PHARMACOGNOSTIC SPECIFICATIONS AND COUMARIN CONTENTS OF *ALYXIA REINWARDTII* AND *EUPATORIUM STOECHADOSMUM*

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ลักษณะทางเภสัชเวทและปริมาณวิเคราะห์สารคูมารินของชะลูดและสันพร้าหอม

นางสาวกัญญารัตน์ เป็งงำเมือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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กัญญารัตน์ เป็งงำเมือง : ลักษณะทางเภสัชเวทและปริมาณวิเคราะห์สารคูมารินของชะลูดและสันพร้าหอม (PHARMACOGNOSTIC SPECIFICATIONS AND COUMARIN CONTENTS OF *ALYXIA REINWARDTII* AND *EUPATORIUM STOECHADOSMUM*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี, 151 หน้า.

ชะลูดและสันพร้าหอมเป็นเครื่องยาสมุนไพรที่ใช้ในการแพทย์แผนไทยมาเป็นเวลานาน การศึกษานี้มีจุดประสงค์เพื่อจัดทำข้อกำหนดทางเภสัชเวทและวิเคราะห์หาปริมาณสารคูมารินของชะลูดและสันพร้าหอมจาก 15 พื้นที่ในประเทศไทย ประเมินลักษณะทางมหทรรศน์และจุลทรรศน์ของเครื่องยาสมุนไพร ศึกษาคุณสมบัติทางกายภาพและเคมีพบว่าชะลูดมีปริมาณน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด ปริมาณสารสกัดด้วยน้ำ ปริมาณสารสกัดด้วยเอทานอล ปริมาณน้ำและน้ำมันระเหยเท่ากับร้อยละ 8.9±0.2, 7.7±0.1, 1.4±0.1, 16.5±0.1, 8.9±0.3, 11.9±0.2 และ 0 โดยน้ำหนัก ตามลำดับ คุณสมบัติทางกายภาพและเคมีของสันพร้าหอม มีปริมาณน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด ปริมาณสารสกัดด้วยน้ำ ปริมาณสารสกัดด้วยเอทานอล ปริมาณน้ำ และปริมาณน้ำมันระเหยเท่ากับร้อยละ 8.7±0.1, 9.5±0.3, 2.6±0.2, 27.8±0.4, 9.4±0.6, 13.2±0.3 และ 0.1±0.0 โดยน้ำหนัก ตามลำดับ วิเคราะห์ปริมาณสารคูมารินในสารสกัดไดคลอโรมีเทนของชะลูดและสันพร้าหอมด้วยเทคนิคทินเลเยอร์โครมาโทกราฟีโดยมีแผ่นซิลิกาเจล60 F_{254} เป็นวัฏภาคคงที่ กรณีของชะลูดใช้ตัวทำละลายเฮกเซนและเอทิลแอซิเทต (1:1) เป็นวัฏภาคเคลื่อนที่ ในกรณีของสันพร้าหอมขั้นตอนแรกพัฒนาแผ่นทีแอลซีด้วยตัวทำละลายคลอโรฟอร์ม ทิ้งให้แห้งและพัฒนาต่อในตัวทำละลายโทลูอีนเอทิลอะซิเทต และกรดอะซิติก (97:10:3) วิเคราะห์ปริมาณสารคูมารินโดยวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรีโดยใช้เครื่อง CAMAG TLC Scanner ร่วมกับโปรแกรม winCATS และวิธีการวิเคราะห์เชิงภาพทางทินเลเยอร์โครมาโทกราฟีโดยใช้โปรแกรม ImageJ สารคูมารินในเครื่องยาชะลูดมีปริมาณ 0.77±0.04 และ 0.75±0.01 กรัม/100 กรัม โดยวิธีทั้งสอง ตามลำดับ สารคูมารินในเครื่องยาสันพร้าหอมมีปริมาณ 0.44±0.02 และ 0.45±0.04 กรัม/100 กรัม โดยวิธีทั้งสอง ตามลำดับ การเปรียบเทียบปริมาณสารคูมารินระหว่าง 2 วิธี ถูกทดสอบโดยใช้สถิติ paired *t*-test พบว่าปริมาณสารคูมารินโดยทั้งสองวิธีไม่แตกต่างกัน ($p>0.05$) ประเมินวิธีวิเคราะห์ตามหลักการของ ICH guideline พบว่าวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรี และการวิเคราะห์เชิงภาพโดยใช้โปรแกรม ImageJ ที่ใช้มีความถูกต้องและมีความน่าเชื่อถือในการวิเคราะห์หาปริมาณสารคูมารินในเครื่องยาทั้งสองชนิดนี้ วิเคราะห์องค์ประกอบเคมีของน้ำมันระเหยของสันพร้าหอมทั้ง 15 แหล่งด้วยวิธีแกสโครมาโทกราฟี-แมสสเปกโทรเมทรี พบว่าองค์ประกอบหลักคือ Binapacry (ร้อยละ 16.74±5.38) วิเคราะห์จัดกลุ่มสันพร้าหอมตามองค์ประกอบทางเคมีของน้ำมันระเหยด้วยวิธี UPGMA แล้วเขียนแผนภูมิเดนโดรแกรม พบว่าสามารถแบ่งออกได้เป็น 2 กลุ่ม จากผลการศึกษานี้สามารถจัดทำเป็นข้อกำหนดทางเภสัชเวทของสมุนไพรชะลูดและสันพร้าหอม ซึ่งจะเป็นประโยชน์ต่อการควบคุมคุณภาพวัตถุดิบสมุนไพรและการศึกษาวิจัยพัฒนาเครื่องยานี้ต่อไป

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

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KEYWORDS: ALYXIA REINWARDTII BLUME / EUPATORIUM STOECHADOSMUM HANCE / PHARMACOGNOSTIC SPECIFICATION / COUMARIN / TLC-DENSITOMETRY / TLC IMAGE ANALYSIS

KANYARAT PENG-NGUMMUANG: PHARMACOGNOSTIC SPECIFICATIONS AND COUMARIN CONTENTS OF *ALYXIA REINWARDTII* AND *EUPATORIUM STOECHADOSMUM* . ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 151 pp.

Alyxia reinwardtii Blume inner bark and *Eupatorium stoechadosmum* Hance whole plants have been used in traditional Thai medicine for a long time. This study was carried out to investigate the pharmacognostic specifications by qualitative and quantitative analyses as well as coumarin contents of *A. reinwardtii* inner bark and *E. stoechadosmum* whole plants. The samples were collected from 15 different sources throughout Thailand. Macroscopic and microscopic characteristics of two crude drugs were illustrated. The physicochemical properties of *A. reinwardtii* dried inner bark including loss on drying, total ash, acid- insoluble ash, water soluble extractive, ethanol soluble extractive, moisture and volatile oil contents were found to be 8.9±0.2, 7.7±0.1, 1.4±0.1, 16.5±0.1, 8.9±0.3, 11.9±0.2 and 0 % by weight, respectively. The physicochemical propoties of *E. stoechadosmum* whole plants were found to be 8.7±0.1, 9.5±0.3, 2.6±0.2, 27.8±0.4, 9.4±0.6, 13.2±0.3 and 0.14±0.0 % by weight, respectively. Coumarin in dichloromethane extract of *A. reinwardtii* dried inner bark and *E. stoechadosmum* dried whole plants were analyzed by thin layer chromatography (TLC) using silica gel 60 GF_{254} as stationary phase. Hexane and ethyl acetate (1:1) were used as mobile phase for *A. reinwardtii*. In case of *E. stoechadosmum* TLC procedure, first step developed with chloroform, and then with toluene, ethyl acetate and acetic acid (97:10:3). The coumarin contents were evaluated both TLC-densitometry performed using winCATS software and image analysis using imageJ software. The coumarin contents in *A. reinwardtii* crude drugs were found to be 0.77±0.04 and 0.75±0.01 g/100 g by those methods respectively. The coumarin contents in *E. stoechadosmum* crude drugs were found to be 0.44±0.02 and 0.45±0.04 g/100 g by those methods respectively. The coumarin contents between both methods were not statistically significant different ($p>0.05$) by paired *t*-test. According to ICH guideline, TLC-densitometry and TLC image analysis using ImageJ software showed validity and reliability for coumarin quantitations in *A. reinwardtii* and *E. stoechadosmum* crude drugs. The chemical compositions of *E. stoechadosmum* volatile oils from 15 sources were analyzed by GC-MS. The result showed that the main component was binapacry (16.74±5.38%). *E. stoechadosmum* from 15 sources could be divided into two groups based on the dendrogram of volatile oil compositions constructed by UPGMA cluster analysis. This study provided scientistic evidences for identification, authentication, and quality control of *A. reinwardtii* and *E. stoechadosmum* crude drugs.

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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS

°C	Degree celsius
cm	Centimetre
DPPH	2, 2 –diphenyl-1-picryl hydrazyl
g	Gram
GC	Gas chromatography
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
ICH	International Conference on Harmonization
kg	Kilogram
l	Litre
LOD	Limit of detection
LOQ	Limit of quantitation
m	Metre
mg	Miligram
min	Minute
ml	Millilitre
nm	Nanometre
OH	Hydroxyl group
R^2	Coefficient of determination

RSD	Relative standard deviation
SD	Standard deviation
TLC	Thin layer chromatography
UPGMA	Unweighted pair group method with arithmetic average
UV	Ultraviolet
v/v	Volume in a volume
WHO	World health organization
µg	Microgram
µl	Microlitre

CHAPTER I
INTRODUCTION

Background and rationale

The uses of medicinal plants have long history throughout the world. Herbal preparations, including herbal extracts, can be found in the pharmacopeias of numerous countries [1]. World Health Organization (WHO) defines "herbal medicine" as a plant preparation with therapeutic or other human health benefits which contain either raw or processed ingredients from plants [2]. Nowadays, standardization of the plant material is necessary. Several pharmacopeias containing monographs of the plant materials describe only the physicochemical characters. The modern methods describing the identification and quantification of active constituents in the plant material may be valuable for proper standardization of herbs and its formulation. WHO has emphasized the need to ensure the quality of medicinal plant products by using modern controlled techniques and applying suitable standards [3].

Alyxia reinwardtii Blume is mentioned as a folk medicinal plant. The various parts of this plant have been used in function, flavor and medicine. *A. reinwardtii* is used against influenza, cough and fever. Additionally it is used for perfume, tobacco products and is a constituent of several therapeutic recipes [4]. *A. reinwardtii* is one of the plant drugs are used in "jamu". A Jamu prescription is a traditional Indonesia herbal medicine that has been used for many centuries in the Indonesians community to maintain good health and heal diseases. These plant are also used for treating fever, diarrhea, candidiasis and aphthous ulcer [5, 6]. National lists of essential medicines, noticed, "Yar Horm Teph Pa Jitt", traditional Thai medicine recipe which consists of *A. reinwardtii* and another herbal medicines, has been used for treatment of abnormal blood circulation due to wind element imbalance;

moreover, another herbal medicine remedy, “Yar Horm Nao Wa Goad” has also been used for cure of dizziness, faint, nausea, palpitation and uncomfortable stomach [7].

Eupatorium stoechadosmum Hance is an ingredient of “Yar Keaw Horm” remedy which is nostrum. *E. stoechadosmum* presents in another numerous folk remedies in every zones of Thailand. It can be used for antipyretic agent, abdominal distension, cardiac supplement, nourishment, haematic and menstrual abnormality. Generally, the whole plant is used in many different ways, for example, crushing to get an oily components then inhalation the volatile oil for anticonvulsants, chewing for cure refrigerant, boiling in water and oral administration for energy stimulation [8].

Thin layer chromatography (TLC) is particular valuable for qualitative analysis of chemical composition in plant materials. By this technique, very minute quantities of test sample can be used. It is highly sensitive and effective technique. It requires inexpensive equipment. This technique is frequently used for evaluating medicinal plant materials and their preparations [9]. Combination of TLC with TLC image analysis or TLC scanner and processing software offers quantitative study of medicinal plant constituent. The herbal developments seriously need to start by the good standardization to further developmental process. Thus, the purpose of this study is to investigate the standardization parameters by qualitative and quantitative analyses of *A. reinwardtii* inner bark and *E. stoechadosmum* whole plants in Thailand. Their coumarin contents and essential oil compositions are also examined. There are needs of the modern control techniques to insure the quality of medicinal plant materials and herbal medicine [10].

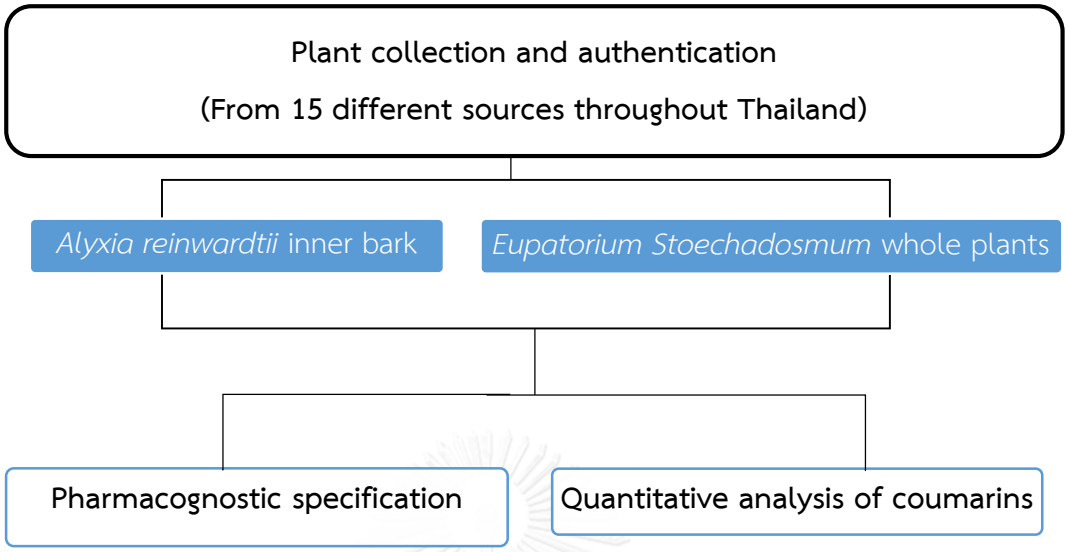
Research problems

The quality parameters as well as coumarin contents of *A. reinwardtii* and *E. stoechadosmum* crude drugs in Thailand have never been established.

Objectives

1. To provide the pharmacognostic specification of *A. reinwardtii* inner bark and *E. stoechadosmum* whole plants.
2. To determine the coumarin contents in *A. reinwardtii* inner bark and *E. stoechadosmum* whole plants by TLC image analysis using ImageJ software compared to TLC densitometry.
3. To investigate the chemical composition of *A. reinwardtii* and *E. stoechadosmum* essential oils.

The conceptual framework



- Macroscopic and microscopic evaluation
- Determination of water content
- Determination of loss on drying
- Determination of total ash
- Determination of acid insoluble ash
- Determination of water extractive value
- TLC fingerprint
- Determination of volatile oil
- Chemical compounds analysis of volatile oil by GC-MS

- Coumarin extraction in dicholomethane by soxhlet apparatus
- TLC image analysis by ImageJ software
- TLC densitometry
- Method validation

CHAPTER II

REVIEW LITERATURES

ALYXIA REINWARDTII BLUME

Botanical classification

Domain: Eukaryota

Kingdom: Plantae

Division: Manoliophyta

Class: Magnoliopsida

Order: Gentianales

Family: Apocynaceae

Genus: *Alyxia*

Species: *Alyxia reinwadtii*

Botanical name: *Alyxia reinwardtii* Blume

Synonyms

Alyxia lucida Wallich

Alyxia stellata auct. non (J.R. Forster & G. Forster) Roem. & Schult.

Alyxia pumila Hook.f.

Alyxia forbesii King & Gamble

Vernacular names [11]

English: Forbes alyxia

China: Chang Hua Lian Zhu Teng

Indonesia: Adas pulasari, Akar mempelas hari, Arevy palasari, Arevy pulasari, Balasari, Calapari, Calpari, Das plasare, Empwlas hari, Mempelas hari, Palasari, Pulasari, Purasane, Talatari

Vietnam: ng[oo]n d[aa]y v[as]t

Thailand: Cha lood, Luut, Nuut, Cha lood khao

Distribution

From southern China, through Burma (Myanmar), Bangladesh, Indonesia, Philippines, Thailand, Laos, Cambodia, Vietnam, southward to the Peninsular Malaysia, Sumatra, Java, Bali, Borneo and Palawan.

Plant description

A. reinwardtii is a brush wood that widely found in Thailand, Indonesia, Malaysia, Philippines, and Vietnam. It is commonly known in Thai as "Chalood". The plant is in the *Alyxia* genus which belongs to the Apocynaceae family. "*A. reinwardtii is a liana woody which grows up to 3 m in tall, glabrous except for inflorescences. Young branchlets are triangular, later terete. Leaves are opposite to whorl of 5; petiole to 5 mm; leaf blade narrowly elliptic or oblong, sub-leathery to leathery, abaxially glabrous, apex narrowly long acuminate, lateral veins obscure abaxially. Cymes are fascicled, pubescent, 3-5 cm; peduncle 1-1.3 cm; bracteoles very narrowly ovate. Sepals are ovate or narrowly 1-2 mm, obtuse or acute, pubescent to subglabrous. Petals are yellowish white, lobes overlapping to the left in bud, mature Petal salverform, tube 8.5 mm; lobes ovate 4 mm. Ovary of 2 separate carpels united into a common style; ovary pubescent all over or only a base; style filiform. A drupe is usually moniliform with one or more subglobose articles, frequently redueced to*

one in mature fruit; one seed per article. Seeds are ovoid with a horny and deeply ruminate endosperm" [12, 13].

Figure 1 *Alyxia reinwardtii* Blume [13]

1. Flowering branch and Inflorescence, 2. Inner bark

Medicinal Uses

The crush stem is used in the production of incense and the other aromatic product. According to traditional Thai medicinal practice guidelines, the leaves and fruits of this plant can be used for reducing fever, and the flowers are effective in treating mental confusion, hallucination associated with high fever, hiccup stopper and correcting unspecified gall bladder illness. The stems are used for treated fainting, heart failure and abdominal discomforts due to gaseous distention or other unspecified causes. The roots are effective in reducing fever [11].

An infusion of the leaves is used as a vermifuge. The bark, leaves and flowers can be taken as infusions to treat gonorrhea. All parts are used as an antipyretic and cardiotonic. The plant is burnt and the smoke used to treat cephalalgia. The bitter sap is used as an emetic. The bark is used as an ingredient in medicines in Indonesia, combined with *Foeniculum vulgare*. This mixture is frequently used in Javanese traditional medicine to treat various illnesses, to improve flavor and to enhance odor of the other ingredients.

A. reinwardtii is a principle ingredient in about 18 different manufactured medicines from Central Java. These are used as antispasmodics and for treating stomach-ache, flatulence, colic and dysentery; moreover, *A. reinwardtii* are used as a carminative and for sprue. The leaves and flowers taste spicy and rather bitter when still fresh and are sometimes used instead of the bark.

The bark extraction of *A. reinwardtii* combined with *Amaranthus spinosus*, *Usnea* spp., and the bark of *Cinnamomum cassia* can be used in the treatment of bronchitis. The juice which extracted from the bark has been pounded with onion, can be used for thrush treatment. The bark of *A. reinwardtii* which combined with the leaves of

Polygonum flaccidum and aniseed of *Pimpinella anisum*, is used as an emmenagogue [14].

Edible Uses

The bark and leaves of *A. reinwardtii* are employed as a flavoring in the manufacture of rum. Local wine industries sometimes use it for flavor their product. The bark is used like cinnamon bark as a spice.

Other Uses

The aromatic bark is put among clothes for its fragrance. Bundles of the dried twigs can be placed in cupboards to perfume them. The dried and finely powdered bark is used as an ingredient in the manufacture of incense in Java. Many species throughout the range of the genus are used in personal adornment and it is thought that the name *Alyxia* comes from the Greek 'halusis' meaning a chain, in reference to the making of leis in the Pacific. Leis are chains of leaves and the bark after it has been stripped off the wood. These are twisted around each other to form a decorative and scented chain for use on festive occasions [14].

Chemical compounds of *A. reinwardtii*

In previous study, various chemical constituents isolated from *A. reinwardtii* are reported. Alyxialactone, 4-*epi*-alyxialactone were isolated from the leaves of *A. reinwardtii* [4]. The inner bark of *Alyxia reinwardti* var. *lucida* contains 3-hydroxycoumarin glycoside 1 and 3-hydroxycoumarin glycoside 2 [15]. The presence of iridoid and trimeric-iridoid diglucoside substances was also reported [14, 16]. The petrol extract of *Alyxia lucida* dried stem yielded coumarin, 3-hydroxycoumarins, triterpenes, and the chloroform extract yielded scopoletin, 8-hydroxycoumarin and 5-hydroxycoumarin [17]. Ethyl acetate and methanolic extract of dried bark yielded

coumarin, 8-hydroxycoumarin, 5-hydroxycoumarin, (-)-pinoresinol and (+)-pinoresinol [6]. The chemical compounds and chemical structures are shown in Figure 2.

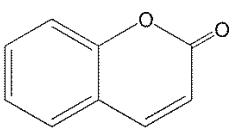
Pharmacological investigations of *A. reinwardtii*

Antioxidant activity study

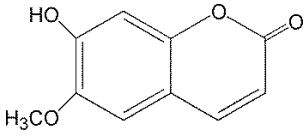
The dichloromethane and ethyl acetate crude extracts from *A. reinwardtii* stem showed antioxidant activity potential. The isolated compounds were evaluated by various *in vitro* model assays which included DPPH radical scavenging activity, xanthine oxidase-related activity and lipid peroxidation inhibitory activity [16]. Methanolic extract of *Alyxia lucida* was assayed for xanthine oxidase inhibitory activity. The IC_{50} values was 63.19 µg/ml [18]. Pinoresinol was isolated from *A. reinwardtii* dried bark did not have a protective effect against H_2O_2-mediated DNA-damage but had less cytotoxic while studies with H4IIE hepatoma cells. In the dichlorofluorescein assay, an antioxidative effect was detectable [6].

Toxicity study

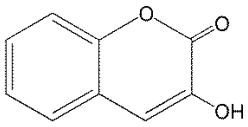
In the acute toxicity study, the 50% ethanolic extract of *A. reinwardtii* was given to the rat by oral administration and subcutaneous injection with 10 g/kg. The study showed that there was no acute toxicity effect in rat.



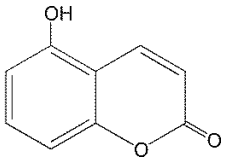
Coumarin



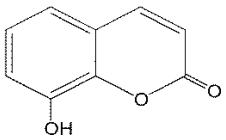
Scopoletin



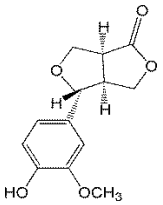
3-hydroxycoumarin



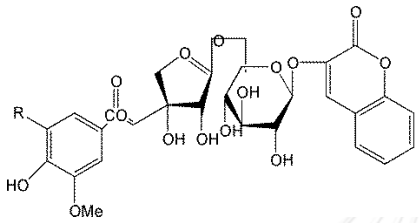
5-hydroxycoumarin



8-hydroxycoumarin

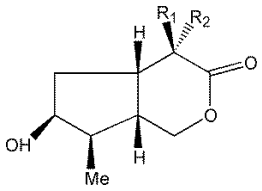


Salicifoliol



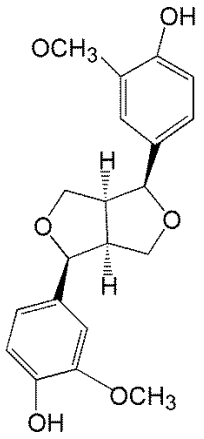
3-hydroxycoumarin glycoside I; R=H

3-hydroxycoumarin glycoside II; R=Ome

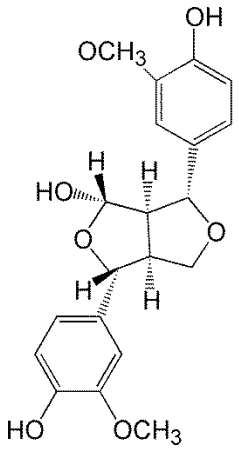


Alyxialactone; R_1=H, R_2=CH_2OH

4-*epi*-alyxialactone; R_1=CH_2OH, R_2=H



Pinoresinol



9-α-hydroxypinoresinol

Figure 2 Chemical compounds of *Alyxia reinwardii* Blume [7, 8, 15, 19-23]

EUPATORIUM STOECHADOSMUM HANCE

Botanical classification

Domain: Eukaryota

Kingdom: Plantae

Division: Manoliophyta

Class: Magnoliopsida

Family: Asteraceae

Genus: *Eupatorium*

Species: *Eupatorium stoechadosmum*

Botanical name: *Eupatorium stoechadosmum* Hance

Synonyms

Eupatorium fortunei [24]

Eupatorium caespitosum Mig.

Eupatorium chinense var *tripatum* Mig.

Eupatorium japonicum Thunb. ex Murray

Vernacular names

China: Tse- Lan, Zelan

Japan: Fuji-Bakama

English: Boneset, Chinese Eupatorium

Thailand: Kiang Pa Yai, Por Kee, Cha Pe, Mog Pa (Northern Thailand), San Pra Hom [8]

Distribution

Honshu (west of Kanto region), Shikoku, and Kyushu in Japan, China, Korea, Thailand, Indo-China, China, Taiwan, Philippines

Plant description

E. stoechadosmum is the plant in *Eupatorium* genus which belongs to the Asteraceae family. It is widely found in Asia, and also known as "San Pra Hom" in Thailand. "*E. stoechadosmum are perennial which grow up to 0.5 m tall. Reddish brown rhizomes are procumbent. Stems are green or reddish purple color, few branched or apically inflorescence branched, sparsely puberulent, more densely on inflorescences and peduncles. Median stem leaves large, apex acuminate; lateral lobes identical to terminal lobe but smaller, pinnately veined; margin coarsely toothed or irregularly finely toothed; lower stem leaves gradually smaller; radical leaves withered by anthesis. Capitula are numerous in apical compound corymbs; inflorescence 3-6(-10) cm in diameter. Involucre campanulate, 6-7 mm; phyllaries 2- or 3-seriate, imbricate, outer phyllaries short, ovate-lanceolate; median and inner phyllaries gradually longer, narrowly elliptic, 7 mm; all phyllaries purple-red, without hairs and glands, apex obtuse; Petals white or reddish, 5 mm, eglandular. Achenes*

black-brown, elliptic, 3-4 mm, 5-angled, glabrous and eglandular; pappus white, 5 mm. 2n = 40" [25].

Figure 3 *Eupatorium stoechadosmum* Hance 1. Twig of *E. stoechadosmum*, 2.and 3. Inflorescence, 4. Crude drug

Chemical compounds of *Eupatorium stoechadosmum* Hance

E. stoechadosmum consists of coumarins, quinone, epatin, rinderine, 7-acetylrinderine and pyrrolizidine alkaloid: lindelofine, supinine [26-29]. The chemical compounds and chemical structure are shown in figure 2. The chemical constituents of leaf oil have been analyzed. The essential oils were obtained from steam distillation, and were subjected to GC-MS analysis for compounds identification. It was found that most of them were terpenic compounds [30]. Thymohydroquinone dimethyl ether occurred as the main constituent among the predominant compounds [31]; beta-caryophyllene, selina-4,11-diene [32], camphene, amyrene, longifolene, a-muurolene, b-caryophyllene, b-farnesene, a-farnesene, carene, b-pinene, myrcene, isobornyl acetate, a-guaiene, b-guaiene, neryl acetate, a-cedrene [33], thymohydroquinone, caryophyllene, carbamodithioic acid, 7-methoxy-2H-1-benzopyran-2-one and 2,3-Dihydro-2,3-dimethyl-1,4-phthalazinedione [34]. In previous study has reported that rel-(1R,2S,3R,4R,6S)-p-menthane-1,2,3,6-tetrol, rel(1R,2R,3R,4S,6S)-p-menthane-1,2,3,6-tetrol, 9-hydroxythymol 3-O-angelate, and (3b,20R)-20hydroxylanost-25-en-3-yl palmitate were isolated from the ethyl acetate and methanolic extracts of *Eupatorium fortunei* [35].

Pharmacological investigations of *Eupatorium stoechadosmum* Hance

E. stoechadosmum is a native plant of Thailand, Japan, Korea and China. It is a medicinal plant of the Chinese Pharmacopoeia. Leaves and stems were collected before flowering are used as decoctions in cases of cold, influenza, cerebral stroke, uterine bleeding, oliguria, and vomiting [29]. In Southern China, it is used to relieve syndromes caused by hot weather, such as headache, fatigue, flatulence, anorexia, diarrhea, nausea, and vomiting. It also applied as an analgesic agent and a nervous sedative. Japanese uses *E. stoechadosmum* for incense and diuretic. In Thailand, the whole plant has been used for treatment of headache, hyperthermia, flatulence, colic distention and also used as sexual stimulant drugs as well. Root has been used as decoction for antidote and treatment menstrual disorder. Leaves have been used for healing common cold and heart nourish.

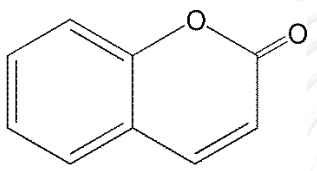
rel-(1*R*,2*S*,3*R*,4*R*,6*S*)-*p*-Menthane-1,2,3,6-tetrol, *rel*(1*R*,2*R*,3*R*,4*S*,6*S*)-*p*-menthane-1,2,3,6-tetrol, acetone thymol-8,9-diyl ketal and 8-methoxy-9-hydroxythymol 3-*O*-angelate which were isolated from *E. fortunei* showed cytotoxic activity against human leukemia cells (HL-60) [35]. *E. fortunei* showed positive antioxidant capacities using the ferric-reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) assays [36].

Coumarin isolated from *Eupatorium japonicum* leaves has been a larval growth inhibitor against *Drosophila melanogaster*. The aerial parts of *E. japonicum* were extracted with 60% ethanol that has been showed histamine- release inhibitory activity (inhibition > 61.3%, 100 μg/ml) and not detectable in nitric oxide- production inhibition test at the same concentrations [37].

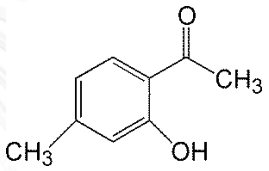
E. fortunei is an ingredient of a Chinese multi-herb remedy which is used for the treatment and prevention of SARS. The study showed that the compounds

isolated from these remedy had robust inhibitory effect on complement system through the classical pathway and alternative pathway of hemolytic assays [38].

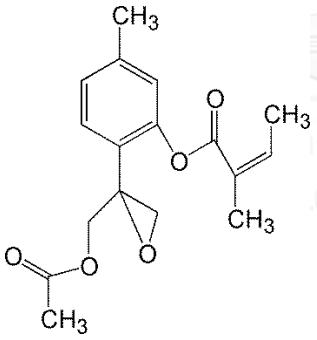
The previous research demonstrated that the ethanolic extract of *E. stoechadosmum* leaves had no potential to be calcium signaling inhibitor by yeast cell growth based assay. Small- molecule inhibitors of calcium signaling pathway in humans are medical importance, since calcium signaling in mammalian cell plays pivotal roles in regulation of diverse cellular processes, including T- cell activation, secretion, motility and apoptosis; in addition, several cell- cycle inhibitors have potential as anticancer agents [39].



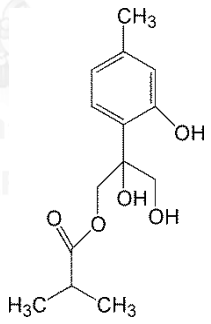
Coumarin



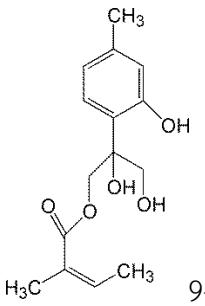
2-hydroxy-4-methylacetophenone



8,10-epoxy-9-acetoxythymolangelate



9-isobutyryloxy-8,10-dihydroxythymol



9-angeloyloxy-8,10-dihydroxythymol

Figure 4 Chemical compounds of *Eupatorium stoechadosmum* Hance [40, 41]

Coumarins

Coumarins are phenolic substances which have been found in plants. Simple coumarin consists of bezene and alpha-pyrone rings. Coumarin has been identified as secondary metabolites from plants, bacteria, and fungi. Simple coumarin was initially found in tonka bean (*Dipteryx odorata* Wild) and was reported in about 150 different species distributed over nearly 30 different families, for instance, Rutaceae, Umbelliferae, Guttiferae, Caprifoliaceae, Oleaceae, and Apiaceae. Biosynthesis of coumarin is reviewed by Bourgaud *et al*. There are types of coumarins found in nature due to various permutations such as substitutions and conjugations [42]. Natural coumarins are mainly classified into six types based on the chemical structure of the compounds illustrated in Table 1.

Use of coumarin

Coumarin is widely distributed in the plant kingdom, but mostly synthetic product has been used in commercial for many years. It is used as odor enhancer to achieve a long lasting effect when combination with natural essential oils such as lavender, citrus, and rosemary. It has several industrial applications such as in the perfumery and cosmetic.

Coumarin is also used in toothpastes, hand soaps, bath products, body lotions, face creams, hair sprays, shampoos, fragrant creams, detergents, antiperspirant deodorants, and perfumes. In addition, it has been used in food industry, associated with vanillin for flavoring cream, chocolates and bake goods [43].

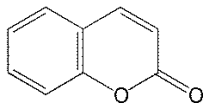
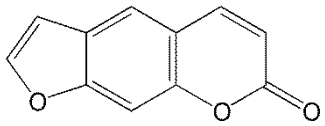
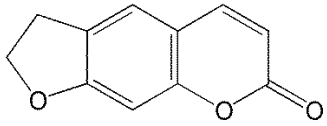
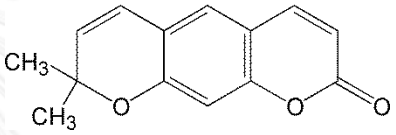
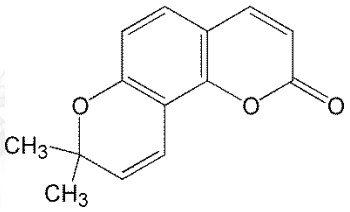
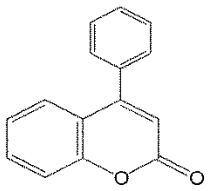
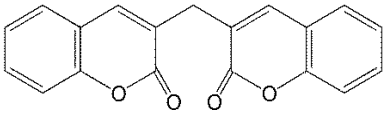
Pharmacological investigations of coumarin [44]

Anti-Inflammatory activity: Coumarin has presented anti-inflammatory property. It has been used for treating edema. This can remove protein and edema fluid from injured tissue by stimulating phagocytosis, enzyme production, and proteolysis. Patients who were postmastectomy lymphoedema of the arm responded to coumarin. The arm gradually decrease swelling over 10 months; whereas, who without coumarin treatment , the swelling was continued increase [45]. Coumarin was also shown significant ability to reduce edema in the rat paw carrageenan test and other inflammatory rodent models at doses of 5 to 50 mg/kg; whereas its metabolite 7-hydroxycoumarin was inactive [46]. In a double-blind randomized matched-group trial on a large group of patients with chronic filiaritic lymphoedema and elephantiasis in India, coumarin can reduce all grades of the disease significantly [47].

Anticoagulant activity: Coumarin has also been the substrate molecule of warfarin which is acted as a vitamin K antagonist that produce their anticoagulant effect by interfering with the cyclic interconversion of vitamin K [48]. Warfarin was a clinically useful anticoagulant and widely employed rodenticide [49].

Antibacterial activity: Coumarin has a very low antibacterial activity. Long chain hydrocarbon substitutions of coumarin such as ammoresinol showed activity against Gram positive bacteria such as *Bacillus megaterium*, *Micrococcus luteus*, *Micrococcus lysodeikticus*, and *Staphylococcus aureus* [50].

Table 1 Coumarins classification

Types of coumarins	Structure
Simple coumarin	
Furano coumarins	
Dihydrofurano coumarins	
Pyrano coumarins are two types Linear type	
Angular type	
Phenyl coumarins	
Bicoumarins	

Anticancer activity: Coumarin which was isolated form cassia leaf oil exhibited cytotoxic activity [51]. Coumarin possesses both immune modulatory and direct antitumor activity. It is presently undergoing clinical trial for treatment of lymphoma following breast cancer treatment and in treatment of lung cancer, kidney cancer and melanoma [43].

Antihypertensive activity: Vasodilatory effects of the coumarin were reported on cultured myocardial cells as well [52]. Scopoletin was isolated form the fruits of *Tetrapleura tetraptera* Taub (Mimosaceae), and it was produced hypotension *in vitro* and *in vivo* through its smooth muscle relaxant activity [53].

Carcinogenicity data: Coumarin has been adequately tested by administration in two experiments in mice and rats. In experimental of mice, it produced increases in lung tumors (adenomas and carcinomas) of both males and females. In rat study, coumarin produced a low incidence of renal tubule adenomas.

Genetic and related effects: Coumarin induced gene mutations in *Salmonella typhimurium* strain TA100 with metabolic activation. A positive result was reported in *Salmonella* strain TA7002. Coumarin failed to induce gene mutations in other strains of this type. Coumarin also found induced sister chromatid exchanges in Chinese hamster cell *in vitro* without exogenous metabolic activation. It did not induce micronuclei in rat primary hepatocytes. The two positive chromosome responses (aberrations, micronuclei) occurred at the highest dose tested, and there was no clear dose response. Coumarin did not induce gene mutations at Hprt locus in Chinese hamster ovary cells. It also failed to induce unscheduled DNA synthesis in human liver slices *in vitro* [43, 54, 55].

Quality control methods for herbal material [10, 56, 57]

Standardized herbal products of consistent quality and containing well-defined constituents are required for reliable clinical trials and provide consistent beneficial therapeutic effects. Development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of bioactive compounds and other major constituents, is a major challenge to scientists. The standardization of crude drug material includes the following steps.

Authentication

The first stage is identification of the plant species or botanical verification by the currently accepted Latin binomial name and synonyms.

Macroscopic examination

Macroscopic identity of medicinal plants is material study on shape, size, color, surface characteristics, texture, fracture characteristics and appearance of cut surface. However, since these characteristics are judged subjectively, substitute or adulterants may closely resemble the genuine material. It is often necessary to substantiate the findings by microscopy and/or physicochemical analysis.

Microscopic examination

Microscopic inspection of medicinal plant materials is indispensable for the identification of broken or powdered materials. This method can be investigated the anatomical and histological characters under microscope. The tissue section and powders are mounted in water onto a glass slide. The specimen may have to be treated with chemical regents. Any additional useful information for preparation or analysis should also be included in the test procedures for individual plant materials,

for example, the determination of leaf constants such as palisade ratio. However, an examination by microscopy alone cannot always provide complete identification.

Determination of water content

Moisture content has been used for measurement of water in each medicament. The moisture content from each plant materials is different because water compositions in each plant cells are various. The high moisture content can reduce quality and effectiveness of the plant materials, and the enzymatic activity may encourage the growth of yeast and mold during storage; as the result, it was defined as an important parameter in herbal drug quality control. The moisture content determination consists of the loss on drying and the volumetric azeotropic distillation which adoption depends on the characteristics of the drug, such as constituent [58].

Loss on drying is the loss of mass expressed as percent w/w. Loss on drying is tested for determination water and volatile matter in the crude drug. It can be carried out either by heating at 100- 105 ℃ or in dessicator reduced pressure at room temperature.

Azeotropic volumetric distillation (toluene distillation method) gives a direct measurement of water present in crude drug. When the sample is heated, the water in the sample is distilled together with an immiscible solvent, such as toluene or xylene. Water and solvent are separated in the receiving tube on cooling. If the solvent is anhydrous, water may remain absorbed in it leading to false results. It is therefore advisable to saturate the solvent with water before use. The volume of water distilled over can be read and the percentage initially present in the sample can be calculated in percentage.

Determination of total ash

The ash remaining following incineration of medicinal plant materials is determined by three different methods. The total ash method is designed to measure the total amount of materials remaining after incineration which are inorganic compounds in plant materials such as phosphorus, alumina, calcium, magnesium, sodium, silica, and iron. The total ash value from the medicinal plant materials may be unequal to another which results from the difference of chemical components in each of medicinal plant materials. Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. If the plant material has a higher ash value than specification, it means that there are some contaminations, adulterations or carelessness in the material preparation.

Determination of solvent extractive values

This method determines amount of active constituents extracted with solvents from a given amount of medicinal plant material. The determination of water soluble and alcohol soluble extractives are used as means of evaluating crude drug which are not readily estimated by other means. The extraction of any crude drug with a particular solvent yields a solution containing different phytochemical constituents. The single of solvent can be the means of provided preliminary information on the quality of particular drug sample.

Determination of volatile oil

Volatile oils are characterized by their odor, oil like appearance and ability to volatilize at room temperature. They are composed of various chemical components. Aromatic compounds are predominated in certain volatile oils. Because they are

considered to be the “essence” of the plant material and often biologically active, they are also known as “essential oils”. In order to determine the volume of oil, the plant material is distilled with water and distillate is collected in graduated tube. The aqueous portion separates automatically and returned to distillation flask.

Chromatographic analysis

Various method for coumarin analysis are chromatography (paper chromatography, thin layer chromatography, gas chromatography, and high-performance liquid chromatography), titrimetric and spectrophotometric methods. In this study used thin layer chromatography (TLC).

Thin-layer chromatography (TLC) is a chromatographic technique which used to identify and separate organic compounds. The sample solution must be enough concentrated so that analysis can be detected in the applied volume. The solvent for dissolve the sample must be proper to viscosity and volatility. Application of samples must be accurate, precise volumes and not damage the surface of TLC plate. The polarity of the solvent used for extraction should be similar with the compound mixture to be separated and analyzed [9, 15].

TLC plates are coated with thin layers stationary phase such as silica, alumina, cellulose, and polyamides on glass, plastic or aluminum sheet supporter. Mobile phase is a mixture of two to five different solvents experimentally selected for separations. Samples can be detected on TLC plate under the ultraviolet light with 254 and 366 nm wavelengths [15, 23].

An important qualitative parameter, which characterizes the position of a spot on TLC plate, is the retardation factor (R_f) value. It is describes as [23] :

$$R_f = \frac{\text{Distance of the compound from original spot travelled to the developed spot}}{\text{Distance of the solvent from original line travelled to the developed line}}$$

TLC is frequently used as a qualitative and quantitative method. Qualitative method can be determined by the number of compounds in a mixture and identified substances. Whereas, quantitative method is used for content determination of require testing substances [23].

Fingerprint is a method for the quality control of herbal samples that has been accepted by WHO. It's suitable for detect adulterations and identify plant species. Chromatographic methods consist of TLC, HPLC and GC is commonly used for fingerprint. TLC is the most popular method used for identify and authenticate compounds in herbal medicines and its derivative for obtains a fingerprint profile [23, 59].

Quantitative analysis can be performed with data from scanning densitometry or image analysis method. Scanning densitometry use optimal wavelength to measure the difference in absorbance or fluorescence signal between a separated zone and the empty plate background [60, 61]. ImageJ is one method of the several image analysis software. The images from camera are required for analysis [40]. ImageJ is an open source which is developed in Java programs, that users can develop program and fix the program. It is used in many fields such as biological microscopy and medical researches. It can be used in both Windows and Macintosh, available free download from website of the US National Institute of Mental Health [62]. TLC-densitometry has been extensively developed over the past decade and has been considered essential for both the accurate identification of the spot position and the precise quantitative estimation of its content. In most tools, the plate surface can be examined employing either reflect light or fluorescent light. The incident light may be absorbed, diffusely

scattered or transmitted through the plate. The normal procedure is measured the light scatter, reflect or generate by fluorescence from the spot and compare it electronically with light from a part of the plate where no sample has passed. When measuring either the adsorbed light, the sensitivity will be inversely related to the scan rate. The relationship between the adsorbed light and the concentration of solute in the spot is not linear, and so either calibration curves must be constructed or the signal must be electronically modified in an appropriate manner to render the output linearly related to solute concentration. In any events, standard solutions must be run for calibration purposes.

Validation of analytical procedures

Method validation is a process used to confirm and demonstrate the performance characteristics of an analytical methodology. The purpose of method validation is to ensure that the methodology is accurate, specific, reproducible, and robust. According to the ICH guidelines and in this study, the methods were evaluated including accuracy, precision, detection limit, quantitation limit, linearity and robustness which are used for validate quantitative methods [63].

Accuracy

The accuracy is expressed the closeness of the test results obtained by the analytical procedure to agreement between the value which was accepted either as a conventional true value or an accepted reference value and the value found. Accuracy is reported as percent recovery by the assay of spiked sample with known amount of analyze.

Specificity

Specificity is an ability to determine impurities in analyze. Purity test is commonly used to certify that all the analytical methods performed allow an accurate statement of the impurity content of an analytical.

Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability (intra-day assay), intermediate precision (inter-day assay) and reproducibility (inter-laboratory assay).

Linearity

Linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyze in the sample. According to ICH guideline, a minimum of 5 concentration levels is recommended for founding of linearity.

Range

Range of procedure is the interval between the upper and lower concentration of analyze in the sample. Range was demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Limit of detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyst in a sample which can be detected but not necessary quantitated as an extract value. LOD can be determined based on the SD of the blank, the residual SD of a regression line, or the SD of y- intercepts of a regression lines.

Limit of quantitation (LOQ)

The quantitation limit of an individual analytical procedure is lowest amount of analyst in a sample which can be quantitative determined with suitable precision and accuracy. LOQ can be determined based on the SD of the blank, the residual SD of a regression line, or the SD of y- intercepts of a regression lines.

Robustness

Robustness of an analytical procedure is an assessment of its capacity to remain an affected by small deliberate variations in method parameters and provides an indication of its reliability during normal usage.

CHAPTER III

MATERIALS AND MATHODOLOGY

Chemical and reagents

1. Acetic acid, glacial grade (BDH chemicals Ltd, Poole, English)
2. Coumarin (CAS no. 91-64-5, purity ≥99 %) (Sigma-Aldrich, St. Louis, MO, USA)
3. Chloroform, HPLC grade (J.T. Baker Chemical, Phillipsburg, USA)
4. Dichloromethane, A.R. grade (RCI Labscan Limited, Thailand)
5. Ethanol (Liquor Distillery Organization Excise Department, Thailand)
6. Ethyl acetate, A.R. grade (RCI Labscan Limited, Thailand)
7. Hexanes, A.R. grade (RCI Labscan Limited, Thailand)
8. Hydrochloric acid, A.R. grade (RCI Labscan Limited, Thailand)
9. Methanol, A.R. grade (RCI Labscan Limited, Thailand)
10. Methanol, HPLC grade (RCI Labscan Limited, Thailand)
11. Toluene (RCI Labscan Limited, Thailand)
12. Water (Ultrapure)

Materials

1. Cover glasses (Menzel-Glaser, Germany)
2. Filter-paper No.4 (Whatman™, UK)
3. Filter-paper NO.40 ashless (Whatman™, UK)
4. Microscope slide (Sail Brand, China)

5. TLC silica 60 GF254 20 × 10 cm and 10 × 10 cm, layer thickness 0.2 mm. (Merck, Germany)

Instrumentations

1. Azeotropic apparatus
2. Canon Power shot A650 IS camera (Canon Marketing (Thailand) Co. Ltd., Thailand)
3. Clevenger apparatus
4. Free tree software (version 0.9.1.50) (Adam Pavlieek, JaroslavFlegr 1998-1999)
5. Gas chromatograph (Trace GC Ultra, Thermo Finnigan, USA) equipped with MS detector (DSQ, Thermo Finnigan, USA)
6. Hot air oven (WTC Binder, Germany)
7. ImageJ software (version: 1.46r) (National Institutes of Health, USA)
8. Linomat 5 applicator (CAMAG, Switzerland)
9. Micropipette (Brand Tech Scientific, Inc., Germany)
10. Microscope (Carl Zeiss model Axio Lab, Germany)
11. Rotary vacuum evaporator (BUCHI Labortechnik AG, Switzerland
12. Sample concentrator (Brinkmann, USA)
13. Shaker (Adolf Kuhner AG, Saitzerland)
14. Soxhlet apparatus
15. Spectrophotometer (PG Instruments Limited, UK)
16. Tree view software (version 1.6.6) (Roderic D. M. Page, 2001)

17. TLC chamber (CAMAG, Switzerland)

18. TLC scanner 3 (CAMAG, Switzerland)

19. TLC visualizer (CAMAG, Switzerland)

20. Ultra-pure water purification (NW20VF, Heal Force, China)

21. Ultrasonic bath (Analytical Lab Science Co., LTD, Thailand)

22. UV fluorescence analysis cabinet (CAMAG, Switzerland)

23. Water bath (Brinkman, USA)

24. winCATS software (version: 1.4.6.2002) (CAMAG, Switzerland)

Methods

Collection of sample

A. reinwardtii inner bark and *E. stoechadosmum* whole plants were collected from various sources of Thailand. The plants were identified and authenticated by Ruangrungsi N. Voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. The fresh parts of plants were for macromorphological and microscopical studies; whereas the dried powder was used for determination of powder microscopy, physicochemical, characterization and phytochemical analysis.

Standardization of *A. reinwardtii* inner bark and *E. stoechadosmum* whole plants

Dried inner bark of *A. reinwardtii* and whole plants of *E. stoechadosmum* were examined. All foreign matters were removed. The clean crude drugs were pulverized for further studies.

Plant description

Whole plant characters were observed and recorded. The drawing outlines of whole plants were illustrated in proportion size which related to real size.

Macroscopic examination

Each plant material of *A. reinwatdtii* and *E. stoechadosmum* was identified by visual examination of the organoleptic characterization such as color, size, texture, odour, and other visuality. The pictures of *A. reinwatdtii* and *E. stoechadosmum* crude drugs were taken with camera and illustrated by proportional scale related to real size.

Microscopic examination

The tissue section and powders of *A. reinwardtii* and *E. stoechadosmum* were studied for anatomical and histological characters under microscope with 10x, 20x, and 40x objective lens magnifications and 10x eyepiece lens. The sample was mounted in water onto a glass slide. Photograph was taken with digital camera, then was expressed the cell characteristics by line drawing.

Determination of water content (Azeotropic method)

Fifty grams of the ground sample in 200 ml of water-saturated toluene were distilled with azeotropic apparatus. When the water was completely distilled, transferred the heat, allowed the receiving tube to cool to room temperature and dislodged any droplets of water adhering to the walls of the receiving tube. The water and toluene layers were separated afterwards read off the volume of water. The content of water was calculated in percentage. Each sample was done in triplicate.

Determination of loss on drying

Three grams of ground sample were accurately weighed in the dried pre-weighed crucible. The sample was dried at 105 ℃ in an oven for 6 hours and weighed to constantly weight, calculated the loss of weight in percentage. Each sample was done in triplicate.

Determination of total ash

Three grams of ground sample were accurately weighed in the dried pre-weighed crucible, then incinerated at 500 ℃ until completely ashing (its color turn to white) then cool the crucible in a desiccator. The content of ash was weighed and calculated the content of total ash in percentage. Each sample was done in triplicate.

Determination of acid insoluble ash

The crucible that containing the total ash from determination of total ash was added with 25 ml of hydrochloric acid (70 g/l), then covered with a watch-glass and gently boiled for 5 minutes, after that, rinsed the watch-glass with 5 ml of hot water and added this liquid to the crucible. Keep insoluble matters on an ashless filter-paper No. 40 and washed with hot water until the filtrate was neutral. The filter-paper that contained insoluble matter was transferred to the previous crucible, then dried on a hot plate and incinerated to constant weight. Then the residue was cooled in a desiccator and weighed. The content of acid-insoluble ash was calculated in percentage. Each sample was done in triplicate.

Determination of alcohol soluble extractive value

Five grams of ground sample were accurately weighed in a glass stopped conical flask and were macerated with 70 ml of 95% ethanol for 6 hours in shaking bath. After that, it were stood for 18 hours, filtered quickly, washed the marc and adjusted the filtrate with 95% ethanol to 100 ml. Twenty milliliters of the filtrate were transferred to pre-weighed small beaker, evaporated to dryness on water-bath. It were dried at 105 °C in an oven, and then cooled in a desiccator for 30 minutes and weighed. Each sample was done in triplicate.

Determination of water soluble extractive value

Five grams of ground sample were accurately weighed in a glass stopped conical flask and were macerated with 70 ml of water for 6 hours in shaking bath after that it were stood for 18 hours. The extract was filtered quickly through Whatman No. 4, washed the marc and adjusted the filtrate with water to 100 ml. Transferred 20 ml of the filtrate in pre-weighed small beaker, evaporated to dryness on water-bath. The

sample was dried at 105 °C in an oven, and then cooled in a desiccator for 30 minutes and weighed. Each sample was done in triplicate.

Determination of volatile oil

The volatile oil of *A. reinwardtii* and *E. stoechadosmum* were determined using Clevenger apparatus. One hundred grams of the ground samples in 600 ml water were subjected to distillation. The volatile oil was completely distilled in 6 hours. The volatile oil and water layers were separated in the receiving tube at least 10 minutes then the volume of volatile oil was read off and calculated as a percentage of dried material. Each sample was done in triplicate. The volatile oil from each sample was collected for chemical compositions study by GC/MS.

Thin layer chromatographic fingerprint

Ethanolic extract of *A. reinwardtii and E. stoechadosmum* were dissolved with ethanol to concentration of 1 mg/ml. Three microliters of each crude extract was applied on the silica gel 60 GF_{254} TLC plate by micropipette. The TLC plates were developed in the saturated TLC chamber with suitable solvents (Toluene: Ethyl acetate 75: 25 for *A. reinwardtii*, Toluene: Ethyl acetate 93: 7 for *E. stoechadosmum*). After development, the plates were removed and allowed it to dry at room temperature. The spot on the plate was observed under short wave (254 nm) and long wave (365 nm) ultraviolet light. The plates were stained by anisaldehyde staining reagent and heated in an oven at 105 °C for 3 minutes.

Analysis of chemical compositions of volatile oil by GC-MS

The volatile oil of *E. stoechadosmum* whole plants extracted by hydro-distillation was diluted (1:100) with methanol (HPLC-grade) and was investigated by capillary gas chromatography-mass spectrometry (GC-MS) using Finnigan trace GC ultra with Finnigan DSQ Quadrupole detector and BPX5 fuse silica column (30 m x 0.25 mm, 0.25 um film thickness). The injector temperature was 180 ℃. One microliter of sample was injected (split ratio 100:1) into capillary column. The oven temperature was 60 ℃ for 1 min., and then ramped to 240 ℃ with the rate of 3 ℃/min. Helium was used as carrier gas (flow rate at 1 ml/min). MS was performed by EI positive mode at 70 eV ionization voltages. The constituents of the oil were identified by matching their mass spectra and retention indices with Adams Essential oil MS library and NIST05 MS library. The percentage compositions were measured from GC peak areas. All the compounds were with content > 0.1% were scored as 0 or 1 for the absence or presence of compounds, respectively. The similarity index was calculated from the data that was generated using Jaccard similarity index coefficient. The dendrogram was constructed base on the similarity matrix data by the unweight pair group method with arithmetic averages (UPGMA), clustering using FreeTree software (Pavlieck, Hrda, &Flegr, 1999). To perform the strength of the resulting branches, bootstrap probabilities were calculated by 100 bootstrap resampling data with this software.

Quantitative analysis of coumarin

Standard preparation

The stock solution of standard coumarin was prepared in dichloromethane containing 10% methanol. The solution was appropriately diluted to obtain working standard solutions at concentration of 0.075, 0.15, 0.3, 0.6, 0.9 and 1.2 mg/ml. These solutions were stored in refrigerator at 4 ºC.

Sample preparation

Five grams powder of *A. reinwardtii* dried inner bark and *E. stoechadosmum* dried whole plants were exhaustively extracted with dichloromethane by Soxhlet apparatus. The extract were filtered and concentrated under reduced pressure at 40 ºC. The *A. reinwardtii* and *E. stoechadosmum* extracts were dissolved with in dichloromethane containing 10% methanol to get the concentration of 5.0 mg/ml and 20 mg/ml respectively. These extracts were further used for TLC-densitometry and TLC image analysis.

TLC densitometric analysis

For the study of *A. reinwardtii*, five microliters of 15 dichloromethane extracts and 6 coumarin standard solutions were applied onto the silica gel 60 GF_{254} TLC plate of 0.2 mm thickness by CAMAG Linomat 5 applicator. Sample bands were set at 10.0 mm. The plate developed in saturated TLC chamber that containing a mixture of hexane and ethyl acetate (1:1) as mobile phase.

For the study of *E. stoechadosmum*, five microliters of 15 dichloromethane extracts and 5 coumarin standard solutions were applied onto the silica gel 60 GF_{254} TLC plate of 0.2 mm thickness by CAMAG Linomat 5 applicator. Sample band was set

at 10.0 mm. The plate developed in saturated TLC chamber. First step developed with chloroform, allowed the developed plate to dry. Second developed with a mixture of toluene, ethyl acetate and acetic acid (97:10:3).

After development, TLC plates were allowed to dry at room temperature. The spots on TLC plates were scanned with CAMAG TLC scanner 3 under optimal wavelength at 285 nm. The calibration curves of coumarin were prepared by plotting peak areas *versus* concentrations of coumarin applied. All of chromatograms were performed by winCATS software. The quantitative study was operated in triplicate.

TLC imageJ analysis

The developed TLC plate photos were photographed under UV 254 nm in ultraviolet fluorescence analysis cabinet using CAMAG TLC visualizer and saved as TIFF files. The spot on TLC plate was analyzed using ImageJ software. The calibration curve of coumarin was arranged by plotting peak areas *versus* concentrations of coumarin applied.

Method validation

Accuracy

The accuracy of the method was tested by adding three known different levels of standard solution (low, medium, high) into samples. Three examinations were operated at each level. Each test was performed in triplicate. The accuracy was determined as recovery of coumarin in percentage following: %Recovery= ((Cs-C)/Ca))×100

Where, Cs = the amount of coumarin that found after spiking standard solution

C = the amount of coumarin that found before spiking standard solution

Ca = the amount of reference standard actually spiked to sample

Precision

The precision was verified by repeatability (intra-day) and intermediate precision (inter-day) studies. Intra-day and inter-day precision were performed by analyzing the sample solution in 3 different concentrations (3 replicates) on the same day and three different days respectively. The contents were calculated by peak area measurement and expressed in terms of % relative standard deviation following: %RSD = (SD/Mean)×100

Calibration range

The calibration range of the method was calculated by plotting peak areas *versus* the concentrations of coumarin standard. Regression line and coefficient of determination of the calibration curve were established by Microsoft Excel 2010.

Limit of detection

The limit of detection (LOD) was calculated based on the residual standard deviation of regression lines (SD) (residual standard deviation = square roots $\Sigma(y-y_{est})^2/(n-2)$) and the slope of the calibration curve (*S*) following the formula: LOD = 3.3(SD)/*S*.

Where, SD = the standard deviation of regression line

S = the slope of the calibration curve

Limit of quantitation

The limit of quantitation (LOQ) will be calculated based on the residual standard deviation of regression lines (SD) and the slope of the calibration curve (*S*) following the formula: LOD= 10(SD)/*S*.

Where, SD = the standard deviation of regression line

S = the slope of the calibration curve

Robustness

The suitable mobile phase ratio in the experiment was slightly changed. Mobile phase having various compositions of hexane and ethyl acetate was performed as 5:5 v/v, 5:4.9 v/v, 4.9:5 v/v in *A. reinwardtii* experiment and a little difference in a mixture ratio of toluene, ethyl acetate and acetic acid was performed as 97:10:3 v/v, 96.9:10:2.95 v/v, 97:9.9:3.05 v/v in *E. stoechadosmum* analysis. The coumarin in plant materials were analyzed in this method. Each test was performed in triplicate. The robustness was interpreted as %RSD of peak areas.

Specificity

The specificity was performed by peak identity checking which was tested by matching UV absorbance spectra of coumarin standard with coumarin in each sample using TLC scanner 3.

Data analysis

The parameters due to standardization were expressed as grand mean ± pooled standard deviation (SD). The coumarin contents between TLC image analysis and TLC-densitometry were compared by paired *t*-test statistical analysis.

CHAPTER IV

RESULTS

Alyxia reinwardtii Blume

Macroscopic examination

The Figure 5 showed white, cream to brown color of *A. reinwardtii* inner bark. The whole plant of *A. reinwardtii* was demonstrated in Figure 6.

Figure 5 *Alyxia reinwardtii* Blume inner bark

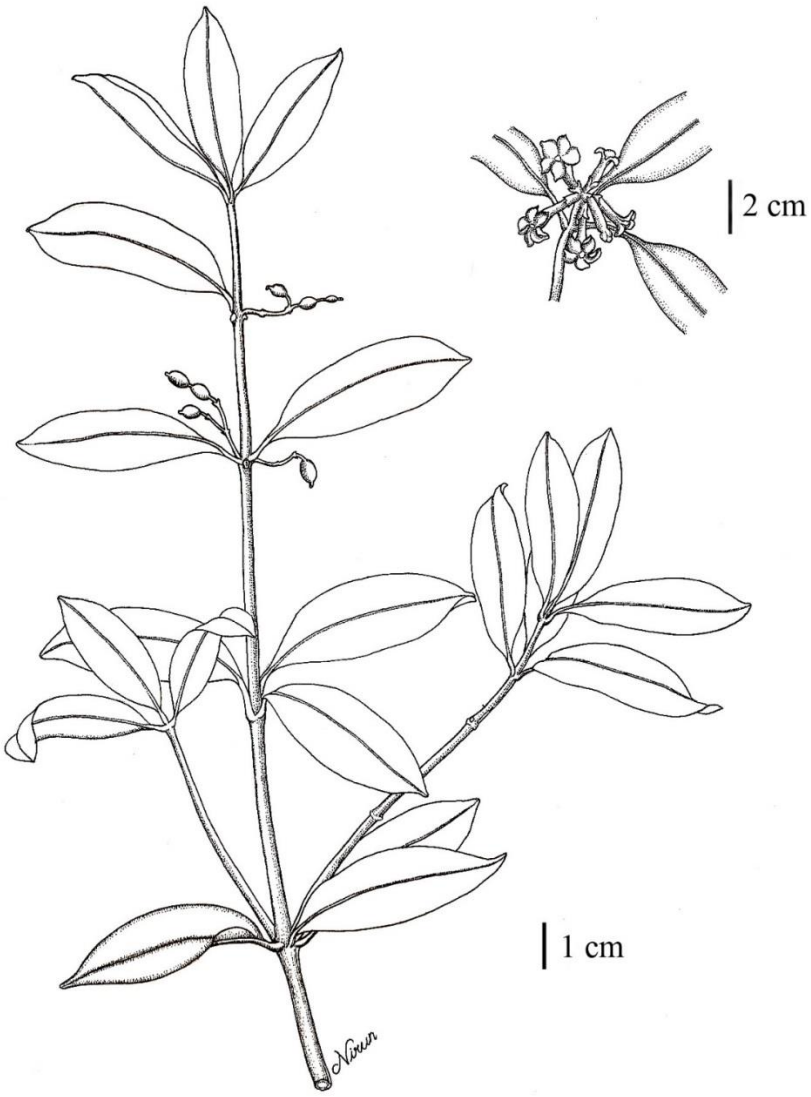


Figure 6 The whole plant of *Alyxia reinwardtii* Blume

Microscopic examination

The histological investigation of *A. reinwardtii* inner bark powdered was shown in Figure 7. Several histological characters including fragment of fibers, fragment of pitted vessel, fragment of reticulated vessel, starch granule, prism crystals of calcium oxalate, fragment of parenchyma, and sclereid were found in *A. reinwardtii* inner bark. The anatomical study of *A. reinwardtii* bark was illustrated in the Figure 8.

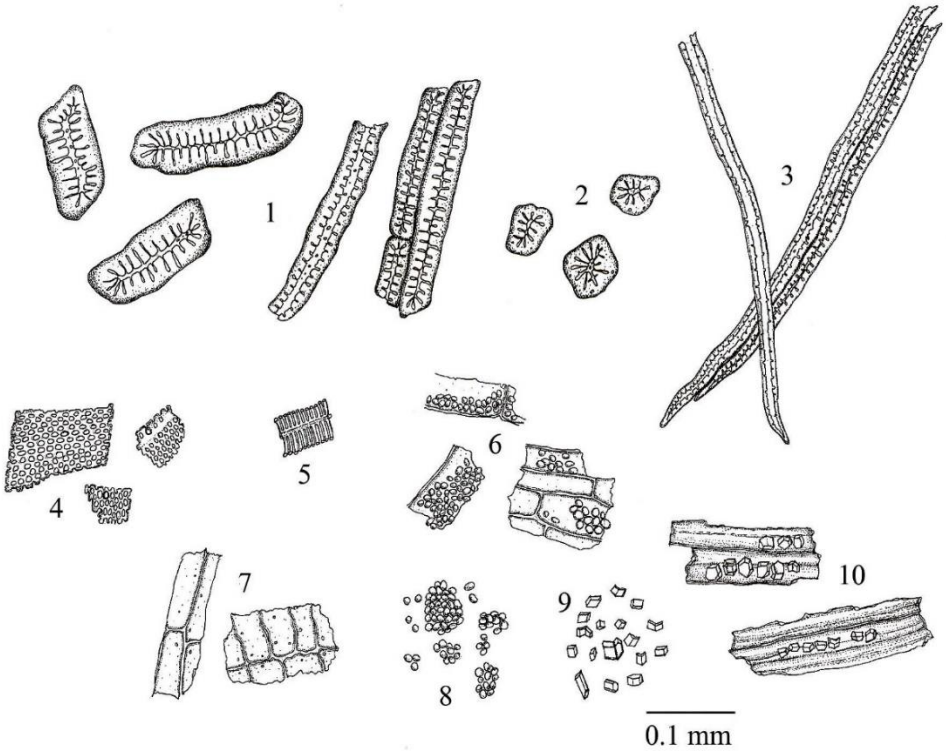


Figure 7 Powder of the bark of *Alyxia reinwardtii* Blume

1. Sclereid in longitudinal view 2. Sclereid in transverse view 3. Fragment of fiber 4. Fragment of pitted vessel 5. Fragment of reticulated vessel 6. Parenchyma containing starch granules 7. Fragment of parenchyma 8. Starch granules 9. Prism crystals of calcium oxalate 10. Calcium oxalate prism sheath

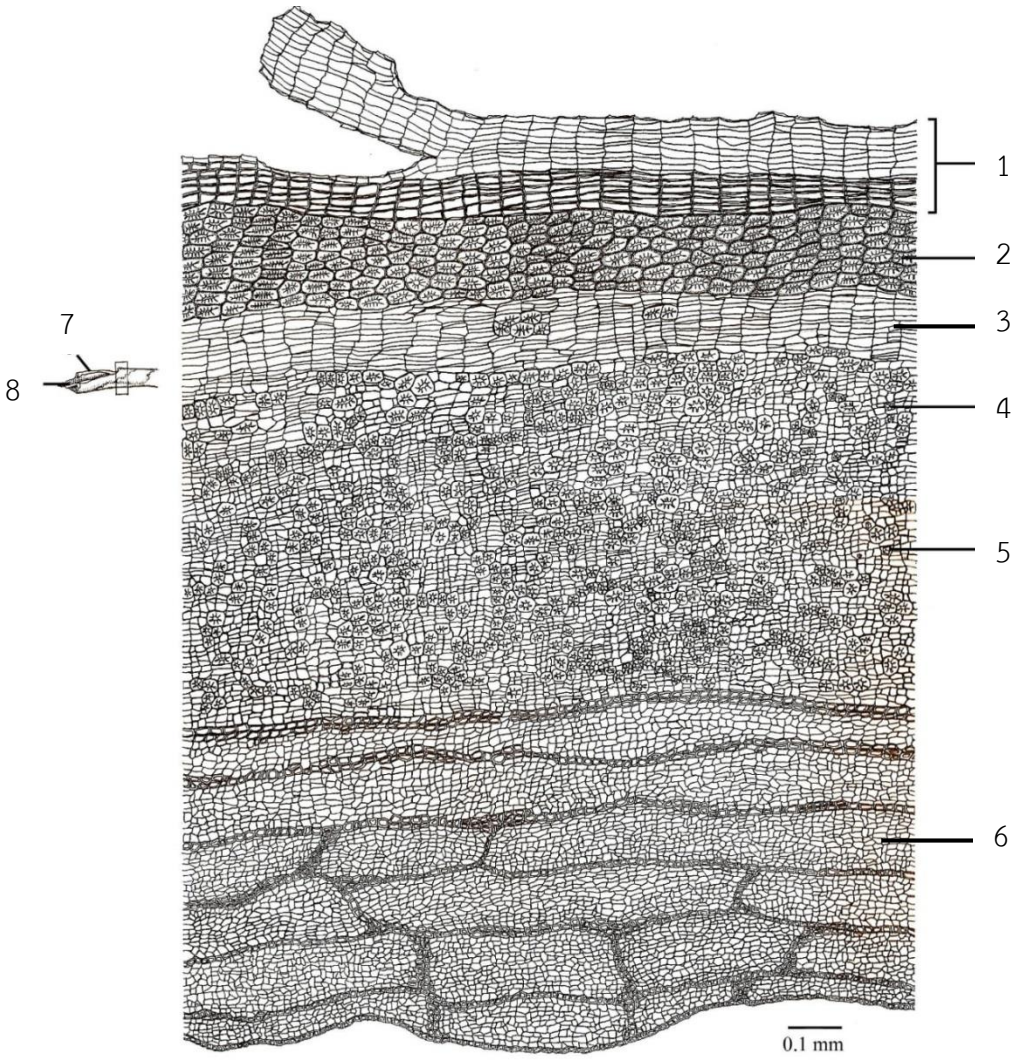


Figure 8 Transverse section of the bark of *Alyxia reinwardtii* Blume

1. Periderm 2. Remnant of epidermis 3. Cork cambium 4. Secondary Phloem 5. Parenchyma containing prism crystal of calcium oxalate 6.secondary xylem 7. Outer bark 8. Inner bark

Physicochemical evaluation

Physicochemical parameters including total ash, acid insoluble ash, loss on drying, water content and extractive value parameters were performed to evaluate the pharmacognostic specification of *A. reinwardtii* inner bark.

The physicochemical parameters of *A. reinwardtii* inner bark from 15 different sources throughout Thailand were illustrated in Table 2.

Table 2 Physicochemical specification (% by weight) of *A. reinwardtii* inner bark

Content	Mean ± SD*	Range**
Total ash	7.65 ± 0.10	7.35 – 7.94
Acid insoluble ash	1.37 ± 0.11	1.03 – 1.70
Loss on drying	8.90 ± 0.15	7.64 – 8.54
water	11.91 ± 0.22	11.25 – 12.57
Ethanol soluble extractive	8.92 ± 0.33	7.39 – 9.91
Water soluble extractive	16.50 ± 0.11	16.16 – 16.83
Volatile oil	0	0

*The parameters were shown as grand mean ± pooled SD. **mean ± 3SD, sample were from 15 different sources throughout Thailand. Each sample was performed in triplicate.

Thin layer chromatographic identification

TLC fingerprint of *A. reinwardtii* inner bark ethanol extract was shown in figure 9.

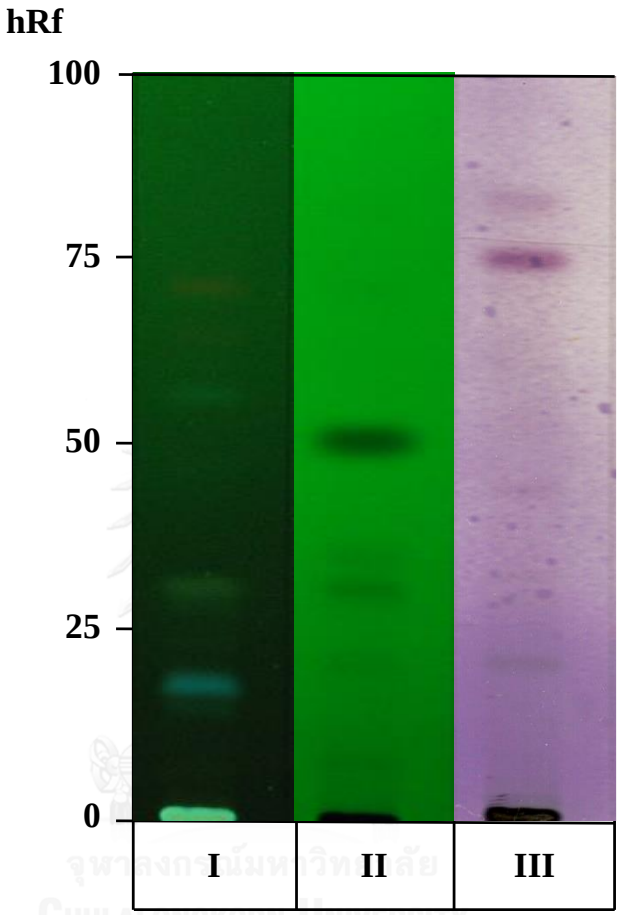


Figure 9 TLC fingerprint of *A. reinwardtii* inner bark ethanol extract

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

Dichloromethane extract of *Alyxia reinwardtii* Blume

The dried powders of *A. reinwardtii* inner bark from 15 sources were extracted with dichloromethane by Soxhlet apparatus. The percent yields of crude extracts were exhibited in Table 3. The average percent yield of *A. reinwardtii* dichloromethane extract was 13.10 ± 3.18 g/100g by dry weight.

Quantitative analysis of coumarin content of *A. reinwardtii* by TLC densitometry

The amounts of coumarin in dichloromethane extract were evaluated by the regression equation: $y = -32139x^2 + 62584x + 8429$ (Figure 10) and reported as grams of coumarin per 100 grams of dried *A. reinwardtii* inner bark (Table 3). The average coumarin content in 100 g of dried *A. reinwardtii* crude drug was found to be 0.77 ± 0.04 g.

Method validation

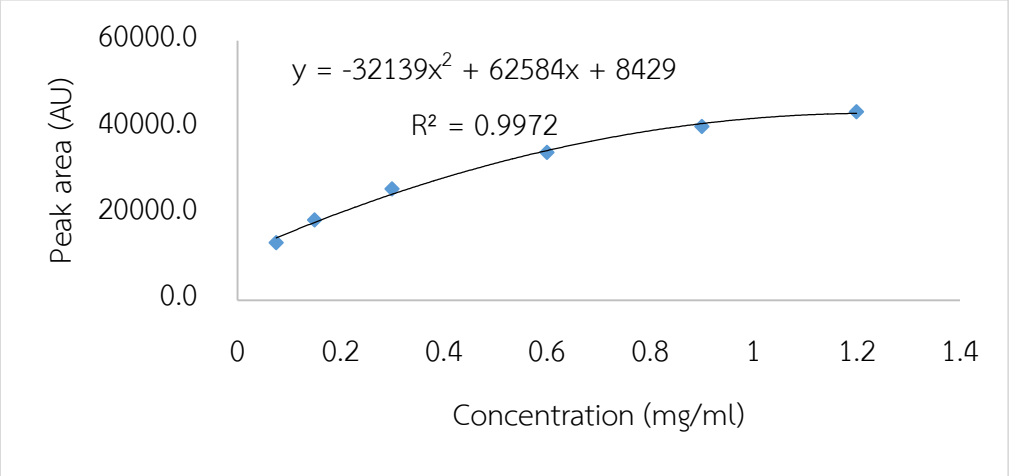
The calibration range, LOD, LOQ, precision, accuracy, specificity and robustness were investigated for the validation of an analytical method followed by ICH guideline.

The calibration curves of standard compounds were polynomial in the range of 0.075-1.2 mg/ml. LOD and LOQ were calculated based on the residual standard deviation of a regression line and the slope of the calibration curve. The LOD value, regard as the lowest concentration of analyte in a sample which can be detected was found to be 0.052 mg/ml. The LOQ values, which regard as the lowest concentration of analyte in a sample which can be quantitatively determined was 0.159 mg/ml.

Table 3 The amount of coumarin contents of *A. reinwardtii* extracts from 15 different sources throughout Thailand in % by weight (TLC densitometry)

Source	coumarin in dichloromethane extract (mg/mg)		yield of the dichloromethane extract (g/100 g of dried crude drug)	Coumarin in *A. reinwardtii* (g/100 g of dried crude drug)	
	mean	SD		mean	SD
1	0.03	0.00	13.78	0.41	0.03
2	0.04	0.00	11.64	0.42	0.02
3	0.05	0.00	11.59	0.54	0.03
4	0.04	0.00	12.57	0.49	0.02
5	0.05	0.00	11.68	0.56	0.03
6	0.06	0.00	10.07	0.61	0.04
7	0.08	0.01	14.92	1.13	0.20
8	0.05	0.00	9.19	0.42	0.01
9	0.05	0.00	22.27	1.12	0.09
10	0.10	0.00	9.23	0.92	0.01
11	0.03	0.00	12.03	0.36	0.01
12	0.11	0.01	14.50	1.60	0.11
13	0.05	0.00	14.50	0.73	0.03
14	0.06	0.00	14.18	0.85	0.04
15	0.09	0.00	14.39	1.30	0.03
			Average	**0.77 ± 0.04**	

The recovery assay was used to validate the accuracy of coumarin quantitation. Standard coumarin was spiked into the sample matrix for additional concentrations of 0.125, 0.25 and 0.5 mg/ml. The recovery values were 92.15 – 97.35 % recovery as demonstrated in Table 4.



*Five microliters of each standard and sample solutions were applied on the silica gel 60 F_{254} TLC plate

Figure 10 The calibration curve of coumarin in *A. reinwardtii* by TLC densitometric method

Table 4 Accuracy of quantitation of coumarin in *A. reinwardtii* by TLC densitometry (n=3)

Coumarin added (mg/ml)	Coumarin found (mg/ml)	% Recovery
-	0.31 ± 0.01	-
0.125	0.42 ± 0.01	97.35
0.250	0.51 ± 0.01	91.58
0.500	0.75 ± 0.03	92.15

The precision of coumarin quantitative analysis by TLC densitometric method was conducted as % RSD, taken as the error of the method was determined of 4 concentrations × 3 replicates at the same and three different days of tests. The results of precision were analyzed from peak area. The repeatability and intermediate precision were between 1.17 – 3.76 % RSD (Table 5).

Table 5 Repeatability and intermediate precision of coumarin in *A. reinwardtii* by TLC densitometry

Coumarin observed (mg/ml)	**% RSD**	
	Repeatability precision (n=3)	**Intermediate precision (n=9)**
0.31	3.76	3.59
0.42	1.57	1.69
0.51	1.17	2.64
0.75	3.43	2.06

The specificity was performed using peak identity checking by matching UV absorbance spectra of standard coumarin with coumarin band in sample (Figure 11).

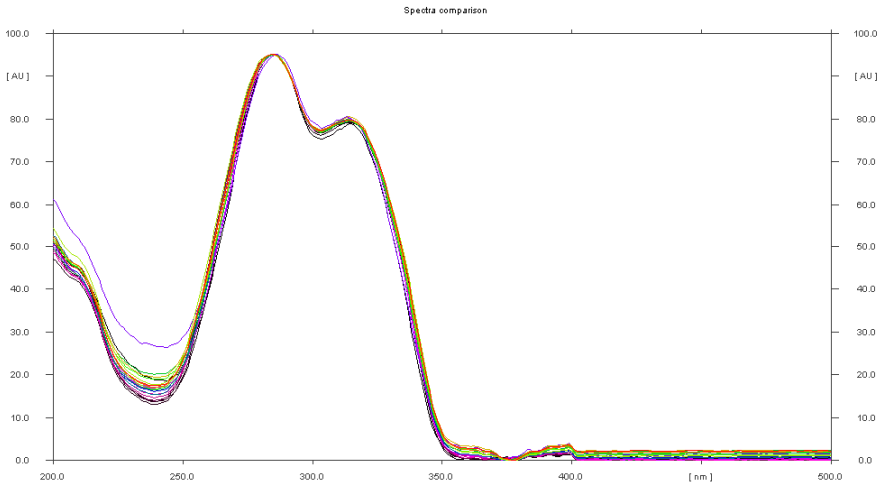


Figure 11 UV absorbance spectra of coumarin standard and coumarin in *A. reinwardtii* 15 sources

The robustness of coumarin quantitation in *A. reinwardtii* by TLC densitometric analysis was determined in three mobile phase ratios. The result of robustness was 0.97 % RSD of peak area (Table 6).

Table 6 Robustness investigation of coumarin in *A. reinwardtii* by TLC densitometry

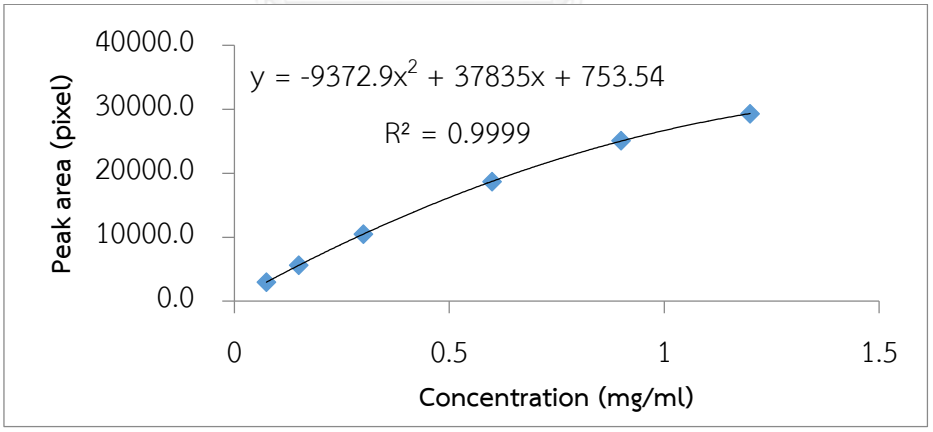
Mobile phase ratio (v/v)		Coumarin peak area (AU)
Hexane	**Ethyl acetate**	
4.9	5	30905.63 ± 125.83
5	5	31318.10 ± 196.35
5	4.9	31495.03 ± 341.63
	mean	31239.59
	SD	302.44
	% RSD	0.97

Quantitative analysis of coumarin content of *A. reinwardtii* by TLC image analysis

The amounts of coumarin in dichloromethane extract were evaluated by the regression equation: $y = -9372.9x^2 + 37835x + 753.54$ and reported as grams of coumarin per 100 grams of dried *A. reinwardtii* inner bark (Table 7). The average coumarin content in 100 g of dried *A. reinwardtii* crude drug was found to be 0.75 ± 0.01 g.

Method validation

The calibration curves of coumarin ranged from 0.075 – 1.2 mg/ml were shown in Figure 12. LOD and LOQ were calculated based on the residual standard deviation of a regression line and the slope of the calibration curve. The LOD was found to be 0.016 mg/ml. The LOQ value was 0.047 mg/ml respectively.



*Five microliters of each standard and sample solutions were applied on the silica gel 60 F_{254} TLC plate

Figure 12 The calibration curve of coumarin in *A. reinwardtii* by TLC image analysis method

Table 7 The amount of coumarin contents of *A. reinwardtii* extracts from 15 different sources throughout Thailand in % by weight (TLC image analysis)

Source	coumarin in dichloromethane extract (mg/mg)		yield of the dichloromethane extract (g/100 g of dried crude drug)	Coumarin in *A. reinwardtii* (g/100 g of dried crude drug)	
	mean	SD		mean	SD
1	0.03	0.00	13.78	0.42	0.02
2	0.03	0.00	11.64	0.40	0.00
3	0.05	0.00	11.59	0.54	0.01
4	0.04	0.00	12.57	0.46	0.01
5	0.04	0.00	11.68	0.50	0.00
6	0.06	0.00	10.07	0.58	0.02
7	0.08	0.01	14.92	1.18	0.01
8	0.04	0.00	9.19	0.40	0.03
9	0.05	0.00	22.27	1.05	0.01
10	0.10	0.00	9.23	0.91	0.01
11	0.03	0.00	12.03	0.40	0.01
12	0.11	0.00	14.50	1.61	0.01
13	0.05	0.00	14.50	0.79	0.00
14	0.06	0.00	14.18	0.85	0.02
15	0.09	0.00	14.39	1.22	0.03
			Average	**0.75 ± 0.01**	

The recovery assay was used to validate the accuracy of coumarin quantitation. Standard coumarin was spiked into the sample matrix for additional concentrations of 0.125, 0.25 and 0.5 mg/ml. The recovery values were 96.78 – 107.31 % recovery as demonstrated in Table 8.

Table 8 Accuracy of quantitation of coumarin in *A. reinwardtii* by TLC image analysis (n=3)

Coumarin added (mg/ml)	**Coumarin found (mg/ml)**	**% Recovery**
-	0.26 ± 0.02	-
0.125	0.41 ± 0.01	107.31
0.250	0.50 ± 0.01	99.47
0.500	0.73 ± 0.02	96.78

The precision of coumarin quantitative analysis by TLC image analysis method was conducted as % RSD, taken as the error of the method was determined of 4 concentrations × 3 replicates at the same and three different days of tests. The repeatability and intermediate precision were between 0.80 – 4.32 % RSD (Table 9).

Table 9 Repeatability and intermediate precision of coumarin in *A. reinwardtii* by TLC image analysis method

Coumarin observed (mg/ml)	% RSD	
	Repeatability precision (n=3)	Intermediate precision (n=9)
0.26	3.10	2.35
0.41	1.86	0.55
0.50	0.80	0.83
0.73	2.71	4.32

The robustness of coumarin quantitation in *A. reinwardtii* by TLC image analysis was determined in three mobile phase ratios. The result of robustness was 0.31 % RSD of peak area (Table 10).

Table 10 Robustness investigation of coumarin in *A. reinwardtii* by TLC image analysis

Mobile phase ratio (v/v)		Coumarin peak area (pixel)
Hexane	Ethyl acetate	
4.9	5	16418.87 ± 132.20
5	5	16340.32 ± 260.82
5	4.9	16324.25 ± 295.50
	mean	16361.15
	SD	50.63
	% RSD	0.31

The comparison of coumarin content between TLC densitometry and TLC image analysis

The comparison of coumarin content between TLC densitometry and TLC image analysis was analyzed by paired *t*-test (Table 11). The result showed that the coumarin content from two methods were not significantly different (p=0.07).

Table 11 The comparison of coumarin contents between TLC densitometry and TLC image analysis

Soure	% Coumarin content	
	TLC- densitometry	TLC image analysis
1	0.41	0.42
2	0.42	0.40
3	0.54	0.54
4	0.49	0.47
5	0.56	0.51
6	0.61	0.58
7	1.13	1.18
8	0.42	0.40
9	1.12	1.05
10	0.94	0.91
11	0.40	0.40
12	1.60	1.61
13	0.77	0.79
14	0.90	0.85
15	1.23	1.22

Table 12 Validity of coumarin quantitative analysis in *A. reinwardtii* inner bark by TLC densitometry and TLC image analysis

Parameter	TLC densitometry	TLC image analysis
Regression equation	$y = -32139x^2 + 62584x + 8429$	$y = -9372.9x^2 + 37835x + 753.54$
Coefficient of determination	0.99	0.99
Range	0.075-1.2 mg/ml	0.075-1.2 mg/ml
Accuracy	92.15-97.35 %recovery	96.78-107.31 %recovery
Precision	1.17-3.76 %RSD	0.80-4.32 %RSD
Robustness	0.97 %RSD	0.31 %RSD
Limit of detection	0.052 mg/ml	0.016 mg/ml
Limit of quantitation	0.159 mg/ml	0.047 mg/ml

***Eupatorium stoechadosmum* Hance**

Macroscopic examination

Figure 13 showed *E. stoechadosmum* crude drug. The whole plant of *E. stoechadosmum* was illustrated in Figure 14.

Figure 13 *Eupatorium stoechadosmum* Hance crude drug

Figure 14 The whole plant of *Eupatorium stoechadosmum* Hance

Microscopic examination

The histological investigation of *E. stoechadosmum* dried powder was shown in Figure 15. Several histological characters including stellate hair, fragment of pitted vessel, brownish mass, sclereid, prism crystal of calcium oxalate, fragment of epidermal cell with stomata, spiral vessel, parenchyma, fragment of fiber, multicellular trichome were found in *E. stoechadosmum*. The anatomical study of *E. stoechadosmum* stem was illustrated in the Figure 16.

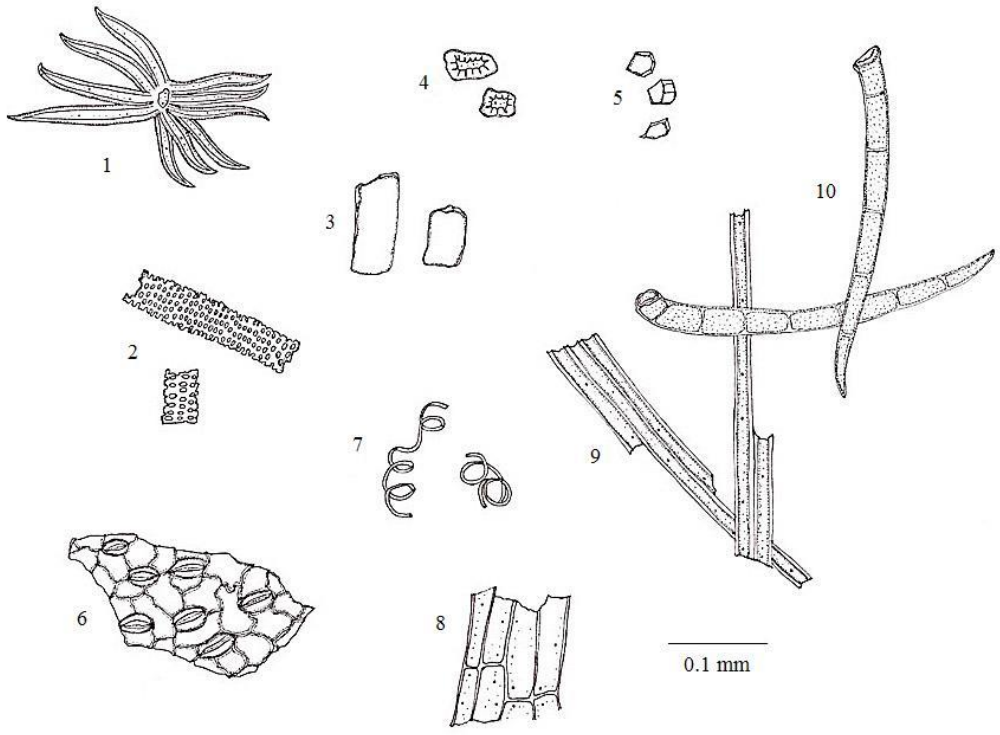


Figure 15 Powder of *Eupatorium stoechadosmum* Hance

1. Stellate hair 2. Fragment of pitted vessel 3. Brownish mass 4. Sclereid 5. Prism crystal of calcium oxalate 6. Fragment of epidermal cell with stomata 7. Spiral vessel 8. Parenchyma, longitudinal view 9. Fragment of fiber 10. Multicellular trichome

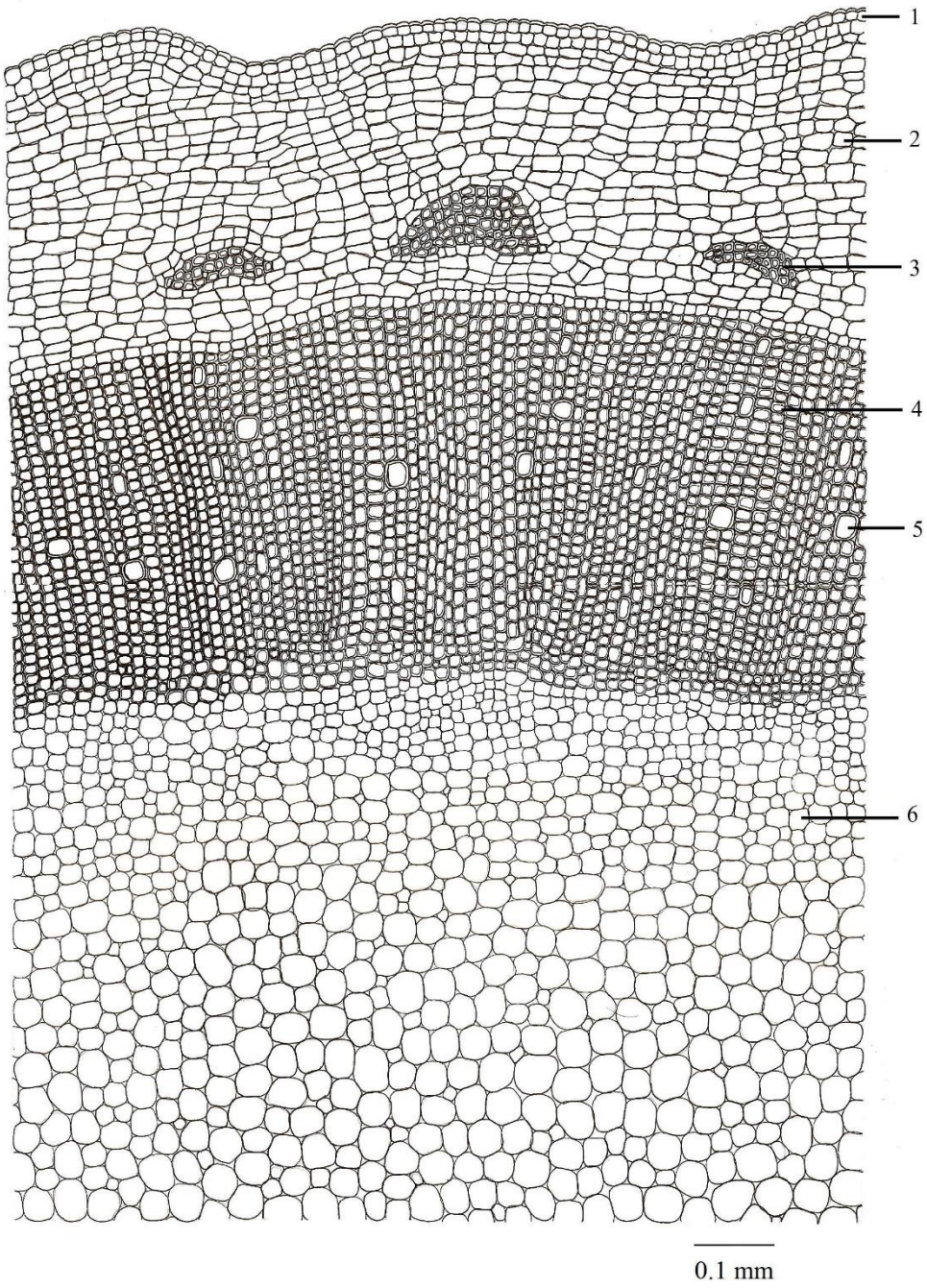


Figure 16 Transverse section of the stem of *Eupatorium stoechadosmum* Hance 1. Epidermis 2. Parenchyma of cortex 3. Group of fiber 4. Xylem fiber 5. Xylem vessel 6. Ground of parenchyma

Physicochemical evaluation

The physicochemical parameters of *E. stoechadosmum* from 15 various sources throughout Thailand were illustrated in Table 13.

Table 13 Physicochemical specification (% by weight) of *E. stoechadosmum* crude drug

Content	Mean ± SD*	Range**
Total ash	9.51 ± 0.31	8.57 – 10.45
Acid insoluble ash	2.64 ± 0.24	1.93 – 3.35
Loss on drying	8.66 ± 0.13	8.27 – 9.05
water	13.19 ± 0.33	12.21 – 14.16
Ethanol soluble extractive	9.42 ± 0.55	7.77 – 11.08
Water soluble extractive	27.78 ± 0.42	26.51 – 29.06
Volatile oil	0.14 ± 0.02	0.08 - 0.20

*The parameters were shown as grand mean ± pooled SD. **mean ± 3SD, sample were from 15 different sources throughout Thailand. Each sample was performed in triplicate.

Thin layer chromatographic identification

TLC fingerprint of *E. stoechadosmum* ethanol extract was shown in figure 17.

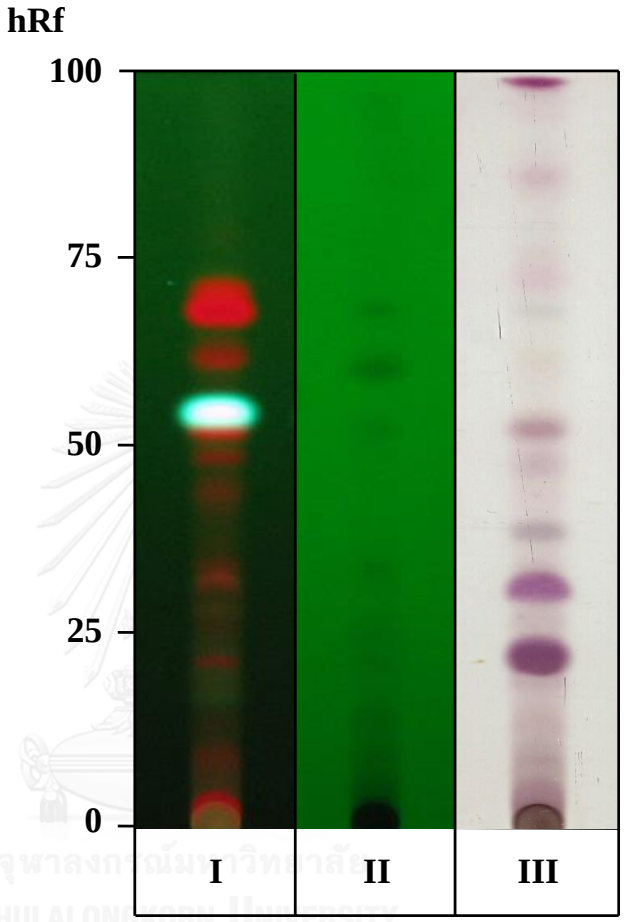


Figure 17 TLC fingerprint of *E. stoechadosmum* ethanol extract

Solvent system Toluene: Ethyl acetate 93: 7

Detection

I = Detection under UV light 365 nm

II = Detection under UV light 254 nm

III = Detection with anisaldehyde staining reagent

Dichloromethane extract of *Eupatorium stoechadosmum* Hance

The dried powders of *E. stoechadosmum* from 15 sources were extracted with dichloromethane by Soxhlet apparatus. The percent yields of crude extracts were exhibited in Table 14. The average percent yield of *E. stoechadosmum* dichloromethane extract was 22.00 ± 5.99 g/100g by dry weight.

Quantitative analysis of coumarin content of *E. stoechadosmum* by TLC densitometry

The amounts of coumarin in dichloromethane extract were evaluated by the regression equation: $y = -24417x^2 + 54568x + 3641.5$ and reported as grams of coumarin per 100 grams (Table 14). The average coumarin content in 100 g of dried *E. stoechadosmum* crude drug was found to be 0.44 ± 0.02 g.

Method validation

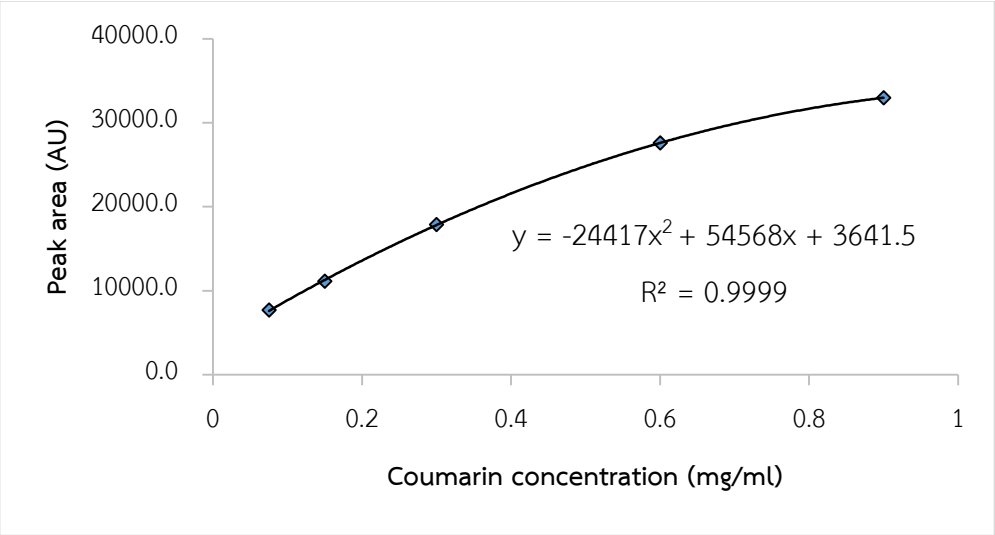
The calibration range, LOD, LOQ, precision, accuracy, specificity and robustness were investigated for the validation of an analytical method followed by ICH guideline.

The calibration curve of standard compounds were polynomial in the range of 0.075-0.9 mg/ml. LOD and LOQ were calculated based on the residual standard deviation of a regression line and the slope of the calibration curve. The LOD value was found to be 0.009 mg/ml. The LOQ value was 0.027 mg/ml respectively.

The recovery assay was used to validate the accuracy of coumarin quantitation. Standard coumarin was spiked into the sample matrix for additional concentrations of 0.125, 0.25 and 0.5 mg/ml. The recovery values were 90.40 – 95.16 % recovery as demonstrated in Table 15.

Table 14 The amount of coumarin contents of *E. stoechadosmum* extracts from 15 different sources throughout Thailand in % by weight (TLC densitometry)

Source	coumarin in dichloromethane extract (mg/mg)		yield of the dichloromethane extract (g/100 g of dried crude drug)	Coumarin in *E. stoechadosmum* (g/100 g of dried crude drug)	
	mean	SD		mean	SD
1	0.01	0.00	25.91	0.32	0.00
2	0.01	0.00	17.11	0.20	0.00
3	0.01	0.00	16.74	0.14	0.01
4	0.02	0.00	18.96	0.40	0.01
5	0.02	0.00	26.18	0.46	0.01
6	0.02	0.00	26.80	0.54	0.02
7	0.03	0.00	14.96	0.38	0.00
8	0.01	0.00	34.40	0.45	0.02
9	0.02	0.00	28.33	0.59	0.02
10	0.03	0.00	23.22	0.64	0.03
11	0.02	0.00	18.95	0.41	0.00
12	0.03	0.00	13.39	0.37	0.02
13	0.03	0.00	15.51	0.42	0.02
14	0.03	0.00	24.71	0.55	0.03
15	0.01	0.00	24.88	0.74	0.03
			Average	**0.44 ± 0.02**	



*Five microliters of each standard and sample solutions were applied on the silica gel 60 F_{254} TLC plate

Figure 18 The calibration curve of coumarin in dried crude drug of *E. stoechadosmum* by TLC densitometric method

Table 15 Accuracy of quantitation of coumarin in *E. stoechadosmum* by TLC densitometry (n=3)

Coumarin added (mg/ml)	Coumarin found (mg/ml)	% Recovery
-	0.22 ± 0.02	-
0.125	0.33 ± 0.00	95.16
0.250	0.44 ± 0.00	93.00
0.500	0.66 ± 0.03	90.40

The specificity was tested by matching standard coumarin with coumarin band in sample. The peak identity was illustrated in Figure 19.

The precision of coumarin quantitative analysis by TLC densitometry method was conducted as % RSD, taken as the error of the method was determined of 4 concentrations × 3 replicates at the same and three different days of tests. The repeatability and intermediate precision were between 0.35 – 9.92 % RSD (Table 16).

The robustness of coumarin quantitation in *E. stoechadosmum* by TLC densitometric analysis was determined in three mobile phase ratios. The result of robustness was 1.07 % RSD of peak area (Table 17).

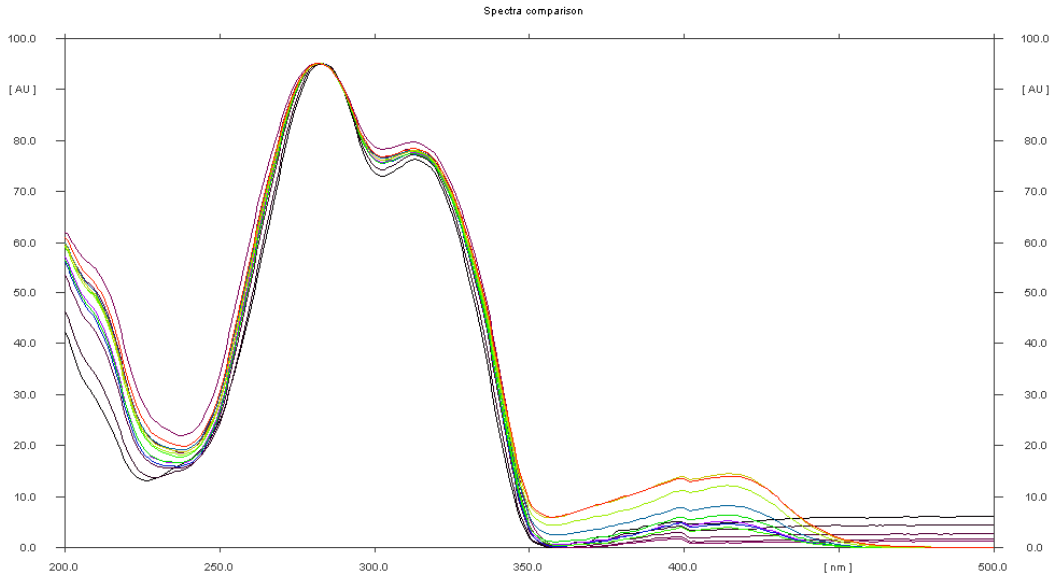


Figure 19 UV absorbance spectra of coumarin standard and coumarin in *E. stoechadosmum* 15 sources

Table 16 Repeatability and intermediate precision of coumarin in *E. stoechadosmum* by TLC densitometry

Coumarin observed (mg/ml)	% RSD	
	Repeatability precision (n=3)	Intermediate precision (n=9)
0.22	9.01	9.92
0.33	0.35	4.46
0.44	0.69	6.23
0.66	4.54	5.36

Table 17 Robustness investigation of coumarin in *E. stoechadosmum* by TLC densitometry

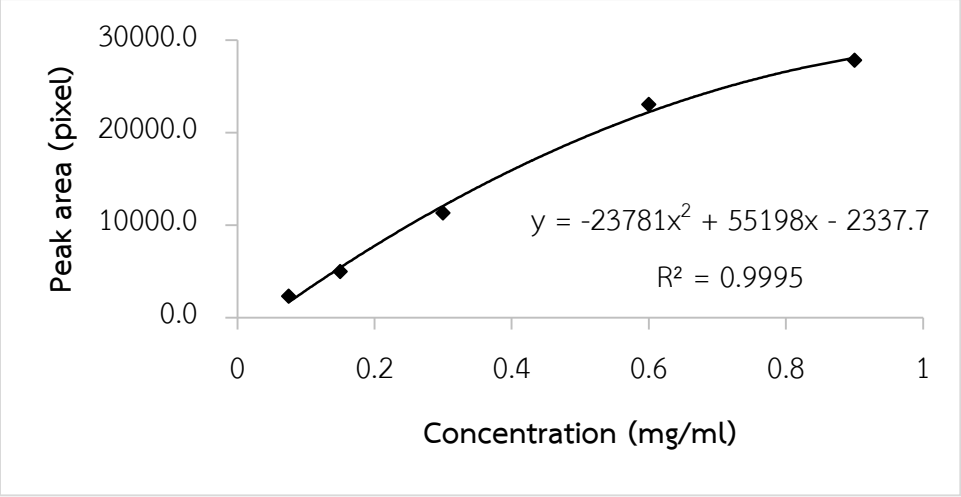
Mobile phase ratio (v/v)			Coumarin peak area (AU)
Toluene	Ethyl acetate	Acetic acid	
96.9	10	2.95	19447.70 ± 366.72
97	10	3	19316.73 ± 591.59
97	9.9	3.05	19724.87 ± 467.13
	mean		19496.43
	SD		208.39
	% RSD		1.07

Quantitative analysis of coumarin content of *E. stoechadosmum* by TLC image analysis

The amount of coumarin in dichloromethane extract was analyzed by the regression equation: $y = -23781x^2 + 55198x - 2337.7$ and reported as grams of coumarin per 100 grams of dried *E. stoechadosmum* whole plants (Table 18). The average coumarin content in 100 g of dried *E. stoechadosmum* crude drug was found to be 0.45 ± 0.04 g.

Method validation

The calibration curve of coumarin ranged from 0.075 – 0.900 mg/ml was shown in Figure 20. LOD and LOQ were calculated based on the residual standard deviation of a regression line and the slope of the calibration curve. The LOD was found to be 0.021 mg/ml. The LOQ value was 0.064 mg/ml respectively.



*Five microliters of each standard and sample solutions were applied on the silica gel 60 F_{254} TLC plate

Figure 20 The calibration curve of coumarin in *E. stoechadosmum* by TLC image analysis method

Table 18 The amount of coumarin contents of *E. stoechadosmum* extracts from 15 different sources throughout Thailand in % by weight (TLC image analysis)

Source	coumarin in dichloromethane extract (mg/mg)		yield of the dichloromethane extract (g/100 g of dried crude drug)	Coumarin in *E. stoechadosmum* (g/100 g of dried crude drug)	
	mean	SD		mean	SD
1	0.01	0.00	25.91	0.31	0.02
2	0.01	0.00	17.11	0.22	0.03
3	0.01	0.00	16.74	0.18	0.01
4	0.02	0.00	18.96	0.38	0.01
5	0.02	0.00	26.18	0.53	0.05
6	0.02	0.00	26.80	0.58	0.06
7	0.03	0.00	14.96	0.39	0.04
8	0.02	0.00	34.40	0.54	0.08
9	0.02	0.00	28.33	0.58	0.05
10	0.03	0.00	23.22	0.64	0.04
11	0.02	0.00	18.95	0.44	0.03
12	0.03	0.00	13.39	0.35	0.02
13	0.03	0.00	15.51	0.40	0.04
14	0.02	0.00	24.71	0.57	0.07
15	0.03	0.00	24.88	0.70	0.02
			Average	**0.45 ± 0.04**	

The recovery assay was used to validate the accuracy of coumarin quantitation. Standard coumarin was spiked into the sample matrix for additional concentrations of 0.125, 0.25 and 0.5 mg/ml. The recovery values were 89.46 – 91.75 % recovery as demonstrated in Table 19.

Table 19 Accuracy of quantitation of coumarin in *E. stoechadosmum* by TLC image analysis (n=3)

Coumarin added (mg/ml)	**Coumarin found (mg/ml)**	**% Recovery**
-	0.26 ± 0.03	-
0.125	0.34 ± 0.02	89.46
0.250	0.46 ± 0.01	91.75
0.500	0.68 ± 0.05	89.85

The precision of coumarin quantitative analysis by TLC image analysis method was conducted as % RSD, taken as the error of the method was determined of 4 concentrations × 3 replicates at the same and three different days of tests. The repeatability and intermediate precision were between 2.07 – 11.87 % RSD (Table 20).

The robustness of coumarin quantitation in *E. stoechadosmum* by TLC image analysis was determined in three mobile phase ratios. The result of robustness was 1.17 % RSD of peak area (Table 21).

Table 20 Repeatability and intermediate precision of coumarin in *E. stoechadosmum* by TLC image analysis method

Coumarin observed (mg/ml)	**% RSD**	
	Repeatability precision (n=3)	**Intermediate precision (n=9)**
0.26	11.87	8.78
0.34	2.07	7.18
0.46	3.17	4.47
0.68	6.70	4.32

Table 21 Robustness investigation of coumarin in *E. stoechadosmum* by TLC image analysis

Mobile phase ratio (v/v)			**Coumarin peak area (AU)**
Toluene	**Ethyl acetate**	**Acetic acid**	
96.9	10	2.95	14362.93 ± 593.38
97	10	3	14529.98 ± 651.05
97	9.9	3.05	14194.29 ± 147.09
	mean		14362.1
	SD		167.85
	% RSD		1.17

The comparison of coumarin content between TLC densitometry and TLC image analysis

The comparison of coumarin content between TLC densitometry and TLC image analysis was analyzed by paired *t*-test (Table 22). The result showed that the coumarin content from two methods were not significantly different (p=0.99).

Table 22 The comparison of coumarin contents between TLC densitometry and TLC image analysis

Source	% Coumarin content	
	TLC- densitometry	TLC image analysis
1	0.32	0.31
2	0.20	0.03
3	0.14	0.18
4	0.41	0.38
5	0.46	0.53
6	0.54	0.58
7	0.38	0.40
8	0.45	0.54
9	0.59	0.58
10	0.64	0.64
11	0.41	0.44
12	0.37	0.35
13	0.42	0.40
14	0.55	0.57
15	0.74	0.70

Table 23 Validity of coumarin quantitative analysis in *E. stoechadosmum* by TLC densitometry and TLC image analysis

Parameter	TLC densitometry	TLC image analysis
Regression equation	$y = -24417x^2 + 54568x + 3641.5$	$y = -10981x^2 + 37432x + 247.38$
Coefficient of determination	0.99	0.99
Range	0.075-0.900 mg/ml	0.075-0.900 mg/ml
Accuracy	90.40-95.16 %recovery	89.46-91.75 %recovery
Precision	0.35-9.92 %RSD	2.07-11.87 %RSD
Robustness	1.07 %RSD	1.17 %RSD
Limit of detection	0.009 mg/ml	0.021 mg/ml
Limit of quantitation	0.027 mg/ml	0.064 mg/ml

Chemical constituent analysis of volatile oil by GC/MS

The chemical compounds of *E. stoechadosmum* volatile oil analyzed by GC/MS consisted of at least 30 compounds as shown in Table 24.

Table 24 The chemical constituents of the *E. stoechadosmum* volatile oil

compound name	RT	KI[a]	Area%[b]
Cumin aldehyde	17.48	1241	0.99 ± 0.96
Carvacrol,methy ether	18.18	1244	0.11 ± 0.27
Thymol	20.05	1290	1.48 ± 1.31
Carvacrol,ethyl ether	20.44	1298	0.47 ± 0.58
Carvacrol	20.71	1299	8.21 ± 6.97
2(3H)-Naphthalenon,4,4a,5,6-tetrahydro-7-methyl-	23.30	-	1.31 ± 1.20
Cyperene	25.15	1398	0.08 ± 0.22
Caryophyllene *(E-)*	25.95	1419	4.13 ± 5.93
Cymene<2,5-dimethoxy-para>	26.07	1426	5.67 ± 6.20
Bergamotene <alpha-*trans*->	26.60	1434	0.48 ± 0.59
Acoradiene<alpha->	27.32	1466	0.23 ± 0.39
Unidentify A	28.53	-	4.49 ± 3.02
Sesquiphellandrene<beta->	30.08	1522	3.14 ± 1.73
Unidentify B	30.17	-	0.54 ± 0.63
Nerolidol<-*E*->	31.60	1563	0.78 ± 1.07
Caryophyllene oxide	32.34	1583	7.28 ± 2.32
Binapacry	33.36	-	16.74 ± 5.38

Table 24 The chemical constituents of the *E. stoechadosmum* volatile oil (Cont.)

compound name	RT	KI[a]	Area%[b]
Caryophylla-4(12),8(13)-dien-5-beta-ol	34.33	1640	5.14 ± 2.14
Guaiene	34.75	-	0.54 ± 0.75
Caryophyllene<14-hydroxy-(*Z*)->	35.11	1667	5.58 ± 2.69
Caryophyllene<14-hydroxy-9-*epi*-€->	35.59	1668	6.77 ± 2.84
Unidentify C	36.24	-	1.22 ± 1.55
Unidentify D	36.78	-	0.34 ± 0.47
Curcumen-15-al<ar->	37.07	1713	0.24 ± 0.40
Khusimol	37.43	1742	0.22 ± 0.51
Unidentify E	38.49	-	1.50 ± 1.54
Unidentify F	38.99	-	1.19 ± 0.98
Unidentify G	39.48	-	0.43 ± 0.53
Unidentify H	40.56	-	1.60 ± 1.33
Unidentify I	41.57	-	0.73 ± 1.60
Cuparenal	44.35	1753	9.06 ± 9.74
Phyto acetate<*E*->	49.96	2218	0.59 ± 0.69

[a] The parameters were shown as mean ± SD. Sample were from 15 different sources throughout Thailand. [b] Kovats index: Retention indices determined relative to *n*-alkanes (C_6-C_{24}) on ZP-5 GC column

The main component was binapacry (16.74±5.38 % peak area)

Table 25 Similarity indices (Jaccards coefficient of *E. stoechadosmum* from 15 sources)

	SPH 01	SPH 02	SPH 03	SPH 04	SPH 05	SPH 06	SPH 07	SPH 08	SPH 09	SPH 10	SPH 11	SPH 12	SPH 13	SPH 14	SPH 15
SPH 01	1.00														
SPH 02	0.82	1.00													
rSPH03	0.78	0.79	1.00												
SPH 04	0.60	0.62	0.59	1.00											
SPH 05	0.68	0.77	0.74	0.63	1.00										
SPH 06	0.50	0.52	0.45	0.71	0.52	1.00									
SPH 07	0.48	0.50	0.48	0.68	0.50	0.71	1.00								
SPH 08	0.79	0.80	0.84	0.67	0.68	0.52	0.50	1.00							
SPH 09	0.67	0.75	0.79	0.50	0.63	0.46	0.50	0.67	1.00						
SPH 10	0.65	0.67	0.59	0.67	0.56	0.69	0.73	0.66	0.55	1.00					
SPH 11	0.63	0.70	0.81	0.59	0.65	0.50	0.53	0.75	0.77	0.63	1.00				
SPH 12	0.40	0.48	0.41	0.54	0.42	0.50	0.61	0.48	0.42	0.54	0.46	1.00			
SPH 13	0.71	0.73	0.77	0.52	0.67	0.37	0.41	0.64	0.73	0.46	0.68	0.43	1.00		
SPH 14	0.64	0.72	0.76	0.54	0.74	0.45	0.48	0.77	0.79	0.53	0.81	0.41	0.70	1.00	
SPH 15	0.57	0.58	0.50	0.65	0.46	0.61	0.52	0.52	0.52	0.58	0.45	0.57	0.55	0.39	1.00

The dendrogram (Figure 21) showed the division of *E. stoechadosmum* into two clusters. Cluster I consisted of sample from Bangkok 3, Ubon Ratchathani, Bangkok 1, Nakorn sawan, Nahon Si thammarat, Surat thani, Nongkhai, Kalasin and Lopburi. Cluster II consisted of sample from Ratchaburi, Uthaithani, Bangkok 2, Nakhon Pathom, Chiangmai, Rayong.

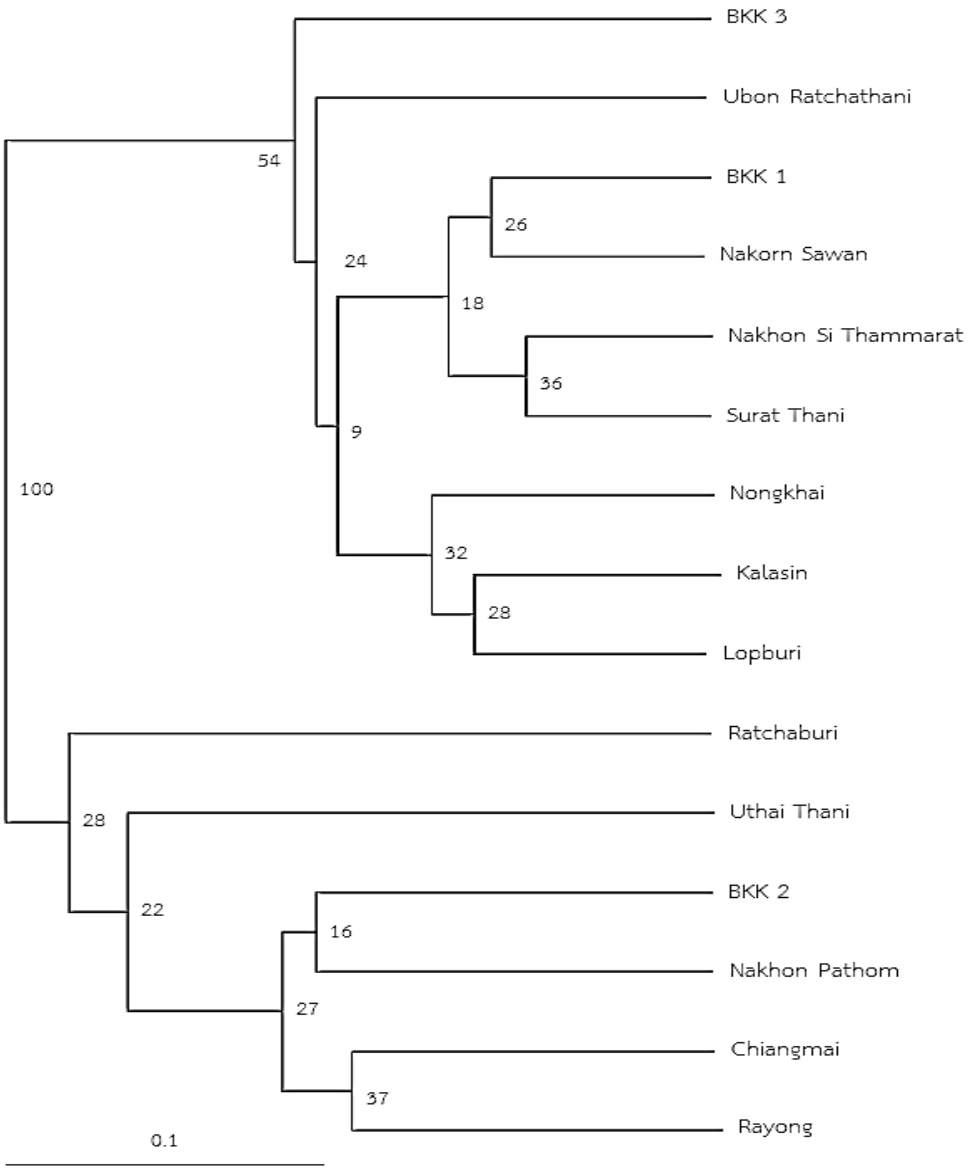


Figure 21 Dendrogram based on volatile oil compositions of *E. stoechadosmum*

GC fingerprint of *E. stoechadosmum* oil

GC chromatogram by MS detector of Lopburi and Nakhon Pathom provinces were shown in Figure 21.

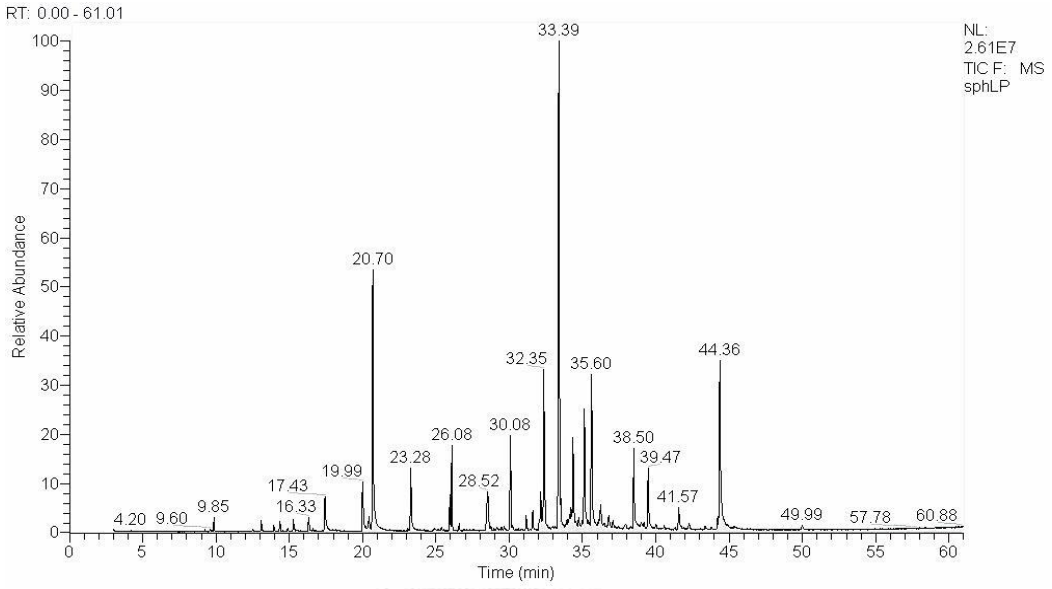


Figure 22 GC fingerprint of *E. stoechadosmum* dried whole plant oil from Lopburi province (cluster I)

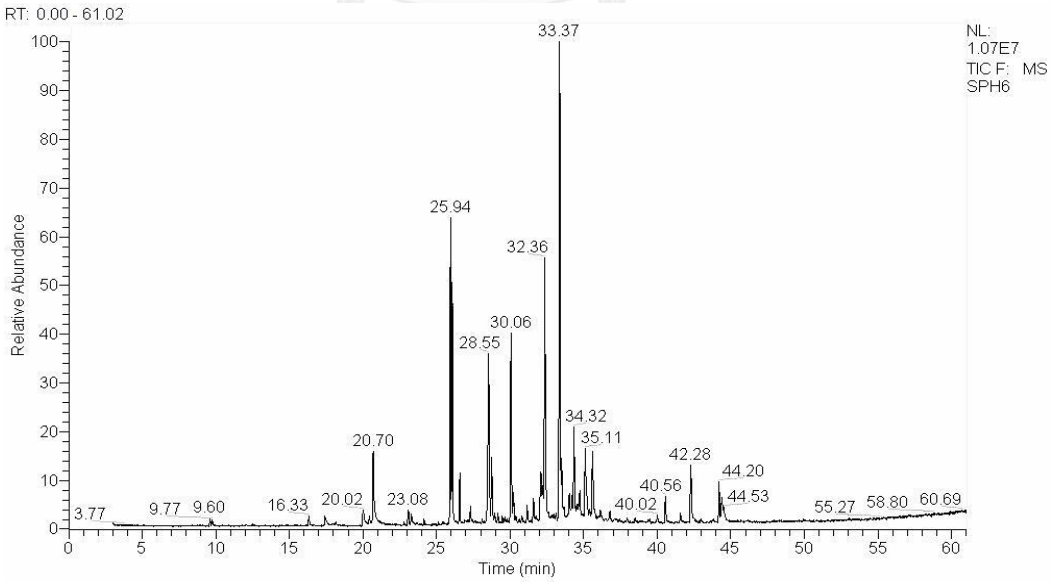


Figure 23 GC fingerprint of *E. stoechadosmum* dried whole plant oil from Nakhon Pathom province (cluster II)

CHAPTER V
DISCUSSION AND CONCLUSION

Alyxia reinwardtii Blume is commonly used in traditional medicine for treatment of fever. It is a component of “Yar Horm Teph Pa Jit” and “Yar Horm Nao Wa Goad”, traditional Thai medicine from the National lists of Herbal Medicine Product A.D. 2006. *Eupatorium stoechadosmum* Hance whole plants have been used for incense and diuretic. *E. stoechadosmum* is also an ingredient of “Yar Keaw Horm”, traditional Thai medicine recipe. The majority of the information on the quantity and quality of herbal medicine can be procured from its macroscopic, microscopic examination, physicochemical specification and TLC fingerprint [64]. Additionally, TLC fingerprint construction has become an important quality control tool for herbal materials. Macroscopic and microscopic investigations are the cheapest and simplest method to provide referential information for the medicinal plant authentication. The physicochemical evaluation of herbal medicine is applied to control the extraction process or to study the quality of plant material and a finished product [65]. The ash examination is useful to determine the purity and quality of powdered crude drug. From the result of pharmacognostic specifications, total ash values of *A. reinwardtii* inner bark and *E. stoechadosmum* dried whole plants were found to be high. Acid-insoluble ash values were low in all samples. An abundantly total value indicates the medicinal plant material contains more of inorganic components such as calcium oxalate crystals, phosphorus, alumina and magnesium. If the plant materials contain a high number of calcium oxalate crystals, the amount of substance remaining after acid treatment should be quite less [66]. The results suggested that the inorganic matter is less in these two plant materials. Extractive values are beneficial to assess the chemical components which present in plant materials and to help in estimation of specific component or group of specific soluble components in particular solvent. Water-

soluble extractive and ethanol-soluble extractive showed high content of polar compounds. If the extractive matter from specified solvent is fewer than this current research study, it indicated inferiority of crude drug. Loss on drying value is used to determine the content of water content and volatile matters in crude drug [10]. Water content plays an important role in stability of plant materials. It should be minimized for protection from chemical degradation as well as microbial contamination and enzymatic activity enhancer [58]. This study proposed the first report of pharmaognostic specifications of *A. reinwardtii* inner bark and *E. stoechadosmum* whole plants in Thailand. The pharmacognostic examinations in this study can be useful for authentication and quality control of these crude drugs.

The coumarin content in medicinal plant was analyzed by thin layer chromatographic technique. It is a proper chromatographic technique for separation and quantification of chemical compositions in the samples. The suitable mobile phase and solvent extraction are important factor for separation of target component. The process optimization such as suitable of mobile phase, concentration of sample was performed following the literature reviews and the chemical properties of the substance.

TLC- densitometry is widely used for quantitative analysis of important substance in herbal medicines. It is easy to perform with automated instruments. However, all of equipment is rather expensive that some laboratories cannot afford. The coumarin contents in *A. reinwardtii* inner bark and *E. stoechadosmum* whole plant from different sources were evaluated by combination of TLC with TLC scanner and TLC image analysis using ImageJ software. The results from both methods were compared using paired *t*-test.

Quantitative analysis using TLC- densitometry and TLC image analysis were validated in terms of accuracy, precision, specificity, calibration range, LOD, LOQ and

robustness which were used to confirm the analytical procedure employed reliable and correct information.

The accuracy was performed by recovery of spiking known three concentrations of standard coumarin in sample matrix. In case of *A. reinwardtii* study, the recovery values of TLC-densitometry were in the range of 92.15 - 97.35% and TLC image analyzes were in the range of 96.78-107.31%.

In *E. stoechadosmum* study, the recovery values of TLC-densitometry were in the range of 90.40 – 95.16% and TLC image analysis were in the range of 89.46 -91.75%. The results from this study exhibited acceptable limits of both methods. The repeatability (intra-day precision) and the intermediate precision (inter-day precision) were conducted as %RSD in all cases [67].

In this study, the specificity of the TLC procedure was performed by peak identity checking which was examined by matching UV absorbance spectra of coumarin standard with coumarin in each sample. The results expressed the identical absorption spectra and showed maximum absorbance at 285 nm. Therefore, this is optimal wavelength for scanning developed TLC plates in this study that accurately quantified coumarin. The calibration curves of both methods were polynomial relationships with good correlation coefficients = 0.99 in concentrations ranges of 0.075 - 1.2 mg/ml in *A. reinwardtii* study and 0.075 - 0.9 mg/ml in case of *E. stoechadosmum* study. An analytical procedure is acceptable, if the correlation coefficient value obtained is 0.99 or better. The great result is acquired when the sample concentration is within the concentration range estimated [67]. The robustness should be examined during the analysis of TLC-densitometry and TLC image analysis. It should demonstrate the reliability of analysis with deliberate variation in the parameters of the method [63]. The robustness was estimated by analysis of results obtained after deliberate variation of mobile phase ratio. This study displayed that there were no differences in the peak

area of coumarin in sample matrix. These results demonstrated that the combination of TLC with densitometer and image analysis method proved to be robust for coumarin analyzed, under the condition evaluates.

The chemical marker as coumarin was chosen for quantitative analysis of *A. reinwardtii* inner bark. The coumarin content of *A. reinwardtii* was analyzed by TLC-densitometry and TLC image analysis which were respectively found to be 0.769 ± 0.045 % dry weight and 0.754 ± 0.013 % dry weight. According to previous study, the dried stems (4.8 kg) of *A. reinwardtii* were macerated with dichloromethane and evaporated to dryness. The crude extract was separated by vacuum liquid chromatography (VLC) over silica gel (Merck Art 7730) using ethyl acetate - dichloromethane (4:6 to 6:4) as mobile phase. From VLC fraction yielded 2.15 g of coumarin which was 0.045% by weight [16]. Various parts of the plant and altered extractive methods might reveal different coumarin contents. The coumarin content of *E. stoechadosmum* dried whole plants was analyzed by TLC-densitometry and TLC image analysis which were respectively found to be 0.442 ± 0.002 % dry weight and 0.454 ± 0.038 % dry weight.

The coumarin content of *A. reinwardtii* inner bark from 15 different sources throughout Thailand that obtained from TLC-densitometry and TLC image analysis were not significantly different ($p>0.05$) by paired *t*-test statistical analysis. The coumarin content of *E. stoechadosmum* dried whole plants from 15 different sources throughout Thailand that obtained from TLC-densitometry and TLC image analysis were compared using paired *t*-test statistical analysis. The results indicated that coumarin contents from both methods were not significantly different ($p>0.05$). From these results, it indicated that TLC image analysis is reliable and efficient technique for quantitative analysis of coumarin in these two plant materials. Furthermore, this study proposed that combination of TLC with image analysis can be used as alternative

method for any laboratory because it is easy, fast, and low price [68]. Image analysis is an analytical data from image that is applied in many areas such as medical engineering, astronomy and metallography. ImageJ is one method of image analysis software that has enabled simple and effective use of TLC as a quantitative analysis.

Gas chromatography-mass spectrometry is common implement for the essential oil analysis [69, 70]. Approximately 0.14 percent yields of essential oil were obtained from *E. stoechadosmum* dried whole plants. The oil is a yellowish liquid with mixed green and sweet odor. At least 30 essential compositions were identified in *E. stoechadosmum* oil. The major constituents were binapacry (16.74±5.38 % peak area), cuparenal (9.06±9.74 % peak area), carvacrol (8.21±6.97 % peak area), caryophyllene oxide (7.28±2.32 % peak area) and caryophyllene (6.77±2.84 % peak area). This result was rather similar to the previous studies [31, 32, 34]. The essential oils were obtained from stream distillation that was subjected to GC-MS analysis for compound identification. It was found that most of them were terpenic compounds [30]. In the previous study, *E. stoechadosmum* fresh leaf (300 g) from one source was distilled using wet-steam distillation method that yielded 0.12 percent of essential oil [31]. The result showed that thymol methyl ether and thymohydroquinone dimethyl ether were identified as predominant compounds in *E. stoechadosmum* [31]. Thymol is a natural monoterpene phenol derivative of cymene, isometric with carvacrol [71]. In this study, all of these compounds were also found. A dendrogram obtained from the cluster analysis based on essential oil composition of *E. stoechadosmum* is shown in Figure 22. The dendrogram showed that the accessions which belong to the same species from different location were grouped together. The dendrogram showed the division of *E. stoechadosmum* into two clusters. Cluster I consisted of sample from Bangkok 3, Ubon Ratchathani, Bangkok 1, Nakorn sawan, Nahon Si thammarat, Surat thani, Nongkhai, Kalasin and Lopburi. This cluster was characterized by the presence of

carvacrol methy ether. Unidentify E (RT: 38.49) was found in all member of this cluster but not common in another cluster. Cluster II consisted of sample from Ratchaburi, Uthaithani, Bangkok 2, Nakhon Pathom, Chiangmai, Rayong. This cluster was characterized by the presence of bergamotene, acoradiene and guaiene.

In conclusion, this research provides pharmacognostic specifications and coumarin contents of *A. reinwardtii* inner bark and *E. stoechadosmum* whole plants that could be set for basis quality control of plant materials in Thai herbal pharmacopeia. This study also demonstrates the chemical constituents of *E. stoechadosmum* whole plant oils in Thailand.

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APPENDICES

APPENDIX A

Data of Physicochemical parameters of *A. reinwardtii* inner bark

Table 26 Details of the plant samples used in the study

Voucher no	Locality
1	Nakhon Pathom
2	Chiang Mai
3	Surat Thani
4	Rayong
5	Bangkok 1
6	Bangkok 2
7	Ratchaburi
8	Ubon Ratchathani
9	Nongkhai
10	Nakhon Si Thammarat
11	Lop Buri
12	Nakhon Sawan
13	Uthai Thani
14	Bangkok 3
15	Bangkok 4

Table 27 Physicochemical parameters of *A. reinwardtii* inner bark from 15 sources (% by dried weight)

sources	Loss on drying	Total ash content	Acid insoluble ash content	Ethanol extractive value	Water extractive value	Water content
1	8.14 ± 0.23	8.18 ± 0.05	1.73 ± 0.17	9.07 ± 0.02	17.42 ± 0.01	10.87 ± 0.23
2	8.00 ± 0.02	7.21 ± 0.08	0.92 ± 0.05	10.43 ± 0.09	17.35 ± 0.09	11.00 ± 0.00
3	8.43 ± 0.21	7.79 ± 0.03	1.38 ± 0.08	9.88 ± 0.20	17.92 ± 0.06	13.00 ± 0.00
4	7.81 ± 0.17	7.78 ± 0.12	1.46 ± 0.19	8.89 ± 0.46	16.67 ± 0.14	13.00 ± 0.00
5	7.54 ± 0.10	2.48 ± 0.31	0.48 ± 0.05	5.91 ± 0.26	10.39 ± 0.23	10.33 ± 0.58
6	7.97 ± 0.21	8.55 ± 0.05	1.55 ± 0.12	4.88 ± 0.40	13.11 ± 0.06	11.00 ± 0.00
7	7.46 ± 0.09	7.54 ± 0.06	1.72 ± 0.12	9.24 ± 0.07	17.26 ± 0.09	11.13 ± 0.12
8	8.40 ± 0.10	7.48 ± 0.02	1.27 ± 0.09	10.11 ± 0.18	17.50 ± 0.11	11.00 ± 0.00
9	8.37 ± 0.20	7.71 ± 0.05	1.28 ± 0.12	6.99 ± 0.19	15.97 ± 0.04	11.00 ± 0.00
10	7.83 ± 0.05	8.44 ± 0.02	1.36 ± 0.12	9.95 ± 0.07	16.83 ± 0.16	12.00 ± 0.00
11	8.45 ± 0.22	7.49 ± 0.06	1.31 ± 0.05	8.95 ± 0.46	16.09 ± 0.15	12.00 ± 0.00
12	7.88 ± 0.15	8.69 ± 0.01	1.60 ± 0.12	9.30 ± 0.25	16.77 ± 0.07	10.33 ± 0.58
13	8.83 ± 0.07	8.67 ± 0.04	1.82 ± 0.06	9.94 ± 0.74	19.02 ± 0.07	14.00 ± 0.00
14	8.04 ± 0.10	8.38 ± 0.08	1.15 ± 0.11	11.31 ± 0.44	18.87 ± 0.06	14.00 ± 0.00
15	8.15 ± 0.11	8.29 ± 0.01	1.44 ± 0.12	8.98 ± 0.25	16.37 ± 0.08	14.00 ± 0.00

APPENDIX B

Data of quantitative analysis of coumarin content of *A. reinwardtii* inner bark

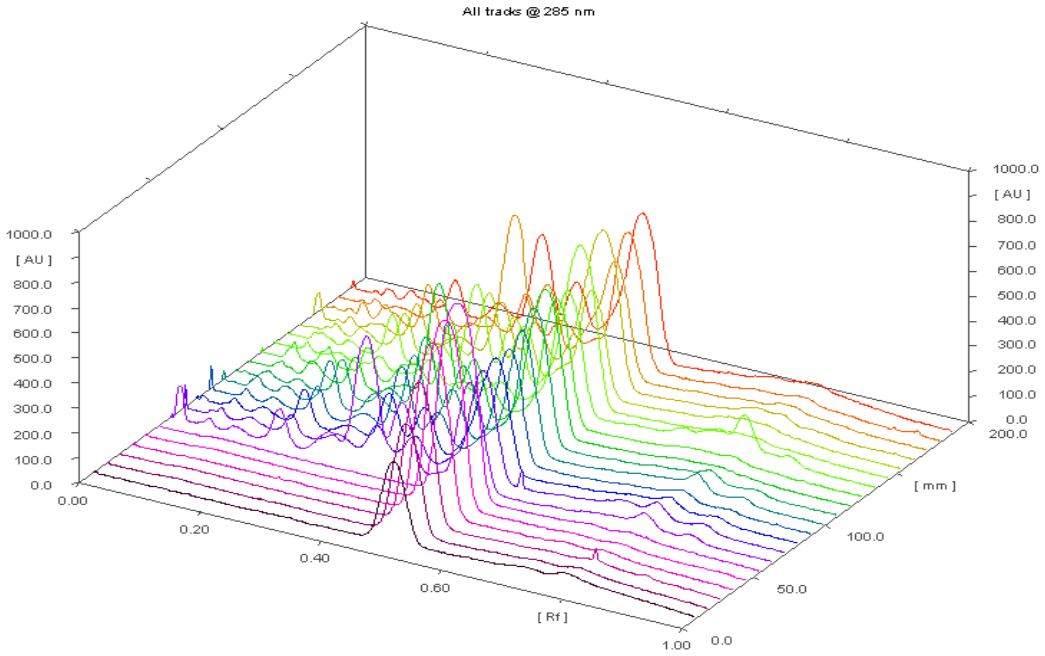


Figure 24 3D TLC- densitograms (Plate A-sample NO.1-15)

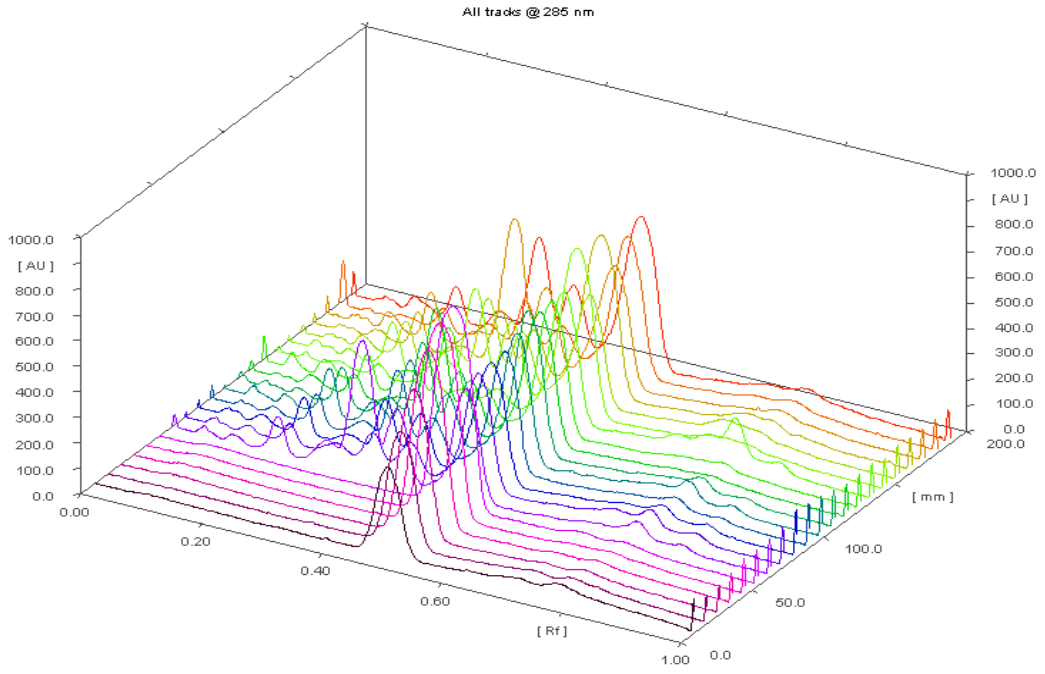


Figure 25 3D TLC- densitograms (Plate B-sample NO.1-15)

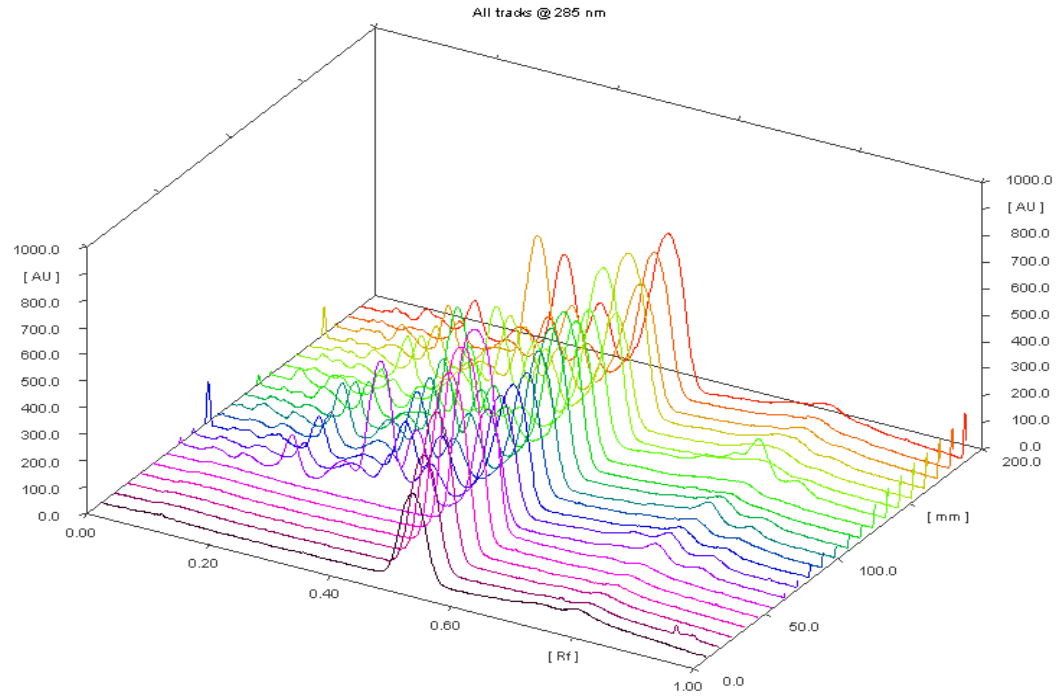


Figure 26 3D TLC- densitograms (Plate C-sample NO.1-15)

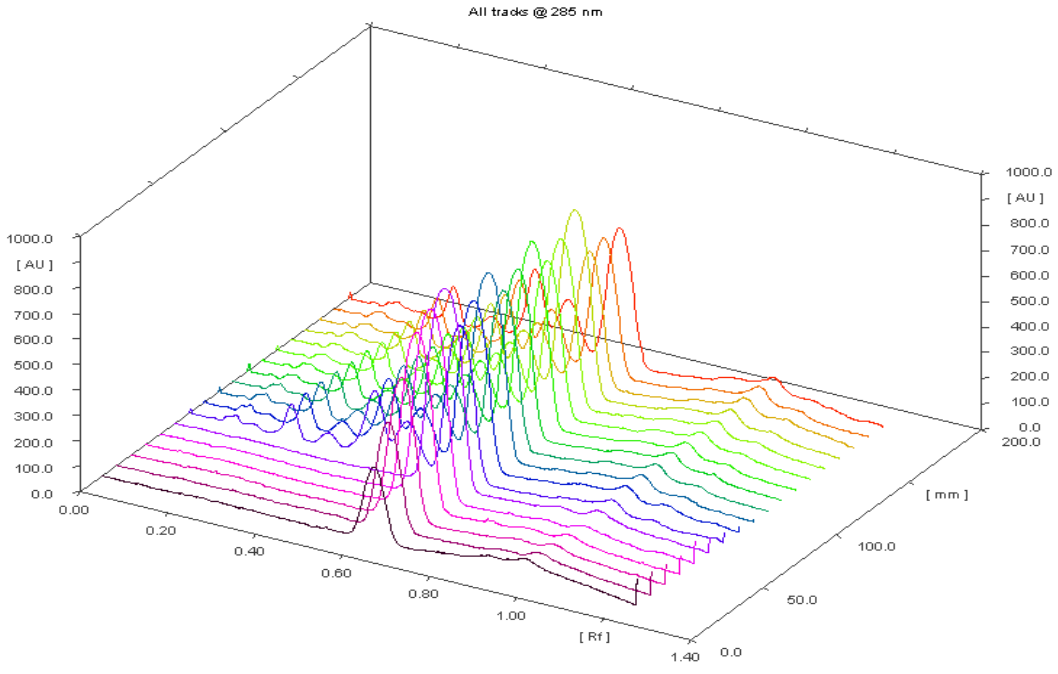


Figure 27 3D TLC- densitograms (Plate D-Accuracy)

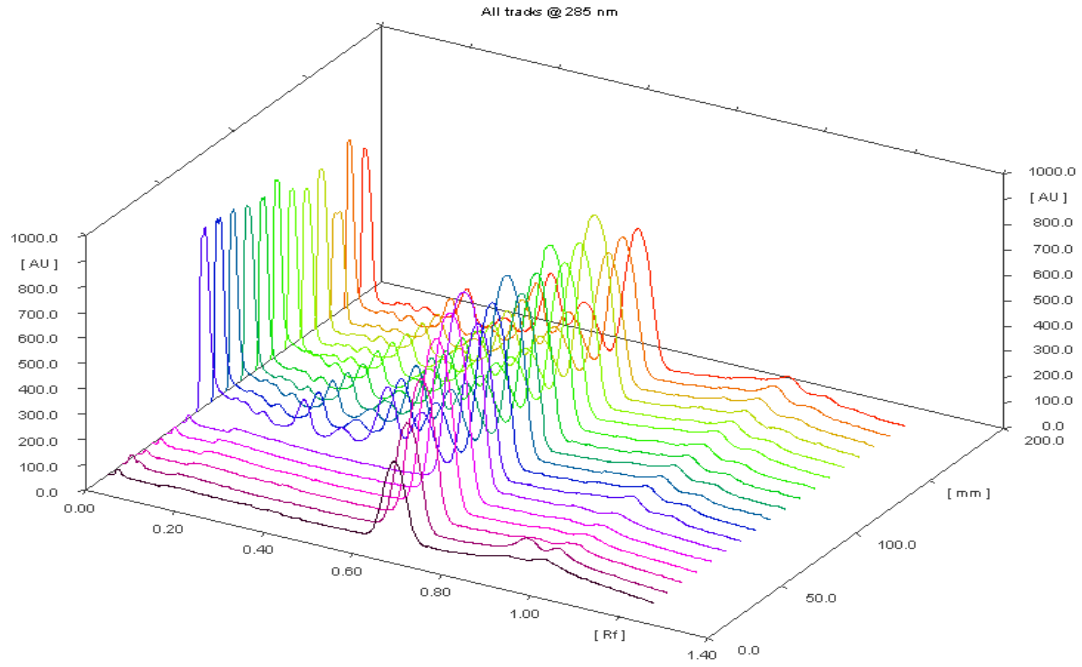


Figure 28 3D TLC- densitograms (Plate E-Precision NO.1)

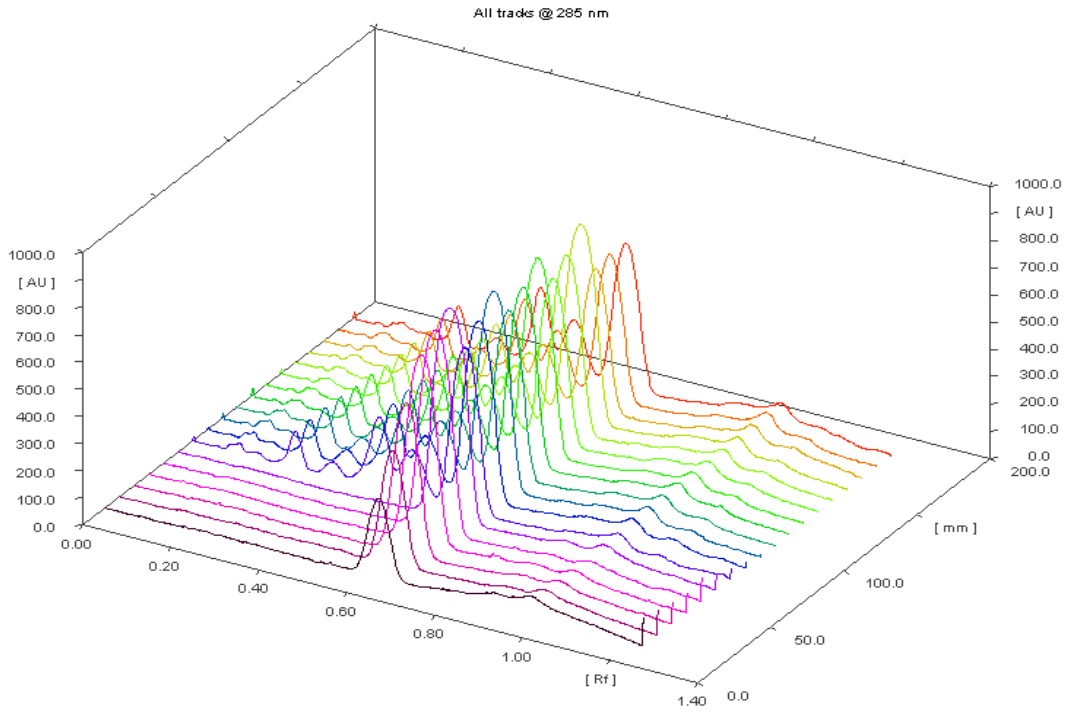


Figure 29 3D TLC- densitograms (Plate F-Precision NO. 2)

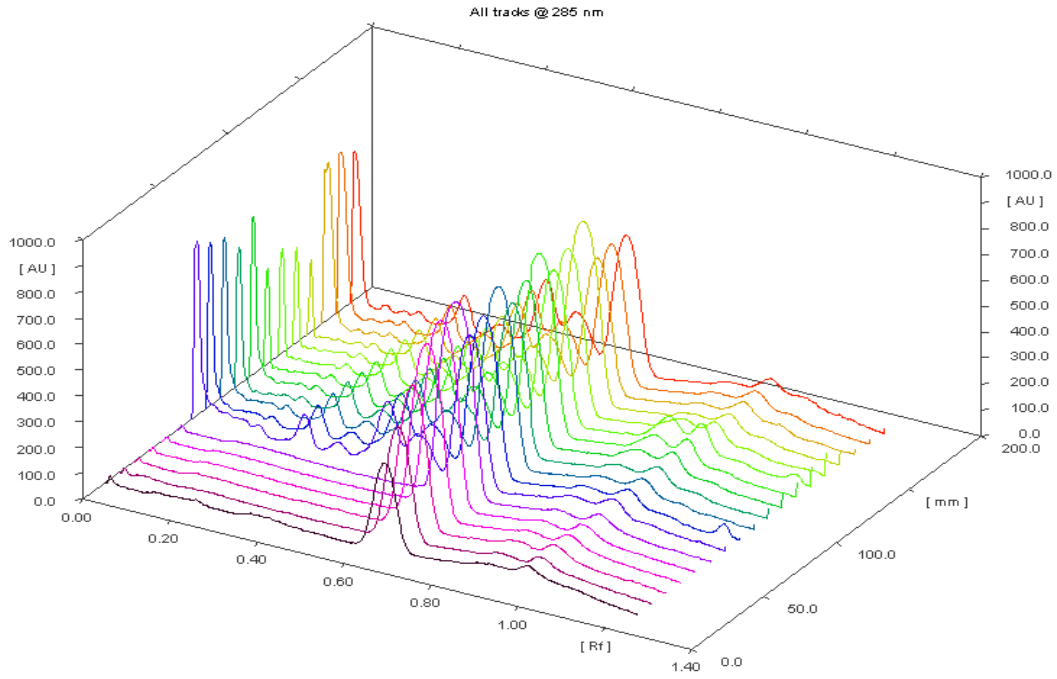
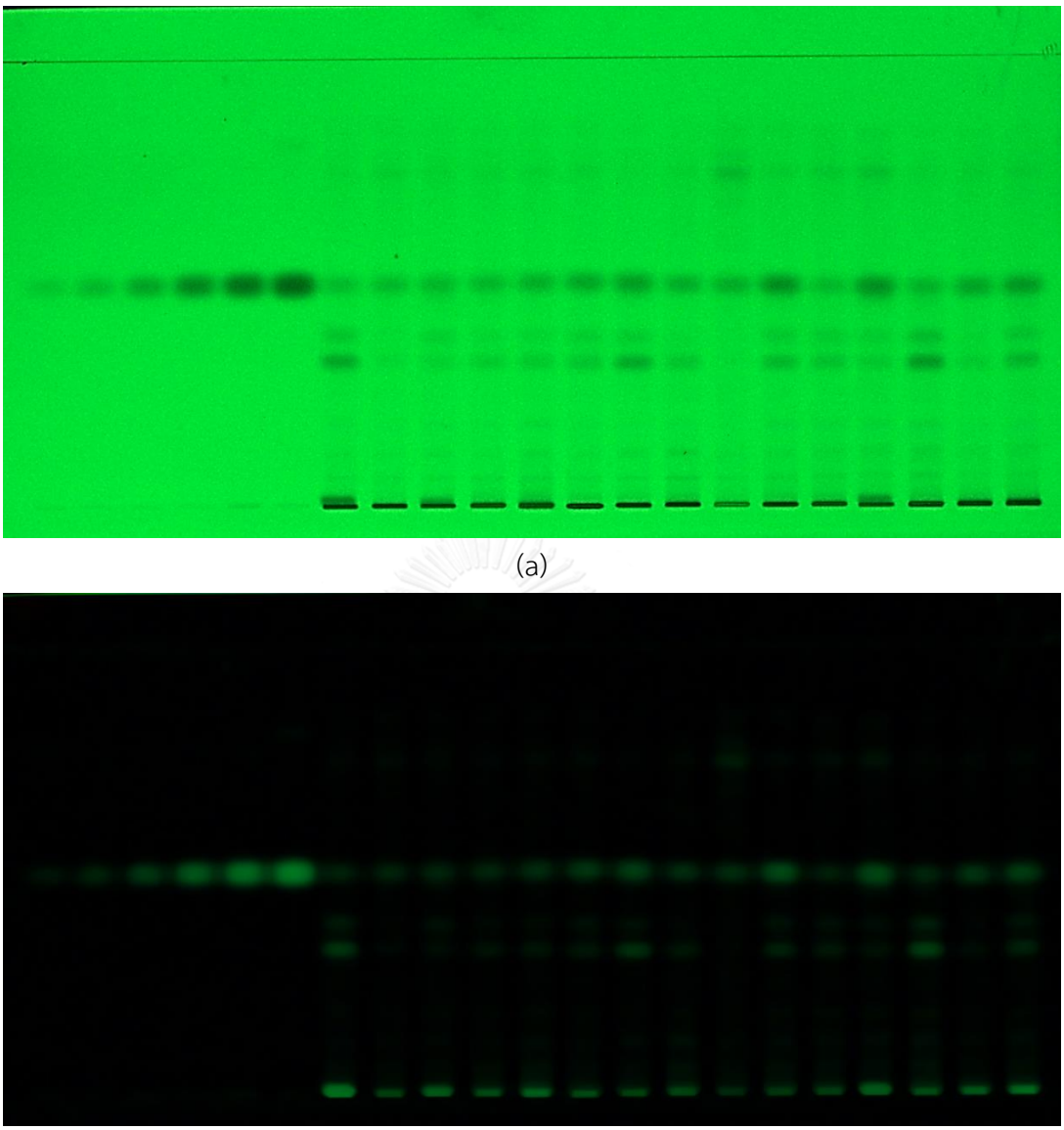


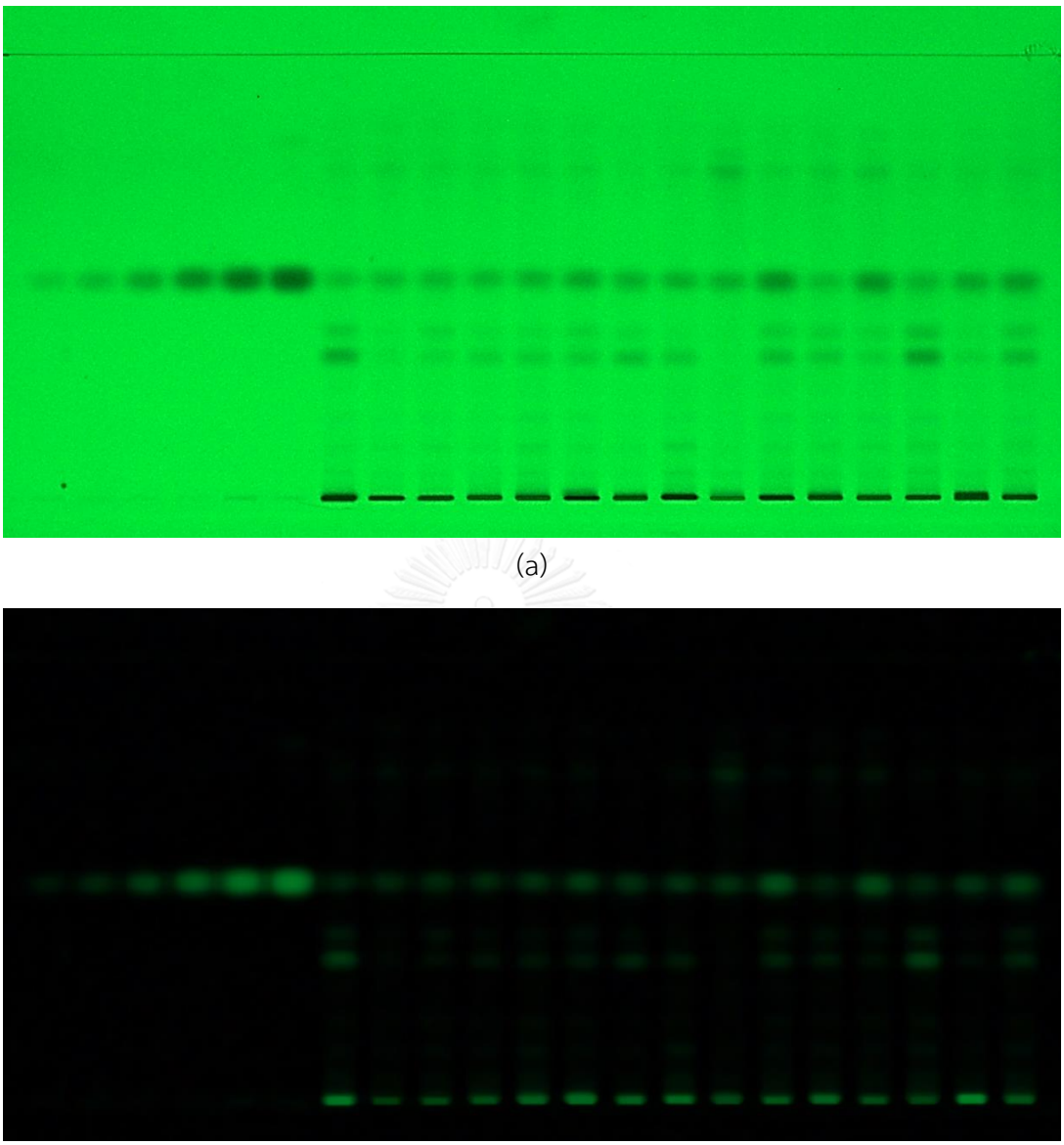
Figure 30 3D TLC- densitograms (Plate G-Precision NO. 3)



(a)

(b)

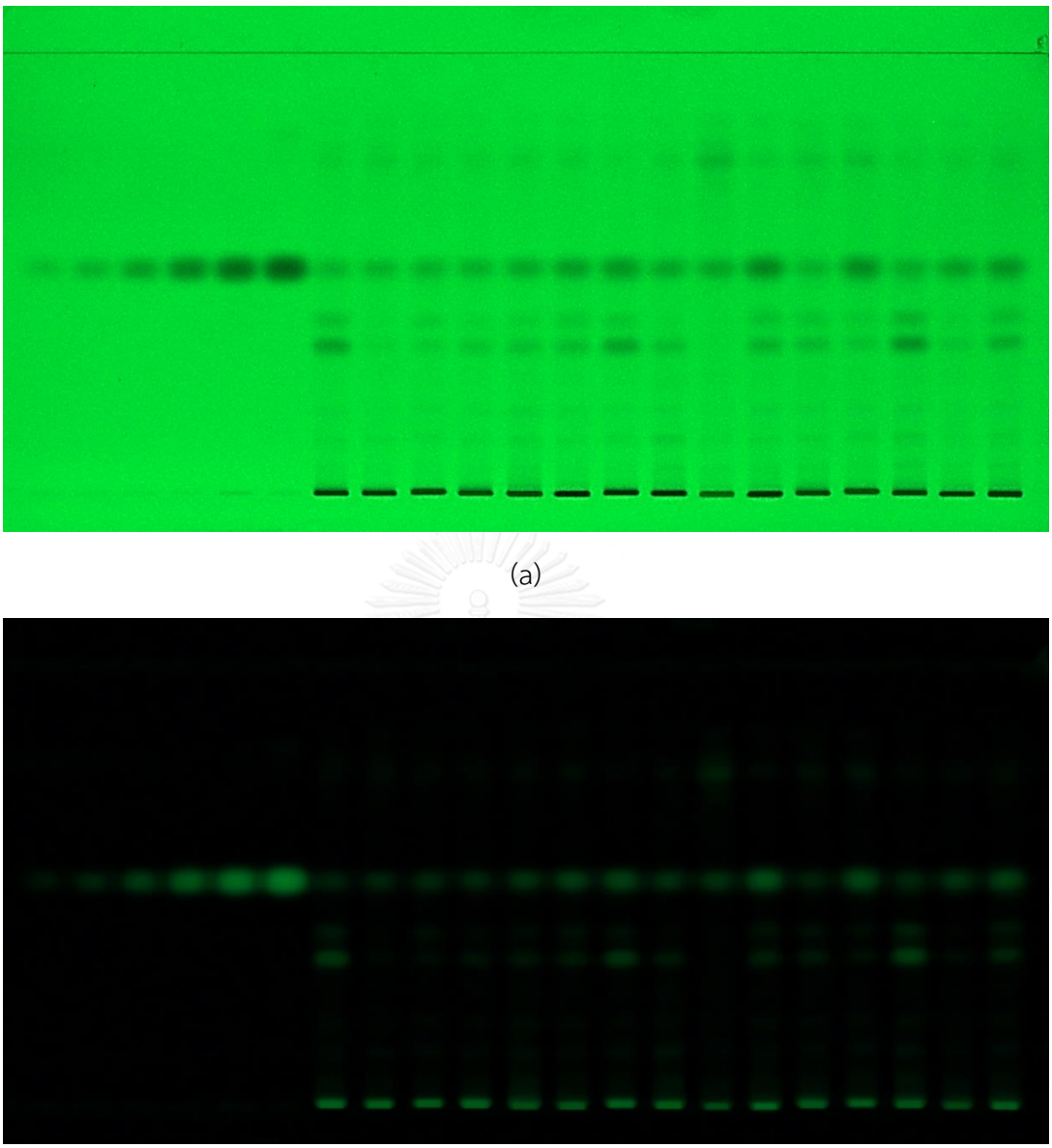
Figure 31 The TLC Plate (A-sample NO.1-15) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

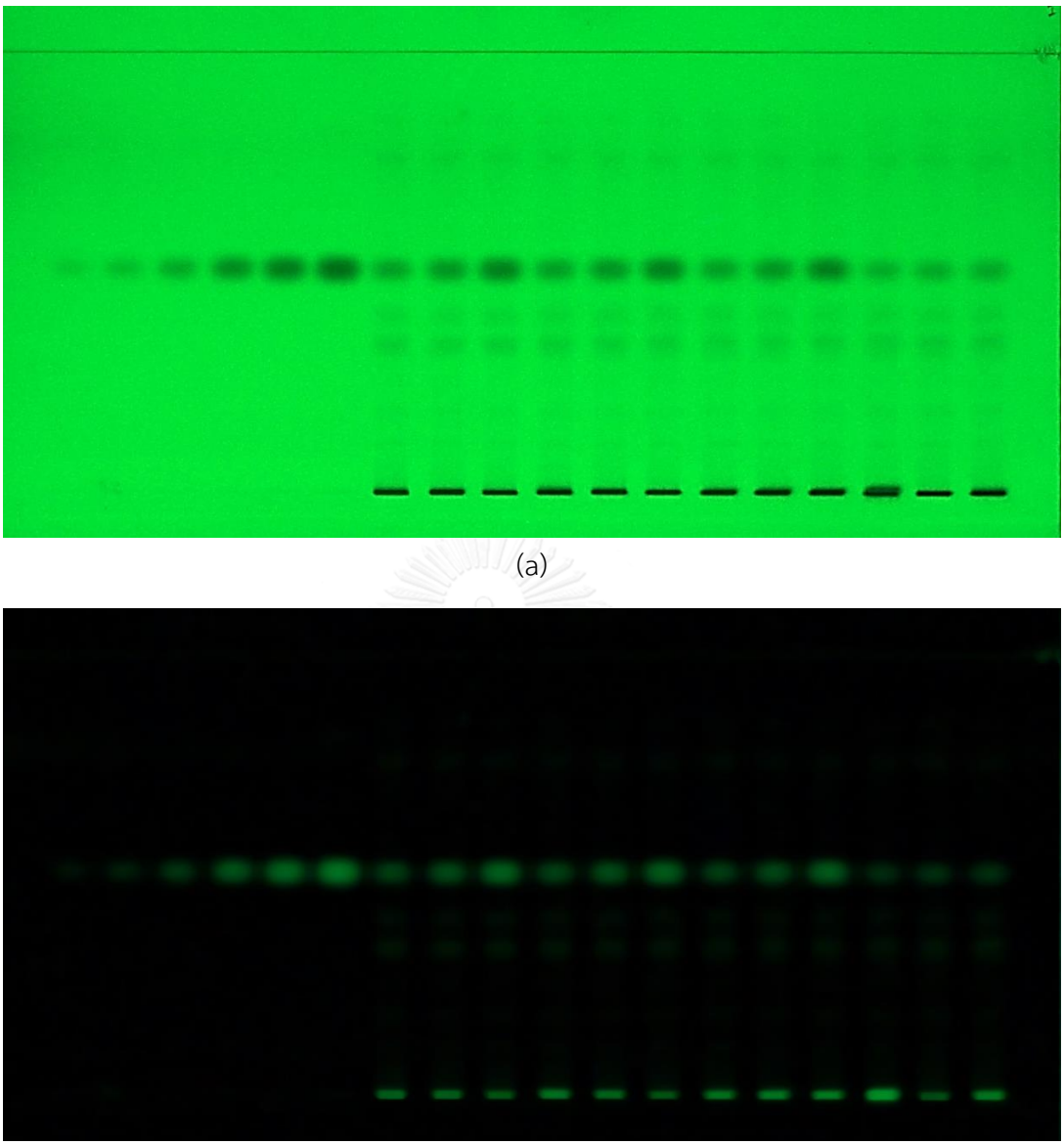
Figure 32 The TLC Plate (B-sample NO.1-15) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

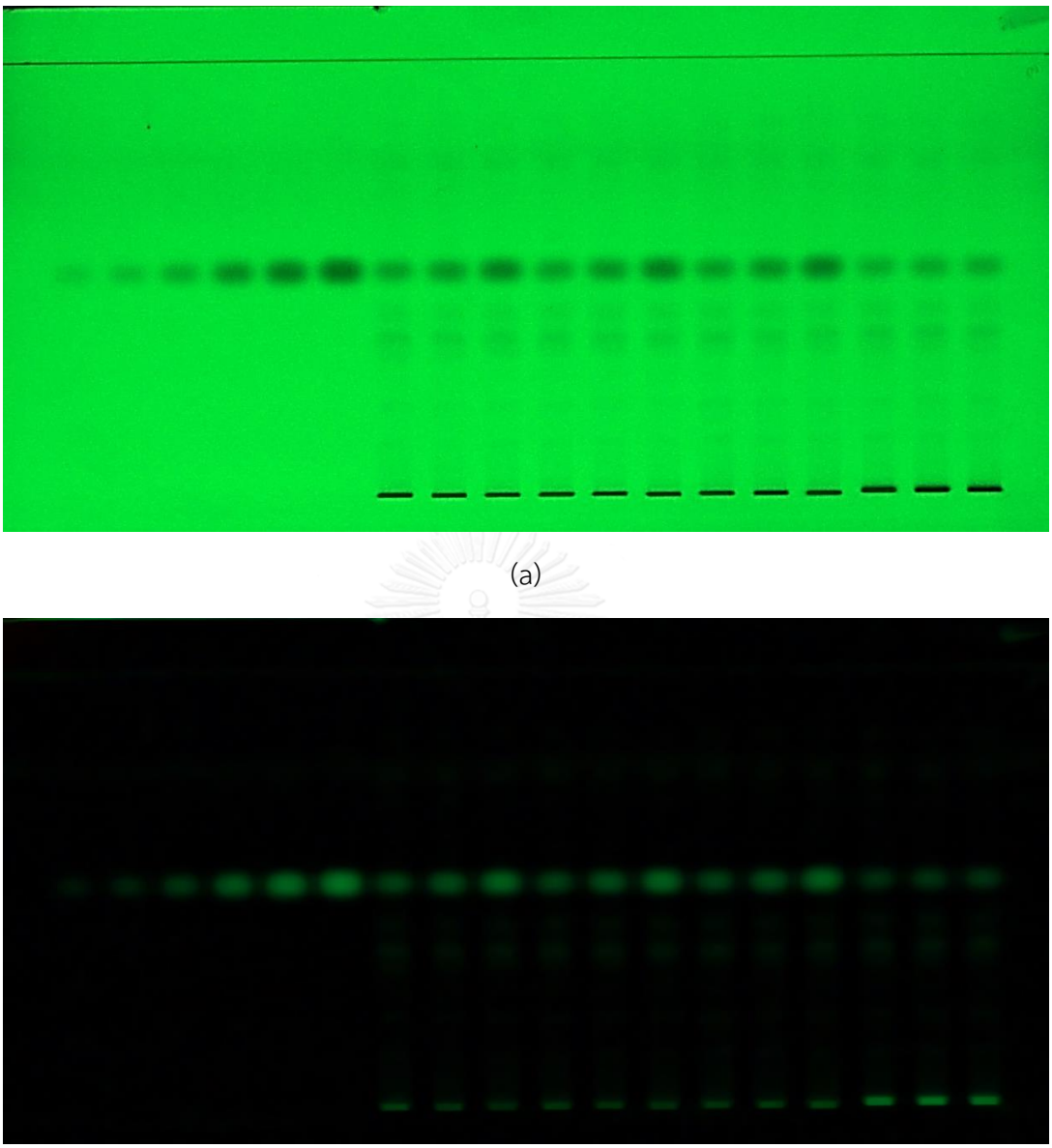
Figure 33 The TLC Plate (C-sample NO.1-15) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

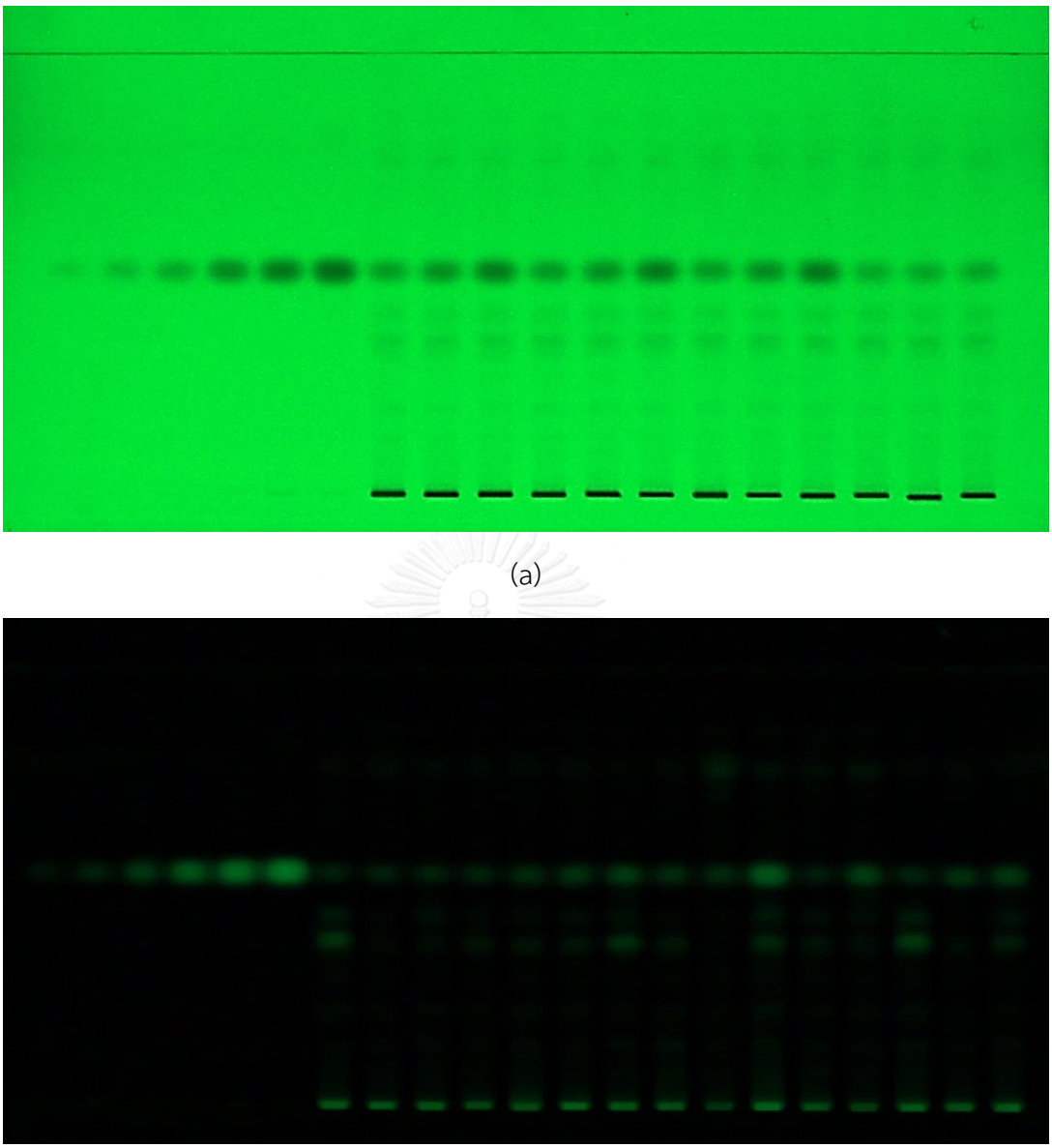
Figure 34 The TLC Plate (D-Accuracy) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

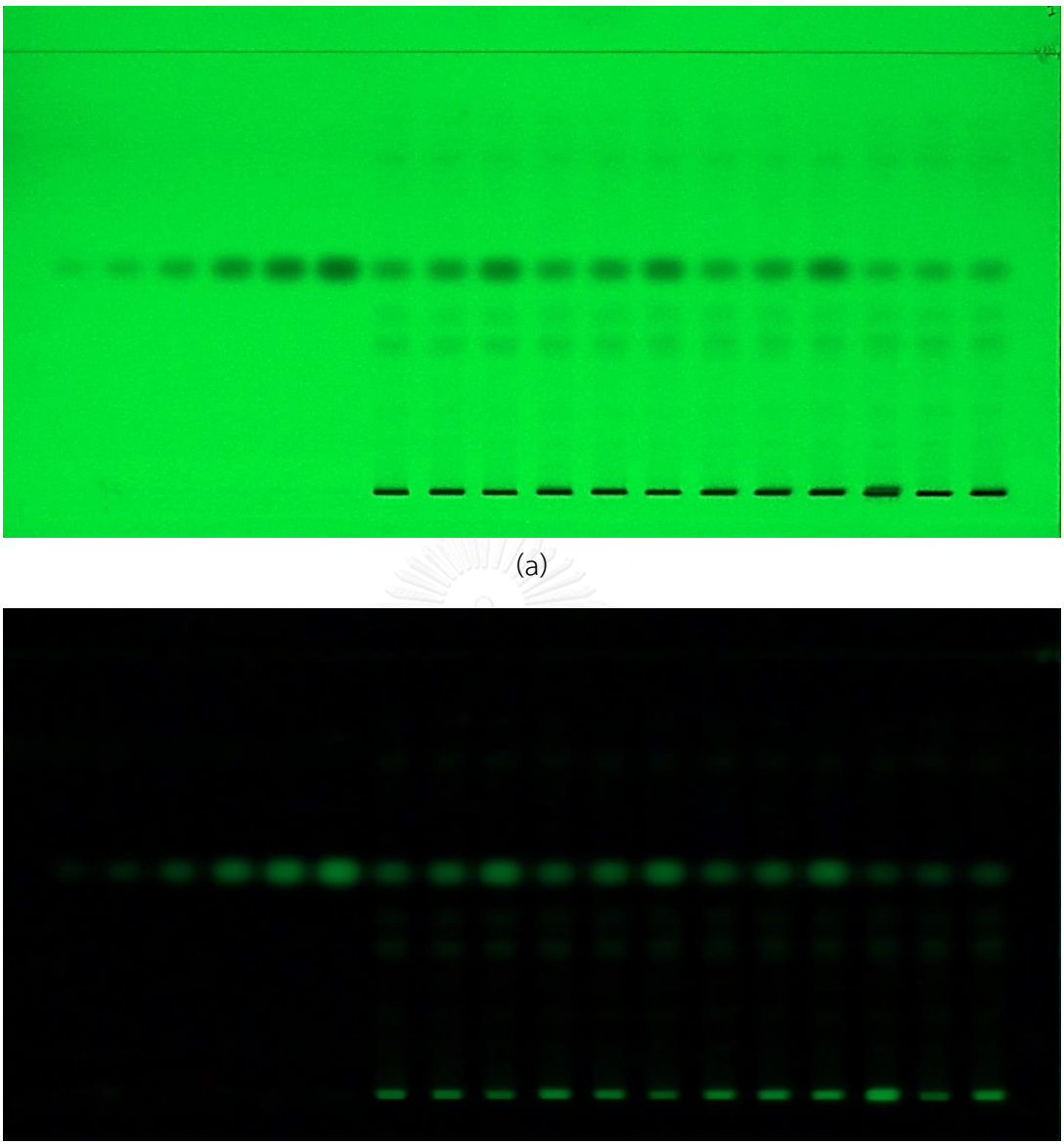
Figure 35 The TLC Plate (E-Precision) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

Figure 36 The TLC Plate (F-Precision) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

Figure 37 The TLC Plate (G-Precision) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)

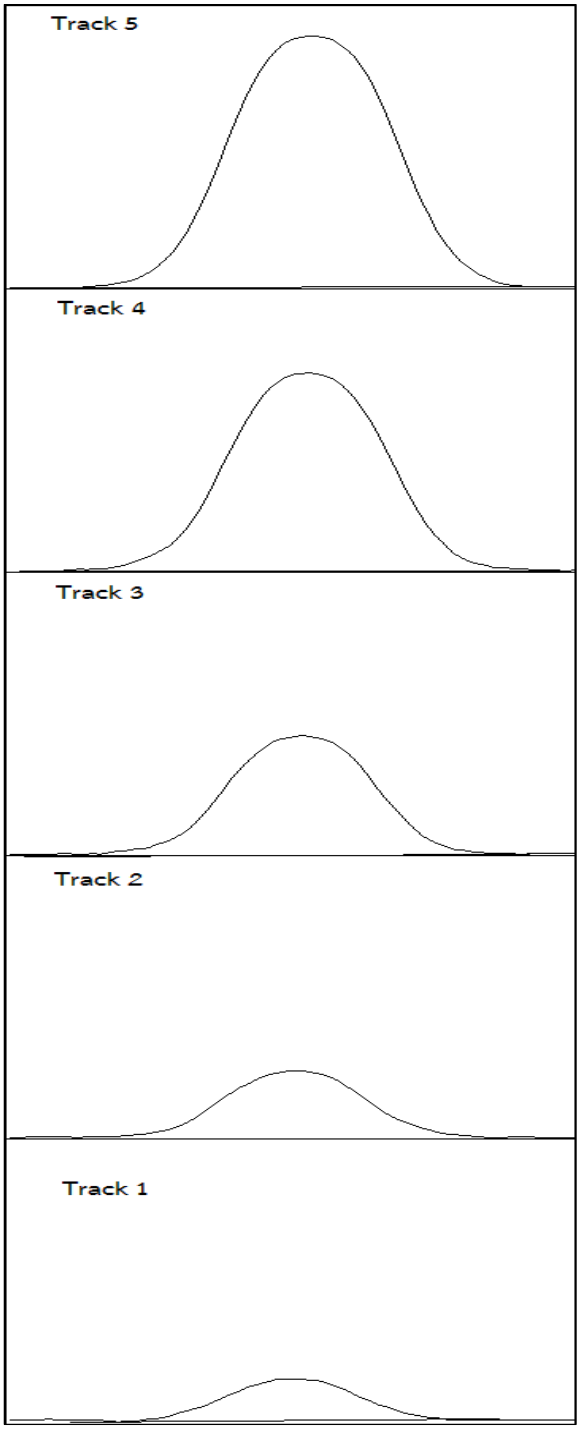


Figure 38 TLC chromatogram of TLC image analysis (ImageJ software); Track 1 was standard coumarin 0.075 mg/ml, Track 2 was standard coumarin 0.15 mg/ml, Track 3 was standard coumarin 0.3 mg/ml, Track 4 was standard coumarin 0.6 mg/ml, Track 5 was standard coumarin 0.9 mg/ ml

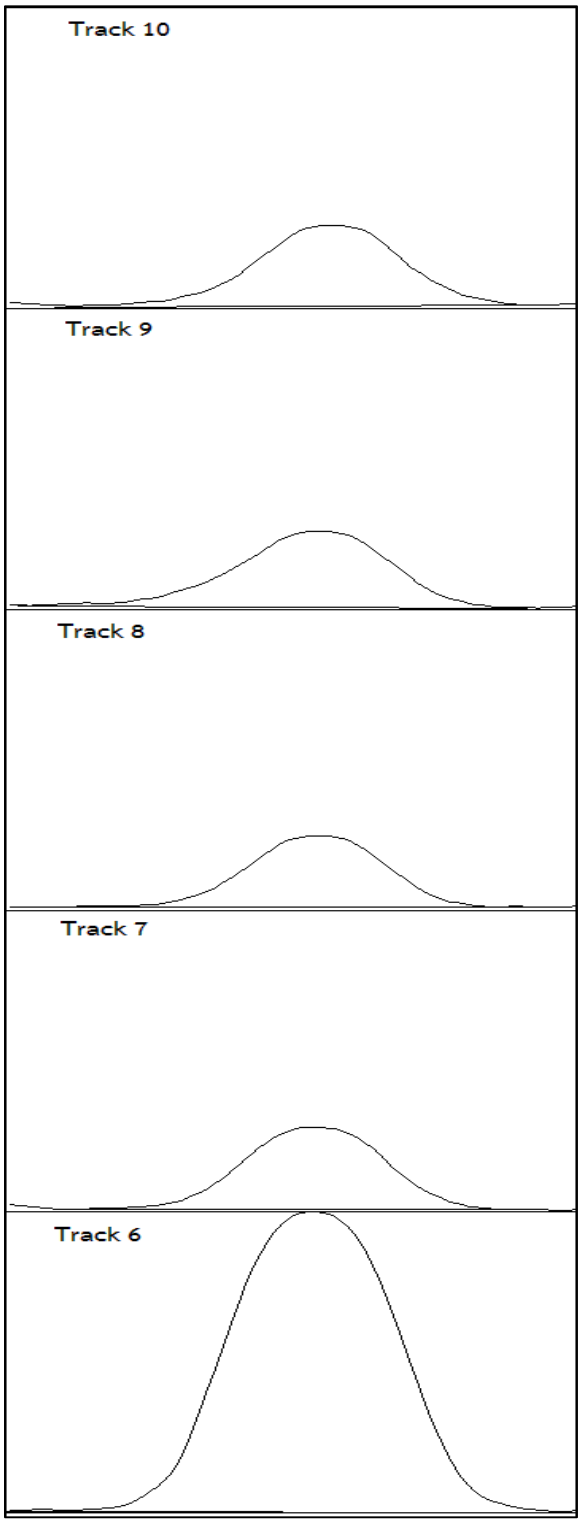


Figure 39 TLC chromatogram of TLC image analysis (Image software); Track 6 was standard coumarin 1.2 mg/ml, Track 7 was sample No.1 5 mg/ml, Track 8 was sample No.2 5 mg/ml, Track 9 was sample No. 3 mg/ml, Track 10 was sample No. 4 mg/ml

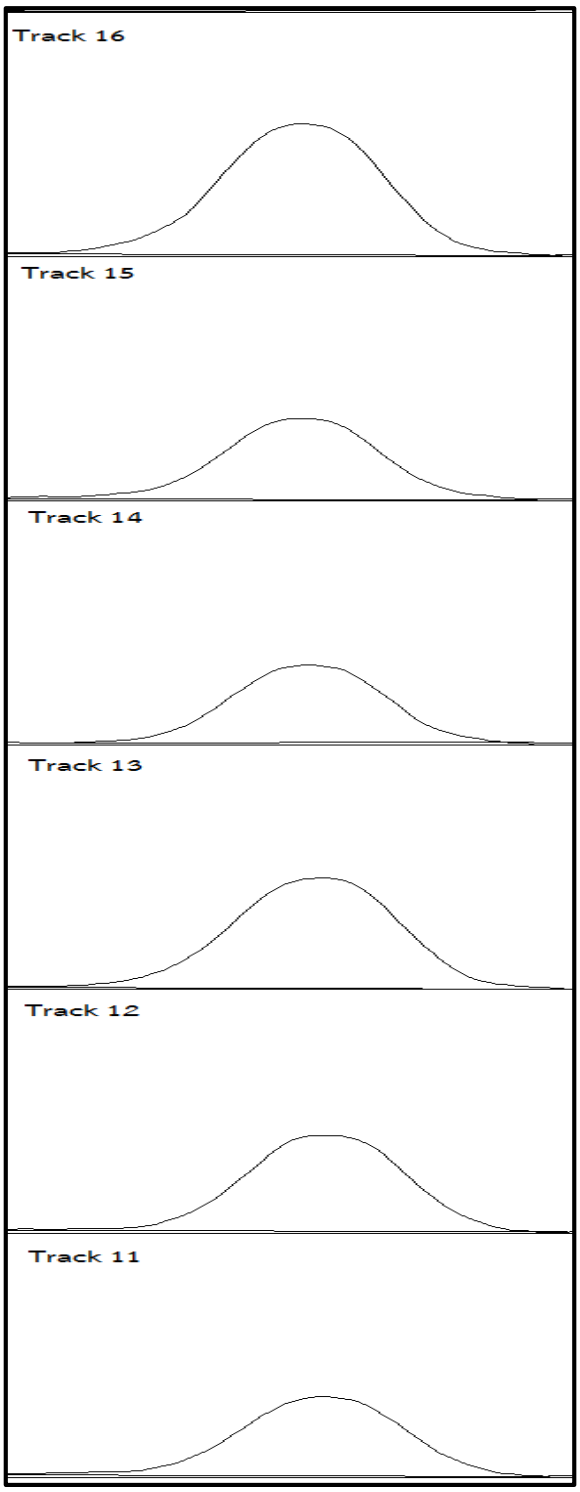


Figure 40 TLC chromatogram of image analysis (Image software); Track 11 was sample (No. 5) 5 mg/ml, Track 12 was sample (No.6) 5 mg/ml, Track 13 was sample (No.7) 5 mg/ml, Track 14 was sample (No. 8) 5 mg/ml, Track 15 was sample (No. 9) 5 mg/ml, Track 16 was sample (No. 10) 5 mg/ml

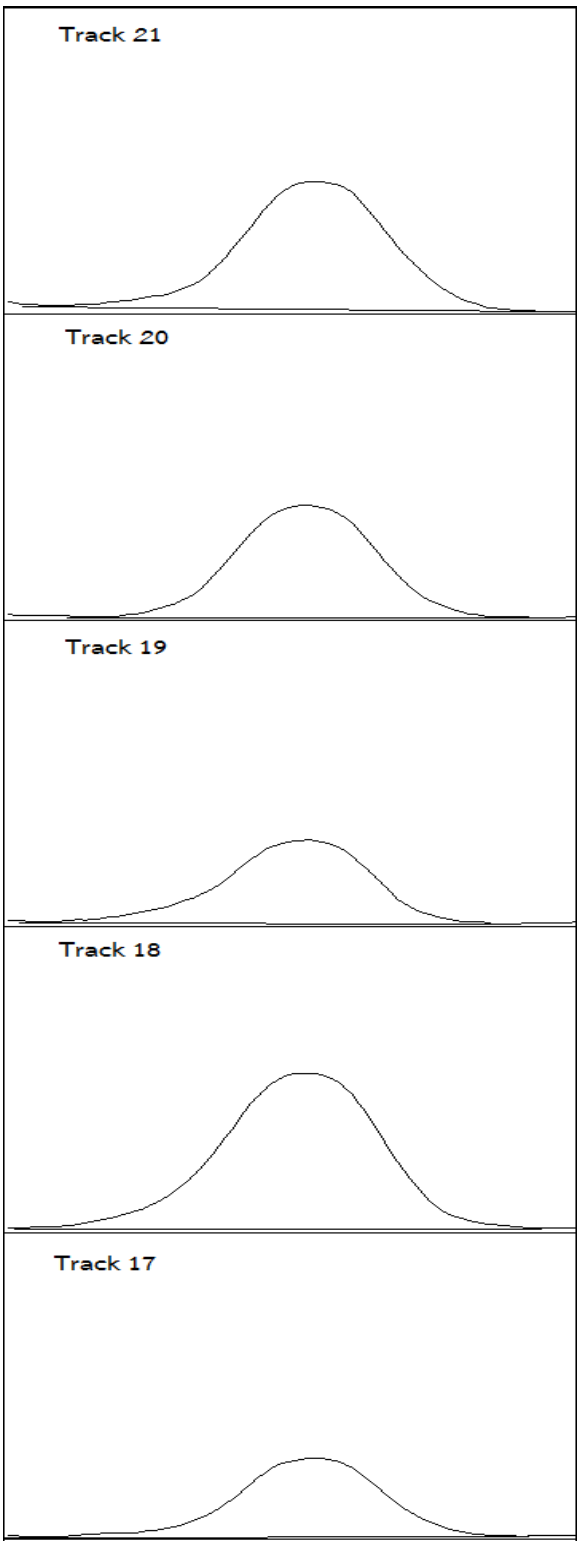


Figure 41 TLC chromatogram of TLC image analysis (Image software); Track 17 was sample (No. 11) 5 mg/ml, Track 18 was sample (No.12) 5 mg/ml, Track 19 was sample (No.13) 5 mg/ml, Track 20 was sample (No. 14) 5 mg/ml, Track 21 was sample (No. 15) 5 mg/ml

APPENDIX C

Data of Physicochemical parameters of *E. stoechadosmum* whole plants

Table 28 Details of the plant samples used in the study

Voucher no	Code	Locality
1	SPH01	Bangkok 1
2	SPH02	Nakorn Sawan
3	SPH03	Surat Thani
4	SPH04	Bangkok 2
5	SPH05	Bangkok 3
6	SPH06	Nakhon Pathom
7	SPH07	Rayong
8	SPH08	Nakhon Si Thammarat
9	SPH09	Nongkhai
10	SPH10	Chiang Mai
11	SPH11	Lopburi
12	SPH12	Ratchaburi
13	SPH13	Ubon Ratchathani
14	SPH14	Kalasin
15	SPH15	Uthai Thani

Table 29 Physicochemical parameters of *E. stoechadosmum* from 15 sources (% by dried weight)

Sources	Loss on drying	Total ash content	Acid insoluble ash content	Water content
1	8.91 ± 0.06	11.66 ± 0.09	3.32 ± 0.25	11.83 ± 0.29
2	8.09 ± 0.20	9.46 ± 0.38	2.75 ± 0.29	11.17 ± 0.29
3	8.46 ± 0.07	10.99 ± 0.31	2.62 ± 0.16	12.07 ± 0.12
4	8.50 ± 0.06	9.93 ± 0.39	2.33 ± 0.05	13.47 ± 0.46
5	8.76 ± 0.13	6.34 ± 0.14	2.02 ± 0.09	12.67 ± 0.58
6	7.91 ± 0.35	8.36 ± 0.39	1.99 ± 0.19	13.27 ± 0.23
7	9.23 ± 0.04	7.06 ± 0.14	1.68 ± 0.10	11.33 ± 0.29
8	8.33 ± 0.05	9.70 ± 0.39	2.37 ± 0.14	11.47 ± 0.06
9	10.78±0.18	8.88 ± 0.39	2.03 ± 0.51	15.53 ± 0.06
10	8.95 ± 0.05	10.02 ± 0.28	3.24 ± 0.20	13.67 ± 0.58
11	9.45 ± 0.05	11.08 ± 0.19	3.24 ± 0.25	15.20 ± 0.52
12	7.85 ± 0.09	8.03 ± 0.36	2.39 ± 0.15	14.17 ± 0.29
13	7.96 ± 0.09	12.12 ± 0.34	4.92 ± 0.29	14.07 ± 0.12
14	8.76 ± 0.04	10.41 ± 0.43	2.97 ± 0.33	13.00 ± 0.00
15	7.97 ± 0.07	8.60 ± 0.18	1.72 ± 0.13	14.93 ± 0.12

Table 29 Physicochemical parameters of *E. stoechadosmum* from 15 sources (% by dried weight) (Cont.)

Sources	Ethanol extractive value	Water extractive value	Volatile oil content
1	11.62 ± 0.36	32.53 ± 0.28	0.25 ± 0.02
2	9.66 ± 0.35	23.92 ± 0.25	0.18 ± 0.06
3	13.28 ± 1.27	28.35 ± 0.11	0.23 ± 0.01
4	5.84 ± 0.05	21.79 ± 0.36	0.15 ± 0.02
5	6.51 ± 0.19	12.12 ± 0.43	0.10 ± 0.07
6	10.93 ± 0.82	28.25 ± 0.33	0.17 ± 0.02
7	10.59 ± 0.30	39.41 ± 1.07	0.11 ± 0.01
8	10.76 ± 0.26	30.63 ± 0.28	0.14 ± 0.03
9	13.45 ± 0.83	16.97 ± 0.70	0.11 ± 0.01
10	11.65 ± 0.90	32.53 ± 0.34	0.06 ± 0.02
11	10.28 ± 0.28	39.43 ± 0.40	0.16 ± 0.03
12	9.58 ± 0.19	31.37 ± 0.15	0.16 ± 0.02
13	4.95 ± 0.13	18.19 ± 0.10	0.11 ± 0.01
14	4.63 ± 0.19	16.62 ± 0.30	0.08 ± 0.02
15	7.54 ± 0.43	24.62 ± 0.15	0.12 ± 0.01

APPENDIX D

Data of quantitative analysis of coumarin content of *E. stoechadosmum*

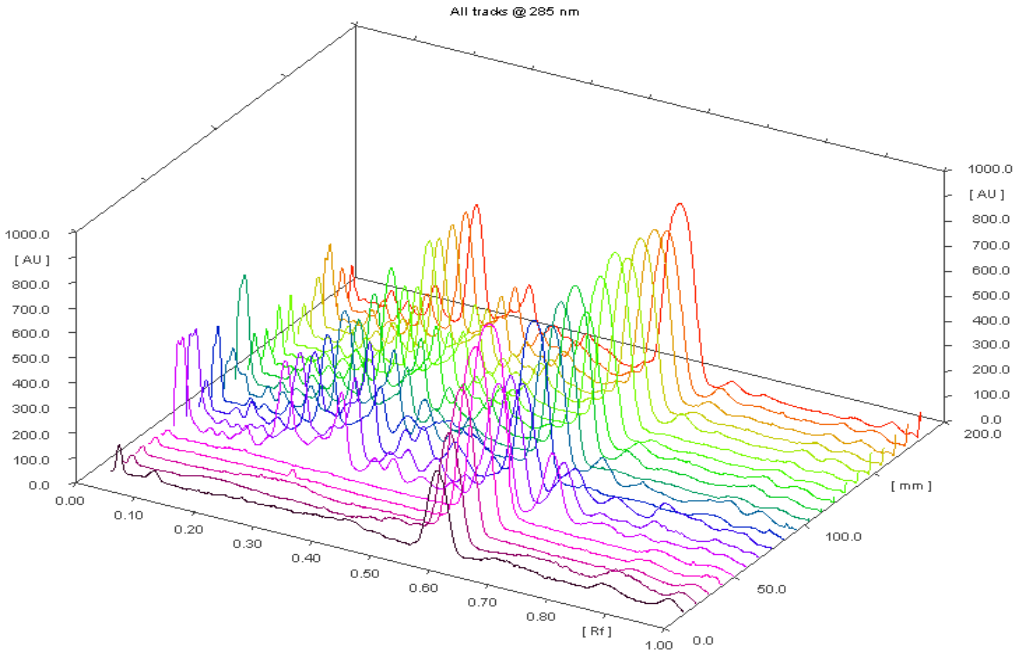


Figure 42 3D TLC- densitograms (Plate H-sample NO.1-15)

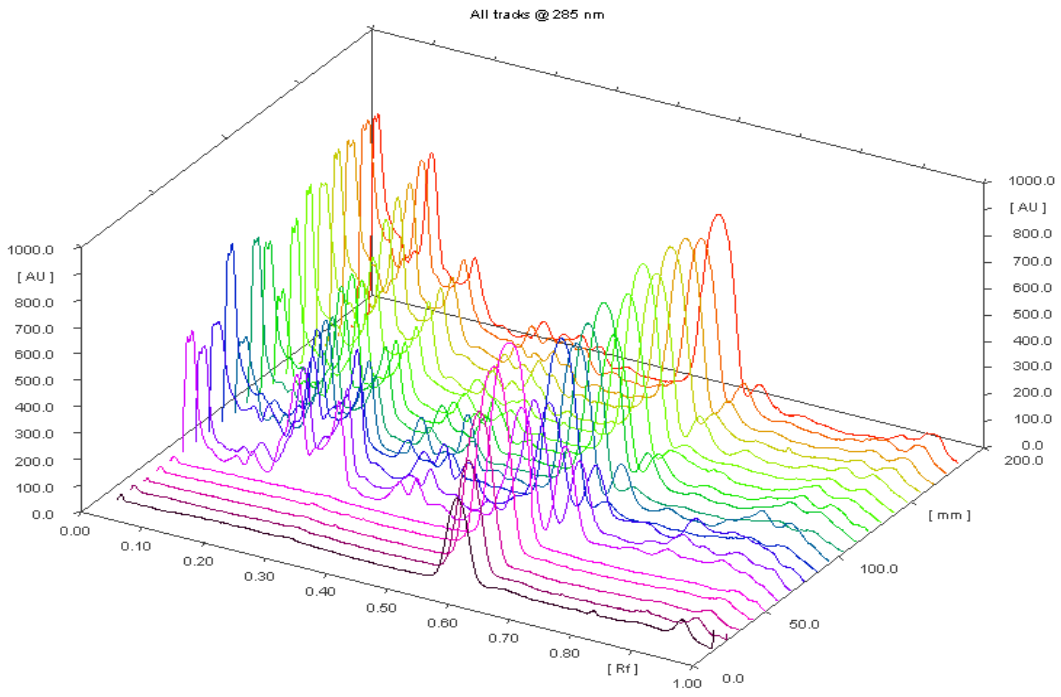


Figure 43 3D TLC- densitograms (Plate I-sample NO.1-15)

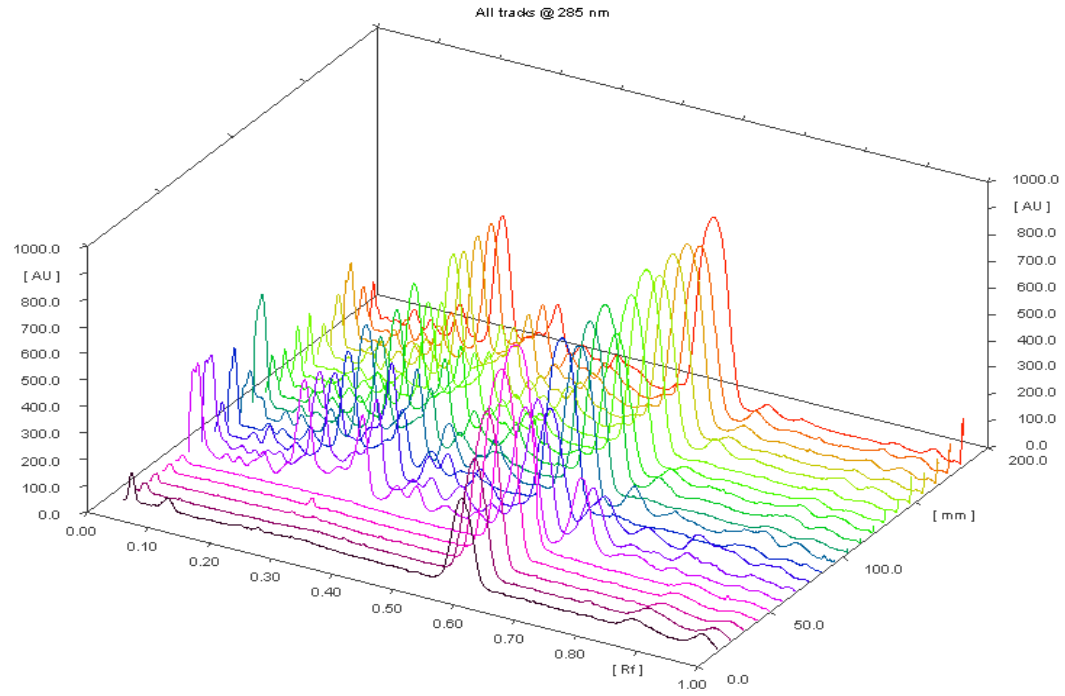


Figure 44 3D TLC- densitograms (Plate J-sample NO.1-15)

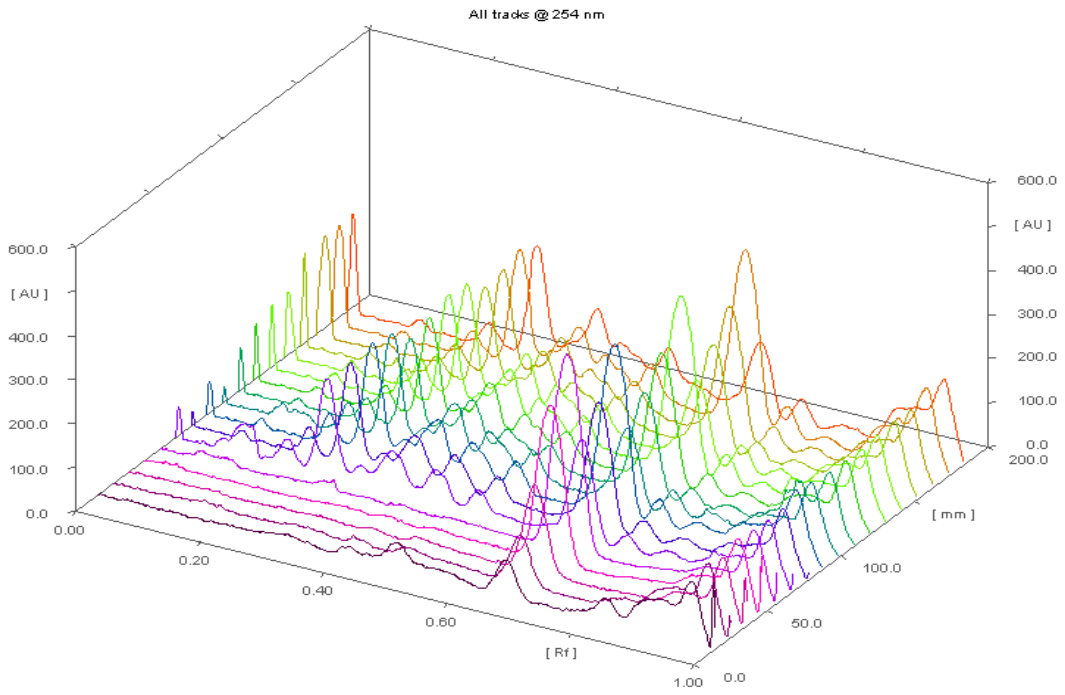


Figure 45 3D TLC- densitograms (Plate K-Accuracy)

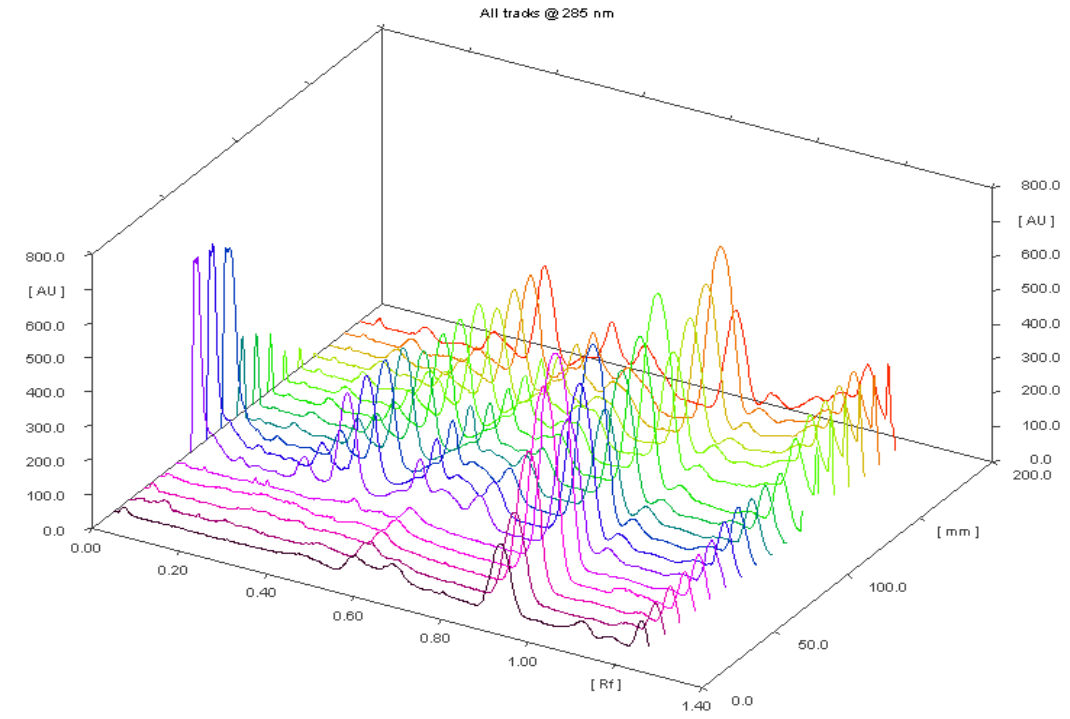


Figure 46 3D TLC- densitograms (Plate L-Precision NO. 1)

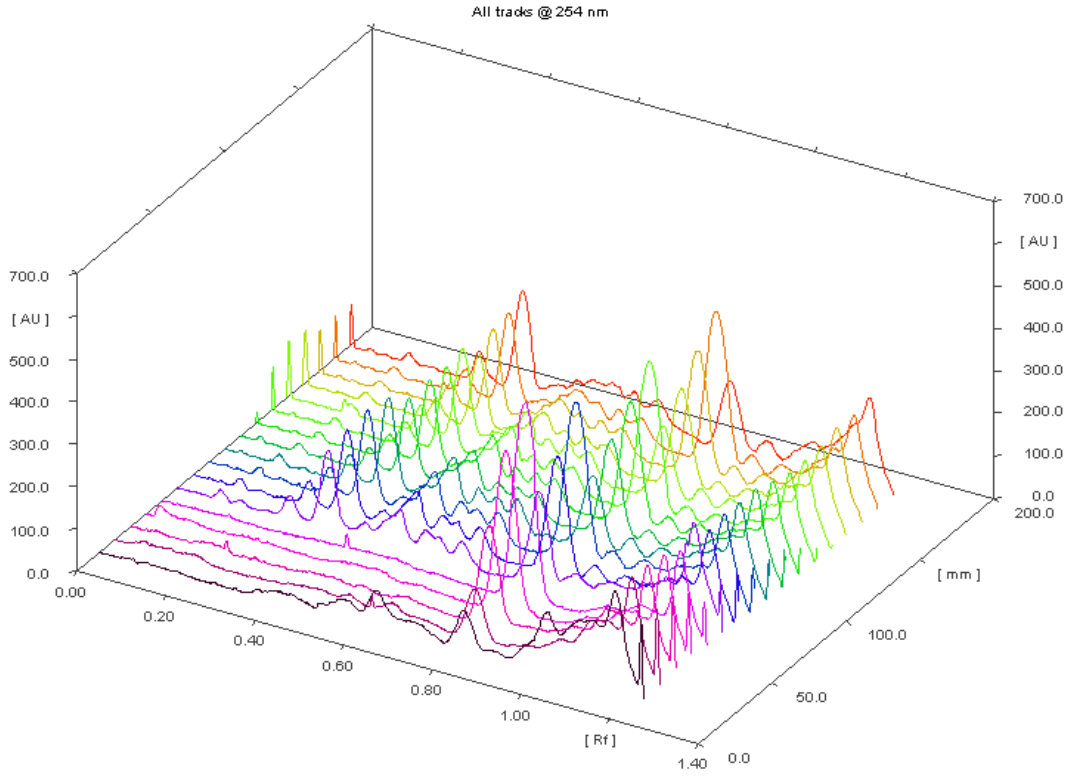


Figure 47 3D TLC- densitograms (Plate M-Precision NO. 2)

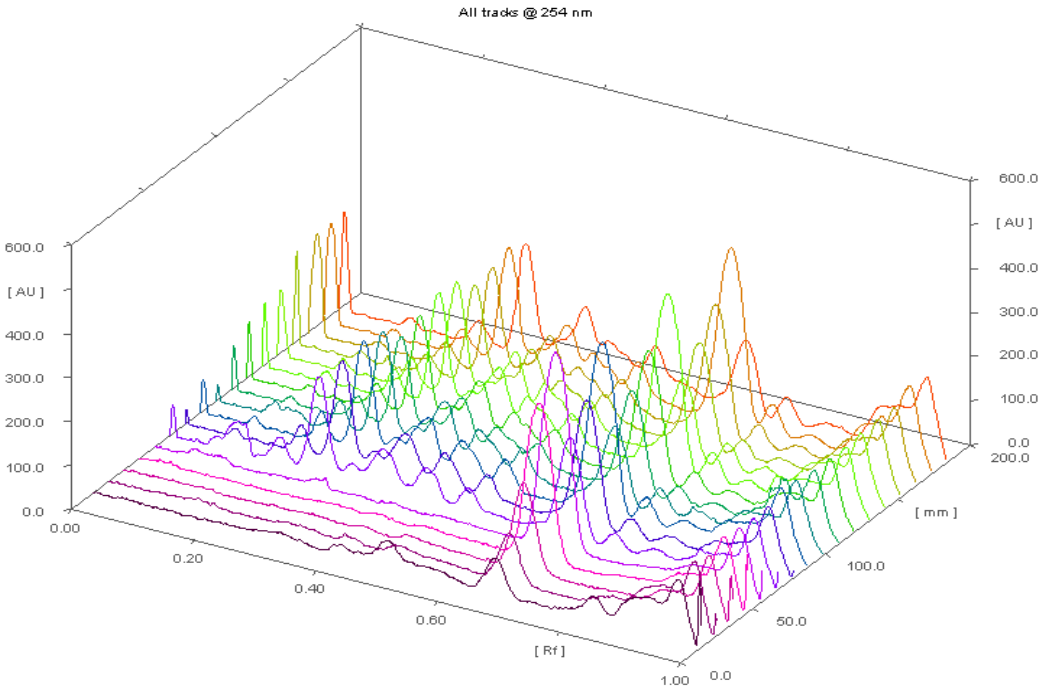
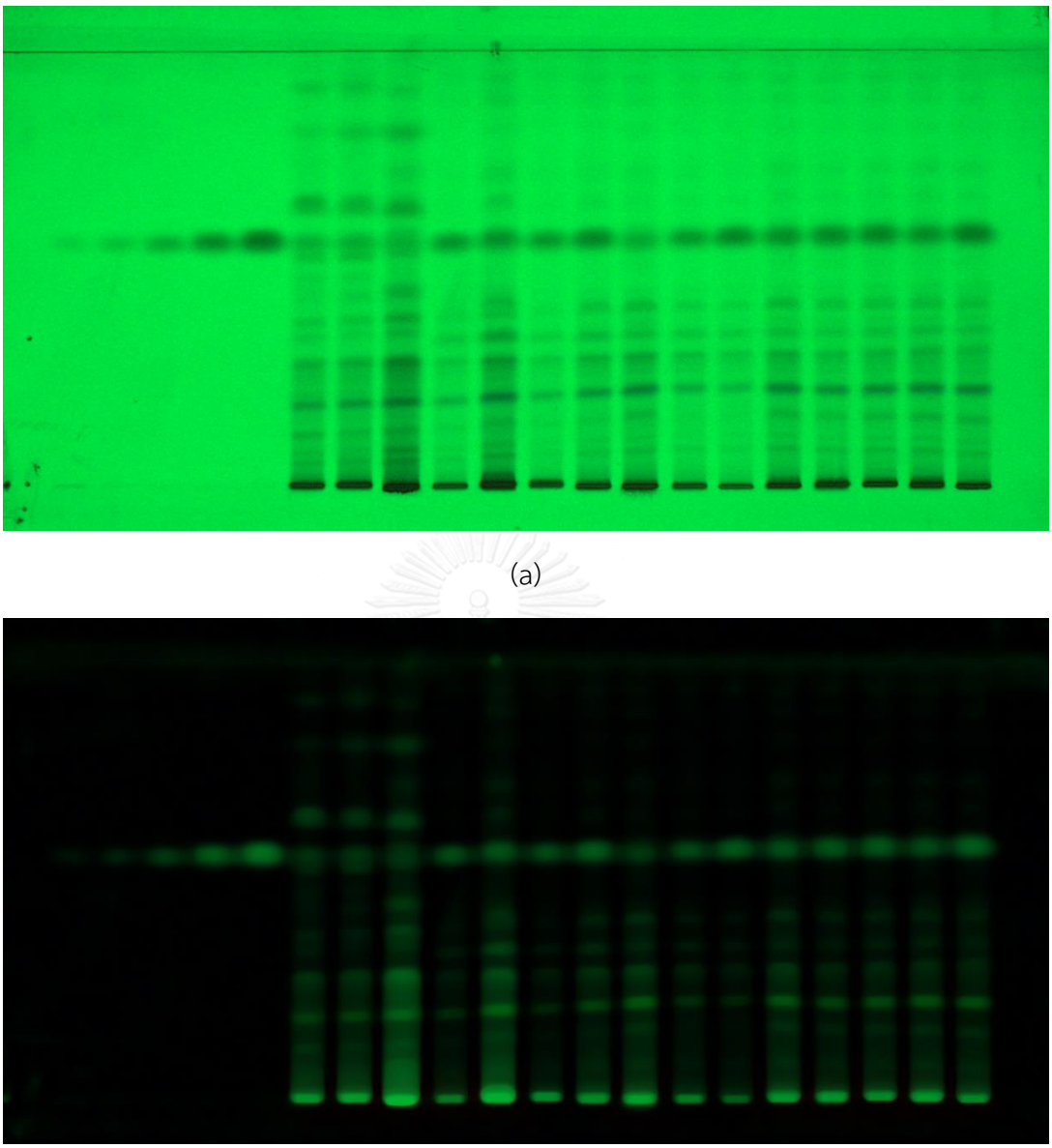


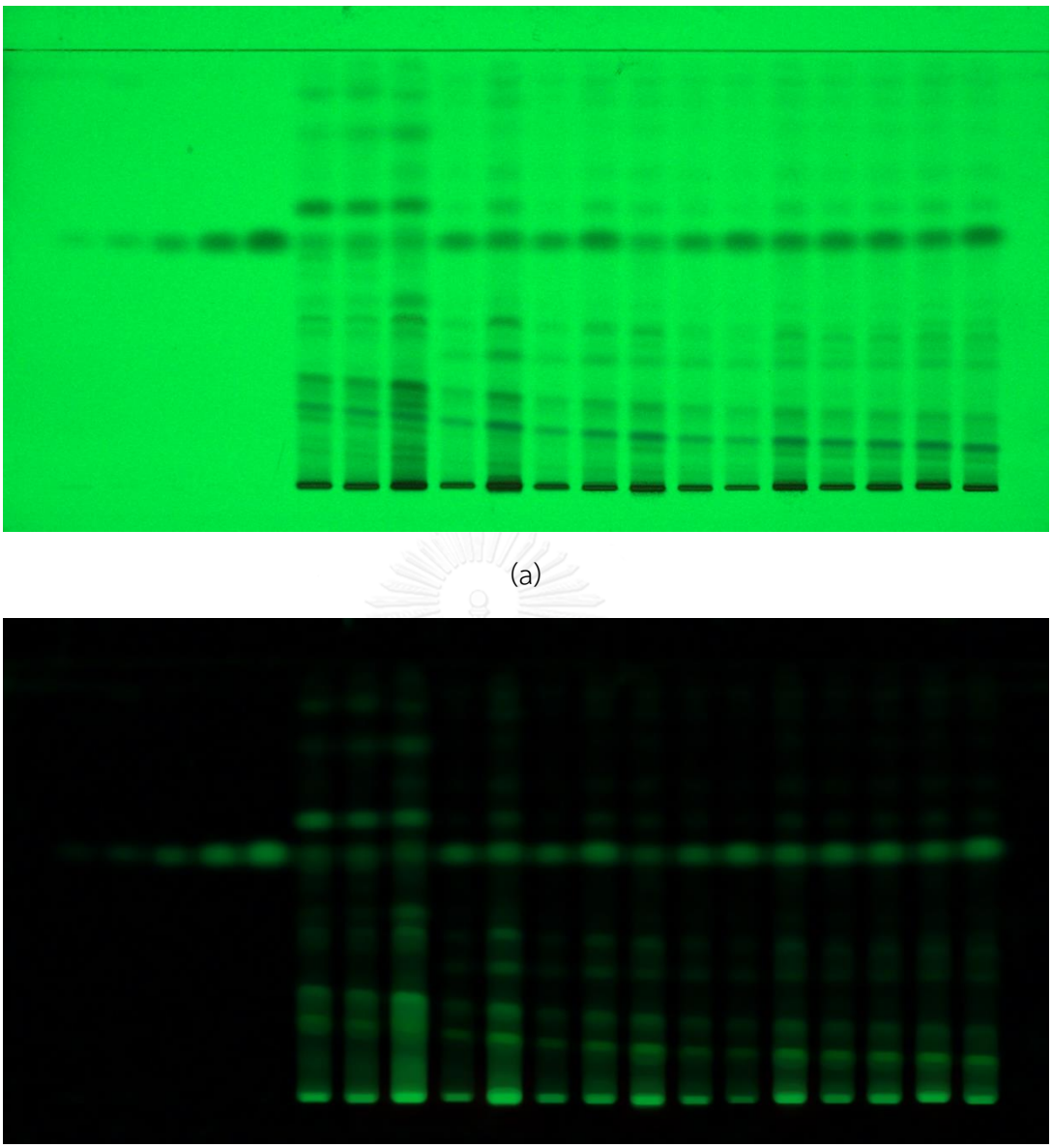
Figure 48 3D TLC- densitograms (Plate N-Precision NO. 3)



(a)

(b)

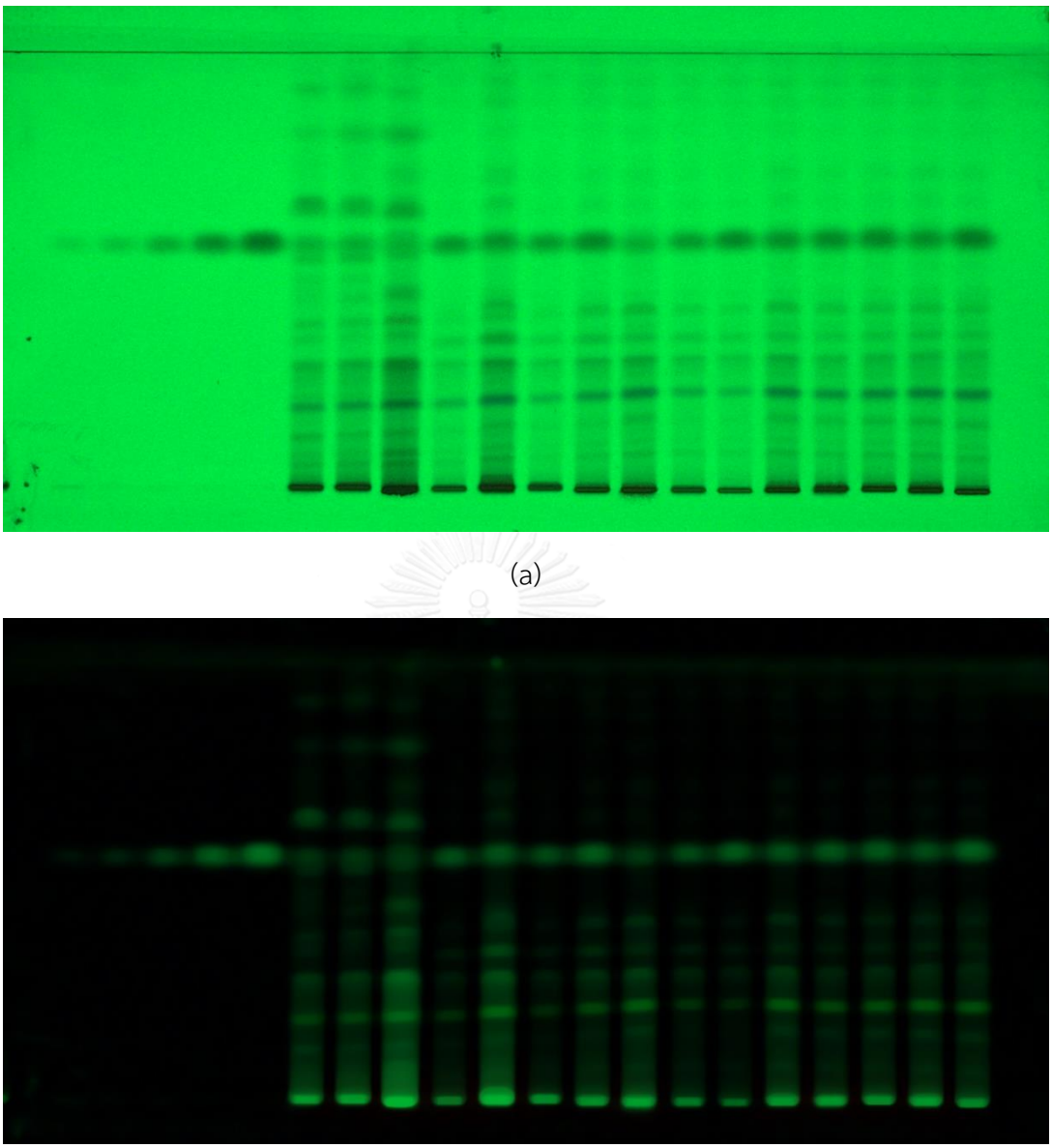
Figure 49 The TLC Plate (H-sample NO.1-15) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

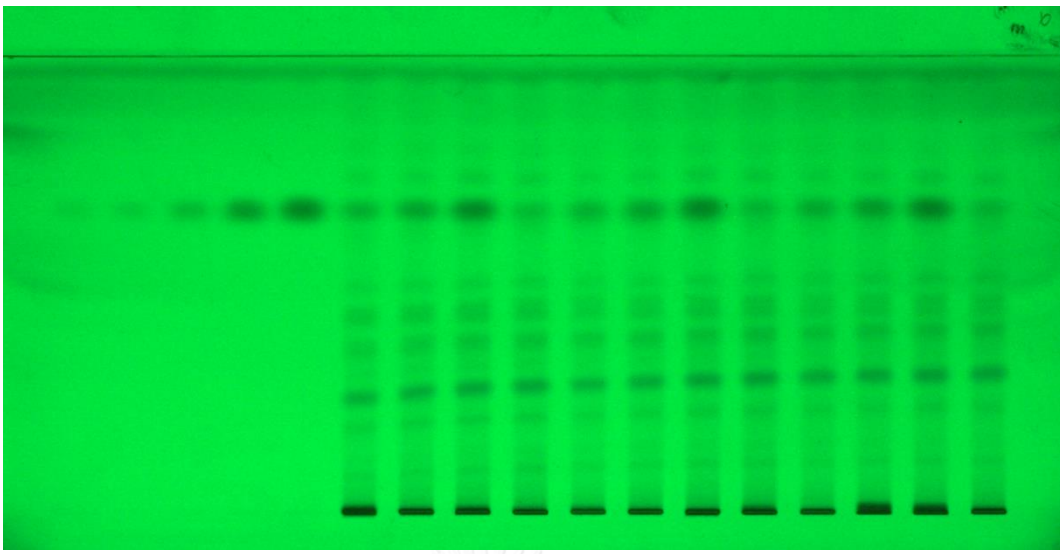
Figure 50 The TLC Plate (I-sample NO.1-15) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



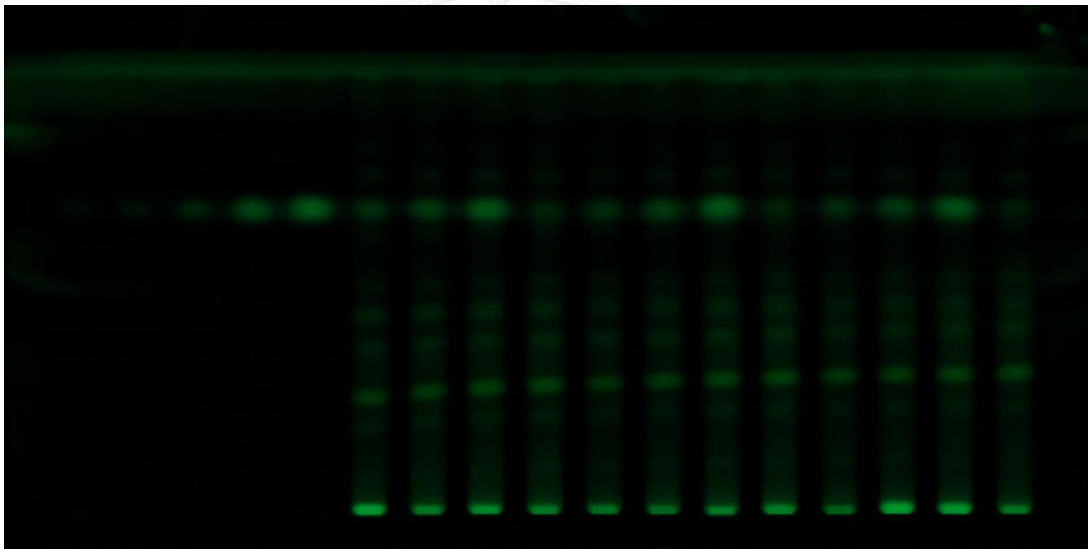
(a)

(b)

Figure 51 The TLC Plate (J-sample NO.1-15) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)

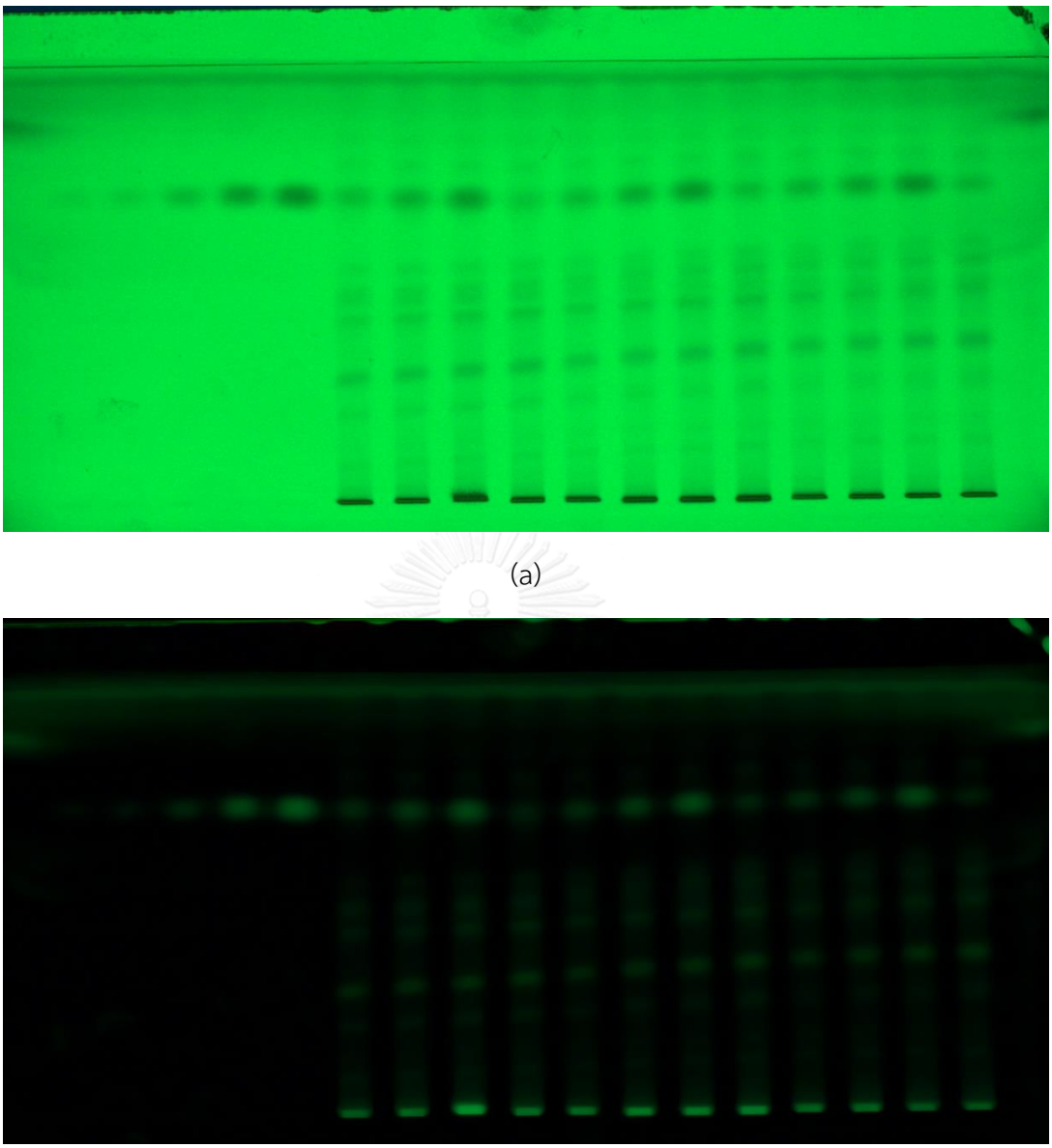


(a)



(b)

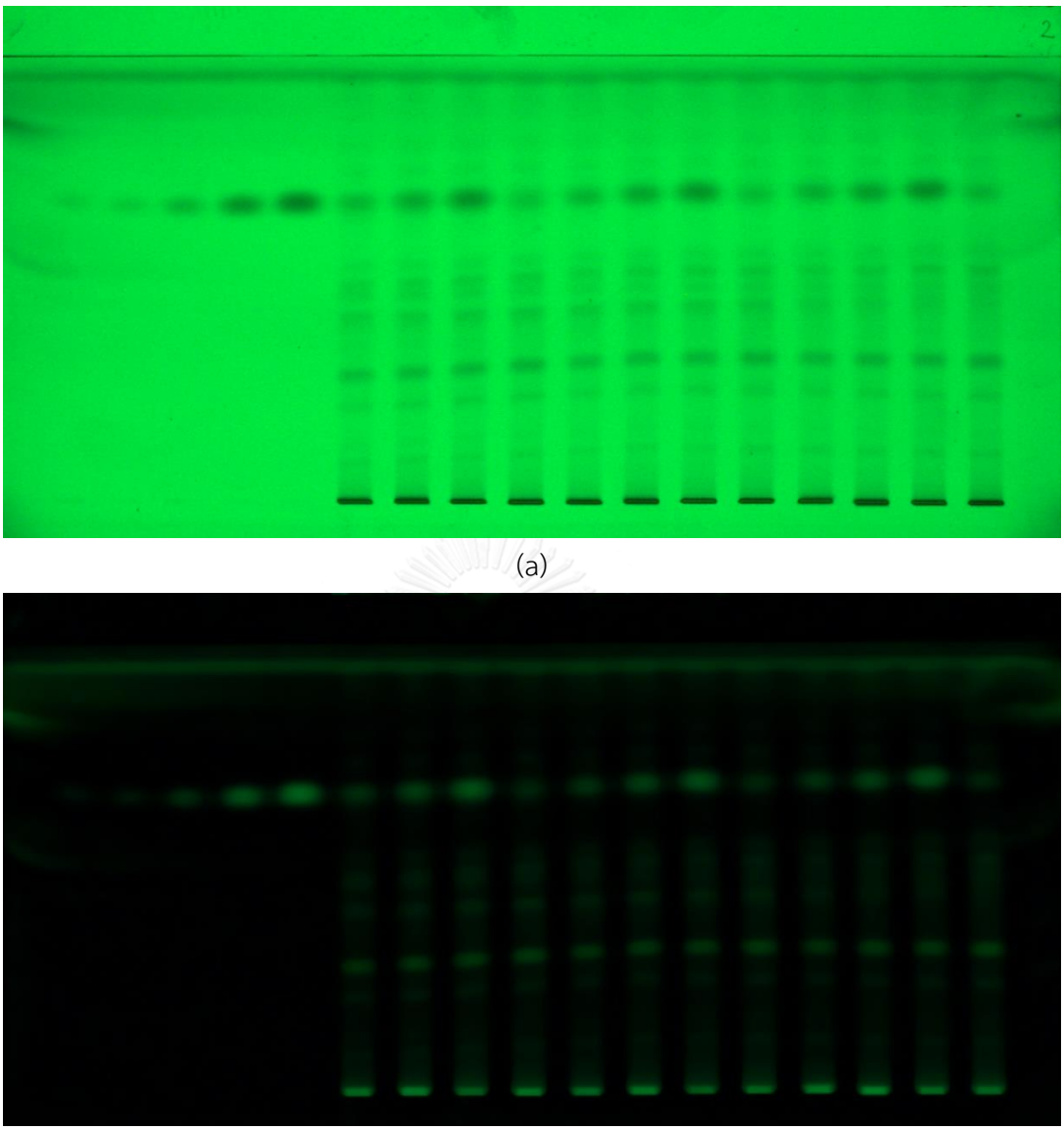
Figure 52 The TLC Plate (K-Accuracy) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

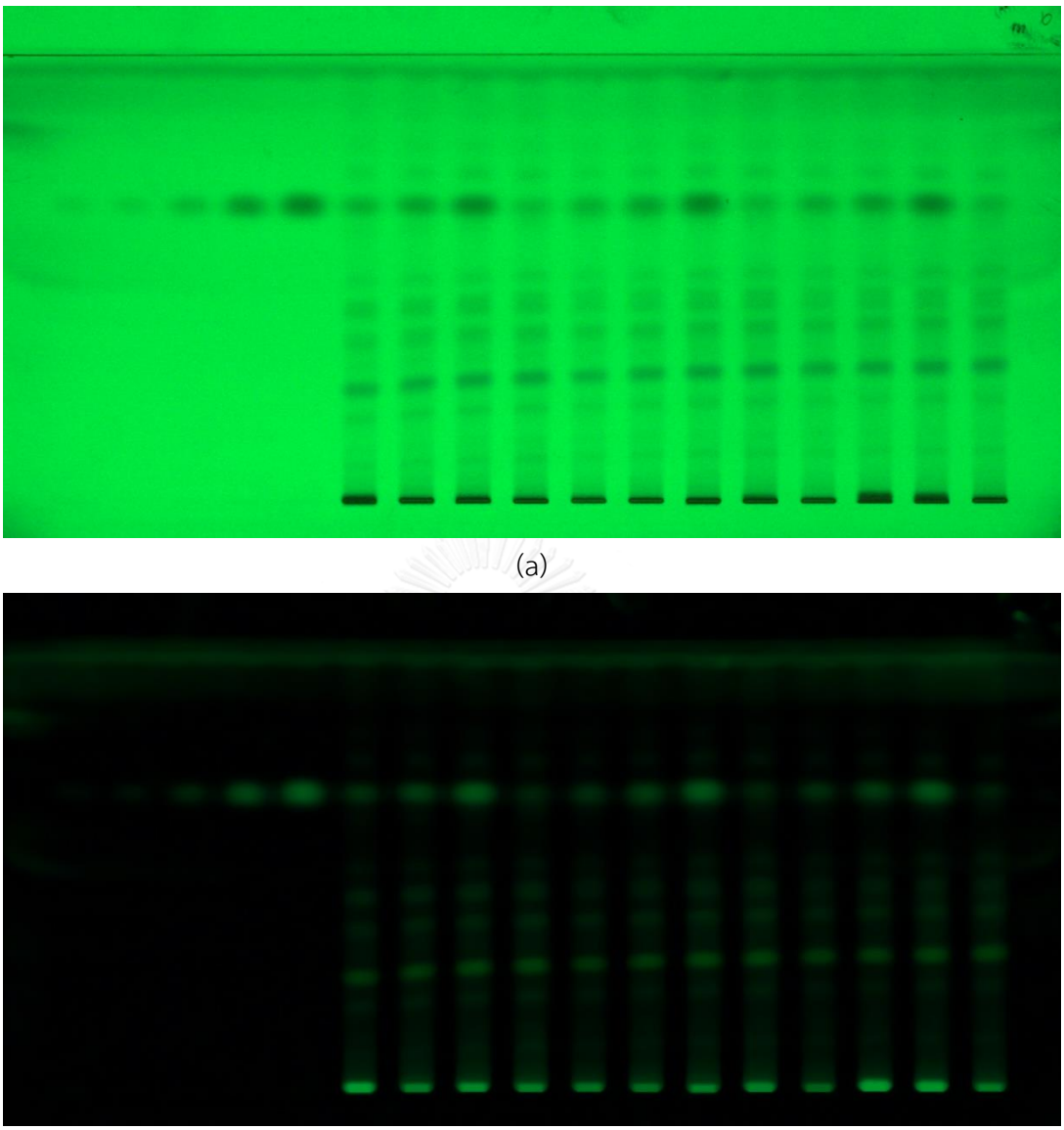
Figure 53 The TLC Plate (L-Precision No.1) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

Figure 54 The TLC Plate (M-Precision No.2) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)



(a)

(b)

Figure 55 The TLC Plate (N-precision No.3) visualized under UV 254 nm (a), with invert and subtracts background by ImageJ software (b)

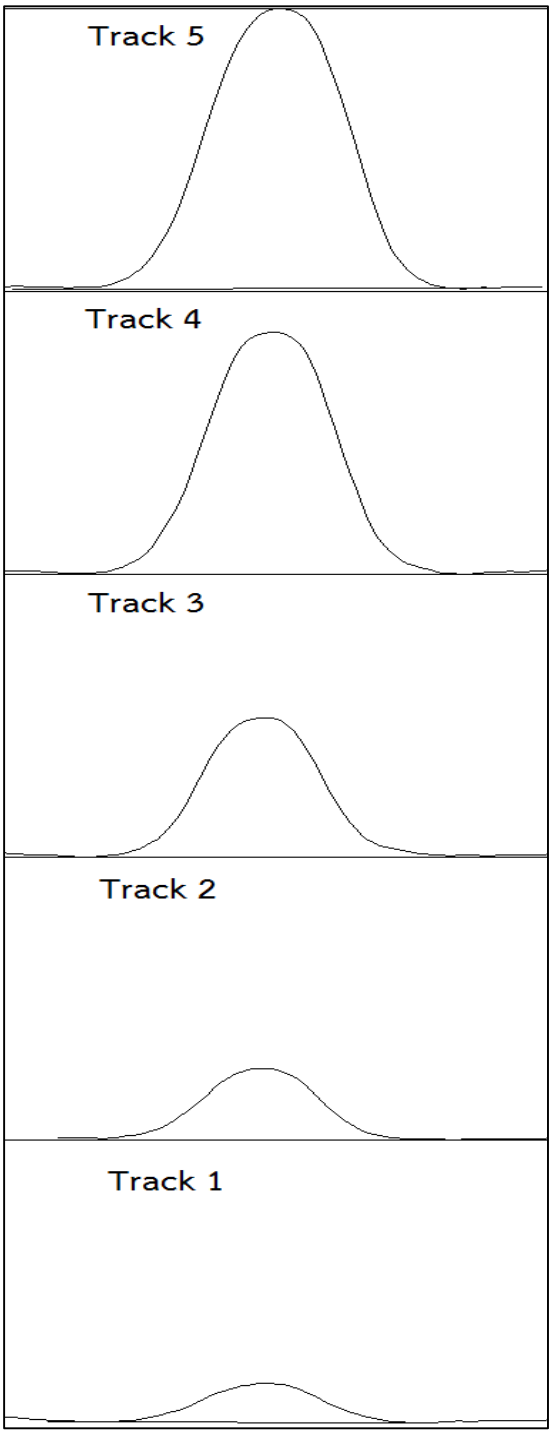


Figure 56 TLC chromatogram of TLC image analysis (ImageJ software); Track 1 was standard coumarin 0.075 mg/ml, Track 2 was standard coumarin 0.15 mg/ml, Track 3 was standard coumarin 0.3 mg/ml, Track 4 was standard coumarin 0.6 mg/ml, Track 5 was standard coumarin 0.9 mg/ ml

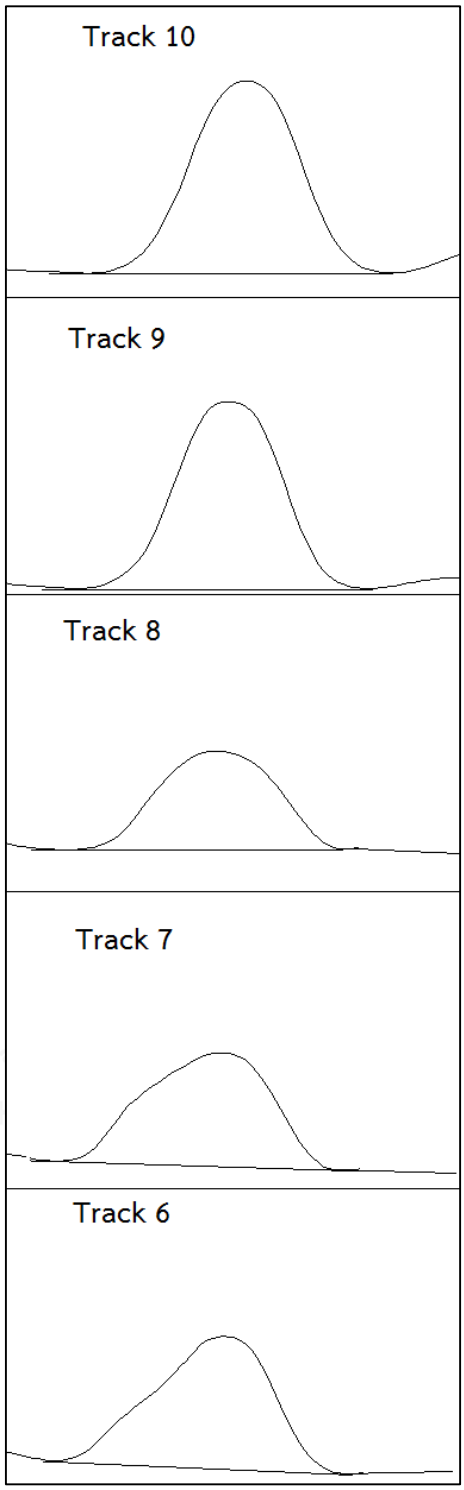


Figure 57 TLC chromatogram of TLC image analysis (Image software); Track 6 was sample (No.1) 20 mg/ml, Track 7 was sample (No.2) 20 mg/ml, Track 8 was sample (No.3) 20 mg/ml, Track 9 was sample (No. 4) 20 mg/ml, Track 10 was sample (No. 5) 20 mg/ml

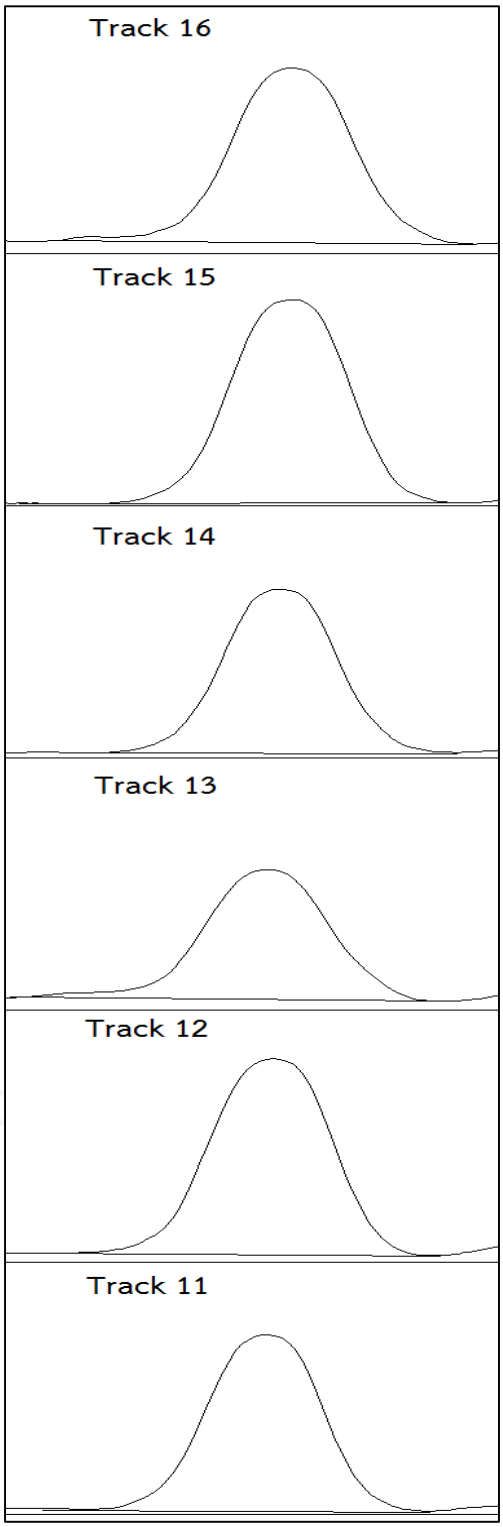


Figure 58 TLC chromatogram of TLC image analysis (Image software); Track 11 was sample (No. 6) 20 mg/ml, Track 12 was sample (No.7) 20 mg/ml, Track 13 was sample (No.8) 20 mg/ml, Track 14 was sample (No. 9) 20 mg/ml, Track 15 was sample (No. 10) 20 mg/ml

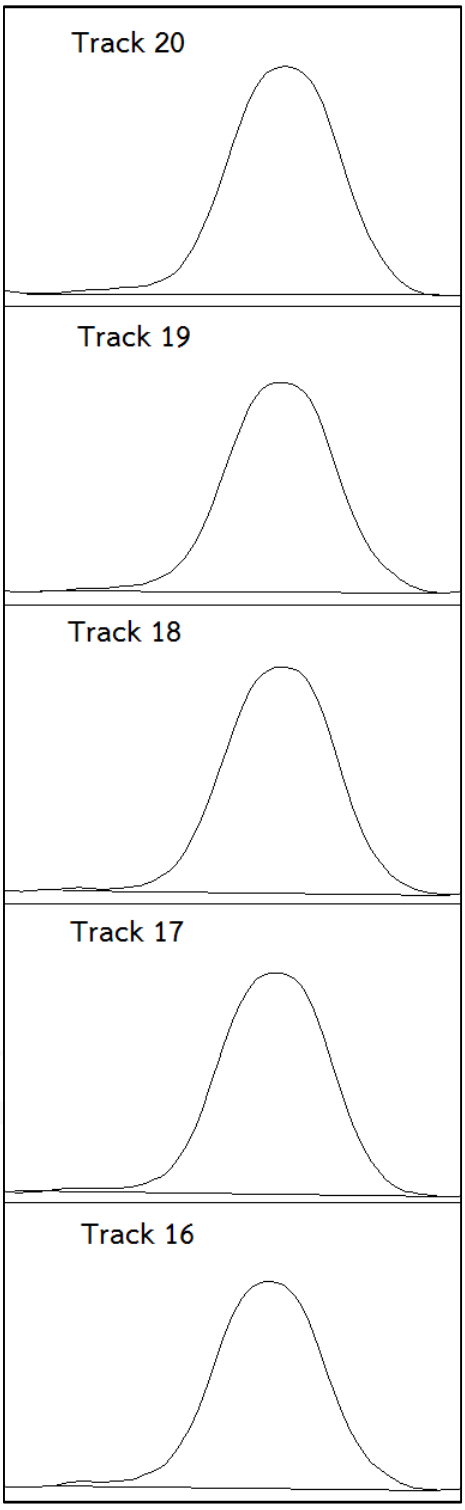


Figure 59 TLC chromatogram of TLC image analysis (Image software); Track 16 was sample (No. 11) 20 mg/ml, Track 17 was sample (No.12) 20 mg/ml, Track 18 was sample (No.13) 20 mg/ml, Track 19 was sample (No. 14) 20 mg/ml, Track 20 was sample (No. 15) 20 mg/ml

APPENDIX E

GC chromatograms of *E. stoechadosmum* oil

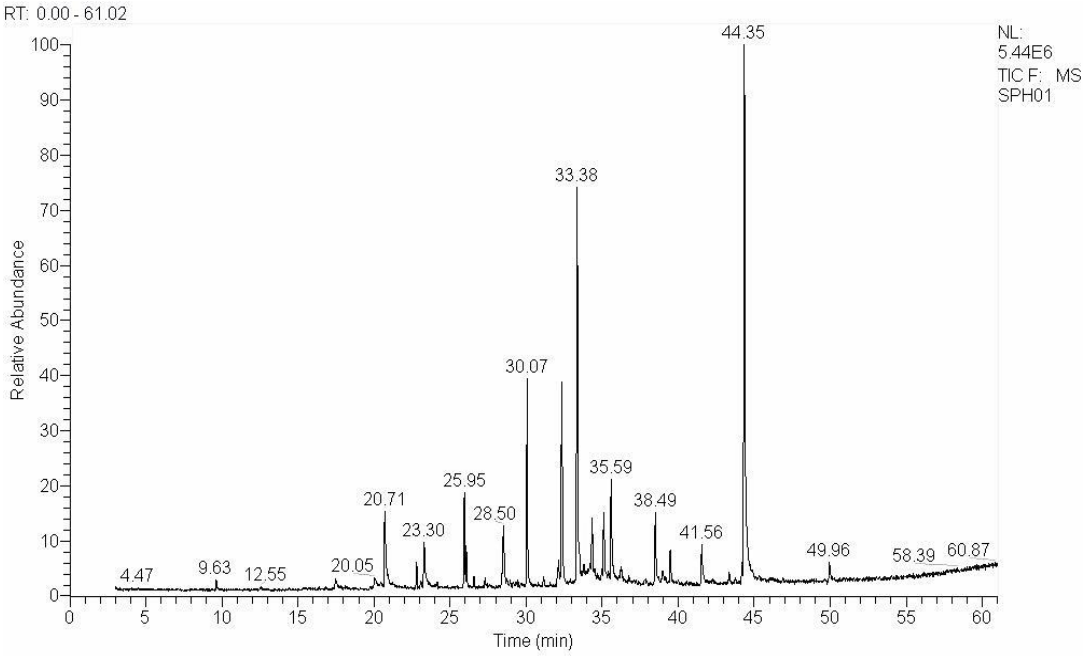


Figure 60 GC chromatogram of *E. stoechadosmum* oil from Bangkok 1

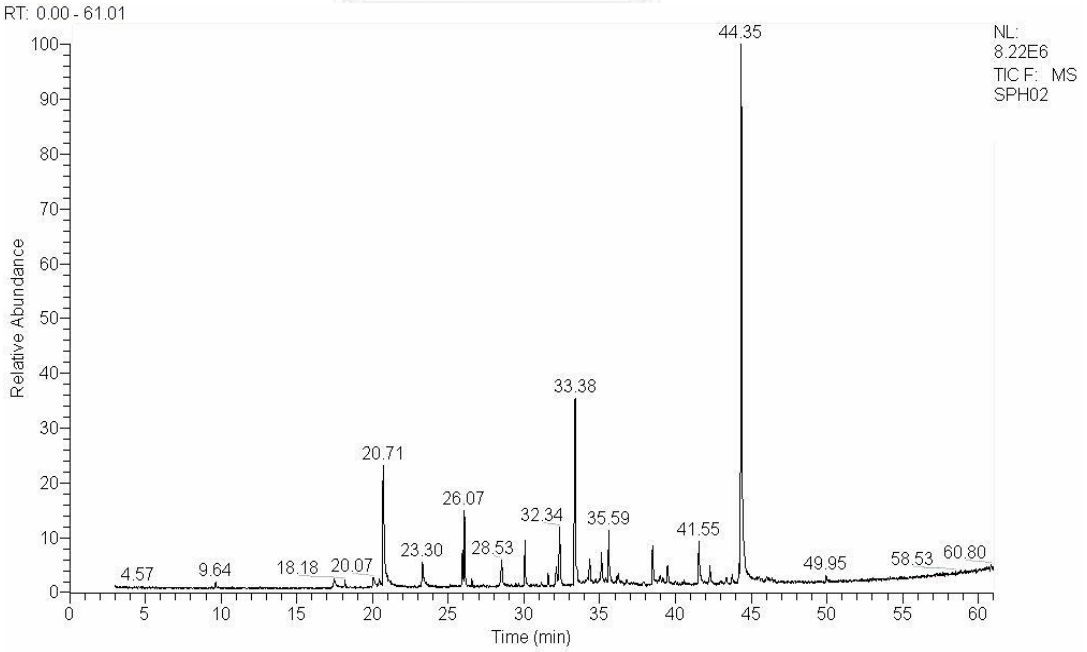


Figure 61 GC chromatogram of *E. stoechadosmum* oil from Nakorn Sawan

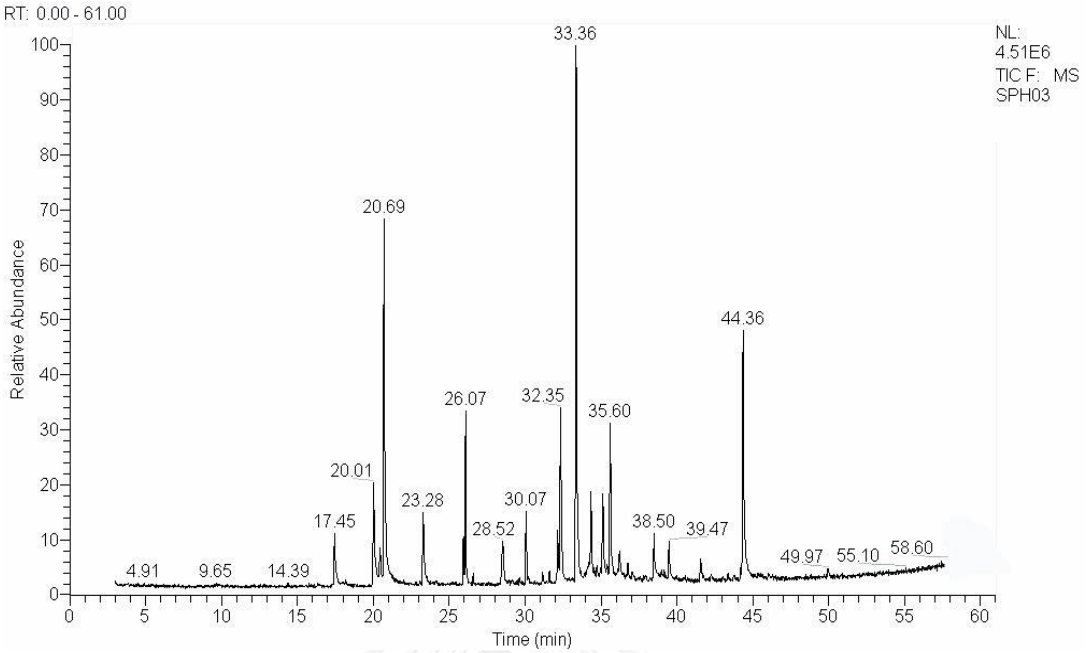


Figure 62 GC chromatogram of *E. stoechadosmum* oil from Surat Thani

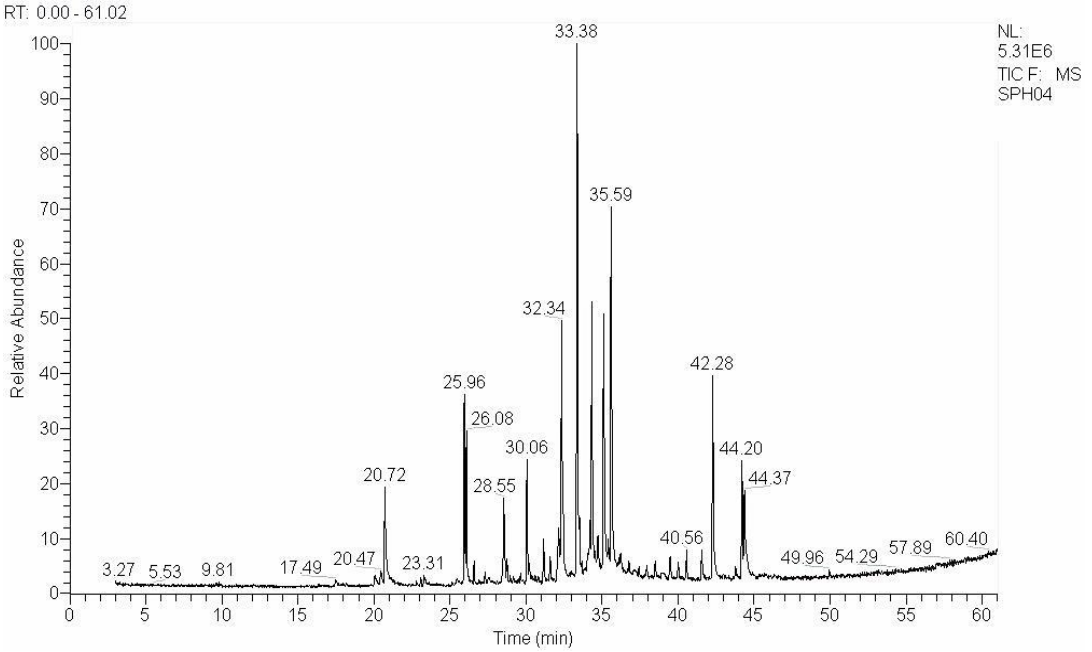


Figure 63 GC chromatogram of *E. stoechadosmum* oil from Bangkok 2

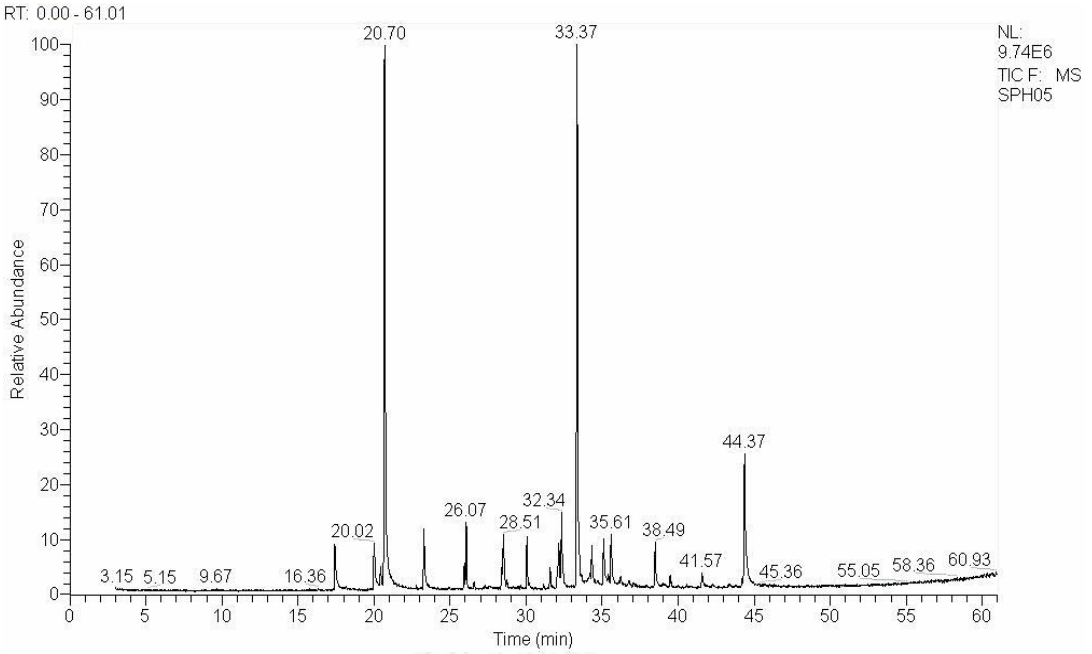


Figure 64 GC chromatogram of *E. stoechadosmum* oil from Bangkok 3

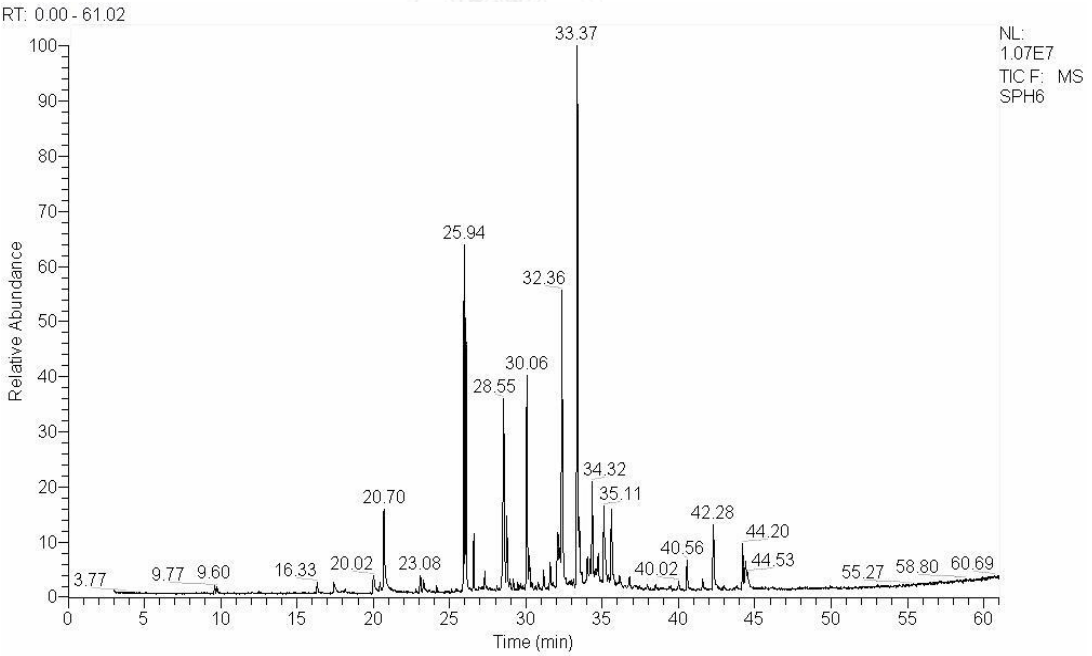


Figure 65 GC chromatogram of *E. stoechadosmum* oil from Nakorn Pathom

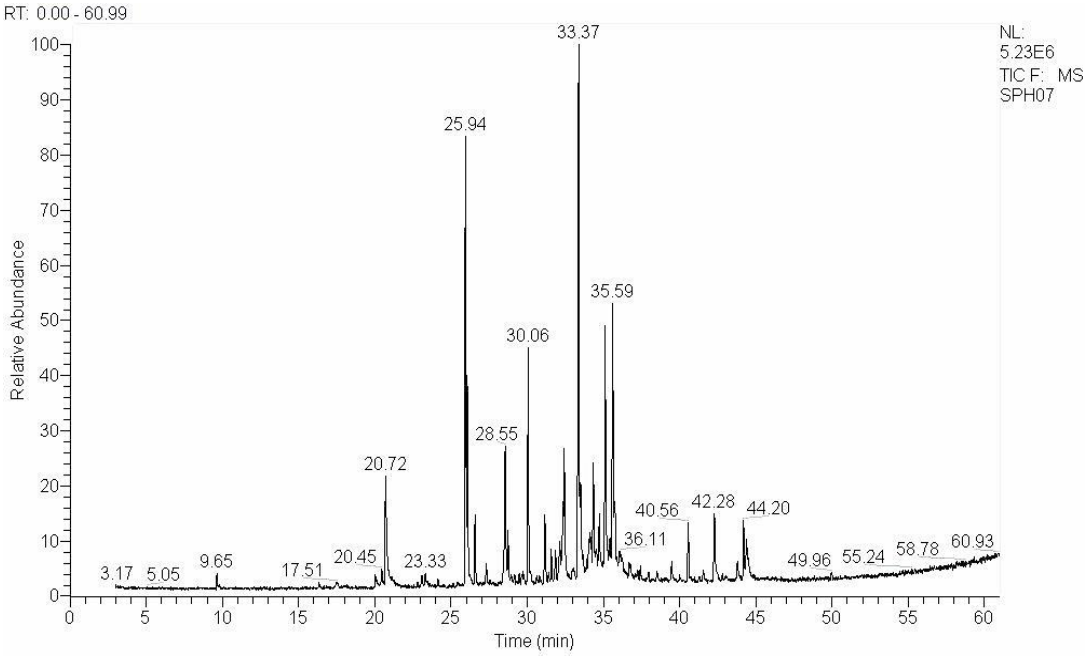


Figure 66 GC chromatogram of *E. stoechadosmum* oil from Rayong

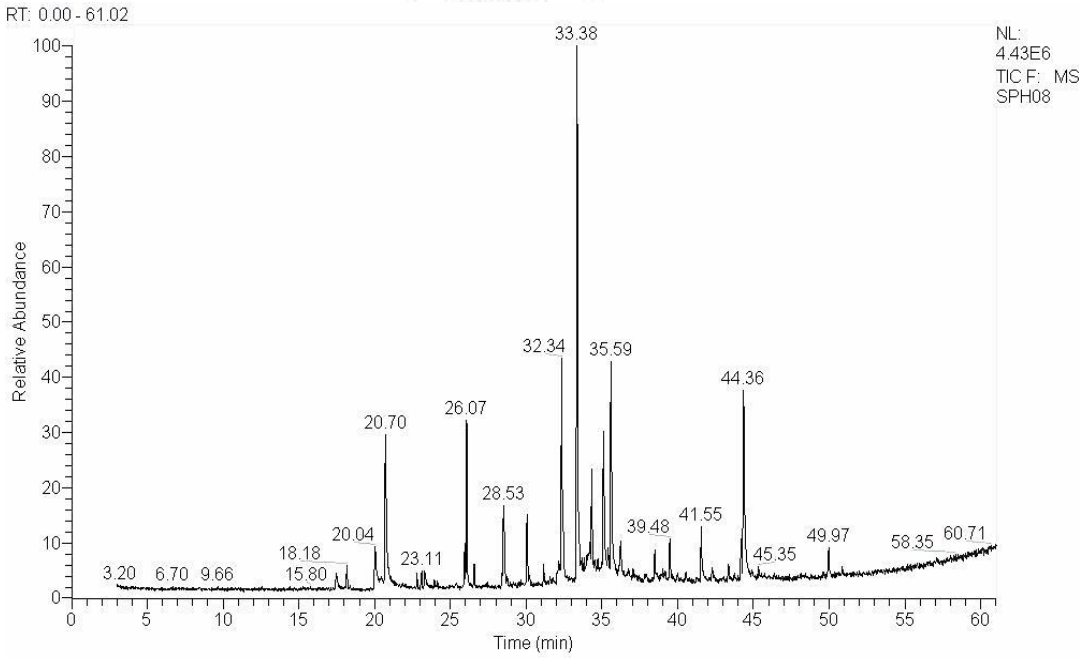


Figure 67 GC chromatogram of *E. stoechadosmum* oil from Nakhon Si Thummarat

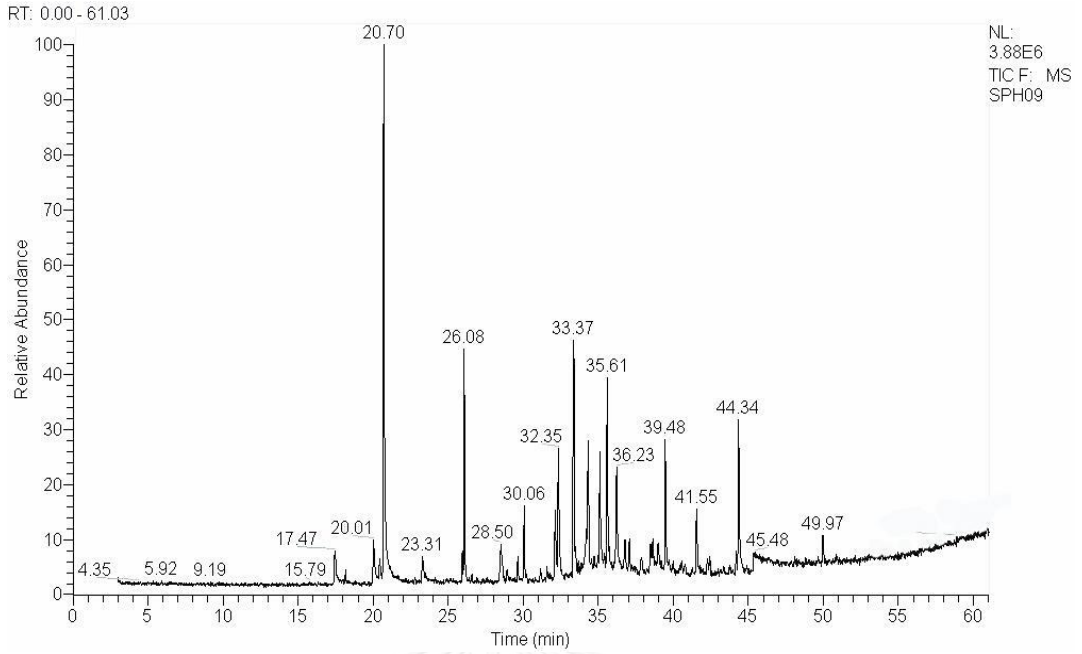


Figure 68 GC chromatogram of *E. stoechadosmum* oil from Nongkhai

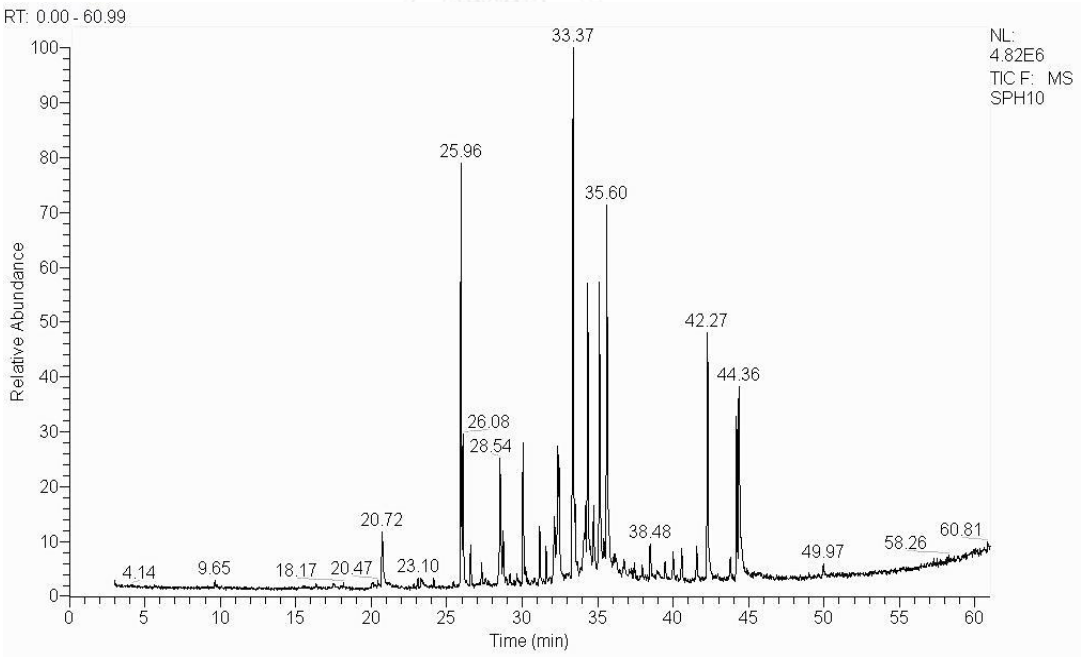


Figure 69 GC chromatogram of *E. stoechadosmum* oil from Chaingmai

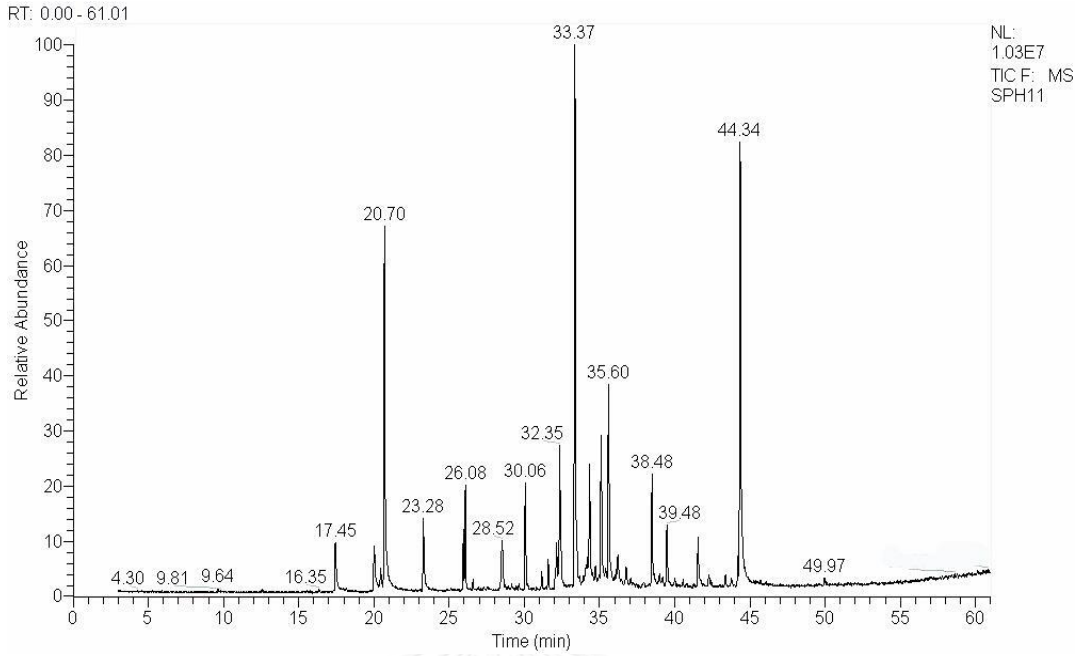


Figure 70 GC chromatogram of *E. stoechadosmum* oil from Lopburi

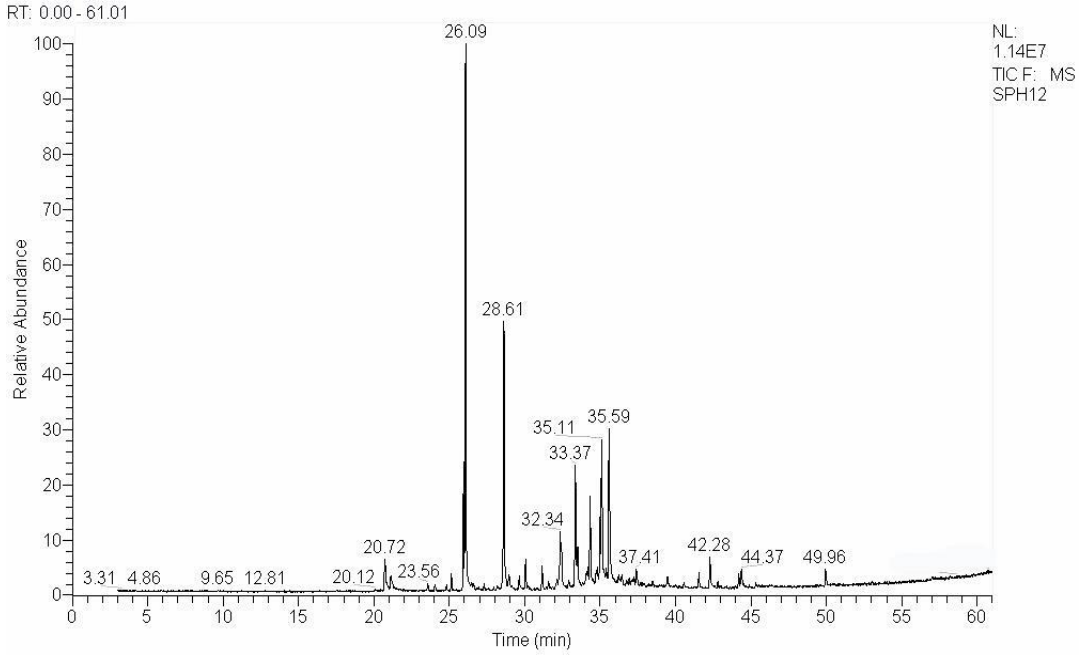


Figure 71 GC chromatogram of *E. stoechadosmum* oil from Ratchaburi

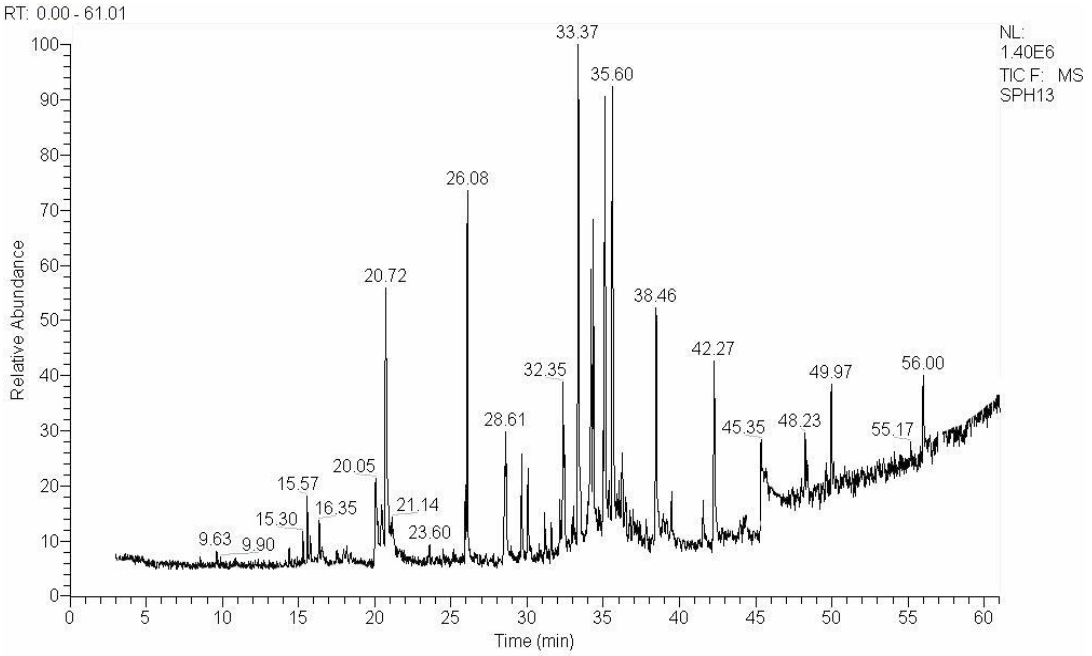


Figure 72 GC chromatogram of *E. stoechadosmum* oil from Ubon Ratchathani

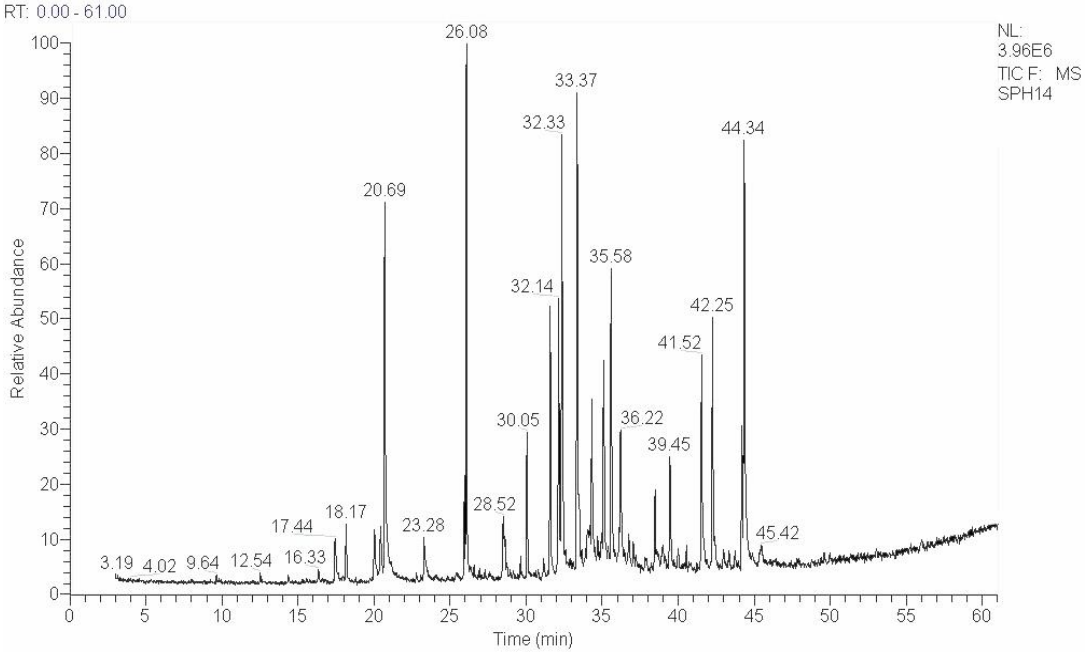


Figure 73 GC chromatogram of *E. stoechadosmum* oil from Kalasin

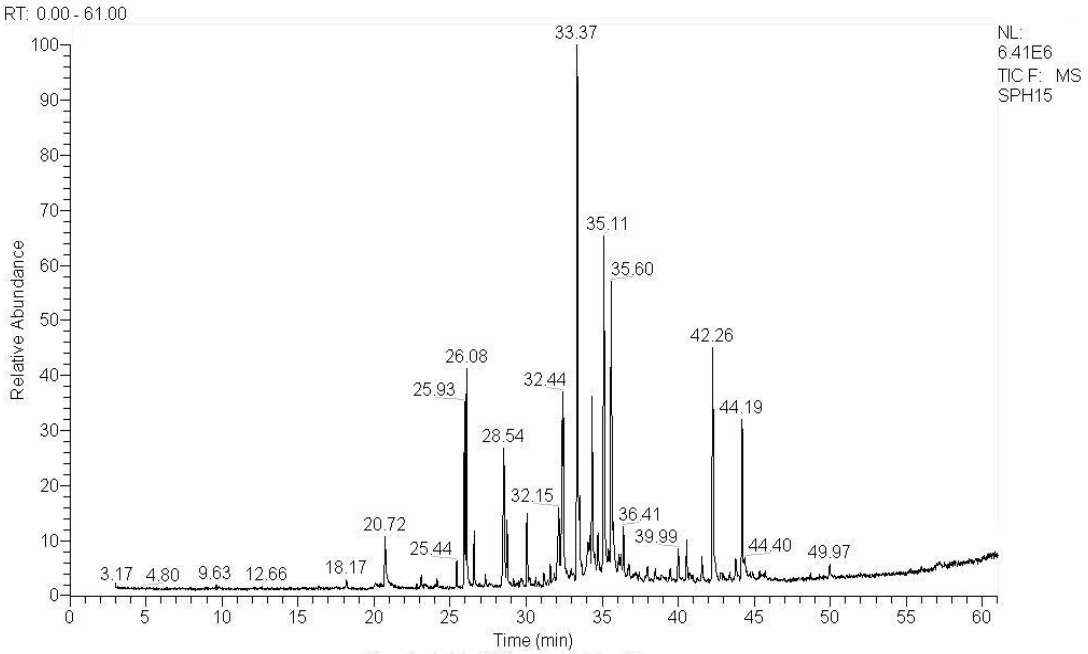


Figure 74 GC chromatogram of *E. stoechadosmum* oil from Uthai Thani

APPENDIX F

Chemical compositions found in the volatile oils of *E. stoechadosmum*

Table 30 Chemical compounds found in the essential oils of *E. stoechadosmum*

Compound name	RT	KI[a]	Occurrence in the oil[b]				
			SPH 01	SPH 02	SPH 03	SPH 04	SPH 05
Cumin aldehyde	17.48	1241	1	1	1	0	1
Carvacrol,methy ether	18.18	1244	0	0	0	0	0
Thymol	20.05	1290	1	1	1	1	1
Carvacrol,ethyl ether	20.44	1298	0	0	1	0	1
Carvacrol	20.71	1299	1	1	1	1	1
2(3H)-Naphthalenon,4,4a,5,6-tetrahydro-7-methyl-	23.30	-	1	1	1	1	1
Cyperene	25.15	1398	0	0	0	0	0
Caryophyllene (E-)	25.95	1419	1	1	1	1	1
Cymene<2,5-dimethoxy-para>	26.07	1426	0	1	1	1	1
Bergamotene <alpha-trans->	26.60	1434	1	0	0	1	0
Acoradiene<alpha->	27.32	1466	0	0	0	1	0
Unidentify A	28.53	-	1	1	1	1	1
Sesquiphellandrene<beta->	30.08	1522	1	1	1	1	1
Unidentify B	30.17	-	0	0	1	1	0
Nerolidol<-E->	31.60	1563	0	1	0	1	1

Table 30 Chemical compounds found in the essential oils of *E. stoechadosmum* (Cont.)

compound name	RT	KI[a]	Occurrence in the oil[b]				
			SPH 01	SPH 02	SPH 03	SPH 04	SPH 05
Caryophyllene oxide	32.34	1583	1	1	1	1	1
Binapacry	33.36	-	1	1	1	1	1
Caryophylla-4(12),8(13)-dien-5-beta-ol	34.33	1640	1	1	1	1	1
Guaiene	34.75	-	0	0	0	1	0
Caryophyllene<14-hydroxy-(Z)->	35.11	1667	1	1	1	1	1
Caryophyllene<14-hydroxy-9-epi-€->	35.59	1668	1	1	1	1	1
Unidentify C	36.24	-	1	1	1	0	0
Unidentify D	36.78	-	0	0	0	0	0
Curcumen-15-al<ar->	37.07	1713	0	0	1	0	0
Khusimol	37.43	1742	0	0	0	0	0
Unidentify E	38.49	-	1	1	1	0	1
Unidentify F	38.99	-	0	1	1	1	1
Unidentify G	39.48	-	1	1	1	1	0
Unidentify H	40.56	-	0	0	0	0	0
Unidentify I	41.57	-	0	1	0	0	0
Cuparenal	44.35	1753	1	1	1	1	1
Phyto acetate<E->	49.96	2218	1	1	1	0	0

Table 30 Chemical compounds found in the essential oils of *E. stoechadosmum* (Cont.)

Compound name	RT	KI[a]	Occurrence in the oil[b]				
			SPH 06	SPH 07	SPH 08	SPH 09	SPH 10
Cumin aldehyde	17.48	1241	1	0	1	1	1
Carvacrol,methy ether	18.18	1244	0	0	1	0	0
Thymol	20.05	1290	1	1	1	1	0
Carvacrol,ethyl ether	20.44	1298	0	1	0	1	0
Carvacrol	20.71	1299	1	1	1	1	1
2(3H)-Naphthalenon,4,4a,5,6-tetrahydro-7-methyl-	23.30	-	0	1	1	1	1
Cyperene	25.15	1398	0	0	0	0	0
Caryophyllene (E-)	25.95	1419	1	1	1	0	1
Cymene<2,5-dimethoxy-para>	26.07	1426	0	0	1	1	0
Bergamotene <alpha-tran->	26.60	1434	1	1	1	0	1
Acoradiene<alpha->	27.32	1466	1	1	0	0	1
Unidentify A	28.53	-	1	1	1	1	1
Sesquiphellandrene<beta->	30.08	1522	1	1	1	1	1
Unidentify B	30.17	-	1	1	1	0	1
Nerolidol<-E->	31.60	1563	1	1	1	0	1
Caryophyllene oxide	32.34	1583	1	1	1	1	1
Binapacry	33.36	-	1	1	1	1	1

Table 30 Chemical compounds found in the essential oils of *E. stoechadosmum* (Cont.)

compound name	RT	KI[a]	Occurrence in the oil[b]				
			SPH 06	SPH 07	SPH 08	SPH 09	SPH 10
Caryophylla-4(12),8(13)-dien-5-beta-ol	34.33	1640	1	1	1	1	1
Guaiene	34.75	-	1	1	0	0	1
Caryophyllene<14-hydroxy-(Z)->	35.11	1667	1	1	1	1	1
Caryophyllene<14-hydroxy-9-epi-€->	35.59	1668	1	1	1	1	1
Unidentify C	36.24	-	0	0	1	1	0
Unidentify D	36.78	-	1	1	0	1	1
Curcumen-15-al<ar->	37.07	1713	0	0	1	1	0
Khusimol	37.43	1742	0	1	0	0	1
Unidentify E	38.49	-	0	0	1	0	1
Unidentify F	38.99	-	0	0	0	1	0
Unidentify G	39.48	-	0	1	1	1	1
Unidentify H	40.56	-	0	1	0	1	1
Unidentify I	41.57	-	1	1	0	1	1
Cuparenal	44.35	1753	0	0	1	1	1
Phyto acetate<E->	49.96	2218	0	0	1	1	1

Table 30 Chemical compounds found in the essential oils of *E. stoechadosmum* (Cont.)

Compound name	RT	KI[a]	Occurrence in the oil[b]				
			SPH 11	SPH 12	SPH 13	SPH 14	SPH 15
Cumin aldehyde	17.48	1241	1	0	0	1	0
Carvacrol,methy ether	18.18	1244	0	0	0	1	0
Thymol	20.05	1290	1	0	1	1	0
Carvacrol,ethyl ether	20.44	1298	1	0	1	1	0
Carvacrol	20.71	1299	1	1	1	1	1
2(3H)-Naphthalenon,4,4a,5,6-tetrahydro-7-methyl-	23.30	-	1	0	0	1	0
Cyperene	25.15	1398	1	1	0	0	0
Caryophyllene (E-)	25.95	1419	0	0	0	0	1
Cymene<2,5-dimethoxy-para>	26.07	1426	1	1	1	1	1
Bergamotene <alpha-tran->	26.60	1434	0	1	0	0	1
Acoradiene<alpha->	27.32	1466	0	0	0	0	0
Unidentify A	28.53	-	1	1	1	1	1
Sesquiphellandrene<beta->	30.08	1522	1	1	1	1	1
Unidentify B	30.17	-	1	1	0	0	0
Nerolidol<-E->	31.60	1563	1	1	0	1	0
Caryophyllene oxide	32.34	1583	1	1	1	1	1
Binapacry	33.36	-	1	1	1	1	1

Table 30 Chemical compounds found in the essential oils of *E. stoechadosmum* (Cont.)

compound name	RT	KI[a]	Occurrence in the oil[b]				
			SPH 11	SPH 12	SPH 13	SPH 14	SPH 15
Caryophylla-4(12),8(13)-dien-5-beta-ol	34.33	1640	1	1	1	1	1
Guaiene	34.75	-	1	0	0	0	1
Caryophyllene<14-hydroxy-(Z)->	35.11	1667	1	1	1	1	1
Caryophyllene<14-hydroxy-9-epi-€->	35.59	1668	1	1	1	1	1
Unidentify C	36.24	-	1	0	1	1	0
Unidentify D	36.78	-	1	0	0	1	0
Curcumen-15-al<ar->	37.07	1713	1	0	0	1	0
Khusimol	37.43	1742	0	1	0	0	0
Unidentify E	38.49	-	1	0	1	1	0
Unidentify F	38.99	-	1	0	0	1	0
Unidentify G	39.48	-	0	1	0	0	1
Unidentify H	40.56	-	1	0	1	1	1
Unidentify I	41.57	-	0	0	0	0	1
Cuparenal	44.35	1753	1	0	1	1	0
Phyto acetate<E->	49.96	2218	1	0	1	0	1

[a] KI: Retention indices determined relative to *n*-alkanes (C_6-C_{24}) on a ZP-5 GC column

[b] 0: absence, 1: presence

VITA

Miss Kanyarat Peng-ngummuang was born on September 18, 1989 in Surin, Thailand. She got a Bachelor's degree of Applied Thai Traditional Medicine with first class honor from Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand in 2012.

Publications

1. Peng-ngummuang, K., Palanuvej, C., and Ruangrungsi, R. Pharmacognostic Specification and coumarin content of Alyxia reinwardtii Inner Bark. Proceedings of The 7th Thailand-Japan International Academic Conference, pp. 252-255, Tokyo, 2014.

2. Peng-ngummuang, K., Thitikornpong, W., Palanuvej, C., and Ruangrungsi, R. Pharmacognostic Specification and coumarin content of Eupatorium stoechadosmum. Proceedings of The 2nd International Conference on Advanced Pharmaceutical Research Strategies and Innovation in Pharmaceutical Research: Safety, Efficacy and Quality, pp. 157-163, Pathumthani, 2015.

3. Peng-ngummuang, K., Palanuvej, C., and Ruangrungsi, R. Pharmacognostic Specification and coumarin content of Alyxia reinwardtii Inner Bark. Engineering Journal, 2015. 19(3): p. 15-20