ANTIMICROBIAL ACTIVITY AND CHEMICAL COMPOSITION OF ESSENTIAL OIL FROM MA SANG (*Feroniella lucida* (Scheff.) Swingle.) FRUIT

Tawan Nopanitaya

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ฤทธิ์ยับยั้งการเจริญของจุลินทรีย์และองค์ประกอบทางเคมีของน้ำมันหอมระเหยจากผลมะสัง (*Feroniella lucida* (Scheff.) Swingle.)

นางสาวตะวัน นพนิตย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2561

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

(Assoc. Prof. Dr. Griangsak Eumkeb)
Chairperson

(Asst. Prof. Dr. Sureelak Rodtong)
Member (Thesis Advisor)

(Assoc. Prof. Dr. Jirawat Yongsawatdigul)
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(Assoc. Prof. Dr. Worawat Meevasana)
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ตะวัน นพนิตย์ : ฤทธิ์ยับยั้งการเจริญของจุลินทรีย์และองค์ประกอบทางเคมีของน้ำมันหอมระเหยจากผลมะสัง (*Feroniella lucida* (Scheff.) Swingle.) (ANTIMICROBIAL ACTIVITY AND CHEMICAL COMPOSITION OF ESSENTIAL OIL FROM MA SANG (*Feroniella lucida* (Scheff.) Swingle.) FRUIT) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุรีลักษณ์ รอดทอง, 101 หน้า.

มะสัง *Feroniella lucida* (Scheff.) Swingle. เป็นพืชพื้นบ้านที่พบเจริญทั่วไปในประเทศไทย โดยเฉพาะอย่างยิ่งในภาคตะวันออกเฉียงเหนือ ใบ ดอก และผลของมะสังมีรสเปรี้ยวมักใช้ปรุงอาหารของคนท้องถิ่น ผลสดเต็มวัยของมะสังที่ศึกษามีเส้นผ่าศูนย์กลางโดยเฉลี่ย 5.73 ถึง 7.01 เซนติเมตร มีชั้นเปลือกนอก เปลือกแข็ง และเนื้อในติดกับเมล็ด เป็นสัดส่วนร้อยละ 9.64 และ 7.01 ของน้ำหนักผลสดทั้งหมด และมีความชื้นร้อยละ 51.21 57.15 และ 78.00 ตามลำดับ ชั้นเปลือกนอกมีการสะสมของต่อมน้ำมันหอมระเหย จากการศึกษาฤทธิ์การยับยั้งการเจริญจุลินทรีย์ และองค์ประกอบทางเคมีของน้ำมันหอมระเหยจากผลมะสังสดจากต้นที่พบเจริญตามธรรมชาติในจังหวัดบุรีรัมย์ ประเทศไทย พบว่าผลผลิตน้ำมันหอมระเหยที่สกัดด้วยน้ำจากผิวของผลมะสังสดที่เก็บจากต้นและผลร่วงโดยธรรมชาติ ซึ่งเก็บรวบรวมในเดือนกุมภาพันธ์ มีนาคม และกันยายน ไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) โดยให้ผลผลิตน้ำมันหอมระเหยในช่วงร้อยละ 0.59 ถึง 0.77 องค์ประกอบทางเคมีของน้ำมันหอมระเหยที่พบในทุกชุดของการสกัด มีสารหลักคือ ดีซิลอะซิเตท (ร้อยละ 58.61 ถึง 66.73) และเดคคานาล (ร้อยละ 7.79 ถึง 22.49) และยังพบสารอื่น ได้แก่ โดดีซิลอะซิเตท (ร้อยละ 6.81 ถึง 12.93) เดคานอล (ร้อยละ 3.95 ถึง 7.68) ออคตานอล (ร้อยละ 0.10 ถึง 0.46) และอัลฟา คาริโอไฟลีน (ร้อยละ 0.7 ถึง 5.7) น้ำมันหอมระเหยจากผลมะสังที่เก็บในเดือนกันยายนมีฤทธิ์การยับยั้งจุลินทรีย์ทดสอบได้ดีที่สุด โดยเฉพาะอย่างยิ่งแบคทีเรียแกรมบวก และยีสต์ เมื่อใช้น้ำมันหอมระเหย 8 มิลลิกรัมต่อแผ่นดิสก์ ทดสอบด้วยวิธีแพร่ผ่านเนื้อวุ้น พบบริเวณยับยั้ง *Bacillus cereus* TISTR 687 *Staphylococcus aureus* ATCC 1466 *Staphylococcus aureus* ATCC 29213 *Staphylococcus aureus* TISTR 517 *Staphylococcus epidermidis* TIRTS 518 *Staphylococcus xylosus* JCM 2418 และ *Saccharomyces cerevisiae* TISTR 5343 ที่มีเส้นผ่านศูนย์กลาง 7.25 14.25 11.22 12.20 18.45 25.93 และ 10.75 มิลลิเมตร ตามลำดับ และพบว่ามีความเข้มข้นของน้ำมันหอมระเหยระดับต่ำสุดที่สามารถยับยั้งการเจริญของจุลินทรีย์ทดสอบ และค่าความเข้มข้นต่ำสุดของน้ำมันหอมระเหยที่สามารถฆ่าจุลินทรีย์ทดสอบกลุ่มดังกล่าวข้างต้น ในช่วง 0.125 ถึง 2.0 และ 2.0 ถึง 16.0 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ ความเข้มข้นต่ำสุดของน้ำมันหอมระเหยที่ยับยั้งการเจริญของแบคทีเรียทดสอบชนิด

B. cereus TISTR 687 และ *S. aureus* ATCC 29213 ที่เท่ากับ 0.5 และ 0.125 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ และความเข้มข้นต่ำสุดที่ฆ่าการเจริญของจุลินทรีย์ทดสอบดังกล่าวข้างต้นที่ 4.0 และ 2.0 มิลลิกรัมต่อมิลลิตร ตามลำดับ ที่ความเข้มข้นดังกล่าวน้ำมันหอมระเหยจากผลมะสังแสดงการยับยั้งการเจริญ และฆ่าแบคทีเรียทดสอบทั้งสองชนิดภายในเวลา 2 ชั่วโมง และส่งผลก่อให้เกิดการรั่วไหลของโปรตีน และโพแทสเซียมไอออน ซึ่งแสดงให้เห็นถึงว่าน้ำมันหอมระเหยสามารถแทรกซึมเข้าสู่เซลล์ และรบกวนความสามารถในการซึมผ่านได้ของเยื่อหุ้มเซลล์แบคทีเรียทั้งสองชนิดได้ อย่างไรก็ดีน้ำมันหอมระเหยนี้ไม่มีผลต่อการก่อให้เกิดการรั่วไหลของสารโมเลกุลใหญ่ เช่น กรดนิวคลีอิก และเอทีพี ผลการเปลี่ยนแปลงรูปร่างของเซลล์แบคทีเรียทดสอบชนิด *S. aureus* ATCC 29213 จากการศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด เป็นลักษณะรอยย่น และหลุมบนผิวเซลล์ ยืนยันฤทธิ์ของน้ำมันหอมระเหยจากผลมะสังที่รบกวนการเจริญของเซลล์ และส่งผลให้เกิดการตายของเซลล์ในที่สุด จากผลการศึกษาดังกล่าวแสดงถึงการใช้ประโยชน์ผลมะสังเป็นแหล่งของน้ำมันหอมระเหย และเป็นแนวทางในการใช้ประโยชน์พืชชนิดนี้ต่อไป

สาขาวิชาปรีคลินิก
ปีการศึกษา 2561

ลายมือชื่อนักศึกษา_____
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

TAWAN NOPANITAYA : ANTIMICROBIAL ACTIVITY AND CHEMICAL COMPOSITION OF ESSENTIAL OIL FROM MA SANG (*Feroniella lucida* (Scheff.) Swingle.) FRUIT. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 101 PP.

FERONIELLA LUCIDA (SCHEFF.) SWINGLE/ ESSENTIAL OIL/ CHEMICAL COMPOSITION/ ANTIMICROBIAL ACTIVITY/MODE OF ACTION

Ma Sang, *Feroniella lucida* (Scheff.) Swingle., is a native plant widely found in Northeastern Thailand. The leaves, flowers, and fruits are sour taste and commonly used as food seasoning by local people. In the study, the mature Ma Sang fruits had the average diameters of 5.73-7.00 cm composing of peel, hard pericarp, and pulp with seeds at the proportions of 9.0, 64.0, and 27.0% of total wet weight. The moisture contents of each part of the fruit were 56.21, 57.15, and 78.00%, respectively. Only the peel was found to have essential oil glands. From the study of antimicrobial activity and chemical composition of essential oil from mature Ma Sang fruits collected from trees in their natural habitat in Satuek District, Buriram Province, it was found that yields of the essential oil extracted from the peel of mature fresh fruits collected from the tree and natural fallen fruits in different months of sampling lots using hydro-distillation were similar, which was around 0.59-0.77% (v/ww) ($p>0.05$). The chemical compositions of essential oil from all lots of samples were found to be decyl acetate (58.61-66.73%) and decanal (7.79-22.49%) followed by dodecyl acetate (6.81-12.93%), decanol (3.95-7.68%), 1-octanol (0.10-0.46%) and α-caryophyllene (0.7-5.7%) which were minor components. The oil obtained from the sampling lot in

September showed the greatest ability to inhibit the following test microorganism particularly, Gram-positive bacteria and yeast by disk diffusion assay using 8 mg/disc, *Bacillus cereus* TISTR 687, *Staphylococcus aureus* ATCC 1466, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* TISTR 517, *Staphylococcus epidermidis* TIRTS 518, *Staphylococcus xylosus* JCM 2418 and *Saccharomyces cerevisiae* TISTR 5343 with the inhibition zones of 7.25, 14.25, 11.22, 12.20, 18.45, 25.93, and 10.75 mm, respectively. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the essential oil against the test microorganisms were found in the range of 0.125-2.0 and 2.0-16.0 mg/mL, respectively. The most sensitive microorganisms were bacteria, *B. cereus* TISTR 687 and *S. aureus* ATCC 29213 which showed the lowest MIC of 0.5 and 0.125 mg/mL and MBC at 4.0 and 2.0 mg/mL, respectively. The essential oil had a significant effect to inhibit growth of the tested bacteria within two hours. The oil caused protein and potassium leakage resulting in the disruption of bacterial cell membrane permeability. However, the oil had no effect on the leakage of large molecules such as nucleic acids and adenosine triphosphates (ATP). The effect of the essential oil on morphological alteration of bacterial cells was tested for *S. aureus* ATCC 29213. Under SEM observation, cells with crease and furrow were detected. The alteration interfered growth and ultimately resulted in cell death. These findings reveal that Ma Sang fruits are the important source of essential oil providing chances for further application of the plant.

School of Preclinic

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Advisor's Signature _____

Co-advisor's Signature _____

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LIST OF ABBREVIATIONS

α	Alpha
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
ATCC	American Type Culture Collection
β	Beta
BSA	Bovine serum albumin
°C	Degree Celsius
CFU	Colony forming unit
CO_2	Carbon dioxide gas
cm	Centimeter
Co., Ltd.	Limited company
DNA	Deoxythymidine triphosphate
et al.	et alia (and others)
e.g.	For example
g	Gram
γ	Gamma
GC-MS	Gas chromatography–mass spectrometry
h	Hour
HCl	Hydrochloric acid

LIST OF ABBREVIATIONS (Continued)

JCM	Japan Collection of Microorganisms
kg	Kilogram
μg	Microgram
μL	Microliter
MBC	Minimum inhibitory concentration
MIC	Minimum bactericidal concentration
min	Minute
mg	Milligram
mL	Milliliter
mm	Millimeter
ng	Nanogram
nm	Nanomater
%	Percentage
PBS	Phosphate buffer saline
RLU	Relative Light Unit
TISTR	Thailand Institute of Scientific and Technological Research
U.K.	United Kingdom
U.S.A..	United States of America
UV	Ultraviolet
v/ww	Volume by wet weight
w/v	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Ma Sang (*Feroniella lucida* (Scheff.) Swingle.), a native plant commonly found in North-eastern Thailand, belongs to Rutaceae family, Aurantiodeae subfamily. It is also a native plant in Indonesia, Cambodia, and Laos. The plant is a medium tree having 5-10 m high found in particularly in deciduous dipterocarp and dry evergreen forests. Fruits and leaves are sour taste used as an ingredient for cooking soup and chili paste by local people. Root and young leaves have been known to be used as Thai traditional medicine for cure fever, diarrhea, latulence ulcers, cancer and cardiovascular diseases (Supudompol, 2009). Currently, Ma Sang trees were popular grown as bonsai for ornamental plants. Ma Sang trees were also well known among gardeners to plant for grafting on citrus plants for increasing growth rate and decreasing virus infection during citrus growth as confirmed in the study of Yoshida (1996). The inorganic chemical extracts of twigs, barks, and root of Ma Sang were studied for their chemical compounds and biological activity, for example; lucidenal and lucidafuranocoumarins from twigs showed potential cytotoxicity against cancer cell, feronielloside from root had significantly as an inhibitor of acetylcholinesterase (Phoopichayanun *et al*., 2008; Sriyatep *et al.*, 2012; Sriyatep *et al.*, 2014). The leaves of Ma Sang were used to extract essential oil for anti-mycobacterial activity against *Mycobacterium tuberculosis* (Supudompol, 2009).

There are limited studies available regarding using Ma Sang fruits in term of essential oil particularly the study its chemical composition and antimicrobial activity. From our previous investigation, the estimation of yield of Ma Sang fruit based on a community in Isaan (Satuek District, Buriram Province), was come up with approximately 700 kg in each crop for each tree. The Ma Sang fruit composed of the peel, hard pericarp, and pulp with seeds. The essential oil was extracted from each separated part of the fruit by hydro-distillation. Only peel could provide oil of 0.37% by wet weigh. In order to increase the use of Ma Sang fruits, this research aims to determine the antibacterial activity of essential oil from Ma Sang fruits and their mode of antimicrobial action of the oil to selected standard test microorganisms.

1.2 Research objectives

1) To determine antimicrobial activity of essential oil from Ma Sang fruit.

2) To detect the chemical composition of the essential oil.

3) To investigate mode of antimicrobial action of the essential oil on test microorganisms.

1.3 Research hypotheses

Essential oil could be extracted from mature Ma Sang (*Feroniella lucida* (Scheff.) Swingle.) fruits, and its chemical composition and major components in the essential oil could be analyzed. Antimicrobial activity of the essential oil could be determined as the minimum inhibition concentration (MIC), and the minimum bactericide concentration (MBC). The Ma Sang essential oil could perform killing rate mode of action, and cell morphology changes of the treated bacteria.

1.4 Scope and limitations of the study

Ma Sang fruits were collected from the available area in North-eastern Thailand. The essential oils were then extracted from the peel. Chemical compositions of the essential oil were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The essential oil was tested for antimicrobial activity against selected standard test microorganisms (Gram-positive and Gram-negative bacteria and fungi). The minimum inhibition concentration (MIC) and the minimum bactericide concentration (MBC) of the essential oils were determined. Mode of antibacterial actions of the essential oil was examined by detecting of permeability and integrity of cell wall and cell membrane.

1.5 Expected results

Data supporting the extraction, yield, and application of essential oil from Ma Sang (*Feroniella lucida* (Scheff.) Swingle.) fruit and in term of antimicrobial activity, chemical composition, and mode of action of the oil were achieved. Results from this study could also contribute to the development of products from Ma Sang fruits.

CHAPTER II

LITERATURE REVIEWS

2.1 Ma Sang (*Feroniella lucida* (Scheff.) Swingle.)

2.1.1 Distribution and characteristics

Ma Sang (*Feroniella lucida* (Scheff.) Swingle.) found in Asian countries, particulary Indonesia, Cambodia, Thailand, Laos, and Vietnam Different common names of the plant were called such as Canthan in Vietnam, Ka Sang in Cambodia, and Ma Sang in Thailand (Backer *et al.*, 1965). Ma Sang grows wild as a small tree having 5 to 10 m high in North-Eastern Thailand or Isaan region, particularly in deciduous dipterocarp forest, dry evergreen forest, and rice field. The plant belongs to the genus *Feroniella* genus in the Rutaceae family, Aurantioideae subfamily, Citreae tribe, Balsamocitrinae subtribe (Swingle *et al.*, 1967). The classification of Aurantiodeae subfamily was recognized by Swingle and Reece in 1967. The subtribe Balsamocitrinae had no pulp-vesicles but had a hard woody exocarp. The subtribe was found in tropical Africa and Asia from India to Indo-China and the northern Philippines. (Swingle *et al.*, 1967). “The Botany of Citrus and Its Wild Relatives” Swingle (1967) described characteristics of *Feroniella lucida* in the book as follows, the species usually grow at less than 400 m altitudes. It is a medium-sized tree, 10 to 15 m tall, with a straight trunk 20 to 30 cm in diameter. The characteristic of the plant was shown in Figure 2.1.

Twigs are slender with gray color, and young tips usually have small hairs. Twigs are slender with gray color, and young tips usually have small hairs. Leaves often have 2 to 5 pairs. Leaflets are the elliptic or obovate shape having obtuse at the apex. Large oil glands present near margin. Panicles of the flower are loose with 3 flowers. The cymes often contain two or three groups in the axils having small hairs. Flowers are usually a hermaphrodite flower with large size.

2.1.2 Application of Ma Sang plant

Several parts of Ma Sang plant have been reported to be useful. These parts are as follows:

A) Leaves and fruits

In Cambodia and Indonesia, *Feroniella lucida* used to propagate as fruit tree. The pulps of the raw fruits are commonly used as an ingredient for soup by local people. The fruit pericarps are applied as medicinal ingrediends (Srichaiwong *et al.*, 2013). Essential oils from leaves of *F. lucida* have been reported to compose of various compounds including β-caryophyllene (26.6%), dodecanal (18.5%), decanal (16.4%) and decyl acetate (13.3%). The oils exhibited a weak anti-mycobacterium (Supudompol, 2009).

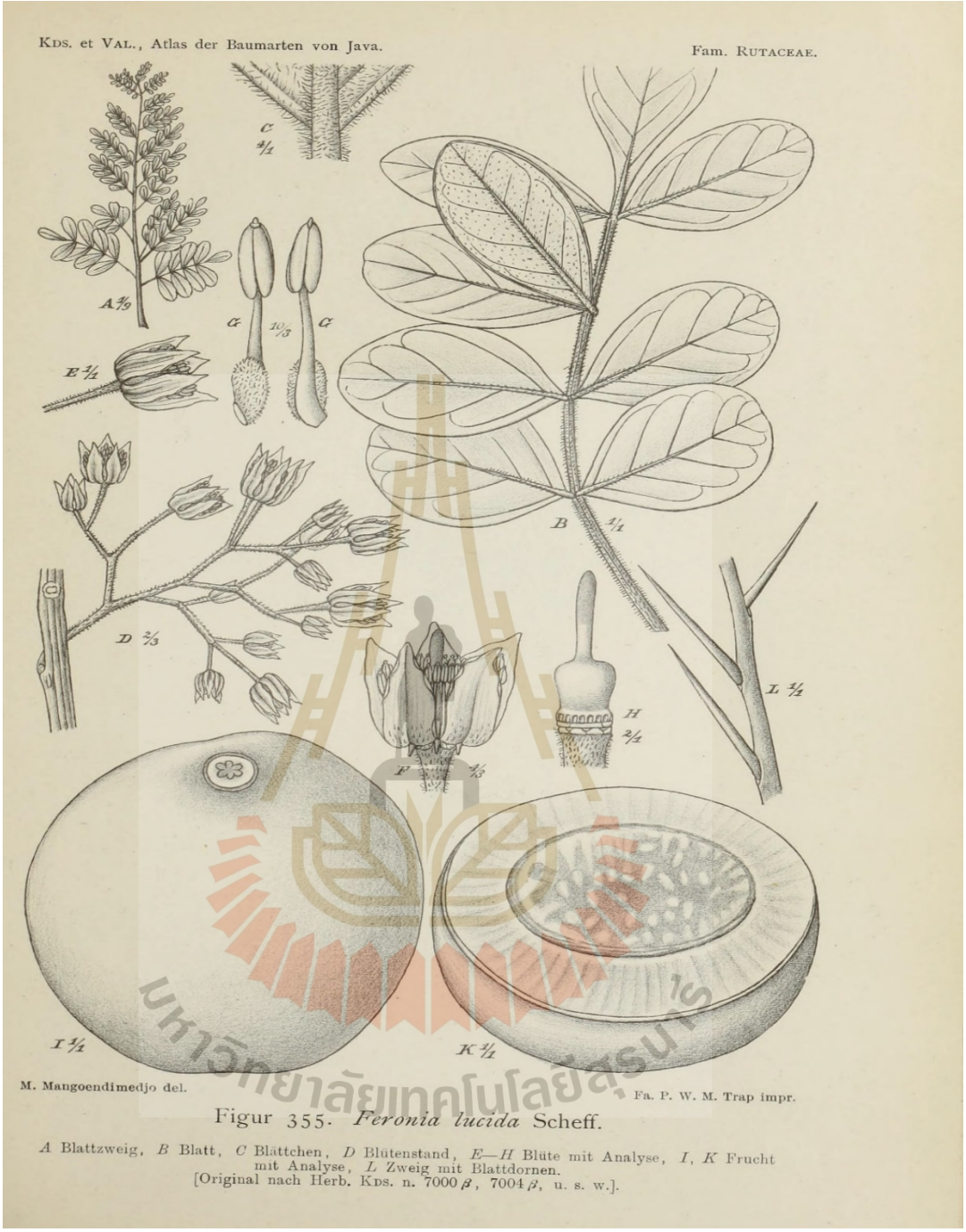

Figure 2.1 Illustration of morphological characteristics of Ma Sang (*Feroniella lucida* (Scheff.) Swingle.) by Mangoendimedjo M: leaf arrangement (A), leaves (B), leaf base (C), inflorescences (D), flower(E), flower arrangement (F), androecium (G), gynoecium (H), fruit (I), fruit cross section (K), and twig (L) (Koorders and Valeton, 1914).

B) Twig and bark

The dichloromethane (CH_2Cl_2) extract of the twigs of *Feroniella lucida* was reported to contain two ligands, lucidenal and more 26 identified components. Lucidenal showed moderate cytotoxicity against several types of cancer cell lines, HuCCA-1 (human cholangiocarcinoma), A549 (human lung carcinoma), MOLT-3 (acute lymphoblastic leukemia), and HepG2 (human hepatocellular liver carcinoma). The 2',3'-epoxyanisolactone which obtained from the same extract also exhibited cytotoxic activity to MOLT-3 cell (lymphoblastic leukemia), and inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* (Sriyatep *et al.*, 2014). Moreover, the acetone extract of the twigs was reported to contain lucidafuranocoumarins B and C together with bergamottin, anisolactone, 2,3-dihydroxyanisolactone, imperatorin, and umbelliferone. Lucidafuranocoumarins B, C, and umbelliferone showed cytotoxicity to KB (oral cavity cancer), MCF-7 (breast cancer) and NCI-H187 (small cell lung cancer) (Sripisut *et al.*, 2012). The dichloromethane extract of the bark *Feroniella lucida* contained feroniellides D and E compounds which represented cytotoxicity to human KB carcinoma (Phuwapraisirisan *et al.*, 2013).

C) Root

The root of the plant has been considerably for extracting various compounds which might be used as a remedy for antiulcer, cardiovascular diseases, and cancer (Supudompol, 2009). For example, furanocoumarin; feroniellins A and B, these compounds were isolated from methanol extract of the plant root. The components showed cytotoxic effect, *in vitro*, on human KB carcinoma and human HeLa carcinoma cells (Phoopichayanun *et al.*, 2008). Phuwapraisirisan *et al.* (2007) reported

feroniellin B from similar extraction that could inhibit human platelet aggregation. In addition, feronielloside extracted from the root plant had significantly effect as an inhibitor of acetylcholinesterase, enzyme inducing Alzheimer's disease (Phoopichayanun *et al.*, 2008). Sripisut, Cheenpracha and Laphookhieo (2011) reported the finding of lucidafuranocoumarin coumarins and alkaloids in root extract which had strong cytotoxicity against KB human cancer cell lines with IC_{50} of 0.637 μg/mL and antimalarial activity against *Plasmodium falciparum* and *Mycobacterium tuberculosis*.

D) Tree

Yoshida (1996) reported that the plant could decrease the rate of citrus tristeza virus infection which usually infects citrus species also increase the rate of citrus growth when was grafted on the citrus plant. Currently, Ma Sang is increasingly interested in growing as a bonsai and ornamental plant (Phoopichayanun *et al.*, 2008).

2.2 Essential oils

2.2.1 Properties of essential oils

Essential oil is oily liquid substances composed of mixture aromatic compounds of low molecular weight which less than 500 daltons and characterized by a strong odor (Burt *et al.*, 2004; Raut and Karuppayil, 2014). Properties of essential oil are hydrophobic which insoluble in water and soluble in alcohol, ether, and natural oils and sensitive to oxygen, heat, or light (Mahato *et al.*, 2017). They are natural products obtained from various parts of certain plants such as leaves, barks, stems, roots, flowers, seeds, and fruits that commonly stored in secretory cells, cavities, canals, epidermis cells or glandular trichomes. Plants produced essential oils as secondary metabolites for

playing role as antibacteria, antivirals, antifungals, insecticides, and also attracting pollinators. (Bakkali *et al.*, 2008; Calo *et al.*, 2015). Plants in various families have been reported to be rich of essential oils, for example; Myrataceae (tea tree, eucalyptus, clove and spices), Laurenceae (cinnamon), Lamiaceae (lavender, rosemary, majoram, basil and pepermint), Apiaceae (anise, cumin,dill and fennel), Zingiberaceae (ginger) and Rutaceae (bergamot, lemon and oranges) (Figueiredo *et al.*, 2008). Many were methods used for isolation and extraction of essential oils such as distillation, pressure, or extraction with organic solvents or supercritical carbon dioxide (Bakkali *et al.*, 2008). However, stream distillation is the preferred method for extraction of essential oil in commercial-scale (Bakkali *et al.*, 2008; Raut and Karuppayil, 2014).

Consequently, chemical compositions of essential oil are analyzed by gas chromatography or gas chromatography and mass spectroscopy (Burt, 2004). On the other hand, substituents of other plant extracts might be examined by phytochemical screening tests for alkaloids, tannins, flavonoids, saponins, glycosides, sterpids and coumarins (Farnsworth, 1966). The chemical compositions of essential oil are normally terpenoids, phenylpropanoids such as monoterpenes and sesquiterpenes which represent around 90% of essential oils reported. Aromatic and aliphatic constituents such as aldehyde, alcohol, phenols, methoxy derivatives, and methylene dioxy compounds are also detected (Raut and Karuppayil, 2014 and Chavez-Gonzalez *et al.*, 2016).

2.2.2 Chemical compositions of essential oils

Chemical compositions of essential oils vary from twenty to hundreds of different substances (Bakkali *et al*., 2008). Major components are characterized by one or two compounds which represented a high proportion in rage 20 to 70% in total oils.

Other substances are minor components. The oil compositions are usually analyzed by gas chromatography (GC) or gas chromatography and mass spectroscopy (GC-MS) (Burt, 2004). Some substituents of plant extracts might be determined by phytochemical screening tests, such as alkaloids, tannins, flavonoids, saponins, glycosides, sterpids, and coumarins (Farnsworth, 1966). Typically, the chemical compositions of essential oil comprise of monoterpene, sesquiterpenes, alcohols, acids, esters, epoxides, aldehydes, ketones, amines, and sulphides. The oil compound can be divided into two groups, terpene and its derivatives compounds and aroma compounds as (Figure 2.2) (Bakkali *et al.*, 2008; Calo *et al.*, 2015). In another group can be followed separated by volatile properties. Volatile compounds are further categorized as alcohols, ethers, aldehydes, ketones, esters, amines, amides, phenols, heterocycles, and terpenes. The non-volatile compounds include long chain hydrocarbons, fatty acids, sterols, carotenoids, and oxygenated heterocyclic compounds (Mahato *et al.*, 2017). Chemical compositions of essential oils are the important part to determine the use of essential oils. These compositions depend on the plant itself (species, variety, development stage, part of plant used). Plant cultivation, environment, harvest time and extraction methods (Ribeiro-Santos *et al.*, 2017; Mahato *et al.*, 2017).

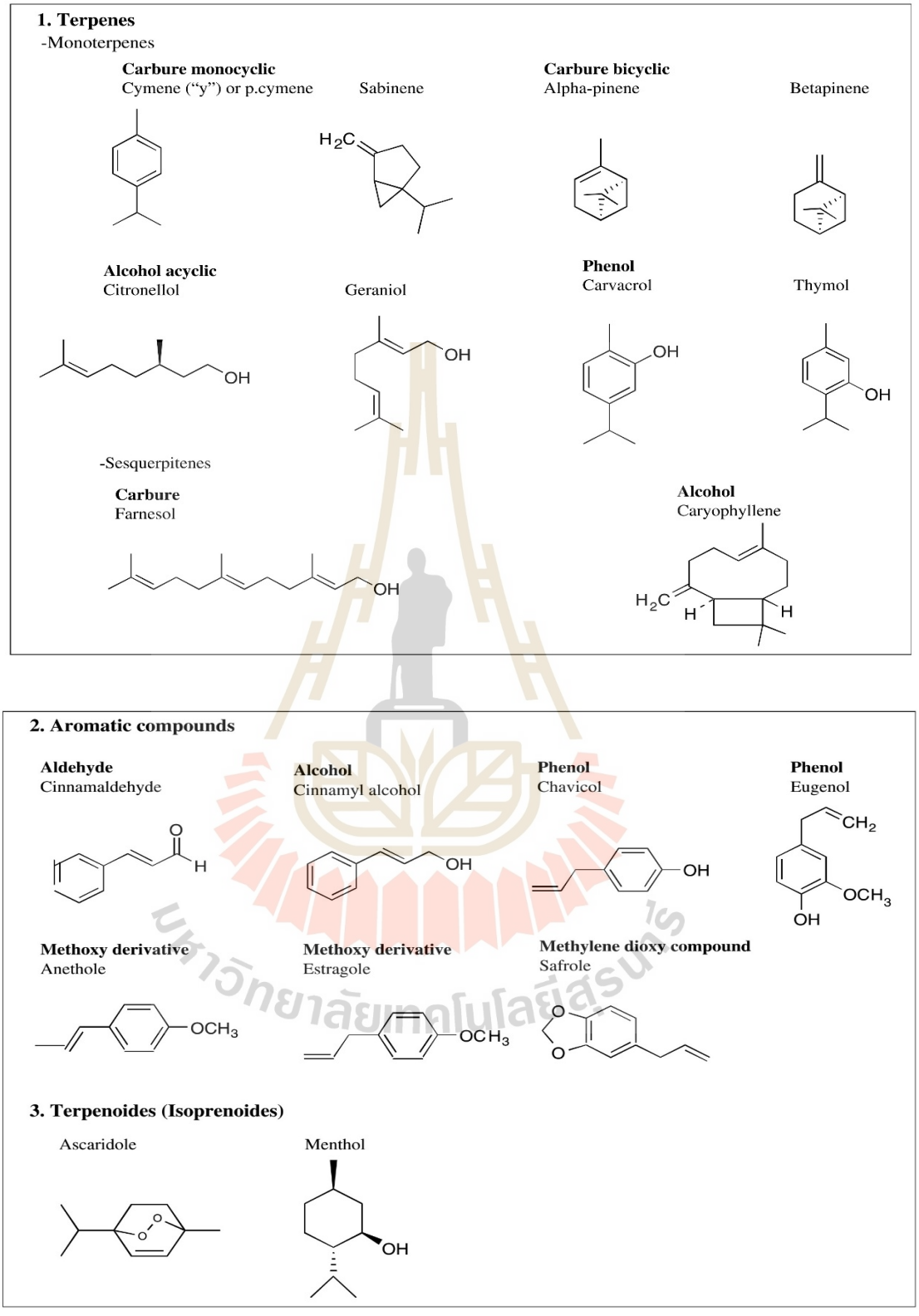

Figure 2.2 Chemical structure of selected components of essential oil (Bakkali *et al.*, 2008).

2.2.3 Antimicrobial activity of essential oils

Currently, there has been a significant increase in the occurrence of antibiotic-resistant bacterium strains, which have made the antibiotic options for unlimited and expensive infection control (Li and Webster, 2018). Essential oils could be applied as one natural and effective antimicrobial against several foodborne and human pathogens with a bored range of activity resulting from their complex mixture components (Burt, 2004; Swamy *et al.*, 2016). The essential oil of plant gets attention as a natural antimicrobial substance. The oil has been useful for several applications such as chemical preservatives in the control and inactivation of pathogens in commercially produced food systems and effective agents for the control of plant pathogens. For a long period of time, natural therapies have been ruled in maintaining human health. In Brazil, the plant compounds have been being used for pharmaceutical purposes (Nascimento *et al.*, 2000). According to the World Health Organization (WHO), medicinal plants may be the best source to obtain a variety of drugs because people increase concern about chemical antibacterial agents causing health risks in human (Fleming-Jones and Smith, 2003). Thus, natural plant components are leading choice for safe antimicrobial agents in both food and human (Fisher and Phillips, 2008; Tajkarimi, Ibrahima, and Cliver, 2010). Studies on the effects of essential oils have begun since 1977 with the investigation of Australian tea tree oil that showed an ability to inhibit pathogenic *Escherichia coli* of animal (Hayes *et al.*, 1997). In Argentina, a research project tested 122 known plant species extracted compounds, twelve plants were found to have great ability to inhibit the growth of *Staphylococcus aureus*, ten plants inhibited *Escherichia coli*, and four plants inhibited *Aspergillus nige*r (Anesini and Perez, 1993).

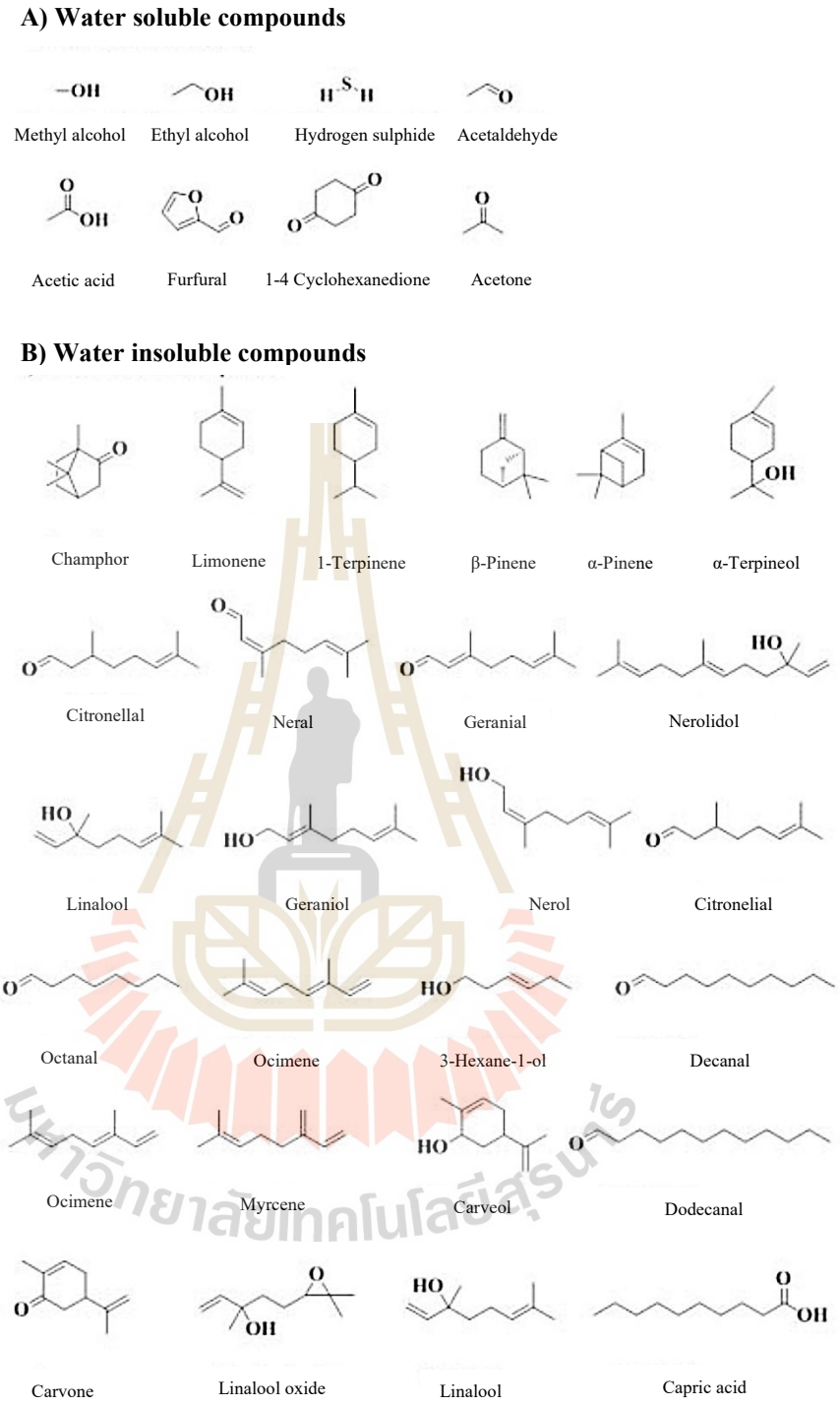

Figure 2.3 Major water-soluble (A) and water-insoluble (B) aroma producing compound present in citrus essential oil (Mahato *et al.*, 2017).

Currently, the study of essential oils from spices has received continuous attention. The essential oils showed strong effective activity to a wide range of bacterial pathogens, for example, essential oil of ginger root, jasmine, carrot seed, celery seed, and orange bitter showed activity against *Campylobacter jejuni* while oregano, bay leaf, clove bud, lemongrass, and allspice oils have been revealed effective against *Esherichia coli* O157:H7, and marjoram showed its ability to inhibit *Salmonella enterica* (Calo *et al.*, 2015). Cinnamon, clove, and lime oils were found to be able to inhibit both Gram-positive and Gram-negative bacteria (Prabuseenivasan *et al.*, 2006). The essential oil from coriander (*Coriandrum sativum* L.) has been to inhibit several potential spoilage bacteria such as *Klebsiella pneumoniae*, *Bacillus megaterium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Escherichia cloaca*, *Enterococcus faecalis*, and a potential to inhibit important oral fungus pathogen, *Candida* spp. with minimum inhibition concentrations (MICs) ranging 15.6 to 31.2 µg/mL, and minimum fungicidal concentrations (MFCs) 31.2 to 62.5 µg/mL (Mandal and Mandal, 2015). Cinnamon and clove were the famous source of essential oils that showed strong effect to inhibit various pathogens including *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 14990, *Enterococcus faecalis* ATCC 29212, *Streptococcus pyogenes* ATCC 1915, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Aeromonas hydrophila* ATCC 7966, *Proteus mirabilis* ATCC 10005, *Klebsiella pneumoniae* ATCC 13883, and *Candida albicans* ATCC 10231 and their biofilms production (Condò *et al*., 2018).

The antibacterial agents from essential oils are the most useful chemical preservatives to control and inactivate pathogens in commercially produced food systems, and useful as the effective agents for controlling of animal and plant

pathogens. (Kalemba and Kunicka, 2003). The necessary sources for producing essential oils were plant. Currently, over 3000 essential oils were known whereas particularly 300 using in commercially pharmaceutical, agroeconomic, cosmetic, sanitary and perfume industry (Bakkali *et al*., 2008). Study and development of a variety of plants using in term of essential oils has been incessantly found. Prévost *et al*. (2018) reported interesting rich of phytol and n-octanol essential oils from *Morinda lucida* fruits showing good antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* within inhibition zones and MIC at the range of 11.6 to 24.3 mm and 32 to 256 µg/mL, respectively. The essential oil extracted from fresh fruits of *Senna occidentalis* exhibited better antimicrobial activity at MIC 78–312 µg/mL against *Escherichia coli*, *Staphylococcus aureus, Bacillus subtilis*, and *Aspergillus niger* (Essien *et al*., 2019). The study reported about the essential oil from *Eucalyptus globulus* fruit exhibited an interesting antibacterial activity against pathogenic and spoilage microorganisms, *Staphylococcus aureus* and *Pseudomonas aerugenosa*, which are known for their resistance to several antibiotics (Bey-Ould Si Said *et al.*, 2016). The essential oil from *Nandina domestica* Thunb was found to have high potential to be used as alternative that synthetic preservatives (Bajpai *et al.*, 2008). Eldahshan and Halim (2016) reported that the essential oil from leaves of Egyptian navel orange trees showed greater effective as an antimicrobial agent on *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Aspergillus fumigatus* than essential oils extracted from its branches. In addition, the study of functional activities of cold-pressed and water distilled peel essential oil of *Citrus paradisi* and *Citrus grandis* (L.) Osbeck revealed that *Citrus grandis* oils were rich in oxygenated or nitrogenated compounds which might be involved in reducing

cardiovascular diseases or enhancing sleep effectiveness. The antimicrobial activities of distilled *Citrus grandis* oil could inhibit *Escherichia coli* and *Salmonella enterica* (Ou *et al.*, 2015).

Many essential oils also have been reported for their ability to inhibit fungi. Boubaker *et al.* (2016) presented that *Thymus leptobotrys* essential oil displayed completely inhibiting the spore germination of *Geotrichum citri-aurantii*, *Penicillium digitatum*, and *Penicillium italicum*. Neroli oil exhibited a very strong antifungal activity (Haj Ammar *et al.*, 2012). Examples of various plants and thier essential oils as antibacterial agents against several microorganisms were showed in Table 2.1. Besides, the study in 2015 suggested the possible efficacy of alternative therapies by photoactivation of citrus essential oil with fluconazole and indocyanine green to increase the killing capability *Candida albicans* and *Trichophyton rubrum*, two common mucocutaneous fungal infections by 10-13% (Fekrazad et al., 2015).

Table 2.1 Chemical composition of various essential oils and their antimicrobial activity against several microorganisms.

Plant	Part used	Major component	Inhibited microorganisms	MIC (µg/mL)	Reference
Bergamot (*Citrus bergamia*)	Peel	1) Limonene	*Bacillus cereus*	0.125	Fisher and Phillips (2006).
		2) Linalyl acetate	*Listeria monocytogenes*	0.125	
		3) Linalool	*Campylobacter jejuni*	>4	
			Escherichia coli O157	0.5	
Black pepper	Seed	1) Caryophyllene	*Staphylococcus aureus*	125	Karsha and Lakshmi (2010)
		2) Limonene	*Bacillus cereus*	250	Menon and Padmakumari (2005)
		3) β-Pinene	*Streptococcus*	500	
Clove (*Syzygium aromaticum*)	Bud	1) Eugenol	*Escherichia coli*	0.40–2.50	Friedman *et al*. (2002).
		2) Eucalyptol	*Listeria monocytogenes*	0.20	
Fennel (*Foeniculum vulgare* L.)	Seed	3) Anethole	*Escherichia coli* O157:H7	80	Dadalioğlu and Evrendilek (2004).
Key lime (*Citrus aurantifolia* (Christm.) Swingle)	Peel	1) Limonene	*Bacillus subtilis*	0.25	Costa *et al.* (2014)
		2) γ-Terinenel	*Enterococcus durans*	1.00	
		3) β-pinen	*Staphylococcus epidermidis*	0..25	
			Escherichia coli	1.00	
Lemongrass (*Cymbopogon citratus* (DC) Stapf)	Leaves	1) Geranial	*Listeria monocytogenes*	0.2	Smith-Palmer *et al.* (1998)
			Salmonella typhimurium	2.5	Hammer *et al.* (1999)
			Staphylococcus aureus	0.6	

Table 2.1 (Continued) Chemical composition of various essential oils and their antimicrobial activity against several microorganisms.

Plant	Part used	Major component	Inhibited microorganisms	MIC (µg/mL)	Reference
Oregano (*Origanum minutiflorum*)	Leaves and aerial parts	1) Carvacrol 2) p-Cymene	*Escherichia coli* O157:H7	50	Burt and Reinders (2003) Dadalioğlu and Evrendilek (2004).
Thyme (*Thymus vulgaris*)	Aerial parts	1) Camphor 2) Camphene	*Escherichia col*	0.45–1.25	Burt and Reinders (2003)
Rosemary (*Rosmarinus officinalis* L.)	Aerial parts	1) α-Pinene 2) Camphor	*Escherichia coli* *Salmonella typhimurium* *Bacillus cereus* *Staphylococcus aureus*	4.5 ->10 >20 0.2 0.4–10	Pintore *et al.* (2002)
Greek sage (*Salvia fruticose)*	Aerial parts	1) 1,8-Cineole 2) α and β-thujone 3) Camphor 4) Caryophyllene	*Fusarium oxysporum* *Fusarium solani*	50-2000 µg/mL	Pitarokili *et al.* (2003)
Aegle marmelos	Leaves	1) γ-Cadinene 2) δ-Carene 3) α-Pinene	*Candida albicans* *Aspergillus niger*	nd	Ibrahim *et al.* (2015)
Coriandrum sativum	Leaves	1) 2E-Decenal 2) Decanal 3) Decanol	*Candida albicans*	nd	Begnami *et al.* (2010)

2.2.4 Mode of action of essential oils

Mechanisms of action of essential oils are still not completely understood over the years studied. Several components of essential oil and their antimicrobial activity cannot be confirmed based only on the action of one compound due to mixture components contained in the essential oils (Bajpai *et al.*, 2012; Burt, 2004). The attribution to penetrate through bacterial membrane to the interior of the cell has been proposed to be the main factor relate to the exhibit inhibitory activity on the functional properties of the cell and to their lipophilic properties of the bacterial cell (Bajpai *et al.*, 2012; Fisher and Phillips, 2009). Hydrophobicity and chemical components of the oils are one of the important properties allowing the effect to bacterial cell by separating the lipid of the bacterial cell membrane and mitochondria resulting in the inhibition of cell bacterium growth (Burt, 2004; Friedly *et al.*, 2009). Phenolic compound is the main nature of essential oils that has revealed the serious effect to bacterial cell by disrupting cell membrane resulting in the inhibition of bacterial growth, and eventually causing leakage of the internal contents of the cell leakage such as protein, potassium ion, and ATP (Bajpai *et al.*, 2012). The mechanisms of action may relate to the ability of phenolic compounds to alter microbial cell permeability, damage cytoplasmic membrane, interfere with cellular energy (ATP) generation system, and disrupt the proton motive force, which is one of these can result in cell death (Bajpai *et al.*, 2012; Burt, 2004; Friedly *et al.*, 2009; Li *et al.*, 2011). Essential oils have the effect of inhibiting the growth of some Gram-positive and Gram-negative of bacteria. Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus* are more susceptible to essential oils than Gram-negative bacteria such as *Escherichia coli* and *Salmonella* Enteritidis (Chorianopoulos *et al.*, 2004). It is

generally mechanism of essential oils could be more effective against Gram-positive bacteria due to the direct interaction of the cell membrane with hydrophobic components of the essential oil and components of Gram-positive membrane (Chao and Young, 2000; Cimanga *et al.*, 2002). Conversely, properties of the hydrophilic cell wall of Gram-negative are not conducive to the influx of hydrophobic molecule as essential oils to the cell resulting more resistant that Gram-positive bacteria (Kim *et al.*, 2011). On the other hand, essential oils can coagulate the cytoplasm leading to alterations in several compartments of cell structure (Gustafson *et al.*, 1998; Burt, 2004). The composition of essential oils appears to act on proteins embedded on the cell membrane of cytoplasm. Enzymes such as ATPases, are known to be bordered by lipid molecules which lipophilic hydrocarbons could act on by accumulating in the bilayer and distorting the lipid-protein interaction. Alternatively, the a,direct interaction of the lipophilic compounds with hydrophobic parts of the protein is possible (Burt, 2004).

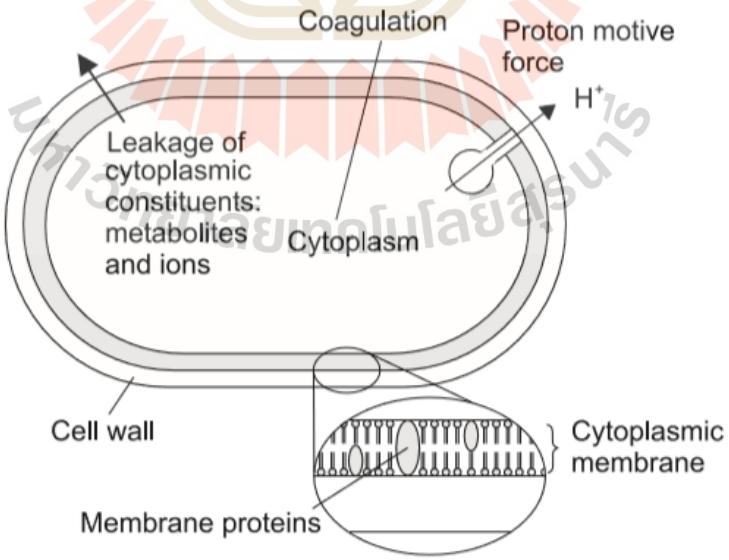

Figure 2.4 Locations in a bacterial cell and mechanisms of action for essential oil components in the cell (Burt, 2004).

Carvacrol, eugenol, and thymol are powerful phenolic compounds against foodborne pathogen depending on concentration with a similar mechanism as other phenolic compounds, disturbing cytoplasmic membrane, disturbing proton motive face, electron flow active transport, coagulation of cell content (Burt, 2004). The study of Dorman and Deans (2000) described the relevance of chemical structure and precision of the antibacterial activity of essential oil. Existence of the hydroxyl group in the phenolic ring has influence effective of the compound by relative position does not appear strongly influence the level of antibacterial activity (Dorman and Deans, 2000). In addition, the presence of acetate moiety in the non-phenolic ring has been confirmed to significantly increased activity of the compound, for example, geranyl acetate is more effective in the range of Gram-positive and negative strains than geraniol. Besides, types of alkyl groups found to influence the activity (alkenyl > alkyl) for example limonene that is more effective than *p*-cymene as the chemical structure shown in Figure 2.5.

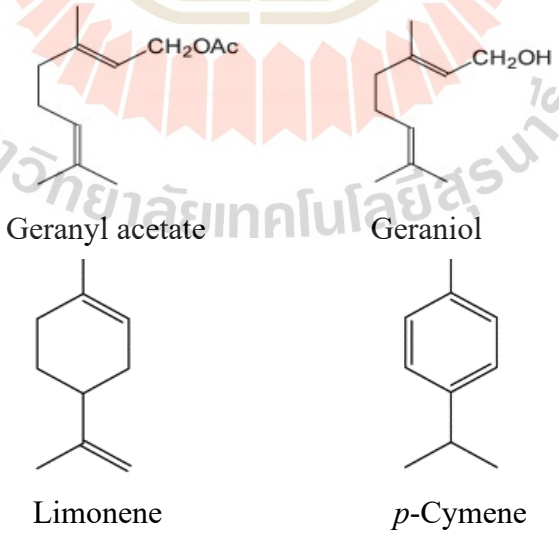

Figure 2.5 The different structure of geranyl acetate and geraniol, limonene and *p*-cymene of essential oil (Chen and Viljoen, 2010; Zhoa *et al.*, 2008).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents, and media

Chemicals, reagents and media used in each step are as follows:

3.1.1 Preparation of essential oil from Ma Sang fruits

Sodium sulfate anhydrous powder (Na_2SO_4) (Hi-Media, India) was used for dehydration of essential oil after extraction from Ma Sang fruits.

3.1.2 Antimicrobial activity of essential oil from Ma Sang fruits

Normal saline (Appendix A2) was used for the preparation of tested microorganism suspensions. Mueller Hinton broth (MHB, Appendix A1.3), tryptic soy broth (TSB, Appendix A1.1), tryptic soy agar (TSA, Appendix A1.1) and yeast extract agar (MY, Appendix A1.2) were used for the cultivation of tested microorganisms and determination of the antimicrobial activity of essential oil from Ma Sang fruits.

3.1.3 Determination of mode of antimicrobial action of the essential oil

Phosphate buffer saline (PBS, 0.1M) (Appendix A2.4) was used for cleaning bacterial cells after centrifugation. Lowry's reagents (Appendix A2.6) were applied for analyzing the leakage of protein.

3.1.4 Examination of bacterial cells changes

Glutaraldehyde (2.5%) and osmic acid (1%) (Sigma-Aldrich, United States) were used for fixed bacteria cells. Acetone (Ajax Finechem, Australia) at several concentrations was used for dehydrating bacterial cells.

3.2 Instrumentation

Instruments required for all of the research activities were located at the Building of the Cassava and Products Research Center, Suranaree University of Technology, and the Instrument Buildings of the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.3 Test microorganisms

The standard test microorganisms composed of Gram-positive bacteria including *Staphylococcus aureus* TISTR 517, *Staphylococcus aureus* ATCC 2913, *Staphylococcus xylosus* JCM 2418, *Staphylococcus epidermidis* TISTR 518 and *Bacillus cereus* TISTR 687, Gram-negative bacteria including *Enterobacter aerogenes* BCC 6719, *Escherichia coli*, *Proteus vulgaris*, *Salmonella* Typhimurium TISTR 292, and yeasts (*Candida albicans* TISTR 5744 *and Saccharomyces cerevisiae* TISTR 5343). The test organisms were obtained from the Microbial Culture Collection and Applications Research Center, Institute of Science, Suranaree University of Technology.

3.4 Plant materials

3.4.1 Collection of Ma Sang fruits

Mature Ma Sang (*Feroniella lucida* (Schiff.) Swingle.) fruits which were dark green color and rough appearance on outer skin, were collected from their natural habitats in Satuek District, Buriram Province, Thailand. The fruits were used for essential oil extraction.

3.4.2 Investigation of Ma Sang fruit used for essential oil extraction

Thirty fresh Ma Sang fruits were measured for their sizes by measuring the fruit dimeters. The average mass of theses Ma Sang fruits was weighted with two-digit digital balance (TP1502, Sartorius, Germany). Then, the fresh fruit structure was extermined by cutting and using blade. Woody pericarp, pulp and seed were observed under stereomicroscope (SZX16, Olympus, Japan).

3.4.3 Measurement of moisture content

The moisture content of peel of Ma Sang fruits was determined according to the standard method (AOAC, 2002). Empty aluminum foil containers were dried in the hot air oven (UF30, Memmert, USA) at 105ºC for 6 h and weighted using analytical balance and recorded, then were used for filling each specimen. The peel, woody pericarp, pulp, and seeds from fresh Ma Sang fruits were filled in weighted by analytical balance and recorded as wet sample mass. The plant samples were dried by hot air oven at 105ºC for 6 h. Each sample was allowed to cool down and stored in a vacuum desiccator. The cooled samples were weighed again and recorded as the dry mass of samples. The formula for determining moisture content is as follows:

$$Mn = \frac{(W1-W2)}{W2} X\ 100$$

Where:

Mn, moisture content (%) of material n

W1, wet mass of the sample

W2, mass of the sample after drying.

3.5 Extraction of essential oil from Ma Sang fruits

The mature Ma Sang fruits were washed and the skin was peeled off. Essential oil was extracted at the ratio 1:3 of fresh peel in water by Clevenger apparatus with metal-cased heating matles (LabHeat®, U.K.) for 3 h. Sodium sulfate anhydrous was used for dehydration of the extracted oil and when was stored at 4ºC until use. Yields of the essential oil were calculated as follows:

$$\text{Yield (\% v/w)} = \frac{\text{Volume of obtained essential oil (mL)}}{\text{Weigh of fresh Ma Sang peel (g)}} \text{ X } 100$$

3.6 Analysis of the chemical compositions of essential oil from Ma Sang fruits

The chemical compositions of essential oil from Ma Sang fruits was analyzed by gas chromatography-mass spectrometry (GC-MS, Bruker® Series 3XD, U.S.A.). One microliter (µL) of the oil sample was injected in a system equipped with Rtx-5MS (30 m x 0.25 mm x fused silica 0.25 µm) capillary column; the injector split ratio of 1:20. Helium was used as carrier gas at a constant flow of 1 mL/min. The injection port was set at 250ºC and the temperature cycle used was initially at 50ºC, ramping at 3ºC/min for 3 min to a final temperature of 250ºC and kept for 15 min with the detector at 280ºC. The MS 320 Bruker operating parameters were transferred line temperature at 240ºC, and electron impact ionization at 70 eV. The components of the essential oil were identified by comparing their mass spectra with the NIST MS search 2.0 (Supudompol, 2009).

3.7 Screening of antimicrobial activity of essential oil from Ma Sang fruits

The antimicrobial activity of essential oil was performed by the disk diffusion method (CLSI, 2006) with some modification, which is normally used as a preliminary screening and selection of bioactive essential oils. Gram-positive and Gram-negative bacteria test organisms were grown in tryptic soy broth (TSB, Appendix A1.1) at 35ºC overnight for preparing the late log phase cultures. The bacterial cultures were diluted with normal saline (0.85%, Appendix A2.2) to yield approximately 10^8 CFU/mL and inoculated on Mueller Hinton agar (MHA, Appendix A1.3) plate by three-dimension swab technique. The sterile blank paper disc (6 mm in diameter, Whatman, U.K.) was impregnated with 10 µL of the sample Ma Sang oil and placed on the inoculated agar, then incubated at 35ºC for 24 h. After that, inoculated agar was measured inhibition zone diameter by the ruler. Each test was carried out in triplicates with controls. The paper disc containing standard streptomycin (10 mcg, Oxoid, CT0047B, U.K.) was used as a positive control. For the antifungal activity assay, the standard strains, *Candida albicans* TISTR 5744 and *Saccharomyces cerevisiae* TISTR 5343, were cultivated in malt yeast extract broth (MY, Appendix A1.2) for 48 h at 30ºC and then adjusted cell concentration to approximately 10^7 CFU/mL to use for three-dimension swab on MY (Appendix A1.2). The sample of Ma Sang essential oils were placed following bacteria section whereas results were examined after incubation for 48 h at 30ºC. One hundred units of standard nystatin (Sigma, U.S.A.) were used as the positive control. All tests were performed in triplicates.

3.8 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oil from Ma Sang fruits

The antimicrobial activity of essential oil was performed by microdilution assay (CLSI, 2012) with some modifications. The microdilution assay was used for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The selected standard test microorganisms used in this section were selected from the results of section 3.7. For bacterial test microorganisms, the active cultures were prepared in tryptic soy broth (TSB, Appendix A1.1) at 35ºC overnight while the tested fungi were cultured in malt yeast broth (MY, Appendix A1.2) at 30ºC overnight. Then, the cultures were cross streaked on TSA (Appendix A1.2) for bacteria or MY agar (Appendix A1.2) for fungi for pure culture colony after confirming Gram-strain (Appendix A2.1). The pure culture was transferred to Mueller Hinton Broth (MHB, Appendix A1.3) and adjusted turbidity with the 0.5 McFarland standard suspension (Appendix A2.5 approximately 10^8 CFU/mL) and dilute with the broth medium for approximately 10^5 CFU/mL. Ma Sang essential oil was dissolved in 0.5% tween 80 (Appendix A2.3) for making tenfold serial dilutions from 0.1 to 16 mg/mL. Twenty microliters of each diluted essential oil were added to each well of 96 well-flat bottom microplates (Thermo Scientific, U.S.A.), then 180 µL of the prepared inoculum in the suitable assay medium, MHB, were added. All treatments were incubated at 35ºC and 30ºC for 20 h for bacteria and yeasts, respectively. The microbial growth was detected by light absorbing using Epoch Microplate Spectrophotometer (BioTek®, U.S.A.) at 530 nm (Appendix B3) for detecting turbidity of the suspensions, then

calculating inhibitory effect (%) according to the equation shown below. To evaluate MBC, ten µL of broth in each well that was no growth of test microorganisms, were enumerated using dropping plate technique on TSA for bacteria and MY agar for fungi with incubation temperature at 35ºC or 30ºC, respectively, for 24 h. The concentration of the essential oil providing no variable cell count was reported as MBC. The inhibitory effect (%) was calculated from turbidity values (OD_{530}) of inoculum, broth, and treatment of each tested microorganism as follows:

$$\text{Inhibitory effect (\%)} = \frac{\text{(Inoculum – Broth) – (Treatment – Broth)}}{\text{Inoculum – Broth}} \times 100$$

The minimum inhibitory concentration (MIC) was defined as the minimum level of essential oil concentration that produced a 90% inhibition of bacterial growth and was determined by the optical density method (Ponce *et al.*, 2003).

3.9 Effect of essential oil from Ma Sang fruits on viable cell counts of the sensitive test bacteria

To follow up the bacterial growth during treatment with essential oil, the method according to Bajpai *et al.* (2013) with some modifications was used. The active culture of the most sensitive test microorganisms to the essential oil (results from section 3.8) was prepared in TSB (Appendix A1.1) with at 35ºC for 6 h. The bacterial concentration of approximately 10^7 CFU/mL was treated with the active essential oil sample. Tween 80 (0.5%, Appendix A2.3) was used as the negative control. The test culture was incubated for 24 h with time interval collection of samples for plate counting on TSA at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 h. The results were performed as time-killing curve.

3.10 Mode of antimicrobial action of essential oil from Ma Sang fruits

3.10.1 Detection of cellular material release

The integrity of cell membrane was monitored by the release of cytoplasmic constituents of the cell according to the methods described by Chimnoi *et al.* (2018) and Bajpai *et al.* (2013) with some modifications. The selected standard test bacteria obtained from section 3.8 were incubated for 8 h in TSB (Appendix A1.1) for late logarithmic growth phase preparation. The cultivated broth was then centrifuged at 10,000 rpm for 10 min at 4ºC and washed twice with 0.1 M Phosphate Buffered Saline (PBS, Appendix A2.4), pH 7.4. The cells were resuspended in 0.1 M PSB (pH 7.4) and adjusted to OD_{620} of 0.3 (approximately 10^7 CFU/mL). Different concentrations of the essential oil at 1xMIC and 2xMIC were added to the cell suspensions, and cell suspension without the essential oil was used as the negative control. The suspensions were further incubated at 35ºC for 0, 30, 60, 90 and 120 min which were immediately removed at each incubation time. The cell suspension was filtered with 0.2 µm pore size syringe filter. The supernatant was detected for its absorbance by SmartSpec™ 3000 Spectrophotometer (Bio-Rad®, U.S.A.) at 260 nm.

3.10.2 Extermination of protein leakage

The cell integrity was also examined by determining the release of proteins into supernatant using the method described by Lowry *et al.* (1951). The microbial cell after microbial cell harvesting (section 3.10.1) was adjusted to OD_{620} of 0.3 (approximately 10^7 CFU/mL), then resuspended in 1% (w/v) peptone water before treating with 1xMIC and 2xMIC of Ma Sang essential oil. The treated cells were incubated at 35ºC for at 0, 30, 60, 90 and 120 min. At each incubation period, the mixture was filtrated through 0.2 µm pore size syringe filter. The concentration of

protein released in supernatant was determined by the method as above and measured at 670 nm (Epoch Microplate Spectrophotometer, BioTek®, U.S.A.).

3.10.3 Determination of potassium ion efflux

The leakage of potassium ion from microbial cells after treating with essential oil from Ma Sang fruits was detected by the method of Paul *et al.* (2011). Cells of the test microorganisms were prepared by the method as mentioned in section 3.10.2, then resuspended in 1% peptone and treated with the essential oil from Ma Sang fruits at concentrations of 1xMIC, 2xMIC and MBC. Tween 80 (0.5%) was used as the negative control. The potassium ion efflux was measured by a photometric procedure using the Potassium kit (Quantofix, Macherey-Nagel GmbH and Co, Germany) after 0, 30, 60, and 120 mins incubation at 35ºC.

3.10.4 Measurement of extracellular ATP concentration

To study the efficiency of essential oil from Ma Sang fruits on the strength of tested bacterial cell membrane, extracellular ATP concentrations were determined according to the method described by Paul *et al.* (2011). Harvested cells of test microorganisms were adjusted to 10^7 CFU/mL in TSB and treated with the essential oil at 1xMIC, 2xMIC and MBC concentrations in 1 mL total volume. The mixtures were maintained at room temperature for 30 min, then centrifuged at 3000 rpm for 5 min. The supernatant was kept on ice to prevent ATP loss until measurement. The extracellular ATP concentrations were measured using an ATP luminescent assay kit (Sigma, U.S.A.) by using Varioskan™ LUX multimode microplate reader (Thermo Fisher Scientific, U.S.A.).

3.11 Morphological changes of bacterial cells analyzed by scanning electron microscope (SEM)

Scanning electron microscope was used to detect morphological change of bacterial cells. The tested bacteria were cultured in TSB (Appendix A1.1) at 35ºC for 8 h, then treated with MIC and MBC of the essential oil except the negative control at 35ºC for 4 h. The microbial cells were collected by centrifugation at 3,000 rpm for 5 min at 4ºC, and washed once with 0.1M PBS (pH 7.4, Appendix A2.4) then prepared for SEM observation according to Souren *et al*. (2011). The harvested cells were fixed with 2.5% glutaraldehyde at 4ºC for 3 h, and washed with 0.1M PBS (pH 7.4). The cells were then fixed with 1% osmic acid at 4ºC for 1.5 h and washed with 0.1M PBS (pH 7.4). The cell pellets were dehydrated using series of acetone concentration as follows: 30, 50, 60, 70, 80, 90, and 100%. Finally, the specimens were sputter-coated with gold under vacuum (NeoCoater, U.S.A.), followed by microscopic examination using scanning electron microscope (SEM, Zeiss® Gemini, Germany).

3.12 Statistical analysis

All data were express as mean ± standard deviation (SD) of three independent replicates. The results from sections 3.4, 3.5, 3.9, and 3.10 were compared by one-way analysis of variance (ANOVA). Tukey's test was used to test significant differences among the means (SPSS statistic version 23). Differences among means at 95% confidence ($p<0.05$) were considered statistically significant.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Plant materials

4.1.1 Characteristics of Ma Sang plant

Ma Sang (*Feroniella lucida* (Scheff.) Swingle.) plant used in this study was naturally found in Sateuk District, Buriram Province, Thailand. Three trees as representatives of the plant were selected for investigation commencing by identifying characteristics of the plant from their habitats according to Swingle *et al*. (1967). From field observation, all three plant trees had similar phenotypic characteristics. The trees were medium size about 10 m high with about 3 m canopy wide, having thick thorns around the trunk, the perpendicular branches to the trunk, and had slender thorns around branches. Some characteristics found were slightly different in each tree such as trunk and branch, and canopy pattern. The trunk of tree no. 1 was noticed to be a straight while the trunks of tree nos. 2 and 3 had branched from the bottom of the trunk. The bottom of trunk of tree no. 3 was destroyed by fungi and termite. Canopy of the three trees was slightly different in pattern, tree no. 1 had canopy expanded as a circle pattern while the others were irregular pattern. However, further investigation is required for conclusion the variation of the plant trees.

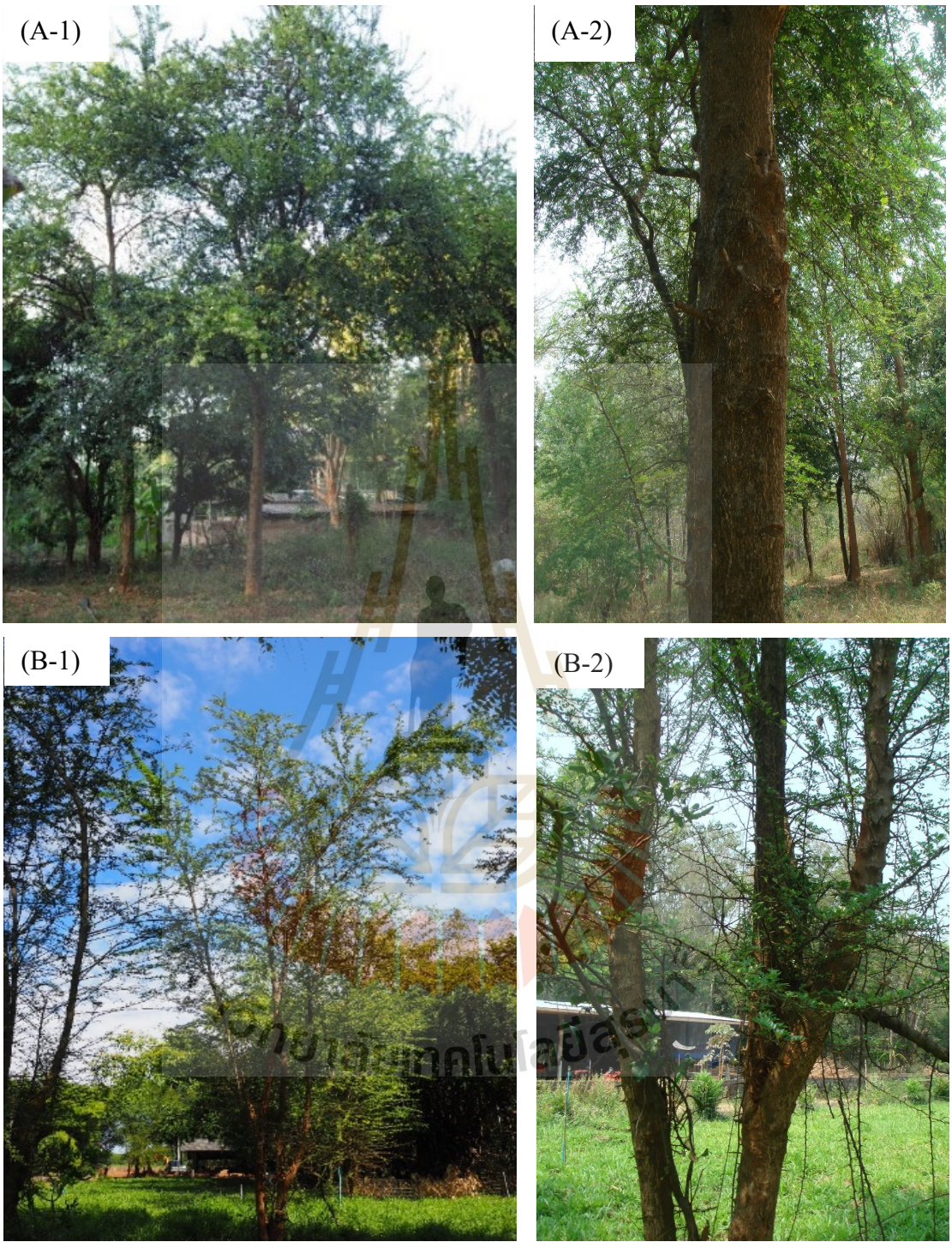

Figure 4.1 The appearance of canopy and trunk of three different Ma Sang trees: canopy of tree no.1 (A-1), tree no.2 (B-1), and no.3 (C-1) from study area in Sateuk District, Buriram Province, Thailand.

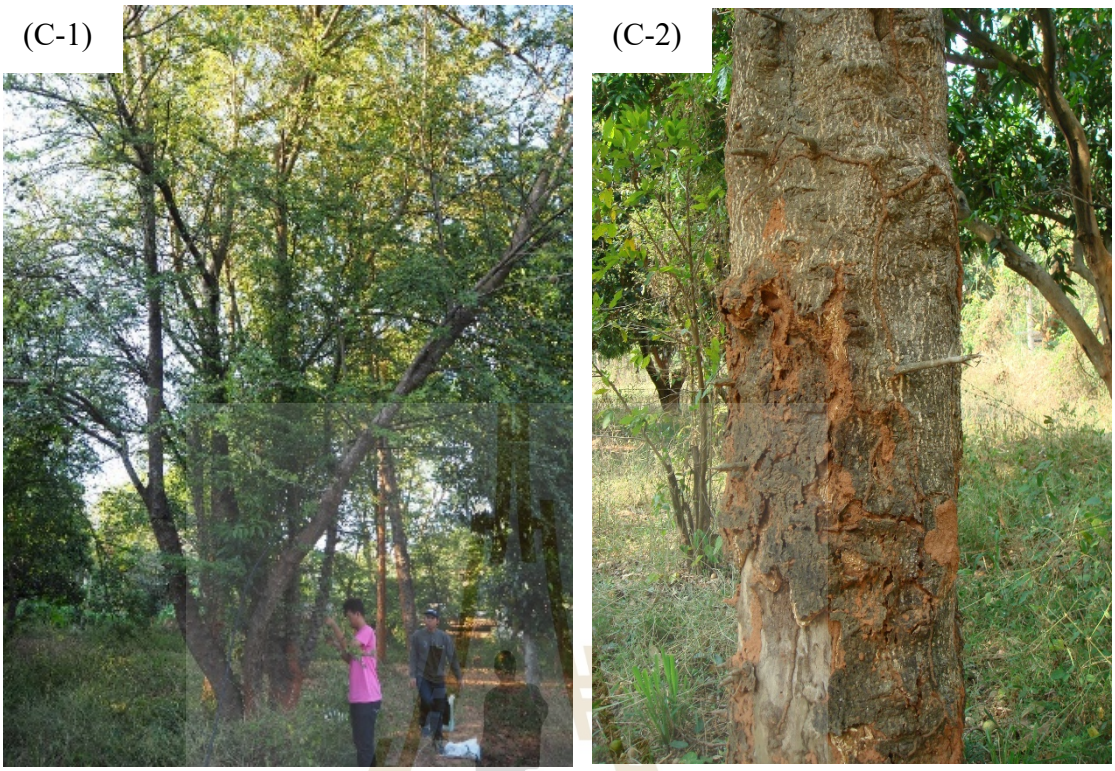

Figure 4.1 (Continued) The appearance of canopy and trunk of three different Ma Sang trees: trunk of tree no.1 (A-2), trunk of tree no.2 (B-2), and no.3 (C-2) from study area in Sateuk District, Buriram Province, Thailand.

For characteristics of Ma Sang leaves of the three plant trees, they were observed to have similar odd-pinnate leave patterns with obovate shape of leaflet and clustered along the branches. The young leaves were normally covered with soft short hairs at the back side and branches while mature leaves had fewer hairs and more glossy than young leaves. Moreover, the oil glands could be seen from the surface of the mature leaves (Figure 4.2). These leaves from the three trees presented significantly difference ($p<0.05$) with width and length (Figure 4.3). The tree no.3 exhibited narrow leaf while tree nos. 2 and 3 showed the wider leaves but were not significant difference. The length of leaves in each tree showed significant difference ($p<0.05$). However, from leaf characteristics of the three trees, they might not be different in species and

the difference in leaf size might cause by variations in the environment such as, soil condition and water, disease, and insect infestation. Further study of the plant characteristics is needed for plant identification confirmation.

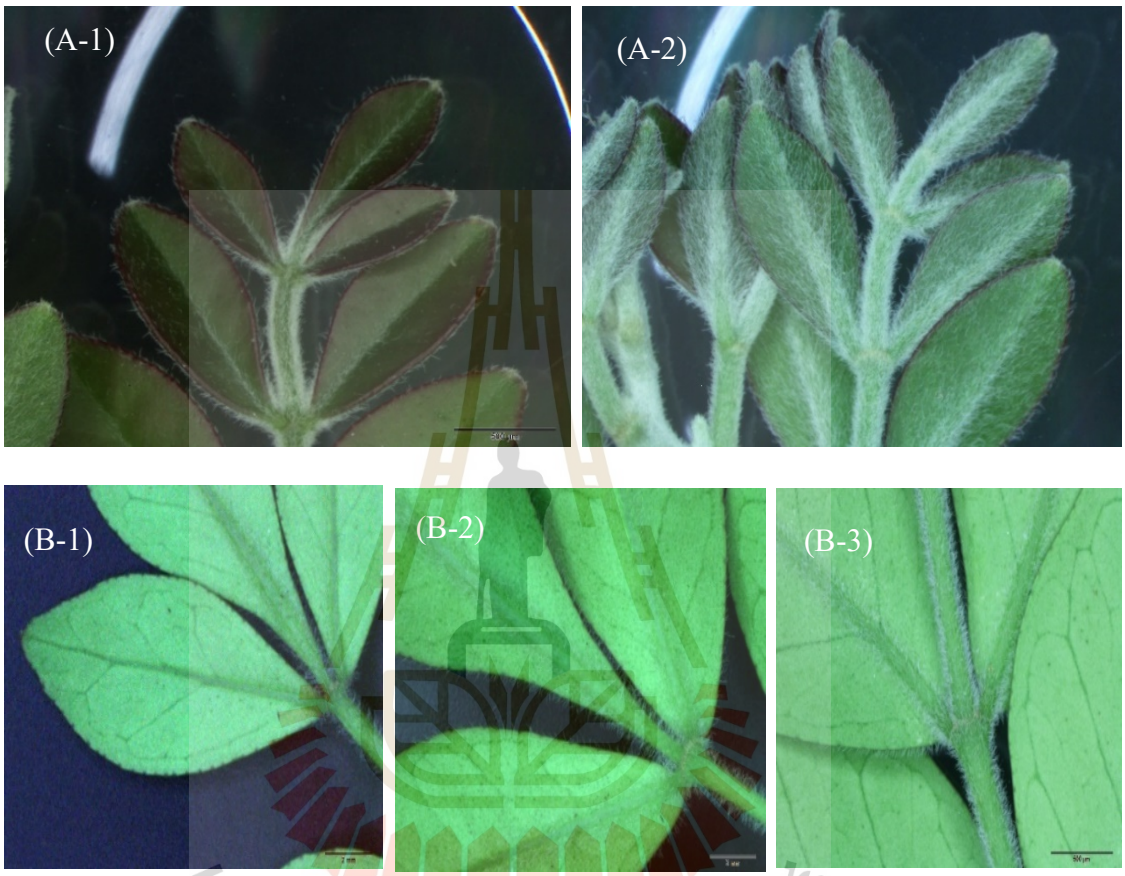

Figure 4.2 Characteristics of Ma Sang leaves of the three plant trees used in this study: young leaves (front) (A-1), young leaves (back) (A-2), leaves of tree nos. 1 to 3 (B-1 to B-3), respectively.

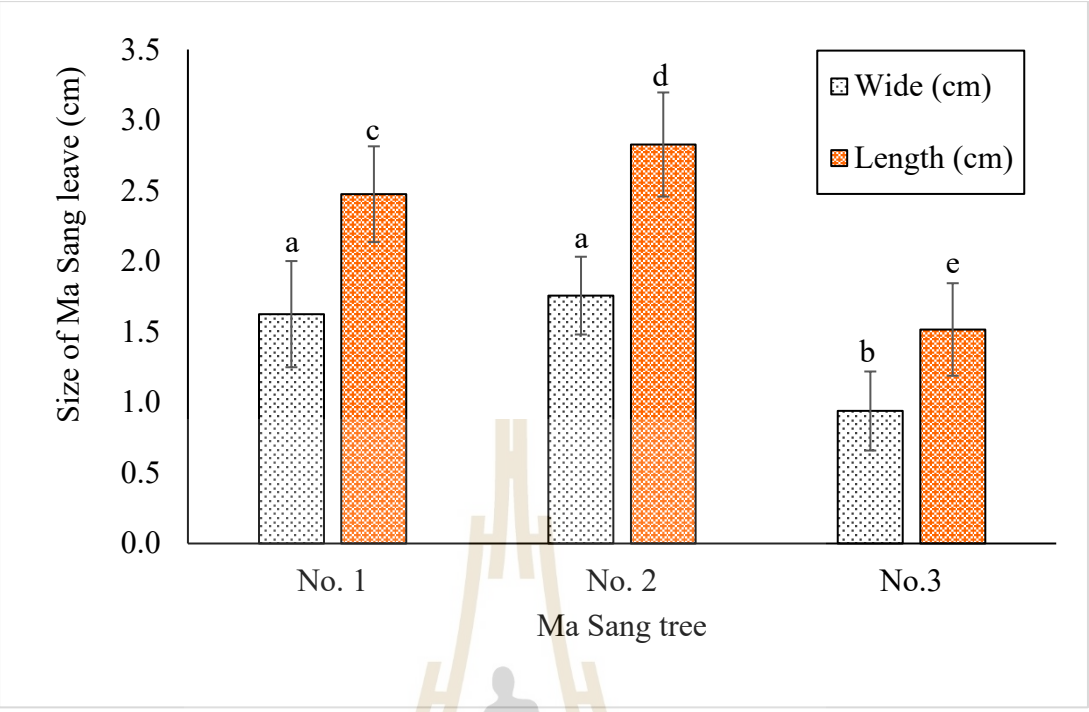

Figure 4.3 Leaf sizes of three Ma Sang trees selected as representatives for this study. A letter on the bar graph indicates the level of significance. Bars denoted by the same letter are not statistically significant ($p>0.05$).

The characteristics and structure of flowers were similar for the different three Ma Sang trees (Figure 4.4). The flowers arranged in loose panicle inflorescence with 10-15 flowers or 2-3 groups in an axil. Each flower presented 5 petals which were linear and curly at the margin with light green color at young flower to white color at the mature flowers. The flower base had many short small hairs. The Ma Sang flowers were hermaphrodite with 12-20 basifixed stamens and 1 pistil with green and round superior ovary. Anthers were oblong with yellow to brownish and connected. The length of style to ovary was about 15 to 21 mm and the stigma deformed or aborted. Based on all of these results, the plants used in this study were *Feroniella lucida* according to Swingle *et al*. (1967).

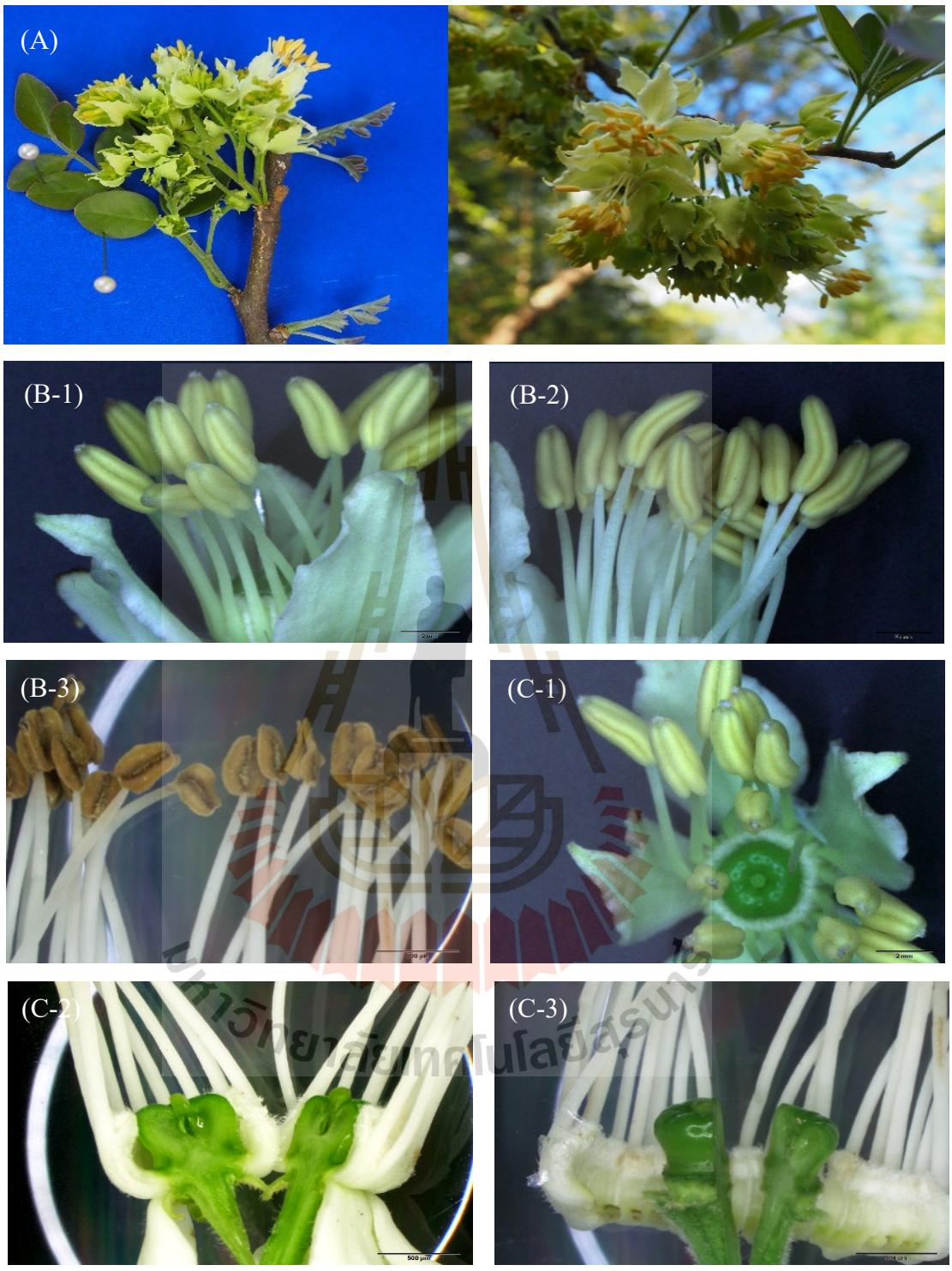

Figure 4.4 Characteristics of Ma Sang flowers (A) composed of stamens of tree no. 1 (B-1), tree no. 2 (B-2), and tree no. 3 (B-3) and ovary of tree no. 1 (C-1), tree no. 2 (C-2) and tree no. 3 (C-3).

4.1.2 Ma Sang fruits

Thirty mature Ma Sang fruits (Figure 4.5) were used as representatives for determining size and weight of the fruits. The fruits had average circumference ranging from 16.0 to 24.4 cm, dimeter of 5.73 to 7.01 cm, and weight ranging from 16.6 to 24.0 g (Table 4.1). The fruit composed of peel, pericarp, and pulp with seed at the proportion of 9, 64, and 27%, respectively of total fruit mass (Figure 4.6). The moisture contents of peel, pericarp, pulp, and seed were 56.21, 57.15, 78.00, and 48.89%, respectively (Figure 4.10). The outermost part of the fruit was the green peel containing many oil glands causing rough skin and unique scent of the fruit (Figures 4.7-4.8). The middle part of the fruit was the woody pericarp consisting woody structure, and the inner part was the white sour pulp with crunchy seeds (Figure 4.9).

Table 4.1 The average size and weight of fresh Ma Sang fruits collected from Sateuk District, Buriram Province, Thailand.

Circumference (cm)	Dimeter (cm)	Weight (g)
20.26±2.58	6.45±0.82	98.83±14.64

Figure 4.5 Ma Sang fruits collected from study area in Sateuk District, Buriram Province, Thailand.

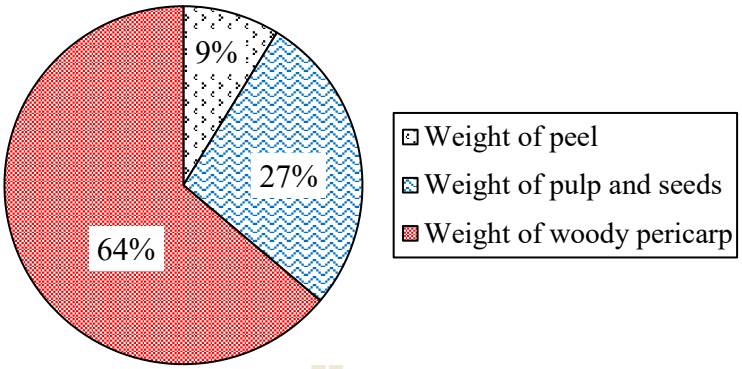

Figure 4.6 The ratio of each part of Ma Sang fruit.

Figure 4.7 The structure of Ma Sang fruit: outer of fruit (A) and inner of fruit (B).

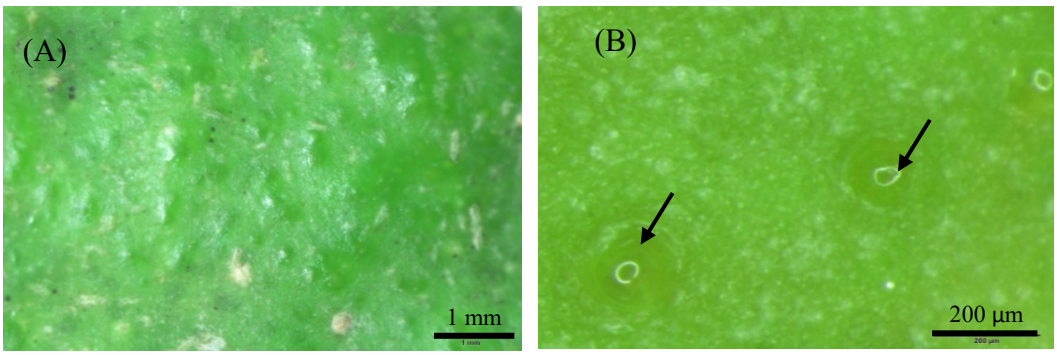

Figure 4.8 Characteristics of outer skin: rough skin of peel (A), and oil gland on peel (B, (arrows) observed under stereomicroscope (SZX16, Olympus, U.S.A.).

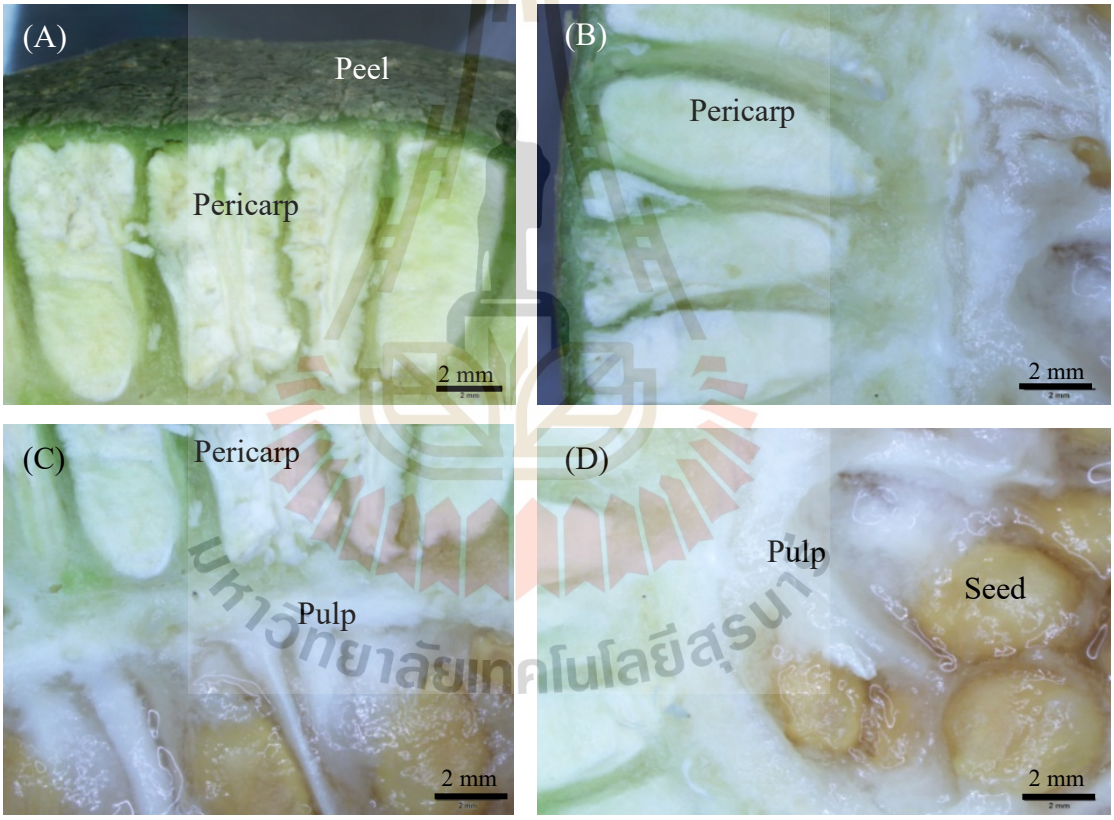

Figure 4.9 9 Characteristics of inner fruit of Ma Sang: peel and pericarp (A), pericarp (B), pericarp and pulp (C), and pulp and seed (D) observed under stereomicroscope (SZX16, Olympus, U.S.A.).

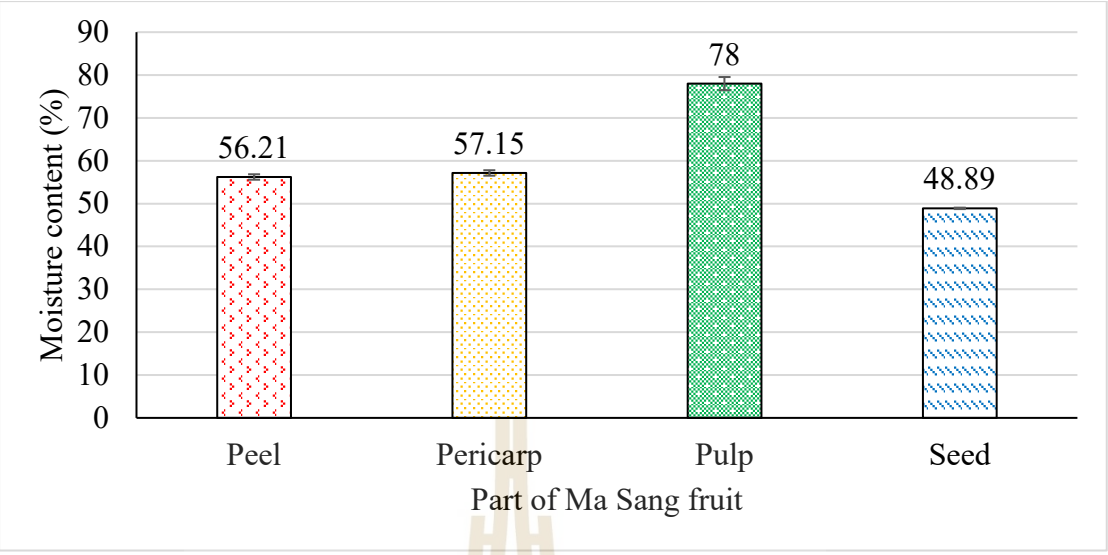

Figure 4.10 The average moisture content of each part of 30 Ma Sang fruit.

4.2 Yield of essential oil from Ma Sang fruits

Ma Sang (*Feroniella lucida* (Scheff.) Swingle.) fruits collected from their natural habitat in Sateuk District, Buriram Province, Thailand in February, March, and September with a vary of the mature fruits and different tree as described as tree nos. 1, 2, and 3. Two groups of fresh Ma Sang fruits were collected in this study. The first group was directly collected from the tree (fresh fruits). Another group was collected in one day after falling from the tree (fallen fruits) (Figure 4.11). From the observation, the woody pericarp became soft structure after store the fruits at room temperature for about 3 days. The skin was then peeled off from the fruit and used for essential oil extraction (Figure 4.12).

Figure 4.11 The mature Ma Sang fruits fresh fruits (A) and fallen fruits (B) (arrows).

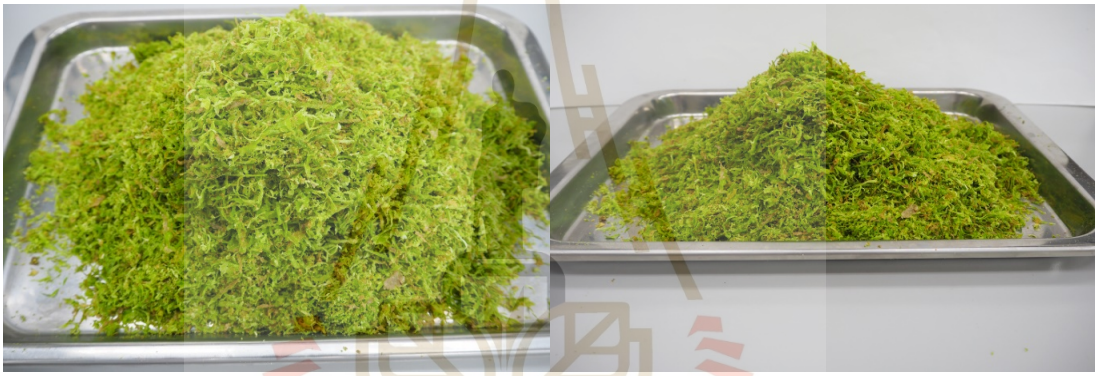

Figure 4.12 Ma Sang peel used for essential oil extraction.

Essential oils from different lots of collected mature fruits, different trees, and different collection times showed quite similar characteristics with strong unique scent and clear yellow color. Yields of essential oil from seven lots of samples of Ma Sang fruits showed slightly different results ranging from 0.59-0.77% (v/ww) as shown in Table 4.2 and Figure 4.13. The two samples of the essential oil from fresh fruits and randomly collected fallen fruits at the same harvest time in March yielded highly percentage of essential oils than other samples of 0.77% (v/ww) based on fresh weight. While, yield of the oil from fresh fruits of tree no. 1 that collected in February obtained

the lowest amount (0.59%) compared to other samples (0.64-0.72%). No significant difference was noted between them (p>0.05). The results confirmed that type of fruits, different harvest times or seasons, and variety the plants were not affected yield of essential oil.

Table 4.2 Sample of Ma Sang fruits and its yield of essential oil (%) based on fresh weight.

Lot.	Sample	Harvest time (Month)	Yield (%) based on fresh weight
1	Tree no. 1, Fresh fruits	February	0.59±0.18
2	Tree no.2, Fresh fruits	February	0.75±0.01
3	Tree no. 3, Fresh fruits	February	0.65±0.16
4	Tree no. 3, Fallen fruits	February	0.64±0.20
5	Tree no.3, Fresh fruits	March	0.77±0.03
6	Random collected fallen fruits	March	0.77±0.03
7	Tree no. 2, Fresh fruits	September	0.72±0.12

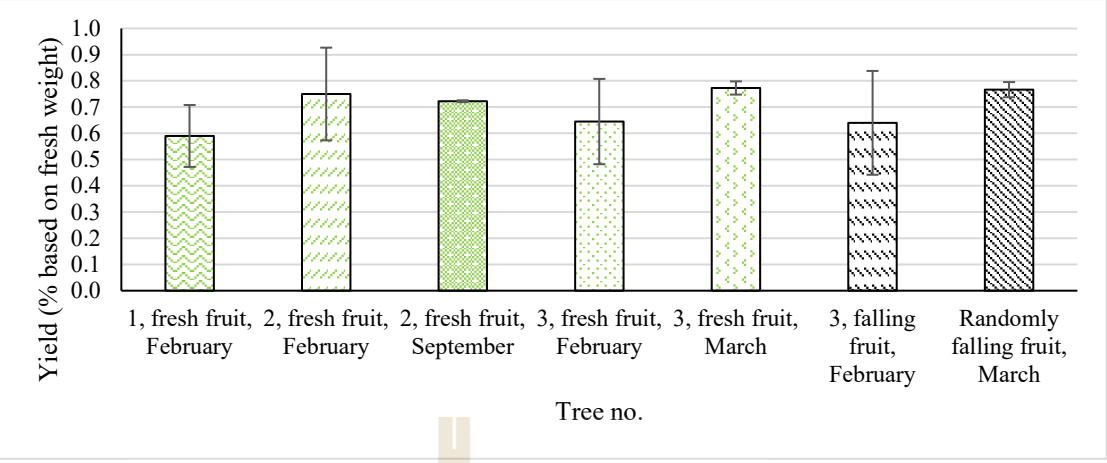

Figure 4.13 Yield (%v/ww) of essential oil from different sampling lots of Ma Sang fruits containing mature fresh and fallen fruits collected in February, March, and September.

Yield of essential oil from fruit was compared with result of Supudompol (2009), who reported the essential oil from *Feroniella lucida* leave in Amnatcharoen District, Thailand. Yield of the oil from fruits (0.59-0.77%) was higher than the oil obtaining from leaves which yielded only 0.12%. In addition, the result also confirmed that the plant part using for extraction of essential oils effected on the yield of essential oil. Perry *et al.* (1999) found similar results in Dalmatian Sage (*Salvia officinalis* L.) oil that extracted from flowers, leaves, and stem. Flowers were provided higher yield (1.56%) than leaves (1.11%) and stems (0.05%). Essential oil of *Blumea balsamifera* (L.) DC., an ancient medicinal herb, from different plant organs were also reported to reveal significant different yield (p<0.05) from different organs, leave, shoot, and stem, but in different harvest month, the yield was not significant different (p>0.05) (Yuan *et al.*, 2016). Bourgou *et al.* (2012) also found the similar results from the study of fruit maturation of four Tunisain citrus species, bitter orange, lemon, orange maltaise, and

mandarin, represented different yields of obtained essential oil. Interesting, the essential oil extracted from Ma Sang fruit, there was no difference of essential oil yields from fallen fruits and fresh fruits collected from the same tree. This makes the fruits harvest more flexible for essential oil extraction.

4.3 Chemical compositions of essential oil from Ma Sang fruits

The total chemical components of essential oil from Ma Sang fruits contained around 20 to 27 compounds (Table 4.3) which could be classified into six groups of components (Table 4.4). Ester was the major group of the oil, which was more than 60% of the total oil followed by aldehyde (7.86-22.75%), alcohol (5.10-9.10%), terpene (1.35-6.64%), epoxide (0.86-1.99%) and saturated fatty acid (0.10-0.22%). Four main major groups of compounds were detected in the oils including decyl acetate (56.2-66.73%), decanal (7.79-22.49%), dodecyl acetate (6.81-12.93%) and decanol (3.95-7.68%) (Figures 4.14 and 4.15). One-octanol (0.10-0.46%), α-caryophyllene (0.7-5.7%), caryophyllene oxide (0.86-1.84%), tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl- (0.44-0.79%), farnesol (0.10-0.34%) and phytol (0.04-0.16%) were recognized as minor compounds in essential oils. Supudompol (2009) reported 15 chemical components found in essential oil from Ma Sang leave with β-caryophyllene as the major compound (26.6%). From the study, 11 compounds found in essential oil from Ma Sang fruits were similar to compounds that were reported to be found in leaves but with difference percentage.

The chemical compounds of essential oil from different sampling lots of Ma Sang fruits showed the different component in two groups of the oil. Essential oil from fallen fruits found to contain seven different compounds more than essential oil from fresh

fruits. There were obviously increased of aldehyde compound and few of ester compound. The compounds were 2-ethylhexyl acetate (3.15%), ethyl ethanoate (0.22%), ethyl laurate (0.12%), β-selinene (0.66%), ethyl decanoate (0.13%), undecanal (0.07%), and nonyl acetate (0.07%). In contrast, the essential oil from fresh Ma Sang fruits found additional nonanal (0.03%), octyl acetate (1.45%), longipinocarvone (0.31%), isoaromadendrene epoxide (0.11%), benzyl benzoate (0.09), 1-tetradecyl acetate (0.08%), 8-hexadecenal-4-methyl-(Z) (0.38%), and 2-dodecene (0.12%) which mostly compounds were ester compound group. However, the major components included decyl acetate, decanal, dodecyl acetate, and decanol were found similarly content in the two oils. The results presented that different collection of Ma Sang fruits were not affected to the changes of the main compounds of the oil but effect to changes in proportion of aldehyde and ester of the oils. In conclude, aldehyde was found increasing in essential oil from fallen fruits than the oil from fresh fruits. The different components found in different mature fruits of Ma Sang relating the change of odor between fresh and fallen fruits of Ma Sang essential oil and might be their biological activity. Aldehyde is the most important flavor and aroma compound in essential oil particularly the saturated aliphatic aldehyde, octanal, nonanal, and decanal which contributed sweet pungent fatty odor (Moshonas, and Lund, 1969).

Table 4.3 Chemical compositions of essential oil from Ma Sang fruits collected from 3 trees at different collection time.

No	RT (min)	Compound	Peak area (%)				
			T1-Feb[1]	T2-Feb[2]	T3-Feb[3]	T3f-Feb[4]	T2-Sep[5]
1	9.183	Octanal	-	-	-	-	0.03
2	12.094	1-Octanol	0.17	0.21	0.38	0.10	0.46
3	13.245	Decane	0.06	0.06	-	-	0.31
4	13.457	Nonanal	0.01	0.03	0.03	-	0.06
5	18.54	Ethyl octanoate	-	-	-	0.22	-
6	18.85	Decanal	10.02	11.09	13.21	7.79	22.49
7	18.926	Octyl acetate	1.04	0.74	1.45	-	0.72
8	19.215	2-Ethylhexyl acetate	-		-	3.15	-
9	21.852	1-Decanol	4.77	3.95	7.18	7.68	6.82
10	23.387	Undecanal	-	-	-	0.07	-
11	23.599	Nonyl acetate	-	-	-	0.07	-
12	27.254	Ethyl decanoate	-	-	-	0.13	-
13	28.698	Decyl acetate	66.73	63.86	60.86	58.61	56.2
14	29.045	α-Bergamotene	-	0.19	-	-	0.05
15	29.642	β-Selinene	-		-	0.66	-
16	29.681	α-Caryophyllene	1.09	1.12	0.7	5.7	0.76
17	30.141	1-Dodecanol	0.46	0.45	0.62	0.88	0.61
18	30.489	β-Farnesene	-	0.23	-	-	0.06

[1]Tree no. 1, Fresh fruits, February

[2]Tree no.2, Fresh fruits, February

[3]Tree no. 3, Fresh fruits, February

[4]Tree no. 3, Fallen fruits, February

[5]Tree no. 2, Fresh fruits, September

Table 4.3 (Continued) Chemical composition of essential oil from Ma Sang fruits collected from 3 trees at different collection time.

No	RT (min)	Compound	Peak area (%)				
			T1-Feb[1]	T2-Feb[2]	T3-Feb[3]	T3f-Feb[4]	T2-Sep[5]
19	31.397	β-Bisabolene	-	0.82	-	-	0.28
20	32.485	α-Bisabolene	-	0.34	-	-	0.14
21	31.818	β-Sesquiphellandrene	-	0.09	-	-	-
22	33.7	Longipinocarvone	0.45	0.34	0.31	-	0.54
23	34.297	Caryophyllene oxide	1.59	1.26	1.29	0.86	1.84
24	35.46	Ethyl laurate	-	-	-	0.12	-
25	35.713	Dodecyl acetate	11.89	11.58	12.26	12.39	6.81
26	36.533	Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-, 4,4-dimethyl-	0.62	0.49	0.46	0.44	0.79
27	37.489	Isoaromadendrene epoxide	0.14	0.12	0.11	-	0.15
28	39.1	Farnesol	0.34	0.1	0.26	0.24	0.10
29	40.578	Benzyl Benzoate	0.12	0.13	0.09	-	-
30	42.024	1-Tetradecyl acetate	0.05	0.06	0.08	-	-
31	43.159	Farnesyl acetate 3	0.17	0.07	0.14	0.14	-
32	47.296	n-Hexadecanoic acid	0.03	0.08	0.07	0.10	0.11
33	51.678	Phytol	0.09	0.16	0.08	0.04	0.04
34	53.14	8-Hexadecenal, 14-methyl-, (Z)-	0.32	1.37	0.38	-	0.17
35	58.671	2-Dodecen-1-yl(-)succinic anhydride	0.10	0.59	0.12	-	0.11

[1]Tree no. 1, Fresh fruits, February

[2]Tree No.2, Fresh fruits, February

[3]Tree no. 3, Fresh fruits, February

[4]Tree no. 3, Fallen fruits, February

[5]Tree no. 2, Fresh fruits, September

Table 4.4 Chemical composition of essential oil from Ma Sang fruits classified by group of components.

Chemical component	Peak area (%) from different sampling lots				
	T1-Feb	**T2-Feb**	**T3-Feb**	**T3f-Feb**	**T2-Sep**
Ester	-		-	-	-
Ethyl octanoare	-		-	0.22	-
Octyl acetate	1.04	0.74	1.45	-	0.72
2-Ethylhexyl acetate	-		-	3.15	-
Nonyl acetate	-		-	0.07	-
Ethyl decanoate	-		-	0.13	-
Decyl acetate	66.47	63.86	60.78	58.61	56.2
Ethyl laurate	-		--	0.12	-
Dodecyl acetate	11.89	11.58	12.26	12.39	6.81
Benzyl Benzoate	0.12	0.13	0.09	-	0.12
1-Tetradecyl acetate	0.05	0.06	0.08	-	-
Farnesyl acetate 3	0.17	0.07	0.14	0.14	-
Aldehyde					
Octanal	-	-	-	-	0.03
Nonanal	0.01	0.03	0.03	-	0.06
Decanal	10.02	11.09	13.21	7.79	22.49
Undecanal	-	-	-	0.07	23.39
8-Hexadecenal, 14-methyl-, (Z)-	0.32	1.37	0.38	-	0.17
Alcohol					
1-Octanol	0.17	0.21	0.38	0.10	0.46
1-Decanol	4.77	3.95	7.18	7.68	6.82
1-Dodecanol	0.46	0.45	0.62	0.88	0.61
Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-, 4,4-dimethyl-	0.62	0.49	0.46	0.44	0.79

[1]Tree no. 1, Fresh fruits, February [2]Tree No.2, Fresh fruits, February [3]Tree no. 3, Fresh fruits, February

[4]Tree no. 3, Fallen fruits, February [5]Tree no. 2, Fresh fruits, September

Table 4.4 (Continued) Chemical composition of essential oil from Ma Sang fruits classified by group of components.

Chemical component	Peak area (%)				
	T2-Sep	T1-Feb	T2-Feb	T3-Feb	T3f-Feb
Terpene					
Decane	0.31	0.06	0.06	-	-
α-Bergamotene	0.05	-	0.19	-	-
β-Selinene	-	-	-	-	0.66
α-Caryophyllene	0.76	1.09	1.12	0.7	5.7
beta-Farnesene	0.06	-	0.23	-	-
β-Bisabolene	0.28	-	0.82	-	-
α-Bisabolene	0.14	-	-	-	-
β-Sesquiphellandrene	-	-	0.09	-	-
Longipinocarvone	0.54	0.45	0.34	0.31	-
Farnesol	0.10	0.34	0.1	0.26	0.24
Phytol	0.04	0.09	0.16	0.08	0.04
Epoxide					
Caryophyllene oxide	1.84	1.59	1.26	1.29	0.86
Isoaromadendrene epoxide	0.15	0.14	0.12	0.11	-
Saturated fatty acid					
n-Hexadecanoic acid	0.11	0.03	0.08	0.07	0.10

[1]Tree no. 1, Fresh fruits, February [2]Tree No.2, Fresh fruits, February [3]Tree no. 3, Fresh fruits, February

[4]Tree no. 3, Fallen fruits, February [5]Tree no. 2, Fresh fruits, September

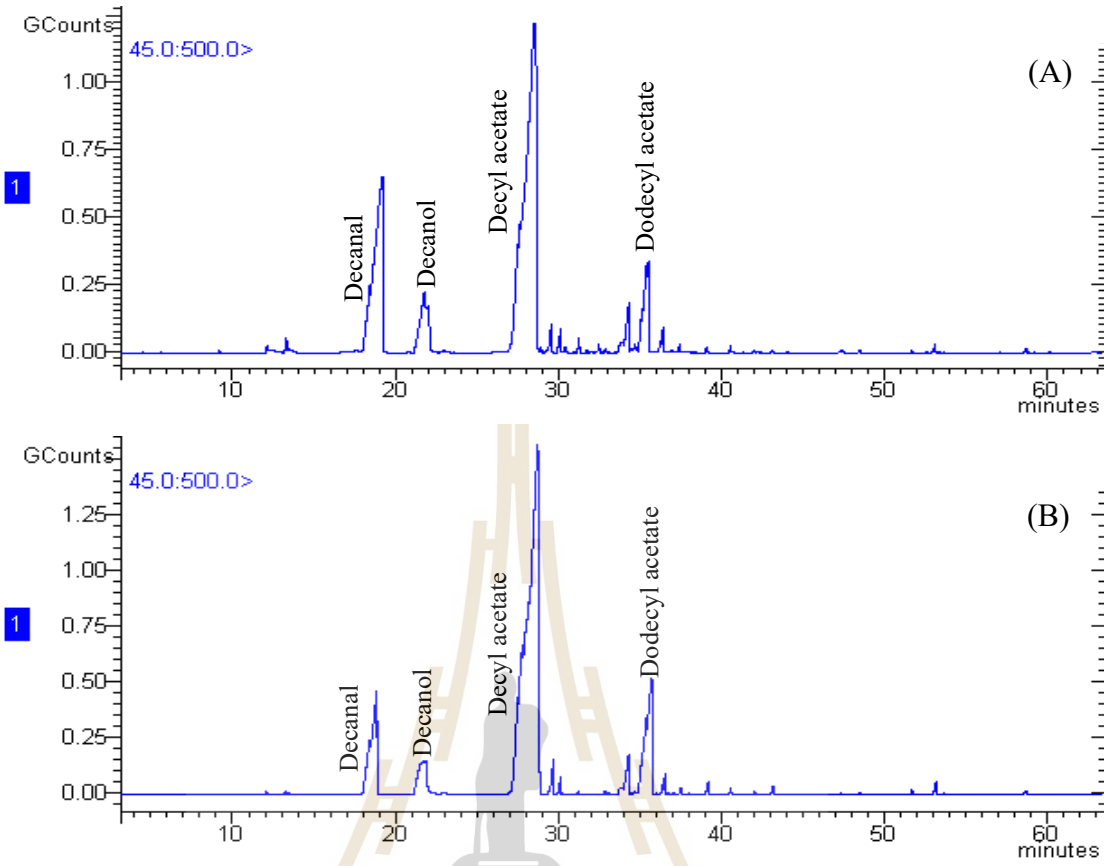

Figure 4.14 GC-MS chromatograms of essential oil from fresh fruit of tree no. 2 in September and (A) February (B).

The chemical compounds of essential oil from Ma Sang fruits found different component in the oil from different tree. The chemical compounds found in essential oil from Ma Sang fruits collected from different tree in February had same 21 components with slightly different each tree as follows: tree no. 1 contained decane (0.06%) and undecanol (23.39%), tree no. 2 also found decane (0.06%), α-bergamotene (0.19%), farnesene (0.23%), β-bisabolene (0.82%) and sesquiphellandrene (0.09%), while tree no. 3 was not found additional substances. The oil obtained from fruits in September had slightly different chemical composition in essential oil from the same tree that collected in February with detecting none of β-Sesquiphellandrene, 1-

tetradecyl acetate, farnesyl acetate, and addition of octanal. Most chemical compounds in this species were similarly found in Citrus species which rich of specific aroma compounds composed of unsaturated aliphatic aldehyde (C_8-C_{14}) and oxygenic mono and sesquiterpene also acetate (Mahato *et al.*, 2017). Main aromatic compounds found in kabusu (*Citrus aurantium* F. Kabusu) were octanal, nonanal, decanal, undecanal, dodecanal which related of our essential oil (Tamura *et al.*, 1993). In general, aldehydes and ester are well known as contributors to citrus flavor that using as flavor and perfume additives (Njoroge *et al.*, 2006). Thus, chemical composition of the essential oil from *Feroniella lucida* could be similar to *Citrus* and *Feroniella* genera.

The results demonstrated that the different months of collection might be involved changes of chemical composition of Ma Sang essential oil. Mature of fruits would affect to existence or increasing in many elements as shown in different compound found in fresh and fallen fruits as different variety of tree collected fruits may be attributed changes of chemical composition of the essential oil. Therefore, these finding results could be confirmed that vary in quality, quantity and in composition according to climate, soil composition, plant organ, age and vegetative cycle stage (Masotti *et al.*; 2003; Angioni *et al.*, 2006).

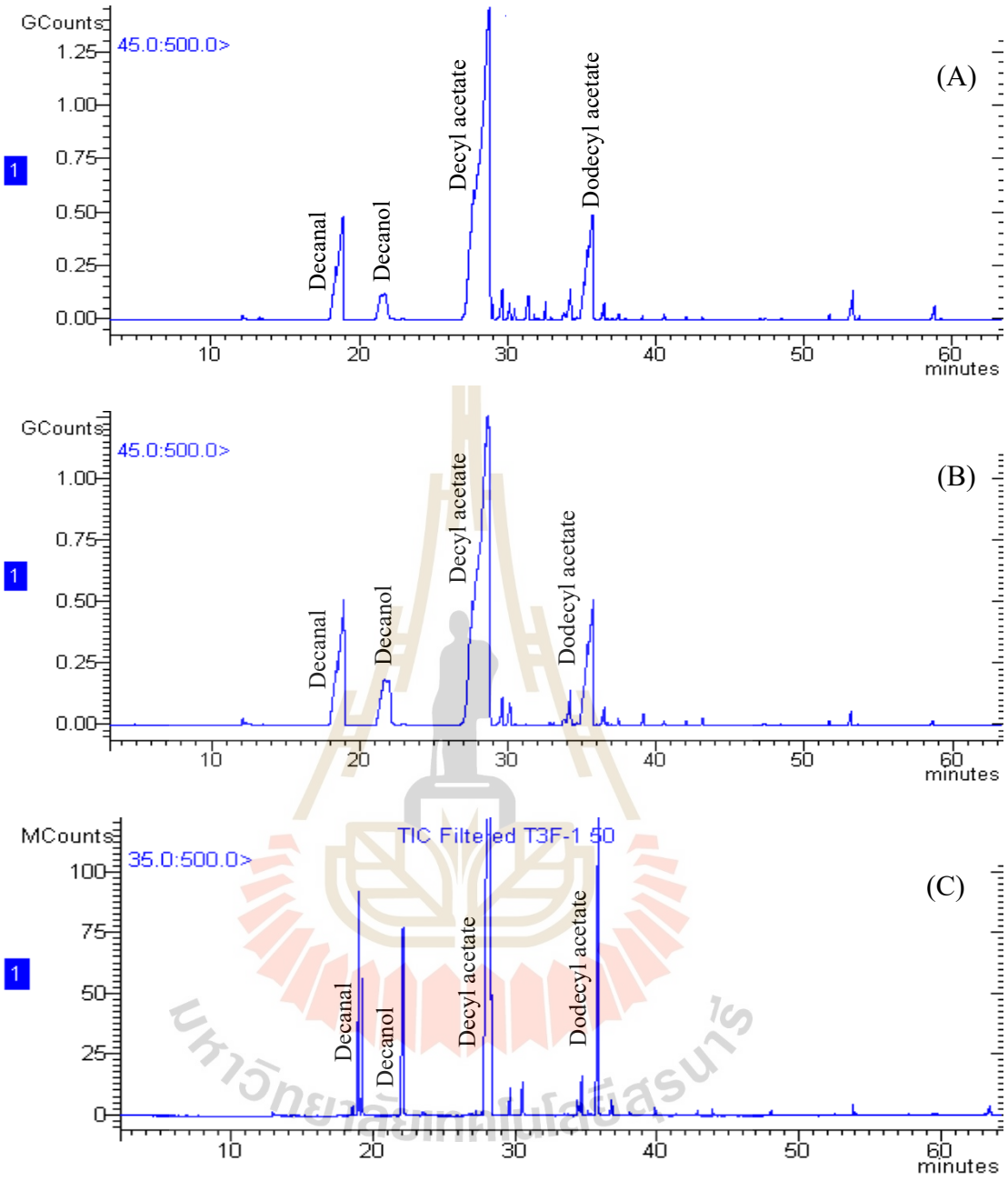

Figure 4.15 GC-MS chromatograms of essential oil from fresh fruit collected in February; tree no.2 (A) and tree no. 3 (B), and falling fruits collected in March; tree no. 3 (C).

4.4 Antimicrobial activity of essential oil from Ma Sang fruits

4.4.1 Inhibitory detection by disk diffusion method

Essential oil from Ma Sang fruits collected in February was selected for antimicrobial activity study (Table 4.5). The results showed that the oil had the potential to inhibit a wide range of tested microorganisms; Gram-positive and Gram-negative bacteria, and yeast. Inhibition zones of tested microorganisms ranged from 10.10-25.93 mm with 50.00 to 84.17% inhibition. Among these test microorganisms, *Staphylococcus xylosus* JCM 2418, *Staphylococcus aureus* TISTR 517, *Staphylococcus epidermidis* TIRTS 518, *Staphylococcus aureus* ATCC 1466, and *Staphylococcus aureus* ATCC 2913 were the most sensitive to the essential oil showing range of inhibitory zone from 11.22 to 25.93 mm with more than 50% of inhibition compared to the standard streptomycin. Similarly, *Saccharomyces cerevisiae* TISTR 5343 showed sensitively with 10.75 mm of inhibition zone and 51.37% of inhibition compared nystatin. However, the essential oil did not have inhibitory effect to some Gram-negative bacteria including *Salmonella* typhimurium TISTR 292, *Escherichia coli* TISTR 780, *Enterobacter aerogenes* BCC6719, *Proteus vulgaris* (environmental isolate), and the yeast *Candida albicans* TISTR 5744. This finding result indicated that the essential oil from Ma Sang fruit had a great inhibitory effect to a wide range of microorganisms particular Gram-positive bacteria and yeast. Essential oils have been reported to exhibit a greater effect of inhibiting the growth of some Gram-positive than Gram-negative bacteria. Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* are more susceptible to essential oils than Gram-negative bacteria such as *Escherichia coli* and *Salmonella* Enteritidis (Chorianopoulos *et al.*, 2004). It is generally mechanism of essential oils should be

more effective against Gram-positive bacteria due to the direct interaction of the cell membrane with hydrophobic components of the essential oil and components of Gram-positive membrane (Cimanga *et al.,* 2002). However, the ability to inhibit growth of microorganisms of essential oils depending on the chemical compounds found in essential oils which those compounds could act as synergistic properties with other compounds or effect alone by main compound in highest proportion in essential oil (Bakkali *et al.*, 2008).

Table 4.5 Antimicrobial activity of essential oil from Ma Sang fruits by disk diffusion method.

Test microorganism	Inhibition zone dimeter (mm)		Inhibition activity (%)
	Ma Sang essential oil (8 mg/disc)	Standard antibiotics[a] (10 mcg)	
Bacillus cereus TISTR 687	7.25	14.50	50.00
Enterobacter aerogenes BCC 6719	-	19.75	-
Escherichia coli[b]	-	1.30	-
Proteus vulgaris[b]	-	14.75	-
Salmonella Typhimurium TISTR 292	-	13.13	-
Staphylococcus aureus ATCC 1466	14.25	26.00	54.81
Staphylococcus aureus ATCC 29213	11.22	12.39	51.20
Staphylococcus aureus TISTR 517	12.20	17.28	70.62
Staphylococcus epidermidis TIRTS 518	18.45	26.90	68.59
Staphylococcus xylosus JCM 2418	25.93	31.38	82.63
Candida albicans TISTR 5744	-	17.13	-
Saccharomyces cerevisiae TISTR 5343	10.75	20.93	51.37

[a], standard streptomycin (Oxoid, U.K.) and nystatin (Sigma, U.S.A.) for testing bacteria and fungi, respectively;
[b], Environmental isolate; and -, No inhibition zone.

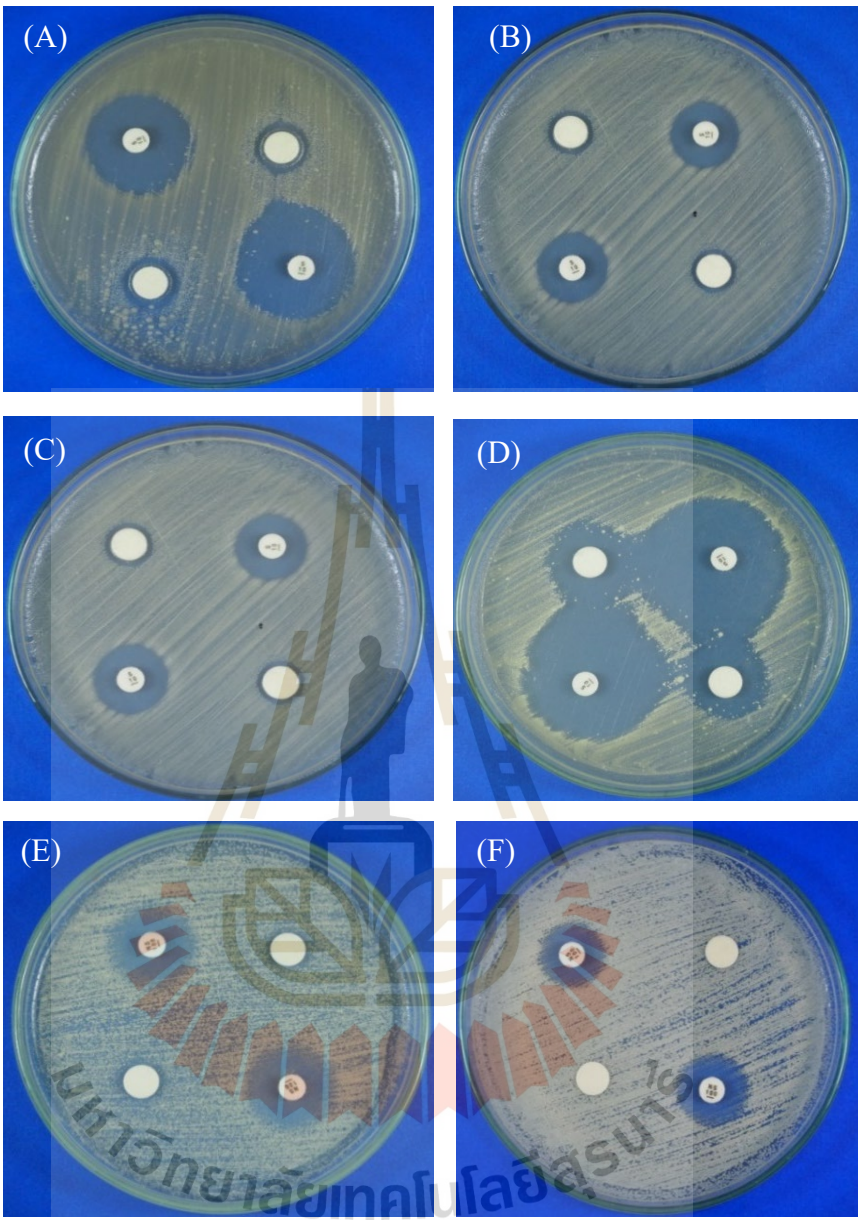

Figure 4.16 Examples of antimicrobial activity of essential oil from Ma Sang fruit against: *Staphylococcus aureus* ATCC 1466 (A), *Staphylococcus aureus* TISTR 517 (B), *Staphylococcus aureus* ATCC 29213 (C), *Staphylococcus xylosus* JCM 2418 (D), *Saccharomyces cerevisiae* TISTR 5343 (E), and *Candida albicans* TISTR 5744 (F).

4.4.2 MIC and MBC

The effect of essential oil from Ma Sang fruits to tested microorganism and efficacy to inhibit and kill the microorganism as MIC and MBC were investigated. The results were shown in Table 4.6, eight samples of essential oil from Ma Sang fresh and fallen fruits, different harvested month, and trees demonstrated different MIC and MBC of the tested microorganism. Essential oil from fresh fruits that harvested in September with different tree had remarkable different activity among other Ma Sang essential oils with lower MIC and MBC value than others. Two samples (different sampling lots) of the oils were found to inhibit *Staphylococcus aureus* ATCC 2913 with MIC ranging from 0.125 to 0.25 mg/mL and MBC at 2 mg/mL. While MIC and MBC of *Staphylococcus aureus* TISTR 517 of 0.5 and 16 mg/mL, respectively. *S. aureus* ATCC 29213 was more sensitive to the essential oil than *S. aureus* TISTR 517. The MIC and MBC of *Staphylococcus epidermidis* TISTR 518 and *Staphylococcus xylosus* JCM 2418 were also found at the range of 0.5 to 2.0 mg/mL, and at 8 to 16 mg/mL, respectively. In addition, essential oil samples of fresh fruits showed the similar inhibitory effect to *Bacillus cereus* TISTR 687 with MIC ranging from 0.5 to 1.0 mg/mL and MBC at 1.0 to 4.0 mg/mL. All samples of essential oil from Ma Sang fruits were detected at low inhibitory activity to *Escherichia coli* TISTR 780 with MIC and MBC at more than 16 mg/mL.

Essential oil was well known to have effect against wide range of microorganisms (Bakkali *et al.*, 2008). However, in this study, Gram-positive bacteria were found to be more susceptible to Ma Sang essential oils than Gram-negative bacteria. The attribution to penetrate through bacterial membranes to the interior of the cell have proposed to be main factor to relate the exhibit inhibitory activity on the

functional properties of the cell, and to their lipophilic properties of the bacteria cell (Bajpai *et al.*, 2012; Fisher and Phillips, 2009). Hydrophobicity of the oils and their chemical components were the of important property allowing the effect to bacteria cell by separating the lipid of bacteria cell membrane and mitochondria resulting inhibit cell growth (Burt, 2004; Friedly *et al.*, 2009). Essential oils have the effect of inhibiting growth of some Gram-positive and Gram-negative of bacteria. Gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* were more susceptible to essential oils than Gram-negative bacteria such as *Escherichia coli* and *Salmonella Enteritidis* (Chorianopoulos *et al.*, 2004). It is generally mechanism of essential oils should be more effective against Gram-positive bacteria due to the direct interaction of the cell membrane with hydrophobic components of the essential oil and components of Gram-positive membrane (Chao and Young, 2000; Cimanga *et al.*, 2002). Conversely, properties of hydrophilic cell wall of Gram-negative were not conducive to the influx of hydrophobic molecule as essential oils to the cell resulting more resistant that Gram-positive bacteria (Kim *et al.*, 2011). In the other hand, essential oils could coagulate the cytoplasm leading to alterations in several compartments of cell structure (Gustafson *et al.*, 1998; Burt, 2004).

Moreover, the activities could be attribute to major components present in the oil such as decyl acetate, decanal and decanol. Standard compounds of decyl acetate, decanal, and dacanol were used for determining MIC and MBC compared Ma Sang essential oil (Table 4.3). Decyl acetate showed the lowest antimicrobial activity with over 16 mg/mL to all tested microorganisms; *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* TISTR 517, *Staphylococcus epidermidis* TISTR 518

Staphylococcus xylosus JCM 2418, *Bacillus cereus* TISTR 687, *Escherichia coli* TISTR 780, and *Candida albicans* TISTR 5779 while decanal had moderate effect as MIC and MBC to *S. aureus* ATCC 29213 at 0.125 and 0.25 mg/mL and 1 mg/mL for both MIC and MBC of *B. cereus* TISTR 687, respectively. In contrast, dodecanol, fatty alcohol, has strongest effect against the tested-bacteria considering by MIC and MBC at 0.016 and 0.032 µg/mL of *S. aureus* ATCC 29213 and 0.04 and 0.08 µg/mL of MIC and MBC for *B. cereus* TISTR 687. From these results, decanol tended to be the important active compound in essential oil for Ma Sang fruits. Kubo at el. (1993) reported the effect of long chain alcohol decanol which had the strongest effect against *Streptococcus mutans* ATCC 25175, Gram-positive cocci bacteria, with MIC and MBC at 6.25 and 12.25 µg/mL, respectively. Decanol also could be strong inhibitory effect to vary Gram-positive bacteria *Bacillus subtilis*, *Propionibacterium acnes*, and *Staphylococcus aureus* with less than 0.05 mg/ml (Kubo at el., 1995). Recently study of essential oil from *Morinda lucida* fruits confirmed existence of alcohol and some terpene compounds, (E)-phytol (14.80%), n-octanol (6.19%) and β-caryophyllene (5.54%) showing good antibacterial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Prévost *et al*., 2018). In addition, there were reported that dodecanol and farnesol had ability to inhibit *Candida albicans* suppressed hypha formation through suppressed via the Ras1-cAMP-Efg1 signalling cascade (Davis-Hanna *et al*., 2007).

In addition, components with C_7-C_{12} carbon chain lengths from the hydrophilic hydroxyl group of long chain and isoprene alcohol with C_7-C_{12} chain lengths affected to activity against *S. mutants* such as 1-octanol (C_9) and farnesol (C_{12}) with MIC at 12.5 and 800 µg/mL, respectively. Recently studies indicated that decanal is one of major

component found in sweet orange oil, and had ability to be both bacteriostatic and bactericidal activity against Gram-positive bacterium (*Staphylococcus aureus*), Gram-negative bacterium (*Escherichia coli*) and fungi (*Saccharomyces cerevisiae* and *Aspergillus niger*) with less than 0.2 mg/mL (Liu *et al.*, 2012). However, the small proportion of the presence of decanol in Ma Sang essential oils, it might be possible synergism with other substances. Essential oil from Ma Sang fresh fruits harvested in September showed slightly different compounds with sesquiterpene such as α-caryophyllen (0.76%), β- bisabolene (0.28%), α-bisabolene (0.14), β-farnesene (0.06%) and α-bergamotene (0.05%). Isomeric caryophyllene; β-caryophyllene and caryophyllene oxide were found in *Zingiber nimmonii* and significant influence activity to wide range of microorganisms (Sabulal *et al.*, 2006). Leave essential oil of *F. lucida* could inhibit *Mycobacterium tuberculosis* with MIC at 0.1 mg/mL with comprising of over then compound found similar to Ma Sang fruits oils (Supudompol, 2009). In other hand, some plant essential oil exhibited strong bacteriostatic activity to *S. aureus*. For example, *Citrus limon* leaf essential oil showed MIC at 2.5 µg/mL due to effect of monoterpene, limonene (Langeveld *et al.*, 2014). Essential oil with terpene and phenolic compounds may contribute a strong antibacterial activity (Liu *et al.*, 2012). However, the ability to inhibit growth of microorganisms of essential oils depended on the chemical compound found in essential oils which those compounds could act as synergistic properties with other compounds or effect alone by main compound in highest proportion in essential oil (Bakkali *et al.*, 2008). The variation of MIC value of crude and the active fraction of coriander essential oils to inhibition effect to *Candida* spp. changing from 15.6-31.2 µg/mL to 31.2-250 µg/mL were confirmed the synergistic activity of the essential oil components (Mandal and Mandal, 2015).

Table 4.6 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oil from Ma Sang fruits.

Tested microorganism	MIC/MBC (mg/mL)											
	Ampicillin*	T1-Sept[1]	T2-Sept[2]	T1-Feb[3]	T2-Feb[4]	T3-Feb[5]	T4-Mar[6]	T3f-Feb[7]	RDF-Mar[8]	Decanol[8]	Decanal	Decyl acetate
Staphylococcus aureus ATCC 29213	0.1	0.125/2	0.5/2	4/8	0.5/16	1/>4	>16/>16	2/>2	>16/>16	0.016/0.032	0.25/2	>16/>16
Staphylococcus aureus TISTR 517	0.1	nd	0.5/16	4/8	16/>16	1/>4	nd	2/>2	>16/>16	nd	nd	>16/>16
Staphylococcus epidermidis TISTR 518	0.1	2/8	1/8	2/4	>8/>8	1/>4	>16/>16	>2/>2	>16/>16	nd	nd	>16/>16
Staphylococcus xylosus JCM 2418	0.1	0.5/16	2/8	2/>2	>8/>8	4/>4	nd	>2/>2	4/nd	nd	nd	>16/>16
Bacillus cereus TISTR 687	0.1	0.5/4	1/4	1/nd	4/nd	nd	0.5/1	nd	1/nd	0.04/0.08	1/1	>16/>16
Escherichia coli TISTR 780	2	>16/>16	>16/>16	>16/>16	>16/>16	>16/>16	>16/>16	>16/>16	>16/>16	nd	nd	>16/>16
Candida albicans TISTR 5779	nd	4/8	nd	nd	nd	nd	nd	nd	0.5	nd	nd	>16/>16

[1]Tree no. 2, fresh fruits [2]Tree no. 1, fresh fruits

[3]Tree no.2, fresh fruits [4]Tree no. 3, fresh fruits

[5]Tree no.4, fresh fruits [6]Tree no. 3, fallen fruits

[7]Randomly fallen fruits

[8]µg/mL

4.5 Killing rate of the sensitive test bacteria by essential oil treatment

The essential oil which provided the strongest antimicrobial activity, fresh fruit in September, were used for studying time-kill curve and mechanism of action against the most sensitive tested microorganism, two Gram-positive bacteria; *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* TISTR 687 (Figure 4.17). One and two-fold of minimum inhibitory concentration (1xMIC and 2xMIC) and minimum bactericidal concentration (MBC) of the essential oil were carried to determine effect of each essential oil concentration to killing rate of the tested bacteria. The results represented both strains displaying different sensitives to the oils. Effect of all concentration of the essential oil demonstrated reducing the viable cell in slightly different trend. For *Staphylococcus aureus* ATCC 29213 particularly at MBC (2 mg/mL) indicated the most decreasing of number of cells within 2 h and none of cells was detected after 2 h. While at 1xMIC (0.125 mg/mL) and 2xMIC (0.25 mg/mL) showed slightly decreasing of cell number in 2 h. At 2xMIC, the concentration showed the higher efficiency to inhibit growth of the strain than 1xMIC. After 6 h incubation, growth of the bacteria was slightly increased and stable at 10^7 and 10^3 CFU/mL until 24 h for 1x and 2xMIC, respectively.

In the treatment of *Bacillus cereus* TISTR 687, it showed moderately different from *S. aureus* ATCC 29213 treatment. At 1xMIC (0.5 mg/mL) and 2xMIC (1.0 mg/mL), the bacteriostatic effect to the strain with inhibition of growth of the bacterium occurred in 24 h. Besides, at MBC (4.0 mg/mL) demonstrated the strongest inhibition of cell in 2 h and no cell growth was observed after that. While at 1xMIC and 2xMIC treatment of the oil, there were not significantly different (p>0.05) over the time. The two treatment concentrations showed effect on growth inhibition of *B. cereus* TISTR 687

by lacking cell growth compared to control. These results confirmed that antibacterial activity of Ma Sang essential oil which significantly effect to the growth of *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* TISTR 687 by depended on concentration and treatment period.

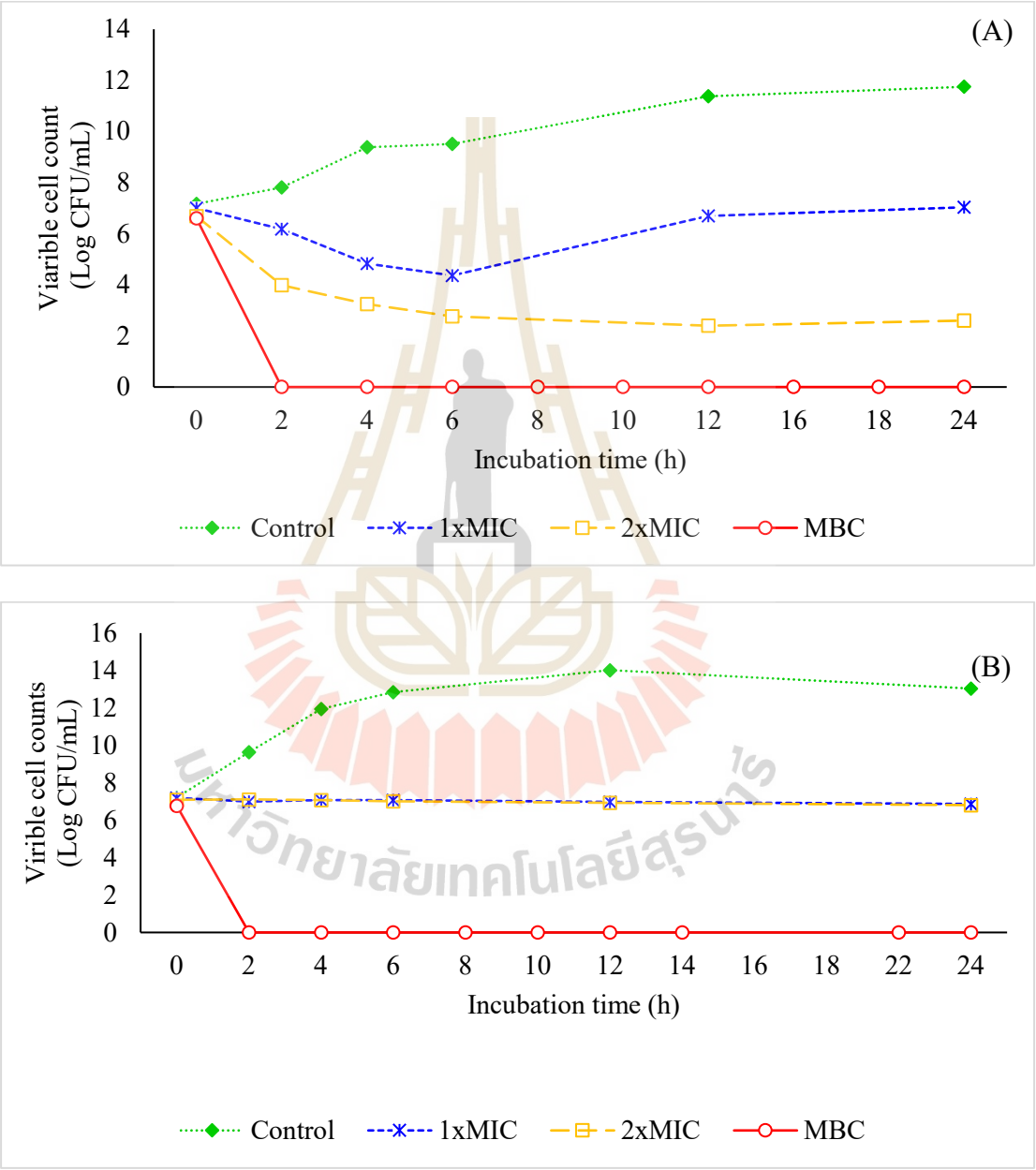

Figure 4.17 Viable cell count of *Staphylococcus aureus* ATCC 29213 (A) and *Bacillus cereus* TISTR 687 (B) after treating with essential oil from Ma Sang fruits at 1xMIC, 2xMIC and MBC for 24 h.

4.6 Mode of antimicrobial action of the essential oil

4.6.1 Cellular material release

The measurement of 260 nm-absorbing materials leakage demonstrated cell lysis by expected leakage of nucleic acids, DNA, and/or RNA (Huang *et al.*, 2018). Further exposing Ma Sang essential oil to cells of *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* TISTR 687 with its 1xMIC, 2xMIC and MBC for 30, 60, 90 and 120 min were found that either treatments comparing control without the essential oil not significantly different. The results indicated that the essential oil was not affected to integrity of the Gram-positive bacterial cell which was not leading to cell broken.

4.6.2 Protein leakage

The release of cellular proteins of *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* TISTR 687 were studied in different condition of Ma Sang essential oil and the results shown in Figure 4.18. For *Staphylococcus aureus* ATCC 29213 treatment, it indicated that at 1xMIC (0.125 mg/mL), 2xMIC (0.25 mg/mL) and MBC (2.0 mg/mL) caused more leakage of cellular protein when increasing concentration of the oil in 30 min comparing control. At 1xMIC showed 75.63 µg/mL of protein in 30 min of treatment and slowly increasing amount of protein to highest at 174.05 µg/mL in 120 min. While at 2xMIC, the concentration demonstrated slightly higher amount of protein than 1xMIC with 107.91 µg/mL in 30 min and to 268.04 µg/mL at the end of treatment time. However, MBC exhibited the strongest effect to *S. aureus* ATCC 29213 cell with 206.32 µg/mL in 30 min and tented to increase until dropped in 90 min with 240.51 µg/mL. The protein leakage of *Bacillus cereus* TISTR 687 after treated with the oil at 1xMIC (0.5 mg/mL) and 2xMIC (1.0 mg/mL) were not different trend from *S.*

aureus ATCC 29213. At 1xMIC (0.5 mg/mL) the protein of 8.54 µg/mL was detected within 30 min after treating and increasing to 42.72 µg/mL in 120 min.

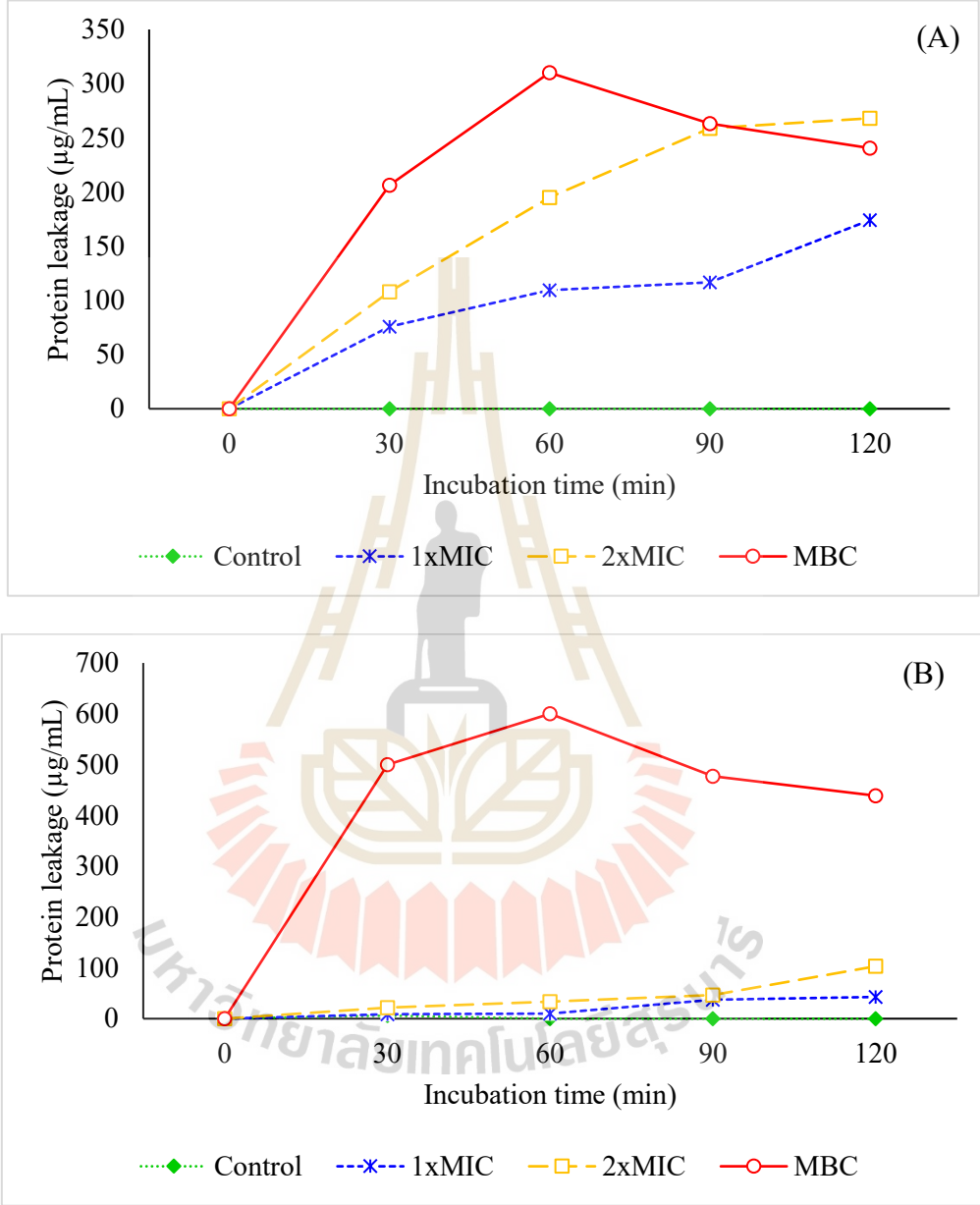

Figure 4.18 Leakage of cellular protein from *Staphylococcus aureus* ATCC 29213 (A) and *Bacillus cereus* TISTR 687 (B) after treated with essential oil from Ma Sang fruits at 1xMIC, 2xMIC, and MBC for 24 h.

Similarly, at concentration of 2xMIC (1.0 mg/mL), the essential oil demonstrated 21.52 µg/mL and increasing to 33.23, 46.20 and 103.16 µg/mL. Protein leakage at MBC of *B. cereus* ATCC 29213 treatment presented a higher amount of protein of 499.91 µg/mL releasing at 30 min and quite dropped at 90 min as shown in *S. aureus* ATCC 29213 treatment. The result of protein leakage represented possibility that the composition of essential oils might appear to act on proteins embedded on cell membrane of cytoplasm but not causing injury of the cell. It may be possible that Ma Sang essential oil binding on cell membrane resulting disturbance of enzymes such as ATPases are known to be bordered by lipid molecules which lipophilic hydrocarbons could act on by accumulate in the bilayer and distort the lipid–protein interaction; alternatively, the direct interaction of lipophilic compounds with hydrophobic parts of the protein is possible (Burt, 2004).

4.6.3 Potassium ion efflux

Essential oil from Ma Sang fruits represented efficiency causing potassium ion changes in cell of *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* TISTR 687 in 30 min at 400 µg/mL and no significant different (p>0.05) changed in 120 min (Figure 4.19).

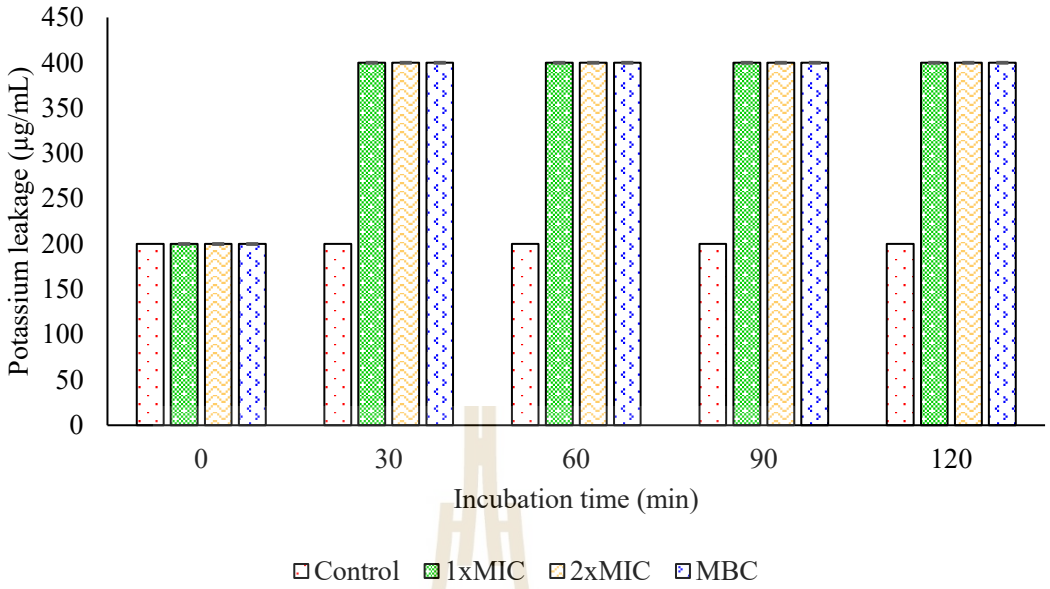

Figure 4.19 Leakage of potassium ion from *Staphylococcus aureus* ATCC 29213 (A) and *Bacillus cereus* TISTR 687 (B) after treating with essential oil from Ma Sang fruits at 1xMIC, 2xMIC and MBC for 24 h.

4.6.4 Extracellular ATP concentration

The effect of essential oil from Ma Sang fruits on extracellular ATP concentration in *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* TISTR 687 cells were presented in Figure 4.20. The extracellular ATP concentration in untreated (control) *S. aureus* ATCC 29213 and *B. cereus* TISTR 687 were 2.22 and 4.51 ng/mL. Cells of *B. cereus* TISTR 687 treated with Ma Sang essential oil at MIC (0.5 mg/mL) and MBC (4 mg/mL) showed no significant difference (p>0.05) in extracellular ATP concentration from control with 4.33 and 3.15 ng/mL, respectively.

In contrast, *S. aureus* ATCC 29213 cells were treated with MIC and MBC of Ma Sang essential oil at 0.125 and 2 mg/mL, and showed significantly (p>0.05) decreased of extracellular ATP concentration to 1.86 to 1.10 ng/mL. The results could indicate that essential oil from Ma Sang fruit not caused a bulky damage of cell

membrane that resulting in leakage of extracellular ATP occur. The reduction of extracellular ATP concentration were found in *S. aureus* ATCC 29213 after treated with Ma Sang essential oil which represented quite different from other reports (Paul *et al.*, 2011; Bajpai *et al.*, 2013). The molecules of essential oil were more concentrated which might result in less flow of large molecules such as ATP out of bacterial cells. Based on the results of measurement, the leakage of various substances from bacterial cells after treated with Ma Sang essential oil were found. Protein, amino acid and small amount of potassium ion were detected when treated *S. aureus* ATCC 29213 and *B. cereus* TISTR 687 with 1xMIC, 2xMIC and MBC. No leakage of cellular materials such as nucleic acid and ATP were presented. Therefore, essential oil from Ma Sang could not cause cell leakage or injury to *S. aureus* ATCC 29213 and *B. cereus* TISTR 687. In the other hand, the essential oil could disturb the bacterial cell wall resulting in unable to grow regularly. However, effects of essential oil were well known including destabilization of the phospholipid bilayer, the destruction of the plasma membrane function and composition, the loss of vital intracellular components and the inactivation of enzymatic mechanisms (Nazzaro *et al.*, 2013).

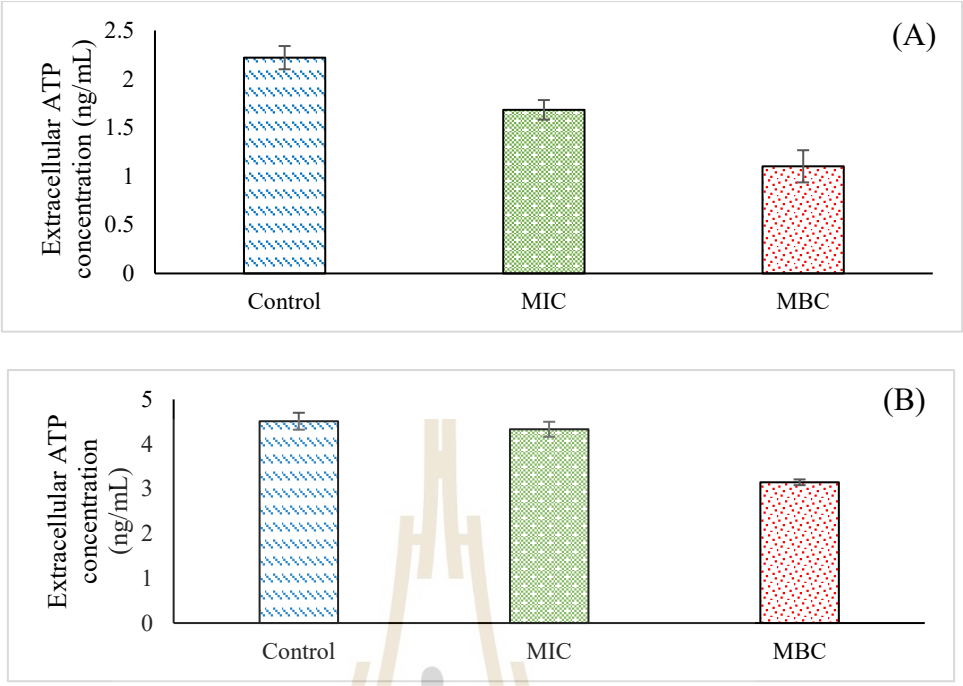

Figure 4.20 Leakage of extracellular ATP concentration from *Staphylococcus aureus* ATCC 29213 (A) and *Bacillus cereus* TISTR 687 (B) after treating with essential oil from Ma Sang fruits at 1xMIC, 2xMIC and MBC for 24 h.

4.7 Effect of essential oil from Ma Sang fruits on bacterial cell morphology disruption

The SEM analysis was used to investigated physical and morphological alterations in cell wall of the sensitive test bacterium, *Staphylococcus aureus* ATCC 29213 (Figure 4.21). Essential oil of Ma Sang fruits caused morphological alterations on the cell wall of the bacterium. Cells of the bacteria were changed with more crease and furrow at concentration of essential at MIC while greater concentration as MBC. Sikkema *et al.* (1995) described effect of the essential oil to alterations in membrane lipid composition, when was thought to be a compensatory mechanism to counter the lipid disordering effects of the treatment agent. Moreover, the hydrophobic molecules

of essential oils also have the ability to bind with cell surface then penetrated into the target area such as membrane-bound enzymes which may cause changes in the cell membrane layer and causing the collapse of the cell wall layer (Nazzaro *et al.*, 2013).

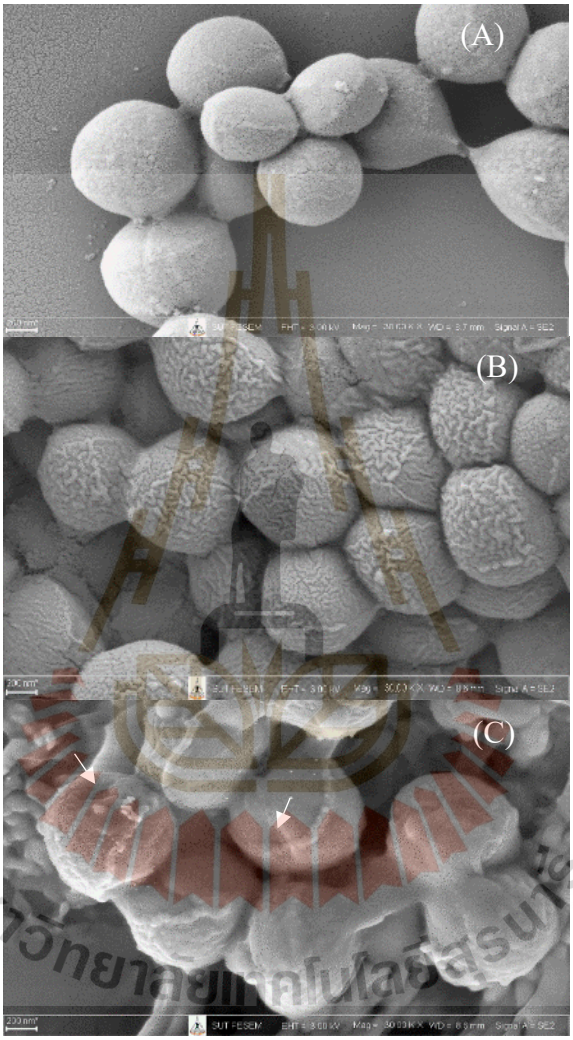

Figure 4.21 SEM micrographs of *Staphylococcus aureus* ATCC 29213 cells without treating with Ma Sang essential oil (A), after treating with the essential oil at 1xMIC (B), and MBC for 4 h (C).

CHAPTER V

CONCLUSION

The plant, Ma Sang (*Feroniella lucida* (Scheff.) Swingle.), is a medium-sized tree belonging to the Rutaceae family which was widely found in Northern and Northeastern Thailand. The young shoots of leaves, flowers, and fruits are commonly used as vegetables and sour taste seasoning by local people. The barks, twigs, leaves, and root of the plant species have been known as sources of abundant bioactive compounds. There are limited studies available regarding using the fruits particularly in terms of essential oil. This study aimed to increase the use of the fruits and study a possibility to extract essential oil from Ma Sang fruits and its chemical compositions, antimicrobial activity, and mode of action.

Ma Sang fruits that collected from natural habitat Satuek District, Buriram Province, Thailand. The fruits composed of peel, pericarp, and pulp with seeds at the proportion of 9.0, 64.0, and 27.0%, respectively of total fruit mass. The moisture contents of peel, pericarp, pulp, and the seed were 56.21, 57.15, 78.00, and 48.89%, respectively. Essential oils were extracted from different lots of collected mature fruits, different trees, and different collection times showed quite similar characteristics with a strong unique scent and clear yellow color. Yields of essential oil from seven lots of Ma Sang fruits samples showed slightly different results ranging from 0.59 to 0.77% (v/ww). Interestingly, there was no difference in essential oil yields from fallen fruits and fresh fruits collected from the same tree. This makes the fruit harvest time more flexible for essential oil extractio

From GC-MS analysis, the total chemical components of essential oil from Ma Sang fruits contained around 20 to 27 compound which could be classified into six groups of compounds. The numbers of compounds had a slightly different within each lot of samples. Ester was the major group of the oil, which was more than 60% of the total oil followed by aldehyde (7.86-22.75%), alcohol (5.10-9.10%), terpene (1.35-6.64%), epoxide (0.86-1.99%), and saturated fatty acid (0.10-0.22%). Four main major groups of compounds were detected in the oil including decyl acetate (56.2-66.73%), decanal (7.79-22.49%), dodecyl acetate (6.81-12.93), and decanol (3.95-7.68). The high proportions of ester and aldehyde were recognized as a strong unique scent of the oil. The different collections of Ma Sang fruits, fresh fruits collected from the tree and falling fruits, were not affected the variation of main compounds of the oil but affected the proportions of aldehyde and ester of the oil. The essential oil from fallen fruits was found to contain higher aldehyde compound and fewer ester compound than fresh fruits. In contrast, the essential oil from fresh Ma Sang fruits was found to be ester compound groups. Similarly, the essential oil from different collection months of the fruits and trees was shown to have different minor components in both fresh fruits from the tree and falling fruits. The oil from Ma Sang fruits collected from different trees in February contained same 21 compounds with slightly different for each tree as follows: trees no. 1 contained decane (0.06%) and undecanol (23.39%), no. 2 also contained decane (0.06%), α-bergamotene (0.19%), farnesene (0.23%), β-bisabolene (0.82%) and sesquiphellandrene (0.09%), while the tree no. 3 was not found in additional substances. The oil obtained from fruits collected in September, had a slightly different chemical composition from the same tree that were collected in February with detecting none of β-sesquiphellandrene, 1-tetradecyl acetate, farnesyl acetate, and octanal. However, the

maturation of fruits would affect the existence or increase in same elements as shown in different compounds found in fresh and fallen fruits more than found in the different months of sampling lots and variety of the trees.

In term of antimicrobial activity, the essential oil from Ma Sang fruit had a great inhibitory effect to a wide range of test microorganisms with 50.00 to 84.17% inhibition especially the greatest effect to Gram-positive bacteria, *Bacillus cereus* TISTR 687, *Staphylococcus aureus* ATCC 1466, *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* TISTR 517, *Staphylococcus epidermidis* TIRTS 518, and *Staphylococcus xylosus* JCM 2418 with 7.25, 14.25, 11.22, 12.20, 18.45, 25.93 mm of inhibition zones, respectively. The oil showed ability against test microorganisms with MIC and MBC in the range of 0.125-16 and 2-16 mg/mL, respectively. The essential oil from fresh fruits that harvested in September had remarkable activity with the lowest MIC at 0.125 and 0.5 mg/mL and MBC at 2 and 4 mg/mL with the most sensitive bacteria, *Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* TISTR 687, respectively while no effect on Gram-negative bacteria. The essential oil had a significant effect to inhibit growth of surviving bacterial cells within two hours resulting in protein and potassium leakage which evaluated the possibility of disruption of the cell wall and cell membrane permeability. The essential oil could disturb the bacterial cell wall resulting in unable to grow regularly leading to the leakage of small molecules such as potassium ion, protein fragments, amino acid l. However, the leakage of larger molecules such as DNA and ATP were not found. The results showed that the essential oil did not affect to the large cell damage whereas might interfere with the cell wall and cytoplasmic membrane which might lead to cell weakness and ultimately resulted in cell death. The study of morphological changes of *S. aureus* ATCC 29213

cells by scanning electron microscope (SEM) was confirmed that the appearance of the bacterial cell surfaces was more wrinkled and the shape changes after treating with a higher concentration of the oil. These results represent a contemporary approach using Ma Sang fruits in the application of non-toxic essential oil for the antimicrobial agent.

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APPENDICES

APPENDIX A

CULTURE MEDIUM AND REAGENT PREPARATION

1. Culture media

1.1 Tryptic soy agar (TSA)

Tryptone (pancreatic digest of casein)	17.00	g
Soya peptone	3.00	g
NaCl	5.00	g
K_2HPO_4	2.50	g
Glucose	2.50	g
Agar	15.00	g

The compositions were dissolved in deionized water, adjusted to pH 7.3±0.02 with HCl or NaOH, and adjusted the volume to 1000 mL with deionized water. Then, the solution was sterilized by autoclaving for 15 min at 121ºC, 15 lb/square inches after preparation.

1.2 Malt yeast agar (MY)

Malt extract	3.00	g
Yeast extract	3.00	g
Peptone	5.00	g
Glucose	10.00	g
Agar	20.00	g

The compositions were dissolved in deionized water, adjusted to pH 7.3 with HCl or NaOH, and adjusted the volume to 1000 mL with deionized water. Then, the solution was sterilized by autoclaving for 15 min at 121ºC, 15 lb/square inches after preparation.

1.3 Mueller Hinton (MHA)

Starch	1.50	g
Beef extract	2.00	g
Tryptone (pancreatic digest of casein)	17.50	g
Agar	15	g

The compositions were dissolved in deionized water, adjusted to pH 7.3 with HCl or NaOH, and adjusted the volume to 1000 mL with deionized water. Then, the solution was sterilized by autoclaving for 15 min at 121ºC, 15 lb/square inches after preparation.

2. Reagents and buffers

2.1 Crystal violet (Gram stain)

Crystal violet	2.00	g
Ethanol (95%)	20.00	mL

Ammonium oxalate (1% Aqueous solution) 80 mL

The components were dissolved in deionized water and then filtrated through Whatman No.1 paper then placed in a stoppered bottle and kept cool in the dark place.

2.2 Normal saline solution

Sodium chloride	8.50	g

The component was dissolved in deionized water and brought volume up to 1000 mL and autoclaved at 121ºC for 15 min, 15 lb/square inches after preparation.

2.3 0.5% Tween 80

Tween 80	0.50	g

The components were dissolved in deionized water and brought volume up to 1000 mL and adjusted pH to 7.0 with HCl or NaOH. The reagent autoclaved at 121ºC for 15 min ,15 lb/square inches after preparation.

2.4 0.1M Phosphate buffer saline (PBS)

NaCl	80.00	g
KCl	2.00	g
Na_2HPO_4	14.40	g
KH_2PO_4	2.40	g

The components were dissolved and adjusted pH to 7.4 with HCl. Then the final volume was adjusted to 1000 ml with deionized water and then, the reagent was autoclaved at 121ºC for 15 min, 15 lb/square inches after preparation.

2.5 0.5 McFarland standard

1.0% (w/v) barium chloride ($BaCl_2$•$2H_2O$)	0.05 mL
1.0% (w/v) sulfuric acid (H_2SO_4)	9.95 mL

The components were mixed together and then, the reagent was autoclaved at 121ºC for 15 min, 15 lb/square inches after preparation.

2.6 Lowry's reagent

2% (w/v) Na_2CO_3	200.00 mL
1% (w/v) $CuSO_4.5H_2O$	20.00 mL
2% (w/v) sodium potassium tartrate	20.00 mL
1N Folin-Ciocalteu reagent	20 µL per well

The components mixed together and rest at room temperature for 10-15 min before using.

APPENDIX B

CHEMICAL ANALYSIS METHODS

1. Total protein

Total protein concentration was estimated by measuring the characteristic blue color at 650 nm (colorimetric assay) according to Lowry *et al.* (1951). The standard curve for calculating the concentration of total protein as follows:

(1) Various concentrations (0, 10, 20, 30, 40, 50, 60 and 70 μg/mL) of bovine serum albumin (BSA) were prepared.

(2) One hundred microliter of BSA solution containing between 10 to 70 μg/mL were pipetted into well of 96 well plate, and 200 μL of biuret reagent containing 2% (w/v) Na_2CO_3, 1% (w/v) $CuSO_4$.5H2O, 2% (w/v) sodium potassium tartrate was added. Then 20 μL of 1N Folin-Ciocalteu reagent were rapidly added and allowed to colour develop for 30 min at room temperature before reading the absorbance of the blue colour solution at 670 nm.

(3) A standard curve was constructed by plotting BSA concentration versus absorbance 670 nm. The amount of total protein could then be determined by reference to the standard curve (Figure B1).

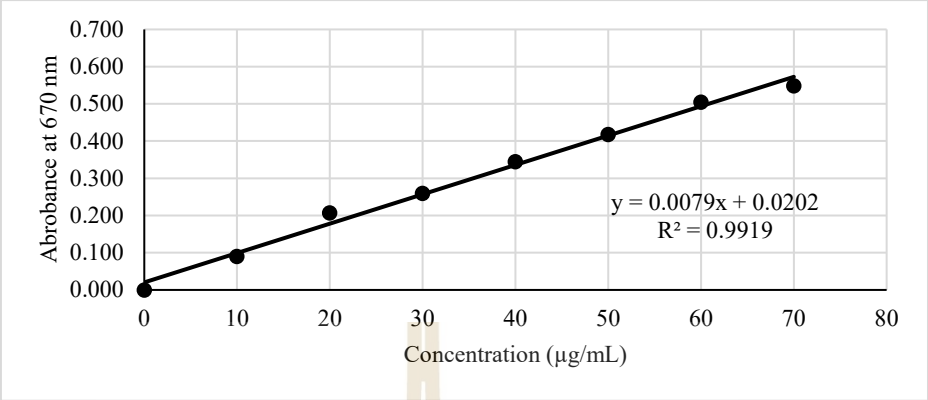

Figure B1. Standard curve of bovine serum albumin (BSA, Sigma) for the determination of protein (Lowry *et al.*, 1951) determined in this study.

2. Total Adenosine triphosphate (ATP)

Adenosine triphosphate (ATP) concentration were calculated from the standard ATP curve which obtained results as RLU; Relative Light Unit. The standard curve for calculating the concentration of ATP was performed as follows:

(1) ATP standard solutions were prepared by tenfold dilution at $3.68x10^{-8}$ to $3.68x10^{-12}$, mol/L from stock solution of 7.25 mol/L.

(2) One hundred of ATP Assay Mix solutions (Sigma, U.S.A.) were added to well of 96-well solid white flat bottom (Corning®. U.S.A.) and allowed to stand at room temperature for 3 min. Then rapidly adding 100 µL of ATP standard, mixed slowly by pipette and immediately measured the amount of light produced with a luminometer.

(3) A standard curve was constructed by plotting ATP concentration versus Relative Light Unit (RLU). The amount of extracellular ATP leakage could then be determined by reference to the standard curve (Figure B2).

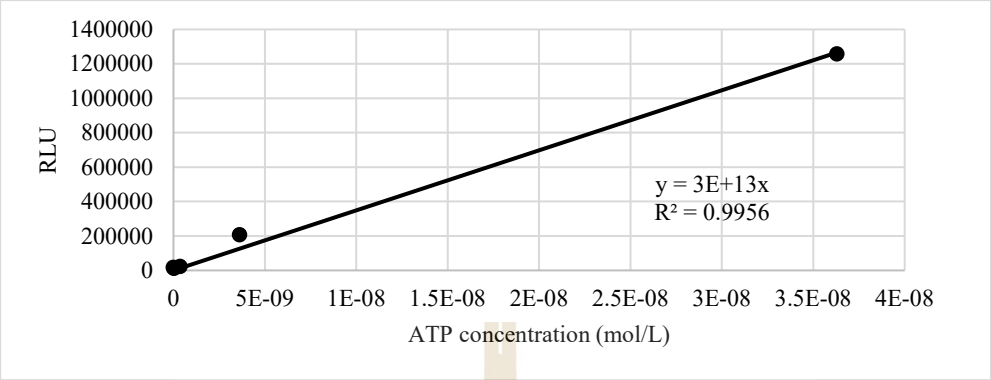

Figure B2. Standard curve of ATP for the determination of ATP concentration in this study.

B3 Lambda max

The result of the optimum wavelengths used for measuring the growth of the test microorganisms in this study was performed as follows:

(1) For bacterial test microorganisms, the active cultures were prepared in tryptic soy broth (TSB, Appendix A1.1) at 35ºC overnight while the fungi were cultured in malt yeast broth (MY, Appendix A1.2) at 30ºC overnight.

(2) Then, the cultures were cross streaked on TSA (Appendix A1.2) for bacteria or MY agar (Appendix A1.2) for fungi for pure culture colony.

(3) The pure culture was transferred to 0.85% normal saline and adjusted turbidity with the 0.5 McFarland standard suspension (Appendix A2.5 approximately 10^8 CFU/mL).

(4) The suspensions were used to scan the lambda max for each test organisms using Epoch Microplate Spectrophotometer (BioTek®, U.S.A.) at 400 to 625 nm.

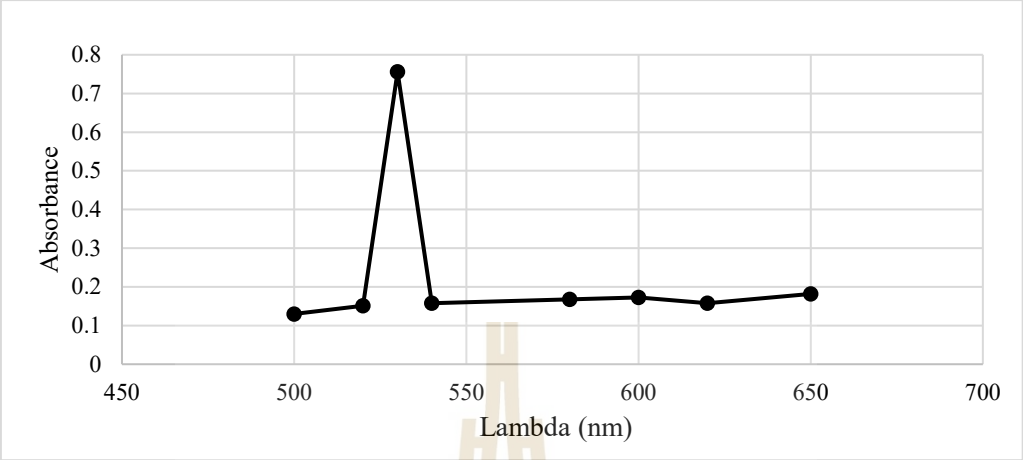

Figure B3. Example of preparing appropriate lambda max at *530* nm for bacterium, Staphylococcus aureus TISTR517 cell turbidity

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APPENDIX C

LIST OF PRESENTATIONS

Poster Presentation

Nopanitaya, T., Pan-anu, A., and Rodtong, S. (2017). Antimicrobial activity of essential oil from Ma Sang fruits. The 12th Conference on Science and Technology of Thailand for Youths. June 3-4, 2017. The Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, Thailand.

Nopanitaya, T., Rodthong, S., and Yongsawatdigul, J. (2019). Antibacterial Activity of Essential Oil from *Feroniella lucida* (Scheff.) Swingle. Fruits against *Staphylococcus aureus.* The 21st Food Innovation Asia Conference. June 13-15, 2019. The Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, Thailand.

ฤทธิ์ยับยั้งจุลินทรีย์ของน้ำมันหอมระเหยจากผลมะสัง

ตะวัน นพนิตย์[1] อัยรา พันอนุ[2] และ สุรีลักษณ์ รอดทอง[2,*]
[1]สาขาวิชาชีววิทยา สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี จ.นครราชสีมา 30000
[2]สาขาวิชาปรีคลินิก สำนักวิชาวิทยาศาสตร์ มหาวิทยาลัยเทคโนโลยีสุรนารี จ.นครราชสีมา 30000
E-mail address: sureelak@sut.ac.th โทรศัพท์ 044-224297

บทคัดย่อ: มะสัง (*Feroniella lucida* (Scheff.) Swingle.) เป็นพืชป่าพบได้ทั่วไปแถบภาคตะวันออกเฉียงเหนือของประเทศไทย ให้ผลสดปริมาณมากที่มีการนำไปใช้ประโยชน์เพียงเพื่อเพิ่มรสเปรี้ยวให้อาหารของคนในท้องถิ่น การศึกษาฤทธิ์ยับยั้งจุลินทรีย์ของน้ำมันหอมระเหยจากผลแก่ของมะสังเป็นแนวทางหนึ่งที่สามารถเพิ่มการใช้ประโยชน์ ผลแก่ของมะสังที่ศึกษามีเส้นผ่านศูนย์กลางโดยเฉลี่ย 5.73-7.0 เซนติเมตร มีชั้นเปลือกนอก เปลือกแข็ง และเนื้อในกับเมล็ด ราวร้อยละ 9, 64 และ 27 ของน้ำหนักผลสดทั้งหมดที่มีความชื้นร้อยละ 56, 57 และ 78 ตามลำดับ พบน้ำมันหอมระเหยเฉพาะที่เปลือกนอกในปริมาณร้อยละ 0.37 ปริมาตรต่อน้ำหนักสด ที่มีสาร Decanoic acid, ethyl ester; 1-Decanol และ Acetic acid, decyl ester เป็นองค์ประกอบหลักโดยเฉลี่ยร้อยละ 45.50, 13.23 และ 13.16 ตามลำดับ และมีฤทธิ์ยับยั้งแบคทีเรีย *Serratia marcescens* TISTR 1354, *Staphylococcus xylosus* JCM 2418, *Staph. aureus* TISTR 517 และยีสต์ *Saccharomyces cerevisiae* TISTR 5343 ผลที่ได้แสดงศักยภาพในการใช้ประโยชน์ผลมะสังต่อไป

คำสำคัญ: *Feroniella lucida*, น้ำมันหอมระเหย, ฤทธิ์ยับยั้งจุลินทรีย์

Antimicrobial Activity of Essential Oil from Ma Sung Fruits

Tawan Nopanitaya[1], Aiyara Pan-anu[2], and Sureelak Rodtong[2,*]
[1]School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
[2]School of Preclinic, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand
E-mail address: sureelak@sut.ac.th Tel. 044-224297

Abstract: Ma Sung or Wood Apple (*Feroniella lucida* (Scheff.) Swingle.) is a native plant commonly found in North-Eastern Thailand. The plant yields a lot of fruits only consumed as a sour ingredient for food by local people. The study of antimicrobial activity of essential oil from ripe Ma Sung fruits could assist to increase the use of the fruits. From this investigation, mature Ma Sung fruits had the average diameters of 5.73-7.0 cm composing of peel, hard pericarp, and pulp with seeds at the proportions of 9, 64, and 27% of wet weight with moisture contents of 56, 57, and 78%, respectively. Only the peel accumulated essential oil around 0.37% v/wet w. The oil mainly contained decanoic acid, ethyl ester; 1-decanol; and acetic acid, decyl ester at the average proportions of 45.50, 13.23, and 13.16%, respectively, and efficiently inhibited the growth of bacteria, *Serratia marcescens*, *Staphylococcus xylosus* JCM 2418, and *Staph. aureus* TISTR 517; and the yeast, *Saccharomyces cerevisiae* TISTR 5343. These results support further application of Ma Sung fruits.

Keywords: *Feroniella lucida*, Essential oil, Antimicrobial activity

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EPJ144

Antibacterial Activity of Essential Oil from *Feroniella lucida* (Scheff.) Swingle. Fruits against *Staphylococcus aureus*

Tawan Nopanitaya1, Sureelak Rodthong1* Jirawat Yongsawatdigul2 (1) School of Priclinic, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, (2) Food Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. *Corresponding author: (sureelak@sut.ac.th)

The facultative anaerobic, Gram-positive, and non-spore-forming bacterium having spherical cells arranged as grape-like clusters, identified as belonging to *Staphylococcus aureus* involves with food spoilage and foodborne illness particularly food intoxication. The safe antibacterial agents are still needed to apply for controlling or inactivating the pathogen. In this study, the essential oil extracted from mature fruits of *Feroniella lucida* (Scheff.) Swingle. by hydro-distillation, was investigated for its antibacterial activity against *Staphylococcus aureus*. Two strains of standard test bacterium, *Staphylococcus aureus* ATCC 29213 and *S. aureus* TISTR 517, were used for demonstrating the antibacterial activity using disk diffusion technique, and the percentages of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). From the disk diffusion test, *S. aureus* TISTR 517 was more sensitive to the oil than another strain, *S. aureus* ATCC 29213. But both strains showed the same values of MIC and MBC, which were at 16 mg/mL. Modes of action of the essential oil by detecting the leakages of cellular materials and protein after treating with 1xMIC (4 mg/mL) and 2xMIC (8 mg/mL) of the essential oil, which revealed the permeability and integrity of the bacterial cells, were then performed. Only the essential oil at 2xMIC exhibited significantly disturbing the permeability of cytoplasmic membrane causing cellular materials to efflux out the cells after 24 h treatment. No leakage of protein was detected. The chemical composition of the essential oil was also analyzed by GC-MS. The major components found were decyl acetate (52.6%), decanal (22.49%), dodecyl acetate (6.81%), and decanol (6.82%). These results support the application of essential oil from *Feroniella lucida* (Scheff.) Swingle fruits to control the pathogenic Staphylococcus aureus.

Keywords: *Feroniella lucida (Scheff.) Swingle, essential oil, Staphylococcus aureus, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC)*

CURRICULUM VITAE

Name Miss Tawan Nopanitaya

Date of Birth 10 June 1995

Place of Birth Surat Thani, Thailand

Education

2013-2017 B.Sc. (Biology), Suranaree University of Technology, Thailand

Scholarship

2013-2017 Scholarships for Talented Student in Science and Technology

2017-Present Graduate Students scholarship under SUT Research and Development Support Fund 2018, Whose Lecturers Are Awarded Research Funding by Outside (OROG)

2018-Present The strategic direction research and innovation: graduate level from National Research Council of Thailand year 2019

Presentation and conferences

Nopanitaya, T., Rodthong, S., and Yongsawatdigul, J. (2019). Antibacterial Activity of Essential Oil from *Feroniella lucida* (Scheff.) Swingle. Fruits against *Staphylococcus aureus.* The 21st Food Innovation Asia Conference. June 13-15, 2019. The Bangkok International Trade and Exhibition Centre (BITEC), Bangkok, Thailand.