedy and its ingredients

Species	Ethanol extract LC_{50} (µg/ml)	Fractionated water extract LC_{50} (µg/ml)
Zingiber zerumbet	531.23	>1,000
Zingiber montanum	>1,000	>1,000
Cymbopogon nardus	>1,000	>1,000
Tree-Phon-Thad Remedy		576.66

Mutagenic assay

Cha Tu Ka La Thad remedy

The results of the mutagenic assay of CKT remedy and root extracts obtained by the Ames test were shown in Figure 54-56 .Most ethanol and fractionated water extract of each root and CKT remedy without nitrite mixture were not mutagenic against *S. typhimurium* strains TA 98and TA 100except the ethanol extract of *A. calamus* root which was mutagenic on both strains by high induced revertant colonies to strain TA98 (1043.67± 22.74, MI= (46.44and strain TA 100 (1157.33± 141.93, MI= (10.16at 3.2mg/plate. The ethanol extract of *P. indica* at 3.2 mg/plate exhibited mutagenicity to strain TA100 with induced revertant colonies of 118.33 ± 22.83 (MI=2.27). The fractionated water extract of *A. calamus* and *P. indica* root also exhibited mutagenicity to strain TA 98with induced revertant colonies of 126.33±) 37MI= (4.29 and 118.33 ± 22.83 (MI= 2.27) revertant colonies, respectively at high dose (Figure 54A-54B).

It was observed that ethanol extract of four root species had mutagenic effects on *S. typhimurium* strains TA98 while the ethanol extract of *A. calamus* and *P. indica* exhibited mutagenic effect on *S. typhimurium* strains TA100 after they were treated with sodium nitrite (nitrosation) under acidic condition. After treating with sodium nitrite in an acidic condition, only the fractionated water extract of *P. indica*

showed dose-response mutagenic effect against *S. typhimurium* TA98 with induced revertant colonies of 132.67 ± 34.47 (MI=2.71) and 213.50 ± 17.04 (MI=4.36) at 1.6 and 3.2 mg/plate, respectively (Figure 55A-55B). However, CKT remedy was no mutagenic effect under the condition with and without nitrite treatment in the Ames test toward both strains of *S. typhimurium* (Figure 56A-56B).

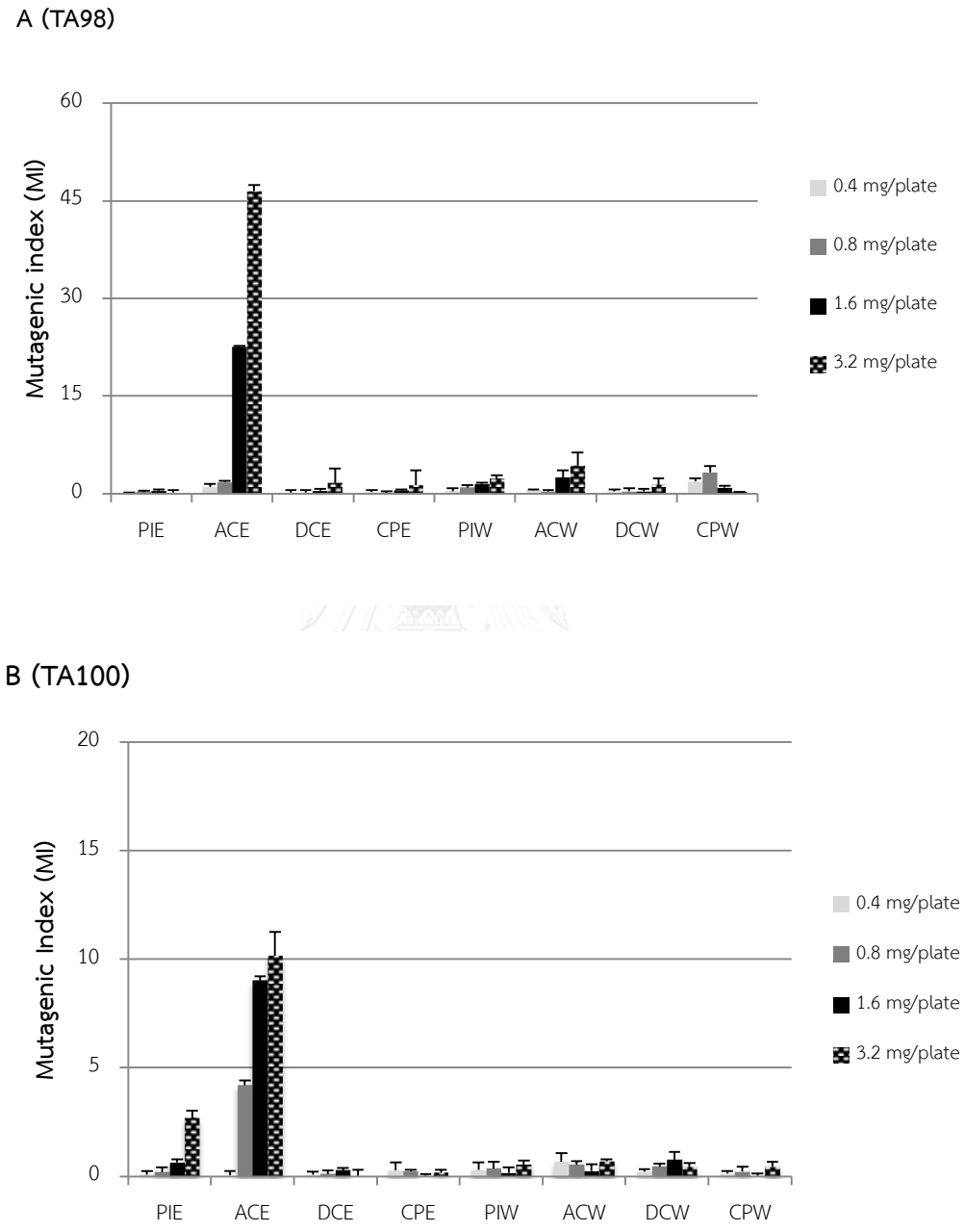


Figure 54 The mutagenic index (MI) induced by the ethanol and fractionated water extract from each plant species of CKT remedy without nitrosation on *S. typhimurium* strains TA98 (A) and TA100 (B) using Ames test; PI = *P. indica*, AC = *A.*

calamus, DC = *D. serrulata,* CP = *C. paniculatum*, E= ethanol extract, W = fractionated water extract

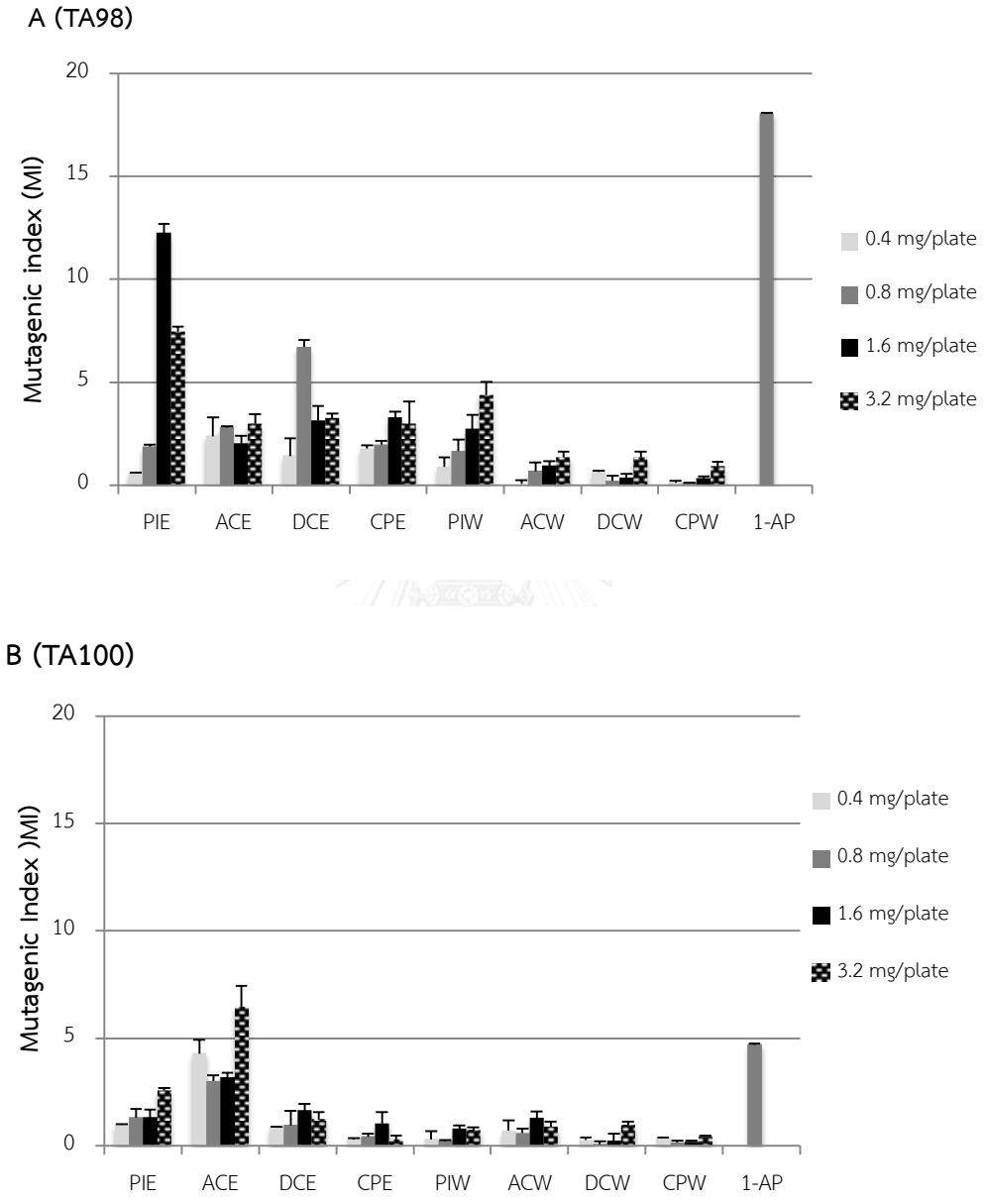


Figure 55 The mutagenic index (MI) induced by the ethanol and fractionated water extract from each plant species of CKT remedy with nitrosation on *S. typhimurium* strains TA98 (A) and TA100 (B) using Ames test; PI = *P. indica*, AC = *A. calamus,* DC =

D. serrulata, CP = *C. paniculatum*, E= ethanol extract, W = fractionated water extract, 1-AP = nitrite treated 1-aminopyrene

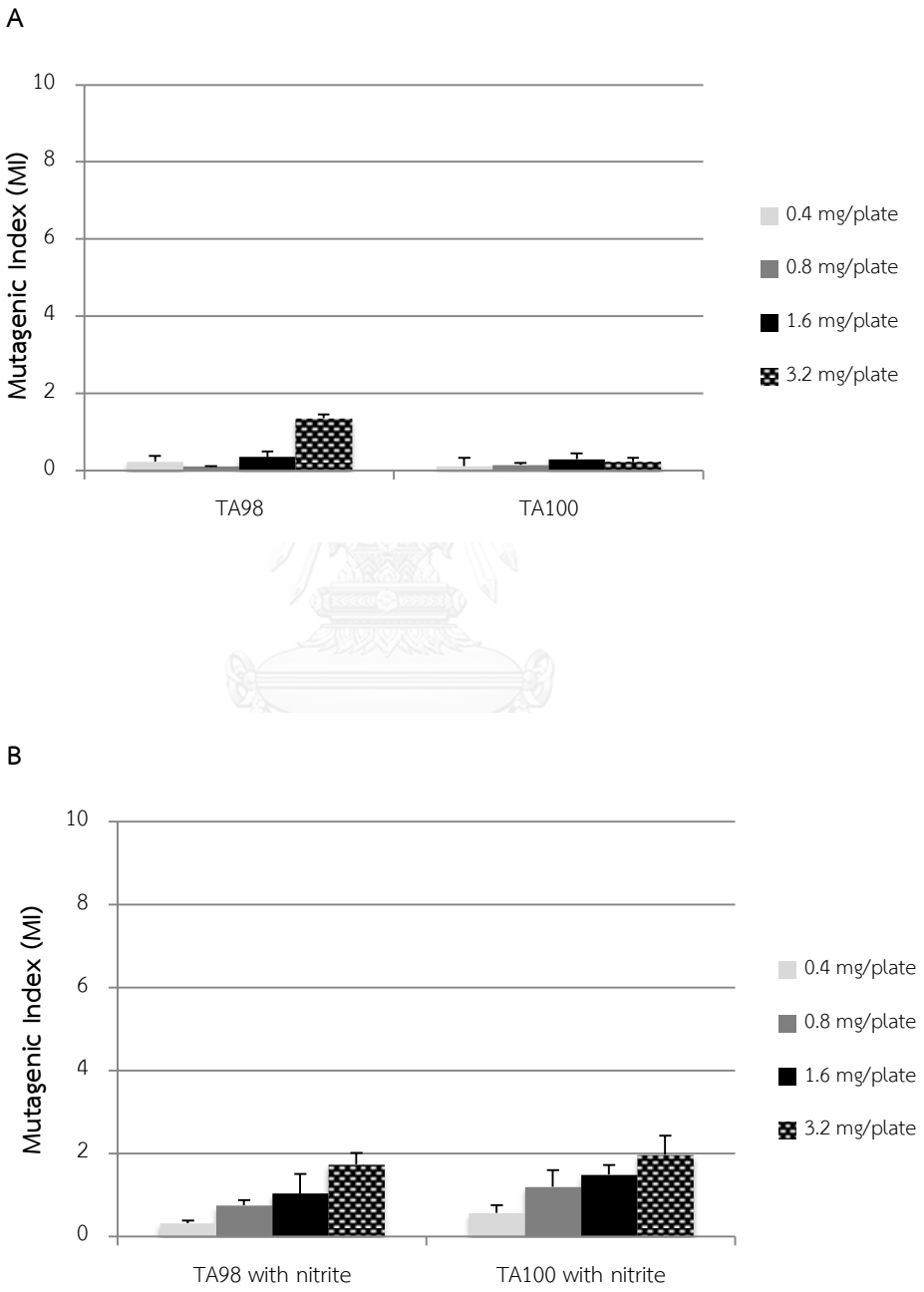


Figure 56 The mutagenic index (MI) induced by CKT remedy on *S. typhimurium* strains TA98 and TA100 (A) without nitrosation and (B) with nitrosation using Ames test

Mutagenicity assay

Tree Phon Thad remedy

The results of the mutagenic assay of TPT remedy and its component extracts obtained by the Ames test were shown in Figure 57-59. The result demonstrated that the extracts and TPT remedy were not directly mutagenic toward *S. typhimurium* strains TA 98and TA100 except the fractionated water extract of *C. nardus* showed slightly mutagenicity toward *S. typhimurium* TA98 by induced revertant colonies of 92.333 ± 16.63 (MI= 2.689) at high dose (Figure 57A).

Most ethanol and fractionated water extract of all rhizomes exhibited their mutagenic effect after they were treated with sodium nitrite (nitrosation) under acidic condition on *S. typhimurium* strains TA 98and TA100. However, there were no mutagenic effects exhibited by the fractionated water of *Z. zerumbet*, *Z. montanum* and *C. nardus* to strain TA100 and TPT remedy extract toward strain TA98 (Figure 58-59).

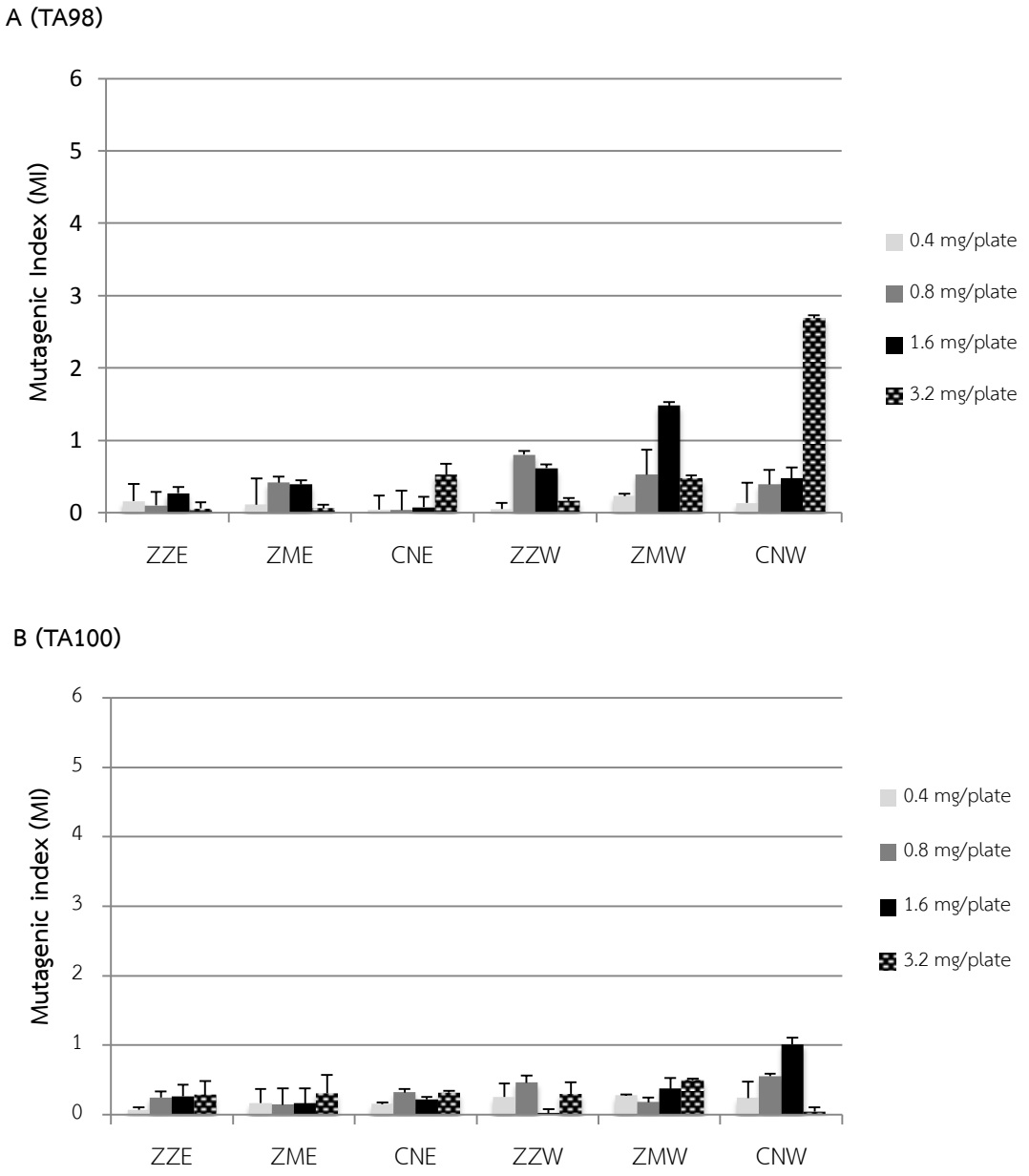


Figure 57 The mutagenic index (MI) induced by the ethanol and fractionated water extract from each plant species of TPT remedy without nitrosation on *S. typhimurium* strains TA98 (A) and TA100 (B) using Ames test; ZZ = *Z. zerumbet*, ZM = *Z. montanum,* CN = *C. nardus,* E= ethanol extract, W = fractionated water extract

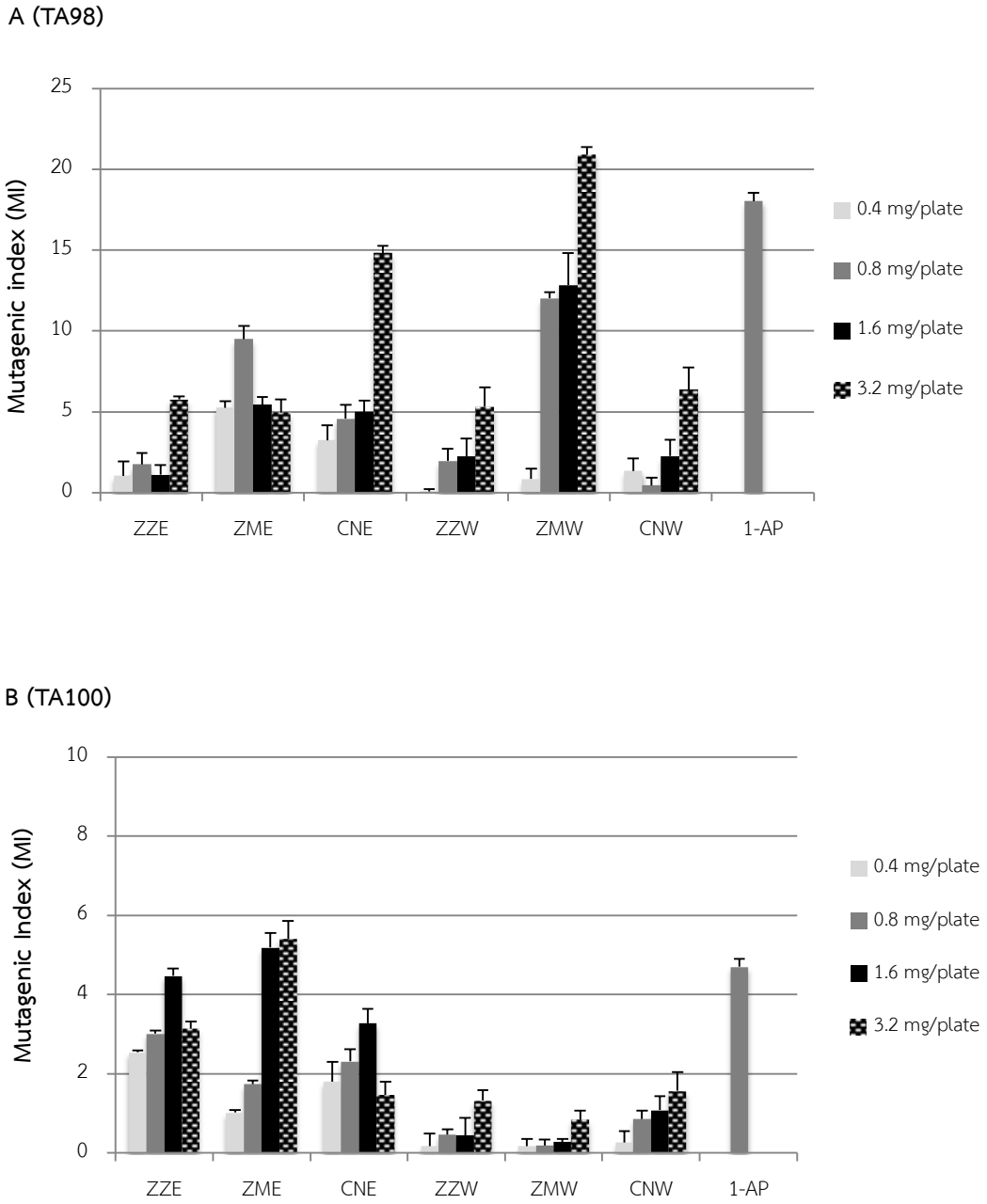


Figure 58 The mutagenic index (MI) induced by the ethanol and fractionated water extracts from each plant species of TPT remedy with nitrosation on *S. typhimurium* strains TA98 (A) and TA100 (B) using Ames test; ZZ = *Z. zerumbet*, ZM = *Z. montanum,* CN = *C. nardus,* E= ethanol extract, W = fractionated water extract

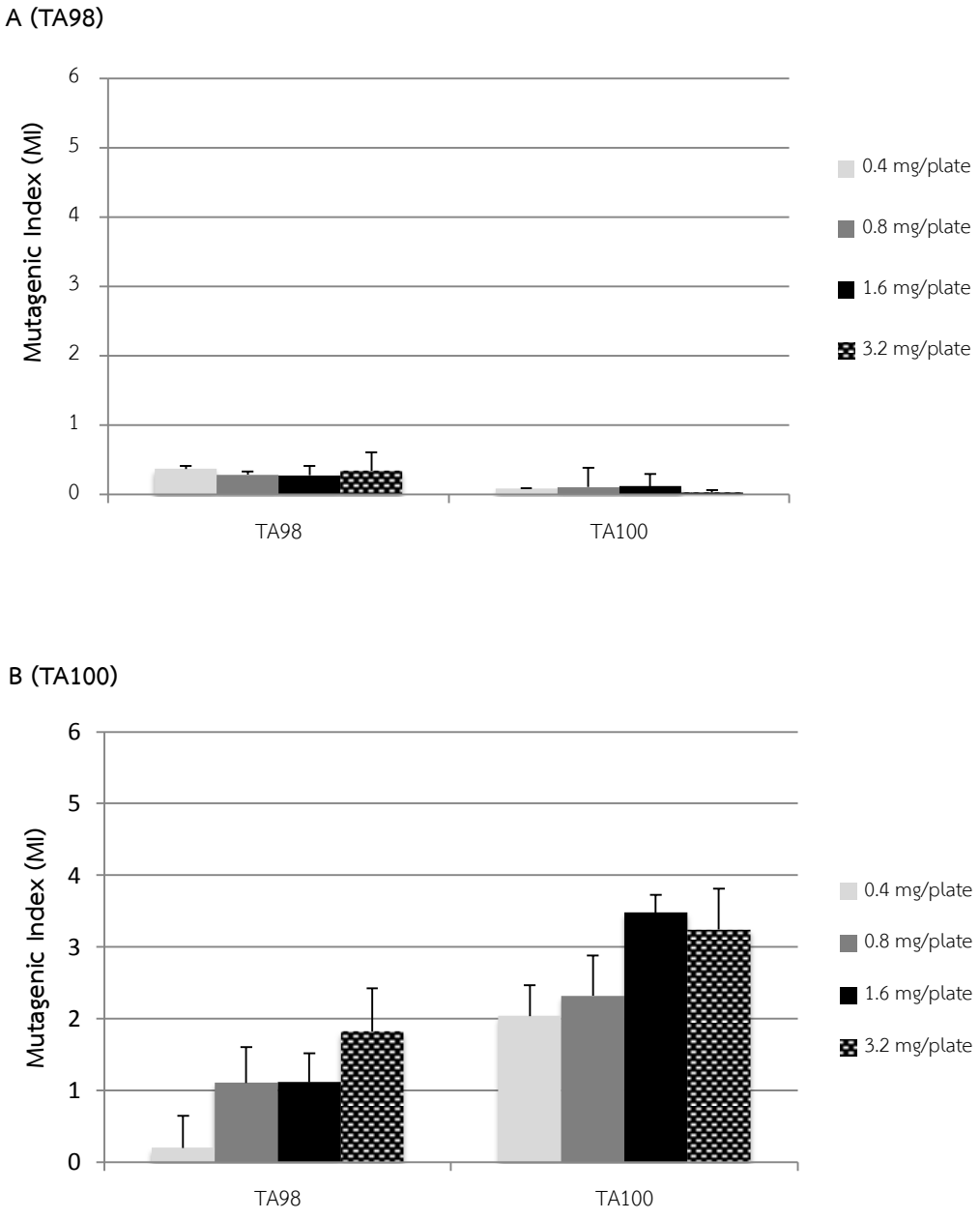


Figure 59 The mutagenic index (MI) induced by TPT remedy on *S. typhimurium* strains TA98 and TA100 (A) without nitrosation and (B) with nitrite treated using Ames test

DNA damage using Comet assay

There are five classes established in terms of tail moment for classification of the comet ranging from 0 (no tail) to 4 (almost all DNA in tail) that give sufficient resolution (Figure 60).

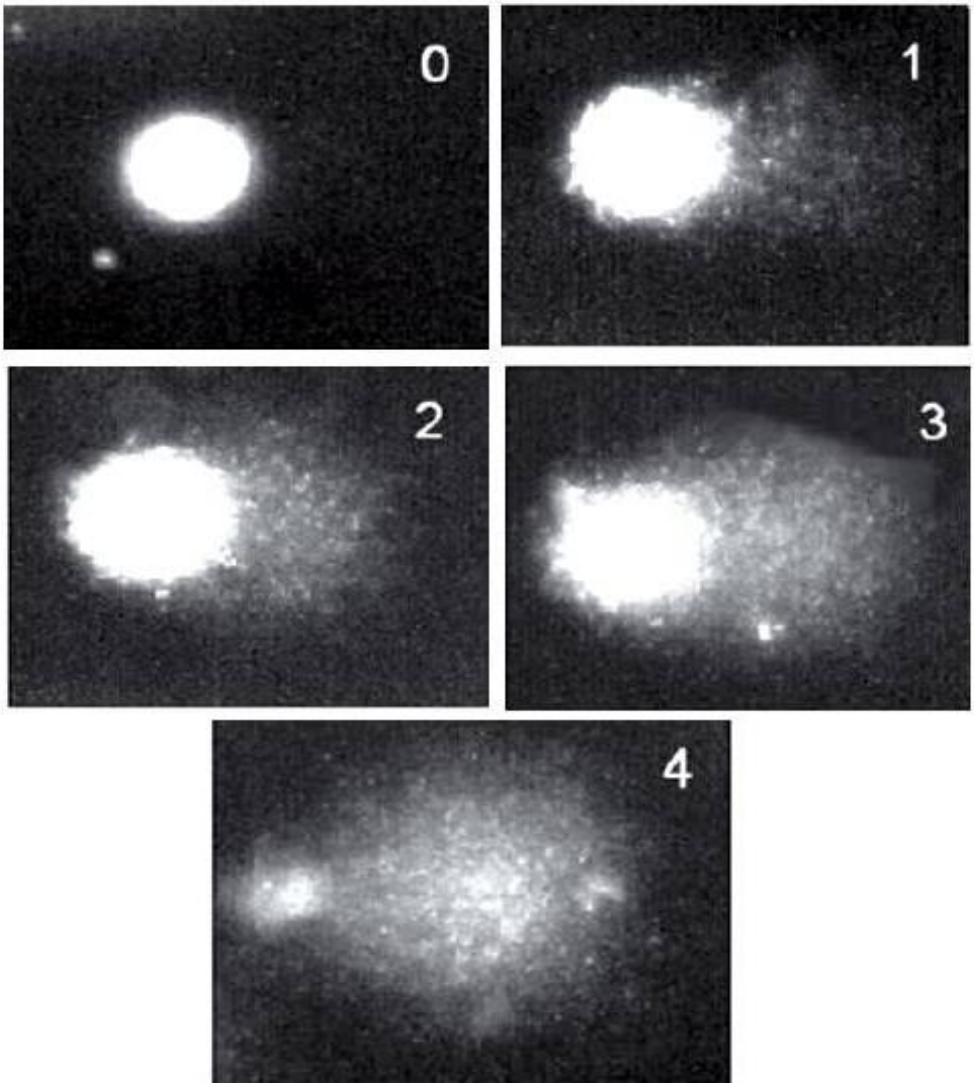


Figure 60 Image of the five classes (0-4) of DNA damage from human lymphocytes for visual scoring classification [156].

Cha Tu Ka La Thad remedy

The results of the DNA damage in human lymphocytes treated with different concentrations of CKT remedy and its ingredients were shown in figure 61-62. Total scores for 100 comets were obtained by multiplying the number of cells in its class. Thus the total score was between 0 (100 cells presenting no damage) and 400 (all cells presenting damage class 4).

Among four root species and CKT remedy extracts, the results demonstrated that the fractionated water extract of *A. calamus* showed the highest DNA damage in human lymphocytes with the total score of 192.00, 222.00 and 233.67 at 25, 50 and 100 µg/ml, respectively. The fractionated water extracts of *P. indica* and *C. paniculatum* presented the total score of 158.33 and 139.33, respectively at 3.2 mg/plate. The ethanol extract of *P. indica* was revealed for the lowest DNA damage in human lymphocytes. H_2O_2 also demonstrated highest damage to DNA in human lymphocytes at 75.33, 141.33 and 377.00, respectively. All samples showed a dose-dependent relationship between the degree of DNA damage and concentration of sample.

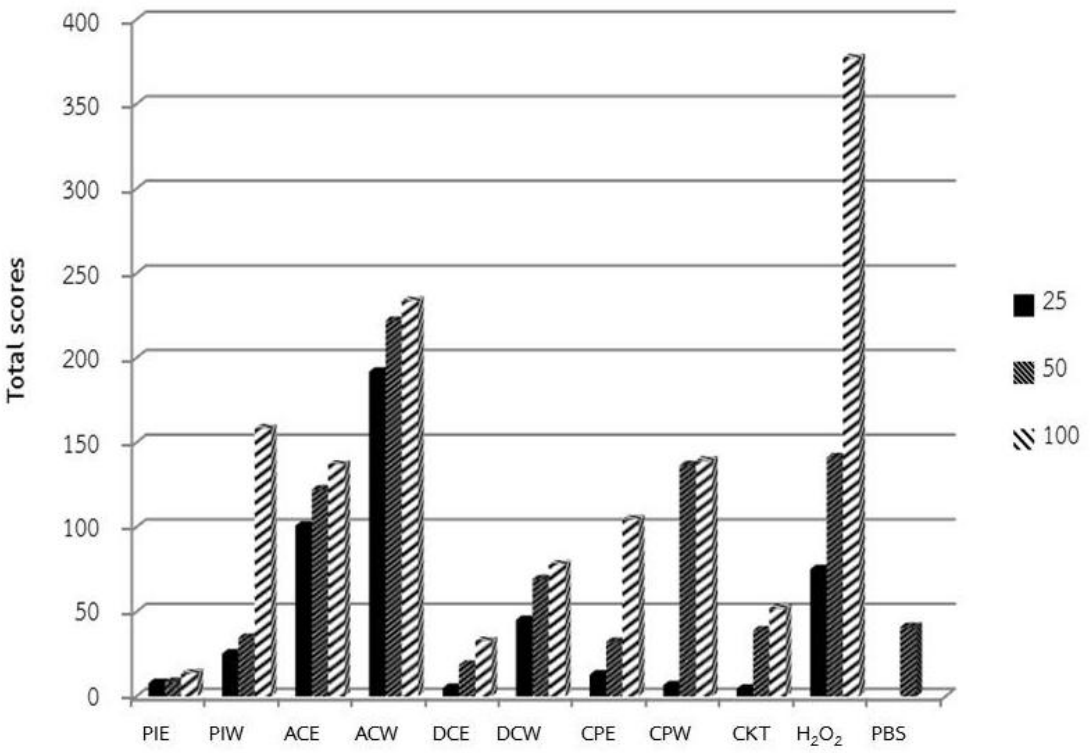
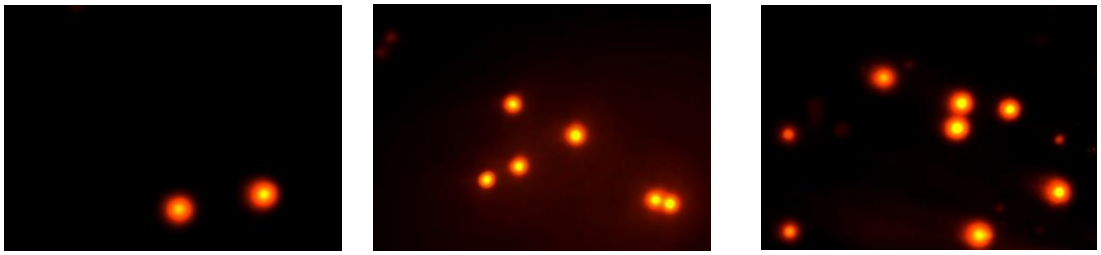


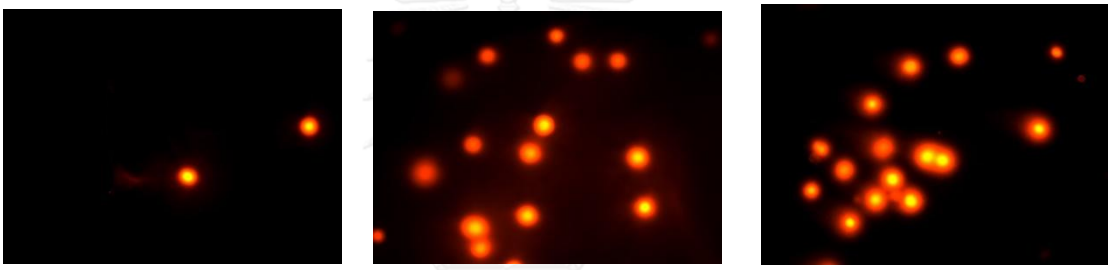
Figure 61 The total scores of DNA damage in human lymphocytes treated with different concentrations of Cha Tu Ka La Thad remedy and four species ingredients; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, E= ethanol extract, W = fractionated water extract, CKT= Cha Tu Ka La Thad remedy, H_2O_2= positive control, PBS= negative control

Figure 62 The DNA damage in human lymphocytes treated with different concentrations of Cha Tu Ka La Thad remedy and four species extracts or controls.



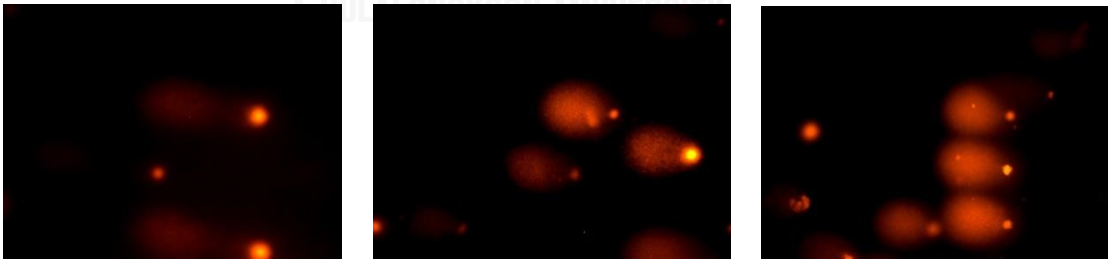
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.1 *Plumbago indica* (fractionated water extract)



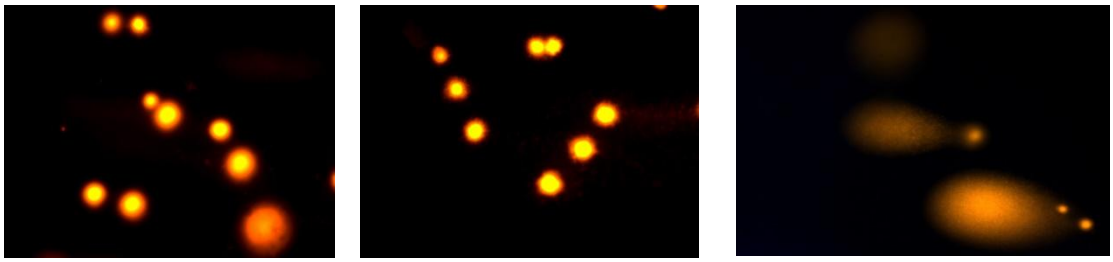
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.2 *Plumbago indica* (Ethanol extract)



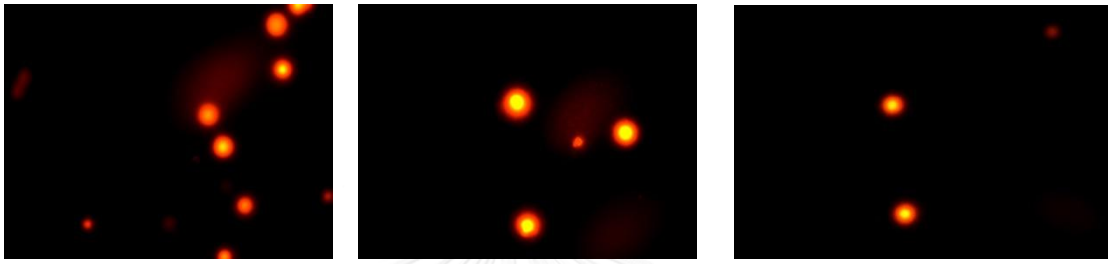
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.3 *Acorus calamus* (fractionated water extract)



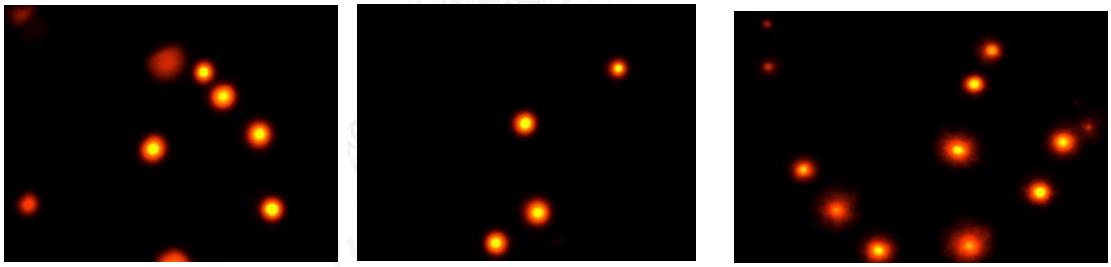
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.4 *Acorus calamus* (Ethanol extract)



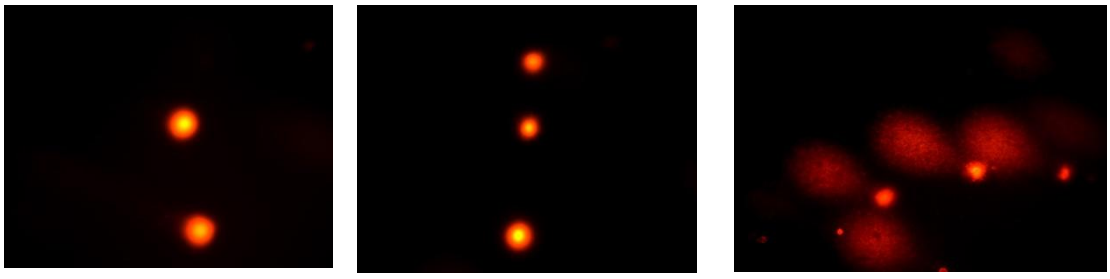
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.5 *Dolichandrone serrulata* (fractionated water extract)



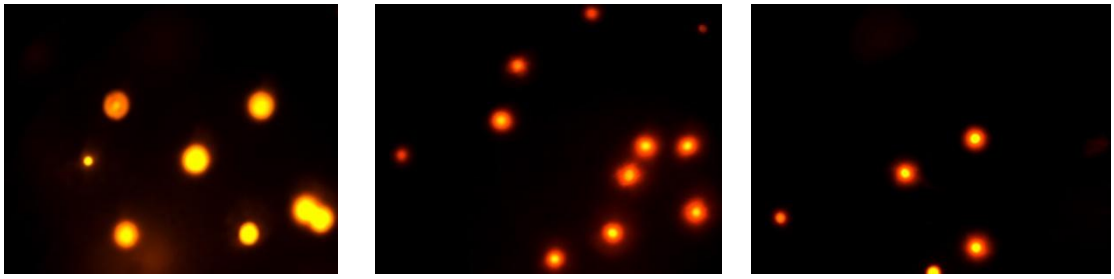
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.6 *Dolichandrone serrulata* (Ethanol extract)



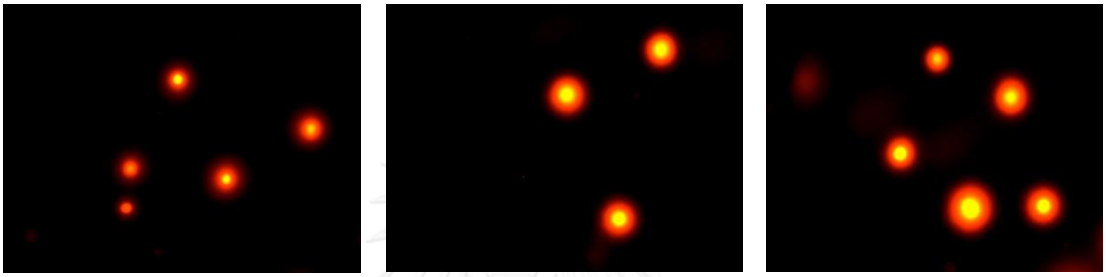
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.7 *Clerodendrum paniculatum* (fractionated water extract)



A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.8 *Clerodendrum paniculatum* (Ethanol extract)



A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.9 CKT remedy

A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 62.10 Positive control : hydrogen peroxide

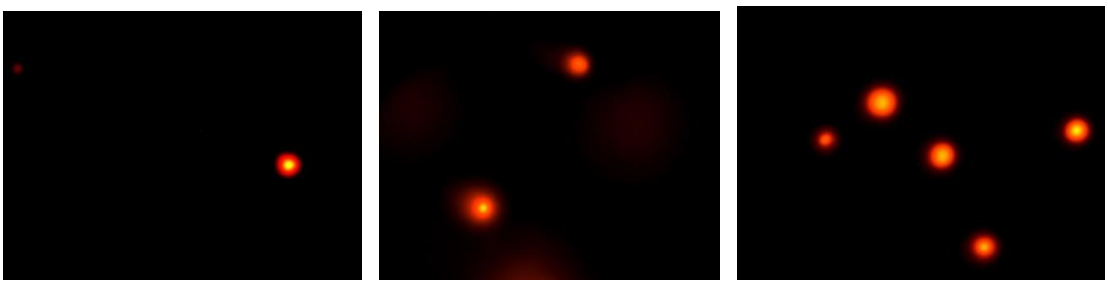


Figure 62.11 Negative control

Tree Phon Thad remedy

The results of the DNA damage in human lymphocytes treated with different concentrations of TPT remedy and its ingredients were shown in figure 63-64. All three species and TPT remedy extracts were revealed for low DNA damage in human lymphocytes as same as the negative control (PBS). Whereas, H_2O_2 showed that the highest damage to DNA in human lymphocytes at 152.64, 271.66 and 299.34, respectively. All samples showed a dose-dependent relationship between the degree of DNA damage and concentration of sample.

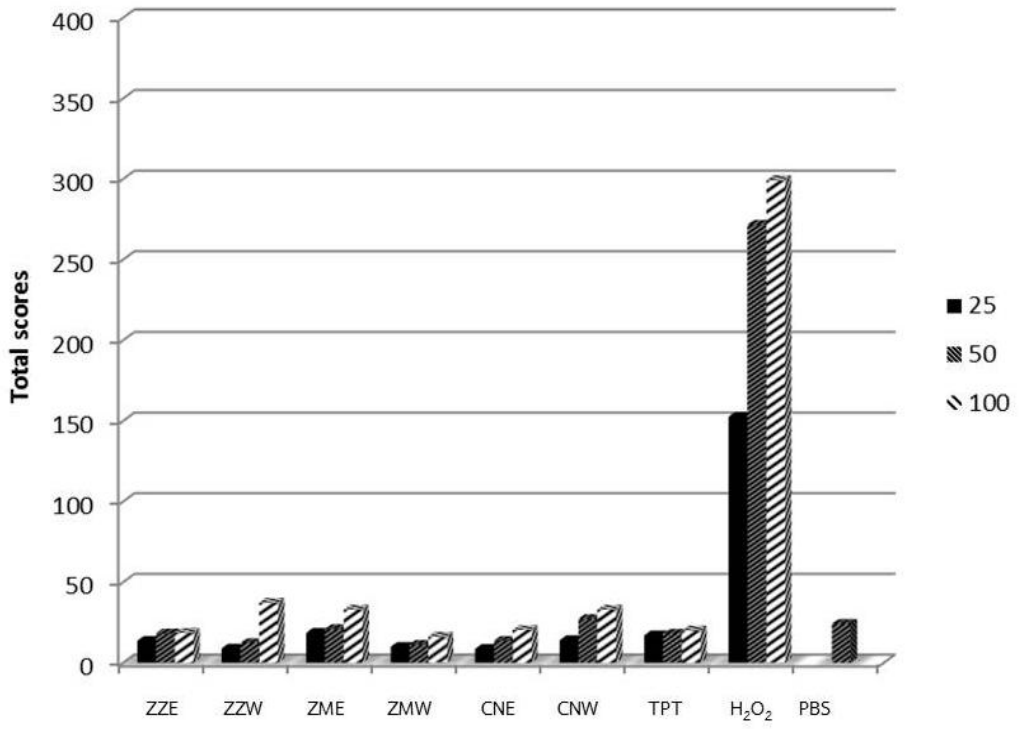
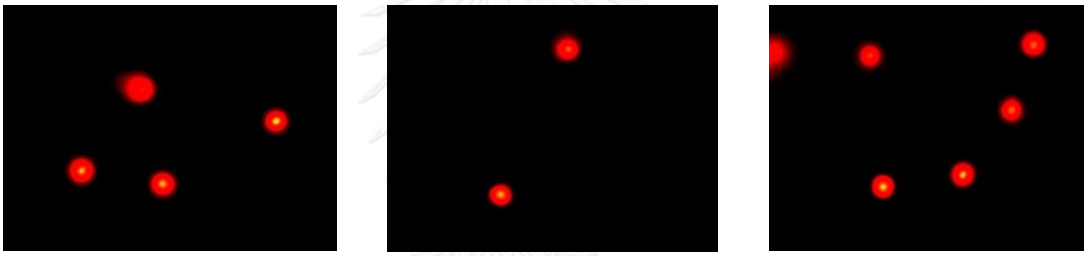


Figure 63 The total scores of DNA damage in human lymphocytes treated with different concentrations of Tree-Phon-Thad remedy and three species ingredients; ZZ = *Z. zerumbet*, ZM = *Z. montanum,* CN = *C. nardus,* E= ethanol extract, W = fractionated water extract, TPT= Tree PHon Thad remedy, H_2O_2= positive control, PBS= negative control

Figure 64 The DNA damage in human lymphocytes treated with different concentrations of Tree Phon Thad remedy and three species extracts or controls.

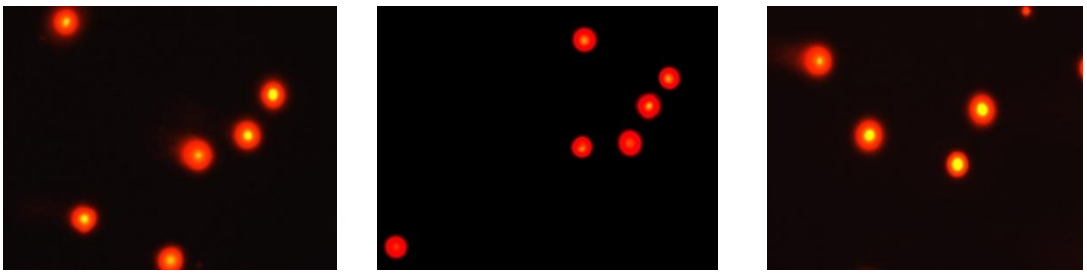
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 64.1 *Zingiber montanum* (fractionated water extract)



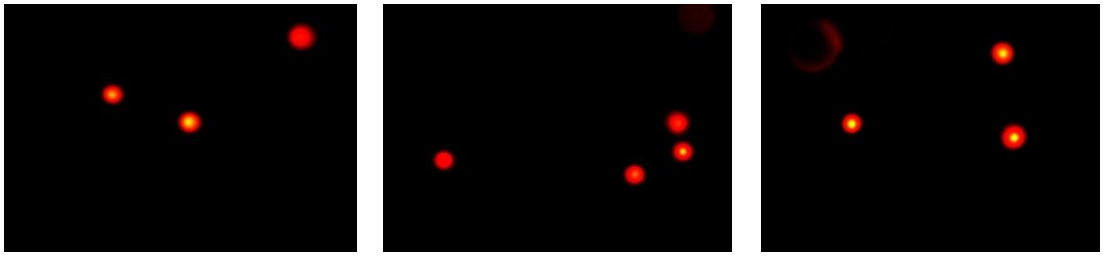
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 64.2 *Zingiber montanum* (Ethanol extract)



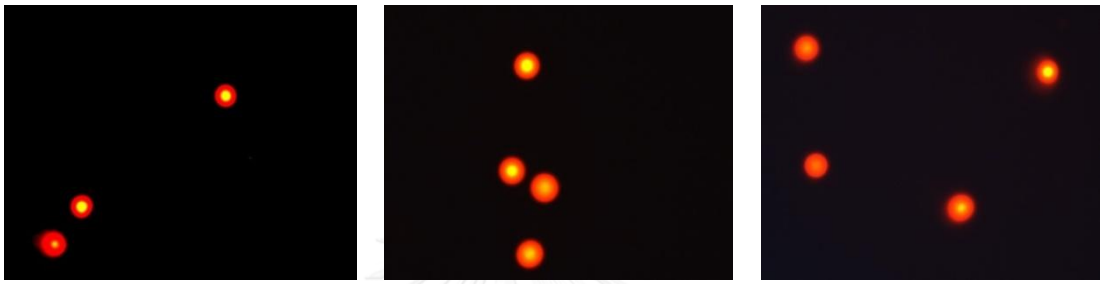
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 64.3 *Cymbopogon nardus* (fractionated water extract)



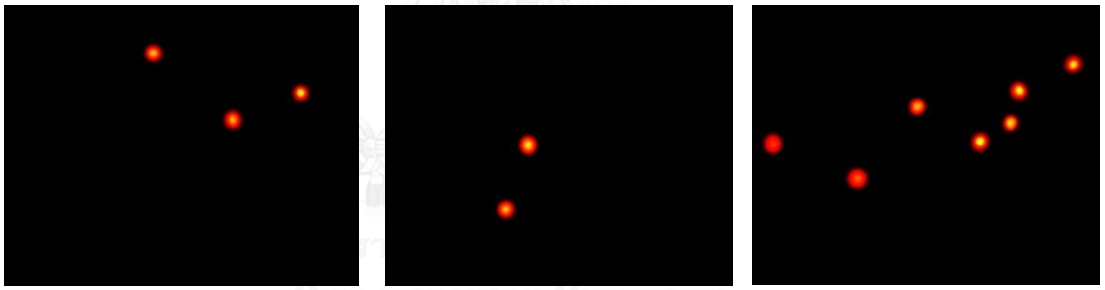
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 64.4 *Cymbopogon nardus* (Ethanol extract)



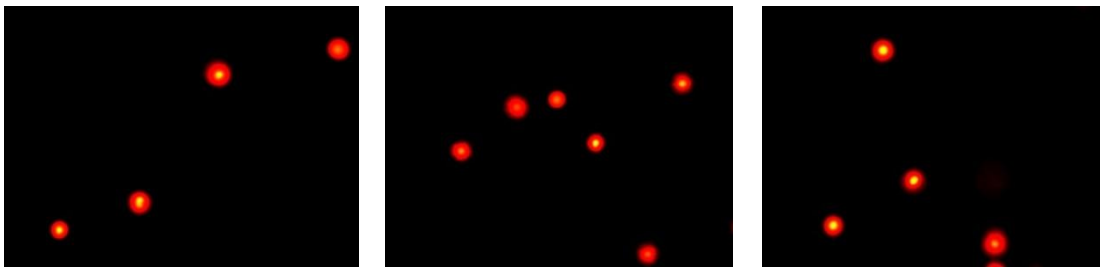
A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 64.5 *Zingiber zerumbet* (fractionated water extract)



A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 64.6 *Zingiber zerumbet* (Ethanol extract)



A. 25 µg/ml B. 50 µg/ml C. 100 µg/ml

Figure 64.7 Tree-Phon-Thad remedy

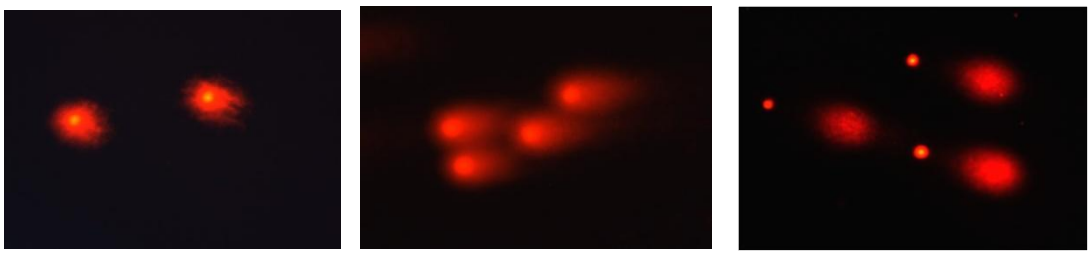


Figure 64.8 Positive control : hydrogen peroxide

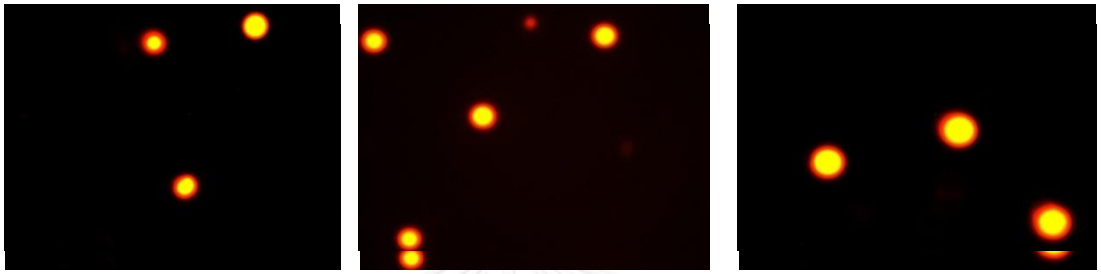


Figure 64.9 Negative control

Efficacy studies

Antimutagenic activity

Cha Tu Ka La Thad remedy

The antimutagenic activity of all root extracts and CKT remedy were shown in Figure 65. All of the extracts had dose-related inhibition effect to the mutagenicity of nitrite treated 1- aminopyrene toward *S. typhimurium* strains TA98 and TA100 in the absence of enzyme activating system. The effects of all extracts ranged from weak (20-40% inhibition) to strongly active (**>** 60% inhibition) except the fractionated water extract of *D. serrulata* root (5 and 10 mg/plate) was negligible inhibitory activity against of sodium nitrite-treated 1-aminopyrene on both strains of *S. typhimurium*.

For the part of ethanol root extracts, all ethanol root extracts demonstrated the strong inhibitory activity. The ethanol extract of *A. calamus* root at 10 mg/plate exhibited the strongest inhibitory activity on *S. typhimurium* TA98 and TA100. The ethanol extracts of *D. serrulata* root exhibited the strong inhibitory activity at 15 mg/plate on *S. typhimurium* TA98 and at 10 mg/plate toward strain TA100. The ethanol extract of *A. calamus* root (15 mg/plate) showed killing effect to strain TA100. The ethanol extract of *P. indica* root showed the strong effects of 63.71%, 95.16% and 97.18% at 5, 10 and 15 mg/plate respectively toward strain TA98, 69.11% and 71.63% at 10 and 15 mg/plate respectively toward strain TA100. Moreover, the strong inhibitory effect was expressed by the ethanol extracts of *D. serrulata* and *C. paniculatum* at 10 and 15 mg/plate toward strain TA98, while at 5 and 10 mg/plate

toward strain TA100. The ethanol extract of *A. calamus* at 5 mg/plate expressed strong inhibitory effect of 71.24% whereas the concentration at 15 mg/plate showed strong enhancement on TA98 strains. The moderate antimutagenic activity was observed on 5 mg/plate of the ethanol extracts of *D. serrulata* and *C. paniculatum* on strain TA98.

For the part of fractionated water extracts, the strong inhibitory activities were observed on 10 and 15 mg/plate of the fractionated water extract of *P. indica* root on both strains, while 15 mg/plate of *C. paniculatum* toward strain TA100. All doses of the fractionated water extract from *C. paniculatum* demonstrated moderate antimutagenic effect on TA98 and at 5 and 10 mg/plate of toward strain TA100. Furthermore, the fractional water extract from *A. calamus* at 15 mg/plate showed negligible enhancement on both strains.

All concentrations of CKT remedy exhibited the strong inhibitory activity on *S. typhimurium* TA98 and TA100 except only at 5 mg/plate showed the moderate inhibitory activity toward strain TA98.

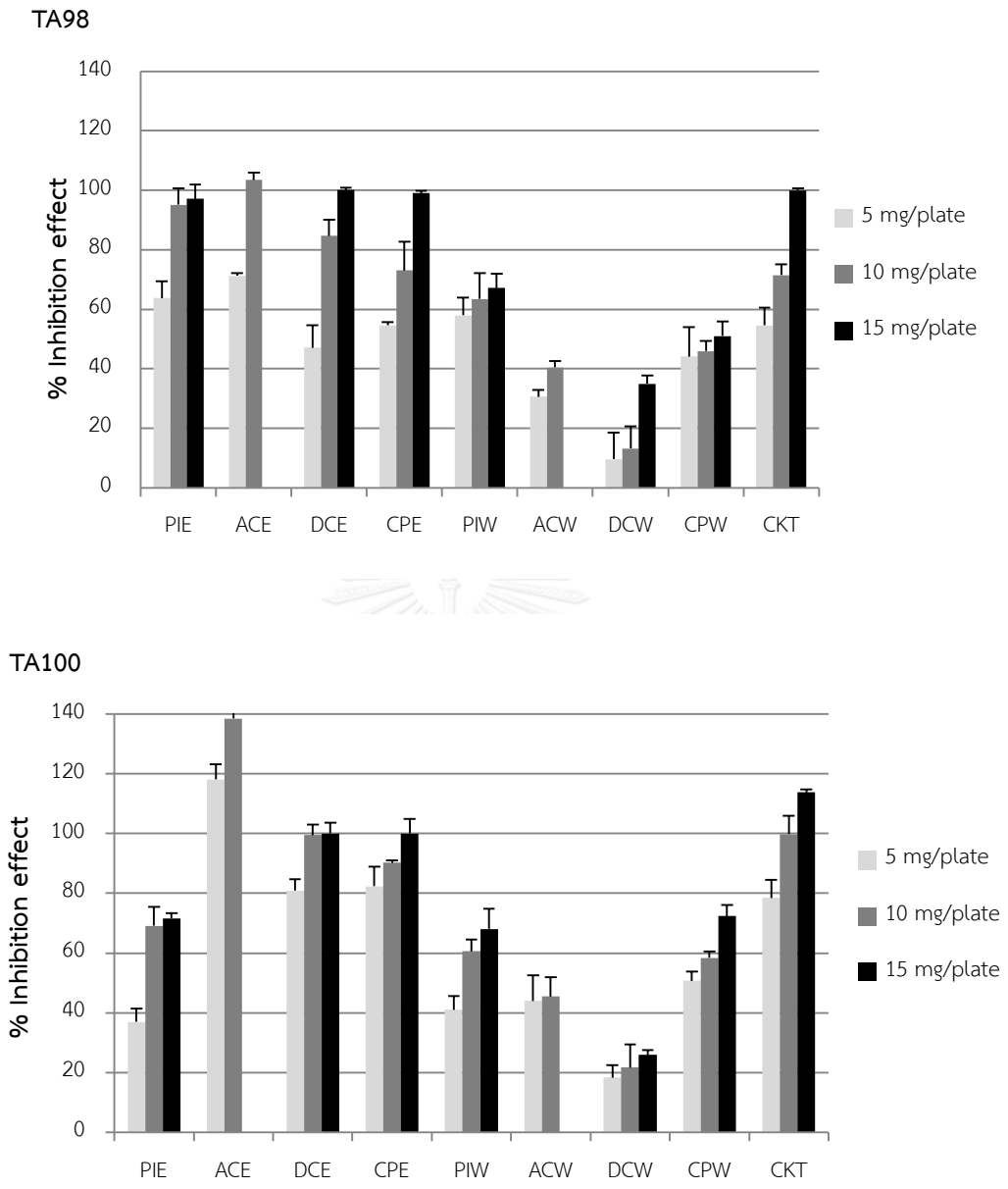


Figure 65 Inhibitory effect of the ethanol and fractionated water extracts from roots and CKT remedy on the mutagenicity of sodium nitrite-treated 1-aminopyrene on *Salmonella typhimurium* strains TA98 and TA100 using Ames test; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy, E = ethanol extract, W = fractionated water extract

Tree Phon Thad remedy

For antimutagenicity activity, all extracts inhibited mutagenicity effect towards *Salmonella typhimurium* strains TA98 and TA100 (figure 66). The effects of all extracts ranged from negligible (0-20% inhibition) to strongly active (> 60% inhibition).

For the part of ethanol extracts, all ethanol extracts demonstrated the strong inhibitory activity toward TA98 and TA100 strain except the ethanol extract of *C. nardus* at 10 mg/plate demonstrated moderate antimutagenic effect and at 5 mg/plate showed negligible inhibitory activity toward strain TA100. The ethanol extract of *Z. zerumbet* rhizome at 5 mg/plate exhibited the strongest inhibitory activity while at 10 and 15 mg/plate showed killing effect to strain TA100. Moreover, at high concentration, the ethanol extract of *Z. zerumbet* also exhibited the strongest inhibitory activity with 98.48% on *S. typhimurium* TA98, followed by the ethanol extract of *C. nardus* and *Z. montanum* at 15 mg/plate with 93.85% and 92.47%, respectively.

For the part of fractionated water extracts, at high concentration of all fractionated water extracts showed strong inhibitory mutagenicity on strain TA98 and TA100 except only the fractionated water extract of *Z. montanum* demonstrated moderate antimutagenic effect on TA100. The moderate inhibitory activity was observed on 5 and 10 mg/plate of the fractionated water extract of *Z. zerumbet*

toward strain TA98. The fractionated water extract of *Z. montanum* at 5 and 10 mg/plate demonstrated negligible antimutagenic effect on strain TA100.

TPT remedy showed strong antimutagenic effect with 72.66% and 104.41% toward TA98 and TA100 strain, respectively at 15 mg/plate.

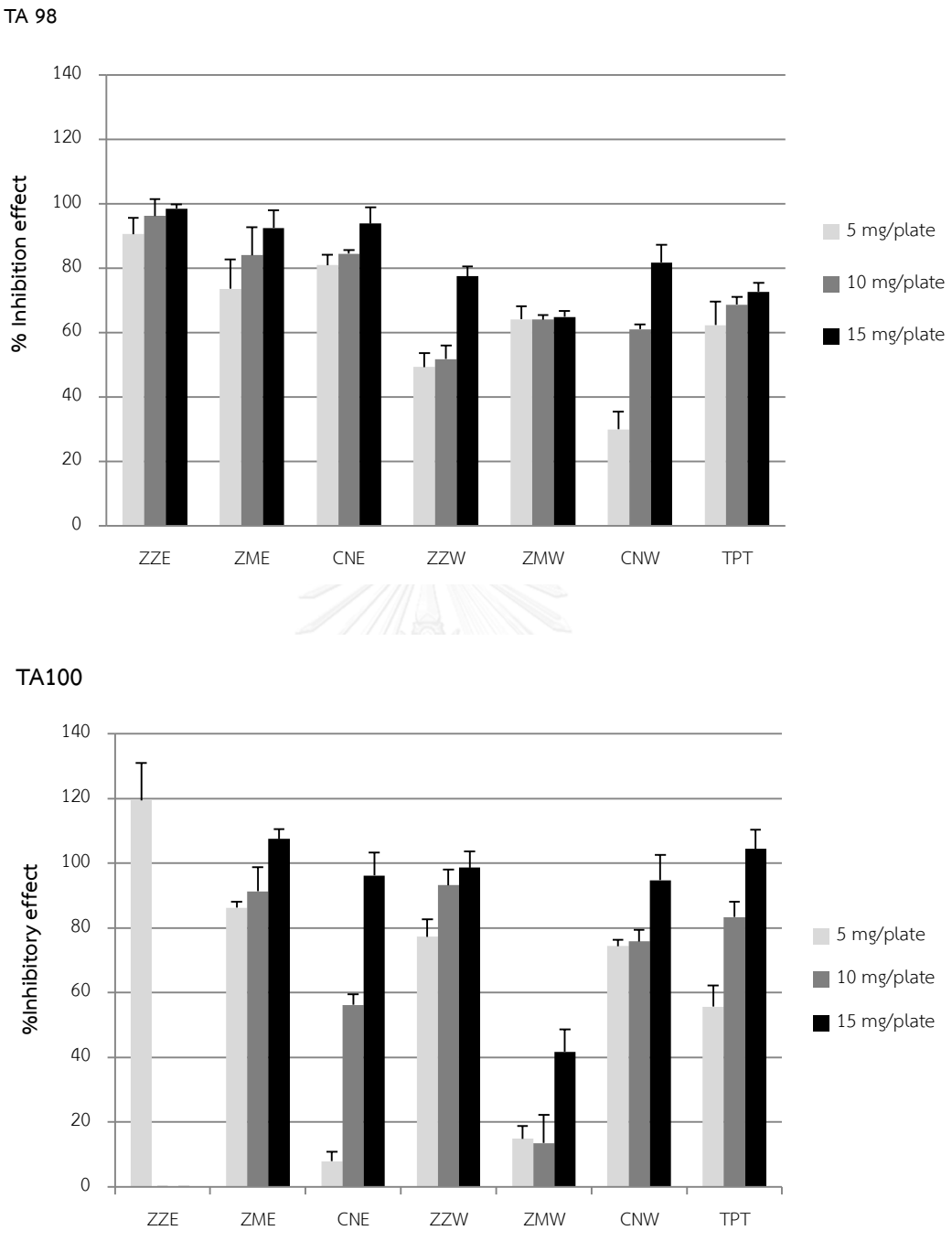


Figure 66 Inhibitory effect of the ethanol and fractionated water extracts from three species and TPT remedy on the mutagenicity of sodium nitrite-treated 1-aminopyrene on *Salmonella typhimurium* strains TA98 and TA100 using Ames test; *ZZ = Z. zerumbet*, ZM = *Z. montanum,* CN = *C. nardus,* E= ethanol extract, W = fractionated water extract, TPT= Tree Phon Thad remedy

Anti-inflammatory activity

Cha Tu Ka La Thad remedy and its ingredients were examined for potential of anti-inflammatory activity in *in vitro* anti-inflammatory models including NO inhibition assay, TNF-α inhibition assay and PGE_2 inhibition assay. Carrageenan-induced paw edema and prostaglandin E2-induced mouse paw edema were performed to detect the anti-inflammatory activity of Tree Phon Thad remedy.

Cha Tu Ka La Thad remedy

Preliminary of cell viability and nitric inhibition assay

The preliminary of cell toxicity of the CKT remedy and its ingredients (ethanol and fractionated water extracts) were determined by resazurin assay. The results revealed that the percentage of cell viability of ethanol extracts and CKT remedy had higher than the fractionated water extracts with concentration ranging from 1-100 µg/ml (Figure 67). Figure 68 showed the effect of the CKT remedy and its ingredients on NO production in LPS-stimulated macrophage cells. All extracts inhibited NO production in LPS-stimulated cells. The percent inhibition of NO production of all ethanol extracts showed higher than the fractionated water extracts. Therefore, CKT remedy and the ethanol extracts were further investigated for the potential of inflammatory activities.

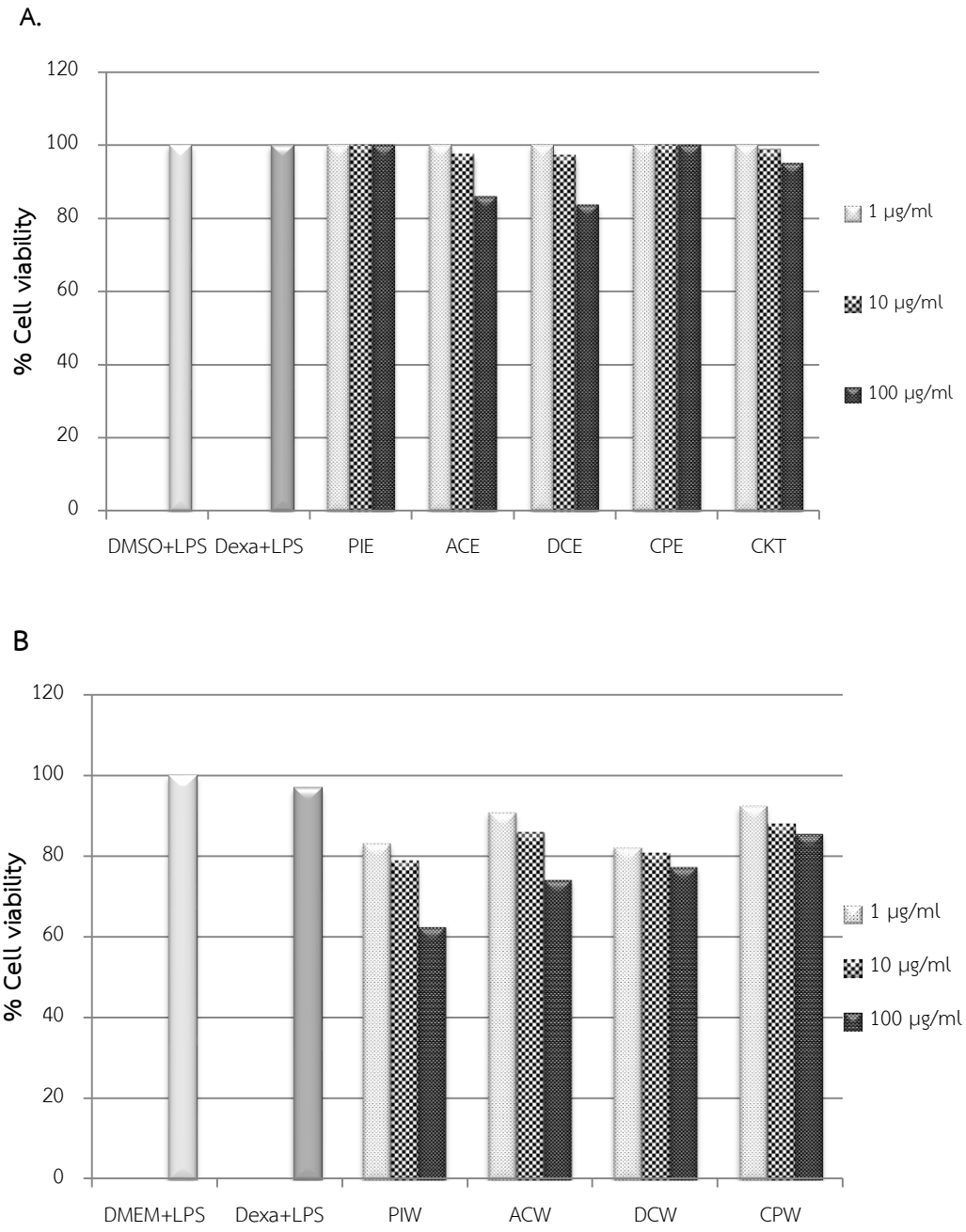


Figure 67 Cell viability of A. the ethanol root extracts and CKT remedy, B. fractionated water extracts; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy, E = ethanol extract, W= fractionated water extract, DEXA= dexamethasone

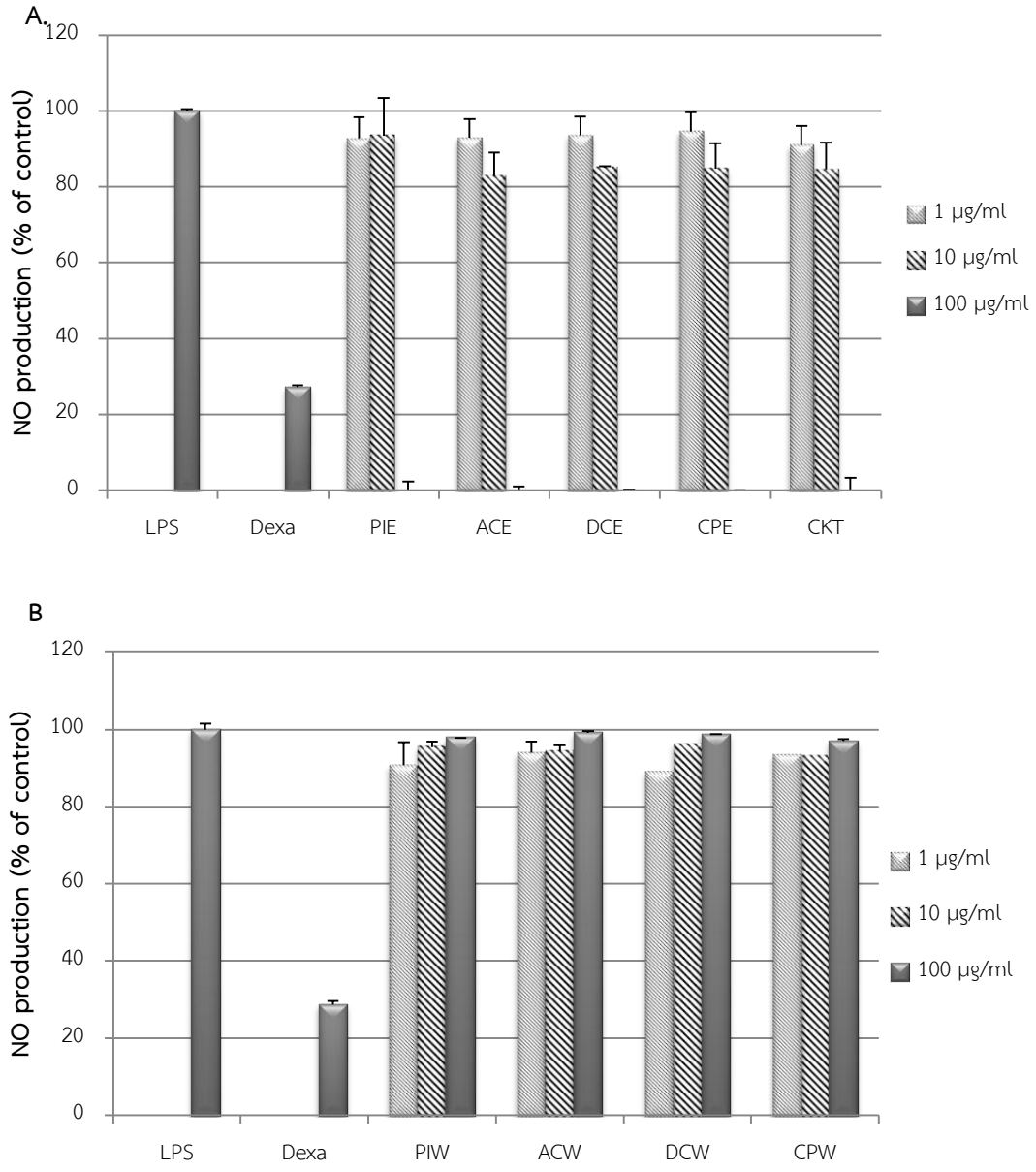


Figure 68 Effect of A CKT remedy and the ethanol extrats, B the fractionated water extracts on LPS-stimulated macrophage inhibition of nitric oxide; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy,E = ethanol extract, DEXA= dexamethasone, Results are presented as the mean ± S.D. (n=6).

Cell viability and NO inhibition assay

The cell toxicity of the ethanol extracts and CKT remedy were determined by resazurin assay. The results revealed that all extracts were non-toxic on J774A.1 cell viability with concentration ranging from 6.25-100 µg/ml (Figure 69). Figure 70 showed the effects of the ethanol root extracts and CKT remedy on NO production in LPS-stimulated macrophage cells. All extracts inhibited NO production in LPS-stimulated cells in a concentration dependent manner. The ethanol root extract of *P. indica*, *A. calamus* and CKT remedy showed the highest percentage inhibition of NO production (100%) at 100 µg/ml. The IC50 values for NO inhibition of *P. indica*, *A. calamus, D. serrulata, C. paniculatum* and CKT remedy were 60.51, 51.95, 59.09, 56.28 and 51.88 µg/ml, respectively.

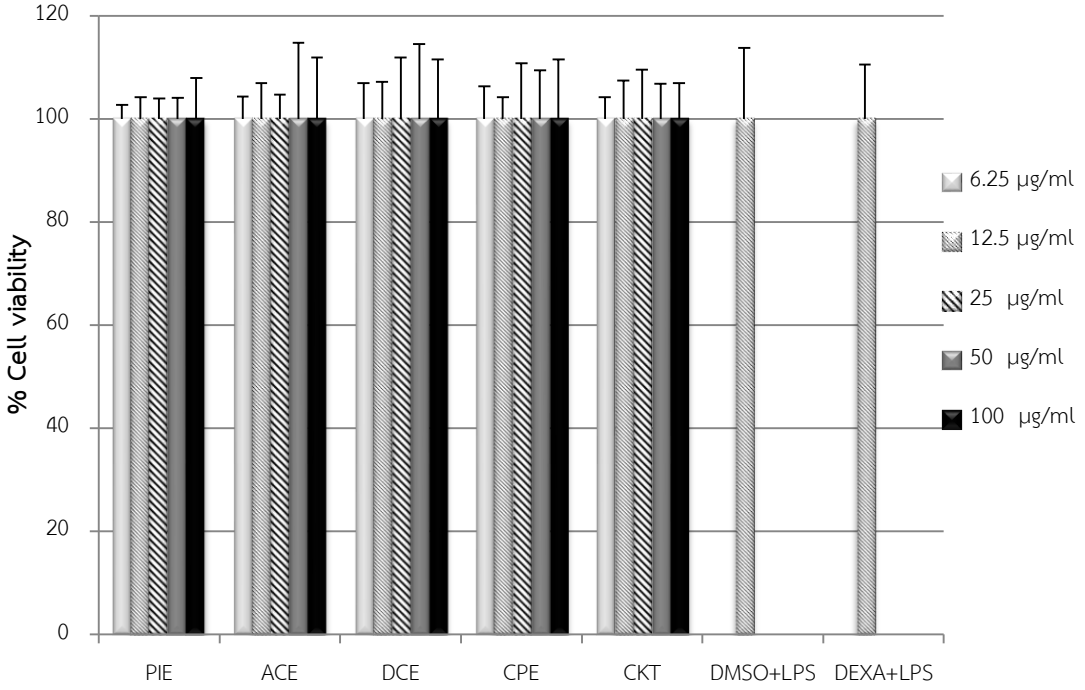


Figure 69 Cell viability of the ethanol root extracts, CKT remedy and control; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy, E = ethanol extract, DEXA= dexamethasone

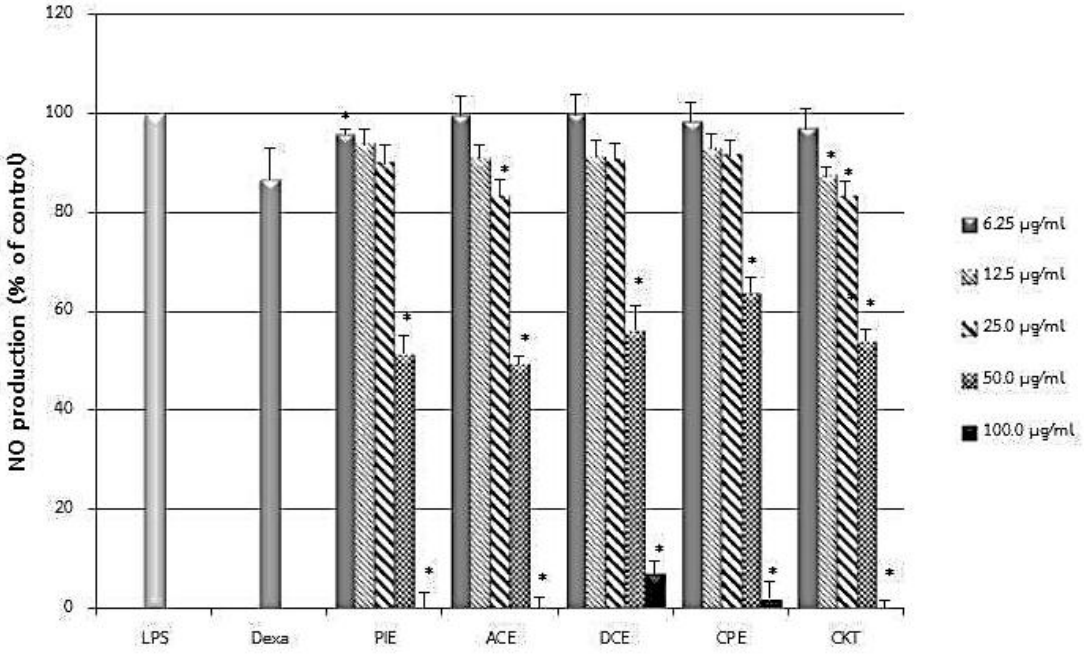


Figure 70 Effect of the ethanol root extracts and CKT remedy on LPS-stimulated macrophage inhibition of nitric oxide; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy, E = ethanol extract, DEXA= dexamethasone; results presented as the mean ± S.D. (n=6), * significant difference compared with the LPS alone ($P < 0.01$)

TNF-alpha inhibition assay

All extracts displayed inhibition of LPS (0.1 μg/ml) induced TNF-α level in J774A.1 macrophage cells in a dose-dependent manner (Figure 71). The ethanol extract of *P. indica* and CKT remedy showed a significant inhibition of TNF-α level at all concentrations. In addition, the significant inhibition of TNF-α level was demonstrated in the ethanol extract of *A. calamus* at 25, 50 and 100 μg/ml as well as the ethanol extracts of *D. serrulata* and *C. paniculatum* at 50 and 100 μg/ml. Maximum inhibition (78.65%) was observed at 100 μg/ml of CKT remedy. Dexamethasone as standard reference drug showed 57.53% inhibition of TNF-α level.

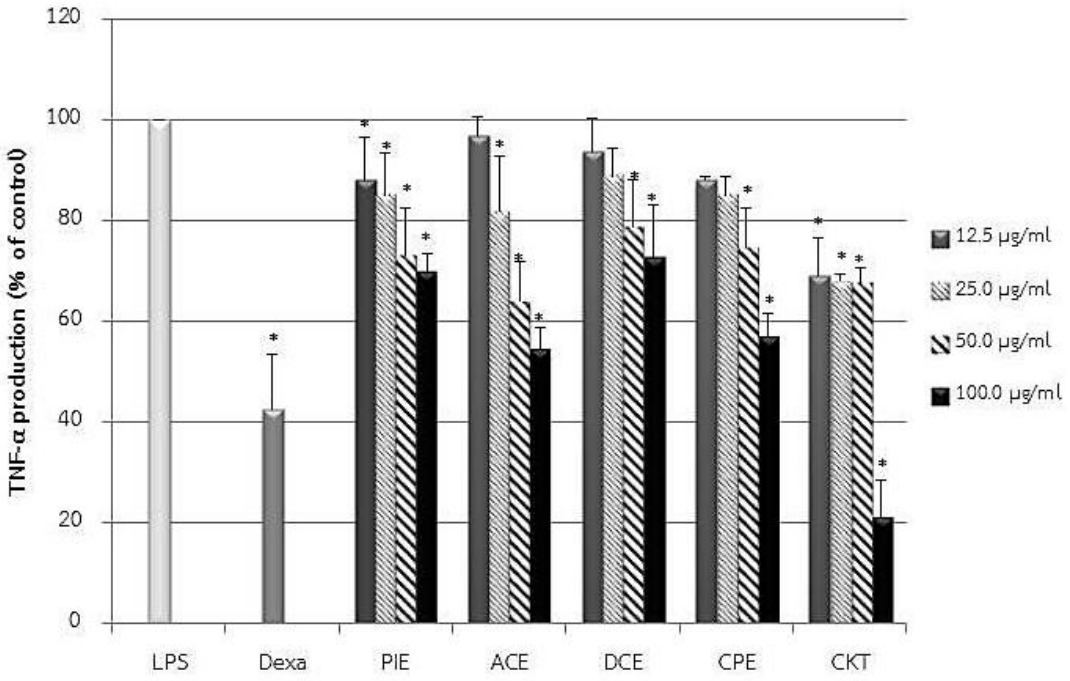


Figure 71 Effect of the ethanol root extracts and CKT remedy on LPS-stimulated macrophage inhibition of TNF-α production; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy, E = ethanol extract, DEXA= dexamethasone; results presented as the mean ± S.D. (n=6), * significant difference compared with the LPS alone ($P < 0.01$)

PGE_2 inhibition assay

The evaluation of inhibition of LPS (0.1 µg/ml) induced PGE_2 production in J774A.1 macrophage cells were demonstrated in figure 72. All extracts inhibited PGE_2 production in LPS-stimulated cells in a concentration dependent manner. At high concentration of CKT remedy showed significant inhibition of PGE_2 production which was 75.37%. The maximum inhibition (89.99%) was observed at 100 µg/ml of *P. indica* extract followed by *D. serrulata* and *C. paniculatum* with 87.81% and 80.38% at 100 µg/ml. The ethanol extract of *A. calamus* revealed less potential of inhibition of LPS induced PGE_2 production (34.44% at 100 µg/ml).

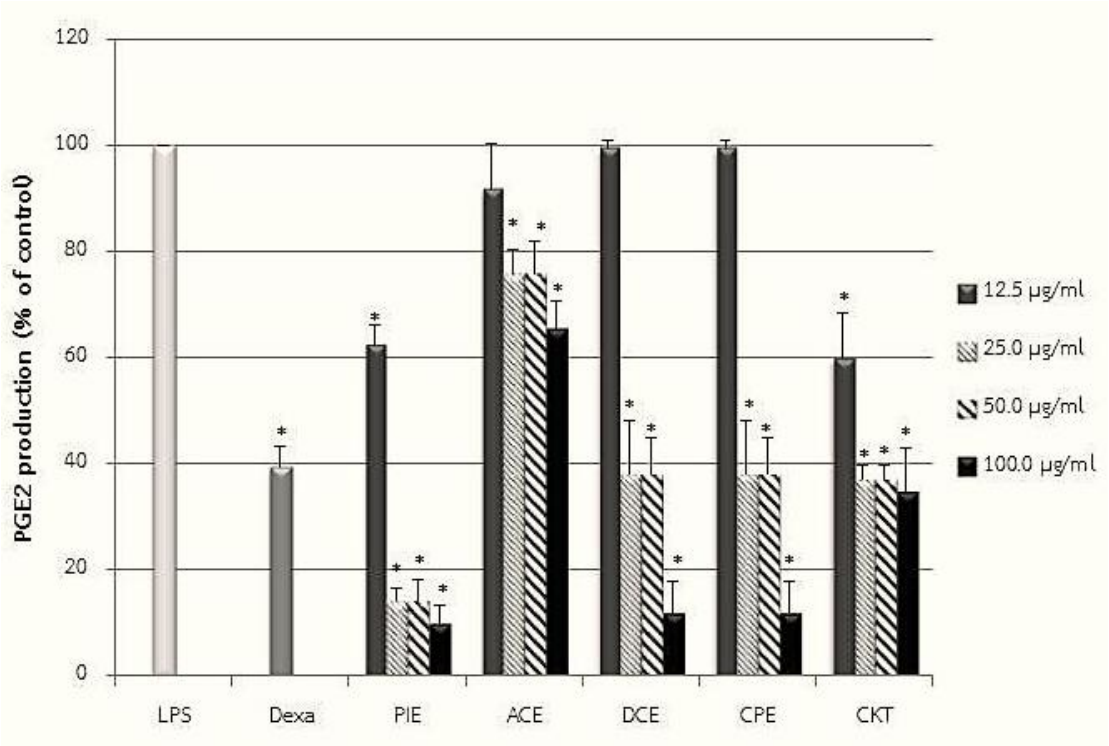


Figure 72 Effect of the ethanol root extracts and CKT remedy on LPS-stimulated macrophage inhibition of PGE_2 production; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy, E = ethanol extract, DEXA= dexamethasone; results presented as the mean ± S.D. (n=6), * significant difference compared with the LPS alone ($P < 0.01$)

Tree Phon Thad remedy

Carrageenan-induced paw edema

The carrageenan-induced mouse paw edema test was performed to examine the anti-inflammatory efficacy of TPT remedy and its ingredients extracts. Each mouse was administered orally with 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg), various doses of TPT (75, 150 and 300 mg/kg) or various doses of *Z. zerumbet*, *Z. montanum,* and *C. nardus* (25, 50, 100, 200, 400 mg/kg).

To demonstrate the validity of the carrageenan-induced mouse paw edema test, IND 10 mg/kg was used as a positive control. As expected, IND significantly decreased paw edema at 2, 3, 4, 5 and 6 h after carrageenan administration compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 95.77% at 6 h. TPT remedy extract at 300mg/kg showed significance of decrease paw edema of %55.95, %72.15, %86.67and %90.14at 3, 4, 5and 6h, respectively after carrageenan administration compared to that of 2% Tween 80. The percentage of inhibition of TPT at 300 mg/kg appears to be time-dependent during 3-6 h and produced a maximum inhibition of paw edema at 6 h (Figure 73).

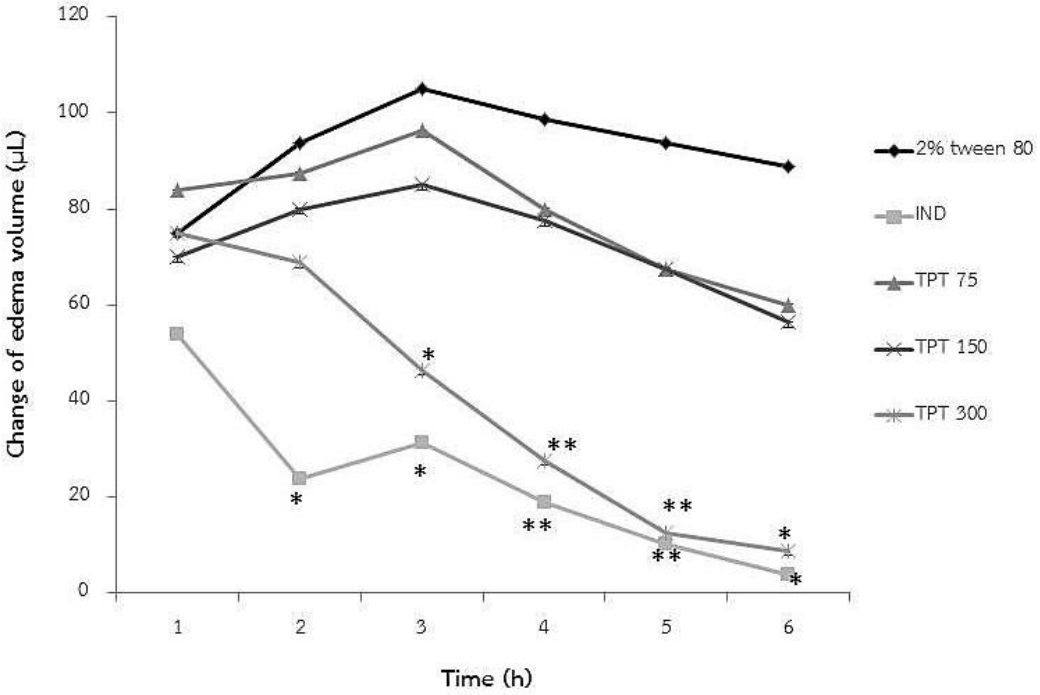


Figure 73 Change of edema volume of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the root extract of Tree Phon Thad remedy (TPT; 75-300 mg/kg) during 1-6 h after carrageenan administration; N=8 for all groups, *p<0.05, **p<0.01 significant difference compared to 2% Tween 80

Z. zerumbet extract at 25 mg/kg of demonstrated the significant inhibition of paw edema of 47.89%, 43.86% and 79.03% at 4, 5 and 6 h, respectively compared to the vehicle control and produced a maximum inhibition of paw edema at 6 h. At doses of 50 and 100 mg/kg, *Z. zerumbet* extract significantly (p<0.01) decreased paw edema at 6 h after carrageenan administration and produced an inhibition of paw edema of 66.13% and 61.29%, respectively.

Z. zerumbet extract at 200 mg/kg produced significant ($p<0.05$) decrease in paw edema of 40.28% and 49.30% at 2 and 4 h, respectively compared to 2% Tween 80. Paw edema induced by carrageenan seems to be unaffected by the highest dose of *Z. zerumbet*. Indomethacin at 10 mg/kg showed significant reduction of hind paw edema at 2, 3, 4, 5 and 6 h after carrageenan injection compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 77.42% at 6 h (Figure 74).

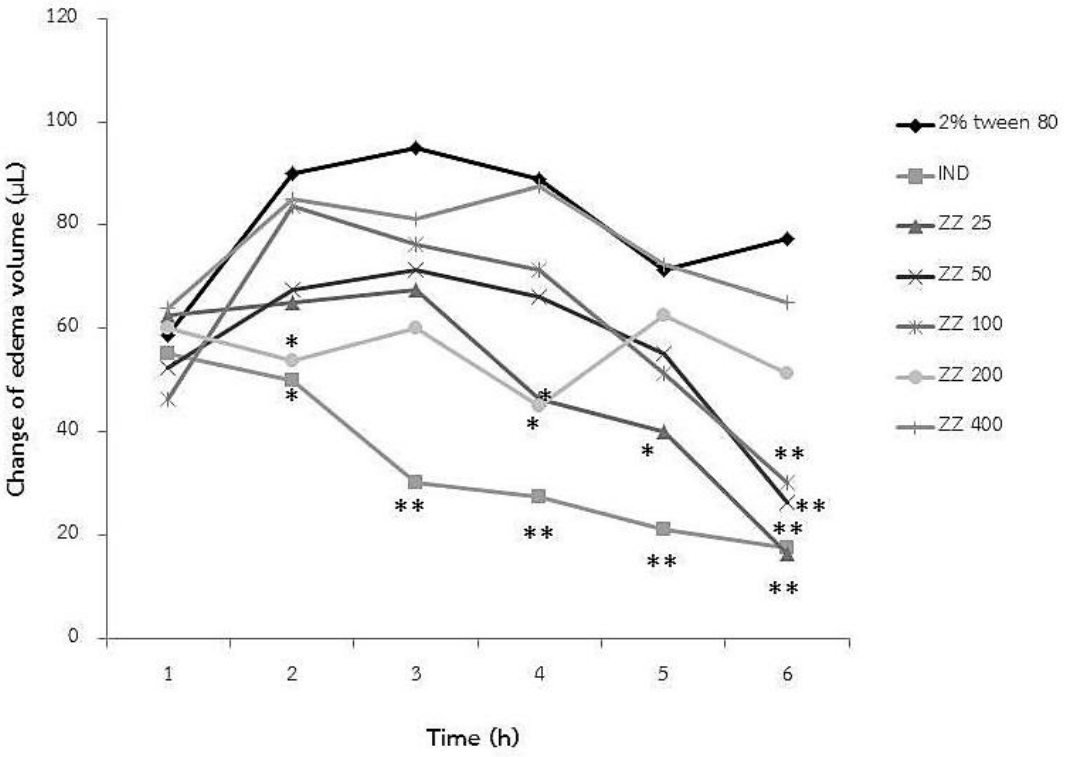


Figure 74 Change of edema volume of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the root extract of *Z. zerumbet* (ZZ; 25-400 mg/kg) during 1-6 h after carrageenan administration; N=8 for all groups, *$p<0.05$, **$p<0.01$ significant difference compared to 2% Tween 80

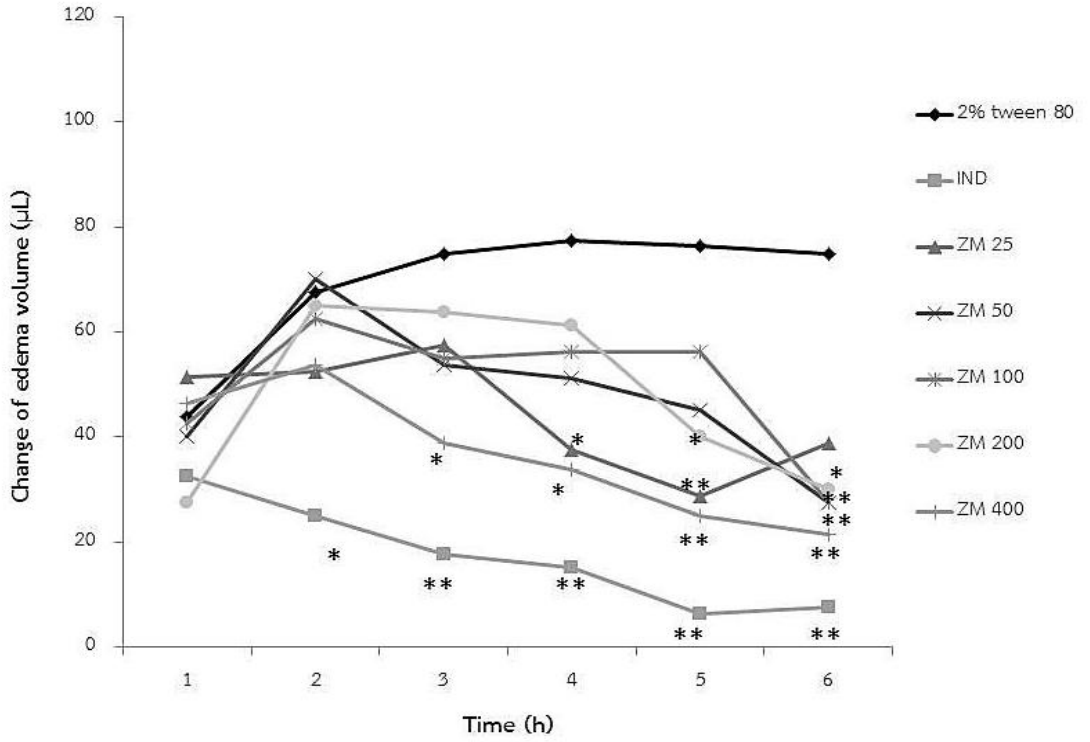


Figure 75 Change of edema volume of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the root extract of *Z. montanum* (ZM; 25-400 mg/kg) during 1-6 h after carrageenan administration; N=8 for all groups, *p<0.05, **p<0.01 significant difference compared to 2% Tween 80

Z. montanum at 25 mg/kg demonstrated a significant inhibition of paw edema of %51.61and %62.30at 4and 5h, respectively after carrageenan administration compared to the vehicle control. *Z. montanum* extract at doses of 50 and 100mg/kg significantly decreased paw edema with %63.33 inhibition at 6h. *Z. montanum* extract at 200mg/kg produced a significant inhibition of paw edema of %47.54and %60.00at 5and 6h, respectively compared to %2Tween .80*Z. montanum* extract at 400mg/kg significantly produced a time-dependent inhibition

of paw edema of %48.33, %56.45, %67.21and %71.67at 3, 4, 5and 6h, respectively. Indomethacin 10mg/kg significantly decreased paw edema at 2, 3, 4, 5 and 6h after carrageenan administration compared to %2Tween 80and showed a maximum inhibition of paw edema of %91.80 at 5h. Indomethacin appeared to have a superior inhibitory activity on carrageenan-induced paw edema compared to *Z. montanum* extract at all doses tested (Figure 75.(

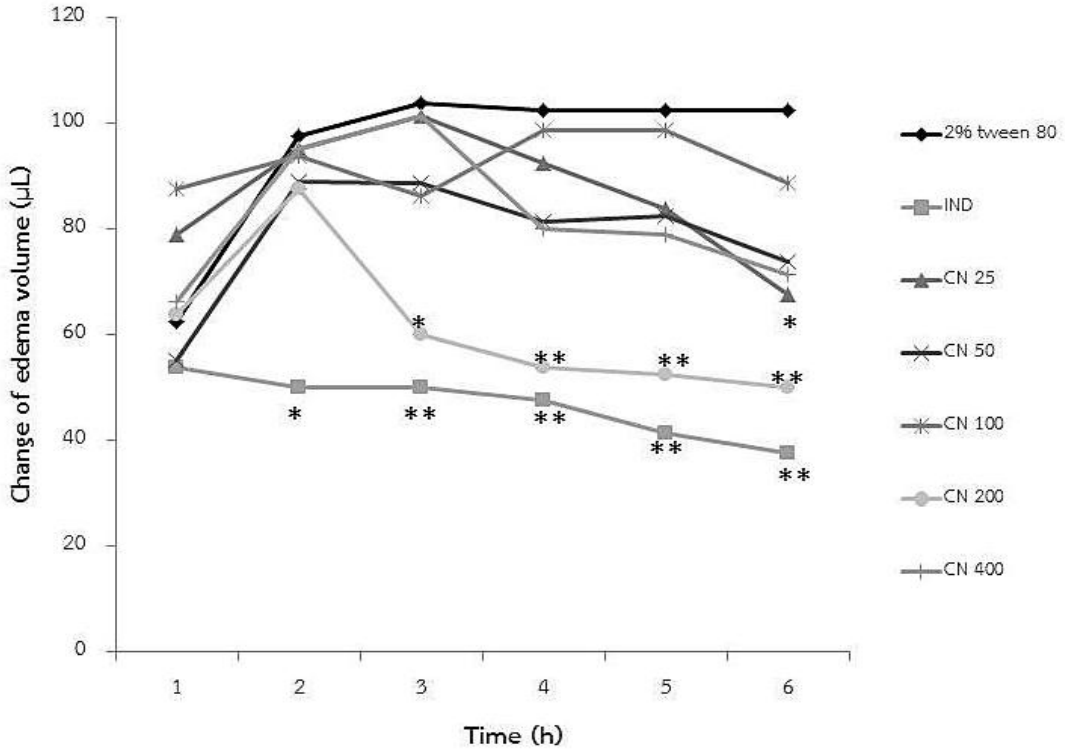


Figure 76 Change of edema volume of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND; 10 mg/kg) and various doses of the root extract of *C. nardus* (CN; 25-400 mg/kg) during 1-6 h after carrageenan administration; N=8 for all groups, *p<0.05, **p<0.01 significant difference compared to 2% Tween 80

C. nardus extract at 25 mg/kg significantly produced an inhibition of paw edema of 34.15% at 6 h after carrageenan administration compared to 2% Tween 80. At 200 mg/kg of *C. nardus* extract significantly produced the inhibition of paw edema of 42.17%, 47.56%, 48.78% and 51.22% at 3, 4, 5 and 6 h, respectively after carrageenan administration compared to 2% Tween 80. The highest dose of *C. nardus* seems to have no efficacy on paw edema induced by carrageenan (Figure 76).

Prostaglandin E2-induced mouse paw edema

The PGE-2induced paw edema test was employed to investigate the mechanism of the component of Tree Phon Thad remedy including *Z. zerumbet, Z. montanum* and *C. nardus* for inhibition inflammation. Mice were administered orally *Z. zerumbet* (25 mg/kg), *Z. montanum* (400mg/kg), *C. nardus* (200 mg/kg), 2% Tween 80 (10 mL/kg) or Indomethacin (10 mg/kg) 1 hour before injection of PGE2 into the plantar surface of the left hind paw.

To demonstrate the validity of the PGE2-induced paw edema test, indomethacin (a prostaglandin inhibitor) was used as a positive control. Indomethacin at 10 mg/kg significantly decreased paw edema at 0.5, 1, 1.5, 2 h after PGE2 administration compared to 2% Tween 80 ($p<0.05$) and showed a maximum inhibition of paw edema of 90.00% at 2 h. *Z. zerumbet* at 25 mg/kg significantly decreased paw edema at 1, 1.5 and 2 h after PGE2 administration compared to 2% Tween 80 ($p<0.05$) and showed a maximum inhibition of paw edema of 95.00% at 2 h. The anti-inflammatory effect of *Z. zerumbet* was comparable to IND at 1, 1.5 and 2 h after PGE2 administration (Figure 77).

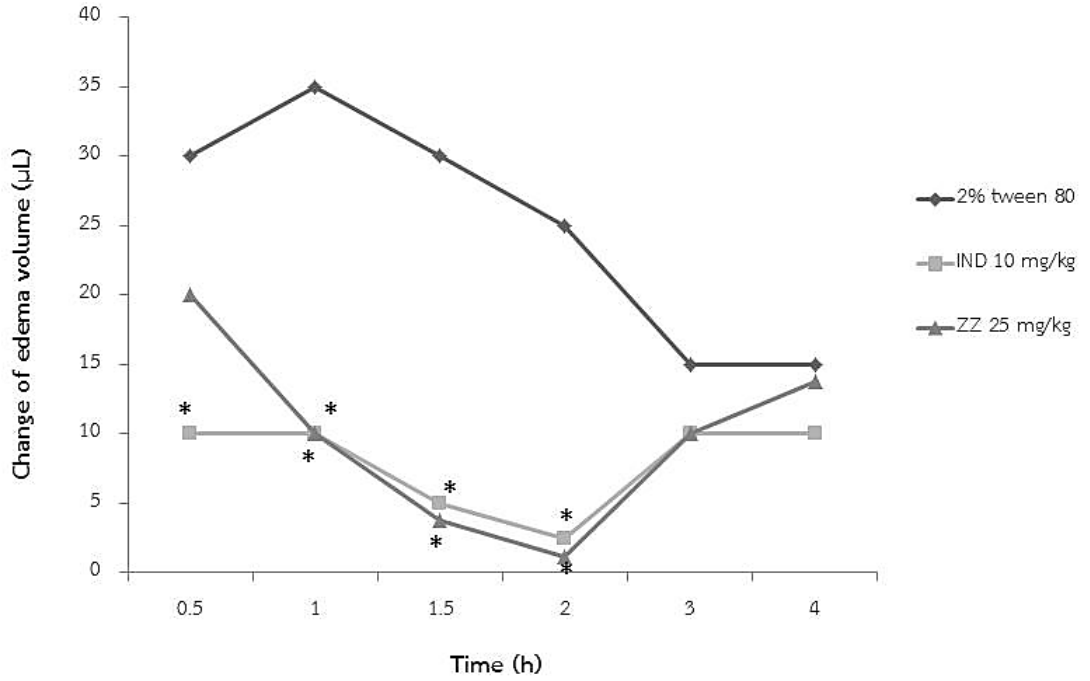


Figure 77 Change of edema volume (µL) of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND, 10 mg/kg) and *Z. zerumbet* root extract (ZZ, 25 mg/kg), during 0.5-4 h after prostaglandin E2 administration; N=8 for all groups, *p<0.05 significant difference compared to 2% Tween 80

Z. montanum rhizome extract at 400 mg/kg significantly decreased paw edema at 1 and 1.5 h after PGE2 administration compared to 2% Tween 80 (p<0.05) and showed a maximum inhibition of paw edema of 88.57% at 1.5 h. Indomethacin at 10 mg/kg significantly decreased paw edema at 0.5, 1, 1.5 and 2 h compared to 2% Tween 80 (p<0.05) and showed a maximum inhibition of paw edema of 93.75% at 2 h. The anti-inflammatory effect of ZM was comparable to indomethacin at 1 and1.5 h after PGE2 administration (Figure 78).

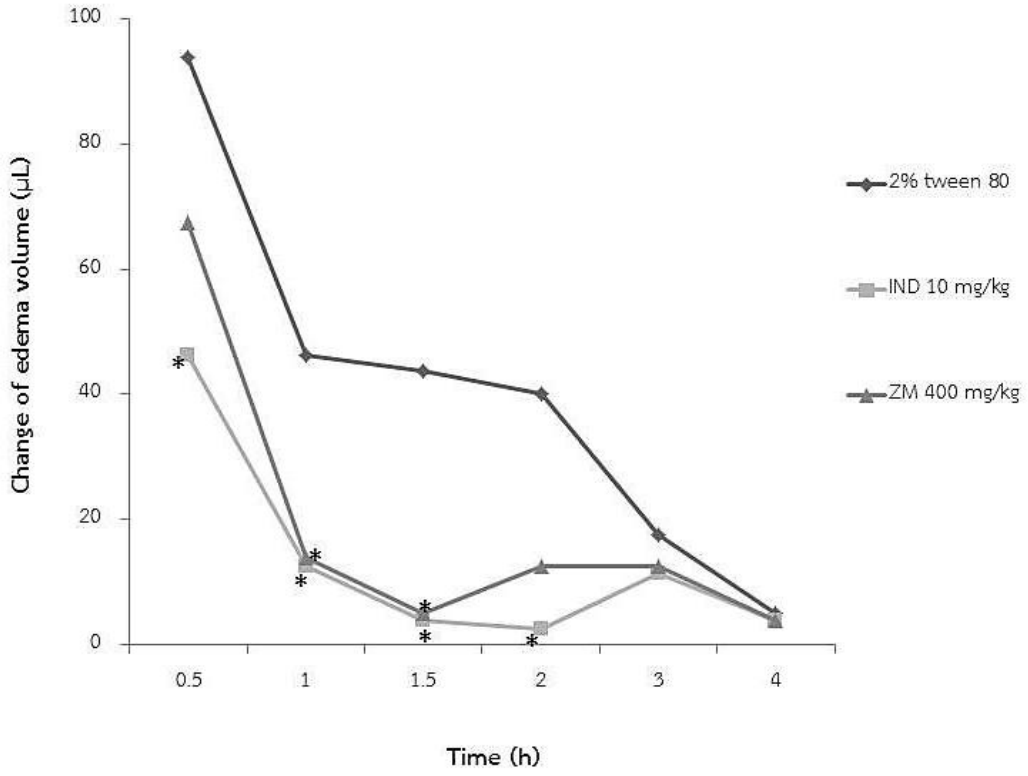


Figure 78 Change of edema volume (µL) of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND, 10 mg/kg) and *Z. montanum* rhizome extract (ZM, 25 mg/kg) during 0.5-4 h after prostaglandin E2 administration; N=8 for all groups, *p<0.05 significant difference compared to 2% Tween 80

C. nardus root extract at 200 mg/kg significantly decreased paw edema at 0.5, 1 and 1.5 h after PGE2 administration compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 86.11% at 1.5 h. Indomethacin at 10 mg/kg significantly decreased paw edema at 0.5, 1 and 1.5 h compared to 2% Tween 80 and showed a maximum inhibition of paw edema of 97.56% at 1 h. The anti-inflammatory effect of indomethacin appears to be higher than *C. nardus* at 1 and 1.5 h after PGE2 administration (Figure 79).

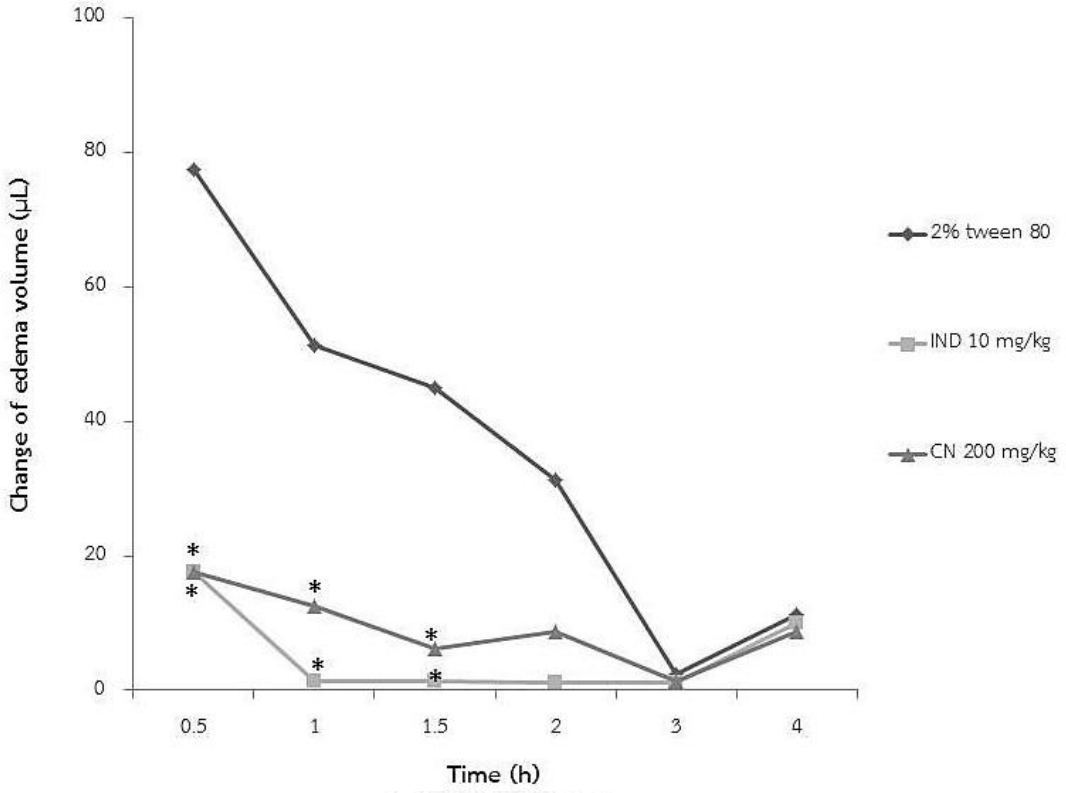


Figure 79 Change of edema volume (µL) of oral administration of 2% Tween 80 (10 mL/kg), indomethacin (IND, 10 mg/kg) and *C. nardus* root extract (CN, 200 mg/kg), during 0.5-4 h after prostaglandin E2 administration; N=8 for all groups, *p<0.05, significant difference compared to 2% Tween 80

Antioxidation activities

Cha Tu Ka La Thad remedy

There were five assays for investigation of the potential of antioxidant activities including total phenolic content, DPPH free radical scavenging activity, ferric ion reducing antioxidant power assay, metal chelating assay and nitric oxide free radical scavenging activity.

Total phenolic content

The extracts of each species and CKT remedy were evaluated for the quantification of total phenolic content by Folin-Ciocalteu reagent. The ethanol extract of *D. serrulata* showed the highest total phenolic content (30.93 μg CE/ 500 mg/ml of the extract), followed by the fractionated water extract of *P. indica* and CKT remedy (28.21 and 24.39 μg CE/ 500 mg/ml of the extract, respectively).

Table 17 Total phenolic content of CKT remedy and its ingredients

Plant name	Total phenolic content (µg CE/ 500 mg/ml)*	
	Ethanol extract	fractionated water extract
Plumbago indica	19.78	**28.21**
Acorus calamus	20.20	13.50
Clerodendrum paniculatum	17.16	3.87
Dolichandrone serrulata	**30.93**	6.64
Cha-Tu-Ka-La-Thad remedy	24.39	

*CE= catechin hydrate

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The results of DPPH radical scavenging activity of four species and CKT remedy extracts were presented in table 18. Quercetin and buthylated hydroxyl toluene (BHT) which were used as positive control in this study showed high radical scavenging activity with IC_{50} of 6.69 and 75.05 µg/ml, respectively. CKT remedy extract demonstrated the IC_{50} of 407.18 µg/ml. Radical scavenging activities of most of ethanol extracts were higher than the fractionated water extracts except the fractionated water extract of *P. indica* (IC_{50} of 426.83 µg/ml). The fractionated water extracts of *D. serrulata* and C. *paniculatum* exhibited weak free radical scavenging activity (IC_{50}>1,000(.

Table 18 DPPH scavenging activity (IC_{50}) of the extracts of CKT remedy and its ingredients

Species	Ethanol extract IC_{50} (µg/ml)	Fractionated water extract IC_{50} (µg/ml)
Plumbago indica	662.35	426.83
Acorus calamus	415.01	951.46
C*lerodendrum paniculatum*	**370.05**	>1,000
Dolichandrone serrulata	377.05	>1,000
Cha Tu Ka La Thad remedy	**407.18**	
Buthylated hydroxyl toluene (BHT)	**75.05**	
Quercetin	**6.69**	

Ferric ion reducing antioxidant power assay

The results of ferric ion reducing antioxidant power assay of four species and CKT remedy extract were presented in table 19. Quercetin and buthylated hydroxyl toluene (BHT) were used as positive control. The fractionated water extract of *P. indica* demonstrated highest reducing power ability with FRAP value of 1.477 mM Fe(ll)/mg dry weight, followed by CKT remedy extract with FRAP value of 0.967 mM Fe(ll)/mg dry weight. The fractionated water extract of *D. serrulata* had lowest reducing power ability with FRAP value of 0.156 mM Fe(ll)/mg dry weight.

Table 19 FRAP values of the extracts of CKT remedy and its ingredients.

Plant	**FRAP value (mM Fe)ll(/mg dry weight)**	
	Ethanol extract	**Fractionated water extract**
Plumbago indica	0.297	1.477
Acorus calamus	0.449	0.680
Clerodendrum paniculatum	0.656	0.211
Dolichandrone serrulata	0.548	0.156
Cha Tu Ka La Thad remedy	0.967	
Quercetin	1.455	
Buthylated Hydroxyl toluene)BHT(	1.287	

Metal chelating activity

The results of metal chelating activity of four species and CKT remedy extracts were presented in table 20. The fractionated water extracts were more efficient in metal ion chelating capacity compared to ethanol extracts. The fractionated water extract of *P. indica* showed highest metal chelating activity with EC_{50} of 64.26 µg/ml, followed by the fractionated water extract of *A. calamus* with EC_{50} of 85.37 µg/ml. The standard EDTA was used as positive control in metal ion chelating capacity with EC_{50} of 53.04 µg/ml.

Table 20 Metal chelating activity (EC_{50}) of CKT remedy and its ingredients

Species	Ethanol extract EC_{50} (µg/ml)	Fractionated water extract EC_{50} (µg/ml)
Plumbago indica	› 1,000	64.26
Acorus calamus	› 1,000	85.37
Clerodendrum paniculatum	› 1,000	198.20
Dolichandrone serrulata	› 1,000	147.59
Cha Tu Ka La Thad remedy	**825.58**	
EDTA	**53.04**	

Nitric oxide free radical scavenging activity

The results of nitric oxide free radical scavenging activity of four species and CKT remedy extracts were presented in table 21. The ethanol extract of *A. calamus* expressed the high potential on nitric oxide scavenging activity with IC_{50} of 375 µg/ml**,** followed by CKT remedy with IC_{50} of 666 µg/ml while ascorbic acid as positive control exhibited the IC_{50} of 482 µg/ml. The fractionated water extract of *C. paniculatum* and *D. serrulata* showed the lowest potential on nitric oxide scavenging activity with IC_{50} › 5,000 µg/ml.

Table 21 Nitric oxide free radical scavenging activity (IC_{50}) of CKT remedy and its ingredients

Species	**Ethanol extract IC_{50} (µg/ml)**	**Fractionated water extract IC_{50} (µg/ml)**
Plumbago indica	1,298	1,278
Acorus calamus	375	4,896
Clerodendrum paniculatum	2,567	› 5,000
Dolichandrone serrulata	2,813	› 5,000
Cha Tu Ka La Thad remedy	**666**	
Ascorbic acid	**482**	

Tree Phon Thad remedy

Total phenolic content

The extracts of each species and TPT remedy were evaluated for quantification of total phenolic content by Folin-Ciocalteu reagent. The ethanol extract of *Z. zerumbet* showed highest total phenolic content (28.31 µg CE hydrate/ 500 mg/ml of the extract), followed by TPT remedy extract and the ethanol extract of *Z. montanum* (27.61 and 24.86 µg CE hydrate/ 500 mg/ml of the extract, respectively).

Table 22 Total phenolic content of TPT remedy and its ingredients

Plant name	Total phenolic content (µg CE/ 500 mg/ml)*	
	Ethanol extract	Fractionated water extract
Zingiber zerumbet	**28.31**	4.37
Zingiber montanum	24.86	8.45
Cymbopogon nardus	20.62	11.67
Tree Phon Thad remedy	**27.61**	

*CE= catechin hydrate

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The results of DPPH radical scavenging activity of the extract of three species and TPT remedies were presented in table 23. Quercetin and buthylated hydroxyl toluene (BHT) which were used as positive control in this study that showed high radical scavenging activity with IC_{50} of 6.69 and 75.05 µg/ml, respectively. Radical scavenging activity of most of ethanol extracts were higher than the fractionated water extracts. The ethanol extract of *Z. montanum* exhibited high free radical scavenging activity with 367.23 µg/ml, followed by TPT remedy extract and the ethanol of *C. nardus* with IC_{50} 533.66 and 610.35, respectively. The fractionated water extracts of *Z. zerumbet* and *C. nardus* exhibited weak free radical scavenging activity (IC_{50}>1,000(.

Table 23 DPPH scavenging activity (IC_{50}) of the extracts of TPT remedy and its ingredients

Species	Ethanol extract IC_{50} (µg/ml)	Fractionated water extract IC_{50} (µg/ml)
Zingiber zerumbet	718.42	>1,000
Zingiber montanum	367.23	763.17
Cymbopogon nardus	610.35	>1,000
Tree Phon Thad remedy	533.66	
Buthylated hydroxyl toluene (BHT)	**75.05**	
Quercetin	**6.69**	

Ferric ion reducing antioxidant power assay

The results of ferric ion reducing antioxidant power assay of three species and TPT remedy extract were presented in table 24. Quercetin and buthylated hydroxyl toluene (BHT) were used as positive control. The ethanol extract of *Z. montanum* showed reducing power ability with FRAP value of 0.662 mM Fe(ll)/mg dry weight, followed by the fractionated water extract of *Z. montanum* and TPT remedy extract showed reducing power ability with FRAP value of 0.612 and 0.530 mM Fe(ll)/mg dry weight, respectively.

Table 24 FRAP values of the extracts of each species of TPT remedy and its ingredients

Plant	**FRAP value (mM Fe)ll(/mg dry weight)**	
	Ethanol extract	**Fractionated water extract**
Zingiber zerumbet	0.174	0.140
Zingiber montanum	0.662	0.612
Cymbopogon nardus	0.352	0.296
Tree Phon Thad remedy	0.530	
Quercetin	1.455	
Buthylated Hydroxyl toluene	1.287	

Metal chelating activity

The results of metal chelating activity of three species and TPT remedy extracts were presented in table 25. The fractionated water extracts of *Z. zerumbet* showed highest metal chelating activity with EC_{50} of 157.15 µg/ml compared to all extracts, followed by the fractionated water extract of *C. nardus* demonstrated that the metal chelating activity with EC_{50} of 358.22 µg/ml. The standard EDTA was used as positive control in metal ion chelating capacity with EC_{50} of 53.04 µg/ml.

Table 25 Metal chelating activity (EC_{50}) of TPT remedy and its ingredients

Species	Ethanol extract EC_{50} (µg/ml)	Fractionated water extract EC_{50} (µg/ml)
Zingiber zerumbet	608.49	157.15
Zingiber montanum	657.84	› 1,000
Cymbopogon nardus	› 1,000	348.22
Tree Phon Thad remedy	798.01	
EDTA	**53.04**	

Nitric oxide free radical scavenging activity

The results of nitric oxide free radical scavenging activity of three species and TPT remedy extracts were presented in table 26. The ethanol extract of *Z. montanum* expressed the high potential on nitric oxide scavenging activity with IC_{50} of 533 µg/ml**,** followed by *Z. zerumbet* and CKT remedy with IC_{50} of 1,501 and 1,650 µg/ml. Ascorbic acid as positive control exhibited the IC_{50} of 482 µg/ml. The fractionated water extract of *Z. montanum* and *C. nardus* showed the lowest potential on nitric oxide scavenging activity with IC_{50} › 5,000 µg/ml.

Table 26 Nitric oxide free radical scavenging activity (IC_{50}) of TPT remedy and its ingredients

Species	**Ethanol extract IC_{50} (µg/ml)**	**Fractionated water extract IC_{50} (µg/ml)**
Zingiber zerumbet	1,501	3,802
Zingiber montanum	533	› 5,000
Cymbopogon nardus	4,429	› 5,000
Tree Phon Thad remedy	1,650	
Ascorbic acid	**482**	

CHAPTER V
DISCUSSION

Pharmacognostic specification

Cha Tu Ka La Thad (CKT) remedy is composed of the roots of *Plumbago indica*, *Acorus calamus*, *Clerodendrum paniculatum* and *Dolichandrone serrulata,* each in equal part by weight. CKT has been used as an antipyretic and anti-inflammatory drug in traditional Thai medicine. Its ingredients is commonly used in the remedies in The List of Herbal Medicine Products of the National List of Essential Drugs A.D. 2006 for example, ประสะกานพลู, ประสะไพล, ธรณีสัณฑฆาต, ยาหอมเนาวโกฐ, บำรุงโลหิต and ไฟห้ากอง.

Pharmacognostic specification plays an important tool for herbal drugs in order to establish the identity, purity, safety and quality. The majority of the information can be obtained from its macroscopy, microscopy, physio-chemical parameters and TLC fingerprint. Macroscopic and microscopic examinations are simple and cheap method for start with establishing the correct authentication of herbal drugs [157]. The macroscopic evaluation of four root species revealed their shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface. Although each species of CKT remedy can easily be distinguished on the basis of whole plant morphology as shown in figure 15, 20, 25 and 30, but it becomes very difficult when the raw materials are in dried crude drugs and in

powdered form. In this study, the result obtained in anatomical and histological characters of four species were a good tool to authenticate the crude drugs. The anatomy of four roots was studied by taking a transverse section. The transverse section of *P. indica* root showed a periderm, cortex, secondary phloem, vascular cambium, second xylem, rays and pith that were in agreement with earlier reported by Ragunathan and Senthamarai for periderm, cork, secondary phloem, secondary xylem, xylem ray, vessel, central empty pith canal and pith cavity [158]. Microscopy of the powder of *P. indica* root showed epidermal cells containing brownish resin, parenchyma, xylem, reticulate vessel , sclerenchymatous sclerids, prism crytals of calcium oxalate, starch grains and fragment of fiber as reported by the previous study in Thailand [159]. The microscopic character of *A. calamus* rhizome was in accordance with the report by the faculty of pharmacy, Mahidol University describing of epidermis, collenchyma, cortical fibers, calcium oxalate crystals, oil glands, vascular bundles and starch granules [160]. The possible identifying characters of anatomical features of root of *D. serrulata* demonstrated that endodermis, xylem vessel, xylem ray, xylem fiber, cortical parenchyma and group of cortical fibers. The microscopic study of *C. paniculatum* root showed epidermis, parenchyma of cortex, sclereid cell, xylem ray, xylem parenchyma, xylem vessel.

The physico-chemical evaluation (including total ash, loss on drying, water content, volatile oil content and extractive values parameters) of crude drug is useful for setting standards of the crude drug and judging the purity and quality of the

crude drug [4]. The determination of ash is useful for detecting inorganic materials such as metallic salts, silica, carbonates, calcium oxalate crystals, sandy and earth matter in crude drug [161]. In addition, a high ash value indicates that contamination, adulteration, substitution and carelessness in preparing the crude drug for marketing.

The determination of water content and loss on drying need to be investigated because the moisture is also an inevitable component of crude drugs which should be eliminated as far as practicable. The loss on drying test is designed to measure the amount of water and volatile matters in a sample when the sample is dried under specified conditions. Excess moisture can result in the breakdown of important constituents by enzymatic activity and may encourage the growth of yeast and fungi during storage. Extractive values indicate the chemical constituents of crude drug soluble in a particular solvent which yields a solution containing different phyto-constituents [162].

The physico-chemical parameters of *P. indica* root showed that loss on drying, total ash, acid-insoluble ash, ethanol-soluble extractive, water-soluble extractive, and water content values were 9, 9, 3, 7, 18 and 11 % of dry weight respectively. The results of loss on drying, total ash and acid-insoluble ash values were less than previous report in 1994 that were not more than 9, 12 and 5 % of dry weight respectively [163]. In 2013, the study in India [158] revealed that the ethanol-soluble extractive and water-soluble extractive values were not less than 5 and 11 % of dry weight respectively which were lower than in this study. It indicated that this

plant contained higher content of water soluble chemical compounds. For *A. calamus*, the total ash and acid insoluble ash were less than previously reported in India which specified the limit at not more than 7 % for total ash and not more than 0.9 % for acid insoluble ash [164]. Ethanol-soluble extractive and water-soluble extractive values specified the limit at not less than 16 and 23 % of dry weight respectively [164]. The physico-chemical parameters of *C. paniculatum* and *D. serulata* indicated that the loss on drying, total ash, acid-insoluble ash, ethanol-soluble extractive, water-soluble extractive, and water content values were 6, 6, 2, 3, 8, 8 and 8, 3, 0.8, 5, 10, 12 % of dry weight respectively. The results indicated that both plants contained higher content of polar compounds.

The fingerprinting analysis is a useful technique for the quality control for separation and authentication of chemical constituents of herbal drug. Thin layer chromatographic fingerprint of ethanol extract of *P. indica*, *A. calamus*, *C. paniculatum* and *D. serrulata* were performed using hexane and ethyl acetate (8 : 2), toluene and ethyl acetate (9 : 1), chloroform and methanol (9 : 1) and Hexane : Diethyl ether (4 : 6), respectively as mobile phase. The bands were clearly detected under ultraviolet light (254 nm and 365 nm) and stain reagent.

Tree Phon Thad remedy (TPT) is used for antipyretic and anti-inflammatory drug in traditional Thai medicine. This remedy is composed of *Zingiber zerumbet*, *Zingiber montanum* and *Cymbopogon nardus,* each in equal part by weight. TPT is also a component of the remedy appointed to be in The List of Herbal Medicine

Products of the National List of Essential Drugs A.D. 2006 (ยาไฟประลัยกัลป์). In this study, the result obtained in macroscopy, microscopy, physio-chemical parameters and TLC fingerprint for setting the standard parameters for authentication and quality control the crude drugs. The transverse section of the rhizome of *Z. zerumbet* showed the epidermis, hypodermis, cork cell, cortical parenchyma containing starch grain, vascular bundle, mass of oleoresin, oil droplet, endodermis and stele parenchyma containing starch grain that were in agreement with the earlier report by Rout *et al* in 2011 which reported of cork cell, cortex, epidermis, air canal, oil cell, parenchyma containing starch grains, vessel and pith [165]. The rhizome powder of *Z. zerumbet* showed the presence of scalariform vessel, reticulate vessel, fragment of fibers, parenchyma in transverse view, masses of oleoresin and starch grains fiber as stated in the previous study [165]. However, the co-oxalate crystals and unicellular trichomes were not found in this study. The microscopic character of *C. nardus* root demonstrated epidermis, parenchyma, oil gland, phloem, xylem vessel, endodermis and pith. The anatomical characters of rhizome of *Z. montanum* showed cork, cork cambium, parenchyma containing starch granules, vascular bundle, pseudoendodermis and parenchyma containing oleoresin.

The previous investigation of physico-chemical parameters of *Z. zerumbet* in India [165] reported low total ash and acid insoluble ash. Additionally, the ethanol and water extractive values represented higher content of chemical constituents than in this study. This may be due to the difference of geography and number of

sample size. The physico-chemical specifications of *C. nardus* reported that total ash, acid-insoluble ash, loss on drying and water content should not be more than 6, 4, 8 and 8 % of dry weight respectively; while volatile oils, ethanol and water soluble extractive values should not be less than 3, 5 and 8 % of dry weight respectively. Previous study on the physico-chemical specifications of *C. nardus* leaves showed that total ash, acid insoluble ash and moisture content should not be more than 6, 2 and 3, respectively. The volatile oil, ethanol and water soluble extractive values should not be less than 0.1, 6 and 8 %, respectively [166]. The roots were found to contain more acid insoluble ash, moisture and volatile oil than the leaves. The physico-chemical parameters of *Z. montanum* indicated that the loss on drying, total ash, acid-insoluble ash, ethanol-soluble extractive, water-soluble extractive, and water content values were 10, 7, 2, 7, 10 and 10 % of dry weight respectively.

Thin layer chromatographic fingerprint of ethanol extracts of *Z. zerumbet*, *C. nardus* and *Z. montanum* were performed using toluene: ethyl acetate (7.5: 2.5), hexane: diethyl ether (4: 6) and chloroform, respectively as mobile phase. The bands were clearly detected under ultraviolet light (254 nm and 365 nm) and stain reagent.

GC/MS analysis

In this study, the volatile oils of *Z. zerumbet*, *C. nardus, Z. montanum,* and *A. calamus* were analyzed by GC/MS. Gas chromatography coupled with mass

spectrometry (GC-MS) is the most common analytical method for chemical characteristics of essential oil. The major components of volatile oil from dried roots of *Z. zerumbet* were zerumbone (51.67 ± 10.23 %) followed by alpha-caryophyllene (14.13 ± 8.05%) and humulene epoxide II (10.85 ± 4.47%). The investigated revealed that the major components were in agreement with previous studies [167, 168]. For example, the major components of volatile oil from rhizome of *Z. zerumbet* in Bangladesh was zerumbone (46.83%), alpha-caryophyllene (19.00%) and humulene epoxide II (4.28%) [168] while the major components of volatile oil from rhizome of *Z. zerumbet* in India was zerumbone (75.2%), alpha-caryophyllene (7.1%), camphene (5.1%), eucalyptol (2.4%), and camphor (3.0%) [167].

Zerumbone and alpha-caryophyllene were the major common components in rhizomes oil of *Z. zerumbet*. Zerumbone which is a sesquiterpene occurring in zingiberaceous plants [169], showed the potential of an anticancer, anti-inflammatory, antinociceptive and antimalarial activities in previous study [169-172]. Alpha-caryophyllene or alpha-humulene is a naturally occurring monocyclic sesquiterpene that is a constituent of many essential oils. There were several previous studies reported the biological activities of alpha-caryophyllene. Alpha-caryophyllene derived from the essential oil of *C. verbenacea* largely prevented both tumor necrosis factor-alpha (TNF-alpha) and interleukin-1beta (IL-1beta) generation and reduced the production of prostaglandin E_2 (PGE_2), as well as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2) expression in

carrageenan-injected rats [173]. Moreover, the alpha-caryophyllene extracted from *Salvia officinalis* essential oil inhibited tumor cell growth by MTT assay [174].

Previous studies of *C. nardus* dried leaf oils indicated monoterpenes as the major components which in Thailand were geraniol (35.7%), *trans*-citral (22.7%) and *cis*-citral (14.2%) [175]; in Togo were citronellal (35.5%), geraniol (27.9%) and citronellol (10.7%) [104] while in Malaysia were citronellal (29.06%) [100]. The major components of volatile oil from dried roots of *C. nardus* were revealed in this study as sesquiterpenes which were elemol (22.87%) and eudesmols (alpha 16.09%, gamma 11.72% and beta 11.33%). Various biological activities of plant extracts consisted of elemol and alpha-eudesmol were reported. The essential oil of *Schinus molle*, which elemol was one of the main compounds, had biological activity as repellent and had to be applied as fumigant for controlling *Sitophilus oriza* [176]. The essential oil of *Nepeta septemcrenata* which consisted of 13.8% elemol acted as antimicrobial against *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* [177]. Moreover, elemol was identified as an active principle responsible for the larvicidal activity of *C. nardus* fractional distillated oil [178]. Alpha-eudesmol was shown for high voltage-gated calcium channel blocker activity which possibly affected on anti-migrain treatment [179]. Furthermore, alpha-eudesmol displayed an ability to attenuate post-ischemic brain injury in rats [180].

Twenty components were identified as constituents of the essential oils of *Z. montanum* rhizomes from 15 different sources summarized in Table 10 and

Figures 50. The major components of volatile oil from rhizome of *Z. montanum* were monoterpenes with terpinen-4-ol (36.48±11.02 %) followed by sabinene (32.14±8.54 %). The major components of the rhizome oil in Thailand were thus found to be similar to that of India and Indonesia previouly reported [83, 96]. The report by Sukatta *et al.* (2009) in Thailand [181] revealed that the main constituents of the essential oil from fresh rhizome were sabinene (36.71- 53.50%) and terpinen-4-ol (21.85-29.96%). However, there were only three samples for analysis in previous study [181]. As terpinen-4-ol is widely employed in the perfumery industry [83], it has potential of antimicrobial antifungal and anti-inflammatory activities [182-185]. The essential oil of *Myristica fragrans* which consisted of 41.7% of sabinene, acted as antimicrobial against *Botrytis cinerea, Staphylococcus aureus 3476, Penicillium frequentance, Sarcina lutea 6589, Aspergillus niger* and *Bacillus subtilis 763* [186]. Furthermore, sabinene derived from the essential oils from Hallabong flowers (*Citrus unshiu, Citrus sinensis, Citrus reticulata*) which was the remedy in South Korean, inhibited the production of the inflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β[187] .

The major chemical constituent of the calamus oil from dried rhizome crude drugs in Thailand were revealed as sesquiterpene with 67.5 ± 8.1 % β-asarone followed by 22.4 ± 7.9 % α-asarone (Figure 53 and Table 11). The previous study reported the content of β-asarone in calamus oil from fresh rhizomes was higher than in this study. Raina *et al.* in 2003 [188] and Wangsittidet *et al*. in 2007 [189]

demonstrated 82.9% and 83.2% of ß-asarone in calamus oil from fresh rhizomes. The minimum and maximum percent content of ß-asarone in calamus oil obtained from 15 rhizome sources were 52.66 % and 78.96% respectively. This high content of ß-asarone confirmed the tetraploid cytotype of *A. calamus* in Thailand [190]. Nevertheless, there were several reports of the different main constituents in calamus oil from dried rhizome. Lui *et al* in 2013 [191] reported that the principal compounds in the essential oil from *A. calamus* rhizomes were alpha-asarone ((%50.09, (E)-methylisoeugenol ((%14.01, and methyleugenol ((%8.59, followed by ß-asarone ((%3.51, alpha-cedrene ((%3.09and camphor ((%2.42 and Venskutonis *et al* in 2003 [192] demonstrated that major constituents of the oil were acorenone (20.86%) followed by isocalamendiol (12.75%). It may be caused by external factors such as growing habitat, geographical condition and processing method. beta-Asarone and alpha-asarone are highly active in antifungal, antimicrobial, anticancer, protective cerebral ischemia and antidiabetes activities [193]. ß-Asarone compound of *A. calamus* had the highest inhibitory effect against *Escherichia coli* strain at various concentration [194]. ß-Asarone and alpha-asarone isolated from *A. calamus* rhizomes has been found to possess the antifungal activity against *Penicillium chrysogenum, Aspergillus niger, Aspergillus flavus, Microsporum canis* and the yeast strain of *Candida albicans, Cryptococcus neoformans*, *Cryptococcus gastricus, Saccharomyces cerevisae* and also against *A. Niger* [195, 196]. ß-Asarone and alpha-Asarone have been reported the anticancerogenic activity against the human

carcinoma cells [197]. The administration of ß-asarone could reduce focal cerebral ischemic/reperfusion injury in rat model [198].

Toxicity study

Brine shrimp (*A. salina*) lethality assay is a preliminary toxicity screening for further experiments on mammalian animal models. It has been used as an indicator for general toxicity of heavy metals, pesticides, medicines especially bioactive natural products [199, 200]. It is rapid, reliable, inexpensive and convenient as an in-house general bioassay tool [134]. The result in brine shrimp lethality assay is determined using LC_{50} value that is good correlation with the *in vivo* test. Perra *et al.* in 2001 reported that *A. salina* was a useful method to predict oral acute toxicity of mice model in plant extracts [201]. For the results, CKT remedy extract was revealed that toxicity to *A. salina,* and the toxicity may be affected from its ingredients, i.e. *A. calamus* and *C. paniculatum*. These results were in accordance with the study of Padmaja *et al.* [202] and Khan and Islam [203] which also found that the ethanol extract of rhizome of *A. calamus* showed cytotoxic activity to *A. salina* with LC_{50} 50 and 217 µg/ml. In contrary with Arunachalam *et al.* [204] who has reported the different results in animal model that the hydro alcoholic extract of rhizome of *A. calamus* showed non-toxic at acute and sub-acute toxicity in mice. Furthermore, the ethanol extract of *A. calamus* did not appear to have toxicity on acute and chronic administration in Wistar rats by Payal *et al.* [205]. The report by

Praveen *et al.* [61] showed a contrast result that the methanol extracts of the leaves of *C. paniculatum* exhibited non-toxicity to brine shrimp. This discrepancy might be due to the different parts used of *C. paniculatum* in present study. Furthermore, the results of both extracts of *D. serrulata* was in accordance with the previous study that indicated the absent of potent cytotoxicity to brine shrimp [206].

TPT remedy also exhibited toxic activity against the brine shrimp, and the toxicity is assumed to be due to in *Z. zerumbet.* Previous related studies showed the effective toxicity of the ethanol extract of *Z. zerumbet* against *A. sallina,* with an LC_{50} of 127.4 µg/ml [207] and also showed the highest cytotoxicity with LC_{50} was 1.24 µg/ml by Shakhawoat *et al.* [79]. The similarly results were reported by Anish *et al.* [208] that reported cytotoxicity of *Z. zerumbet* on lymphocyte cells at concentrations above 320 µg/ml.

Lagarto *et al.* [201] demonstrated that there was a good correlation ($r = 0.85$; $P < 0.05$) between the LC50 of the brine shrimp lethality assay and the LD50 of the acute oral toxicity assay in mice. Brine shrimp LC50 < 10 µg/ml could estimate mouse LD50 between 100 and 1000 mg/kg; LC50 < 20 µg/ml could estimate LD50 between 1000 and 2500 mg/kg, and LC50 > 25 µg/ml could estimate LD50 between 2500 and 8000 mg/kg. This present study found that the extracts exhibited toxic to *A. sallina,* while previous studies in mice or rats model showed non-toxicity. However, this discrepancy might be due to the difference of mechanism between brine shrimp and mice or rats. Therefore, the best toxic study should be confirmed the toxicity

profile using other cytotoxicity assays. Hence further confirmation of the toxicity profile was carried out using resazurin assay for CKT remedy and acute toxicity study in rats for TPT remedy.

The cell toxicity of the CKT remedy and it ingredients (ethanol and fractionated water extracts) were determined by resazurin assay. Resazurin is a redox dye which has been broadly used for investigating cytotoxicity by measure the metabolic activity of living cells [209-212]. Mitochondrial enzymes, as carriers of diaphorase activities, are probably responsible for the transference of electrons from NADPH + H^+ to resazurin (blue or dark blue color), which is reduced to resorufin (pink color) [115]. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent. Nonviable cells rapidly lose metabolic capacity, cannot reduce the indicator dye, and thus do not generate a fluorescent signal [213]. Examination of the cytotoxicity of all extracts in J774A.1 macrophage using resazurin assay indicated that the CKT remedy and it ingredients at 0-100 µg/ml did not affect the viability of J774A.1 cells.

TPT remedy and its ingredients extracts were evaluated for the safety by observed for 72 h and recorded the morbidity or mortality in mice. The results indicated that all extracts had no acute toxicity or mortality after oral administration. Koontongkaew *et al.* [214] and Panyathanya *et al*. [215] found that the ethanol extract of *Z. cassumunar* rhizome had shown no toxicity or mortality to animals on acute and chronic toxicity studies. The similarly results were reported by Chang *et al.*

[216] that the ethanol extract of *Z. zerumbet* rhizomes were non-toxic to Wistar rats in acute and subchronic toxicity studies and Abena *et al.* [217] revealed that the essential oils of *C. nardus* had no toxic effect observed until the dose level of 12 ml/kg. From this toxic study, the results indicated that CKT and TPT remedy extracts showed non-toxic in *in vitro* and *in vivo* study.

Genotoxic activities

Mutagenic activity

The Ames *Salmonella* assay is a short-term *in vitro* screening which has highly efficient in detecting carcinogens and mutagens. It has been tested with a wide variety of carcinogens such as direct alkylating agents, nitrosamines, polycyclic hydrocarbons, fungal toxins, aromatic amines, nitrofuran carcinogens, a variety of antineoplastic agents, and antibiotic carcinogens such as adriamycin, daunomycin, and mitomycin C. [218]

The genotoxicity are investigated by the mutagenic response toward histidine dependent *S. tryphimurium* strains which were deficient in excision repair of DNA damage (*uvrB*), ampicillin-resistant R-factors and presence of *pKM101*. When they receive a particular mutagen, they can revert back to the wild type [219]. In this study, the mutagenic and antimutagenic activities of CKT remedy, TPT remedy and their ingredients were tested in absence of methabolic activation using pre-incubation method of the Ames test based on *S. tryphimurium* strain TA98 (detecting

frameshift mutagens) and TA100 (detecting base-pair substitute mutagens) to observe the response of the extracts in an acidic condition.

Cha Tu Ka La Thad remedy

Most extracts without nitrite treatment were not mutagenic toward both strains except that the extracts of *A. calamus* exhibited mutagenicity to both strains and the water extract of *P.indica* exhibited toward strain TA98. These results indicated that the extracts of *A. calamus* might cause both frameshift and base-substitution mutation and the water extract of *P.indica* may cause only frameshift mutation. The high dose of ethanol extract of *A. calamus* displayed a high mutagenic index to strain TA98 (MI=46.44) and TA100 (MI=10.16) and the fractionated water extract of *A. calamus* also showed mutagenic activity to TA98 (MI= 4.36), whilst the study of Farrukh *et al.* demonstrated that the methanolic extract of *A. calamus* was not mutagenicity toward TA97a, TA100, TA102 and TA104 at 0.025-0.1 mg/plate [220]. However, there were studies showed that α-asarone and β-asarone which were the major chemical constituents [221], had mutagenic and carcinogenic potential [222-224]. At low levels, β-asarone was not mutagenic in the Ames test with and without metabolic activation, but it showed mutagenic activity at high concentration [223]. The result was in accordance with the previous study that at high concentration like high content of β-asarone, *A. calamus* demonstrated the mutagenicity but at low concentrations (0.4 mg/plate) *A. calamus* did not show mutagenic activity on both strains.

The ethanol extract of *P. indica* exhibited non-mutagenicity without nitrite treatment on *S. tryphimurium* strain TA98 and TA100. Our results agreed with earlier finding that the sequentially extract with hexane, chloroform and methanol of *P. indica* were not mutagenic toward either strain TA98 or TA100 [225]. However, the fractionated water extract of *P. indica* showed a slight mutagenic activity without nitrite treatment on *S. tryphimurium* strain TA98 in this study that might be affected from plumbagin (5-hydroxy-2-methyl-1,4-napthoquinone) which was a therapeutically important natural naphthoquinone occurs mainly in the roots of *P. indica* root [226]. Hakura *et al.* [227] and Tikkanen *et al.* [228]demonstrated that plumbagin was mutagenic to strain TA104 and TA2637. In contrast, Durga *et al*. reported plumbagin did not show mutagenic effect in Ames test [229].

Nevertheless, the Ames test was modified by treating the extracts with nitrite in acid solution similar to that occurring during stomach digestion. There were previous studies showed that the plant extracts exhibited mutagenicity after nitrosation [230, 231]. The result demonstrated that ethanol extract of four root species had mutagenic effects on *S. typhimurium* strains TA98 while the ethanol extract of *A. calamus* and *P. indica* exhibited mutagenic effect on *S. typhimurium* strains TA100 after they were treated with sodium nitrite (nitrosation) under acidic condition. There were several studies reported the mutagenic effect of many medicinal plants, remedies and chemical compounds showed direct-acting mutagenicity after nitrite treatment without metabolic activation.

Wongwattanasathien *et al.* [231] reported that some extracts from flowers showed direct-acting genotoxicity in the Ames test after being treated with nitrite. Higashimoto *et al.* [232] found that methanol and water extracts of spices treated with nitrite were mutagenic for strain TA 100 without metabolic activation. In addition, Thai remedies showed mutagenic potential after treating with nitrite on strain TA98 and TA100 [233, 234].

Tree Phon Thad remedy

This study is the first report for investigation of mutagenic activity of TPT remedy and *C. nardus* root. The results of the mutagenic assay of TPT remedy and its component extracts demonstrated that they were not directly mutagenic toward *S. typhimurium* strains TA 98and TA100 except the fractionated water extract of *C. nardus* showed slightly mutagenicity toward *S. typhimurium* TA98. The result was in accordance with previous study that the ethanol extract from the rhizome of *Z. zerumbet* was no mutagenic effect to *S. typhimurium* (strains TA97, TA98, TA100, TA102, and TA1535) at concentrations of 150–5000 μg/plate [235]. There was a research showed that terpinen-4-ol, which was a major component of volatile oil of *Z. montanum* rhizome, was not genotoxic in *in vitro* mammalian cells [236].

This study demonstrated that ethanol and fractionated water extract of all rhizomes exhibited their mutagenic effect after they were treated with sodium nitrite (nitrosation) under acidic condition on *S. typhimurium* strains TA 98and TA100. There were several researches employed *in vitro* assay to determine the genotoxic

carcinogen by treatment with the nitrosation reaction mixture or directly with N-nitroso compounds; similar to the *in vivo* assays of its biological activity [237]. Previous studies revealed that high levels of nitrite increased incidences of gastric and liver cancer. It has been indicated that N-nitroso compounds is an etiology of human cancer [238].

Nitrite is presented in nitrite-preserved meat of fish, spoiled foods, even nitrate mostly found in foods and vegetables. Nitrite is the most important precursor to generate the nitrosating agents [239]. It has been revealed that N-nitroso compounds are formed by the interaction of nitrogenous compounds with nitrosating agents, the most important of which is acid nitrite [240]. Therefore, when the extracts containing the nitrogen trixodie (N_2O_3) or dihitrogen tetroxide (N_2O_4) with primary, secondary, or tertiary amines, or with secondary amide [241] reacts with the sodium nitrite (nitrosating agent precursor), it can be generate the carcinogenic N-nitroso compounds under the acidic condition with gastric pH [242]. Therefore, the co-administration of the extracts with nitrite containing food should be avoided. Although some root extracts showed potential to be mutagenic with and without nitrite treatment, CKT remedy and TPT remedy showed non-mutagenicity with and without nitrite treatment in the Ames test toward both strains of *S. typhimurium.*

However, the Ames test uses prokaryotic cell as the model, which differs from mammalian cell in many factors such as metabolism, chromosome structure and DNA repair processes. Ames test has been used as a basic biological assay to

assess the mutagenic potential of chemical compounds prior to *in vivo* animal models [135].

Comet assay

Comet assay which is a rapid standard test was used to assess the DNA damage activity of compound in eukaryotic cells. It is based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis [243]. This assay is also becoming an important *in vitro* assay for evaluating the genotoxicity potential of compound *in vivo* and used successfully to monitor DNA damage in human populations.

The advantages of the comet assay for evaluation of genotoxicity includes: it identifies DNA damage at single cell level, it is able to detect low levels of DNA damage, it uses only small number of cells for testing in each sample, the assay can investigate with several eukaryotic cell type, it is easy for application, it is a low cost assay and short time needed to perform the assay [243]. In this study, DNA damage was investigated in human lymphocytes exposed to CKT remedy, TPT remedy and their ingredients at different concentrations.

The four root species and CKT remedy extracts, showed a dose-dependent relationship between the degree of DNA damage and concentration of sample. The results demonstrated that only the fractionated water extract of *A. calamus* showed the highest DNA damage, followed by the fractionated water extract of *P. indica* and *C. paniculatu*m and the ethanol extract of *A. calamus* showed the moderate DNA

damage in human lymphocytes. *A. calamus* was found to induce both DNA damage by the comet assay and reverse mutation by Ames test in *S. typhimurium* strains. These findings are in agreement with earlier results which indicated that beta-asarone induced genotoxicity in micronucleus assay [244]. However, all concentrations of CKT remedy extract presented non-genotoxicity as the comet class did not differ from the negative control. When human lymphocytes were exposed of CKT remedy extract, most of the cells tested on slide were undamaged (class 0), few cells showed low level genotoxicity (class 1) and very few showed a large amount of damage (class 2-4). This result suggested that CKT remedy extract had no potentiating effect on DNA damage *in vitro* test.

All three species and TPT remedy extracts showed a dose-dependent relationship between the degree of DNA damage and concentration of sample. The results revealed DNA damage in human lymphocytes as low as negative control (PBS), whereas H_2O_2 showed that the highest damage to DNA in human lymphocytes. Similarly, there was the report in Taiwan showed that ethanol extracts from the rhizome of *Z. zerumbet* did not possess genotoxic potential in the micronucleus assay [235]. TPT remedy extract was no effect on DNA migration in all concentrations and these results were confirmed in three replicates performed. The result of TPT remedy in comet assay is in accordance with the results in the Ames test which indicated non-mutagenicity toward *S. typhimurium* strains.

The results in the *in vitro* genotoxic test including the Ames test and the comet assay suggest non genotoxic potential of CKT and TPT remedy extracts.

Antimutagenic activity

The investigation for antimutagenicity of medicinal plant is significant in the discovery of new effective anticarcinogenic therapeutic drug [138]. 1-Aminopyrene treated with nitrite in acid solution was direct-acting mutagen toward *S. typhimurium* strain TA98 and TA100 [245, 246]. The antimutagenic effects of the root extracts and CKT remedy were evaluated agiants the reaction of 1-aminopyrene treated with nitrite under acidic condition on both strains of *S. typhimurium* in the Ames test.

It revealed that most of the root extracts and CKT remedy extract exhibited antimutagenic potential ranged from negligible (0-20%) to strong (>60%) effects toward both strains of *S. typhimurium.* The killing effect was observed at 15 mg/plate and the partial effect was observed at 5 and 10 mg/plate of the ethanol extract of *A. calamus* on strain TA100. Nevertheless, at the highest dose, CKT remedy demonstrated the partial killing effect on strain TA100. Thus, their results of the antimutagenic potential at high dose might be partly due to the cytotoxic effect of the extracts [247].

The results suggested that the root ethanol extracts were highly antimutagenic more than the root fractionated water extract. The ethanol extract of *D. serrulata*, *C. paniculatum* and CKT remedy at 15 mg/plate were strong

antimutagenic effect against 1-aminopyrene on strains TA98 and at 10 mg/plate on strain TA100. This finding agreed with the study of Manohan *et al.* [206] who found that all doses of the ethanol extract of *D. serrulata* demonstrated strong antimutagenic effect on both strains. Moreover, the present study revealed that at 10 and 15 mg/plate of extracts, *P. indica* also showed the strong inhibition on mutagenic response by the product of 1-aminopyrene with nitrite in both strains. In an earlier study, the organic solvent extracts from *P. indica* could strongly inhibit the mutagenicity of alflatoxin B_1 [225]. Kuma *et al*. [248] reported that plumbagin showed strong antimutagenic activity against ultraviolet and ethyl methanesulfonate and it reduced significantly the mutagenic effect of 4-nitrophenylene diamine, phenyl hydrazine and sodium azide in the Ames test [229]. Furthermore, the CKT remedy had the highest antimutagenic potential against 1-aminopyrene on both strains of *S. typhimurium* in the Ames test.

The antimutagenic effect of the TPT remedy and its ingredients extracts revealed that most of the extracts exhibited antimutagenic potential ranged from negligible (0-20% inhibition) to strongly active (**>** 60% inhibition) effects toward both *S. typhimurium* strains. These extracts showed a dose-dependent by inhibitory effect to both *S. typhimurium* strains. All ethanol extracts demonstrated the strong inhibitory activity toward TA98 and TA100 strain. The overall antimutagenic potential of the TPT remedy and its ingredients extracts was found to be in order of *Z. zerumbet* > *Z. montanum* > *C. nardus* > Tree Phon Thad to strain TA98 and *Z.*

zerumbet > *Z. montanum* > Tree Phon Thad > *C. nardus* to strain TA100. The ethanol extract of *Z. zerumbet* rhizome at 5 mg/plate exhibited the strongest inhibitory activity while at 10 and 15 mg/plate showed killing effect to strain TA100. This data agreed with the previous studies that zerumbone showed strong antimutagenic activity against sodium azide induced mutation in Ames test [249]. The similar result demonstrated that zerumbone isolated from *Z. zerumbet* was suggested to be chemopreventive and antimutagenic agent [250]. Therefore, the results of this study indicate that the CKT remedy, TPT remedy and their ingredients extracts have antimutagenic potential and can improve to be an antimutagenic and anticancer agent.

Anti-inflammatory activities

Cha Tu Ka La Thad remedy and its ingredients were examined for potential of anti-inflammatory activity in *in vitro* anti-inflammatory models including NO inhibition assay, TNF-alpha inhibition assay and PGE_2 inhibition assay. Carrageenan-induced paw edema and prostaglandin E2-induced mouse paw edema were performed to detect the anti-inflammatory activity of Tree Phon Thad remedy.

***In vitro* anti-inflammatory test**

This study evaluated the inhibitory effect of root extracts and CKT remedy on LPS-induced NO, TNF-alpha and PGE_2 production in J774A.1 cells. LPS is a gram-negative bacteria which has an endotoxin and a constituent of the outer membrane.

LPS stimulates innate immunity by regulating the production of inflammatory mediators such as NO, TNF- alpha and PGE_2. Tumor necrosis factor- alpha is a member of pro-inflammatory cytokines family which can stimulate the synergistic induction of NO synthesis in LPS-stimulated macrophage [251]. NO and PGE_2 are important inflammatory mediator in the inflammatory process. NO is produced by NO synthase and PGE_2 is produced from the conversion of arachidonic acid by cyclooxygenase-2 (cox-2) after are stimulated by LPS [252].

Examination of the cytotoxicity of all extracts in J774A.1 macrophage using resazurin assay indicated that all extracts at 0-100 µg/ml did not affect the viability of J774A.1 cells. Thus, the inhibition of LPS-induced mediator inflammation by all extracts were not the result of a possible cytotoxic effect on these cells. This study demonstrated that all extracts significantly reduced the production of NO, TNF-alpha and PGE_2. The ethanol extract of *P. indica*, *A. calamus*, and CKT remedy showed the highest percentage inhibition of NO production at high dose. Moreover CKT remedy had a maximum inhibition of TNF-alpha production and the ethanol extract of *P. indica* possessed the highest PGE_2 inhibition. Similarly previous studies were reported by Sibi *et.al* [253] suggested the aqueous extracts of *P. indica* had potent anti-inflammatory activity in carrageenan induced paw edema assay and Tingyu *et al.* [254] demonstrated that plumbagin significantly inhibited the expression of TNF-alpha, IL-1beta, IL-6 and iNOS in LPS-stimulated RAW 264.7 cells. Earlier reports revealed that the butanolic fraction of *A. calamus* showed strong inhibition on

arachidonic acid metabolism and platelet aggregation through multiple pathways [255]. The study of Hyeri *et al.* [256] suggested that the water extract of *A. calamus* leaves inhibited the production of pro-inflammatory cytokines that might be an effective anti-inflammatory agent. In addition, the petroleum ether and chloroform extracts of *C. paniculatum* leaves possessed significant anti-inflammatory activity by *in vitro* and *in vivo* assay as evidenced by the work of Joseph *et al.* [257]

In conclusion, the present study indicates that CKT remedy possesses high anti-inflammation potential by reduced release of inflammatory mediator (NO, PGE_2) and pro-inflammatory cytokines (TNF-alpha). Our findings support the ethnomedicinal use of root extracts and CKT remedy in the management of antimutagenic and anti-inflammatory activities. However, the further investigation is needed to detect the specific active compound in the plant extracts and remedy that are responsible for these biological activities and to estimate their bioavailability and efficacy *in vivo* screening.

In vivo anti-inflammatory test

There were several researches that revealed the potential of anti-inflammatory activities of the components of plant species of Tree Phon Thad remedy in *in vitro* testings [78, 96, 97]. Therefore, *in vivo* anti-inflammatory testings of Tree Phon Thad remedy and its ingredients were investigated in this study by Carrageenan-induced paw edema and prostaglandin E2-induced mouse paw edema tests.

Carrageenan-induced paw edema

The carrageenan-induced mouse paw edema test was performed to examine the anti-inflammatory efficacy of TPT remedy and its ingredients extracts (ethanol/fractionated water extract).

The carrageenan-induced paw edema test is a standard model for assessment of anti-inflammatory drugs and is widely used for evaluating the acute inflammatory activities of natural compounds [258]. Carrageenan-induced rat paw edema was first described by Winter et al., 1962 [259] and carrageenan-induced mouse paw edema test was established by Levy, 1969 [260]. Since then, the mouse paw edema test has been increasingly used to evaluate the anti-inflammatory drugs candidates [261]. Multiple physical and behavior responses can be followed to assess the extent of injury and its prevention or reversal by NSAIDs [262]. Carrageenan induced edema is a biphasic response. The first phase is mediated through the release of histamine, serotonin and kinins whereas the second phase is related to the release of prostaglandin and slow reacting substances which peak at 3 h after intraplantar injection of carrageenan [263]. More recently, the second phase has also been attributed to involvement of free radicals, nitric oxide and cyclooxygenases in the hind paw exudate [264]. The inflammatory response is usually quantified by increase in paw size (edema) which is maximal around 5 h post-carrageenan injections and is

modulated by inhibitors of specific molecules within the inflammatory cascade. Indomethacin is a clinically useful example [265].

IND, a positive control, significantly reduced paw edema from 2-6 h (second phase) after carrageenan administration. IND is a cyclooxygenase inhibitor and contributes to the reduction of prostaglandin synthesis. The results are consistent with the previous report that IND caused strong inhibition of the second phase without affecting the development of the first phase [263].

Only the highest dose of TPT remedy (300 mg/kg) significantly decreased mouse paw edema at 3-6 h after carrageenan injection. *Z. zerumbet* at doses of 25-200 mg/kg had this effect at 2-6 h after carrageenan injection. The result of *Z. zerumbet* in this study is consistent with the previous study of Zakaria that showed the antiedema effect of the methanol extract of *Z. zerumbet* given subcutaneously at all doses tested (25-100 mg/kg) [266]. The onset of antiedema effect of the methanol extract of *Z. zerumbet* was faster when compared to *Z. zerumbet* (1.5-2.5 h vs. 2-6 h) after carrageenan injection. All doses of *Z. montanum* (25-400 mg/kg) showed significant reduction of paw edema at 3-6 h after carrageenan injection. *C. nardus* at doses of 25 and 200 mg/kg significantly decreased mouse paw edema at 3-6 h after carrageenan injection. These results indicate that TPT remedy, *Z. zerumbet*, *Z. montanum* and *C. nardus* exert anti-inflammatory actions during the second phase of inflammation which involves prostaglandin synthesis. The mechanism

underlying these activities of all herbal root extracts may be mediated through the inhibition of prostaglandin synthesis.

Prostaglandin E2-induced mouse paw edema

In a further attempt to explore the possible explanation, in addition to the COX-inhibitory effect during the second phase of inflammation induced by carrageenan, the effect of three herbal roots extracts on PGE_2-induced mouse paw edema was investigated. PGE_2 is generally considered as a key pro-inflammatory mediator and it is synthesized by many cells and tissues throughout the body. High levels of PGE_2 have been found in inflammatory exudates and injection of PGE_2directly into tissue has been shown to induce a number of classical signs of inflammation [267]. PGE_2 has long been considered as the principal prostaglandin in acute inflammation, as well as in arthritic diseases such as rheumatoid and osteoarthritis [268].

IND, a positive control, significantly inhibited paw edema induced by $PGE_2$from 0.5 h and last for 2 h after PGE_2 injection. *Z. zerumbet*, *Z. montanum* and *C. nardus* significantly inhibited paw edema induced by PGE_2 at the same period after PGE_2 administration indicating that these effects were due to PGE_2degradation and/or inactivation. The result of *Z. zerumbet* (25 mg/kg) in this study is consistent with the previous study of Nhareet that showed anti-inflammatory effect of intraperitoneally administered aqueous extract of *Z. zerumbet* (50 and 100 mg/kg), while the

ethanolic extract was without effect. However, the duration of antiedema action of the aqueous extract of *Z. zerumbet* (4 h) was longer than *Z. zerumbet* (2 h) after PGE_2injection [269].

Claudino *et al*., 2006 [267] demonstrated that edematogenic response elicited by PGE_2in mice are mediated by E-prostanoid (EP_3) receptor activation and are largely prevented by a selective EP_3 receptor antagonist. They also involve the stimulation of phospholipase C (PLC), protein kinase C (PKC) and mitogen protein-activated kinase (MAPKs) pathways and include the participation of vanilloid receptor (TRPV1) and neurokinin (NK_1) receptors. The reduction of PGE_2–induced paw edema could explained, at least in part, the inhibitory effect of all three root extracts on acetic acid-induced writhing and formalin tests. These results complement those obtained from other models involving inflammatory mediators, including acetic acid-induced writhing and formalin licking responses.

In conclusion, the results of this study demonstrated that TPT, *Z. zerumbet*, *Z. montanum* and *C. nardus* possessed anti-inflammatory properties in an acute inflammation model. The anti-inflammatory mechanisms of action of all three herbal root extracts were due to PGE_2 degradation and/or inactivation. TPT and all three herbal root extracts demonstrated similar results in all animal models, indicating that the anti-inflammatory effects of TPT remedy were resulted from all three herbal root extracts. The anti-inflammatory effects of the root extracts of *Z. zerumbet* and *Z. montanum* were in agreement with the previous studies, while the anti-inflammatory

effects of the root extract of *C. nardus* has never been reported. Therefore, this is the first study that demonstrates other properties of *C. nardus*. Further studies are required to understand the mechanisms of action of these effects. However, the results clarify the pharmacological actions of TPT remedy and all three herbal root extracts and highlight the potential use of these natural compounds for treating inflammatory disorders.

Antioxidant activities

Oxidative stress is caused, in part, by highly free radical including reactive oxygen species or ROS (superoxide anions, hydrogen peroxide and singlet oxygen) and reactive nitrogen species or RNS (nitric oxide and peroxynitrites) that can lead to many illnesses such as cardiovascular diseases, type 2 diabetes, inflammation, degenerative diseases, cancer, metabolic syndrome and neurodegenerative disease [270, 271]. The reactive oxygen species propagate inflammation by stimulating release of cytokines such as interleukin-1, tumor necrosis factor- alpha and interferon-gramma which stimulate recruitment of increase neutrophils and macrophages. Free radicals are important mediators that can motivate or encourage inflammatory process and consequently, their neutralization by antioxidant and radical scavenger can mitigate inflammation [272]. Therefore, the assessments of antioxidant properties of natural compounds from medicinal plants are very important because they have beneficial effects to prevent and treat disease in human health. The antioxidant capacities are influenced by many factors which

cannot be fully described by only one method. It is necessary to investigate the various mechanisms of antioxidant action. Therefore, the antioxidant activities of CKT remedy, TPT remedy and their ingredients extracts were assessed on the basis of total phenolic contents, radical scavenging activity against DPPH free radical and nitric oxide, ferric reducing antioxidant power assay and metal chelating activity.

DPPH radical scavenging activity

DPPH (2,2-Diphenyl-1-picrylhydrazyl) is a stable free radical which used for estimation of the electron donation ability of medicinal plants or natural products [273]. DPPH is a stable nitrogen-centered free radical which has violet color with maximum absorbance at 515-520 nm [274]. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution resulting into reduction of deep violet colour converts to yellow colour. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [275].

All extracts of CKT remedy and its ingredients exhibited weak scavenging of the DPPH radical compared to quercetin and buthylated hydroxyl toluene (BHT) which were used as positive control. The DPPH radical scavenging activity of CKT remedy and its ingredients extracts can be ranked as CPE> DCE > CKT > ACE > PIW > PIE > ACW > CPW and DCW (Table 18). Previous study showed that the methanol

extract of leaf and root of *C. paniculatum* are good antioxidants by enzymatic and non-enzymatic methods [276]. The result of *D. serrulata* extracts exhibited weak scavenging of the DPPH radical which is different with early previous study of Manohan *et al.* and Phomkaivon *et al.* [277, 278]. It might be related to the different location of collecting the sample and processing of extraction and other environment factors [279].

For the results of TPT remedy and its ingredients, the radical scavenging activities of most ethanol extracts were higher than the fractionated water extracts. The DPPH radical scavenging activity of TPT remedy and its ingredients extracts can be ranked as ZME > TPT > CNE > ZZE > ZMW and ZZW, CNW (Table 23). The ethanol extract of *Z. montanum* exhibited high free radical scavenging activity when compared to all extracts. Previous study of the ethanol extract of rhizome *Z. montanum* from the north in Thailand showed the highest scavenging DPPH radical [280].

Nitric oxide free radical scavenging activity

Nitric oxide is a free radical or reactive nitrogen species (RNS) which is an important effecter molecule in physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation, especially in various inflammatory processes and regulation of cell mediated toxicity [281]. The nitric oxide generated from sodium nitroprusside which thereby interacts with oxygen to produce nitrate ions that can be estimated by use of Greiss reagents. The extract

inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide [282].

For the results of CKT remedy and its ingredients extracts, the ethanol extracts had more potent nitric oxide scavenging activity than the fractionated water extracts. The nitric oxide scavenging activity of CKT remedy and its ingredients extracts can be ranked as ACE > CKT > PIW > PIE > CPE > DCE > ACW and CPE, DCE (Table 21). The ethanol extract of *A. calamus* (IC_{50} 375 µg/ml) showed the highest nitric oxide scavenging activity when compared to all extracts. In addition, it had more potent nitric oxide scavenging activity than the standard L-ascorbic acid (IC_{50} 482 µg/ml). The results of the present study are similar to Verma *et al.* [283] who reported that the rhizomes extract of *A. calamus* showed prominent IC50 value of 376.2 µg/ml by nitric oxide assay. Chandana *et al.* [284] reported that the ethanol extract of whole plant of *A. calamus* showed the highest nitric oxide scavenging activity.

For the result of TPT remedy and its ingredients extracts, the ethanol extracts had more potent nitric oxide scavenging activity than the fractionated water extracts. The nitric oxide scavenging activity of TPT remedy and its ingredients extracts could be ranked as ZME > ZZE > TPT > CNE > ZZW > and ZMW, CNW (Table 26). Only the ethanol extract of *Z. montanum* showed high nitric oxide scavenging activity and had IC50 value of 533 µg/ml which comparable to the reference standard indicating a

good nitric oxide scavenging potential. Previous study similarly reported that the ethanol extract of *Z. montanum* exhibited potent inhibitory effect of nitric oxide production [285].

Ferric ion reducing antioxidant power assay

FRAP assay is an inexpensive reagents, simple prepared, highly reproducible and linearly related to molar concentration of the antioxidants [286]. The reducing ability of an antioxidant is measured by changing from a ferric tripyridyltriazine [$Fe3^{+}$-TPTZ] complex to a coloured ferrous tripyridyltriazine [$Fe2^{+}$-TPTZ] [150]. FRAP value is based on reducing ferric ion by antioxidants that are reducing agent. The reducing properties are associated with the presence of compounds which exert their actions by breaking the free radical chain by donating a hydrogen atom [287]. High FRAP value indicated that the compound in sample can break free radical chain as a reductant in a redox-linked colorimetric reaction [288].

In the present study, CKT remedy and its ingredients extracts showed concentration-dependent increase in ferric reducing power. The fractionated water extract of *P. indica* demonstrated highest reducing power ability, followed by CKT remedy extract. The reducing power capacity of fractionated water extract of *P. indica* expressed the FRAP value of 1.477 mM Fe(ll)/mg dry weight which was higher than the standard quercetin and BHT. Hence, the high value of fractionated water

extract of *P. indica* indicated that some compounds may act as electron donors and could react with free radicals.

For the results of TPT remedy and its ingredients extracts, both extracts of *Z. montanum* showed the highest reducing power ability compared to all extracts. However it was lower than standard quercetin and BHT. Both extracts of *Z. zerumbet* had lowest reducing power ability. These results were different from previous study [289]. It may be affected by the quality of extracted compounds which depended on storage time, geographic origin, harvesting date, environment and technological factors. Besides that, the temperature and light also contribute to antioxidant activity change during storage [290].

Metal chelating activity

Iron and other transition metals (copper, cobalt, vanadium, cadmium, chromium, arsenic and nickel) can stimulate lipid peroxidation by Fenton reaction. Metal ion chelating capacity is important because it reduces the concentration of the transition metal that catalyzes lipid peroxidation [291]. Chelating activities of all extracts were compared to chelating standards, EDTA.

According to the results of CKT and TPT remedy, the fractionated water extracts of *P. indica* showed high metal chelating activity as same as the activity of the standard EDTA. The metal chelating effects of the extracts were dose-dependent.

All investigated fractionated water extracts were capable of chelating Fe^{2+} ions. It is interesting to note that the fractionated water extracts displayed more chelating activity than the ethanol extracts. The results were in accordance with previous study that polar compounds had strong chelating activity and it could be concluded that water was more suitable for extraction of such strong chelating ability substances [292]. Previous studies reported a high correlation between total flavonoids and Fe-chelating ability [293, 294]. Hence, high chelating activity of fractionated water extracts may be due to high content of flavonoids. However, the contents of flavonoids was not investigated in study.

Total phenolic contents

Phenolic compounds are considered secondary metabolites that be synthesized by plants. They are derived from phenylalanine and tyrosine [295]. Phenolic compounds of plants are also very important because their hydroxyl groups as able to scavenge free radicals. For the results of CKT remedy and its ingredients, the ethanol extract exhibited high total phenolic contents than the fractionated water extracts that is in agreement with other reports [296]. The ethanol extract of *D. serrulata* showed the highest total phenolic content followed by the fractionated water extract of *P. indica* and CKT remedy. The results were in accordance with the study of Phomkaivon *et al.* [286] that the ethanol extract of the flower of *D. serrulata* had the total phenolics of 13.25 ± 0.28 mg/ GAE/g dry basis.

For the results of TPT remedy and its ingredients, the ethanol extract of *Z. zerumbet* showed the highest total phenolic content followed by TPT remedy extract and the ethanol extract of *Z. montanum.* The ethanol extract exhibited high total phenolic contents than the fractionated water extracts that is in agreement with report of Sreevani *et al.* which the ethanol extract of *Z. zerumbet* showed the highest of total phenolic content compared to other extracts [297].

Several previous studies indicated the relationships between antioxidant activities of medicinal plants and their phenolic contents [297, 298]. This study is in agreement with the literatures that the ethanol extracts containing high phenolic contents showed high potential of free radical scavenging activity and reducing power ability.

Table 27 Summary of safety and efficacy of four species and Cha Tu Ka La Thad remedy

	Experiment	PI		AC		DC		CP		CKT
		E	W	E	W	E	W	E	W	
Safty study	Brine shrimp lethality testing	-	-	+++	-	-	-	-	+	++
	Cytotoxicity (macrophage)	-	+	-	+	-	-	-	-	-
	Mutagenicity assay (without nitrite)	+	-	+++	-	-	-	-	-	-
	Mutagenicity assay (with nitrite)	+++	+	++	-	++	-	+	-	-
	Comet assay	-	++	++	+++	-	+	++	++	-
Efficacy study	Antimutagenic activity	+++	+++	+++	++	+++	++	+++	++	+++
	Anti-inflamatory activity (*In vitro*)									
	NO inhibition assay	+++		+++		+++		+++		+++
	TNF-α inhibition assay	+		++		+		++		+++
	PGE2 inhibition assay	+++		+		+++		+++		++
	Total phenolic	++	+++	+++	++	+++	-	++	-	++
	DPPH assay	+	++	++	-	++	-	++	-	++
	FRAP assay	-	+++	+	+	+	-	+	-	++
	Methal chelating assay	-	+++	-	+++	-	++	-	++	+
	Nitric oxide scavenging assay	+	+	+++	-	-	-	-	-	++

+++ High effect, ++ Moderate effect, + Mild effect, - Negative effect; PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thad remedy , E = ethanol extract, W = fractionated water extract

Table 28 Summary of safety and efficacy of three species and Tree Phon Thad remedy

Experiment		ZZ		ZM		CN		TPT
		E	W	E	W	E	W	
Safty study	Brine shrimp lethality testing	++	-	-	-	-	-	++
	Acute toxicity (animal model)	-	-	-	-	-	-	-
	Mutagenicity assay (without nitrite)	-	-	-	-	-	+++	-
	Mutagenicity assay (with nitrite)	+++	+++	+++	+++	+++	+++	++
	Comet assay	-	-	-	-	-	-	-
Efficacy study	Antimutagenic activity	+++	+++	+++	+++	+++	+++	+++
	Anti-inflamatory activity (animal model)	++		++		+++		+++
	Total phenolic	+++	-	+++	-	+++	+	+++
	DPPH assay	-	-	++	-	-	-	++
	FRAP assay	-	-	++	++	+	+	++
	Methal chelating assay	-	++	-	-	-	++	-
	Nitric oxide scavenging assay	+	-	+++	-	-	-	+

+++ High effect, ++ Moderate effect, + Mild effect, - Negative effect; ZZ = *Z. zerumbet*, ZM = *Z. montanum,* CN = *C. nardus,* TPT= Tree Phon Thad remedy, E= ethanol extract, W = fractionated water extract

Regarding to the results of the present study, the pharmacognostic investigations can be used to set the standard parameters of the four species in Cha Tu Ka La Thad remedy and three species in Tree Phon Thad remedy which is useful for authentication and quality control of each crude drug. In addition, these studies provide important clues to help understand the mechanism underlying the toxicities and biological activities of Cha Tu Ka La Thad remedy, Tree Phon Thad remedy and their components which support in further clinical researches. Additionally, the obtained results also support the validity of the traditional use of both remedies against inflammatory disorders. These remedies can be interesting sources of anti-inflammation and antioxidant agents.

REFERENCES

1. Sahoo, N., P. Manchikanti, and S. Dey, *Herbal drugs: Standards and regulation.* Fitoterapia 2010. **81**: p. 462-71.
2. World Health Organization, *Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines*. 1993, Manila: Regional Office for the Western Pacific.
3. Erin Howe, B.K. and I.M. Khan, *Traditional Medicine and Medicinal Plants: Utilization, Policy and Research in Thailand.* Journal of Public Health and Development, 2004. **2**(1).
4. Willow, J.H. and Lui, *Traditional herbal medicine research methods: Identification, Analysis, Bioassay and Pharmaceutical and Clinical Studies*. 2011, United State: A John Wiley & Sons, Inc.Hoboken New Jersey.
5. Pulok K and Mukherjee, *Quality control of herbal drugs: an approach to evaluation of botanicals*. Vol. 2. 2007, New Delhi: Business horizons.
6. Service, N.R.C. *Plumbago indica L.* . 2013 May 03, 2014; Available from: http://plants.usda.gov/core/profile?symbol=PLIN8
7. India, F.o. *Lal Chitrak*. 2013 May 03, 2014; Available from: http://www.flowersofindia.net/catalog/slides/Lal%20Chitrak.html
8. Names, C.P. *Plumbago indica Linn.* 2014 May 03, 2014; Available from: http://www.efloras.org/florataxon.aspx?flora_id=3&taxon_id=200017526.
9. Paiva, S.R., et al., *Antimicrobial activity in vitro of Plumbagin isolated from Plumbago Species.* Memorias do Instituto Oswaldo Cruz, 2003. **98**: p. 959-61.
10. Nayana, S., et al., *A simple method for isolation of plumbagin from roots of Plumbagin rosea.* Summary Pharmaceutical Biology 2005. **43**: p. 551-5.
11. Dorni, A.I.C., et al., *Antiinflammatory microbial of Plumbago capensis.* Phcog Mag, 2006. **2**: p. 239-43.
12. Mista, M.K., et al., *Medicinal plants used by the Kandhas of Kandhamal district of Orissa.* Ind J Trad Knowledge, 2006. **5**: p. 519-28.

13. Nath, S.C. and J. Purkayastha, *Biological activity of ethonomedicine claim of some plant species of Assam.* Ind J Trad Knowledge, 2005. **5**: p. 229-36.
14. Van, D., *Distribution of plumbagin in the Plumbaginaceae.* Phytochemistry, 1974. **11**: p. 3247-48.
15. plants, P.m. *Plumbago rosea Linn*. 2013 [cited 2014 May 03]; Available from: http://stuartxchange.com/Laurel.html
16. Panyaphu, K., et al., *Phytochemical antioxidant and antibacterial activities of medicinal plants used in Northern Thailand as postpartum herbal bath recipes by the Mien (Yao) community.* Phytopharmacology, 2012. **2**: p. 92-105.
17. Paiva, S.R., et al., *Antimicrobial activity in vitro of Plumbagin isolated from Plumbago Species.* Memorias do Instituto Oswaldo Cruz, 2003. **98**: p. 959-61.
18. Atjanasuppat, K., et al., *In vitro screening for anthelmintic and antitumour activity of ethnomedicinal plants from Thailand. .* Journal Ethonopharmacology 2009. **123**: p. 475-82.
19. Devi, P., F. Solomon, and A. Sharada, *In vivo tumor inhibitory and radiosensitizing effects of an Indian medicinal plant, Plumbago rosea on experimental mouse tumors.* Indian Journal Experimental Biology, 1994: p. 532-38.
20. Dibyajyoti, S. and P. Swati, *Pharmacognostic Studies of Aerial Part of Methanolic Extract of Plumbago indica L. .* Asian Pharma Press Sciences Journal, 2012. **2**: p. 88-90.
21. Joshi, S.B. and D.C. Jain, *Antifertility Activity of Stems of Plumbago rosea in Female Albino Rats.* Pharmaceutical Biology, 2008. **46**: p. 920-927.
22. Binil, E., N. Vicente , and M.S. Latha, *Evaluation of Phytochemical Constituents and In vitro Antioxidant Activities of Plumbago indica Root Extracts.* Journal of Pharmacognosy and Phytochemistry, 2013. **2**: p. 157-161.
23. Swati, P. and S. Dibyajyoti, *Analgesic Activity of Methanol Extract of Plumbago Indica (L.) by Acetic Acid induced writhing method.* Asian J. Pharm. Tech., 2012. **2**: p. 74-76.

24. Ittiyavirah, J.K.V., et al., *Antiinflammatory and analgesic activities of plumbago capensis and plumbago indica.* Adv. Pharmacol. Toxicol 2012. **13**: p. 47-52.
25. Sharma, L., D. Gusain, and V. Dixit, *Hypolipidaemic and antiatherosclerotic effects of plumbagin in rabbits.* Ind J Physiol Pharmacol, 1991. **35**(1): p. 10-14.
26. Emerson, F., A.C. Sharada, and D. Uma, *Toxic effects of crude root extract of Plumbago Rosea (Rakta chitraka) on mice and rats. .* .Journal of Ethonopharmacology 1993. **38**: p. 79-84.
27. Hashmat, I., et al., *Sweet flag (Acorus calamus Linn.): An incredible medicinal herb.* International Journal of Green Pharmacy, 2013. **7**: p. 288-296.
28. Sylvan, T. and A.F. Runkel, *Wildflowers of Iowa Woodlands*. Vol. 2. 2009, Iowa, China: April Leidig-Higgins.
29. Balakumbahan, R., K. Rajamani, and K. Kumanan, *Acorus calamus: An overview.* J Med Plant Res 2010. **4**: p. 2740-45.
30. Paithankar, V., S. Belsare, and R. Charde, *Acorus calamus: An overview.* Int J Biomed Sc., 2011. **2**: p. 518-529.
31. Mukherjee, P.K., et al., *Acorus calamus: Scientific validation of ayurvedic tradition from natural resources. .* J. Pharmaceutical Biology, 2007. **45**: p. 651-666.
32. AG, P.m.p. *Acorus gramineus: SWEET FL*. 2013 [cited 2014 May 03]; Available from: http://www.stuartxchange.org/Lubigan.html
33. Manikandan, S., et al., *In-vitro antibacterial activity of aqueous and ethanolic extracts of Acorus calamus.* Int J Appl Biol Pharm Technol 2010. **1**: p. 1072-75.
34. Begum, J., et al., *Antifungal activity of forty higher plants against phytopathogenic Fungi.* Bangladesh J Microbiol 2007. **24**: p. 76-78.
35. Abhijit, M., et al., *Synergistic Anthelmintic activity of rhizomes of Acorus calamus and roots of Vitex negundo.* Pharmacologyonline 2011. **3**: p. 209-12.
36. Farrukh, A., Z. Maryam, and Iqbal, *Antimutagenic activity of methanolic extracts of four ayurvedic medicinal plants.* Indian Journal of Experimental Biology, 2008. **46**: p. 668-72.

37. Rajkumar, V., et al., *Evaluation of cytotoxic potential of A. calamus rhizome.* Ethnobotanical Leaflets 2009. **13**: p. 832-39.

38. Prisilla, D., R. Balamurugan, and H. Shah, *Antidiabetic activity of methanol extract of Acorus calamus in STZ induced diabetic rats.* Asian Pac J Trop Biomed, 2012. **2**: p. 941-6.

39. Tripathi, A.K. and R.H. Singh, *Clinical study on an indigenous drug vaca (Acorus calamus) in the treatment of depressive illness.* J Res Ayur Siddha 1995. **16**: p. 24-36.

40. Subathraa, K. and T. Poonguzhali, *In vitro studies on antioxidant and free radical scavenging activities of aqueous extract of Acorus calamus L.* Int J Curr Sci 2012. **1**: p. 69-73.

41. Patel, P., et al., *Antihypertensive effect of rhizome part of Acorus calamus on renal artery occlusion induced hypertension in rats.* J. Asian Pac J Trop Dis, 2012. **2**: p. 6-10.

42. Ahad, A.M. and I.M. Torequl, *Analgesic and cytotoxic activity of A. calamus L., Kigelia pinnata L., Mangifera indica L.and Tabernaemontana divaricata L.* Journal of Phamacy and BioAllied Sciences, 2012. **4**: p. 149-154.

43. Kim, H., T. Han, and S.G. Lee, *Anti-inflammatory activity of a water extract of Acorus calamus L. leaves on keratinocyte HaCaT cells.* J Ethnopharmacol 2009. **122**: p. 149-156.

44. Panyal, D.S., et al., *Toxicity study of ethanolic extract of A. calamus rhizome.* International Journal Green Pharmacy, 2012. **6**: p. 29-35.

45. Madan, B.R., R.B. Arora, and K. Kapila, *Anticonvulsant, antiveratrinic and antiarrhythmic actions of Acorus calamus Linn.: an Indian indigenous drug.* Pharmacodyn. ThOr., 1960. **124**: p. 201.

46. Ahad, A., M., and I.M. Torequl, *Analgesic and cytotoxic activity of A. calamus L., Kigelia pinnata L., Mangifera indica L.and Tabernaemontana divaricata L.* Journal of Phamacy and BioAllied Sciences, 2012. **4**: p. 149-154.

47. China, F.o. *Dolichandrone serrulata (Wall. ex DC.) Seem.* 1870 [cited 2014 May, 04]; Available from: http://www.tropicos.org/Name/100182549?projectid=8.

48. Sinaphet, B., et al., *Dolichandroside, a new phenolic triglycoside from Dolichandrone serrulata (DC.) Seem.* J Nat Med, 2006. **60**: p. 251-4.
49. Bounmy, S., et al., *Dolichandroside, a new phenolic triglycoside from Dolichandrone serrulata (DC.) Seem.* J Nat Med 2006. **60**: p. 251-254.
50. Bansuttee, S., et al., *Antipyretic effect of BEN-CHA-MOON-YAL remedy.* Journal health research, 2010. **24**: p. 181-5.
51. Kiratipaiboon C., et al., *Antinociceptive and anti-inflammatory effects of BEN-CHA- MOON-YAL remedy.* Journal health research 2012. **26**.
52. Pancharoen, O. and R. Klomkaew, *A new cyclohexylathanoid from Dolichandrone serrulata, Proceedings of the 1st Kamphaengaen International ,*. 2010, Bangkok, Thailand,: Natural Products Symposium.
53. Wetwitayalung, P., et al., *The study of antioxidant activities of edible flower extracts*, in *International Workshop on Medicine and Aromatic Plants*, I.A. Horticulturae, Editor. 2007: Thailand. p. 185-91.
54. Phomkaivon, N. and V. Areekul, *Screening for antioxidant activity in selected Thai wild plants.* Asian Journal of Food and Agro-Industry, 2009. **2**: p. 433-440.
55. Suppliers, P.E. *Clerodendrum paniculatum*. 2012 [cited 2014 May 03]; Available from: http://www.floridata.com/ref/c/cler_pan.cfm
56. Toxicology centre. *Medicinal Plants Used For Snake Treatment*. 2012 [cited 2014 May 03]; Available from: http://www.toxicologycentre.com/English-/plants/Botanical/-cheemaperigalam.html
57. William, W., *Handy Pocket Guide to Tropical Flowers*. Periplus Editions (HK) Ltd. ed. 2012, Tai Seng, Singpore,: PT Java Books.
58. Praveen, M., et al., *Preliminary Phytochemical, antimicrobial and toxicity studies on Clerodendrum paniculatum Linn leaves.* Hygeia.J.D.Med, 2012. **4**: p. 41-50.
59. Jeenu, J., A.R. Bindhu, and N.A. Aleykutty, *In vitro and in vivo Antiinflammatory Activity of Clerodendrum paniculatum Linn. Leaves.* Indian J Pharm Sci, 2013. **75**: p. 376-9.
60. Flora, M. *Clerodendrum paniculatum Lin Pagoda flowers*. 2010 [cited 2014 May 03]; Available from:

http://www.mynicegarden.com/2010/06/clerodendrum-paniculatum-pagoda-flower.html

61. Praveen, M., et al., *Preliminary Phytochemical, antimicrobial and toxicity studies on Clerodendrum paniculatum Linn leaves.* Journal for drugs and medicines 2012. **4**: p. 41-50.
62. Jeenu, J., A.R. Bindhu, and N.A. Aleykutty, *Antimicrobial activity of Clerodendrum paniculatum Linn. leaves.* International journal of research in Ayurveda & Pharmacy, 2011. **2**: p. 1003-4.
63. Leena, P.N. and N.A. Aleykutty, *Comparitive Study on Antibacterial Activities of Clerodendron infortunatum Linn and Clerodendron paniculatum Linn. Root Extract.* International journal of advances in Pharmacy, biology and chemistry 2012. **1**: p. 325-7.
64. Jeril, J., et al., *In vitro anticancer study of Clerodendron paniculatum. .* Pharmacologyonline, 2010. **3**: p. 384-91.
65. Jeril, J., et al., *Free radical scavenging and anticancer activity of Clerodendron paniculatum.* Pharmacologyonline, 2008. **2**: p. 730-43.
66. Arun, P.V., S. Sachin, and P. Suganyadevi, *Antioxidant potential of Clerodendrum paniculatum. .* Journal of Pharmacy Research, 2011. **4**: p. 1796-9.
67. Jeenu, J., A.R. Bindhu, and N.A. Aleykutty, *In vitro and in vivo anti-inflammatory activity of Clerodendrum paniculatum Linn. Leaves.* Indian J Pharm Sci, 2013. **75**: p. 376-9.
68. Encyclopedia of life. *Zingiber zerumbet :Classifications* 2010 [cited 2014 May 03]; Available from: http://eol.org/pages/987220/names?all=1
69. Miller, G., *ZINGIBER.* Flora of China 2000. **24**: p. 323-33.
70. Yob, N.J., et al., *Zingiber zerumbet (L.) Smith: A Review of Its Ethnomedicinal,Chemical, and Pharmacological Uses.* Hindawi Publishing Corporation Evidence-Based Complementary and Alternative Medicine., 2011. **1**: p. 1-12.

71. Sigma-Aldrich. *Zerumbone* 2010 [cited 2014 May 03]; Available from: http://www.sigmaaldrich.com/catalog/product/sigma/z3902?lang=en&region=TH

72. Hamid, A., et al., *Role Of Oxidative Stress In The Protective Effects Of Zingiber Zerumbet Smith Ethyl- Acetate Extract Against Paracetamol-Induced Hepatotoxicity In Sprague-Dawley Rats.* Australian Journal of Basic and Applied Sciences 2011. **5**: p. 1519-25.

73. Abdul, A.B.H., et al., *Anticancer activity of natural compound (Zerumbone) extracted from Zingiber zerumbet in Human HeLa Cervical Cancer cells.* International Journal of pharmacology 2008. **4**: p. 160-8.

74. Abdul, H.Z., et al., *Nephroprotective effects of Zingiber zerumbet Smith ethyl acetate extract against paracetamol-induced nephrotoxicity and oxidative stress in rats.* Journal of Zhejiang University Sciences B, 2012. **13**: p. 176-185.

75. Somchit, M.N., et al., *Anti-pyretic and analgestic activity of Zingiber zerumbet.* International Journal of pharmacology, 2005. **1**: p. 277-280.

76. Sulaiman, M.R., et al., *Preliminary analysis of the antinociceptive activitity of Zerumbone.* Fitoterpia 2009. **80**: p. 230-2.

77. Sulaiman, M.R., et al., *Antinociceptive activity of the essential oil of Zingiber zerumbet.* Planta med, 2010. **76**: p. 106-12.

78. Sulaiman, M.R., et al., *Anti-inflammatory effect of zerumbone on acute and chronic inflammation models in mice.* Fitoterapia, 2010. **81**: p. 855-8.

79. Shakhawoat, H., et al., *Cytotoxicity of the rhizome of medicinal plants.* Asian Pacific Journal of Tropical Biomedicine, 2012: p. 125-7.

80. Rumiza, A.R. and H.L.P. Azimahtol, *The antiproliferative effects of Zingiber zerumbet extracts and fractions on the growth of human breast carcinoma cell lines.* Malaysian Journal of Pharmaceutical Sciences 2005. **3**: p. 45-52.

81. United States Department of Agriculture. *Zingiber montanum (J. Koenig) Link ex A. Dietr.:Cassumunar ginger*. 2012 [cited 2014 May 04]; Available from: http://plants.usda.gov/core/profile?symbol=ZIMO2

82. Global Information Hub On Integrated Medicine. *Zingiber cassumunar*. 2010 [cited 2014 May 04]; Available from:

http://www.globinmed.com/index.php?option-=com_content&view=article&id=79238:zingibercassumunar&catid=199-&Itemid=139

83. Ajit, K.B., S. Jaroslava, and A.L. Piet, *Essential Oils of Zingiber cassumunar Roxb. from Northeast India.* Journal of Essential Oil Research 1999. **11**: p. 441-5.

84. Rattima, J., et al., *Anti-inflammatory activity of (E)-1-(3,4-dimethoxyphenyl) butadiene from Zingiber cassumunar Roxb.* Journal of Ethnopharmacology, 2003. **87**: p. 143-8.

85. Hans, W., *Phytochemistry and pharmacology of plants from the ginger family, Zingiberaceae.* Southern Cross University ePublications@SCU, 2008: p. 49-51.

86. compound, P. *Sabinene* 2013 [cited 2014 May 04,]; Available from: http://pubchem.ncbi.nlm.nih.gov/rest/chemical/sabinene

87. Sigma-Aldrich. *(−)-Terpinen-4-ol*. 2013 [cited 2014 May 04,]; Available from: http://www.sigmaaldrich.com/catalog/product/aldrich/11584?lang=en&region=TH.

88. Pubchem compound. *cassumunin A*. 2014 [cited 2014 May 04]; Available from: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=10460395

89. Pubchem compound. *cassumunin B*. 2013 [cited 2014 May 04]; Available from: http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=10054109

90. Saowaluck, B. and P. Yingyong, *Essential Oil and Antioxidant Activity of Cassumunar Ginger(Zingiberaceae: Zingiber montanum (Koenig) Link ex Dietr.) Collected from Various Parts of Thailand.* Nat. Sci., 2009. **43**: p. 467-75.

91. Udomlak, S., et al., *Chemical Composition and Physical Properties of Oil from Plai (Zingiber cassumunar Roxb.) Obtained by Hydro Distillation and Hexane Extraction.* Kasetsart J. (Nat. Sci.) 2009. **43**: p. 212-7.

92. Mohammad, N., et al., *Volatile constituents of essential oils isolated from leaf and rhizome of Zingiber cassumunar Roxb.* Bangladesh Journal of Pharmacology, 2008. **3**.

93. Saowaluk, B.I. and Yingyong, *Essential oil and antioxidant activity of Cassumunar Ginger (Zingiberaceae: Zingiber montanum (Koenig) Link ex*

Dietr.) collected from various parts of Thailand. Kasetsart Journal 2009. **43**: p. 467-75.

94. Pithayanukul, P., J. Tubprasert, and M. Wuthi-Udomlert, *In vitro antimicrobial activity of Zingiber cassumunar (Plai) oil and a 5% Plai oil gel.* Phytotherapy research, 2007. **21**: p. 146-9.
95. Koba, K., et al., *In vitro cytotoxic activity of Cymbopogon citrates L. and Cymbopogon nardus L. essential oils from Togo.* A Journal of the Bangaladesh Pharmacological Society 2009. **4**: p. 29-34.
96. Taroeno, B.J.J. and J.H.L. Zwaving *Analysis of the Essential Oil of Zingiber cassumunar Roxb. from Indonesia.* FLAVOUR AND FRAGRANCE JOURNA, 1991. **6**: p. 161-3.
97. Vimala S., A.W. Norhanom, and M. Yadav, *Anti-tumour promoter activity in Malaysian ginger rhizobia used in traditional medicine.* Br J Cancer, 1999. **80**: p. 110-6.
98. Flora of China. *Cymbopogon nardus*. 1899 [cited 2014 May 04]; Available from: http://efloras.org/florataxon.aspx?flora_id=2&taxon_id=200025100
99. Encyclopedia of life. *Cymbopogon nardus*. 2010 [cited 2014 May 03]; Available from: http://eol.org/pages/1114868/names?all=1
100. Lee, S.W. and W. Wendy, *Chemical composition and antimicrobial activity of Cymbopogon nardus citronella essential oil against systemic bacteria of aquatic animals.* Iranian Journal of microbiology 2013. **5**: p. 147-52.
101. Sigma-Aldrich. *(S)-(−)-Citronellal*. 2013 [cited 2014 May 03]; Available from: http://www.sigmaaldrich.com/catalog/product/aldrich/373753?lang=en&region=TH. .
102. Luangnarumitchai, S., S. Lamlertthon, and W. Tiyaboonchai, *Antimicrobial activity of essential oils against five strais of Propionibacterium acnes.* Mahidol University Journal of Pharmaceutical Sciences, 2007. **34**: p. 60-64.
103. Kazuhiko, N., et al., *Chemical composition and antifungal activity of Essential oil from Cymbopogon nardus (citronella Grass).* Japan International Research Center for Agricultural Sciences, 2003. **37**: p. 249-52.

104. Nakahara, K., et al., *Chemical Composition and Antifungal Activity of Essential Oil from Cymbopogon nardus (Citronella Grass).* Japan Agricultural Research Quarterly, 20. **37**: p. 549-53.

105. Organization, *W.H.O. Quality control methods for medicinal plant materials*. 1998, Geneva: document WHO/EDM/TRM/98.1.

106. Sherma, J. and B. Fried, *Handbook of Thin Layer Chromatography*. 1991, New York: Marcel Dekker.

107. McMaster, M. and McMaster C. , *GC/MS: a practical user's guide*. 1998, New York: VCH Publishers.

108. Montanher, A.B.P., M.G. Pizzolatti, and I.M.C. BRIGHENTE, *Application of the Brine Shrimp Bioassay for General Screening of Brazilian Medicinal Plants.* An Acta Farm. Bonaerense, 2002. **21**: p. 175-8.

109. Ames, B.N., D.L. Frank, and W. E.D., *An improved bacterial test system for the detection and classification of mutagens and carcinogens.* Proc. Natl. Acad. Sci. , 1973. **70**: p. 782-6.

110. Levin, D.E., et al., *A new Salmonella tester strain (TA102) with A:T base pairs at the site of mutation detects oxidative mutagens.* Proc. Natl. Acad. Sci., 1982. **79**: p. 7445-9.

111. Levin, D.E., E. Yamasaki, and B.N. Ames, *A new Salmonella tester strain for the detection of frameshift mutagens: a run of cytosines as a mutational hot-spot.* Mutation Research, 1982. **94**: p. 315-30.

112. Liao, W., A.M. Mcnutt, and W. Guo Zhu, *The comet assay: A sensitive method for detecting DNA damage in individual cells.* Methods 2009. **48**: p. 46-53.

113. Toprasri, P., C. Chinpaisal, and T. Phaechamud, *Comet Assay to Test Antioxidative Effects of Extracts from Different Parts of Areca catechu L. .* Thai Pharm Health Sci J, 2008. **3**(3): p. 309-15.

114. Singh, N.P., et al., *A simple technique for quantitation of low levels of DNA damage in individual cells.* Cell Res 1988. **175**: p. 184-191.

115. Ricardo, C.B., et al., *The comet assay: A simple method to measure cell viability in proliferation and cytotoxicity assays.* Braz Oral Res, 2009. **23**: p. 255-62.

116. Hassain, E., C.S. Mandal, and Gupta JK., *Phytochemical screening and in-vivo antipyretic activity of the methanol leaf extract of Bombax Malabonium DC (Bombacaceae).* Tropical Journal of Pharmaceutical Research, 2011. **10**(1): p. 55-60.

117. Jie, S., et al., *Measurement of Nitric Oxide Production in Biological Systems by Using Griess Reaction Assay.* Sensors 2003. **3**: p. 276-84.

118. Laboratories, I., *ELISA Technical Guide*. 2013, Maine, USA.

119. Abcam company, *ab46105 – TNF alphaMouse ELISA Kit.* 2013, UK.

120. Eagle Biosciences, *Human Interleukin 1 Alpha (IL-1α) ELISA Assay Kit*. 2014, Inc Nashua: United States.

121. Invitrogen Corporation, *Human IL-1α*. 2014, Camarillo.

122. BIOTECH, C. *Mouse Prostaglandin E2 (PGE2) ELISA Kit*. 2014 [cited 2014 May 03]; Available from: http://www.cusabio.com/

123. Gentaur Molecular product, *Mouse Prostaglandin E2(PGE2) ELISA Kit*. 2014, Kampemhour, Belguim.

124. Laupattarakasem, P., et al., *An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis.* Journal of Ethnopharmacology 2003. **85**: p. 207-15.

125. Santos, F. and V. Rao, *A study of the anti-pyretic effect of quinine, an alkaloid effective against cerebal malaria, in fever induced by bacterial endotoxin and yeast in rats.* J of pharmacy and pharmacology 1998. **50**(2): p. 225-9.

126. Rafaela, F.C., et al., *Pharmacological and Molecular Characterization of the Mechanisms Involved in Prostaglandin E2-Induced Mouse Paw Edema.* The Journal Of Pharmacology And Experimental Therapeutics. , 2006. **318**: p. 611-8.

127. PERCIVAL M., *Antioxidants* CLINICAL NUTRITION INSIGHTS, 1998. **10**: p. 1-4.

128. Andressa, B., et al., *Application and Analysis of the Folin Ciocalteu Method for the Determination of the Total Phenolic Content from Limonium Brasiliense L.* Molecules 2013. **18**: p. 6852-5.

129. Milan, S.S. and Kragujevac, *Total Phenolic Content, Flavonoid Concentration And Antioxidant Activity Of Marrubium Peregrinum L. Extracts.* J. Sci., 2011. **33**: p. 63-72.

130. Michael, A., et al., *Methods for testing antioxidant activity.* Analyst 2002. **127**: p. 183-198.

131. Dharmendra, S., et al., *Nitric Oxide radical scavenging assay of bioactive compounds present in methanol Extract of Centella asiatica.* International Journal of Pharmacy and Pharmaceutical Science Research, 2012. **2**(3): p. 42-44.

132. Ince, I., et al., *Effects of Pinus brutia bark extract and Pycnogenol® in a rat model of carrageenan induced inflammation.* Phytomedicine 2009. **16**(12): p. 1101-4.

133. Chandra, M.S., et al., *Metal ion chelating activity and hydrogen peroxide scavenging activity of medicinal plant Kalanchoe pinnata.* Journal of Chemical and Pharmaceutical Research, 2012. **4**(1): p. 197-202.

134. Meyer, B.N., et al., *Brine shrimp: A convenient General Bioassay for Active Plant Constituents.* Journal of Medicinal Plant Research 1982. **45**: p. 31-34.

135. Kristien, M.E.Z., *The Ames Salmonella/microsome mutagenicity assay.* Mutation Research 2000. **455**: p. 29-60.

136. Levin, D.E., E. Yamasaki, and B.N. Ames, *A new Salmonella tester strain for the detection of frameshift mutagens: a run of cytosines as a mutational hot-spot.* Mutation Research 1982. **94**: p. 315-30.

137. Maron, D. and B.N. Ames, *Revised methods for the Salmonella mutagenicity test.* Mutation Research, 1983. **113**: p. 173-215.

138. Reid, K.A., et al., *Mulholland and L. Verschaeve. Evaluation of the mutagenic and antimutagenic effects of South African plants.* Journal of Ethnopharmacology 2006. **106**: p. 44-50.

139. Kotaro Aoyama, K.I.N.M., *Application of Euglena gracilis cells to comet assay: evaluation of DNA damage and repair.* Mutation Research 2003. **538**: p. 155-162.

140. Peggy, L.O. and P.B. Judit, *The comet assay: a method to measure DNA damage in individual cells.* Nature Protocol 2006. **1**(1): p. 23-29.

141. Vongnam, T., et al., *Inhibitory effect of Derris reticulate ethanol extract on LPS-induced macrophage activation.* Asian Biomedicine, 2013. **7**: p. 89-95.

142. Anoopkumar, D.S., et al., *Resazurin assay of radiation response in culture cells.* Br J Radiol 2005. **78**: p. 945-7.

143. Chandrasekaran, C.V., A. Gupta, and A. Agarwal, *Effect of an extract of Andrographis paniculata leaves on inflammatory and allergic mediators in vitro.* Journal of Ethnopharmacology, 2010. **129**: p. 203-7.

144. Winter, C., E. Risley, and G. Nuss, *Carrageenin-induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs*, P.o.t.S.f.E.B.a. Medicine, Editor. 1962. p. 544-7.

145. Dongmo, A.B., et al., *Anti-inflammatory and analgesic properties of the stem bark extract of Mitragyna ciliate in rats.* Journal of Ethnopharmacology, 2003. **84**: p. 17-21.

146. Goldenberg, M.M. and A.C. Ilse, *Anti-inflammatory activity of EU-2972.* Achives of International Pharmacodynamics, 1977. **228**: p. 153-61.

147. Chu, D. and B.A. Kovacs, *Anti-inflammatory activity in Oakgal extracts. .* Achives of International Pharmacodynamics 1977. **230**: p. 166-76.

148. Sachindra, N., et al., *Radical scavenging and singlet oxygen quenching activity of extracts from Indian seaweeds.* J Food Sci Technol, 2010. **47**: p. 94-9.

149. Brand-Williams, W., M. Cuvelier, and C. Berset, *Use of a free radical method to evaluate antioxidant activity.* Lebensm Wiss u Technol 1995. **28**(1): p. 25-30.

150. Benzie, I.F. and J.J. Strain, *The ferric reducing ability of plasma (FRAP) as a measure of avtioxidant power : the FRAP assay.* Analytical biochemistry, 1996. **239**: p. 70-6.

151. Chaea, S.Y., *Protection of insulin secreting cells from nitric oxide induced cellular damage by crosslinked hemoglobin.* Biomaterials, 2004. **25**: p. 843-50.

152. Dawson, T.M. and V.L. Dawson, *Nitric oxide: Actions and pathological roles.* The Neuroscientist, 1995. **1**: p. 7-18.

153. Sun, J., et al., *Measurement of Nitric Oxide Production in Biological systems by using Griess Reaction assay.* Sensors, 2003. **3**(8): p. 276-284.

154. Ramil, S., K.I. Harada, and N. Ruangrungsi, *Antioxidant, antimicrobial and cytotoxicity activities of Acacia farnesiana (L.) Wild. leaves ethanol extract.* Pharmacognosy Journal, 2011. **3**: p. 50-58.

155. Robert, P.A., *Identification essential oil components by Gas Chromatrography/ Mass Spectrometry*. Vol. 4. 2007, Illonoise: Allured Publishing Corporation.

156. Andrew, R.C., *The Comet Assay for DNA Damage and Repair: Principles, Applications, and Limitations.* Molecular Biotechnology, 2004. **26**: p. 249-61.

157. Saurabh, S., M. Lalit, and M.G. Chauhan, *Pharmacognostic study of male leaves of Trichosanthes dioica Roxb. with special emphasis on microscopic technique.* Journal of Pharmacognosy and Phytotherapy 2010. **2**(5): p. 71-5.

158. Ragunathan, M. and R. Senthamarai, *Comprehensive Anatomical Investigation of Root of Plumbago Roseus Linn.* Iranian Journal of Pharmaceutical Sciences. , 2013. **9**(3): p. 45-54.

159. Chuakul, W., P. Saralamp, and Supatarawanich P., *Pharmacognostic Characters of Plumbago indica Linn. .* Mahidol University Journal of Pharmaceutical Sciences, 1994. **21**.

160. Aimon, S., *Specification of Thai medicinal plants*, Faculty of Pharmacy, Editor. 1986: Mahidol University

161. Wallis, T.E., *Textbook of pharmacognosy*, ed. 5. 2005, New Delhi: CBS pudlisher $ Distributors.

162. Llanchezhian, R., et al., *Pharmacognostical and physicochemical analysis of Bhallataka (Semecarpus anacardium Linn.)* Fruit. Phcog J. , 2011. **3**(20): p. 9-16.

163. Sciences, D.o.M., *Chemical specification of Thai herbal drugs*. Vol. 1. 1993, Bangkok: Ministry of Public Health.

164. Kaushal, A. and R. Arora, *Pharmacognostic Study of Acorus Calamus.* Int J Ayu Pharm Chem, 2015. **2**(1).

165. Rout, O.P., et al., *Pharmacognostical and phytochemical studies of Zingiber zermbet (L.) SM. Rhizome.* IJRAP, 2011. **2**(3): p. 689-703.

166. นิรอปิยะด์, น. การทำมาตรฐานของสมุนไพรตะไคร้หอมและน้ำมันตะไคร้หอม. in *Thailand research symposium 2010 proceedings poster presentation*. 2010. ศูนย์ประชุมบางกอกคอนเวนชันเซ็นเตอร์ เซ็นทรัลเวิลด์, กรุงเทพมหานคร: หจก. อรุณการพิมพ์.

167. Chingakham B.S., et al., *Chemical composition and biological activity of the essential oil of rhizome of Zingiber zerumbet (L.) Smith. .* Pharmacognosy and Phytochemistry 2014. **3**(3).

168. Nazrul, I.B., U.C. Jasim, and B. Jaripa, *Chemical investigation of the leaf and rhizome essential oils of Zingiber zerumbet (L.) Smith from Bangladesh.* Bangladesh J Pharmacol 2009. **4**: p. 9-12.

169. Murakami, A., M. Miyamoto, and H. Ohigashi, *Zerumbone, an anti-inflammatory phytochemical, induces expression of proinflammatory cytokine genes in human colon adenocarcinoma cell lines.* Biofactors, 2004. **21**((1-4)): p. 95-101.

170. Radhakrishnan, R., et al., *Antinociceptive properties of zerumbone, a natural compound found in ginger.* The journal of pain, 2012. **13**(4): p. S72.

171. Sulaiman, M.R., et al., *Anti-inflammatory effect of zerumbone on acute and chronic inflammation models in mice.* Fitoterapia, 2010. **81**: p. 855-8.

172. Uraiwan, S., et al., *Antimalarial activity and cytotoxicity of zerumbone derivatives.* ScienceAsia, 2013. **39**: p. 95-9.

173. Elizabeth, S.F., et al., *Anti-inflammatory effects of compounds alpha-humulene and (–)-trans-caryophyllene isolated from the essential oil of Cordia verbenacea.* European Journal of Pharmacology, 2007. **569**(3): p. 228-36.

174. Adil, H., et al., *Cytotoxic activity of α-humulene and transcaryophyllene from Salvia officinalis in animal and human tumor cells.* An. R. Acad. Nac. Farm., 2010. **76**(3): p. 343-56.

175. Koba, K., et al., *Chemical Composition and in vitro Cytotoxic Activity of Essential Oils from Two Tropical Lamiaceae: Aeollanthus pubescens Benth.*

and Ocimum gratissimum L. A Journal of the Bangaladesh Pharmacological Society, 2009. **4**: p. 29-34.

176. Benzi, V., N. Stefanazzi, and A.A. Ferrero, *Biological activity of essential oils from leaves and fruits of pepper tree (Schinus molle L.) to control rice weevil (Sitophilus oryzae L.).* Chilean journal of agricultural research, 2009. **69**: p. 154-9.
177. El-Moaty, H.I.A., *Essential oil and iridoide glycosides of Nepeta septemcrenata Erenb. .* Journal of Natural Products, 2010. **3**: p. 103-11.
178. Ranaweera, S.S. and Dayananda. K.R., *MOSQUITO-LARVICIDAL ACTIVITY OF CEYLON CITRONELLA (Cymbopogon nardus L. Rendel oil fractions).* J. Natn. Sci. Coun. Sri Lanka, 1996. **24**: p. 247-252.
179. Asakura, K., et al., *alpha-eudesmol, a P/Q-type Ca(2+) channel blocker, inhibits neurogenic vasodilation and extravasation following electrical stimulation of trigeminal ganglion.* Brain Res. , 2000. **873**(1): p. 94-101.
180. Asakura, K., et al., *omega-agatoxin IVA-sensitive Ca(2+) channel blocker, alpha-eudesmol, protects against brain injury after focal ischemia in rats.* Eur J Pharmacol., 2000. **394**(1): p. 57-65.
181. Sukatta, U., et al., *Chemical composition and physical properties of oil from plai (Zingiber Cassumunar Roxb.) obtained by hydro distillation and hexane extraction.* Kasetsart J. (Nat. Sci.), 2009. **43**: p. 212-7.
182. Francesca, M., et al., *In vivo activity of terpinen-4-ol, the main bioactive component of Melaleuca alternifolia Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic Candida species.* BMC Infectious Diseases 2006. **6**: p. 158.
183. Giwanon, R., et al., *Antimicrobial activity of terpinen-4-ol and sabinene.* Thai J Pharm Sci, 2000. **24**.
184. Kotchaphan, C., et al., *Dermal Pharmacokinetics of Terpinen-4-ol Following Topical Administration of Zingiber cassumunar (plai) Oil.* Planta Med, 2012.
185. Wasuwat, S., et al., *Studies on antimicrobial and antifungal activities of terpinen-4-ol extracted from Zingiber cassumunar Roxb.* , in *Thailand Institute of Scientific and Technological Research* 1989. p. 30-32.

186. Mahesh, P., et al., *Composition and anti-microbial activity of essential oil of Myristica fragrans from Andaman Nicobar Island. .* Int. J. of Pharm. & Life Sci. (IJPLS), 2011. **2**(10): p. 1115-7.

187. Min-Jin, K., et al., *Chemical composition and anti-inflammatory effects of essential oil from hallabong flower.* EXCLI Journal 2013. **12**: p. 933-942.

188. Raina, V.K., S.K. Srivastava, and K.V. Syamasunder, *Essential oil composition of Acorus calamus L. from the lower region of the Himalayas.* Flavour Fragr. J. , 2003. **18**.

189. Wangsittidet, T., et al., *Chemical constituent and biological activities of essential oil from Eugenia aromatic, Kaemferia galangal and Acorus calamus.* Thai Journal of Pharmeceutical Sciences, 2007. **31**: p. 23.

190. Guriev, A.M., S.M. Yusubov, and A.V. Tkachev, *Chemical study of essential oil from Acorus calamus L.* International conference on Natural Products and Physiologiccally Active Subtance, 2004. **1**.

191. Liu, X., et al., *Identification of insecticidal constituents of the essential oil of Acorus calamus rhizomes against Liposcelis bostrychophila Badonnel.* Molecules, 2013. **18**(5): p. 5684-96.

192. Venskutonis, P.R. and A. Dagilyte, *Composition of Essential Oil of Sweet Flag (Acorus calamus L.) Leaves at Different Growing Phases.* Journal of Essential Oil Research, 2003. **15**(5): p. 313.

193. Divya, G., et al., *Pharmacological Activities of Acorus calamus: A Review. .* Asian Journal of Biochemical and Pharmaceutical Research, 2011. **1**(4).

194. Tiwari, N., et al., *Antimicrobial activity of ß-asarone from Acorus calamus leaves.* International Journal of Chemical and Analytical Science, 2010. **1**(9): p. 211.

195. Devi, A. and D. Ganjewala, *Antimicrobial activity of Acorus calamus (L.) rhizome and leaf extract.* Acta Biol Szeged, 2009. **53**(1): p. 45.

196. Phongpaichit, S., et al., *Antimicrobial activities of the crude methanol extract of Acorus calamus Linn.* Songklanakarin J. Sci.Technol, 2005. **27**.

197. Pascal, U., F. Matthias, and Melzig, *Comparative Study of the Cytotoxicity and Genotoxicity of Alpha- and Beta-Asarone.* Sci Pharm., 2012. **80**(3): p. 663-668.

198. Yuan-Xiao, Y., et al., *Beta-asarone, a major component of Acorus tatarinowii Schott, attenuates focal cerebral ischemia induced by middle cerebral artery occlusion in rats.* BMC Complementary and Alternative Medicine 2013. **2013**(13): p. 236.

199. Lilybeth, F., Olowa, and M.N. Olga, *Brine Shrimp Lethality Assay of the Ethanolic Extracts of Three Selected Species of Medicinal Plants from Iligan City, Philippines.* International Research Journal of Biological Sciences, 2013. **2**(11): p. 74-7.

200. Price, K.S., G.T. Waggy, and R.A. Conway, *Brine shrimp bioassay and seawater BOD of petrochemicals.* BJ Water Pollut Control Fed, 1974. **46**(1): p. 63-77.

201. Logarto, P., et al., *Comparative study of the assay of Artemia salina L. and the estimate of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts.* Phytomedicine, 2001. **8**(5): p. 395-400.

202. Padmaja, R., et al., *Brine shrimp lethality bioassay of selected Indian medicinal plants.* Fitoterapia, 2002. **73**: p. 508-10.

203. Khan, M. and M. Islam, *Analgesic and cytotoxic activity of Acorus calamus L., Kigelia pinnata L., Mangifera indica L. and Tabernaemontana divaricata L.* J Pharm Bioallied Sci. , 2012. **4**(2): p. 149-54.

204. Arunachalam, M. and S. Nirmal, *Acute and sub-acute oral toxicity profile of Acorus calamus (Sweet flag) in rodents.* Asian Pacific Journal of Tropical Biomedicine, 2012: p. S1017-S1023.

205. Payal, D.S., et al., *Toxicity study of ethanolic extract of Acorus calamus rhizome.* IJGP, 2012. **6**(1): p. 29-35.

206. Manohan, R., C. Palanuvej, and N. Ruangrungsi, *Genotoxic and cytotoxic effects of Ben-Cha-Moon-Yai remedy and it's ingredients.* Research Journal of Pharmaceutical, Biological and Chemical, 2013. **4**: p. 1144-55.

207. Augusto, B., et al., *Evaluation of larvicidal activity and brine shrimp toxicity of rhizome extracts of Zingiber zerumbet (L.) Smith.* Rev. Soc. Bras. Med. Trop. , 2013. **46**(3).

208. Anish, N., B. Maumita, and M. Anita, *Antioxidant Activities and Cytotoxicity of Zingiber zerumbet (L.) Smith Rhizome.* Journal of Pharmacognosy and Phytochemistry 2013. **2**(3): p. 102-8.

209. Hai-xia, Z., D. Guan-hua, and Z. Jun-tian, *Assay of mitochondrial functions by resazurin in vitro.* Acta Pharmacol Sin., 2004. **25**(3): p. 385-9.

210. Nakayama, G.R., et al., *Assessment of the Alamar Blue assay for cellular growth and viability in vitro.* J Immunol Methods., 1997. **204**(2): p. 205-8.

211. Nociari, M., et al., *A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity.* J Immunol Methods., 1998. **213**(2): p. 157-67.

212. O'Brien, J., et al., *Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity.* Eur J Biochem., 2000. **267**(17): p. 5421-6.

213. Voytik-Harbin, S.L., et al., *Application and evaluation of the Alamar Blue assay for cell growth and survival of fibroblasts. In Vitro Cell. .* Dev. Biol. Animal 1998. **34**(239-46).

214. Seewaboon, S., et al., *Safety Evaluation of Zingiber cassumunar Roxb. Rhizome Extract: Acute and Chronic Toxicity Studies in Rats.* Hindawi Publishing Corporation International Scholarly Research Notices 2014.

215. Panyathanya, R., et al., *Study of Acute and Chronic Toxicity of Phai (Zingiber Cassumunar Roxb)in Rats. .* Siriraj Hospitol Gazette, 1986. **38**: p. 413-6.

216. Chia Ju, C., et al., *Acute and 28-Day Subchronic Oral Toxicity of an Ethanol Extract of Zingiber zerumbet (L.) Smith in Rodents. .* Evidence-Based Complementary and Alternative Medicine 2012. **2012**.

217. Abenaa, A.A., et al., *Comparative chemical and analgesic properties of essential oils of cymbopogon nardus (l) rendle of benin and congo.* Afr. J. Trad. CAM, 2007. **4**(2): p. 267-72.

218. Joseph, J., A.R. Bindhu, and N.A. Aleykutty, *In vitro and in vivo anti-inflammatory activity of clerodendrum paniculatum Linn.* Indian J Pharm Sci, 2013. **75**: p. 376-9.

219. White, R.D., et al., *An evaluation of acetone extracts from six plants in the Ames mutagenicity test. .* Toxicol Lett, 1983. **15**: p. 25-31.

220. Farrukh, A., Z. Maryam, and A. Iqbal, *Antimutagenic activity of methanolic extracts of four ayurvedic medicinal plants.* Indian Journal of Experimental Biology 2008. **46**: p. 668-72.

221. Hashmat, I., et al., *Sweet flag (Acorus calamus Linn.): An incredible medicinal herb.* International Journal of Green Pharmacy 2013. **17**(4): p. 288-96.

222. Chamarro, G., et al., *Dominant lethal study of α-asarone in male mice.* Toxicology Letters, 1998. **95**: p. 71-72.

223. Hsia, M., J. Adamovic, and B. Kreamer, *Microbial mutagenicity studies of insect growth regulators and other potential in salmonella typhimurium.* Chemosphere, 1979. **8**(521-9).

224. Keller, K. and E. Stahi, *Composition of the essential oil from beta-asarone free calamus.* Plant Medica, 1983. **47**: p. 71-4.

225. Rojanapo, W., A. Tepsuwan, and P. Siripong, *Mutagenic and antimutagenicity of Thai medicinal plants.* Basic life sci 1990. **52**: p. 447-52.

226. Lenora, R., et al., *Investigation of plumbagin content in Plumbago indica Linn. Growm under different growing system.* Pharmacologia 2012. **3**(2): p. 57-60.

227. Hakura, A., et al., *Mutagenicity and cytotoxicity of naphthoquinones for Ames Salmonella tester strains. .* Chem Res Toxicol 1994. **7**(4): p. 559-67.

228. Tikkanen, L., et al., *Mutagenicity of natural napthoquinones and benzoquinones in the salmonella/microsome test.* Mutat Res 1983. **124**(1): p. 25-34.

229. Durga, R., P. Sridhar, and H. Polasa, *Antimutagenic activity of plumbagin in Ames Salmonella typhimurium test.* Indian J Med Res 1992. **96**: p. 143-5.

230. Duanghatai, P. and K. Keaw, *Roles of Mangosteen, Durian Products and Their Combinations in Reduction of Mutagenesis and Mutagen Formation: Studies on Nitrite Treated 1-Aminopyrene.* Thai J Toxicology, 2009. **24**(1): p. 7-16.

231. Wongwattanasathien, O., K. Kangsadalampai, and L. Tongyonk, *Antimutagenicity of some flowers grown in Thailand.* Food and Chemical Toxicology, 2010. **48**: p. 1045-51.

232. Higashimoto, M., et al., *Mutagenicity and antimutagenicity of extracts of three spices and a medicinal plant in Thailand.* Mutat. Res. , 1993. **303**: p. 135-142.

233. Linna, T., et al., *Mutagenicity and anti-mutagenicjty of thai traditional medicine: Ya-Rid-Si-Duang-Mahakal.* Thai J Health Res, 2006. **20**: p. 155-168.

234. Preeyakamol, M., et al., *Effects of selected Thai ancient remedies extracts on mutagenicity and antimutagenicity using ames test.* J Health Res, 2012. **26**: p. 173-7.

235. Chia Ju, C., et al., *Absence of Genotoxic andMutagenic Effects of Zingiber zerumbet (L.) Smith (Zingiberaceae) Extract.* Hindawi Publishing Corporation Evidence-Based Complementary and Alternative Medicine. **2012**.

236. Pereira, T.S., et al., *In vitro genotoxicity of Melaleuca alternifolia essential oil in human lymphocytes.* J Ethnopharmacol., 2014. **151**(2): p. 852-7.

237. Brambilla, G. and A. Martelli, *Genotoxic and carcinogenic risk to humans of drug-nitrite interaction products.* Mutat Res, 2007. **635**: p. 17-52.

238. Mirvish, S., *Blocking the formation of N-nitroso compounds with ascorbic acid in vitro and in vivo.* Cancer Res 1994. **54**: p. 175-80.

239. Sugimura, T. and S. Sato, *Bacterial mutagenicity of natural materials, pyrolysis products and additives in foodstuffs and their association with genotoxic effects in mammals.* Dev Toxicol Environ Sci, 1983. **11**: p. 115-33.

240. Kyrtopoulos, S.A., *Ascorbic acid and the formation of N-nitroso compounds: possible role of ascorbic acid in cancer prevention.* Am J Clin Nutr. , 1987. **45**: p. 1344-50.

241. Mirvish, S.S., *Formation of N-nitroso compounds: chemistry, kinetics and in vivo occurrence.* ToxicoL Appl. Pharmacol 1975. **31**: p. 325-51.

242. Bagwe, A.N., et al., *Evaluation of the mutagenicity of 'pan masala', a chewing substitute widely used in India.* Mutat Res., 1990. **241**(4): p. 349-54.

243. Wenjuan, L., et al., ***The comet assay: A sensitive method for detecting DNA damage in individual cells.*** Methods, 2009. **48**: p. 46-53.

244. Pascal, U., F. Matthias, and Melzig, *Comparative Study of the Cytotoxicity and Genotoxicity of Alpha- and Beta-Asarone.* Sci Pharm., 2012. **80**(3): p. 663-8.

245. Kangsadalampai, K., C. Butryee, and K. Manoonphol, *Direct mutagenicity of the polycyclic aromatic hydrocarbon-containing fraction of smoked and charcoalbroiled foods treated with nitrite in acid solution.* Food Chem. Toxicol, 1996. **35**: p. 213-8.

246. Kato, T., et al., *Transformation of arylamines into direct-acting mutagens with nitrite.* Mutat. Res. , 1991. **249**: p. 243-54.

247. Cherdshewasart, W., et al., *The mutagenic and antimutagenic effects of the traditional phytoestrogen-rich herbs, Pueraria mirifica and Pueraria lobata.* Braz J Med Biol Res, 2009. **42**(9): p. 816-23.

248. Kumar, S., S. Gautam, and A. Sharma, *Antimutagenic and antioxidant properties og plumbagin and other napthoquinones.* Mutat Res, 2013. **755**(1): p. 30-41.

249. Santosh, K., et al., *Antibacterial and antimutagenic activities of novel zerumbone analogues. .* Food Chemistry, 2013. **141**: p. 1097-1103.

250. Tanaka, T., et al., *Chemoprevention of azoxymethane-induced rat aberrant crypt foci by dietary zerumbone isolated from Zingiber zerumbet.* Life Sci., 2001. **69**(16): p. 1935-45.

251. Jung, C.L., S.D. Jeng, and S.C. Chuan, *Anti-inflammatory activities of Cinnamomum cassia constituents in vitro and in vivo.* Evidence-Based Complementary and Alternative Medicine, 2012. **1**: p. 1-12.

252. Jinfeng, W.U., D.U. Juan, and X.U. Changqing, *In vivo and in vitro anti-inflammatory effect of a novel derivative of icariin.* Immunopharmacology and Immunotoxicology 2011. **33**(1): p. 49-54.

253. Sibi, P.I., K.V. Jobin, and M.S. Jissa, *Anti-inflammatory and analgesic activities of Plumbago capensis and Plumbago indica.* Adv. Pharmacol. Toxicol 2012. **13**(1): p. 47-52.

254. Tinyu, W., W. Feihua, and J. Zhigui, *Plumbagin inhibit LPS-induced inflammation through the inactivation of the nuclear factor-kappa B and mitogen activated protein kinase signaling pathways in RAW 264.7 cells. .* Food and Chemical Toxicology, 2014. **64**: p. 177-83.

255. Sagheer, A., et al., *Pharmacological basis of the use of Acorus calamus L. in inflammatory diseases and underlying signal transduction pathways. .* Boletin Latinoamericano y del Caribe de Plantas Medicinales Aromaticas 2014. **13**(1): p. 38-46.

256. Hyeri, K., H.H. Tae, and G.L. Seong, *Anti-inflammatory activity of a water extract of Acorus calamus L. leaves on keratinocyte HaCaT cells.* Journal of Ethnopharmacology, 2009. **122**: p. 149-56.

257. Joseph, J., A.R. Bindhu, and N.A. Aleykutty, *In vitro and in vivo anti-inflammatory activity of clerodendrum paniculatum Linn.* Indian J Pharm Sci 2013. **75**: p. 376-9.

258. Di Rosa, M., J.P. Giroud, and D.A. Willoughby, *Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine.* The Journal of pathology. , 1971. **104**(1): p. 15-29.

259. WINTER, C.A., E. RISLEY, and G.W. NUSS, *Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs.* Proc Soc Exp Biol Med. , 1962. **111**: p. 544-7.

260. Levy, L., *Carrageenan paw edema in the mouse.* Life Sci, 1969. **8**(11): p. 601-6.

261. Posadas, I., et al., *Carrageenan-induced mouse paw oedema is biphasic, age-weight dependent and displays differential nitric oxide cyclooxygenase-2 expression.* Br J Pharmacol., 2004. **142**(2): p. 331-8.

262. François, N., et al., *Distribution and regulation of cyclooxygenase-2 in carrageenan-induced inflammation.* British Journal of Pharmacology 1999. **128**(4): p. 853-9.

263. Vinegar, R., W. Schreiber, and R. Hugo, *Biphasic development of carrageenin edema in rats.* J Pharmacol Exp Ther., 1969. **166**(1): p. 96-103.

264. Masahiro, I., et al., *Involvement of Nitric Oxide in a Rat Model of Carrageenin-Induced Pleurisy.* Mediators of Inflammation, 2010.

265. Paul, G., et al., *Inflammation Protocols.* Methods in Molecular Biology 2003. **225**.

266. Zakaria, Z.A., et al., *Antiinflammatory and antinociceptive activities of Zingiber zerumbet methanol extract in experimental model systems.* Med Princ Pract. , 2010. **19**(4): p. 287-94.

267. Claudino, R., et al., *Pharmacological and molecular characterization of the mechanisms involved in prostaglandin E2-induced mouse paw edema.* J Pharmacol Exp Ther., 2006. **18**(2): p. 611-8.

268. Park, J.Y., M. Pillinger, and S.B. Abramson, *Prostaglandin E2 synthesis and secretion: the role of PGE2 synthases.* Clin Immunol., 2006. **119**(3): p. 229-40.

269. Nhareet, S. and S. Nur, *Anti inflammatory property of ethanol and water extracts of zingiber zerumbet.* Indian Journal of Pharmacology 2003. **35**: p. 181-2.

270. Fredric, J. and Pashkow, *Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention?* SAGE-Hindawi Access to Research International Journal of Inflammation, 2011. **1**: p. 1-9.

271. Kira, G.H., et al., *Modeling inflammation and oxidative stress in gastrointestinal disease development using novel organotypic culture systems.* Stem Cell Research & Therapy, 2013. **4**(1): p. 1-5.

272. Filomena, C., et al., *In vivo anti-inflammatory and in vitro antioxidant activities of Mediterranean dietary plants.* Journal of Ethnopharmacology 2008. **116**: p. 144-51.

273. Nunes, P., et al., *Biological oxidations and antioxidant activity of natural products, Phytochemicals as nutraceuticals.* Global Approaches to Their Role in Nutrition and Health, 2012.

274. Mohana, C., et al., *Anti-oxidant and anti-inflammatory activity of leaf extracts and fractions of Mangifera indica.* Asian Pacific Journal of Tropical Medicine, 2013. **6**(4): p. 311-4.

275. Krishnaiah, D., R. Sarbatly, and R. Nithyanandam, *A review of the antioxidant potential of medicinal plant species.* Food Bioprod Process, 2011. **89**: p. 217-33.

276. Arun, V.P., S. Sachin, and P. Suganyadevi, *Antioxidant potential of Clerodendrum paniculatum.* Journal of Pharmacy Research, 2011. **4**(6): p. 1796.

277. Manohana, R., C. Palanuveja, and Ruangrungsi. N., *Safety efficacy and quality assessments of Ben Cha Moon Yai remedy*, in *College of Public Health Sciences*. 2012, Chulalongkorn University.

278. Naraporn, P. and A. Varipat, *Screening for antioxidant activity in selected Thai wild plants.* As. J. Food Ag-Ind. , 2009. **2**(4): p. 433-40.

279. Prior, R.L. and G. Cao, *Antioxidant phytochemicals in fruits and vegetables: diet and health implications.* HortScience, 2000. **35**(4): p. 588-92.

280. Bua-in, S. and P. Yingyong, *Essential Oil and Antioxidant Activity of Cassumunar Ginger (Zingiberaceae: Zingiber montanum (Koenig) Link ex Dietr.) Collected from Various Parts of Thailand.* Kasetsart J. (Nat. Sci.) 2009. **43**: p. 467-75.

281. Hagerman, A.E., et al., *High molecular weight plant polyphenolics (tannins) as biological antioxidants.* J. Agric. And Food Chem., 1998. **46**: p. 1887-1892.

282. Bibhabasu, H., B. Santanu, and M. Nripendranath, *Antioxidant and free radical scavenging activity of Spondias pinnata.* BMC Complementary and Alternative Medicine, 2008. **8**: p. 63.

283. Verma, A., A.K. Ahirwar, and N. Singh, *PHYTOCHEMICAL SCREENING OF METHONOLIC RHIZOMES EXTRACT OF ACORUS CALAMUS (FAMILY-ARACEAE) FOR ITS-VITRO ANTIOXIDANT ACTIVITY.* URJC, 2015. **3**(01): p. 3-8.

284. Chandana, C.B., et al., *Comparative study of the in vitro antioxidant property of different extracts of Acorus calamus Linn* J.Nat. Prod. Plant Resour., 2014. **4**(1): p. 8-18.

285. Anuthakoengkun, A. and A. Itharat, *Inhibitory effect on nitric oxide production and free radical scavenging activity of Thai medicinal plants in osteoarthritic knee treatment.* J Med Assoc Thai., 2014. **97**(8): p. 116-24.

286. Hodzic, Z., et al., *The influence of total phenols content on antioxidant capacity in the whole grain extracts.* European Journal of Scientific Research, 2009. **28**: p. 471-7.

287. Duh, P., P. Du, and G. Yen, *Action of methanolic extract of mung bean hull as inhibitors of lipid peroxidation and non-lipid oxidative damage.* Food & Chem Toxicol 1999. **37**: p. 1055-61.

288. Guo, C., et al., *Antioxidant activities of peel, pulp and seed fractions as determined by FRAP assay.* Nutrition Research, 2003. **23**: p. 1719-26.

289. Naga, S., et al., *Studies on antioxidant activity of Zingiber zerumbet spent and its constituents through in vitro models.* Journal of Food Technology, 2013. **1**(3): p. 48-55.

290. Moure, A., et al., *Natural antioxidants from residual sources.* Food Chemistry, 2001. **72**: p. 145-71.

291. Marijana, Z.K., et al., *Antiradical, Chelating and Antioxidant Activities of Hydroxamic Acids and Hydroxyureas.* Molecules, 2011. **16**: p. 6232-42.

292. Guilong, Y., et al., *Antioxidant Activities of Extracts and Fractions from Eupatorium lindleyanum DC.* Molecules 2011. **13**: p. 5998-6009.

293. Monica, R., et al., *Radical scavenging, antioxidant and metal chelating activities of Annona cherimola Mill. (cherimoya) peel and pulp in relation to their total phenolic and total flavonoid contents.* Journal of Food Composition and Analysis 2012. **25**: p. 179-84.

294. Pietta, P., *Flavonoids as antioxidants.* J Nat Prod., 2000. **63**(7): p. 1035-42.

295. Marian, N. and S. Fereidoon, *Extraction and analysis of phenolics in food.* Journal of Chromatography A, 2004. **1054**: p. 95-111.

296. Ao, C., et al., *Evaluation of antioxidant and antibacterial activities of Ficus microcarpa L. fill extract.* . Food Contrl, 2008. **19**: p. 940-8.

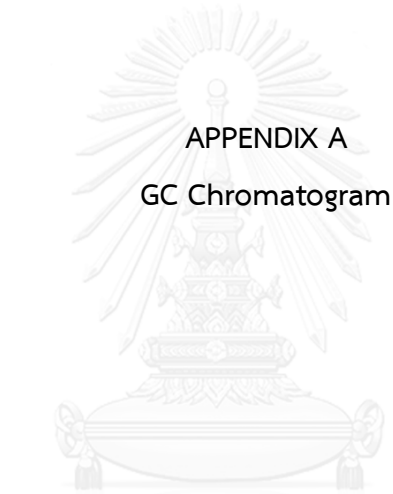
297. Ravipati, A., et al., *Antioxidant and anti-inflammatory activities of selected Chinese medicinal plants and their relation with antioxidant content.* BMC Complementary and Alternative Medicine, 2012. **12**: p. 173.

298. Velioglu, Y.S., et al., *Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products.* Journal ofAgricultural and Food Chemistry, 1998. **46**: p. 4113-7.

APPENDICES

APPENDIX A

GC Chromatogram



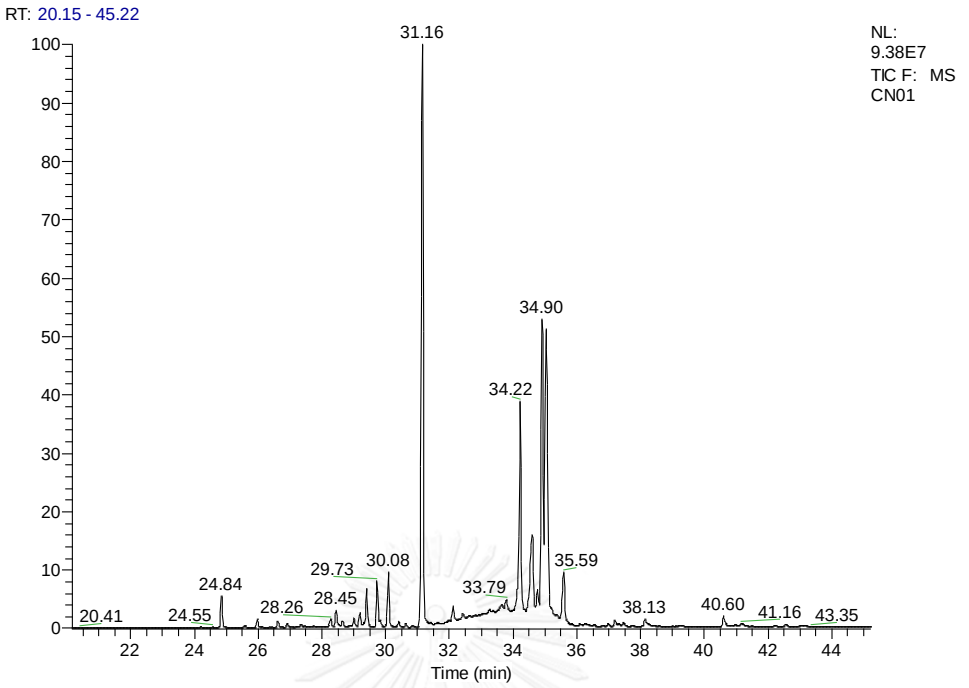


Figure 80 GC chromatogram of the volatile oil of *C. nardus* dried root (1)

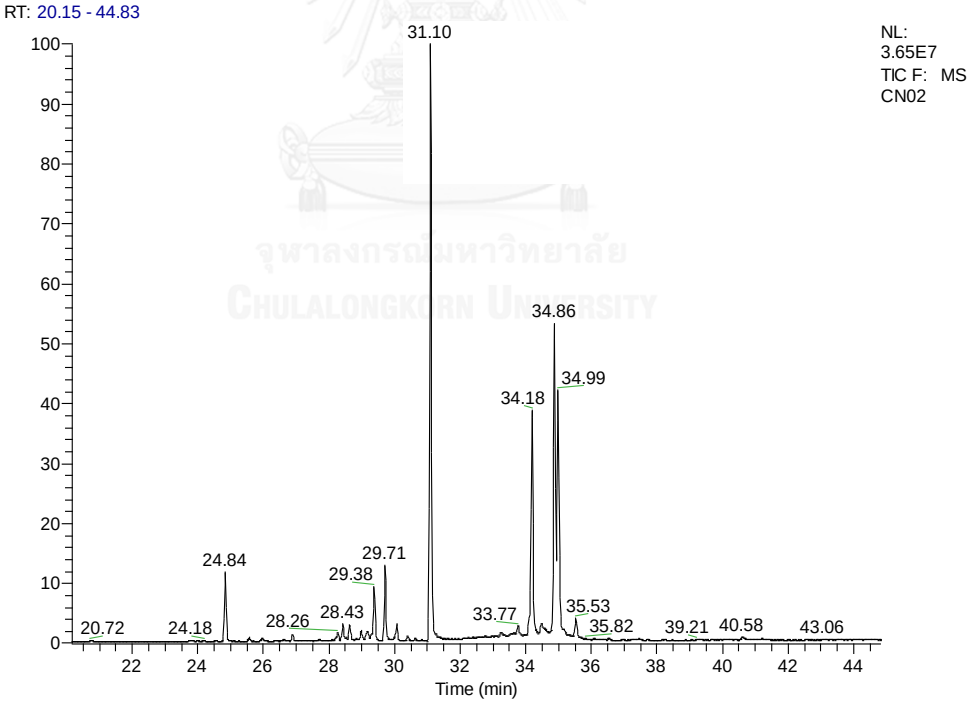


Figure 81 GC chromatogram of the volatile oil of *C. nardus* dried root (2)

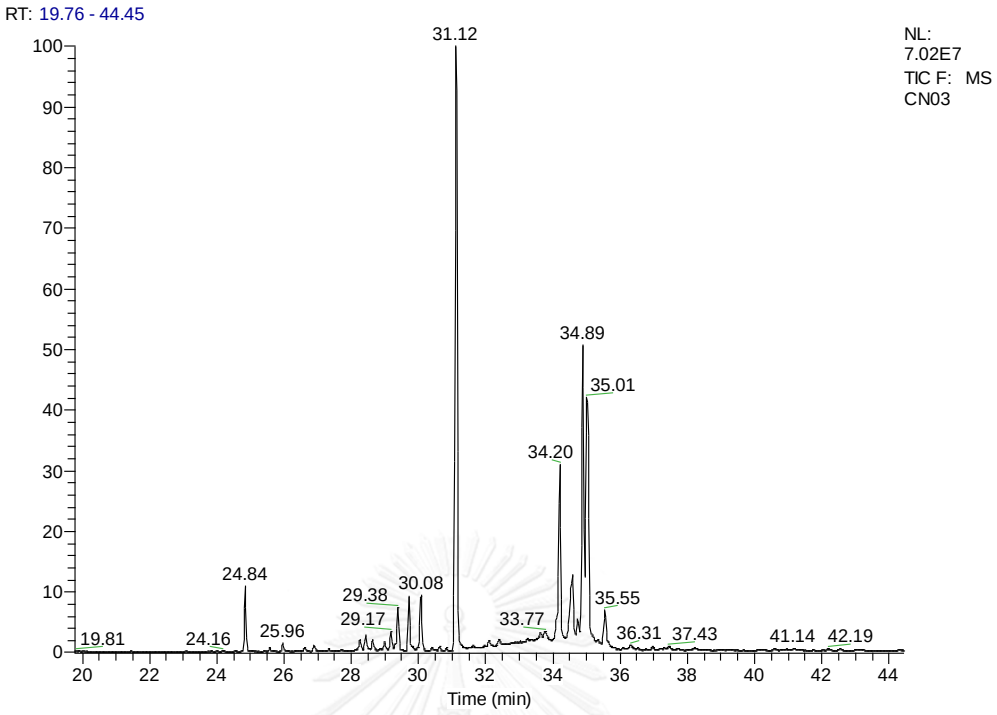


Figure 82 GC chromatogram of the volatile oil of *C. nardus* dried root (3)

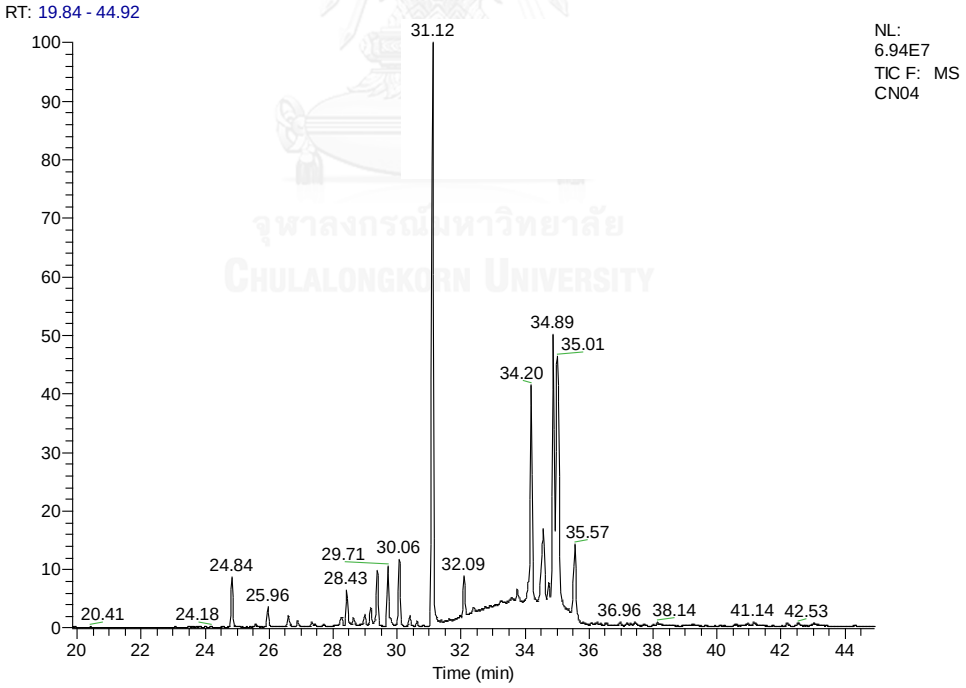


Figure 83 GC chromatogram of the volatile oil of *C. nardus* dried root (4)

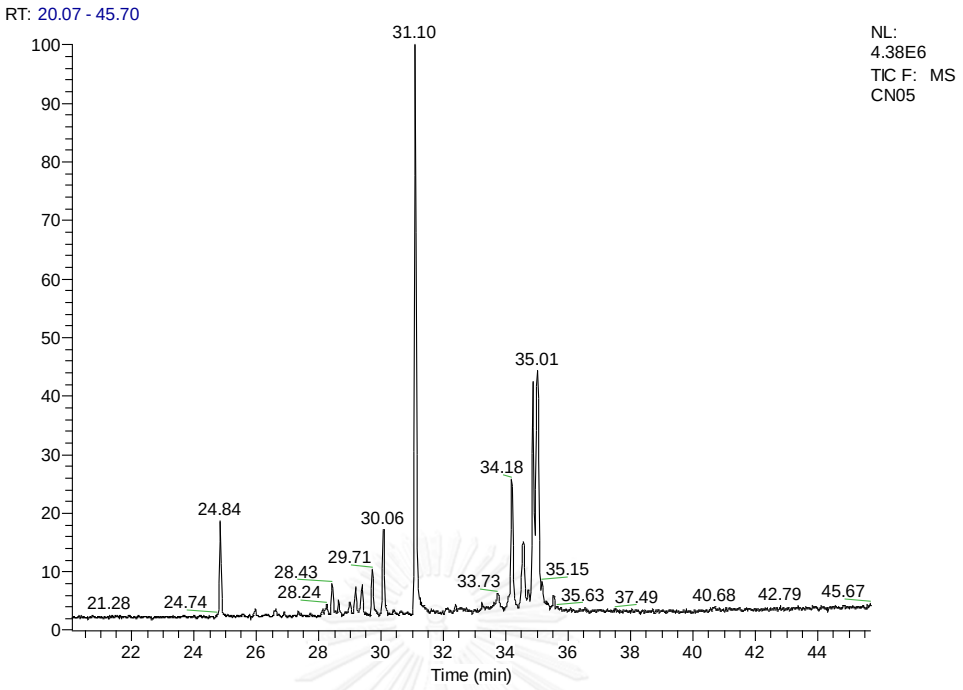


Figure 84 GC chromatogram of the volatile oil of *C. nardus* dried root (5)

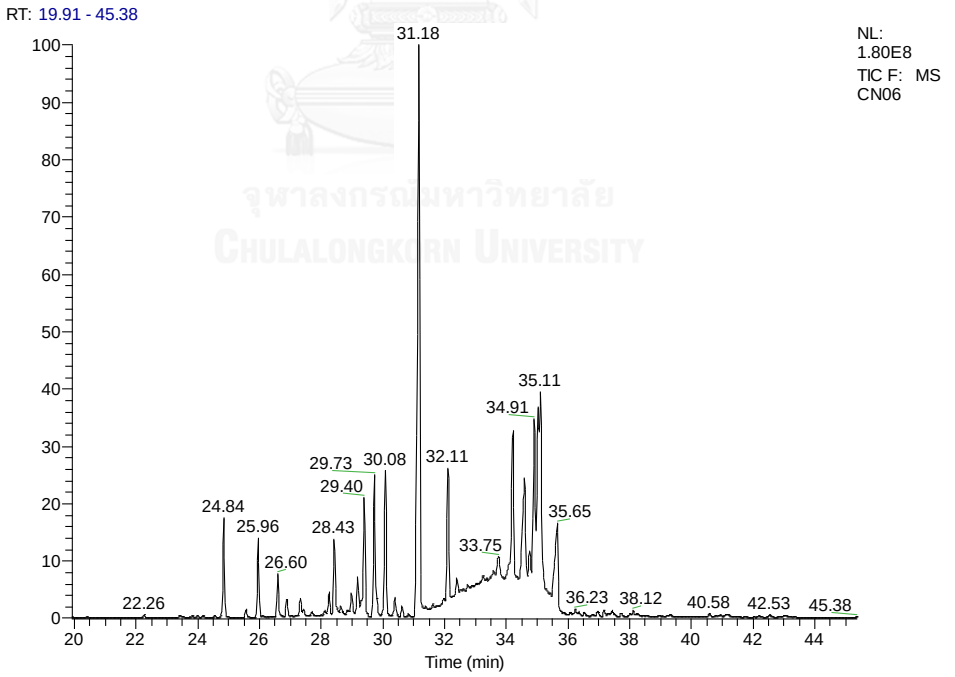


Figure 85 GC chromatogram of the volatile oil of *C. nardus* dried root (6)

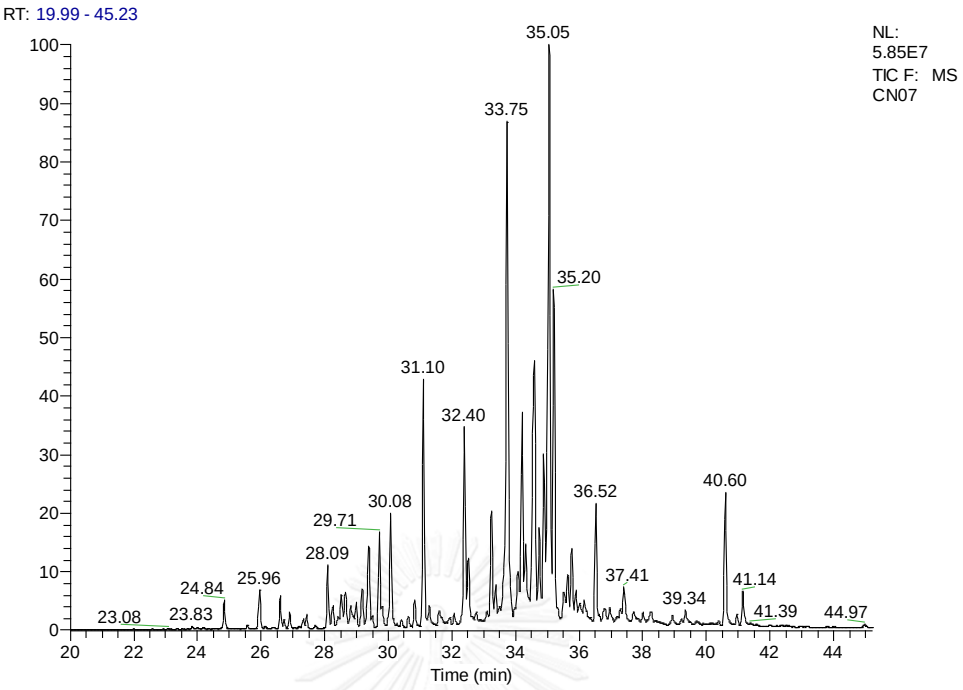


Figure 86 GC chromatogram of the volatile oil of *C. nardus* dried root (7)

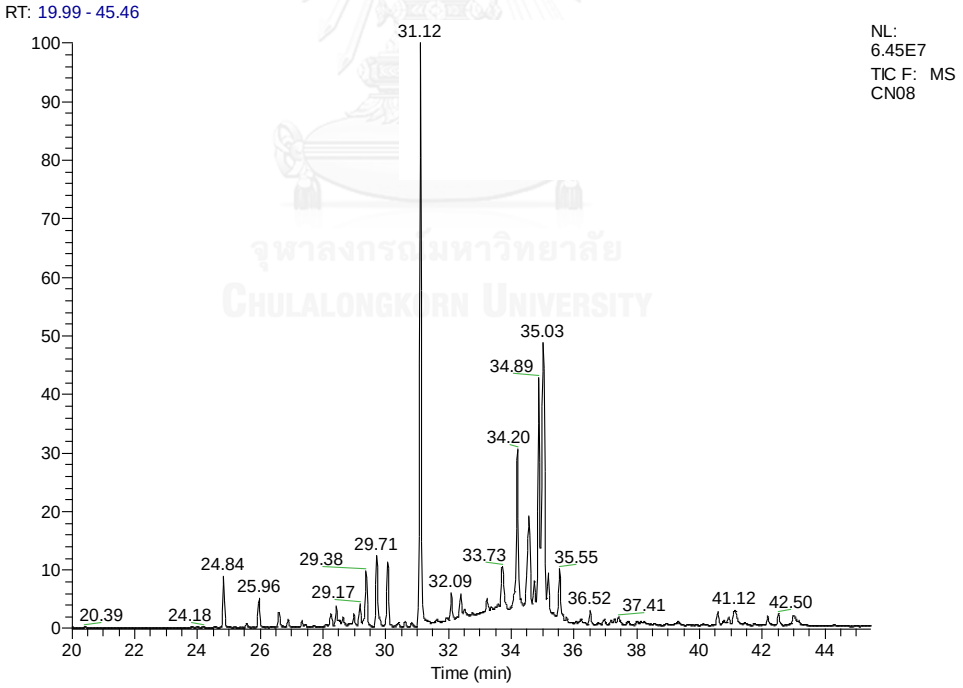


Figure 87 GC chromatogram of the volatile oil of *C. nardus* dried root (8)

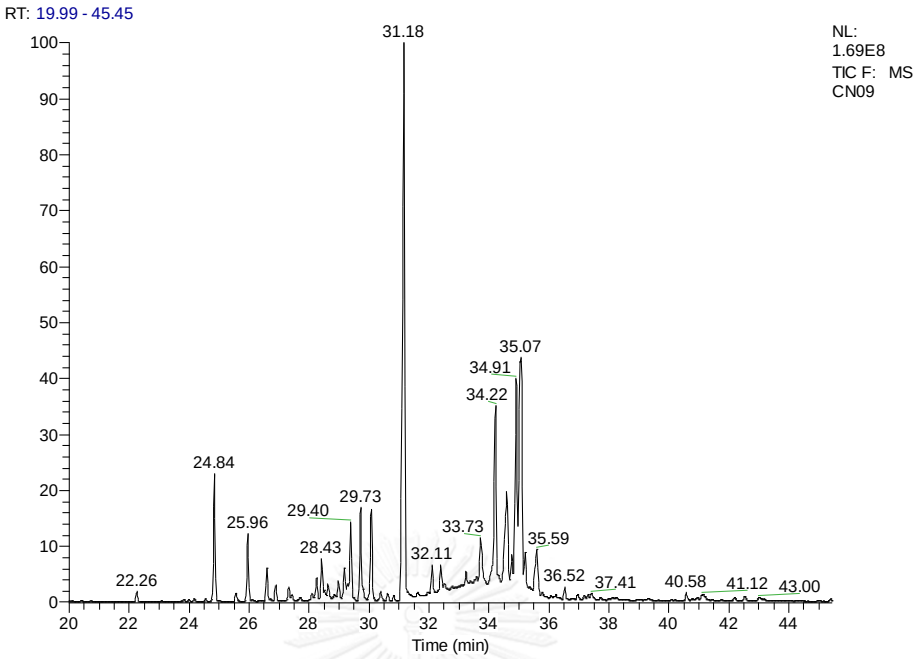


Figure 88 GC chromatogram of the volatile oil of *C. nardus* dried root (9)

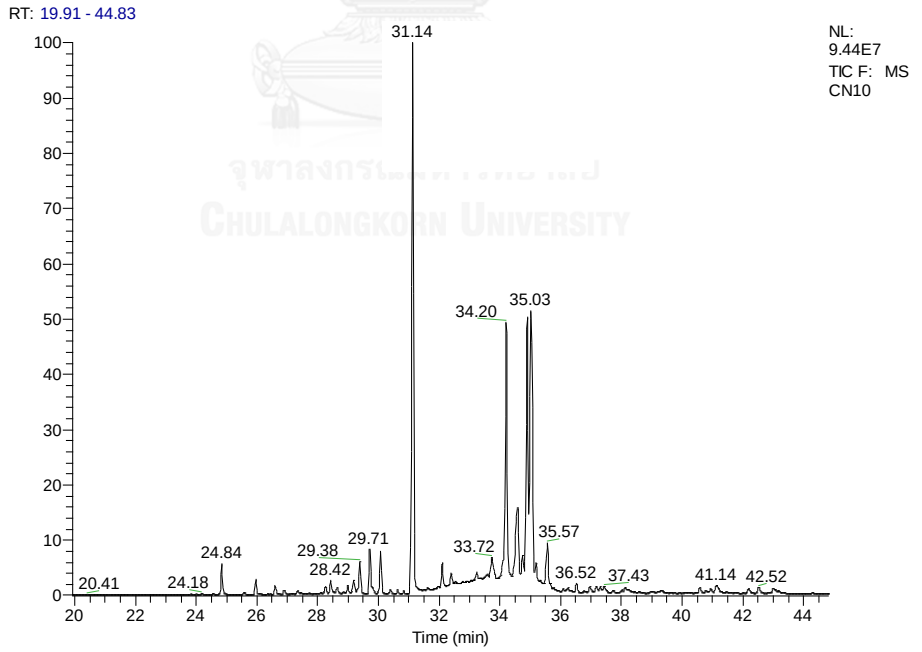


Figure 89 GC chromatogram of the volatile oil of *C. nardus* dried root (10)

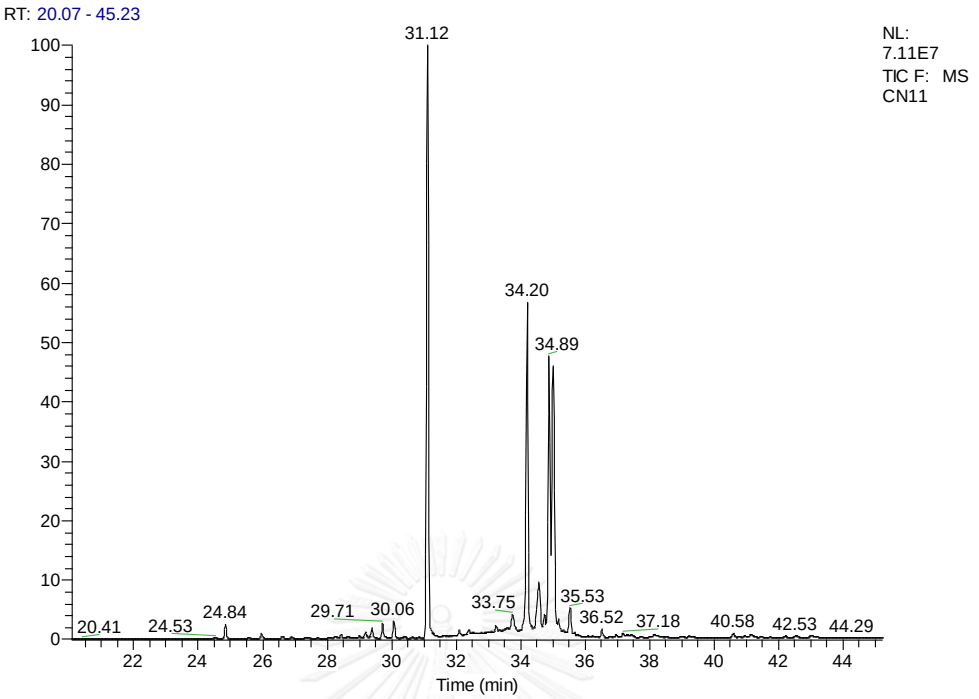


Figure 90 GC chromatogram of the volatile oil of *C. nardus* dried root (11)

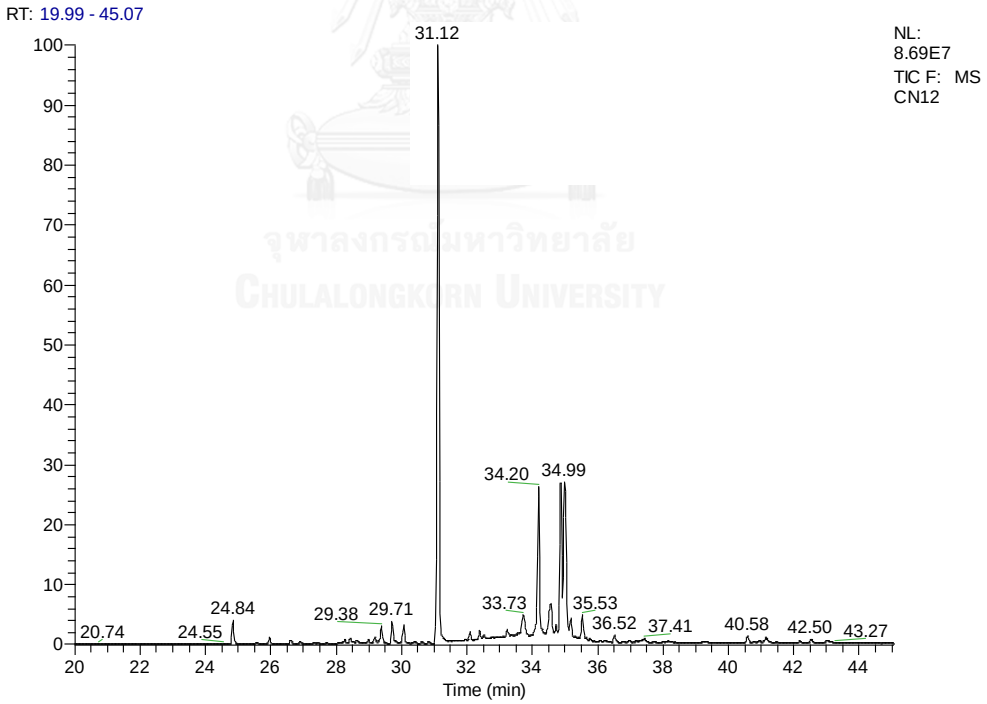


Figure 91 GC chromatogram of the volatile oil of *C. nardus* dried root (12)

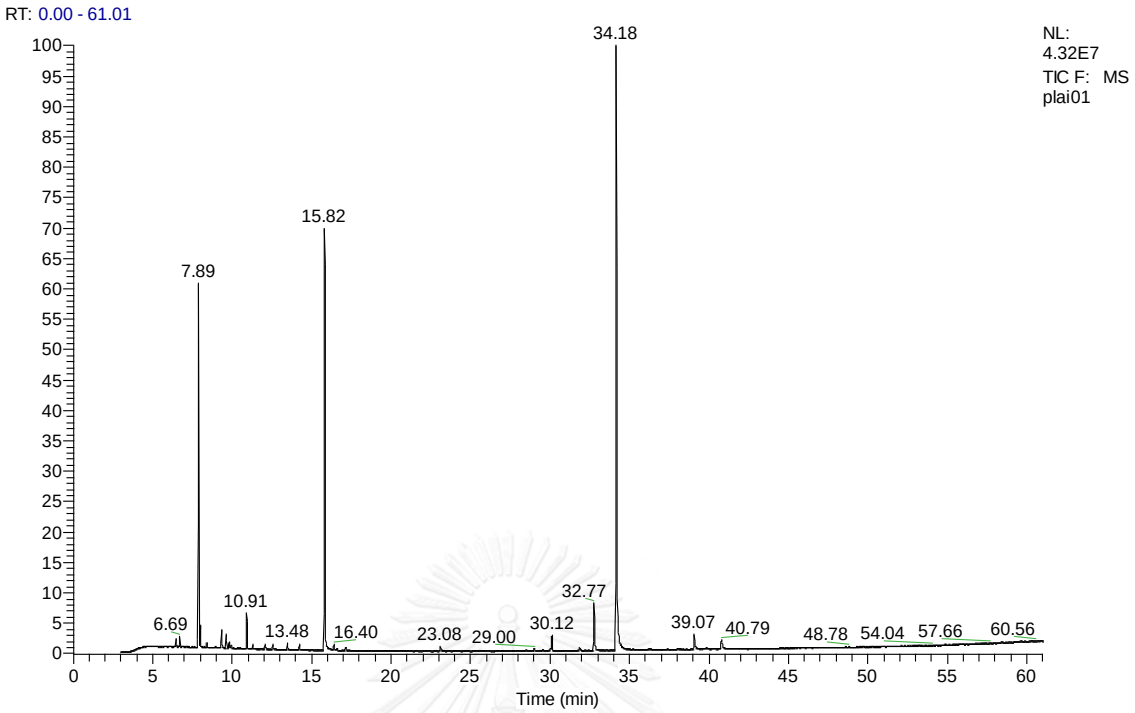


Figure 92 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (1)

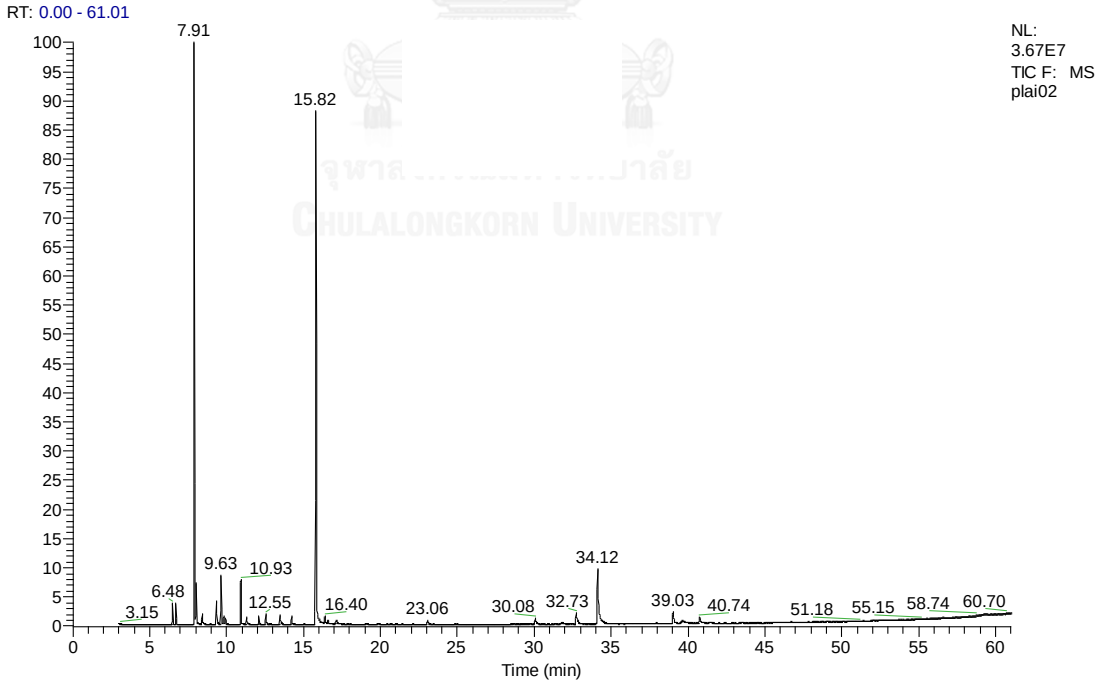


Figure 93 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (2)

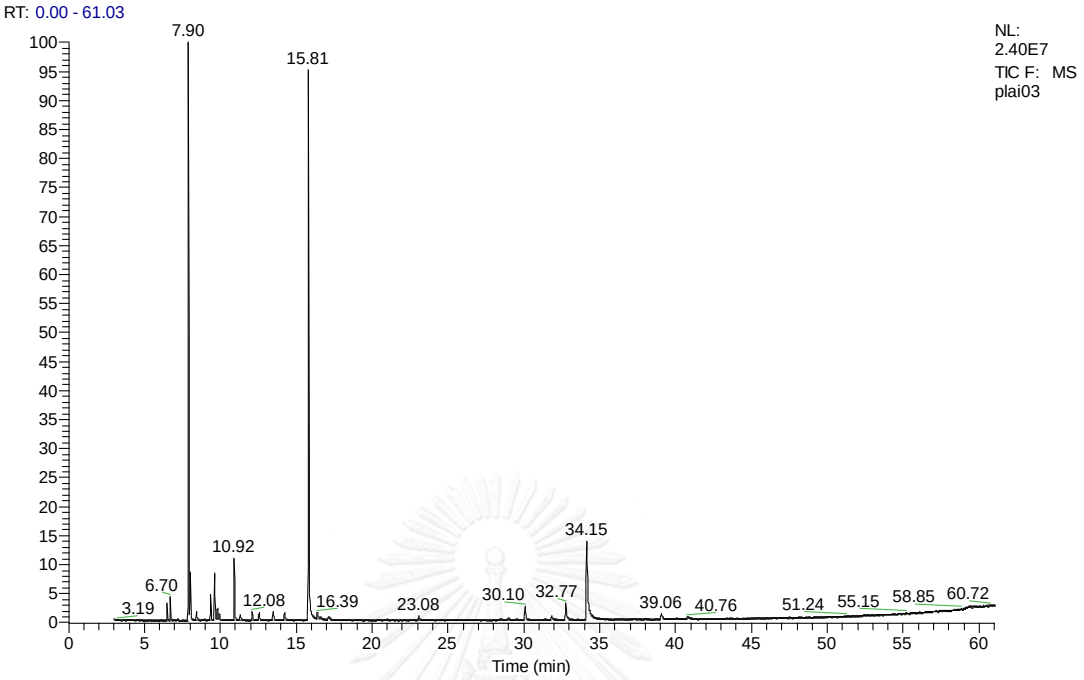


Figure 94 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (3)

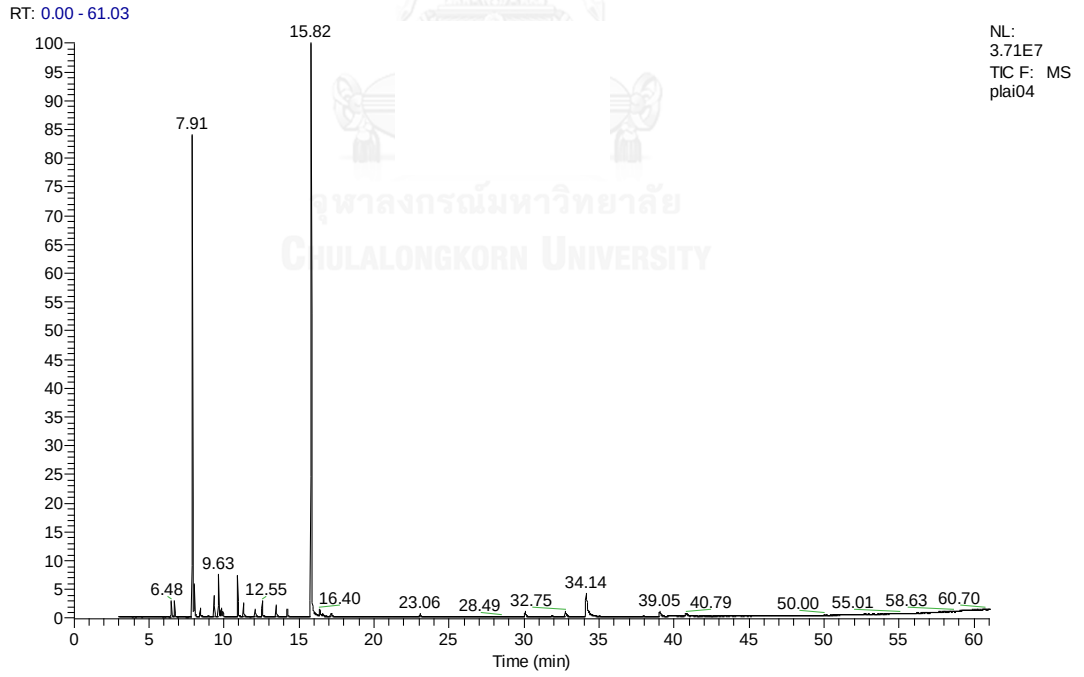


Figure 95 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (4)

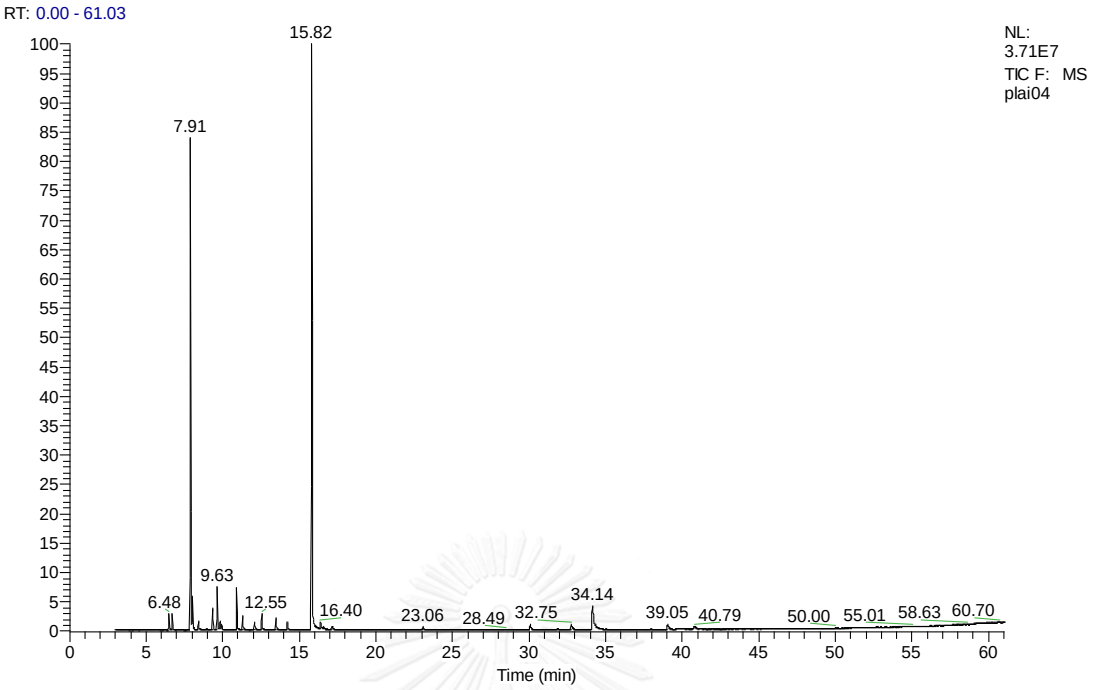


Figure 96 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (5)

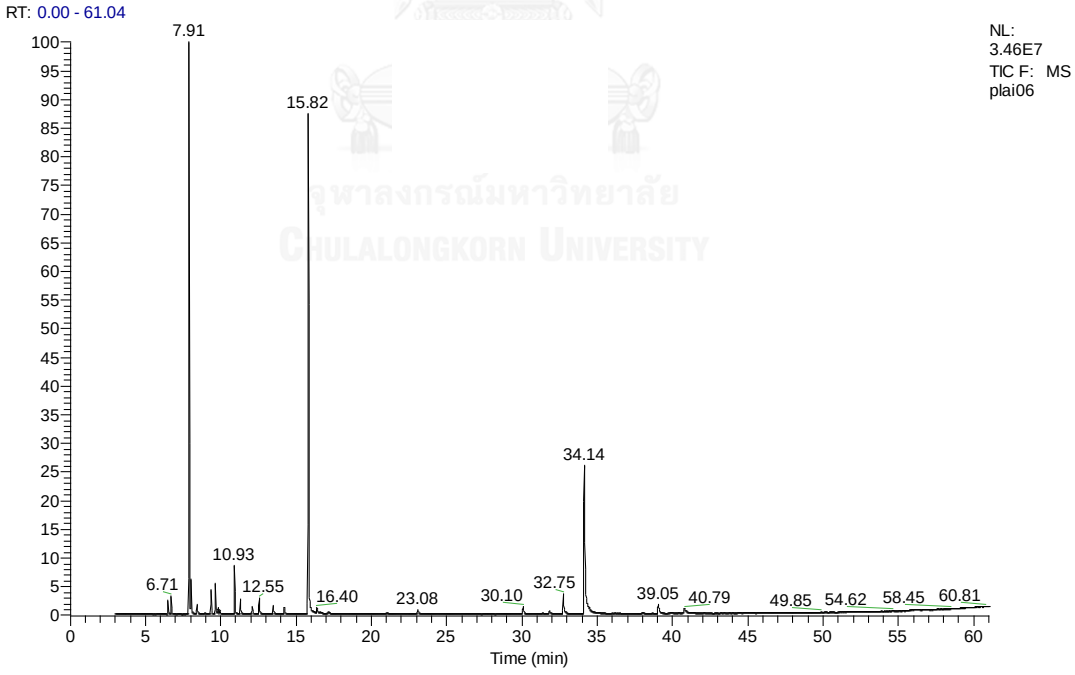


Figure 97 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (6)

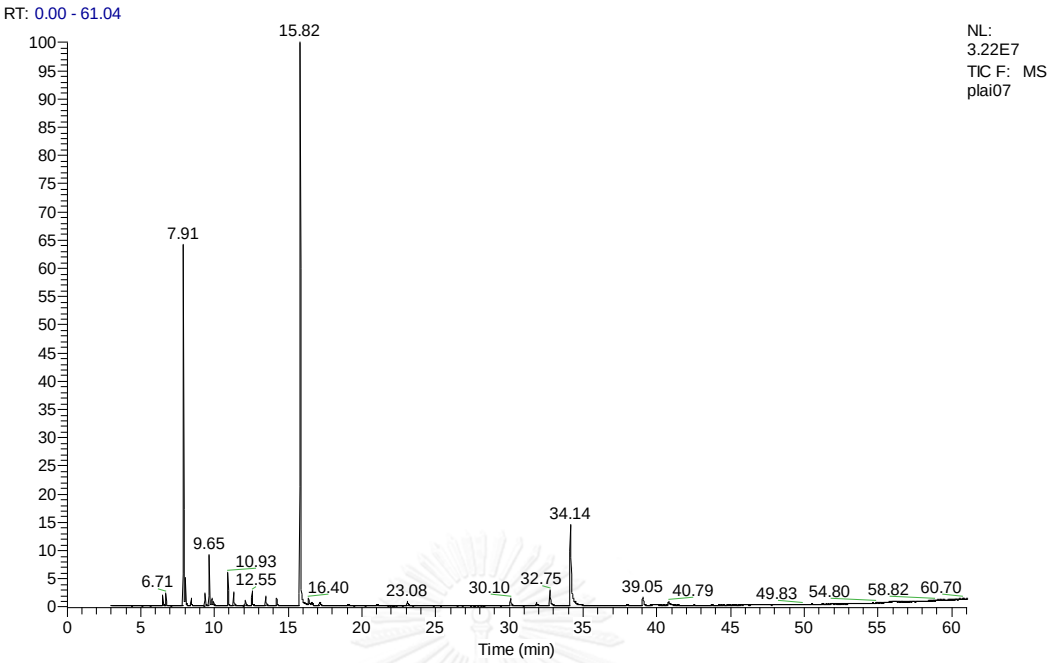


Figure 98 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (7)

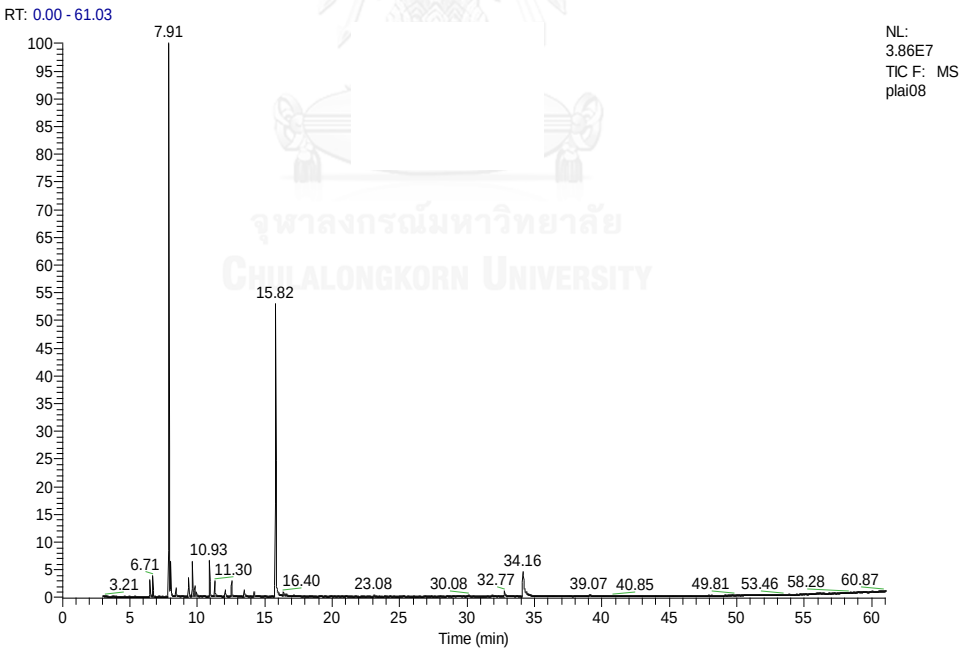


Figure 99 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (8)

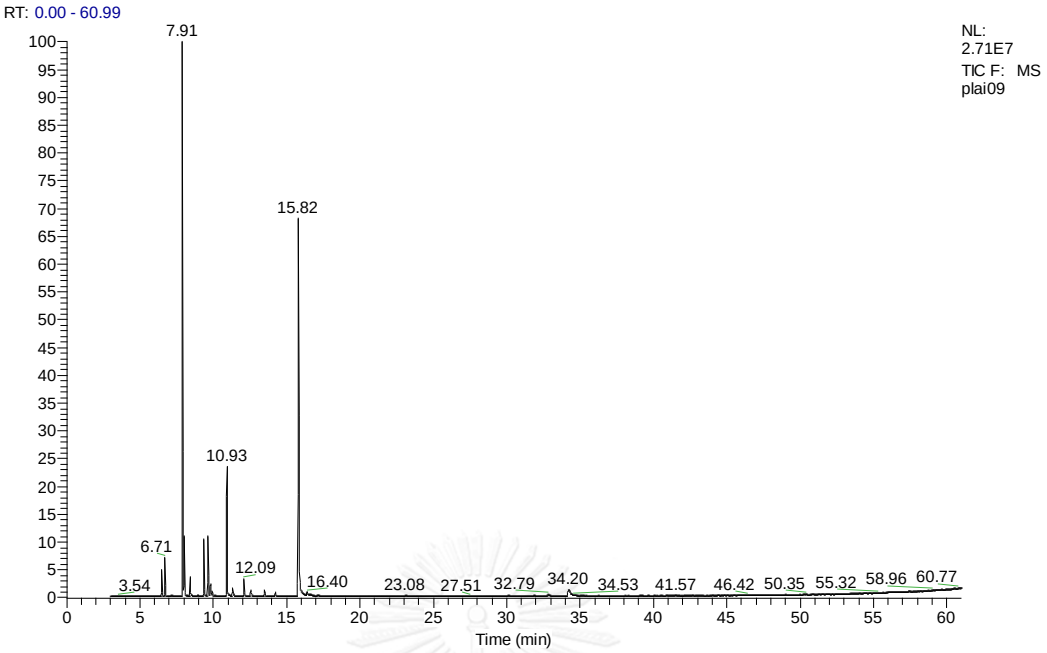


Figure 100 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (9)

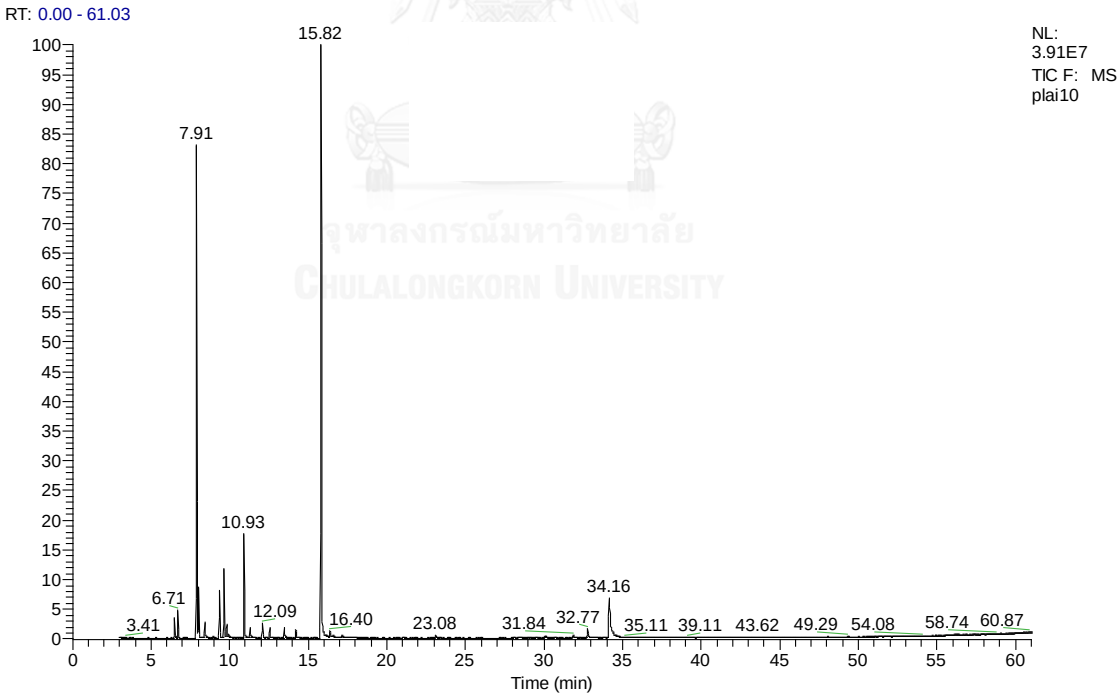


Figure 101 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (10)

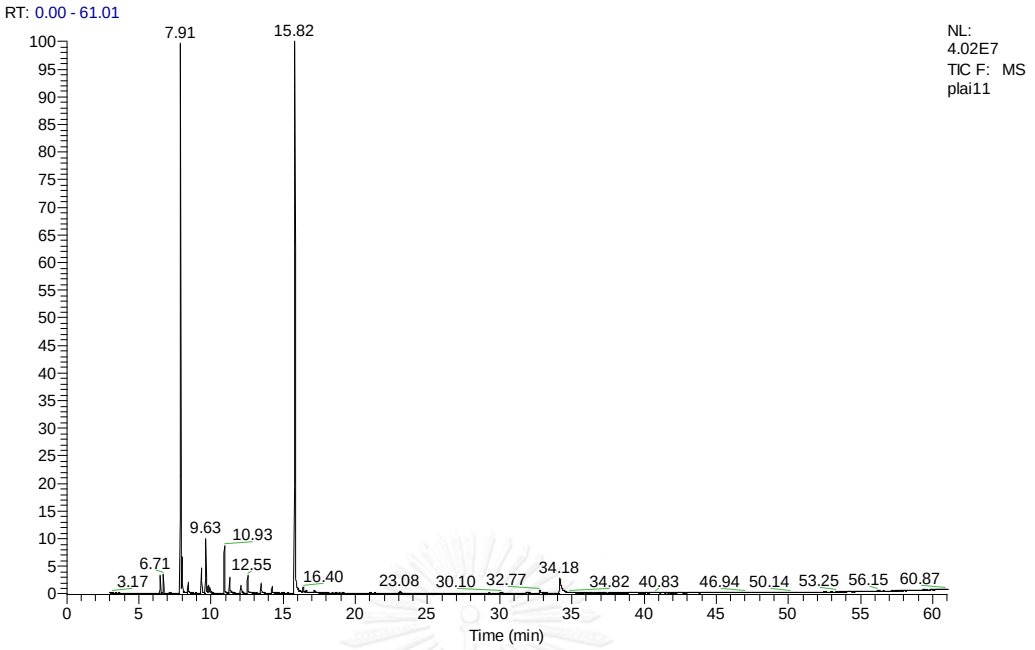


Figure 102 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (11)

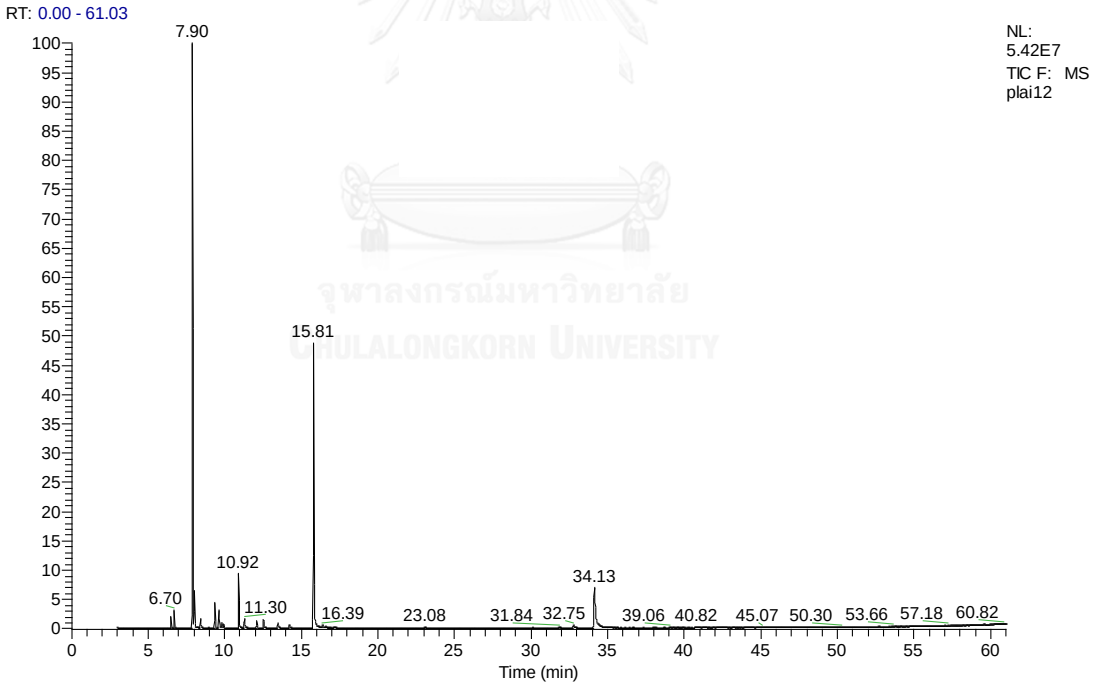


Figure 103 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (12)

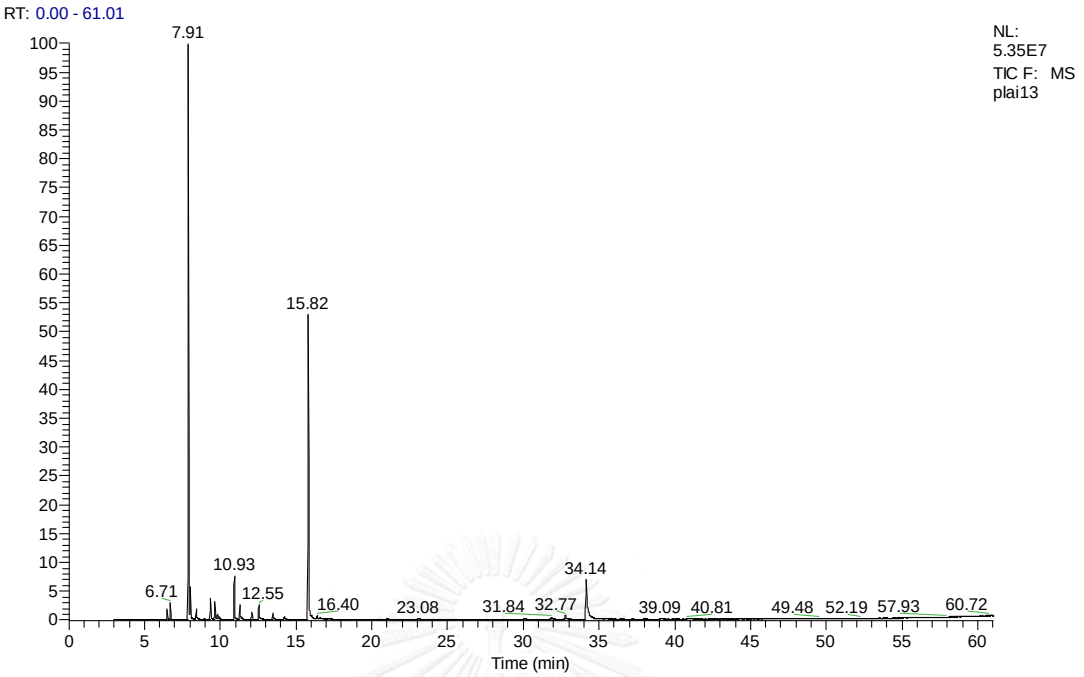


Figure 104 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (13*)*

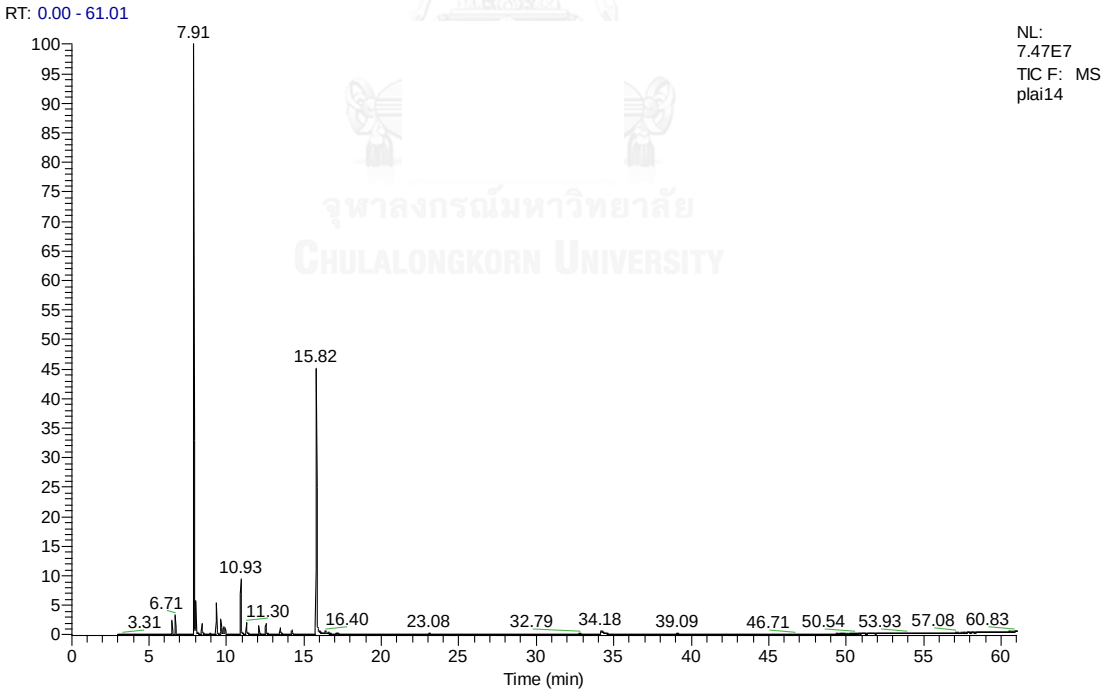


Figure 105 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (14)

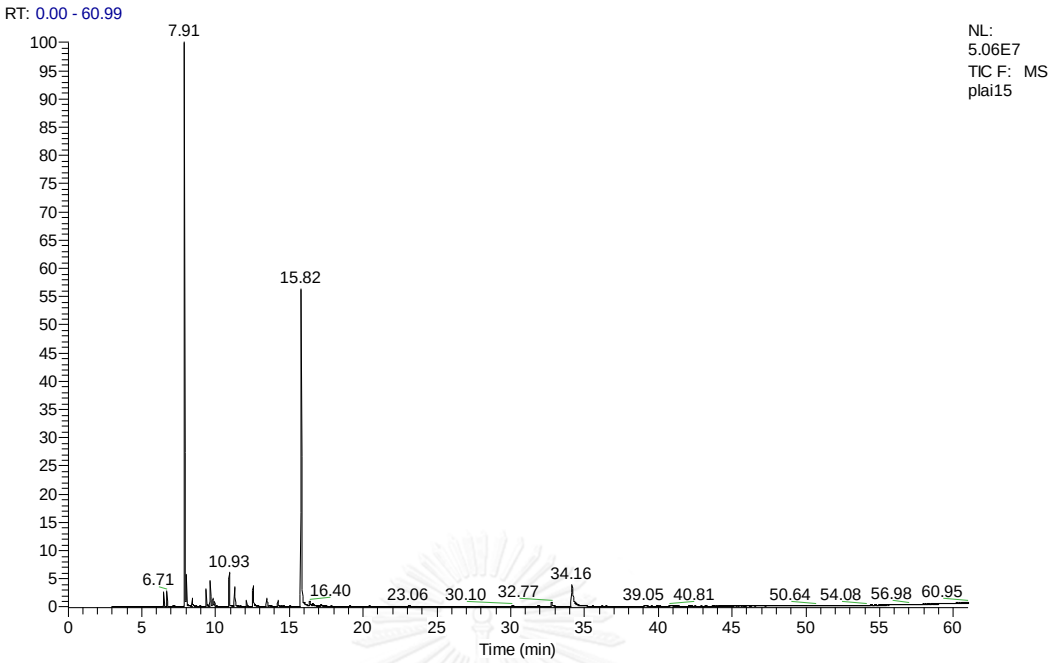


Figure 106 GC chromatogram of the volatile oil of *Z. montanum* dried rhizome (15)

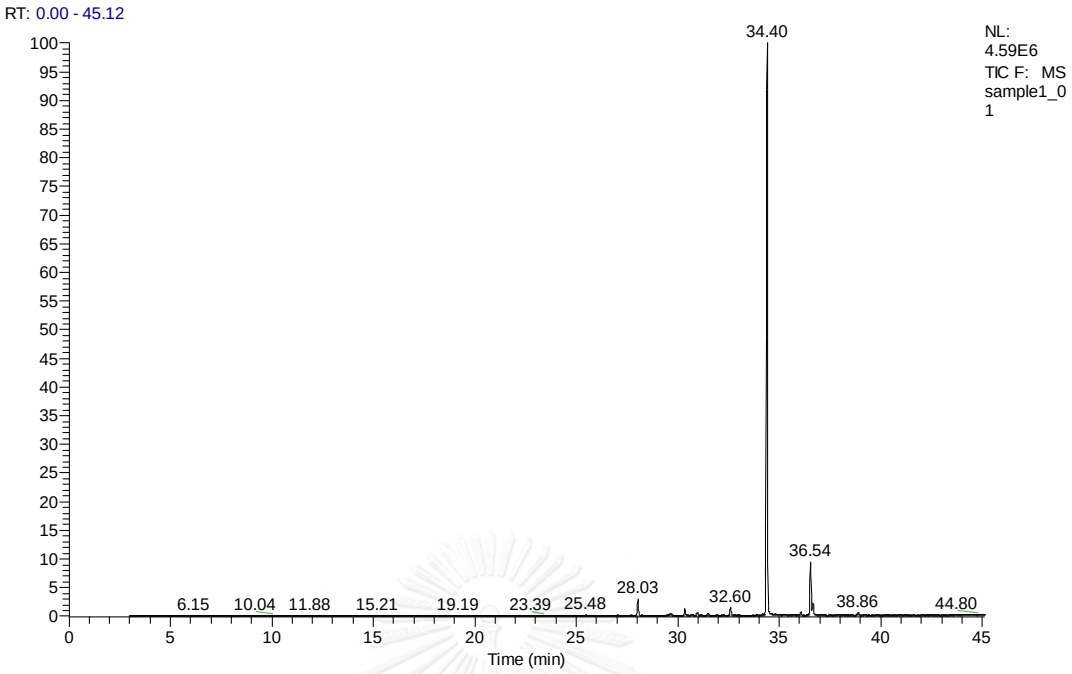


Figure 107 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (1)

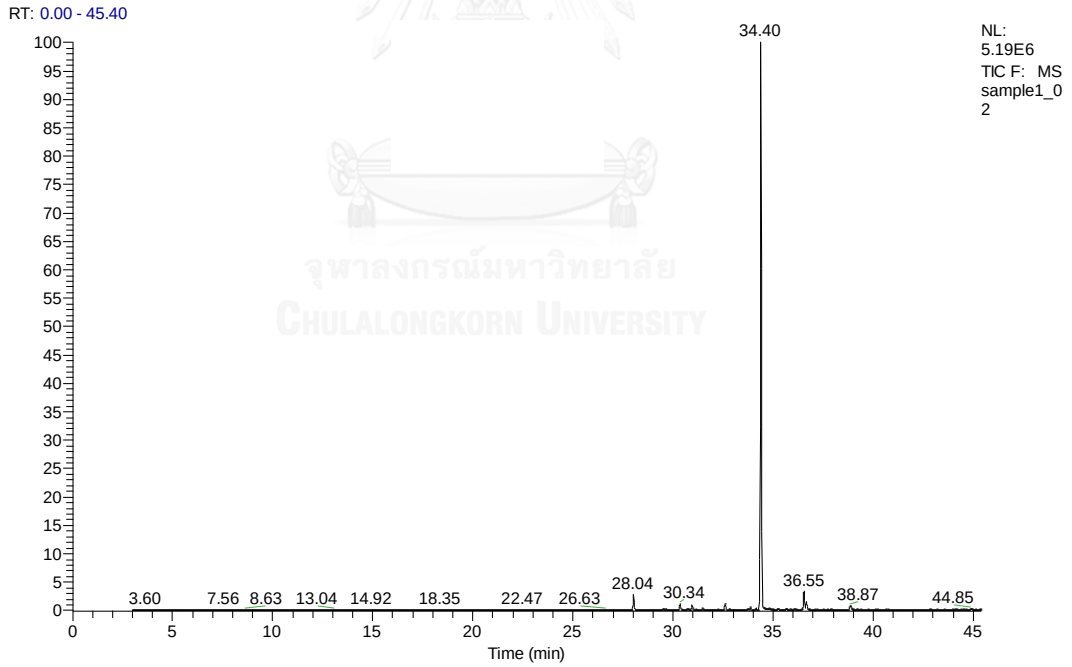


Figure 108 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (2)

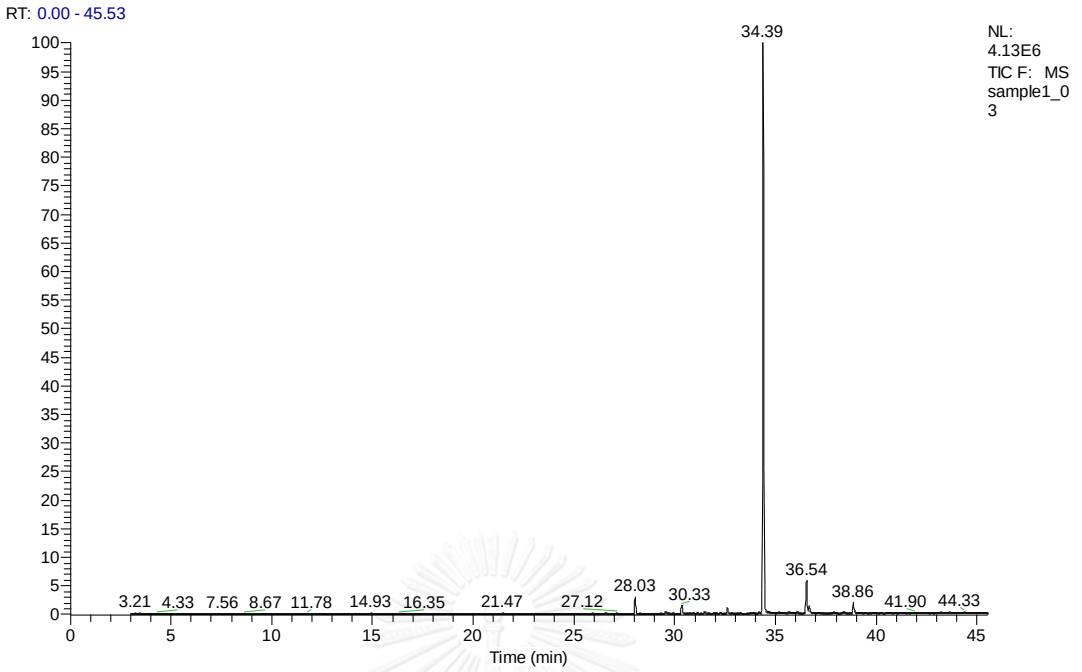


Figure 109 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (3)

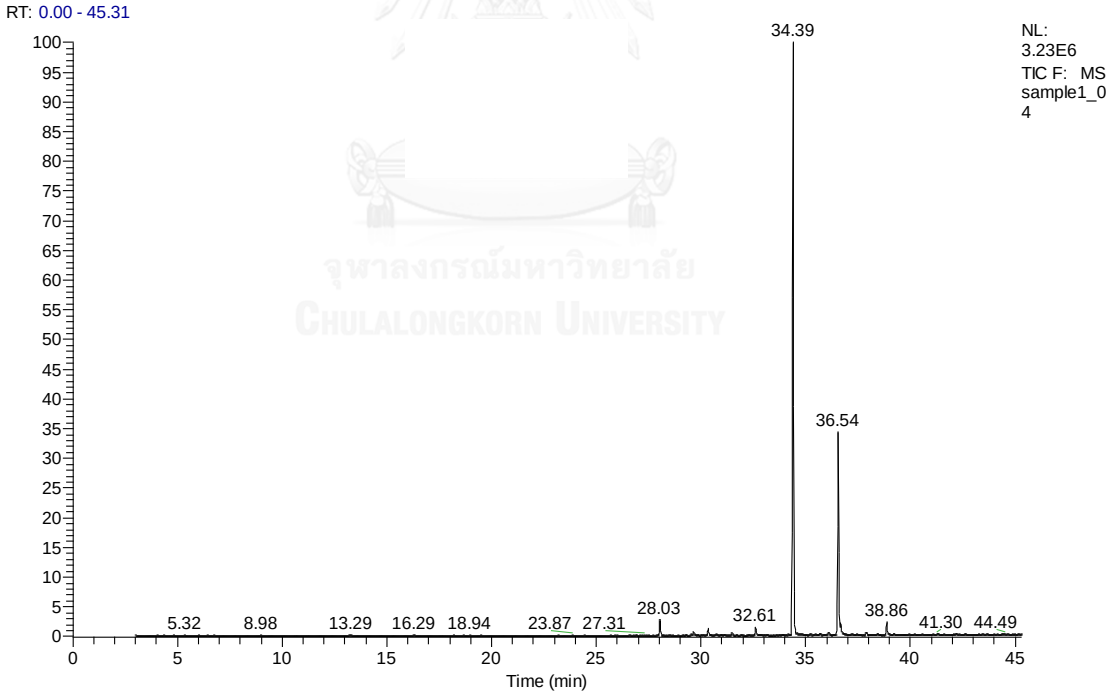


Figure 110 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (4)

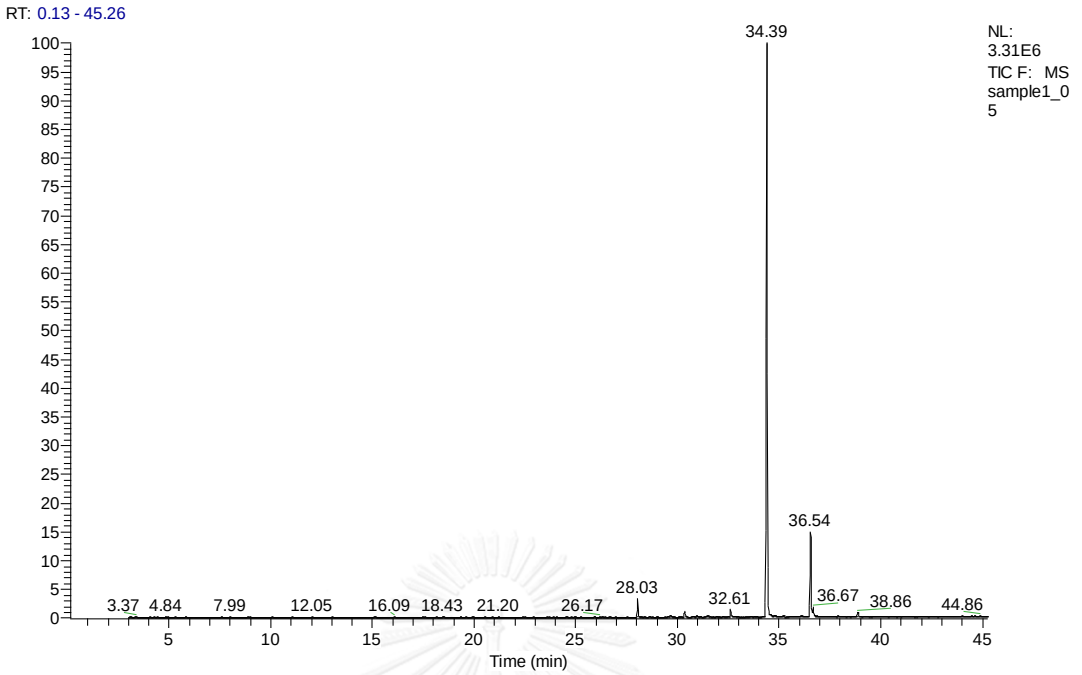


Figure 111 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (5)

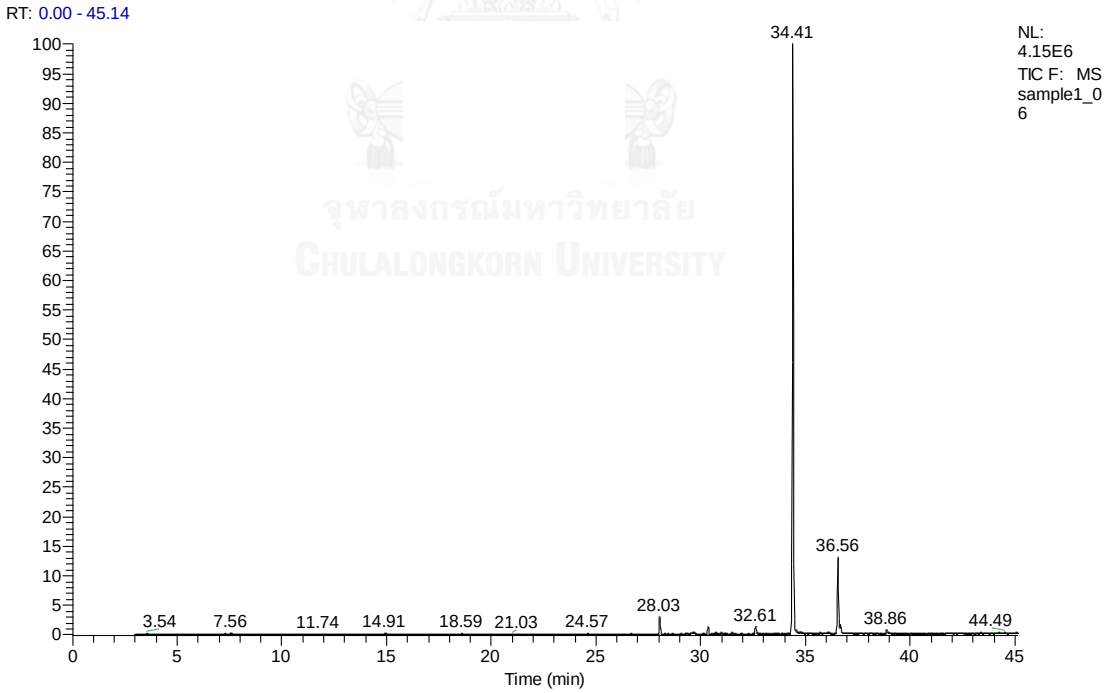


Figure 112 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (6)

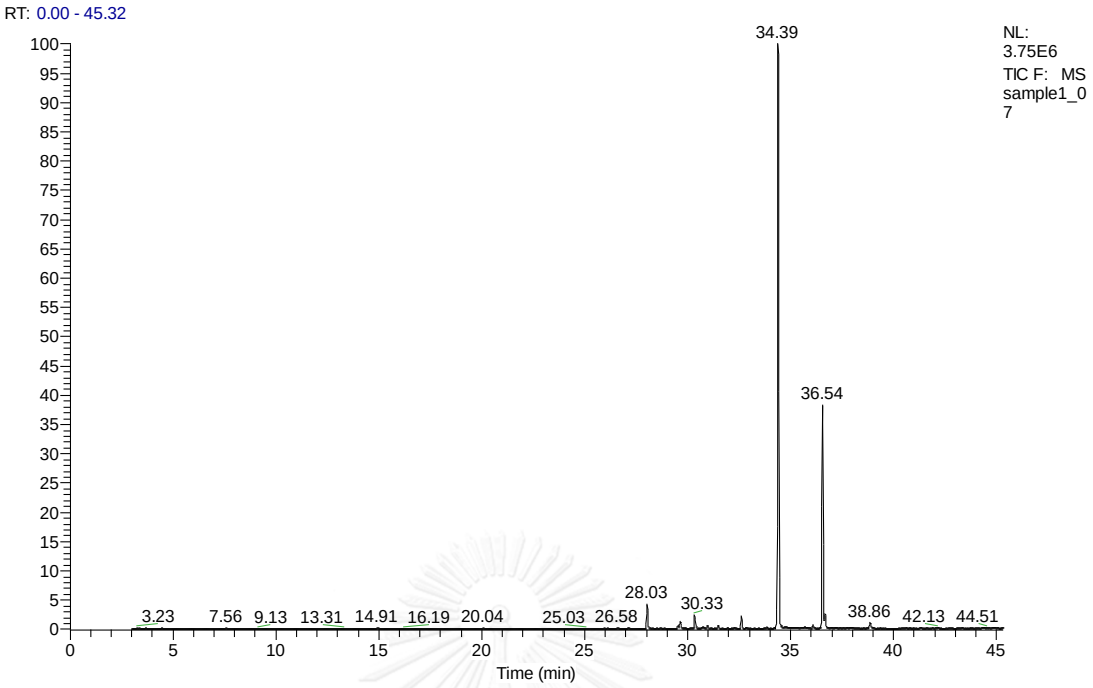


Figure 113 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (7)

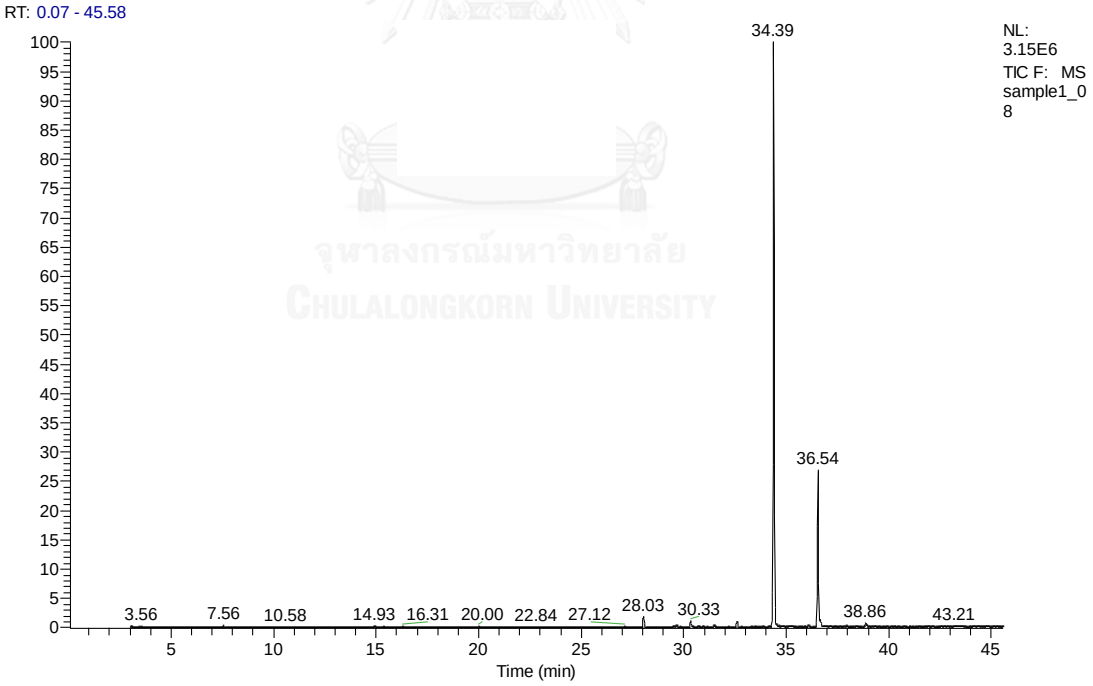


Figure 114 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (8)

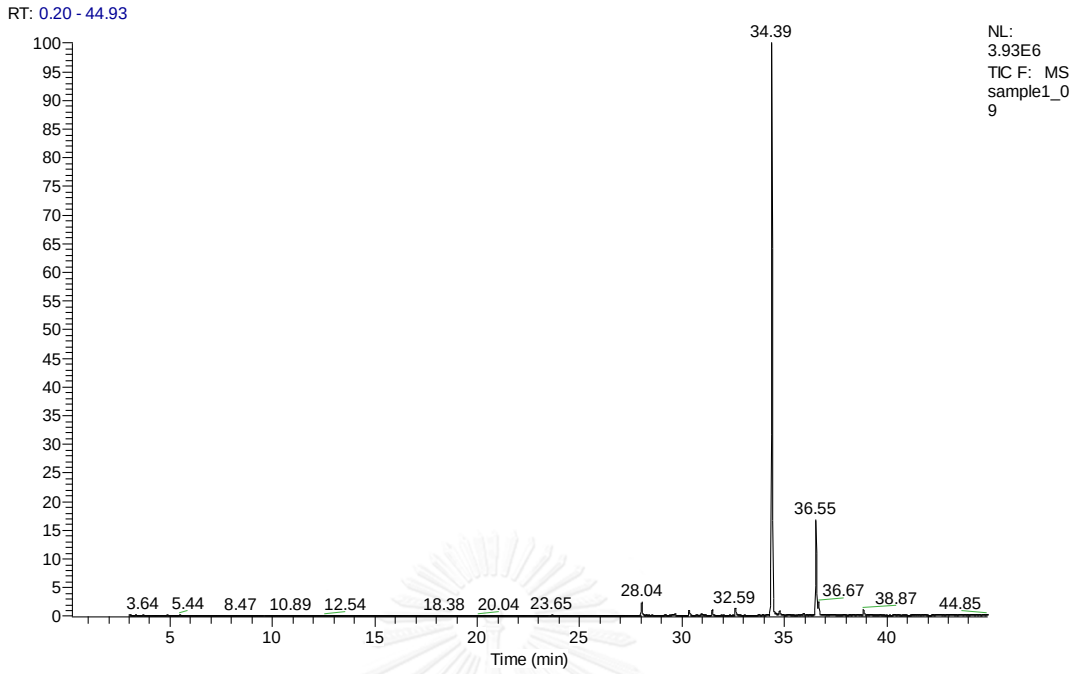


Figure 115 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (9)

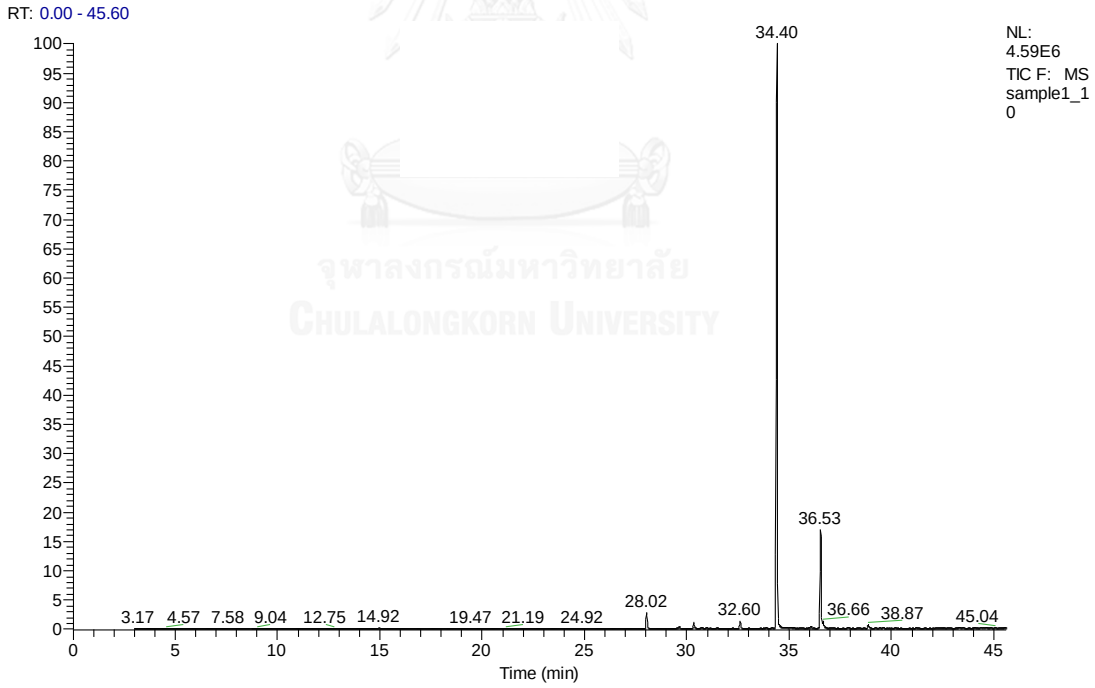


Figure 116 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (10)

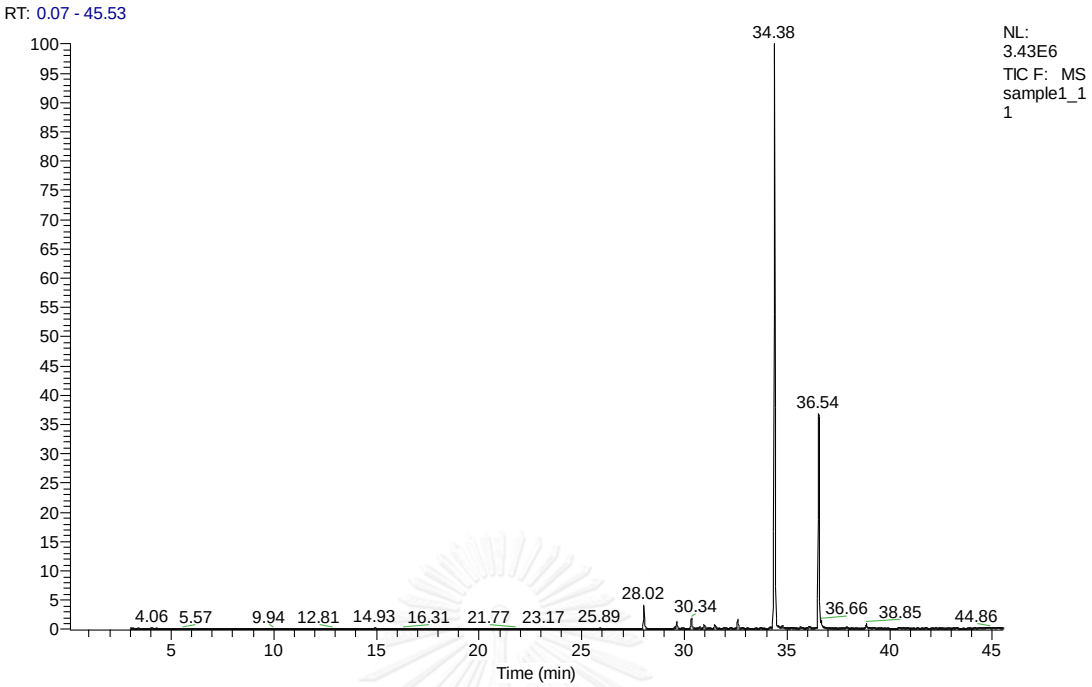


Figure 117 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (11)

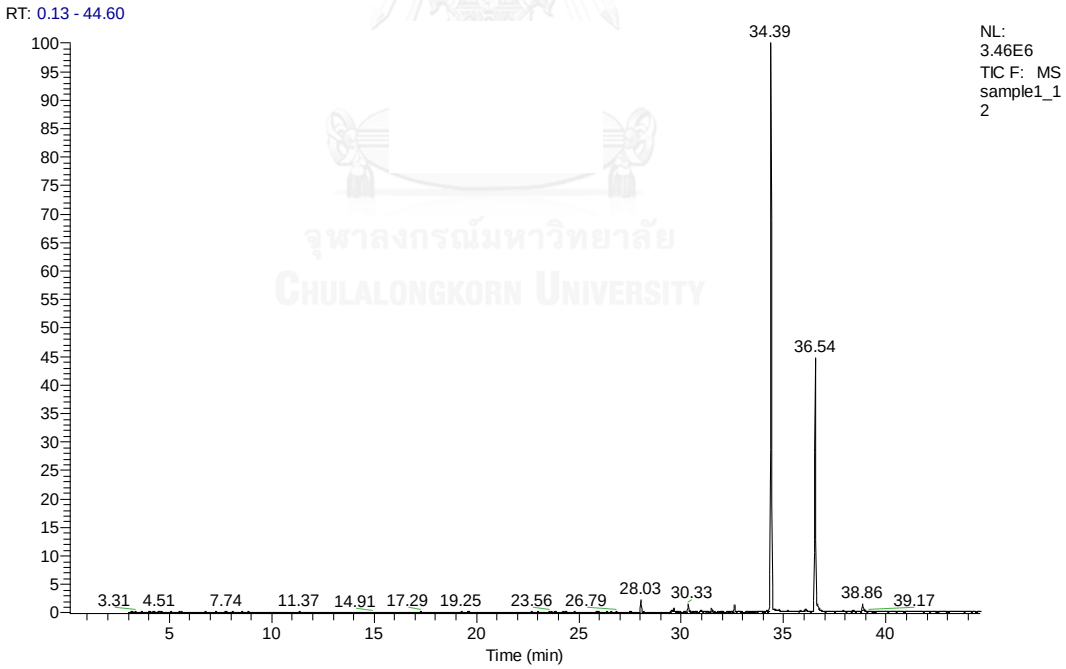


Figure 118 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (12)

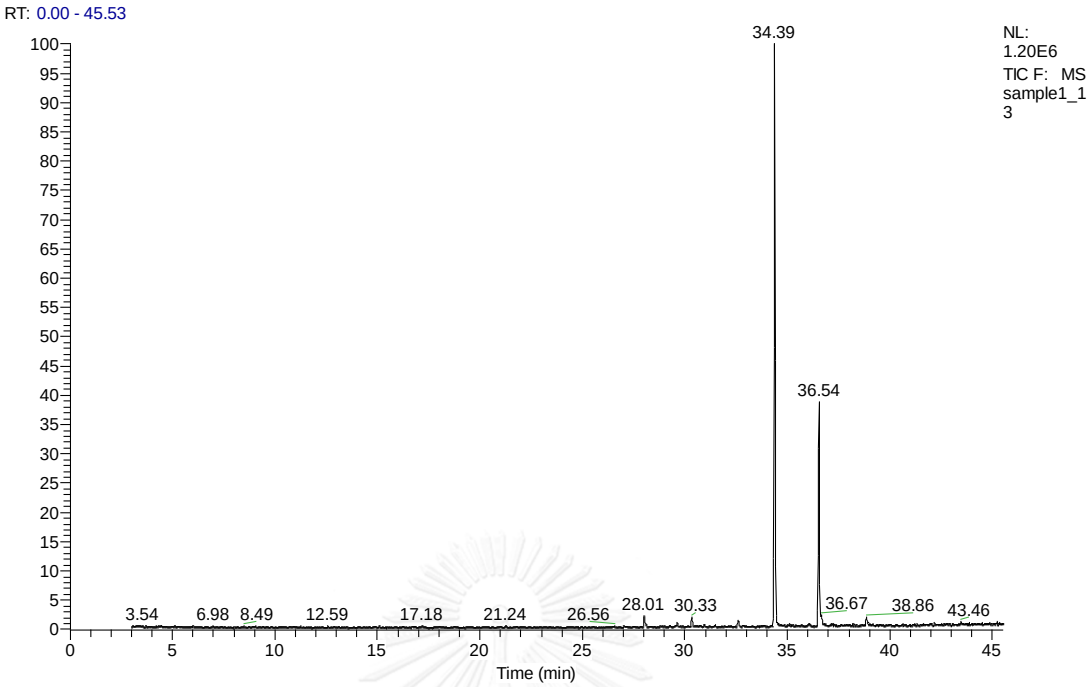


Figure 119 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (13)

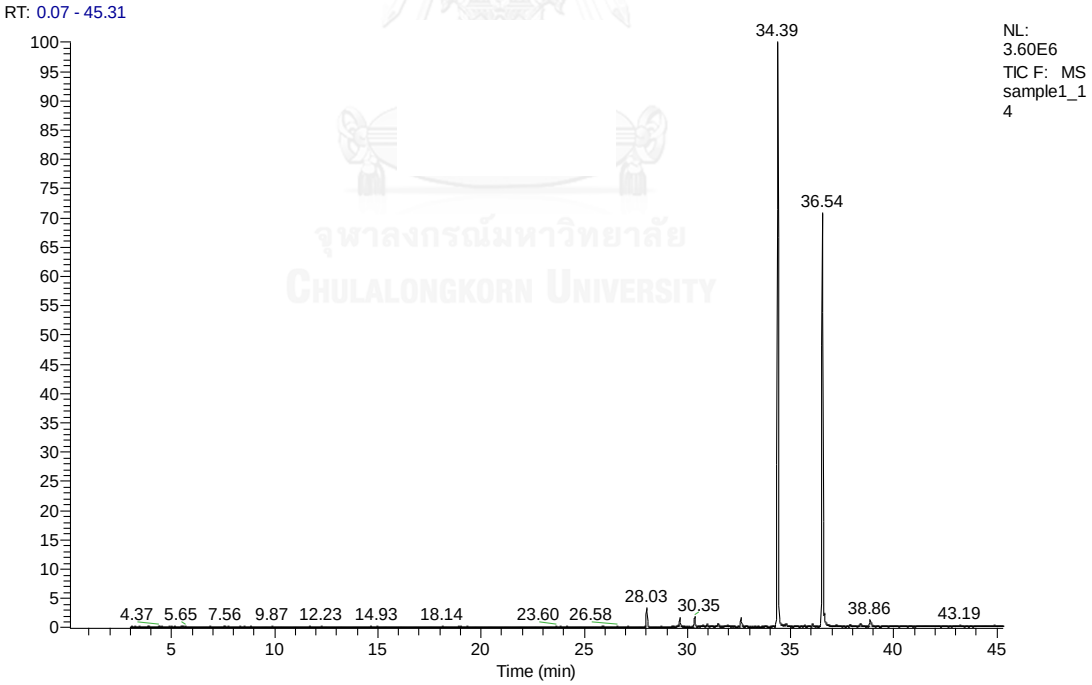


Figure 120 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (14)

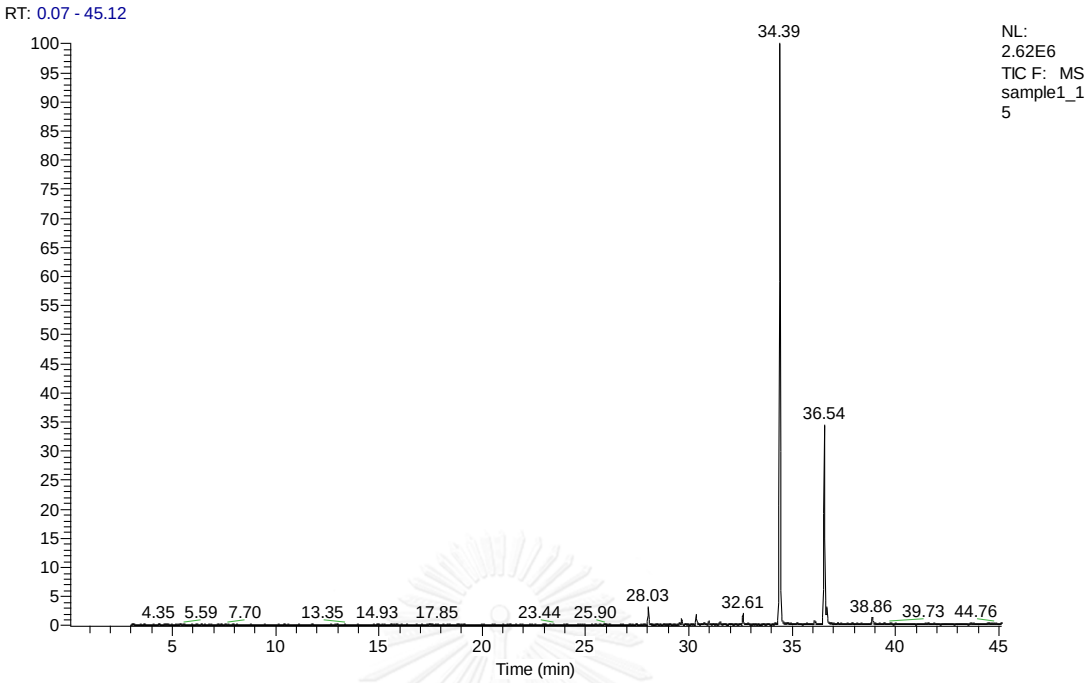


Figure 121 GC chromatogram of the volatile oil of *A. calamus* dried rhizome (15)

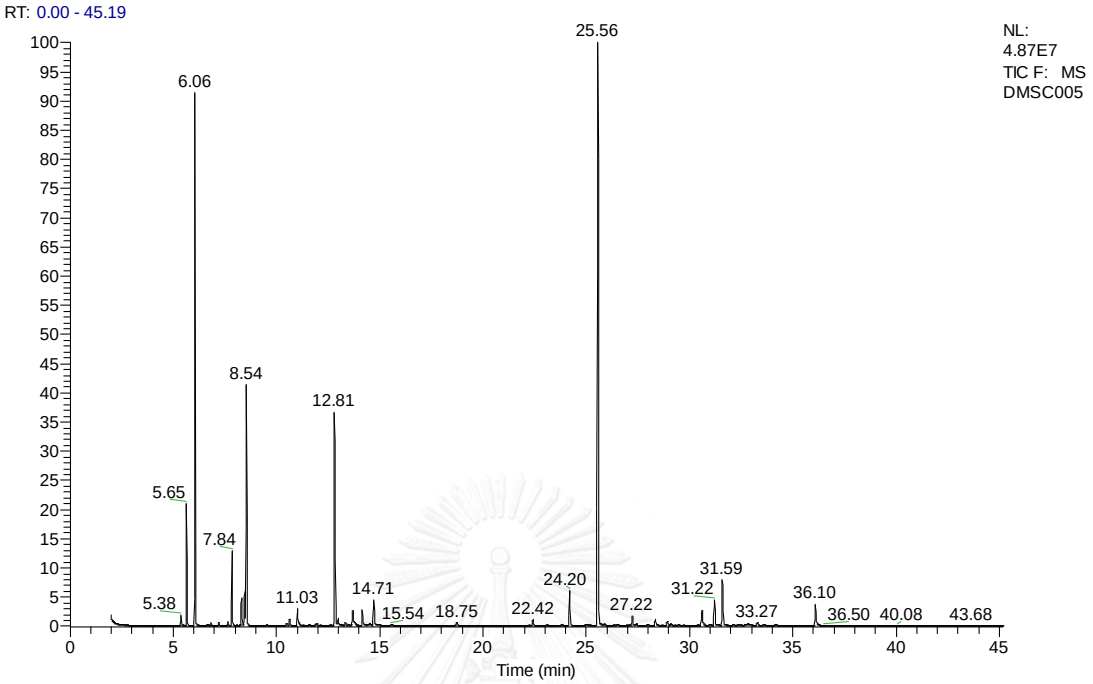


Figure 122 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (1)

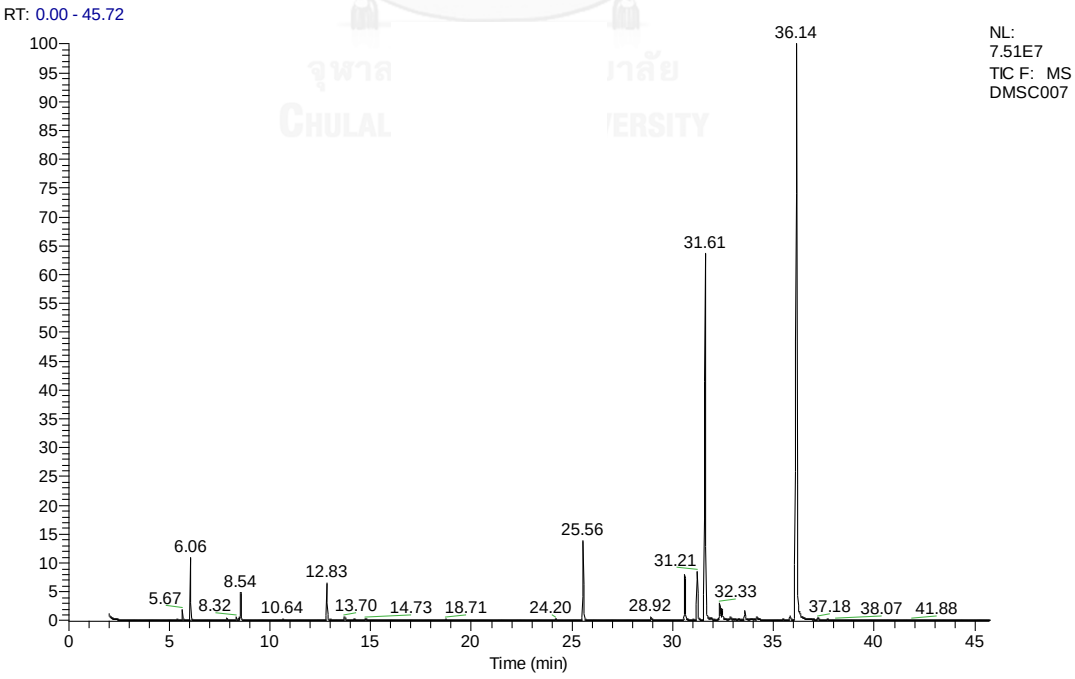


Figure 123 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (2)

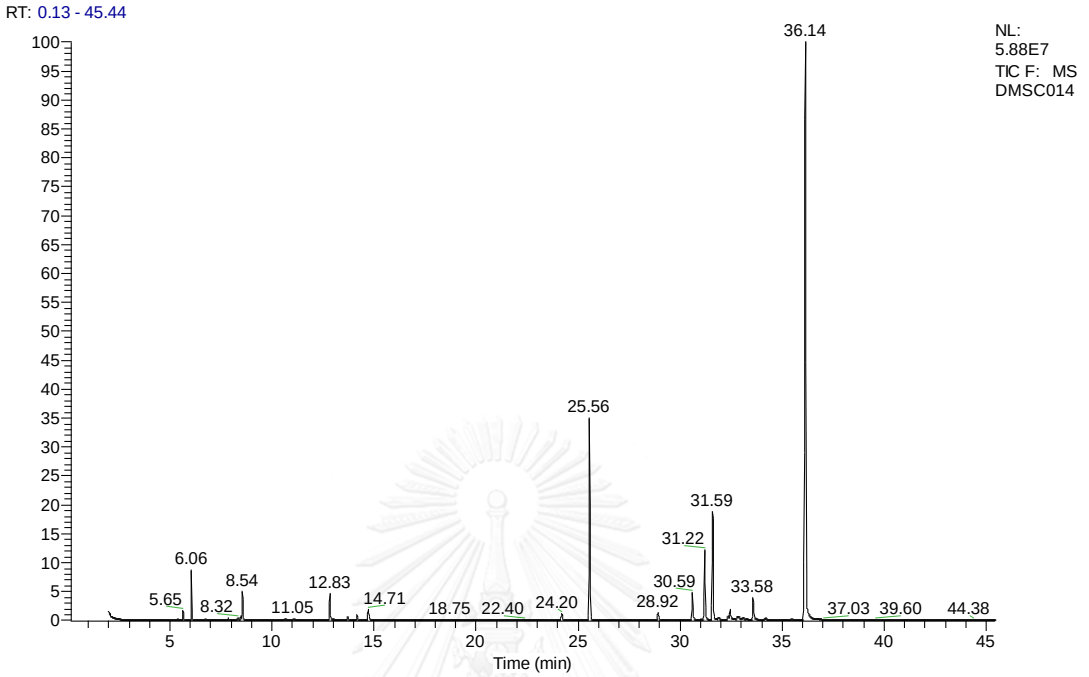


Figure 124 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (3)

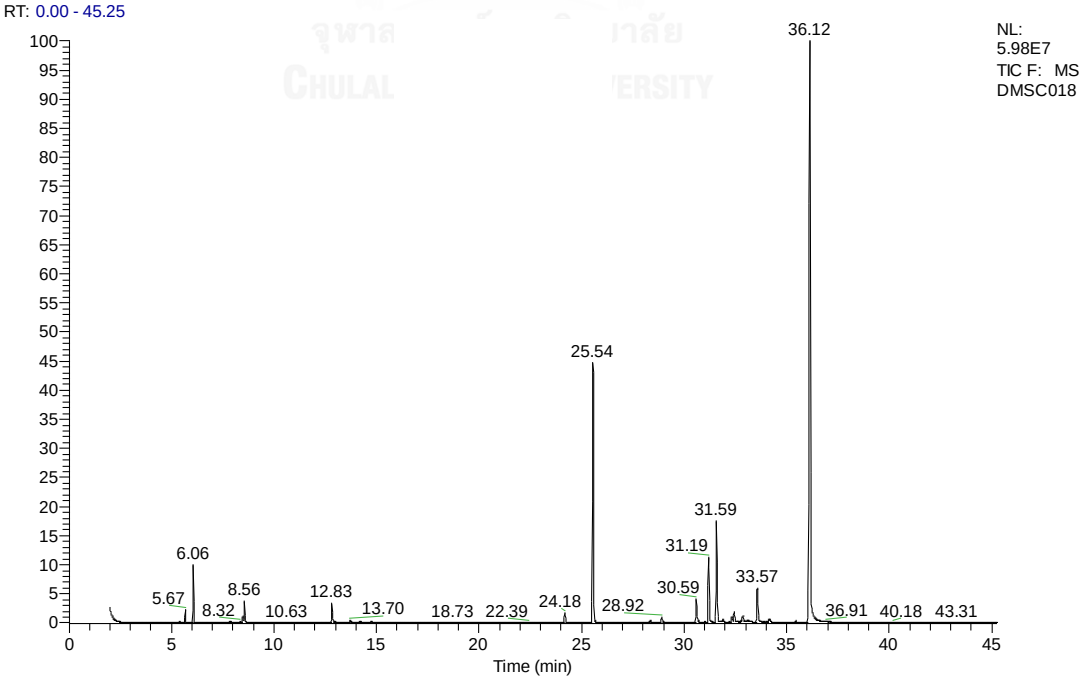


Figure 125 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (4)

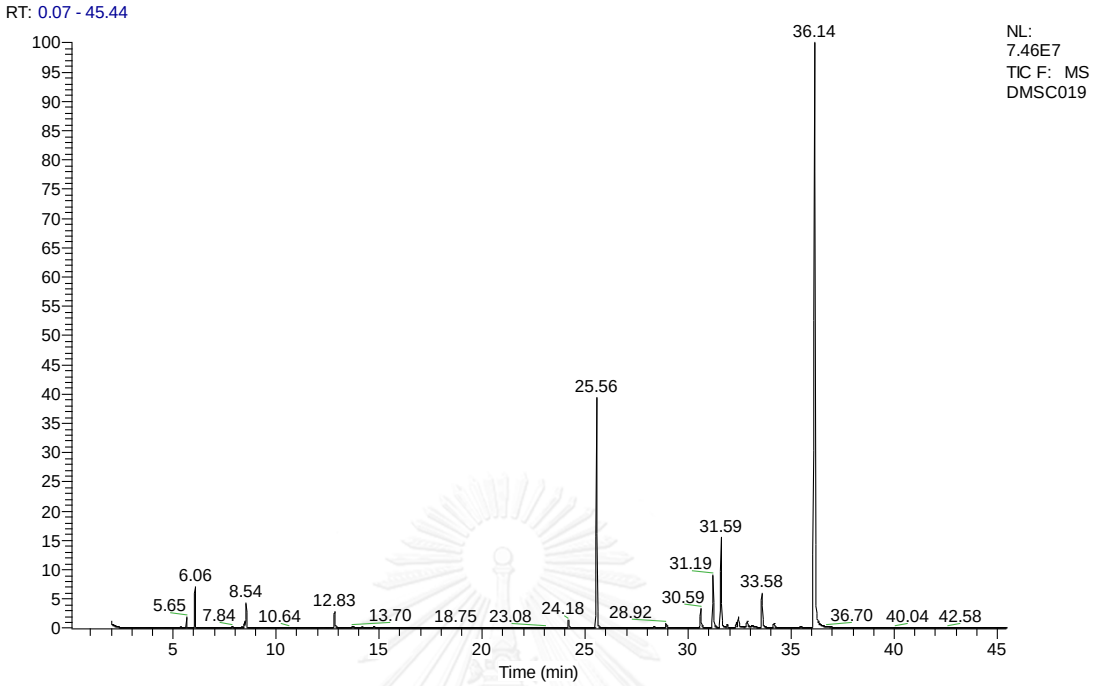


Figure 126 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (5)

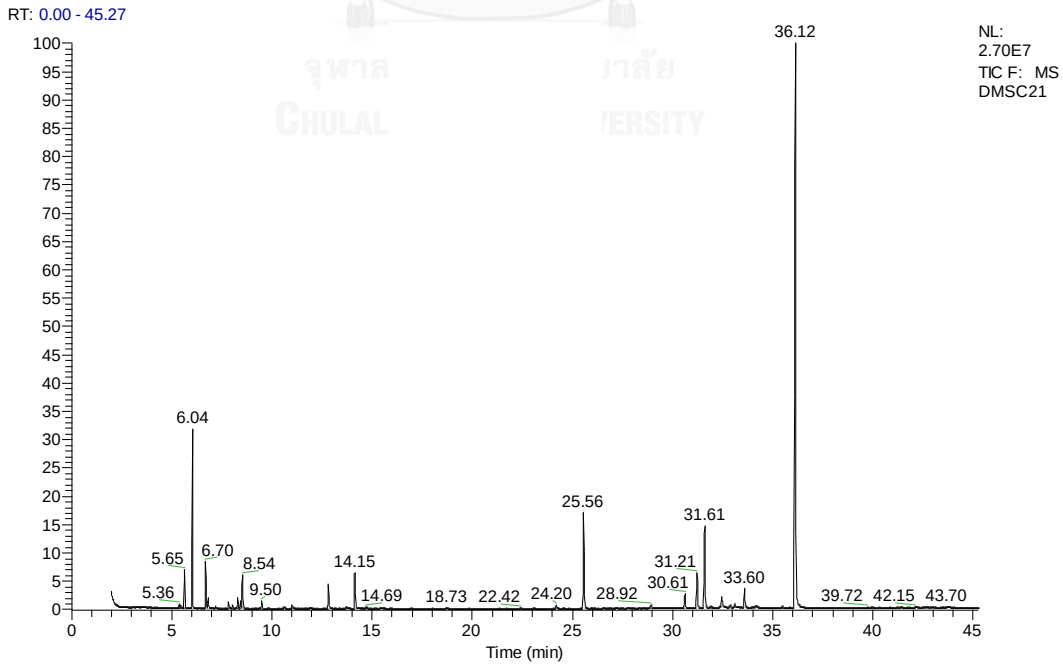


Figure 127 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (6)

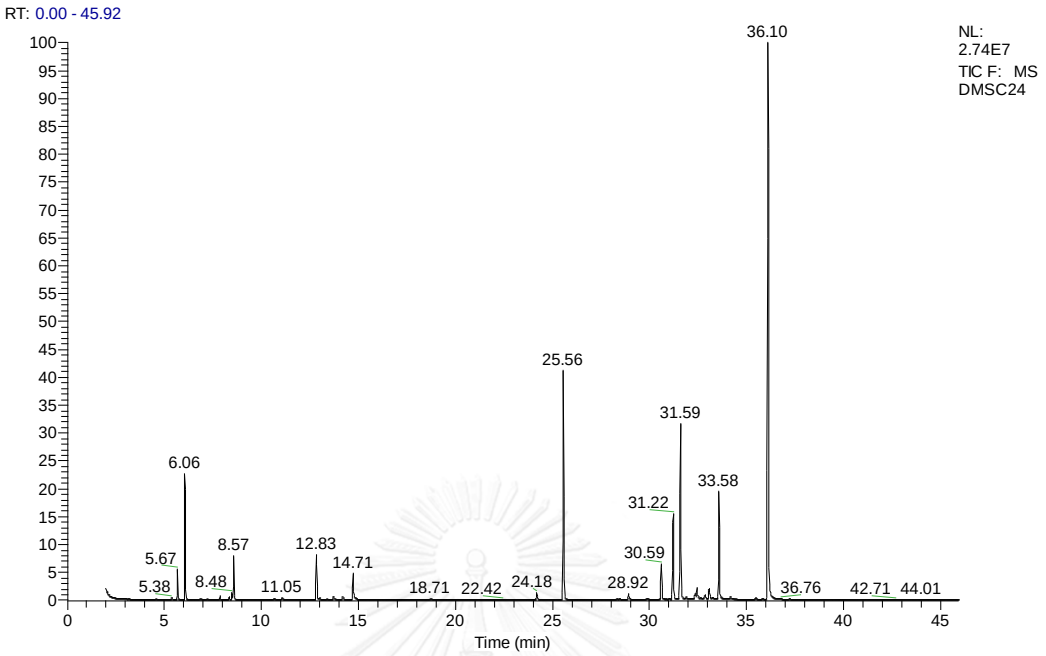


Figure 128 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (7)

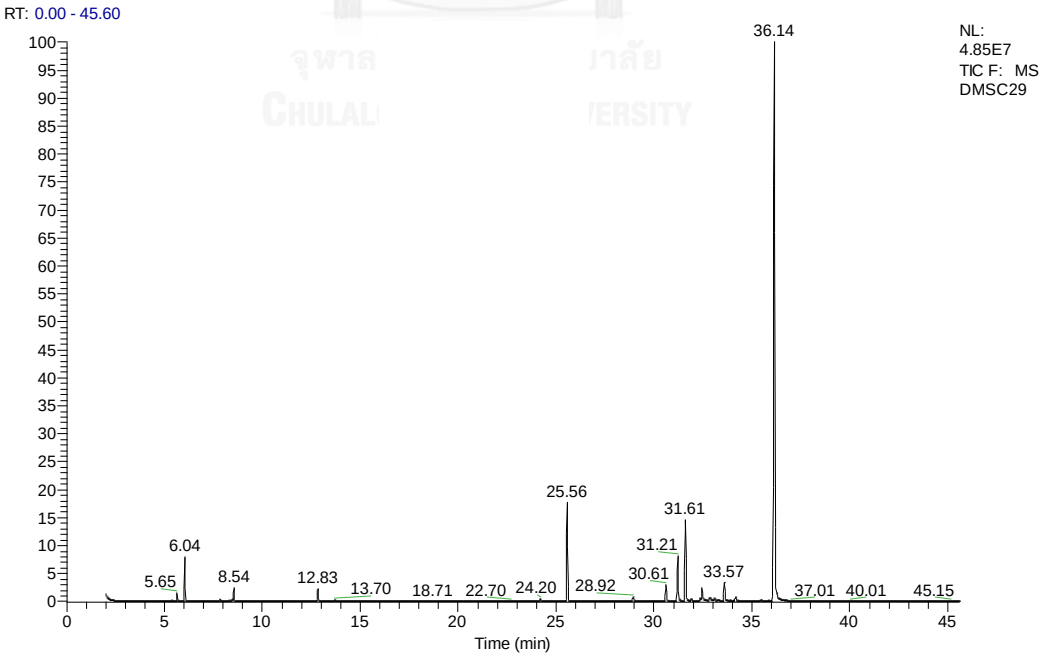


Figure 129 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (8)

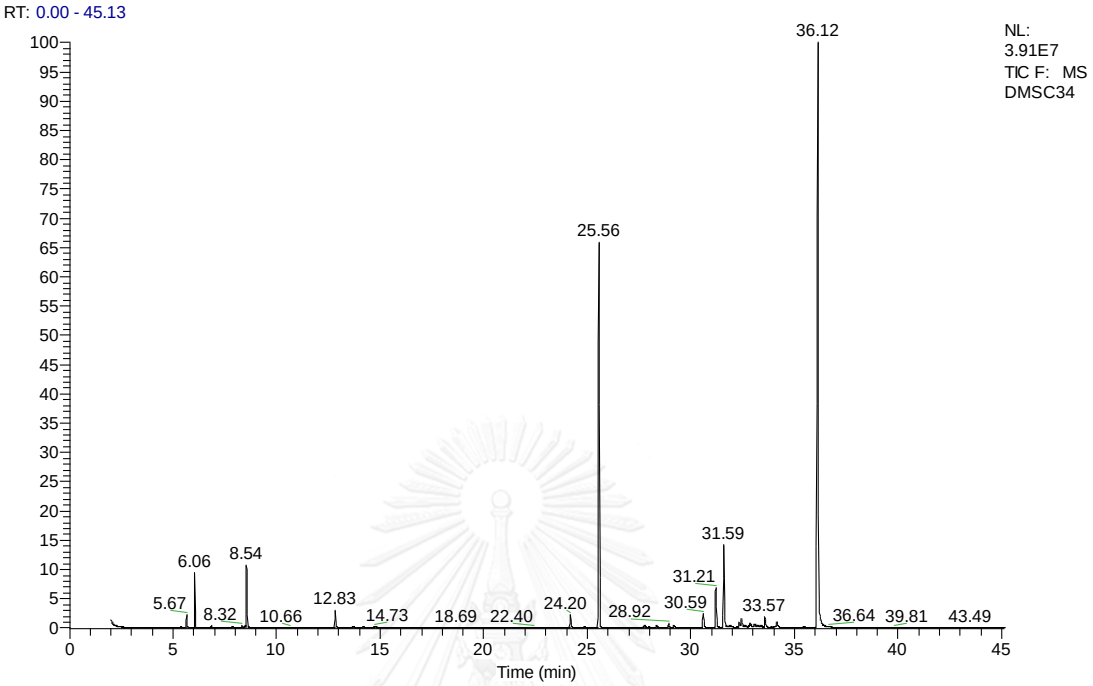


Figure 130 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (9)

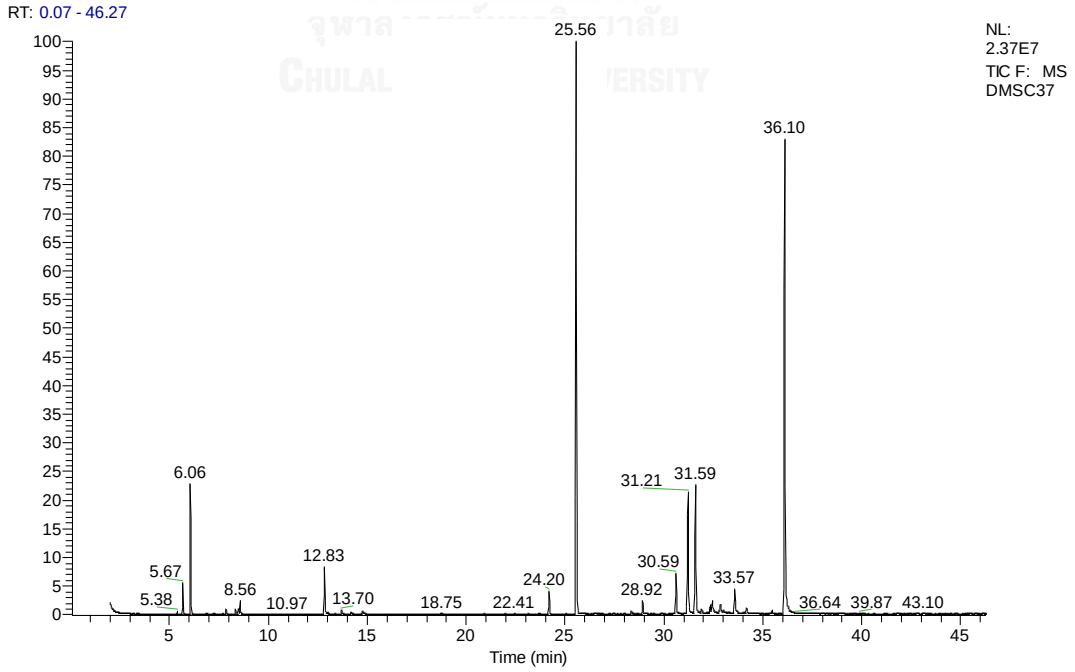


Figure 131 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (10)

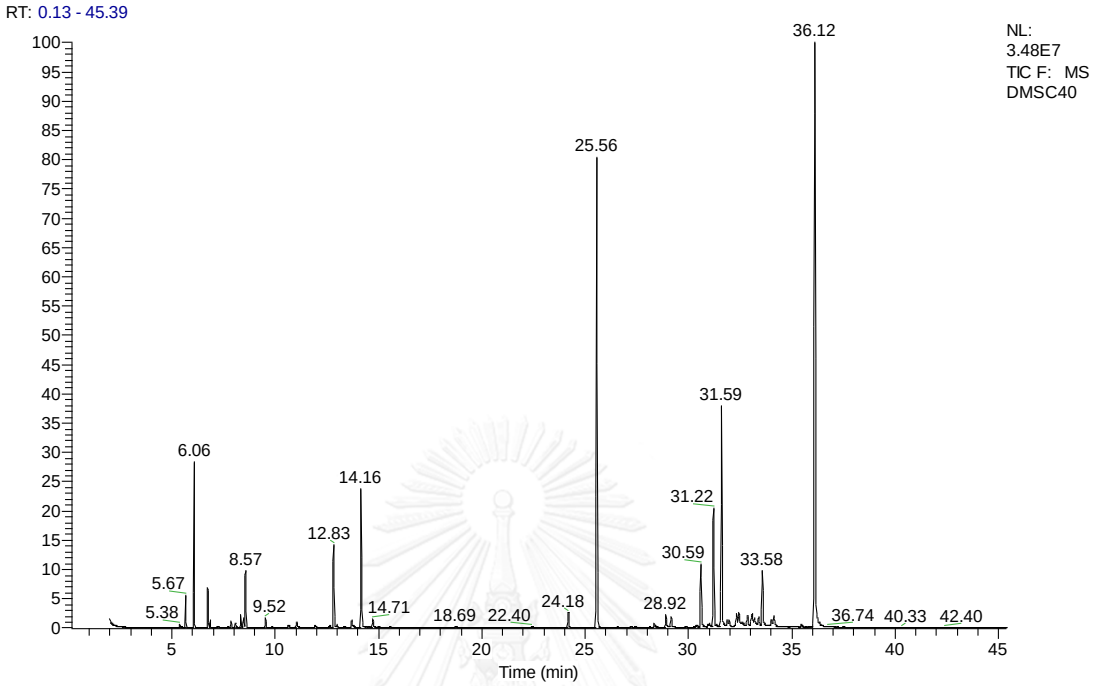


Figure 132 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (11)

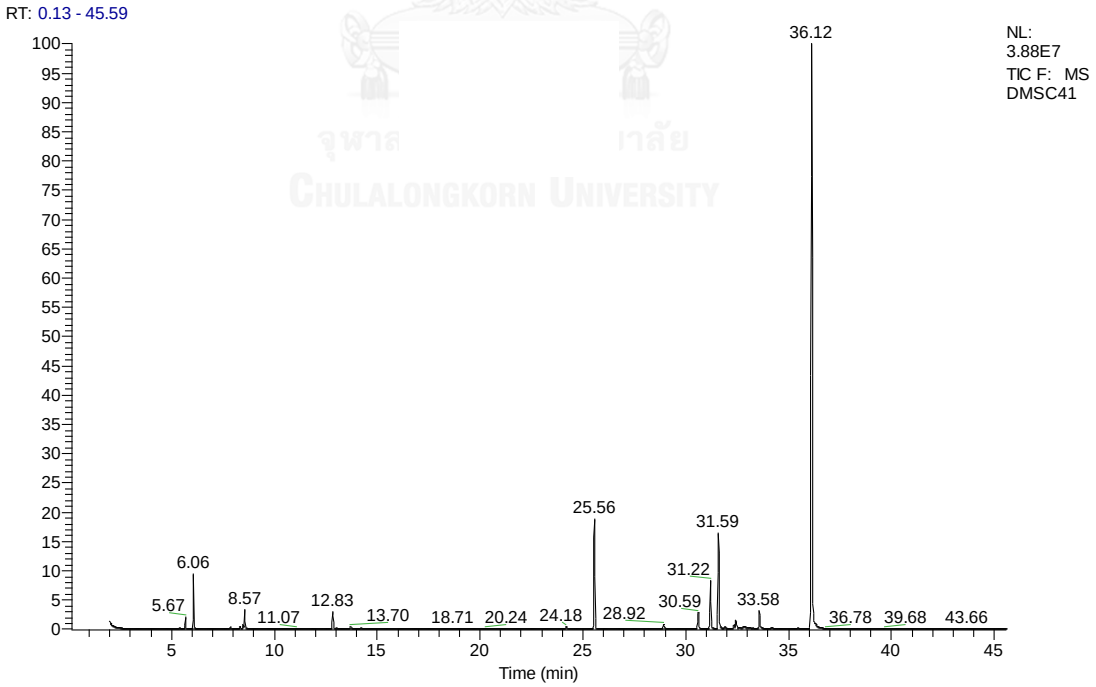


Figure 133 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (12)

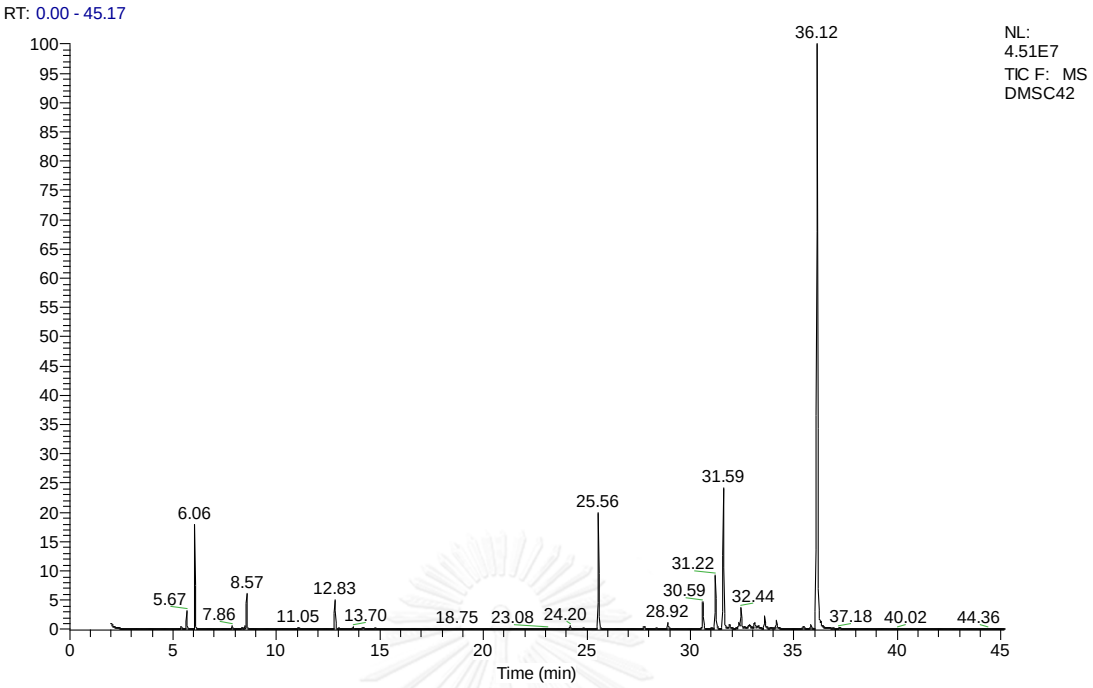


Figure 134 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (13)

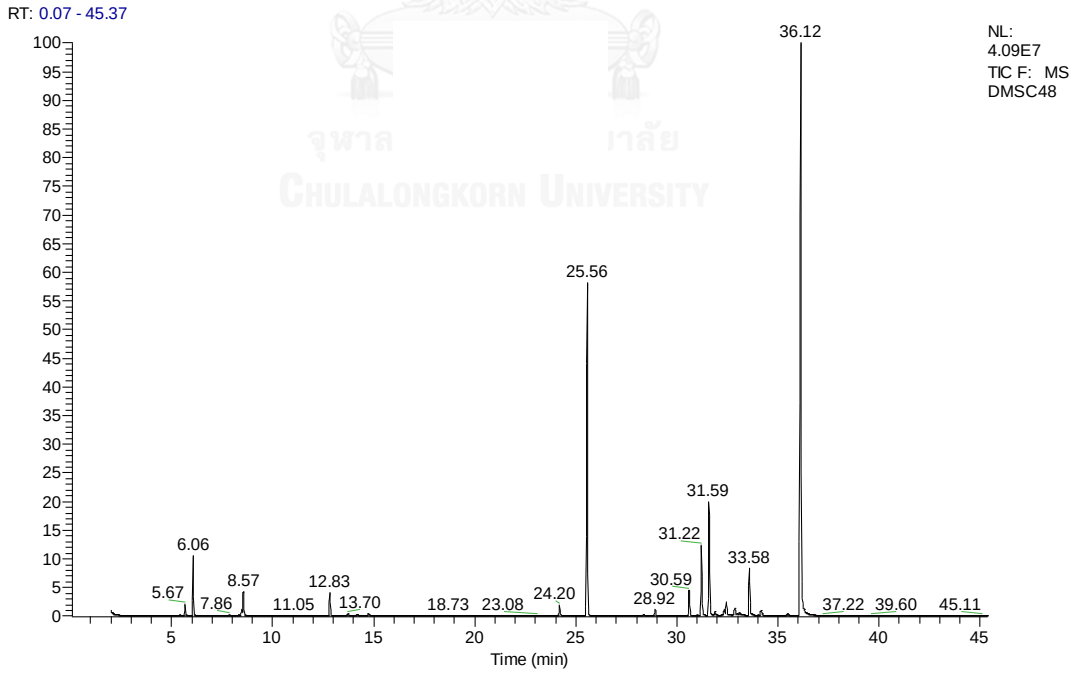


Figure 135 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (14)

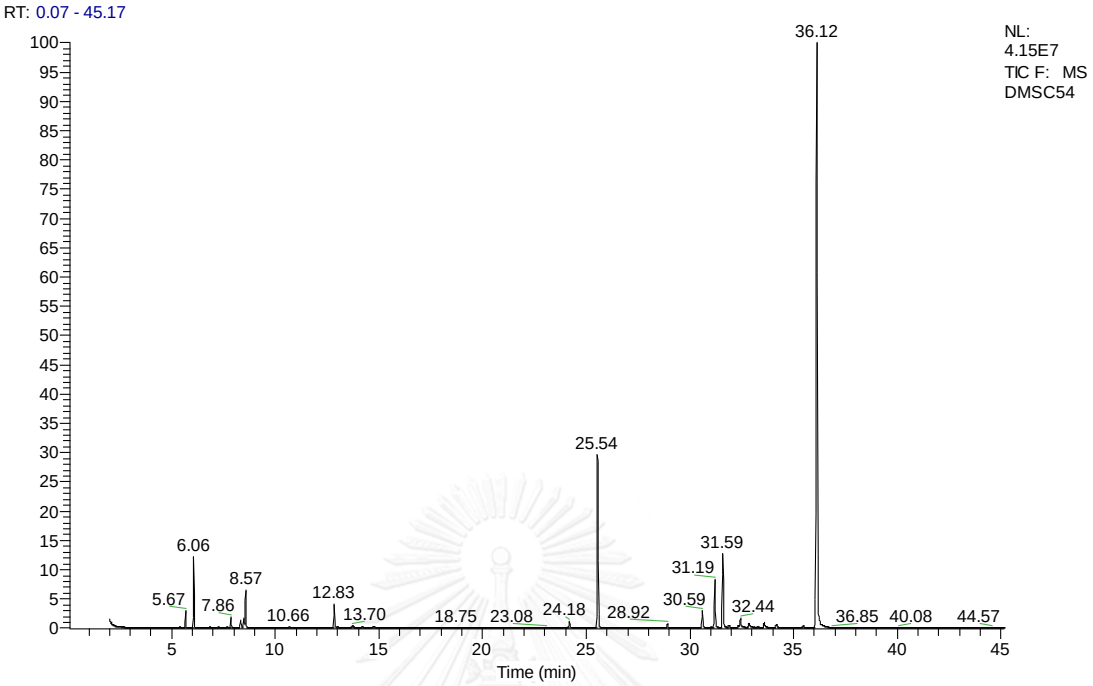


Figure 136 GC chromatogram of the volatile oil of *Z. zerumbet* dried rhizome (15)

APPENDIX B

Antixidant activities

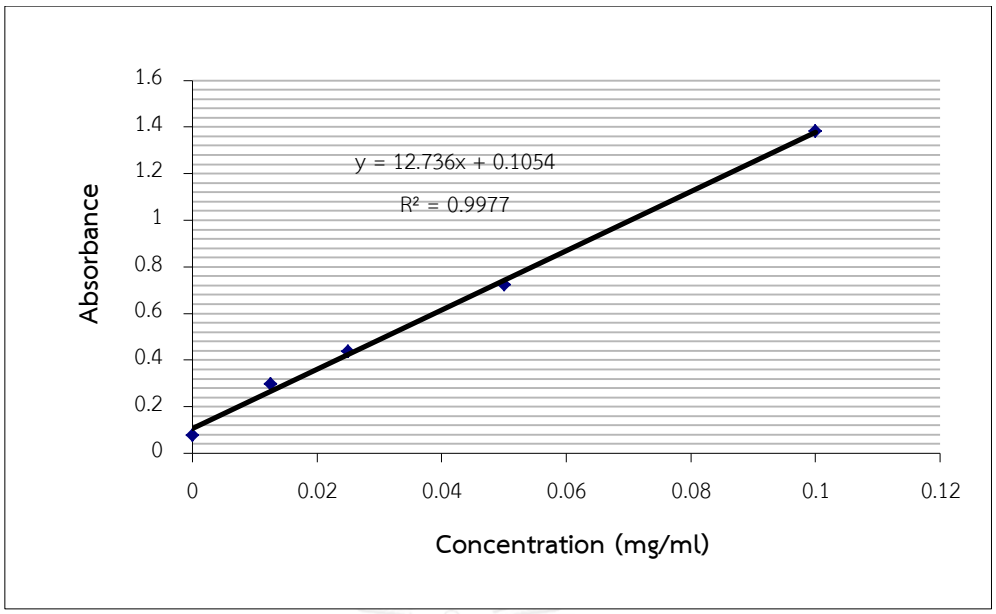


Figure 137 Calibation curve of catechin hydrate that used for calculate total phenolic content of all extracts

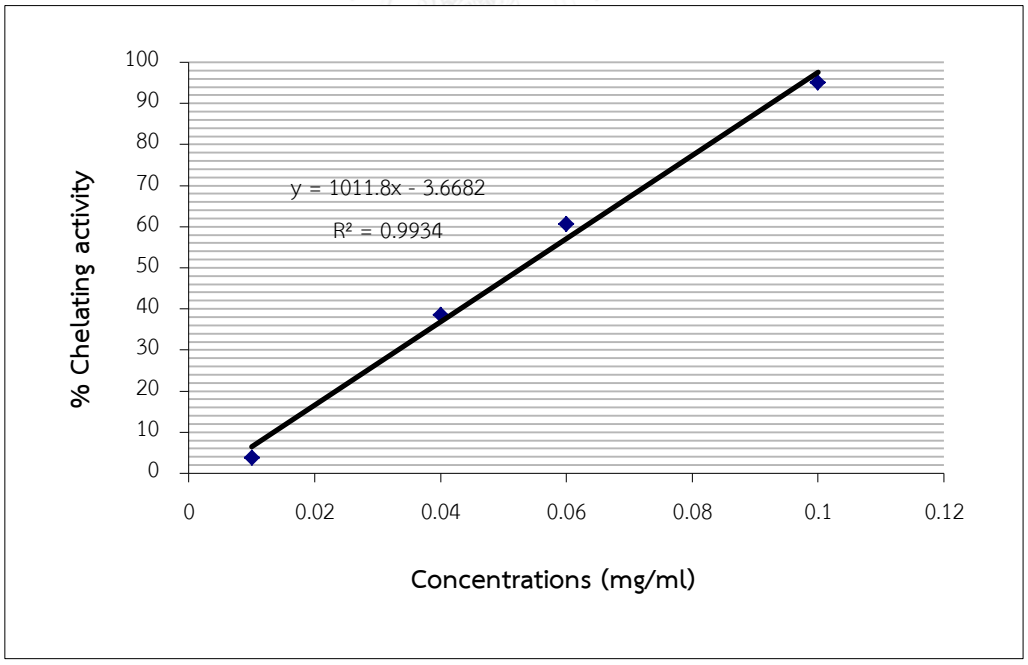


Figure 138 Calibation curve of standard EDTA in metal chelating assay

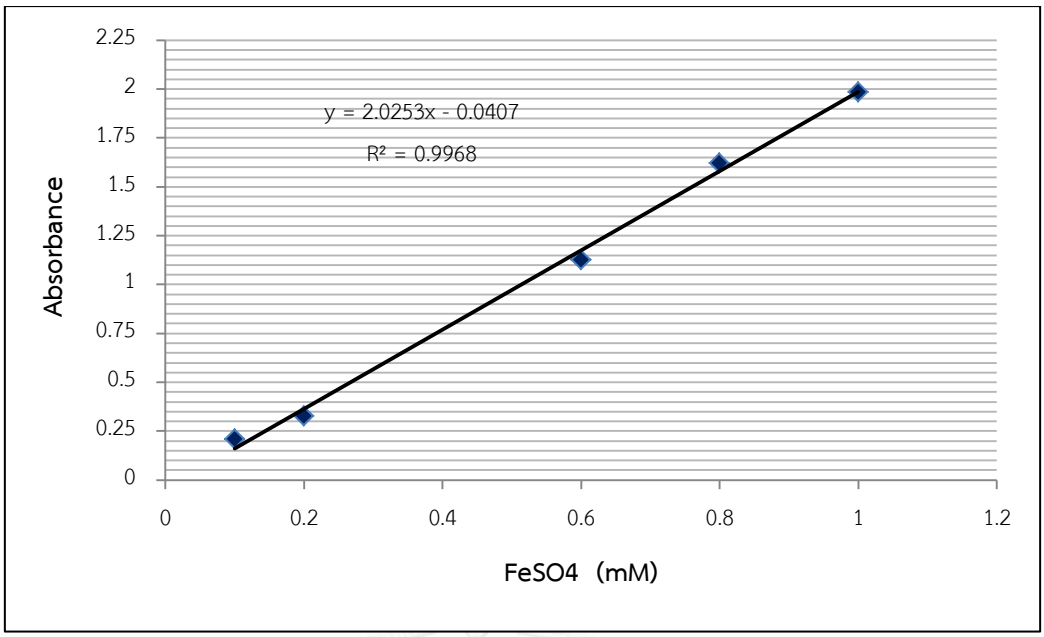


Figure 139 Calibation curve of $FeSO_4$ that used for calculate the FRAP value of all extracts

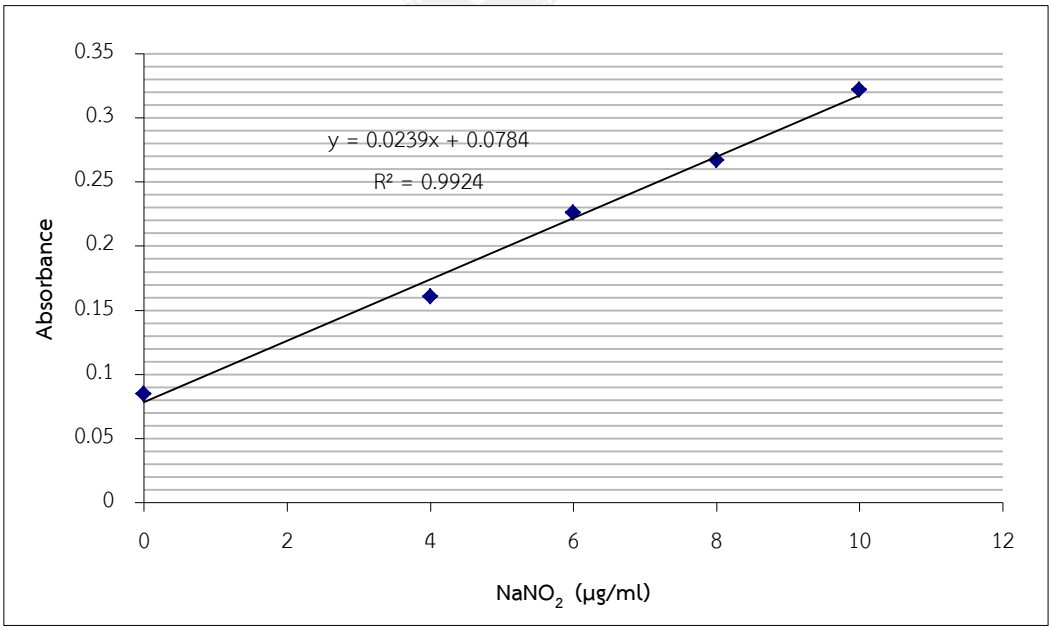


Figure 140 Calibation curve of sodium nitrite that used for calculate nitric oxide content of all extracts.

APPENDIX C

Mutagenic activity

Table 29 Mutagenic assays for the ethanol extracts of each plants species and CKT remedy based on without the nitrite mixture and with the nitrite mixture using *S. typhimurium* TA98

Sample (mg/plate)	TA 98 Without nitrite treatment		TA 98 With nitrite treatment	
	Number of revertant colonies	MI	Number of revertant colonies	MI
DMSO	21.00 ± 3.95	-	45.83 ± 1.47	-
PIE 0.4	23.00 ± 0.63	0.10 ± 0.03	48.33 ± 5.01	0.06 ± 0.19
0.8	27.33 ± 3.01	0.30 ± 0.14	131.17 ± 3.97	1.86 ±0.09
1.6	30.00 ± 4.05	0.43 ± 0.19	606.67 ± 158.60	12.24 ± 3.46
3.2	23.00 ± 8.44	0.14 ± 0.40	387.00 ± 99.07	7.44 ± 2.16
DMSO	22.00 ± 4.98	-	56.33 ± 8.48	-
ACE 0.4	43.83 ± 10.38	0.99 ± 0.47	190.00 ± 52.29	2.37 ± 0.93
0.8	36.33 ± 17.95	1.75 ± 0.17	256.67 ± 85.65	2.81 ± 0.06
1.6	517.33 ± 4.27	22.52 ± 0.19	170.17 ± 21.23	2.02 ± 0.38
3.2	1043.67 ± 22.74	46.44 ± 1.03	214.50 ± 3.27	2.97 ± 0.46
DMSO	16.00 ± 2.76	-	56.33 ± 8.48	-
DCE 0.4	17.83 ± 6.74	0.12 ± 0.42	136.00 ± 47.59	1.41 ± 0.85
0.8	18.33 ± 6.02	0.15 ± 0.38	433.83 ± 19.68	6.70 ± 0.35
1.6	23.00 ± 3.85	0.44 ± 0.29	232.17 ± 40.88	3.12 ± 0.73
3.2	28.00± 4.00	1.60 ± 2.25	239.67 ± 12.79	3.25 ± 0.23
DMSO	22.00 ± 2.53	-	56.33 ± 8.48	-
CPE 0.4	27.67 ± 6.71	0.26 ± 0.31	156.00 ± 9.40	1.77 ± 0.17
0.8	25.50 ± 5.89	0.16 ± 0.22	166.50 ± 10.39	1.96 ± 0.18
1.6	33.17 ± 2.64	0.51 ± 0.12	240.83 ± 16.01	3.28 ± 0.28
3.2	31.00 ± 6.69	0.41 ± 0.30	222.83 ± 61.41	2.96 ± 1.10
DI	41.33 ± 19.21	-	49.00 ± 14.97	-
PIW 0.4	58.00 ± 19.37	0.40 ± 0.47	92.167 ± 23.33	0.88 ± 0.48
0.8	81.33 ± 14.07	0.97± 0.34	129.67 ± 27.82	1.65 ± 0.57

1.6	99.17 ± 12.66	1.40 ± 0.31	181.67 ± 34.47	2.71 ± 0.70
3.2	118.33 ± 22.83	2.27 ± 0.58	254.33 ± 17.04	4.36 ± 0.65
DI	35.33 ± 15.62	-	49 ± 14.97	-
ACW 0.4	55.83 ± 3.66	0.58 ± 0.10	51.67 ± 9.52	0.05 ± 0.19
0.8	47.17 ± 6.85	0.34 ± 0.19	82.17 ± 19.83	0.68 ± 0.41
1.6	121.50 ± 41.35	2.44 ± 1.17	94.33 ± 10.63	0.93 ± 0.22
3.2	161.67 ± 37.40	4.29 ± 1.99	114.67 ± 13.26	1.34 ± 0.27
DI	41.33 ± 19.21	-	49.00 ± 14.97	-
DCW 0.4	55.33 ± 11.52	0.34 ± 0.28	79.67 ± 3.01	0.63 ± 0.06
0.8	55.33 ± 20.84	0.34 ± 0.50	58.67 ± 12.45	0.20 ± 0.25
1.6	55.67 ± 16.55	0.35 ± 0.40	66.17 ± 10.36	0.35 ± 0.21
3.2	84.67 ± 14.08	1.34 ± 1.00	114.67 ± 13.26	1.34 ± 0.27
DI	41.33 ± 19.21	-	49.00 ± 14.97	-
CPW 0.4	117.83 ± 21.15	1.85 ± 0.51	49.17 ±4.88	0.00 ± 0.10
0.8	177.00 ± 39.92	3.28 ± 0.97	49.33 ± 1.03	0.01 ± 0.02
1.6	77.67 ± 12.49	0.88 ± 0.30	50.33 ± 6.09	0.03 ± 0.12
3.2	48.33 ± 3.62	0.17 ± 0.08	53.50 ± 11.85	0.09 ± 0.24
DMSO	24.00 ± 2.10	-	56.33 ± 8.48	-
CKT 0.4	29.50 ± 3.67	0.23 ± 0.15	73.50 ± 3.27	0.31 ± 0.06
0.8	24.33 ± 0.52	0.01 ± 0.02	98.17 ± 7.20	0.74 ± 0.13
1.6	32.33 ± 3.67	0.35 ± 0.15	114.33 ± 9.40	1.03 ± 0.17
3.2	56.50 ± 2.43	1.35 ± 0.10	154.00 ± 15.54	1.73 ± 0.28

Data are reported as means ± SD of three independent replicates and the mutagenic index (MI). Abbreviations including: PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thaก remedy, E = ethanol extract.

Table 30 Mutagenic assays for the ethanol extracts of each plants species and CKT remedy based on without the nitrite mixture and with the nitrite mixture using *S. typhimurium* TA100

Sample (mg/plate)	TA 100 Without nitrite treatment		TA 100 With nitrite treatment	
	Number of revertant colonies	MI	Number of revertant colonies	MI
DMSO	118.33 ± 7.97	-	155.67 ± 12.08	-
PIE 0.4	9.50 ± 19.51	0.08 ± 0.17	149.67 ± 5.13	0.96 ± 0.03
0.8	22.00 ± 26.17	0.19 ± 0.22	203.67 ± 61.74	1.31 ± 0.40
1.6	74.83 ± 16.75	0.63 ± 0.14	202.67 ± 58.56	1.30 ± 0.38
3.2	106.39 ± 49.08	2.69 ± 0.34	397.67 ± 19.75	2.56 ± 0.13
DMSO	103.67 ± 17.01	-	143.00 ± 38.84	-
ACE 0.4	3.67 ± 19.47	0.04 ± 0.19	614.00 ± 89.54	4.29 ± 0.63
0.8	434.67 ± 23.67	4.19 ± 0.23	429.00 ± 39.42	3.00 ± 0.28
1.6	934.83 ± 20.96	9.02 ± 0.20	452.83 ± 33.00	3.17 ± 0.23
3.2	1053.33 ± 141.93	10.16 ± 1.11	923.00 ± 141.37	6.46 ± 0.99
DMSO	108.67 ± 6.98	-	134.00 ± 7.04	-
DCE 0.4	8.00 ± 13.87	0.07 ± 0.13	117.67 ± 1.63	0.88 ± 0.01
0.8	11.67 ± 15.73	0.11 ± 0.15	127.33 ± 90.73	0.95 ± 0.68
1.6	30.67 ± 11.84	0.28 ± 0.11	218.67 ± 42.96	1.63 ± 0.32
3.2	0.83 ± 3.67	0.01 ± 0.03	163.83 ± 44.96	1.22 ± 0.34
DMSO	99.33 ± 9.69	-	134.00 ± 7.04	-
CPE 0.4	26.67 ± 36.03	0.27 ± 0.36	40.67 ± 5.05	0.30 ± 0.04
0.8	22.33 ± 5.68	0.23 ± 0.06	56.67 ± 19.66	0.42 ± 0.15
1.6	1.83 ± 7.68	0.02 ± 0.08	138.00 ± 70.54	1.03 ± 0.53
3.2	15.50 ± 13.47	0.16 ± 0.14	37.33 ± 25.03	0.28 ± 0.19
DI	139.00 ± 16.63	-	210.00 ± 23.01	-
PIW 0.4	40.33 ± 46.92	0.29 ± 0.34	62.33 ± 80.15	0.30 ± 0.38
0.8	50.67 ± 40.96	0.37 ± 0.30	44.67 ± 9.00	0.21 ± 0.04
1.6	20.33 ± 34.48	0.15 ± 0.25	162.67 ± 30.72	0.78 ± 0.15
3.2	75.33 ± 26.33	0.54 ± 0.19	151.33 ± 24.12	0.72 ± 0.12

DI	139.00 ± 16.63	-	210.00 ± 23.01	-
ACW 0.4	95.00 ± 53.74	0.68 ± 0.39	146.67 ± 99.81	0.70 ± 0.48
0.8	75.67 ± 21.79	0.54 ± 0.16	121.33 ± 41.74	0.58 ± 0.20
1.6	32.17 ± 45.56	0.23 ± 0.33	269.33 ± 67.56	1.28 ± 0.32
3.2	96.33 ± 10.82	0.69 ± 0.08	183.00 ± 52.24	0.87 ± 0.25
DI	139.00 ± 16.63	-	187.33 ± 9.85	-
DCW 0.4	29.67 ± 16.18	0.21 ± 0.12	49.33 ± 21.45	0.26 ± 0.12
0.8	63.00 ± 18.63	0.45 ± 0.13	13.33 ± 26.37	0.07 ±0.14
1.6	105.00 ± 49.36	0.76 ± 0.36	39.00 ± 29.16	0.21 ± 0.34
3.2	59.00 ± 25.71	0.42 ± 0.19	182.00 ± 29.28	0.97 ± 0.16
DI	139.00 ± 16.63	-	210.00 ± 23.01	-
CPW 0.4	16.00 ± 18.82	0.12 ± 0.13	77.33 ± 3.27	0.37 ± 0.02
0.8	27.33 ± 34.74	0.20 ± 0.25	26.83 ± 19.17	0.13 ± 0.09
1.6	2.33 ± 13.78	0.02 ± 0.10	33.67 ± 15.67	0.16 ± 0.08
3.2	60.00 ± 32.37	0.43 ± 0.23	87.00 ± 14.63	0.41 ± 0.07
DMSO	136.00 ± 13.49	-	161.50 ± 41.20	-
CKT 0.4	15.33 ± 30.40	0.11 ± 0.22	90.50 ± 29.39	0.56 ± 0.18
0.8	5.83 ± 6.80	0.04 ± 0.05	191.50 ± 64.21	1.19 ± 0.40
1.6	39.17 ± 20.45	0.29 ± 0.15	239.17 ± 38.94	1.48 ± 0.24
3.2	29.67 ± 14.25	0.22 ± 0.11	317.17 ± 76.48	1.96 ± 0.47

Data are reported as means ± SD of three independent replicates and the mutagenic index (MI). Abbreviations including: PI = *P. indica*, AC = *A. calamus,* DC = *D. serrulata,* CP = *C. paniculatum*, CKT = Cha-Tu-Ka-La-Thaก remedy, W = ethanol extract.

Table 31 Mutagenic assays for the ethanol extracts of each plants species and TPT remedy based on without the nitrite mixture and with the nitrite mixture using *S. typhimurium* TA98

Sample (mg/plate)	TA 98 Without nitrite treatment		TA 98 With nitrite treatment	
	Number of revertant colonies	MI	Number of revertant colonies	MI
DMSO	15.00 ± 3.03	-	23.67 ± 8.50	-
ZZE 0.4	17.33 ± 3.62	0.16 ± 0.24	48.33 ± 21.35	1.04 ± 0.90
0.8	16.50 ± 2.74	0.10 ± 0.18	64.83 ± 16.81	1.74 ± 0.71
1.6	19.00 ± 1.23	0.27 ± 0.08	49.33 ± 14.58	1.09 ±0.62
3.2	15.83 ± 1.33	0.56 ± 0.09	160.00 ± 62.50	5.76 ± 2.64
DMSO	19.67 ± 7.47	-	23.67 ± 8.50	-
ZME 0.4	21.83 ± 7.08	0.11 ± 0.36	147.83 ± 102.72	5.25 ± 4.34
0.8	27.83 ± 19.28	0.42 ± 0.98	248.50 ± 42.81	9.50 ± 1.81
1.6	27.33 ± 12.75	0.39 ± 0.65	152.17 ± 35.35	5.43 ± 1.49
3.2	20.83 ± 7.22	0.06 ± 0.37	141.00 ± 43.26	4.96 ± 1.83
DMSO	13.33 ± 2.34	-	23.33 ± 4.27	-
CNE 0.4	13.83 ± 3.49	0.04 ± 0.27	99.00 ± 18.87	3.24 ± 0.81
0.8	13.83 ± 3.49	0.04 ± 0.26	129.67 ± 20.03	4.56 ± 0.86
1.6	14.33 ± 5.35	0.08 ± 0.40	140.00 ± 17.58	5.00 ± 0.75
3.2	20.33 ± 6.89	0.53 ± 0.52	269.50 ± 10.60	14.84 ± 0.45
DMSO	20.67 ± 0.52	-	23.67 ± 8.50	-
ZZW 0.4	21.67 ± 1.86	0.05 ± 0.09	25.50 ± 2.67	0.08 ± 0.11
0.8	37.17 ± 11.97	0.80 ± 0.58	69.83 ± 17.76	1.96 ± 0.75
1.6	33.33 ± 10.33	0.61 ± 0.50	76.33 ± 26.54	2.23 ± 1.12
3.2	24.00 ± 0.89	0.16 ± 0.04	149.33 ± 59.76	5.31 ± 2.53
DI	10.17 ± 1.33	-	11.33 ± 3.45	-
ZMW 0.4	12.50 ± 3.99	0.23 ± 0.39	20.83 ± 7.23	0.84 ± 0.64
0.8	15.50 ± 3.51	0.53 ± 0.35	147.50 ± 50.75	12.02 ± 4.48
1.6	59.00 ± 5.76	4.80 ± 0.57	156.83 ± 31.45	12.84 ± 2.78
3.2	15.00 ± 4.82	0.48 ± 0.47	237.00 ± 29.08	20.91 ± 2.57

DI	34.33 ± 13.08	-	18.83 ± 8.01	-
CNW 0.4	38.83 ± 9.75	0.13 ± 0.28	43.83 ± 15.09	1.33 ± 0.80
0.8	47.67 ± 15.67	0.38 ± 0.46	27.00 ± 8.99	0.43 ± 0.48
1.6	50.67 ± 5.01	0.48 ± 0.15	61.33 ± 28.02	2.26 ± 1.49
3.2	126.67 ± 16.63	2.69 ± 0.48	138.67 ± 26.21	6.36 ± 1.39
DI	19.33 ± 7.66	-	22.33 ± 6.81	-
TPT 0.4	26.50 ± 8.69	0.37 ± 0.45	22.83 ± 10.07	0.02 ± 0.45
0.8	24.83 ± 10.87	0.28 ± 0.56	47.00 ± 19.45	1.10 ± 0.87
1.6	24.67 ± 2.33	5.33 ± 2.73	48.00 ± 21.57	1.15 ± 0.97
3.2	19.50 ± 5.09	0.01 ± 0.26	63.00 ± 24.38	1.82 ± 1.09

Data are reported as means ± SD of three independent replicates and the mutagenic index (MI). Abbreviations including: ZZ = *Z. zerumbet*, ZM = *Z. montanum,* CN = *C. nardus,* TPT = Tree Phon Thad, E= ethanol extract, W = fractionated water extract

Table 32 Mutagenic assays for the ethanol extracts of each plants species and TPT remedy based on without the nitrite mixture and with the nitrite mixture using *S. typhimurium* TA100

Sample (mg/plate)	TA 100 Without nitrite treatment		TA 100 With nitrite treatment	
	Number of revertant colonies	MI	Number of revertant colonies	MI
DMSO	67.00 ± 14.34	-	109.17 ± 17.08	-
ZZE 0.4	71.50 ± 2.73	0.07 ± 0.04	386.33 ± 63.09	2.54 ± 0.58
0.8	83.17 ± 5.98	0.24 ± 0.09	437.83 ± 31.27	3.01 ± 0.84
1.6	84.50 ± 11.62	0.26 ± 0.17	597.50 ± 149.58	4.47 ± 1.37
3.2	85.83 ± 17.54	0.28 ± 0.26	451.83 ± 197.49	3.14 ± 1.81
DMSO	65.50 ± 18.82	-	109.17 ± 17.08	-
ZME 0.4	76.33 ± 22.18	0.17 ± 0.34	220.17 ± 7.20	1.02 ± 0.07
0.8	74.67 ± 22.77	0.14 ± 0.35	298.83 ±103.26	1.74 ± 0.95
1.6	75.83 ± 14.47	0.16 ± 0.22	675.33 ± 40.68	5.19 ± 0.37
3.2	85.33 ± 17.77	0.30 ± 0.27	699.17 ± 159.37	5.41 ± 1.46
DMSO	74.83 ± 5.60	-	105.00 ± 14.06	-
CNE 0.4	86.00 ± 24.32	0.15 ± 0.33	294.33 ± 94.51	1.80 ± 0.90
0.8	98.67 ± 42.24	0.32 ± 0.56	348.17 ± 31.98	2.32 ± 0.30
1.6	91.00 ± 29.36	0.22 ± 0.39	448.67 ± 37.58	3.27 ± 0.36
3.2	98.17 ± 21.66	0.31 ± 0.29	258.83 ± 35.09	1.47 ± 0.33
DMSO	107.33 ± 2.51	-	133.17 ± 22.43	-
ZZW 0.4	134.33 ± 23.93	0.25 ± 0.22	156.50 ± 41.53	1.18 ± 0.31
0.8	156.67 ± 10.65	0.46 ± 0.10	194.33 ± 17.31	0.46 ± 0.13
1.6	109.17 ± 6.68	0.02 ± 0.06	192.83 ± 58.03	0.45 ± 0.44
3.2	139.00 ± 18.22	0.30 ± 0.17	308.50 ± 34.65	1.32 ± 0.26
DI	65.50 ± 17.82	-	128.67 ± 2.94	-
ZMW 0.4	83.33 ± 1.21	0.27 ± 0.02	150.50 ± 22.41	0.17 ± 0.17
0.8	77.00 ± 4.38	0.18 ± 0.07	152.67 ± 18.83	0.19 ± 0.15
1.6	89.67 ± 10.91	0.37 ± 0.16	164.83 ± 8.59	0.28 ± 0.07
3.2	97.83 ± 1.47	0.49 ± 0.02	237.17 ± 28.59	0.84 ± 0.22

DI		94.50 ± 25.17	-	124.33 ± 23.65	-
CNW	0.4	117.17 ± 21.90	0.24 ± 0.24	156.67 ± 35.56	0.26 ± 0.29
	0.8	146.50 ± 42.36	0.55 ± 0.45	231.17 ± 26.26	0.86 ± 0.21
	1.6	189.83 ± 31.37	1.01 ± 0.33	258.17 ± 44.86	1.08 ± 0.36
	3.2	98.50 ± 6.03	0.04 ± 0.06	319.17 ± 58.83	1.57 ± 0.47
DI		61.50 ± 19.50	-	112.50 ± 19.46	-
TPT	0.4	66.67 ± 11.34	0.08 ± 0.18	341.67 ± 48.82	2.04 ± 0.43
	0.8	68.00 ± 17.18	0.11 ± 0.28	373.17 ± 63.93	2.32 ± 0.57
	1.6	69.00 ± 10.66	0.12 ± 0.17	504.00 ± 27.80	3.48 ± 0.25
	3.2	63.50 ± 2.07	0.03 ± 0.03	477.33 ± 84.39	3.24 ± 0.75

Data are reported as means ± SD of three independent replicates and the mutagenic index (MI). Abbreviations including: ZZ = *Z. zerumbet*, ZM = *Z. montanum,* CN = *C. nardus,* TPT = Tree Phon Thad, E= ethanol extract, W = fractionated water extract

APPENDIX D

In vitro anti-inflammatory activities

Nitric oxide inhibition assay

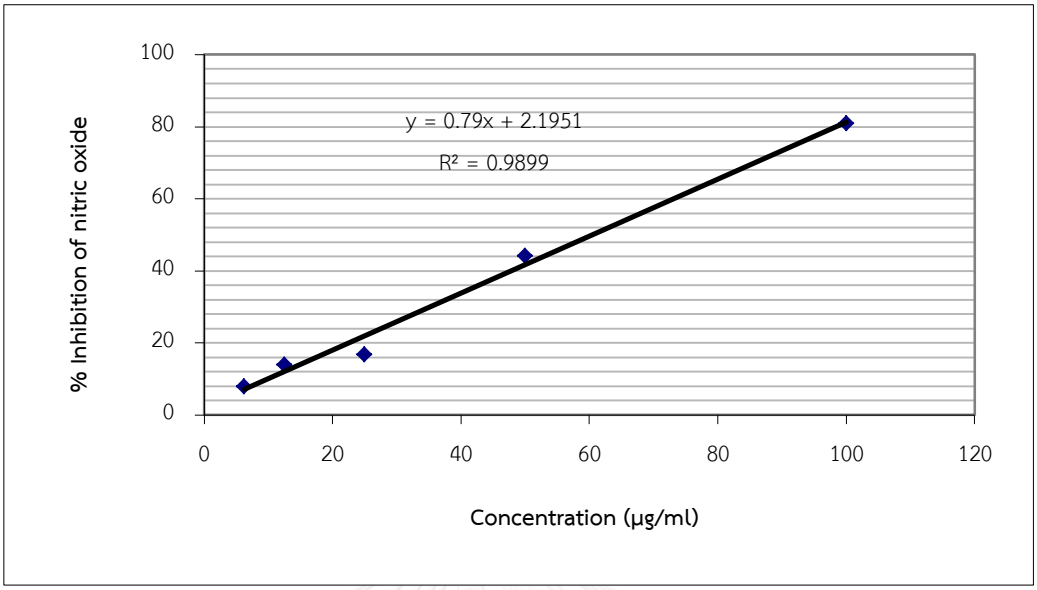


Figure 141 Calibration curve of the ethanol extract of *P. indica* root

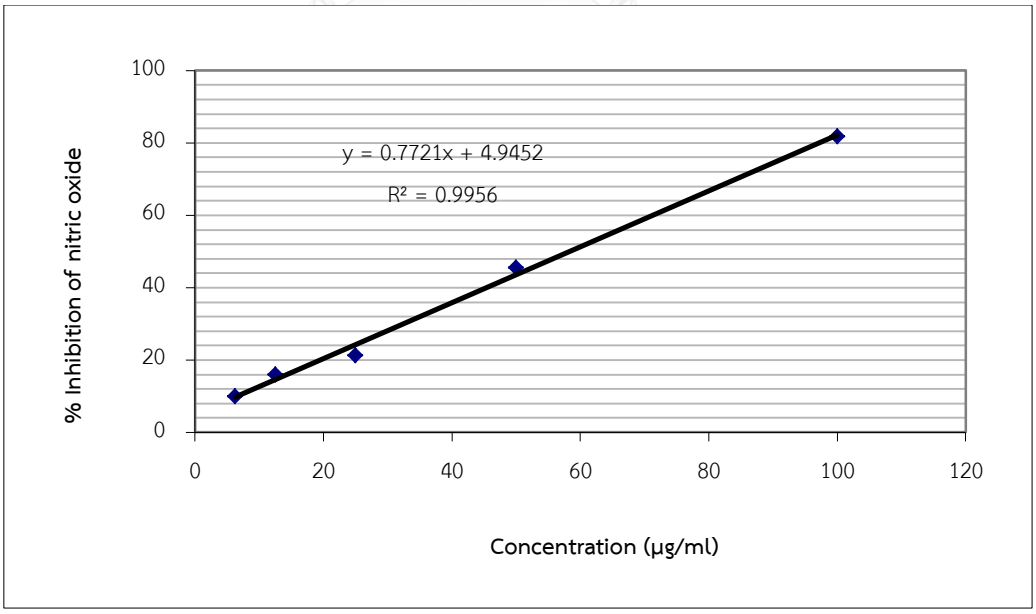


Figure 142 Calibration curve of the ethanol extract of *A. calamus* rhizome

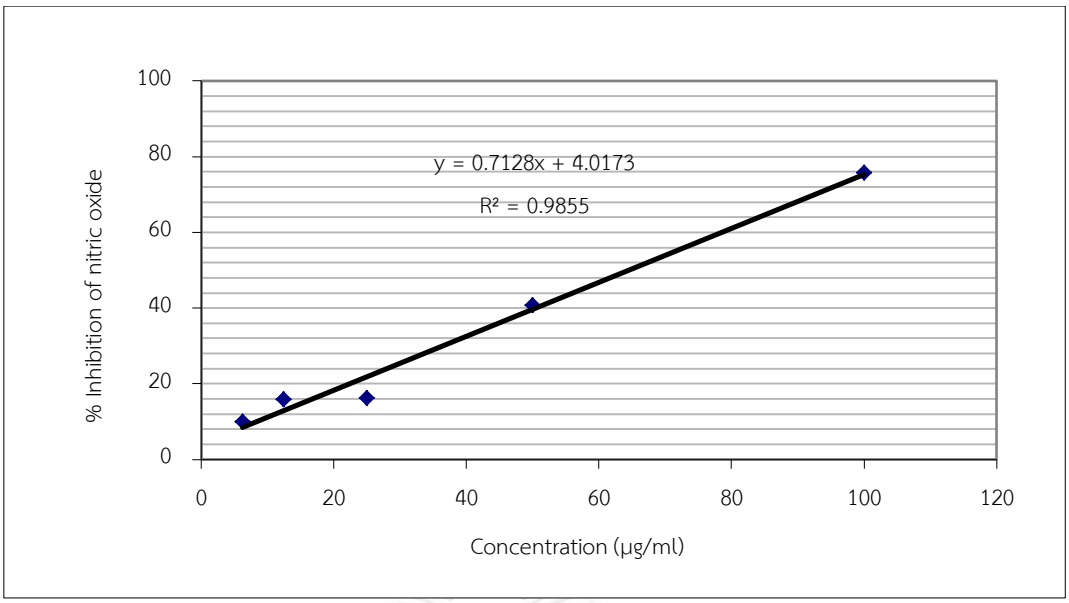


Figure 143 Calibration curve of the ethanol extract of *D. serrulata* root

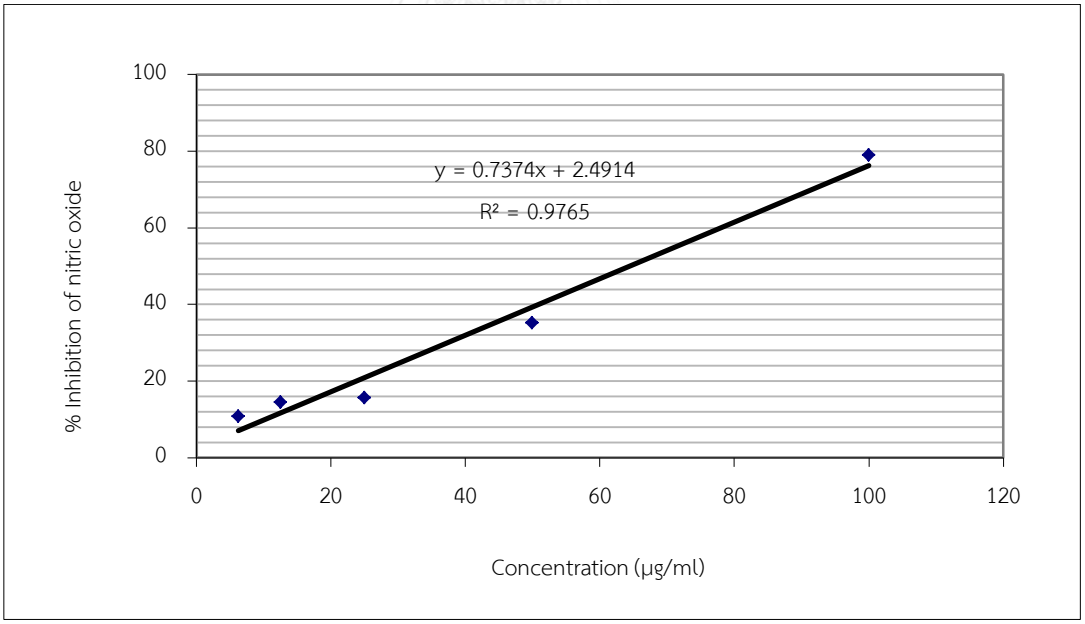


Figure 144 Calibration curve of the ethanol extract of *C. paniculatum* root

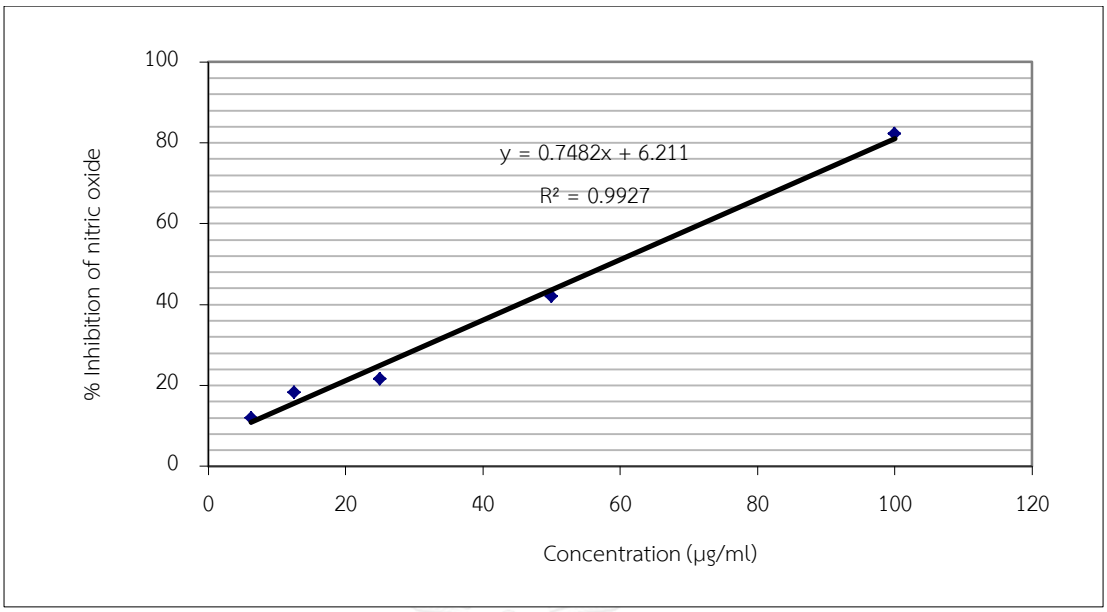


Figure 145 Calibration curve of the CKT extract

TNF-alpha inhibition assay

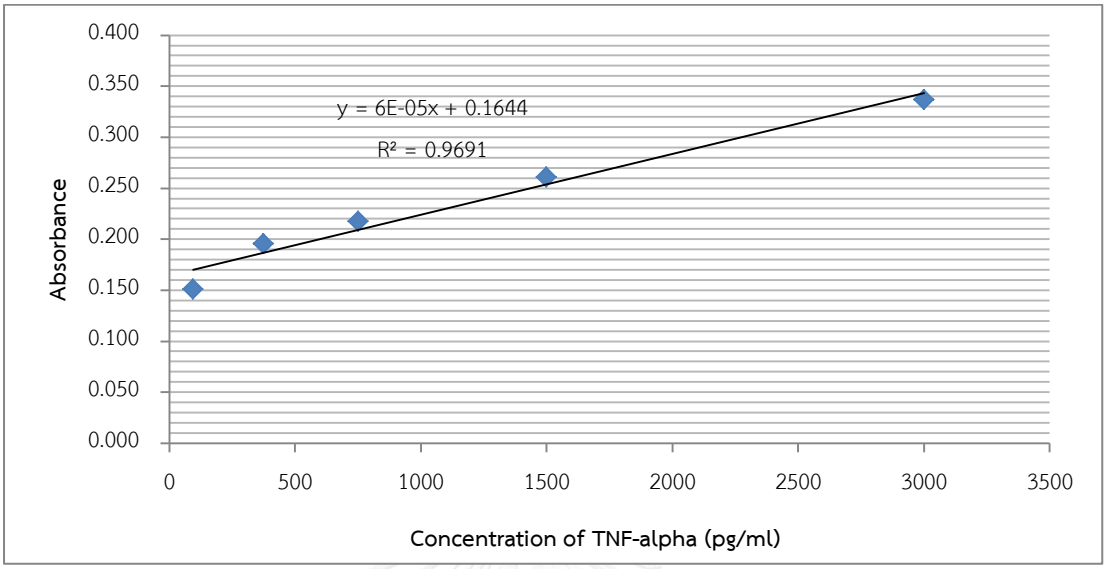


Figure 146 Calibration curve of Standard TNF-alpha for calculated the TNF-alpha content in CKT remedy and its ingredients

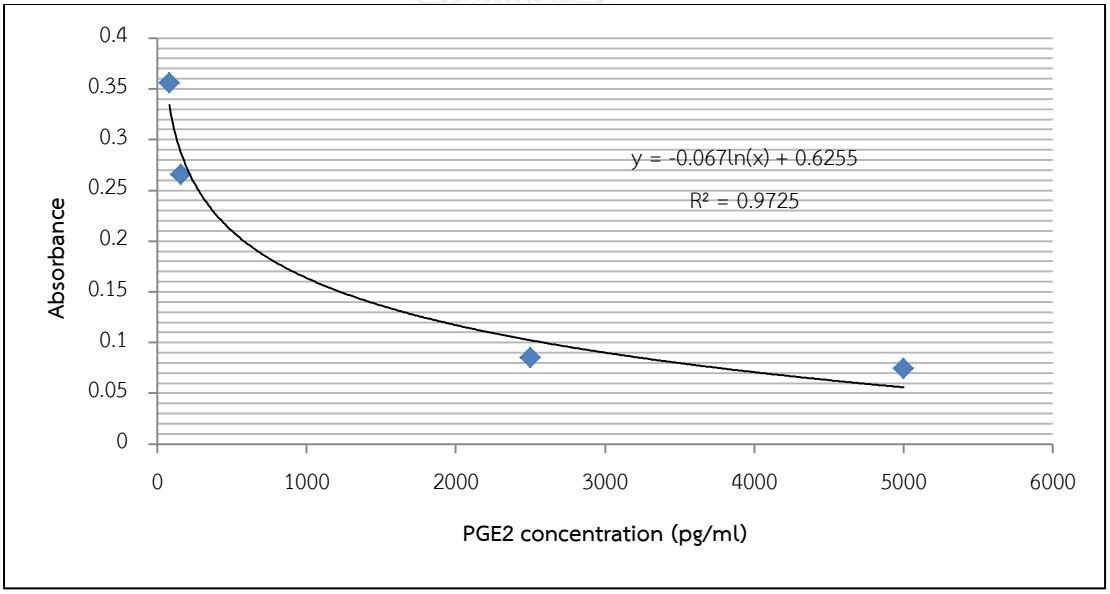


Figure 147 Calibration curve of Standard TNF-alpha for calculated the TNF-alpha content in CKT remedy and its ingredients

VITA

Miss Pravaree Phuneerub was born on December 27, 1987 in Prajinburi, Thailand. She got a Bachelor's degree of Applied Thai Traditional Medicine with first class honor from School of Health Sciences, Mae Fah Luang University, Thailand in 2009.

Publications

1. Phuneerub, P., Palanuvej, C. and Ruangrungsi, N. Pharmacognostic specification, mutagenic and antimutagenic properties of Cymbopogon nardus roots in Thailand. Journal of Chemical and Pharmaceutical Research 6 (2014): 389-396.

2. Phuneerub, P., Limpanasithikul, W., Palanuvej, C. and Ruangrungsi, N. In vitro anti-inflammatory, mutagenic and antimutagenic activities of ethanolic extract of Clerodendrum paniculatum root. Journal of Advanced Pharmaceutical Technology & Research 6 (2015): 48-52.

3. Sunisa, D., Pravaree, P., Chanida, P., Nijsiri, R., and Pasarapa, T. Antinociceptive and anti-inflammatory activities of Tree-Phon-Thad remedy. The Thai Journal of Pharmaceutical Sciences 38 (2014): 156-209.

Poster presentation

1. Phuneerub, P., Palanuvej, C. and Ruangrungsi, N. "Pharmacognostic specification and antioxidant activities of C. nardus roots in Thailand" The 6th Thailand-Japan International Academic Conference, November 9th, 2013, Osaka University, Osaka, Japan.

Oral presentations

1. Phuneerub, P., Palanuvej, C. and Ruangrungsi, N. "Anatomical and Histological Characters of Cymbopogon nardus Roots and Its Mutagenic Properties" XIII International Conference on Pharmaceutical Sciences and Pharmacology, January 23-24, 2015, Paris, France.

2. Phuneerub, P., Palanuvej, C. and Ruangrungsi, N. "Mutagenic and antimutagenic activities of Clerodendrum paniculatum root" The 2nd Internationnal Conference on Advanced Pharmaceutical Research, March 12, 2015, Rangsit University, Phatumthani, Thailand.