PHARMACOGNOSTIC SPECIFICATIONS AND ANTIMICROBIAL ACTIVITIES OF *HOUTTUYNIA CORDATA*

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ข้อกำหนดทางเภสัชเวทและฤทธิ์ต้านจุลชีพของต้นพลูคาว

นางสาวพิมพ์พรรณ ฉัตรชินรัตน์

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Field of Study	Public Health Sciences
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พิมพ์พรรณ ฉัตรชินรัตน์ : ข้อกำหนดทางเภสัชเวทและฤทธิ์ต้านจุลชีพของต้นพลูคาว (PHARMACOGNOSTIC SPECIFICATIONS AND ANTIMICROBIAL ACTIVITIES OF *HOUTTUYNIA CORDATA*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี, 123 หน้า.

พลูคาวหรือคาวตองมีชื่อทางวิทยาศาสตร์คือ *Houttuynia cordata Thunb*. เป็นผักพื้นบ้านนิยมรับประทานทางภาคเหนือของประเทศไทย การศึกษานี้ได้เก็บรวบรวมต้นพลูคาวจาก 12 พื้นที่ในประเทศไทย โดยมีวัตถุประสงค์เพื่อจัดทำข้อกำหนดทางเภสัชเวท วิเคราะห์หาปริมาณสารเควอซีทินและองค์ประกอบทางเคมีของน้ำมันระเหยจากต้น พลูคาว จากผลการศึกษาแสดงให้เห็นถึงเอกลักษณ์ทางกายภาพและเคมีของต้นพลูคาว พบว่าน้ำหนักที่หายไปเมื่อทำให้แห้ง ปริมาณเถ้ารวม เถ้าที่ไม่ละลายในกรด ปริมาณสารสกัดที่ละลายด้วยน้ำ ปริมาณสารสกัดด้วยเอทานอล ปริมาณความชื้นและปริมาณน้ำมันระเหยมีค่าเป็นร้อยละ 7.22±0.34, 14.31±0.15, 4.11±0.36, 14.17±1.03, 4.69±0.48, 11.70±1.18, 0.08±0.01 โดยน้ำหนักแห้ง ตามลำดับ วาดภาพลายเส้นแสดงลักษณะทางมหทรรศน์และจุลทรรศน์ การศึกษาด้วยเทคนิคทางทินเลเยอร์โครมาโทกราฟีใช้ตัวทำละลายโทลูอีนต่อเอทิล อะซีเตทต่อเมทานอลและกรดฟอร์มิก (5: 5: 3: 2) ตรวจวัดภายใต้แสงขาว แสงอัลตราไวโอเลตความยาวคลื่น 254 และ 366 นาโนเมตร และทำปฏิกิริยาเกิดสีกับเนเซอรัลโปรดักส์-โพลีเอทิลีนไกลคอล วิเคราะห์หาปริมาณสารเควอซีทินในสิ่งสกัดเอทานอลของต้นพลูคาวทั้ง 12 แหล่ง โดยวิธีทินเลเยอร์โครมาโทกราฟีเดนซิโทเมทรีและการวิเคราะห์เชิงภาพทาง ทินเลเยอร์โครมาโทกราฟี วิธีวิเคราะห์ทั้งสองวิธีมีความเที่ยงและเชื่อถือได้จากการประเมินโดยใช้แนว ทางของไอซีเอช (ICH guideline) ปริมาณสารเควอซีทินในสารสกัดต้นพลูคาวเกือบทุกแหล่งพบปริมาณน้อยกว่าค่าขีด จำกัดของการหาปริมาณ ยกเว้นแหล่งเดียวที่พบปริมาณสารเควอซีทิน 0.0049 กรัม/กรัมของต้นแห้ง โดยวิธีทินเลเยอร์โครมาโทกราฟีเดนซิโทเมทรี และ 0.0031 กรัม/กรัม ของต้นแห้ง โดยการวิเคราะห์เชิงภาพทางทินเลเยอร์โครมาโทกราฟี สกัดน้ำมันระเหยจากต้นพลูคาวโดยวิธีการกลั่นด้วยน้ำ วิเคราะห์องค์ประกอบทางเคมีโดยวิธีแกสโครมาโทกราฟี-แมสสเปกโทเมทรี พบองค์ประกอบทางเคมีอย่างน้อย 30 ชนิด องค์ประกอบหลักคือ decanoic acid (ร้อยละ 40.40±27.21), 2-undecanone (ร้อยละ 26.43±14.54) และ myrcene (ร้อยละ 19.21±15.09) การศึกษาฤทธิ์ต้านจุลชีพพบว่าน้ำมันระเหยต้นพลูคาวมีฤทธิ์ต้านเชื้อ แบคทีเรียแกรมบวก (*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Kocuria rhizophila, Straphylococcus epidermidis*) และเชื้อรา (*Candida albicans, Saccharomyces cerevisiae*)

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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PIMPAN CHATCHINARAT: PHARMACOGNOSTIC SPECIFICATIONS AND ANTIMICROBIAL ACTIVITIES OF *HOUTTUYNIA CORDATA* . ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 123 pp.

Houttuynia cordata Thunb. (Plu-Klao) is a perennial plant that belongs to Saururaceae Family. Fresh leaves of *H. cordata* are popular vegetable eaten with main course in the northern region of Thailand and all parts were used for immune stimulization. The aims of this study were to investigate the pharmacognostic specifications, analyses of the content of quercetin and volatile oil chemical constituents of *H. cordata* whole plants. *H. cordata* were collected from 12 different locations in Thailand. The results demonstrated the content of loss on dying, total ash, acid insoluble ash, water-soluble extractive value, ethanol- soluble extractive value, moisture and volatile oil content as 7.22 ±0.34, 14.31±0.15, 4.11±0.36, 14.17±1.03, 4.69±0.48, 11.70±1.18, 0.08±0.01 % by dried weight, respectively. The macroscopic and microscopic characteristics were illustrated in detail. Thin layer chromatographic fingerprints of ethanolic extract were performed using toluene: ethyl acetate: methanol: formic acid (5: 5: 3: 2) as mobile phase and visualization under daylight, UV 254 nm, UV 366 nm and staining with natural product–polyethylene glycol reagent. Quercetin contents in 12 ethanolic extracts were determined by TLC-densitometry and TLC image analysis using imageJ software. Both methods were valid and reliable according to ICH guideline. The quantitative analysis revealed that quercetin contents in most extracts were less than the limit of quantitation except only one source which was found to be 0.0049 g/g crude drugs by TLC densitometry and 0.0031 g/g crude drugs by TLC image analysis. *H. cordata* volatile oil was extracted by hydrodistillation. At least 30 chemical constituents of volatile oil were identified by GC/MS. The major constituents were decanoic acid (40.40±27.21%), 2-undecanone (26.43±14.54%) and myrcene (19.21±15.09%). The volatile oil was investigated for antimicrobial activity by agar diffusion assay and broth microdilution assay and was shown positive against gram positive bacteria (*Bacillus subtilis*, *Bacillus cereus, Staphylococcus aureus*, *Kocuria rhizophila*, *Straphylococcus* epidermidis) and fungi (*Candida albicans*, *Saccharomyces cerevisiae*).

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

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LIST OF ABBREVIATIONS

ºC	= Degree Celsius
cm	= Centimetre
DDPH	= 1, 1-diphenyl-2-picrylhyzyl
GC	= Gas chromatography
g	= Gram
HCI	= Hydrochloric acid
ICH	= International Conference on Harmonization
LOD	= Limit of detection
LOQ	= Limit of quantitation
MS	= Mass spectrometry
mg	= milligram
min	= Minute
ml	= milliliter
nm	= nanometer
R^2	= Correlation coefficients
R_f	= Retention factors
RSD	= Relative standard deviation
SD	= Standard deviation
TLC	= Thin layer chromatography
UV	= Ultraviolet

v/v = Volume in a volume

WHO = World Health Organizations

µg = microgram

µl = microliter

α = Alpha

β = Beta

CHAPTER I
INTRODUCTION

Background and rationale

Herbal medicine consists of herbs, herbal materials, herbal preparation and finished herbal product. This is the oldest healthcare that has been used in all cultures throughout history. People learned to treat an illness using simple method prepared from parts of plants around them. A compilation of this knowledge has been transmitted from generation to generation. During the past decade, people are interested in healthcare and pay attention to use herbal medicine as an alternative of health promotion, prevention and treatment of illnesses which make herbal medicine is popular in many countries [7]. Evaluation and assurance of quality, safety and efficacy become an important issue for herbal products. The World Health Organization (WHO) encourages standardization and quality control of herbal materials by modern analytical techniques [2].

Saururaceae is a plant family in Piperales order, comprising four genera and six species of aromatic herbaceous flowering plants, native to eastern and southern Asia as well as North America, sometime known as the lizard's-tail family. *Houttuynia cordata* Thunb. Is only species that can be found in Thailand [3].

Houttuynia codata Thunb. is a perennial plant with fishy smelling characteristics known in English as “Heartleaves” or “Fishwort”, in Thailand, known as “Plu-khao”

or “Khow-tong”. *H. cordata* is widely used in traditional and alternative medicine for long times ago. Fresh leaves of *H. cordata* are popular vegetable eaten with main course in the northern region of Thailand, all parts are used for immune stimulization. Traditional Chinese medicine used only *H. cordata* or combination with other herbs for respiratory tract treatment such as sinus inflammation, pulmonary abscess and cough. In Ayuravedic medicine, people eat both raw and cooked leaves of *H. cordata* for treating tuberculosis and drink a juice to treat gastritis and peptic ulcer [4, 5]. In Japan, “Dokudami cha”, a tea from *H. cordata* is drunk to quench thirst. It is also believed that it keeps the skin beautiful and slows down the effects of aging. The leaves are crushed and rubbed into the nostrils to relieve congestion and detox. In Korea, the leaves have been used to treat dropsy, cough, pneumonia, bronchitis, dysentery, leucorrhea, eczema, acne, chronic sinusitis, nasal polyps, herpes simplex, and used as an ingredient in cosmetics to reduce wrinkles, and anti-aging [6, 7]. *H. cordata* was reported for a lot of active compound groups such as flavonoid, alkaloid, ketone, fatty acid, and volatile oil. Flavonoid is a group of phytochemicals which can be found in *H. cordata*; for example, quercetin, rutin, quercitrin, kaemferol, isoquercetin and hyperin. It has many benefits in health promotion and care.

Quercetin is one of the six members of flavonoid that can be found in fruits and vegetables including onion, green apple, berries, broccoli and many other foods.

Pharmacological study reports that quercetin has many benefits such as antimicrobial activity, anti-inflammation, reducing risk for tumor and strong antioxidation. Volatile oil is natural compound that found in *H. cordata*, it is a substance with a unique smell and flavor. The plant produces the oil and stores in the cell walls in various parts of the plant such as seeds, flowers, leaves, fruits, bark or roots. *H. cordata* oil has been demonstrated to exhibit antibacterial, anti-inflammatory activities and has been used for antiseptic agent [8-10].

Medicinal plant materials could be adulterated with other plant parts or substances with low quality. Although *H. cordata* is widely used in many counties through various same or different methods, there is no pharmacognostic specification available for the standardization of this medicinal plant. The quality control of herbal medicine is important, therefore this study involves the qualitative and quantitative analyses of *H. cordata* with the special reference to quercetin marker.

Research Gap

The quality parameters as well as quercetin content of *H. cordata* crude drug in Thailand have never been established and the scientific evidence of antimicrobial activity of *H. cordata* volatile oil is limited.

Objectives of the study

1. To develop the standardization parameters of *H. cordata* crude drug.

2. To determine the quercetin content in *H. cordata* by ImageJ free software compared to TLC densitometry.

3. To evaluate the antimicrobial activities of *H. cordata* volatile oil.

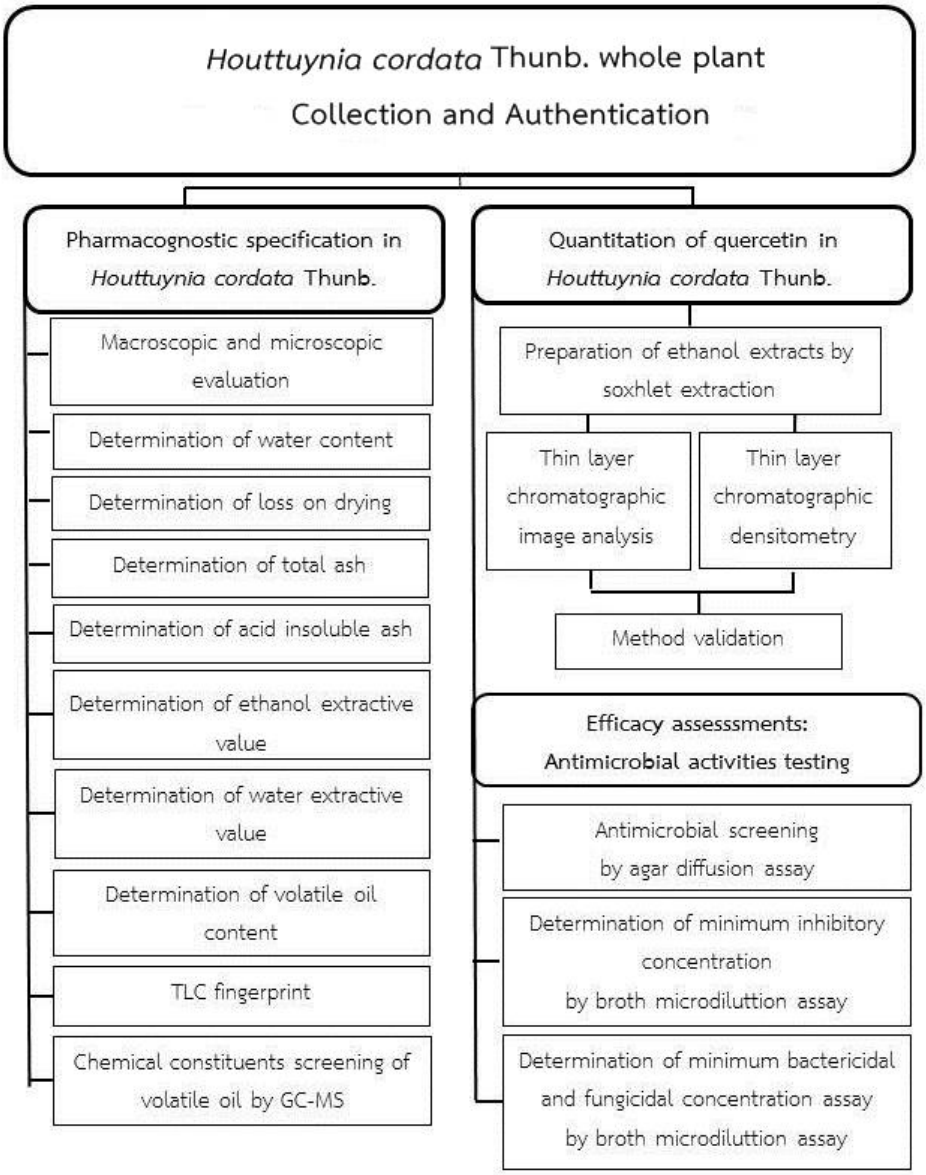

Figure 1 The conceptual framework

CHAPTER II

LITERRATURE REVIEWS

Description of Saururaceae family

Herbs perennial, aromatic, Stems erect, ascending, or prostrate, obviously jointed. Leaves alternate, simple; stipules interpetiolately connate or adnate to petiole, forming a sheath. Inflorescence a dense spike or raceme, sometimes resembling a single flower when large involucral petaloid bracts are present at base; bracts evident or inconspicuous. Flowers bisexual; perianth absent. Stamens usually 3, 6, or 8, free or adnate to ovary base; anthers 2-loculed, longitudinally dehiscent. Pistils (2 or) 3- or 4-carpelled; carpels distinct or connate, if distinct: each carpel with 2-4 ovules, if connate: ovary 1-loculed, placentation parietal, each placenta with 6-13 ovules; styles free. Fruit a schizocarp or apically dehiscent capsule. Seed 1 or many; endosperm scanty; perisperm abundant; embryo minute. Four genera and ca. six species in East and South Asia, North America [11] and one species in Thailand [12].

Houttutnia Cordata Thunb.

Common names : Chameleon Plant, Heartleaf

Family : Saururaceae

Synonyms : *Polypara cochinchinensus* Lours. *Houttuynia foetida* Loudon

Common names : Plu-khao or Khao-Thong (Thailand), Chameleon-plant, Dokudami (Japan), Yu xing cao (China), Giấp cá (Vietnam)

Figure 2 *Hottuynia cordata* Thunb.

Taxonomy

Kingdom Plantae – Plants

Subkingdom Tracheobionta – Vascular plants

Superdivision Spermatophyta – Seed plants

Division Magnoliophyta – Flowering plants

Class Magnoliopsida – Dicotyledons

Subclass Magnoliidae

Order Piperales

Family Saururaceae – Lizard's-tail family

Genus *Houttuynia Thunb. – houttuynia*

Description and distribution

Herbs, 30-60 cm high; rhizomes creeping, thin. Basal part of stems creeping, rooted in whorls at nodes, apical part erect, glabrous or pubescent on nodes, sometimes purplish red. Stipular sheath (0.5-)1-2.5 cm, 1/4-1/2 as long as petiole, usually ciliate, base enlarged and slightly clasping; petiole (0.7-) 1-3.5(-4) cm, glabrous; leaf blade broadly ovate or ovate-cordate, (1.5-)4-10 × (1.8-)2.5-6 cm, thinly papery, densely glandular, usually glabrous, sometimes pubescent at vein axils, usually purplish abaxially, base cordate, apex shortly acuminate; veins 5-7, basal or innermost pair arising ca. 5 mm above base, if 7-veined, then outermost pair very slender or inconspicuous; reticulate veins ± conspicuous. Inflorescences (0.4-) 1.5-2.5(-2.7) cm × (2-) 5-6 mm; peduncles 1.5-3 cm, subglabrous; involucral bracts oblong or obovate, (5-)10-15 × (3-)5-7 mm, apex rounded. Bract beneath each flower linear, terete, inconspicuous. Stamens longer than ovary. Capsule 2-3 mm, with persistent styles [11]. *H. cordata* distribute in shaded places, shallow water, low light situations and moist loamy soils, can be found in China, Taiwan, Himalayas, Cambodia, Vietnam, India, Japan, Korea, Thailand, Nepal and Myanmar [13].

Medicinal uses

In China, *H. cordata* leaves have been used to treat of urethritis, cough and sore from insect bites. In Japan and Korea, drinking *H. cordata* tea can treat stomach ulcers, infection and be as diuretics, *H. cordata* has been used for the treatment of

respiratory tract including cough, pneumonia, bronchitis, dysentery, dropsy and leucorrhea. It is ground and heaped on wound or eczema, herpes simplex, acne and inhaled for treatment of nasal polyps, chronic sinusitis, and mixed in cosmetic or anti-aging and skin care products. The shoot has been used for the freshness, heart disorders and good sleep in the Eastern Himalayan region of India. In Thailand, people eat fresh leaves as side dish, it has been used for immune stimulation and anticancer agent [14, 15].

Biological activities of *H. cordata*

H. cordata is reported for many chemical components such as flavonoids, alkaloids, fatty acid, and volatile oil.

Antioxidant activities

Nuengchamnong reported antioxidant activities of *H. cordata* methanolic extract determined by off-line DPPH assays compared to on-line DPPH radical scavenging technique coupled with a liquid chromatography–electrospray ionization mass spectrometer (LC–ESI–MS). The antioxidant compounds were identified as catechin, quinic acid derivative, quercetin hexoside, caffeic acid derivatives, neo-chlorogenic acid, crypto-chlorogenic acid, procyanidin B and chlorogenic acid. *H. cordata* expressed as EC_{50} value of 341.5 ± 17.2 µg/ml [15]. In 2011, Seal reported antioxidant from root extract of *H. cordata* by DPPH assay, The IC_{50} was 317.75 ± 2.75

μg/ml and total phenolic was 24.60 ± 0.44 expressed as gallic acid equivalents (GAE) in miligram per gram (mg/g) [16].

Antimicrobial activities

Chomnawang investigated *H. cordata* crude extract based on antimicrobial susceptibility testing against *Propionibacterium acnes* and *Staphylococcus epidermidis* which had been accepted as pus-forming bacteria triggering inflammation in acne. *H. cordata* crude extract showed MIC of 0.039 mg/ml and MBC of 2.5 mg/ml in *Propionibacterium acnes* and MIC of 1.25 mg/ml and MBC > 5 mg/ml in *Staphylococcus epidermidis* [17].

Hot water extract of *H. cordata* has been tested for cytotoxicity by XTT-based colorimetric assay. Cells were infected with herpes simplex virus (HSV) and then were cultured with hot extract. The results showed that *H. cordata* had the effect at a concentration of 250 μg/ml. The ED_{50} of HSV type 1 (HSV-1) and HSV type 2 (HSV-2) of *H. cordata* extract were 822.4 μg/ml and 362.5 μg/ml respectively [6].

Anti-inflammatory activities

H. cordata supercritical extract inhibited the release of LPS-induced PGE (2) from mouse peritoneal macrophages with IC_{50} of 44.8 μg/ml. The result could be suggested that *H. cordata* inhibited COX-2 enzyme activity and affected related gene and protein expression. *H. cordata* worked that a mechanism similar to NSAIDs [18].

In 2012, Dajeong assessed the aerial part of *H. cordata,* extracted under CO_2 supercritical conditions for 2 hours, in six-week-old male Sprague-Dawley rats for the anti-inflammatory effects of by analyzing mediators in the two major pathways of inflammation. In comparison with the synthetic steroid, dexamethasone and a non-steroidal anti-inflammatory drug (NSAID), indomethacin, the results indicated that *H. cordata* exhibits anti-inflammatory effects by inhibiting both TNF-α-NO and cyclooxygenase-2-PGE2 pathways [19].

Anticancer activities

The study of 50% ethanolic extracts of *H. cordata* in a dose 0, 125, 250 and 500 µg/ml on inhibition of cancer cell growth was performed. After treatment with *H. cordata* (250 µg/ml) for 24 hours, cells exhibited chromatin condensation (an apoptotic characteristic). *H. cordata* increased reactive oxygen species (ROS) production and decreased the mitochondrial membrane potential in examined human primary colorectal cancer cells. Mitochondria-dependent apoptotic signaling pathway was shown to be involved as determined by increase in the levels of cytochrome c, Apaf-1, and caspase-3 and -9. The decrease in the level of the mitochondrial membrane potential was shown. Based on the results, *H. cordata* induced apoptotic cell death in human primary colorectal cancer cells through a mitochondria-dependent signaling pathway [20].

Volatile oils

The volatile oil is one of natural products extracted from plant. Recent studies have been reported many components in volatile oil of *H. cordata.* These compositions were decanoic acid, myrcene, caryophyllene, pinene, camphene, limonene, bornyl acetate, bornyl acetate, decanol, terpineol and others. Pharmacological studies indicated *H. cordata* volatile oil component effects on anti-inflammatory, anti- bacterial and antiviral activities [10, 21, 22].

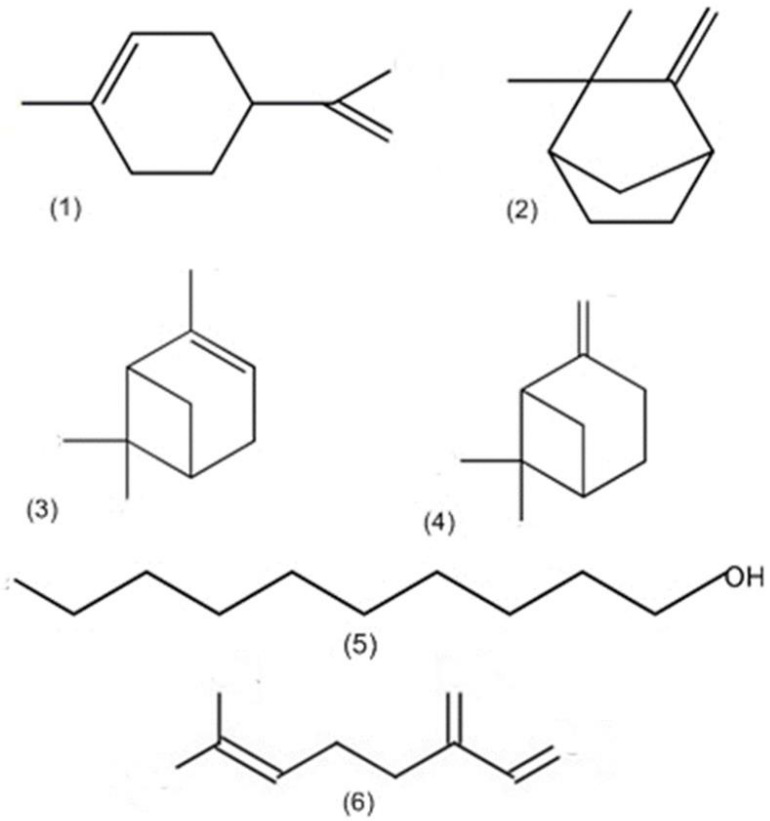

Figure 3 Stuctures of chemical compounds found in *H. cordata* volatile oil; (1) limonene, (2) camphene (3) α-pinene, (4) β- pinene, (5) decanol, (6) myrcene

Biological activities of *H. cordata* volatile oil

Antimicrobial activities

In 2006, Hongmei indicated that *H. cordata* volatile oil had abilities to inhibit microoganisms, *in vitro* studies reported activities against *Staphylococcus aureus* (MIC=0.25×10^{-3} ml/ml), *Sarcina ureae* (MIC=0.0625×10^{-3} ml/ml), *Propionibacterium acnes* (MIC=0.039 mg/ml, MBC= 2.5 mg/ml) and *Staphylococcus epidermidis* (MIC= 1.25 mg/ml, MBC > 5 mg/ml) [10, 17].

Chiang reported that the steam distillate of *H. cordata* showed viral inhibitory activities against herpes simplex virus type 1 (HSV-1), influenza virus, and human immunodeficiency virus type 1 (HIV-1) [23].

Quercetin

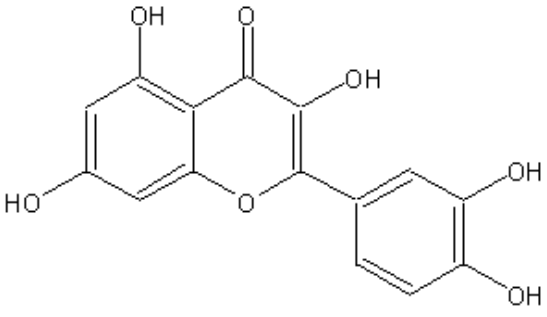

Figure 4 Structure of quercetin

IUPAC name: 3, 3', 4', 5, 7-pentahydroxy-2-phenylchromen-4-one

Molecular formula : $C_{15}H_{10}O_7$

Molecular Weight : 302.23

Physical description : yellow crystalline powder

Melting Point : 310 - 317 °C

Boiling Point : Sublime

Classification : Flavonoid, Flavonol

Quercetin is the aglycone in flavonoid groups, including rutin, quercitrin, isoquercetin, and hyperoside. These molecules are derivatives of quercetin for their specific sugar molecule attached in place of hydroxyl group at position 3, 5, 7, 3' or 4', which dramatically changes the activity of the molecule. Quercetin is widely found in food dietary including berries, onion, apples, tea, grape as well as nuts, barks and leaves [24].

Biological activities of quercetin

Antioxidant activities

The antioxidant properties of quercetin have exhibited some successes in preventing free radical damage. In 2011, DPPH assay studies from Poland reported

that 0.1 μg of quercetin showed more antioxidant activity than vitamin c and trolox [25].

Antidiabetic effect

In 2003, Vessal reported the effects of intraperitoneal injection of quercetin in streptozocin-induced diabetic rats compared to normal rat. Although quercetin had no effect on plasma glucose level of normal animals, it significantly and dose-dependently decreased the plasma glucose level of streptozocin-induced diabetic rats [26].

Anticancer activities

Quercetin was reported for antiviral activity against reverse transcriptase of HIV and other retroviruses, and reduction in the infectivity and cellular replication of Herpes simplex virus. Over the past decade, substantial progress has been made in researches on the natural products for the treatment of AIDS. Several plants and their products including *H. cordata* have been shown anti-HIV activity [14]. Pratheeshkumar concluded that quercetin could can inhibit cancerous cell growth in cancers such as prostate cancer, according to the down regulation of protein kinases that regulate cell proliferation including protein kinase B (or AKT), Mammalian target of rapamycin (mTOR) and P70S6K expressions [27].

Quality control method for herbal material [2]

WHO publishes “Quality control method for herbal material” guideline that describes various information of analytical tests for evaluation of the quality of plant materials. The following methods facilitate to examine the quality of herbal material by using modern analytical techniques.

Macroscopic and microscopic examination

An examination to assign these characteristics is the first process towards establishing the identity and degree of purity of such materials, and should be carried out before any further tests are undertaken. Macroscopic entity of herbal materials is based on size, shape, 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 water content

This constant parameter is important for plant material specification. An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. The azeotropic distillation gives a direct measurement of the water present in the material being examined. The sample is distilled together with a water immiscible solvent, such as toluene or xylene, the water and the solvent are distilled together and separated in the

receiving tube on cooling. Solvent should be saturated with water before use to avoid water absorption in solvent.

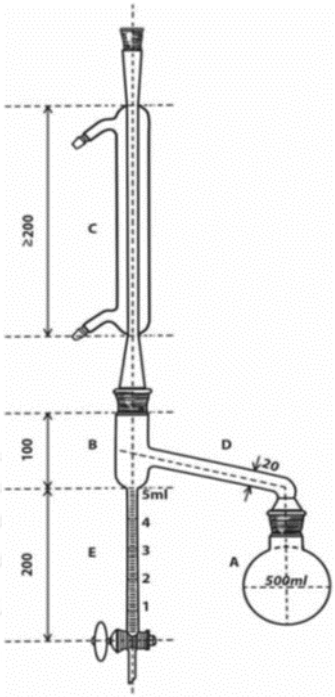

Figure 5 Azeotropic apparatus for determination of water content (dimentions in mm), (A) a glass flask, (B) a cylindrical tube, (C) a refluk condenser, (D) a receiving tube, (E) a graduated receiving tube [2]

Determination of loss on drying

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

Determination of total ash and acid in soluble ash

Ash values are helpful in determination of the purity and quality of plant materials. Total ash is determined to measure the total amount of inorganic components in remaining herbal materials after complete incineration at about 500

°C. Adulteration or contamination with adhering materials to the plant e.g. sand and soil affects total ash increasing. Acid-insoluble ash is the residue obtained after boiling the total ash with 70g/l hydrochloric acid, and incinerating the remaining insoluble matters. This measures the amount of silica present, especially as sand and siliceous earth.

Determination of volatile oil

Volatile oils are characterized by their odor, oil-like appearance and ability to volatilize at room temperature. They are composed of mixture of chemical compounds, for example, monoterpenes, sesquiterpenes and their oxygenated derivatives. Aromatic compounds predominate in certain volatile oils. In order to determine the volume of oil, the plant material is distilled with water and the distillate is collected in a graduated tube. The aqueous portion separates automatically and is returned to the distillation flask. If the volatile oils possess a mass density higher than or near to that of water, or are difficult to separate from the aqueous phase owing to the formation of emulsions, a solvent with a low mass density and a suitable boiling-point such as xylene may be added to the measuring tube. The dissolved volatile oils will then float on top of the aqueous phase.

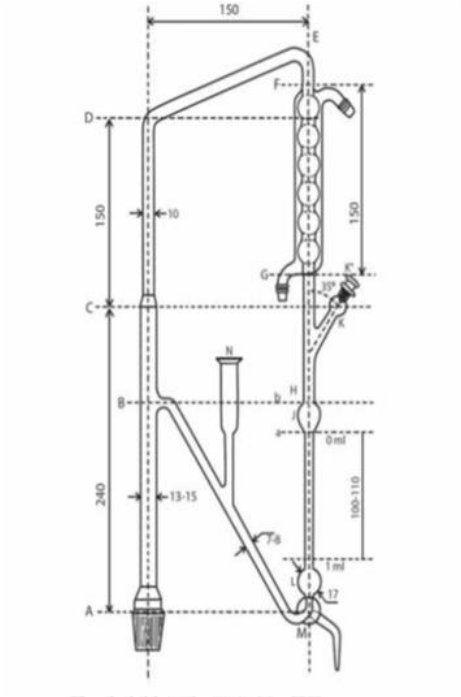

Figure 6 Clevenger apparatus for determination of volatile oil content (dimensions in mm), (AC) a vertical tube, (CDE) a bent tube, (FG) a bulb-condenser, (GH) a tube, (HK) a side-arm tube, (K) a tube and (K') a vented ground-glass stopper, (J) a pear-shaped bulb with a volume of 3 ml, (JL) a tube with a volume of 1 ml, (L) a bulb-like swelling with a volume of about 2 ml, (M) a three-way tap, (BM) a connecting tube, (N) a security tube [2]

Gas Chromatography/ mass spectrometry (GC/MS)

The gas chromatography (GC) is a technique used for the separation and analysis of the mixture of volatile compounds. Compounds with a lower molecular weight will elute out earlier than compounds with higher molecular weights due to differences in boiling points. The mobile phase is inert carrier gas such as helium, nitrogen, hydrogen and argon. The stationary phase is a usually chemical that can

selectively interact with the components in a sample mixture. Mass Spectrometer (MS) is a kind of detector machine which uses electron or chemical to ionize the chemical compound and measures the mass-to charge ratio of ions based on the details of motion of the ions as they transit through electromagnetic fields [21, 28, 29].

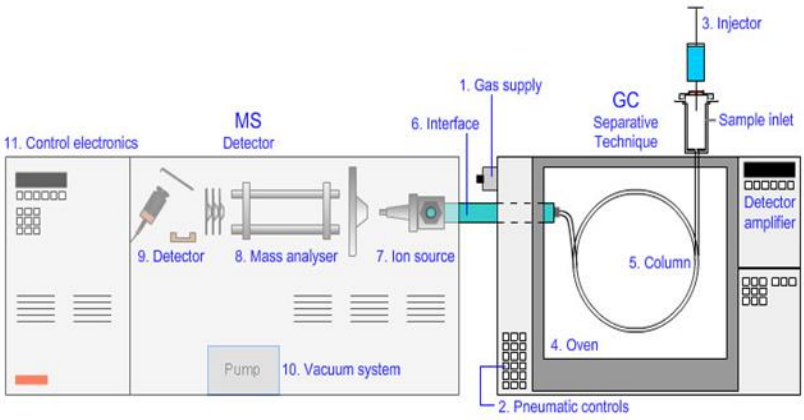

Figure 7 GC/MS accessory: (1) gas supply, (2) pneumatic controls, (3) injector, (4) oven, (5) column, (6) interface, (7) ion source, (8) mass analyzer, (9) detector, (10) vacuum system, (11) control electronics.

Thin layer chromatographic identification (TLC fingerprint) [30, 31]

Chromatography is general technique for separation of mixture compounds. Mixture of substances can be separated base on difference of physico-chemical properties, for example, polarity and proton donor/acceptor capacity. Thin layer

chromatography which is also called planar chromatography is solid- liquid adsorption. The stationary phase is solid usually silica or alumina.

TLC can be used to identify compounds by comparison with known samples, to check the purity of a compound, or to monitor the progress of a reaction, an extraction, or a purification procedure.

Retention factor

The R*f* is calculated by dividing the distance the compound traveled from the original position by the distance the solvent travelled from the original position (the solvent front).

$$hR_f = \frac{\text{Distance of center of spot from starting point}}{\text{Distance of solvent front from starting point}} \times 100$$

The R*f* value is a constant for each component only under identical experimental condition. TLC fingerprint is a methodology for the quality control of herbal materials. It is appropriate for adulteration detection and plant identification and has been accepted by WHO.

Quantitative analysis of quercetin in *H. cordata*

Quantitative analysis can be performed with data from scanning image analysis and scanning densitometry. The amount of components separated on TLC plate were measured based on the intensity of pixel by image analysis or intensity the UV/Vis absorption, fluorescence emission or fluorescence quenching by densitometry

ImageJ analysis

ImageJ is a public domain Java image processing and analytic program provided by NIH. It runs, either as an online or a downloadable application. It can calculate area and pixel value statistics of user-defined selections. It can measure distances and angles. Program created density histograms and line profile plots. It supports standard image processing functions such as contrast manipulation, smoothing, sharpening, edge detection and median filtering. It does geometric transformations such as rotation, scaling and flips. The pixel of sample bands and background on developed TLC plate photographed under short ultra violet light at 254 nm or 366 nm can be transformed to peak chromatogram by ImageJ software. ImageJ can be downloaded from

http://imagej.nih.gov/ij/download.html [32].

TLC Densitometry

Densitometer can measure the amount of a substance that is on the TLC plate based on the intensity of the absorption or fluorescent emission and then converts the signal into densitogram or peak chromatogram. It can scan wavelength ranging 190-800 nm. The analysis is accurate and available for qualitative analysis [33].

Figure 8 Densitometer

Efficacy evaluation: antimicrobial activities testing [10, 17, 34]

Disc diffusion method

Disc diffusion is most commonly used for antimicrobial testing, recommended by the CLSI (the Clinical and Laboratory Standards Institute). This method uses the diffusion of substances. A filter paper disc with specified diameter containing sample substances is placed on suitable agar surface for substance diffusion. Sample with antimicrobial activity will demonstrate zone of inhibition of microbial growth around the disc.

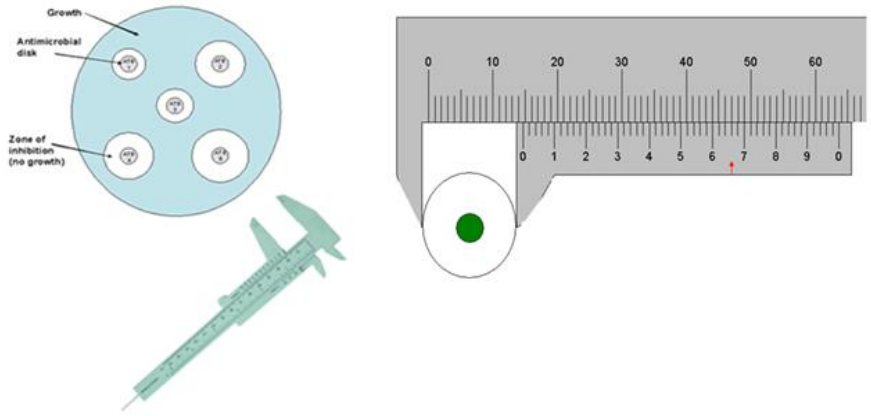

Figure 9 Inhibition zone measurement using a caliper

Broth dilution method and minimum inhibitory concentration (MIC)

Broth dilution testing is used to determine the minimal concentration of substance to inhibit the growth of microorganism. This can be achieved by serial dilution of substance in broth culture and determining microbial growth inhibition. . Turbidity change of broth culture indicates the ability to inhibit the growth of microorganisms. MIC is the lowest concentration where no growth is visually observed.

Minimum bactericidal / minimum fungicidal concentration (MBC/MFC)

MBC/MFC test determines the lowest concentration at which an antimicrobial agent will kill a microorganism. After MIC test has been completed, subculture all aforementioned broth with no growth observed to agar medium. MBC/MFC as the minimum concentration that kill microorganism, resulting in no microbial colony appeared.

CHAPTER III

MATERIALS AND METHODOLOGY

Chemicals

Chloroform	J.T Baker Chemical Co., Phillipsburg, USA
Dimethyl sulfoxide	Merch KGaA, Germany
Ethanol	J.T Baker Chemical Co., Phillipsburg, USA
Ethyl acetate	RCI Labscan Limited, Bangkok, Thailand
Formic acid	RCI Labscan Limited, Bangkok, Thailand
Methanol	RCI Labscan Limited, Bangkok, Thailand
Meuller Hinton agar and broth	Merck, Darmstadt, Germany
Quercetin	Sigma-Aldrich., St. Louis, USA
Sabouraud Dextrose agar and broth	Merck, Darmstadt, Germany
Toluene	RCI Labscan Limited, Bangkok, Thailand

All of chemicals and reagents were analytical grade.

Materials

Filter paper No.4	Whatman™ Paper, UK
Filter paper No.40 ashless	Whatman™ Paper, UK
Microscope slide	Sali Band, China

TLC silica gel 60 GF_{254} MERCK, LTD, USA

- 20 x 10 cm. 0.2 mm thickness

96-well microtiter plates Constar, USA

Instruments and equipments

Ashing Furnance Carbolite, UK

Balance readability 0.01 g Pioneer™ Ohaus Crop. Pine Brook, NJ, USA

Balance readability 0.0001 g Adventure™ Ohaus Crop. Pine Brook, NJ, USA

CAMAG TLC Chamber CAMAG, Switzerland

CAMAG Visualizer CAMAG, Switzerland

Cannon, PowerShot A650 IS camera Cannon Marketing (Thailand) Co., LTD.Bangkok

Clevenger appatatus Becthai Bangkok Equipment and Chemical Co., LTD, Bangkok

Hot air oven WTC Binder, Germany

Gas Chromatography TRACE™ Ultra Gas chromatography, USA

Microscope Carl Zeiss model Axio Lab, Germany

Masss-spectrometry Thermo DSQ™, USA

Rotary vacuum evaporator Buchi, Switzerland

Soxhlet apparatus

TLC Chamber	CAMAG, Switzerland
TLC-densitometry instrument	CAMAG, Switzerland
Ultrasonic bath	Analytical Lab Science Co., LTD, Thailand
UV fluorescence analysis cabinet	Spectronics Corporation, USA

Computer software

ImageJ softwere (Version: 1.46r)	The National Institute of Mental Health, USA
winCATS software	CAMAG, Switzerland

Plant Materials

The whole plant of *Houttuynia cordata* Thunb. were collected from 12 sources throughout Thailand and then were authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi. The voucher specimen was deposited at College of Public Health Sciences, Chulalongkorn University. They were dried in an oven at 45 °c and peverized for further investigation. Fresh leaves were used for anatomical characteristic examination.

Methodology

Pharmacognostic specification [2]

Macroscopic and microscopic examinations

Visual characteristics of sample were examined. Whole plants was illustrated botanically by line drawing. Microscopic evaluation of *H. cordata* leaf was carried out under the appropriate scale using a photomicroscope connected with digital camera. Transverse sectional midrib of fresh leaf and powders of dried whole plants were examined under microscope by wet mounted in water. The anatomical and histological characters were drawn related by the original size.

Determination of loss on drying

Powdered *H. cordata* was accurately weighed (3.0 g) in pre-weighed crucible and was dried with 105 °C in an oven. The crucible was allowed to cool in desiccator was weighed and was calculated for the loss of weight in percentage. Each sample was done in triplicate.

Determination of total ash

Accurated 3 g of *H. cordata* powder were weighed in pre-weighed crucible and were incinerated at about 500°C until complete ashing and were cooled in a desiccator. The total ash was calculated in percentage. Each sample was done in triplicate.

Determination of acid insoluble ash

At this stage, the crucible of the previous step, added 25.00 mL of hydrochloric acid (70 g/l), covered with watch glass and boiled for 5 minutes. After that ash was rinsed with 5.0 ml of hot water and was added the liquid to the crucible. Collected the insoluble matters on an ashless filter-paper No.40 and washed with hot water until the filtrate was neutral, was dried on hot-plate and was incinerated at 500°C to constant weight, was cooled in desiccator and was calculated the amount of acid insoluble ash in percentage of air-dried material.

Determination of water soluble extractive value

Accurated 3 g of powdered *H. codata*, were added with 70 ml of water, were macerated for 6 hours under shaking and let stand for 18 hours. Carefully filtered to avoid loss of water, washed the marc and adjusted the volume to 100 ml. Transferred 20 ml of the filtrate to pre weighed small beaker, evaporated on water-bath, dried for 6 hours at 105 °c and cooled in desiccator, weighted and calculated the content in percentage. Each sample was done in triplicate.

Determination of ethanol soluble extractive value

Accurated 3 g of powdered *H. codata*, were added with 70 ml of ethanol, were macerated for 6 hours under shaking and were left standing for 18 hours. Filtered carefully to avoid loss of ethanol, washed the marc and adjusted the volume to 100 ml. Transferred 20 ml of the filtrate to pre weighed small beaker, evaporated on water-bath, dried for 6 hours at 105 ° c and cooled in desiccator,

weighed and calculated the content in percentage. Each sample was done in triplicate.

Determination of water content

Accurated 50 g of powdered *H. cordata* were poured into round bottom flask, 200 ml of water-saturated toluene were added and boiled by azeotopic distillation. The water distillated was measured and recorded in percentage. Each sample was done in triplicate.

Determination of volatile oil

Accurated 100 g of powdered *H. cordata* were poured into round bottom flask, were added 600 ml of water and were boiled in Clevenger type apparatus (Figure 5.), the volatile oil distillated was measured and calculated in percentage and was storage at 4 °C. Each sample was done in triplicate.

Chemical constituent analysis of volatile oil by GC-MS

H. cordata oil extracted by hydro-distillation using Clevenger apparatus was diluted to 1:100 v/v with methanol and was investigated by capillary gas chromatography-mass spectrometry (GC-MS) connected to Finnigan trace GC ultra with Finnigan DSQ Quadrupole detector and BPX5 was fused silica column (30 m x 0.25 mm, 0.25 µm, film thickness). The injector temperature was 180 ℃. The oil solution in methanol was injected with splitter ratio of 100:1. The oven temperature was 60 ℃ for 1 min., then ramped to 240 ℃ with the rate of 3 ℃/min. Helium was

used as carrier gas (flow rate at 1 ml/min). MS was performed by EI positive mode at 70 eV ionization voltages. The constituents of the oil were identified by matching their mass spectrum, retention indices with Adams Essential oil MS library and the amount of each component was computed as GC peak area ratio in percentage.

Thin-layer chromatographic identification (TLC fingerprint)

Transferred another 20 ml of the ethanolic filtrated mentioned above and evaporated to dryness, re-dissolved the residue in 1 ml of ethanol. Applied 3 µl on TLC siliga gel 60 GF_{254}. Developed in the chamber containing the suitable solvent system, for example mixture of toluene, ethyl acetate, methanol, formic acid (5: 5: 3: 2). After development, the plate was dried and visualized under UV light at 254 nm, 365 nm and were dipped the TLC plate with natural products (NP) polyethylene glycol (PEG) reagents.

Quantitative analysis of quercetin in *H. cordata*

Preparation of standard solutions

The 1.0 mg of quercetin was dissolved in 1 ml of 95% ethanol and diluted to obtain the series of standard solutions with concentration of 0.2, 0.4, 0.8, 1.6 and 3.2 µg/spot.

Preparation of ethanol extracts of *H. cordata*

Accurated 3.0 g of powdered *H. cordata* were extracted with 200 ml of ethanol by soxhlet apparatus. The exhaustively extracted was filtered and evaporated by rotary evaporator. Thirty milligrams of the extract were dissolved in 1 ml of 95% ethanol for TLC densitometry and TLC image analysis.

TLC densitometry

Three microliters of the ethanolic extract solution and quercetin standard solutions were applied onto the silica gel 60 GF_{254} TLC plate. The plate was developed to a distance of 8.0 cm in chamber that contained the mobile phase. After development, the plate was scanned by CAMAG TLC scanner under wavelength of maximum absorbance for quantitative analysis. The intensity of band was performed to the chromatographic peak by WinCATS software. The calibration curve of quercetin was obtained by plotting peak areas *versus* concentration of quercetin in µg/spot.

TLC image analysis by ImgeJ software

An image under UV 254 or 365 nm was taken by a digital camera. The image saved as .Tiff format, the ImageJ software was used to analyze and quantitate the quercetin spot on TLC plate. The quercetin content was determined by plotting peak area *versus* concentration of quercetin in mg/spot.

Method validation [35]

Calibration range

Regression line of peak area *versus* quercetin concentration and correlation coefficient were determined by Excel 2010 program.

Accuracy

The accuracy of quantitative analysis was determined by spike method. Three differences concentration (0.2, 0.6, 1 µg/spot) of quercetin spiked into the extract. The accuracy was calculated as percent recovery following formula:

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

Where A = the amount of quercetin test in spike sample extract

B = the amount of quercetin test un-spike sample extract

C = the amount of quercetin standard actually add to sample

Precision

The precision of quantitative analysis was determined by repeatability (intra-day) and intermediate precision (inter-day) studies. Intra-day and inter-day precision were performed by analyzing sample solution of three concentrations (each one in triplicate) on the same day and three different days respectively. The relative standard deviation (RSD) was calculated in percentage by the following formula;

%RSD = SD x 100/ Mean

Limit of detection (LOD)

The limit of detection (LOD) was the lowest concentration that can be detected but not surely known the quantitative amount of quercetin. LOD was determined from the calibration curve using following formula:

$$LOD = 3.3\ \sigma/S$$

Where, σ = the standard deviation of regression-line

S = the slope of calibration curve

Limit of quantitative (LOQ)

The limit of quantitation (LOQ) was the lowest concentration that could be detected. LOQ was determined from the calibration curve using following formula:

$$LOQ = 10\ \sigma/S$$

Where, σ = the standard deviation of regression-line

S = the slope of calibration

Robusness

Mobile phase compositions was selected for robustness parameter in this study. A little variation in a mixture ratio of mobile phase including toluene: chloroform: acetone: formic acid (4.5: 3.1: 3.5: 1.1), (4.4: 3: 3.4: 1), (4.5: 3: 3.5: 1). The

robustness were represented by %RSD of peak area and was calculated for RSD in percentage.

Specificity

Specificity of TLC quantitative analysis was operated by identification method. The identification method was performed by comparison of absorption spectra of quercetin standard and all samples using CAMAG TLC scanner.

Efficacy evaluation: antimicrobial activities testing [10, 17, 34]

Microorganisms

The microorganisims were used in agar diffusion method were included two spore forming gram-positive bacteria; *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778. Three non-spore forming gram-positive bacteria; *Staphylococcus aureus* ATCC 6538P, *Kocuria rhizophila* ATCC 9341, *Straphylococcus epidermidis* (clinical isolate). Non-spore froming gram-negative bacteria; *Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella typhi* (clinical isolate), *Salmonella typhimurium* (clinical isolate) and *Shigella ssp.* (clinical isolate) and two fungi strains; *Candida albicans* ATCC 10230 and *Saccharomyces cerevisiae* ATCC 9763. They are obtained from the Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Department of Microbiology, Faculty of Sciences, Chulalongkorn University and Department of

Microbiology, Faculty of Sciences and Technology, Suan Sunandha Rajabhat University.

Disc diffusion method

The agar diffusion method was used a two-layer agar technique. The bacterial and fungal strains was grown on Mueller Hinton agar (MHA) and Sabouraud Dextose agar (SDA) respectively. After incubated at 37^{o}C for 24 hours, the cultures were suspended in sterile 0.85% NaCl to obtain the turbidity equivalent to 0.5 McFarland standards (1 x 10^{8} CFU/ml). One hundred microliters of the suspension were mixed with 3 ml of sterile seeds agar and pour to sterile base agar. The plates were allow to dry at room temperature. Twenty microliters of *H. cordata* volatile oil (200 mg/ml in dimethyl sulfoxide (DMSO) were added to each paper disc. Ampicillin and Amikacin (1 mg/ml, 20 µl) were used as positive control and DMSO (20 µl) was used as a negative control. The plates were incubated at 37^{o}C for 24 hours and the diameters of inhibition zones were measured in millimeters. Each sample was tested in triplicate.

Determination of the minimum inhibitory concentration (MIC)

The method was performed in 96 well microtiter plates, *H. cordata* volatile oil was serially diluted in DMSO. Fifty µl of 0.5 McFaland microbial suspension in broth were added to each well were containing fifty µl of volatile oil solution, positive control and negative control respectively. Incubated at 37 °C for 18-24 hours

(for bacteria) and 24-48 hours (for fungi). The least concentration of the volatile oil with no turbidity observed was recorded as MIC.

Determination of minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

All the MIC wells, which do not show any turbidity, was streak on agar plates. The lowest concentrations of sample that were not permit any visible growth on the plates after incubated at 37 °C 18-24 hours (for bacteria) or 24-48 hours (for fungi), were recorded as MBC and MFC.

Data analysis

The parameters due to standardization were calculated as grand mean ± pooled standard deviation (SD).

CHAPTER IV

RESULTS

Pharmacognostic specifications

Houttuynia cordata Thunb. (Figure 10) dried whole plants (Figure 11) have been used as herbal crude drug.

The transverse section of *H. cordata* leaf showed the anatomical characteristics of upper epidermis, palisade mesophyll, multiicellular trichome, spongy mesophyll, collenchyma, vascular tissue, parenchyma and lower epidermis as illustrared in Figure 12.

The histological characteristics of powders of *H. cordata* crude drug including epidermis, multicellular trichomes, fibers, paracytic stoma and xylem vessel, were shown in Figure 13.

Thin layer chromatographic fingerprint of ethanolic extract of *H. cordata* was shown in Figure 14.

The physico-chemical constant numbers due to quality of *H. cordata* were demonstrated in Table 1.

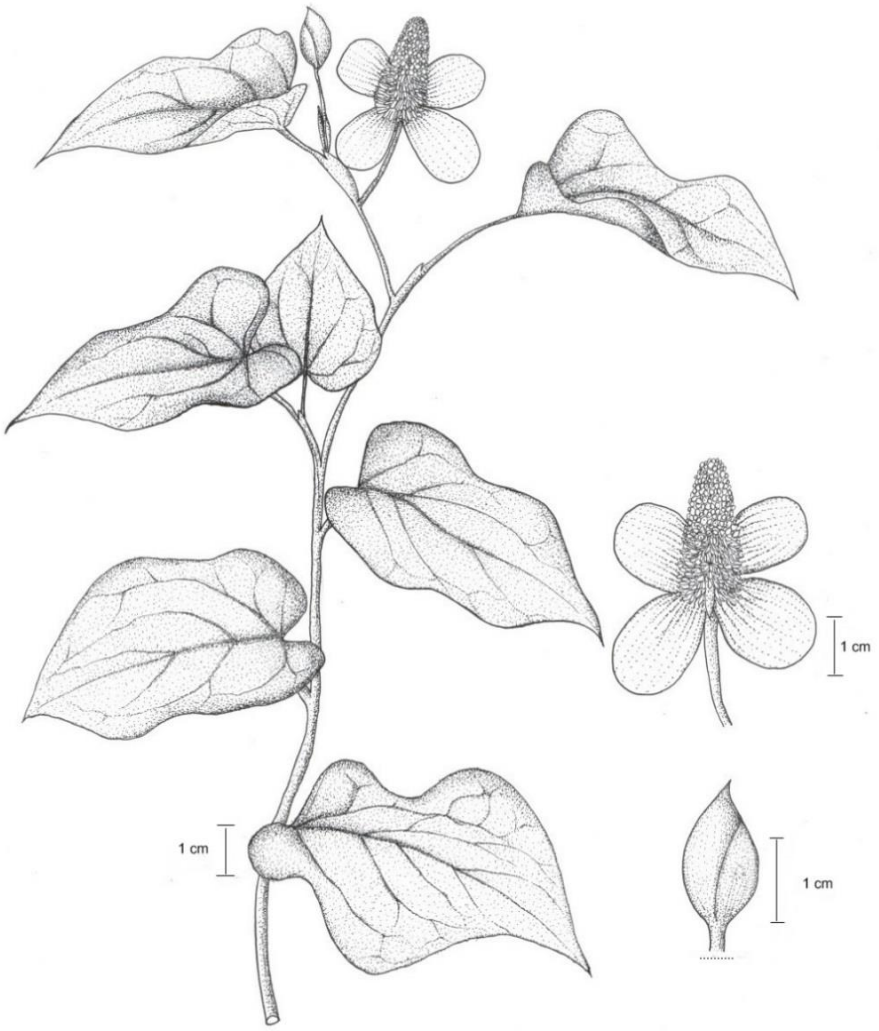

Figure 10. *Houttuynia cordata* Thunb.

Figure 11. Dried whole plants of *Houttuynia codata* Thunb.

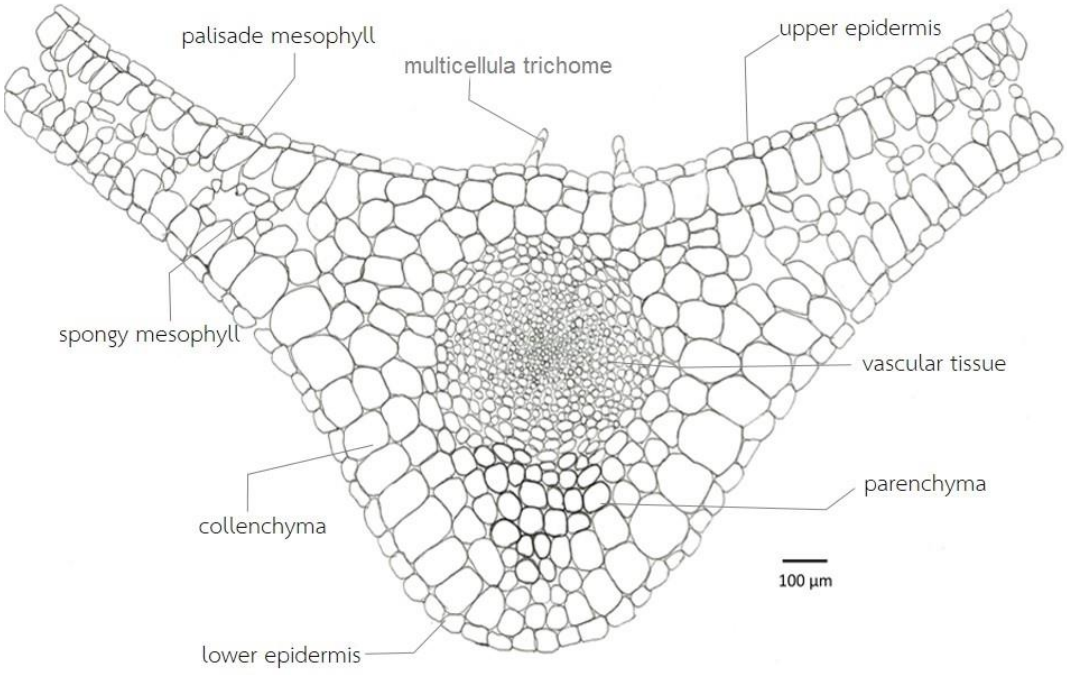

Figure 12. Transverse section of the leaf of *Houttuynia cordata* Thunb.

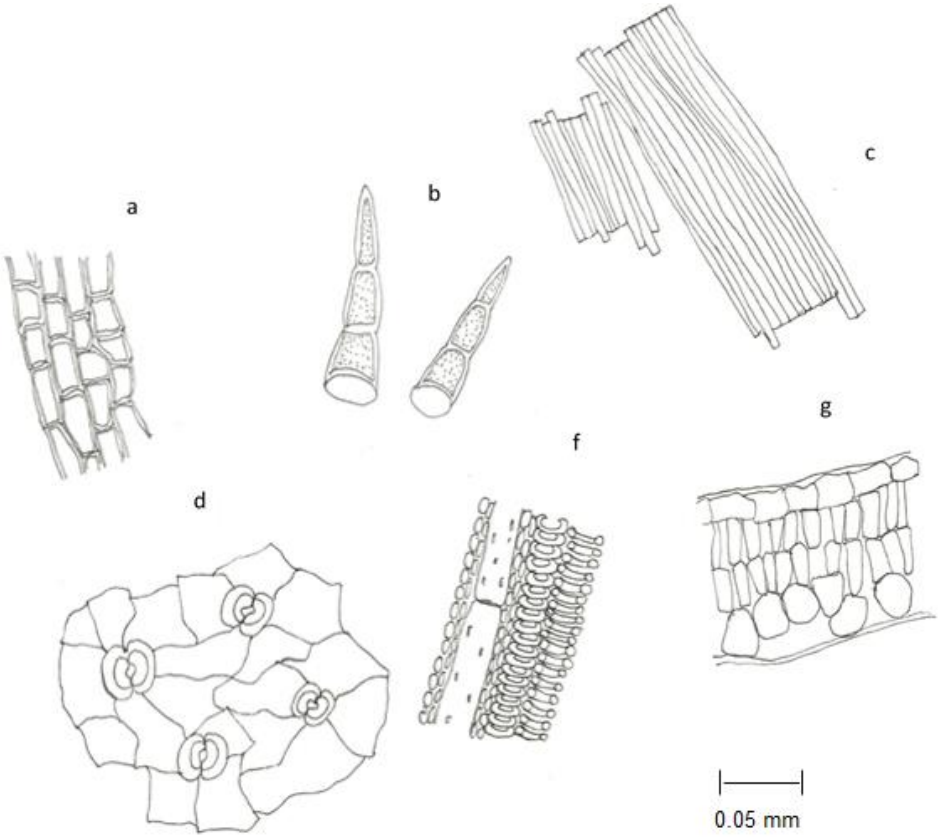

Figure 13 Powder of dried aerial part of *Houttuynia cordata* Thunb. a. Epidermis b. Multicellular trichome c. Fiber d. Paracytic stomata f. Xylem vessel g. The upper epidermis with palisade cells and part of spongy mesophyll

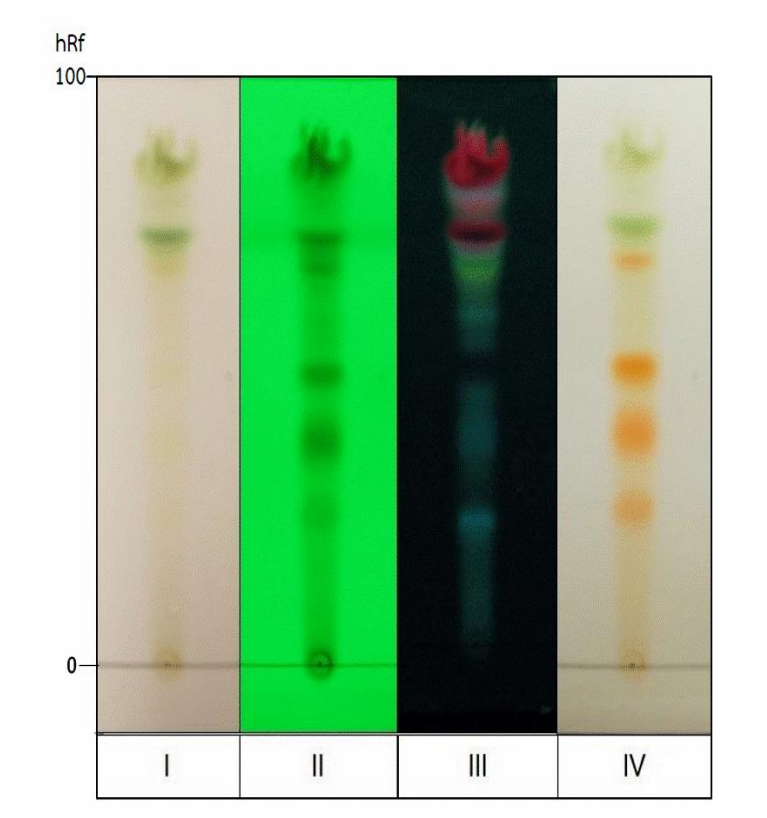

Figure 14. TLC fingerprint of ethanol extract of *Houttuynia cordata* Thunb. whole plants

Solvent system: Toluene: Ethyl acetate: Methanol: Formic acid, 5: 5: 3: 2

Detection I = detection under daylight

II = detection under UV 254 nm

III = detection under UV 365 nm

IV = detection with natural product / polyethylene glycol staining reagent

Table 1. The constant numbers due to quality parameters of *H. cordata*

Specification (% by weight)	**Mean ± SD[a]**	**Range***
Loss on drying	7.22 ± 0.34	6.21 - 8.23
Total ash	14.31 ± 0.15	13.86 - 14.74
Acid-insoluble ash	4.11 ± 0.36	3.04 - 5.18
Ethanol-soluble extractives	4.69 ± 0.48	3.40 – 6.06
Water-soluble extractives	14.17 ± 1.03	11.07 – 17.26
Water content	11.70 ± 1.18	8.17 – 15.23
Volatile oil content*	0.08 ± 0.01	0.05 – 0.11

[a] The parameters were shown as grand mean ± pooled SD. * mean ± 3SD, Sample were from 12 different sources throughout Thailand. Each sample was done in triplicate.

Ethanolic extracts of *H. cordata* dried whole plants

The percent yield of ethanol extracts of *H. cordata* crude drug were shown in table 2. The average yield was 16.36 ± 6.08% by dried weight.

Table 2. The percent yield of ethanol extract of dried *H. cordata*

Source	Crude drug (g)	weight of extractive value (g)	% yield
1	5.0069	1.4578	29.12
2	5.0147	1.0297	20.53
3	5.0099	0.8092	16.15
4	5.0107	0.8647	17.26
5	5.0038	0.9412	18.81
6	5.0033	0.5224	10.44
7	5.0002	0.4988	9.98
8	5.0042	0.5305	10.60
9	5.0044	0.9679	19.34
10	5.0081	1.1148	22.26
11	5.0175	0.6489	12.93
12	5.0055	0.4473	8.94
Average	**5.0074**	**0.8194**	**16.36**

The quantitation of quercetin in *H. cordata* crude drugs

Method validation of TLC densitometry

Calibration curve

The calibration curve of quercetin by TLC densitometric method was shown in Figure 15. The calibration range of quercetin was 0.2 - 3.2 µg/spot.

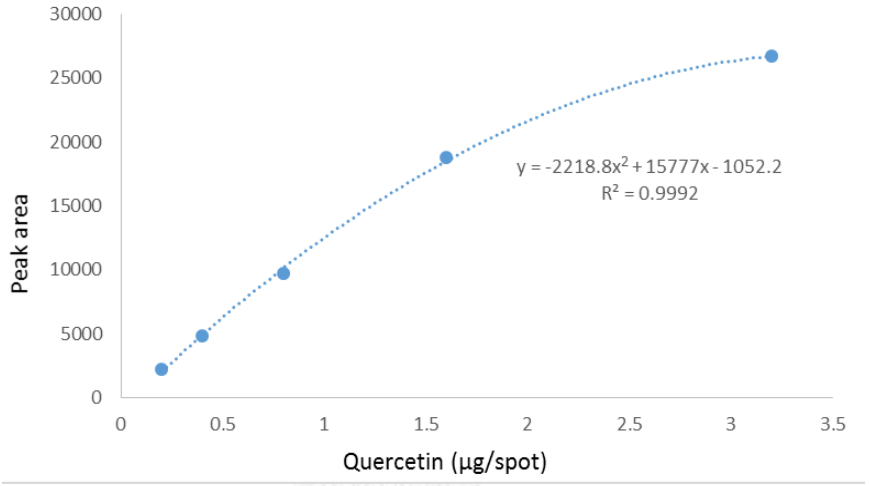

Figure 15. The calibration curve of quercetin by TLC densitometric method

Accuracy

The accuracy of quercetin quantitation by TLC densitometric method was assessed in percentage of recovery. The recovery of quercetin spiked into the extract at three different concentrations were between 94.22 - 95.75 % (Table 3).

Table 3 Recovery of quercetin by TLC densitometric method (n=3)

Quercetin added (µg/ml)	Quercetin found (µg/ml)	%Recovery
0.0	1.177 ± 0.044	
0.2	1.299 ± 0.048	94.36
0.6	1.702 ± 0.040	95.75
1.0	2.052 ± 0.102	94.22

Precision

The precision of quercetin quantitation by TLC densitometric method was performed in triplicate at the concentrations of 0.2, 0.6 and 1 µg/spot. The result was shown as the percentage of relative standard deviation which represented the error of the method. The repeatability was evaluated on the same day. The intermediate precision was determined on three different days. The repeatability and intermediate precision were shown between 2.36 – 4.98 %RSD and 2.63 – 4.88 %RSD respectively.

Table 4 Repeatability and intermediate precision of quercetin quantitative analysis in *H. cordata* crude drug by TLC densitometry

Quercetin Content (µg/spot)	Repeatability (%RSD)(n=3)	Intermediate precision (%RSD)(n=9)
0.2	3.68	4.63
0.6	2.36	2.63
1.0	4.98	4.88

Limit of detection (LOD) and Limit of quantitation (LOQ)

For this study, LOD and LOQ in TLC densitometry were computed based on the slope of calibration curve and the residual standard deviation of a regression line. The LOD and LOQ values for quercetin analysis by TLC densitometry were 0.04 µg/spot and 0.13 µg/spot respectively.

Robustness

Robustness of TLC densitometric method was performed by introducing small changes in the mobile phase mixture (toluene: chloroform: acetone: formic acid). The robustness values was 4.94 %RSD. The peak area of quercetin were between 14104.85- 15420.17 (Table 5).

Table 5. Robustness of quercetin quantitation in *H. cordata* crude drug by TLC densitometric method (n=3)

mobile phase ratio (v/v)	Peak area of quercein (AU)
4.5: 3.1: 3.5: 1.1	14104.85
4.5: 3: 3.5: 1	15420.17
4.4: 3: 3.4: 1	15346.27
Mean ± SD	14957.1 ± 738.99
%RSD	4.94

Specificity

The absorption spectra of quercetin in sample and standard were identical with the maximum absorption at 388 nm which represented the method specificity.

Quercetin quantitation by TLC densitometry

The quercetin contents in *H. cordata* extracts from 12 sources which determined in triplicate by TLC densitometry were shown in Table 6. The quercetin contents in *H. cordata* crude drugs were evaluated (Table 6).

Table 6 The content of quercetin in *H. cordata* crude drug by TLC densitometry (n=3)

Source	Quercetin in ethanol extract (g/g)	yield of ethanol extract (g/100g of dried crude drug)	Quercetin in crude drug (g/100g)
	Mean		Mean
1	Trace*	20.53	Trace*
2	Trace*	19.34	Trace*
3	Trace*	29.12	Trace*
4	Trace*	16.15	Trace*
5	Trace*	17.26	Trace*
6	0.0049	22.26	0.1092
7	Trace*	18.81	Trace*
8	Trace*	10.44	Trace*
9	Trace*	12.93	Trace*
10	Trace*	8.94	Trace*
11	Trace*	9.98	Trace*
12	Trace*	10.60	Trace*
Average	**$0.0049 \pm 2.2 \times 10^{-5}$**	**16.36**	**0.1092**

***** Less than the LOQ

Method validation of TLC image analysis

Calibration curve

The calibration curve of quercetin by TLC image analysis was shown in Figure 16. The calibration range of quercetin was 0.2 - 3.2 µg/spot.

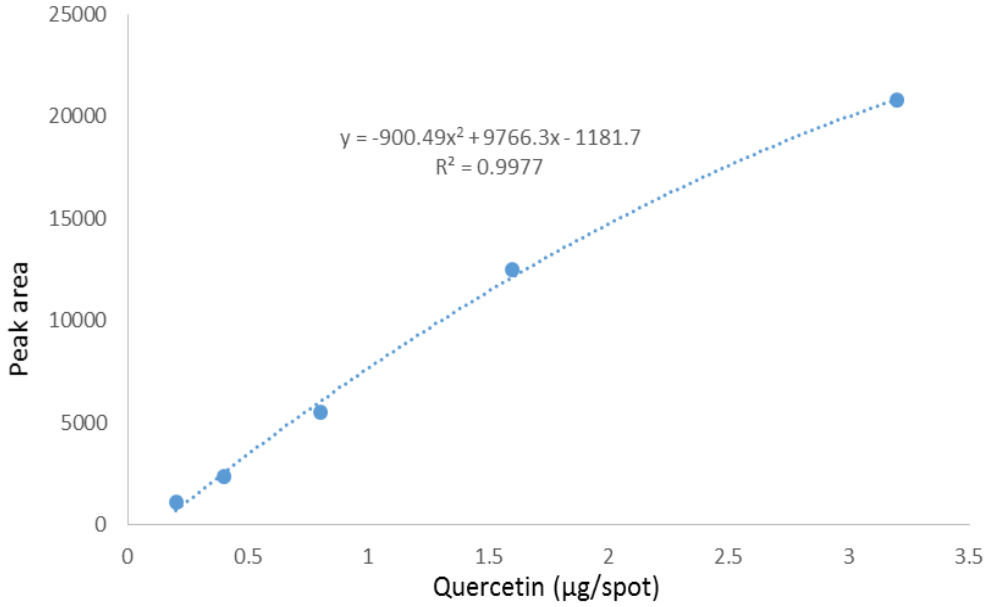

Figure 16 The calibration curve of TLC image analysis

Accuracy

The accuracy of quercetin quantitation by TLC image analysis was performed using recovery studies. The recovery of quercetin spiked into the extract at three different concentrations were between 85.00 – 100.32 % (Table 7).

Table 7 Recovery of quercetin (n=3) by TLC image analysis

Quercetin added (µg/ml)	Quercetin found (µg/ml)	%Recovery
0.0	0.860 ± 0.098	
0.2	1.059 ± 0.033	100.32
0.6	1.440 ± 0.121	98.60
1.0	1.581 ± 0.099	85.00

Precision

Reapeatability and intermediate precision by TLC image analysis method were performed in triplicate with sample solutions that contained three different concentrations of quercetin. The repeatability and intermediate precision were in range 3.15 – 8.43 %RSD and 6.79 – 12.49 %RSD respectively.

Table 8 Repeatability and intermediate precision of quercetin quantitation in *H. cordata* crude drug by TLC image analysis (n=3)

Quercetin Content (µg/spot)	Repeatability (%RSD)(n=3)	Intermediate precision (%RSD)(n=9)
0.2	3.15	6.79
0.6	8.43	12.49
1.0	6.29	6.88

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ by TLC image analysis were computed based on the slope of calibration curve and the residual standard deviation of a regression line .The LOD

and LOQ values for TLC image analysis were 0.12 µg/spot and 0.38 µg/spot respectively.

Robustness

Robustness of TLC image analysis was performed by introducing small changes in the mobile phase mixture (toluene: chloroform: acetone: formic acid). The robustness values was 11.13 %RSD. The peak area of quercetin were between 4476.71– 5437.58 (Table 9).

Table 9. Robustness of quercetin in *H. cordata* crude drug by TLC image analysis

mobile phase ratio (v/v)	Peak area of quercein (Pixel)
4.5: 3.1: 3.5: 1.1	4476.71
4.5: 3: 3.5 :1	5437.58
4.4:3: 3.4: 1	4543.49
Mean ± SD	4819.26 ± 536.52
%RSD	11.13

Quercetin quantitation by TLC image analysis using ImgeJ software

The quercetin contents in *H. cordata* extracts from 12 sources which determined in triplicate by TLC image analysis were shown in Table 10. Quercetin contents in *H. cordata* crude drugs were evaluated (Table 10)

Table 10 The content of quercetin in *H. cordata* crude drug by TLC image analysis (n= 3)

Source	Quercetin in ethanol extract (g/g)	yield of ethanol extract (g/100g of dried crude drug)	Quercetin in crude drug (g/100g)
	Mean		Mean
1	Trace*	20.53	Trace*
2	Trace*	19.34	Trace*
3	Trace*	29.12	Trace*
4	Trace*	16.15	Trace*
5	Trace*	17.25	Trace*
6	0.0031	22.26	0.0696
7	Trace*	18.81	Trace*
8	Trace*	10.44	Trace*
9	Trace*	12.93	Trace*
10	Trace*	8.94	Trace*
11	Trace*	9.98	Trace*
12	Trace*	10.60	Trace*
Average	**$0.0031 \pm 2.2 \times 10^{-5}$**	**16.36**	**0.0696**

* Less than the LOQ

Quercetin contents in *H. cordata* crude drugs by TLC densitometry and TLC image analysis

The ethanolic extracts of 12 *H. cordata* crude drugs were investigated for their quercetin contents. It was found that quercetin contents in *H. cordata* extracts were less than the LOQ by both TLC densitometry and TLC image analysis. The quercetin content in one crude drug from source 6 was found to be 0.0049 g/g extract (0.11 g/100 g crude drug) by TLC densitometry and 0.0031 g/g extract (0.07 g/100 g) by TLC image analysis (Table 6, 10).

Chemical constituent analysis of *H. cordata* volatile oil by GC/MS

The chemical components of *H. cordata* oil analyzed by GC/MS consists of at least 30 compounds as shown in Table 11. The main component was decanoic acid 40.40±27.21 %.

Table 11 The chemical constituents of *H .cordata* volatile oil

Compound name	RT	KI[a]	Peak area%[b]
Thujene	6.50	930	1.20 ± 0.83
α-pinene	6.71	939	2.28 ± 1.02
Camphene	7.12	954	1.57 ± 1.95
β-pinene	8.01	979	2.21 ± 1.41
Myrcene	8.40	990	19.21 ± 15.09
Cymene	9.60	1026	1.00 ± 0.40
Limonene	9.77	1029	0.97 ± 0.79
Eucalyptol	9.87	-	0.62 ± 0.00
Ocimene	10.08	1050	2.19 ± 1.08
Terpinene	10.93	1059	1.54 ± 0.84
Borneol	15.28	1169	1.05 ± 0.40
Arginine	15.55	-	1.31 ± 0.18
4-Terpeneol	15.78	1177	3.46 ± 2.11
Estragole	16.66	-	14.56 ± 18.16
Decanol	16.91	1269	0.91 ± 0.31
Carvyl acetate	19.81	-	1.15 ± 0.57
Bonyl acetate	20.39	1288	4.39 ± 2.55
2-Undecanone	20.70	1294	26.43 ± 14.54
Unidentify A	21.96	-	0.76 ± 0.23

Table 11 The chemical constituents of *H .cordata* volatile oil (Cont.)

Compound name	RT	KI[a]	Peak area%[b]
Decanoic acid	24.05	1361	40.40 ± 27.21
Linalyl butyrate	24.45	-	3.34 ± 4.01
Cystine	24.97	-	0.92 ± 0.52
Caryophyllene	25.94	1410	1.93 ± 0.79
Bergamotene	26.75	1412	1.20 ± 0.25
2-Tridecanone	28.94	1495	2.53 ± 1.89
Selinene	29.68	1498	1.33 ± 0.93
Cadinene	29.72	1523	1.01 ± 0.53
Dodecanoic acid	31.47	1566	1.19 ± 0.57
Benzenethanamine, 2-5-difloro-a', 3-4-trihydroxy-Nmethyl	32.13	-	2.05 ± 1.74
Caryophyllene oxide	32.35	1583	2.61 ± 1.93
Unidentify B	34.53	-	2.09 ± 1.05

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

[b] The parameters were shown as mean ± SD. Sample were from 12 different sources.

The GC chromatogram showed that *H. cordata* volatile oil could be divided into two clusters. Cluster 1 consisted of sample from Pathumtani, Nakhonpathom1, Nakhonpathom2, Lampang1, Bangkok and Nontaburi. Cluster2 consisted of sample from Khonkaen, Chiangmai, Chaingrai1, Lampang2, Loei and Chiangrai2.

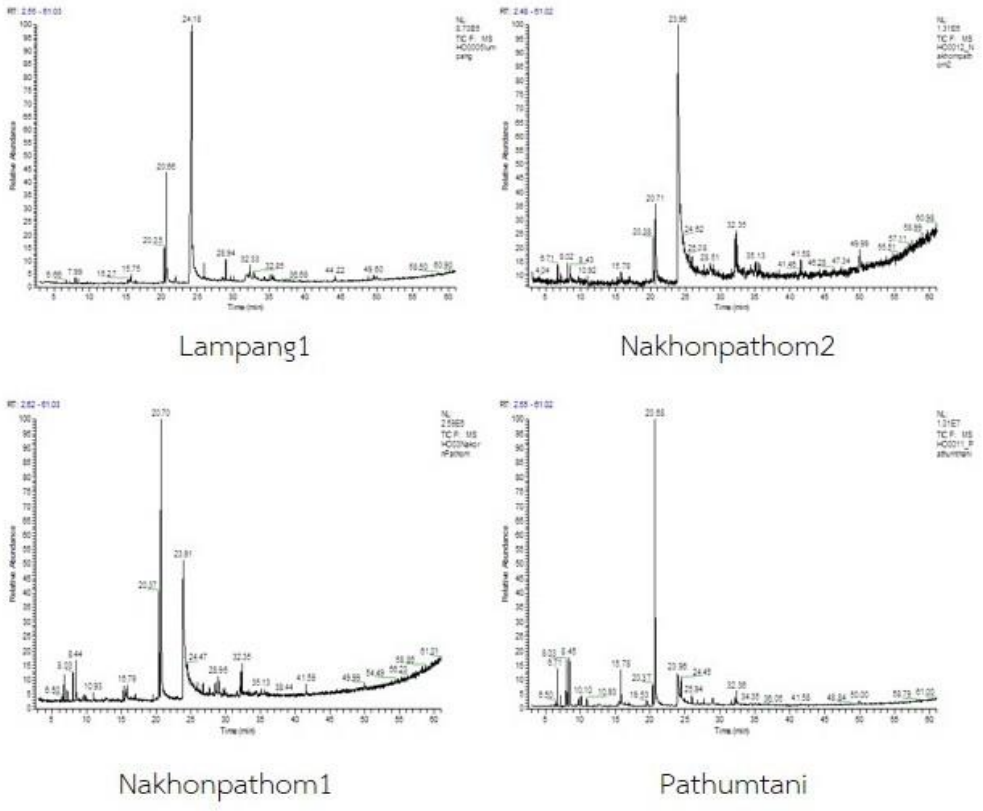

Figure 17 GC chromatogram of *H. cordata* volatile oil cluster 1

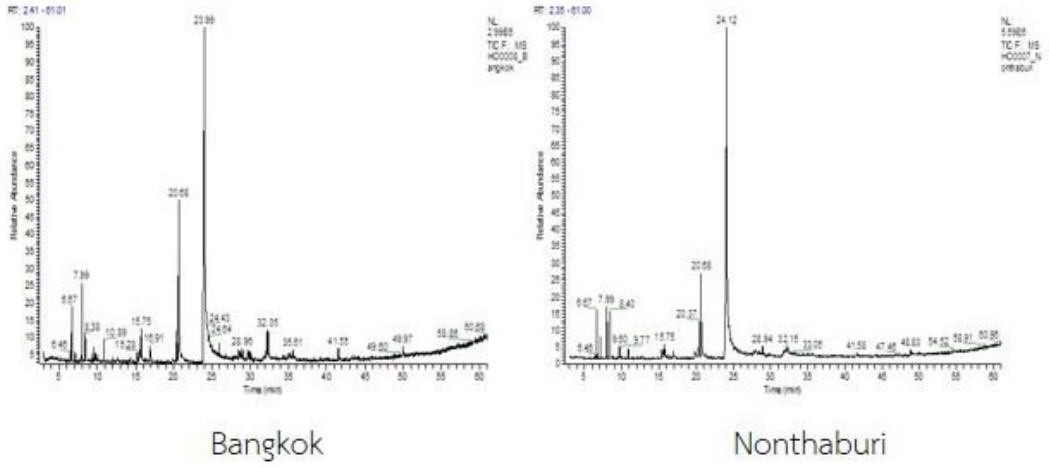

Figure 17 GC chromatogram of *H. cordata* volatile oil cluster 1 (Cont.)

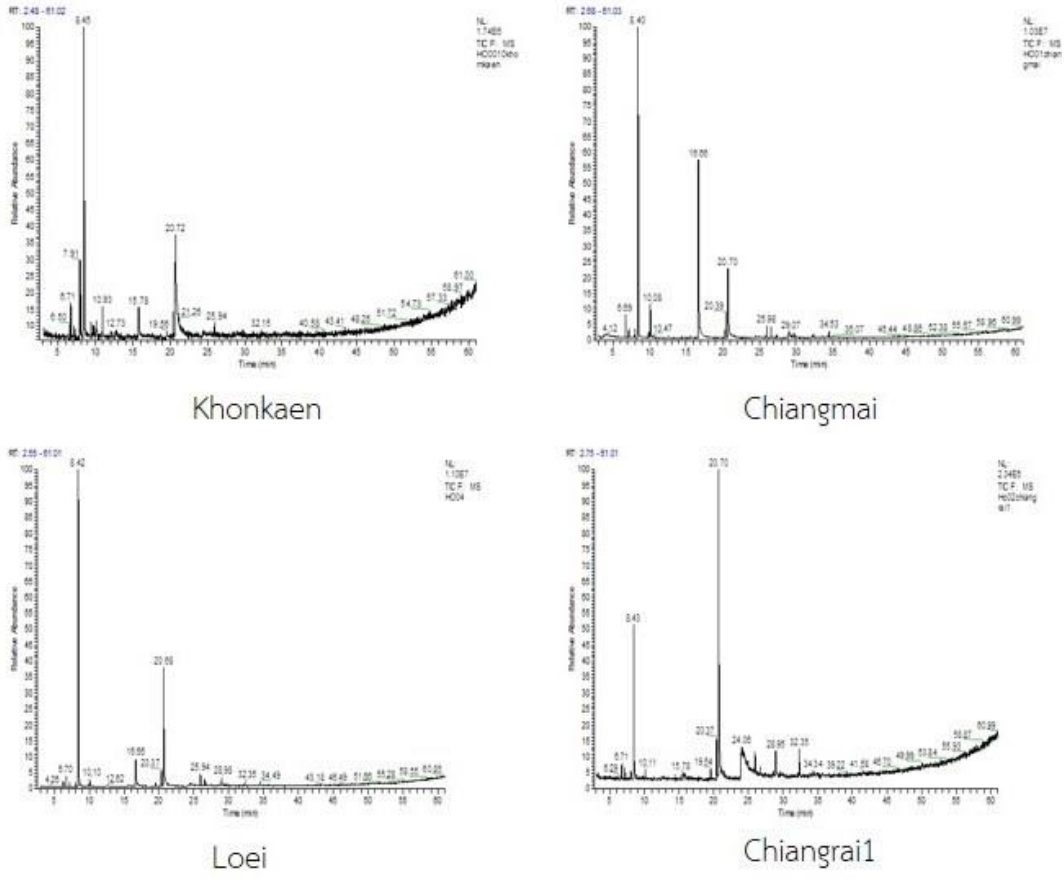

Figure 18 GC chromatogram of *H. cordata* volatile oil cluster 2

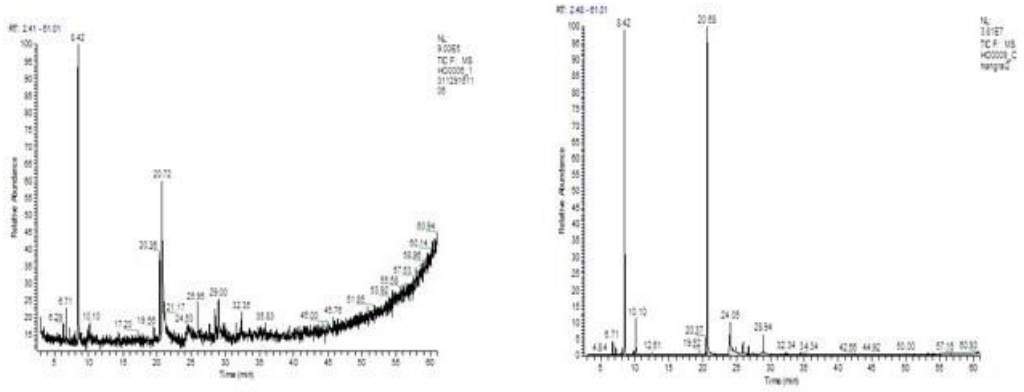

Figure 18 GC chromatogram of *H. cordata* volatile oil cluster 2 (Cont.)

Antimicrobial activities of *H. cordata* volatile oil

The volatile oil of *H. cordata* whole plants and its dilutions in 50 %v/v DMSO were determined for antimicrobial activities by agar diffusion method. *H. cordata* oil showed inhibition zone in Table 12

Table 12 Inhibition zone by agar diffusion method

Microorganism	Inhibition zone (mm)*		
	***H. cordata* oil**	**Ampicillin**	**Amikacin**
Basillus cereus	9.67 ± 1.15	17.33 ± 1.15	16.33 ± 1.15
Bacillus subtilis	13.67 ± 0.58	18.00 ± 0.00	16.00 ± 1.00
Staphylococcus aureus	11.33 ± 0.58	37.33 ± 1.55	14.60 ± 1.00
Staphylococcus epidermidis	11.67 ± 0.58	27.00 ± 0.00	23.33 ± 1.53
Kocuria rhizophila	16.67 ± 0.58	42.00 ± 0.00	19.67 ± 2.52
Pseudomanas aeruginosa	7.00 ± 0.00	NA	12.00 ± 0.87
Enterobacter aerogrnes	7.67 ± 0.58	7.00 ± 0.00	9.83 ± 0.76
Salmonella typhi	7.00 ± 0.00	25.00 ± 0.00	11.00 ± 1.00

*mean ± SD, NA = no activity, Ø 6 mm of disc. The tests were done in triplicate.

Table 12 Inhibition zone by agar diffusion method (Cont.)

Microorganism	Inhibition zone (mm)*		
	H. cordata oil	Ampicillin	Amikacin
Salmonella typhimurium	9.33 ± 0.58	29.33 ± 0.58	12.33 ± 1.15
Shigella Spp.	9.67 ± 0.58	24.33 ± 0.58	9.83 ± 0.58
Escherichia coli	8.33 ± 1.15	20.67 ± 1.15	11.67 ± 0.58
Candida albicans	13.67 ± 0.58	NA	NA
Saccharomyces cerevisiae	16.67 ± 0.58	NA	NA

*mean ± SD, NA = no activity, Ø 6 mm of disc. The tests were done in triplicate.

MIC and MBC or MFC tests represented bactericidal or fungicidal potentials of *H. cordata* oil against microbial organisms were shown in Table 13.

Table 13 MIC and MBC or MFC by Broth microdilution method

Microoraganism	*H. cordata* oil		Ampicillin		Amikacin	
	MIC (µl/ml)	MBC/MFC (µl/ml)	MIC (µg/ml)	MBC/MFC (µg/ml)	MIC (µg/ml)	MBC/MFC (µg/ml)
Basillus cereus	5.000	10.000	0.125	1.000	0.125	1.000
Bacillus subtilis	10.000	20.000	1.000	1.000	0.250	1.000
Staphylococcus aureus	5.000	10.000	0.031	1.000	1.000	1.000
Staphylococcus epidermidis	5.000	10.000	0.250	1.000	0.500	0.500
Kocuria rhizophila	5.000	10.000	0.008	0.031	0.078	0.250
Pseudomanas aeruginosa	20.000	>20.000	NA	NA	0.500	1.000
Enterobacter aerogrnes	20.000	>20.000	1.000	1.000	0.125	1.000
Escherichia coli	20.000	>20.000	1.000	1.000	1.000	1.000
Salmonella typhi	20.000	>20.000	0.500	1.000	0.500	0.500

The tests were done in triplicate.

Table 13 MIC and MBC or MFC by Broth microdilution method (Cont.)

Microoraganism	*H. cordata* oil		Ampicillin		Amikacin	
	MIC (µl/ml)	MBC/MFC (µl/ml)	MIC (µg/ml)	MBC/MFC (µg/ml)	MIC (µg/ml)	MBC/MFC (µg/ml)
Salmonella typhimurium	20.000	>20.000	0.031	1.000	0.125	1.000
Shigella Spp.	20.000	>20.000	0.250	1.000	0.250	1.000
Candida albicans	0.625	1.250	NA	NA	NA	NA
Saccharomyces cerevisiae	0.625	1.250	NA	NA	NA	NA

The tests were done in triplicate.

CHAPTER V
DISCUSSION AND CONCLUSION

The quality control method are fundamental and important in maintaining the safety and efficacy of herbal medicines. The quality control of herbal medicine contains standardization which include processes namely, macroscopic and microscopic identification, physiochemical parameters, chromatographic fingerprint and quantification of chemical marker. These performings provide the evidence data about purity and quality of the crude drug.

Houttuynia cordata Thunb. is one of the medicinal plants used in many countries. In Thailand, Plu-khao are vegetable as side dish and also used to treat inflammation. The present study provides the information of pharmacognostic specifications, quercetin content of *H. cordata* whole plants and antimicrobial activities of *H. cordata* volatile oil.

The characteristic specifications are the first step that can help to identify and authenticate plant materials. *H. cordata* was illustrated for its macroscopic structures as its shape, size, color, margin, length and wide of leaf and flower. The leaf was transverse sectioned and the crude drug were poverized. Both were examined under microscope for cellular structures. This step was necessary for screening purity of materials and identification of correct plant species in powder form.

The physico-chemical parameters are important to ensure identity, purity, and quality of plant materials. The powders of *H. cordata* whole plant crude drug were subjected for determination of physico-chemical parameters such as loss on drying, ethanol extractives, water extractives, ash values, water content and volatile oil content. Total ash and acid-insoluble ash contents usually represent the non-volatile inorganic matters remaining after incineration of plant materials and is an important quality parameter. This specification is beneficial to control crude drug adulteration, contamination or unconcern in preparing the crude drug for marketing. The total ash indicates phosphate, oxalate, carbonates, silicates and silica in plant tissues whereas acid insoluble ash indicates mostly silica which can be admixture from sand and siliceous earth. The total ash values of whole *H. cordata* should not more than 14.31 ± 0.15 %by weigh [1, 2].

The ethanol and water extractive values represented the amount of active components of the extracted with solvents from a given amount of plant materials and obtained from mean of assessing the crude drug should not less than 4.69 ± 0.48 and 14.17 ± 1.03 % respectively. Water extractive values of this study (14.17 ± 1.03 %) related with previous studied (14.9 %) but ethanol extractive values 4.69 ± 0.48 % was found less than previous studied (12.8 %) [38].

TLC is simple technique used to obtain a fingerprint profile and present pattern of components in medicinal plants. Each compound can be separated and showed different R_f value which can be used as marker for quality control.

This study demonstrated TLC fingerprint of *H. cordata* crude drug using toluene, ethyl acetate, methanol, formic acid as mobile phase. Visualization was performed under daylight, ultraviolet light at the wavelength of 254 nm, 366 nm and staining with natural product and polyethylene glycol reagent.

For the determination of quercetin content in *H. cordata* crude drug, TLC densitometry and TLC image analysis by ImageJ software were used for quantitative analysis. The methods were validated in terms of specificity, accuracy, precision, LOD, LOQ and robustness. Both methods were accurate with % recovery between 80-120%.

The precisions were acceptable i.e. 2.36-4.98 %RSD for TLC densitometry and 3.15-12.49 %RSD for TLC image analysis. The LOD and LOQ values of TLC-densitometry were 0.04 and 0.13 µg/spot respectively and the LOD and LOQ of TLC image analysis were 0.12 and 0.38 µg/spot respectively. The robustness of TLC densitometry and TLC image analysis were 4.94 %RSD and 11.13 %RSD of peak area respectively. These results indicated that small changes in mobile phase ratio did not affected the methods.

The ethanolic extract of *H. cordata* crude drugs from 12 sources were investigated for their quercetin contents by both methods. Only one extract from sources 6 (Khonkaen) could be quantitatively determined. The sample from the other sources contained quercetin in the amount of less than the LOQ.

Furthermore, the quercetin content in the extract from source 6 was found to be 0.0049 g/g by TLC densitometry but 0.0031 g/g by TLC image analysis. This difference might be according to the fact that, by densitometry, the amount of quercetin was evaluated from the intensity of its light absorption of 388 nm but in image analysis, pixel intensity was used instead. In addition, the image was obtained from visualization of TLC plate under 254 nm. It can be concluded that quercetin quantitatively analysis in *H. cordata* crude drug by TLC image analysis is not suitable and cannot be as alternative method to TLC densotometry detected for some flavonoids in herbal materials.

Gas chromatography-mass spectrometry is an instrumental technique, comprising a gas chromatograph (GC) coupled to a mass spectrometer (MS), by which complex mixtures of components can be separated, identified and quantified.

At least 30 chemical compounds were identified in *H. cordata* whole plant oil. The major constituents were decanoic acid (40.40 ± 27.21%), 2- undecanone (26.43 ± 14.54%), myrcene (19.21 ± 15.09%) respectively. The result was related to

the previous studies of volatile oil of *H cordata* in China which reported myrcene (16.03%) decanoic acid (18.67%) and 2-undecanone (1.80%) [10, 36, 37].

The chemical compositions and GC fingerprint patterns of *H. cordata* volatile oils were shown in Figure 19, 20. The chromatogram showed that *H. cordata* volatile oil could be classified into two clusters. Cluster 1 consisted of samples from Lampang1, Nakhonpathom1, Nakhonpathom2, Pathumtani, Bangkok and Nontaburi. This cluster dominated in decanoic acid and 2-undecanone (Figure 19). Cluster 2 consisted of samples from Khonkaen, Chiangmai, Loei, Chiangrai1, Lampang2, and Chaingrai2. This cluster dominated in myrcene, 2-undecanone and estragole (Figure 20).

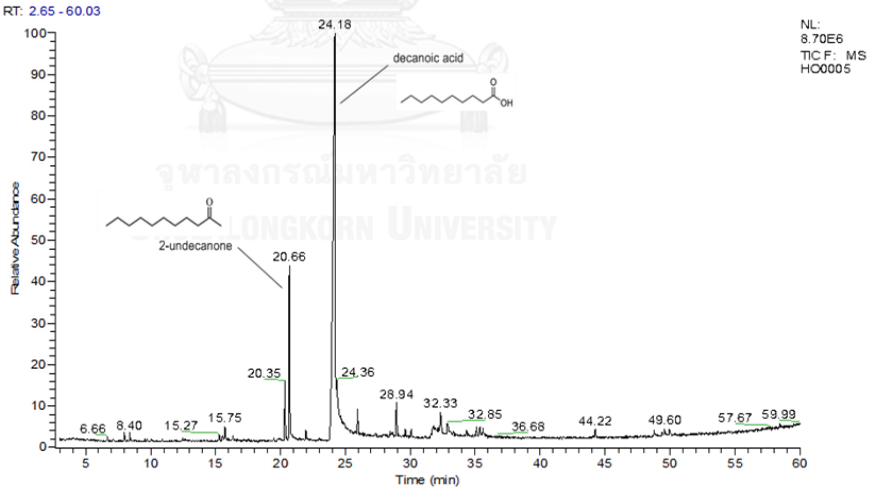

Figure 19 GC fingerprint of *H. cordata* oil GC of Lampang1 province (cluster 1)

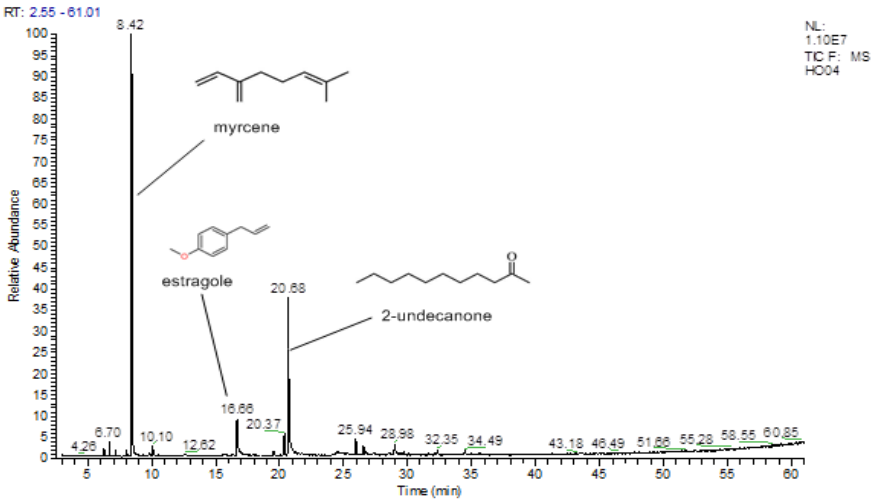

Figure 20 GC fingerprint of *H. cordata* oil GC of Loei province (cluster 2)

Antimicrobial activities of volatile oil from *H. cordata* whole plants were determined against eleven bacteria and two fungi. *H. cordata* oil showed inhibition zone against eleven bacterials and two fungi. The large inhibition zone of 16.67±0.58 mm was found against *Kocuria rhizophila* and *Saccharomyces cevevisiae*. MIC and MBC/MFC tests showed that the oil had the promising bactericidal activity against tested five gram-positive bacteria and especially two fungi. MBC/MFC tests showed no activity in gram-negative bacteria. The recent study also showed that *H. cordata* oil was active against *Staphylococus aureus* (MIC= $0.25x10^{-3}$ ml/ml) and *Staphylococcus epidermidis* (MIC= 1.25 mg/ml, MBC > 5 mg/ml) [10].

The pharmacognostic specifications of *H. cordata* whole plants established in this study could be used as guide marker for quality control in standardization of *H. cordata* in Thailand. The chemical components and antimicrobial activities of *H. cordata* oil were demonstrated.

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APPENDICES

APPENDIX A

Data of pharmacognostic specifications of *H. cordata* whole plant

Table 14 Determination of water content (%by weight) of *H. cordata* from 12 different sources.

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
1	Chiangmai	1	10.40		
		2	9.20	9.73	0.61
		3	9.60		
2	Chiangrai1	1	9.60		
		2	10.40	10.4	0.8
		3	11.20		
3	Lampang1	1	10.00		
		2	10.00	10.53	0.92
		3	11.60		
4	Lampang2	1	10.80		
		2	8.80	9.33	1.29
		3	8.40		
5	Loei	1	10.40		
		2	8.00	9.47	1.29
		3	10.00		
6	Nakhonpathom 1	1	14.00		
		2	14.80	15.07	1.22
		3	16.40		
7	Nonthaburi	1	13.60		
		2	12.00	12.53	0.92
		3	12.00		
8	Chiangrai2	1	14.80		
		2	16.00	14.53	1.62
		3	12.80		

Table 14 Determination of water content (%by weight) of *H. cordata* from 12 different sources (Cont.)

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
9	Nakhonpathom2	1	12.40		
		2	14.80	13.60	1.20
		3	13.60		
10	Patumthani	1	8.80		
		2	11.60	9.47	1.89
		3	8.00		
11	Bangkok	1	14.40		
		2	14.80	14.00	1.06
		3	12.80		
12	Khonkaen	1	12.40		
		2	11.20	11.73	0.61
		3	11.60		
	Grand average			**11.70**	**1.18**

Table 15 Determination of loss on drying (% by weight) of *H. cordata* from 12 different sources

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
1	Chiangmai	1	6.98		
		2	6.94	6.93	0.06
		3	6.86		
2	Chiangrai1	1	8.84		
		2	8.90	8.88	0.03
		3	8.90		
3	Lampang1	1	9.13		
		2	8.92	9.02	0.1
		3	9.02		
4	Lampang2	1	6.02		
		2	6.09	6.06	0.04
		3	6.08		
5	Loei	1	8.10		
		2	8.92	8.33	0.52
		3	7.97		
6	Nakhonpathom1	1	11.28		
		2	10.85	10.96	0.28
		3	10.74		
7	Nonthaburi	1	4.84		
		2	4.78	4.81	0.03
		3	4.79		
8	Chiangrai2	1	6.67		
		2	6.74	6.79	0.15
		3	6.96		

Table 15 Determination of loss on drying (% by weight) of *H. cordata* from 12 different sources (Cont.)

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
9	Nakhonpathom2	1	6.56		
		2	6.55	6.53	0.04
		3	6.48		
10	Patumthani	1	6.73		
		2	6.62	6.64	0.09
		3	6.56		
11	Bangkok	1	5.77		
		2	5.82	5.79	0.02
		3	5.79		
12	Khonkaen	1	5.38		
		2	7.01	5.89	0.98
		3	5.27		
	Grand average			**7.22**	**0.34**

Table 16 Determination of total ash (% by weight) of *H. cordata* from 12 different sources

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
1	Chiangmai	1	8.64		
		2	8.58	8.62	0.04
		3	8.64		
2	Chiangrai1	1	16.54		
		2	16.68	16.61	0.07
		3	16.61		
3	Lampang1	1	13.64		
		2	13.58	13.60	0.04
		3	13.56		
4	Lampang2	1	19.05		
		2	18.84	18.78	0.31
		3	18.44		
5	Loei	1	17.15		
		2	16.80	16.98	0.17
		3	16.99		
6	Nakhonpathom1	1	13.14		
		2	12.97	13.02	0.10
		3	12.96		
7	Nonthaburi	1	14.75		
		2	14.71	14.81	0.14
		3	14.98		
8	Chiangrai2	1	14.79		
		2	15.16	14.99	0.19
		3	15.00		

Table 16 Determination of total ash (% by weight) of *H. cordata* from 12 different sources (Cont.)

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
9	Nakhonpathom2	1	13.07		
		2	12.90	13.02	0.1
		3	13.07		
10	Patumthani	1	11.98		
		2	11.85	11.96	0.1
		3	12.05		
11	Bangkok	1	13.27		
		2	13.07	13.27	0.21
		3	13.48		
12	Khonkaen	1	16.04		
		2	16.03	16.01	0.04
		3	15.97		
	Grand average			**14.31**	**0.15**

Table 17 Determination of acid insoluble (% by weight) of *H. cordata* from 12 different sources

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
1	Chiangmai	1	2.12		
		2	1.74	2.06	0.29
		3	2.31		
2	Chiangrai1	1	4.52		
		2	5.13	4.90	0.33
		3	5.04		
3	Lampang1	1	3.29		
		2	3.02	3.13	0.14
		3	3.08		
4	Lampang2	1	6.17		
		2	6.25	6.11	0.19
		3	5.90		
5	Loei	1	4.87		
		2	4.77	4.89	0.12
		3	5.02		
6	Nakhonpathom1	1	2.92		
		2	2.9	3.18	0.46
		3	3.71		
7	Nonthaburi	1	4.60		
		2	4.43	4.50	0.09
		3	4.49		
8	Chiangrai2	1	3.65		
		2	4.09	3.74	0.32
		3	3.47		

Table 17 Determination of acid insoluble (% by weight) of *H. cordata* from 12 different sources (Cont.)

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
9	Nakhonpathom2	1	3.42		
		2	3.35	3.43	0.08
		3	3.51		
10	Patumthani	1	4.59		
		2	3.83	3.99	0.54
		3	3.54		
11	Bangkok	1	3.23		
		2	3.10	3.20	0.08
		3	3.26		
12	Khonkaen	1	5.94		
		2	5.58	6.21	0.79
		3	7.10		
Grand average				**4.11**	**0.36**

Table 18 Determination of ethanol-soluble extractive (% by weight) of *H. cordata* from 12 different sources

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
1	Chiangmai	1	6.72		
		2	6.65	6.60	0.14
		3	6.45		
2	Chiangrai1	1	4.59		
		2	4.18	5.03	1.14
		3	6.33		
3	Lampang1	1	3.46		
		2	3.2	3.31	0.13
		3	3.27		
4	Lampang2	1	3.18		
		2	2.94	3.39	0.59
		3	4.06		
5	Loei	1	3.99		
		2	4.45	4.17	0.24
		3	4.09		
6	Nakhonpathom1	1	2.36		
		2	1.77	2.01	0.31
		3	1.92		
7	Nonthaburi	1	1.86		
		2	2.43	1.98	0.4
		3	1.66		
8	Chiangrai2	1	9.11		
		2	9.04	8.96	0.21
		3	8.72		

Table 18 Determination of ethanol-soluble extractive (% by weight) of *H. cordata* from 12 different sources (Cont.)

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
9	Nakhonpathom2	1	9.11		
		2	9.04	8.96	0.21
		3	8.72		
10	Patumthani	1	3.89		
		2	4.01	4.11	0.29
		3	4.45		
11	Bangkok	1	3.89		
		2	4.01	4.11	0.29
		3	4.45		
12	Khonkaen	1	3.89		
		2	4.01	4.11	0.29
		3	4.45		
Grand average				**4.73**	**0.44**

Table 19 Determination of water-soluble extractive (% by weight) of *H. cordata* from 12 different sources

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
1	Chiangmai	1	14.46		
		2	12.74	14.12	1.25
		3	15.17		
2	Chiangrai1	1	16.98		
		2	18.52	17.53	0.86
		3	17.08		
3	Lampang1	1	15.67		
		2	13.97	14.84	0.85
		3	14.88		
4	Lampang2	1	18.48		
		2	21.57	20.32	1.63
		3	20.9		
5	Loei	1	17.79		
		2	16.72	17.82	1.11
		3	18.95		
6	Nakhonpathom1	1	13.57		
		2	16.18	15.17	1.40
		3	15.76		
7	Nonthaburi	1	12.46		
		2	12.88	12.35	0.60
		3	11.7		
8	Chiangrai2	1	10.38		
		2	10.28	9.52	1.40
		3	7.9		

Table 19 Determination of water-soluble extractive (% by weight) of *H. cordata* from 12 different sources (Cont.)

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
9	Nakhonpathom2	1	10.29		
		2	9.5	9.85	0.40
		3	9.76		
10	Patumthani	1	10.23		
		2	9.81	10.05	0.22
		3	10.11		
11	Bangkok	1	13.85		
		2	14.75	14.68	0.81
		3	15.45		
12	Khonkaen	1	12.84		
		2	14.38	13.79	0.83
		3	14.16		
	Grand average			**14.17**	**1.03**

Table 20 Determination of volatile oil content (% by weight) of *H. cordata* from 12 different sources

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
1	Chiangmai	1	0.12		
		2	0.14	0.13	0.01
		3	0.14		
2	Chiangrai1	1	0.08		
		2	0.08	0.07	0.01
		3	0.06		
3	Lampang1	1	0.08		
		2	0.08	0.08	0.00
		3	0.08		
4	Lampang2	1	0.04		
		2	0.04	0.05	0.01
		3	0.06		
5	Loei	1	0.10		
		2	0.10	0.10	0.00
		3	0.10		
6	Nakhonpathom1	1	0.06		
		2	0.08	0.07	0.01
		3	0.08		
7	Nonthaburi	1	0.10		
		2	0.10	0.09	0.01
		3	0.08		
8	Chiangrai2	1	0.10		
		2	0.10	0.09	0.01
		3	0.08		

Table 20 Determination of volatile oil content (% by weight) of *H. cordata* from 12 different sources (Cont.)

Source	Location name	Crude drug sample extracts no.	Amount (% by weight)	Mean	SD
9	Nakhonpathom2	1	0.02		
		2	0.02	0.02	0.00
		3	0.02		
10	Patumthani	1	0.1		
		2	0.06	0.07	0.02
		3	0.06		
11	Bangkok	1	0.04		
		2	0.04	0.04	0.00
		3	0.04		
12	Khonkaen	1	0.12		
		2	0.12	0.12	0.00
		3	0.12		
Grand average				**0.08**	**0.01**

APPENDIX B

Quantitative analysis of quercetin content in *H. cordata*

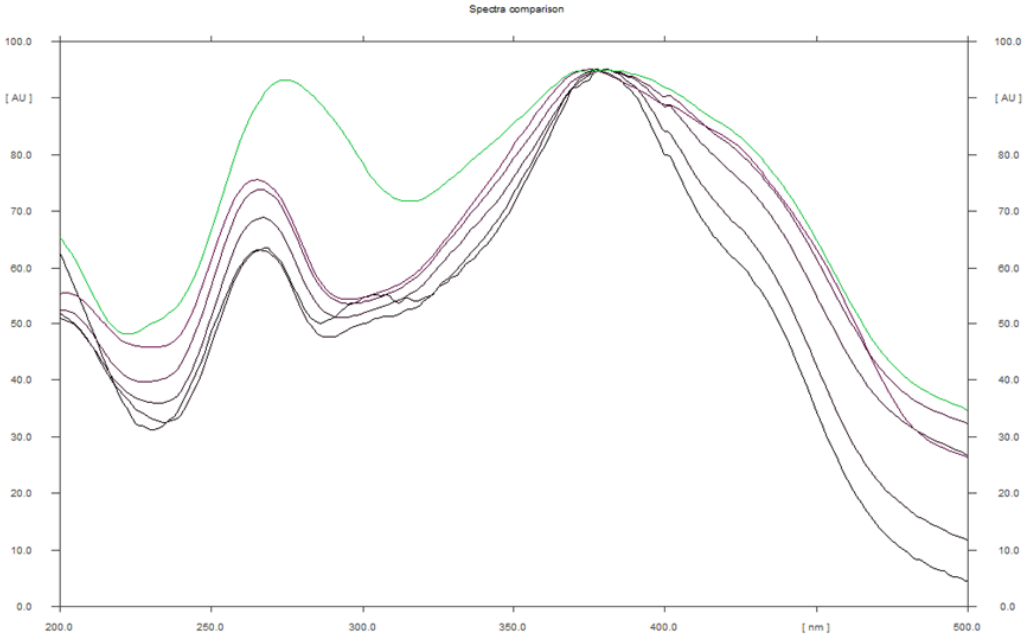


Figure 21 The maximum wavelength of standard quercetin and sample

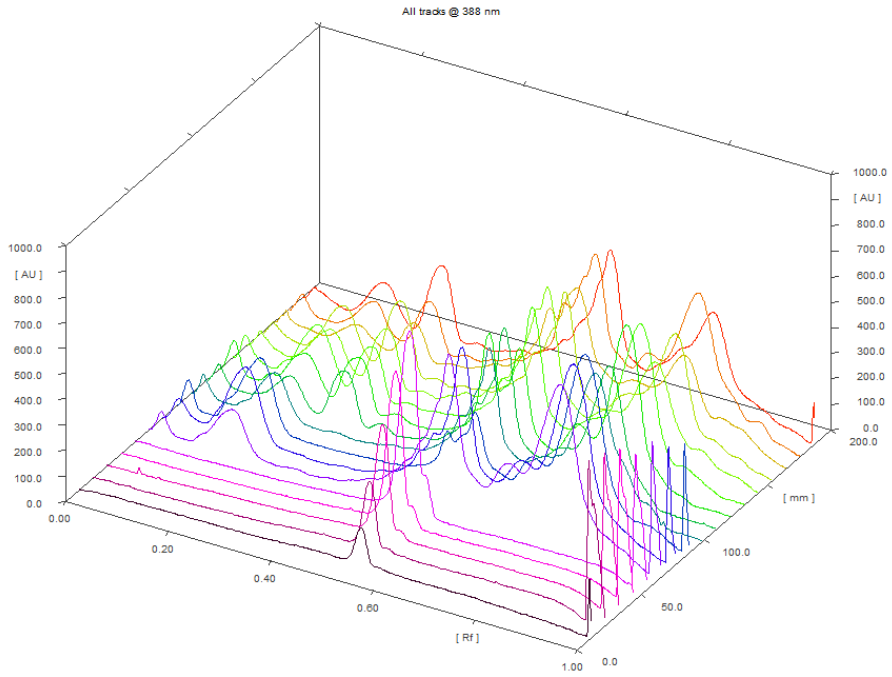


Figure 22 3D TLC densitometry chromatogram of quercetin standard and sample extracts (Plate 1)

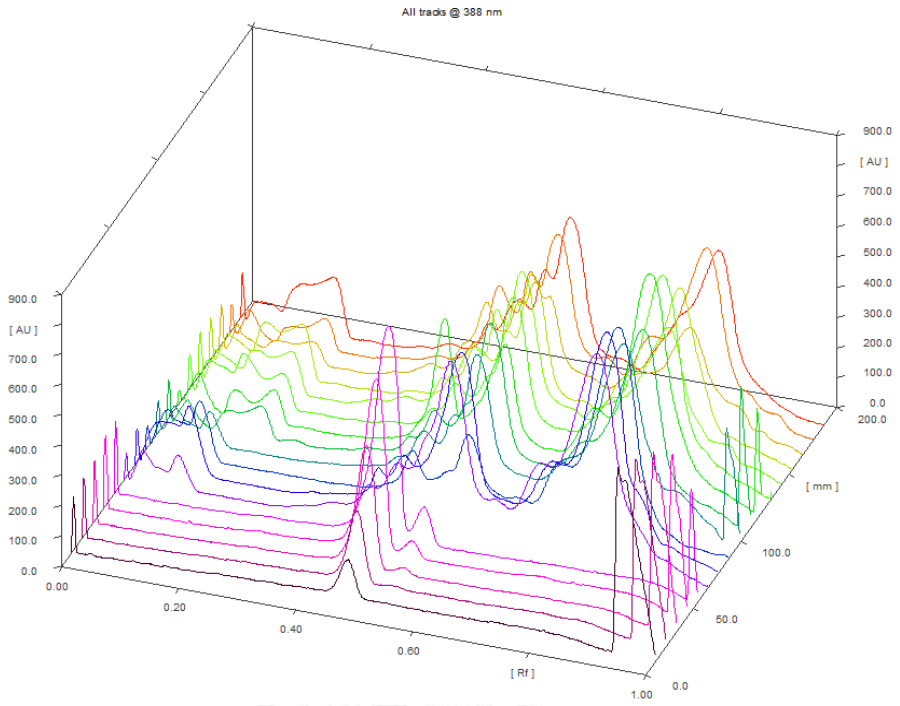


Figure 23 3D TLC densitometry chromatogram of quercetin standard and sample extracts (Plate 2)

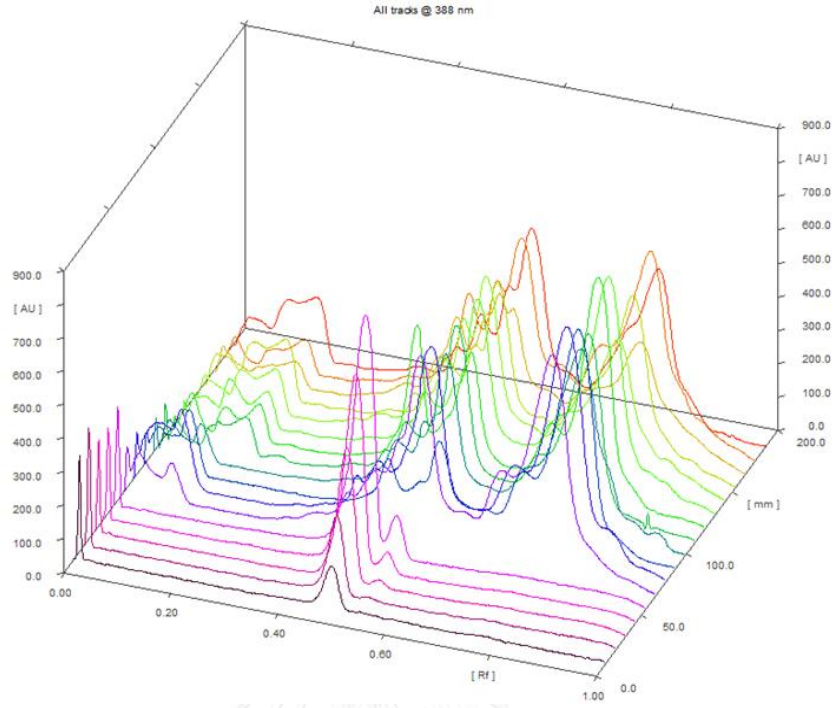


Figure 24 3D TLC densitometry chromatogram of quercetin standard and sample extracts (Plate 3)

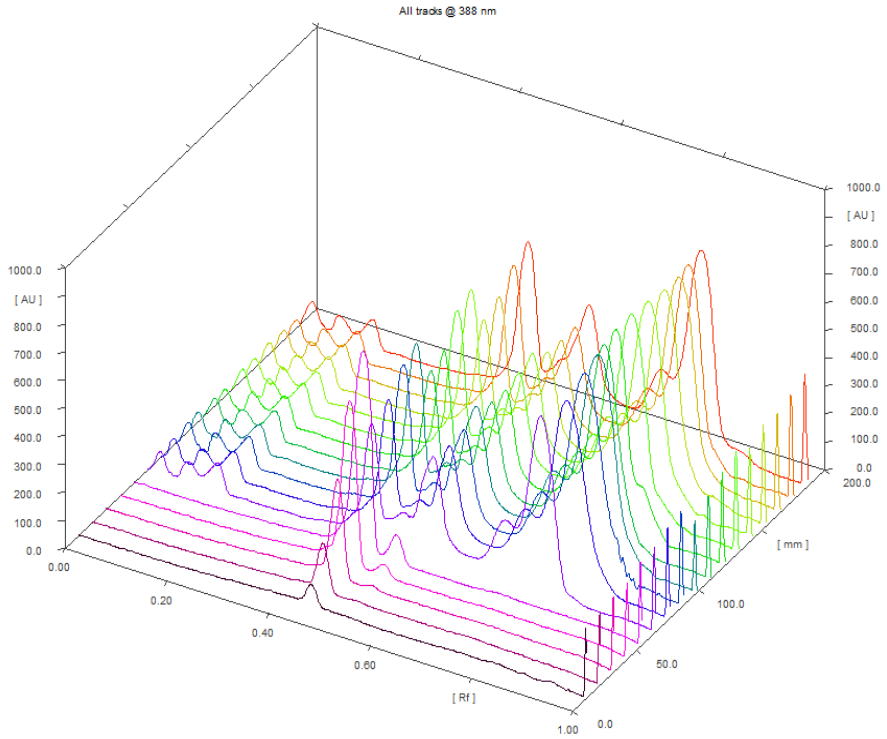


Figure 25 3D TLC densitometry chromatogram of accuracy method (Plate 1-accuracy and sample extracts No.6)

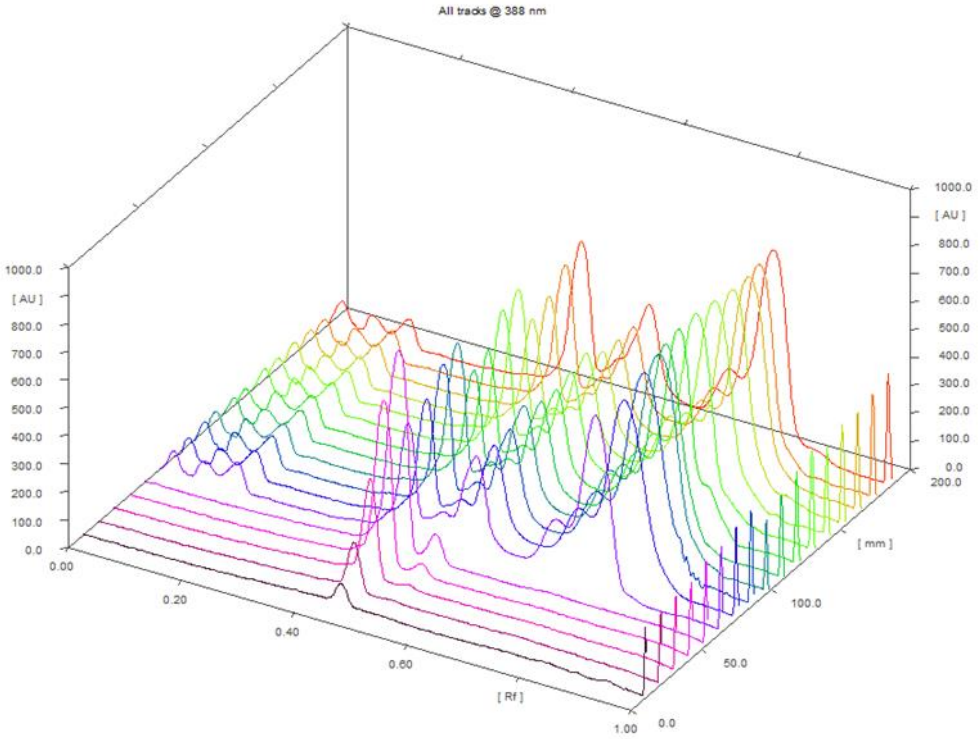


Figure 26 3D TLC densitometry chromatogram of precision method (Plate 1-precision and sample extracts No.6)

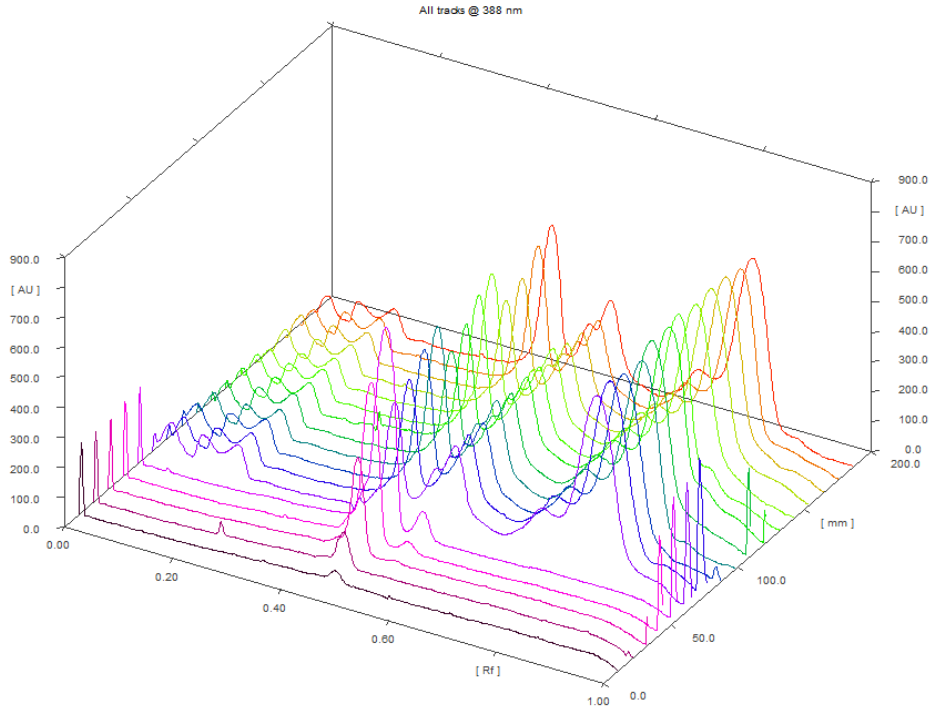


Figure 27 3D TLC densitometry chromatogram of precision method (Plate 2-precision and sample extracts No.6)

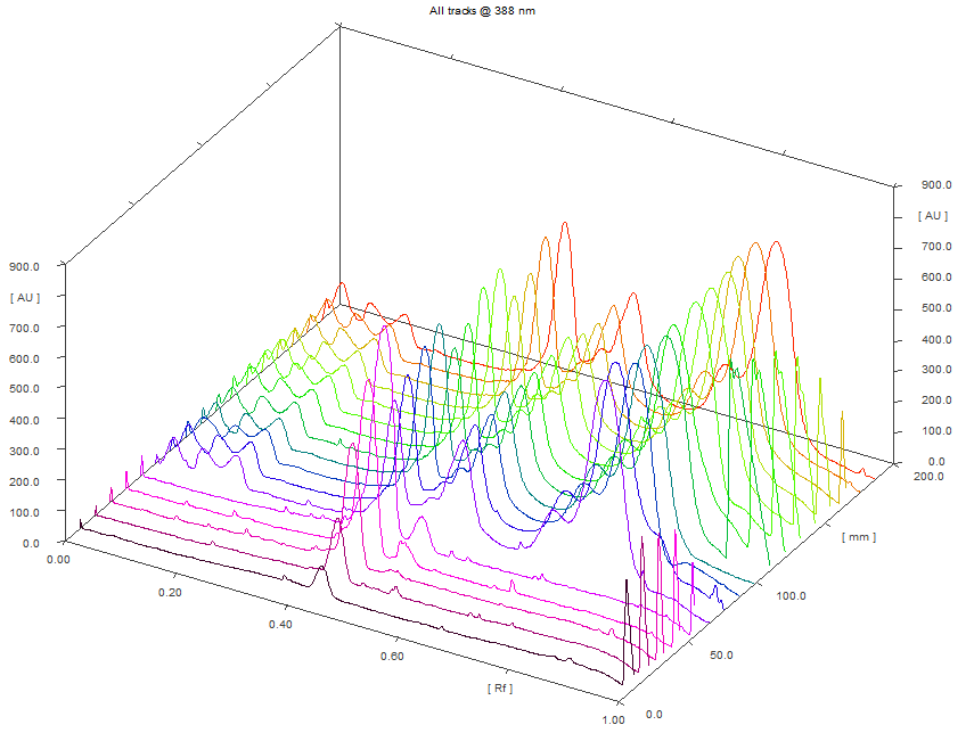
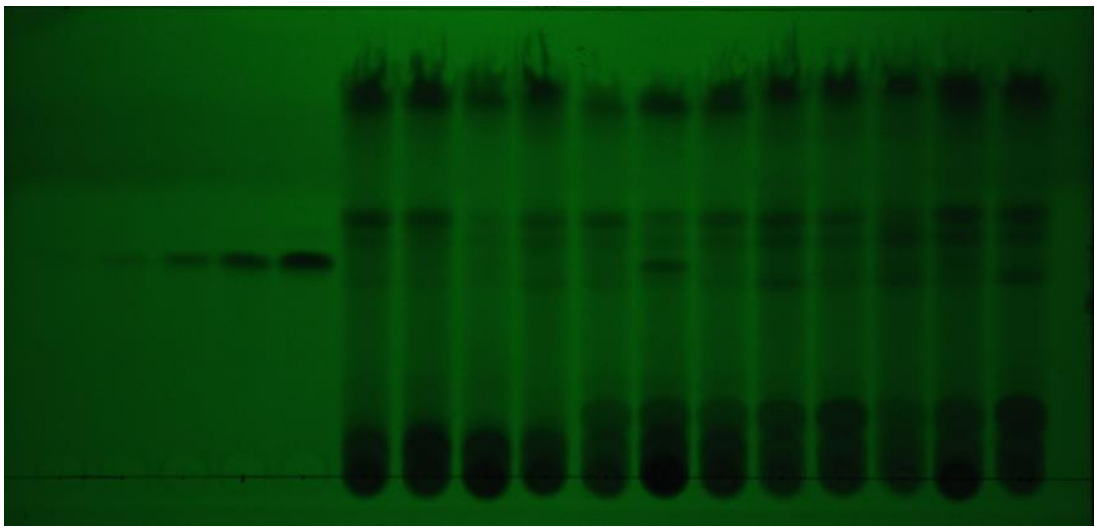
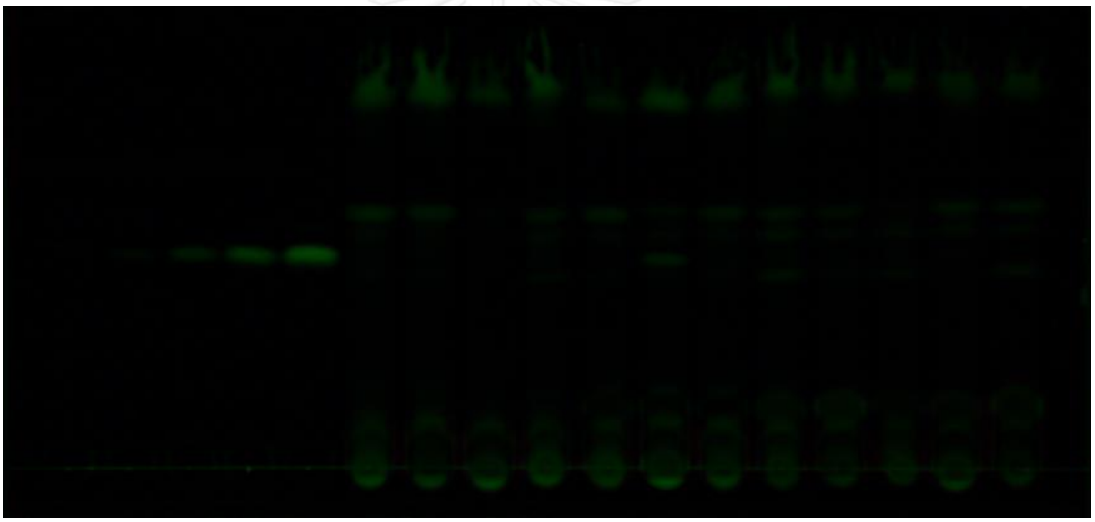


Figure 28 3D TLC densitometry chromatogram of precision method (Plate 3-precision and sample extracts No.6)

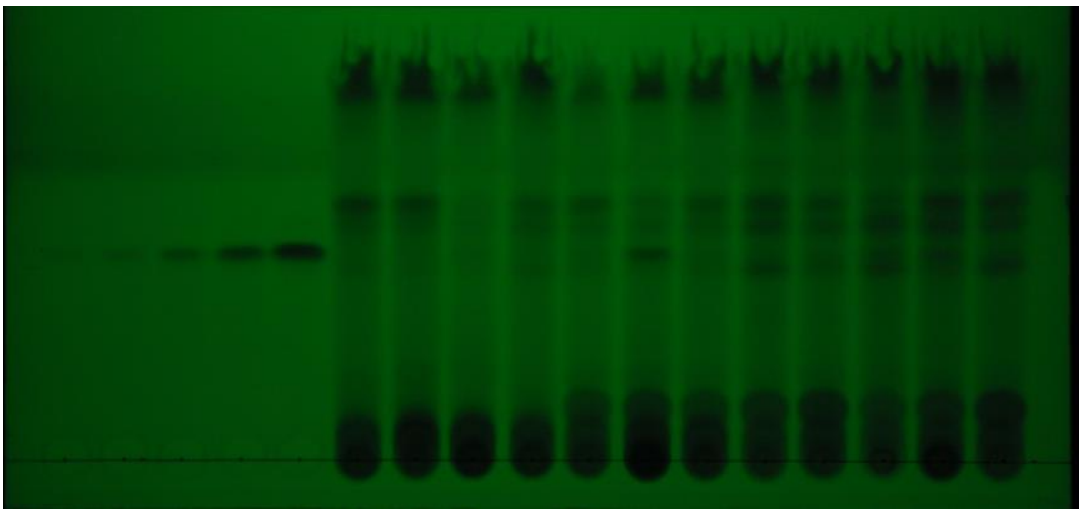


(A)

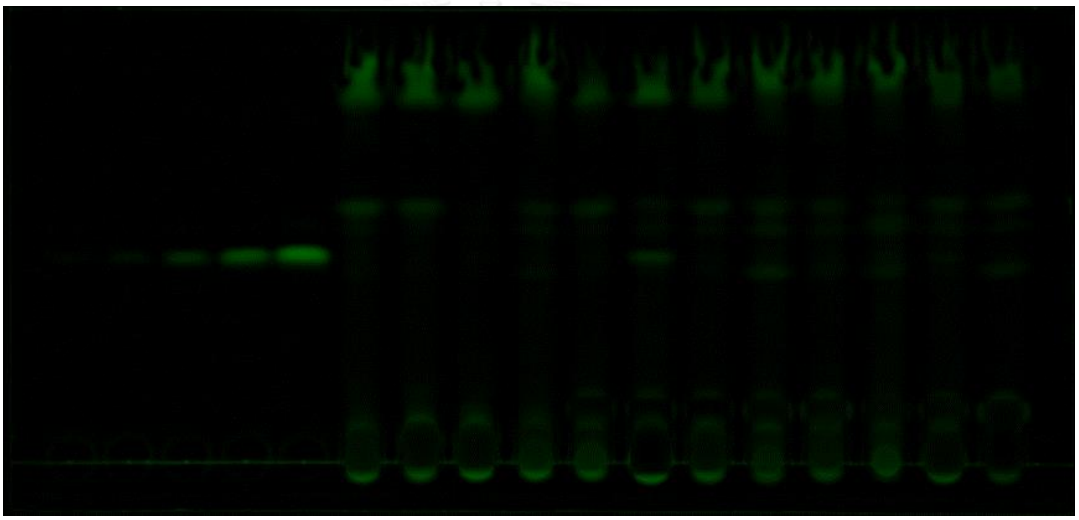


(B)

Figure 29 TLC plate 1 of quercetin standard (0.025-0.4 mg/ml) and all samples extracts (12 sources); TLC plate visual with 254 nm (A), TLC plate subtract background by imageJ software (B)

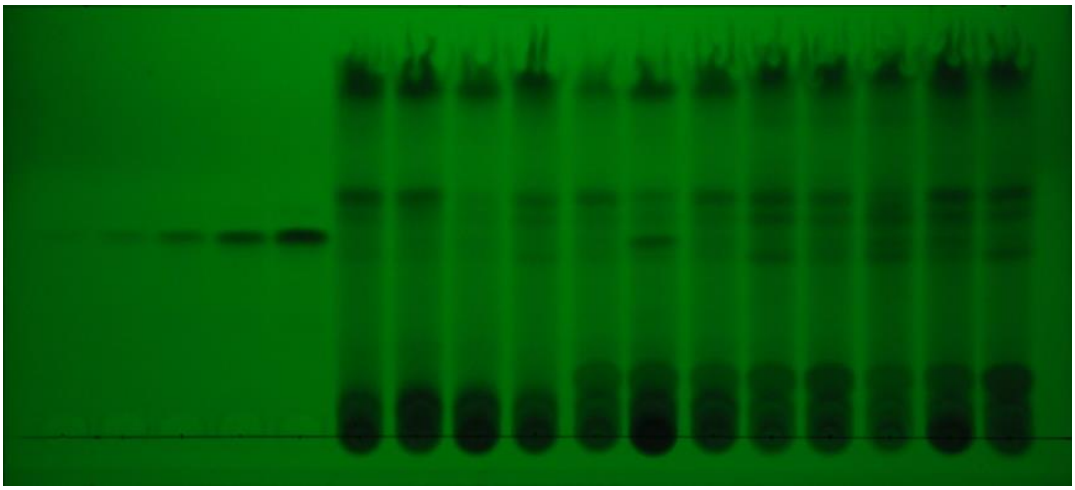


(A)

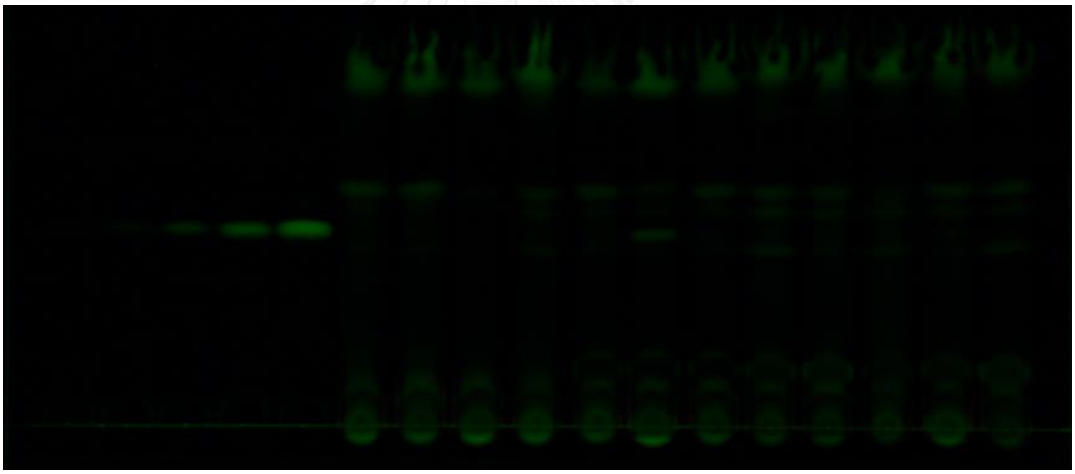


(B)

Figure 30 TLC plate 2 of quercetin standard (0.025-0.4 mg/ml) and all samples extracts (12 sources); TLC plate visual with 254 nm (A), TLC plate subtract background by imageJ software (B)

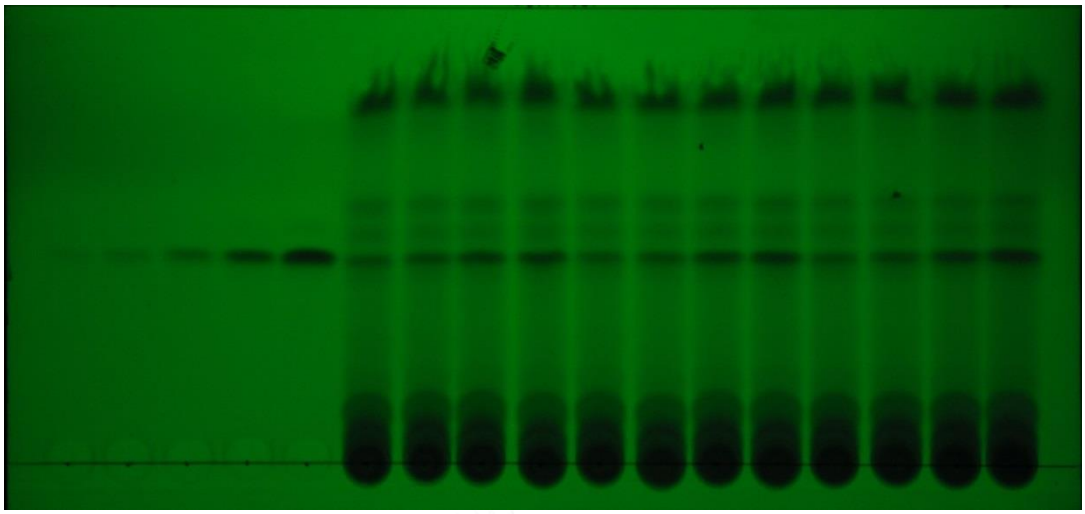


(A)

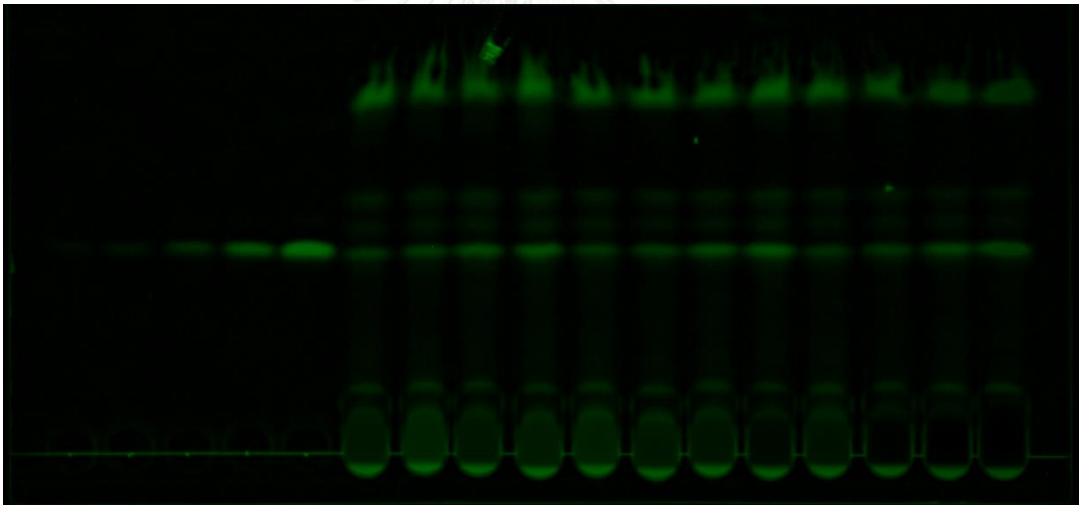


(B)

Figure 31 TLC plate 3 of quercetin standard (0.025-0.4 mg/ml) and all samples extracts (12 sources); TLC plate visual with 254 nm (A), TLC plate subtract background by imageJ software (B)



(A)



(B)

Figure 32 TLC plate 1 of accuracy method; TLC plate visual with 254 nm (A), TLC plate subtract background by imageJ software (B)

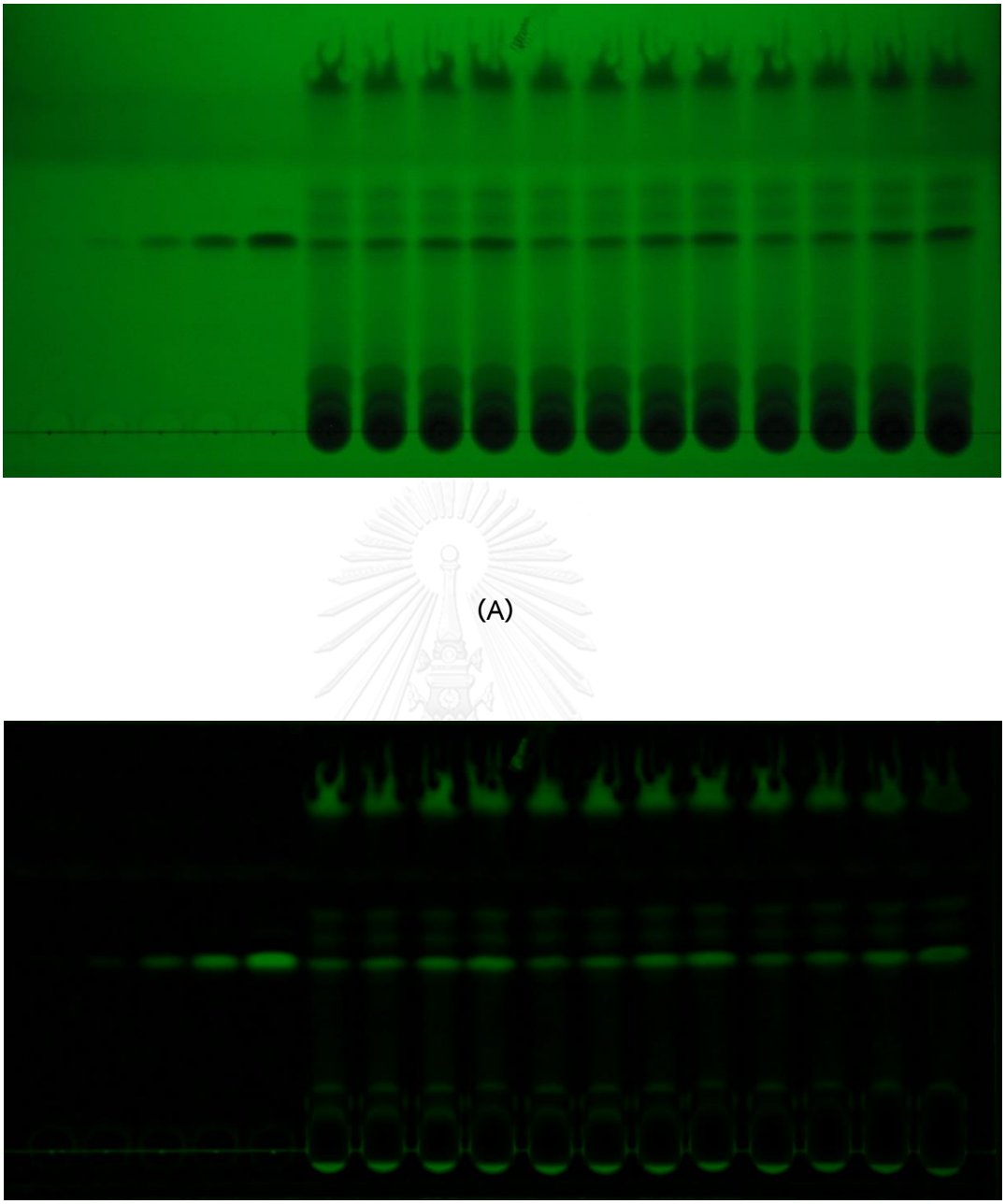
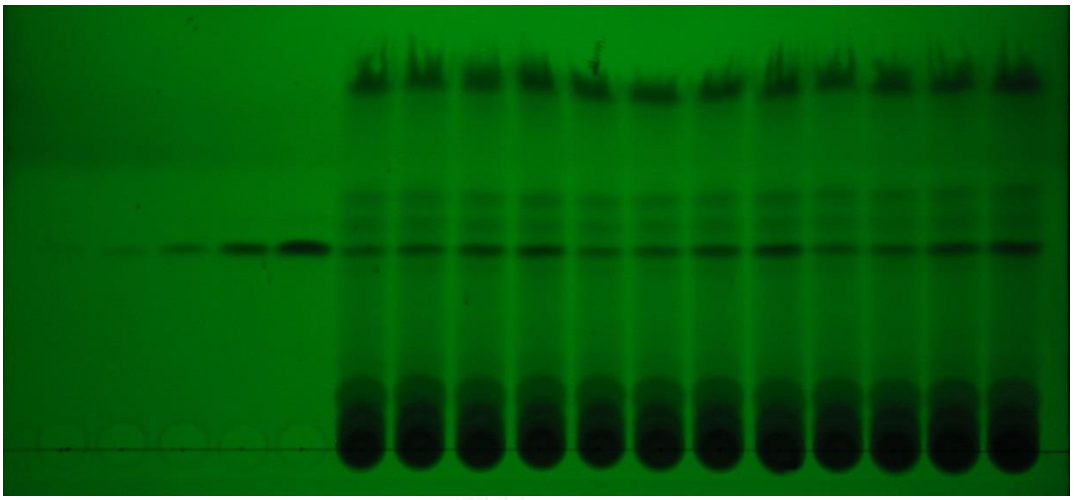
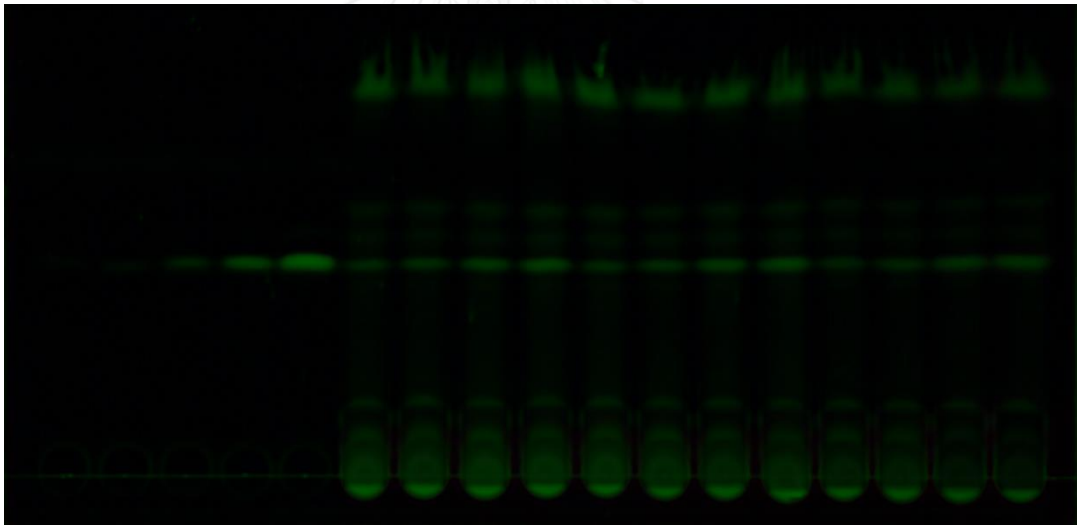


Figure 33 TLC plate 1 of precision method; TLC plate visual with 254 nm (A), TLC plate subtract background by imageJ software (B)

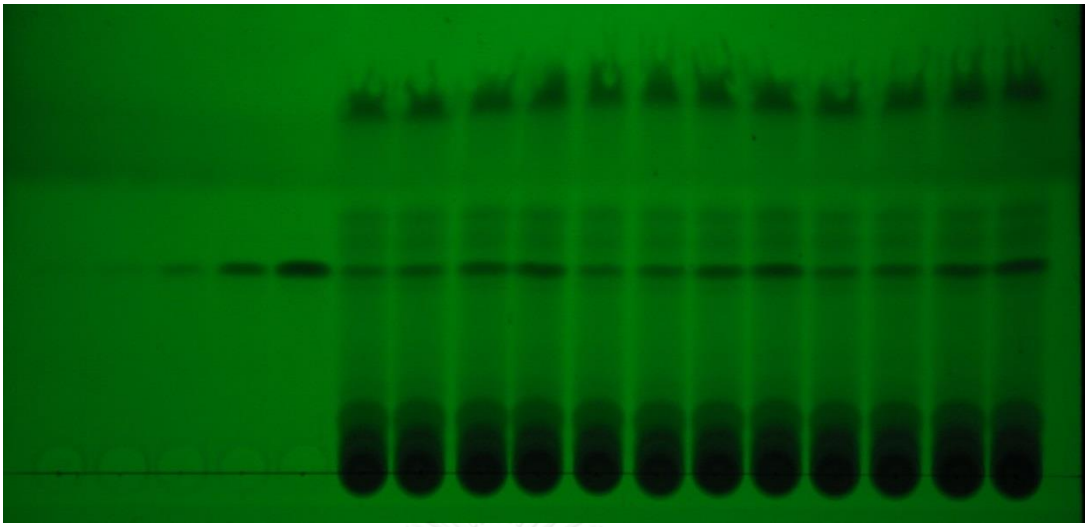


(A)

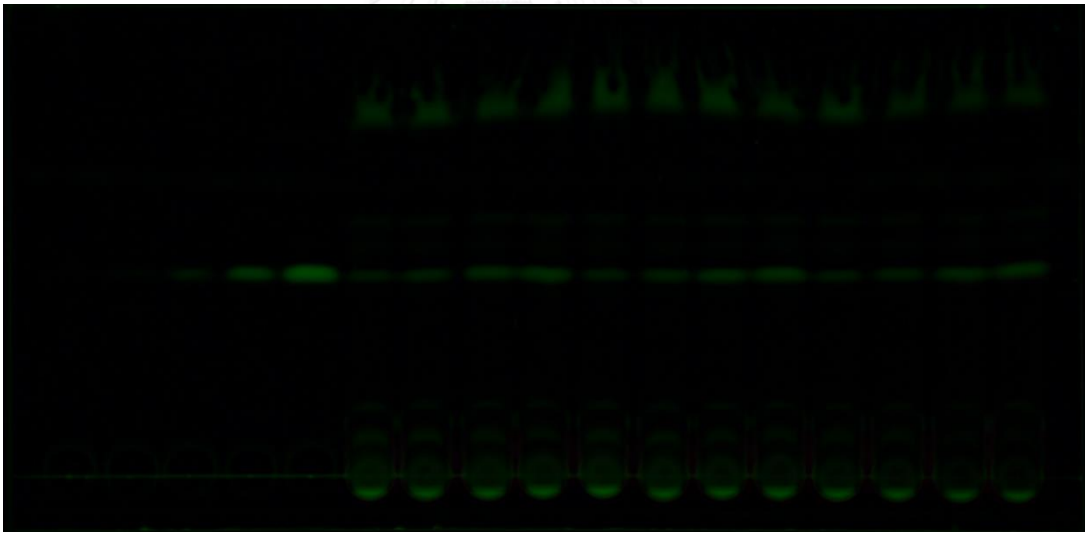


(B)

Figure 34 TLC plate 2 of precision method; TLC plate visual with 254 nm (A), TLC plate subtract background by imageJ software (B**)**



(A)



(B)

Figure 35 TLC plate 3 of precision method; TLC plate visual with 254 nm (A), TLC plate subtract background by imageJ software (B)

APPENDIX C

GC chromatogram of *H. cordata* volatile oil

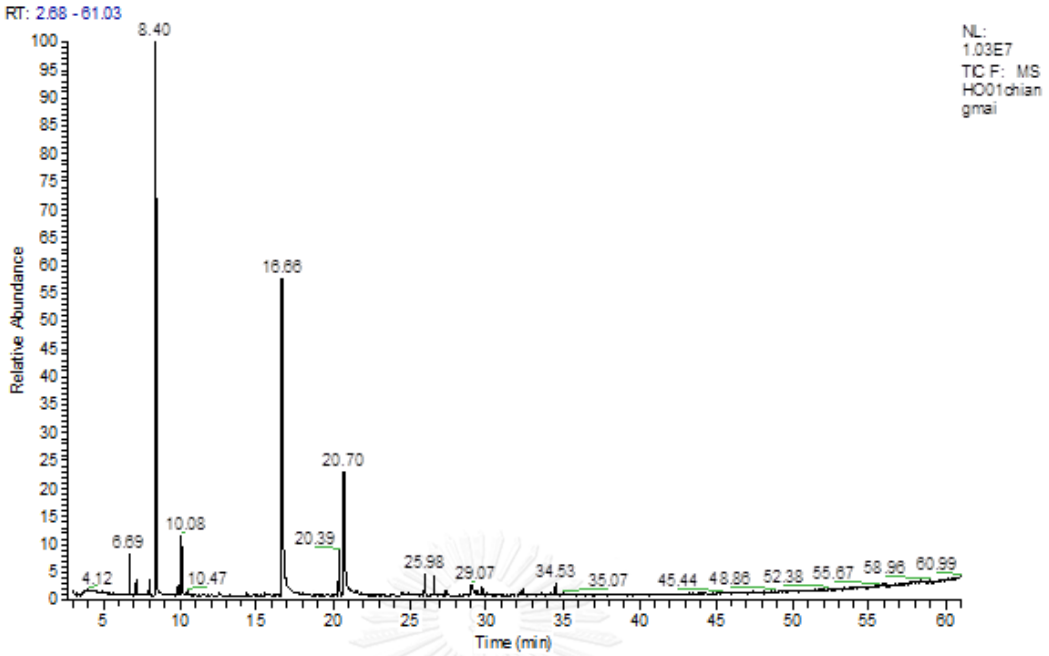


Figure 36 GC chromatogram of *H. cordata* oil from Chiangmai (Source 01)

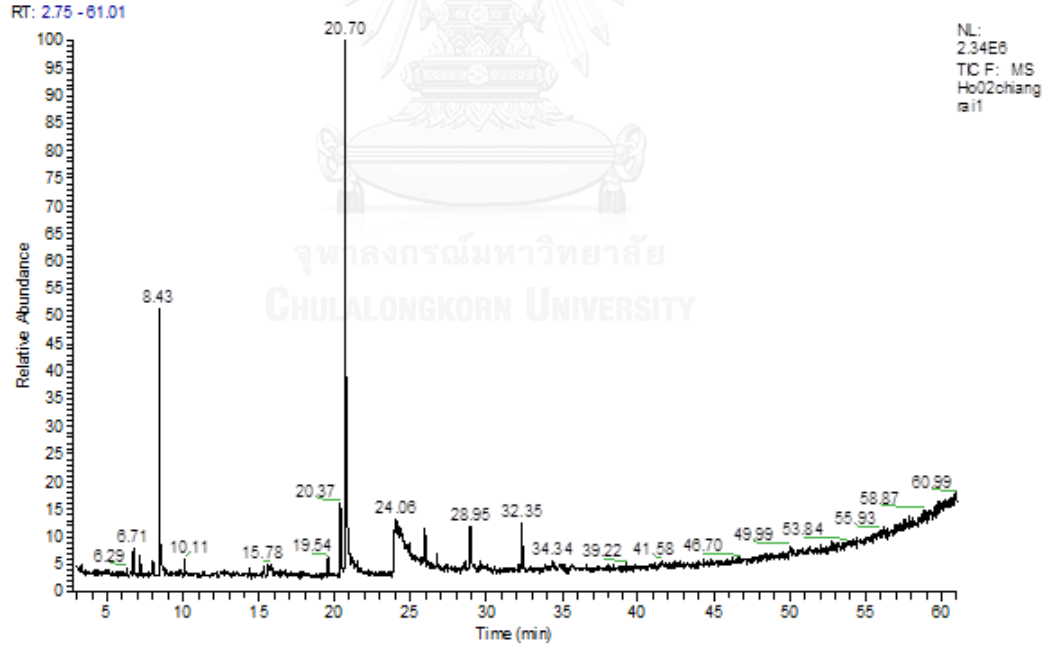


Figure 37 GC chromatogram of *H. cordata* oil from Chiangrai1 (Source 02)

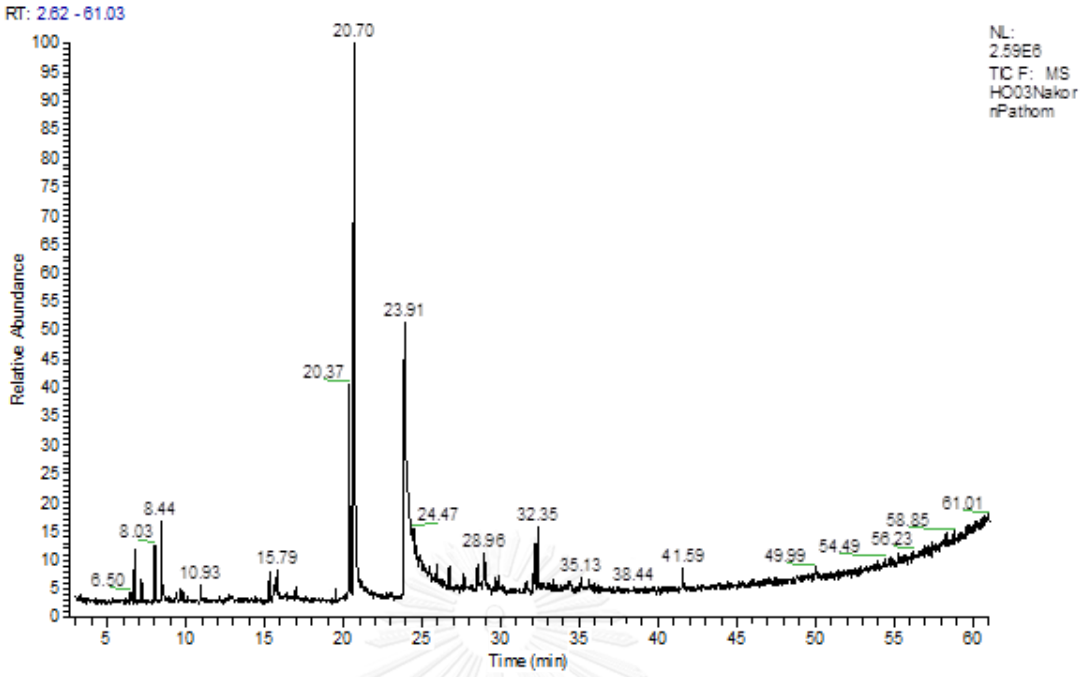


Figure 38 GC chromatogram of *H. cordata* oil from Nakhonpathom1 (Source 03)

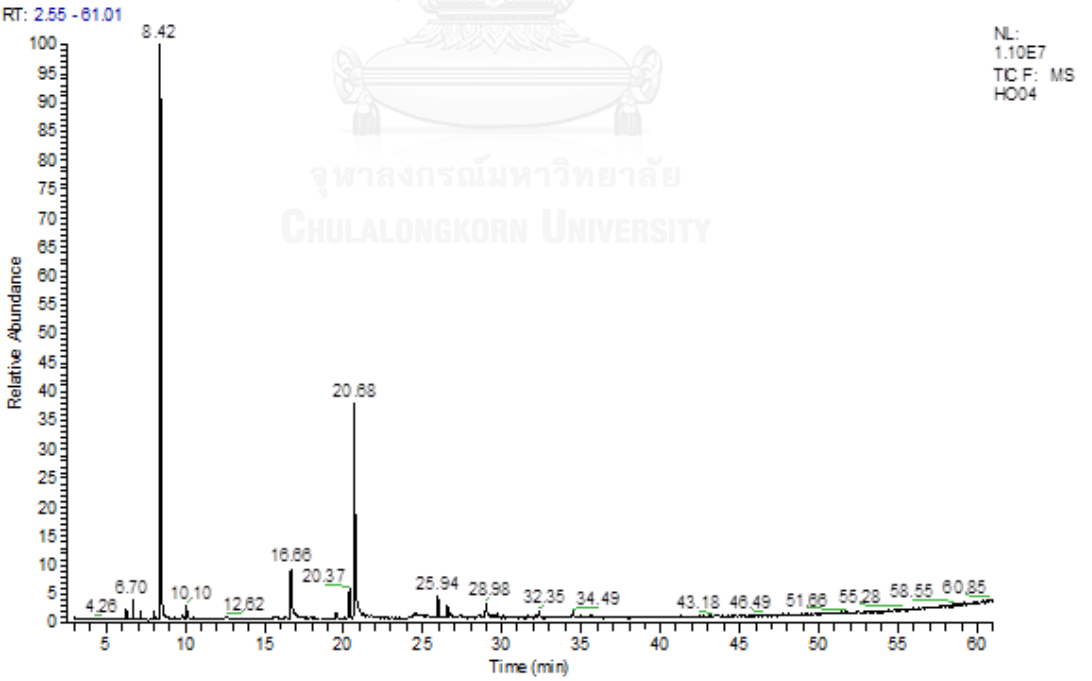


Figure 39 GC chromatogram of *H. cordata* oil from Loei (Source 04)

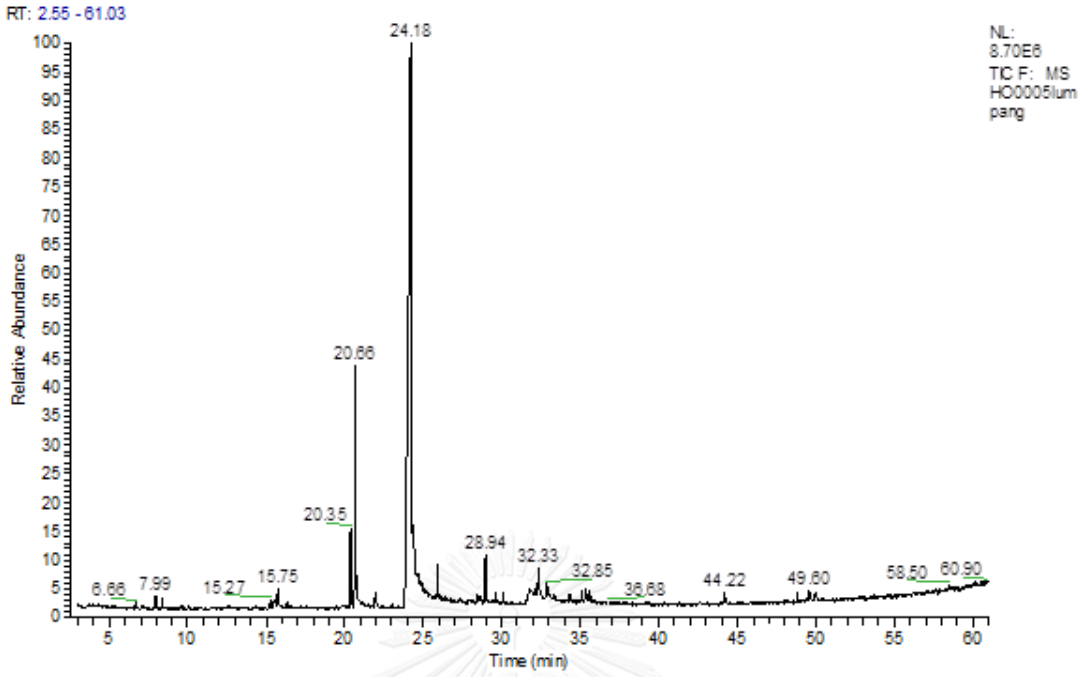


Figure 40 GC chromatogram of *H. cordata* oil from Lampang1 (Source 05)

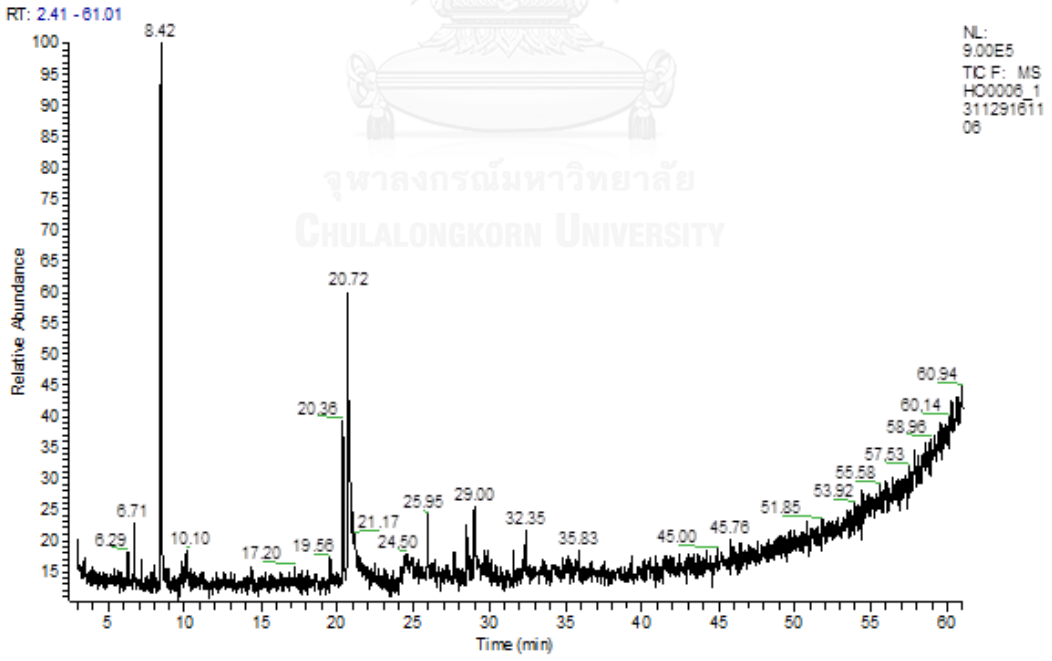


Figure 41 GC chromatogram of *H. cordata* oil from Lampang2 (Source 06)

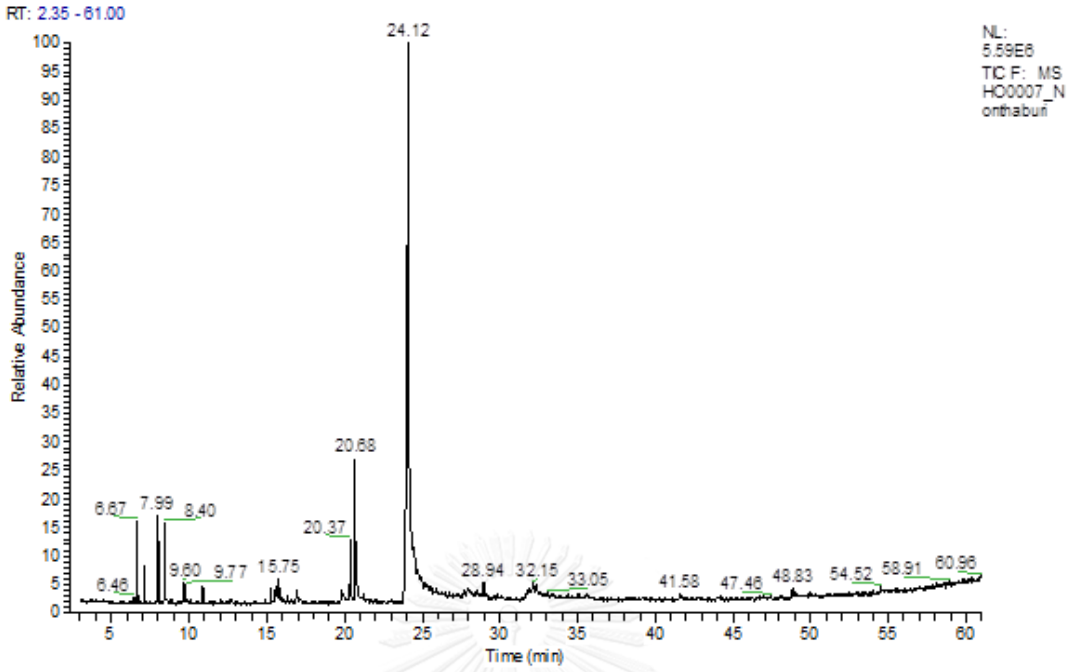


Figure 42 GC chromatogram of *H. cordata* oil from Nontaburi (Source 07)

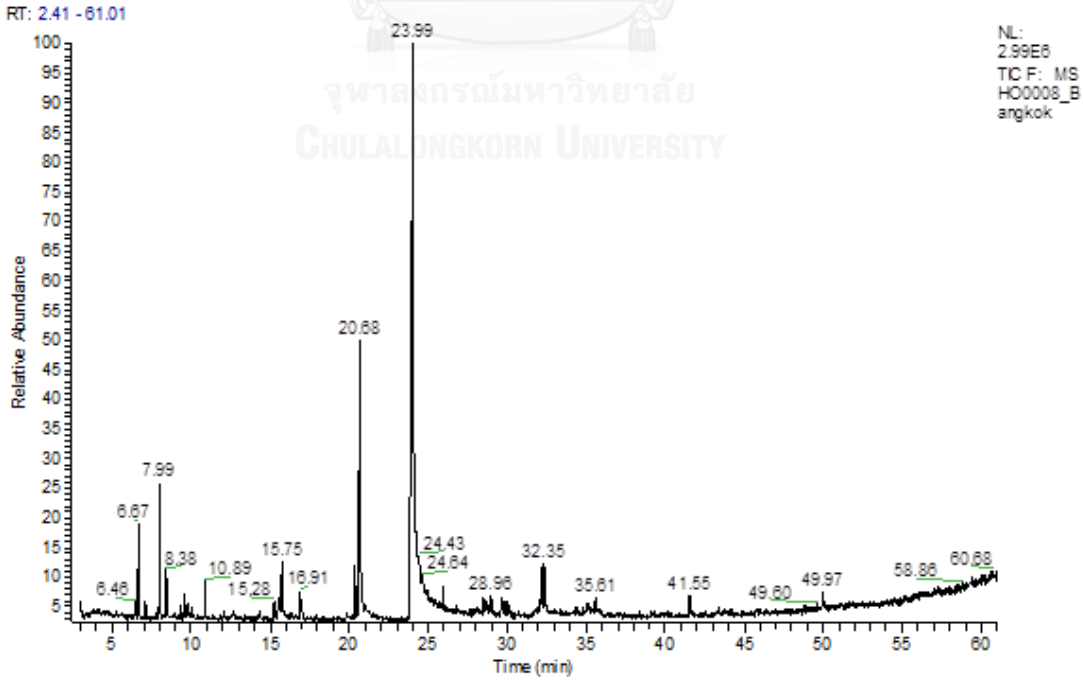


Figure 43 GC chromatogram of *H. cordata* oil from Bangkok (Source 08)

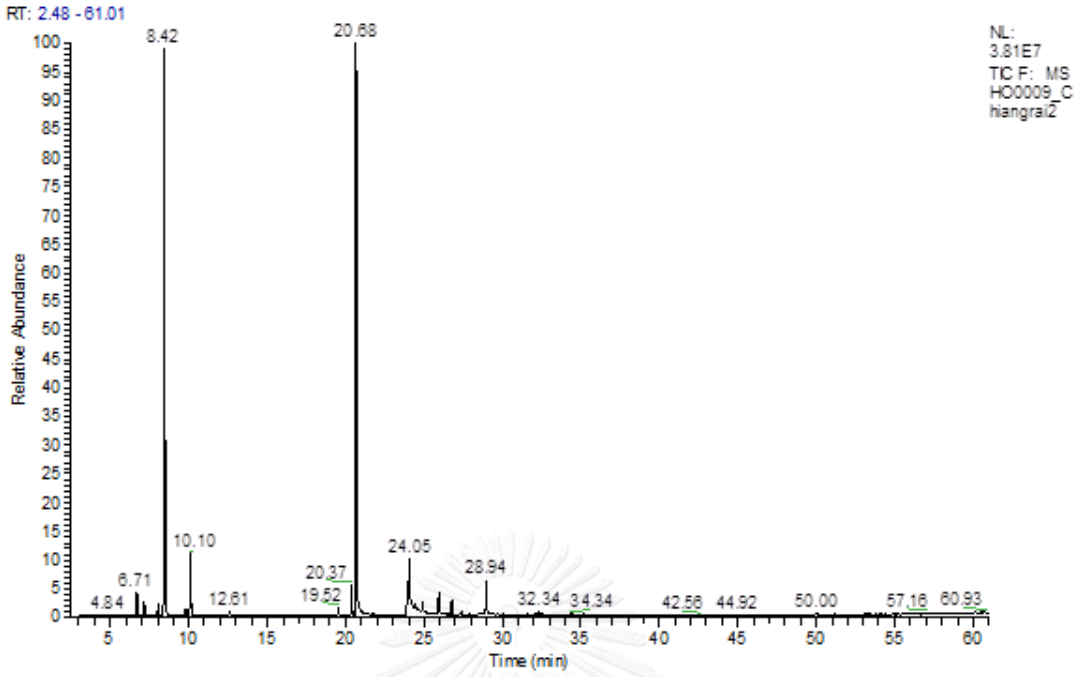


Figure 44 GC chromatogram of *H. cordata* oil from Chiangrai2 (Source 09)

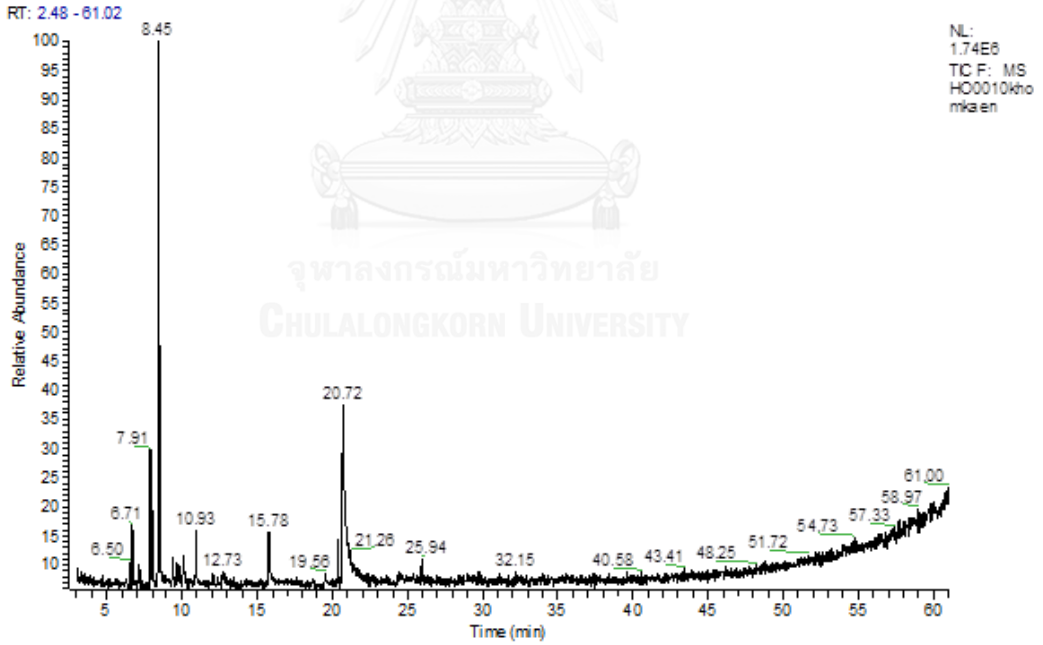


Figure 45 GC chromatogram of *H. cordata* oil from Khonkaen (Source 10)

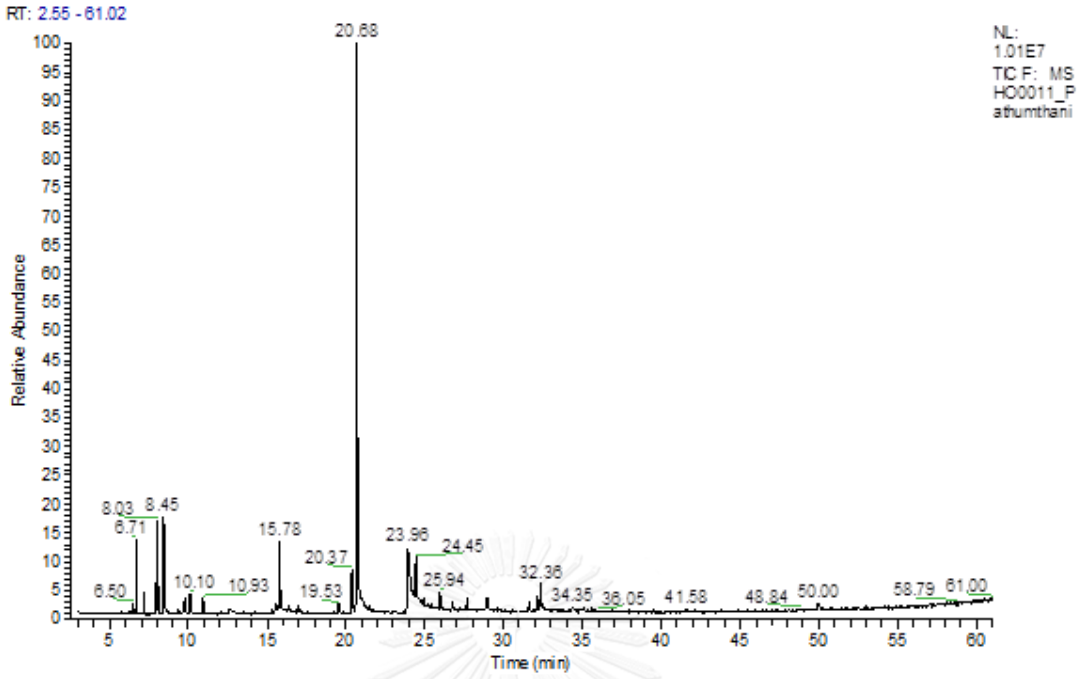


Figure 46 GC chromatogram of *H. cordata* from Pathumthani (Source 11)

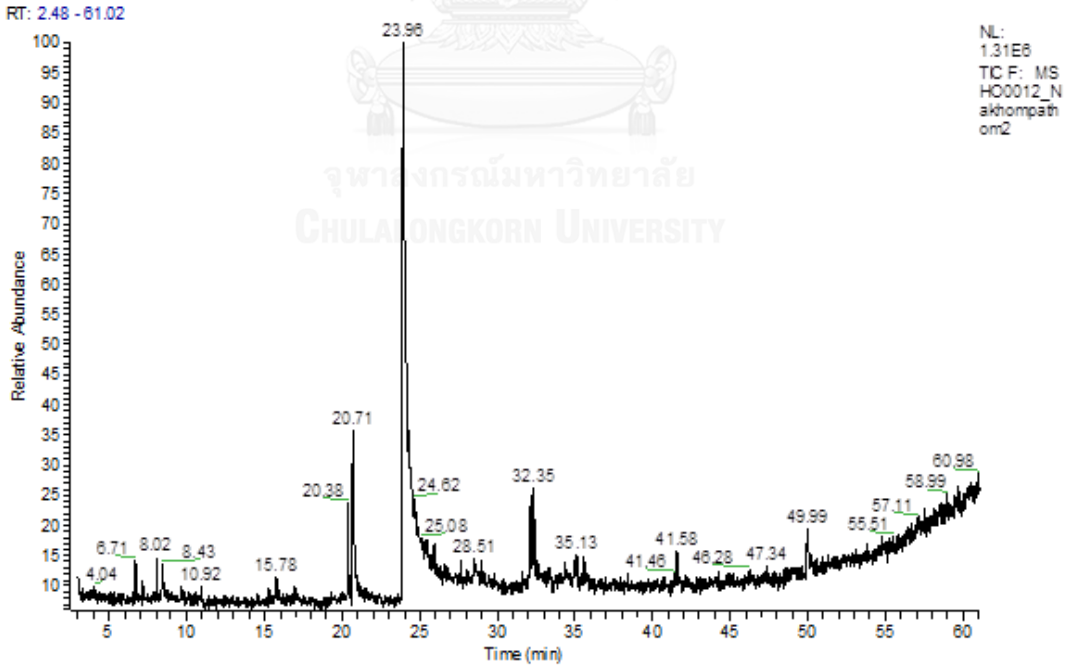


Figure 47 GC chromatogram of *H. cordata* from Nakhonpathom2 (Source 12)

Table 21 The composition of *H. cordata* oil

Compound name	RT	KI[a]	Occurrence in the oil					
			01	02	03	04	05	06
Thujene	6.50	930	0.00	0.00	0.00	0.61	0.00	0.00
α-pinene	6.71	939	1.82	1.26	1.85	1.23	0.00	3.79
Camphene	7.12	954	0.93	1.08	0.86	0.70	0.00	0.00
β-pinene	8.01	979	0.84	0.00	1.89	0.78	0.00	0.00
Myrcene	8.40	990	29.26	15.76	3.74	42.14	0.00	28.13
Cymene	9.60	1026	0.00	0.00	0.00	0.00	0.00	0.00
Limonene	9.77	1029	0.53	0.00	0.00	0.72	0.00	0.00
Ocimene	10.08	1050	3.63	1.57	0.00	1.28	0.00	0.00
Eucalyptol	9.87	-	0.62	0.00	0.00	0.00	0.00	0.00
Terpinene	10.93	1059	0.00	0.00	0.00	0.00	0.00	0.00
Borneol	15.28	1169	0.00	0.00	1.51	0.00	0.00	0.00
Arginine	15.55	-	0.00	0.00	0.00	0.00	1.34	0.00
4-Terpeneol	15.78	1177	0.00	0.00	3.00	0.00	0.94	0.00
Estragole	16.66	-	35.06	0.00	0.00	8.11	0.00	0.00
Decanol	16.91	1269	0.00	0.00	1.01	0.00	0.55	0.00
Carvyl acetate	19.81	-	0.00	1.80	0.00	0.00	0.00	0.00
Bonyl acetate	20.39	1288	3.07	5.28	8.99	3.39	3.56	10.01
2-undecanone	20.70	1294	14.94	52.29	33.98	31.07	11.21	31.12
Unidentify A	21.96	-	0.00	0.00	0.00	0.00	0.76	0.00
Decanoic acid	24.05	1361	0.00	10.02	28.26	0.00	69.39	0.00
Bergamotene	26.75	1412	1.47	0.00	0.00	1.14	0.00	0.00
2-dodecanone	27.52	1470	0.00	0.00	0.00	0.00	0.00	0.00

Table 21 The composition of *H. cordata* oil (Cont.)

Compound name	RT	KI[a]	Occurrence in the oil					
			01	02	03	04	05	06
2-Tridecanone	28.94	1495	1.64	3.62	1.09	2.67	2.51	7.30
Selinene	29.68	1498	0.00	0.00	1.83	0.59	0.77	2.74
Cadinene	29.72	1523	1.75	0.00	0.00	0.93	0.51	0.00
Cystine	30.06	-	0.36	0.00	0.00	0.00	0.61	0.00
Caryophyllene	32.35	1419	2.40	2.59	0.00	2.53	1.58	3.39
Dodecanoic acid	31.47	1566	0.00	0.00	0.00	0.00	0.79	0.00
Benzenethanamine, 2-5-difloro-a', 3-4-trihydroxy-Nmethyl	32.13	-	0.00	0.00	0.00	0.00	0.85	2.19
Caryophyllene oxide	32.35	1583	0.56	4.72	3.79	1.26	1.70	1.94
UniduntifyB	34.53	-	1.24	0.00	0.00	0.93	0.85	0.00

Table 22 The composition of *H. cordata* oil

Compound name	RT	KI[a]	Occurrence in the oil					
			07	08	09	10	11	12
Thujene	6.50	930	0.00	0.00	0.00	1.78	0.00	0.00
α-pinene	6.71	939	2.37	2.35	1.09	3.63	3.43	0.00
Camphene	7.12	954	1.07	0.00	0.64	6.76	0.94	1.16
β-pinene	8.01	979	2.47	3.93	0.60	3.17	4.03	0.00
Myrcene	8.40	990	3.16	1.41	30.39	33.08	5.03	0.00
Cymene	9.60	1026	0.79	0.76	0.00	1.46	0.00	0.00
Limonene	9.77	1029	0.68	0.00	0.46	2.56	0.88	0.00
Eucalyptol	9.87	-	0.00	0.00	0.00	0.00	0.00	0.00
Ocimene	10.08	1050	0.00	0.00	3.37	2.17	1.12	0.00
Terpinene	9.78	1059	0.68	1.37	0.00	2.69	1.43	0.00
Borneol	15.28	1169	0.87	0.77	0.00	0.00	0.00	0.00
Arginene	15.55	-	1.49	1.34	0.00	0.00	1.05	0.00
4-Terpeneol	15.78	1177	1.85	3.08	0.00	6.49	5.42	0.00
Estragole	16.66	-	0.51	0.00	0.00	0.00	0.00	0.00
Decanol	16.91	1269	0.79	1.28	0.00	0.00	0.00	0.00
Carvyl acetate	19.81	-	0.93	0.00	0.73	0.00	0.00	0.00
Bonyl acetate	20.39	1288	2.83	2.27	2.12	3.29	3.20	4.64
2-undecanone	20.70	1294	8.46	13.31	40.84	27.23	41.99	10.74
UnidentifyA	21.96	-	0.00	0.00	0.53	0.00	0.99	0.00
Decanoic acid	24.05	1361	68.14	56.89	10.23	0.00	14.47	65.82
Bergamotene	25.10	1412	0.00	0.00	0.99	0.00	0.00	0.00
2-dodecanone	27.52	1470	0.00	0.00	0.91	0.00	0.00	0.00

Table 22 The composition of *H. cordata* oil (Cont.)

Compound name	RT	KI[a]	Occurrence in the oil					
			07	08	09	10	11	12
2-Tridecanone	28.94	1495	1.64	3.62	1.09	2.67	2.51	7.30
Selinene	29.68	1498	0.00	0.00	1.83	0.59	0.77	2.74
Cadinene	29.72	1523	1.75	0.00	0.00	0.93	0.51	0.00
Cystine	30.06	-	0.36	0.00	0.00	0.00	0.61	0.00
Caryophyllene	32.35	1419	2.40	2.59	0.00	2.53	1.58	3.39
Dodecanoic acid	31.47	1566	0.00	0.00	0.00	0.00	0.79	0.00
Benzenethanamine, m2-5-difloro-a',3-4-trihydroxy-Nmethyl..	32.13	-	0.00	0.00	0.00	0.00	0.85	2.19
caryophyllene oxide	32.35	1583	0.56	4.72	3.79	1.26	1.70	1.94
Unidentify B	34.53	-	0.00	0.00	0.00	0.00	0.00	0.00

APPENDIX D

Antimicrobial activities of *H. cordata* volatile oil

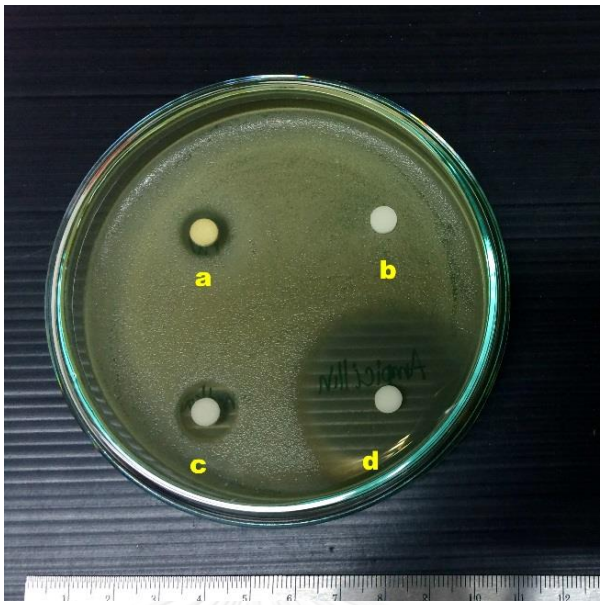


Figure 48 The inhibition zone of *Bacillus cereus* from: a*. H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

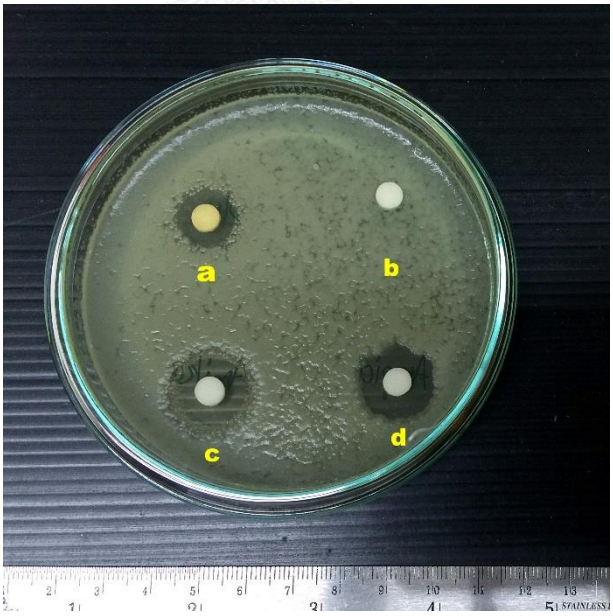


Figure 49 The inhibition zone of *Bacillus subtilis* from: a*. H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

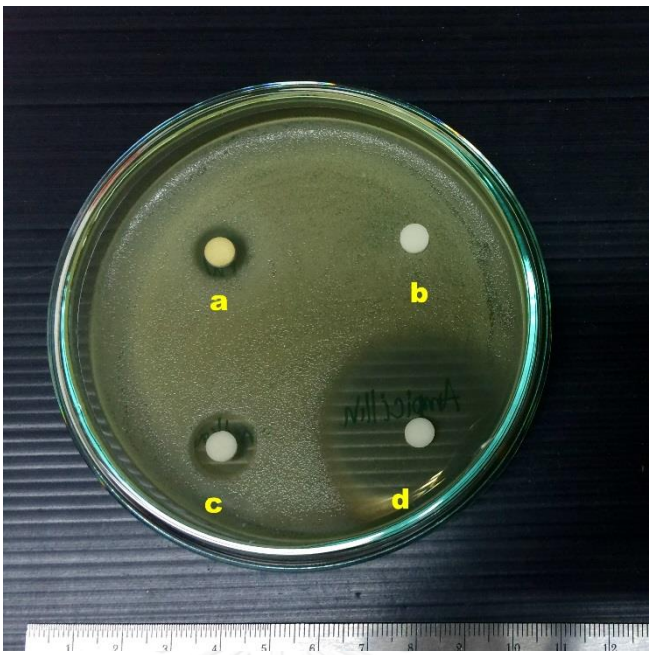


Figure 50 The inhibition zone of *Staphylococcus aureus* from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

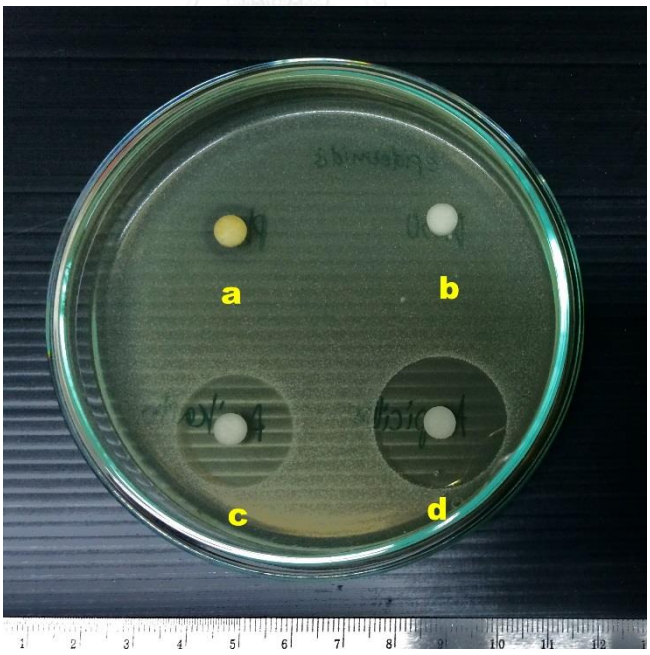


Figure 51 The inhibition zone of *Staphylococcus epidermidis* (Clinical isolate) from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

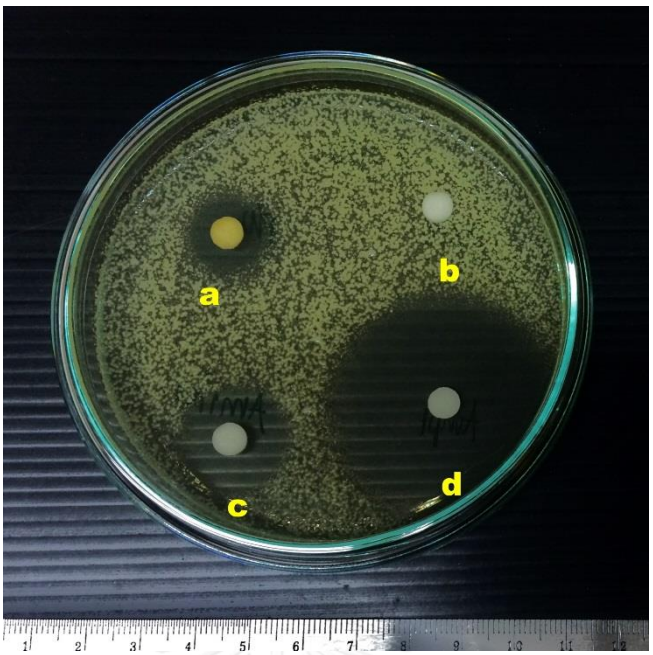


Figure 52 The inhibition zone of *Kocuria rhizophila* from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

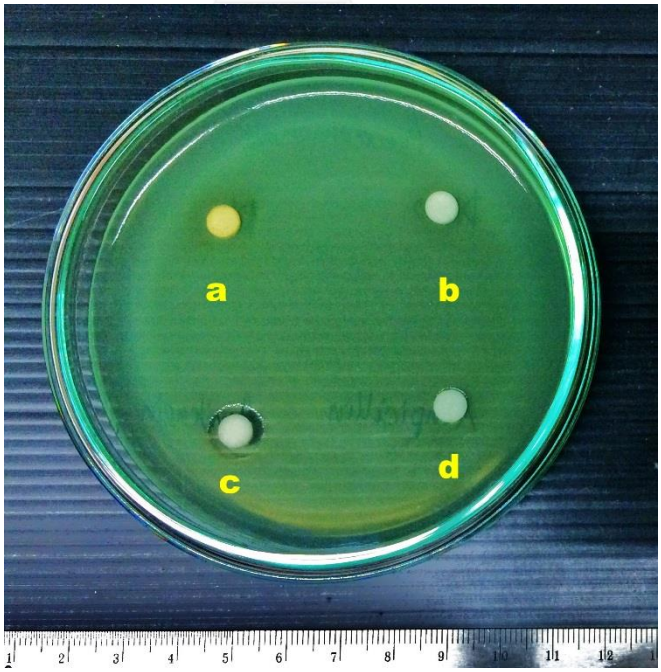


Figure 53 The inhibition zone of *Pseudomanas areruginosa* from: a*. H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

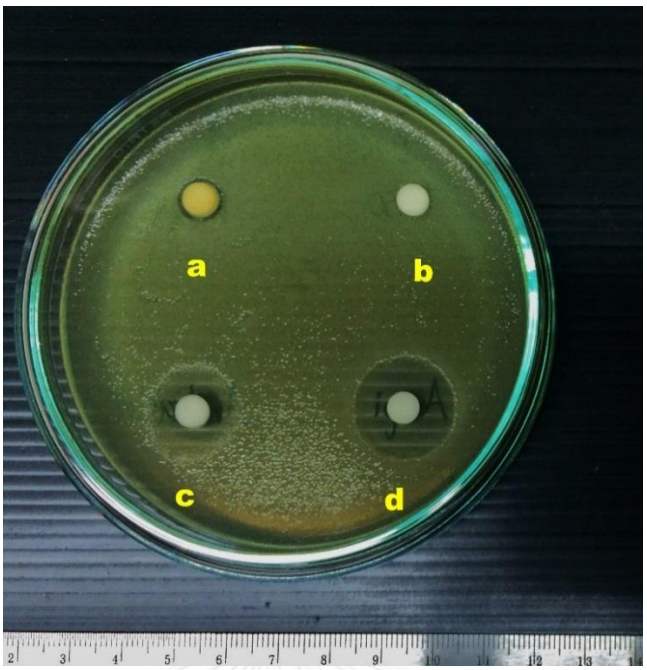


Figure 54 The inhibition zone of *Escherichia coli* from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

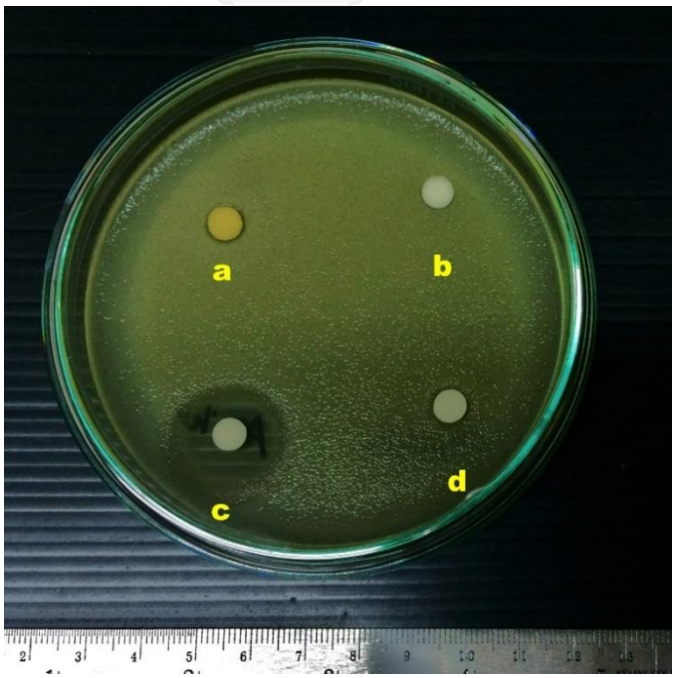


Figure 55 The inhibition zone of *Enterobacter aerogenes* from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

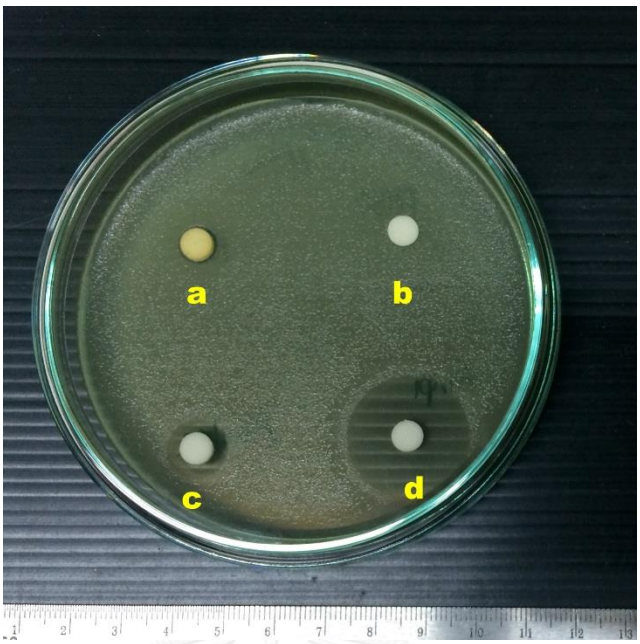


Figure 56 The inhibition zone of *Salmonella typhi* (Clinical isolates) from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

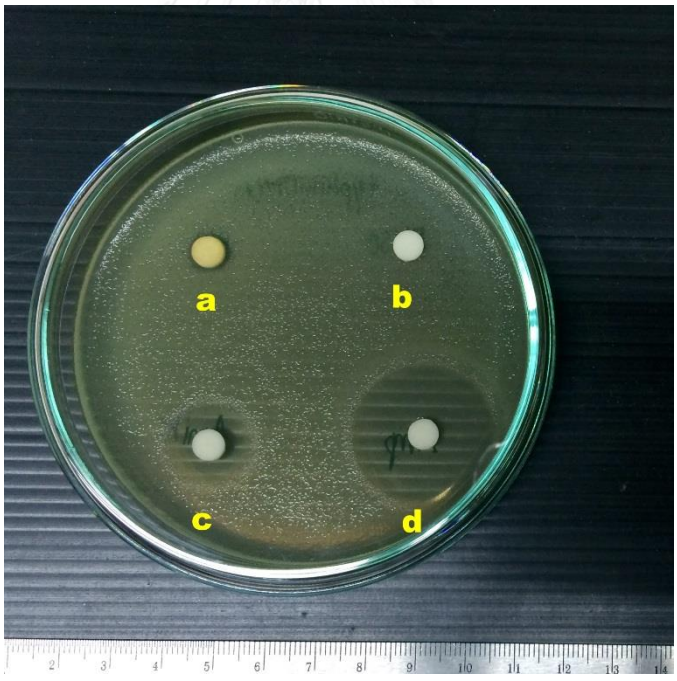


Figure 57 The inhibition zone of *Salmonella typhimurium* from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

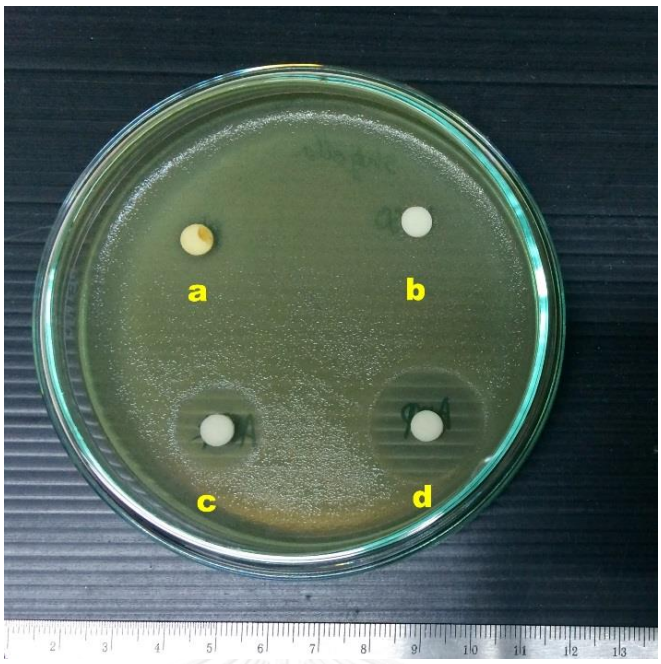


Figure 58 The inhibition zone of *shigella sp.* (Clinical isolates) from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

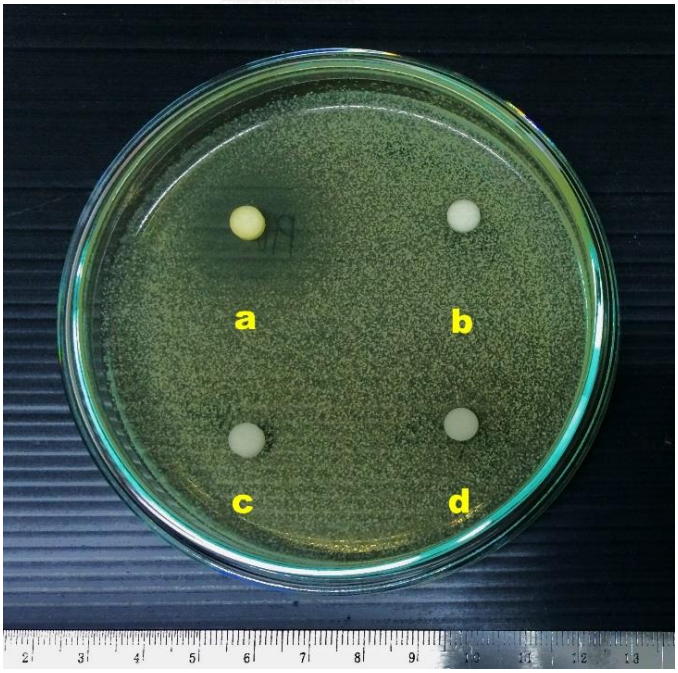


Figure 59 The inhibition zone of *Candida albicans* from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

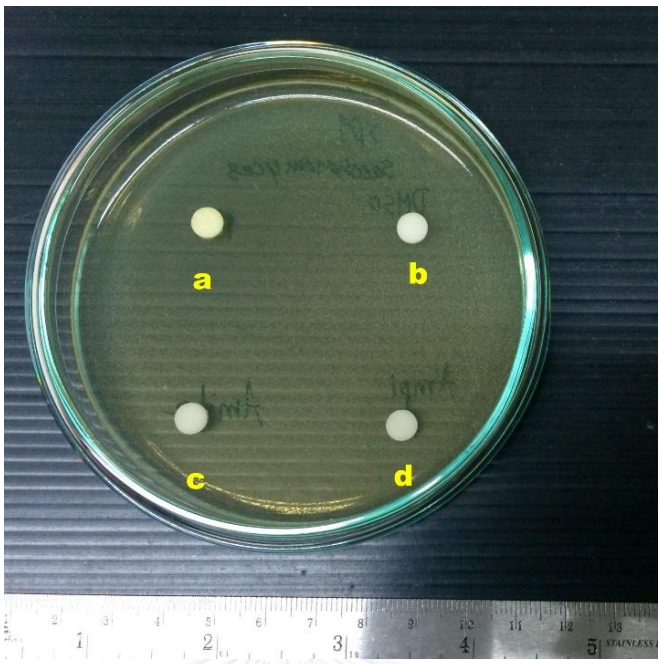


Figure 60 The inhibition zone of *Saccharomyces cerevisiae* from: a. *H. cordata* oil, b. DMSO, c. Amikacin sodium, d. Ampicillin sulfate

VITA

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Publication

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