STANDARDIZATION OF *CHROMOLAENA ODORATA, KAEMPFERIA PARVIFLORA* AND *ZANTHOXYLUM PIPERITUM*

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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สาบเสือมีชื่อทางวิทยาศาสตร์ว่า *Chromolaena odorata* (L.) R.M. King & H. Rob. ซึ่งจัดอยู่ในวงศ์ Asteraceae ต้นสาบเสือสามารถนำมาใช้ในการรักษาโรคผิวหนัง โรคเบาหวาน รวมไปถึงป้องกันแมลงกัดต่อย*Kaempferia parviflora* Wall. ex Baker หรือกระชายดำ จัดอยู่ในวงศ์ Zingiberaceae ซึ่งมักนำไปใช้เพื่อบำรุงร่างกาย รักษาโรคระบบทางเดินอาหาร และต้านการอักเสบ *Zanthoxylum piperitum* (L.) DC. จัดอยู่ในวงศ์ Rutaceae ซึ่งเป็นที่รู้จักกันในชื่อไทยว่าพริกหอม ทุกส่วนของต้นพริกหอมใช้ในการรักษาอาการปวดท้อง อาเจียนและท้องร่วง กรดคลอโรจีนิก 5,7-ไดเมทอกซีฟลาโวน และแซนโทไซลิน เป็นสารออกฤทธิ์ทางชีวภาพที่อยู่ในใบสาบเสือ เหง้ากระชายดำ และผลพริกหอมตามลำดับ และสารเหล่านี้ถูกนำมาใช้เป็นตัวบ่งชี้มาตรฐานในการศึกษาครั้งนี้ สำหรับการวิเคราะห์เชิงปริมาณพบว่าปริมาณกรดคลอโรจีนิก 5,7-ไดเมทอกซีฟลาโวน และแซนโทไซลิน ที่วิเคราะห์โดยทั้งวิธีทินเลเยอร์โครมาโทกราฟี-เด็นซิโทเมทรีและวิธีทินเลเยอร์โครมาโทกราฟีโดยวิเคราะห์ภาพถ่ายไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($P > 0.05$) สำหรับการทดสอบฤทธิ์ทางชีวภาพในหลอดทดลองพบว่าสารสกัดเอทานอลและสารออกฤทธิ์ของพืชแต่ละชนิดมีฤทธิ์ในการต้านออกซิเดชันและการต้านเบาหวาน สารสกัดเอทานอลของเหง้ากระชายดำพบความเป็นพิษต่อไรทะเล ในขณะที่สารสกัดเอทานอลของใบสาบเสือและผลพริกหอมพบความเป็นพิษต่อไรทะเลเพียงเล็กน้อย จากผลการทดสอบความเป็นพิษต่อเซลล์มะเร็งโดยวิธีเอ็มทีทีพบว่าสารทดสอบทั้งหมดไม่พบความเป็นพิษต่อเซลล์มะเร็งและเซลล์ปกติ จากการวิเคราะห์น้ำมันหอมระเหยโดยวิธีแกสโครมาโทกราฟี-แมสสเปคโทเมทรีแสดงให้เห็นถึงส่วนประกอบสำคัญของน้ำมันหอมระเหยในพืชแต่ละชนิด นอกจากนี้ยังได้มีการจัดทำข้อกำหนดทางเภสัชเวทของเหง้ากระชายดำ โดยประเมินลักษณะทางมหทรรศน์และจุลทรรศน์ ลักษณะทางเคมี-ฟิสิกส์ และเอกลักษณ์ทางเคมีของเหง้ากระชายดำ

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YAMON PITAKPAWASUTTHI: STANDARDIZATION OF *CHROMOLAENA ODORATA, KAEMPFERIA PARVIFLORA* AND *ZANTHOXYLUM PIPERITUM*. ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., CO-ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., 200 pp.

Chromolaena odorata (L.) R.M. King & H. Rob. or Sabsue is a species in family Asteraceae. In Thailand, it is locally used to treat skin diseases, diabetes, as well as insect bites. *Kaempferia parviflora* Wall. ex Baker or Krachai Dum belongs to Zingiberaceae family. In herbal medicine, it is generally used to promote health, to cure gastrointestinal disorder and anti-inflammation. *Zanthoxylum piperitum* (L.) DC., belonging to the Rutaceae family, is known in Thai name as Prig Horm. All parts of *Z. piperitum* are used to treat abdominal pain, vomiting and diarrhea. Chlorogenic acid, 5,7-dimethoxyflavone and xanthoxylin are the active components in *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits respectively and these compounds were used as a standard marker in this study. For quantitative analysis, the results indicated that the chlorogenic acid, 5,7-dimethoxyflavone and xanthoxylin contents quantified by TLC-densitometry and TLC image analysis were not statistically significantly different ($P > 0.05$). For *in vitro* biological activities, the results revealed that each extract and its active constituents showed its antioxidant and anti-diabetic potentials. *K. parviflora* rhizome showed potent toxicity whilst *C. odorata* leaf and *Z. piperitum* fruit ethanolic extract showed weak toxicity against brine shrimp nauplii. As the result of cell viability activity, all tested samples exhibited no significant cytotoxic activity against 5 human cancer and 1 normal cell lines. For GC-MS analysis, each plant samples showed its major components of essential oil. Furthermore, the pharmacognostic specification of *K. parviflora* rhizomes were established in order to provide macroscopic and microscopic evaluation, physico-chemical parameters and chemical fingerprints.

Field of Study: Public Health Sciences Student's Signature .....

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LIST OF ABBREVIATIONS

BHT	Buthylated hydroxytoluene
BT-474	Ductal carcinoma breast
Chago-K1	Undifferentiated lung carcinoma
°C	Degree Celsius
cm	Centimetre
DMSO	Dimethyl sulfoxide
DPPH	2, 2- diphenyl-1 picryl hydrazyl
Fe^{2+}	Iron (II)
Fe^{3+}	Iron (III)
$FeSO_4$	Iron (II) sulfate
FRAP	Ferric reducing antioxidant power
g	Gram
GAE	Gallic acid equivalent
GC	Gas chromatography
HCl	Hydrochloric acid
Hep-G2	Liver hepatoblastoma
hrs	Hours
IC_{50}	Half maximal inhibitory concentration
ICH	International Conference on Harmonization
KATO-III	Gastric carcinoma
kg	Kilogram
l	Litre
LOD	Limit of detection
LOQ	Limit of quantitation
m	Metre
MBC	Minimum bactericidal concentration
MFC	Minimum fungicidal concentration
mg	Milligram

MIC	Minimal inhibitory concentration
min	Minute
ml	Millilitre
mM	Millimolar
nm	Nanometer
NSS	Normal saline solution
OH^-	Hydroxyl group
pH	Potential of hydrogen ion
R	Correlation coefficient
Rf	Retention factor
rpm	Revolutions per minute
RSD	Relative standard deviation
SD	Standard deviation
SW-620	Colon adenocarcinoma
TLC	Thin layer chromatography
TPTZ	2, 4, 6-tripyridyl-S-triazine
UV	Ultraviolet
v/v	Volume in a volume
WHO	World Health Organization
Wi-38	Lung fibroblast
µg	Microgram
µl	Microlitre

CHAPTER I
INTRODUCTION

Background and significance of the study

Traditional medicine is all of the knowledge, practices and skills based on the theories, experiences and beliefs indigenous to different cultures. It is used to diagnose, prevent, improve or treat the mental and physical ailments. In Thailand, it has historically demonstrated the efficacy and safety of traditional medicine regarding the uses of many experiences and practices from the past generation to present. However, the scientific researchers need to provide the scientific evidences in quality control which indicates the quality, efficacy and safety evaluations of herbal medicines and make them more reliable [1]. The quality control needs to measure the phytochemical compounds in medicinal plants for ensuring the quality reliability of natural products obtained from plant sources. The chemical compounds in medicinal plant can be varied depending on many factors such as botanical sources, harvest seasons or drying processes. Therefore, one of a major problem for the quality control of herbal medicines is the insufficient of the markers which are both primary and secondary compounds in plants. Moreover, the American Herbal Pharmacopoeia has discussed that a single or several chemical markers are necessarily used for assuring the quality control [2].

Chromolaena odorata (L.) R.M. King & H. Rob. (syn. *Eupatorium odoratum* L.), known in common name as Siam Weed, Christmas Bush, or Common Floss Flower, is a species in family Asteraceae. This plant is widely distributed in Asia, Africa and the Pacific [3, 4]. In terms of medicinal uses, leaf extracts are used in the treatment of colds and sometimes sore throats when used with salt. Commercially, it is used as a scent in aromatic baths [5]. Evidently, it has been reported that *C. odorata* extract shows antimicrobial property against *Neisseria gonorrhoeae* which can cause gonorrhea in men [6]. Also, it has been revealed to accelerate blood clotting [7]. In Thailand, *C. odorata* is locally used to treat skin diseases, diabetes, as well as insect bites. From the literatures, the leave extracts of *C. odorata* are found to be more beneficial than

the other parts. Phenolic compounds are one type of natural products that widely found in plants. Structurally, chlorogenic acid is a phenolic natural compound which is an ester of quinic acid and caffeic acid [8]. This active compound which isolated from this plant may be responsible for its pharmacological activities. *C. odorata* has showed its pharmacological activities such as anti-inflammatory, antimicrobial, blood coagulating, insecticidal and antioxidant activities [9].

Kaempferia parviflora Wall. ex Baker is known in common name as Krachai Dum, Thai Ginseng, Black Turmeric and Black Galingale, which belongs to Zingiberaceae family. It is an herbaceous plant found in the upper Northeastern regions of Thailand. Since ancient time, *K. parviflora* has been used for medicinal purposes in Thailand. In herbal medicine, it is generally used to promote health and to cure gastrointestinal disorder and anti-inflammation [10]. It is also used as an aphrodisiac for stimulating sexual performance in male. It has traditionally been used to improve vitality and treat of metabolic ailments [11]. According to Wasuntarawat *et al.* in 2010, *K. parviflora* could be eaten either fresh or dry rhizome before physical performance to improve physical work capacity [12]. The previous quantitative analysis using gas chromatographic method revealed that *K. parviflora* had 11 flavonoid constituents; thereby, 5,7,4′-trimethoxyflavone and 5,7-dimethoxyflavone were considered to be main constituents [10]. Additionally, the quantitative analysis of methoxyflavones in *K. parviflora* ethanolic extract using HPLC assay indicated that *K. parviflora* ethanolic extract contained several methoxyflavones. Some methoxyflavones such as 5,7,4′-trimethoxyflavone, 5,7-dimethoxyflavone, and 3,5,7,3′,4′-pentamethoxyflavone had been identified as the major components [13].

Zanthoxylum piperitum (L.) DC., belonging to the Rutaceae family, is known in common name as Japanese pepper, Sichuan pepper, Szechwan pepper, Japanese pricklyash, and Prig Horm in Thai. It is a deciduous aromatic shrub found in East Asia, North China, Japan, and Korea. It is broadly rounded to upright, spiny, deciduous or evergreen trees and shrubs. It has small green or yellow-green flowers appear in spring or summer, followed by small fruits that split to reveal seeds attached by short threads [14]. The active compound obtained from *Z. piperitum* fruits is

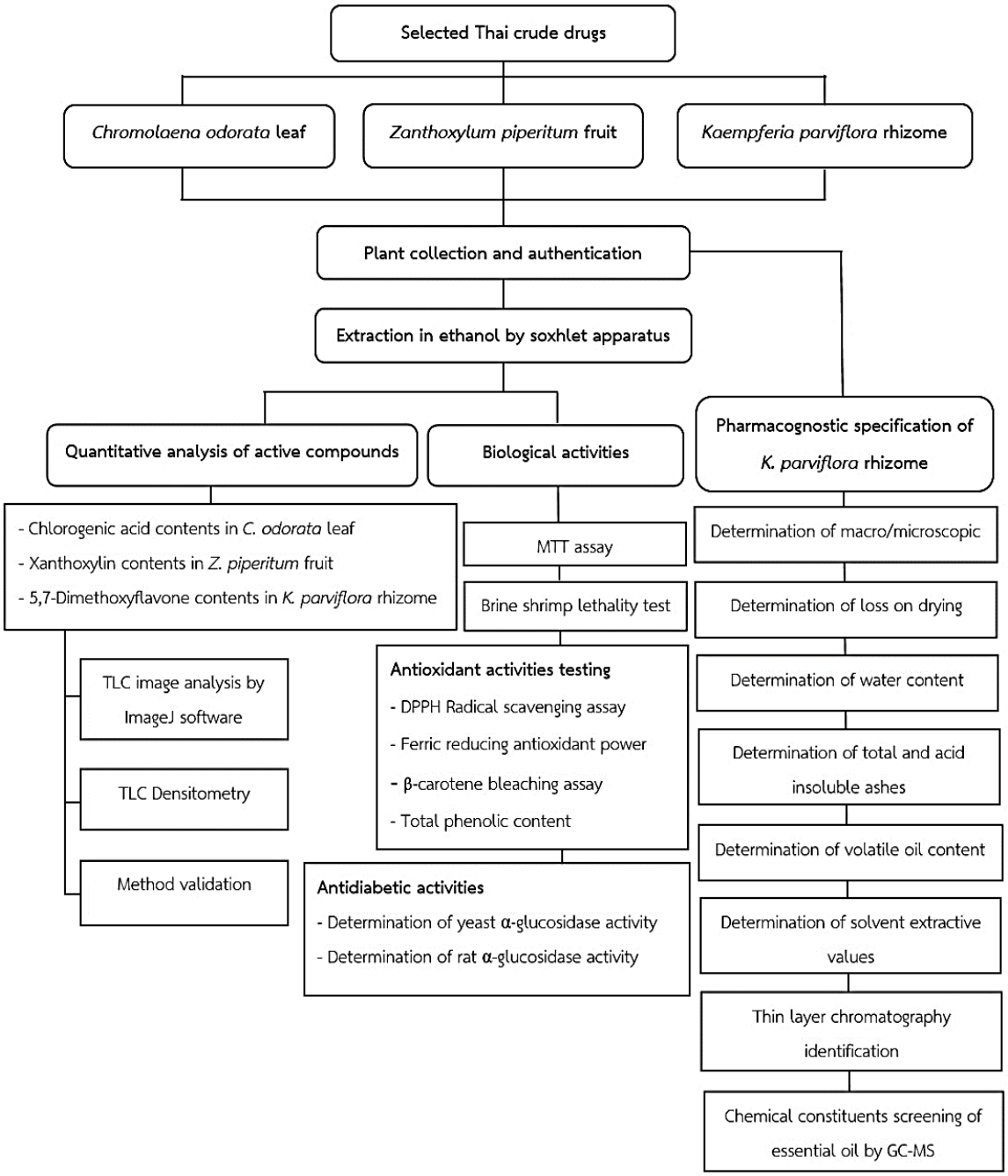
xanthoxylin which containing a methoxy group and ketone attached to the phenol structure [15]. In addition, the young leaves and fruits are commonly used as a spice or an ingredient of some spice mixtures in Japan because of their strong pungent taste and pleasant odor [16]. All parts of *Z. piperitum* are used to treat abdominal pain, vomiting and diarrhea [17].

Recently, there are many scientific researches which have reported on the application of several analytical methods for quantitation of active compounds in the plant materials. However, there have been no reported about the content of chlorogenic acid, xanthoxylin and 5,7-dimethoxyflavone in *C. odorata* leaves*, Z. piperitum* fruits, and *K. parviflora* rhizomes, respectively. For quantitative analysis, TLC-densitometry as well as TLC image analysis were developed. The screening of bioactive compounds from the herbal extract, the standardization, biological activities and quality control of raw herbal materials are more important to herbal medicine development [18]. Moreover, the Department of Thai traditional and alternative medicine has reported that *K. parviflora* has been selected as one of the five champion herbal products that has been promoted to make income for the country [19]. Even though *K. parviflora* has been widely used in Thai traditional medicine for a long time, the quality parameters of *K. parviflora* crude drug in Thailand have never been established. This study aimed to investigate the standardization parameters, to analyze chemical constituents of essential oil by gas chromatography - mass spectrometry (GC-MS), to determine the active component in all samples by thin layer chromatography (TLC), and to provide the scientific evidences in efficacy evaluation of these crude drug.

Objectives of the study

1. To evaluate the active constituents (chlorogenic acid, xanthoxylin and 5,7-dimethoxyflavone) in *C. odorata* leaves*, Z. piperitum* fruits, and *K. parviflora* rhizomes by TLC-densitometry compared to TLC image analysis.
2. To evaluate selected *in vitro* biological activities of ethanolic extract of *C. odorata* leaves*, Z. piperitum* fruits, and *K. parviflora* rhizomes.
3. To analyze chemical constituents of essential oil by gas chromatography - mass spectrometry (GC-MS) of *C. odorata* leaves*, Z. piperitum* fruits, and *K. parviflora* rhizomes.
4. To establish the pharmacognostic specification of *K. parviflora* dried rhizomes in Thailand.

Conceptual framework



CHAPTER II

LITERATURE REVIEW

Chromalaena odorata (L.) R.M. King & H. Rob.

Taxonomy

Kingdom: Plantae

Division: Tracheophyta

Class: Magnoliopsida

Order: Asterales

Family: Asteraceae

Genus: *Chromolaena*

Species: *Chromolaena odorata* (L.) R.M. King & H. Rob.

Plant description

A spreading, much-branched, tangled, thicket-forming, perennial shrub, up to 3 m tall, scrambling up to 7 m. Root system rather superficial, upper part of the root growing horizontally and swollen, but taproot growing deep and massive. Stem profusely branched, herbaceous when young, tough and semi-woody when older, cylindrical, finely striate, yellowish, shortly hairy or nearly glabrous; branches slightly ridged longitudinally, pubescent. Leaves simple, opposite; petiole 1-3 cm long or more, glabrous or sparingly pubescent; blade ovate-triangular, conspicuously 3(-5)-veined, 5-14 cm × 2-8 cm, acuminate, margins toothed, dotted with glands, sparsely hispid-hairy to glabrous, often purple when young. Inflorescence a homogamous, 10-35-flowered head, arranged in corymbose clusters arising from the axils of upper leaves; peduncle 1-2 cm long; involucre cylindrical, 8-10 mm × 3-4 mm, bracts in 5 or 6 rows, closely overlapping, oblong, increasing in size upwards, up to 10 mm × 3 mm, straw-coloured to greenish; corolla tubular, 5 mm long, 5-lobed, pale mauve, pale blue or whitish, protruding from the involucre; stigma with a long, exserted arm. Fruit a narrow achene, linear, angular, 3-5 mm long, brown or black, with short, white, stiff hairs along the edges; pappus white, consisting of rough bristles, 4-5 mm long. Seed minute [20].


Figure 1 Leaves and flowers of *Chromolaena odorata*

Distribution

Chromolaena odorata is native in the tropical areas of Asia as well as Central and South America to Argentina [3, 4].

Common names

Siam Weed

Christmas Bush

Devil Weed

Common Floss Flower

Vernacular name

Sab Suea (Thai)

Synonyms

Eupatorium odoratum L.

Eupatorium conyzoides M. Vahl

Traditional uses

It is sometimes grown as an ornamental and medicinal plant. It is widely used as a traditional medicine in Thailand, Indonesia and parts of Africa including Nigeria. In Thailand, *C. odorata* is locally used to treat skin diseases, diabetes, as well as insect bites. The resulting liquid from the crushed leaves can be used in the treatment of skin wounds.

In terms of medicinal uses, leaf extracts is used in the treatment of colds and sometimes sore throats when used with salt. Commercially, it is used as a scent in aromatic baths [5]. Evidently, it has been reported that *C. odorata* extract shows antimicrobial property against *Neisseria gonorrhoeae* which can cause gonorrhea in men [6]. Also, it has been revealed to accelerate blood clotting [7]. A decoction of the leaf is used in the treatment of malaria when used with lemon grass and guava leaves. Traditionally, fresh leaves or a decoction of *C. odorata* have been used throughout Vietnam for many years as well as in other tropical countries for the treatment of burn wounds, soft tissue wounds, leech bite, skin infection and dento-alveolitis. The poultice or juice pressed out of leaves is traditionally applied to stop bleeding and promote healing. It is also applied topically as an antidote against the sting from the spine of the common sea catfish. An aqueous decoction of the roots is used as an antipyretic and analgesic remedy [21, 22].

Chemical Constituents of *C. odorata*

The previous studies of *C. odorata* have revealed the presence of essential oils, steroids triterpenes and flavonoids [23-25]. The isolated compounds of this plant are shown in Table 1.

Table 1 Chemical constituents studies of *C. odorata*

Part of plant	Chemical constituents	References
Whole plant	2'-hydroxy-3,4,4',5',6'-pentamethoxy-chalcone 2',4-dihydroxy-4',5',6'-trimethoxychalcone Scutellarein tetramethyl ether Sinensetin 2'-hydroxy-4,4',5',6'-tetramethoxychalcone Aromadendrin 4' methyl ether Eriodicytol 7,4'-dimethyl ether Naringenin 4'-methyl ether Taxifolin 4'-methyl ether; taxifolin 7-methyl ether Quercetin 7,4'-dimethyl ether	[24, 26, 27]

	Kaempferol 4'-dimethyl ether Quercetin 3-O-rutinoside Quercetin 4'-methyl ether Quercetin 7-methyl ether Essential oils; Alpha-pinene Limonene *p*-Cymene cadinene Beta-caryophyllene Camphor Cardinal Germacrene D	
Leaves	Isosakuranetin 2'-hydroxy-4, 4', 5', 6'-tetramethoxy chalcone Tamarixetin Trihydroxymonomethoxyflavanone Pentaethoxyflavanone Dihydroxytrimethoxychalcone Eupatillin; 5,6,7,4'-Tetramethoxyflavanone 5-Hdroxy6,7,3',4'-tetramethoxyflavone Kaempferide Protocatechuic acid *p*-Coumaric acid *p*-Hydroxybenzoic acid Ferulic acid Vanillic acid Chlorogenic acid Sinensetin Rhamsetin Tetrahydroxymonomethoxyflavanone	[23, 28]

Flowers	Isosakuranetin Persicogenin Acacetin Luteolin Akuranetin 5,6,7,4'-tetramethoxyflavanone 4'-hydroxy-5,6,7-trimethoxyflavanone 2'-hydroxy-4, 4', 5', 6' tetramethoxychalcone 4,2'-dihydroxy-4', 5',6'-trimethoxychalcone 3,5,4'-trihydroxy-7-methoxyflavanone 5,7,3'-trihydroxy-5'-methoxyflavanone 3,5,7-trihydroxy-4'-methoxyflavanone 5, 7-dihydroxy-6-4'-dimethoxyflavanone	[24, 29, 30]
Roots	eupatoric acid Poriferasterol Octadecane butyrospermol acetate bis(2- ethylhexyl) phthalate chrysophanol physcion palmitic acid	[31]

Pharmacological activities of *Chromolaena odorata*

Anthelmintic activity

The anthelmintic potential of *C. odorata* whole plant was evaluated. The ethanolic extract at various concentrations of 10, 50 and 100 mg/ml were tested on *Pheretima posthuma.* Time of paralysis and time of death of the worms were investigated. The result showed that the ethanolic extract exhibited significant anthelmintic activity at highest concentration of 100 mg/ml. Piperazine citrate (10

mg/ml) and 1% gum acacia in normal saline were used as a reference standard and control, respectively [32].

Analgesic activity

The analgesic activity of ethanolic extract, petroleum ether extract and chloroform extract of *C. odorata* leaves was carried out. Aspirin was used as a standard drug. The ethanolic extract at a dose of 300 mg/kg exhibited the maximum analgesic activity while chloroform extract and petroleum ether extract exhibited moderate analgesic activity at the same dose [33].

Anti-inflammatory, antipyretic and antispasmodic properties

The anti-inflammatory and antipyretic activity of methanolic extract of *C. odorata* leaves were investigated. Additionally, the effects of the extract on intestinal transit of charcoal meal and castor oil-induced diarrhoea were also evaluated. The study showed that the extract at the dose of 50-200 mg/kg inhibited paw edema in rats and produced significant reduction in rectal temperature of mice rendered hyperthermic using yeast suspension. Antidiarrhoeal and antimotility effects were produced by the extract in intact mice [34].

Antimicrobial activity

Four flavones were isolated from *C. odorata* flowers and tested for antimycobacterial activity against *Mycobacterium tuberculosis*. The results showed that isosakuranetin had moderate antimycobacterial activity with the MIC value of 174.8 μM, while 4-hydroxy -5, 6, 7-trimethoxyflavanone, acacetin and luteolin had weak antimycobacterial activity with the MIC values of 606.0, 704.2 and 699.3 μM respectively. Moreover, acacetin exhibited moderate cytotoxicity against human small cell lung cancer (NCI-H187) cells with the MIC value of 24.6 μM, while luteolin showed moderate toxicity against NCI-H187 cells and week toxicity against human breast cancer cells with the MIC values of 19.2 and 38.4 μM, respectively [35].

Chomnawang et al. conducted the antimicrobial study of *C. odorata* against *Propionibacterium acnes* and *Staphylococcus epidermidis* using disc diffusion and broth dilution methods. The results of disc diffusion method demonstrated that *C.*

odorata showed good inhibition against *Propionibacterium acnes*. The MIC values were found to be 0.039 mg/ml for both bacterial strains, while the MBC values were found to be 0.039 and 0.156 mg/ml against *Propionibacterium acnes* and *Staphylococcus epidermidis* respectively [36].

Insecticidal activity

The essential oil extracts from *C. odorata* leaves were used to determine the efficacy on the mortality against maize grain weevil (*Sitophilus zeamais*). The LD_{50} of essential oil extract of *C. odorata* was found to be 6.78% which indicated the significant mortality of insect. The result revealed that the essential oils of *C. odorata* leaves might be used to take advantage for insect control in stored products [37].

Antioxidant properties

The antioxidant activity and free radical scavenging of ethanolic and methanolic extract of *C. odorata* leaves were evaluated. The percent DPPH radical inhibition of ethanolic extract, methanolic extract and ascorbic acid were found to be 59.10, 52.13 and 81.12 % respectively. Likewise, ethanolic and methanolic extract also revealed significant free radical scavenging potential and antioxidant properties against hydroxyl radical and nitric oxide [38].

Antigonorrhoeal activity

The alcoholic extracts of *C. odorata* leaves were used to determine the *in vitro* antigonorrhoeal activity against *Neisseria gonorrhoeae* isolated from symptomatic patients. The plants were macerated in 50% alcohol and the tincture and tested by standard bacteriological procedures. As the result, *C. odorata* inhibited five strains of *N. gonorrhoea* [39].

Anti-inflammatory activity

The aqueous extract of *C. odorata* was used to evaluate the anti-inflammatory activity using the cotton pellet granuloma, carrageenan-induced oedema and formalin-induced oedema methods. The different doses of extracts at 25, 50, 100 and 200 mg/kg were administered to the rats by oral route. In the cotton pellet method, granuloma

weight was significantly reduced from 14 ± 0.1 to 9.0 ± 0.1 mg. In the carrageenan method, the paw oedema was significantly reduced by all the doses of the extract administered, which the dose at 200 mg/kg produced the highest oedema inhibition (80.5%). In the formaldehyde induced arthritis, the extract inhibited the oedema during the 10-day period [40].

Hemostatic properties

Akomas *et al*. conducted the effects on bleeding and clotting times of ethanol extracts of *C. odorata* leaves in albino rats. The different doses of extracts at 150 mg/kg and 300 mg/kg body weight were administered orally in rats daily for 14 days. Aspirin (5mg/kg) was used as control drug in a non- treated group and another group. The bleeding and clotting times were investigated at the end of the treatment period. The ethanol extract of *C. odorata* showed a significant decrease in bleeding and clotting times. The results indicated that the leaves of *C. odorata* had a potent hemostatic properties [41].

***Kaempferia parviflora* Wall. ex Baker**

Taxonomy

Kingdom: Plantae

Division: Tracheophyta

Class: Liliopsida

Order: Zingiberales

Family: Zingiberaceae

Genus: *Kaempferia*

Species: *Kaempferia parviflora* Wall. ex Baker

Plant description of *Kaempferia parviflora*

It is an herbaceous plant with 50-70 cm tall. Rhizome is underground with purplish-black underneath the outer layer. The leaves are simple leaf with deep violet color, oval/elliptical shape, 5-10 cm wide, 10-15 cm length. The flowers are inflorescence and appear between the stem and the base of leaf, stalk 5-6 cm, petals fuse at base forming a tube, length 3-3.2 cm, split at tip. Stamens are sterile, white, oblong, 3 mm wide, 10-13 mm long and purple lip [42].

Figure 2 Leaves and rhizomes of *Kaempferia parviflora*

Distribution

Kaempferia parviflora is found in India, Myanmar, Thailand (Loei, Tak, Kanchana Buri and other northern provinces).

Common names

Black Turmeric

Black Galingale

Thai Ginseng

Vernacular name

Krachai Dum (Thai)

Synonyms

Kaempferia rubromarginata (S.Q.Tong) R.J.Searle

Stahlianthus rubromarginatus S.Q.Tong

Traditional uses

Since ancient time, its rhizome has been used for medicinal purposes in Thailand. In herbal medicine, they are applied as general health promoting agents, anti-inflammatory agents and gastrointestinal disorder drug [10]. They are also used as an aphrodisiac compound for stimulate sexual performance in male. It has traditionally been used to improve vitality and treat of metabolic ailments [11]. According to Wasuntarawat *et al.* in 2010, *K. parviflora* can be eaten either fresh or dry rhizome before physical performance to improve physical work capacity [12]. To ingest *K. parviflora*, the ground powder will be prepared in hot water or as an alcoholic solution. Even though the rhizome can be mixed with other herbs to make a nourishing tonic, the Thai traditional medicine institute suggested that the daily dose is ranged between 1.2 g of dried crude drug and 20 g of fresh rhizome [43].

Chemical constituents studies

The previous study on the quantitative analysis using gas chromatographic method revealed that *K. parviflora* had 11 flavonoid constituents, thereby 5,7-dimethoxyflavone and 5,7,4′-trimethoxyflavone were considered to be the major constituents [10]. The quantitative analysis of methoxyflavones in *K. parviflora* ethanolic extract using HPLC assay indicated that the *K. parviflora* ethanolic extract contained several methoxyflavones. Some methoxyflavones such as 3,5,7,3′,4′-

pentamethoxyflavone, 5,7-dimethoxyflavone and 5,7,4′-trimethoxyflavone had been identified as the major component [13]. *K. parviflora* mainly contains methoxyflavone which has a respectable *in vitro* antioxidant profile [44].

Pharmacological activities of *Kaempferia parviflora*

Antiplasmodial, antifungal and antibacterial activities

From the previous studies, *K. parviflora* has been shown beneficial on pharmacological activities. According to Yenjai *et al.* in 2004, the flavonoids 3,5,7,4′-tetramethoxyflavone and 5,7,4′-trimethoxyflavone which were isolated from *K. parviflora* showed mild antifungal and antimycobacterial activities. The flavonoid 5,7,4′-trimethoxyflavone also exhibited antiplasmodial against *Plasmodium falciparum* [45].

According to Waungsintaweekul *et al*., the volatile oils and the methanol extracts of *K. parviflora* were determined for susceptibility testing against eight bacteria and three fungi. The results reported that *K. parviflora* did not show any antimicrobial activity against tested organisms [46].

Anti-inflammatory activity

For anti-inflammatory activity, 5-hydroxy 3,7,3′,4′ tetramethoxyflavone compounds which isolated from *K. parviflora* rhizomes exhibited the inhibitory activity against the nitric oxide (NO) production with IC_{50} value of 16.1 μM. 5-Hydroxy 3,7,3′,4′ tetramethoxyflavone compound was also investigated on prostaglandin E_2 (PGE_2) and tumour necrosis factor-alpha (TNF-α) production. The results showed that this compound possessed a potent inhibitory effect on PGE_2 production, but a mild effect on TNF-α with IC_{50} of 16.3 μM and more than 100 μM respectively [47].

Aphrodisiac activity

The ethanolic extract, hexane extract and water extract of *K. parviflora* were conducted to examine the effects of feeding male rats for 3–5 weeks on the reproductive organs, aphrodisiac activity, fertility, sperm motility, and blood flow to the testis. As the result, three different extracts of *K. parviflora* had no effect on sperm motility or fertility. Whereas, the ethanolic extract of *K. parviflora* had an aphrodisiac activity which produced a significant increase blood flow in the testis of male rats [48].

Similar study was conducted to investigate the phosphodiesterase 5 (PDE5) and phosphodiesterase 6 (PDE6) inhibitory activities of *K. parviflora* rhizome extract and its 7-methoxyflavone compound using the two-step radioactive assay. Eight chemical compounds isolated from *K. parviflora* rhizomes including tectochrysin, 5,7-dimethoxyflavone, 5-hydroxy-7,4′-dimethoxyflavone, 5,7,4′-trimethoxyflavone, 5-hydroxy-3,7-dimethoxyflavone, 3,5,7-trimethoxyflavone, 5-hydroxy-3,7,4′-trimethoxyflavone and 3,5,7,3′,4′-pentamethoxyflavone were further examined on inhibitory activity against PDE5 and PDE6. The results demonstrated that 7-methoxyflavones from *K. parviflora* inhibited toward both enzymes. Especially, the compound 5,7-dimethoxyflavone had potent inhibitory activity against PDE5 inhibitor with IC_{50} value of 10.64 ± 2.09 μM. Structurally, the activity indicated that the relationship of methoxyl group at C-5 position of 7-methoxyflavones was needed for PDE5 inhibition [49].

Antioxidant activity

The antioxidant capacity of Krachai-Dum wines was investigated by DPPH scavenging assay and FRAP assay. Antioxidant capacity of wine samples by both methods showed similar results. The antioxidant activities in Krachai-Dum wine prepared by fermented peeled or unpeeled Krachai-Dum with tamarind juice are higher than those prepared by Krachai-Dum extracts. The total phenolic content was found to be 107.00 ± 9.27 to 306.62 ± 6.21 mgGAE/l. Krachai-Dum wines demonstrated potent antioxidant capacity on free radical scavenging activity and ferric reducing antioxidant power correlated with total phenolic content [50, 51].

According to Waungsintaweekul *et al*., The methanol extract of *K. parviflora* rhizome was conducted to investigate the antioxidant property by DPPH radical scavenging assay and lipid peroxidation assay. For DPPH assay, the methanolic extract of *K. parviflora* exhibited the radical scavenging activity with IC_{50} of 61.5 μg/ml. The result of lipid peroxidation assay exhibited that a reference standard (fisetin) at concentration of 50 μg/ml could inhibit lipid peroxide at the level of 10%, while methanolic extracts of *K. parviflora* exhibited less activity [46].

Anti-gastric ulcer effect

K. parviflora ethanolic extract exerts an anti-gastric ulcer activity on rat models. The extract was administered orally at doses of 30, 60 and 120 mg/kg. These extracts significantly inhibited gastric ulcer formation induced by indomethacin, HCl/EtOH and water immersion restraint-stress in rats. The gastric wall mucus was significantly protected by the extract pretreatment at doses of 60 and 120 mg/kg when the ulcerated rats were induced by ethanol. The results showed that *K. parviflora* ethanolic extract had gastroprotective potential to preserve gastric mucus secretion [50].

Anti-allergic activity

The seven methoxyflavone derivatives isolated from *K. parviflora* ethanolic extract were examined on anti-allergic activity. The compound 5-hydroxy-3,7,3′,4′-tetramethoxyflavone had highest anti-allergic activity against antigen-induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells with IC_{50} value of 8.0 μM, followed by 5-hydroxy-7-methoxyflavone and 5-hydroxy-7,4′-dimethoxyflavone with IC_{50} value of 20.6 and 26.0 μM respectively, while the other compounds exhibited moderate anti-allergic activity with IC_{50} value ranged from 37.5 to 66.5 μM.

Zanthoxylum piperitum (L.) DC.

Taxonomy

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Sapindales

Family: Rutaceae

Genus: *Zanthoxylum*

Species: *Zanthoxylum piperitum* (L.) DC.

Plant description

Zanthoxylum are broadly rounded to upright, spiny, deciduous or evergreen trees and shrubs. They have aromatic bark and leaves. Sprays of small green or yellow-green flowers appear in spring or summer, followed by small fruits that split to reveal seeds attached by short threads. It is a deciduous aromatic shrub. In spring plants bloom with yellow or green axillary flower clusters approximately 5 mm in diameter. Dioecious with female plants bearing 5 mm diameter green berries with a single large black seed. Berries turn scarlet in autumn and burst or spilt open to release the seed. Branches have pairs of long sharp thorns and odd, pinnately compound leaves alternately arranged, with 5 - 9 pairs of ovate leaflets having crenate (slightly serrated) margins [52].

Figure 3 Fruits of *Zanthoxylum piperitum*

Distribution

Zanthoxylum piperitum is native from East Asia to North China, Japan and Korea.

Common names

Japanese pepper

Sichuan pepper

Szechwan pepper

Japanese pricklyash

Vernacular name

Prig Horm (Thai)

Synonyms

Fagara piperita L.

Traditional uses

Since ancient time, they are applied as seed cooked. It is pulverized into a powder and used as a pepper substitute. The fruit can also be used. It is often heated in order to bring out its full flavor and can be mixed with salt for use as a table condiment. The dry-roasted fruit is an ingredient of the Chinese called “five spice powder”. The bark and leaves are used as a spice. Young leaves can also be used (raw or cooked). They are used in soups or as a flavoring in salads. The essential oils of green and ripe fruits and dried pericarp of the Japanese pepper, are also commonly used in Japanese dishes as spices. In herbal medicine, all parts of the plant are used to heal vomiting, diarrhea, and abdominal pain which the herbal drugs are administrated by oral route (macerated or decoction powder) [14].

Chemical constituents studies

Two serotonin derivatives including *N,N*-dimethylserotonin 5-*O*-*β*-glucoside and *N*-methylserotonin 5-*O*-*β*-glucoside were isolated from immature *Z. piperitum* seeds. The quantities were reaching up to 0.29% and 0.15% respectively. The spectroscopic

analyses and multi-step conversion reactions were used to determine their structures [53].

Tannins were also found in the fruit peels of *Z. piperitum* in the form of polymeric proanthocyanidins. These ones showed also certain capacity to suppress the antibiotic resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) [54].

Aliphatic acid amides have been isolated from the pericarp of *Z. piperitum* fruits and their chemical structures determined by MS and NMR spectroscopic. Structurally, these compounds contained a ketone and/or hydroxyl groups in the unsaturated aliphatic acid moiety of the structure of the amides [55].

Dried pericarp, green and ripe fruits of *Z. piperitum* were conducted to determine the essential oils using GC-MS and aroma extract dilution analysis. Oxygenated terpenes containing geranial, geraniol and citronellal were the major essential oil compositions in ripe fruit. Citronellal and geranial were found to be 20% of the essential oil. In the dried pericarp, the terpenoids seemed to induce the flavor character of the dried pericarp to be milder than that of the ripe fruit [56].

Pharmacological Activities of *Zanthoxylum piperitum*

Antioxidant activity

The antioxidant activity of methanol extract of *Z. piperitum* fruit were investigated. The major antioxidants of methanol extract of *Z. piperitum* fruit were found to be hyperoside and quercitrin. Their chemical structures determined by UV/Vis spectroscopy, mass spectrometry, HPLC, and TLC. Hyperoside and quercitrin from *Z. piperitum* fruit were conducted to evaluate the radical scavenging capacities using DPPH assay. The results showed that hyperoside and quercitrin possessed the radical scavenging activity with IC_{50} of 16 and 18 μM, respectively [57].

Similar study was conducted to investigate the antioxidant activity of dried pericarp and seed of *Z. piperitum*. The methanol extract from the seed and the ethyl acetate extract from the pericarp demonstrated antioxidant potential against linoleic

acid *via* the ferric thiocyanate and thiobarbituric acid (TBA) methods. The main antioxidants from the extract were identified to be arbutin and magnoflorine as determined by instrumental analyses which magnoflorine could be found only in the seed and not in the pericarp. For the DPPH method, ascorbic acid, BHT and trolox were used as reference standard. The results showed that arbutin and magnoflorine exhibited lower radical scavenging activity against the DPPH radical when compared with the standard [16].

Antibacterial activity

The essential oils of *Z. piperitum* were evaluated on the anitibacterial activity against foodborne pathogens using the agar well diffusion susceptibility method. The results showed that the essential oil (10 µg/well) of *Z. piperitum* were effective against all foodborne pathogens tested except *Salmonella choleraesuis*. Among all tested foodborne pathogens, the most susceptible to *Z. piperitum* essential oil were *B. subtilis* and *B. cereus.* Among the essential oil compositions of *Z. piperitum*, α-pinene and limonene possessed the potent antibacterial activity especially α-pinene. The MIC values of *Z. piperitum* essential oil were found to be 1.25 mg/ml, similar for all test foodborne pathogens. The MBC values were found to be 2.5 mg/ml for *B. subtilis* and *B. cereus.* The MBC of *Z. piperitum* essential oil were investigated to be lower on *L. monocytogenes*, *S. aureus*, *A. hydrophila* and *V. parahaemolyticus* with MBC values of 10 mg/ml than that of *S. choleraesuis*, *S. enterica* and *V. vulnificus* with MBC values of 20 mg/ml [58].

Tyrosinase activity

Two flavonols including quercitrin and quercetin isolated from *Z. piperitum* leaves were determined for the tyrosinase activity. Quercetin exhibited significant inhibition against mushroom tyrosinase with IC_{50} value of 3.8 µg/ml, and appeared to inhibit the polyphenol oxidase activity of tyrosinase in a competitive manner when L-tyrosine was used as a substrate [59].

Mosquito repellent

According to Kamsuk *et al*., 2007, the protective effects of *Z. piperitum* for repellency against mosquitoes were investigated under laboratory and field conditions using synthetic repellent standard, N,N-diethyl-3-methylbenzamide (DEET). As a result, the essential oil of *Z. piperitum* with 5% vanillin was the better repellency against a wide range of natural mosquito populations when compare to 25% DEET with 5% vanillin [60].

Antinociceptive activity

The essential oil of *Z. piperitum* was tested for antinociceptive activity and anti-inflammatory effect using animal models (formalin-, capsaicin-, and glutamate-induced paw licking and hot plate) and carrageenan-induced leukocyte migration into the subcutaneous air pouch (SAP) with measurement of cytokines respectively. The essential oil of *Z. piperitum* showed antinociceptive effect on formalin-induced licking, glutamate, and hot plate tests for the first phase, but it had no effect on capsaicin-induced paw licking and carrageenan-induced inflammation [61].

Phenolic compounds

Phenolic compounds are the biggest group of phytochemicals and they are chemical compounds that have one or more hydroxyl groups (OH) attached directly to an aromatic ring. Hence, plant constituents that possesses a phenol group; an aromatic ring bearing hydroxyl groups are classified as phenolic compounds. Plants produce phenolic compounds as secondary metabolites to interact with the environment [62].

Chlorogenic acid

Chlorogenic acid, one of the most abundant polyphenol compounds in the human diet, is a group of phenolic secondary metabolites produced by certain plant species and is an important component of coffee. Structurally, chlorogenic acid is a phenolic natural compound which is an ester of quinic acid and caffeic acid (Table 2). The common form of chlorogenic acid is also known as 5-O-caffeoylquinic acid (5-CQA) [63]. Coffee is a complex mixture of chemical compounds that provides significant amounts of chlorogenic acid. According to epidemiological research, the results of coffee ingestion has been claimed to prevent many chronic diseases such as Parkinson's disease, type 2 diabetes mellitus and liver disease (cirrhosis and hepatocellular carcinoma). Furthermore, chlorogenic acid are also known as an antioxidant which also slows the release of glucose into the bloodstream after a meal [64-66]. Lipid metabolic homeostasis and intracellular glucose are vital for maintaining basic life activities of a cell or an organism. Glucose and lipid metabolic disorders are closely related with the occurrence and progression of diabetes, obesity, hepatic steatosis, cardiovascular disease, and cancer. Recently, the roles and applications of chlorogenic acid, particularly in relation to glucose and lipid metabolism have been highlighted [65].

Chlorogenic acid and caffeic acid were investigated on the beneficial effects using *in vitro* and *in vivo* antioxidant experiments. The measurement of superoxide anion and the radical chain-breaking activity assay were carried out. For the *in vivo* study, Caco-2 cells were used as an intestinal model and the intestinal ischemia–

reperfusion model was used to evaluate antioxidant activities. For the *in vitro* study, caffeic acid had stronger antioxidant activity than that of chlorogenic acid. The uptake of chlorogenic acid by Caco-2 cells was much less than that of caffeic acid. The results found that both chlorogenic acid and caffeic acid had effects on intestinal ischemia–reperfusion injury [64].

Commercial whole coffee fruit extracts were analyzed for chlorogenic acids, caffeine and antioxidant activities. Chlorogenic acid and caffeine were characterized by HPLC and LC–MS and quantified by UV absorbance. The antioxidant capacities including peroxyl radical scavenging capacity (ORAC assay), hydroxyl radical scavenging capacity (HORAC assay), peroxynitrite scavenging capacity (NORAC assay), superoxide anion scavenging assay (SORAC assay) and singlet oxygen scavenging assay (SOAC assay) were investigated. The results indicated that total antioxidant activity of whole coffee fruit extracts displayed strong correlation to chlorogenic acid content [67].

The anti-inflammatory, antinociceptive and antipyretic activities of chlorogenic acid in rats was evaluated. The results demonstrated that chlorogenic acid at doses of 50 and 100 mg/kg inhibited carrageenan-induced paw edema beginning at the 2nd hour of the experimental procedure when compared to control. Moreover, chlorogenic acid at doses of 50 and 100 mg/kg also inhibited the number of flinches in the late phase of formalin-induced pain test. On the other hand, even at the highest tested dose (200 mg/kg), chlorogenic acid did not inhibit the febrile response induced by lipopolysaccharide (LPS) in rats [68].

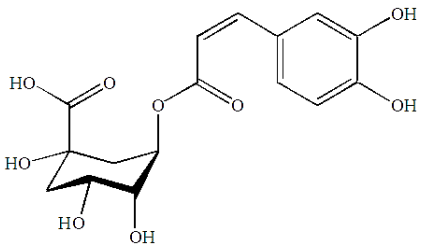
The antibacterial activity and mechanism of action of chlorogenic acid against bacteria were investigated. The results showed that chlorogenic acid effectively inhibited the growth of all tested bacterial pathogens and the MIC values were ranging from 20 to 80 µg/ml. An investigation on action mode of chlorogenic acid against the pathogen indicated that chlorogenic acid significantly increased the outer and plasma membrane permeability resulting in the loss of the barrier function, even inducing slight leakage of nucleotide. The leakage of cytoplasmic contents was also observed by electron micrographs. These results supported that chlorogenic acid bound to the

outer membrane, disrupted the membrane, exhausted the intracellular potential, and released cytoplasm macromolecules which led to cell death [69].

Three major constituents of an extract of mulberry leaves (*Morus alba* L.) including chlorogenic acid, rutin and isoquercitrin were determined the *in vivo* anti-diabetic activity. Quantities of the three constituents of interest in the extract were determined by HPLC-DAD. Activity was determined using a type II diabetic rat model. After 11 days of *per oral* administration of 250 and 750 mg/kg of extract and the corresponding amounts of each individual compound, a dose dependent decrease of non-fasting blood glucose levels were found for mulberry leaves, chlorogenic acid and rutin, but not for isoquercitrin [70].

The efficacy of chlorogenic acid on altering body fat in high-fat diet induced-obese mice compared to caffeic acid was investigated. Both caffeic acid and chlorogenic acid significantly lowered body weight, visceral fat mass and plasma leptin and insulin levels compared to the high-fat control group. They also lowered triglyceride (in plasma, liver and heart) and cholesterol (in plasma, adipose tissue and heart) concentrations. Triglyceride content in adipose tissue was significantly lowered, whereas the plasma adiponectin level was elevated by chlorogenic acid supplementation compared to the high-fat control group. Body weight was significantly correlated with plasma leptin and insulin levels, respectively. These results suggested that caffeic acid and chlorogenic acid improve body weight, lipid metabolism and obesity-related hormones levels in high-fat fed mice. Chlorogenic acid seemed to be more potent for body weight reduction and regulation of lipid metabolism than caffeic acid [71].

Table 2 Chemical descriptions of chlorogenic acid [72]



Chemical name: Chlorogenic acid

IUPAC name: (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5 trihydroxycyclohexane-1-carboxylic acid

Synonym: 3-Caffeoylquinic acid

Molecular weight: 354.311 g/mol

Molecular formula: $C_{16}H_{18}O_9$

Melting point: 205 to 209 °C

5,7-Dimethoxyflavone

Flavonoids constitute one of the most important groups of phenolic compounds in plant. Flavonoids have the C_6-C_3-C_6 (C_{15}) general structural backbone in which the two C6 units are phenolic nature. According to the hydroxylation pattern and variations in the chromane ring, flavonoids can be further divided into different sub-groups such as flavones, flavanones, flavonols and anthocyanins [73].

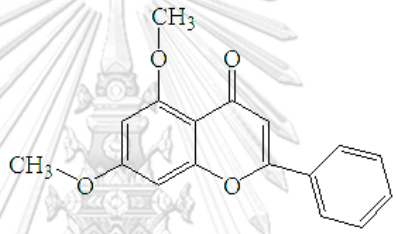
5,7-Dimethoxyflavone is a constituent that found in tea such as *Leptospermum scoparium* (red tea). It is flavonoids with methoxy groups attached to the C_7 atom of the flavonoid backbone which belongs to the class of organic compounds known as 7-O-methylated flavonoids [74]. The structure and chemical descriptions of 5,7-dimethoxyflavone are shown in Table 3.

The compound 5,7-dimethoxyflavone was investigated for anti-inflammatory activity using rat paw edema model. Aspirin was used as reference drugs. This compound showed no inhibitory effect on cotton pellet-induced granuloma formation. However, 5,7-dimethoxyflavone showed an antiexudative effect and inhibited prostaglandin biosynthesis, as well as interfered with leukocyte migration [75].

Identification and evaluation of anti-inflammatory compounds from *Kaempferia parviflora* were carried out. The specific compounds from the hexane extract of *K. parviflora* were investigated by gas chromatography-mass spectrometry. There were 5,7-dimethoxyflavone (DMF), 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (TMF), 3,5,7-trimethoxyflavone, 5-hydroxy-7,4′-dimethoxyflavone and 3,5,7,4′-tetramethoxyflavone. The anti-inflammatory activities were tested in rat basophilic leukemia (RBL-2H3) cells stimulated with an IgE antigen and a calcium ionophore. The results found that DMF and TMF more potently inhibited antigen-induced degranulation than nobiletin, a well-known anti-inflammatory agent. In addition, when compared to RBL-2H3 cells stimulated with a calcium ionophore, those treated with DMF and TMF showed more marked inhibition of the degranulation and the production and mRNA expression of inflammatory mediators [76].

The vascular effects of DMF, isolated from the rhizomes of *Kaempferia parviflora*, on rat isolated aortic rings and its possible mechanisms were investigated. The results demonstrated that DMF caused endothelium-dependent relaxation. Additionally, DMF-induced responses are mainly due to increasing K^+ efflux, and inhibition of Ca^{2+} influx from the extracellular space. The vasodilator effects of DMF provided experimental support for the potential use of *K. parviflora* as a medical plant in the treatment of cardiovascular diseases [77].

Table 3 Chemical descriptions of 5,7–dimethoxyflavone [74]



Chemical name: 5,7-Dimethoxyflavone

IUPAC name: 5,7-dimethoxy-2-phenylchromen-4-one

Synonym: Dimethylchrysin

Molecular weight: 282.295 g/mol

Molecular formula: $C_{17}H_{14}O_4$

Melting point: 154 °C

Xanthoxylin

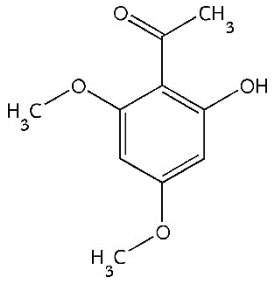
Xanthoxylin is generally found in fats and oils. It is obtained from *Zanthoxylum piperitum* (Japanese pepper tree) and *Sapium sebiferum* (Chinese tallowtree). This compound belongs to methoxyphenols family and the class of chemical compounds known as alkyl-phenylketones which containing a ketone substituted by one alkyl group, and a phenyl group attached to the phenol structure [15]. The structure and chemical descriptions of xanthoxylin are shown in Table 4.

The effects of xanthoxylin on melanogenesis was investigated by measure melanin content at 405 nm, mRNA expression of regulatory melanogenesis proteins using RT-PCR and dendriticity in mouse B16F10 melanoma cells using photograph from microscope. The results revealed that xanthoxylin increased melanin content, mRNA expression of regulatory melanogenesis proteins (tyrosinase and Mitf expressions) as well as increases dendrites of mouse B16F10 melanoma cells. However, xanthoxylin had no effect on viability of mouse B16F10 melanoma cells [78].

Xanthoxylin was tested for fungicidal and fungiostatic effects against yeast and mycelial fungi which presented as MIC and MFC values. The tested compounds were effective against *C. albicans, M. canis, T. rubrum, A. flavus, A. parasiticum* and *Penicillium.* However, the xanthoxylin isolated from leaves and stems of *Sebastiania schottiana* displayed the highest activity against *M. canis* (72-T) with MIC and MFC values of 31.2 and 62.5 µg/ml, respectively [79].

The synthetic chalcones obtained from xanthoxylin were investigated for antinociceptive effects using writhing test and visceral pain model in mice. Aspirin and acetaminophen were used as reference standard. The study reported that four chalcones displayed significant antinociceptive activities which were more effective than reference drugs [80].

Table 4 Chemical descriptions of xanthoxylin [15]



Chemical name: xanthoxylin

IUPAC name: 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone

Synonym: 2-hydroxy-4,6-dimethoxyacetophenone

Molecular weight: 196.202 g/mol

Molecular formula: $C_{10}H_{12}O_4$

Melting point: 85-88 °C

Quality control method for herbal material [81]

Macroscopic and microscopic examination

An examination of macroscopic is the first process to determine the characteristics, identity and degree of purity of medicinal plant materials, and should be carried out before any further tests are undertaken. Macroscopic identity of herbal materials is based on shape, size, color, texture, surface characteristics, fracture characteristics and appearance of the cut surface. Microscopic inspection of herbal materials is investigation for the identification of broken or powdered materials.

Determination of extractable matter

Extractable matters represent the amount of active constituents from plant materials. The plant material is extracted with specified solvents such as water and ethanol. Ethanol is used for the slightly non-polar substances whereas water was used for the polar substances.

Determination of water content

Azeotropic method is specifically used for measurement of water content in plant material specification. An excess of water in herbal materials will encourage microbial growth, the presence of insects or fungi, and deterioration following hydrolysis. The azeotropic distillation method gives a direct measurement of the water present in the material being examined. The plant material is distilled together with a water immiscible solvent such as toluene or xylene. Solvent should be saturated with water before use to avoid water absorption in solvent for accurate result.

Determination of loss on drying

The test for loss on drying determines both water and volatile matters. Drying can be carried out by heating in oven at 100-105°C and cooling in desiccator.

Determination of total ash and acid insoluble ash

Ash values are helpful in determination of the purity and quality which indicate the inorganic substances in plant materials. Total ash method determines the total

amount of non-volatile inorganic matters remaining in herbal materials after complete incineration at 500°C.

The remaining insoluble matter is acid insoluble ash which is mainly silica (silicon dioxide) obtained after boiling the total ash with 70 g/l hydrochloric acid and incinerating.

Determination of volatile oil

Volatile oils are characterized by their odor, oil-like appearance and ability to volatilize at room temperature. The chemical compounds of volatile oils are usually comprised of monoterpene, sesquiterpenes and their oxygenated derivatives. Aromatic compounds predominate in certain volatile oils. In order to determine the volatile oil, the plant material is distilled in water using Clevenger apparatus (Figure 4) [81].

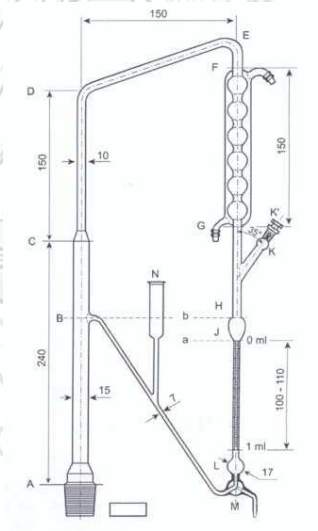


Figure 4 Clevenger apparatus for volatile oil extraction

Gas chromatography-mass spectrometry (GC-MS)

The gas chromatography (GC) is the widely used analytical equipment for plant materials by reason of their high resolving power and low limit of detection, good accuracy and high reproducibility. GC is used to separate the mixture of compounds that are naturally volatile or that can be converted to volatile derivatives. Compounds with a lower molecular weight will elute out earlier than compounds with higher molecular weights due to differences in boiling points. Mass spectrometer (MS) is a

kind of detector instrument which uses electron or chemical to ionize the chemical compound and measures the mass to charge (m/z) ratio of ions based on the details of motion of the ions as they transit through electromagnetic fields. For the process, the sample solution is injected into the GC inlet where it is vaporized and swept onto a chromatographic column by the carrier gas. The inert carrier gas is used as mobile phase such as helium, nitrogen, hydrogen and argon. The sample flows through the column and the compounds comprising the mixture of interest are separated by virtue of their relative interaction with the coating of the column (stationary phase) and the carrier gas (mobile phase). The latter part of the column passes through a heated transfer line and ends at the entrance to ion source where compounds eluting from the column are converted to ions and detected according to their mass to charge ratio (Figure 5) [82, 83].

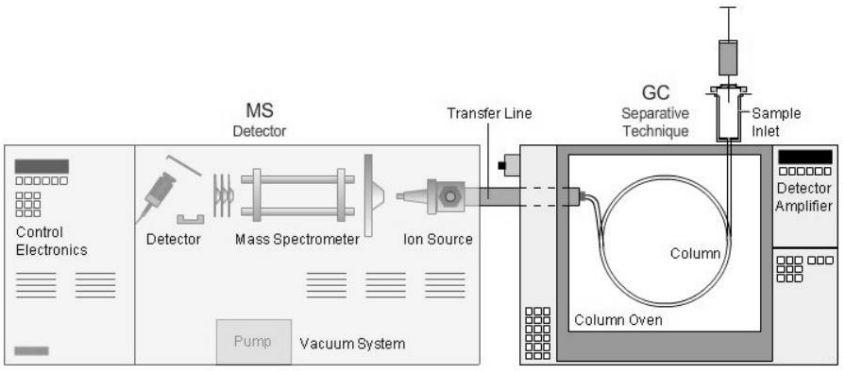


Figure 5 Schematic diagram of GC-MS [83]

Thin layer chromatography

Thin layer chromatography (TLC) is a planar chromatographic technique that is a simple and fast method to separate and to identify the substances. It can be used to check purity of compounds and used for quantitative analysis. TLC includes of spotting, development and visualization steps. The advantages of this technique are easy to use, inexpensive, short time for analysis. TLC system consists of mobile phase and stationary phase. During the procedure, a mobile phase (eluent) distributes the compounds present in a mixture over a stationary phase (adsorbent). The result of TLC can be detected when the spots are visualized under UV light or with suitable detection reagents [84].

The retention factor (Rf) is a calculated value for the distance of the spots from compound appear from origin in TLC plates and the distance moved of the solvent from origin. The Rf value can be used for identify the compounds under the same conditions. The Rf values can be calculated using the following formula:

$$Rf = \frac{distance\ of\ compound\ from\ origin}{distance\ of\ solvent\ front\ from\ origin}$$

TLC- Densitometry

The compound separated by TLC are quantified using TLC densitometer. Densitometry is the quantitative and qualitative measurement of absorbed visible, UV light or emitted fluorescence upon excitation with UV light [85]. The TLC densitometer (CAMAG TLC scanner 4) contains a single wavelength, multiple wavelengths up to 31 selected wavelengths or a combination of measurements in absorption and fluorescence detection mode. It transforms the selected compound on TLC plate into digital computer data using winCATS software. It can evaluate a reflection in absorbance or fluorescence mode with the spectrums range from 190-900 nm. There is three light sources including of deuterium lamp (190-450 nm), halogen-tungsten lamp in the visible region (350-900 nm) and high pressure mercury lamp (254-578 nm) [86].

TLC-image analysis

ImageJ is a public domain Java image processing and analysis program developed at the National Institutes of Health. It is a free software which can quantitate and calculate pixel intensity in digital image of TLC spot and transform to chromatographic peak. It can calculate area and pixel value statistics of defined selections by user [87].

Antioxidant activities

DPPH radical scavenging assay

DPPH (2, 2 diphenyl-1-picrylhydrazyl) is a stable free radical form which gives characteristics of deep purple color with a maximum absorbance of 517 nm. This method evaluates the ability to scavenge DPPH radical by antioxidant compounds. The delocalization of DPPH molecule due to the donation of hydrogen or electron to quench the DPPH radical. The color turns from purple to yellow when receives hydrogen atom from antioxidant (Figure 6) [88].

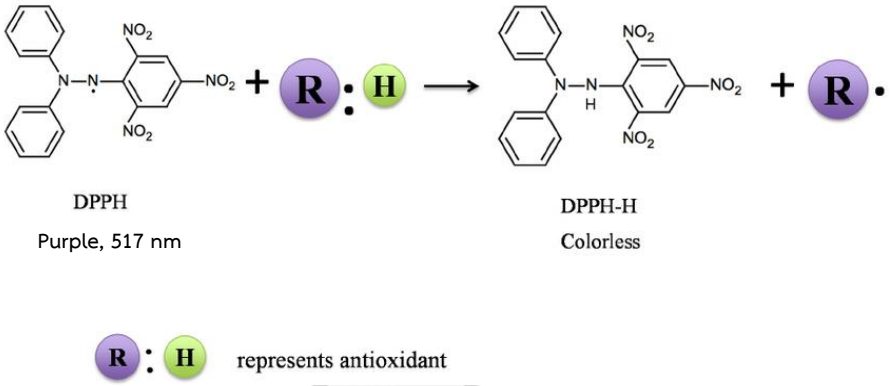


Figure 6 DPPH and antioxidant reaction

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay is used to measure the ability of an antioxidant compound to reduce ferric ions into ferrous complex. This method uses ferric tripyridyltriazine, complex compound of iron as the tested compounds. Iron atom of ferric ion (Fe^{3+} -TPTZ) is reduced by the antioxidant and produces a complex compound of ferrous ion (Fe^{2+} -TPTZ) that gives the blue color at the maximum absorbance of 593 nm [89].

ß-carotene bleaching assay

The ß-carotene bleaching assay is the determination of the antioxidant ability to inhibit lipid peroxidation which delay the bleaching of ß-carotene and linoleic acid emulsion. Bleaching is based on the loss of the yellow color of ß-carotene due to its

reaction with radicals, which are formed by linoleic acid oxidation in an emulsion. The rate of ß-carotene bleaching can be slowed down in the presence of antioxidants [90].

Total phenolic content

Colorimetric reactions are widely used in the UV/VIS spectrophotometric method, which is easy to perform, rapid and applicable in routine laboratory use, and low-cost. However, it is important that colorimetric assay need to use a reference substance. Then this method measures the total concentration of phenolic hydroxyl groups in the plant extract using the Folin-Ciocalteu assay and a phenolic acid such as gallic acid to set up a calibration curve. This assay is based on the oxidation of a phenolate ion from the sample and the reduction of the phosphotungstic-phosphomolybdic reagent (Folin-Ciocalteu reagent) to form a blue complex of phosphotungstic-phosphomolybdenum in the alkaline solution that can be quantified by visible-light spectrophotometry [91].

***In vitro* α-glucosidase inhibitory activity**

Diabetes is described as a metabolic diseases in a person which has high blood sugar, either because the body doesn't produce enough insulin or cells do not respond to the insulin that is produced. This high blood sugar will produce the classical symptoms of polyuria, polydipsia and polyphagia. There are two major types of diabetes.

Type 1 diabetes used to be known as insulin-dependent diabetes, or juvenile-onset diabetes as it often begins in childhood. Type 1 diabetes is an autoimmune condition where the immune system wrongly identifies and subsequently attacks the pancreatic cells that produce insulin, leading to little or no insulin production.

Type 2 diabetes used to be known as non-insulin dependent diabetes and adult onset diabetes, but it is commonly increase in children, mostly due to children being more likely to be overweight or obese. In this condition, the body usually still produces some insulin, but this is not enough to meet demand and the body's cells do not properly respond to the insulin. The latter effect is called insulin resistance,

where consistently elevated blood glucose has caused cells to be overexposed to insulin, making them less responsive or unresponsive to the hormonal messenger [92].

Alpha-glucosidases are two complex enzymes located in the brush border of the small intestine that acts upon alpha 1, 4-glycosidic bonds. Inhibition of this enzyme system reduces the rate of digestion of starch. Less glucose is absorbed because the starch are not broken down into glucose molecules. Starch is digested by salivary and pancreatic α-amylase to form oligosaccharide-dextrins. Alpha-glucosidases cleave dextrins to absorbable glucose in small intestine. The synthetic or natural α-glucosidase inhibitors are interested as therapeutics to delay postprandial hyperglycemia in Type 2 diabetes [93]. Anti-diabetic drugs orally used for diabetes mellitus type 2 such as acarbose, miglitol, nojirimycin and 1-deoxynojirimycin act as competitive inhibitors of α-glucosidase [94].

Brine shrimp lethality assay

Brine shrimp (*Artemia salina*) is an aquatic species. It always uses for toxicological activity test of the natural products because it is a sensitive indicator species. The cytotoxic activity can be screened by the concentration of medicinal plant extracts and brine shrimp lethality relationship. In several studies, brine shrimp lethality assay has been an authentic assay to estimate toxicity of the compounds or the extracts [95-97]. For interpretation and data analysis of the extract, the results of brine shrimp lethality assay are interpreted as LC_{50} values: LC_{50} values > 1000 µg/ml (non - toxicity), ≥ 500 ≤ 1000 µg/ml (weak toxicity) and < 500 µg/ml (toxic) [98].

MTT cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay has been used to quantitate cell survival and proliferation in macrophage-mediated cytotoxicity. Colorimetric assay is used based on the capacity of succinate dehydrogenase in mitochondria of viable cells to transform the MTT tetrazolium salt into MTT formazan (5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan). The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The advantages of this method is savings in costs, reagents and equipment,

reducing labor through the elimination of sample processing steps for liquid scintillation and gamma counting as well as avoiding use of radioisotopes. The result will be expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value). According to the American NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with IC_{50} ≤20 µg/ml, while this value was deemed at ≤4 µg/ml for a pure compound [99].

CHAPTER III

MATERIALS AND METHODOLOGY

Chemicals and reagents

2, 2-diphenyl-1-picylhydrazyl (DPPH)	(Sigma-Aldrich, St. Louis, USA)
2, 4, 6-Tripyridyl-s-triazine (TPTZ)	(Sigma-Aldrich, St. Louis, USA)
3, 5-Di-tert-4-butylhydroxytoluene (BHT)	(Sigma-Aldrich, St. Louis, USA)
5, 7-dimethoxyflavone	(Sigma-Aldrich, St. Louis, USA)
Acarbose	(Sigma-Aldrich, St. Louis, USA)
Acetone	(Merck, Darmstadt, Germany)
Alpha-glucosidase from Saccharomyces cerevisiae	(Sigma-Aldrich, St. Louis, USA)
Beta-carotene	(Fulka, USA)
Chloroform, AR grade	(RCI Labscan, Thailand)
Chlorogenic acid	(Sigma-Aldrich, St. Louis, USA)
Dimethyl sulfoxide (DMSO)	(Merck, Darmstadt, Germany)
Di-Sodium hydrogen phosphate (Na_2HPO_4)	(Sigma-Aldrich, St. Louis, USA)
Doxorubicin	(Sigma-Aldrich, Germany)
Ethanol, AR grade	(RCI Labscan, Thailand)
Ethyl acetate, AR grade	(RCI Labscan, Thailand)
Fetal calf serum	(Biochrom Gmbh, Germany)
Folin-Ciocalteu reagent	(Merck, Darmstadt, Germany)
Formic acid	(Fisher Scientific, Leicestershire, UK)
Hydrochloric acid 37%, AR grade	(RCI Labscan, Thailand)
Intestinal acetone powders from rat	(Sigma-Aldrich, St. Louis, USA)
Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$)	(Sigma-Aldrich, St. Louis, USA)
Iron (III) chloride ($FeCl_3 \cdot 6H_2O$)	(Ajax Finechem, New Zealand)
Linoleic acid	(Sigma-Aldrich, St. Louis, USA)
Methanol, AR grade	(RCI Labscan, Thailand)
MTT	(Invitrogen, USA)

Normal saline	(General Hospital Product Public Co., Ltd., Thailand)
p-nitrophenyl-α-D-glucopyranoside (PNP-G)	(Sigma-Aldrich, St. Louis, USA)
Potassium chloride (KCl)	(Merck, Darmstadt, Germany)
Potassium dihydrogen phosphate (KH_2PO_4)	(Merck, Darmstadt, Germany)
Quercetin hydrate	(Sigma-Aldrich, St. Louis, USA)
RMPI 1640	(Biochrom Gmbh, Germany)
Sodium bicarbonate (Na_2HCO_3)	(Sigma-Aldrich, St. Louis, USA)
Sodium carbonate (Na_2CO_3)	(QRëC, New Zealand)
Sodium chloride (NaCl)	(Ajax Finechem, New Zealand)
Toluene	(RCI Labscan, Thailand)
Tween 20	(Merck, Darmstadt, Germany)
Trypsin	(Sigma-Aldrich, Germany)
Xanthoxylin	(Sigma-Aldrich, St. Louis, USA)

Materials

Microtiter plate with 96 wells	(BRAND plates, Wertheim®, Germany)
Filter paper No.4	(WhatmanTM paper, UK)
Filter paper No.40 ashless	(WhatmanTM paper, UK)
Tissue culture flask 25 cm2	(Constar, USA)
Tissue culture plate 96 well	(Constar, USA)
TLC Silica gel 60 F254	(Merck, Darmstadt, Germany)

Instruments and equipments

Autoclave (Model: HVE-50)	(hirayama, Tokyo, Japan)
AxioVision40 software (V 4.6.3.0)	(Zeiss Inc., Germany)
CAMAG TLC Plate Heater III	(CAMAG, Switzerland)
CAMAG TLC Chamber	(CAMAG, Switzerland)
CAMAG TLC Scanner 4	(CAMAG, Switzerland)

CO_2 incubator forma series II	(Thermo Electron Corporation, USA)
Refrigerated centrifuge	(Sigma, Germany)
Ashing furnaces	(Carbolite, Scientific Promotion, Bangkok, Thailand)
Digital balance (Model: SI-234)	(Denver Instrument, New York, USA)
Digital camera (Canon PowerShot A640)	(Canon Inc., Japan)
Digital orbital shaker (Model: SHO-2D)	(Daihan Scientific, Korea)
Gas chromatography (GC) / mass spectrometry	(Thermo Finnigan model Trace GC Ultra equipped with Finnigan DSQ MS detector, USA)
Hemocytometer deep 1/10 nm	(BOECO, Germany)
Hot air oven	(WTB binder, Scientific Promotion, Bangkok, Thailand)
Image J software	(National Institutes of Health, USA)
Incubator	(Memmert, Germany)
Invert microscope	(Nikon, Japan)
Laminar hood (Model: Class II BSC)	(ESCO, Singapore)
Microplate reader (Anthos Zenyth 200 RT)	(Biochrom, England)
Microplate reader Multiskan FC 540 nm	(Thermo Scientific, USA)
Microscope (Axio imager A2)	(Zeiss Inc., Germany)
Plate mixer SH30	(Bio-Active Co., Ltd.)
Rotary evaporation	(Buchi, Switzerland)
TLC syringe	(Hamilton Company, USA)
Ultra-pure water purification NW20VF	(Heal Force, China)
Ultrasonic bath	(Analytical Lab Science Co., LTD, Bangkok, Thailand)
UV viewing cabinet	(Spectronics Corp., USA)
Vortex mixer (K-550-GE)	(Scientific Industries, Inc., USA)

Water bath	(Brinkmann, USA)
winCATS software	(CAMAG, Switzerland)

Cell lines

Five human cancer cell lines

BT-474 (Ductal carcinoma breast)	(ATCC, USA)
CHAGO-K1 (Undifferentiated lung carcinoma)	(ATCC, USA)
Hep-G2 (Liver hepatoblastoma)	(ATCC, USA)
KATO-3 (Gastric carcinoma)	(ATCC, USA)
SW-620 (Colon adenocarcinoma)	(ATCC, USA)

One human normal cell line

Wi-38 (Lung fibroblast)	(ATCC, USA)

Sample collection

Ten samples of *Chromolaena odorata* leaves were collected from 10 different locations throughout Bangkok Metropolis of Thailand. Fifteen samples of *Kaempferia parviflora* rhizomes and *Zanthoxylum piperitum* fruits were purchased from 15 Thai traditional drug stores throughout Thailand. All sets of crude drug were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi. The voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand. After removal of any foreign matters, each authentic sample was air dried and pulverized into powders.

Plant extraction

Plant materials were be pulverized and exhaustively extracted with ethanol by Soxhlet apparatus. The extract was filtered through Whatman No.4 filter paper and evaporated to dryness *in vacuo*. The yield was recorded and the extract was stored at -20°C.

Quantitative analysis of 5,7-dimethoxyflavone in *Kaempferia parviflora* rhizomes

Preparation of standard solution of 5,7-dimethoxyflavone

One milligram of standard 5,7-dimethoxyflavone was dissolved in 1 ml of 95% ethanol. The stock solution was diluted to obtain the series of standard solution range from 0.2 to 1 mg/ml. These solutions were stored in refrigerator at 4°C.

Preparation of ethanol extracts of *Kaempferia parviflora* rhizomes

Five grams of *K. parviflora* powder was exhaustively extracted with 95% ethanol by Soxhlet apparatus, filtered and evaporated to dryness. The extract was dissolved with ethanol to get the concentration of 2 mg/ml. These extract were stored in refrigerator at 4°C and further used for TLC densitometry and TLC image analysis.

TLC-densitometry

Three microliters of 15 ethanol extracts and standard solutions were applied onto the Silica gel 60 GF_{254} TLC plate by Linomat 5 applicator. The plate was developed in a TLC chamber that contained a mixture of toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2), then the plate was removed and allowed to dry at room temperature. After that, the same TLC plate was developed again for two more times to increase the distance of the band. After development, the plate was dried and scanned with CAMAG TLC Scanner 4 (CAMAG, Switzerland) under wavelength of maximum absorbance (265 nm). The calibration curve of 5,7-dimethoxyflavone was prepared by plotting peak areas versus concentrations of 5,7-dimethoxyflavone in µg/spot.

TLC image analysis by ImageJ software

The 5,7-dimethoxyflavone spots on the developed TLC plates were photographed under short wave ultraviolet light (254 nm) by a digital camera. Peak area of each spot was quantitated using ImageJ software. The content of 5,7-dimethoxyflavone was determined by comparing peak area to the calibration curve obtained from the same TLC plate.

Quantitative analysis of chlorogenic acid in *Chromolaena odorata* leaves

Preparation of standard solution of chlorogenic acid

One milligram of standard chlorogenic acid was dissolved in 1 ml of 95% ethanol. The stock solution was diluted to obtain the series of standard solution range from 0.05 to 0.45 mg/ml. These solutions were stored in refrigerator at 4°C.

Preparation of ethanolic extracts of *Chromolaena odorata* leaves

Five grams of *C. odorata* powder was exhaustively extracted with 95% ethanol by Soxhlet apparatus, filtered and evaporated to dryness. The extract was dissolved with ethanol to get the concentration of 20 mg/ml. These extract were stored in refrigerator at 4°C and further used for TLC densitometry and TLC image analysis.

TLC-densitometry

Three microliters of 10 ethanol extracts and standard solutions were applied onto the Silica gel 60 GF_{254} TLC plate by Linomat 5 applicator. The plate was developed in a TLC chamber that contained a mixture of ethyl acetate: water: formic acid (17: 3: 2). After that, the plate was dried and scanned with CAMAG TLC Scanner 4 (CAMAG, Switzerland) under wavelength of maximum absorbance (330 nm). The peak area of each band was determined and calculated the content of chlorogenic acid.

TLC image analysis by ImageJ software

The chlorogenic acid spots on the developed TLC plates were photographed under short wave ultraviolet light (254 nm) by a digital camera. Peak area of each spot was quantitated using ImageJ software. The content of chlorogenic acid was determined by comparing peak area to the calibration curve obtained from the same TLC plate.

Quantitative analysis of xanthoxylin in *Zanthoxylum piperitum* fruits

Preparation of standard solution of xanthoxylin

One milligram of standard xanthoxylin was dissolved in 1 ml of methanol. The stock solution was diluted to obtain the series of standard solution range from 0.2 to 1 mg/ml. These solutions were stored in refrigerator at 4°C.

Preparation of ethanolic extracts of *Zanthoxylum piperitum* fruits

Five grams of *Z. piperitum* powder was exhaustively extracted with 95% ethanol by Soxhlet apparatus, filtered and evaporated to dryness. The extract was dissolved with methanol to get the concentration of 5-60 mg/ml. These extract were stored in refrigerator at 4°C and further used for TLC densitometry and TLC image analysis.

TLC-densitometry

Three microliters of 15 ethanol extracts and standard solutions were applied onto the Silica gel 60 GF_{254} TLC plate by Linomat 5 applicator. The plate was developed in a TLC chamber that contained a mixture of toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2). After that, the plate was dried and scanned with CAMAG TLC Scanner 4 (CAMAG, Switzerland) under wavelength of maximum absorbance (291 nm). After scanning, the peak area of each band was determined and calculated the content of xanthoxylin.

TLC image analysis by ImageJ software

The xanthoxylin spots on the developed TLC plates were sprayed with 1% aluminium chloride in ethanol then heated at 100°C for 10 minutes. After that, the plate was photographed under ultraviolet light (365 nm) by a digital camera. Peak area of each spot was quantitated using ImageJ software. The content of xanthoxylin was determined by comparing peak area to the calibration curve obtained from the same TLC plate.

Method validation

According to the ICH guidelines [100], the method validation including calibration range, specificity, accuracy, precision, LOD, LOQ and robustness were performed.

Calibration range

The calibration range was determined by plotting peak areas *versus* concentrations of standard applied and correlation coefficient was determined by Excel program.

Specificity

The specificity of quantitative analysis of tested compound in the plant samples was determined by comparing absorption spectra under the range of 200-700 nm of all sample spots to that of standard compound using CAMAG TLC Scanner 4, as well as comparison of the absorption spectra at up-slope, apex and down-slope of the peak.

Accuracy

The accuracy was examined by carrying out recovery studies at different spike levels. Standard solution was spiked into the extract to obtain three different levels that would be low, medium and high in calibration range. The spiked and un-spiked sample were analyzed under the same conditions in triplicate. The accuracy was determined as percent recovery using following formula:

$$\%\ \text{Recovery} = \frac{A}{B + C} \times 100$$

Where, A = amount of spiked sample

B = amount of un-spiked sample

C = amount of standard spiked into the recovery sample

Precision

The precision of the method was examined by repeatability (intra-day) and intermediate (inter-day) precision. The method was performed by analyzing sample

solution of 3 concentrations (3 replicates) on the same day and three different days respectively, and expressed in terms of % Relative standard deviation (% RSD) by following formula:

$$\% \text{ RSD} = \frac{\text{SD}}{\text{Mean}} \times 100$$

Where, SD = the standard deviation of each measurement

Limit of detection

The limit of detection (LOD) was determined from the calibration curve using following formula:

$$\text{LOD} = \frac{3.3\ (\text{SD})}{\text{S}}$$

Where, SD = the residual standard deviation of regression line

S = the slope of regression line

Limit of quantitation

The limit of quantitation (LOQ) was determined from the calibration curve using following formula:

$$\text{LOD} = \frac{10\ (\text{SD})}{\text{S}}$$

Where, SD = the residual standard deviation of regression line

S = the slope of regression line

Robustness

The robustness was determined for variations in a mixture ratio of mobile phase and calculated for %RSD of peak area to evaluate whether the mobile phase variations alter the results of quantitative analysis.

Antioxidant activities

DPPH radical scavenging assay

The antioxidant activity was assessed by observing the method as described by Brand-William et al., 1995 [101]. One hundred microliters of various concentrations of the extract and positive control (BHT, Quercetin) in methanol was added to 100 µl of DPPH radical methanolic solution (120 µM) in 96 well microplate. The plate was incubated at room temperature for 30 minutes in the dark. The absorbance was measured at the wavelength of 517 nm. Each sample was done in triplicate. The radical scavenging activity was evaluated from the inhibition of decreasing in absorbance value at 517 nm as the following formula.

$$\% \text{ Inhibition} = \frac{[\text{Absorbance control} - \text{Absorbance sample}]}{\text{Absorbance control}} \times 100$$

The radical scavenging activity was expressed as IC_{50} value which indicated the concentration of sample required to scavenge 50% of DPPH free radical.

Ferric reducing antioxidant power (FRAP) assay

The antioxidant power was measured following Benzie and Strain, 1996 with minor modification [102]. FRAP working reagent was freshly prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of TPTZ in 40 mM HCl (2,4,6 -tripyridyl-s-triazine) and 20 mM of $FeCl_3.H_2O$ in ratio of 10:1:1. Twenty-five microliters of the extract and positive control (BHT, Quercetin) was mixed with 175 µl of FRAP working reagent in a 96 well plate. The plate was incubated at room temperature for 30 minutes. The absorbance was measured at 593 nm with a 96 well microplate reader. Three replicates were made for each test sample. The reducing antioxidant power was evaluated as the amount of mM of ferrous iron (Fe (II)) per mg extract obtained from ferrous sulphate calibration curve.

ß-carotene bleaching assay

ß-carotene Bleaching Assay was examined following Jayaprakasha et al., 2002 with minor modification [103]. This assay was performed in 96 well plate. One milliliter of ß-carotene solution (2 mg/ml in chloroform) was added with 20 µl of linoleic acid

and 200 µl of Tween 20 in 96 well microplate. Chloroform was removed at 40 °C under vacuum. Ultra-pure water (50 ml) was added and shaken to form an emulsion. Aliquots (200 µl) of the emulsion were transferred into the 96 well plates containing 10 µl of the various concentrations of extract, standard compound or positive controls (BHT and quercetin) and heated at 50 °C. Absorbance at wavelength 470 nm was recorded at 30 minutes intervals for 120 minutes. Each sample was done in triplicate. The antioxidant activity was evaluated in the bleaching of the ß-carotene using the following formula.

$$\% \text{ Antioxidant activity} = \left(1 - \frac{\mathbf{A0-A120}}{\mathbf{C0-C120}}\right) \times 100$$

Where A0 = the absorbance values measured at zero time of sample

A120 = the absorbance values measured at end time of sample

C0 = the absorbance values measured at zero time of control

C120 = the absorbance values measured at end time of control

Total phenolic content

Twenty-five microliters of extracts (0.5 mg/ml) was mixed with 125 µl of 10% Folin-Ciocalteu's phenol reagent in with a 96 well plate. After 5 min, 100 µl of a 7.5% Na_2CO_3 solution was added to the mixture then incubation at room temperature for 60 min, after which the absorbance was measured at 756 nm using a microplate reader. The total phenolic content was calculated from the calibration curve of gallic acid and the data was expressed as gallic acid equivalents per mg extract. Three replicates were made for each test sample [104].

Brine shrimp lethality assay

Brine shrimp lethality assay was evaluated according to Mayer *et al*., 1982 [105]. The eggs of *A. salina* were hatched in artificial sea water. Artificial sea water at the concentration of 36.66 % (w/v) was prepared and aerated for 24 hours in brine shrimp hatching box under illumination. The brine shrimp cysts were added and incubated at room temperature. After 48 hours, ten brine shrimps were transferred using pasture

pipette to each vials filled with 5 ml of saline water. The various concentration of extracts in methanol were pipetted into a small filter paper and left until methanol was dried. Then the prepared filter paper was placed into each vials containing the brine shrimp. Each concentration was performed in five replicate. The percent death of nauplii at 6, 12, 18 and 24 hours was counted, recorded and calculated for the LD_{50}.

MTT cell viability assay

Five human cancer and 1 human normal cell lines including BT-474 (breast ductal carcinoma), CHAGO-K1 (undifferentiated lung carcinoma), SW-620 (colon adenocarcinoma), KATO-3 (gastric carcinoma), Hep-G2 (Liver hepatoblastoma) and Wi-38 (Lung fibroblast) respectively were used in cytotoxic assay. Cell survival was measured using the MTT method.

The crude ethanolic extract was dissolved in DMSO to obtain the concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 10 and 100 µg/ml. Five milligrams of doxorubicin positive control drug were dissolved in normal saline to obtain the concentrations of 0.001, 0.01, 0.1, 1 and 10 µg/ml. MTT was dissolved in normal saline at 5 mg/ml, stored in the dark at 4 °C and used within 1 month.

The cell line was cultured in tissue culture flask in RpMI-1640 supplemented with 5% (v/v) fetal calf serum and incubated at 37°C in 5% CO_2 for 3 days. Two hundred microliters of cell were seeded in a 96-well culture plates at a density of $1x10^4$ cells/well and cultured in 5% CO_2 incubator at 37°C and 100% relative humidity for 24 hours.

The sample solution (2 µl) was dispensed into the appropriate wells. This analysis was performed in 4 replicates (control cells group, n=4; each sample treatment group, n=4; Doxorubicin/DMSO n=4). Culture plates were incubated for 72 hours prior to the addition of MTT solution (10 µl). Plates were incubated for 4 hours at 37 °C in 5% CO_2 incubator. After incubation, the formazan produced in the cell was captured as dark crystal in the bottom of the wells. The supernatant from culture

medium was removed from wells. DMSO (150 µl) was added to dissolve the resulting formazan and mixed for 2-3 minutes on an orbital shaker. Following formazan solubilization, the absorbance was measured using a microplate reader at the wavelength of 540 nm. The cell survival was calculated in percentage using the following formula:

$$\text{Percentage of cell survival} = \frac{\text{Mean absorbance in test wells}}{\text{Mean absorbance in control wells}} \times 100$$

The cytotoxic activity was expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value).

Antidiabetic activities

Inhibition of yeast alpha-glucosidase activity

The activity of alpha-glucosidase from *Saccharomyces cerevisiae* using 1 mM of *p*-nitrophenyl-α-D-glucopyranosiade as substrate was performed according to Wan *et al*. with minor modifications [106]. The extract solutions and positive control (acarbose) were prepared in DMSO to obtain different concentrations. In 96 well plate, 10 µl of the extracts and positive control was added into 120 µl of 0.1 M sodium phosphate buffer (pH 6.9) and 20 µl of 0.5 U/ml α-glucosidase. The plates were incubated at 37°C for 15 minutes. After that, 20 µl of substrate was added and incubated again at 37°C for 30 minutes. Then, 80 µl of 0.2 µM Na_2CO_3 was added to stop the reaction. The absorbance was measured at 405 nm using microplate reader. All tested samples were done in triplicate. The percent inhibition was calculated by the following formula.

$$\% \text{ Inhibition} = \frac{[\text{Absorbance control} - \text{Absorbance sample}]}{\text{Absorbance control}} \times 100$$

Inhibition of rat alpha-glucosidase activity

The activity of plant extracts against rat intestinal alpha-glucosidase was performed on 96 well plates using 1 mM of *p*-nitrophenyl-α-D-glucopyranosiade as

substrate, following the method of Hemalatha *et al*. and Lordan *et al*. [107, 108] with modifications. Rat intestinal acetone powder (30 mg/ml) in 0.1 M sodium phosphate buffer (pH 6.9) was sonicated for 20 minutes. The suspension was centrifuged at 3500 rpm for 30 minutes to remove particulate matter. Then the resulting supernatant was used as enzyme solution. The extract solutions and positive control (Acarbose) were prepared in DMSO and diluted to obtain different concentrations. In 96 well plate, 50 µl of the extracts and positive control was added into 100 µl of 1 mM of *p*-nitrophenyl-α-D-glucopyranosiade and 50 µl of the enzyme. The plate was incubated at 37°C for 30 minutes. The absorbance was measured at 405 nm using microplate reader. All tested samples were done in triplicate. The percent inhibition was calculated by the following formula.

$$\% \text{ Inhibition} = \frac{[\text{Absorbance control} - \text{Absorbance sample}]}{\text{Absorbance control}} \times 100$$

Pharmacognostic specification of *Kaempferia parviflora*

The standardization parameters were examined by standard methods of World Health Organization (WHO) [81].

Macroscopic examination

Macroscopic examination of plant materials was carried out for the size, color, texture, surface characteristics and other visual inspection.

Microscopic examination

The transverse section and powder were examined under microscopic with the objective lens magnification of 10X, 20X and 40X and the eyepiece lens magnification of 10X. The tissue section and powder were mounted onto a slide in water for observation cell and tissue structures.

Determination of loss on drying

Three grams of ground sample was weighed into a pre-weighed crucible and then dried at 105°C for 6 hours until constant weight. After that, the crucible was left

to cool at room temperature. The loss on drying was weighed and calculated in a percentage of dried material.

Determination of total ash

Three grams of plant materials was added into a previously weighed crucible and incinerated at 500°C for 5 hours until it is become white which indicating the absence of carbon. The crucible was left to cool in a desiccator. The total ash was weighed and calculated in percentage.

Determination of acid-insoluble ash

Twenty-five milliliters of hydrochloric acid (70 g/L) was added into the crucible which contained the total ash and boiled for 5 minutes. The insoluble matters were collected on an ashless filter-paper No.40, transferred to the original crucible, dried on a hotplate and incinerated to ash again. The crucible was left to cool in desiccator and weighed. The content of acid-insoluble ash was calculated in percentage.

Determination of water content

Fifty grams of plant materials was added with 200 ml of water-saturated toluene and heated by azeotropic distillation. The toluene and water layer were separated then the volume of water was measured and calculated in percentage.

Determination of volatile oil content

One hundred grams of plant materials was added with 600 ml of water and distilled by Clevenger apparatus. When volatile oil completely distilled, allow the receiving tube to cool in room temperature. The volatile oil and water layers were separated, then the volume of volatile oil was measured and calculated in percentage.

Determination of ethanol extractive value

Five grams of plant materials was macerated with 70 ml of 95% ethanol in a conical flask under shaking for 6 hours and standing for 18 hours. The marc was washed and the filtrate was transferred through Whatman No.4 into a pre-weighed beaker and evaporated to dryness. The extract was dried in oven at 105°C for 6 hours and cooled

in desiccator for 30 minutes. The ethanol soluble extractive matter was weighed and calculated in percentage.

Determination of water extractive value

Five grams of plant materials was macerated with 70 ml of distilled water in conical flask under shaking for 6 hours and standing for 18 hours. The marc was washed and the filtrate was transferred through Whatman No.4 into a pre-weighed beaker and evaporated to dryness. The extract was dried in oven at 105℃ for 6 hours and cooled in desiccator for 30 minutes. The ethanol soluble extractive matter was weighed and calculated in percentage.

Thin layer chromatography fingerprint

The ethanol extract of plant materials was dissolved with 1 ml of 95% ethanol. Three microliters of sample solution was spotted on TLC silica gel 60 GF_{254} plate and developed in appropriate solvent system. After development, the plate was removed and allowed to dry and examined under ultraviolet light (254, 365 nm). The spots were detected by dipping in suitable reagent.

Gas chromatography-mass spectrometry of the volatile oil

The volatile oil was analyzed by a Finnigan Trace GC ultra with DSQ Quadrupole detector. BPX5 fuse silica column (30m x 0.25 mm, 0.25μm film thicknesses) was used as stationary phase. The oven temperature started from 60 °C to 240°C with a constant rate of 3°C/min. The carrier gas was helium with the flow rate of 1ml/min. One microliter of the oil (1:100 in HPLC grade methanol) was injected by Finnigan Autoinjector AI3000 with split ratio of 100:1. MS was performed by EI positive mode at 70 electron volts. The chemical constituents were identified by matching mass spectra and retention time indices with Adams Essential Oils Mass Spectral library and NIST05 Mass Spectral library. Peak area was shown in percentage.

Data analysis

The parameters of standardization were represented by grand mean ± pooled standard deviation. The quantitative analysis between TLC image analysis and TLC-densitometry were compared by paired T-test statistical analysis. The results of antioxidant, antidiabetic and MTT cell viability activities were expressed as IC_{50}. The total phenolic content and FRAP assay were evaluated from a standard curve of gallic acid and ferrous sulphate respectively. The results were expressed as mg gallic acid equivalents (GAE) per gram extract in total phenolic content and as mM of ferrous iron (Fe(II)) per milligram extract in FRAP assay.

CHAPTER IV

RESULTS

Quantification by TLC-densitometry and TLC image analysis

Chromolaena odorata leaves

Ethanolic extraction of *C. odorata* leaves

The dried powders of *C. odorata* leaves from 10 different sources in Thailand were extracted with 95 % ethanol by Soxhlet apparatus. The average percent yield of *C. odorata* leaves ethanolic extract was 27.46 ± 2.21 % by weight (Table 5).

Table 5 The percent yield of ethanolic extract of *C. odorata* leaves from 10 different sources in Thailand

Sources	Weight of sample (g)	Weight of extractive matter (g)	% yield
Bangkok 1	5.00	1.27	25.45
Bangkok 2	5.00	1.29	25.71
Bangkok 3	5.00	1.34	26.77
Bangkok 4	5.00	1.26	25.18
Bangkok 5	5.00	1.29	25.86
Bangkok 6	5.00	1.36	27.10
Bangkok 7	5.00	1.56	31.24
Bangkok 8	5.00	1.35	27.04
Bangkok 9	5.00	1.50	30.08
Bangkok 10	5.00	1.51	30.14
		Average	27.46 ± 2.21
		Min	25.18
		Max	31.24

Quantitative analysis of chlorogenic acid contents in *Chromolaena odorata* leaves by TLC-densitometry

Standard chlorogenic acid and the ethanolic extracts were developed in ethyl acetate: water: formic acid (17: 3: 2). TLC plate was scanned by CAMAG TLC scanner under 330 nm. TLC densitograms of chlorogenic acid standards and samples of *C. odorata* leaves performed under wavelength of 330 nm were demonstrated in Figure 7. The peak areas were computed by winCATS software. The ethanolic extracts of *C. odorata* leaves were determined for the chlorogenic acid content in triplicate by TLC-densitometry and found to be 0.12 ± 0.03 g/100g of the crude drug (Table 6).

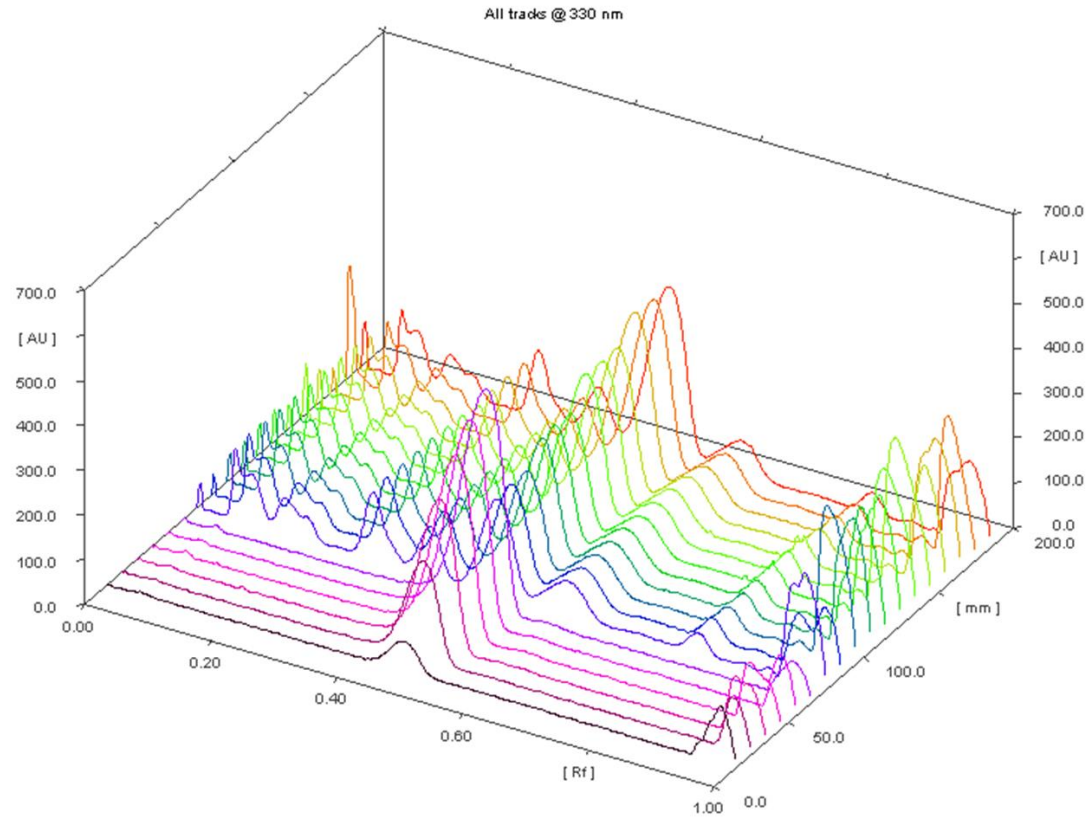


Figure 7 TLC densitograms of chlorogenic acid standards and samples of *C. odorata* leaves under UV 330 nm

Table 6 The amount of chlorogenic acid in *C. odorata* leaves from 10 sources in Thailand by TLC-densitometry

Source	Chlorogenic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100g of dried crude drug)	Chlorogenic acid in *C. odorata* leaves (g/100g of dried crude drug)
1	0.005	25.452	0.132
2	0.005	25.709	0.124
3	0.005	26.771	0.130
4	0.006	25.183	0.154
5	0.002	25.864	0.064
6	0.006	27.104	0.173
7	0.004	31.244	0.137
8	0.004	27.038	0.113
9	0.003	30.080	0.092
10	0.004	30.139	0.113
	Average		**0.123 ± 0.031**

Method validation (TLC-densitometry)

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

Calibration range

The calibration curve of standard chlorogenic acid was polynomial in the range of 0.05-0.45 µg/spot with the regession equation of $y = -72082x^2 + 90517x - 1153.3$. The coefficient of determination (R^2) of chlorogenic acid was 1.0000 (Figure 8).

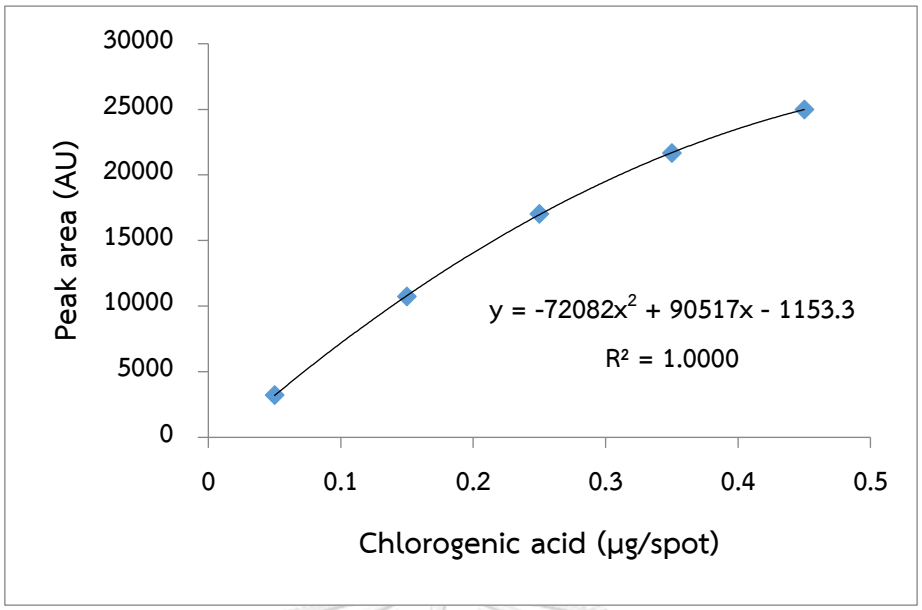


Figure 8 The calibration curve of standard chlorogenic acid by TLC-densitometry

Detection limit and quantitation limit

The detection limit and quantitation limit determination were calculated based on the standard deviation of regression line and the slope of the calibration curve. The LOD value, considered as the lowest concentration for analyte in a sample that could be detected was found to be 0.003 µg/spot. The LOQ value, the lowest concentration of analyte in a sample that could be quantitatively defined was found to be 0.008 µg/spot.

Accuracy

The recovery analysis was applied to validate the accuracy of chlorogenic acid quantitation in the ethanolic extract of *C. odorata* leaves. Standard chlorogenic acid was spiked into the extract to have three different levels of chlorogenic acid (low, medium, high). The recovery values were 85.94 – 94.13 % as demonstrated in Table 7.

Table 7 Accuracy of quantitation of chlorogenic acid in *C. odorata* leaves by TLC-densitometry (n=3)

Chlorogenic acid added (µg/spot)	Chlorogenic acid found (µg/spot)	% Recovery
0.000	0.146 ± 0.009	-
0.055	0.189 ± 0.011	94.130 ± 2.406
0.165	0.267 ± 0.016	85.940 ± 2.457
0.275	0.366 ± 0.034	86.823 ± 6.157
	Average	**88.964 ± 4.495**

Precision

The precision was examined by repeatability (intra-day) and intermediate precision (inter-day). This method was represented as %RSD at four concentrations of chlorogenic acid in the extracts on the same day and three different days respectively. The results of repeatability and intermediate precision were 0.81 and 2.74 %RSD respectively as demonstrated in Table 8.

Table 8 Repeatability and intermediate precision of chlorogenic acid in *C. odorata* leaves by TLC-densitometry (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	**% RSD**	**Amount (µg/spot)**	**% RSD**
0.140 ± 0.002	1.337	0.136 ± 0.003	2.000
0.180 ± 0.001	0.583	0.174 ± 0.005	2.989
0.246 ± 0.001	0.551	0.238 ± 0.006	2.702
0.310 ± 0.002	0.754	0.304 ± 0.010	3.254
Average	**0.806 ± 0.365**		**2.736 ± 0.540**

Specificity

Peak identity and peak purity

The specificity was confirmed by comparing light absorption spectrum of the peak at apex among standard chlorogenic acid and all samples which representing peak identity (Figure 9) and comparing the absorption spectrum of the sample peak at up-slope, apex and down-slope which representing peak purity (Figure 10). The maximum absorbance of chlorogenic acid was at the wavelength of 330 nm.

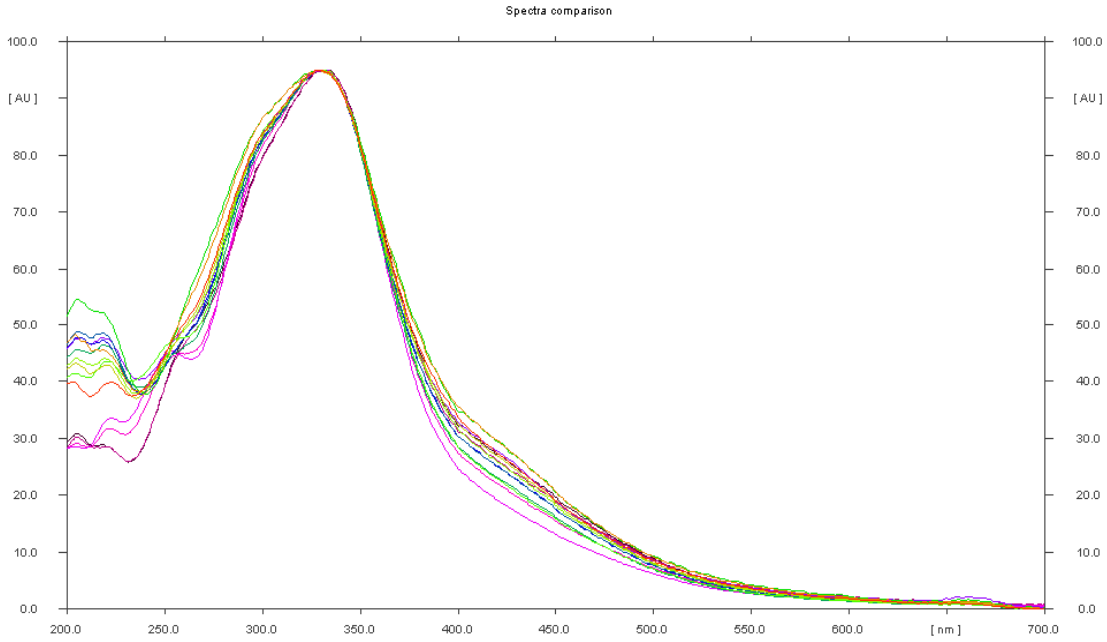


Figure 9 The absorbance spectra of chlorogenic acid in *C. odorata* extracts from 10 different sources and standard chlorogenic acid representing peak identity

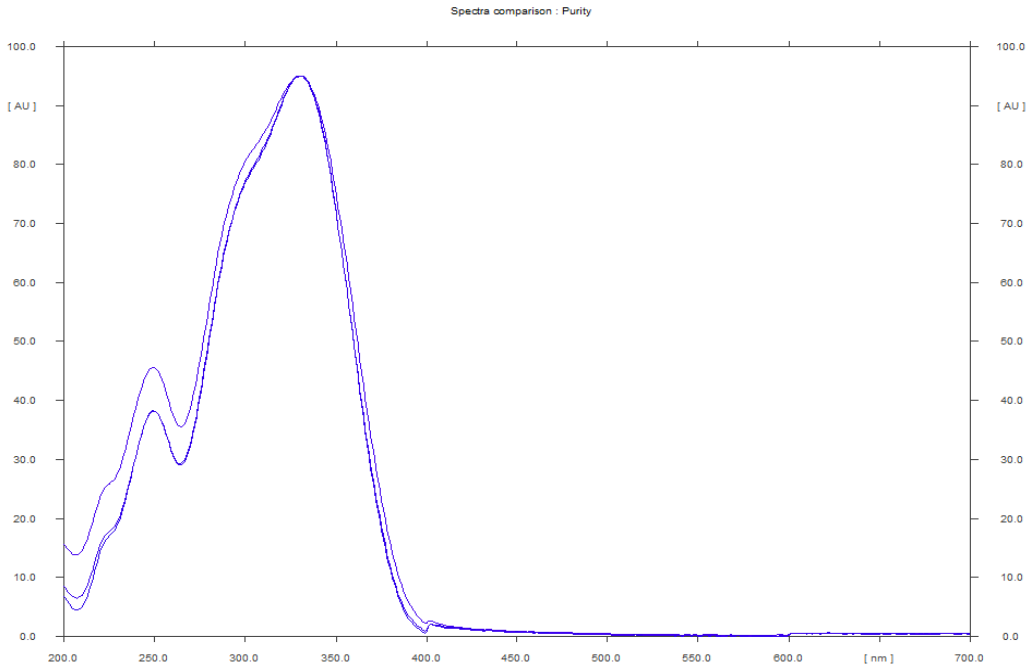


Figure 10 Peak purity measurement using up-slope, apex and down-slope of the peak

Robustness

The robustness of chlorogenic acid quantitation in *C. odorata* leaves by TLC densitometric analysis was performed by adjusting the mobile phase ratio. The selected mobile phase ratio was shown in Table 9. The result of robustness was 0.99 %RSD of peak area.

Table 9 Robustness of chlorogenic acid in *C. odorata* leaves by TLC-densitometry

Mobile phase composition **Ethyl acetate: Water: Formic acid**	**Peak area**
17.0 : 3.0 : 2.0	23273.51
17.1 : 2.9 : 2.0	23723.76
16.9 : 3.1 : 2.0	23399.54
Mean ± SD	**23465.60 ± 232.28**
%RSD	**0.99**

Quantitative analysis of chlorogenic acid contents in *Chromolaena odorata* leaves by TLC image analysis

TLC plate containing standard chlorogenic acid and the ethanolic extracts which were developed in ethyl acetate: water: formic acid (17: 3: 2) were photographed under UV 254 nm by a digital camera. The image of TLC plate was analyzed for chlorogenic acid peak areas by ImageJ software. The amounts of chlorogenic acid were found to be 0.12 ± 0.03 g/100g of *C. odorata* leaves crude drug (Table 10).

Table 10 The amount of chlorogenic acid in *C. odorata* leaves by TLC image analysis (% by weight)

Source	Chlorogenic acid in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100g of dried crude drug)	Chlorogenic acid in *C. odorata* leaves (g/100g of dried crude drug)
1	0.005	25.452	0.132
2	0.005	25.709	0.116
3	0.005	26.771	0.124
4	0.006	25.183	0.143
5	0.003	25.864	0.068
6	0.007	27.104	0.185
7	0.004	31.244	0.134
8	0.004	27.038	0.114
9	0.003	30.080	0.088
10	0.004	30.139	0.114
	Average		**0.122 ± 0.032**

Method validation (TLC image analysis)

In the same way as TLC-densitometry validation, the validity of TLC image analysis was as follows:

Calibration range

The calibration curve of standard chlorogenic acid was polynomial in the range of 0.05-0.45 µg/spot with the regession equation of $y = -5275.8x^2 + 31682x + 675.37$. The coefficient of determination (R^2) of chlorogenic acid was 0.9998 (Figure 11).

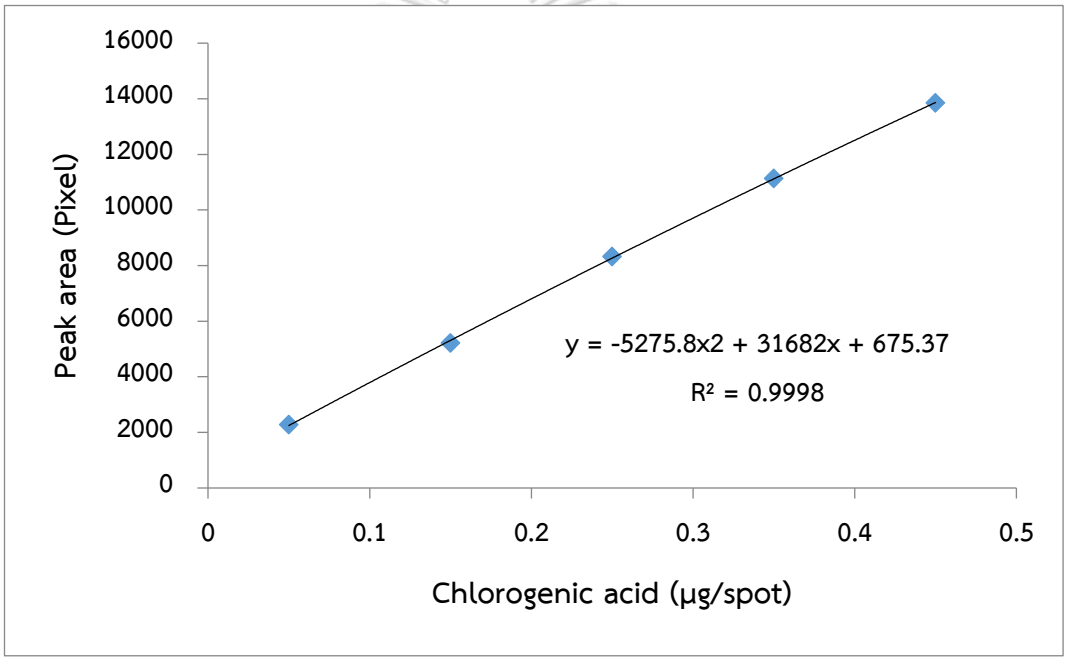


Figure 11 The calibration curve of chlorogenic acid in *C. odorata* leaves by TLC image analysis

Detection limit and quantitation limit

The LOD and LOQ values were found to be 0.008 and 0.023 µg/spot, respectively.

Accuracy

The recovery values were 84.69 – 103.99 % as demonstrated in Table 11.

Table 11 Accuracy of quantitation of chlorogenic acid in *C. odorata* leaves by TLC image analysis (n=3)

Chlorogenic acid added (µg/spot)	Chlorogenic acid found (µg/spot)	% Recovery
0.000	0.129 ± 0.041	-
0.055	0.189 ± 0.026	103.986 ± 9.959
0.165	0.267 ± 0.035	90.813 ± 2.505
0.275	0.341 ± 0.023	84.686 ± 4.441
	Average	**93.162 ± 9.862**

Precision

The results of repeatability and intermediate precision were 1.85 and 5.00 %RSD respectively as demonstrated in Table 12.

Table 12 Repeatability and intermediate precision of quantitation of chlorogenic acid in *C. odorata* leaves by TLC image analysis (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	**% RSD**	**Amount (µg/spot)**	**% RSD**
0.128 ± 0.007	5.548	0.135 ± 0.012	9.020
0.191 ± 0.002	1.001	0.197 ± 0.013	6.520
0.266 ± 0.001	0.447	0.260 ± 0.009	3.387
0.325 ± 0.001	0.409	0.324 ± 0.003	1.054
Average	**1.851 ± 2.479**		**4.995 ± 3.495**

Robustness

The robustness of chlorogenic acid quantitation in *C. odorata* leaves by TLC image analysis performed by adjusting the mobile phase ratio was 2.62 %RSD of peak area (Table 13).

Table 13 Robustness of chlorogenic acid in *C. odorata* leaves by TLC image analysis

Mobile phase composition **Ethyl acetate: Water: Formic acid**	**Peak area**
17.0 : 3.0 : 2.0	6841.52
17.1 : 2.9 : 2.0	7175.15
16.9 : 3.1 : 2.0	6879.29
Mean ± SD	**6965.32 ± 182.70**
%RSD	**2.62**

Method comparison

The contents of chlorogenic acid determined by TLC-densitometry and TLC image analysis were analyzed by paired t-test. The result showed that the chlorogenic acid contents from two methods were not statistically significantly different ($P > 0.05$) (Table 14).

Table 14 Chlorogenic acid contents in *C. odorata* leaves by TLC-densitometry and TLC image analysis

Source	Chlorogenic acid contents (g/100g of dried crude drug)	
	TLC-densitometry	TLC image analysis
1	0.132	0.132
2	0.124	0.116
3	0.130	0.124
4	0.154	0.143
5	0.064	0.068
6	0.173	0.185
7	0.137	0.134
8	0.113	0.114
9	0.092	0.088
10	0.113	0.114
Average	**0.123 ± 0.031**	**0.122 ± 0.032**

***Kaempferia parviflora* rhizomes**

Ethanolic extraction of *K. parviflora* rhizomes

The dried powders of *K. parviflora* rhizomes from 15 different sources in Thailand were extracted with 95 % ethanol by Soxhlet apparatus. The average percent yield of *K. parviflora* rhizomes ethanolic extract was 9.57 ± 1.49 % by weight (Table 15).

Table 15 The percent yield of ethanolic extract of *K. parviflora* rhizomes from 15 different sources in Thailand

Sources	Weight of sample (g)	Weight of extractive matter (g)	% yield
Tak	5.00	0.45	9.06
Phitsanulok	5.01	0.55	11.04
Phetchabun	5.00	0.55	10.91
Chiang Mai	5.00	0.55	10.96
Loei	5.00	0.53	10.68
Nakhon Pathom	5.01	0.27	5.47
Suphan Buri	5.00	0.49	9.78
Nakhon Sawan	5.00	0.53	10.50
Petchaburi	5.01	0.45	9.07
Trang	5.00	0.45	8.90
Songkhla	5.00	0.44	8.81
Krabi	5.00	0.42	8.36
Rayong	5.00	0.56	11.14
Yasothon	5.00	0.44	8.89
Bangkok	5.01	0.50	9.97
		Average	9.57 ± 1.49
		Min	5.47
		Max	11.14

Quantitative analysis of 5,7-dimethoxyflavone contents in *Kaempferia parviflora* rhizomes by TLC-densitometry

Standard 5,7-dimethoxyflavone and the ethanolic extracts were developed in toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2). TLC plate was scanned by CAMAG TLC scanner under 265 nm. TLC densitograms of 5,7-dimethoxyflavone standards and samples of *K. parviflora* rhizomes performed under wavelength of 265 nm were demonstrated in Figure 12. The peak areas were computed by winCATS software. The ethanolic extracts of *K. parviflora* rhizomes were determined for the 5,7-dimethoxyflavone content in triplicate by TLC-densitometry and found to be 2.15 ± 0.64 g/100g of the crude drug (Table 16).

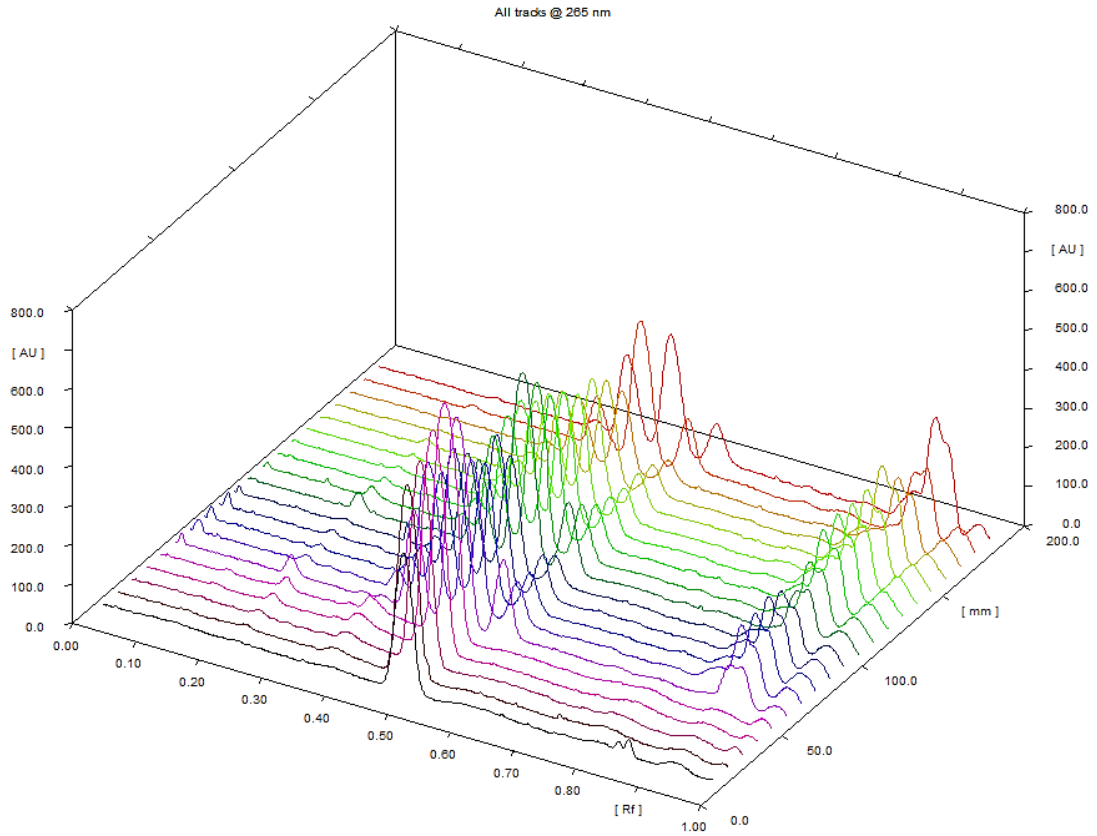


Figure 12 TLC densitograms of 5,7-dimethoxyflavone standards and samples of *K. parviflora* rhizomes under UV 265 nm

Table 16 The amount of 5,7-dimethoxyflavone in *K. parviflora* rhizomes from 15 sources in Thailand by TLC-densitometry

Source	5,7-Dimethoxyflavone in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100g of dried crude drug)	5,7-Dimethoxyflavone in *K. parviflora* rhizomes (g/100g of dried crude drug)
1	0.39	9.06	3.52
2	0.20	11.04	2.19
3	0.16	10.91	1.79
4	0.18	10.96	1.94
5	0.12	10.68	1.30
6	0.41	5.47	2.24
7	0.29	9.78	2.82
8	0.21	10.50	2.16
9	0.18	9.07	1.61
10	0.19	8.90	1.72
11	0.23	8.81	2.00
12	0.20	8.36	1.71
13	0.15	11.14	1.63
14	0.39	8.89	3.43
15	0.21	9.97	2.13
	Average		**2.15 ± 0.64**

Method validation (TLC-densitometry)

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

Calibration range

The calibration curve of standard 5,7-dimethoxyflavone was polynomial in the range of 0.6 - 3.0 µg/spot with the regession equation of $y = -1133.7x^2 + 8361.8x + 5277.2$. The coefficient of determination (R^2) of 5,7-dimethoxyflavone was 0.9998 (Figure 13).

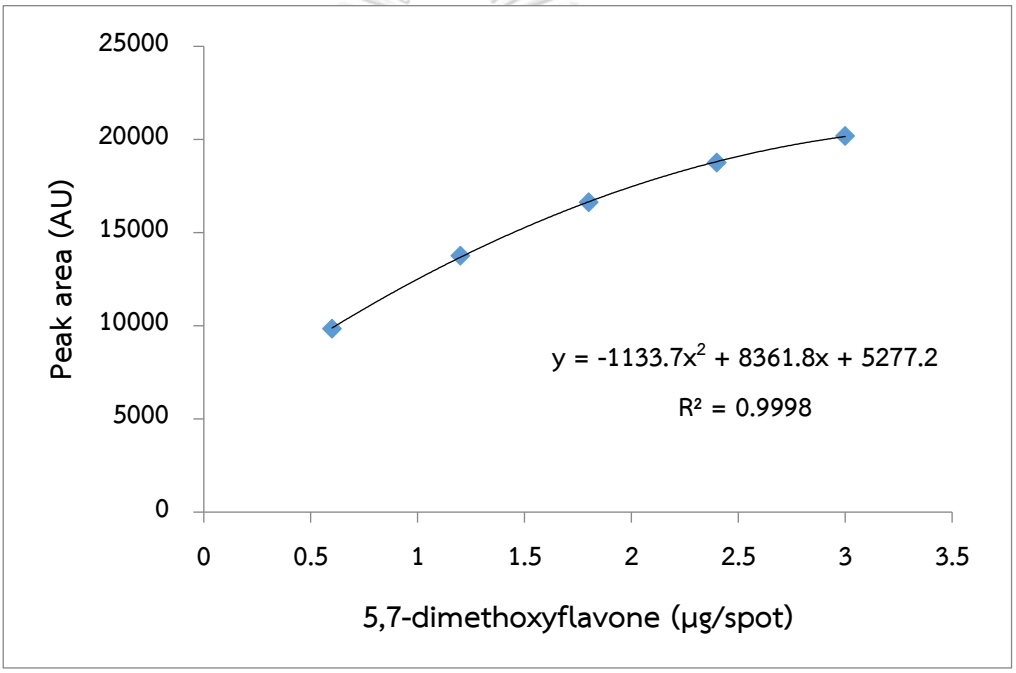


Figure 13 The calibration curve of standard 5,7-dimethoxyflavone by TLC-densitometry

Detection limit and quantitation limit

The lowest concentration of 5,7-dimethoxyflavone that could be detected was found to be 0.03 µg/spot. The lowest concentration that could be quantitatively defined was found to be 0.10 µg/spot.

Accuracy

The recovery of 5,7-dimethoxyflavone spiked into the ethanolic extract of *K. parviflora* rhizomes were 96.64 – 99.20 % as demonstrated in Table 17.

Table 17 Accuracy of quantitation of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC- densitometry (n=3)

5,7-Dimethoxyflavone added (µg/spot)	5,7-Dimethoxyflavone found (µg/spot)	% Recovery
0.00	0.94 ± 0.01	-
0.15	1.05 ± 0.01	96.64 ± 1.03
0.75	1.68 ± 0.06	99.20 ± 3.05
1.50	2.39 ± 0.09	97.82 ± 4.11
	Average	**97.89 ± 1.28**

Precision

The repeatability and intermediate precision were 2.29 and 2.72 %RSD respectively as demonstrated in Table 18.

Table 18 Repeatability and intermediate precision of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC-densitometry (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	**% RSD**	**Amount (µg/spot)**	**% RSD**
0.94 ± 0.01	1.06	0.97 ± 0.03	3.21
1.05 ± 0.01	0.55	1.08 ± 0.03	2.77
1.68 ± 0.06	3.64	1.68 ± 0.01	0.41
2.39 ± 0.09	3.89	2.27 ± 0.10	4.47
Average	**2.29 ± 1.73**		**2.72 ± 1.70**

Specificity

Peak identity and peak purity of 5,7-dimethoxyflavone absorption spectra were demonstrated in Figure 14 and 15. The maximum absorbance of 5,7-dimethoxyflavone was at the wavelength 265 nm.

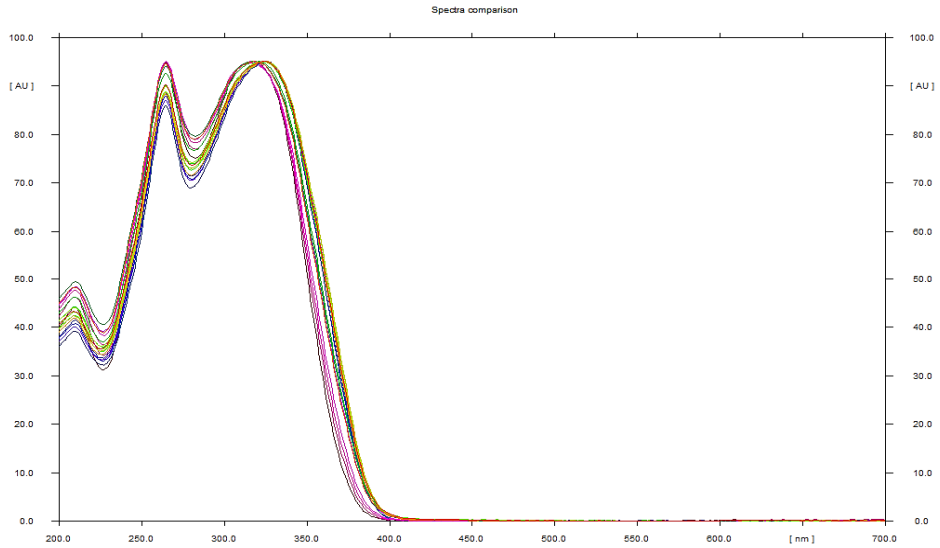


Figure 14 The absorbance spectra of 5,7-dimethoxyflavone in *K. parviflora* extracts from 15 different sources and standard 5,7-dimethoxyflavone representing peak identity

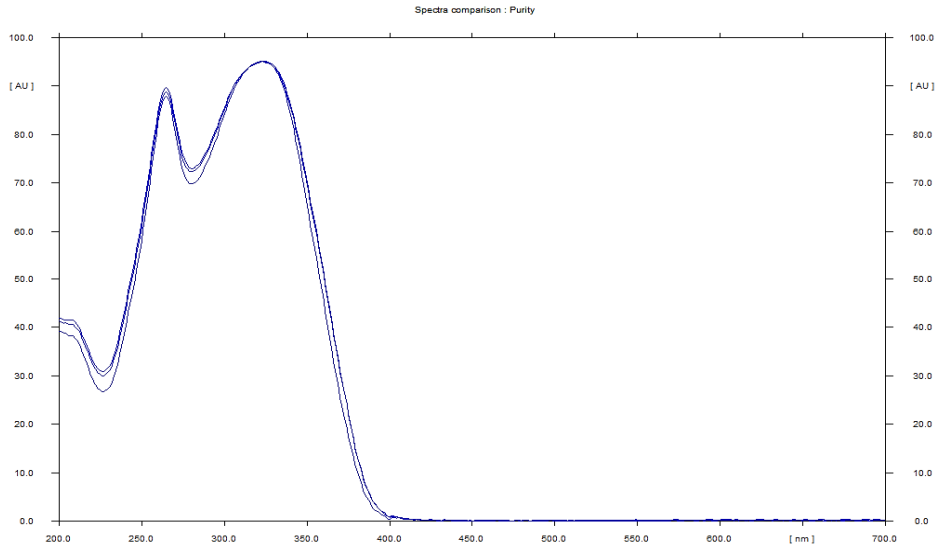


Figure 15 Peak purity measurement using up-slope, apex and down-slope of the peak

Robustness

The robustness of 5,7-dimethoxyflavone quantitation in *K. parviflora* rhizomes by TLC densitometric analysis performed by adjusting the mobile phase ratio was shown in Table 19. The result of robustness was 0.76 %RSD of peak area.

Table 19 Robustness of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC-densitometry

Mobile phase composition **Toluene: Chloroform: Acetone: Formic acid**	**Peak area**
10.0 : 8.0 : 2.0 : 0.4	13461.10
10.1 : 7.9 : 2.1 : 0.3	13521.23
9.9 : 8.1 : 1.9 : 0.5	13662.00
Mean ± SD	**13548.11 ± 103.11**
%RSD	**0.76**

Quantitative analysis of 5,7-dimethoxyflavone contents in *Kaempferia parviflora* rhizomes by TLC image analysis

TLC plate containing standard 5,7-dimethoxyflavone and the ethanolic extracts which were developed in toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2) were photographed under UV 254 nm by a digital camera. The image of TLC plate was analyzed for 5,7-dimethoxyflavone peak areas by ImageJ software. The amounts of 5,7-dimethoxyflavone were found to be 1.96 ± 0.51 g/100g of *K. parviflora* rhizomes crude drug (Table 20).

Table 20 The amount of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC image analysis (% by weight)

Source	5,7-Dimethoxyflavone in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100g of dried crude drug)	5,7-Dimethoxyflavone in *K. parviflora* rhizomes (g/100g of dried crude drug)
1	0.35	9.06	3.15
2	0.20	11.04	2.18
3	0.15	10.91	1.58
4	0.13	10.96	1.40
5	0.15	10.68	1.60
6	0.29	5.47	1.58
7	0.26	9.78	2.57
8	0.19	10.50	2.04
9	0.18	9.07	1.61
10	0.19	8.90	1.71
11	0.21	8.81	1.88
12	0.19	8.36	1.58
13	0.20	11.14	2.27
14	0.30	8.89	2.64
15	0.16	9.97	1.57
	Average		**1.96 ± 0.51**

Method validation (TLC image analysis)

Calibration range

The calibration curve of standard 5,7-dimethoxyflavone was polynomial in the range of 0.6-3.0 µg/spot with the regession equation of $y = -2959.6x^2 + 20545x - 532.97$. The coefficient of determination (R^2) of 5,7-dimethoxyflavone was 0.9991 (Figure 16).

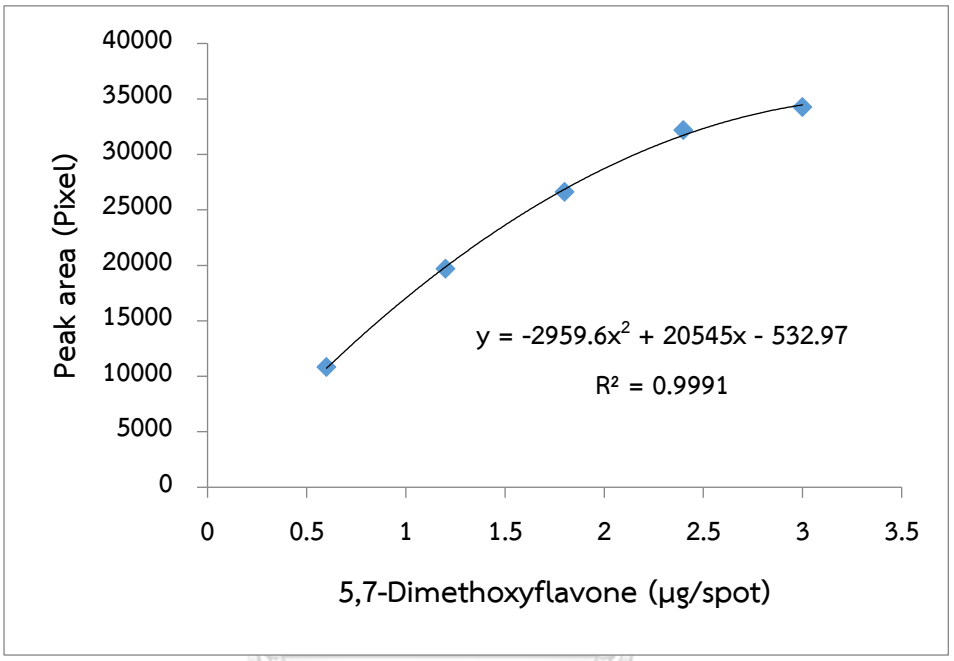


Figure 16 The calibration curve of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC image analysis

Detection limit and quantitation limit

The detection limit and quantitation limit determination calculated based on the standard deviation of regression line and the slope of the calibration curve were found to be 0.08 and 0.23 µg/spot, respectively.

Accuracy

The recovery values of spiked known amount of 5,7-dimethoxyflavone in the ethanolic extract of *K. parviflora* rhizomes were 85.31 – 100.56 % by TLC image analysis as demonstrated in Table 21.

Table 21 Accuracy of quantitation of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC image analysis (n=3)

5,7-Dimethoxyflavone added (µg/spot)	5,7-Dimethoxyflavone found (µg/spot)	% Recovery
0.00	0.90 ± 0.02	-
0.15	1.01 ± 0.03	96.19 ± 4.84
0.75	1.66 ± 0.03	100.56 ± 3.14
1.50	2.05 ± 0.12	85.31 ± 4.92
	Average	**94.02 ± 7.86**

Precision

The repeatability and intermediate precision by TLC image analysis were 3.26 and 4.49 %RSD respectively as demonstrated in Table 22.

Table 22 Repeatability and intermediate precision of quantitation of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC image analysis (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	% RSD	Amount (µg/spot)	% RSD
0.90 ± 0.02	2.46	0.90 ± 0.04	4.82
1.01 ± 0.03	3.02	1.02 ± 0.04	3.94
1.66 ± 0.03	1.84	1.57 ± 0.09	5.56
2.05 ± 0.12	5.73	2.04 ± 0.07	3.65
Average	**3.26 ± 1.71**		**4.49 ± 0.87**

Robustness

The robustness of 5,7-dimethoxyflavone quantitation in *K. parviflora* rhizomes by TLC image analysis was 2.38 %RSD of peak area (Table 23).

Table 23 Robustness of 5,7-dimethoxyflavone in *K. parviflora* rhizomes by TLC image analysis

Mobile phase composition **Toluene: Chloroform: Acetone: Formic acid**	**Peak area**
10 : 8 : 2 : 0.4	32612.62
10.1 : 7.9 : 2.1 : 0.3	31705.08
9.9 : 8.1 : 1.9 : 0.5	31107.75
Mean ± SD	**31808.48 ± 757.75**
%RSD	**2.38**

Method comparison

The result showed that the 5,7-dimethoxyflavone contents from two methods were not statistically significantly different (paired t-test, $P > 0.05$) (Table 24).

Table 24 5,7-Dimethoxyflavone contents in *K. parviflora* rhizomes by TLC-densitometry and TLC image analysis

Source	5,7-Dimethoxyflavone contents (g/100g of dried crude drug)	
	TLC-densitometry	TLC image analysis
1	3.52	3.15
2	2.19	2.18
3	1.79	1.58
4	1.94	1.40
5	1.30	1.60
6	2.24	1.58
7	2.82	2.57
8	2.16	2.04
9	1.61	1.61
10	1.72	1.71
11	2.00	1.88
12	1.71	1.58
13	1.63	2.27
14	3.43	2.64
15	2.13	1.57
Average	**2.15 ± 0.64**	**1.96 ± 0.51**

Zanthoxylum piperitum fruits

Ethanolic extraction of *Z. piperitum* fruits

The dried powders of *Z. piperitum* fruits from 15 different sources in Thailand were extracted with 95 % ethanol by Soxhlet apparatus. The average percent yield of *Z. piperitum* fruits ethanolic extract was 26.72 ± 5.60 % by weight (Table 25).

Table 25 The percent yield of ethanolic extract of *Z. piperitum* fruits from 15 different sources in Thailand

Sources	Weight of sample (g)	Weight of extractive matter (g)	% yield
Phuket	5.01	1.56	31.08
Rayong	5.01	1.14	22.73
Songkhla	5.00	1.55	30.99
Nakhon Sawan	5.00	1.17	23.39
Petchaburi	5.01	0.71	14.25
Krabi	5.05	1.15	22.84
Lampang	5.00	1.53	30.54
Chanthaburi	5.00	1.30	26.05
Surat Thani	5.00	1.42	28.45
Udon Thani	5.00	1.64	32.77
Nakhon Ratchasima	5.01	1.53	30.61
Bangkok	5.08	1.32	26.10
Nonthaburi	5.00	0.88	17.57
Lampang2	5.00	1.61	32.11
Ubon Ratchathani	5.00	1.57	31.34
		Average	26.72 ± 5.60
		Min	14.25
		Max	32.77

Quantitative analysis of xanthoxylin contents in *Zanthoxylum piperitum* fruits by TLC-densitometry

Standard xanthoxylin and the ethanolic extracts were developed in toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2). TLC plate was scanned by CAMAG TLC scanner under 291 nm. TLC densitograms of xanthoxylin standards and samples of *Z. piperitum* fruits performed under wavelength of 291 nm were demonstrated in Figure 17. The peak areas were computed by winCATS software. The ethanolic extracts of *Z. piperitum* fruits were determined for the xanthoxylin content in triplicate by TLC-densitometry and found to be 0.51 ± 0.43 g/100g of the crude drug (Table 26).

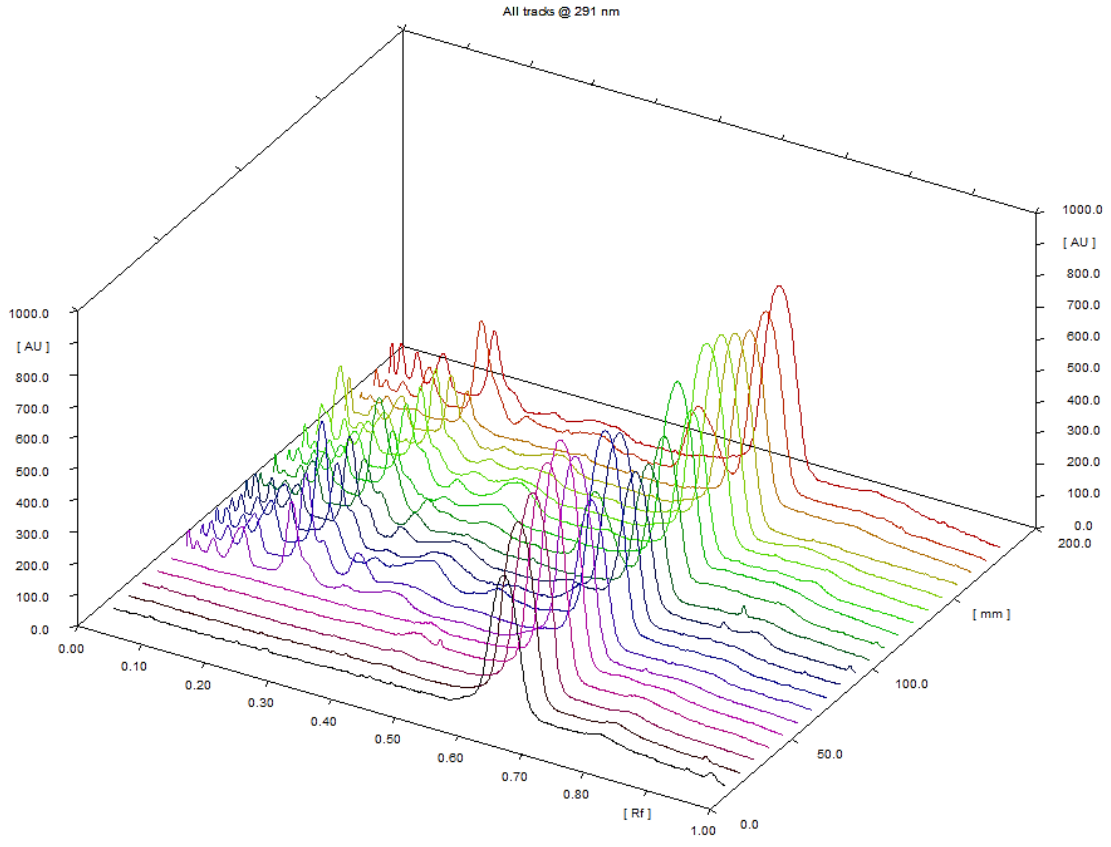


Figure 17 TLC densitograms of xanthoxylin standards and samples of *Z. piperitum* fruits under UV 291 nm

Table 26 The amount of xanthoxylin in *Z. piperitum* fruits from 15 sources in Thailand by TLC-densitometry

Source	Xanthoxylin in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100g of dried crude drug)	Xanthoxylin in *Z. piperitum* fruits (g/100g of dried crude drug)
1	0.040	31.077	1.240
2	0.006	22.734	0.127
3	0.017	30.986	0.535
4	0.016	23.395	0.366
5	0.005	14.253	0.066
6	0.005	22.845	0.108
7	0.005	30.535	0.161
8	0.029	26.053	0.765
9	0.005	28.446	0.151
10	0.018	32.768	0.604
11	0.016	30.608	0.500
12	0.028	26.099	0.719
13	0.089	17.568	1.556
14	0.010	32.113	0.322
15	0.015	31.343	0.469
	Average		**0.513 ± 0.427**

Method validation (TLC-densitometry)

Calibration range

The calibration curve of standard xanthoxylin was polynomial in the range of 0.6 - 3.0 µg/spot with the regession equation of $y = -2452.4x^2 + 17022x + 4720$. The coefficient of determination (R^2) of xanthoxylin was 0.9990 (Figure 18).

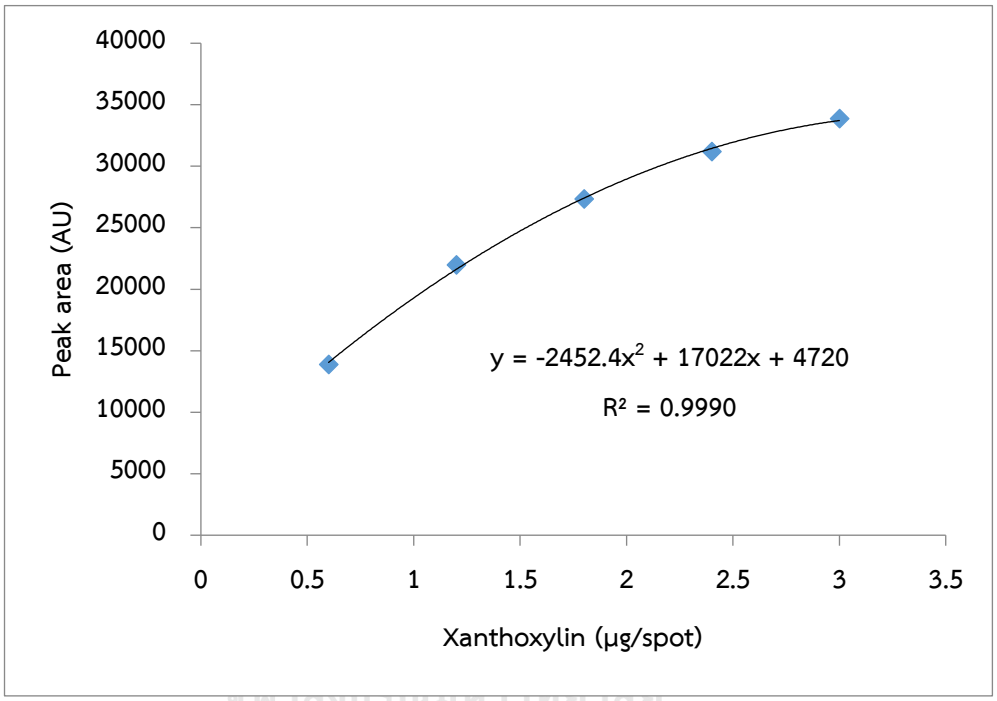


Figure 18 The calibration curve of standard xanthoxylin by TLC-densitometry

Detection limit and quantitation limit

The LOD and LOQ values were found to be 0.07 and 0.21 µg/spot, respectively.

Accuracy

The recovery of xanthoxylin spiked into the ethanolic extract of *Z. piperitum* fruits were 94.60 – 102.64 % as demonstrated in Table 27.

Table 27 Accuracy of quantitation of xanthoxylin in *Z. piperitum* fruits by TLC-densitometry (n=3)

Xanthoxylin added (µg/spot)	Xanthoxylin found (µg/spot)	% Recovery
0.00	0.98 ± 0.03	-
0.15	1.16 ± 0.01	102.64 ± 2.19
0.90	1.78 ± 0.04	94.60 ± 2.83
1.80	2.74 ± 0.04	98.60 ± 2.20
	Average	**98.62 ± 4.02**

Precision

The repeatability and intermediate precision were 1.70 and 2.64 %RSD respectively as demonstrated in Table 28.

Table 28 Repeatability and intermediate precision of xanthoxylin in *Z. piperitum* fruits by TLC-densitometry (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	% RSD	Amount (µg/spot)	% RSD
0.98 ± 0.03	2.83	0.92 ± 0.03	3.10
1.16 ± 0.01	0.58	1.13 ± 0.03	2.34
1.78 ± 0.04	2.03	1.85 ± 0.04	2.07
2.74 ± 0.04	1.37	2.75 ± 0.08	3.05
Average	**1.70 ± 0.96**		**2.64 ± 0.52**

Specificity

Peak identity and peak purity of xanthoxylin absorption spectra were demonstrated in Figure 19 and 20. The maximum absorbance of xanthoxylin was at the wavelength 291 nm.

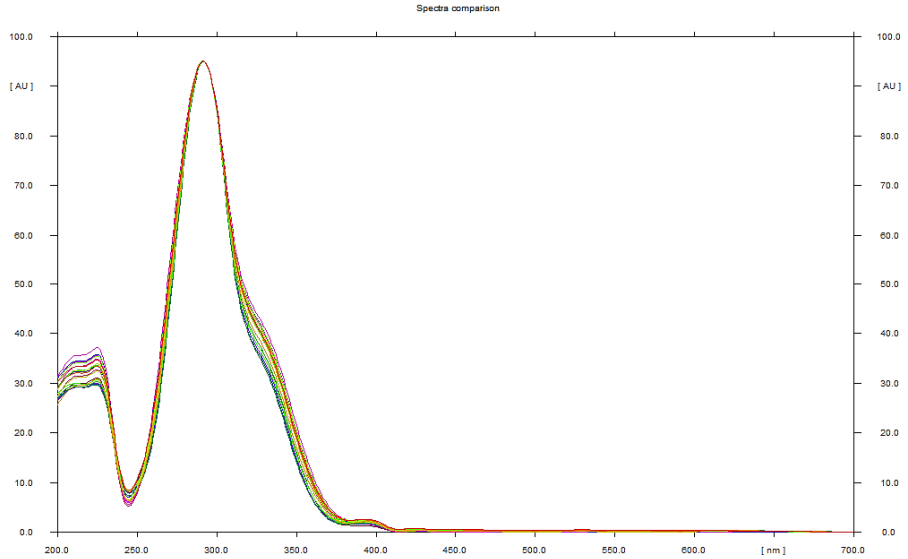


Figure 19 The absorbance spectra of xanthoxylin in *Z. piperitum* extracts from 15 different sources and standard xanthoxylin representing peak identity

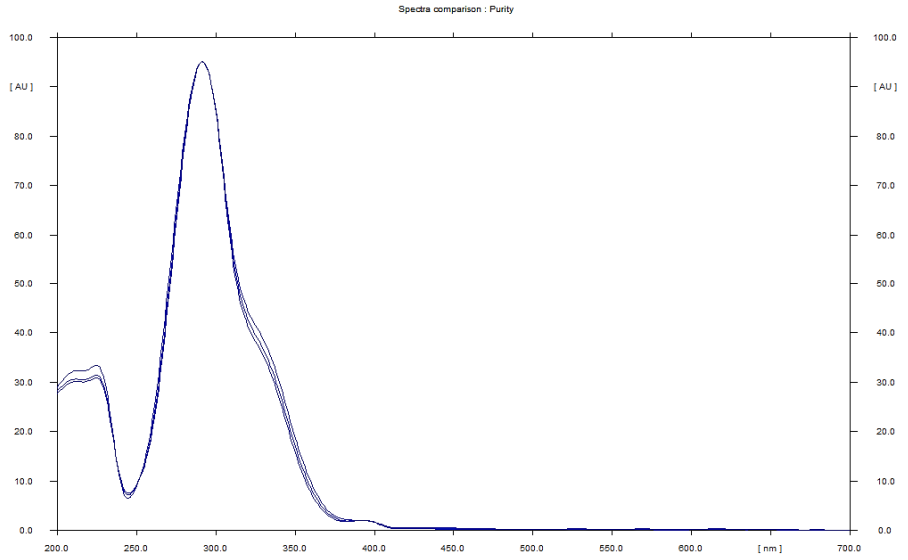


Figure 20 Peak purity measurement using up-slope, apex and down-slope of the peak

Robustness

The robustness of xanthoxylin quantitation in *Z. piperitum* fruits by TLC densitometric analysis performed by adjusting the mobile phase ratio was shown in Table 29. The result of robustness was 1.79 %RSD of peak area.

Table 29 Robustness of xanthoxylin in *Z. piperitum* fruits by TLC-densitometry

Mobile phase composition **Toluene: Chloroform: Acetone: Formic acid**	**Peak area**
10.0 : 8.0 : 2.0 : 0.4	26638.16
10.1 : 7.9 : 2.1 : 0.3	26488.96
9.9 : 8.1 : 1.9 : 0.5	25757.65
Mean ± SD	**26294.92 ± 471.24**
%RSD	**1.79**

Quantitative analysis of xanthoxylin contents in *Zanthoxylum piperitum* fruits by TLC image analysis

The developed TLC plates containing standard xanthoxylin and the ethanolic extracts were sprayed with 1% aluminium chloride in ethanol and photographed under UV 365 nm by a digital camera. The image of TLC plate was analyzed for xanthoxylin peak areas by ImageJ software. The amounts of xanthoxylin were found to be 0.52 ± 0.45 g/100g of *Z. piperitum* fruits crude drug (Table 30).

Table 30 The amount of xanthoxylin in *Z. piperitum* fruits by TLC image analysis (% by weight)

Source	Xanthoxylin in the ethanolic extract (g/g)	Yield of the ethanolic extract (g/100g of dried crude drug)	Xanthoxylin in *Z. piperitum* fruits (g/100g of dried crude drug)
1	0.034	31.077	1.046
2	0.008	22.734	0.182
3	0.017	30.986	0.522
4	0.015	23.395	0.346
5	0.005	14.253	0.078
6	0.005	22.845	0.107
7	0.006	30.535	0.182
8	0.024	26.053	0.634
9	0.009	28.446	0.250
10	0.017	32.768	0.566
11	0.019	30.608	0.580
12	0.031	26.099	0.809
13	0.105	17.568	1.841
14	0.011	32.113	0.367
15	0.011	31.343	0.345
	Average		**0.524 ± 0.453**

Method validation (TLC image analysis)

Calibration range

The calibration curve of standard xanthoxylin was polynomial in the range of 0.6-3.0 µg/spot with the regession equation of $y = -3589.6x^2 + 26482x - 4.5114$. The coefficient of determination (R^2) of xanthoxylin was 0.9990 (Figure 21).

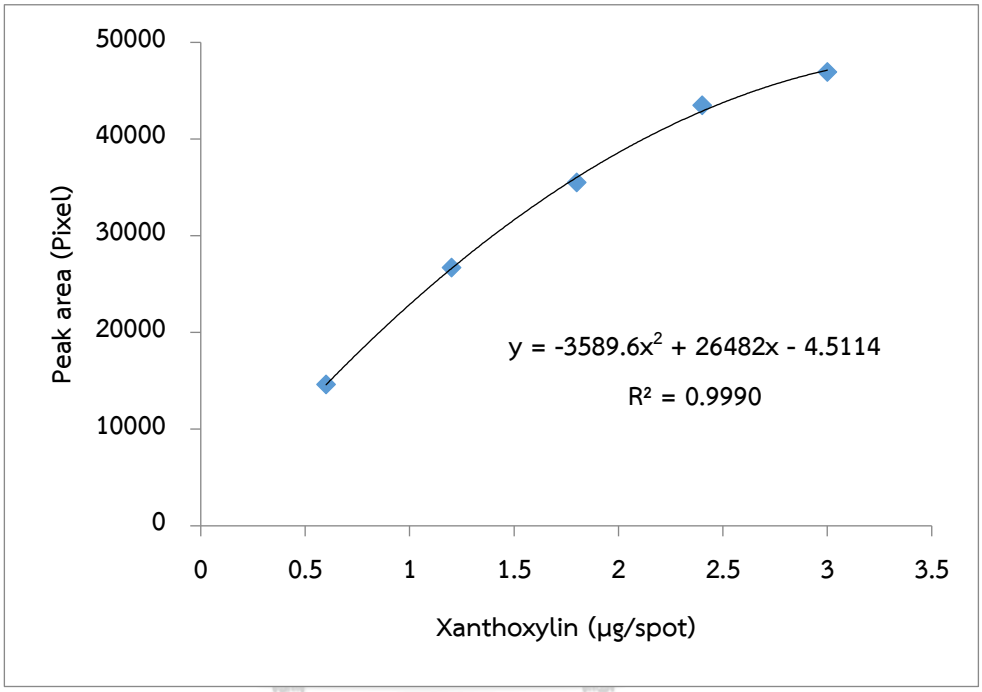


Figure 21 The calibration curve of xanthoxylin in *Z. piperitum* fruits by TLC image analysis

Detection limit and quantitation limit

The detection limit and quantitation limit determination calculated based on the standard deviation of regression line and the slope of the calibration curve were found to be 0.08 and 0.24 µg/spot, respectively.

Accuracy

The recovery values of spiked known amount of xanthoxylin in the ethanolic extract of *Z. piperitum* fruits were 98.14 – 100.20 % by TLC image analysis as demonstrated in Table 31.

Table 31 Accuracy of quantitation of xanthoxylin in *Z. piperitum* fruits by TLC image analysis (n=3)

Xanthoxylin added (µg/spot)	Xanthoxylin found (µg/spot)	% Recovery
0.00	0.84 ± 0.01	-
0.15	0.98 ± 0.05	99.62 ± 5.79
0.90	1.71 ± 0.05	98.14 ± 3.39
1.80	2.64 ± 0.02	100.20 ± 0.65
	Average	**99.32 ± 1.06**

Precision

The repeatability and intermediate precision by TLC image analysis were 2.45 and 2.95 %RSD respectively as demonstrated in Table 32.

Table 32 Repeatability and intermediate precision of quantitation of xanthoxylin in *Z. piperitum* fruits by TLC image analysis (n=3)

Repeatability		Intermediate precision	
Amount (µg/spot)	**% RSD**	**Amount (µg/spot)**	**% RSD**
0.84 ± 0.01	1.39	0.84 ± 0.01	0.79
0.98 ± 0.05	4.79	1.03 ± 0.01	0.66
1.71 ± 0.05	2.85	1.71 ± 0.12	9.98
2.64 ± 0.02	0.77	2.77 ± 0.09	3.39
Average	**2.45 ± 1.79**		**2.95 ± 2.97**

Robustness

The robustness of xanthoxylin quantitation in *Z. piperitum* fruits by TLC image analysis was 2.90 %RSD of peak area (Table 33).

Table 33 Robustness of xanthoxylin in *Z. piperitum* fruits by TLC image analysis

Mobile phase composition **Toluene: Chloroform: Acetone: Formic acid**	**Peak area**
10 : 8 : 2 : 0.4	35463.69
10.1 : 7.9 : 2.1 : 0.3	35416.47
9.9 : 8.1 : 1.9 : 0.5	33687.63
Mean ± SD	**34855.93 ± 1012.05**
%RSD	**2.90**

Method comparison

The result showed that the xanthoxylin contents from two methods were not statistically significantly different (paired t-test, P > 0.05) (Table 34).

Table 34 The comparison of xanthoxylin contents in *Z. piperitum* fruits by TLC-densitometry and TLC image analysis

Source	Xanthoxylin contents (g/100g of dried crude drug)	
	TLC-densitometry	TLC image analysis
1	1.24	1.05
2	0.13	0.18
3	0.54	0.52
4	0.37	0.35
5	0.07	0.08
6	0.11	0.11
7	0.16	0.18
8	0.77	0.63
9	0.15	0.25
10	0.60	0.57
11	0.50	0.58
12	0.72	0.81
13	1.56	1.84
14	0.32	0.37
15	0.47	0.35
Average	**0.51 ± 0.43**	**0.52 ± 0.45**

In vitro biological activities

Antioxidant activities

DPPH radical scavenging activity

The results of DPPH radical scavenging activity of each extract and its active constituent were demonstrated in Table 35. BHT and quercetin which were used as positive control showed IC_{50} of 32.55 and 3.82 µg/ml, respectively (Figure 22). The chlorogenic acid (IC_{50} = 10.59 µg/ml) exhibited more potent free radical scavenging activity than BHT (IC_{50} = 32.55 µg/ml).

Table 35 DPPH radical scavenging activity (IC_{50}) of the ethanolic extract of *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits

Samples	IC_{50} (µg/ml)
The ethanolic extract of *C. odorata* leaves	72.23
Chlorogenic acid	10.59
The ethanolic extract of *K. parviflora* rhizomes	226.10
5,7-Dimethoxyflavone	> 1000.00
The ethanolic extract of *Z. piperitum* fruits	95.00
Xanthoxylin	27497.00
Butylated hydroxyl toluene (BHT)	32.55
Quercetin	3.82

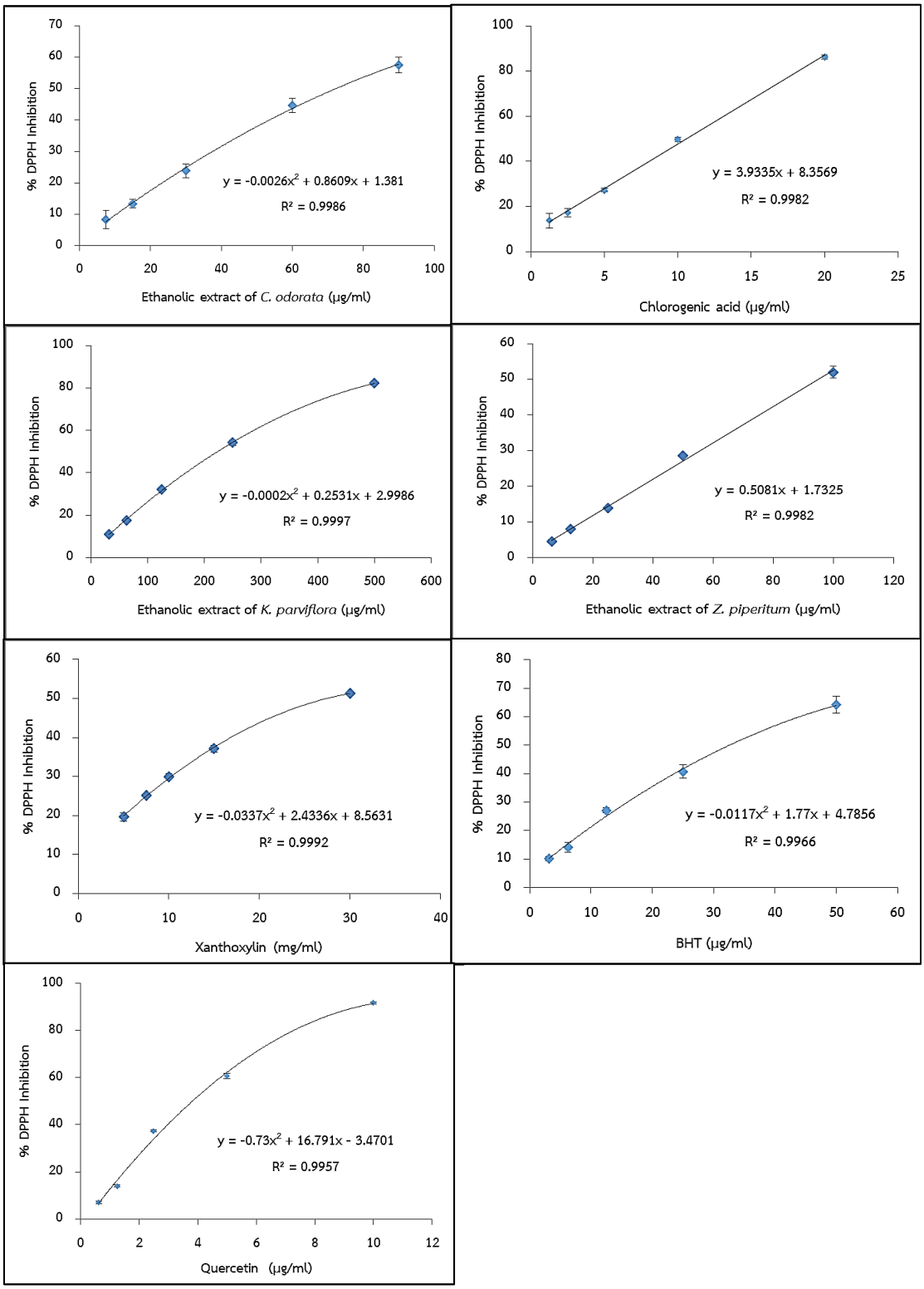


Figure 22 DPPH inhibition of tested samples

Ferric ion reducing antioxidant power (FRAP)

The ferrous sulphate ion concentration was calculated according to the equation of standard curve of ferrous sulphate (Figure 23). BHT and quercetin were used as positive control. The results of tested samples were presented in Table 36. The ethanolic extract of *C. odorata* leaves demonstrated the highest reducing power ability with FRAP value of 0.992 mM Fe(II)/mg, followed by chlorogenic acid, *Z. piperitum* ethanolic extract, *K. parviflora* ethanolic extract and xanthoxylin with FRAP value of 0.956, 0.637, 0.339 and 0.040 mM Fe(II)/mg, respectively. On the other hand, the 5,7-dimethoxyflavone had the lowest reducing power ability with FRAP value of 0.012 mM Fe(II)/mg.

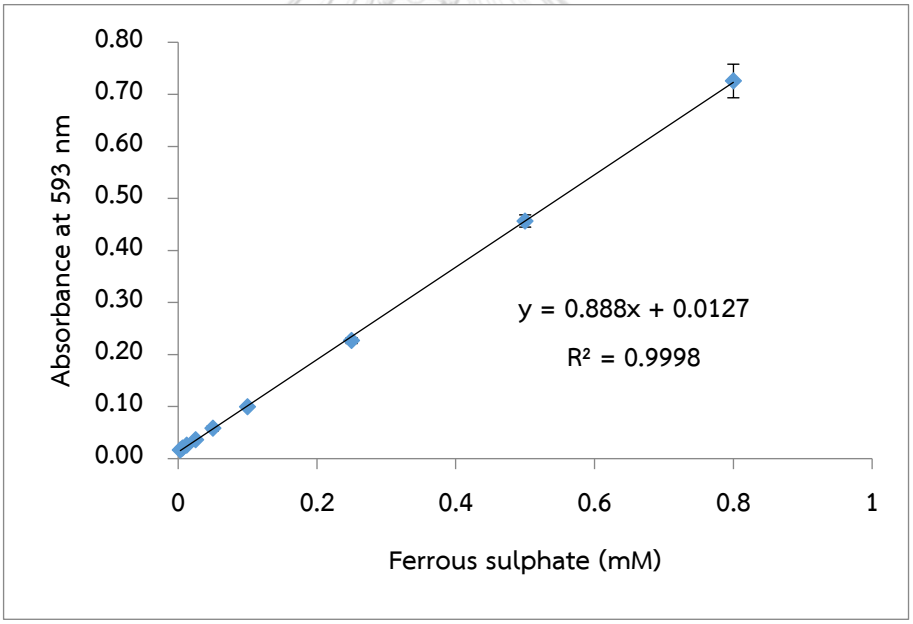


Figure 23 Standard curve for determination of antioxidant capacity by ferric ion reducing antioxidant power

Table 36 FRAP value of *C. odorata* ethanolic extract, chlorogenic acid, *K. parviflora* ethanolic extract, 5,7-dimethoxyflavone, *Z. piperitum* ethanolic extract, xanthoxylin, quercetin and BHT

Samples	FRAP value (mM Fe(II)/mg)
The ethanolic extract of *C. odorata* leaves	0.992
Chlorogenic acid	0.956
The ethanolic extract of *K. parviflora* rhizomes	0.339
5,7-Dimethoxyflavone	0.012
The ethanolic extract of *Z. piperitum* fruits	0.637
Xanthoxylin	0.040
Butylated hydroxyl toluene (BHT)	0.985
Quercetin	0.973

Beta-carotene bleaching inhibition

The peroxidation inhibition in the beta-carotene bleaching assay were evaluated and demonstrated in Table 37. *C. odorata* ethanolic extract, *K. parviflora* ethanolic extract and *Z. piperitum* ethanolic extract at 2 mg/ml showed 69.46 %, 62.15 % and 48.09 % antioxidant activity compared to 60.82 %, 3.03 % and 3.74 % of chlorogenic acid, 5,7-dimethoxyflavone and xanthoxylin at the same concentration, respectively. BHT and quercetin which were used as positive control showed peroxidation inhibition of 93.77 % and 87.98 %, respectively. The antioxidant activities of these extracts, standard compounds and positive controls demonstrated the dose-response relationship (Figure 24).

Table 37 Beta-carotene bleaching inhibition of *C. odorata* leaf, *K. parviflora* rhizome, *Z. piperitum* fruit, chlorogenic acid, 5,7-dimethoxyflavone, xanthoxylin and positive controls at the concentration of 2 mg/ml

Tested samples	**Beta-carotene bleaching inhibition (%)**
C. odorata leaf ethanolic extract	69.46 %
K. parviflora rhizome ethanolic extract	62.15 %
Z. piperitum fruit ethanolic extract	48.09 %
Chlorogenic acid	60.82 %
5,7-Dimethoxyflavone	3.03 %
Xanthoxylin	3.74 %
Butylated hydroxytoluene (BHT)	93.77 %
Quercetin	87.98 %

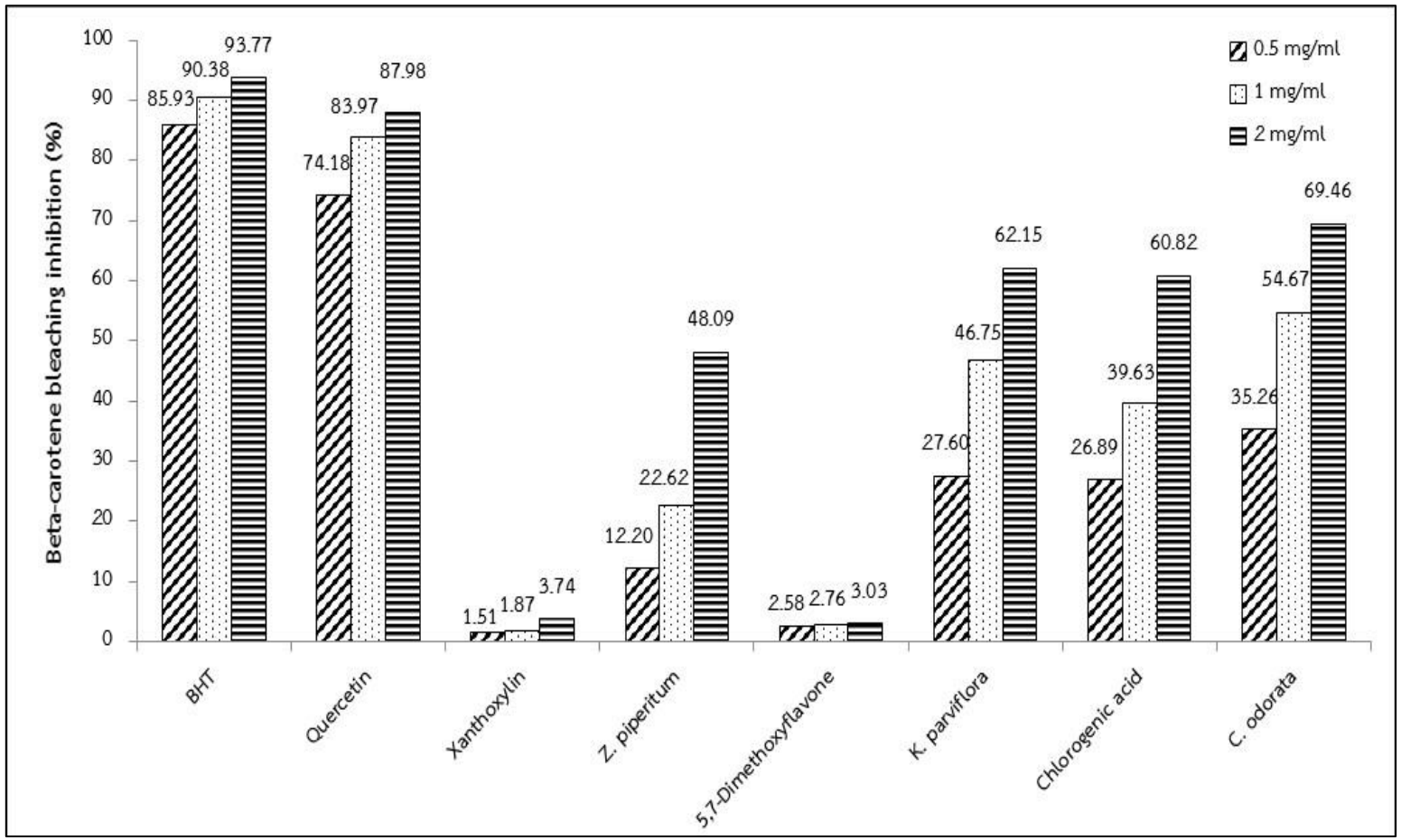


Figure 24 The antioxidant activity of varying concentrations of tested samples compared to BHT and quercetin by beta-carotene bleaching assay

Total phenolic content

The ethanolic extract of *C. odorata*, *K. parviflora* and *Z. piperitum* were evaluated for the total phenolic content using Folin-Ciocalteu reagent. Gallic acid was used to set up a calibration curve (Figure 25). The total phenolic contents of the ethanolic extract of *C. odorata*, *K. parviflora* and *Z. piperitum* were presented in Table 38. The ethanolic extract of *C. odorata* leaves showed the highest total phenolic content (70.06 mg GAE/g extract), followed by *Z. piperitum* ethanolic extract and *K. parviflora* ethanolic extract with total phenolic contents of 37.28 and 22.08 mg GAE/g extract, respectively.

Table 38 Total phenolic content of the ethanolic extract of *C. odorata*, *K. parviflora* and *Z. piperitum*, which calculated using the equation from standard curve of gallic acid

Samples	Gallic acid equivalent (mg GAE/g extract)
The ethanolic extract of *C. odorata* leaves	88.36 ± 1.71
The ethanolic extract of *K. parviflora* rhizomes	49.09 ± 1.92
The ethanolic extract of *Z. piperitum* fruits	61.53 ± 2.46

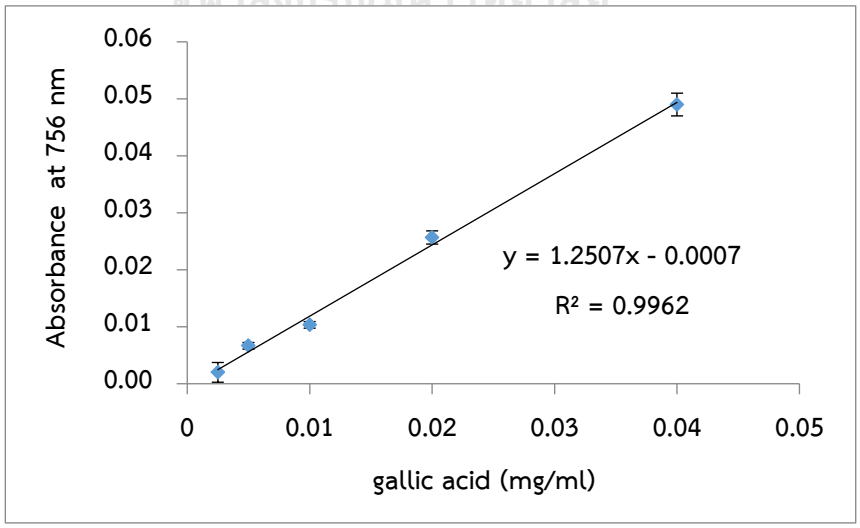


Figure 25 Gallic acid calibration curve for total phenolic quantification

Brine shrimp lethality activity

The results of brine shrimp lethality testing of *C. odorata* leaves, *K. parviflora* rhizomes, and *Z. piperitum* fruits were shown in Table 39 and expressed as LC_{50} values: LC_{50} values > 1000 µg/ml (non - toxicity), ≥ 500 ≤ 1000 µg/ml (weak toxicity) and < 500 µg/ml (toxic) [98]. It was found that the ethanolic extract of *K. parviflora* rhizomes exhibited the highest toxicity against brine shrimp nauplii with LC_{50} of 41.28 µg/ml, whereas the ethanolic extract of *C. odorata* leaves and *Z. piperitum* fruits showed LC_{50} of 874.46 µg/ml and 654.00 µg/ml respectively (Figure 26).

Table 39 Brine shrimp lethality (LC_{50}) of the ethanolic extract of *C. odorata*, *K. parviflora* and *Z. piperitum*

Samples	Brine shrimp lethality (24 hrs) LC_{50} (µg/ml)	Toxicity
C. odorata leaf ethanolic extract	874.46	weak
K. parviflora rhizome ethanolic extract	41.28	toxic
Z. piperitum fruit ethanolic extract	654.00	weak

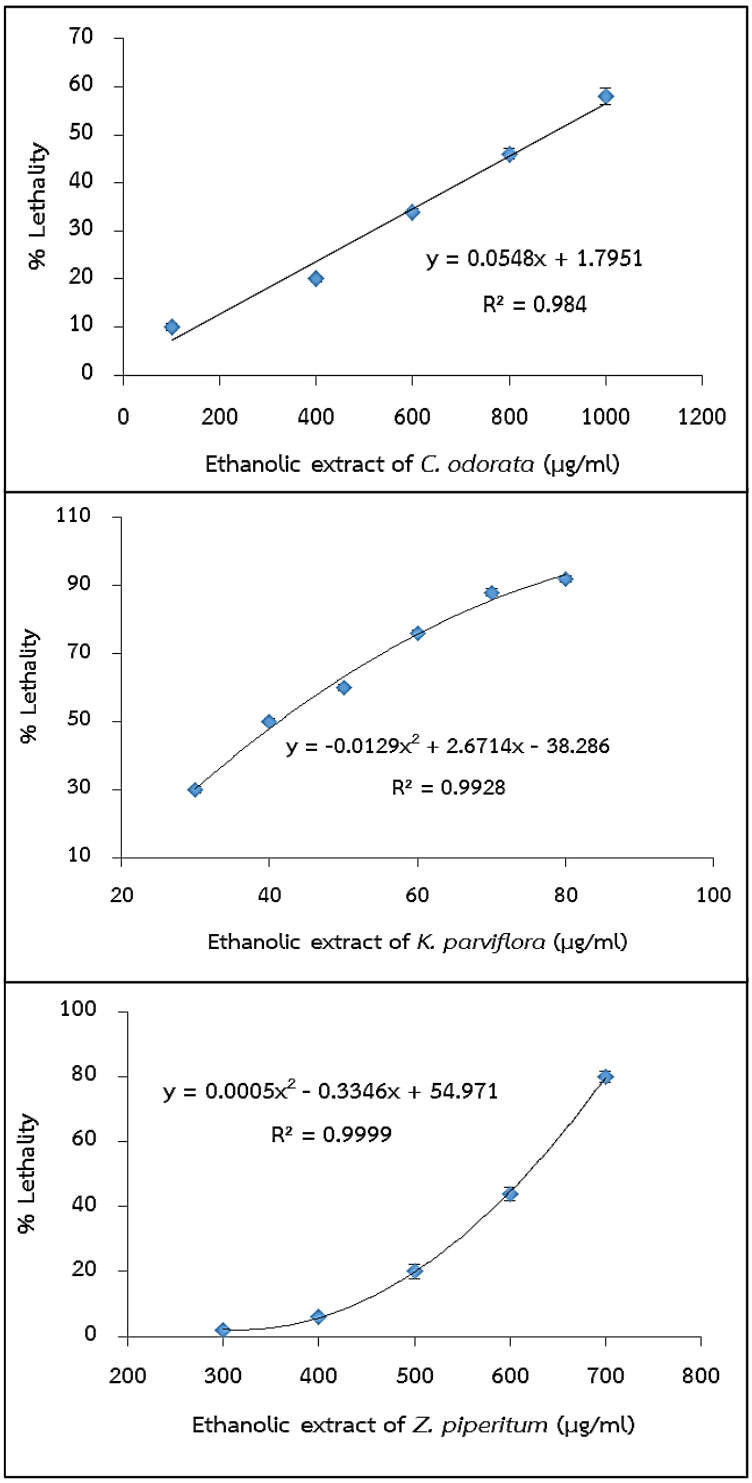


Figure 26 Cytotoxic activity of tested samples due to brine shrimp lethality testing

MTT cell viability activity

The ethanolic extract *C. odorata*, *K. parviflora*, *Z. piperitum*, standard compounds and positive control were tested for *in vitro* cytotoxic activity against 5 human cancer cell lines and 1 normal cell line. Doxorubicin was used as a positive control. The results were shown in Table 40. The criteria of cytotoxicity for the crude extract and pure compound which established by the U.S. National Cancer Institute (NCI), is an IC_{50} < 20 µg/ml and < 4 µg/ml respectively [99]. As the result, all tested samples exhibited no significant cytotoxic activity against six cell lines with IC_{50} more than standard criteria (20 µg/ml). However, *C. odorata* ethanolic extract, 5,7-dimethoxyflavone and xanthoxylin showed more cytotoxic potential against hepatocarcinoma (HEP-G2) with IC_{50} of 30.02, 21.27 and 23.12 µg/ml respectively, whereas the ethanolic extract of *K. parviflora* rhizome showed more cytotoxic potential against breast ductal carcinoma (BT474) with IC_{50} of 30.13 µg/ml.

Table 40 IC_{50} of *C. odorata* ethanolic extract, *K. parviflora* ethanolic extract, *Z. piperitum* ethanolic extract, standard compounds and positive control on 5 human cancer cell lines and 1 normal cell line

Samples	IC_{50} (µg/ml)					
	BT474	CHAGO-K1	HEP-G2	KATO-III	SW620	Wi-38
C. odorata leaf	41.81	39.59	30.02	33.48	41.71	61.65
Chlorogenic acid	>100	>100	>100	>100	>100	>100
K. parviflora rhizome	30.13	58.54	40.11	46.66	34.57	38.67
5,7-Dimethoxyflavone	29.66	29.49	21.27	24.46	27.90	24.91
Z. piperitum fruit	>100	>100	>100	>100	>100	>100
Xanthoxylin	28.56	24.30	23.12	24.32	38.47	38.45
Doxorubicin	0.40	0.50	0.07	0.71	0.23	0.17

IC_{50} : The concentration at which cell viability was reduced by 50%

Antidiabetic activities

The results of antidiabetic activities of tested samples were shown in Table 41. The antidiabetic activities of these extract, standard compounds and positive controls demonstrated the dose-response relationship (Figure 27, 28).

For yeast α-glucosidase, the ethanolic extract of *Z. piperitum* fruit and xanthoxylin showed the highest potential effect on yeast α-glucosidase inhibition with IC_{50} of 4.42 and 8.71 mg/ml respectively when compared to acarbose which used as positive control (IC_{50} = 11.63 mg/ml). On the contrary, the ethanolic extract of *K. parviflora* rhizome showed the lowest potential effect on yeast α-glucosidase inhibition (IC_{50} = 29.99 mg/ml).

For rat α-glucosidase, these extracts and standard compounds showed the potential effect on rat α-glucosidase inhibition less than positive control (acarbose). The ethanolic extract of *C. odorata* leaf, *K. parviflora* rhizome and *Z. piperitum* fruit exhibited the IC_{50} values of 10.23, 9.22 and 14.10 mg/ml, respectively.

Table 41 Antidiabetic activities of *C. odorata* ethanolic extract, *K. parviflora* ethanolic extract, *Z. piperitum* ethanolic extract, standard compounds and positive control

Tested samples	IC_{50} (mg/ml)	
	Yeast α-glucosidase inhibition	Rat α-glucosidase inhibition
C. odorata leaf ethanolic extract	17.48	10.23
Chlorogenic acid	16.54	11.47
K. parviflora rhizome ethanolic extract	29.99	9.22
5,7-Dimethoxyflavone	> 1.00	> 1.00
Z. piperitum fruit ethanolic extract	4.42	14.10
Xanthoxylin	8.71	> 40.00
Acarbose	11.63	0.30

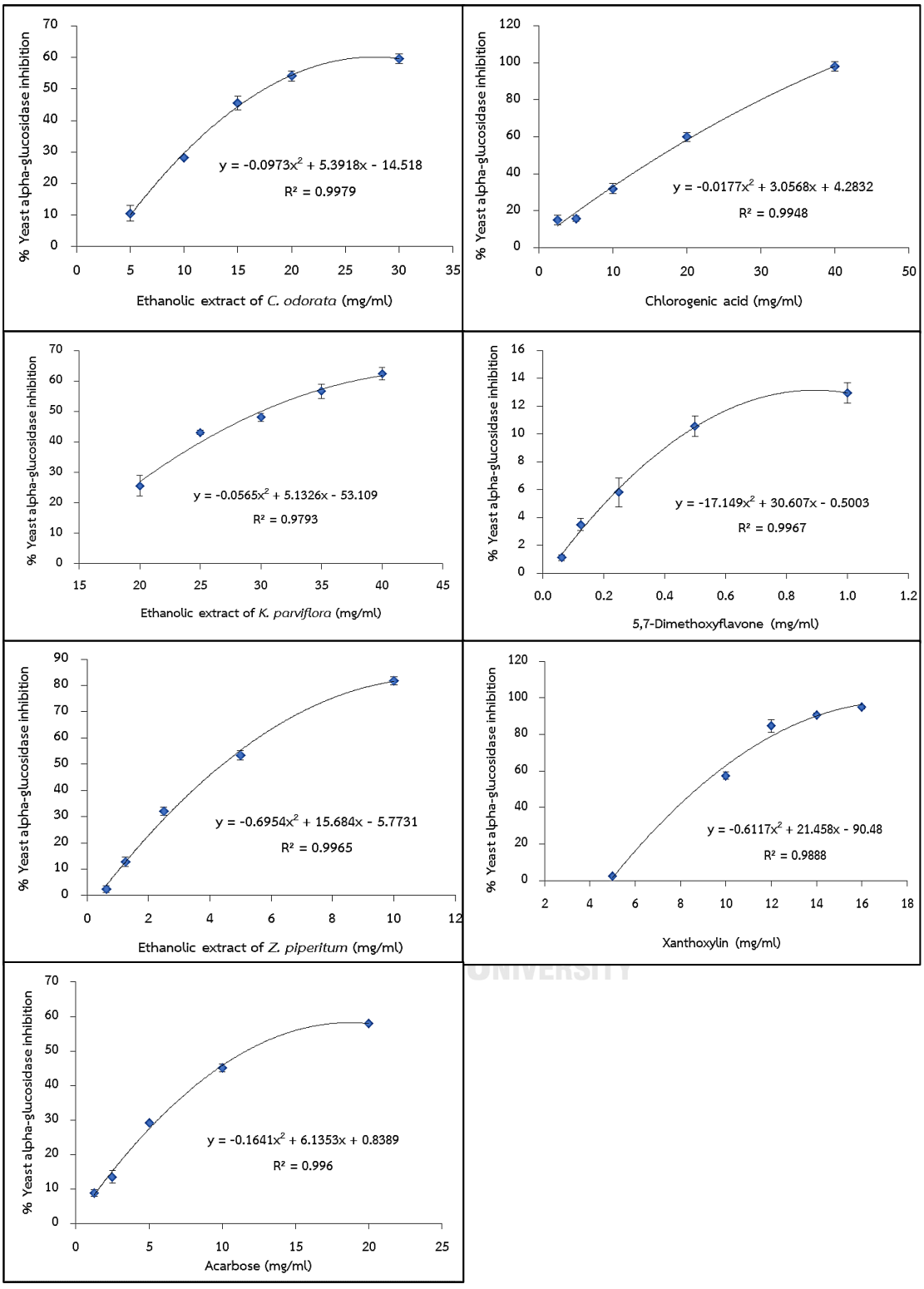


Figure 27 Yeast alpha-glucosidase inhibition of tested samples varying in concentrations

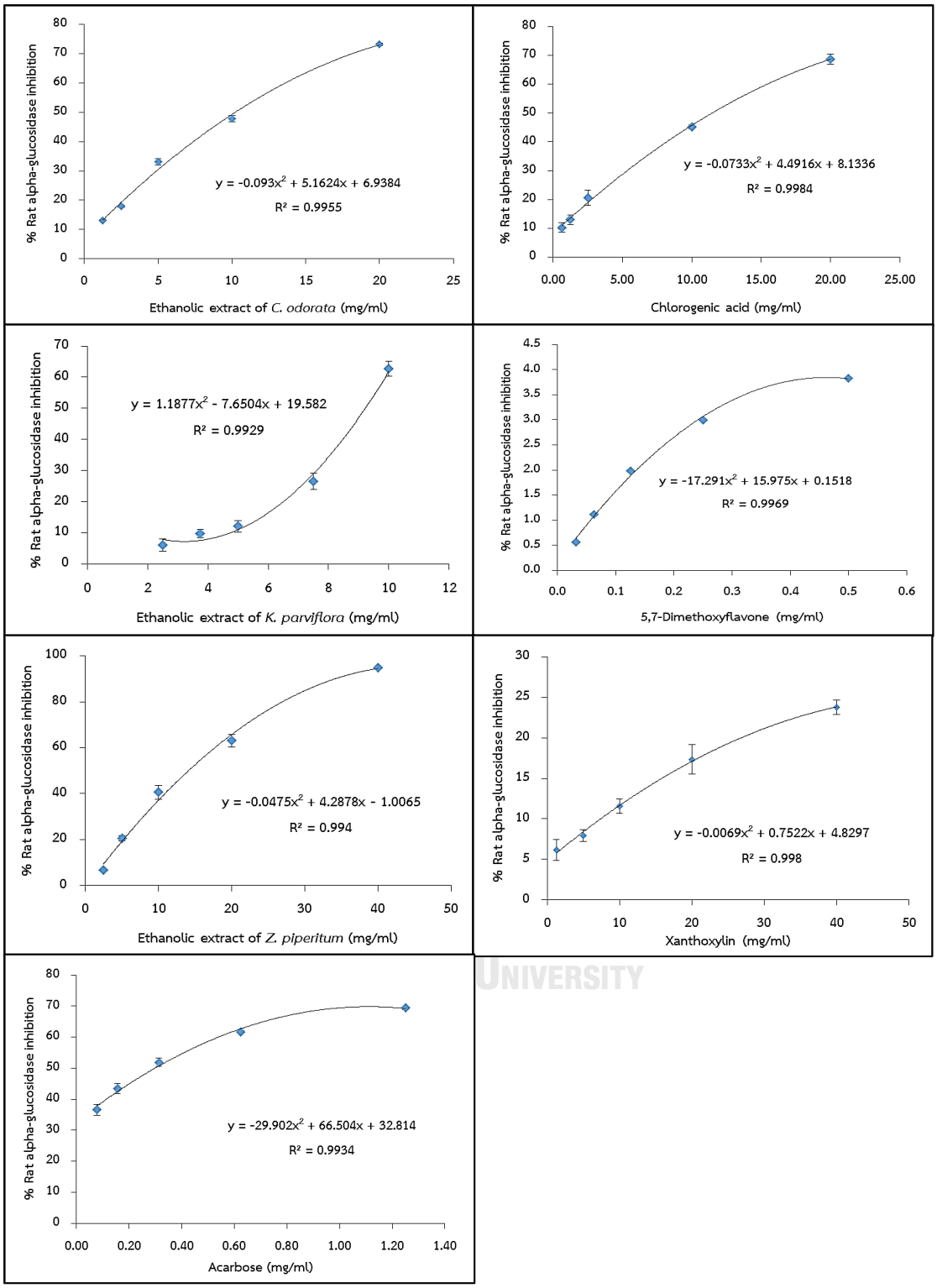


Figure 28 Rat alpha-glucosidase inhibition of tested samples varying in concentrations

Chemical constituents of *Chromolaena odorata* essential oil by GC-MS

The percent yield of *C. odorata* leaves essential oil was found to be 0.03 % by weight. The essential oils of *C. odorata* leaves were analyzed by GC-MS and at least 20 compounds were detected as shown in Table 42. GC chromatogram of *C. odorata* essential oil was shown in Figure 29. Pregeijerene, dauca-5,8-diene, α-pinene, (E)-caryophyllene and β-pinene were found as major components of the essential oil. Their quantities were 40.60%, 16.75%, 9.67%, 6.11% and 5.37% respectively.

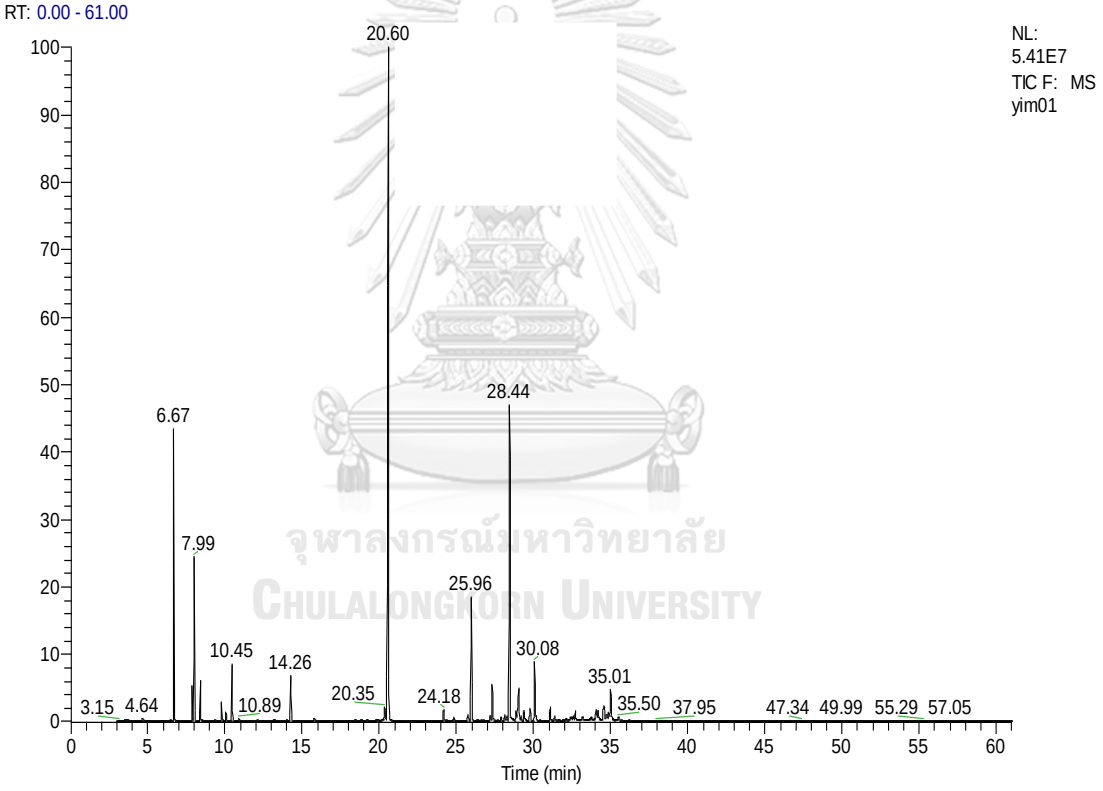


Figure 29 GC chromatogram of *C. odorata* essential oil

Table 42 The chemical constituents of *C. odorata* essential oil

Compound name	Retention time (min)	Peak area %	Kovat's index*
α-Pinene	6.67	9.67	939
Sabinene	7.87	1.10	975
β-Pinene	7.99	5.37	979
Myrcene	8.38	1.54	990
Sylvestrene	9.77	0.77	1030
(E)-β-Ocimene	10.45	2.20	1050
Geijerene	14.26	1.90	1143
Cogeijerene	20.35	0.80	1285
Pregeijerene	20.60	40.60	1287
(E)-Caryophyllene	25.96	6.11	1419
α-Humulene	27.33	1.77	1454
Dauca-5,8-diene	28.44	16.75	1472
γ-Muurolene	28.98	0.86	1479
Bicyclogermacrene	29.04	1.65	1500
α-Bulnesene	29.37	0.67	1509
γ-Cadinene	29.77	0.85	1513
Δ-Cadinene	30.08	3.21	1523
Elemol	31.07	0.77	1549
α-Muurolol	34.55	1.25	1646
α-Cadinol	35.01	2.19	1654

*Kovat's index: retention indices determined relative to *n*-alkanes (C_6-C_{24}) on ZP-5 GC column

Chemical constituents of *Kaempferia parviflora* essential oil by GC-MS

The percent yield of *K. parviflora* rhizome essential oil was found to be 0.028 ± 0.012 % by weight. The essential oil of *K. parviflora* dried rhizomes consisted of at least 20 compounds as shown in Table 43. GC chromatogram of *K. parviflora* essential oil was shown in Figure 30. It was found that *K. parviflora* dried rhizome oils among 15 habitats in Thailand contained similar pattern of chemical constituents but varied in the content ratio. The major components of *K. parviflora* essential oil were α-copaene (11.68%), dauca-5,8-diene (11.17%), camphene (8.73%), β-pinene (7.18%), borneol (7.05%) and linalool (6.58%), respectively.

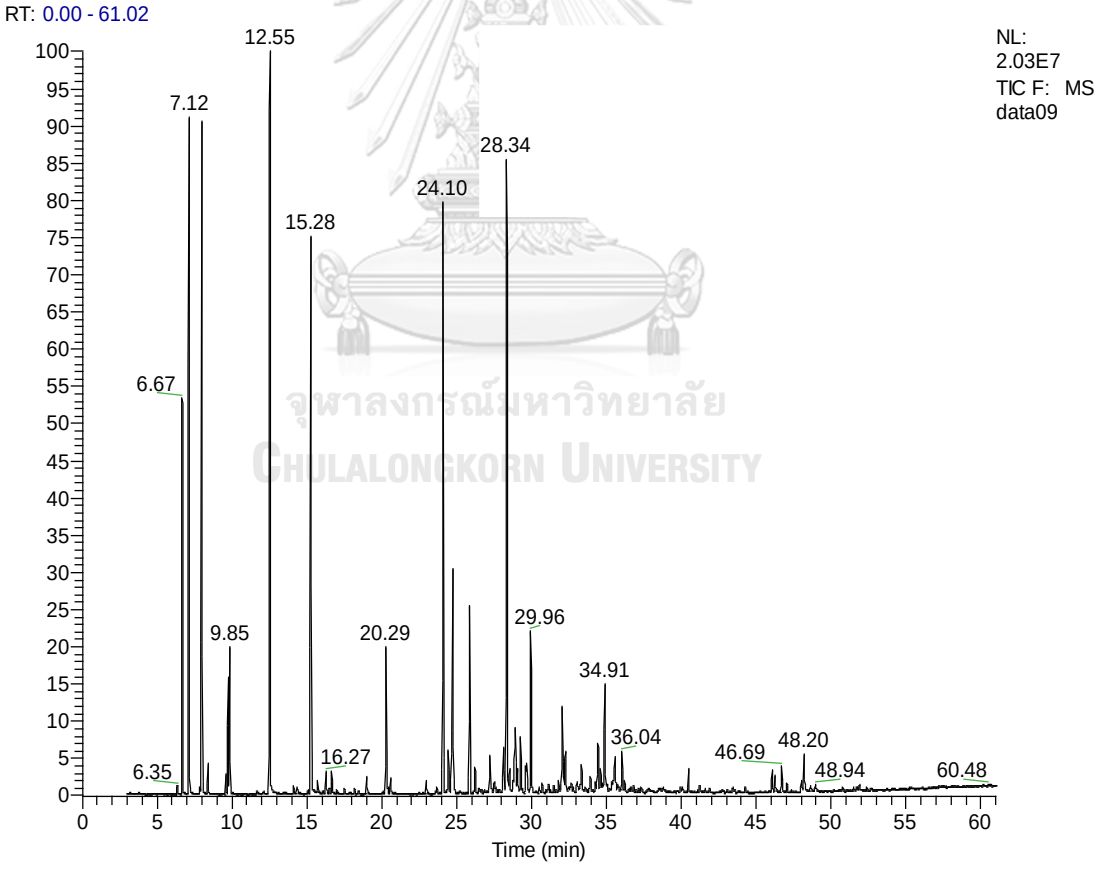


Figure 30 GC chromatogram of *K. parviflora* essential oil

Table 43 The chemical constituents of *K. parviflora* essential oil

Compound name	Retention time (min)	Peak area %[a]	Kovat's index[b]
α-Pinene	6.67	5.51 ± 2.75	939
Camphene	7.12	8.73 ± 4.70	954
β-Pinene	7.99	7.18 ± 3.81	979
Limonene	9.75	1.80 ± 0.16	1029
Linalool	12.48	6.58 ± 4.64	1096
Borneol	15.24	7.05 ± 2.70	1169
Bornyl acetate	20.29	3.75 ± 2.18	1288
α-Copaene	24.10	11.68 ± 3.98	1376
β-Elemene	24.74	5.83 ± 2.86	1390
(E)-Caryophyllene	25.85	6.03 ± 2.33	1419
α-Humulene	27.22	1.88 ± 0.43	1454
Dauca-5,8-diene	28.34	11.17 ± 5.21	1472
γ- Gurjunene	28.94	2.78 ± 1.13	1477
β-Salinene	29.30	2.05 ± 0.72	1490
Δ-Cadinene	29.96	3.89 ± 1.33	1523
Spathulenol	32.05	2.21 ± 0.90	1578
Caryophyllene oxide	32.25	2.95 ± 1.85	1583
Epi-α-muurolol	34.45	2.25 ± 0.82	1642
α-Cadinol	34.90	4.00 ± 1.82	1654
Longiborneol acetate	35.56	1.93 ± 0.81	1685

[a] The peak area were shown as mean ± SD in percentage. Samples were from 15 different sources. [b] Kovat's index: retention indices determined relative to *n*-alkanes (C_6-C_{24}) on ZP-5 GC column

Chemical constituents of *Zanthoxylum piperitum* essential oil by GC-MS

The percent yield of *Z. piperitum* fruit essential oil was found to be 1.27 ± 0.52 % by weight. The essential oils of *Z. piperitum* dried fruits were analyzed by GC-MS and at least 20 compounds were detected as shown in Table 44, 45. GC chromatogram of *Z. piperitum* essential oil was shown in Figure 31, 32. Limonene (18.81 %), methylchavicol (13.31 %), sabinene (7.77 %) and myrcene (4.70 %) were found as major components in cluster 1, whereas limonene (22.77 %), β-phellandrene (18.27 %), sabinene (15.65 %) and terpinen-4-ol (9.53 %) were found as major components in cluster 2.

The GC chromatogram showed that *Z. piperitum* essential oil could be divided into two clusters (Figure 31, 32). Cluster 1 consisted of sample from Khonkaen, Lampang, Lampang2 and Trang. Cluster 2 consisted of sample from Phuket, Rayong, Songkhla, Nakhon Sawan, Petchaburi, Chanthaburi, Surat Thani, Nakhon Ratchasima, Udon Thani, Bangkok and Nonthaburi.

Table 44 The chemical constituents of *Z. piperitum* essential oil (Cluster 1)

Compound name	RT (min)	Peak area %[a]	KI[b]
α-Thujene	6.04	2.82 ± 2.79	930
α-Pinene	6.27	3.95 ± 1.58	939
Sabinene	7.41	7.77 ± 0.35	969
β-Pinene	7.58	2.50 ± 3.39	979
Myrcene	7.89	4.70 ± 2.18	990
α-Phellandrene	8.49	2.55 ± 3.98	1002
α-Terpinene	8.86	3.20 ± 3.81	1017
o-Cymene	9.13	3.57 ± 3.74	1026
Limonene	9.29	18.81 ± 6.64	1029
(Z)-β-Ocimene	9.50	2.25 ± 0.77	1037
(E)-β-Ocimene	9.92	0.49 ± 0.12	1050
γ-Terpinene	10.35	2.52 ± 1.79	1059
Terpinolene	11.41	0.79 ± 0.39	1088
Linalool	11.97	1.10 ± 0.83	1096
Terpinen-4-ol	15.32	3.95 ± 2.92	1177
α-Terpineol	15.94	0.80 ± 0.00	1188
Methylchavicol	16.02	13.31 ± 23.36	1196
Piperitone	18.40	0.51 ± 0.12	1252
α-Terpinyl acetate	22.34	0.62 ± 0.09	1349
2,4-dimethylether phloroacetophenone	34.74	1.13 ± 0.00	1668

[a] The peak area were shown as mean ± SD in percentage. Samples were from 15 different sources. [b] Kovat's index: retention indices determined relative to *n*-alkanes (C_6-C_{24}) on ZP-5 GC column

Table 45 The chemical constituents of *Z. piperitum* essential oil (Cluster 2)

Compound name	RT (min)	Peak area %[a]	KI[b]
α-Thujene	6.04	1.70 ± 0.45	930
α-Pinene	6.27	6.37 ± 2.07	939
Sabinene	7.41	15.65 ± 9.49	969
β-Pinene	7.58	1.41 ± 0.45	979
Myrcene	7.89	5.38 ± 1.32	990
α-Phellandrene	8.49	0.69 ± 0.15	1002
α-Terpinene	8.86	2.70 ± 0.80	1017
o-Cymene	9.13	3.60 ± 1.33	1026
Limonene	9.29	22.77 ± 15.04	1029
β-Phellandrene	9.36	18.27 ± 2.81	1029
(Z)-β-Ocimene	9.50	4.03 ± 1.47	1037
(E)-β-Ocimene	9.92	0.86 ± 0.29	1050
γ-Terpinene	10.35	5.50 ± 1.76	1059
Terpinolene	11.41	1.54 ± 0.44	1088
Linalool	11.97	2.44 ± 1.26	1096
Terpinen-4-ol	15.32	9.53 ± 2.72	1177
α-Terpineol	15.94	1.29 ± 0.49	1188
Piperitone	18.40	1.87 ± 0.67	1252
α-Terpinyl acetate	22.34	1.29 ± 0.60	1349
2,4-dimethylether phloroacetophenone	34.74	2.48 ± 1.09	1668

[a] The peak area were shown as mean ± SD in percentage. Samples were from 15 different sources. [b] Kovat's index: retention indices determined relative to *n*-alkanes (C_6-C_{24}) on ZP-5 GC column

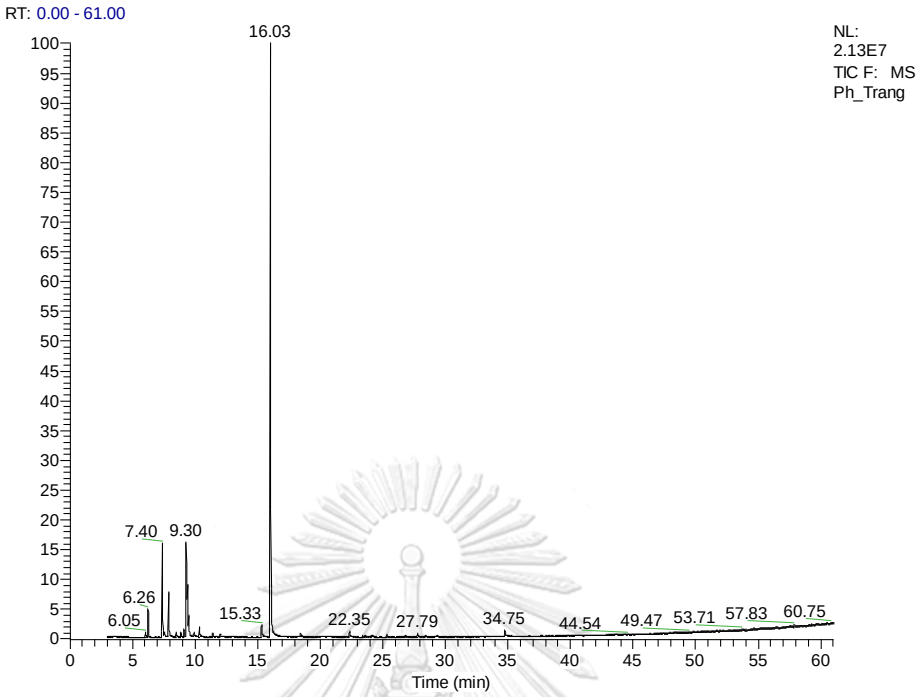


Figure 31 GC chromatogram of *Z. piperitum* essential oil from Trang province (cluster 1)

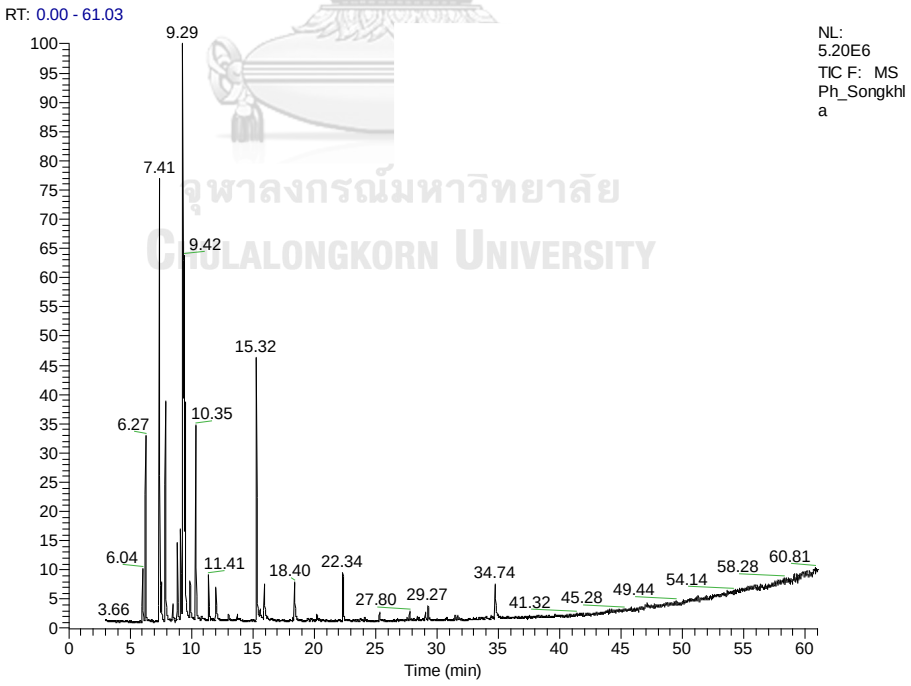


Figure 32 GC chromatogram of *Z. piperitum* essential oil from Songkhla province (cluster 2)

Pharmacognostic specification of *Kaempferia parviflora*

Scientific Name *Kaempferia parviflora* Wall. ex Baker

Common name Krachaidum

Family Zingiberaceae

Distribution India, Myanmar, Thailand (the upper Northeastern regions)

Used part Rhizome

Macroscopic evaluation

Herbaceous plant with 50-70 cm tall. Rhizome purplish-black. Simple leaf with deep violet color, oval/elliptical shape, 5-10 cm wide, 10-15 cm length. Flowers are inflorescence and appear between the stem and the base of leaf, stalk 5-6 cm, petals fuse at base forming a tube, length 3-3.2 cm, split at tip. Stamens are sterile, white, oblong, 3 mm wide, 10-13 mm long and purple lip. The botanical drawing of *K. parviflora* was shown in Figure 33. The dried rhizome of *K. parviflora* was purplish-black color, 1-2 cm in width and 4-5 cm in length (Figure 34).

Microscopic evaluation

The transverse section of *K. parviflora* rhizome showed the anatomical characteristics of epidermis, hypodermis, cork cell, mass of oleoresin, cortical parenchyma, oil droplet, vascular bundle, endodermis, stele parenchyma and starch grain in parenchyma (Figure 35). The histological characteristics of powder of *K. parviflora* rhizome demonstrated starch grain, parenchyma containing starch grain, mass of oleoresin, cork in surface view, fragment of reticulated vessel and parenchyma in longitudinal view (Figure 36).

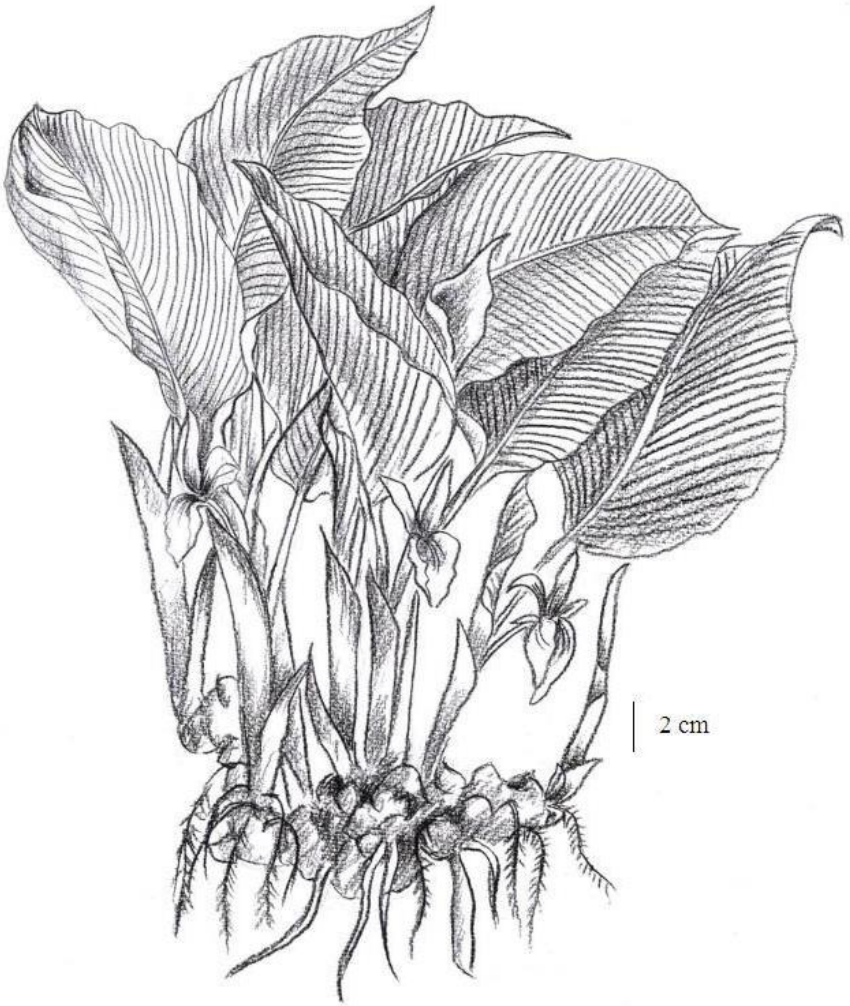


Figure 33 Whole plant of *K. parviflora*

Figure 34 Dried rhizome of *K. parviflora*

Anatomical Character

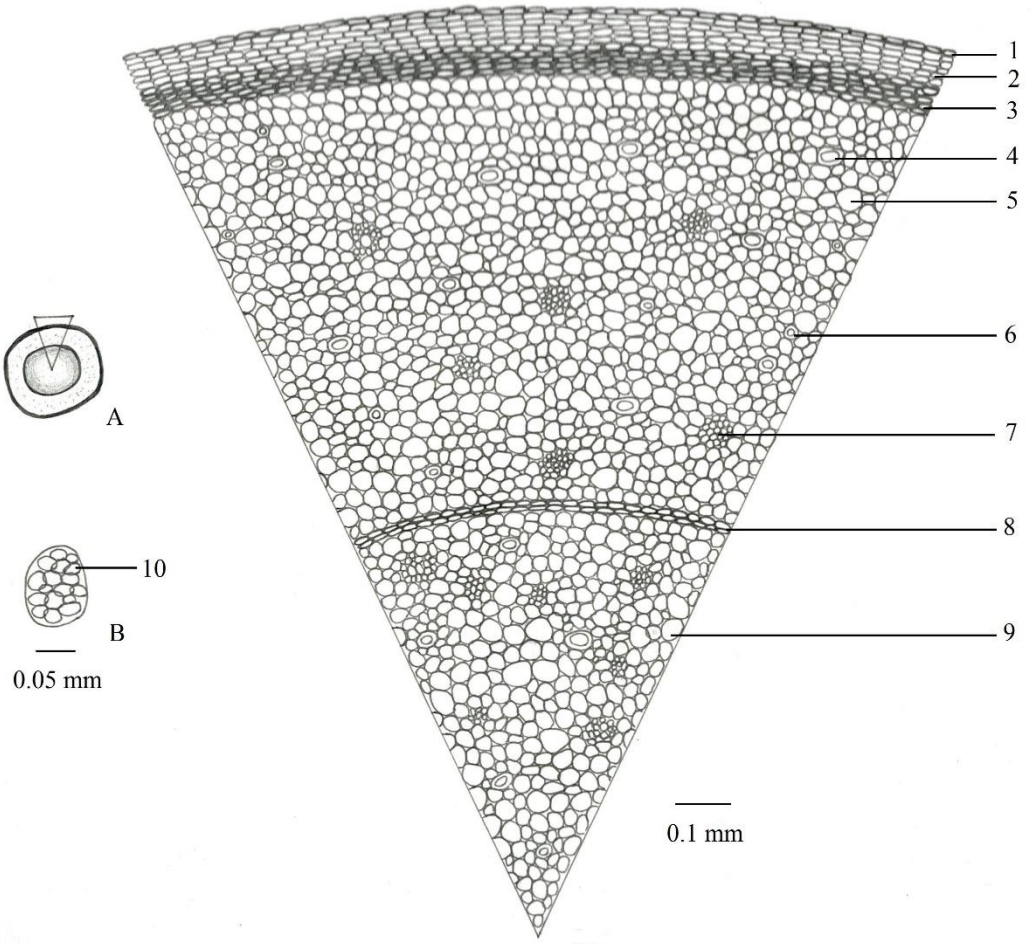


Figure 35 Transverse section of *K. parviflora* rhizome

A. Diagram of transverse section

B. Cortical parenchyma

1. Epidermis
2. Hypodermis
3. Cork cell
4. Mass of oleoresin
5. Cortical parenchyma
6. Oil droplet
7. Vascular bundle
8. Endodermis
9. Stele parenchyma
10. Starch grain in parenchyma

Histological Character

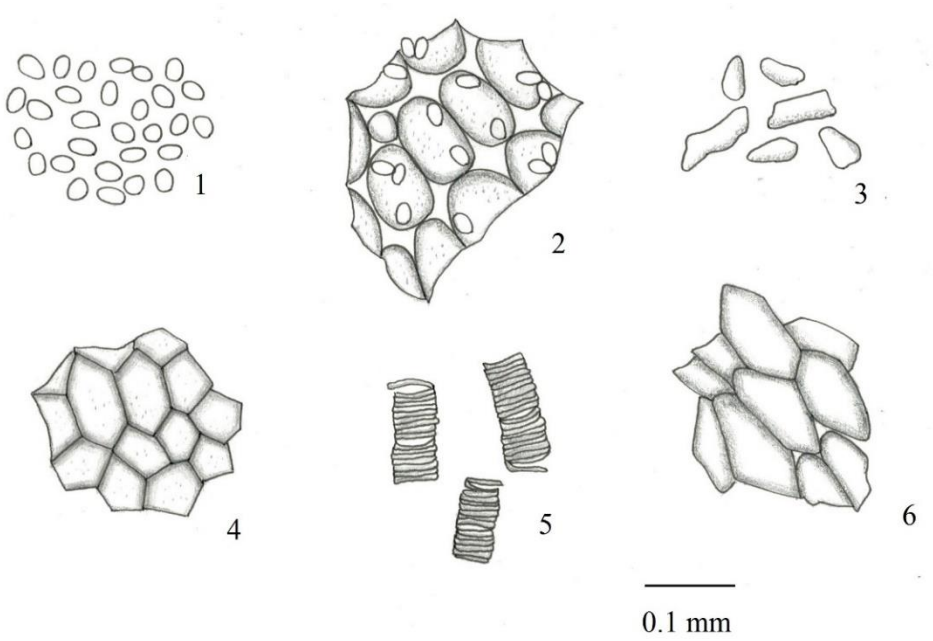


Figure 36 Histological characters of *K. parviflora* rhizome in powdered form

1. Starch grain
2. Parenchyma containing starch grain
3. Mass of oleoresin
4. Cork, surface view
5. Fragment of reticulated vessel
6. Parenchyma, longitudinal view

Thin layer chromatographic fingerprint

The ethanolic extract of *K. parviflora* was spotted on TLC silica gel 60 GF_{254} plate developed in toluene: chloroform: acetone: formic acid (5: 4: 1: 0.2) and observed under ultraviolet light (254, 365 nm) and dipped with anisaldehyde reagent (Figure 37).

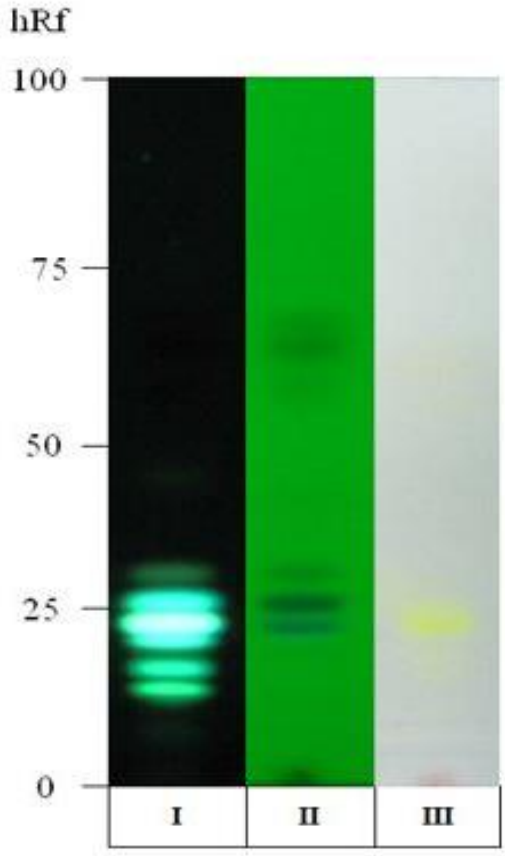


Figure 37 TLC fingerprint of ethanolic extract of *K. parviflora* rhizome

I = detection under UV light 365 nm

II = detection under UV light 254 nm

III = detection with anisaldehyde reagent

Physico-chemical parameters of dried *Kaempferia parviflora* rhizome

The contents of physico-chemical parameters of *K. parviflora* rhizome were shown in Table 46. The loss on drying, total ash, acid insoluble ash and water content should be not more than 9.0, 5.1, 2.2 and 9.3 % of dry weight respectively. The volatile oil content, ethanol soluble extractive matter and water soluble extractive matter should not be less than 0.03, 5.14 and 8.25 % by dry weight respectively.

Table 46 Physico-chemical content of *K. parviflora* rhizome (% by weight)

Specification	Content (% dry weight)*
Loss on drying	8.979 ± 0.041
Total ash	5.127 ± 0.060
Acid insoluble ash	2.174 ± 0.092
Water content	9.291 ± 0.458
Volatile oil content	0.028 ± 0.003
Ethanol soluble extractive matter	5.138 ± 0.092
Water soluble extractive matter	8.254 ± 0.191

*The parameters were shown as grand mean ± pooled SD. Samples were collected from 15 different sources in Thailand. Each sample was tested in triplicate.

CHAPTER V
DISCUSSION AND CONCLUSION

Quantitative analysis by TLC-densitometry and TLC image analysis

The quality control needs to measure the phytochemical compounds in medicinal plants for ensuring the quality reliability of natural products obtained from plant sources. The chemical compounds in medicinal plant can change depending on many factors such as botanical sources, harvest seasons or drying processes. Therefore, one of a major problem for the quality control of herbal medicines is the insufficient of the markers which are both primary and secondary compounds in plants. Moreover, the American Herbal Pharmacopoeia has discussed that a single or several chemical markers are necessarily used for assuring the quality control [2]. In order to achieve the quality control of herbal medicine, the conventional practice of selecting a single marker for testing has been determined for active components of plant materials based on the applicability of analytical techniques. Quantitative analysis of multi-components by single marker was proposed and accepted as a method to reflect the internal quality of medicinal plants. Therefore, reliable methods are required to assay the amounts of toxic or active compounds in medicinal plants to ensure the safety and internal quality control of herbal medicine [109]. For quantitative analysis, TLC-densitometry as well as TLC-image analysis were developed.

In this study, the active compound contents in medicinal plants were analyzed by thin layer chromatographic technique. The advantages of this technique are easy to use, inexpensive, short time for analysis. It can be used to check purity of compounds and used for quantitative analysis [84]. The compound separated by TLC are quantified using TLC densitometer. Densitometry is the quantitative and qualitative measurement of absorbed visible, UV light or emitted fluorescence upon excitation with UV light [85]. ImageJ is a free software which can quantitate and calculate pixel intensity in digital image of TLC spot and transform to chromatographic peak [87]. In this study, *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits were determined by TLC–densitometry and TLC image analysis. Chlorogenic acid, 5,7-

dimethoxyflavone and xanthoxylin are the active components in *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits respectively and these compounds were used as a standard marker for quantitative analysis. Quantitative analysis using TLC-densitometry and TLC image analysis were validated in terms of accuracy, precision, specificity, calibration range, LOD, LOQ and robustness following ICH guideline which were used to confirm the analytical procedure for reliable, consistent and accurate data.

For method validation, developed TLC-densitometric analysis of active compound contents in the *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits were found to be valid. The quantitative analysis of active compound in *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits by TLC image analysis was also developed and its validity was demonstrated. The specificity was confirmed by comparing light absorption spectrum of the peak at apex among standard compounds and all samples which representing peak identity and comparing the absorption spectrum of the sample peak at up-slope, apex and down-slope which representing peak purity. The absorption spectrum of chlorogenic acid in this study showed the maximum absorbance at the wavelength of 330 nm which in accordance to the previous studies that the maximum UV spectrum of chlorogenic acid could be detected at 330 nm [110, 111]. The maximum absorbance of 5,7-dimethoxyflavone and xanthoxylin in this study was at a wavelength of 265 and 291 nm, respectively. The UV absorption at 265 and 291 nm were the same as the reported data for 5,7-dimethoxyflavone (264 nm) and xanthoxylin (287 nm) in previous studies [112, 113]. Thus, these are optimal wavelength for quantitative analysis which accurately quantified active compound contents in this study. The recovery was determined to evaluate the accuracy by spiking known three concentrations of standard compound in a sample and found to be within acceptable limits (80-115%) [114]. The repeatability and the intermediate precision were determined on the same day and in three different days. The repeatability and the intermediate precision were expressed as %RSD in all cases and found to be < 10 %RSD for both methods. The calibration curves of both methods of these standard compounds (chlorogenic acid, 5,7-

dimethoxyflavone and xanthoxylin) were polynomial relationships with good correlation coefficients (R^2 > 0.999). The detection limit and quantitation limit were calculated based on the standard deviation of regression line and the slope of the calibration curve. In this study, the LOD and LOQ values showed the lowest concentration for analyte in a sample which could be detected and quantitatively defined and displayed sufficient sensitivity of the methods. The robustness of this study was performed by adjusting the mobile phase ratio. The results demonstrated that there were no differences in peak area of active compounds in sample matrix. As the results of method validation, TLC-densitometry and TLC image analysis were efficient and reliable to quantitate the active compound contents in *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits.

The chlorogenic acid content of *C. odorata* leaves determined by TLC-densitometry and TLC image analysis were 0.123 ± 0.031 and 0.122 ± 0.032 g/100 g of dry leaves, respectively. The quantitative analysis of 5,7-dimethoxyflavone in *K. parviflora* rhizomes determined by TLC-densitometry and TLC image analysis were found to be 2.15 ± 0.64 and 1.96 ± 0.51 g/100 g of dry rhizomes, respectively. *K. parviflora* dried rhizomes from 12 different origins were macerated in 95% ethanol for 4 days. The ethanolic extracts were analyzed by gas chromatography with flame ionization detector. The result showed 5,7-dimethoxyflavone contents ranged from 0.62 to 2.17 g/100g of dried powder which was similar to this study [10]. The xanthoxylin content of *Z. piperitum* fruits determined by TLC-densitometry and TLC image analysis were found to be 0.51 ± 0.43 and 0.52 ± 0.45 g/100 g of dry fruits, respectively. For image analysis, the spot of xanthoxylin on developed TLC plate could not be clearly visualized under UV 254 nm because of the minimum absorbance of xanthoxylin at 254 nm as shown in Figure 19. Therefore, the developed TLC plate was sprayed with 1% aluminium chloride in ethanol and the spot of xanthoxylin was obviously inspected under UV 365 nm as the result of bathochromic shift of absorption and the background was clear. The shift of absorption to a longer wavelength is due to substitution or solvent effect [115]. The addition of $AlCl_3$ leads to the formation of an aluminium chloride-dihydroxyl complex resulting in a bathochromic shift [116].

Eventually, the results indicated that the chlorogenic acid, 5,7-dimethoxyflavone and xanthoxylin contents quantified by both methods were not significantly different ($P > 0.05$) using paired t-test. TLC-densitometry is a quantitative technique with high reliability due to measuring the chemical compound related to its optical characteristics [85]**.** Besides this, TLC chromatogram can be saved as image file by TLC visualizer or even digital camera but only in the specified UV wavelengths (254, 365 nm) and the visible range, then quantified the compound band or spot related to the image pixel intensity. TLC image analysis is inexpensive and convenient technique and is demonstrated to be used as an alternative method for quantitative analysis of active compound contents in *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits.

Antioxidant activities

Antioxidants are a group of natural compounds that can be found in the food, medicinal plant materials or supplementation. The natural antioxidants are known to minimize the adverse effects of free radicals in living system. Free radicals cause damage to the cells that lead to poor function of the immune system. Antioxidants are the compounds that can destroy free radicals or oxidants. Important characteristics of antioxidant agents are the ability to donate electron to oxidant or reactive oxygen substances and inhibit oxidative stress reaction [117]. There were four assay for investigation of the antioxidant potential in this study including DPPH free radical scavenging activity, ferric ion reducing antioxidant power assay, beta-carotene bleaching inhibition assay and total phenolic content.

DPPH radical scavenging activity

DPPH radical scavenging assay evaluates the ability to scavenge DPPH radical by antioxidant compounds. The delocalization of DPPH molecule due to the donation of hydrogen or electron to quench the DPPH radical. The color turns from purple to yellow when receives hydrogen atom from antioxidant compounds [88].

In this study, the scavenging activity of *C. odorata* ethanolic extract (IC_{50} = 72.23 µg/ml) was less potent than quercetin, chlorogenic acid and BHT with IC_{50} of 3.82,

10.59 and 32.55 µg/ml respectively. Additionally, this ethanolic extract was more potent than the chloroform extract with IC_{50} value of 0.31 mg/ml [118]. DPPH radical scavenging activity of chlorogenic acid was previously reported the IC_{50} of 7.83 µg/ml, whereas quercetin and BHT showed the DPPH radical scavenging activity with IC_{50} of 4.84 and 24.82 µg/ml, respectively [119]. DPPH radical scavenging activity of the ethanolic extract of *C. odorata* leaves in India was reported the IC_{50} of 780 µg/ml which was less potent than this study [120]. In the same way, Omoregie *et al*. reported IC_{50} of aqueous and ethanol extracts of *C. odorata* leaves in Nigeria as 0.07 and 0.42 mg/ml, respectively [121]. The ethyl acetate, methanolic and aqueous fraction of the root bark of *C. odorata* which defatted with petroleum ether were shown the IC_{50} of 24.47, 12.28 and 170.71 µg/ml, respectively [122]. The different types of solvent extracts of *C. odorata* had different degrees of DPPH radical scavenging potential. Moreover, the DPPH radical scavenging activity of *C. odorata* from different parts and localities expressed the individual potential free radical scavenging capacity.

The ethanolic extract of *K. parviflora* dried rhizomes in this study was able to decolorized DPPH radical with IC_{50} of 226.10 µg/ml compared to quercetin, BHT and 5,7-dimethoxyflavone with the IC_{50} of 3.82, 32.55 and > 1000 µg/ml, respectively. The previous study also reported DPPH radical scavenging activity of methanol extract of *K. parviflora* fresh rhizomes with IC_{50} of 61.5 µg/ml which was more potent than this study [46]. The results demonstrated that *K. parviflora* consisted of a variety of flavonoids which play an important role as radical scavengers. According to the study of Butkhup and Samappito, *K. parviflora* dried rhizomes extracted with 50 ml of 60% aqueous methanol containing 1.2 M HCl showed more potent scavenging activity on the DPPH radical than this study with IC_{50} of 0.02 mg/ml. From the result, they suggested that *K. parviflora* could be used as a potential source of natural antioxidants with pharmaceutical applications [123]. However, the results might be related to many factors such as the different location of collecting the samples, solvent or processing of extraction and other environment factors.

The ethanolic extract of *Z. piperitum* fruits in this study could scavenge DPPH free radical with IC_{50} of 95 µg/ml. The scavenging activity of *Z. piperitum* ethanolic

extract was less potent than quercetin and BHT with IC_{50} of 3.82 and 32.55 µg/ml respectively, whereas the radical scavenging activity of *Z. piperitum* ethanolic extract was more potent than xanthoxylin with IC_{50} of 27497 µg/ml. In previous study, DPPH radical scavenging activity of methanolic extract of leaf, root, fruit and stem of *Z. piperitum* demonstrated the IC_{50} of 7.41, 15.29, 15.66 and 22.04 µg/ml, respectively which was more potent than this study. *Z. piperitum* showed strong DPPH radical-scavenging activities which has been used widely since ancient times to treat several pathological conditions as well-known as a cure for gastrointestinal disorders, an antiphlogistic and an antidote [124].

Ferric reducing antioxidant power

FRAP assay is used to measure the ability of an antioxidant compound to reduce ferric ions (Fe^{3+} -TPTZ) into ferrous complex (Fe^{2+} -TPTZ) which gives the blue color at the maximum absorbance of 593 nm. This method uses ferric tripyridyl triazine, complex compound of iron as the tested compounds. Iron atom in this compound is reduced by the antioxidant and produces a complex compound of ferrous Fe^{2+} -TPTZ that gives the blue color [102]. The ferrous ion concentration was calculated according to the standard curve of ferrous sulphate. Quercetin and BHT were used as positive control.

In this study, the ethanolic extract of *C. odorata* leaves demonstrated the highest reducing power ability with FRAP value of 0.992 mM Fe(II)/mg extract, followed by chlorogenic acid with FRAP value of 0.956 mM Fe(II)/mg substance. The result indicated that *C. odorata* ethanolic extract and chlorogenic acid showed high reducing potential when compared with the reference control (quercetin, BHT). In addition, the ethanolic extract of *C. odorata* leaves was a significant source of natural antioxidant. The ferric reducing antioxidant power of chlorogenic acid in previous study found to be 1.17 ± 0.02 mM $FeSO_4$/mg substance which was similar to this study [119]. From another previous study, the ferric reducing activity of three extracts (ethyl acetate, methanol and aqueous extracts) of *C. odorata* root bark at the concentration of 100 µg/ml were determined. The results indicated that the methanol extract had the highest absorbance (2.14 ± 0.03) which the absorbance value was somehow equal to

the standard ascorbic acid (2.34 ± 0.07), followed by ethyl acetate extract (1.89 ± 0.07) and aqueous extract (0.77 ± 0.08) [122]. The ethanolic extract of *K. parviflora* rhizomes in this study demonstrated the moderate reducing power ability with FRAP value of 0.339 mM Fe(II)/mg extract. Among the tested samples, the 5,7-dimethoxyflavone had the lowest reducing power ability with FRAP value of 0.012 mM Fe(II)/mg substance. In previous study, the methanolic extract of *K. parviflora* rhizomes showed a value equivalent to 126.47 ± 2.72 mmol Fe(II)/g extract [125]. The ethanolic extract of *Z. piperitum* fruit in this study showed reducing power ability with FRAP value of 0.64 mM $FeSO_4$/mg extract, whereas xanthoxylin showed FRAP values of 0.04 mM $FeSO_4$/mg substance. From previous study, the ethanolic extract of *Z. piperitum* fruit exhibited high antioxidant capacity with FRAP value of 461.50 ± 7.94 µmol TE/g DW [126]. In this study, the reducing power of quercetin and BHT which were used as the positive control had a value equivalent to ferrous sulphate 0.97 and 0.99 mM $FeSO_4$/mg substance, respectively. In the same way, quercetin and BHT were previously reported the FRAP values of 1.48 ± 0.06 and 1.51 ± 0.01 mM $FeSO_4$/mg substance, respectively [119]. Eventually, the reducing power determination of *C. odorata* extract demonstrated good reductive capabilities when compared with positive control, followed by *Z. piperitum* and *K. parviflora* extracts. Increase in absorbance indicates increase in the reducing property was observed in the plant extracts. However, the differences in antioxidant potential vary with the different solvents used.

Beta-carotene bleaching inhibition

The beta-carotene bleaching assay is used for the determination of the antioxidant ability to inhibit lipid peroxidation which delay the bleaching of beta-carotene and linoleic acid emulsion. When beta-carotene exposed to radicals or to oxidizing species, the compound loses their color because their double bond is interrupted by oxidation [127]. In this assay, linoleic acid is used to produce hydrogenperoxide. The bleaching of yellow colour of beta-carotene is due to peroxide free radicals. The rate of beta-carotene bleaching can be slowed down in the presence of antioxidants [103]. Hence the absorbance decreased rapidly in samples without

antioxidant, whereas in the presence of an antioxidant, samples retained their color and thus absorbance for a longer time.

In this study, the results indicated that all of the extracts showed higher ability to inhibit the bleaching of beta-carotene by scavenging linoleate-derived free radicals than negative control and their active compounds in plant samples. Both synthetic antioxidant such as quercetin and BHT showed the highest ability to prevent the bleaching of beta-carotene than the extracts. The antioxidant activities of these extract, standard compounds and positive controls demonstrated the dose-response relationship. The results of beta-carotene bleaching inhibition of chlorogenic acid (39.63 %), quercetin (83.97 %) and BHT (90.38 %) in this study were in accordance with the previous study which showed antioxidant activity of 40.18 %, 78.12 % and 91.81 % at a concentration at 1 mg/ml, respectively [119].

Total phenolic content

This method measures the total concentration of phenolic hydroxyl groups in the plant extract using the Folin-Ciocalteu assay and a phenolic acid such as gallic acid to set up a calibration curve. This assay is based on the oxidation of a phenolate ion from the sample and the reduction of the phosphotungstic-phosphomolybdic reagent (Folin-Ciocalteu reagent) to form a blue complex of phosphotungstic-phosphomolybdenum in the alkaline solution that can be quantified by visible-light spectrophotometry [91].

In this study, the ethanolic extract of *C. odorata* leaves showed the highest total phenolic content (88.36 ± 1.71 mg GAE/g extract), followed by *Z. piperitum* ethanolic extract and *K. parviflora* ethanolic extract with total phenolic content values of 61.53 ± 2.46 and 49.09 ± 1.92 mg GAE/g extract, respectively. From the previous study, it was found that the ethyl acetate extract of *C. odorata* root bark showed maximum phenolic contents (313.31 ± 2.88 mg GAE/g extract) when compared with methanolic (246.16 ± 1.05 mg GAE/g extract) and aqueous extracts (125 ± 2.18 mg GAE/g extract) [122]. Ojiako *et al*. also reported that the total phenolic content of the methanolic extract of *C. odorata* leaves was 7.97 ± 0.20 mg GAE/g dry weight of the

extracts [128]. According to the previous research, it was found that the ethanolic extract of *C. odorata* leaves contained higher total phenolic compounds (536.3 ± 9.17 mg GAE/g of extract) than aqueous extract (379.0 ± 7.00 mg GAE/g of extract) [121]. Moreover, the result of total phenolic content of *K. parviflora* ethanolic extract in present study was more than the previous which showed the amount of total phenolics (24.62 ± 2.79 mg GAE/g extract) [125]. Furthermore, the total phenolic content of *Z. piperitum* ethanolic extract in this study was more than the previous study which showed total phenolic content value of 38.86 ± 2.34 mg GAE/g DW [126]. Chon *et al*. also reported that the total phenolic content of methanolic extract of *Z. piperitum* young sprouts was found to be 194.1 mg/kg of ferulic acid equipvalent (FAE) [129]. However, the values of total phenolic content were found to be different among different sources of the samples. In addition, the effective and strong antioxidant activity were reported to be present in different parts of plants and related to phenolic compounds which played an important role as potential therapeutics against oxidative damages [130]. Therefore, phenolic compounds in plant samples may be the major contributor to their antioxidant capacity [126].

Brine shrimp lethality activity

The preliminary toxicity investigation is brine shrimp lethality testing described by Mayer *et al*., 1982 [105]. Brine shrimp (*Artemia salina*) lethality assay is always used for toxicological activity test of the natural products because it is a sensitive indicator species. It is rapid, reliable, inexpensive and convenient bioassay for testing plant extracts which correlates reasonably well with cytotoxic properties [131]. The cytotoxic activity can be screened by the concentration of medicinal plant extracts and brine shrimp lethality relationship. In several studies, brine shrimp lethality assay has been an authentic assay to estimate toxicity of the compounds or the extracts [95-97].

The results of brine shrimp lethality testing of *C. odorata* leaves, *K. parviflora* rhizomes, and *Z. piperitum* fruits were evaluated and expressed as LC_{50} values: LC_{50} values > 1000 µg/ml (non - toxicity), ≥ 500 ≤ 1000 µg/ml (weak toxicity) and < 500 µg/ml (toxic) [98]. It was found that the ethanolic extract of *K. parviflora* rhizomes exhibited the highest toxicity against brine shrimp nauplii with LC_{50} of 41.28 µg/ml,

whereas the ethanolic extract of *C. odorata* leaves and *Z. piperitum* fruits showed weak toxicity with LC_{50} values of 874.46 and 654.00 µg/ml respectively. Based on the results, the brine shrimp lethality of these extracts demonstrated the concentration dependent manner. This preliminary study can be concluded that all tested samples has cytotoxicity against brine shrimp. In previous study, the ethanolic and aqueous extracts of *C. odorata* leaf from Nigeria were evaluated for toxicity using brine shrimp lethality assay which showed LC_{50} of 392 and 324 µg/ml, respectively [131]. The report by Lilybeth *et al*. showed that the ethanolic extracts of *C. odorata* was potent against brine shrimps with LC_{50} of 10 µg/ml. This assay has been used as an indicator for general toxicity of heavy metals, pesticides, medicines as well as natural products [132]. This activity could be explained by the phytochemical constituents present in the extract such as flavonoids, alkaloids, and phenolics [133]. However, the previous report mentioned that *C. odorata* was investigated as a source of natural pesticides and whose ethanolic extracts were potent fish poison having chalcone as an active constituent [134].

MTT cell viability activity

The MTT assay has been used successfully to quantitate cell survival and proliferation in macrophage-mediated cytotoxicity. Colorimetric assay is used based on the capacity of succinate dehydrogenase in mitochondria of viable cells to transform the MTT tetrazolium salt into MTT formazan (5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan). The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The result expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value). According to the American NCI plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity with IC_{50} ≤20 µg/ml, while this value was deemed at ≤4 µg/ml for a pure compound [99]. Five human cancer lines and 1 human normal cell line were used to evaluate the cytotoxic effect in this study. Different cell lines might be exhibited different sensitivities towards the cytotoxic compounds. Thereby, the use of more than one cell line is considered necessary in the detection of cytotoxic compounds. As the result, all test samples exhibited no significant cytotoxic activity

against six cell lines with IC_{50} more than standard criteria. However, *C. odorata* ethanolic extract, 5,7-dimethoxyflavone and xanthoxylin showed more cytotoxic potential against hepatocarcinoma (HEP-G2) with IC_{50} of 30.02, 21.27 and 23.12 µg/ml respectively, whereas the ethanolic extract of *K. parviflora* rhizome showed more cytotoxic potential against breast ductal carcinoma (BT474) with IC_{50} of 30.13 µg/ml.

In previous study, the ethanolic extract of *K. parviflora* rhizome was investigated for cytotoxic effect against human colon cancer cell line (SW-620) and breast cancer cell line (MCF-7) by MTT test. The results of cytotoxicity of *K. parviflora* ethanolic extract on SW-620 and MCF-7 cell line exhibited weak cytotoxic on cell proliferation with IC_{50} values of 282.29 and 694.92 µg/ml, respectively [135]. Moreover, the previous studies also reported the antiproliferative activities of *K. parviflora* ethanol extract against human promyelocytic leukemic cells (HL-60) and human cholangiocarcinoma cell lines (HuCCA-1 and RMCCA-1) [136, 137]. Another research also reported that 5,7-dimethoxyflavone was isolated from *K. parviflora* rhizomes and was tested for cytotoxicity against various human tumor cell lines including BT474 (breast), CHAGO (lung), HEP-G2 (hepatoma), HS27 (fibroblast), KATO-3 (gastric) and SW620 (colon) by MTT method. The results indicated that 5,7-dimethoxyflavone showed cytotoxicity against KATO-3 cell lines [138]. Li *et al*. studied the anticancer effect of 5,7-dimethoxyflavone against liver cancer cell line (HEP-G2) using MTT assay. It was found that 5,7-dimethoxyflavone showed significant anticancer activity against HEP-G2 cell line with IC_{50} value of 25 µM [139]. In addition, 5,7-dimethoxyflavone screened from the commercially available flavonoids was the most potent anticancer compound against HEP-G2 cells [140].

Rodanant *et al*. mentioned that the dichloromethane extract of *C. odorata* leaves showed the cytotoxic effect against human gingival fibroblasts cells (HGFs) and monocyte cell lines (U937) using MTT assay with IC_{50} values of 461.59 and 64.07 µg/ml, respectively [141]. Yajarla *et al*. studied the anti-proliferative property on a skin cancer cell line (A431). The results indicated that both hexane and ethyl acetate extracts of *C. odorata* leaves showed the cytotoxicity on A431 skin cancer cell lines upon incubation with IC_{50} values of 35 and 40 µg/ml, respectively [142]. Moreover, the

previous study also reported that chlorogenic acid showed no significant cytotoxic activity against six cell lines including BT-474, CHAGO-K1, SW-620, KATO-3, Hep-G2 and Wi-38 [143].

The anticancer activity of *Z. piperitum* methanolic extracts was examined on human pulmonary carcinoma (Calu-6) and human gastric carcinoma cell lines (SMU-601) using MTT assay. It was found that the methanolic extract of *Z. piperitum* showed more inhibition on SNU-601 (IC_{50} = 349.0 µg/ml) cell line than that of Calu-6 (IC_{50} = 470.4 µg/ml) [129]. Zaidi *et al*. studied the cytotoxic effect of xanthoxylin on melanocyte cell line (B16F10). The results showed that xanthoxylin at the lowest concentration significantly increased the cell's viability [144]. However, xanthoxylin triggers significant increase in dendrites formation in B16F10 melanocytes in dose dependent manner as reported by Moleephan *et al*. [145].

Anti-diabetic activity

Alpha-glucosidase is a group of enzymes act for the final step of starch digestion to glucose. Inhibition of these enzymes reduce the rate of digestion and less glucose is absorbed. In this study, alpha-glucosidase inhibition was measured using p-nitrophenol-alpha-D-glucopyranoside as substrate, which was hydrolyzed into p-nitrophenol and glucose by alpha-glucosidase enzyme [106, 146]. The synthetic or natural alpha-glucosidase inhibitors are interested as therapeutics to delay postprandial hyperglycemia in Type 2 diabetes [93]. Anti-diabetic drugs orally used for diabetes mellitus type 2 such as acarbose which act as competitive inhibitors of alpha-glucosidase was used as positive control in this study [94].

This study revealed that the ethanolic extract of *Z. piperitum* fruit and xanthoxylin showed high potential effect on yeast alpha-glucosidase inhibition with IC_{50} of 4.42 and 8.71 mg/ml respectively when compared to acarbose which used as positive control (IC_{50} = 11.63 mg/ml). It was found that *Z. piperitum* ethanolic extract and xanthoxylin exhibited remarkable antidiabetic activity more than a standard antidiabetic drug (acarbose). The ethanolic extract of *C. odorata* leaf and chlorogenic acid showed moderate enzyme inhibition. On the contrary, the ethanolic extract of *K.*

parviflora rhizome showed the lowest potential effect on yeast alpha-glucosidase inhibition, whereas 5,7-dimethoxyflavone showed the IC_{50} value more than 1 mg/ml. For rat alpha-glucosidase, these extracts and standard compounds showed the potential effect on rat alpha-glucosidase inhibition that less effective than positive control (acarbose).

The results indicated that the alpha-glucosidase inhibitory activity of all tested samples were increased in dose dependent manner. Flavonoids have also been reported to suppress glucose level significantly and the typical flavonoid has been found to be a strong inhibitor of alpha-glucosidase [147]. The previous research also studied the crude extracts and active natural compounds isolated from the medicinal plants (antidiabetic plants) which inhibited alpha-glucosidase. It was found that many kinds of the isolated natural products exhibited strong activity such as alkaloids, polyphenol, triterpene, acids (chlorogenic acid, betulinic acid, vanillic acid, bartogenic acid, oleanolic acid, gallic acid), phytosterol, flavonoids, flavonolignans, anthraquinones, anthrones, xanthones, flavanone glucosides, acetophenone glucosides, flavonol, anthocyanin and others [148]. Oboh *et al*. reported that chlorogenic acid inhibited alpha-glucosidase activity in a dose dependent manner with IC_{50} of 9.24 µg/ml [149]. Zheng *et al*. studied the inhibitory effect of chlorogenic acid on the postprandial blood glucose concentration in rats. It was found that chlorogenic acid inhibited the alpha-glucosidase activity and reduced the postprandial blood glucose concentration [150]. Furthermore, chlorogenic acid as well as acarbose, strongly inhibited the activity of alpha-glucosidase and reduced the postprandial blood glucose concentration. It was reported that chlorogenic acid suppresses postprandial hyperglycemia by inhibiting alpha-glucosidase and that its action resembles that of currently available alpha-glucosidase inhibitors such as acarbose, voglibose, and miglitol [151, 152]. The result of acarbose in this study was related to the previous study which showed the inhibition with the IC_{50} of 11.93 and 0.45 mg/ml for yeast alpha-glucosidase and rat alpha-glucosidase, respectively [153].

Onkaramurthy *et al*. investigated the effect of *C. odorata* leaf methanolic extract in reducing hyperglycemia in diabetic rats and they found the hypoglycemic

effect of their extract [154]. Ngwe *et al*. evaluated the alpha-glucosidase inhibition of *C. odrata* leaf from Myanmar. It was found that the ethanolic extract (IC_{50} = 0.31 µg/ml) and aqueous extract (IC_{50} = 0.14 µg/ml) of *C. odrata* leaf showed high potency of alpha-glucosidase inhibition [155]. Azuma *et al*. studied on *in vitro* rat alpha-glucosiadse activities of the extract of *K. parviflora* rhizomes. The dichloromethane extract and ethyl acetate-soluble fraction showed potent alpha-glucosidase inhibitory activities. 5,7,3′,4′-Tetramethoxyflavone and 5,7,4′-trimethoxyflavone showed high inhibitory effects for alpha-glucosidase inhibition. The results indicated that 5,7,4′-trimethoxyflavone was the most principal contributors to the alpha-glucosidase inhibition. The high concentrations of these flavones showed the potency of *K. parviflora* rhizomes as a source of alpha-glucosidase inhibitors [156]. Kusirisin *et al*. also reported that in the study of 30 medicinal plants traditionally used in Thailand as alternative treatments in diabetes, *K. parviflora* exhibited high antioxidative activity and might be used for reducing oxidative stress in diabetes [157].

Moreover, the previous study reported that the inhibition of alpha-glucosidase might be largely divided into two groups, type 1 (baker's yeast) and type 2 (mammalian small intestinal), based on the difference in primary structure. This leads to assumption that the inhibitory effect against alpha-glucosidase would vary between both groups, although no systematic studies have been performed. Primarily, the effect of alpha-glucosidase origin (baker's yeast and rat, rabbit, or pig small intestines) on the inhibitory activity exhibited a variety of alpha-glucosidase inhibition's behavior according to its origin. Acarbose which are known to be excellent *in vivo* alpha-glucosidase inhibitors had lower capacity to inhibit *in vitro* alpha-glucosidase from baker's yeast relative to that from small intestines [158]. However, the results of alpha-glucosidase inhibition of tested samples might be related to many factors such as the different type of enzymes and substrates, solvent or processing of extraction and other environment factors.

GC-MS analysis

The gas chromatography (GC) is the widely used analytical equipment for plant materials by reason of their high resolving power and low limit of detection, good

accuracy and high reproducibility. GC is used to separate the mixture of compounds that are naturally volatile or that can be converted to volatile derivatives. Compounds with a lower molecular weight will elute out earlier than compounds with higher molecular weights due to differences in boiling points. Mass spectrometer (MS) is a kind of detector instrument which uses electron or chemical to ionize the chemical compound and measures the mass to charge (m/z) ratio of ions based on the details of motion of the ions as they transit through electromagnetic fields [82, 83].

Pregeijerene and dauca-5,8-diene were the main chemical constituents of *C.odorata* essential oil in this study (40.60 and 16.75% respectively). The previous studies in Thailand reported pregeijerene (17.6%) and germacrene D (19.34%) as the major constituents of the essential oil from aerial parts and leaves respectively [159, 160]. The study in Nigeria showed that the main constituents in the stem essential oil were α-pinene (13.60%) and caryophyllene (9.20%) [161]. The chemical compositions of samples from different parts and localities expressed the individual amount of components [162].

The result of *K. parviflora* essential oil in this study showed that α-copaene (11.68%) and dauca-5,8-diene (11.17%) were major constituents. The previous study of essential oil in the hexane extract of *K. parviflora* rhizomes reported the dominant components as borneol (10.24%), β-pinene (8.60%), camphene (7.62%), α-copaene (7.23%), and linalool (6.40%) [163].

For GC-MS analysis of *Z. piperitum* essential oil, the previous study in Thailand reported that the main component of *Z. piperitum* fruit oil was limonene (37.99%), with minor amounts of sabinene (13.30%) and β-myrcene (7.17%) [164]. This study revealed that *Z. piperitum* essential oil could be divided into two clusters (Figure 31, 32). Cluster 1 was characterized by the main presence of limonene (18.81%) and methylchavicol (13.31%), whereas cluster 2 was characterized by the main presence of limonene (22.77%), β-phellandrene (18.27%) and sabinene (15.65%).

Pharmacognostic specification of *K. parviflora* rhizome

K. parviflora has been used for medicinal purposes in Thailand. In herbal medicine, it is generally used to promote health and to cure gastrointestinal disorder and anti-inflammation [10]. It is also used as an aphrodisiac for stimulating sexual performance in male. It has traditionally been used to improve vitality and treat of metabolic ailments [11]. In addition, *K. parviflora* has been selected as one of the five champion herbal products that has been promoted to make income for the country [19]. Even the government organizations have tried to promote the exportation of Thai herbal plant products, the quality control of herbal plants can provide to guarantee the standard of raw materials for manufacturing of traditional medicines, foods, beverages, dietary supplements as well as cosmetic products. Even though *K. parviflora* has been widely used in Thai traditional medicine for a long time and having shown benefits, the standardization in plant materials are still lacked. Therefore, the quality parameters of *K. parviflora* crude drug in Thailand have been established.

Macroscopic and microscopic examination are the first process to determine the characteristics, identity and degree of purity of medicinal plant materials. This evaluation procedure provides the simplest and quickest means to establish the identity and purity and thereby ensure quality of a particular sample. The herbal medicines need to provide the quality control evidences which indicate the quality evaluation of plant materials and make them more reliable. Pharmacognostic specification is primary important tool for identification, authentication and standardization of herbal medicines [81]. The anatomical character in transverse section and histological characters in powdered form of *K. parviflora* rhizome in this study were illustrated (Figure 35, 36). Putiyanan *et al*. reported parenchyma cell with many starch grains and vascular bundle in transverse section which in agreement with this study [165]. Oleoresin and oil droplet were revealed, but the covering trichrome was not found in this study. The pharmacognostic parameters of *K. parviflora* rhizome in Thiland were evaluated. In this study, the results revealed that the loss on drying, total ash, acid-insoluble ash and water content should be not more than 8.98, 5.13, 2.17 and 9.29% of dry weight, respectively. Ash values are helpful in determination of

the purity and quality which indicate the inorganic substances in plant materials after complete incineration such as Ca, Na, K, Cl, etc. The remaining insoluble matter obtained after boiling the total ash with about 2N hydrochloric acid and incinerating is acid insoluble ash which is mainly aluminium and silicon. A high ash value indicates the adulteration, contamination or substitution of plant materials. The determination of loss on drying can carried out both water and volatile matter in crude drugs, whereas the moisture content test can carried out only water that present in plant materials. The humidity of the environment may affect the quality of plant materials among the storage. Excessive of water content in plant materials can encourage bacteria, fungi growth or activate enzymatic systems which leads to make chemical degradation or microbial contamination in plant materials. Thus, the limit of water content should be set for stability of plant materials. Moreover, the ethanol soluble extractive, water soluble extractive values and volatile oil content should be not less than 5.14, 8.25 and 0.03% of dry weight, respectively. Determination of solvent extractive matters is used to determine the amount of active constituents in a given amount of medicinal plant material when extracted with specific solvent. The results showed that water soluble extractive value was higher than ethanol soluble extractives which indicated the high content of polar compounds. However, the presence of the active constituents in plant materials could vary according to extraction conditions and solvent used. For plant extraction in Thai traditional medicine, soaking of plant materials in alcohol or boiling it in water are the commonly used methods. Therefore, ethanol and water, a common solvent used for plant extraction were used as a specific solvent in this study. In previous study, physicochemical properties of *K. parviflora* rhizome in India were screened. The moisture content, total ash, acid-insoluble ash, alcohol and water soluble extractive values were found to be 7.75 ± 0.02, 12.32 ± 0.10, 0.55 ± 0.01, 4.59 ± 0.04 and 17.40 ± 0.03 % w/w, respectively [166]. Furthermore, the quality control needs to measure the phytochemical compounds in medicinal plants for ensuring the quality reliability of natural products obtained from plant sources [2]. Thus, TLC fingerprint demonstrated the pattern of phytochemical characteristic constituents. TLC fingerprint of ethanolic extract of *K. parviflora* rhizome presented good separation of bands on the TLC plate which revealed clearly detected

of bands under ultraviolet light (254, 365 nm) and staining reagent. This TLC fingerprint could be used as reference standard for further identification of plant samples.

Conclusion

In conclusion, this study provides the contents of active components in all plant samples by TLC-densitometry compared to TLC image analysis and the chemical constituents of essential oil by GC-MS of *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits. The present study proposed the scientific evidences in efficacy evaluation including *in vitro* antioxidant, anti-diabetic as well as cytotoxicity activities in *C. odorata* leaves, *K. parviflora* rhizomes and *Z. piperitum* fruits. Furthermore, it provides pharmacognostic specification of *K. parviflora* rhizomes in Thailand which could be used for basic quality control and standardization of plant materials.

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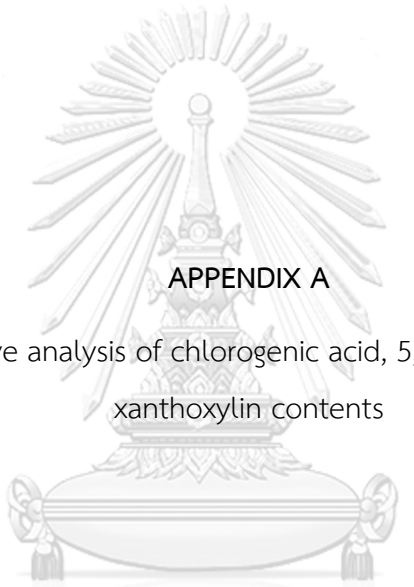
APPENDICES



APPENDIX A

Data of quantitative analysis of chlorogenic acid, 5,7-dimethoxyflavone and xanthoxylin contents

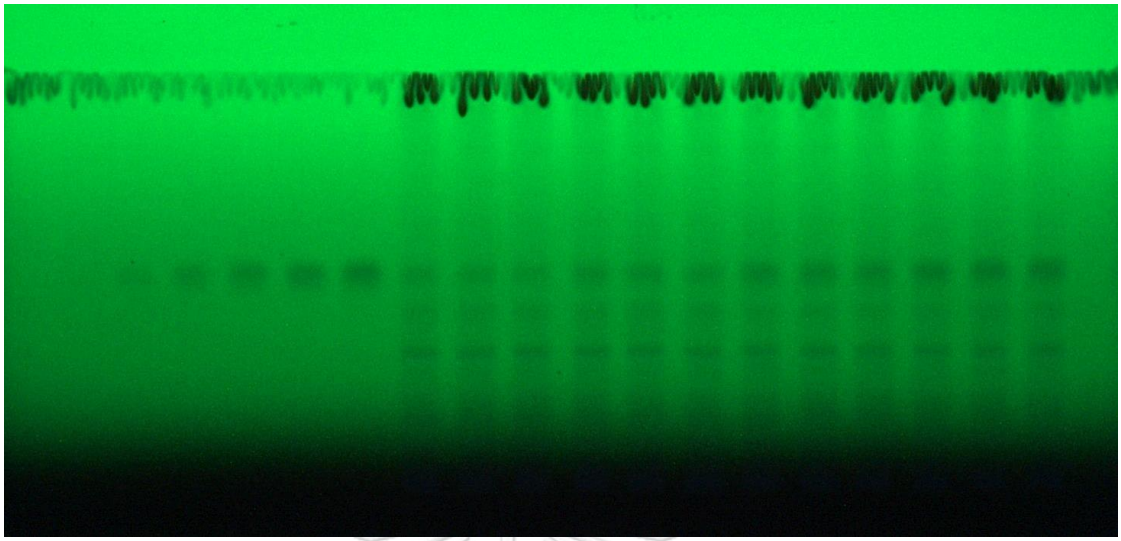


Figure 38 The TLC plate under UV 254; standard chlorogenic acid (track 1 to 5) and *C. odorata* leaf ethanolic extracts from 10 different locations

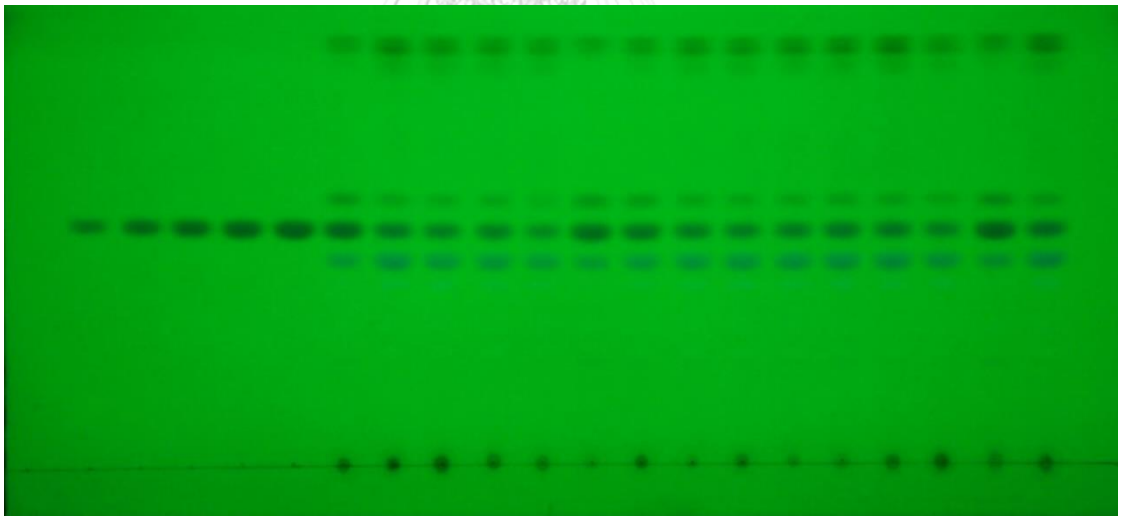


Figure 39 The TLC plate under UV 254 nm; standard 5,7-dimethoxyflavone (track 1–5), and *K. parviflora* rhizome ethanolic extracts from 15 different sources (track 6-20)

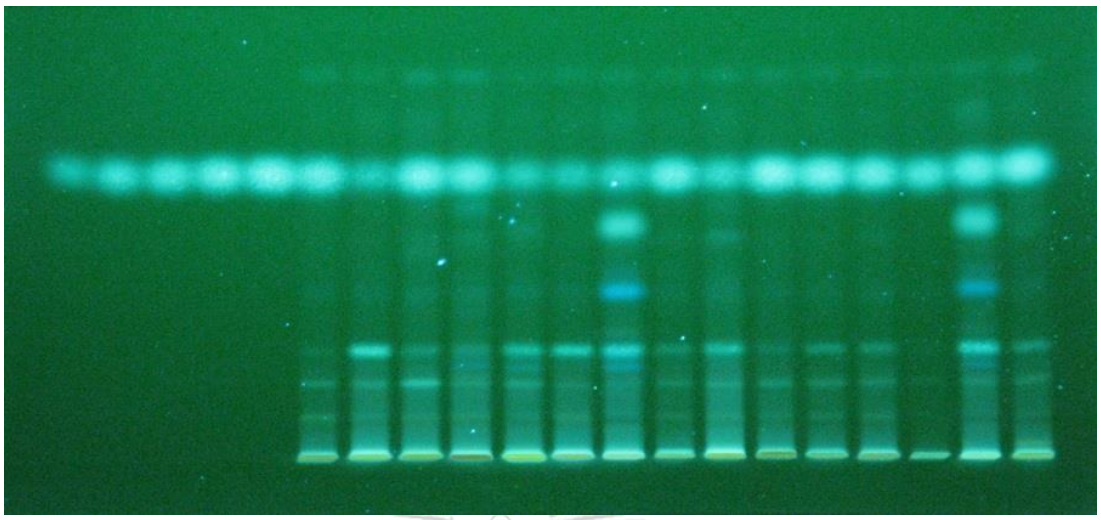


Figure 40 The TLC plate under UV 365 nm; standard xanthoxylin (track 1 to 5) and *Z. piperitum* fruit ethanolic extracts from 15 different locations (track 6 to 20) after spraying with 1% aluminium chloride in ethanol

APPENDIX B

Data of antioxidant activities

DPPH radical scavenging activity

Table 47 DPPH radical scavenging activity of ethanolic extracts of *C. odorata* leaves

Conc.	OD_{517} (reaction mixture)			DPPH inhibition (%)				
(µg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.234	0.236	0.238					
7.50	0.213	0.212	0.224	9.746	10.169	5.085	8.333	2.821
15	0.201	0.205	0.207	14.831	13.136	12.288	13.418	1.295
30	0.174	0.181	0.184	26.271	23.305	22.034	23.870	2.174
60	0.127	0.128	0.137	46.186	45.763	41.949	44.633	2.334
90	0.096	0.098	0.107	59.322	58.475	54.661	57.486	2.483

Table 48 DPPH radical scavenging activity of chlorogenic acid

Conc.	OD_{517} (reaction mixture)			DPPH inhibition (%)				
(µg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.234	0.236	0.238					
1.25	0.195	0.206	0.210	17.373	12.712	11.017	13.701	3.291
2.5	0.191	0.195	0.200	19.068	17.373	15.254	17.232	1.911
5	0.170	0.174	0.171	27.966	26.271	27.542	27.260	0.882
10	0.118	0.121	0.117	50.000	48.729	50.424	49.718	0.882
20	0.030	0.034	0.033	87.288	85.593	86.017	86.299	0.882

Table 49 DPPH radical scavenging activity of ethanolic extracts of *K. parviflora* rhizomes

Conc.	OD_{517} (reaction mixture)			DPPH inhibition (%)				
(µg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.295	0.299	0.295					
31.25	0.263	0.262	0.266	11.149	11.486	10.135	10.923	0.703
62.50	0.247	0.242	0.244	16.554	18.243	17.568	17.455	0.850
125.00	0.201	0.201	0.199	32.095	32.095	32.770	32.320	0.390
250.00	0.140	0.135	0.132	52.703	54.392	55.405	54.167	1.365
500.00	0.056	0.050	0.052	81.081	83.108	82.432	82.207	1.032

Table 50 DPPH radical scavenging activity of 5, 7-dimethoxyflavone

Conc.	OD_{517} (reaction mixture)			DPPH inhibition (%)				
(µg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.294	0.290	0.288					
100	0.306	0.307	0.306	-5.155	-5.498	-5.155	-5.269	0.198
200	0.317	0.315	0.313	-8.935	-8.247	-7.560	-8.247	0.687
400	0.314	0.312	0.312	-7.904	-7.216	-7.216	-7.446	0.397
500	0.309	0.311	0.315	-6.186	-6.873	-8.247	-7.102	1.050
1000	0.312	0.312	0.307	-7.216	-7.216	-5.498	-6.644	0.992

Table 51 DPPH radical scavenging activity of ethanolic extracts of *Z. piperitum* fruits

Conc.	OD_{517} (reaction mixture)			DPPH inhibition (%)				
(µg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.0	0.290	0.292	0.291					
6.3	0.279	0.277	0.277	4.124	4.811	4.811	4.582	0.397
12.5	0.268	0.268	0.266	7.904	7.904	8.591	8.133	0.397
25.0	0.251	0.251	0.250	13.746	13.746	14.089	13.860	0.198
50.0	0.209	0.208	0.207	28.179	28.522	28.866	28.522	0.344
100.0	0.145	0.137	0.137	50.172	52.921	52.921	52.005	1.587

Table 52 DPPH radical scavenging activity of xanthoxylin

Conc.	OD_{517} (reaction mixture)			DPPH inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.0	0.290	0.292	0.291					
5.0	0.237	0.231	0.234	18.557	20.619	19.588	19.588	1.031
7.5	0.219	0.217	0.218	24.742	25.430	25.086	25.086	0.344
10.0	0.206	0.202	0.203	29.210	30.584	30.241	30.011	0.715
15.0	0.185	0.180	0.184	36.426	38.144	36.770	37.113	0.909
30.0	0.142	0.141	0.142	51.203	51.546	51.203	51.317	0.198

Table 53 DPPH radical scavenging activity of positive control (BHT)

Conc. (µg/ml)	OD_{517} (reaction mixture)			DPPH inhibition (%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.234	0.236	0.238					
3.125	0.210	0.213	0.213	11.017	9.746	9.746	10.169	0.734
6.25	0.200	0.200	0.207	15.254	15.254	12.288	14.266	1.712
12.5	0.172	0.170	0.174	27.119	27.966	26.271	27.119	0.847
25	0.141	0.134	0.145	40.254	43.220	38.559	40.678	2.359
50	0.086	0.077	0.091	63.559	67.373	61.441	64.124	3.006

Table 54 DPPH radical scavenging activity of positive control (Quercetin)

Conc. (µg/ml)	OD_{517} (reaction mixture)			DPPH inhibition (%)				
	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0.00	0.234	0.236	0.238					
0.625	0.219	0.218	0.221	7.203	7.627	6.356	7.062	0.647
1.25	0.204	0.202	0.203	13.559	14.407	13.983	13.983	0.424
2.5	0.147	0.147	0.149	37.712	37.712	36.864	37.429	0.489
5	0.094	0.095	0.090	60.169	59.746	61.864	60.593	1.121
10	0.019	0.019	0.021	91.949	91.949	91.102	91.667	0.489

Ferric ion reducing antioxidant power (FRAP)

Table 55 FRAP value of *C. odorata* ethanolic extract, chlorogenic acid, *K. parviflora* ethanolic extract, 5,7-dimethoxyflavone, *Z. piperitum* ethanolic extract, xanthoxylin, quercetin and BHT

Tested samples (0.5 mg/ml)	Absorbance at 593 nm				Ferrous sulphate equivalent (mM)	FRAP value (mM Fe(II)/mg)
	1	2	3	mean ± SD		
C. odorata extract	0.443	0.465	0.452	0.453 ± 0.011	0.496	0.992
chlorogenic acid	0.450	0.441	0.421	0.437 ± 0.015	0.478	0.956
K. parviflora extract	0.155	0.167	0.167	0.163 ± 0.007	0.169	0.339
5,7-dimethoxyflavone	0.020	0.017	0.017	0.018 ± 0.002	0.006	0.012
Z. piperitum extract	0.281	0.308	0.298	0.296 ± 0.014	0.319	0.637
xanthoxylin	0.028	0.033	0.031	0.031 ± 0.003	0.020	0.040
Quercetin	0.437	0.452	0.445	0.445 ± 0.008	0.486	0.973
BHT	0.451	0.453	0.446	0.450 ± 0.004	0.492	0.985

Beta-carotene bleaching inhibition

Table 56 The absorbance of beta-carotene bleaching of tested samples at 0.5 mg/ml

Tested samples	Time	OD_{470} (reaction mixture)				
		exp 1	exp 2	exp 3	Average	SD
BHT	0	0.406	0.409	0.402	0.406	0.004
	30	0.388	0.384	0.387	0.386	0.002
	60	0.373	0.373	0.371	0.372	0.001
	90	0.361	0.362	0.363	0.362	0.001
	120	0.352	0.353	0.354	0.353	0.001
Quercetin	0	0.410	0.410	0.409	0.410	0.001
	30	0.373	0.371	0.370	0.371	0.002
	60	0.349	0.347	0.348	0.348	0.001
	90	0.329	0.329	0.327	0.328	0.001
	120	0.314	0.313	0.312	0.313	0.001
Chlorogenic acid	0	0.409	0.405	0.404	0.406	0.003
	30	0.307	0.306	0.300	0.304	0.004
	60	0.219	0.232	0.221	0.224	0.007
	90	0.163	0.172	0.166	0.167	0.005
	120	0.137	0.133	0.127	0.132	0.005
C. odorata ethanolic extract	0	0.409	0.408	0.405	0.407	0.002
	30	0.324	0.323	0.310	0.319	0.008
	60	0.256	0.257	0.236	0.250	0.012
	90	0.210	0.208	0.187	0.202	0.013
	120	0.174	0.171	0.150	0.165	0.013
5,7-Dimethoxyflavone	0	0.382	0.381	0.382	0.382	0.001
	30	0.148	0.156	0.158	0.154	0.005
	60	0.052	0.051	0.053	0.052	0.001
	90	0.026	0.026	0.028	0.027	0.001
	120	0.017	0.017	0.017	0.017	0.000

Table 56 The absorbance of beta-carotene bleaching of tested samples at 0.5 mg/ml (Cont.)

Tested samples	Time	OD_{470} (reaction mixture)				
		exp 1	exp 2	exp 3	Average	SD
K. parviflora ethanolic extract	0	0.392	0.393	0.391	0.392	0.001
	30	0.305	0.305	0.303	0.304	0.001
	60	0.224	0.217	0.218	0.220	0.004
	90	0.169	0.162	0.163	0.165	0.004
	120	0.125	0.118	0.120	0.121	0.004
Xanthoxylin	0	0.387	0.388	0.388	0.388	0.001
	30	0.165	0.162	0.166	0.164	0.002
	60	0.054	0.053	0.057	0.055	0.002
	90	0.028	0.027	0.029	0.028	0.001
	120	0.018	0.019	0.020	0.019	0.001
Z. piperitum ethanolic extract	0	0.381	0.381	0.382	0.381	0.001
	30	0.230	0.239	0.240	0.236	0.006
	60	0.119	0.132	0.134	0.128	0.008
	90	0.072	0.082	0.085	0.080	0.007
	120	0.045	0.055	0.058	0.053	0.007
Negative control	0	0.388	0.386	0.387	0.387	0.001
	30	0.154	0.158	0.159	0.157	0.003
	60	0.037	0.036	0.038	0.037	0.001
	90	0.019	0.018	0.019	0.019	0.001
	120	0.012	0.013	0.013	0.013	0.001

Table 57 The absorbance of beta-carotene bleaching of tested samples at 1 mg/ml

Tested samples	Time	OD_{470} (reaction mixture)				
		exp 1	exp 2	exp 3	Average	SD
BHT	0	0.402	0.401	0.400	0.401	0.001
	30	0.389	0.389	0.387	0.388	0.001
	60	0.379	0.379	0.379	0.379	0.000
	90	0.372	0.373	0.372	0.372	0.001
	120	0.365	0.366	0.364	0.365	0.001
Quercetin	0	0.408	0.409	0.409	0.409	0.001
	30	0.381	0.381	0.380	0.381	0.001
	60	0.368	0.369	0.369	0.369	0.001
	90	0.354	0.358	0.356	0.356	0.002
	120	0.348	0.348	0.350	0.349	0.001
Chlorogenic acid	0	0.402	0.402	0.402	0.402	0.000
	30	0.323	0.325	0.320	0.323	0.003
	60	0.258	0.269	0.262	0.263	0.006
	90	0.211	0.216	0.210	0.212	0.003
	120	0.174	0.179	0.175	0.176	0.003
C. odorata ethanolic extract	0	0.408	0.408	0.407	0.408	0.001
	30	0.349	0.350	0.348	0.349	0.001
	60	0.303	0.304	0.306	0.304	0.002
	90	0.262	0.266	0.265	0.264	0.002
	120	0.237	0.239	0.238	0.238	0.001
5,7-Dimethoxyflavone	0	0.383	0.380	0.381	0.381	0.002
	30	0.148	0.151	0.149	0.149	0.002
	60	0.046	0.046	0.045	0.046	0.001
	90	0.024	0.023	0.023	0.023	0.001
	120	0.017	0.016	0.019	0.017	0.002

Table 57 The absorbance of beta-carotene bleaching of tested samples at 1 mg/ml (Cont.)

Tested samples	Time	OD_{470} (reaction mixture)				
		exp 1	exp 2	exp 3	Average	SD
K. parviflora ethanolic extract	0	0.389	0.389	0.388	0.389	0.001
	30	0.326	0.326	0.331	0.328	0.003
	60	0.272	0.272	0.266	0.270	0.003
	90	0.230	0.228	0.222	0.227	0.004
	120	0.192	0.191	0.185	0.189	0.004
Xanthoxylin	0	0.387	0.387	0.388	0.387	0.001
	30	0.159	0.163	0.160	0.161	0.002
	60	0.056	0.058	0.055	0.056	0.002
	90	0.030	0.030	0.028	0.029	0.001
	120	0.019	0.021	0.020	0.020	0.001
Z. piperitum ethanolic extract	0	0.387	0.386	0.386	0.386	0.001
	30	0.292	0.296	0.277	0.288	0.010
	60	0.195	0.176	0.185	0.185	0.010
	90	0.142	0.158	0.131	0.144	0.014
	120	0.103	0.095	0.092	0.097	0.006
Negative control	0	0.388	0.386	0.387	0.387	0.001
	30	0.154	0.158	0.159	0.157	0.003
	60	0.037	0.036	0.038	0.037	0.001
	90	0.019	0.018	0.019	0.019	0.001
	120	0.012	0.013	0.013	0.013	0.001

Table 58 The absorbance of beta-carotene bleaching of tested samples at 2 mg/ml

Tested samples	Time	OD_{470} (reaction mixture)				
		exp 1	exp 2	exp 3	Average	SD
BHT	0	0.405	0.405	0.405	0.405	0.000
	30	0.396	0.398	0.398	0.397	0.001
	60	0.393	0.392	0.391	0.392	0.001
	90	0.386	0.386	0.385	0.386	0.001
	120	0.382	0.381	0.382	0.382	0.001
Quercetin	0	0.412	0.412	0.411	0.412	0.001
	30	0.393	0.393	0.392	0.393	0.001
	60	0.383	0.384	0.382	0.383	0.001
	90	0.373	0.375	0.372	0.373	0.002
	120	0.367	0.367	0.366	0.367	0.001
Chlorogenic acid	0	0.405	0.405	0.405	0.405	0.000
	30	0.357	0.356	0.353	0.355	0.002
	60	0.316	0.314	0.310	0.313	0.003
	90	0.285	0.283	0.280	0.283	0.003
	120	0.258	0.261	0.256	0.258	0.003
C. odorata ethanolic extract	0	0.417	0.415	0.414	0.415	0.002
	30	0.376	0.379	0.376	0.377	0.002
	60	0.345	0.347	0.345	0.346	0.001
	90	0.319	0.324	0.323	0.322	0.003
	120	0.298	0.303	0.302	0.301	0.003
5,7-Dimethoxyflavone	0	0.387	0.384	0.385	0.385	0.002
	30	0.164	0.173	0.165	0.167	0.005
	60	0.056	0.060	0.058	0.058	0.002
	90	0.030	0.030	0.033	0.031	0.002
	120	0.020	0.023	0.024	0.022	0.002

Table 58 The absorbance of beta-carotene bleaching of tested samples at 2 mg/ml (Cont.)

Tested samples	Time	OD_{470} (reaction mixture)				
		exp 1	exp 2	exp 3	Average	SD
K. parviflora ethanolic extract	0	0.395	0.395	0.394	0.395	0.001
	30	0.351	0.353	0.353	0.352	0.001
	60	0.313	0.313	0.310	0.312	0.002
	90	0.282	0.283	0.278	0.281	0.003
	120	0.255	0.255	0.249	0.253	0.003
Xanthoxylin	0	0.384	0.383	0.383	0.383	0.001
	30	0.170	0.172	0.172	0.171	0.001
	60	0.067	0.067	0.066	0.067	0.001
	90	0.036	0.036	0.034	0.035	0.001
	120	0.023	0.024	0.022	0.023	0.001
Z. piperitum ethanolic extract	0	0.391	0.392	0.393	0.392	0.001
	30	0.332	0.330	0.333	0.332	0.002
	60	0.273	0.276	0.269	0.273	0.004
	90	0.234	0.226	0.238	0.233	0.006
	120	0.199	0.189	0.205	0.198	0.008
Negative control	0	0.388	0.386	0.387	0.387	0.001
	30	0.154	0.158	0.159	0.157	0.003
	60	0.037	0.036	0.038	0.037	0.001
	90	0.019	0.018	0.019	0.019	0.001
	120	0.012	0.013	0.013	0.013	0.001

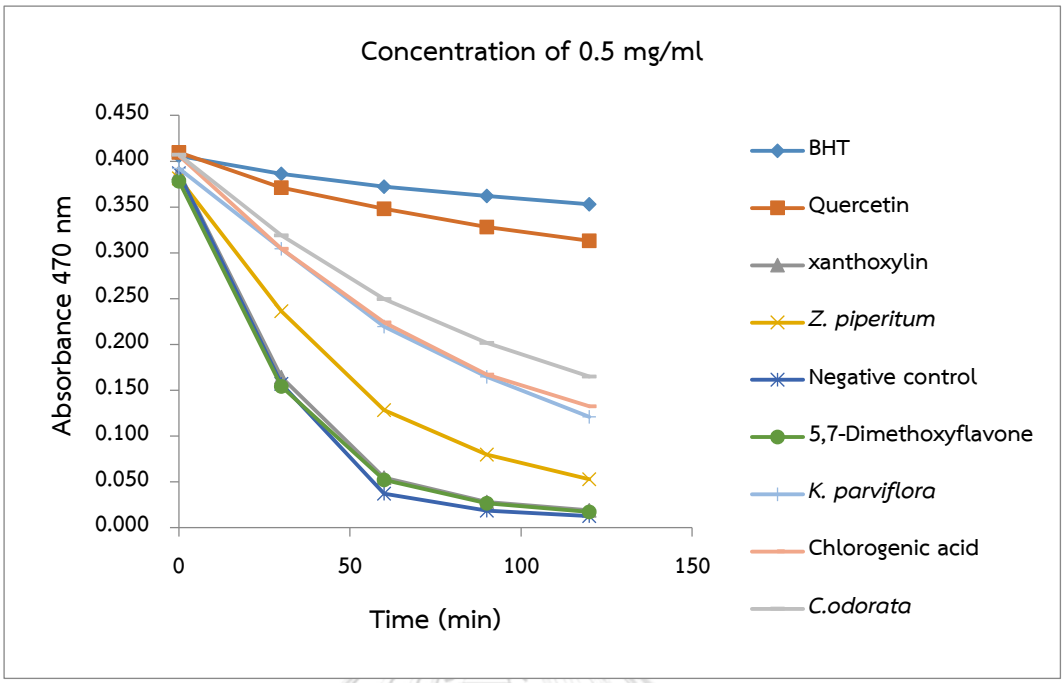


Figure 41 The absorbance of beta-carotene bleaching of tested samples at 0.5 mg/ml

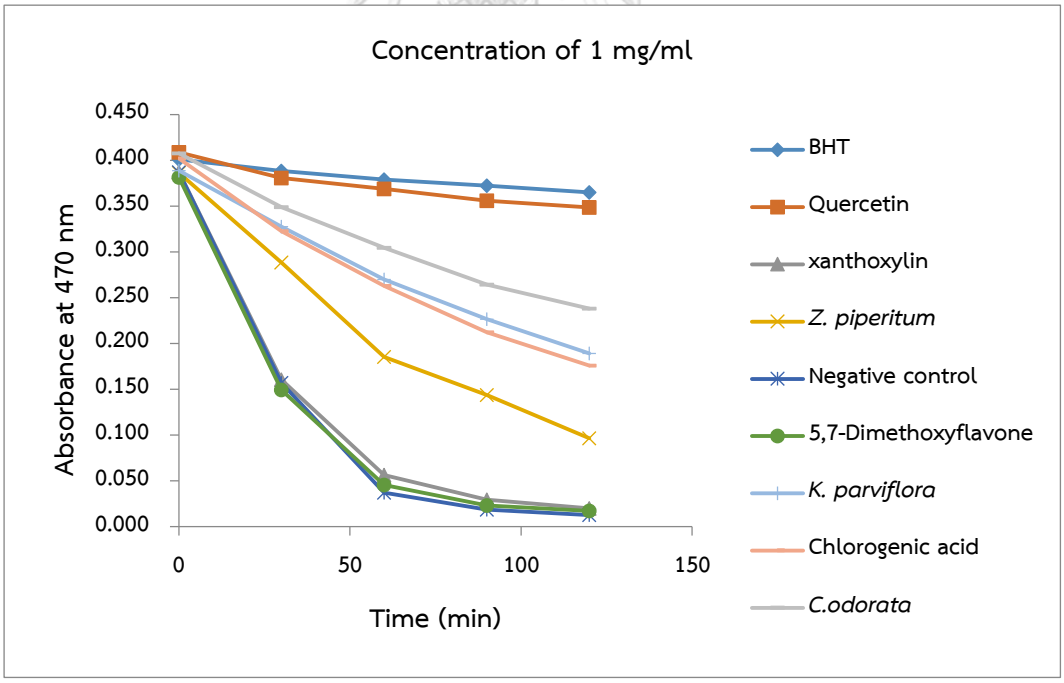


Figure 42 The absorbance of beta-carotene bleaching of tested samples at 1 mg/ml

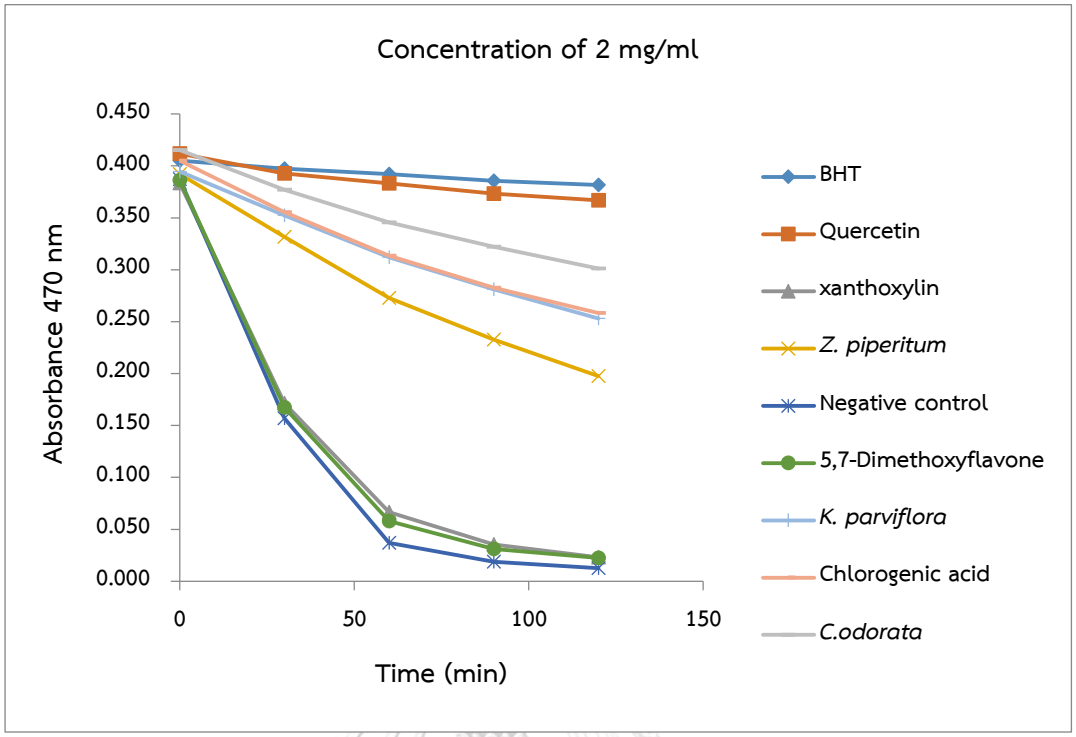


Figure 43 The absorbance of beta-carotene bleaching of tested samples at 2 mg/ml

Total phenolic content

Table 59 Total phenolic content of *C. odorata* ethanolic extract

Samples (0.5 mg/ml)	Absorbance at 756 nm				Gallic acid equivalent (mg GAE/g extract)
	1	2	3	mean ± SD	
C. odorata (1)	0.062	0.048	0.050	0.053 ± 0.008	86.40
C. odorata (2)	0.062	0.053	0.051	0.055 ± 0.006	89.60
C. odorata (3)	0.060	0.049	0.056	0.055 ± 0.006	89.07
mean ± SD					**88.36 ± 1.71**

Table 60 Total phenolic content of *K. parviflora* ethanolic extract

Samples (0.5 mg/ml)	Absorbance at 756 nm				Gallic acid equivalent (mg GAE/g extract)
	1	2	3	mean ± SD	
K. parviflora (1)	0.027	0.030	0.029	0.029 ± 0.002	46.96
K. parviflora (2)	0.033	0.030	0.030	0.031 ± 0.002	50.69
K. parviflora (3)	0.029	0.033	0.030	0.030 ± 0.002	49.63
mean ± SD					**49.09 ± 1.92**

Table 61 Total phenolic content of *Z. piperitum* ethanolic extract

Samples (0.5 mg/ml)	Absorbance at 756 nm				Gallic acid equivalent (mg GAE/g extract)
	1	2	3	mean ± SD	
Z. piperitum (1)	0.035	0.037	0.036	0.036 ± 0.001	58.69
Z. piperitum (2)	0.039	0.037	0.040	0.039 ± 0.002	62.95
Z. piperitum (3)	0.038	0.038	0.040	0.039 ± 0.001	62.95
mean ± SD					**61.53 ± 2.46**

APPENDIX C

Data of brine shrimp lethality and MTT cell viability activities

Table 62 Number of survivor nauplii at each time among various concentrations of *C. odorata* ethanolic extract

Conc. (µg/ml)	Time (hr)																								%Lethality
	6						12						18						24						
	1	2	3	4	5	mean	1	2	3	4	5	mean	1	2	3	4	5	mean	1	2	3	4	5	mean	SD	
1000	9	9	10	10	10	9.6	6	4	8	4	7	5.8	6	2	6	6	4	4.8	6	2	4	6	3	4.2	1.8	58
800	10	9	9	10	10	9.6	9	9	9	8	10	9	7	7	7	5	8	6.8	7	5	6	4	5	5.4	1.1	46
600	9	9	9	9	10	9.2	7	9	9	7	9	8.2	6	7	6	7	7	6.6	6	7	6	7	7	6.6	0.5	34
400	9	10	10	10	10	9.8	9	8	9	10	10	9.2	8	8	7	9	9	8.2	9	8	7	8	8	8.0	0.7	20
200	10	10	10	10	10	10	10	8	10	10	10	9.6	8	8	8	10	9	8.6	8	7	8	9	9	8.2	0.8	18
100	10	10	10	10	10	10	9	10	10	10	9	9.6	9	8	9	9	10	9	9	8	9	9	10	9.0	0.7	10
Negative control	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0.0	0

Table 63 Number of survivor nauplii at each time among various concentrations of *K. parviflora* ethanolic extract

Conc. (µg/ml)	Time (hr)																								%Lethality
	6						12						18						24						
	1	2	3	4	5	mean	1	2	3	4	5	mean	1	2	3	4	5	mean	1	2	3	4	5	mean	SD	
80	10	10	10	10	9	9.8	3	4	3	3	3	3.2	2	0	1	1	3	1.4	1	0	1	1	1	0.8	0.4	92
70	10	9	9	10	10	9.6	3	4	5	6	4	4.4	2	3	4	3	3	3	1	2	2	1	0	1.2	0.8	88
60	10	10	10	10	10	10	4	6	5	10	7	6.4	2	4	5	4	5	4	1	3	3	3	2	2.4	0.9	76
50	10	10	10	10	10	10	8	6	8	7	9	7.6	6	3	6	7	7	5.8	4	3	5	4	4	4.0	0.7	60
40	10	10	10	10	10	10	10	9	9	10	9	9.4	6	6	8	7	5	6.4	4	6	6	5	4	5.0	1.0	50
30	10	10	10	10	10	10	10	10	10	10	10	10	9	10	10	10	8	9.4	7	7	7	6	8	7.0	0.7	30
Negative control	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0.0	0

Table 64 Number of survivor nauplii at each time among various concentrations of *Z. piperitum* ethanolic extract

Conc. (µg/ml)	Time (hr)																								%Lethality
	6						12						18						24						
	1	2	3	4	5	mean	1	2	3	4	5	mean	1	2	3	4	5	mean	1	2	3	4	5	mean	SD
800	6	2	1	2	5	3.2	5	1	1	0	2	1.8	5	1	1	0	0	1.4	5	1	1	0	0	1.4	2.1	86
700	2	2	1	2	10	3.4	2	2	1	2	9	3.2	2	1	1	1	5	2.0	2	1	1	1	5	2.0	1.7	80
600	5	4	5	6	10	6	5	4	5	6	9	5.8	4	4	5	6	9	5.6	4	4	5	6	9	5.6	2.1	44
500	10	6	5	10	10	8.2	10	6	5	10	10	8.2	10	6	5	9	10	8.0	10	6	5	9	10	8.0	2.3	20
400	10	10	10	10	10	10	10	10	10	10	10	10	9	10	10	10	9	9.6	9	10	9	9	10	9.4	0.5	6
300	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	9	10	10	9.8	0.4	2
Negative control	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	0.0	0

Table 65 Cytotoxicity effect of *C. odorata* leave ethanolic extract by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.119	0.142	0.166	0.201	0.157	25
50	0.196	0.240	0.233	0.294	0.241	38
10	0.584	0.614	0.543	0.715	0.614	98
1	0.664	0.615	0.592	0.670	0.635	101
0.1	0.621	0.624	0.653	0.711	0.652	104
0.01	0.805	0.744	0.609	0.609	0.692	111
DMSO	0.636	0.575	0.542	0.750	0.626	100

Concentration	Chago-K1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.117	0.110	0.194	0.152	0.143	26
50	0.187	0.147	0.265	0.212	0.203	36
10	0.459	0.451	0.610	0.473	0.498	89
1	0.575	0.494	0.653	0.486	0.552	99
0.1	0.518	0.555	0.634	0.631	0.585	104
0.01	0.597	0.547	0.667	0.738	0.637	114
DMSO	0.581	0.545	0.579	0.536	0.560	100

Concentration	Hep-G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.127	0.143	0.169	0.154	0.148	13
50	0.205	0.258	0.369	0.361	0.298	27
10	0.787	0.822	0.949	0.924	0.871	78
1	0.916	0.869	0.978	0.835	0.900	81
0.1	0.792	0.928	1.011	0.742	0.868	78
0.01	0.903	0.905	0.915	0.972	0.924	83
DMSO	1.161	1.207	0.990	1.108	1.117	100

Table 65 Cytotoxicity effect of *C. odorata* leave ethanolic extract by MTT cell viability (Cont.)

Concentration	KATO-III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.227	0.284	0.237	0.238	0.247	18
50	0.346	0.345	0.445	0.426	0.391	29
10	0.990	1.142	1.249	1.091	1.118	83
1	1.089	1.138	1.244	1.176	1.162	87
0.1	1.069	1.074	1.235	1.191	1.142	85
0.01	1.235	1.184	1.374	1.378	1.293	96
DMSO	1.308	1.328	1.385	1.349	1.343	100

Concentration	SW-620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.119	0.126	0.134	0.121	0.125	10
50	0.426	0.422	0.519	0.657	0.506	39
10	1.230	1.396	1.185	1.037	1.212	93
1	1.359	1.285	1.304	1.174	1.281	98
0.1	1.297	1.393	1.308	1.218	1.304	100
0.01	1.513	1.310	1.359	1.473	1.414	108
DMSO	1.416	1.217	1.278	1.321	1.308	100

Concentration	Wi-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.126	0.157	0.219	0.184	0.172	16
50	0.522	0.659	0.666	0.684	0.633	58
10	1.130	1.213	1.216	1.090	1.162	107
1	1.088	1.251	1.289	1.144	1.193	109
0.1	1.090	1.022	1.088	1.005	1.051	96
0.01	1.094	1.041	1.139	1.034	1.077	99
DMSO	1.220	1.132	0.931	1.080	1.091	100

Table 66 Cytotoxicity effect of chlorogenic acid by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.660	0.555	0.570	0.631	0.604	87
50	0.414	0.580	0.698	0.680	0.593	85
10	0.547	0.560	0.582	0.667	0.589	85
1	0.490	0.632	0.632	0.592	0.587	84
0.1	0.615	0.744	0.858	0.667	0.721	104
0.01	0.490	0.541	0.565	0.711	0.577	83
DMSO	0.672	0.711	0.674	0.728	0.696	100

Concentration	Chago-K1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.536	0.524	0.458	0.436	0.489	95
50	0.652	0.528	0.517	0.560	0.564	110
10	0.532	0.518	0.467	0.505	0.506	98
1	0.381	0.447	0.470	0.544	0.461	89
0.1	0.487	0.455	0.438	0.514	0.474	92
0.01	0.449	0.456	0.487	0.493	0.471	92
DMSO	0.497	0.535	0.549	0.480	0.515	100

Concentration	Hep-G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.951	0.902	0.847	0.876	0.894	104
50	1.160	1.025	0.816	0.917	0.980	113
10	1.139	1.070	1.030	0.907	1.037	120
1	0.989	0.954	0.946	0.841	0.933	108
0.1	0.892	0.961	0.963	0.892	0.927	107
0.01	0.695	0.993	0.993	0.867	0.887	103
DMSO	0.825	0.854	0.833	0.939	0.863	100

Table 66 Cytotoxicity effect of chlorogenic acid by MTT cell viability (Cont.)

Concentration	KATO-III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	1.263	1.107	0.880	0.891	1.035	107
50	1.147	0.944	0.815	0.869	0.944	97
10	1.098	1.001	0.880	0.856	0.959	99
1	1.041	1.069	0.900	0.870	0.970	100
0.1	1.170	1.024	0.927	0.920	1.010	104
0.01	1.179	1.073	0.951	0.893	1.024	106
DMSO	0.912	0.973	0.955	1.030	0.968	100

Concentration	SW-620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	1.274	1.184	1.047	1.070	1.144	98
50	1.253	1.161	1.117	1.131	1.166	100
10	1.302	1.211	1.117	1.135	1.191	102
1	1.196	1.141	1.139	1.103	1.145	98
0.1	1.226	1.161	1.161	1.056	1.151	99
0.01	1.224	1.215	1.138	1.128	1.176	101
DMSO	1.189	1.204	1.101	1.177	1.168	100

Concentration	Wi-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	1.205	1.197	0.989	1.053	1.111	117
50	1.160	1.120	1.040	0.918	1.060	112
10	0.993	0.977	0.971	0.882	0.956	101
1	1.018	0.914	0.850	0.846	0.907	95
0.1	0.888	0.926	0.936	0.863	0.903	95
0.01	1.002	0.947	0.956	0.930	0.959	101
DMSO	0.888	0.957	0.996	0.957	0.950	100

Table 67 Cytotoxicity effect of *K. parviflora* ethanolic extract by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.075	0.073	0.070	0.066	0.071	10
50	0.114	0.124	0.093	0.107	0.110	16
10	0.518	0.630	0.610	0.691	0.612	88
1	0.581	0.792	0.748	0.692	0.703	101
0.1	0.711	0.751	0.652	0.633	0.687	99
0.01	0.677	0.641	0.645	0.677	0.660	95
DMSO	0.672	0.711	0.674	0.728	0.696	100

Concentration	Chago-K1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.066	0.066	0.066	0.067	0.066	13
50	0.341	0.279	0.289	0.307	0.304	59
10	0.499	0.473	0.476	0.438	0.472	92
1	0.569	0.552	0.504	0.532	0.539	105
0.1	0.514	0.529	0.549	0.475	0.517	100
0.01	0.505	0.462	0.500	0.529	0.499	97
DMSO	0.497	0.535	0.549	0.480	0.515	100

Concentration	Hep-G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.068	0.068	0.068	0.069	0.068	8
50	0.353	0.302	0.287	0.341	0.321	37
10	0.810	0.783	0.718	0.723	0.759	88
1	0.987	0.924	0.949	0.873	0.933	108
0.1	0.933	0.914	0.892	0.852	0.898	104
0.01	0.940	0.933	0.887	0.881	0.910	105
DMSO	0.825	0.854	0.833	0.939	0.863	100

Table 67 Cytotoxicity effect of *K. parviflora* ethanolic extract by MTT cell viability (Cont.)

Concentration	KATO-III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.085	0.076	0.074	0.074	0.077	8
50	0.453	0.444	0.506	0.366	0.442	46
10	0.902	0.902	0.808	0.827	0.860	89
1	1.049	1.034	1.031	1.006	1.030	106
0.1	0.827	0.849	0.887	0.902	0.866	89
0.01	0.849	0.824	0.899	0.981	0.888	92
DMSO	0.912	0.973	0.955	1.030	0.968	100

Concentration	SW-620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.066	0.066	0.067	0.068	0.067	6
50	0.213	0.265	0.340	0.313	0.283	24
10	1.087	1.211	1.035	1.184	1.129	97
1	1.398	1.141	1.227	1.320	1.272	109
0.1	1.099	1.195	1.107	1.239	1.160	99
0.01	1.139	1.170	1.039	1.153	1.125	96
DMSO	1.189	1.204	1.101	1.177	1.168	100

Concentration	Wi-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.074	0.076	0.072	0.073	0.074	8
50	0.313	0.280	0.347	0.370	0.328	34
10	0.939	0.970	0.752	0.747	0.852	90
1	1.205	1.047	0.932	0.869	1.013	107
0.1	0.947	1.079	1.041	0.915	0.996	105
0.01	0.920	0.968	1.002	0.946	0.959	101
DMSO	0.888	0.957	0.996	0.957	0.950	100

Table 68 Cytotoxicity effect of 5,7-dimethoxyflavone by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.059	0.058	0.058	0.057	0.058	9
50	0.060	0.056	0.056	0.056	0.057	9
10	0.476	0.635	0.715	0.695	0.630	101
1	0.510	0.635	0.685	0.681	0.628	100
0.1	0.736	0.755	0.578	0.662	0.683	109
0.01	0.631	0.613	0.550	0.577	0.593	95
DMSO	0.636	0.575	0.542	0.750	0.626	100

Concentration	Chago-K1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.070	0.068	0.068	0.068	0.069	12
50	0.068	0.064	0.067	0.064	0.066	12
10	0.403	0.426	0.546	0.539	0.479	85
1	0.565	0.469	0.563	0.559	0.539	96
0.1	0.631	0.554	0.648	0.542	0.594	106
0.01	0.614	0.719	0.685	0.505	0.631	113
DMSO	0.581	0.545	0.579	0.536	0.560	100

Concentration	Hep-G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.060	0.069	0.067	0.063	0.065	6
50	0.063	0.063	0.063	0.065	0.064	6
10	0.734	0.738	0.705	0.559	0.684	61
1	1.162	1.116	1.009	0.939	1.057	95
0.1	0.931	0.941	0.972	0.938	0.946	85
0.01	0.904	0.822	0.860	0.733	0.830	74
DMSO	1.161	1.207	0.990	1.108	1.117	100

Table 68 Cytotoxicity effect of 5,7-dimethoxyflavone by MTT cell viability (Cont.)

Concentration	KATO-III (OD_{540})					Percent
(μg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.070	0.073	0.077	0.075	0.074	5
50	0.069	0.067	0.075	0.072	0.071	5
10	0.0859	1.124	1.017	1.027	1.007	75
1	1.116	1.318	1.270	1.277	1.245	93
0.1	1.165	1.202	1.346	1.325	1.260	94
0.01	1.115	1.175	1.315	1.258	1.216	91
DMSO	1.308	1.328	1.385	1.349	1.343	100

Concentration	SW-620 (OD_{540})					Percent
(μg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.079	0.063	0.064	0.061	0.067	5
50	0.063	0.061	0.062	0.062	0.062	5
10	1.223	1.223	1.106	1.248	1.200	92
1	1.450	1.249	1.264	1.442	1.351	103
0.1	1.363	1.468	1.327	1.409	1.392	106
0.01	1.420	1.456	1.136	1.167	1.295	99
DMSO	1.416	1.217	1.278	1.321	1.308	100

Concentration	Wi-38 (OD_{540})					Percent
(μg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.066	0.066	0.065	0.067	0.066	6
50	0.066	0.066	0.068	0.069	0.067	6
10	0.718	0.858	0.822	0.797	0.799	73
1	0.837	1.052	1.124	1.045	1.015	93
0.1	1.156	1.113	1.123	1.007	1.100	101
0.01	0.954	1.041	0.988	0.934	0.979	90
DMSO	1.220	1.132	0.931	1.080	1.091	100

Table 69 Cytotoxicity effect of *Z. piperitum* fruit ethanolic extract by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.339	0.477	0.482	0.511	0.452	65
50	0.572	0.598	0.646	0.557	0.593	85
10	0.583	0.750	0.725	0.686	0.686	99
1	0.653	0.618	0.619	0.738	0.657	94
0.1	0.686	0.680	0.665	0.819	0.713	102
0.01	0.713	0.709	0.674	0.670	0.692	99
DMSO	0.672	0.711	0.674	0.728	0.696	100

Concentration	Chago-K1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.341	0.399	0.425	0.493	0.415	80
50	0.456	0.492	0.504	0.498	0.488	95
10	0.498	0.526	0.475	0.506	0.501	97
1	0.512	0.490	0.500	0.543	0.511	99
0.1	0.527	0.510	0.507	0.500	0.511	99
0.01	0.511	0.503	0.509	0.444	0.492	95
DMSO	0.497	0.535	0.549	0.480	0.515	100

Concentration	Hep-G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.797	0.882	0.850	0.861	0.848	98
50	0.822	0.921	0.949	0.878	0.893	103
10	0.847	0.889	0.848	0.900	0.871	101
1	0.817	0.912	0.878	0.824	0.858	99
0.1	0.890	0.874	0.824	0.845	0.858	99
0.01	0.805	0.858	0.858	0.893	0.854	99
DMSO	0.825	0.854	0.833	0.939	0.863	100

Table 69 Cytotoxicity effect of *Z. piperitum* fruit ethanolic extract by MTT cell viability (Cont.)

Concentration	KATO-III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.967	0.923	0.906	0.932	0.932	96
50	0.830	0.937	0.942	1.012	0.930	96
10	0.878	0.927	0.949	0.981	0.934	96
1	0.884	0.884	0.900	0.983	0.913	94
0.1	0.832	0.954	0.949	0.924	0.915	94
0.01	0.919	0.971	1.015	0.966	0.968	100
DMSO	0.912	0.973	0.955	1.030	0.968	100

Concentration	SW-620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.895	0.844	0.906	0.902	0.887	76
50	0.998	1.092	1.166	1.246	1.126	96
10	1.004	1.054	1.157	1.309	1.131	97
1	0.981	0.923	1.117	1.213	1.059	91
0.1	1.069	1.213	1.096	1.258	1.159	99
0.01	1.123	1.204	1.004	1.212	1.136	97
DMSO	1.189	1.204	1.101	1.177	1.168	100

Concentration	Wi-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.863	0.918	0.942	0.894	0.904	95
50	0.856	0.895	0.968	0.977	0.924	97
10	0.854	0.952	0.920	0.997	0.931	98
1	0.921	0.945	0.897	0.917	0.920	97
0.1	0.957	1.094	1.010	0.926	0.997	105
0.01	0.938	0.993	0.997	0.973	0.975	103
DMSO	0.888	0.957	0.996	0.957	0.950	100

Table 70 Cytotoxicity effect of xanthoxylin by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.093	0.075	0.074	0.068	0.078	12
50	0.094	0.075	0.082	0.091	0.086	14
10	0.524	0.479	0.425	0.712	0.535	85
1	0.518	0.583	0.538	0.665	0.576	92
0.1	0.528	0.546	0.501	0.650	0.556	89
0.01	0.696	0.495	0.674	0.746	0.653	104
DMSO	0.636	0.575	0.542	0.750	0.626	100

Concentration	Chago-K1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.073	0.070	0.073	0.074	0.073	13
50	0.095	0.074	0.071	0.089	0.082	15
10	0.524	0.324	0.457	0.477	0.446	80
1	0.232	0.363	0.473	0.590	0.415	74
0.1	0.340	0.378	0.439	0.437	0.399	71
0.01	0.325	0.513	0.553	0.568	0.490	87
DMSO	0.581	0.545	0.579	0.536	0.560	100

Concentration	Hep-G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.102	0.075	0.076	0.072	0.081	7
50	0.179	0.083	0.109	0.092	0.116	10
10	0.911	0.923	0.922	0.753	0.877	79
1	0.873	0.799	0.935	0.914	0.880	79
0.1	0.832	0.910	0.877	0.824	0.861	77
0.01	1.012	0.907	0.847	0.897	0.916	82
DMSO	1.161	1.207	0.990	1.108	1.117	100

Table 70 Cytotoxicity effect of xanthoxylin by MTT cell viability (Cont.)

Concentration	KATO-III (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.089	0.147	0.107	0.111	0.114	8
50	0.254	0.170	0.183	0.177	0.196	15
10	1.115	0.952	0.996	1.036	1.025	76
1	1.014	1.094	1.137	1.072	1.079	80
0.1	1.212	1.041	1.113	0.929	1.074	80
0.01	1.005	1.079	1.066	1.025	1.044	78
DMSO	1.308	1.328	1.385	1.349	1.343	100

Concentration	SW-620 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.151	0.173	0.188	0.142	0.164	13
50	0.592	0.447	0.423	0.372	0.459	35
10	1.232	1.151	1.123	1.150	1.164	89
1	1.260	1.219	1.178	1.240	1.224	94
0.1	1.299	1.207	1.172	1.283	1.240	95
0.01	1.145	1.381	1.342	1.384	1.313	100
DMSO	1.416	1.217	1.278	1.321	1.308	100

Concentration	Wi-38 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
100	0.249	0.225	0.180	0.195	0.212	19
50	0.322	0.271	0.402	0.437	0.358	33
10	1.055	1.063	1.102	1.010	1.058	97
1	1.038	1.059	1.098	1.025	1.055	97
0.1	1.143	1.139	1.190	0.793	1.066	98
0.01	1.052	1.085	1.067	0.984	1.047	96
DMSO	1.220	1.132	0.931	1.080	1.091	100

Table 71 Cytotoxicity effect of doxorubicin by MTT cell viability

Concentration	BT-474 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.103	0.106	0.092	0.085	0.097	15
1	0.126	0.127	0.124	0.108	0.121	19
0.1	0.563	0.535	0.537	0.385	0.505	78
0.01	0.687	0.664	0.587	0.590	0.632	98
0.001	0.782	0.805	0.674	0.692	0.738	115
Control	0.647	0.550	0.717	0.661	0.644	100

Concentration	Chago-K1 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.079	0.086	0.085	0.088	0.085	11
1	0.138	0.146	0.148	0.125	0.139	19
0.1	0.603	0.664	0.635	0.653	0.639	87
0.01	0.865	0.815	0.741	0.738	0.790	107
0.001	0.778	0.754	0.802	0.660	0.749	102
Control	0.634	0.772	0.779	0.761	0.737	100

Concentration	Hep-G2 (OD_{540})					Percent
(µg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.325	0.397	0.407	0.363	0.373	34
1	0.086	0.071	0.074	0.092	0.081	7
0.1	0.581	0.565	0.573	0.523	0.561	51
0.01	1.078	1.263	1.138	1.164	1.161	105
0.001	1.193	1.043	1.219	1.378	1.208	110
Control	1.068	1.144	1.016	1.185	1.103	100

Table 71 Cytotoxicity effect of doxorubicin by MTT cell viability (Cont.)

Concentration	KATO-III (OD_{540})					Percent
(μg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.532	0.487	0.496	0.519	0.509	27
1	0.564	0.534	0.580	0.564	0.561	29
0.1	0.698	0.942	0.920	1.125	0.921	48
0.01	1.937	2.011	1.876	2.175	1.999	105
0.001	1.707	1.558	2.089	2.392	1.937	102
Control	1.778	1.954	1.901	1.994	1.907	100

Concentration	SW-620 (OD_{540})					Percent
(μg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.088	0.096	0.095	0.087	0.092	4
1	0.214	0.213	0.221	0.189	0.209	8
0.1	0.551	0.646	0.483	0.445	0.531	21
0.01	1.867	1.861	2.033	1.769	1.883	74
0.001	2.649	2.981	2.695	2.350	2.669	105
Control	2.193	2.616	2.494	2.817	2.530	100

Concentration	Wi-38 (OD_{540})					Percent
(μg/ml)	Exp 1	Exp 2	Exp 3	Exp 4	Mean	survival
10	0.269	0.289	0.332	0.293	0.296	34
1	0.232	0.200	0.250	0.225	0.227	26
0.1	0.506	0.514	0.607	0.562	0.547	62
0.01	0.888	0.930	0.902	0.820	0.885	101
0.001	1.066	0.782	0.792	0.766	0.852	97
Control	0.856	0.858	0.842	0.965	0.879	100

APPENDIX D

Data of anti-diabetic activities

Yeast alpha-glucosidase inhibitory activity

Table 72 Yeast alpha-glucosidase inhibition of acarbose (positive control)

Conc.	OD_{405} (reaction mixture)			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.258	0.271	0.276					
1.25	0.238	0.246	0.250	7.752	9.225	9.420	8.799	0.912
2.5	0.218	0.239	0.240	15.504	11.808	13.043	13.452	1.881
5	0.183	0.191	0.196	29.070	29.520	28.986	29.192	0.288
10	0.145	0.147	0.150	43.798	45.756	45.652	45.069	1.102
20	0.108	0.113	0.117	58.140	58.303	57.609	58.017	0.363

Table 73 Yeast alpha-glucosidase inhibition of ethanolic extract of *C. odorata* leaves

Conc.	OD_{405} (reaction mixture)			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.265	0.266	0.268					
5	0.230	0.239	0.246	13.208	10.150	8.209	10.522	2.520
10	0.189	0.191	0.193	28.679	28.195	27.985	28.287	0.356
15	0.138	0.147	0.150	47.925	44.737	44.030	45.564	2.075
20	0.117	0.122	0.127	55.849	54.135	52.612	54.199	1.619
30	0.104	0.106	0.113	60.755	60.150	57.836	59.580	1.541

Table 74 Yeast alpha-glucosidase inhibition of chlorogenic acid

Conc.	OD_{405} (reaction mixture)			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.403	0.403	0.420					
2.5	0.331	0.347	0.364	17.866	13.896	13.333	15.032	2.471
5	0.337	0.347	0.349	16.377	13.896	16.905	15.726	1.607
10	0.261	0.283	0.290	35.236	29.777	30.952	31.988	2.873
20	0.151	0.169	0.172	62.531	58.065	59.048	59.881	2.347
40	0.002	0.003	0.021	99.504	99.256	95.000	97.920	2.532

Table 75 Yeast alpha-glucosidase inhibition of ethanolic extract of *K. parviflora* rhizomes

Conc.	OD_{405} (reaction mixture)			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.238	0.241	0.247					
20	0.168	0.186	0.186	29.412	22.822	24.696	25.643	3.396
25	0.135	0.139	0.139	43.277	42.324	43.725	43.109	0.716
30	0.120	0.127	0.130	49.580	47.303	47.368	48.084	1.296
35	0.097	0.108	0.110	59.244	55.187	55.466	56.632	2.266
40	0.084	0.092	0.097	64.706	61.826	60.729	62.420	2.054

Table 76 Yeast alpha-glucosidase inhibition of 5,7-dimethoxyflavone

Conc.	OD_{405} (reaction mixture)			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.443	0.448	0.452					
0.063	0.439	0.443	0.446	0.903	1.116	1.327	1.115	0.212
0.125	0.429	0.433	0.434	3.160	3.348	3.982	3.497	0.431
0.25	0.412	0.424	0.429	6.998	5.357	5.088	5.814	1.034
0.5	0.395	0.398	0.408	10.835	11.161	9.735	10.577	0.747
1	0.386	0.393	0.390	12.867	12.277	13.717	12.953	0.724

Table 77 Yeast alpha-glucosidase inhibition of ethanolic extract of *Z. piperitum* fruits

Conc.	OD_{405} (reaction mixture)			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.368	0.370	0.383					
0.63	0.359	0.366	0.369	2.446	1.081	3.655	2.394	1.288
1.25	0.314	0.325	0.339	14.674	12.162	11.488	12.775	1.679
2.5	0.245	0.258	0.259	33.424	30.270	32.376	32.023	1.606
5	0.166	0.171	0.186	54.891	53.784	51.436	53.370	1.764
10	0.063	0.065	0.076	82.880	82.432	80.157	81.823	1.461

Table 78 Yeast alpha-glucosidase inhibition of xanthoxylin

Conc.	OD_{405} (reaction mixture)			Yeast alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.335	0.348	0.351					
5	0.329	0.338	0.342	1.791	2.874	2.564	2.410	0.558
10	0.137	0.147	0.157	59.104	57.759	55.271	57.378	1.945
12	0.045	0.047	0.068	86.567	86.494	80.627	84.563	3.409
14	0.027	0.035	0.036	91.940	89.943	89.744	90.542	1.215
16	0.015	0.017	0.022	95.522	95.115	93.732	94.790	0.938

Rat alpha-glucosidase inhibitory activity

Table 79 Rat alpha-glucosidase inhibition of acarbose (positive control)

Conc.	OD_{405} (reaction mixture)			Rat alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	1.485	1.494	1.486					
0.078	0.972	0.933	0.927	34.545	37.550	37.618	36.571	1.755
0.156	0.845	0.820	0.864	43.098	45.114	41.857	43.356	1.644
0.313	0.729	0.696	0.724	50.909	53.414	51.279	51.867	1.352
0.625	0.570	0.565	0.574	61.616	62.182	61.373	61.724	0.415
1.250	0.463	0.454	0.450	68.822	69.612	69.717	69.384	0.490

Table 80 Rat alpha-glucosidase inhibition of ethanolic extract of *C. odorata* leaves

Conc.	OD_{405} (reaction mixture)			Rat alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	1.322	1.292	1.330					
1.25	1.144	1.124	1.159	13.464	13.003	12.857	13.108	0.317
2.5	1.087	1.063	1.087	17.776	17.724	18.271	17.924	0.302
5	0.889	0.850	0.901	32.753	34.211	32.256	33.073	1.016
10	0.678	0.668	0.709	48.714	48.297	46.692	47.901	1.068
20	0.357	0.341	0.359	72.995	73.607	73.008	73.203	0.350

Table 81 Rat alpha-glucosidase inhibition of chlorogenic acid

Conc.	OD_{405} (reaction mixture)			Rat alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.845	0.896	0.872					
0.63	0.745	0.820	0.780	11.834	8.482	10.550	10.289	1.691
1.25	0.748	0.763	0.762	11.479	14.844	12.615	12.979	1.712
2.50	0.666	0.738	0.672	21.183	17.634	22.936	20.584	2.701
10	0.459	0.497	0.476	45.680	44.531	45.413	45.208	0.601
20	0.248	0.294	0.276	70.651	67.188	68.349	68.729	1.763

Table 82 Rat alpha-glucosidase inhibition of ethanolic extract of *K. parviflora* rhizomes

Conc.	OD_{405} (reaction mixture)			Rat alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	1.861	1.844	1.913					
2.50	1.786	1.699	1.786	4.030	7.863	6.639	6.177	1.958
3.75	1.696	1.636	1.739	8.866	11.280	9.096	9.747	1.332
5.00	1.631	1.590	1.719	12.359	13.774	10.141	12.091	1.831
7.50	1.399	1.299	1.427	24.825	29.555	25.405	26.595	2.580
10.00	0.675	0.656	0.764	63.729	64.425	60.063	62.739	2.344

Table 83 Rat alpha-glucosidase inhibition of 5,7-dimethoxyflavone

Conc.	OD_{405} (reaction mixture)			Rat alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	1.945	1.948	1.958					
0.03	1.936	1.939	1.943	0.463	0.462	0.766	0.564	0.175
0.06	1.893	1.945	1.948	2.674	0.154	0.511	1.113	1.363
0.13	1.900	1.901	1.934	2.314	2.413	1.226	1.984	0.659
0.25	1.859	1.905	1.912	4.422	2.207	2.349	2.993	1.239
0.50	1.861	1.880	1.886	4.319	3.491	3.677	3.829	0.434
1.00	1.89	1.903	1.905	2.828	2.310	2.707	2.615	0.271

Table 84 Rat alpha-glucosidase inhibition of ethanolic extract of *Z. piperitum* fruits

Conc.	OD_{405} (reaction mixture)			Rat alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	1.535	1.549	1.571					
2.5	1.436	1.438	1.471	6.450	7.166	6.365	6.660	0.440
5.0	1.212	1.215	1.266	21.042	21.562	19.414	20.673	1.121
10.0	0.879	0.897	0.988	42.736	42.092	37.110	40.646	3.079
20.0	0.537	0.553	0.628	65.016	64.300	60.025	63.114	2.698
40.0	0.078	0.079	0.081	94.919	94.900	94.844	94.888	0.039

Table 85 Rat alpha-glucosidase inhibition of xanthoxylin

Conc.	OD_{405} (reaction mixture)			Rat alpha-glucosidase inhibition (%)				
(mg/ml)	exp 1	exp 2	exp 3	exp 1	exp 2	exp 3	Mean	SD
0	0.938	0.972	0.959					
1.25	0.868	0.913	0.912	7.463	6.070	4.901	6.145	1.283
5.00	0.868	0.887	0.886	7.463	8.745	7.612	7.940	0.701
10.00	0.822	0.858	0.857	12.367	11.728	10.636	11.577	0.875
20.00	0.756	0.81	0.806	19.403	16.667	15.954	17.341	1.821
40.00	0.707	0.74	0.74	24.627	23.868	22.836	23.777	0.899

APPENDIX E

Data of physico-chemical parameters of *K. parviflora* rhizomes

Table 86 Physico-chemical parameters of *K. parviflora* rhizomes

Sources	No.	% by weight						
		Water content	Loss on drying	Total ash	Acid insoluble ash	Ethanol extractive value	Water extractive value	Volatile oil content
Tak	1	10.00	9.76	5.04	1.90	4.43	6.82	0.03
	2	9.33	9.73	5.01	1.94	4.29	6.83	0.03
	3	9.33	9.65	4.91	1.68	4.39	6.12	0.03
Phitsanulok	1	7.66	9.51	3.36	1.02	5.00	6.41	0.05
	2	7.65	9.41	3.40	1.22	4.83	6.60	0.04
	3	8.65	9.49	3.37	1.11	4.82	6.44	0.05
Phetchabun	1	6.66	7.86	5.42	1.82	4.41	9.20	0.03
	2	6.32	7.85	5.47	1.87	4.58	8.84	0.03
	3	7.32	7.81	5.49	1.66	4.44	9.06	0.03
Chiang Mai	1	6.65	7.36	4.26	1.47	4.97	7.07	0.04
	2	7.33	7.47	4.26	1.46	4.84	7.71	0.03
	3	6.32	7.38	4.26	1.34	4.68	7.34	0.04
Loei	1	10.00	8.55	4.46	1.87	6.41	6.49	0.03
	2	10.99	8.57	4.44	1.77	6.38	6.02	0.02
	3	9.98	8.45	4.42	1.86	6.33	6.20	0.02
Nakhon Pathom	1	10.66	10.30	4.70	1.90	3.12	7.54	0.01
	2	10.99	10.34	4.64	1.85	3.04	7.34	0.01
	3	10.99	10.33	4.70	1.79	3.07	7.27	0.01
Suphan Buri	1	9.32	8.65	5.60	2.69	6.17	9.40	0.01
	2	9.66	8.64	5.58	2.75	6.22	9.58	0.01
	3	8.66	8.59	5.62	2.82	6.27	9.74	0.01
Nakhon Sawan	1	10.33	9.79	6.55	3.27	6.38	9.13	0.03
	2	11.33	9.76	6.51	3.28	6.31	8.87	0.03
	3	10.99	9.75	6.57	3.27	6.30	9.04	0.03
Petchaburi	1	9.00	9.09	5.27	2.15	5.03	10.01	0.02
	2	9.33	9.08	5.36	2.23	5.02	9.82	0.02
	3	9.66	9.04	5.22	2.13	5.04	9.97	0.02
Trang	1	9.00	9.29	4.86	2.03	4.78	9.84	0.05
	2	9.67	9.34	4.82	1.86	4.80	9.85	0.04
	3	9.67	9.26	4.89	1.95	4.74	10.01	0.05
Songkhla	1	9.33	9.16	4.41	2.25	5.21	6.47	0.01
	2	9.66	9.16	4.50	2.43	5.29	6.51	0.01
	3	9.66	9.22	4.35	2.20	5.33	6.44	0.01

Table 86 Physico-chemical parameters of *K. parviflora* rhizomes (Cont.)

Sources	No.	% by weight						
		Water content	Loss on drying	Total ash	Acid insoluble ash	Ethanol extractive value	Water extractive value	Volatile oil content
Krabi	1	10.00	9.75	3.89	1.37	4.98	7.68	0.04
	2	10.66	9.77	3.88	1.34	5.11	7.81	0.04
	3	10.00	9.70	3.88	1.36	5.12	7.79	0.04
Rayong	1	9.00	7.94	6.19	2.87	5.88	10.29	0.03
	2	8.00	7.85	6.23	2.78	5.91	10.39	0.03
	3	8.00	7.83	6.20	2.84	5.76	10.55	0.03
Yasothon	1	9.33	8.58	5.97	2.16	4.95	9.66	0.03
	2	8.66	8.54	5.88	2.12	4.98	9.84	0.03
	3	9.33	8.56	5.81	2.17	4.81	10.10	0.03
Bangkok	1	10.66	9.31	6.88	3.96	5.62	7.62	0.03
	2	10.67	9.29	6.97	3.80	5.57	7.93	0.02
	3	11.66	9.29	7.20	4.21	5.62	7.81	0.02

Formulas:

$$\text{Grand mean} = \frac{\bar{x}_1 n_1 + \bar{x}_2 n_2 + \ldots + \bar{x}_k n_k}{n_1 + n_2 + \ldots + n_k}$$

$$\text{Pooled SD} = \sqrt{\frac{((n_1 - 1) \times SD_1^2) + ((n_2 - 1) \times SD_2^2) + \ldots + ((n_k - 1) \times SD_k^2)}{(n_1 + n_2 + \ldots + n_k) - k}}$$

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Publications

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Poster Presentation

Yamon Pitakpawasutthi, Chanida Palanuvej and Nijsiri Ruangrungsi. "Chlorogenic acid content, essential oil compositions, and in vitro antioxidant activities of Chromolaena odorata leaves". The 3rd Global Summit on Herbals & Traditional Medicine, October 18-20, 2017 at Osaka, Japan.

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