# Supercritical Carbon Dioxide Extracted - Volatile Oils from Rutaceous Plants for Aromatherapy

**Porchaya Chumsuwan**

**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of**

**Master of Science in Herb Sciences (International Program)**

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**Major Program** Herb Sciences (International Program)

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**ชื่อวิทยานิพนธ์** น้ำมันหอมระเหยของพืชในวงศ์ Rutaceae จากการสกัดด้วยคาร์บอนไดออกไซด์วิกฤตยิ่งยวดเพื่อใช้ในสุคนธบำบัด

**ผู้เขียน** นางสาว ปอชญา ชุมสุวรรณ

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**ปีการศึกษา** 2554

## บทคัดย่อ

สุคนธบำบัดเป็นธรรมชาติบำบัดที่มีการใช้น้ำมันหอมระเหยเพื่อการรักษาโรค ในการแพทย์แผนโบราณนั้นมีการใช้น้ำมันหอมระเหยของพืชในวงศ์ Rutaceae โดยเฉพาะอย่างยิ่งในพืชสกุล *Citrus* ในการขับลม รักษาอาการนอนไม่หลับ และบรรเทาอาการไอ น้ำมันจากพืชตระกูลนี้มีกลิ่นที่ทำให้รู้สึกสดชื่น มีฤทธิ์ฆ่าเชื้อ กระตุ้นจิตใจ และส่งผลต่อระบบย่อยอาหารทั้งระบบ การศึกษาครั้งนี้สกัดน้ำมันหอมระเหยจากพืชสกุลนี้โดยการสกัดด้วยคาร์บอนไดออกไซด์วิกฤตยิ่งยวด วิธีการนี้ใช้อุณหภูมิต่ำและแก้ปัญหาของการใช้สารละลายในการสกัดและลดการปนเปื้อน

จากการศึกษาการสกัดน้ำมันหอมระเหยจากพืชในวงศ์ Rutaceae 4 ชนิด อันประกอบด้วยเปลือกส้ม *Citrus reticulata* 4 สายพันธุ์ ได้แก่ เขียวหวาน สายน้ำผึ้ง โชกุนและหัวจุก เปลือกส้มโอ *Citrus maxima* 4 สายพันธุ์ ได้แก่ หอมหาดใหญ่ ทองดี ขาวใหญ่และขาวน้ำผึ้ง เปลือกมะนาว *Citrus aurantifolia* และเปลือกส้มจี๊ด *Fortunella japonica* พบว่าการสกัดด้วยคาร์บอนไดออกไซด์วิกฤตยิ่งยวดสามารถเตรียมน้ำมันหอมระเหยได้ปริมาณแตกต่างกันที่ 0.28-6.91 % v/w และส้มธุ์สายน้ำผึ้งมีปริมาณน้ำมันหอมระเหยมากที่สุด (6.91%) จากการวิเคราะห์องค์ประกอบของน้ำมันหอมระเหยโดยโครมาโตกราฟีพบว่าสารหลักของน้ำมันหอมระเหยของพืชทั้งสิบสายพันธุ์เหมือนกันคือ *d*-limonene แต่มีปริมาณแตกต่างกันไป จากการศึกษาฤทธิ์ต้านเชื้อจุลชีพพบว่า น้ำมันมะนาวมีฤทธิ์ยับยั้งและฆ่าเชื้อดีที่สุด โดยสามารถยับยั้งและฆ่าเชื้อ *Staphylococcus aureus* และ *S. epidermidis* ได้ที่ความเข้มข้น 0.63 mg/ml และเชื้อ *Trichophyton rubrum* ได้ที่ 0.04 mg/ml สำหรับการทดสอบฤทธิ์ต้านการอักเสบนั้น พบว่าน้ำมันมะนาว ส้มจี๊ดและส้มโอหอมหาดใหญ่สามารถยับยั้งการสร้าง NO ได้ดีโดยมีค่า $IC_{50}$ = 10.2, 12.1 และ 20.3 µg/ml ตามลำดับ ในขณะที่ชนิดอื่นสามารถยับยั้งได้ปานกลางและมีค่า $IC_{50}$ ใกล้เคียงกับ *d*-limonene และไม่พบความเป็นพิษต่อเซลล์ของน้ำมันหอมระเหย

ทั้ง 10 ที่ความเข้มข้น 100 μg/ml และการศึกษาครั้งนี้ไม่พบฤทธิ์ต้านอนุมูลอิสระโดยวิธี DPPH radical scarvenging assay

การศึกษาก่อนการตั้งตำรับของน้ำมันมะนาว ส้มจี๊ด และส้มโอสายพันธุ์หอมหาดใหญ่ที่มีฤทธิ์ยับยั้งเชื้อจุลชีพและต้านการอักเสบพบว่า ลักษณะน้ำมันแต่ละตำรับมีความเป็นเนื้อเดียวกันแต่ไม่ปรากฏฤทธิ์การยังยั้งเชื้อจุลชีพ ต้านการอักเสบ และต้านอนุมูลอิสระ อาจสืบเนื่องมาจากความจำกัดในปริมาณของน้ำมันหอมระเหยที่ใช้ ผลการศึกษาความคงตัวจากอุณหภูมิและแสงพบว่า แสงสว่างไม่มีผลต่อคุณสมบัติทางกายภาพของตำรับเมื่อเวลาผ่านไปสามเดือน แต่ที่อุณหภูมิสูง (60 องศาเซลเซียส) มีผลทำให้ความหนืดของน้ำมันเปลี่ยนไปและมีกลิ่นหืน

**Thesis Title** Supercritical carbon dioxide extracted - volatile oils from Rutaceous plants for aromatherapy

**Author** Ms. Porchaya Chumsuwan

**Major Program** Herb Sciences (International program)

**Academic Year** 2011

## ABSTRACT

Aromatherapy is one of the natural therapies that are the use of essential oils for therapeutic or medical purposes. Rutaceous volatile oils, especially *Citrus* genus are used as carminative, treatment for insomnia, and antitussive effect in the traditional medicine. They have a refreshing aroma and antiseptic, stimulating and tonic, having significant effects on the whole of the digestive tract. In the present study, supercritical carbon dioxide extraction ($SCO_2$) is appropriate to solve the problems of the other methods. It could extract essential oils with low temperature and no solvent used resulting in the improvement of the extract efficiency and the reduction of impurities.

In this study, ten Rutaceous peel were selected including *Citrus reticulata* varied in 4 cultivars (Keaw-hwarn, Sai-nam-pueng, Sho-kun, and Neck orange), *Citrus maxima* varied in 4 cultivars (Hom-hat-yai, Tong-dee, Kaw-yai, Kaw-nam-pueng), *Citrus aurantifolia* or Lime and *Fortunella japonica* or Round Kamquat. The $SCO_2$ method produced a various yield of Rutaceous oils at 0.28-6.91 % v/w and *C. reticulata* cv. Sai-nam-pueng produced the most yield of volatile oil (4.55-6.91%). The essential oil composition was determined by Gas Chromatography – Mass Spectroscopy (GC-MS) analysis. The major component of the whole oils was *d*-limonene, but the amounts were different. Antimicrobial activity evaluation using broth micro dilution technique, the *C. aurantifolia* was shown the best activity. It could inhibit and kill *Staphylococcus aureus* and *S. epidermidis* at 0.63 mg/ml and 0.04 mg/ml for *Trichophyton rubrum.* The oils were investigated for the anti-inflammatory effect. The results showed that *C. aurantifolia*, *F. japonica* and *C. maxima* cv. Hom-Hat-Yai volatile oils had a potent inhibitory effect on the release of NO with $IC_{50}$ values of 10.2, 12.1 and 20.3 µg/ml, respectively, whereas the other oils had a moderate effect. The oils that showed a moderate effect on the release of NO established $IC_{50}$ close to *d*-limonene. Cytotoxic effects were not observed in this

experiment at 100 μg/ml. For antioxidant activity, Rutaceous volatile oil which extracted by $SCO_2$ had no antioxidant effect by DPPH radical scarvenging assay.

Three volatile oils which exhibited the best anti-microbial and anti-inflammatory activities including Lime, Round Kamquat and Hom-Hat-Yai were selected to study pre-formulation. The results showed the blended oils were homogeneous but the formulas did not exhibit the antimicrobial, anti-inflammatory, and antioxidant activities probably because of the limitation of essential oil quantity. The effect of temperature and light on stability of blended oils was examined. The result showed that after 3 months all tested light did not affect either the physical property of the oils, whereas the tested temperature oils did not affect either the physical properties except the condition at 60 °C which were rancid and the viscosity had changed.

## ACKNOWLEDGEMENTS

This study was carried out at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. I wish to express my deepest appreciation and grateful thanks to my thesis advisor, Assistant Professor Dr. Chatchai Wattanapiromsakul for his invaluable guidance, suggestion and encouragement. My sincere thanks are expressed to my thesis co-advisor, Assistant Professor Dr. Thanaporn Amnuaikit, for her kindness and helpful suggestions throughout the course of this work.

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Porchaya Chumsuwan

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## LIST OF ABBREVIATIONS AND SYMBOLS

| | | |
|---|---|---|
| g | = | Gram |
| µg | = | Microgram |
| mg | = | Milligram |
| Kg | = | Kilogram |
| L | = | Liter |
| µl | = | Microliter |
| ml | = | Milliliter |
| mm | = | Millimeter |
| µM | = | Micromolar |
| No. | = | Number |
| $IC_{50}$ | = | 50% Inhibitory concentration |
| MIC | = | Minimum inhibitory concentration |
| MBC | = | Minimum bactericidal concentration |
| LPS | = | Lipopolysaccharide |
| min | = | Minute |
| h | = | Hour |
| S.E.M. | = | Standard error of the Mean |
| v/w | = | Volume by weight |
| º C | = | Degree celsius |
| / | = | Per |
| cps | = | centipoises |
| rpm | = | Round per minute |

# CHAPTER 1

# INTRODUCTION

## 1. Background and Rational

In Thailand, there are many sources of essential oil especially plants in Rutaceae. Especially *Citrus* oils are used as carminative, treatment for insomnia, antitussive effect, and demulcent properties in the traditional medicine. The *Citrus* peel oils have a refreshing aroma and are antiseptic, stimulating and tonic, having significant effects on the whole of the digestive tract. They have been used as flavour in foods, beverages and pharmaceutical products. They also have been used as fragrance in perfumes, cosmetic and aromatherapy. The aroma of *Citrus* oil is classified in top notes and commonly used in spa.

The extraction methods of *Citrus* essential oil are important, since these oils are very sensitive to heat. The old classical method used only for *Citrus* peel extract is cold pressing method. The smell of cold press oil is natural, but contains a lot of impurities. The second classical method for essential oil extraction is steam distillation. This method is universal for most volatile oil extraction. It is an easy method and gives pure essential oil separated from water. However, this method is used with high temperature which some chemicals in essential oil may be changed their structure by heat.

In the present study, supercritical carbon dioxide extraction is use to solve the problems of the classical methods. It could extract essential oils with low temperature and no solvent used resulting in the improvement of the extract efficiency and the reduction of impurities.

## 2. Review of literature

### 2.1 Aromatherapy

The word aromatherapy comes from a combination of two words: “aroma”, which means smell or fragrance, and “therapy”, which means a treatment for the body or mind. Many people misunderstand about meaning of aromatherapy. It does not mean smell or smell for therapy but aromatherapy is one of the natural therapies that are the use of essential oils for therapeutic or medical purposes (Buckle, 2004). Aromatherapy is both science and arts. It is ruleless and depend on use and satisfaction of individual. The art of aromatherapy has been practiced for thousands of years. The first evidence of its use comes from China, 4500 years B.C. The use of essential oils continued to blossom around the European world, like England, Germany, and France. Present day, aromatherapists have continued to refine and develop the ancient art of using essential oils (Meadows, 1999).

There are a number of different ways to use essential oils in aromatherapy. These range from massage, through baths including hand bath, foot bath, and inhalation to sprays and compresses for specific healing needs. Each essential oil gives different biological activities such as antiseptic, antibacterial, antiviral, stimulating, warming, expectorant and antidepressant. When each activity is tested in different formulation, it shows vary potencies such as previous study in 2001. Orafidiya and team reported the formulation of an effective topical antibacterial product containing *Ocimum gratissimum* leaf essential oil and they found that the methanolic solutions of the oil exhibited higher antibacterial effects than corresponding liquid paraffin solutions.

### 2.2 Essential oil

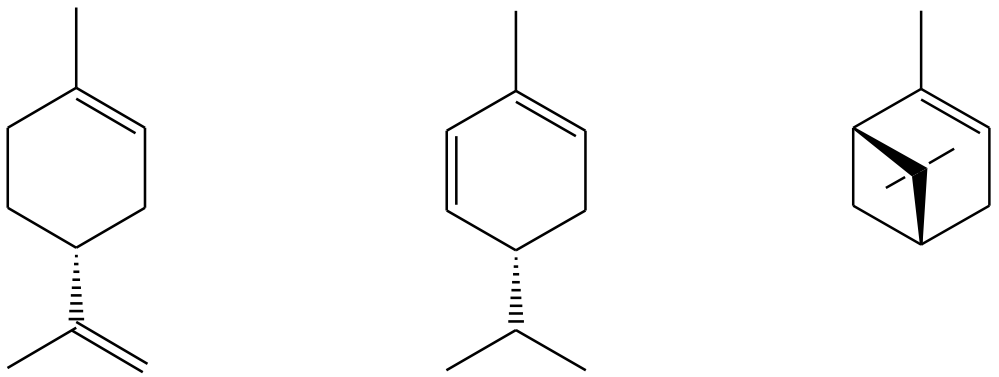
Essential oil is highly volatile organic compounds that plants produce and keep in cell wall. They are called essential oils because they represent the characteristic essence of their origin. The responsible chemical for the flavor or aroma are organoleptic compounds, which affect the sense organs. They present in their sources at various concentration levels ranging from part per billion to part per hundred. These compounds have molecular weights normally below 300 amu and are relatively volatile. There are many parts of plant contain the oil such as petals, fruits, peels, seeds, leaves, barks, woods, roots, rhizomes and latex (คมสันต์ หุตะแพทย์, 2549). Essential oils or aroma chemicals have greatly different in their chemical constitutions, but they have some common characteristic physical properties, such as refractive index, optical activity, immiscibility with water, and sufficient solubility to impart aroma to water. They are soluble in ether, alcohol, organic solvents, as well as supercritical carbon dioxide.

Nowadays, essential oils are widely used in spa. Their smell is classified in three groups by scent characteristics; top notes, middle notes, and base notes. The essential oils that are considered as top notes normally evaporate very fast. Top notes are highly volatile, fast acting, and give the first impression of the blend. Middle notes have balancing effect. They are normally warm and soft fragrances. They are not always immediately evident and may take a couple of minutes to establish their scent. The essential oils that are classified as base notes are normally very heavy. It will be present for a long time and slow down the evaporation of the other oils. They are normally rich and relaxing in nature.

### 2.3 Chemical constituents of essential oil

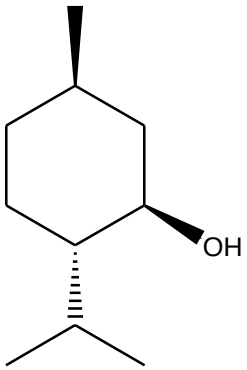
Essential oils are volatile oils that differ from nonvolatile fixed oils, i.e., glycerides of fatty acids. Generally, essential oils consist of chemical compounds which have hydrogen, carbon and oxygen in their building blocks. The primary functional groups of the essential oils used in aromatherapy are alcohol volatile oils, aldehyde volatile oils, ketone volatile oils, phenol volatile oils, ether volatile oils, oxide volatile oils and ester volatile oils.

Terpene hydrocarbons (monoterpenes and sesquiterpenes) are the hydrocarbons derive from isoprene unit (2-methyl butadiene) having molecular formula of $(C_5H_8)_n$ (monoterpene hydrocarbons contain n = 2 and sesquiterpene hydrocarbon contain n=3). They are the unsaturated compounds that can decompose by hydrolysis or photolysis and change to the other compounds. The odor and taste of essential oils are mainly determined by these oxygenated constituents, which some extent is soluble in water, and most of them are soluble in alcohol (Giacomo and Giacomo, 2002) (Mukhopadhyay, 2000).

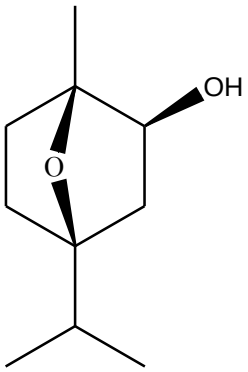


*d*-limonene alpha-phellandrene (-)-alpha-pinene

**Figure 1** Hydrocarbon volatile oils

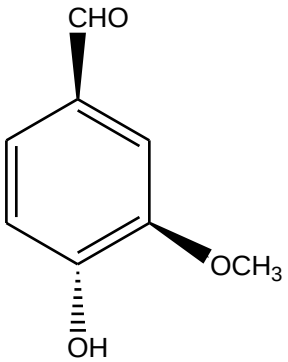



(+)-menthol

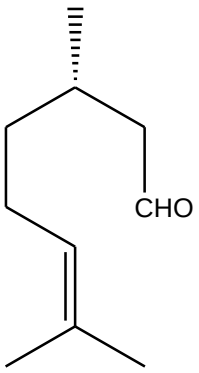



2-*endo*-hydroxy-1,8-cineole

**Figure 2** Alcohol volatile oils

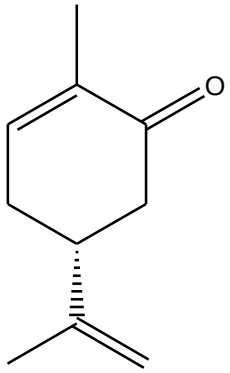



(-)-citral

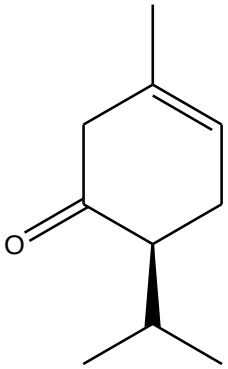



(+)-citronellal

**Figure 3** Aldehyde volatile oils

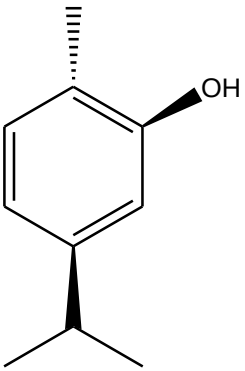



(+)-carvone

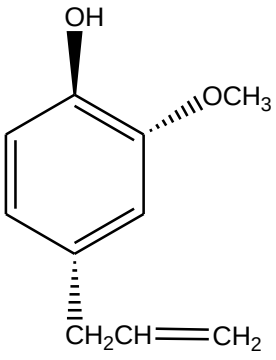



(+)-piperitone

**Figure 4** Ketone volatile oils

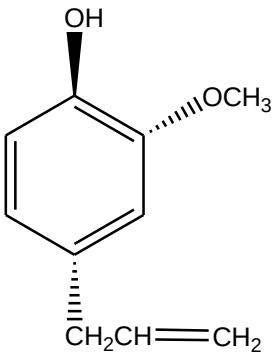



(+)-carvacrol

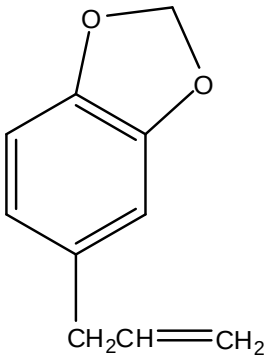



(+)-eugenol

**Figure 5** Phenol volatile oils

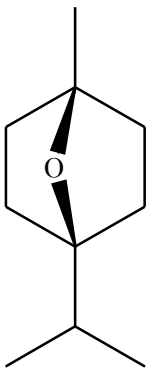



(+)-eugenol



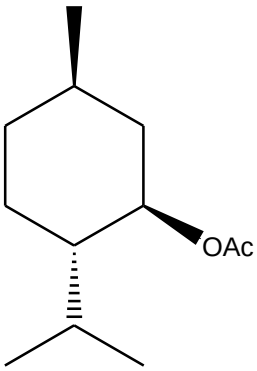

Safrol

**Figure 6** Phenolic ether volatile oils




1,8 cineol

**Figure 7** Oxide volatile oils

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*l*-menthyl acetate

**Figure 8** Ester volatile oils

### 2.4 Essential oil properties

Essential oils vary in their natural qualities and constituencies. Most oils are not greasy and are thin in texture, with the exception of a few oils that become thicker in consistency over time. The color of the oil varies from clear to color, though pale to dark yellow, amber, pink, reddish brown, and pale to dark olive green to blue. Each essential oil, volatile by nature, which means it will evaporate quickly when coming into contact with the atmosphere and does not make permanent grease spot on the paper.

### 2.5 Essential oil absorption

The essential oils are absorbed into the body with two main ways, inhalation and skin absorption. When an essential oil is inhaled, it activates the nerve cells in the nasal cavity which send impulses that stimulate the brain and produce positive feelings and emotions, while at the same time substances of the essential oils are drawn into the lungs and then quickly absorbed into the bloodstream providing physiological therapeutic benefits. Moreover, when an essential oil is applied to skin (never undiluted), it penetrate the skin and enter the bloodstream this way (Price, 2007).

### 2.6 Extraction methods

There are several ways of extraction the volatile components from plants such as distillation (water distillation, water and steam distillation, steam distillation, or steam and vacuum distillation), expression, enfleurage, solvent extraction, and carbon dioxide extraction (Price, 2000).

#### 2.6.1 Cold pressing method

Cold pressing, also known as expression, is used exclusively for citrus oils. This is a very mild and gentle pressing treatment in which the outer layer of the peel is ruptured and the oil is then pressed out. Cold-pressed citrus oils are the same composition as in the plant itself. Nevertheless, during the process, the abrasion of the peel allows the oil to ooze out from the outside surface in the form of liquid that is separated rapidly after standing into three layers. The upper layer is practically pure essential oil; the intermediate layer contains emulsified oil and includes colloidal particles in suspension; and the lower aqueous layer contains no oil and cannot be used. Recovering of the essential oil could be done by centrifugation. The essential oil that gains from this method may contain traces of water, which will induce slow hydrolysis. The water should be eliminated by treating with anhydrous sodium sulphate. In many essential oil factories the peel is steam distilled after expression, which releases even more oil, though of a poorer quality.

### 2.6.2 Distillation method

The technique called distillation is the main way to extract essential oils from plants. Steam is passed over the leaves or flowers that have been placed in a still. The steam or vapor then passes into a condenser where it produces a liquid that contain both oil and water. The oil is then easily separated because depending upon the weight of the oil, it either rests on the surface of the water or sinks to the bottom. Distillation is the preferential method to extract essential oil because it is comfortable and savable. However, essential oils which are difficult to volatile in steam are mostly left behind in the still with the botanical matter, while some very volatile chemicals may be lost in the distillation process. The steam distillation process is the most widely acceptable process for large scale of essential oils production. However, an obvious drawback of this process may be the induction of chemicals changes by oxidation and hydrolysation reaction, so that, the recovered oils are often difference from those presence in the original source. Furthermore, if distillation is carried out under reduced pressure, vacuum steam distillation, the consequent lowering of the boiling point significantly helps to avoid, or reduce, harmful reactions to the integrity of the original composition of the essential oil and allows recovery of components which are not distilled under ordinary pressure condition.

### 2.6.3 Solvent extraction

Enfleurage is a gentle method used to extract the oil from delicate petals and blossoms. Pomades were obtained from the enfleurage process used long ago, when petals or leaves were laid on trays of animal fat for many days, being replaced regularly until the fat used as a solvent was saturated with the plant extracts.

### 2.6.4 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) is a fairly new method of extracting essential oils. SFE has become an alternative to more conventional extraction procedures, because the dissolving power of supercritical fluids can be adjusted by regulating the pressure and temperature conditions employed. The technology calls for very expensive, complicated equipment, which utilizes carbon dioxide ($CO_2$) at very high pressures and extremely low temperatures. The aroma of the resultant oil is more like the essential oil in the plant, as many terpenes in a distilled oil seem to form during the distillation process, which also breaks down some of the acetates (esters) in the plant material. $CO_2$ is the most widely used fluid for supercritical extraction, because supercritical conditions are readily attained. $CO_2$ extracted essential oils are pure and stable and no residue of $CO_2$ left in them. Furthermore, $CO_2$ extracted essential oils are pure and chemically stable, non-toxic, non-flammable, and no solvent residue in the extract.

## 2.7 Rutaceous plants

In Thailand, there are many sources of essential oil contained in plants especially plants in Rutaceae. Leaves, barks and fruits of Rutaceous plants have glands (pellucid dot), and often have strongly fragrant essential oils (Stone, 1973). Many kinds of volatile oil from plants in Rutaceae are used for aromatherapy including; lime oil, orange oil, neroli oil, etc. Some of them exhibit medical values such as neroli oil is prescribed for asthma, control temper balance including anxiety, depression and exhaustion (เพ็ญนภา ทรัพย์เจริญ และคมสันต์ หุตะแพทย์, 2549).

*Citrus* is one of genera in Rutaceae which including many species. The *Citrus* species are famous for the source of essential oils. The aroma of *Citrus* oil is classified in top notes and commonly used in spa. The *Citrus* flower oils have the relaxing and hormone balancing effects which have been used in aromatherapy and perfumery (Giacomo and Giacomo, 2002).

### 2.7.1 *Citrus maxima*

*Citrus maxima* Merr. (pomelo, ส้มโอ) is one of the famous fruits in Thailand, a native plant of Asia, which is best cultivated in China, southern Japan, Vietnam, Malaysia, Indonesia and Thailand (วิจิตต์ วรรณชิต, 2544). This, the largest citrus fruit, is known in the western world mainly as the principal ancestor of the grapefruit. As a luscious food, it is famous in its own right in its homeland, the Far East. The other scientific or synonyms of pomelo are *Aurantium maximum* Burm. ex Rumph, *Citrus aurantium* L. var *grandis* L., *Citrus decumana* L., *Citrus grandis* Osbeck and *Citrus pamplemos* Risso.

The pomelo is a medium sized tree, may be 16 to 50 ft (5-15 m) tall, with a somewhat crooked trunk 4 to 12 in (10-30 cm) thick, and low, irregular branches. The young branchlets are angular and often densely hairy, and there are usually spines on the branchlets, old limbs and trunk. Its leaves have the small winged petioles. The flowers are bisexual and sweet smell. The tree can flower when its age is four years old. In Thailand, the flowering in December – February is called Som-Pee when it produces lots of flower, and the flowering in August – September is called Som-Ta-Wai. The fruit ranges from nearly round to oblate or pear-shaped and big size, 4 to 12 in (10-30 cm) wide. Fruit peel has the 3 layers: the outer layer is called flavedo or epicarp, clinging or more or less easily removed, may be greenish-yellow or pale-yellow, minutely hairy, which has the oil glands, 1.25-2 cm thick, the medium layer is called albedo or mesocarp which is white or pink and has plenty of spongy cells, and the inner layer is called endocarp which is the edible portion of the fruit, the juice sags. Pulp varies from greenish-yellow or pale-yellow to pink or red. Generally, there are only a few, large, yellowish-white seeds, white inside, though some fruits may be quite seedy (Morton, 1987). The fruit of pomelo is commonly eaten fresh or made as juice. It is also popular for jam and syrup. In traditional medicine, the fruit peel has been used for cough, swellings, and epilepsy, because of the effectiveness of the volatiles (Sawamura, 2005).

In Thailand, pomelo was developed to plenty of cultivar for their pleasant taste and their names were depended on the cultivated areas. For example, Khao-Tong-Dee, Khao-Num-Pueng, Khao-Hom, Khao-Poung, Khao-Pan and Khao-Yai varieties are found in the Central: Nakhon-Pathom,

Samutsakhon, Samutsongkarm and Rachaburi provinces. While Khao-Tang-Gwar, Ta-Koy varieties are found in the North: Chainat, Nakhonsawan, Uthaithani, Phichit and Phitsanulok provinces. There were some varieties in the south such as Hom-Hat-Yai in Songkhla province (มงคล หลิม, 2535 และ วิจิตต์ วรรณชิต, 2544).

### 2.7.2 *Citrus reticulata*

*Citrus reticulata* Blanco (mandarin, orange, ส้ม) is one of the famous fruits in Thailand and the fruit of mandarin is commonly eaten fresh or made as juice (มงคล หลิม, 2535) similar to pomelo. Mandarin is a group name for a class of oranges with thin, loose peel, which have been dubbed "kid-glove" oranges. The name "tangerine" could be applied as an alternate name to the whole group, but, in the trade, is usually confined to the types with red-orange skin. In the Philippines all mandarin oranges are called *naranjita.* Spanish-speaking people in the American tropics call them *mandarina*. The mandarin orange is considered a native of south-eastern Asia and the Philippines. It is most abundantly grown in Japan, southern China, India, and the East Indies, and is esteemed for home consumption in Australia. It gravitated to the western world by small steps taken by individuals interested in certain cultivars. The mandarin tree may be much smaller than that of the sweet orange or equal in size, depending on variety. With great age, some may reach a height of 7.5 m with a greater spread. The tree is usually thorny, with slender twigs, broad-or slender-lanceolate leaves having minute, rounded teeth, and narrowly-winged petioles. The flowers are borne singly or a few together in the leaf axils. The fruit is oblate, the peel bright-orange or orange-yellow, glossy, rough and puffy, pulp orange-yellow, of rich, sweet flavor, separating easily from the segments. Seeds are small, pointed at one end, green inside (Morton, 1987).

Mandarin oranges of all kinds are primarily eaten out-of-hand, or the sections are utilized in fruit salads, gelatins, puddings, or on cakes. Very small types are canned in syrup. The essential oil expressed from the peel is employed commercially in flavoring hard candy, gelatins, ice cream, chewing gum, and bakery goods (Morton, 1987).

### 2.7.3 *Citrus aurantifolia*

*Citrus aurantifolia* Swingle (Lime, มะนาว) is native to the Indo-Malayan region. Lime continues to be cultivated more or less on a commercial scale in India, Egypt, Mexico, the West Indies, tropical America, and throughout the tropics of the Old World. Because of its special bouquet and unique flavor, it is ideal for serving in half as a garnish and flavoring for fish and meats, for adding zest to cold drinks, and for making limeade. Throughout Malaysia, this lime is grown mainly to flavor prepared foods and beverages. Commercially bottled lime juice is prized the world over for use in mixed alcoholic drinks. The lime tree is exceedingly vigorous, may be shrubby or range from 6 1/2 to 13 ft (2-4 m) high, with many slender, spreading branches, and usually has numerous, very sharp, axillary spines to 3/8 in (1 cm) long. The evergreen, alternate leaves are pleasantly aromatic, densely set, elliptic or oblong-ovate, rounded at the base, 2 to 3 in (5-7.5 cm) long, leathery, light purplish when young, dull dark-green above, paler beneath, when mature, with minute, rounded teeth and narrowly-winged petioles. The fruit, borne singly or in 2's or 3's (or sometimes large clusters), at the twig tips, is round, obovate, or slightly elliptical, sometimes with a slight nipple at the apex, the base rounded, 1 to 2 in (2.5-5 cm) in diameter, peel is green and glossy when immature, pale-yellow when ripe, somewhat rough to very smooth, the pulp is greenish-yellow in 6 to 15 segments which do not readily separate, aromatic, juicy, very acid and flavorful, with few or many small seeds, green inside (Morton, 1987).

The oil derived from the lime is obtained by three different methods in the West Indies, by hand-pressing in a copper bowl studded with spikes. This method yields oil of the highest quality but it is produced in limited amounts. It is an important flavoring for hard candy. The second is by machine pressing, cold expression, of the oil from the spent half-shells after juice extraction, or simultaneously but with no contact with the juice. The third is by distillation from the oily pulp that rises to the top of tanks in which the washed, crushed fruits have been left to settle for 2 weeks to a month. This yields the highest percentage of oil. With terpenes and sesquiterpenes removed, it is extensively used in flavoring soft drinks, confectionery, ice cream, sherbet, and other food products. The settled juice is marketed for beverage manufacturing. The residue can be processed to recover citric acid (Morton, 1987).

### 2.7.4 *Fortunella japonica*

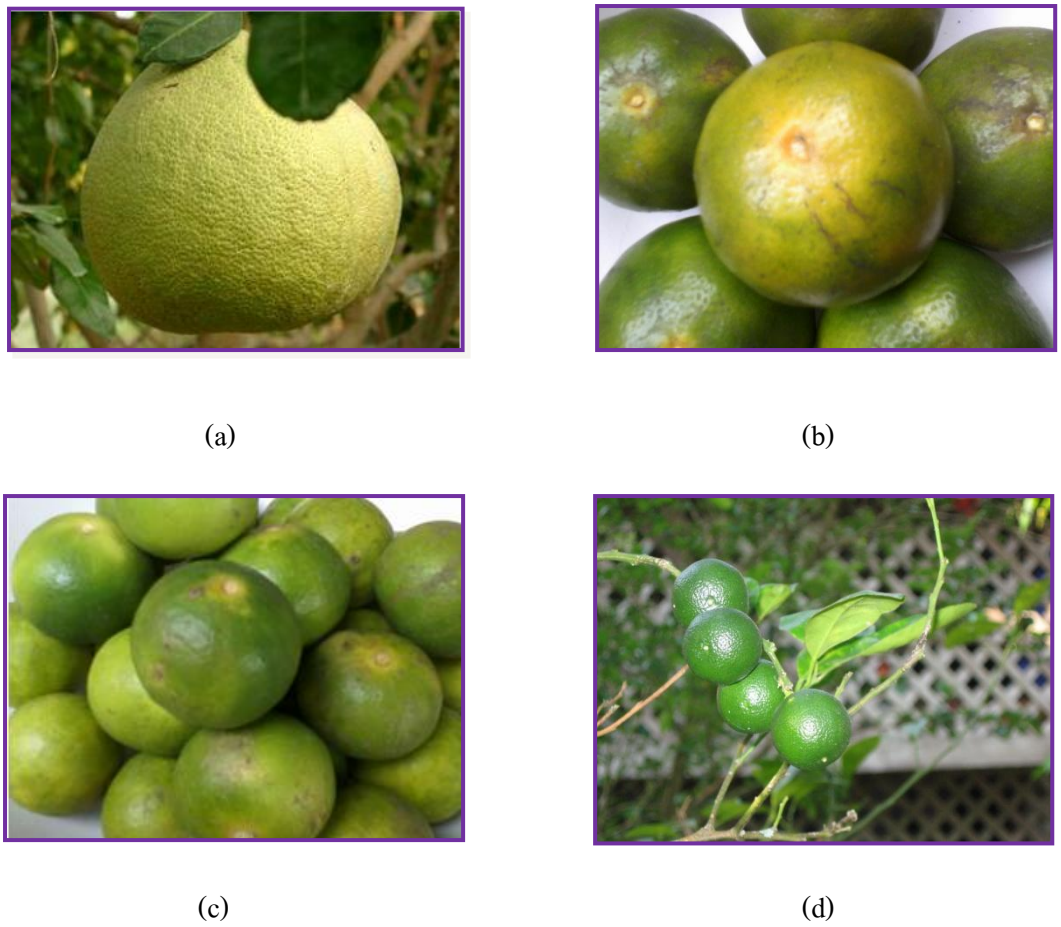
*Fortunella japonica* (Thunb.) Swingle, or *Citrus japonica* Thunb. (Round Kamquat, ส้มจี๊ด) is close to the *Citrus* genus. Kumquats have been called "the little gems of the citrus family". They were included in the genus *Citrus* until about 1915 when Dr. Walter T. Swingle set them apart in the genus *Fortunella,* which embraces six Asiatic species. The kumquat tree is slow-growing, shrubby, compact, 8 to 15 ft (2.4-4.5 m) tall, the branches light-green and angled when young, thornless or with a few spines. The apparently simple leaves are alternate, lanceolate, 1 1/4 to 3 3/8 in (3.25-8.6 cm) long, finely toothed from the apex to the middle, dark-green, glossy above, lighter beneath. Sweetly fragrant, 5-parted, white flowers are borne singly or 1 to 4 together in the leaf axils. The fruit is oval-oblong or round, 5/8 to 1 1/2 in 1.6-4 cm wide; peel is golden-yellow to reddish-orange, with large, conspicuous oil glands, fleshy, thick, tightly clinging, edible, the outer layer spicy, the inner layer sweet; the pulp is scant, in 3 to 6 segments, not very juicy, acid to subacid; contains small, pointed seeds or sometimes none; they are green within (Morton, 1987).

**Table 1** Morphological property of *Citrus maxima* fruits cultivar Hom-Hat-yai, Tong-Dee, Kaw-Yai, and Kaw-Nam-Pueng

| Cultivation | Size/Diameter (cm.) | Texture color | | |
|---|---|---|---|---|
| | | Epicarp | Mesocarp | Endocarp/Pulp |
| Hom-Hat-Yai | 18-22 | 1-1.5 cm thick, light green | pink | pink |
| Tong-Dee | 15-20 | 1 cm thick, yellow green | pink | red |
| Kaw-Yai | 20-25 | 1.25-2 cm thick, light green | white/light green | white/ pale-yellow |
| Kaw-Nam-Pueng | 18-22 | 1-1.5 cm thick, light green | white/light green | white/ pale-yellow |

**Table 2** Morphological property of *Citrus reticulata* fruits cultivar Keaw-Hwarn, Sai-Nam-Pueng, Sho-Kun, and Neck Orange

| Cultivation | Size/Diameter (cm.) | Texture color | | |
|---|---|---|---|---|
| | | Epicarp | Mesocarp | Endocarp/Pulp |
| Keaw-Hwarn | 5-6 | ~0.1cm thick, green-yellow | thin, white | orange-yellow |
| Sai-Nam-Pueng | 5-6 | 0.1-0.15 cm thick, bright-orange | thin, white | orange-yellow |
| Sho-Kun | 5-6 | 0.1-0.2 cm thick, dark green-yellow | thin, white | orange-yellow |
| Neck Orange | 6.5-7 | 0.3-0.4 cm thick, dark green | 0.1-0.3 thick, white | pale-yellow |



(a) (b)

(c) (d)

**Figure 9** The fruit of *C. maxima* (a), *C. reticulata* (b), *C. aurantifolia* (c), and *F. japonica* (d)

### 2.8 *Citrus* peel oils

*Citrus* oils are derived from three different parts of the plant. There are peels, leaves and flowers. The obtained *Citrus* peel oils from bergamot, grapefruit, lemon, and orange for aromatherapy are not distilled, but mechanically expressed. They contain large molecules which would not come over in distillation, including colour and waxes, and the latter can precipitate if the oils are stored incorrectly or kept for a long time. The waxes do no harm and may be removed by filtration. *Citrus* essences are especially susceptible to oxidation and the precious active aldehydes may degrade into acid. To prevent this, nitrogen gas is used to displace the air as the oil is decanted (Price and Price, 2007).

## 2.9 Biological activity of *Citrus* oil

The *Citrus* oil is used as carminative, treatment for insomnia, antitussive effect, and demulcent properties in the traditional medicine. The *Citrus* peel oils have a strong and desirable aroma with refreshing effect. (Giacomo and Giacomo, 2002). They were reported as antiseptic, stimulating and tonic, having significant effects on the whole of the digestive tract. This is especially true of bergamot and bitter orange, which are stomach antispasmodics (Price and Price, 2007).

### *Citrus maxima*

*C. maxima* have lots of bioactivities, especially antioxidant and anti-microbial activities (Lertsatitthanakorn, 2006). The previous reports of *C. maxima* peel essential oil showed the anti-bacterial against *Staphylococcus aureus* and *Pseudomonas aeruginosa* with the concentration of 20.0 µl/agar disc (Ontengco, *et al*., 1995) and the anti-fungal against *Trichophyton mentagrophytes* and *Microsporum audounil* with MIC 500 of ppm using agar plate method (Yadav and Dubey, 1994). In traditional medicine, the fruit peel has been used for cough, swellings, and epilepsy, because of the effectiveness of the volatiles (Sawamura, 2005). In the Philippines and Southeast Asia, decoctions of the leaves, flowers, and rind are given for their sedative effect in cases of epilepsy, chorea and convulsive coughing. The hot leaf decoction is applied on swellings and ulcers. The fruit juice is taken as a febrifuge. The seeds are employed against coughs, dyspepsia and lumbago. Gum that exudes from declining trees is collected and taken as a cough remedy in Brazil (Morton, 1987).

### *Citrus reticulata*

Viuda-Martos, *et al*., (2008) reported about the effect of the essential oils of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) on the growth of moulds commonly associated with food spoilage: *Aspergillus niger*, *Aspergillus flavus*, *Penicillium chrysogenum* and *Penicillium verrucosum*, using the agar dilution method. All the oils showed antifungal activity against all the moulds. Especially, mandarin essential oil

was most effective at reducing the growth of *Aspergillus flavus*. Although, mandarin has been used widely, there is no study of the mandarin oil extracted by supercritical $CO_2$ extraction method.

***Citrus aurantifolia***

Lime juice dispels the irritation and swelling caused by mosquito bites. In 2001, Ezeonu *et al.* reported statistical studies using the randomised complete block design with four replicates showed that volatile extracts of two species of orange peel - *Citrus sinensis* (sweet orange) and *Citrus aurantifolia* (lime) had insecticidal activity against mosquito, cockroach and housefly. Insecticidal activity was better after 60 min than at 30 min spraying of rooms. In Malaya, the juice is taken as a tonic and to relieve stomach ailments. Mixed with oil, it is given as a vermifuge. The pickled fruit, with other substances, is poulticed on the head to allay neuralgia. In India, the pickled fruit is eaten to relieve indigestion. The juice of the Mexican lime is regarded as an antiseptic, tonic, an antiscorbutic, an astringent, and as a diuretic in liver ailments, a digestive stimulant, a remedy for intestinal hemorrhage and hemorrhoids, heart palpitations, headache, convulsive cough, rheumatism, arthritis, falling hair, bad breath, and as a disinfectant for all kinds of ulcers when applied in a poultice (Morton, 1987).

***Fortunella japonica***

*Fortunella japonica* or Round kumquat has a typical citrus flavor character. Peel of round kumquat is used as carminative in the traditional medicine. There are few reports on essential oil properties from this plant. Most of research was the studies on chemical constituents. Koyasako and Bernhard (1983), who identified 71 volatile compounds in the oil of kumquat, reported that limonene was the most abundant compound comprising 93%. In 1994 Katumi *et al.* identified volatile compounds isolated from round kumquat. Volatile constituents of round kumquat fruit were isolated by steam distillation and simultaneous purging/extraction (SPE) methods. The isolated volatiles were identified with gas chromatography (GC) and gas chromatography/mass spectrometry (GC-MS). From this study, they found that *d*-limonene was the most abundant compound, comprising 87% of the sample from steam distillation and 97% of the sample from SPE. Resembling to Choi' study in 2005, he determined the composition of kumquat cold-pressed peel oil and determined for volatile components that

responsible for the aroma of this oil. Eighty-two compounds were identified in the oil with GC and GC-MS. The major compounds were limonene (93.73%), myrcene (1.84%) and ethyl acetate (1.13%). However, there is little information available on bioactivities and therapies, and also no report on essential oil extraction from kumquat by supercritical $CO_2$ extraction.

### 2.10 Supercritical carbon dioxide extraction ($SCO_2$) (Mukhopadhyay, 2000)

The extraction methods of *Citrus* essential oil are important, since these oils are very sensitive to heat. There are two old classical medthods for *Citrus* essential oil extraction. The first method is cold pressing method. This method is used only for *Citrus* peel extract. The smell of cold press oil is natural, but contains a lot of impurities. The second classical method is steam distillation. This method is universal for most volatile oil extraction. It gives pure essential oil separated from water. However, this method is used high temperature which some chemicals in essential oil may be decomposed, especially *Citrus* oils. Therefore the steam distillation is not the suitable method for *Citrus* essential oil extraction. *Citrus* essential oil extraction needs low temperature method which gives low impurities contamination. (Thavanapong, 2006)

At present, supercritical carbon dioxide extraction is appropriate to solve the problems of the classical methods. It could extract essential oils with low temperature and no solvent used resulting in the improvement of the extraction efficiency and the reduction of impurities. When a gas is compressed to a sufficient high pressure, it becomes liquid. If the gas is heated to a specific temperature, at the specific pressure, the hot gas will become supercritical fluid. This temperature is called the critical temperature and the corresponding vapor pressure is called the critical pressure. The values of the temperature and pressure are defined as critical point which is unique to a given substance. These states of the substances are called supercritical fluid. This fluid now takes on several of gas and liquid properties. Supercritical fluid is the region where the maximum solvent capacity and the largest variations in solvent properties can be achieved with small changes in temperature and pressure. It offers very attractive extraction characteristics, owing to its favorable diffusivity, viscosity, surface tension and other physical properties. Its diffusivity is one to two orders of magnitude higher than those of liquids.

The diffuseness facilitates rapid mass transfer and faster completion of extraction than conventional liquid solvents. The low viscosity and surface tension enable it to easily penetrate the botanical materials from which the active components are extracted. The gas-like characteristics of supercritical fluid provide ideal conditions for extraction of solutes giving a high degree of recovery in a short period of time.

The most desirable supercritical fluid solvent for extraction of natural products is carbon dioxide ($CO_2$). It is an inert, inexpensive, easily available, odorless, tasteless, environment-friendly, and generally regards as safe solvent. In the supercritical fluid processing with $CO_2$, there is no solvent residue in the extract, because it becomes gas in the ambient condition. Its near-ambient critical temperature, 31.1 ºC, makes it ideally suitable for thermolabile natural products extraction. Due to its low latent heat of vaporization, low energy input is required for the extraction separation system. $SCO_2$ produces the most natural smelling extracts, since the hydrolysis does not occur in the process (Giacomo and Giacomo, 2002).

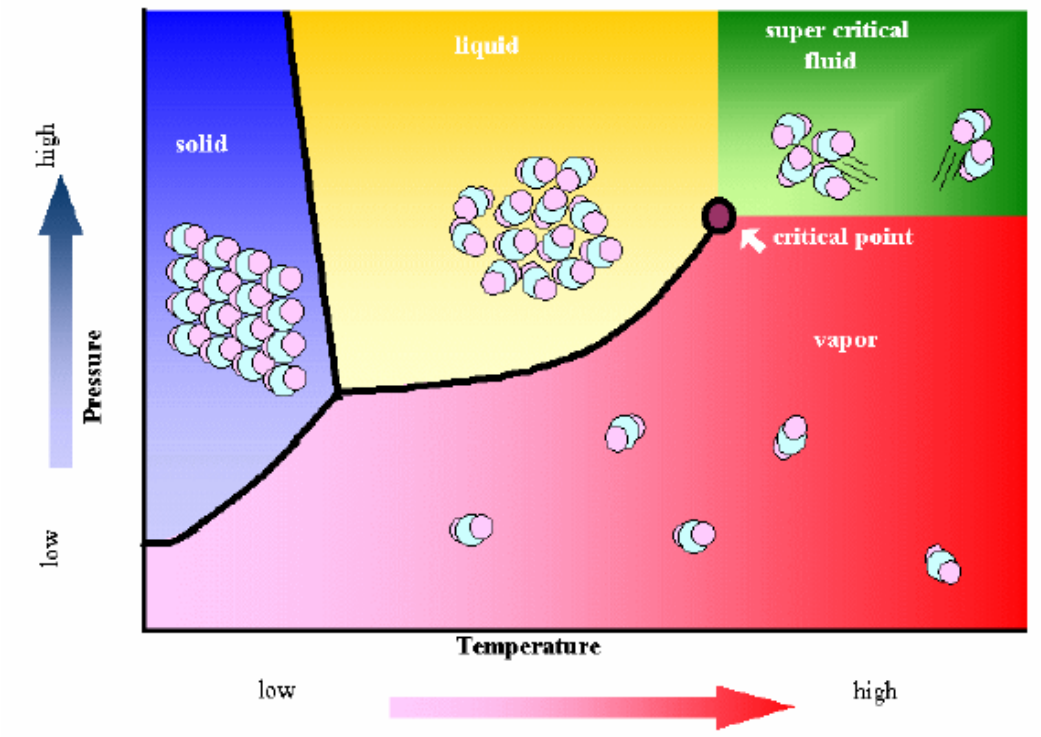



**Figure 10** Pressure-temperature phase diagram (Department of Applied Chemistry, Chemical Engineering and Biomolecular Engineering, School of Engineering, Tohoku University, 2009)

### 2.11 Inflammation

The definition of inflammation is the body's response to tissue injury (Gould, 2002). Inflammation is a defense reaction of the organism and its tissue to injurious stimuli that leads to the local accumulation of plasmatic fluid and blood cells. Although it is a defense mechanism, the complex events and mediators involved in the inflammatory reaction can be included, maintained or aggravated by many diseases. The aim is to repair the damage or at least to limit it and also to remove the cause, for example, bacteria or foreign bodies (Gupta *et al*., 2003).

Inflammation is associated with many different types of tissue injury. Causes include direct damage (cuts, sprains), chemicals (acids), ischemia and cell necrosis or infarction, allergic reactions, physical agents (thermal injuries or burns, radiation), foreign bodies (splinters or dirt) and infection (Gould, 2002). Inflammation is grouped into two basic forms. Acute inflammation is of relatively short duration, lasting from a few minutes up to a few days, and is characterized by fluid and plasma protein exudation, and by a predominantly neutrophilic leukocyte accumulation. Chronic inflammation is of longer duration (days to years) and is manifested histologically by influx of lymphocytes and macrophages and by tissue destruction and repair; the latter is associated with vascular proliferation and fibrosis (Kumar et al., 1997).

### 2.12 Biological significance of nitric oxide

Nitric oxide (NO) is a simple, inorganic, gaseous free radical whose predominant functions are that of a messenger and effector molecules. In mammals, NO is synthesized by a family of enzymes referred as the nitric oxide synthases (NOS). NO is physiologically significant for its role in regulating vascular tone and in signaling neurotransmission (Moncada *et al*., 1991). Nitric oxide is also an important component of the antineoplastic and antimicrobial armament of macrophages (Coleman *et al*., 2001). This highly labile and noxious gas is produced in large and sustained quantities by macrophages following exposure to a variety of immunologic and inflammatory mediators.

The high-output production of nitric oxide is dependent on induction and expression of the inducible nitric oxide synthase (iNOS) expressed in macrophages (Fang *et al*., 1997). Endothelial cells and neurons also express unique forms of nitric oxide synthase as eNOS and nNOS, respectively. However, the expressions of both enzymes are constitutive and nitric oxide is produced at lower steady state levels than iNOS (Hevel *et al*., 1991). It is this low level of production that is biological significance, while overproduction may lead to circulatory shock, chronic inflammation and carcinogenesis (Hidaka *et al*., 1997). NO and its functions have been shown to be more and more complex in physiological and pathological processes, for example, NO can regulate vascular tone, smooth muscle cell relaxation, neurotransmission, neuromodulation, apoptosis (Kim *et al*., 2004) and modulate mitochondrial energy generation (Moncada and Erusalimsky, 2002). NO also has been implicated in different mechanisms of diseases such as atherosclerosis (Barton and Haudenschild, 2001), asthma, neurologic disorders and septic shock (Thiemermann *et al*., 1997).

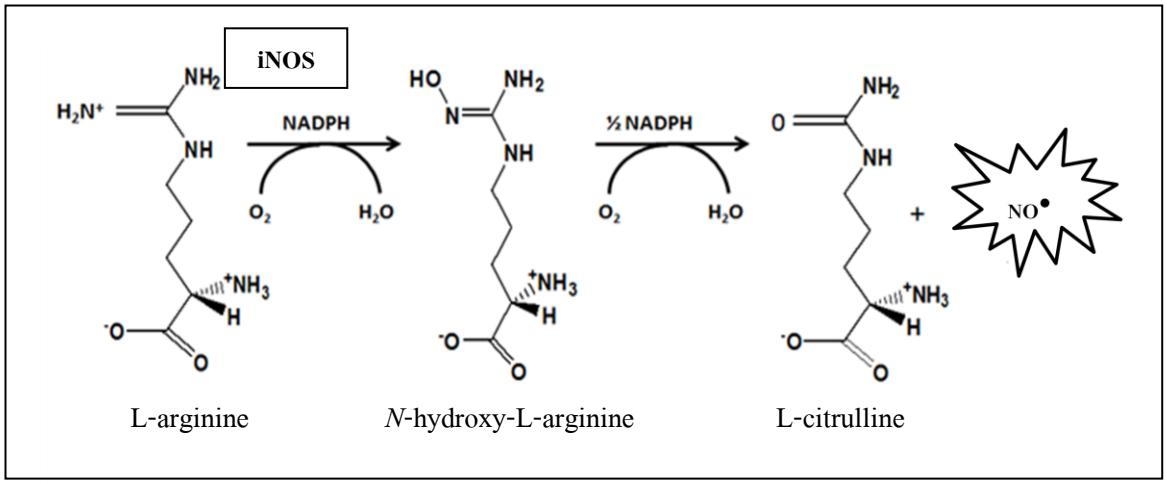



**Figure 11** Nitric oxide generation from L-arginine by iNOS (Eijk et al., 2006)

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs. This inorganic free radical has been implicated in physiologic and pathologic processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. NO is produced by the oxidation of L-arginine by NO synthase (NOS). In the family of NOS, inducible NOS (iNOS) is involved in pathological aspects, and can be expressed in response to pro-inflammatory agents such as tumor necrosis factor-$\alpha$ (TNF- $\alpha$), interleukin 1-$\beta$ (IL-1$\beta$) and lipopolysaccahride (LPS) in various cell types including macrophages. NO acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities. However, excessive production of this free radical is pathogenic to the host tissue itself, since NO can bind with other superoxide radicals and acts as a reactive radical which directly damages the function of normal cell (Tewtrakul and Itharat, 2007).

### 2.13 Free radicals

Free radicals are normal biochemical intermediates of any metabolic reactions. They consist of any chemical species (atom, ions or molecules) that contain one or more unpaired electrons in there outer atomic or molecular orbital. This makes them highly unstable and violently reactive (Benedetto, 1998 and Wickens, 2001). The most free radicals found are reactive oxygen species (ROS) which include oxygen free radicals or oxygen-centered free radicals and non radical species.

### 2.14 Defense mechanism of skin against oxidative damage

The epidermis of the skin possesses an extremely efficient antioxidant activity that is superior to most tissues (Jenkins, 2002). There are two types of antioxidants, the enzymatic and non enzymatic antioxidants. The enzymatic antioxidants including superoxide dismutases (SOD), catalase, glutathione peroxidase (GSHP) and glucose-6-phosphate dehydrogenase (G-6-PD), protect cell by hastening biochemical reaction. The non enzymatic antioxidants including ascorbic acid (vitamin C), $\alpha$-tocopherol (vitamin E), $\beta$-carotene and glutathione, help dissipate intracellular oxidants or ROS by acting as tree radical scavengers (Benedetto, 1998).

### 2.15 Carrier oils

Essential oils are too strong to be applied neat to the skin so when used in massage they should be mixed with a carrier or base oil. Carrier oils are generally cold-pressed vegetable oils derived from the fatty portions of the plant. Unlike essential oils that evaporate and have a concentrated aroma, carrier oils do not evaporate or impart their aroma as strongly as essential oils. Here are some of the oils most commonly used in an Asian tropical spa (Sullivan, 1991):

**Almond:** This vaguely aromatic oil is gentle and rich in proteins and vitamins. It is nourishing, light and softening for dry hands, eczema and irritated skin. It is a good lubricant, so blends well with other oils as an excellent massage base.

**Avocado:** This is a rich, heavy oil with high vitamin content. It is often blended for a velvet-like consistency. It also contains a mild sunscreen.

**Coconut:** Traditionally, this was the main carrier oil in tropical Asia because of its abundance. It is a saturated-oil with its own district smell. Coconut oil aids tanning and is reputed to help filter the sun's ray. It is emollient on hair and skin. It remains stable for a long period and is particularly nourishing in hair treatments.

**Grapeseed:** This is an extremely fine and pure oil, so light it absorbs immediately into the skin. It is good for helping the essential oils penetrate quickly. It leaves a satiny, not stick, coating.

**Jojoba:** This is a natural fluid wax rather than an oil. It has a fine consistency (similar to collagen) which effectively penetrates the skin. It reputedly nourishes hair and prevents hair loss.

**Macadamia:** The acids in this oil are natural components of skin sebum. It has a rich nutty aroma and consistency. Its emollient qualities make it a good all round moisturizer, particular for dry and mature skin.

**Olive:** Rich in proteins and vitamins, this oil is rapidly absorbed by the skin although it has a strong aroma and is often blended with other oils. It is a naturally warming oil, so it is good for massage in cold weather or in treatments for muscular pains.

**Wheatgerm:** This is rich, dark oil, high in vitamin E but sometimes thought too heavy and aromatic to use alone. It is an antioxidant: it stabilizes essential oils and other carrier oils, making them last longer. It also benefits scarring.

In this study, four *Citrus spp*. are selected including *Citrus reticulata* Blanco varied in 4 cultivars, *Citrus maxima* Burm. Merrill varied in 4 cultivars, *Citrus aurantifolia* Swingle and *Fortunella japonica* (Thunb.) Swingle. It is true that in Thailand, there are lots of cultivars of *C. maxima*. The *C. maxima* is peeled for their fresh pulp and juice that are sold both in Thailand and foreign countries. The high demand of pomelo in the market causes around one metric ton of the peel left as by products in each day. These wastes could be served as raw materials for essential oil industries. So the study of *C. maxima* peel essential oil could increase the value of the useless waste. Because of *C. maxima* was developed to plenty of cultivar and color, smell and test of each cultivar are different, they may give the different chemical constituents and biological activities evaluation in different cultivars. It is the same as *C. reticulata*, there are lots of cultivar, and the color of pulp and peel are very different, especially, *C. reticulata* Blanco cv. Neck Orange, a native plant of southern Thailand.

Although there are many studies about chemical characteristic and pharmacological activities of orange, pomelo and lime, there are very few studies in different cultivation and $SCO_2$ technique especially round kumquat. In addition, *Citrus reticulata* Blanco cv. Neck Orange, a native plant of southern of Thailand, which is a rare plant at present day. There are lots of oil glands in their fruit peel, so the study of Neck Orange peel essential oil could increase the value of the useless waste. The gardeners may be interested in cultivation improved in case this study shows the values of this plant. Furthermore, the worth of this study is to maintain variety and biodiversity. Whereas, the Neck Orange hasn’t been admired, the successful study might keep in existence of Neck Orange cultivation.

## 3. Objectives

1. To extract essential oils from Rutaceous plants including 4 cultivars of *Citrus reticulata* Blanco, 4 cultivars of *Citrus maxima* Burm. Merrill, *Citrus aurantifolia* Swingle and *Fortunella japonica* (Thunb.) Swingle by supercritical carbon dioxide extraction.
2. To screen antimicrobial, antioxidant, and anti-inflammatory activities of the oils from Rutaceous plants.
3. To study the chemical constituent of the oils from Rutaceous plants.
4. To do pre-formulation study of aromatherapy preparation

# CHAPTER 2

# MATERIALS AND METHODS

## 1. Plant material

Ten Rutaceous fruits were collected from the south and central part of Thailand in December 2008 for their peel including 4 cultivars of *Citrus reticulata* (orange), 4 cultivars of *Citrus maxima* (pomelo), *Citrus aurantifolia* (Lime) and *Fortunella japonica* (Round Kamquat). The fruits of *C. maxima* cultivar Kaw–Yai and Kaw-Nam-Pueng were collected from Amphawa District, Samutsongkarm Province, while the cultivar Tong-Dee were collected from Pra Tew District, Chumphon Province, and cultivar Hom-Hat-Yai were collected from Hat-Yai District, Songkhla Province. The fruits of *C. reticulata* cultivar Neck Orange were collected from Hat-Yai District, Songkhla Province, cultivar Sho-Kun were collected from Betong District, Yala Province, and cultivar Keaw-Hwarn and Sai-Nam-Pueng were collected from Khlong Lan District, Kamphaeng Phet Province. The fruits of *C. aurantifolia* (Lime) were collected from Ban Lat District, Phetchaburi Province. The fruits of *F. japonica* (Round Kamquat) were collected from from Hat-Yai District, Songkhla Province. The specimens were deposited in the herbarium of Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-yai, Songkhla, Thailand. The peels were washed and dried with hot air oven (50 ºC) and milled with a cutting mill (Taian Hkeb-11, D.O.L. Magnetic starter) to minimize the approximate particle size 0.5 cm.

## 2. Chemicals, reagents, microbial, media and antibiotic

### 2.1 Anti-microbial activity

#### (1) Microbial

| Microbial | Source |
|---|---|
| - *Staphylococcus aureus* ATCC 25923 | - Department of Pathology, Faculty of Medicine, Prince of Songkla University |
| - *Staphylococcus epidermidis* TISTR 517 | - Thailand Institute of Scientific and Technology Research |
| - *Trichophyton rubrum* DMST 30263 | - Department of Pathology, Faculty of Medicine, Prince of Songkla University |

#### (2) Media

| Media | Source |
|---|---|
| - Mueller Hinton Agar (MHA) | - Difco, Bacto Dickinson and Company, Spark USA. |
| - Mueller Hinton Broth (MHB) | - Difco, Bacto Dickinson and Company, Spark USA. |
| - Sabouraud's Dextrose Agar (SDA) | - Difco, Bacto Dickinson and Company, Spark USA. |
| - Sabouraud's Dextrose Broth (SDB) | - Difco, Bacto Dickinson and Company, Spark USA. |

**(3) Antibiotic**

| Antibiotic | Source |
|---|---|
| - Tetracycline | - Sigma, Sigma-Aldrich, Germany |
| - Clotrimazole | - Sigma, Sigma-Aldrich, Germany |

**2.2 Anti-oxidant activity**

| Reagent | Source |
|---|---|
| Absolute ethanol, AR grade | - Merk, Darmstadt, Germany |
| 1,1-Diphenyl-2-picrylhydrazyl (DPPH) | - Sigma, St.Louis, USA |

**2.3 Anti-inflammatory activity assay**

| Reagent | Source |
|---|---|
| - Lipopolysaccharide (LPS, from *Escherichia coli*) | - Gibco, USA |
| - RPMI 1640 medium | - Gibco, USA |
| - 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazodium bromide (MTT) | - Gibco, USA |
| - L-nitroarginine (L-NA) | - Gibco, USA |
| - Indomethacin | - Sigma, USA |
| - Phosphate-buffered saline (PBS) | - Sigma, USA |
| - Foetal calf serum (FCS) | - Gibco, USA |
| - Trypan blue | - Gibco, USA |
| - Trypsin-EDTA | - Gibco, USA |
| - Dimethyl sulfoxide (DMSO) | - Lab scan Asia, Thailand |
| - Isopropanol | - Lab scan Asia, Thailand |

## 3. Equipment and instruments

The equipments used in this study were listed in Table 3

**Table 3** General information of equipments

| Instrument | Model | Company |
|---|---|---|
| Autoclave | Model HA-3D | Hirayama, Japan |
| Centrifuge | Kubota model 5922 | Kubota Coporation, Tokyo |
| $CO_2$ incubator | Shel LAB | GIBTHAI, Thailand |
| Digital water bath | SB-1000 | EYELA, Japan |
| GC-MS | Thermo Electron Corporation | Fortune Scientific, Thailand |
| Hot air over | DIN 12880-KI | Memmert, Germany |
| Laminar air flow | Faster Ultrasafe 48 | FASTER, Italy |
| Magnetic Stirrer | G-560E | Scientific industries, USA |
| Multi-channel pipette | CyberScan 510 | - |
| Auto-pipette | Transferpettt-8 (30-300 µl) | Germany |
| Pipette | Accu-jet | Germany |
| Pipette | Pipetman (2, 200, 1000 µl) | Gilson, France |
| Microplate | 96-well | Nunc, Denmark |
| Pilot scale $SCO_2$ | Guangzhou Masson New Separation Technology | Masson Group Company Limited, Chaina |
| Refractometer | ABBE60 standard model | Bellingham & Stanley Limited, England |
| UV-Visible Spectrophotometer | Spectronic Genesys 6 | BECTHAI, Thailand |
| Viscometer | Brookfield DV-III Ultra Programmable Rheometer | Brookfield Engineering Laboratories, USA |

## 4. Extraction procedure

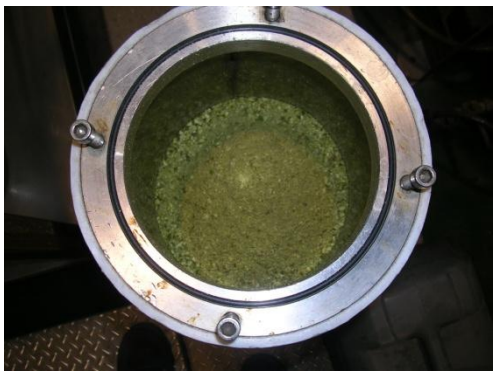
### Supercritical carbon dioxide extraction ($SCO_2$)

This research used the pilot scale $SCO_2$ (Guangzhou Masson New Separation Technology, China) which was belong to Thai Traditional and Herbal Development Center (TDC), where located at module 1, Biotechnology Pilot Plant, Thailand Science Park, Klong 1, Klongluang, Pathumtani, Thailand. The plant materials were loaded into a high pressure stainless steel extractor tank and the extract-laden carbon dioxide were sent to the two separator tanks. Samples (3-8 kg) of prepared peel as described above, were extracted with supercritical carbon dioxide. The best established temperature and pressure conditions (40 ºC and 15 MPa) were selected and used in the experiments for separation the essential oil from plant materials. Other parameters were kept constant as carbon dioxide flow rate (1.0 ml/min), time for equilibrium condition (30 min) and extraction time (3.0 hr). From this method the extraction condition gave the oleoresin deposit in the first separator tank and the second separator tank contained two immiscible phase of essential oils and water. The essential oils were separated and filtered, and then anhydrous sodium sulphate was added for elimination of trace water. All of the oil samples are kept in air tight, protected from light containers and placed in refrigerated at 4 ºC prior to analysis. (Thavanapong, 2006)

**Figure 12** The supercritical carbon dioxide extractor


(a)


(b)



(c)

**Figure 13** Extractor tank (a), separator tank (b), and milled sample (c)

**5. Physical characteristic**

The physical characteristic of essential oils was determined including physical properties (Avis, Lieberman, and Lachman, 1992), refractive index and solubility.

**5.1 Physical properties**

**5.1.1 Density**

The density of essential oils was determined by calculation. The density of a substance is a measure of how much mass is present in a given unit of volume. The formula was shown below:

$$\text{Density} = \text{mass} / \text{volume} \; ; D = m / V$$

**5.1.2 Viscosity**

The viscosity of essential oils was measured by using the viscometer (Brookfield DV-III Ultra Programmable Rheometer) at the Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkla University. This study used LV type of Brookfield Programable Rheometer. The spindle nummer was SC4-31. Shear rate was 85.00 1/sec at room temperature (25 ºC), and the programmed speed was 250 RPM. All samples were determined in triplicate.

**5.1.3 Sedimentation**

The sedimentation of essential oils was evaluated by observation. The obtained oils were placed in refrigerated at 4 ºC. After 3 months the oils were observed the precipitate.

### 5.1.4 Clearness and color

The color and clearness of essential oil were recorded by description.

## 5.2 Refractive index

The refractive index of all essential oils at 25 ºC was measured by using the Refractometer (ABBE60 standard model) at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The determination was performed in triplicate.

## 5.3 Solubility (British Pharmacopoeia Commission, 2001)

The essential oils were accurately weighed to 10 mg and placed in a vessel of at least 100 ml capacity. The vessel was placed in a constant temperature device, maintained at a temperature of 25 ± 0.2 ºC. Various solvents (water, methanol, ethyl acetate, chloroform and hexane) were examined by adding of the solvents by increments of 10 µl, shaking frequently and vigorously for 10 minutes. The volume of solvent was recorded when a clear solution is obtained. If the solution became cloudy or non-dissolve, the solvent will be continuously added until 10 ml. After addition of 10 ml of solvents, the sample or parts of it remain non-dissolve; the experiment had to be repeated in a 100 ml volumetric flask. At lower solubility, the time required to dissolve a substance can be considerably longer, at least 24 hours should be allowed.

Descriptive term of solubility and approximate volume of solvents required to completely dissolve a solute (in milliliters per gram of solute) were drawn as follow.

**Table 4** Solubility criteria of the extract in various solvents

| Solubility term | Volume of solvent required to dissolve 1 g of solute (ml) |
|---|---|
| Very soluble | less than 1 |
| Freely soluble | from 1 to 10 |
| Soluble | From 10 to 30 |
| Sparingly soluble | from 30 to 100 |
| Slightly soluble | from 100 to 1000 |
| Very slightly soluble | from 1000 to 10,000 |
| Practically insoluble | more than 10,000 |

**6. Chemical Characteristic**

**Gas Chromatography-Mass Spectrometry (GC-MS)**

Chemical identification and quantification of essential oils were investigated by Gas Chromatography - Mass Spectrometry (Thermo Electron Corporation) at Thai Traditional and Herbal Development Center (TTHD), where located at module 1, Biotechnology Pilot Plant, Thailand Science Park, Klong 1, Klongluang, Pathumtani, Thailand. The column (30 m × 0.32 mm, 0.25 μm film thickness) was ZB-5ms. Helium (TIG) was used as the carrier gas at a flow rate of 1 ml/min. The programmed column temperature was ramped from 60°C to 250°C at a route of 3°C/min, and hold at 60°C for an additional 5 min. The temperature at injector port was 200°C, and at detector port was 270°C. Detection was based on electron ionization (EI) mass spectrometry. Peak identification was carried out by comparison with those available in Wiley 7n.L Database.

## 7. Antimicrobial activity

The antimicrobial activity determination was determined using the broth micro dilution technique (Lorian, 1996; Norrell and Messley, 1997).

### 7.1 Broth micro dilution technique

#### 7.1.1 Microorganisms preparation

Mueller Hinton Agar (MHA, Difco$^{TM}$) and Muller Hinton broth (MHB, Difco$^{TM}$) were used for culturing the bacteria, and Sabouraud Dextrose Agar (SDA, Difco$^{TM}$) and Sabouraud Dextrose broth (SDB, Difco$^{TM}$) were used for culturing the fungus. The targeted microorganisms in this study were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* TISTR517, and *Trichophyton rubrum* DMST 30263. The bacteria were preinoculated on MHA (35°C, 18-24 hr), whereas the fungus was cultured on SDA (35°C, 7-10 days).

A bacterium from stock was streaked and incubated follow under the condition. A colony from culture agar plate was taken to suspend in 0.85% NaCl solution. The cell suspension was dilution with 0.85% NaCl solution to achieve optical density (OD) with 0.08-0.1 by spectrophotometer at 625 nm (approximately to $10^8$ CFU/ml) or comparable to McFarland suspension No.5 (0.5% $BaCl_2$ in 0.56 N in $SO_4$ v/v), turbidity standard, and further diluted to be $10^6$ CFU/ml using media (MHB) as diluents. For fungus, a colony from culture agar slant was taken to suspend in 0.85% NaCl solution. The cell suspension was dilution with 0.85% NaCl solution to achieve optical density (OD) with 0.11-0.13 by spectrophotometer at 530 nm (approximately to $10^6$ CFU/ml).

### 7.1.2 Sample preparation

The essential oil was prepared in dimethylsulfoxide (DMSO) and diluted with media (MHB/SDB) to the concentration of 40 mg/ml. Tetracycline and Clotrimazole as a positive control of bacteria and fungus, was diluted in sterile water distillation (water was filtered through 0.45 micron sterile filter paper) to a concentration 500 µg/ml. The growth control is 2% DMSO in media and contamination control is only media.

## 7.2 Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was defined the lowest of compound to inhibit the growth of microorganisms. The essential oils were determined the MIC by Broth micro-dilution method. MHB was used as nutrient sources for the test bacteria and SDB was used as nutrient sources for the test the fungus. To overcome the insolubility of the oils in the broth, the assay was performed in 2% DMSO of MHB/SDB. The test was performed in 96-well plate two-fold dilutions were prepared directly in wells, as follow: 50 µL of the working solution of positive control or sample was added to well 1 and 2 of the dilution series. To each remaining well, 50 µL of MHB/SDB was added. With a sterile pipette, 50 µL was transferred from well 2 to well 3. After thorough mixing, 50 µL was transferred (with a separate pipette for this and each succeeding transfer) to well 4. This process was continued to the next well until to the last well, from which 50 µL was removed and discarded. The solvent control was added with 50 µL of 2% DMSO. The contamination control and growth control were added with 100 and 50 µL MHB/SDB, respectively. The stock solution of Tetracycline and Clotrimazole were diluted with MHB/SDB to give the concentration of 250 to 0.1220 µg/ml. The stock solution of the sample was diluted with MHB/SDB to give the concentration of 20 to 0.0098 mg/ml. The 50 µL of the adjusted inoculum was added to positive control, sample, and growth control wells. The final concentration of bacteria and fungus in each well was $5 \times 10^5$ CFU/ml. The cultures were then incubated at37 °C, 24 hr for and 3-4 days for fungus.

### 7.3 Measurement of the result

The lowest concentration that turbidity was not observed of microbial was the taken as the MIC, and confirmed with colorimetric method using Alamar blue as indicator, started by adding 5 µL of 1% Alamar blue in sterile water distillation every well and incubated 5-10 hr. The pink color showed growth of microbial, while that still blue color did not show any growth of microbial.

### 7.4 Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration was defined as the lowest concentration of compound to kill microorganisms. The incubation mixtures that showed positive result of inhibitory effect (MIC) were streaked on MHA for bacteria and SDA for fungus then incubated follow under the same condition. The lowest concentration that did not show any growth was taken as the MBC. Each experiment was performed in triplicate.

## 8. Antioxidant activity

### 8.1 DPPH radical scavenging assay

The antioxidant activity of the essential oil was determined according to the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay which was originally described by Blois (1958). DPPH is considered as a stable radical because of the paramagnetism conferred by its odd electron (delocalization of the spare electron over the molecule as a whole). The solution (in absolute ethanol) is appeared as a deep violet colour and showed a strong absorption band at 520 nm. DPPH radical can accept an electron or hydrogen radical to become a stable diamagnetic molecule and has pale violet. If substance for testing antioxidant activity is mixed with DPPH solution and gives rise to yellow, it suggests that this substance has antioxidant effect by mechanism of free radical scavenging

activity. The following assay procedure was modified from those described by Blois (1958) and Yamasaki, *et al.* (1994).

**8.2 Testing procedure and data analysis**

To determine antioxidant activity of Rutaceous oil, the essential oils were dissolved in absolute ethanol (1 mg/ml) as stock solution. The samples were further diluted for at least 5 concentrations in absolute ethanol (two-fold dilution). DPPH solution ($6x10^{-5}$ M) was prepared in absolute ethanol. A portion of sample solution (100 µl) was mixed to an equal volume of DPPH solution in eppendorf tube. The mixture was shaken and stood at the room temperature for 30 minutes in the dark. The mixture was measured the absorbance at 520 nm by UV spectrophotometer, using a mixture of 100 µl sample solution and 100 µl absolute ethanol as blank. Each sample was done in triplicate. Butylated hydroxytoluene (BHT) and vitamin C were used as positive standards in the same system.

The free radical scavenging activity of each sample was determined corresponding to the intensity of quenching DPPH. The result was expressed as the percentage inhibition calculated by the following equation:

$$\% \text{ inhibition} = \frac{\text{control - sample}}{\text{control}} \times 100$$

Where Control : absorbance of DPPH solution (100 µl) in absolute ethanol (100 µl) without sample solution

Sample : absorbance of DPPH solution (100 µl) in absolute ethanol (100 µl) with sample solution

Blank : absolute ethanol

.

Dose-response curve was plotted between % inhibition and concentration. Linear regression analysis was carried out for calculating the effective concentration of sample required to scavenge DPPH radical by 50 % ($ED_{50}$ value).

**9. Anti-inflammatory activity assay**

**Effects on production of nitric oxide (NO) in LPS-stimulated macrophage RAW264.7 cells**

Inhibitory effect on nitric oxide (NO) production by murine macrophage like RAW 264.7 cells was evaluated using a modified method from that previously reported (Tewtrakul and Itharat, 2007). Briefly, the RAW264.7 cells [purchased from Cell Lines Service (CLS)] were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 0.1% sodium bicarbonate, 2 mM glutamine, penicillin G (100 U/ml), streptomycin (100 µg/ml) and 10% fetal cafe serum. The cells were harvested with trypsin-EDTA and diluted to a suspension in fresh medium. The cells were seeded in 96-well plates with $1x10^5$ cells/well and allowed to adhere for 1 hr at 37 ºC in a humidified atmosphere containing 5% $CO_2$. After that the medium was replaced with fresh medium containing 100 µg/ml of lipopolysaccharide (LPS) together with test samples at various concentrations and then incubated for 48 hr. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent. Indomethacin (non-steroidal anti-inflammatory drug, NSAID) was used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the RPMI medium (final DMSO was 1%). Inhibition (%) was calculated using the following formula and $IC_{50}$ was determined graphically ($N$=4).

**10. Determination of cytotoxic effect**

Cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method (Dong *et al*., 2001). The RAW 264.7 cells which were tesed in inhibitory effect on NO production assay were used in this study continuously. Briefly, after 48 hr incubation with test samples, MTT solution (10 µl, 5 mg/ml in phosphate buffered saline) was added to the wells. After 4 hr at 37 ºC incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The absorbance of formazan solution was measured with a microplate reader at 570 nm. When the absorbance at 570 nm of the sample-treated group was less than 80% of that in the control (vehicle-treated) group, the test compound was considered to exhibit cytotoxic effect (Matsuda et al., 2003).

**11. Pre-formulation study**

Essential oils are too strong to be applied neat to the skin. When used in massage they should be mixed with carrier oil or base oil. In this study, after obtain three essential oils which exhibited the best biological activity, the oils were studied in pre-formulation by using blending oil technique.

**12. Blending oil technique**

Coconut oil is generally used in Thailand. This oil is the main carrier oil in tropical Asia because of its abundance. It is a saturated-oil with its own district smell. Coconut oil aids tanning and is reputed to help filter the sun's ray. It is emollient on hair and skin. It remains stable for a long period and is particularly nourishing in hair treatments (Price, 2000). For the aromatherapy oil, the products labeled thus usually consist of a 2% maximum dilution of essential oil in fixed oil (Price and Price, 2007). Nine formulas were determined in this experiment. The three selected oils were mixed and blended with coconut oil as a formula 1, 2 and 3. Essential oil 1 and 2 were mixed and blended

with coconut oil as formula 4. Essential oil 2 and 3 were mixed and blended with coconut oil as formula 5. Essential oil 1 and 3 were mixed and blended with coconut oil as formula 6. Essential oil 1, 2 and 3 were mixed in different proportion and blended with coconut oil as formula 7, 8, and 9.

**Table 5** Essential oil and carrier oil proportion

| Formula | Essential oil 1 (ml) | Essential oil 2 (ml) | Essential oil 3 (ml) | Coconut oil (ml) |
|---|---|---|---|---|
| 1 | 0.5 | - | - | 25 |
| 2 | - | 0.5 | - | 25 |
| 3 | - | - | 0.5 | 25 |
| 4 | 0.25 | 0.25 | - | 25 |
| 5 | 0.25 | - | 0.25 | 25 |
| 6 | - | 0.25 | 0.25 | 25 |
| 7 | 0.2 | 0.2 | 0.1 | 25 |
| 8 | 0.2 | 0.1 | 0.2 | 25 |
| 9 | 0.1 | 0.2 | 0.2 | 25 |

After blending, the blended oils were tested physical properties including density, viscosity, sedimentation, clearness and color. The biological activities of essential oils were evaluated.

**13. Stability studies**

The effect of temperature and light on the physical and chemical stability of blended oils was examined. Stability changes can occur in the form of color change, pH shift, or decomposition. In this study, the stability of essential oil was determined in term of temperature and light.

**13.1 Temperature stability**

The blended oils were transferred 15 ml transparent glass vials and wrapped with aluminium foil and were placed into a constant-temperature place at 4, 25, and 60 ºC for 3 months. Then, the blended oils were parted to study physical properties at intervals of 0, 30, 60, and 90 days. All determinations were performed in triplicate.

**13.2 Light stability**

Two conditions were designed for the study of the effect of light on the blended oils. The first condition, normal lighting condition, the formulae were placed into a constant-light. The second condition, the formulae were protected from light. The blended oils were transferred 15 ml transparent glass vials and positioned 40 cm below the white light (36 W, Philip, Thailand) at 25 ºC compared with the oils that stored in transparent glass vials which were wrapped with aluminium foil for 3 months. All of determinations were performed in triplicate. Samples were collected at intervals of 0, 30, 60, 90 days for analysis. The physical property of the samples was also noted.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 1. Essential oil extraction

The peel of ten Rutaceous plants which were harvested from the south and central part of Thailand were extracted by supercritical carbon dioxide ($SCO_2$) method. From the experiments, the extraction condition gave the oleoresin deposit in the first separator and the second separator contained two immiscible phase of essential oils and water. The essential oils were separated, filtered and then anhydrous sodium sulphate was added for elimination of trace water. The color of essential oil was clear solution with light-green or light-yellow colors. After storage, a small amount of precipitate at the bottom of the container was obserived. The $SCO_2$ method produced a various yield of Rutaceous oils at 0.28-6.91 % v/w. The %Yield was shown in figure 4.1. *C. reticulata* produced more yield of volatile oil (4.55-6.91%) when compared with others. All of the oil samples were kept in air tight, protected from light containers and placed in refrigerated at 4 ºC.

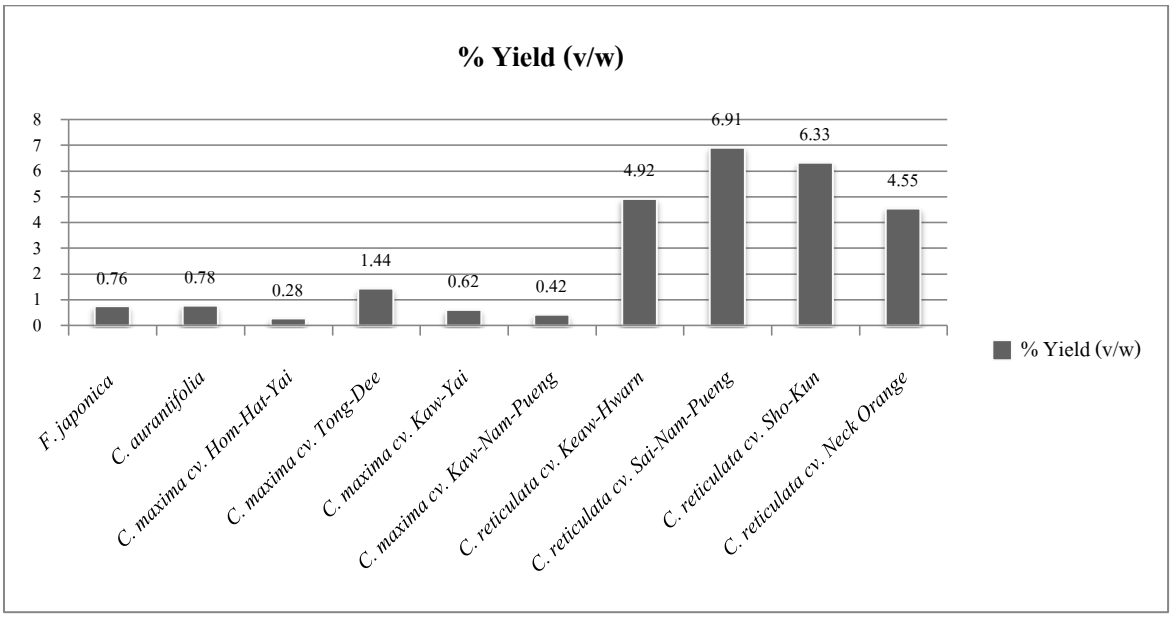



**Figure 14** %Yield (v/w) of essential oils extracted from $SCO_2$ method

**Figure 15** The waxy product (a) and essential oil product (b) of *F. japonica*

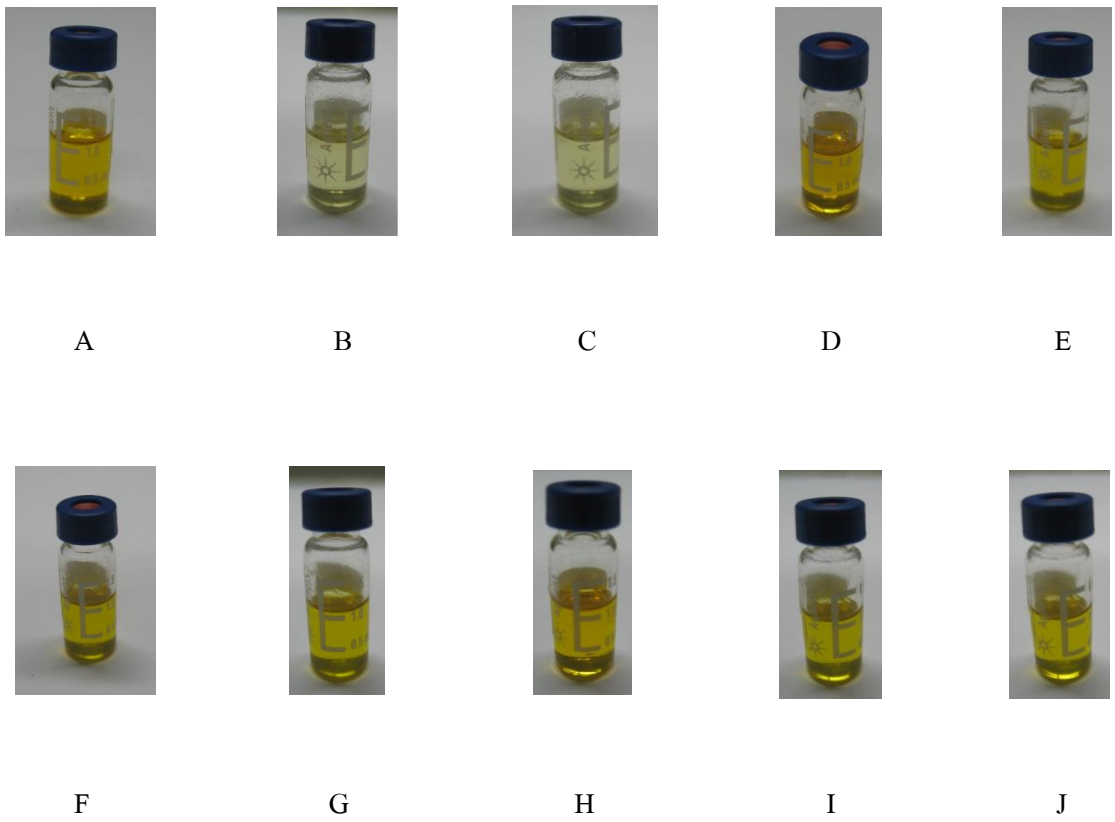



**Figure 16** The $SCO_2$ extracted - volatile oil of *F. japonica* or Round Kamquat, *C. aurantifolia* or Lime, *C. maxima* cv. Hom-Hat-Yai, Tong-Dee, Kaw-Yai and Kaw-Nam-Pueng, and *C. reticulata* cv. Keaw-Hwarn, Sai-Nam-Pueng, Sho-Kun and Neck Orange, respectively. (A-J)

**2. Physical characteristic**

Physical values of the Rutaceous volatile oils were shown in Table 6. The density, viscosity and refractive index of Rutaceous peel oils were close to each other. Their density was around 0.82-0.84 g/ml and their refractive index was about 1.475-1.476. Their viscosity was varied from 1.72 to 2.64 cps. Their color was varied from light yellow to light green in different intensity, no sedimentation. From the values of density, viscosity, and refractive index of all kind of oils that were very close to each other meant that the chemical components in the oils might be nearly the same, but different in proportion.

**Table 6** Physical property of Rutaceous oil extracted by $SCO_2$

| Essential oil | Physical properties | | | |
|---|---|---|---|---|
| | **Density**** (g/ml) | **Viscosity**** (cps) | **Refractive index** | **Clearness & color*** |
| Round Kamquat | 0.84 | 2.64 ± 0.00 | 1.476 | LY +++ |
| Lime | 0.83 | 1.72 ± 0.14 | 1.475 | LG + |
| Hom-hat-yai | 0.83 | 2.04 ± 0.00 | 1.475 | LG + |
| Tong-dee | 0.84 | 2.04 ± 0.00 | 1.475 | LY +++ |
| Kaw-yai | 0.83 | 1.88 ± 0.07 | 1.475 | LY ++ |
| Kaw-nam-pueng | 0.83 | 2.04 ± 0.00 | 1.475 | LY ++ |
| Keaw-hwarn | 0.84 | 1.80 ± 0.00 | 1.475 | LY ++ |
| Sai-nam-pueng | 0.84 | 1.80 ± 0.00 | 1.475 | LY +++ |
| Sho-kun | 0.84 | 1.76 ± 0.07 | 1.475 | LY ++ |
| Neck orange | 0.84 | 2.64 ± 0.00 | 1.476 | LY ++ |

*no sedimentation ; LY : Light-yellow ; LG : Light-green

+ : Dark color ; +,++,+++ : Light to dark color

**Values represent mean ± SD = ± 0.00 (N=3)

**3. Determination of solubility**

Solubility is commonly expressed as a maximum equilibrium amount of a solute that can normally dissolve per amount of solvent or a maximum concentration of a saturated solution. These maximum concentrations are often expressed as grams of solute per ml of solvent. The solubility of the Rutaceous volatile oils was estimated in various solvents. The result showed that the volatile oils of *F. japonica, C. aurantifolia*, *C. maxima* (Hom-Hat-Yai, Tong-Dee, Kaw-Yai, Kaw-Nam-Pueng) and *C. reticulate* (Keaw-Hwarn, Sai-Nam-Pueng, Sho-Kun, Neck Orange) were freely soluble in methanol, ethyl acetate, chloroform and hexane. They were practically soluble in water (Table 7-10). The volatile oils contained most likely moderate and non-polar compounds therefore the suitable solvents for them should be a moderate and non- polar solvents.

**Table 7** Solubility property of *F. japonica* volatile oil

| **Solvent** | **Volume of solvent per 1 g of solute (ml)** | **Level of solubility** |
|---|---|---|
| Methanol | 1 | Freely soluble |
| Ethyl acetate | 1 | Freely soluble |
| Chloroform | 2 | Freely soluble |
| Hexane | 4 | Freely soluble |
| Water | >10,000 | Practically insoluble |

**Table 8** Solubility property of *C. aurantifolia* volatile oil

| **Solvent** | **Volume of solvent per 1 g of solute (ml)** | **Level of solubility** |
|---|---|---|
| Methanol | 1 | Freely soluble |
| Ethyl acetate | 1 | Freely soluble |
| Chloroform | 1 | Freely soluble |
| Hexane | 3 | Freely soluble |
| Water | >10,000 | Practically insoluble |

**Table 9** Solubility property of *C. maxima* volatile oil: Hom-Hat-Yai, Tong-Dee, Kaw-Yai, Kaw-Nam-Pueng (C3, C4, C5, C6)

| **Solvent** | **Volume of solvent per 1 g of solute (ml)** | | | | **Level of solubility** |
|---|---|---|---|---|---|
| | **C3** | **C4** | **C5** | **C6** | |
| Methanol | 1 | 1 | 1 | 1 | Freely soluble |
| Ethyl acetate | 1 | 1 | 1 | 1 | Freely soluble |
| Chloroform | 1 | 2 | 2 | 1 | Freely soluble |
| Hexane | 3 | 3 | 3 | 3 | Freely soluble |
| Water | >10,000 | >10,000 | >10,000 | >10,000 | Practically insoluble |

**Table 10** Solubility property of *C. reticulata* volatile oil: Keaw-Hwarn, Sai-Nam-Pueng, Sho-Kun, Neck Orange (C7, C8, C9, C10)

| Solvent | Volume of solvent per 1 g of solute (ml) | | | | Level of solubility |
|---|---|---|---|---|---|
| | C7 | C8 | C9 | C10 | |
| Methanol | 1 | 1 | 1 | 1 | Freely soluble |
| Ethyl acetate | 1 | 1 | 1 | 1 | Freely soluble |
| Chloroform | 1 | 1 | 1 | 1 | Freely soluble |
| Hexane | 3 | 4 | 3 | 4 | Freely soluble |
| Water | >10,000 | >10,000 | >10,000 | >10,000 | Practically insoluble |

**4. Chemical Characteristic**

The essential oil compositions were determined by GC-MS and showed in table 11-12. Although the major component of the all oils was *d*-limonene, the amounts were different. The % yield of major compound, *d*-limonene, of *C. maxima* cv. Kaw-Yai (91.74) was similar to Thavanapong's study (93.74%) in 2006, and from her study $SCO_2$ technique presented more yields of volatile oil (0.4 % v/w) when compared with cold pressing (0.3 % v/w) and vacuum steam distillation (0.2 % v/w) methods.

Top three major compounds in *C. maxima* oils were *d*-limonene, beta-pinene, and alpha-phellandrene. The quantification of these compounds in three cultivation including Tong-Dee, Kaw-Yai, and Kaw-Nam-Pueng were closed. There were some different proportion of *d*-limonene (68.97%) and beta-pinene (23.36%) in Hom-Hat-Yai. The volatile oil of Hom-Hat-Yai which extracted by $SCO_2$ produced more percent of beta-pinene when compared with other cultivation. For the major compounds of *C. reticulata* volatile oil: Keaw-hwarn, Sai-nam-pueng, Sho-kun, Neck orange, *d*-limonene was a major compound, and the percentage of beta-pinene, delta-3-carene and gamma-terpinene were quite closed.

Major components of *F. japonica* or Round Kamquat volatile oil were *d*-limonene (56.94%) and germacrene D (17.38%). Although the physiology of Round Kamquat was similar to *Citrus* spp., the major components were different. In this study, germacrene D was found in Round Kamquat volatile oil in high proportion. In 1994 Katumi *et al.* report the volatile constituents of Round Kumquat fruit which were isolated by steam distillation and simultaneous purging/extraction (SPE) methods. They found that *d*-limonene was the most abundant compound, comprising 87% of the sample from steam distillation and 97% of the sample from SPE. Resembling Choi' study (2005), he was conducted to determine the composition of Kumquat cold-pressed peel oil. The major compounds were limonene (93.73%), myrcene (1.84%) and ethyl acetate (1.13%). From these, no report the germacrene D to be a major compound whereas the supercritical carbon dioxide extraction in this study could produce the germacrene D in high percentage. For the *C. aurantifolia* or Lime volatile oil, the major component also was *d*-limonene (61.10%). Beta-pinene (9.54%) and gamma-terpinene (6.37%) were inferior, respectively. When compared with Dugo *et al.*'s study (1997) which studied about Lime oil extracted by cold-pressed technique, the major components were similar.

**Table 11** % Yield (v/w) and major components of *F. japonica* or Round Kamquat, *C. aurantifolia* or Lime, *C. maxima* cv. Hom-Hat-Yai, Tong-Dee, Kaw-Yai and Kaw-Nam-Pueng, and *C. reticulata* cv. Keaw-Hwarn, Sai-Nam-Pueng, Sho-Kun and Neck Orange volatile oils

| **Essential oil** | **% Yield (v/w)** | **Major compounds (% Relative area)** | | |
|---|---|---|---|---|
| | | **Compound1** | **Compound2** | **Compound3** |
| Round Kumquat | 0.76 | *d*-limonene (56.94) | Germacrene-D (17.38) | citronellal (4.31) |
| Lime | 0.78 | *d*-limonene (61.10) | beta-pinene (9.54) | gamma-terpinene (6.37) |
| Hom-Hat-Yai | 0.28 | *d*-limonene (68.97) | beta-pinene (23.36) | alpha-phellandrene (0.56) |
| Tong-Dee | 1.44 | *d*-limonene (90.30) | beta-pinene (5.49) | alpha-phellandrene (0.42) |
| Kaw-Yai | 0.62 | *d*-limonene (91.74) | beta-pinene (1.95) | alpha-phellandrene (0.70) |
| Kaw-Nam-Pueng | 0.42 | *d*-limonene (91.66) | beta-pinene (1.39) | alpha-phellandrene (0.82) |
| Keaw-Hwarn | 4.92 | *d*-limonene (86.84) | beta-pinene (2.53) | gamma-terpinene (1.75) |
| Sai-Nam-Pueng | 6.91 | *d*-limonene (95.65) | delta-3-carene (1.48) | beta-pinene (1.09) |
| Sho-Kun | 6.33 | *d*-limonene (95.01) | delta-3-carene (1.52) | beta-pinene (1.48) |
| Neck Orange | 4.55 | *d*-limonene (84.44) | gamma-terpinene (9.32) | delta-3-carene (1.63) |

**Table 12** GC-MS analysis of ten Rutaceous oils extracted by $SCO_2$

| Compound | Retention time (min) | % Relative area | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | C1 | C2 | C3 | C4 | C5 | C6 | C7 | C8 | C9 | C10 |
| alpha-phellandrene | 7.37 | Trace | Trace | 0.56 | 0.42 | 0.70 | 0.82 | Trace | - | - | 0.30 |
| alpha-pinene | 7.64 | - | 1.09 | 0.35 | 0.50 | 0.48 | 0.40 | 0.05 | 0.54 | 0.62 | 1.20 |
| beta-phellandrene | 9.22 | - | 1.75 | 0.18 | 0.25 | 0.26 | 0.23 | 0.51 | 0.33 | 0.42 | 0.19 |
| beta-pinene | 9.41 | Trace | 9.54 | 23.36 | 5.49 | 1.95 | 1.39 | 2.53 | 1.09 | 1.48 | 0.57 |
| sabinene | 9.55 | - | - | 0.05 | 0.45 | 0.41 | 0.26 | - | - | - | - |
| delta-3-carene | 9.95 | 0.84 | 1.00 | - | - | - | - | 1.27 | 1.48 | 1.52 | 1.63 |
| alpha-terpinene | 11.15 | - | Trace | - | - | - | - | - | - | - | 0.24 |
| *d*-limonene | 11.91 | 56.94 | 61.10 | 68.97 | 90.30 | 91.74 | 91.66 | 86.84 | 95.65 | 95.01 | 84.44 |
| beta-ocimene | 12.66 | - | - | 1.95 | 0.40 | 0.17 | 0.12 | - | - | - | - |
| gamma-terpinen | 13.07 | 0.97 | 6.37 | - | - | - | - | 1.75 | - | - | 9.32 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| unknown 1 | 14.25 | - | - | - | - | - | - | Trace | - | - | 0.57 |
| linalool | 15.08 | 1.37 | Trace | 0.11 | 0.08 | 0.14 | 0.17 | 0.27 | 0.11 | 0.18 | 0.43 |
| citronellal | 17.49 | 4.31 | - | - | - | - | - | - | - | - | 0.05 |
| terpinen-4-ol | 18.94 | - | - | 0.10 | 0.03 | - | - | - | - | - | - |
| alpha-terpineol | 19.68 | - | 0.49 | 0.07 | 0.08 | 0.21 | 0.24 | - | - | - | - |
| alpha-terpinyl acetate | 19.50 | Trace | - | - | - | - | - | 0.22 | 0.06 | 0.06 | 0.19 |
| *l*-undecyne | 20.04 | - | - | - | - | - | - | - | - | - | 0.26 |
| geraniol | 20.89 | - | - | - | - | - | - | - | - | - | 0.05 |
| citronellol | 21.05 | Trace | - | - | - | - | - | - | - | - | - |
| *Z*-citral | 21.68 | - | 0.66 | 0.10 | 0.04 | 0.07 | 0.14 | - | - | - | - |
| *E*-citral | 23.06 | - | 0.63 | 0.10 | 0.04 | 0.08 | 0.16 | - | - | - | - |
| (-)-perillaldehyde | 23.11 | - | - | - | - | - | - | - | - | - | 0.05 |
| delta-elemene | 25.73 | 1.74 | 0.56 | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| bicyclogermacrene | 25.75 | - | - | - | - | - | - | - | - | - | Trace |
| alpha-carophyllene | 26.88 | - | - | - | - | - | - | - | - | - | Trace |
| camphene | 27.76 | 1.52 | - | - | - | - | - | - | - | - | - |
| nerol | 27.92 | - | 0.61 | - | - | - | - | - | - | - | - |
| *E*-7-tetradecenol | 29.08 | - | - | - | - | - | - | - | - | - | 0.06 |
| beta-caryophyllene | 29.29 | 0.90 | 2.71 | 0.36 | 0.21 | 0.39 | 0.43 | 0.77 | - | - | - |
| alpha-bergamotene | 30.08 | - | - | - | - | - | - | 0.52 | - | - | - |
| gamma-humulen | 30.09 | - | 1.93 | - | - | - | - | - | - | - | - |
| germacrene D | 31.92 | 17.38 | 2.07 | 3.01 | 0.39 | 1.87 | 2.52 | 0.56 | 0.14 | 0.19 | - |
| alpha-guaiene | 32.18 | 1.01 | - | - | - | - | - | - | - | - | - |
| germacrene B | 32.44 | 1.73 | - | - | - | - | - | - | - | - | - |
| alpha-fanesene | 33.12 | - | 2.80 | - | - | - | - | 1.03 | 0.05 | 0.07 | - |
| alloaromadendrene | 33.20 | - | 3.26 | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| delta-elemene | 33.84 | - | | 0.56 | - | - | - | - | - | - | - |
| valencene | 34.67 | 1.67 | - | 0.33 | 0.14 | 0.20 | 0.23 | - | - | - | - |
| aromadendrene | 34.93 | Trace | - | - | - | - | - | - | - | - | - |
| gamma-gurjunene | 35.09 | - | 1.89 | - | - | - | - | - | - | - | - |
| (-)-sinularene | 35.09 | - | - | - | - | - | - | 0.27 | 0.07 | 0.07 | - |
| gamma-elemene | 35.23 | Trace | Trace | - | - | - | - | - | - | - | - |
| beta-guaiene | 35.70 | Trace | - | - | - | - | - | - | - | - | - |
| aristolene | 37.89 | 0.70 | - | - | - | - | - | - | - | - | - |
| alpha-eudesmol | 38.69 | 2.69 | - | - | - | - | - | - | - | - | - |
| cemcrene | 49.09 | - | - | 0.20 | - | - | - | - | - | - | - |
| palmitic acid | 49.66 | 0.71 | - | - | - | - | - | - | - | - | - |
| unknown 2 | 50.55 | 1.81 | - | - | - | - | - | - | - | - | - |
| unknown 3 | 54.07 | 1.62 | - | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| unknown 4 | 55.04 | 0.66 | - | - | - | - | - | - | - | - | - |
| unknown 5 | 55.74 | 0.84 | - | - | - | - | - | - | - | - | - |

Trace : less than 0.05%

C1 : essential oil of *F. japonica* peel extracted by supercritical carbon dioxide extraction

C2 : essential oil of *C. aurantifolia* peel extracted by supercritical carbon dioxide extraction

C3 : essential oil of *C. maxima* cultivar Hom-Hat-Yai peel extracted by supercritical carbon dioxide extraction

C4 : essential oil of *C. maxima* cultivar Tong-Dee peel extracted by supercritical carbon dioxide extraction

C5 : essential oil of *C. maxima* cultivar Kaw–Yai peel extracted by supercritical carbon dioxide extraction

C6 : essential oil of *C. maxima* cultivar Kaw-Nam-Pueng peel extracted by supercritical carbon dioxide extraction

C7 : essential oil of *C. reticulata* cultivar Keaw-Hwarn peel extracted by supercritical carbon dioxide extraction

C8 : essential oil of *C. reticulata* cultivar Sai-Nam-Pueng peel extracted by supercritical carbon dioxide extraction

C9 : essential oil of *C. reticulata* cultivar Sho-Kun peel extracted by supercritical carbon dioxide extraction

C10 : essential oil of *C. reticulata* cultivar Neck Orange peel extracted by supercritical carbon dioxide extraction

**5. Evaluation of microbial activity of Rutaceous volatile oils against *Staphylococcus aureus, Staphylococcus epidermidis*, and *Trichophyton rubrum***

The result of Rutaceous volatile oils which were evaluated the antibacterial activity was shown in Table 13. Since the oils could volatile, the disc diffusion assay could not indicate the activity. The results from the broth micro dilution assay indicated that three volatile oils showed good activity against *S. aureus, S. epidermidis*, and *T. rubrum*. The essential oil of *C. aurantifolia* or Lime peel was shown the best activity against *S. aureus, S. epidermidis*, and *T. rubrum*. The Lime oil could inhibit and kill *S. aureus* and *S. epidermidis* at 0.63 mg/ml and 0.04 mg/ml for *T. rubrum*. The essential oil of *C. maxima* cv. Hom-Hat-Yai peel could inhibit *S. aureus* and *S. epidermidis* at 1.25 mg/ml and 0.04 mg/ml for *T. rubrum*. As the essential oil of *F. japonica* or Round Kamquat peel could inhibit and kill *S. aureus* and *S. epidermidis* at concentration 2.5 mg/ml, and 0.16 mg/ml for *T. rubrum*. There were some different on the result of the other oils. They were not exhibit the high potent to against the microorganism. As the results indicated, the different antimicrobial activity of the essential oil samples may due to their different chemical compositions. The other oils showed the result closed to *d*-limonene which was a high percent in them.

**6. DPPH radical scavenging assay**

The antioxidant activity of Rutaceous volatile oils were evaluated by DPPH radical scavenging assay for screening test. If substance for testing antioxidant activity is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has antioxidant effect by mechanism of free radical scavenging activity. From the result there were no the volatile oils could give rise to pale violet of DPPH solution, it suggests that these Rutaceous volatile oil which extracted by $SCO_2$ had no antioxidant effect by mechanism of free radical scavenging activity.

**Table13** The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of volatile oils against *S. aureus, S. epidermidis and T. rubrum*.

| **Microorganisms** | ***S. aureus*** | | ***S. epidermidis*** | | ***T. rubrum*** | |
|---|---|---|---|---|---|---|
| **Samples** | **MIC** (mg/ml) | **MBC** (mg/ml) | **MIC** (mg/ml) | **MBC** (mg/ml) | **MIC** (mg/ml) | **MBC** (mg/ml) |
| *C. reticulata* cv. Neck Orange | 10 | 20 | - | - | 1.25 | 1.25 |
| *F. japonica* or Round Kamquat | 2.5 | 2.5 | 2.5 | 2.5 | 0.16 | 0.16 |
| *C. maxima* cv. Hom-Hat-Yai | 1.25 | 1.25 | 1.25 | 2.5 | 0.08 | 0.16 |
| *C. maxima* cv. Kaw-Yai | 10 | 20 | 10 | 10 | 1.25 | 2.5 |
| *C. maxima* cv. Tong-Dee | 10 | 20 | 20 | - | 1.25 | 2.5 |
| *C. maxima* cv. Kaw-Nam-Pueng | 10 | 20 | 10 | 20 | 1.25 | 1.25 |
| *C. reticulata* cv. Keaw-Hwarn | 10 | 20 | 20 | - | 1.25 | 1.25 |
| *C. reticulata* cv. Sai-Nam-Pueng | 20 | 20 | 20 | - | 1.25 | 1.25 |
| *C. reticulata* cv. Sho-Kun | 10 | 20 | 20 | 20 | 0.31 | 1.25 |
| *C. aurantifolia* or Lime | 0.63 | 0.63 | 0.63 | 0.63 | 0.04 | 0.04 |
| Tetracycline | 0.001 | 0.001 | 0.004 | 0.008 | - | - |
| Clotrimazole | - | - | - | - | 0.0004 | 0.0004 |
| *d*- Limonene | 10 | 10 | 10 | 20 | 0.31 | 0.31 |

**7. Inhibition on NO production of Rutaceous volatile oils**

In murine macrophage RAW264.7 cells, LPS alone increased NO production (Yoon et al., 2009). Using the Griess reaction, a spectrophotometric determination of nitrite ($NO_2^-$) was carried out to quantify the levels in the conditioned medium of RAW 264.7 cells treated with LPS. This cell-based assay system has been used for drug screening and the evaluation of potential inhibitors of the pathways leading to the induction of NO production. Our results showed that *C. aurantifolia* or Lime, *F. japonica* or Round Kamquat and *C. maxima* cv. Hom-Hat-Yai volatile oils had a potent inhibitory effect on the release of NO with $IC_{50}$ values of 10.2, 12.1 and 20.3 µg/ml, respectively, whereas the other oils had a moderate effect. The oils that showed a moderate effect on the release of NO established $IC_{50}$ close to *d*-limonene (28.6µg/ml) which was a major compound. Cytotoxic effects were not observed in this experiment. (Table 14)

**Table 14** NO inhibitory effects of essential oils from Rutaceous plants

| **Sample** | **$IC_{50}$ (μg/ml)** |
|---|---|
| *F. japonica* or Round Kamquat | 12.1 |
| *C. aurantifolia* or Lime | 10.2 |
| *C. maxima* cv. Hom-Hat-Yai | 20.3 |
| *C. maxima* cv. Tong-Dee | 28.8 |
| *C. maxima* cv. Kaw-Yai | 28.6 |
| *C. maxima* cv. Kaw-Nam-Pueng | 29.3 |
| *C. reticulata* cv. Keaw-Hwarn | 39.0 |
| *C. reticulata* cv. Sai-Nam-Pueng | 35.6 |
| *C. reticulata* cv. Sho-Kun | 32.7 |
| *C. reticulata* cv. Neck Orange | 27.3 |
| *d*-limonene | 28.6 (209.94 μM) |
| Indomethacin | 38.6 (107.88 μM) |

**8. Pre-formulation study**

Three volatile oils which exhibited the best anti-bacterial and anti-inflammatory activities including *C. aurantifolia* or Lime, *F. japonica* or Round Kamquat and *C. maxima* cv. Hom-Hat-Yai were selected to blend with coconut oil using blending oil technique. After blending, their physical properties and biological activities were evaluated. The physical values of the formulae were shown in Table 16. The density and refractive index of the formulae were close to coconut oil. Their density was around 0.85g/ml and their refractive index was about 1.451-1.453. Their viscosity was varied from 41.03 to 41.75 cps. Their color was varied from no color to light yellow, no sedimentation. From the result, density, and refractive index of all formulae were very close to coconut oil which was the carrier oil.

**Table 15** *C. aurantifolia* or Lime, *F. japonica* or Round Kamquat and *C. maxima* cv. Hom-Hat-Yai volatile oils and coconut oil proportion

| Formula | Lime (ml) | Round Kamquat (ml) | Hom-Hat-Yai (ml) | Coconut oil (ml) |
|---|---|---|---|---|
| 1 | 0.5 | - | - | 25 |
| 2 | - | 0.5 | - | 25 |
| 3 | - | - | 0.5 | 25 |
| 4 | 0.25 | 0.25 | - | 25 |
| 5 | 0.25 | - | 0.25 | 25 |
| 6 | - | 0.25 | 0.25 | 25 |
| 7 | 0.2 | 0.2 | 0.1 | 25 |
| 8 | 0.2 | 0.1 | 0.2 | 25 |
| 9 | 0.1 | 0.2 | 0.2 | 25 |

**Table 16** Physical property of the blended oils

| Formula | Physical properties | | | |
|---|---|---|---|---|
| | **Density**** (g/ml) | **Viscosity**** (cps) | **Refractive index** | **Clearness & color*** |
| 1 | 0.85 | 41.27 ± 0.14 | 1.453 | no color |
| 2 | 0.85 | 41.71 ± 0.00 | 1.451 | LY +++ |
| 3 | 0.85 | 41.39 ± 0.07 | 1.453 | no color |
| 4 | 0.85 | 41.59 ± 0.00 | 1.452 | LY ++ |
| 5 | 0.85 | 41.51 ± 0.07 | 1.452 | no color |
| 6 | 0.85 | 41.75 ± 0.00 | 1.452 | LY ++ |
| 7 | 0.85 | 41.32 ± 0.12 | 1.452 | LY + |
| 8 | 0.85 | 41.15 ± 0.51 | 1.452 | LY + |
| 9 | 0.85 | 41.03 ± 0.07 | 1.452 | LY + |
| Coconut oil | 0.85 | 44.07 ± 0.14 | 1.453 | no color |

*no sedimentation ; LY : Light-yellow ; LG : Light-green

+ : Dark color ; +,++,+++ : Light to dark color

**Values represent mean ± SD = ± 0.00 (N=3)

In the same way to the result of biological activities, the formulae showed the result similar to coconut oil. They could not inhibit *S. aureus, S. epidermidis and T. rubrum*. As Inhibitory effect on NO production, the formulas and coconut oil could not establish anti-inflammatory activity. Their $IC_{50}$ values were more than 100 μg/ml. Since the proportion of volatile oils that blended with carrier oil was small, the volatile oils could not present the real activities in experiment. Two percent of volatile oil after blending was too small amount. For anti-microbial activity, the maximum concentration of samples was 20 mg/ml, the maximum concentration of volatile oil was only 0.4 mg/ml. It was similar to anti-inflammatory activity test, the maximum concentration was 100 μg/ml, the maximum concentration of volatile oil was only 0.2 μg/ml. Therefore the blended oils could not establish the activities.

Although the formulae could not establish the biological activities for *in vitro* study because of the small propotion, it does not mean they were useless. The *in vivo* study should be studied further. However, cell viability in MTT assay showed no poisoning of cells.

Since the Rutaceous volatile oils which extracted by $SCO_2$ could not establish the antioxidant value, the coconut oil which enrich in antioxidant property was determined. From DPPH radical scavenging assay result, the blended oils did not exhibit the antioxidant effect when followed the protocol. However to ensure the antioxidant property, the study of antioxidant activity should be determined in other techniques.

## 9. Stability studies

Stability studies have received considerable attention in recent years because of their importance in development and quality control of pharmaceutical products. There are many parameters that could influence drug stability, including temperature, storage time, drug concentration, sample pH, addition of preservatives and container system among others (Jiménez *et al*., 2004). Consequently, the stability of blended oils study was investigated. Stability testing is performed to ensure that products retain their full quality, efficacy, and safety of the products (Grimm, 1993). The effect of temperature and light on the physical and chemical stability of blended oils was examined. Stability changes can occur in the form of color change, pH shift, or decomposition.

### 9.1 Effect of temperature on stability of blended oils

The effect of temperature on the stability of blended oils was examined under light protected condition at 4 °C, 25 °C, and 60 °C for 3 months. Physical properties of the blended oils were observed and examined at intervals of 0, 30, 60, and 90 days. The results showed that tested temperature did not affect either the physical properties except the condition at 60 °C which the viscosity had changed and was rancid. At 4 °C, 25 °C, their densities were around 0.85 g/ml and their refractive indexs were about 1.451-1.453. Their viscosities were varied from 41.03 to 41.84 cps. Their colors were not changed and had not sedimentation. When compared to the original blended oil in the same condition during 90 days (Table 17), the result indicated that the blended oil was not stable when stored at high temperature. Since, the oleic and linoleic acids which are minor unsaturated fatty acids in coconut oil can lead to oil rancidity because of lipid oxidation (Wongpoowarak, 2008). Hence, they should be kept in closed container and stored at low temperature (room temperature or 4 °C).

### 9.2 Effect of light on stability of blended oils

The effect of light on the stability of blended oils was evaluated. The blended oils were kept in transparent glass vials and stored either under the white light at air condition room (25 ºC) or in transparent glass vials wrapped with aluminium foil for a period of 3 months. Physical properties of the oils were observed and evaluated. The densities were around 0.85 g/ml and their refractive indexs were about 1.451-1.453. Their viscosities were varied from 40.99 to 41.92 cps. Their colors were not changed and had not sedimentation. The results showed that that all tested light did not affect either the physical property of the oils. The oils stored in the presence of light and dark, the color, sedimentation, density, and viscosity of blended oil in both conditions were observed as no change. This showed that the blended oils were no degraded in photolytic condition. Although the result showed the blend oils could resisted to light, they might be degraded for long term.

**Table 17** Effect of temperature on stability of blended oils (60°C)

| **Formula** | **Viscosity (cps)** | | | |
|---|---|---|---|---|
| | 0 month | 1 month | 2 months | 3 months |
| F1 | 41.27 ± 0.14 | 43.39 ± 0.13 | 43.69 ± 0.72 | 43.83 ± 0.43 |
| F2 | 41.71 ± 0.00 | 43.79 ± 0.24 | 44.41 ± 0.39 | 44.24 ± 0.45 |
| F3 | 41.39 ± 0.07 | 42.44 ± 0.16 | 42.53 ± 0.20 | 42.65 ± 0.69 |
| F4 | 41.59 ± 0.00 | 42.55 ± 0.07 | 42.75 ± 0.17 | 42.68 ± 0.40 |
| F5 | 41.51 ± 0.07 | 42.75 ± 0.07 | 42.79 ± 0.07 | 42.82 ± 0.16 |
| F6 | 41.75 ± 0.00 | 42.20 ± 0.29 | 42.22 ± 0.26 | 42.78 ± 0.24 |
| F7 | 41.32 ± 0.12 | 42.62 ± 0.17 | 42.65 ± 0.18 | 42.64 ± 0.11 |
| F8 | 41.15 ± 0.51 | 42.43 ± 0.21 | 42.74 ± 0.35 | 42.45 ± 0.28 |
| F9 | 41.03 ± 0.07 | 43.73 ± 0.17 | 43.54 ± 0.37 | 43.76 ± 0.19 |
| Coconut oil | 44.07 ± 0.14 | 46.00 ± 0.16 | 46.13 ± 0.06 | 46.12 ± 0.16 |

*Values represent means ± SD (N=3)

# CHAPTER 4

# CONCLUSION

From this study, it was found that *Citrus reticulata* produced more yield of volatile oil when compared with *Citrus maxima*, *Citrus aurantifolia*, and *Fortunella japonica*. Their physical properties were similar. Although the major component of the whole oils was *d*-limonene, the amounts were different. The *C. maxima* cv. Hom-Hat-Yai produced less percent of *d*-limonene, as to *C. aurantifolia* and *F. japonica*.

This is the first report on *F. japonica* volatile oil which was extracted by supercritical carbon dioxide extraction and the biological activities, as the study of *C. reticulata* and *C.maxima* in different cultivation in Thailand on anti-inflammatory activity, especially, *C. reticulata* Blanco cv. neck orange, a native plant of southern of Thailand, which is a rare plant in present day.

The Rutaceous volatile oils which were extracted by supercritical carbon dioxide extraction were tested for antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis,* and *Trichophyton rubrum* using broth micro dilution method. The result showed that the volatile oil of *C. aurantifolia* or Lime peel was shown strongest antimicrobial effect, against *S. aureus, S. epidermidis*, and *T. rubrum* with MIC values of 0.63, 0.63 and 0.04 mg/ml, respectively.

The volatile oil of *C. aurantifolia* or Lime exhibited the most potent inhibitory activity against NO production ($IC_{50}$ = 10.2 μg/ml), followed by the *F. japonica* or Round Kamquat ($IC_{50}$ = 12.1 μg/ml), and *C. maxima* cv. Hom-Hat-Yai ($IC_{50}$ = 20.3 μg/ml), respectively. Cytotoxic effects were not observed in this study.

From the result of DPPH radical scavenging assay, the Rutaceous volatile oils did not exhibit the antioxidant activity.

From blending oil technique, the blended oils were homogeneous but the formulae did not exhibit the antimicrobial, anti-inflammatory, and antioxidant activities. They could not against *S. aureus, S. epidermidis and T. rubrum*. As Inhibitory effect on NO production, the formulae and coconut oil could not establish anti-inflammatory activity. Since the proportion of volatile oils that blended with carrier oil was less, the volatile oils could not present the real activities in experiment. The *in vivo* study should be studied further. However, cell viability in MTT assay showed no poisoning of cells.

From DPPH radical scavenging assay result, the blended oils did exhibit the antioxidant effect when followed the protocol. To ensure the antioxidant property, the study of antioxidant activity should be determined in other techniques.

The effect of temperature and light on stability of blended oils, all tested light did not affect either the physical property of the oils, whereas the tested temperature oils did not affect either the physical properties except the condition at 60 °C which were rancid and the viscosity had changed.

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# APPENDIX

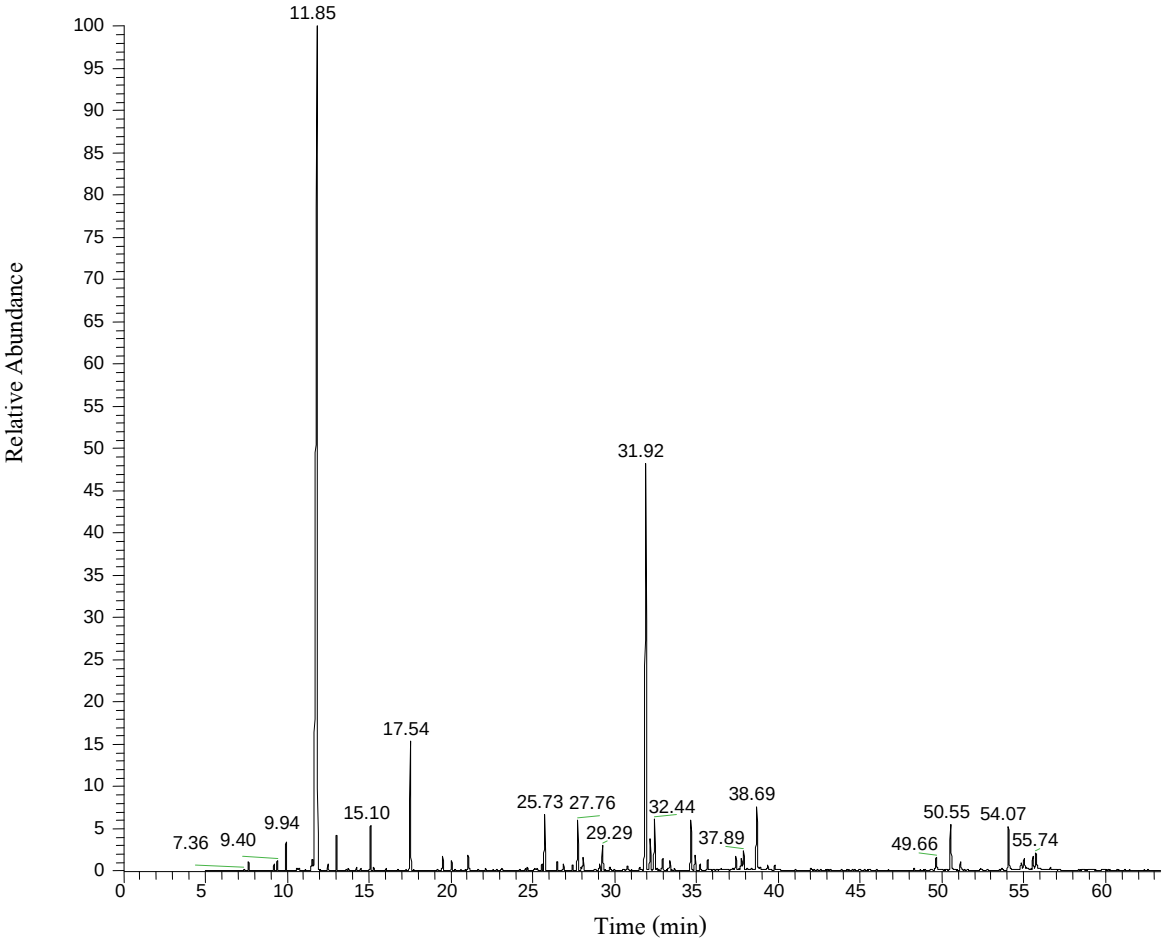


**Figure 17** GC-MS chromatogram of *F. japonica* or Round Kamquat peel oil extracted by Supercritical carbon dioxide method

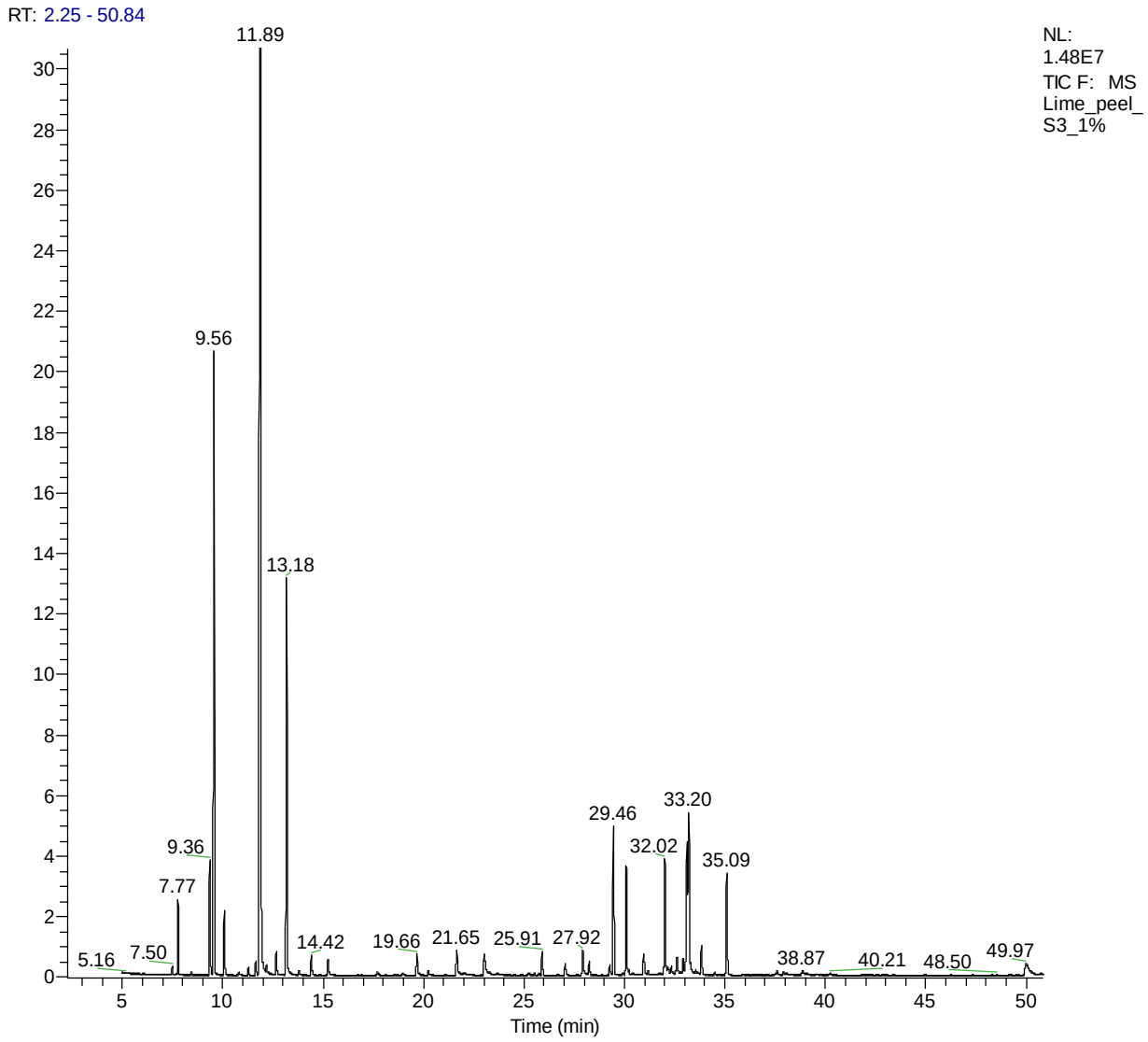


**Figure 18** GC-MS chromatogram of *C. aurantifolia* or Lime peel oil extracted by Supercritical carbon dioxide method

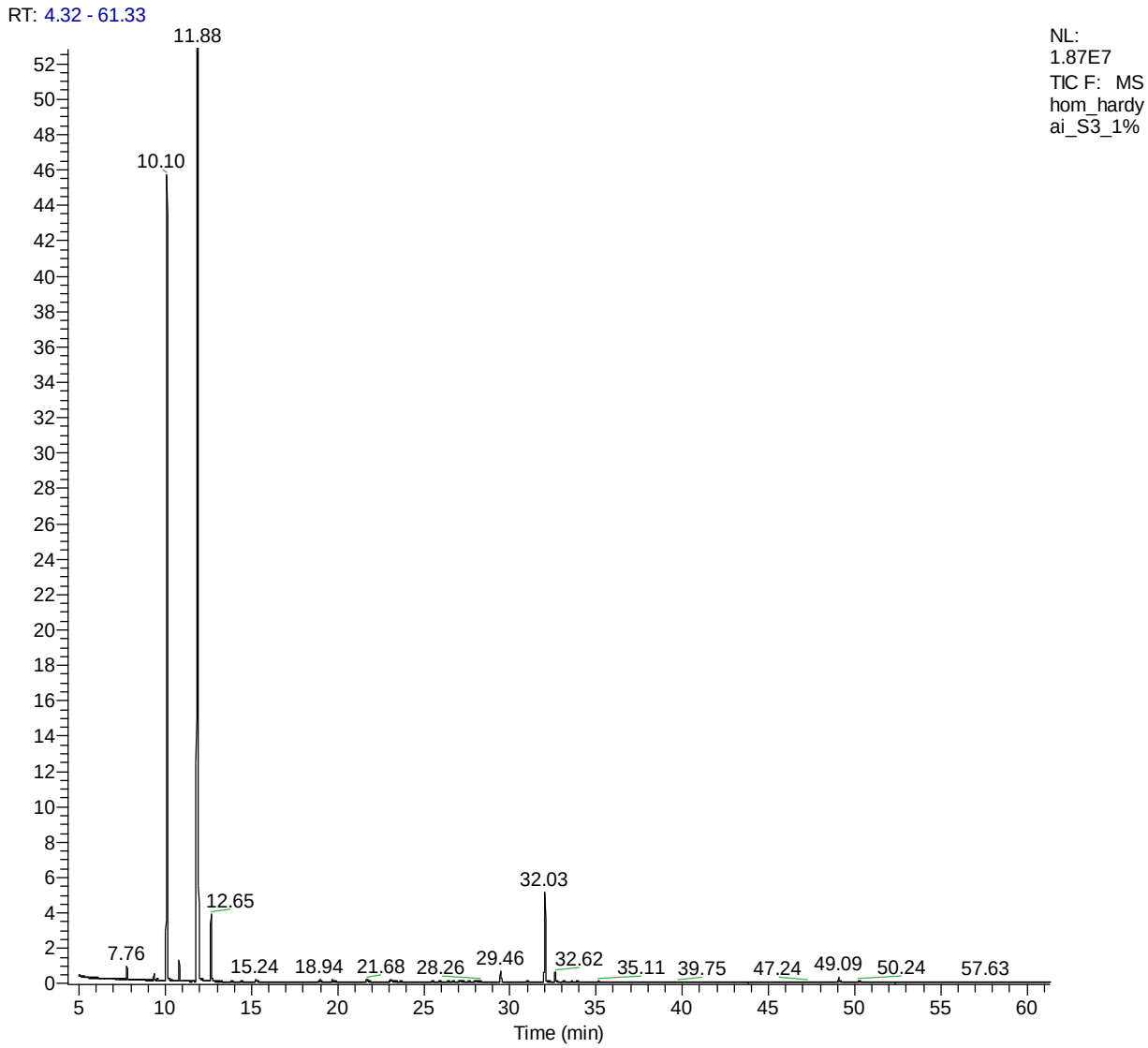


**Figure 19** GC-MS chromatogram of *C. maxima* cv. Hom-hat-yai peel oil extracted by Supercritical carbon dioxide method

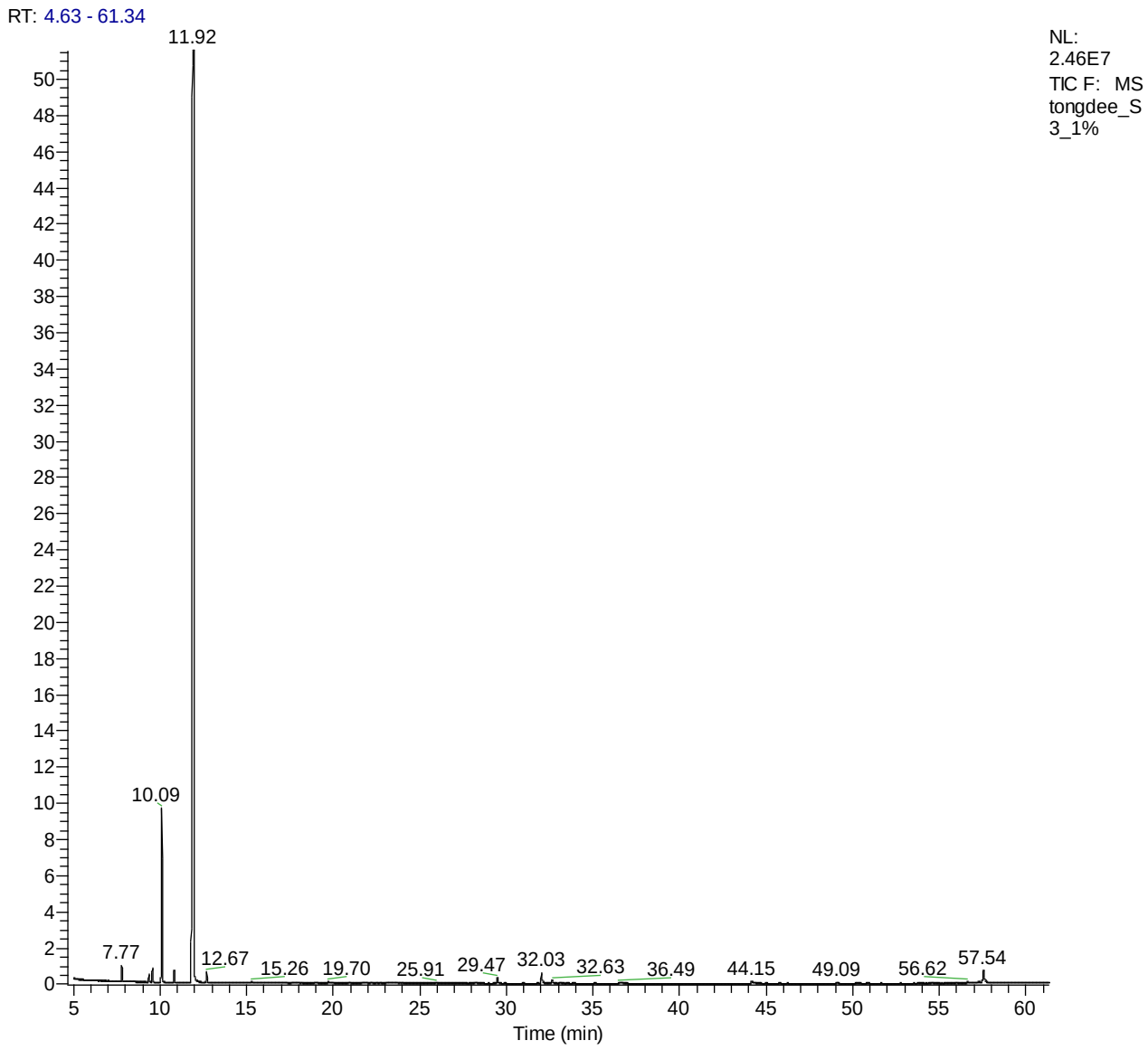


**Figure 20** GC-MS chromatogram of *C. maxima* cv. Tong-dee peel oil extracted by Supercritical carbon dioxide method

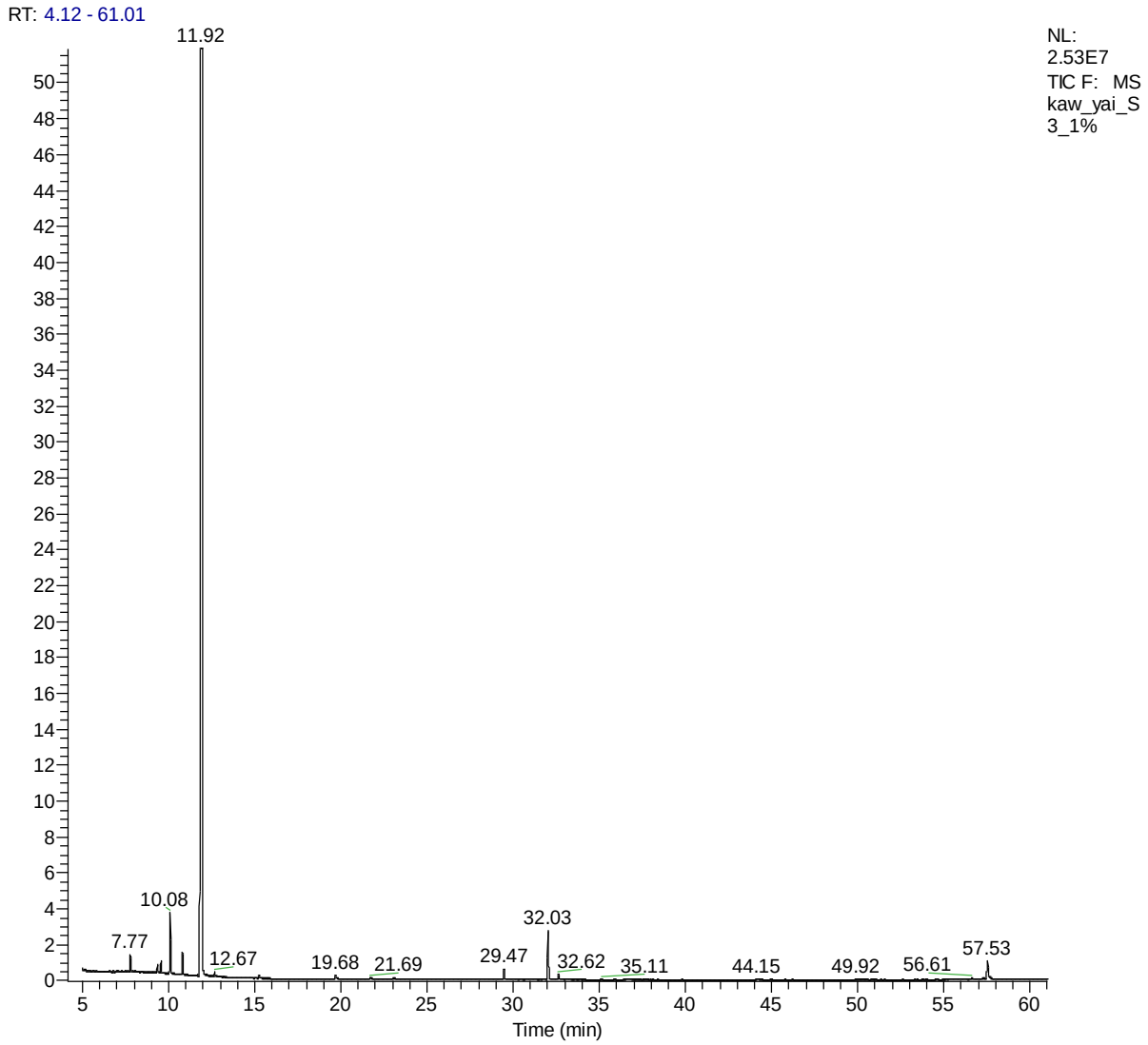


**Figure 21** GC-MS chromatogram of *C. maxima* cv. Kaw-yai peel oil extracted by Supercritical carbon dioxide method

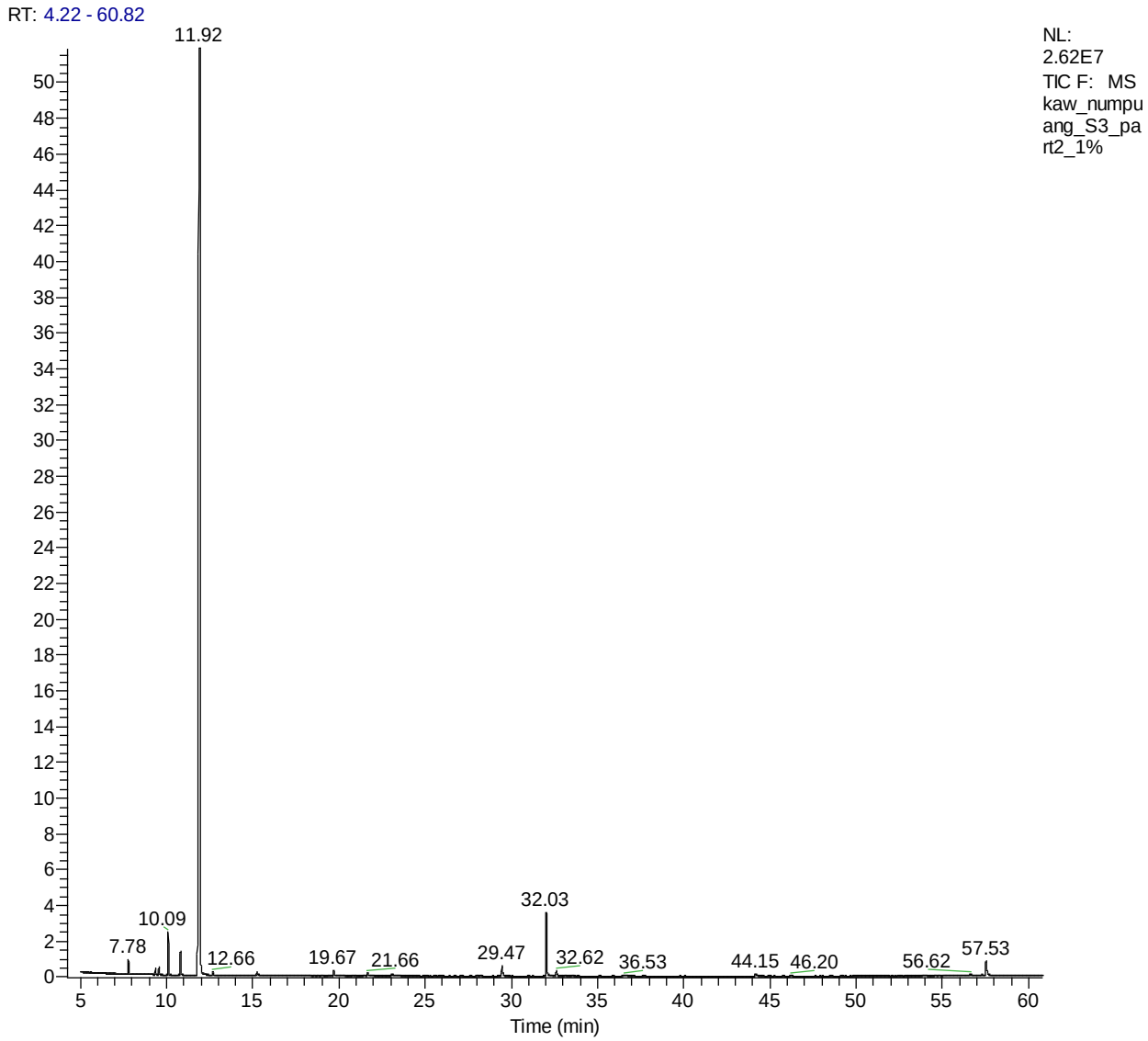


**Figure 22** GC-MS chromatogram of *C. maxima* cv. Kaw-nam-peung peel oil extracted by Supercritical carbon dioxide method

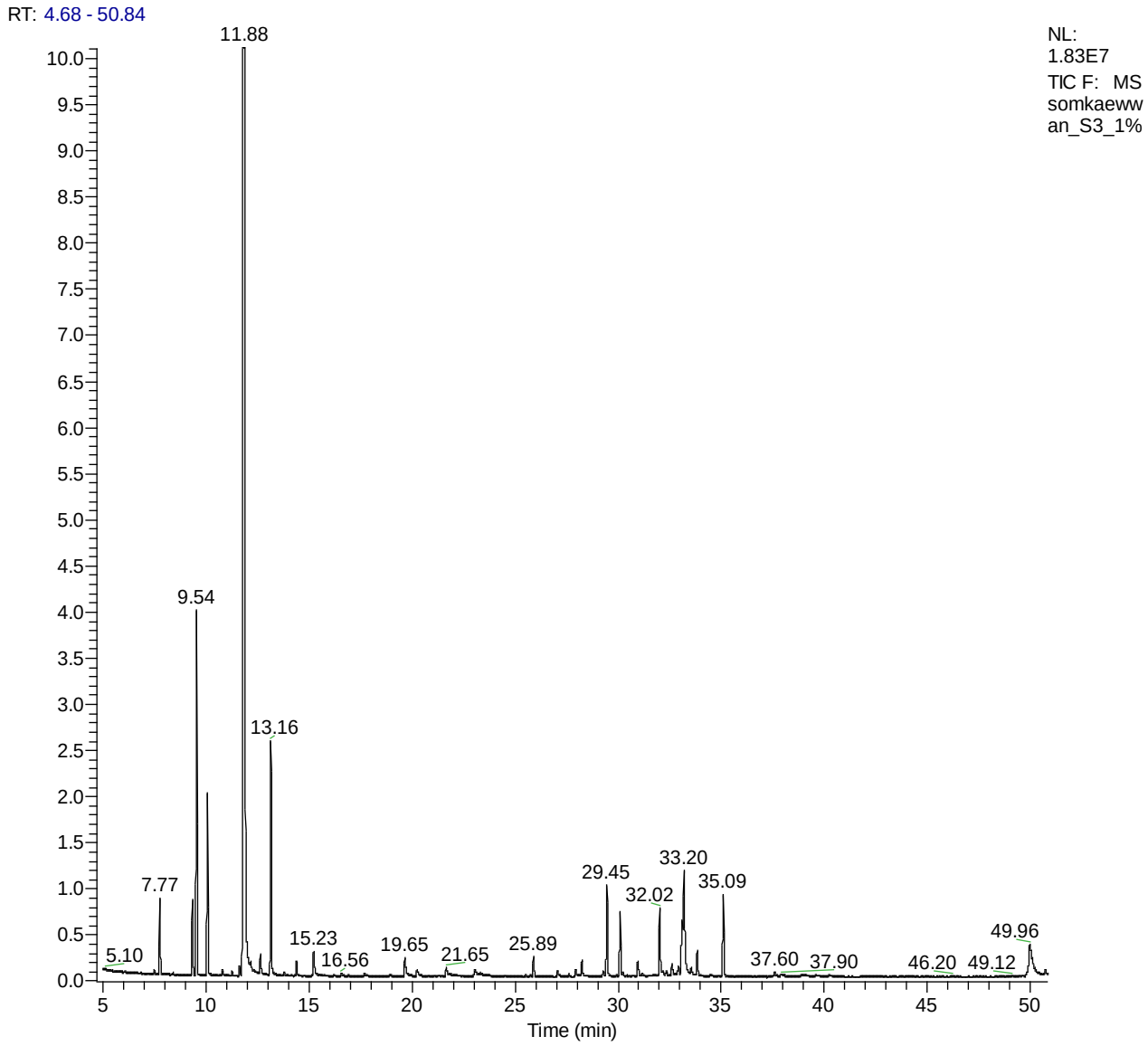


**Figure 23** GC-MS chromatogram of *C. reticulata* cv. Keaw-hwan peel oil extracted by Supercritical carbon dioxide method

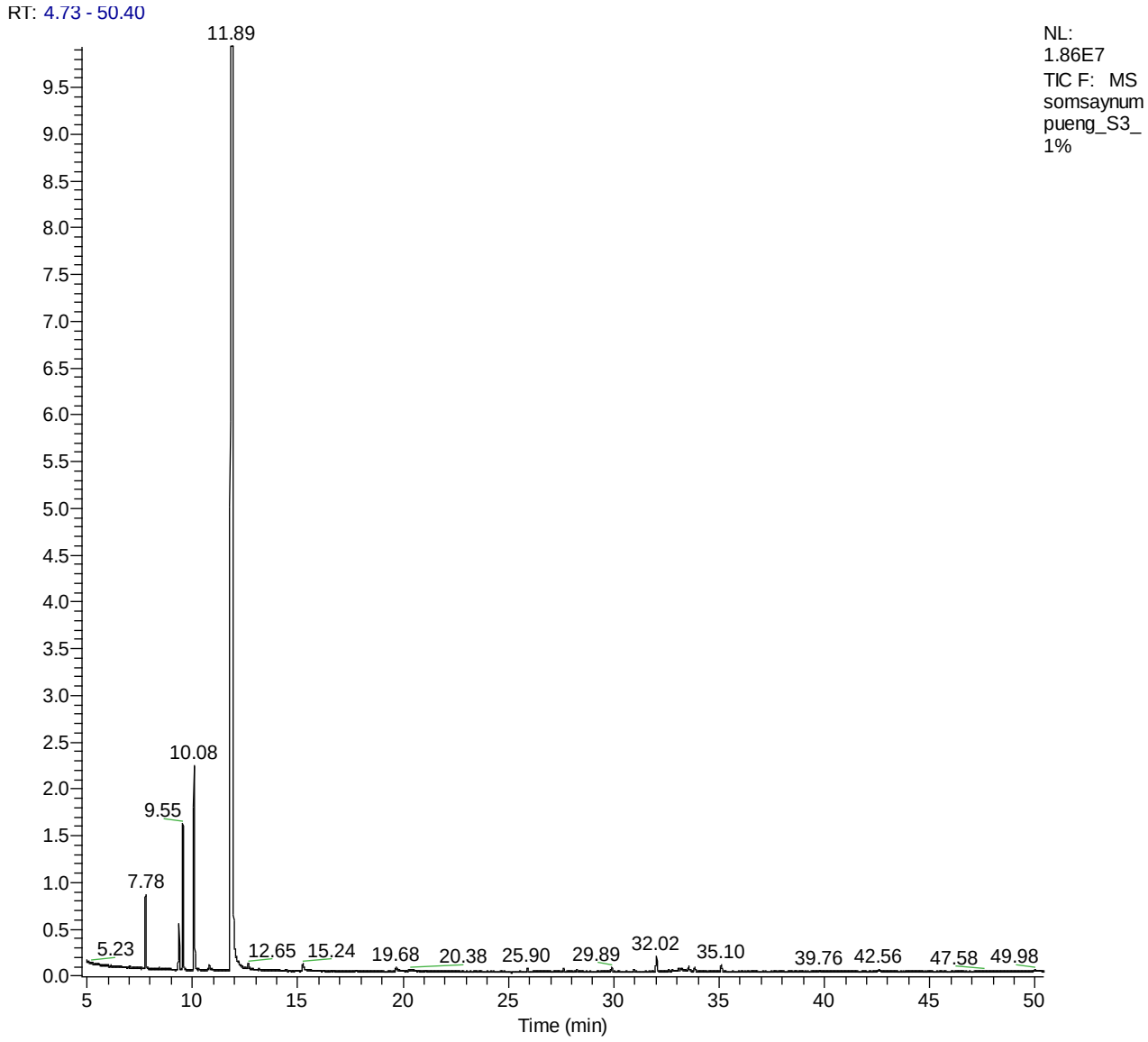


**Figure 24** GC-MS chromatogram of *C. reticulata* cv. Sai-nam-peung peel oil extracted by Supercritical carbon dioxide method

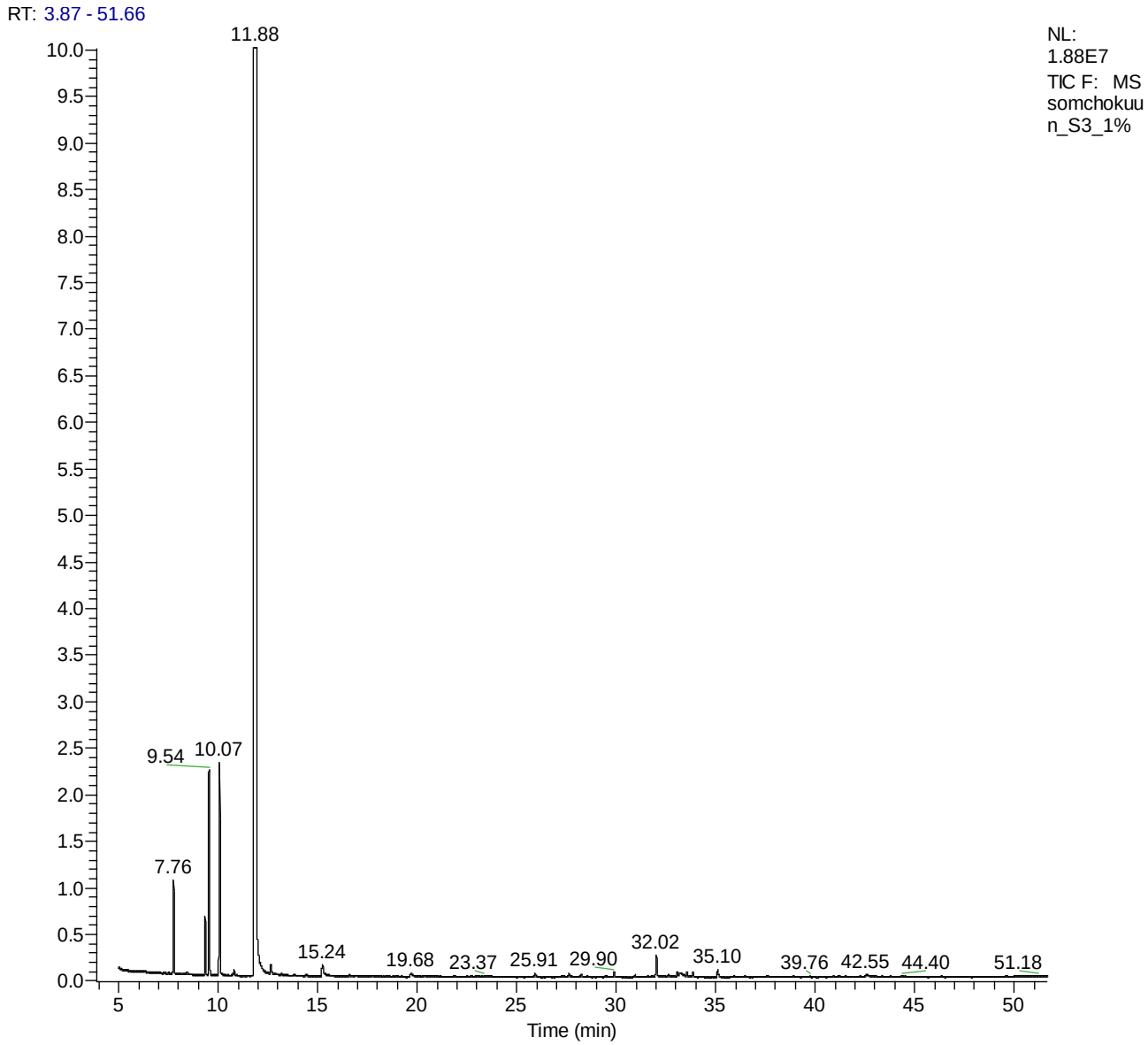


**Figure 25** GC-MS chromatogram of *C. reticulata* cv. Sho-kun peel oil extracted by Supercritical carbon dioxide method

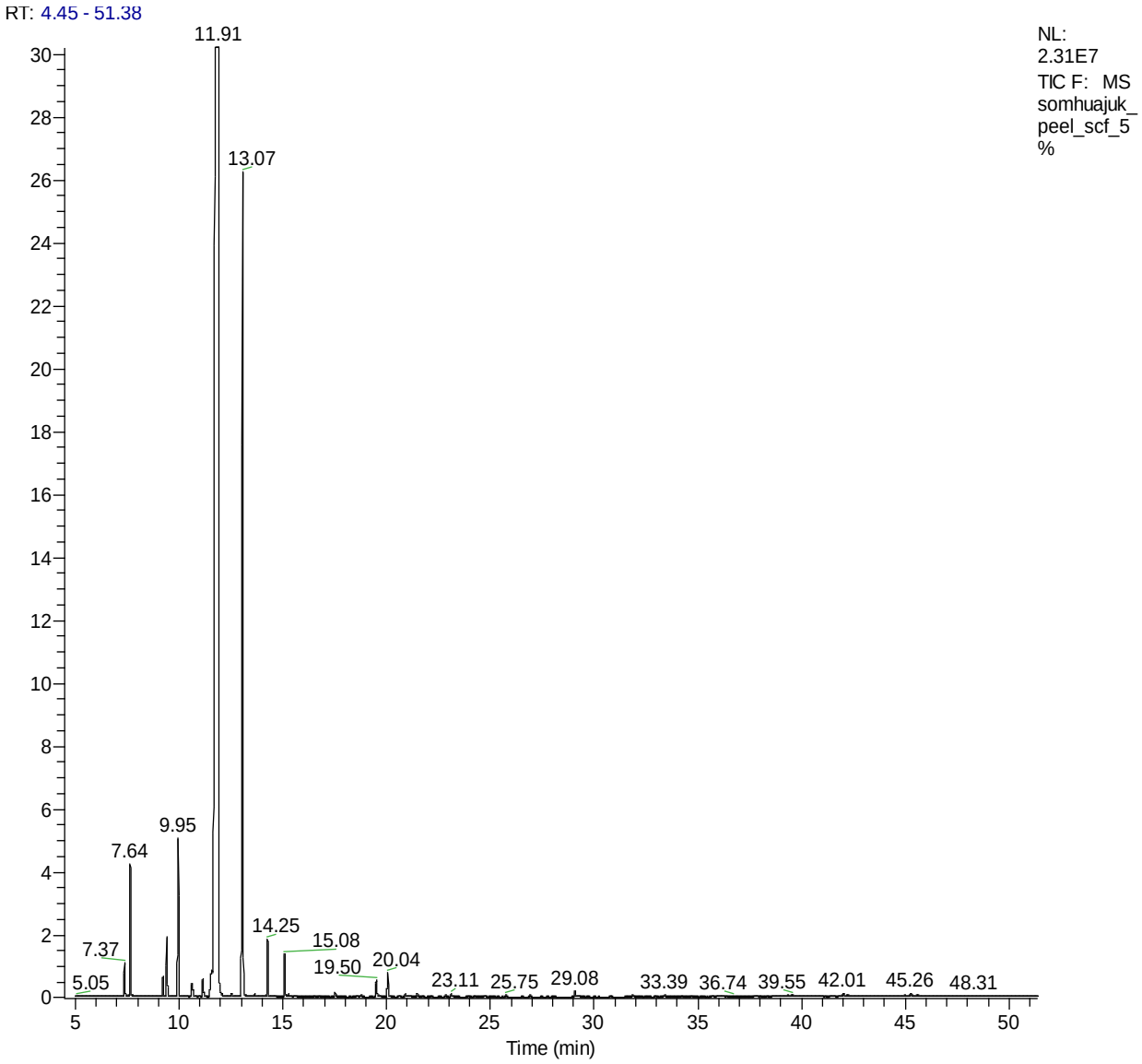


**Figure 26** GC-MS chromatogram of *C. reticulata* cv. Neck orange peel oil extracted by Supercritical carbon dioxide method

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**Proceedings**

Chumsuwan, P. and Wattanapiromsakul, C., 2009. Biological activities of *Fortunella japonica* (Thunb.) Swingle essential oil. *Proceeding of 12th National Graduate Research Conference, Khon Kaen University, Thailand*: 1361-1366.