DEVELOPMENT OF TOPICAL MOSQUITO REPELLENT EMULGELS CONTAINING *ZANTHOXYLUM LIMONELLA* ESSENTIAL OILS

MISS THI THI SAN

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pharmaceutical Technology

Department of Pharmaceutics and Industrial Pharmacy

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic Year 2012

การพัฒนาตำรับยาไล่ยุงสำหรับทาภายนอกที่มีส่วนผสมของน้ำมันจากมะแขว่น

นางสาว ทิ ทิ ซัน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

Thesis Title	DEVELOPMENT OF TOPICAL MOSQUITO REPELLENT EMULGELS CONTAINING *ZANTHOXYLUM LIMONELLA* ESSENTIAL OILS
By	Ms. THI THI SAN
Field of Study	Pharmaceutical Technology
Thesis Advisor	Assistant Professor Walaisiri Muangsiri, Ph.D.
Thesis Co-advisor	Assistant Professor Pornpen Werawatganone, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……..……..……..……..…….Dean of the Faculty of
Pharmaceutical Sciences
(Associate Professor Pintip Pongpech, Ph.D.)

THESIS COMMITTEE

……..Chairman
(Assistant Professor Nontima Vardhanabhuti, Ph.D.)

……..Thesis Advisor
(Assistant Professor Walaisiri Muangsiri, Ph.D.)

……..Thesis Co-advisor
(Assistant Professor Pornpen Werawatganone, Ph.D.)

……..Examiner
(Associate Professor Parkpoom Tengamnuay, Ph.D.)

……..External Examiner
(Associate Professor Narumon Komalamisra, Ph.D.)

ทิ ทิ ซัน : การพัฒนาตำรับยาไล่ยุงสำหรับทาภายนอกที่มีส่วนผสมของน้ำมันจากมะแขว่น เครื่องสำอาง (DEVELOPMENT OF TOPICAL MOSQUITO REPELLENT EMULGEL CONTAINING *ZANTHOXYLUM LIMONELLA* ESSENTIAL OILS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ร.ต.ท. หญิง ดร. วลัยศิริ ม่วงศิริ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.พรเพ็ญ วีระวัฒกานนท์, 104 หน้า.

โรคที่นำโดยยุงอิดีสอิยิบไต และยุงชนิดอื่นกลายเป็นปัญหาสุขภาพทั่วโลก น้ำมันหอมระเหยจากแซนโธไซลัมลิโมเนลลาที่อุดมไปด้วยลิโมนีนมีความเป็นไปได้ในการให้ผลไล่ยุง ในการศึกษานี้ ประสบความสำเร็จในการสกัดน้ำมันหอมระเหยจากผลแห้งด้วยวิธีการกลั่นด้วยไอน้ำ ได้น้ำมันหมอระเหยคิดเป็นผลผลิตร้อยละ 6 ร้อยละปริมาตรต่อมวล ผลจาก GC-MS แสดงว่าน้ำมันหอมระเหยประกอบด้วยพีคหลัก 5 พีคโดยมีลิโมนีนเป็นสารระเหยที่เป็นองค์ประกอบสำคัญ ผลจาก GC-FID แสดงว่าความเข้มข้นของลิโมนีน คิดเป็นร้อยละ 71.07 ร้อยละมวลต่อมวล น้ำมันหอมระเหยถูกนำไปบรรจุในไมโครแคปซูลด้วยวิธีอัดรีดที่ประกอบด้วยการทำให้เกิดอิมัลชันและกระบวนการทำให้เกิดร่างแห โดยใช้โซเดียมอัลจิเนตเป็นพอลิเมอร์มาทริกซ์และใช้แคลเซียมคลอไรด์เป็นสารทำให้เกิดร่างแห ไมโครแคปซูลของแคลเซียมอัลจิเนตที่บรรจุน้ำมันหอมระเหยได้จากการใช้โซเดียมอัลจิเนต (ร้อยละ 1.5) สแปน 80 (ร้อยละ 3) และน้ำมันหอมระเหย (ร้อยละ 20) ทำให้ได้ไมโครแคปซูลที่มีขนาดเส้นผ่านศูนย์กลางน้อยกว่าหรือเท่ากับ 200 ไมโครเมตร ผลผลิตร้อยละและประสิทธิภาพในก ารกักเก็บคิดเป็นร้อยละ 67.29±2.56 และ 29.96±1.65 ในหน่วยร้อยละมวลต่อมวล ตามลำดับ ไมโครแคปซูลของแคลเซียมอัลจิเนตที่บรรจุน้ำมันหอมระเหยหรือน้ำมันหอมระเหยถูกนำไปบรรจุในสูตรตำรับอิมัลเจล อิมัลเจลที่มีส่วนผสมของน้ำมันหอมระเหย น้ำมันโจโจบา ครีโมฟอร์ อาร์เอช40 และ คาร์โบพอล940 เป็นครีมสีขาวขุ่นเหมือนน้ำนม กลิ่นเหมือนกลิ่นมะนาว วานิลินเป็นสารที่ทำให้การระเหยช้าลงถูกเติมในสูตรตำรับอิมัลเจลให้มีความเข้มข้นร้อยละ 5 ของสูตรตำรับ อิมัลเจลมีความคงตัวภายหลังการปั่นเหวี่ยงด้วยความเร็ว 6000 รอบต่อนาที เป็นเวลา 30 นาทีต่อครั้ง จำนวน 8 ครั้ง โดยไม่มีการแยกชั้นของวัฏภาค ไม่พบการเปลี่ยนแปลงของสี กลิ่น ความเป็นกรด-ด่าง และความหนืดภายหลังการนำอิมัลเจลไปผ่านรอบฮีทติ้งคูลลิ่งจำนวน 6 รอบ นอกจากนี้พบว่า ตำรับอิมัลเจลยังคงคุณสมบัติการไหลแบบซูโดพลาสติกตลอดการทดสอบดังกล่าว ตำรับอิ มัลเจลที่มีความคงตัวทางกายภาพถูกนำไปทดสอบการไล่ยุงในอาสาสมัครมนุษย์ ตำรับอิมัลเจลที่มีส่วนผสมของน้ำมันหอมระเหย (ร้อยละ 18) และวานิลิน (ร้อยละ 5) แสดงระยะเวลาในการไล่ยุงลายนาน 3.1-3.4 ชั่วโมง ตำรับอิมัลเจลที่ผ่านการเก็บภายใต้สภาวะ 30 เซลเซียส ความชื้นสัมพัทธ์ร้อยละ 75 หรือที่ 40 ความชื้นสัมพัทธ์ร้อยละ 75 เป็นเวลา 12 สัปดาห์ และทำการตรวจหาปริมาณลิโมนีน และวานิลิน ในสูตรตำรับ พบว่า สูตรตำรับมีความคงตัวที่ 30 เซลเซียส ความชื้นสัมพัทธ์ร้อยละ 75 เป็นเวลา 8 สัปดาห์ ดังนั้น อิมัลเจลที่มีส่วนผสมของน้ำมันหอมระเหยจากมะแขว่นมีความเป็นไปได้ในการถูกนำไปใช้ไล่ยุงได้

ภาควิชา..วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม..... ลายมือชื่อนิสิต…….....

สาขาวิชา...เทคโนโลยีเภสัชกรรม..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก…….

ปีการศึกษา....2555..... ... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม…….....

5376852633 : MAJOR PHARMACEUTICAL TECHNOLOGY
KEYWORDS : LIMONENE / *ZANTHOXYLUM LIMONELLA* / EMULGEL / MICORCAPSULES / TOPICAL MOSQUITO REPELLENT

THI THI SAN : DEVELOPMENT OF TOPICAL MOSQUITO REPELLENT EMULGELS CONTAINING *ZANTHOXYLUM LIMONELLA* ESSENTIAL OILS. ADVISOR : ASST. PROF. WALAISIRI MUANGSIRI, Ph.D., CO-ADVISOR : ASST. PROF. PORNPEN WERAWATGANONE, Ph.D., 104 pp.

Vector-borne diseases caused by *Aedes aegypti* and other mosquitoes have become a global health problem. *Zanthoxylum limonella* essential oils, one of the rich sources of limonene, have a potential mosquito repellent effect. In this current study, the essential oils were successfully extracted from clusters of dried fruits by steam distillation method. The percentage yield of the essential oils was 6 %v/w. GC-MS results suggested that the essential oils composed mainly of five peaks where limonene was found as a major volatile constituent. GC-FID result showed that the concentration of limonene was 71.07 %w/w. The essential oils were prepared as microcapsules using the extrusion method involving emulsification and crosslinking processes where sodium alginate was employed as polymer matrix and calcium alginate as a crosslinking agent. The calcium alginate microcapsules of essential oils were obtained by employing sodium alginate (1.5%), Span 20 (3%) and essential oils (20%). The microcapsules obtained were ≤200 μm in diameter. The percentage yield and percentage entrapment efficiency of alginate microcapsules were 67.29±2.5 %w/w and 29.96±1.65 %w/w, respectively. The microcapsules of essential oils and free essential oils were separately prepared in the emulgels. The emulgels containing microcapsules of essential oils or free essential oils, jojoba oil, cremophorRH 40 and carbopol 940 were whitish milky cream with lemon-like odor. 5% vanillin was also added into the emulgels as a fixative. The emulgels were found as stable under centrifugation at 6000 rpm for 8 cycles with no phase separation. There were no changes in appearance, color, odor, pH and viscosity after emulgels were subjected to 6 cycles of heating-cooling cycle. Moreover, the emulgels showed pseudoplastic behavior throughout the heating-cooling cycle test. The physically stable emulgel preparations were subjected to mosquito repellency test on human volunteers. The emulgel preparations containing essential oils (18%) and vanillin (5%) showed 3.1-3.4 hours of protection against *Ae. aegypti*. The emulgel was stored at 30˚C, 75% RH and 40˚C, 75% RH for 12 weeks and determined the amount of limonene and vanillin. The results showed that limonene and vanillin were stable for 8 weeks at 30˚C. Therefore, emulgel containing *Zanthoxylum limonella* essential oils was a potential mosquito repellent for general public use.

Department: Pharmaceutics and Industrial Pharmacy Student's Signature……..

Field of Study:...Pharmaceutical Technology….……Advisor's Signature……..

Academic Year:...2012……....Co-advisor's Signature……

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my thesis advisor and thesis co-advisor, Assistant Professor Dr. Walaisiri Muangsiri and Assistant Professor Dr. Pornpen Werawatganone for the continuous support in my study and research, for their patience, motivation, enthusiasm and immense knowledge. Their guidance help me throughout my research and writing of this thesis. I could not have imagined having better advisor and mentors for my study.

Also, I cannot find words to express my appreciation to my thesis committee, Assistant Professor Dr. Nontima Vardhanabhuti, Associate Professor Dr. Parkpoom Tengamnuay, and Associate Professor Dr. Narumon Komalamisra for their encouragement and insightful comments on my thesis.

I would like to express a special thank to Dr. Usavadee Thavara, Dr. Apiwat Tawatsin and Dr. Jakkrawarn Chompoosri from Biology & Ecology Section, National Institute of Health, Thailand for offering me the laboratory facilities for the repellency tests.

My sincere thanks also go to all faculty staffs under the Faculty of Pharmaceutical Sciences, Chulalongkorn University and my friends and fellow labmates for supporting me from the initial stage of my studies until the very end.

Last but not least, I would like to thank my family: my parents for giving birth to me in the first place and supporting me mentally and spiritually throughout my life.

CONTENTS

CHAPTER **Page**

Chapter **Page**

Chapter **Page**

Chapter **Page**

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

%	percentage
˚C	degree Celsius
µ	micro (10^{-6})
µg	microgram
µL	microliter
µm	micrometer
et al.	*et alii*, and other
etc.	*et cetera* (and other similar things)
Ae. aegypti	*Aedes aegypti*
DENV-1	Dengue virus 1
DENV-2	Dengue virus 2
DENV-3	Dengue virus 3
DENV-4	Dengue virus 4
g	gram
GC-MS	gas chromatography mass spectrometry
GC-FID	gas chromatography with flame ionization detector
HLB	hydrophilic-lipophilic balance
IC_{50}	Inhibitory concentration 50
k	kilo (10^{3})
kg	kilogram
L	litre (s)
m	milli (10^{-3})
m	meter

mg	milligram
min	minute (s)
MIC	minimum inhibitory concentration
mL	milliliter
mm	milimetre
no.	number
o/w	oil-in-water
ppm	parts per million
pH	the negative logarithm of the hydrogen concentration
R^2	coefficient of determination
RH	relative humidity
rpm	round per minute
RT	room temperature
RRT	relative retention time
S.D.	standard deviation
span®	sorbitan monooleate
tween®	polysorbate
v/w	volume by weight
w/o	water-in-oil
w/w	weight by weight

CHAPTER I

INTRODUCTION

1 Background and significance of the study

Mosquitoes are historically responsible for the transmission of mosquito-borne diseases. These diseases are booming worldwide since the effective vaccines are not available up to now. For decades, physical techniques and chemical substances have been used in order to annihilate, prevent or protect mosquito bites including mosquito nets, insecticides or repellent, respectively (Kyle and Harris, 2008).

The use of topical mosquito repellents is fascinating for individuals to protect against contact with the disease-carrying mosquitoes. With the increase of public concerns on the safety of herbal products, many researchers highlighted the potential of many plants developed as natural ingredients in the repellents. The use of plant-based repellent becomes apparently practical, economical and eco-friendly practice in many communities (Moretti et al., 2002).

Zanthoxylum limonella Alston, a member of the Rutaceae, is commonly known as “Makhwaen” in Thailand. The ripe fruits have been widely recognized as a condiment in curries. The fruit essential oils have long been used as traditional medicine for prevention and improvement of various ailments and disorders (Itthipanichpong et al., 2002). Pharmacological activities of the essential oils include anti-inflammatory (Agshiker et al., 1972; Abraham and Agshiker, 1972), anti-hypertension (Agshiker and Abraham, 1972), smooth muscles stimulant

(Itthipanichpong et al., 2002) and antibacterial effect against cholera (Nayak and Dutta, 1961). Additionally, the fruit essential oils possess a mosquito repellent effect (Das et al., 2003; Trongtokit et al., 2004; Trongtokit et al., 2005).

Zanthoxylum limonella essential oils mainly contain monoterpenes and sesquiterpenes. These volatile components can create a vapor layer on our skin and protect us against mosquito bites (Nerio et al., 2010). Limonene, a monoterpene, is one of the major constituents in the essential oils. Limonene is a common ingredient in soaps, perfumes, and foods (Djordjevic et al., 2008). Limonene also is a registered active ingredient in pesticide products used as insecticides, dog and cat repellents, insect repellent and mosquito repellent (Karr and Coats, 1987). Limonene is also reported to show low mammalian toxicity and can be used as an alternative to synthetic mosquito repellent (Karr and Coats, 1987; Tripathi et al., 2009).

Several mosquito repellents are available on the market in different topical preparations such as sprays, creams and lotions (Moretti et al., 2002). Although essential oils from *Zanthoxylum limonella* have a potential to be used as a mosquito repellent, its poor solubility and tendency to undergo oxidation are major obstacles in product development for commercial purposes as an alternative to synthetic mosquito repellent.

Microencapsulation of essential oils by polymeric materials is expected to slow down the release and volatility of essential oils. Furthermore, microencapsulation can protect the encapsulated material from air, resulting in a longer shelf-life (Maji et al., 2007). According to the microencapsulation technique,

compounds with low water solubility and poor stability are entrapped in liquid droplets or solid particles. The droplet's wall or the particle matrix can be designed to permit controlled release of the encapsulated material under desired conditions. The encapsulated material must be released at a reasonable rate by crashing the capsules during the application (Chang and Dobashi, 2003; Lertsutthiwong et al., 2008).

In the past few decades, the development of emulgel has gained considerable attention. Emulgels are semisolid dosage forms where emulsions, oil-in-water (o/w) or water-in-oil (w/o) emulsion, mix with a gelling agent (Mohamed, 2004; Shahin et al., 2011; Khullar et al., 2012). There has been great interest in the use of novel polymers with complex functions as emulsifiers and thickeners because the gelling capacity of these compounds allow the formulation of stable emulsions and creams by decreasing surface and interfacial tensions while at the same time increasing the viscosity of the aqueous phase (Khullar et al., 2012). The oil phase of emulgels allows incorporation of a hydrophobic therapeutic moiety while the water phase in the presence of a gelling agent gives a pleasant effect following the application (Mohamed, 2004; Shahin et al., 2011; Khullar et al., 2012). The essential oils are hydrophobic compounds and hence emulgel preparation of essential oils for topical application is preferable. Emulgels have several favorable properties suitable for dermatological uses such as greaseless, stainless and an emollient which is spreadable, removable and has a pleasing appearance (Mohamed, 2004; Shahin et al., 2011; Khullar et al., 2012).

2 Objectives of the study

The overall objective was to add a commercial value to Thai herb, *Zanthoxylum limonella*. The specific objectives were as follows:

a. To extract essential oils from *Zanthoxylum limonella*

b. To prepare the emulgel or microcapsules product containing essential oils and determine their stabilities

c. To test mosquito repellency efficacy of preparations on human volunteers.

Chapter II

Literature Review

1 *Aedes aegypti* : a principal dengue vector

Dengue has become a major international public health concern because its incidence is so far increasing in urban and semi-urban areas. According to the WHO estimate, 2.5 billion people are potentially at risk to dengue with the possibility of 50 million dengue infections worldwide every year. In the last few decades, dengue haemorrhagic fever (DHF) was first recognized in the Philippines and Thailand. Both dengue and dengue haemorrhagic fever are still serious health problems in Thailand since there are no approved vaccines for the dengue viruses (Kyle and Harris, 2008).

Four serogroups of dengue viruses, DENV-1, DENV-2, DENV-3 and DENV-4, are closely related and cause very similar diseases in human. They are members of the *Flavivirus* genus, belonging to the Flaviridae family. Recovery from infection by one the of dengue serotypes provides lifelong immunity against that particular serotype. However, cross-immunity to the other serotypes after recovery is considered as only partial and temporary. Subsequent infections by other serotypes increase the risk of developing severe dengue (Kyle and Harris, 2008).

The main vector of dengue viruses is *Aedes aegypti*, a member of genus *Aedes*, subgenus *Stegomya*, family Culicidae and order Diptera. The *Ae. aegypti* mosquitoes lives in urban habitats and breed mostly in man-made containers. *Ae.*

aegypti is known as a daytime feeder and its peak biting periods are early in the morning and in the evening before dusk.

Uninfected female *Ae. aegypti* mosquitoes generally get the viruses while feeding on the blood of an infected person. After 8-10 days of incubation period, the infected mosquitoes are capable of transmitting the viruses for the rest of its lives, approximately 6-8 weeks. The viruses survive in the peripheral blood of infected humans for 2-7 days, prior to showing symptoms of dengue (Beasley et al., 2008).

The life cycle of *Ae. aegypti* involves a complete metamorphosis with an egg, larvae (first, second, third and fourth instars), pupae and adult stage, as shown in **Figure 1**. Larval and pupae are aquatic forms while eggs and adults are terrestrial forms. Adults *Ae. aegypti* have distinct features, the bodies are darker with silvery-white scales presented in the clipeo and in the basis of their anthems. In addition, the dorsal thorax possesses two external sinuous lines and two internal lines with silvery scales which are similar to the musical instrument lyre (Beasley et al., 2008).

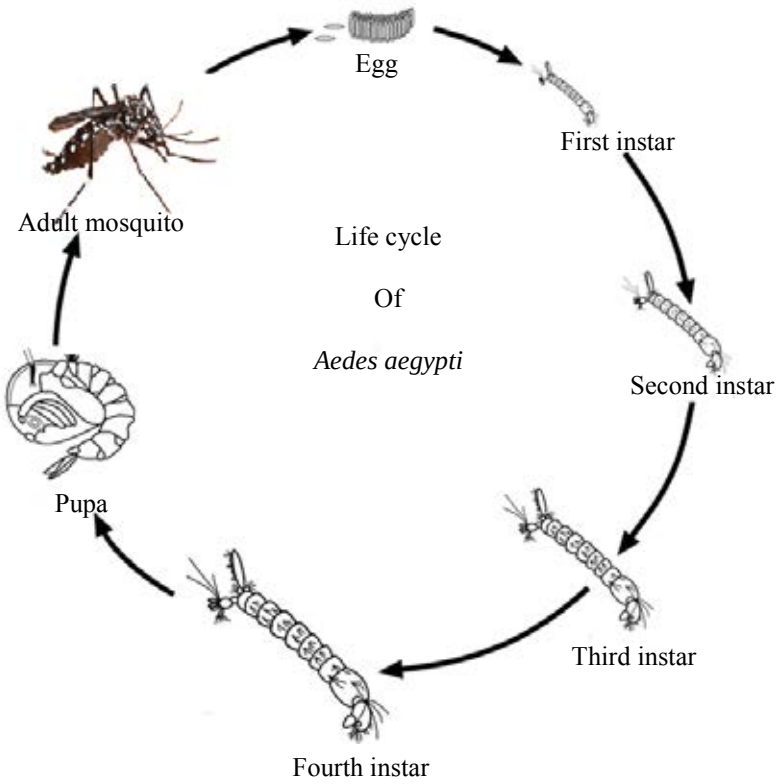


Figure 1 Life cycle of *Aedes aegypti*

The females *Ae. aegypti* obtain nutrients for egg maturation from blood. After 2 days of blood meal, the females lay eggs on damp surfaces. After 2-7 days, these developed eggs arise to the fourth sequencing larval instars that take approximately 8 days. Then larval enters the pupal stage and after 2-3 days. They transform into adult mosquitoes (male 27.14 days and females 42.25 days) for starting a new life cycle (Beasley et al., 2008).

2 Prevention and control

The methods of controlling and preventing the widespread of dengue disease are implemented by community-base management. It includes disposing solid waste properly, filling or drainage of breeding places and covering water containers in order to prevent access by egg-laying females *Ae. aegypti*.

Possible methods for controlling the adult mosquito population include larvicide application, perifocal treatment and space spraying. Larvicides such as organophosphorus, temephos, methoprene and pyriproxyfen (the insect growth regulators), and *Bacillus thuringiensisisraelensis* [H-14] (BTI) are commonly applied for controlling mosquitoes (**Figure 2**). The most commonly used larvicides, temephos and BTI possess extremely low toxicity to mammalian. Perifocal treatment involves the use of hand or power sprayers to apply insecticides such as pyrethrins I and II, permethrin and cypermethrin (Maheswaran and Savarimuthu 2012).

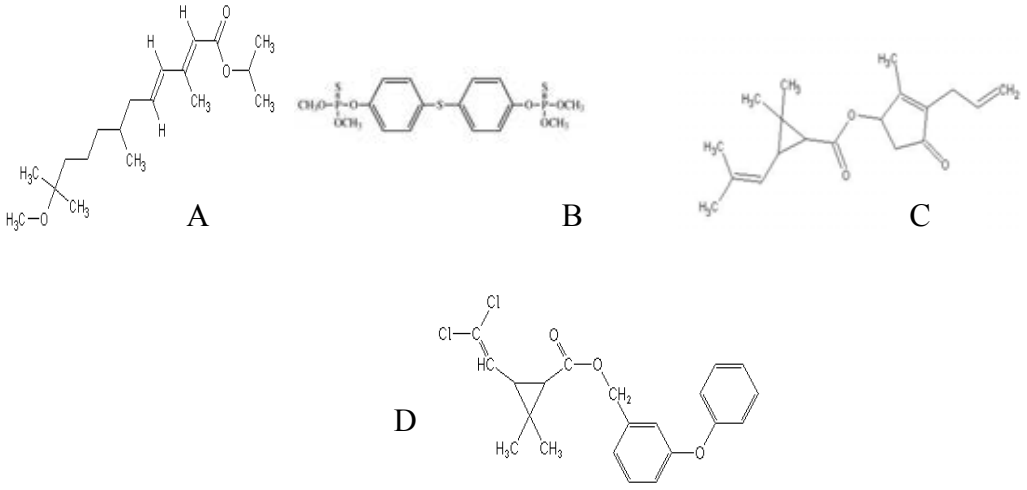


Figure 2 Chemical structures of larvicides such as Methoprene (**A**), Temphos (**B**) and insecticides such as Pyrethrin (**C**), Permethrin (**D**)

The most effective mean of prevention is personal protection against contact with the disease-carrying mosquitoes. Mosquito repellents are substances that act locally or at a distance usually by providing a vapor barrier preventing the mosquito from either landing on the skin or biting (Nerio et al., 2010). Mosquito repellents are useful as a practical and economical means of preventing mosquito-

borne diseases. They are essential not only for local people in disease risk areas but also for tourists and travelers who might be affected by mosquito-borne diseases (Naucke et al., 2007).

2.1. DEET and IR 3535

DEET (N,N – diethyl-meta-toluamide), icaridin or picaridin and IR3535 (ethyl butylactylaminopropionate) are widely used as mosquito repellents (**Figure 3**). Behavioral studies of *Ae. aegypti* with DEET showed that the mosquitoes were able to detect the DEET molecule by lactic acid sensitive neurons in antennae. DEET was considered to have an effect on lactic acid sensitive receptors by inhibiting the lactic acid excited receptor and by stimulating the lactic acid inhibited receptor (Davis and Sokolove, 1976). Lactic acid is a major compound of human sweat, a concentration range from 1 to 5 mg/mL, a major attractant to many kinds of female mosquitoes. However, mosquitoes are not only attracted to lactic acid but also to other unidentified human odor components in a synergistic manner and therefore, further studies are necessary for the better understanding on repellent activity (Song et al., 2013).

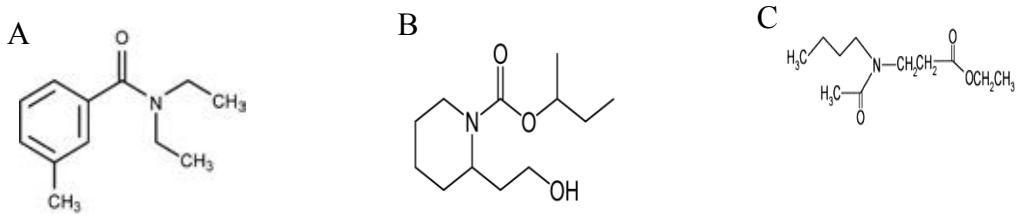


Figure 3 Chemical structures of mosquito repellents such as DEET (**A**), picaridin (**B**) and IR3535 (**C**)

The synthetic mosquito repellents are considered to be safe but unfortunately DEET may cause environmental and human health risks. There were few reports of mild to severe effects such as contact urticaria, skin eruption and systemic toxicity following DEET dermal application. Thus, topical use of DEET on children may cause encephalopathy. IR3535 is regarded as a high margin of safety to humans including children and lack of toxic effect. It is derived from a naturally occurring amino acid β-alanine. Although skin application of IR3535 was less persistent than DEET, IR3535 received approval as repellent for personal use by WHO (Debboun et al., 2006).

2.2. Essential oils

Natural repellents based on essential oils are known as effective and ecofriendly repellents. These compounds have been considered to be good alternatives to conventional synthetic mosquito repellents because of their low toxicities to mammals and other non-target organisms. They are reported to possess a range of effects from lethal toxicity to repellence and oviposition deterrence in mosquitoes. They are volatile, natural, complex compounds formed by aromatic plants as secondary metabolites (Maheswaran and Savarimuthu 2012).

Essential oils are complex mixtures of volatile compounds. Monoterpenes are major constituents (up to 90%) in the essential oils and have a great variety of structures with diverse functions. The monoterpenes used in traditional and

commercial mosquito repellents are listed in **Table 1** and their structures are shown in **Figure 4** (Debboun et al., 2006)**.**

Table 1 Monoterpenes with repellency effect on *Ae. aegypti* (Debboun et al., 2006)

Classification	**Compound**	**Duration of Protection (hour)**
Terpene (Hydrocarbon)	Terpenene	0
Aliphatic aldehyde	Citronellal	< 1
Terpene (Hydrocarbon)	Limonene	≤ 1
Terpene (Hydrocarbon)	Myrcene	≤ 1
Terpene (Hydrocarbon)	α-pinene	≤ 1
Acyclic alcohol	Citronellol	1-2
Aromatic phenol	Eugenol	1-2
Acyclic alcohol	Linalool	1-2
Monocyclic alcohol	β-terpeneol	1-2
Acyclic alcohol	Geraniol	2-3
Aliphatic aldehyde	Citral	2-3

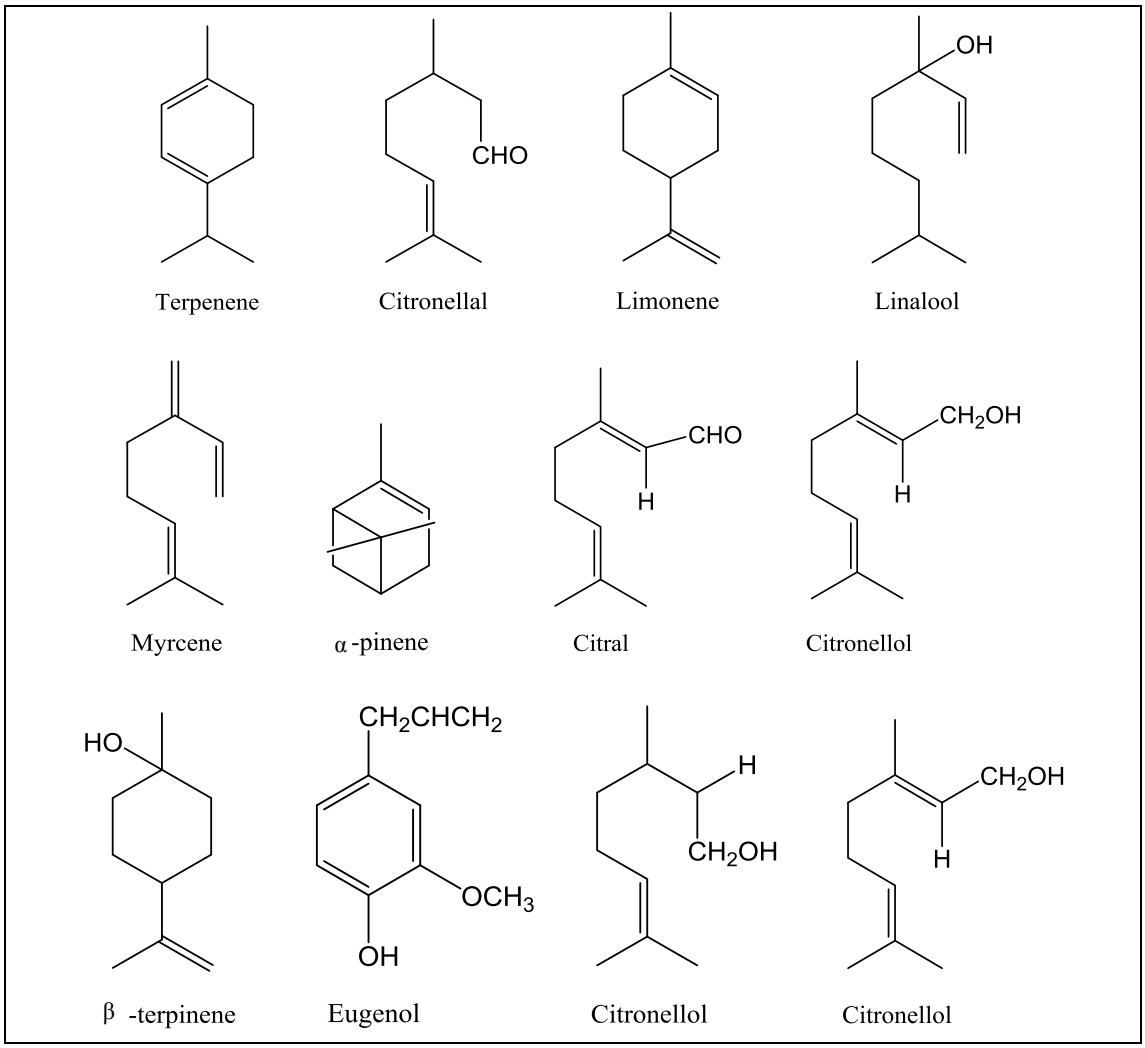


Figure 4 The structure of monoterpenes possessing the repellency effect on *Ae. Aegypti* (Debboun et al., 2006).

Early studies on structure activity relationship between functional groups and repellency noted that monoterpenes containing alcohols, ketones, aldehydes and hydroxyl esters were active whereas parent hydrocarbons and phenols were poor repellents (Bunker and Hirschfelder, 1925). By comparing alcohol (C-O-H) groups with ester ($C=O_2$), phenol (C-O-C), ketone and aldehyde (=C=O), the repellent activity was related to the oxygen moiety (Christophers, 1947). In addition, unsaturated alcohols were better repellents compared to saturated ones and conversion of alcohol to the corresponding ester showed superior repellent

activity (Moore, 1934). The active chemicals, such as 35.6% nitrogen compounds, 16.9% alcohols, 16.9% esters and lactones, and 10% ethers and acetals could provide more than 5 hours of protection against *Ae. aegyti* (Paluch et al., 2010).

Although repellent activity of essential oils is generally attributed to some particular compounds, minor constituents found in low percentages may show synergistic effects on enhancing the effectiveness of the major constituents through a variety of mechanisms. A synergistic phenomenon among these metabolites may result in a higher bioactivity of essential oils compared to the pure compound (Tawatsin et al., 2001; Tawatsin et al., 2006).

3 *Zanthoxylum limonella*

Zanthoxylum limonella Alston, belonging to the family of Rutaceae, is commonly known as prickly ash. *Zanthoxylum budrunga* and *Zanthoxylum rhetsa* are the synonyms of this species (Rahman et al., 2005). It is locally grown in northern part of Thailand and is named Makhwaen.

Many parts of prickly ash including stem bark, leaves, fruits and seeds are used in Thai traditional medicines. The bark is noted for its febrifugal, sudorific and diuretic properties. The chloroform and methanol extracts from the stem bark and leave shows highly potent antibacterial and antifungal activity. The most significant cytotoxic activity is found from the methanol extract of the stem bark at the concentration of 21.12 ppm (Islam et al., 2001). In addition, the methanol extract of *Zanthoxylum limonella* stem bark orally administered to mice, at dose 250 and 500 mg/kg, significantly reduced the abdominal contraction induced by

acetic acid and the diarrheal episodes induced by castor oil in mice (Rahman et al., 2002).

The ripe fruits are used as condiment in curries. The picture of dried *Zanthoxylum limonella* fruits is shown in **Figure 5**. The chloroform extract of dried fruits significantly inhibits seed germination and seedling growth in plants. Moreover, the crude extract shows antiplasmodial activity against *Plasmodium falciparum* with EC_{50} value at 3.3 µg/mL and antituberculous activity against *Mycobacterium tuberculosis* H37 Ra with MIC value at 200 µg/mL (Charoenying et al., 2008).

Figure 5 Picture of dried fruits of *Zanthoxylum limonella*

The essential oil extracted from the fruits is commonly known as mullilam oil. The pharmacological activities of these fruit essential oils include anti-inflammatory (Agshiker et al., 1972; Abraham and Agshiker, 1972), anti-

hypertension (Agshiker and Abraham, 1972), antibacterial effect against cholera (Nayak and Dutta, 1961) and antioxidant properties (Tangjitjaroenkun et al., 2012). In addition, it initiates smooth muscle contraction by a non-specific mechanism and also stimulates muscle movement in the gastrointestinal system (Itthipanichpong et al., 2002).

Additionally, the fruit essential oils are used as a natural, ecofriendly and biodegradable repellent against many species of mosquitoes (Das et al., 2003; Trongtokit et al., 2004). They are effective lavicides against *Ae. aegypti* and *Anopheles dirus* at the LC_{50} values of 24.61 ppm and 57.22 ppm (Pitasawat et al., 2007).

Prickly ash is also known as the economic plant. Dried fruits can be obtained abundantly throughout the year at a reasonable price. The essential oils can be extracted by traditional distillation methods and they are regarded as higher yielding in a range of 5.72 – 12.5% (Itthipanichpomg et al., 2002; Choochote et al., 2005; Tangjitjaroenkun et al., 2012). In total, at least 33 aromatic compounds; 13 monoterpenes, 15 oxygenated monoterpenes, 2 sesquiterpenes and 3 non-terpenoid compounds were analyzed in fruit essential oils. Among them, d-limonene (31-39%), terpin-4-ol (5-14%) and sabinene (9-43%) are identified as major components. The distinct flavor of the essential oils mainly comes from d-limonene which is concentrated in the fruit shell (Itthipanichpomg et al., 2002; Tangjitjaroenkun et al., 2012).

3.1. d-limonene

d-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a major constituent of citrus fruits. d-limonene gives out a lemon-orange fragrance while l-limonene has a piney, turpentine-like odor. It has been generally regarded as safe and is used in food manufacturing and some medicines for many years. It is commonly used in cosmetics, botanical insecticides and insect repellent against German cockroaches and house flies (Karr and Coats, 1987; Tripathi et al., 2009; Djordjevic et al., 2008). d-limonene is also proven to be toxic to all life stages of the cat flea, *Ctenocephalides felis* (Hink and Fee 1986). LC_{50} values of d-limonene against early 4^{th} instar larvae of the mosquito, *Culex quinquefasciatus,* after 24 and 48 hours of exposure are 53.80 ppm and 32.52 ppm, respectively. Limonene treated water is unfavorable for oviposition by female mosquitoes (Karr and Coats, 1987).

d-limonene is a relatively stable compound and can be distilled without decomposition. The boiling point of d-limonene is 176˚C and is stable in acidic conditions but degradation of limonene is greater and faster at the neutral or basic conditions. d-limonene mainly undergoes auto-oxidation that results in the formation of limonene hydroperoxide, carvone and limonene oxide (Djordjevic et al., 2008). Antioxidant such as 2.6-di-*tert*-butyl-4-methylphenol (BHT), are often added to d-limonene to prevent the rapid oxidation reaction. BHT can delay auto-oxidation for a time, but the stability relies on the purity of d-limonene and also on the storage temperature (Nilsson et al., 1996).

One of the important values of used d-limonene as a topical mosquito repellent is its safety profile. The pure limonene is not allergenic but 1-3% of limonene hydroperoxides can cause slight irritation (**Figure 6**). In human volunteers, the skin irritation test of (5-10%) non-oxidized limonene does not show any irritation effect but show very low irritation (no reaction – marginal reaction by visual reading score) at the concentration of 20% (Christensson et al., 2009). It is interesting to note that d-limonene evidently shows less mammalian toxicity than some other essential oil compounds. Lethal toxicity value (LD_{50}) of d-limonene testing on rats is as >4600 mg/kg (Tripathi et al., 2009).

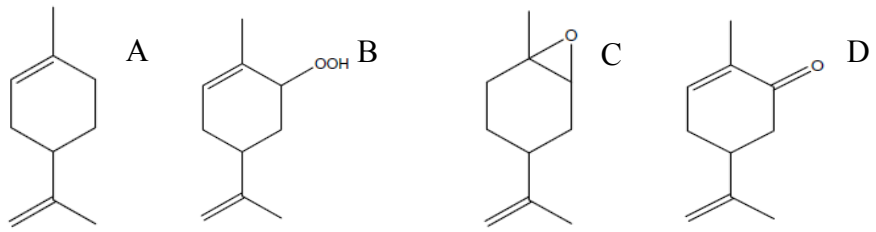


Figure 6 Structures of limonene (**A**), limonene hydroperoxide (**B**), limonene oxide (**C**) and carvone (**D**)

4 Mosquito repellent preparations containing essential oils

Repellent products containing essential oils are commercially available as sprays, lotions, sticks, gels, soaps and impregnated towelettes. The topical mosquito repellent products are over-the-counter products and the formulation has been focused on ease of application and cosmetic acceptance. They are needed to be safe, pleasant and easy to wear off. Moreover, they should be capable of extended protection against mosquito by using simple and cost effective techniques (Debboun et al., 2006).

The repellency of many plant essential oils, mainly composed of α-pinene, β-pinene, d-limonene, cymene, borneol, linalool, eucalyptol, eugenol, citronellol, citronellal and caryophyllene, are screened against *Ae. aegypti*. Citronella oils (25%) or hairy basil oils (25%) provide 3 hours repellency while turmeric oils or kaffir lime oils give only 1 hour repellent time. An addition of 5% vanillin markedly extends the repellency of these oils. However, 5% of vanillin in ethanol solution does not show any repellent effect (Tawatsin et al., 2006). When 10% of essential oils obtained from various plant species are prepared in the presence of ethanol, vanillin, propylene glycol and polyethylene glycol, the repellent times are seen in a range of 1.7-2.8 hours as shown in **Table 2**. The complex formulation of 20% essential oils obtained from *Litsea cubeba, Melalecua quinqenervia* and *Nepeta cataria* with genapol, polyethylene glycol, ethanol and vanillin shows as 8 hours repellency similar to 20% DEET (Amer et al., 2006). The fixative materials such as vanillin, polyethylene glycol, salicylic acid, mustard and coconut oils can improve the repellency of the products (Nerio et al., 2010). It has been reported that the duration of repellency of essential oils is also depended on the concentration of the oils. The higher concentration of oils is thought to extend the protection hour. Moreover, a combination of two or more essential oils provides longer repellency hour than using single oils (Trongtokit et al., 2004).

Table 2 Repellency time of various plant essential oils against *Ae. aegypti* (Amer et al., 2006)

Plant species	Repellency time (hours)
Psidium guajava	2.8
Curcuma longa	2.3
Piper nigrum	2.3
Schefflera leucantha	1.9
Vitex trifolia	1.8
Litsea cubeba	1.7
Zingiber officinale	1.7

Commercial repellent products need to cling on the skin for as long as possible. The essential oils are highly volatile and easily washed out by sweat and abrasion. It could be improved through the development of formulations that would be able to keep the active ingredients onto the skin for longer period (Debboun et al., 2006).

4.1 Microencapsulation of essential oils

Microencapsulation is a process of confining active compounds within a microsphere to achieve desirable properties. Essential oils are encapsulated for various reasons such as conversion of liquid to solid form to facilitate handling, taste or smell masking, protection from evaporation or oxidation, and reduction of skin absorption rate. The capsulated materials should also be released at a reasonable rate by crushing the microcapsules (Chang and Dobashin 2003).

Microencapsulation of essential oils by extrusion method involving emulsification followed by cross-linking process is simple and does not require an elevated temperature. The preparation of microcapsules containing essential oils by emulsification is favorable because emulsifier can be lower the surface tension in coating matrix resulting in spherical microsphere with inner oil core (Yoo et al., 2006).

Sodium alginate can be used as a biopolymer to prepare microcapsules containing essential oils owed to its good biocompatibility, biodegradability, non-toxicity and inexpensive. The extrusion of water soluble sodium alginate into calcium chloride solution results in formation of insoluble calcium alginate microcapsules. Alginate gel structure is relatively stable at acidic pH but it is easily enlarged and disintegrated under mild alkali conditions (Liu et al., 2004). Alginate has been widely studied for particle formation in the size range of 100 nm of 2 mm for pharmaceutical and cosmetic preparations (Lertsutthiwong et al., 2008; Chuah et al., 2009).

4.2 Emulgel preparation containing essential oils

Gel dosage forms are highly patient acceptance possessing since they possess several favorable properties such as being emollient, non-greasy, non-staining, easily spreadable and easily washable. However, gel preparations are unsuitable for hydrophobic compounds. Essential oils are difficult in incorporation directly into gel base. However, essential oils can be easily prepared into emulsion preparations. Emulsion preparations possess a certain degree of elegance and are

easily washed off but emulsions are not stable (Mohamed 2004; Shahin et al., 2011).

Recent development of emulgel dosage forms is of a particular interest for preparation of repellent products containing essential oils. Emulgels possess favorable properties of both gel and emulsion. Emulgels are semisolid preparations in which essential oils containing emulsions are stabilized with gel phase. Gel phase increases the viscosity of the system and provides macromolecular network while having comparatively better loading capacity. Emulgels are simple, cost effective and feasible in production (Mohamed 2004; Shahin et al., 2011).

4 Mosquito repellent test

Recently, there have been many reports concerned with the repellent properties of essential oils. Most of the results came from artificial (in vitro) testing methods using cloth, filter paper, animal membrane or olfactometry. The results from different methods cannot be compared directly because the results are strongly related to the laboratory conditions. The evaluation of repellency is preferably performed on human subjects, because laboratory animals may not accurately mimic the conditions of human skin to which repellents will eventually be applied (Debboun et al., 2006).

In Human-bait technique, laboratory-reared mosquitoes are used to eliminate the risk of pathogen transmission. Volunteers showing history of irritation to mosquito bites are excluded. Skin-mediated effects such as absorption and

penetration of repellent to the skin and physical factors such as evaporation, abrasion, washing of treated surfaces and perspiration can result in the loss of the repellent. The random selection of volunteers, the appropriate use of sample sizes and proper replication of treatments can minimize the experimental error of repellent tests. The use of negative and positive controls favors an objective outcome in repellent tests. A negative control is designed by observation of mosquito biting rate on the untreated forearm. The biting rate on one forearm of a volunteer treated with standard repellent (a positive control) is compared with the biting rate on the remaining forearm treated with a tested sample (Debboun et al., 2006).

It has been reported that the duration of repellency of essential oils is also dependent on many factors. Environmental factors affecting the protection time are light, temperature, wind speed and humidity (Debboun et al., 2006). These factors can be manipulated to desired levels in the laboratory tests. Exposure time in mosquito cage should be 3 minutes because a shorter exposure time may lead to a higher repellency time (Tawatsin et al., 2006). The effect of density of mosquito population on the protection hours depends on the species of mosquito. For example, *Ae. aegypti* mosquito density does not affect the repellent protection time. The level of the mosquitoes' hunger significantly affects the protection time. The behavioral factors such as timing and intensity of mosquito biting activity should be considered as important factors. *Ae. aegypti* is known as a daytime feeder and the timing of the test for this species should be during the daytime from 9:00 – 17:00. In addition, *Ae. aegypti* is said to have a more aggressive biting

behavior than other species. The repellency of essential oils is usually less sensitive to *Ae. aegypti* comparing to *Ae. albopictus* and the other species (Debboun et al., 2006).

CHAPTER III

MATERIALS AND METHODS

1 Materials

1.1 Raw material

1. *Zanthoxylum limonella* dried fruits clusters

1.2 Equipment and instruments

1 Filtering paper (Whatman no.5) (Whatman International Ltd., UK)

2 GC-FID (SHIMAZU GC/2010-model, Japan)with a Flame Ionization Dectector

3 GC-MS (Shimadzu LC-10, Japan)

4 HP-5MS (5% diphenyl dimethyl siloxane, 30 m x 0.25 mm id, film thickness 0.25μm)

5 RTX-5MS (5% diphenyl dimethyl siloxane, 30 m x 0.25 mm id, film thickness 0.25μm)

6 Vortex mixer (Scientific industries, USA)

7 De-ionized water (DI water) system (ELGAStat Option 3B) (ELGA, UK)

8 Disposable syringe 1 mL without needle (NIPRO, USA)

9 Disposable syringe 5 mL with needle (NIPRO, USA)

10 Centrifuge (CENTRIFUGETTE 4206)

11 Balance (AX/MX/UMX, METTLER TOLEDO, Switzerland)

12 Cooler (HETOFRIG, UK)

13 pH meter (PB20, SARTORIUS, USA)

14 High speed refrigerated micro centrifuge (MX305, TOMY, Japan)

15 Centrifuge (CENTRIFUGETTE 4206)

16 Sonicator (Elma, Germany)

17 Water bath (Becthai, Thailand)

18 Vacuum pump (Waters, USA)

19 Rheometer RV1 (HAAKE RotoVisco®1) (Fisher Scientific, USA)

1.3 Chemicals

1 Isopropyl alcohol (HPLC grade, RCI Labscan, Thailand)

2 Ultrapure water (ELGA, UK)

3 Limonene (Sigma-Aldrich, USA)

4 Sodium alginate (Sigma-Aldrich, USA)

5 Calcium chloride dehydrate (Sigma-Aldrich, USA)

6 Carbopol 940 (Namsian, Thailand)

7 Jojoba oil (S. Thong, Thailand)

8 Dimethicone (S. Thong, Thailand)

9 Methylparaben (S. Thong, Thailand)

10 Cetyl alcohol (Chemicals of Highest Quality, Malaysia)

11 Propylene glycol (Chemicals of Highest Quality, Malaysia)

12 Cremophor 40 (S. Thong, Thailand)

13 Hydroxypropyl methylcellulose (S. Thong, Thailand)

14 Isopropyl myristate (S. Thong, Thailand)

15 Mineral oil (S. Thong, Thailand)

16 Dimethicone (S. Thong, Thailand)

17 Tween 80 (S. Thong, Thailand)

18 Span 80 (S. Thong, Thailand)

19 Brij 71 (S. Thong, Thailand)

20 Brij 721 (S. Thong, Thailand)

21 Vanillin (HPLC grade)

22 Polyethylene glycol (Chemicals of Highest Quality, Malaysia)

2 Methods

2.1 Extraction of essential oils from *Zanthoxylum limonella* locally grown in Thailand

The dried fruits clusters were collected from Nan province. The essential oils were extracted from the dried fruit clusters without additional process. Extraction was performed by the direct steam distillation technique using a steel vessel that contained the plant material on a perforated grid and boiling water at the base (Handa et al., 2008). The apparatus was controlled under the optimized temperature of 40˚C and pressure of -7 barr. During processing, the mixture of hot vapor form steam distiller was allowed to pass through a cooling system where condensation of vapor took place. The oil portion was collected and further dried by an addition of a drying agent, anhydrous sodium sulphate. The whole process took about 3 hours. The essential oils

were kept in the airtight amber color bottles at 4˚C for the further studies. The percentage yield of extracted oils was calculated by the following equation:

$$\text{ie ld} \quad \frac{\text{Volume of extracted essential oils m)}}{\text{Mass of raw plant material kg)}} \quad 100 \text{ ---------- Equation 1}$$

2.2 Characterization of *Zanthoxylum limonella* essential oils

The chemical constituents of essential oils were characterized by Gas Chromatography-mass spectrometry (GC-MS) technique. Retention times from gas chromatogram and mass from mass spectrum were used to identify major compounds in the essential oils.

GC-MS conditions were modified from a previously reported method (Tiwary et al., 2007). GC-MS analysis of the oil was performed on an Aligent Technologies GC model 6890N, equipped with Mass Selected Detector (MSD) model 5973 and an injector model 7683. MSD was set to scan mass in a range of 25-300 m/z at Central Instrument Facility Research Division, Faculty of Science at Mahidol University. A separation took place in a fused silica capillary column (30 m x 0.25 mm id, film thickness 0.25μm), coated with 5 diphenyl dimethyl siloxane HP-5MS). Helium was used as carrier gas at a flow rate of 1.0 ml/min; 15:1 split ratio. Oven temperature was programmed from 60 to 200˚C at 2˚C/min and then held for 20 min. The injector and detector temperatures were set at 250˚C.

The extracted essential oils (400 ppm) and standard limonene (428 ppm) were separately dissolved in hexane and analyzed by the GC-MS according to the above conditions. Volatile compounds containing in the essential oils was identified by comparing the retention times in chromatograms and mass in mass spectrums with the

retention time of standard d-limonene along with retention times and masses of other volatile compounds reported in literatures as well as in the library spectra (Wiley7n.l).

2.3 GC-FID analysis of limonene in *Zanthoxylum limonella* essential oils

The percentage of limonene in the essential oils was analyzed by a Gas Chromatography equipped with Flame Ionization Detector (GC-FID).The essential oils were dissolved in pure isopropyl alcohol at a final concentration of 10000 ppm. The stock solution of standard limonene was also prepared in isopropyl alcohol giving the final concentration of 16000 ppm. It was further diluted to obtain the desired concentrations of 8000, 4000, 2000 and 500 ppm for the determination of comparative active ingredient.

The concentration of limonene was determined by using GC-FID method as described in Tiwary et al. (2007) with slight modifications. The analysis of the oils was carried out on GC-FID (SHIMAZU GC/2010-model), equipped with a Flame Ionization Detector at Scientific and Technological Research Equipment Center at Chulalongkorn University. Moreover, a fused silica capillary column (30 m x 0.25 mm id, film thickness 0.25μm), coated with 5% diphenyl dimethyl siloxane (RTX-5MS) was employed. Helium was used as carrier gas at a flow rate of 3.0 ml/min; split ratio 20:1. Oven temperature was programmed from 80 to 200˚C at 2˚C/min and then held for 35 min. The injector and detector temperatures were set at 260˚C.

The concentration of limonene in essential oils was calculated from a linear equation that described a standard curve, a plot of peak areas against standard

limonene concentrations. The coefficient of determination (R^2) was used to determine the linearity. The acceptable R^2 is not less than 0.999.

2.4 Preparation of microcapsules containing *Zanthoxylum limonella* essential oils

The essential oils were encapsulated in alginate microspheres by extrusion method involving emulsification followed by cross-linking with calcium chloride. Various parameters, such as types of emulsifiers (Tween 80, Span 80, Tween 20 and Span 20) and concentrations (1-5 %w/w), concentrations of essential oils (5-30 %w/w), concentrations of sodium alginate (0.5-2.5 %w/w) and stirring rate during cross-linking process (500-1000 rpm), on microcapsule formation and encapsulation efficiency were studied as exhibited in **Table 3**.

Preparation of alginate microcapsules containing essential oils was described herein. Sodium alginate was slowly dispersed in water at room temperature and sonicated for 30 minutes in order to facilitate hydration the alginate polymer. The aqueous solution of sodium alginate was then slowly poured into the essential oils containing an emulsifier blend or an emulsifier. The mixture was homogeneously stirred by using a mechanical stirrer until emulsion was formed. The types of emulsion were classified as mentioned in the section **2.4.1**.The emulsion was extruded dropwise through a syringe (0.25 mm) into 2% calcium chloride solution. During extrusion, the calcium chloride solution was continuously stirred with the mechanical stirrer at a predetermined speed. The dispersion was kept stirring for 30 minutes, a cross-linking time (Liu et al., 2002). The volume of emulsion to the volume of calcium chloride solution was fixed as 1:3 for throughout the study (Chang et al., 2003). Both emulsification and cross-linking processes were carried out in a closed

glass container. The microcapsules were harvested by filtration on a Whatman No.5 filter paper and washing with ultrapure water prior to vacuum dry. The drying time was estimated by weighing the microcapsules until the constant weight was obtained.

The physical characteristics of microcapsules including size, shape, aggregation and physical stability before and after the drying process were evaluated under a light microscope connected with a digital video camera. The selected microcapsule preparation was evaluated for their percentage yield and percentage encapsulation efficiency as mentioned in the section **2.4.2** and **2.4.3**.

Table 3 Formulation of microcapsules containing *Zanthoxylum limonella* essential oils

Formulations / Ingredients	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20
Essential oils	20	20	20	20	20	20	20	20	20	20	20	20	5	10	15	30	20	20	20	20
Tween 80	-	-	-	1	3	5	-	-	-	-	1.2	-	-	-	-	-	-	-	-	-
Span 80	1	3	5	-	-	-	-	-	-	-	1.8	2	-	-	-	-	-	-	-	-
Tween 20	-	-	-	-	-	-	3	-	-	-	-	1	-	-	-	-	-	-	-	-
Span 20	-	-	-	-	-	-	-	1	3	5	-	-	3	3	3	3	3	3	3	3
Sodium alginate	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	0.5	2.5	1.5	1.5
Water	77.5	75.5	72.5	77.5	75.5	72.5	75.5	77.5	75.5	72.5	75.5	75.5	90.5	85.5	80.5	65.5	76.5	74.5	75.5	75.5
Stirring rate during cross-linking (rpm)	750	750	750	750	750	750	750	750	750	750	750	750	750	750	750	750	750	750	500	1000

2.4.1 Identification of emulsion types by dilution test

The obtained emulsion preparations were classified into oil-in-water (o/w) or water-in-oil (w/o) type by dropping 0.5 mL of emulsions into 10 mL of water. o/w emulsions could be very readily dispersed in the water that was a contrary to w/o emulsions.

2.4.2 Percentage yield of vacuum dried calcium alginate microcapsules containing essential oils

Microcapsules after vacuum drying were collected and weighed. Percentage yield of microcapsules was determined using the following equation:

$$\text{ie ld} = \frac{\text{Weight of vacuum dried microcapsules g)}}{\text{Weight of total ingredients without water g)}} \quad 100 \text{ ---------- Equation 2}$$

Where, weight of total ingredients without water = weight of essential oils + weight of emulsifier + weight of sodium alginate

2.4.3 Percentage entrapment efficiency of limonene in microcapsules

The encapsulated amount of limonene from *Zanthoxylum limonella* essential oils was determined according to the previously reported method (Maji and Hussain, 2008). The microcapsules were destructed by the following procedure. The microcapsules (250 mg) were crush by using the mortar and pestle and the essential oils were dissolved and adjusted with isopropyl alcohol (5 mL). The amount of limonene was quantified by GC-FID technique as described in the section **2.3**. Standard curve was constructed by plotting peak areas of standard limonene in isopropyl alcohol with concentrations in a range of 500-16000 ppm. The concentration of limonene entrapped in microcapsules was calculated by using the

constructed standard curve. The percentage entrapment efficiency (%EE) of microencapsulation was calculated using the equation below:

$$= \frac{\text{Concentration of limonene in the microcapsules g)}}{\text{Theoretical concentration of limonene in the microcapsules g)}} \quad 100 \text{ ---------- Equation 3}$$

2.5 Development of emulgel preparations containing microcapsules of essential oils or essential oils

The microcapsules of essential oils or essential oils were separately incorporated in emulgel preparation for further study on product stability and repellent efficacy.

2.5.1 Development of emulgel preparation containing standard limonene

The emulsion and/or emulgel preparations were developed by an addition of an aqueous phase to an oil phase without heat. Herein, various fixed oils (mineral oil, isopropyl myristate, jojoba oil and dimethicone) were selected based on HLB values in a range of 5 to 12. Non-ionic emulsifiers, such as Brij 72 and Brij 721 or Span 80 and Tween 80 or CremophorRH 40, were adjusted to satisfy the HLB value of the oil phase. The gelling agent, hydroxypropyl methylcellulose (HPMC) or Carbopol 940, was dispersed in water and left overnight prior to the addition into the emulsions. In the case of carbopol gel, an appropriate amount of triethanolamine (TEA) was added to adjust the pH to form a gel. The emulgel was prepared by two methods in term of incorporation of the gel phase into the formulations.

2.5.1.1 Method A

The oil phase of emulsion was the mixture of 5% fixed oil with 20% d-limonene and a lipophilic emulsifier (**Table 4**). The aqueous phase contained 5% propylene glycol, 1% paraben concentrate (1.8 g of methyl paraben and 0.2 g of propyl paraben in 100 g of propylene glycol) and a hydrophilic emulsifier dispersing in purified water. The aqueous phase was poured into the oil phase and at the room temperature. The mixtures were kept stirring using a magnetic stirrer at 1200 rpm until emulsion was formed. Then the emulsions were homogenized using a high speed homogenizer at 12,000 rpm for 5 minutes. HPMC (3% or 6%) or Carbopol (1% or 2%) were separately dispersed in water and 50 g of the gel was added into 50 g of emulsion (**Table 5**).

Table 4 Ingredients of emulsion formulations (Method A)

Formulations / Ingredients	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Oil phase (%w/w)										
d-limonene	20	20	20	20	20	20	20	20	20	20
Mineral oil	5	5	5	5						
Isopropyl myristate					5	5				
Jojoba oil							5	5		
Dimethicone									5	5
Brij 72	2.6	5.2								
Span 80			2.3	4.7	1.6	3.2	4.2	8.4	4.7	9.4
Water phase (%w/w)										
Propylene glycol	5	5	5	5	5	5	5	5	5	5
paraben concentrate	1	1	1	1	1	1	1	1	1	1
Brij 72	2.4	4.8								
Tween 80			2.7	5.3	3.4	6.8	0.8	1.6	0.3	0.6
Water	64	59	64	59	64	59	64	59	64	59

Table 5 Ingredients of emulgel formulations (Method A)

Formulations / Ingredients	E11	E12	E13	E14	E15	E16	E17	E18
Oil phase (%w/w)								
Mineral oil	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
d-limonene	10	10	10	10	10	10	10	10
Brij 72								
Span 80	1.15	2.35	1.15	2.35	1.15	2.35	1.15	2.35
Water phase (%w/w)								
Propylene glycol	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
paraben concentrate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Brij 72								
Tween 80	1.35	2.65	1.35	2.65	1.35	2.65	1.35	2.65
HPMC	1.5	1.5	3	3				
Carbopol 940					0.5	0.5	1	1
Water	79.6	78	77.7	76.5	81.5	79	81	78.5

2.5.1.2 Method B

In this method, the emulgel was prepared by addition of an aqueous gel phase to an oil phase. List of the ingredients in emulgel preparations were shown in **Table 6**. The oil phase was the mixture of 5% fixed oils, 20% d-limonene, 5% emulsifier (span 80 and tween 80 or cremophorRH 40), 5% propylene glycol and 1% paraben concentrate. The aqueous gel phase (3% HPMC or 0.5%, 1% or 2% carbopol 940) was added into the oil mixture at room temperature. The mixtures were kept stirring using a magnetic stirrer at 1200 rpm until emulgel was formed.

The emulgel preparations were selected depending on their physical stabilities and feelings after skin application. Physical stabilities were evaluated from their physical appearances after storage at room temperature for a week. Feelings after application was evaluated based on smoothness, stickiness and spreadability of the

emulgel. The preparations having better physical appearance, better skin feeling and better physical stability were selected for further development of emulgel containing essential oils.

Table 6 Ingredients of emulgel formulations (Method B)

Formulations / Ingredients	E19	E20	E21	E22	E23	E24	E25	E26	E27	E28
Oil phase (%w/w)										
d-limonene	20	20	20	20	20	20	20	20	20	20
Mineral oil	5	5	5	5						
Isopropyl myristate					5					
Dimethicone						5				
Jojoba oil							5	5	5	5
Span 80	2.3	4.7	2.3	4.7	1.6	4.2	4.7			
CremophorRH 40								5	5	5
Tween 80	2.7	5.3	2.7	5.3	3.4	0.8	0.3			
Propylene glycol	5	5	5	5	5	5	5	5	5	5
Paraben	1	1	1	1	1	1	1	1	1	1
Water phase (%w/w)										
HPMC	3	3								
Carbopol 940			1	1	1	1	1	1	0.5	2
Water	61	56	63	58	63	63	63	62.5	62	63

2.5.2 Preparation of emulgel containing *Zanthoxylum limonella* essential oils or microcapsules of essential oils

Zanthoxylum limonella essential oils or microcapsules containing essential oils were prepared according to the method B as described in the section **2.5.1.2**. In the case of microcapsules containing essential oils, they are added to the preparation at the final step. The emugel preparation with the desirable properties was further modified by an addition of an antioxidant, butylated hydroxytoluene (BHT) (0.02%), a humectant, polyethylene glycol (3%) and a fixative material, vanillin (5%). In emulgel containing microcapsule of essential oils, stock solution of 2% BHT (2g of

BHT in 100 g of Arlamol HD) at the final concentration of 0.02% BHT was added (**Table 7**). The obtained preparations were further evaluated for the amount of limonene, physical stability, repellent efficacy and chemical stability.

Table 7 Ingredients of emulgel formulations containing essential oils and microcapsule of essential oils

Formulations / Ingredients	E29	E30	E31	E32	E33	E34	E35	E36	E37	E38
Oil phase (%w/w)										
Essential oils	7	9	11	18	22	9	18			
Jojoba oil	5	5	5	5	5	5	5	8	8	8
Arlamol HD								2	2	2
Butylated hydroxytoluene	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
CremophorRH 40	5	5	5	5	5	5	5	5	5	5
Propylene glycol	5	5	5	5	5	5	5	5	5	5
Polyethylene glycol						3	3			
Paraben	1	1	1	1	1	1	1	1	1	1
Water phase (%w/w)										
Microcapsules								18	24	28
Vanillin						5	5			
Carbopol 940	1	1	1	1	1	1	1	1	1	1
Water	75.98	73.98	71.98	64.98	60.98	65.98	56.98	59.98	54.98	49.98

2.5.2.1 Determination of limonene concentration in emulgel

The concentrations of limonene in emulgel E29 – E38 were analysed after the preparation process. In this study, 3 batches of each formulation were prepared. The emulgel containing essential oils (0.4g) was dissolved and adjusted with isopropyl alcohol (5mL) before analysis. The amount of limonene was quantified by GC-FID technique as described in the section **2**.**3**. Standard curve was constructed by plotting peak areas of standard limonene in isopropyl alcohol with concentrations in a range of 500-16000 ppm. The concentration of limonene was calculated by using the constructed standard curve.

2.5.2.2 Physical stability evaluation of emulgel

The centrifugation and heating-cooling cycle were performed in order to evaluate the physical stability of the emulgel preparations.

The centrifugation was conducted to emulgel preparation at 6000 rpm for 30 minutes according to Thai Industrial Standard (152-2539). Each cycle of centrifugation creaming and phase separation behaviors of emulgel were observed.

Physical stability of the emulgels was also evaluated after 6 rounds of heating cooling cycle (Marquardt et al., 1998). Each cycle composed of storage at 40˚Cand for 48 hours and at 4˚C for 48 hours. After each temperature cycle, physical examinations such as color, odor, homogeneity, consistency, and phase separation were inspected. pH values and viscosity were determined using pH meter and rheometer respectively.

The rheology measurement was performed using rheometer using cone and plate. The temperature for all the experiments was set at 30±2˚C. Plate and cone diameter were 35 mm and cone angle was 1˚. Viscosity values were determined at the end of 60 seconds measuring time with a shear rate of 100 1/s. A plot between shear rate versus shear stress, of each formulation was compared. The shear rate was applied in range of 0-6000 1/s and the shear stress was measured accordingly in a pascal (Pa) unit.

2.5.2.3 Repellency efficacy test of emulgels containing *Zanthoxylum limonella* essential oils against *Ae. aegypti*

The repellency of the emulgels was evaluated using the human-bait technique (Tawatsin et al, 2001). Ethical clearance was approved by The Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn Universtiy. Bangkok, Thailand.

The mosquitoes used in this study were laboratory-reared female *Ae. aegypti* according to the standard protocol of the Biology & Ecology Section, National Institute of Health, Thailand. Three to five-day-old females of this specie were used in the repellency test. The test for was performed during the daytime from 9:00 to 17:00. Evaluations were carried out in a 6x6x3 m room, at 25-29°C with relative humidity of 60-80%.

Volunteers (age 25-61 years) were recruited for the laboratory tests. Volunteers who were sensitivity to mosquito bites or ingredients in the preparation, psoriasis, pregnant or lactation and unwilling to continue the experiment were excluded. Individuals were asked to wash their hands and forearms and cleaned with

detergent and dry the wet areas before starting the test. Three to five volunteers were subjected to tested preparations on one forearm while the other volunteer was subjected to a standard chemical, 15% IR3535 in ethanol, on one forearm and considered as a positive control. An area of 30x100 mm on each forearm of volunteers was marked out with a marker. Then, 15% IR3535 in ethanol was applied on the marked area of the forearm of the positive control volunteer and the test sample emulgels E30, E32, E34, E35, E37 and emulgel with standard limonene were applied on the marked areas of the forearms of volunteers. One tenth gram of test samples or the positive control sample were weighed and applied on the marked area on forearms of each volunteer. The forearms were then covered by a paper sleeve with a hole corresponding to the marked area. Before the start of each exposure period, the bare hand of the test person, used as negative control area for each volunteer, was exposed for up to 10 seconds in a mosquito cage (30x30x30 cm), containing 250 host-seeking female mosquitoes. If at least two mosquitoes landed on or bit the hand, the repellency test was then continued. This was done to ensure that the mosquitoes were host seeking. Each volunteer put the test forearms in the mosquito cage and left for three minutes. Numbers of mosquitoes landed and bit in the middle of the marked area was counted as a positive result and recorded in a work sheet, while mosquitoes landed on the margin of the marked area were not accounted for. The test was carried out every 30 min until the second bite occurred. The time between application of test sample and the second bite was recorded as the protection time. The test was carried out for up to 7 hours. An average protection time was computed from the data obtained from each volunteers.

2.5.2.4 Chemical stability evaluation of emulgel

Changes in limonene concentration in the emulgel were monitored at 30˚C, 75 RH and 40˚C, 75 RH for 12 weeks according to Asean Guideline on Stability Study of Drug Product (9th ACCSQ-PPWG Meeting, Philippines, 2005). At appropriate times, samples were analyzed in term of the percentage of limonene using GC-FID as mentioned in the section **3**. The percentage remaining of vanillin was also calculated based on the peak area mentioned in the chromatograms. The physicochemical properties in terms of color, odor, homogeneity, consistency, and phase separation were inspected. pH values and viscosity were monitored over a period of 12 weeks at the predetermined time intervals.

Chapter IV

Results and Discussion

3.1 Extraction of essential oils from *Zanthoxylum limonella* locally grown in Thailand

Zanthoxylum limonella essential oils were extracted by the steam distillation method. The obtained oils were easily separated using separating funnel (**Figure 7**). As the essential oils were less dense than water, oils appeared on top of water. The physical characteristics of essential oils obtained from the dried fruit clusters were clear pale green in color with strong lemon-like odor.

Figure 7 The picture of essential oils separation after distillation process

The percentage yield of the essential oils was 6% v/w. It was reported that the fruit essential oils obtained by hydrodistillation method were in the range of 5.72-12.5% (Itthipanichpomg et al., 2002; Choochote et al., 2005; Tangjitjaroenkun et al., 2012). The variation in the percentage yield and chemical profile of extracted volatile oils could generally depend on several factors. The variability was mainly caused by

herbal quality, region of growth, the time or season of harvesting, preparation of the herb prior to extraction process such as drying and storage, and extraction process.

3.2 Characterization of *Zanthoxylum limonella* essential oils

GC chromatogram of standard limonene showed one major peak with a retention time of 8.41 minutes (**Figure 8**) corresponding to mass to charge (m/z) ratio of 136.1 (**Figure 9**). GC chromatogram showed that the essential oils obtained from *Zanthoxylum limonella* fruit clusters composed of five major peaks and all peaks were well separated from each other as shown in **Figure10**. These peaks were eluted at the relative retention time 5.32, 6.47, 7.53, 8.34 and 8.41 minutes respectively. The mass to charge ratio (m/z) of the peak at the retention time 8.34 minutes was 134.1 (**Figure 14**) while that of the rest peaks was 136.1 (**Figure 11, 12, 13, 15**).

By comparing their retention times of peaks in **Figure 10** with retention time of standard limonene together with mass spectrum and mass to charge ratio in **Figure 8** and **Figure 9**, the major peak with a retention time of 8.41 minutes was limonene. By comparing mass spectra of the essential oils with the library spectra (Wiley7n.l), the results suggested that peaks with relative retention time of 5.32, 6.47, 7.53 and 8.34 minutes were α-pinene, sabinene, α-phellandrene and β-cymene respectively as shown in **Figure 11, 12, 13** and **14**. Based on the peak areas, compositions of *Zanthoxylum limonella* essential oils were d-limonene (area of 80.37%), sabinene area of 9.51), α-phelladrene (area of 6.01%), α-pinene (area of 2.63%) and β-cymene (area of 1.48%).

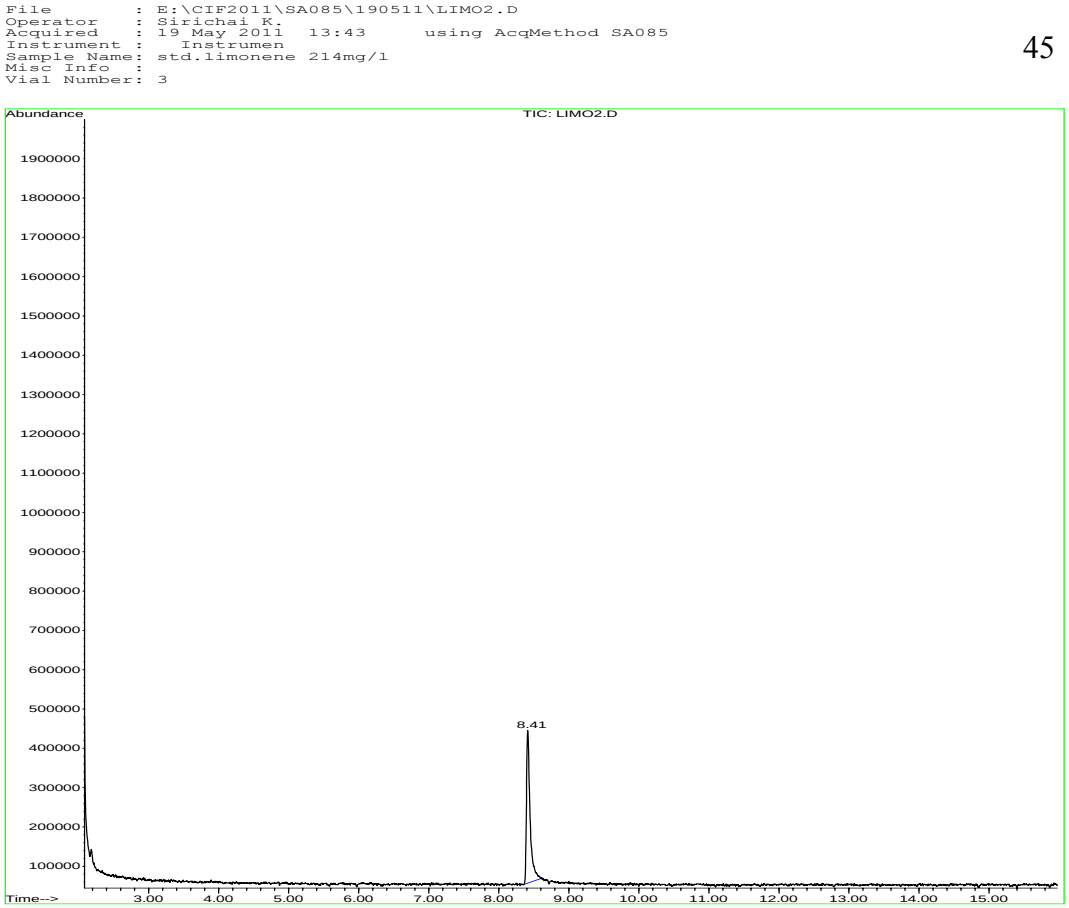


Figure 8 GC chromatogram of standard limonene

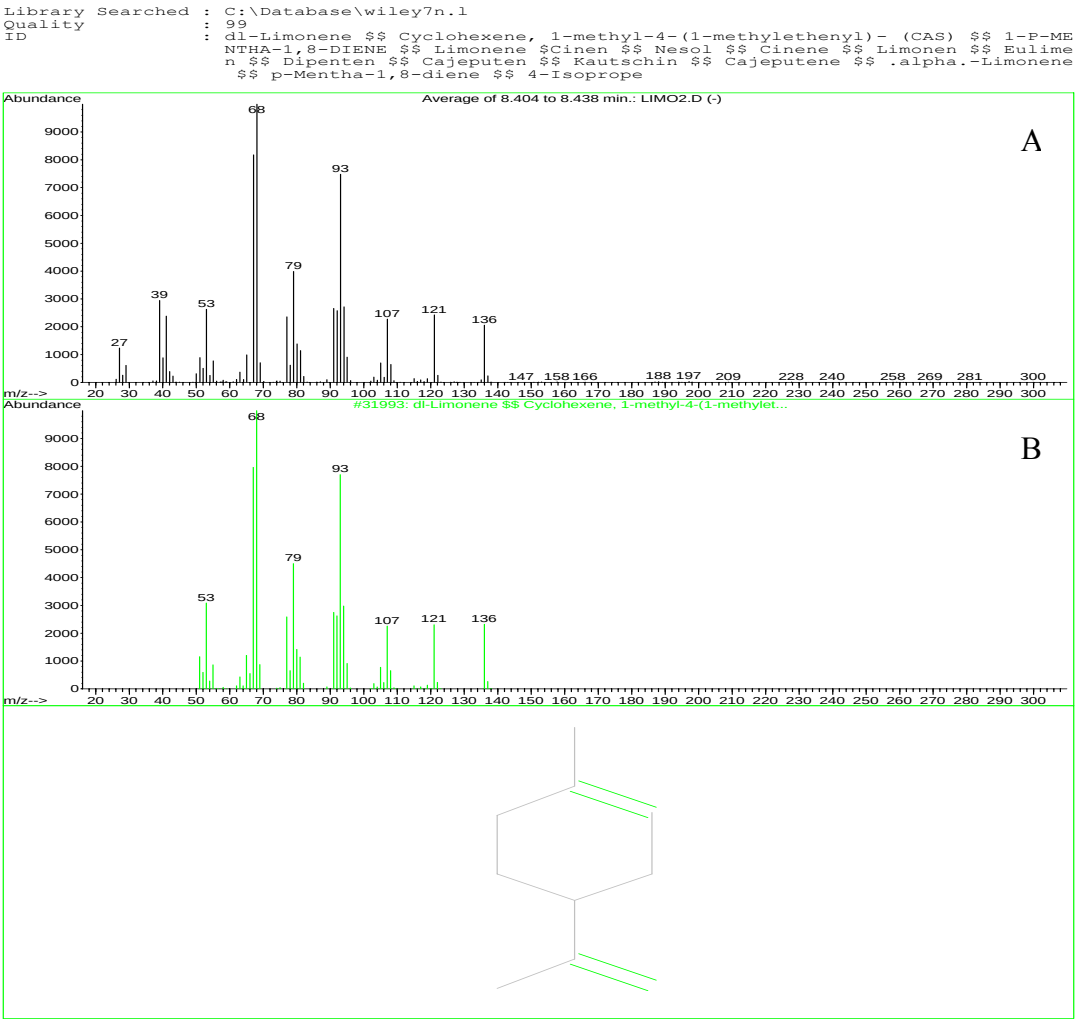


Figure 9 Mass spectra of standard d-limonene (**A**) and its library reference (**B**)

```
File         : E:\CIF2011\SA085\190511\SAMPLE.D
Operator     : Sirichai K.
Acquired     : 19 May 2011   15:25      using AcqMethod SA085
Instrument   :   Instrumen
Sample Name: sample 436mg/1
Misc Info    :
Vial Number: 5
```

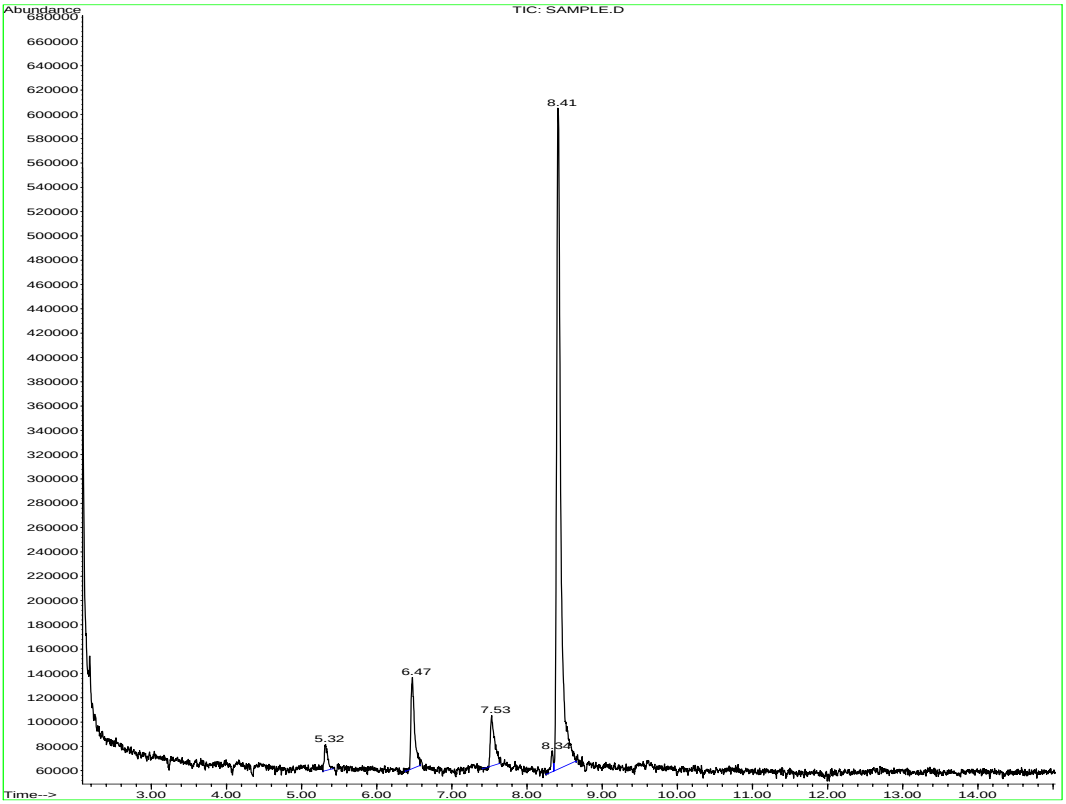


Figure 10 GC chromatogram of *Zanthoxylum limonella* essential oils

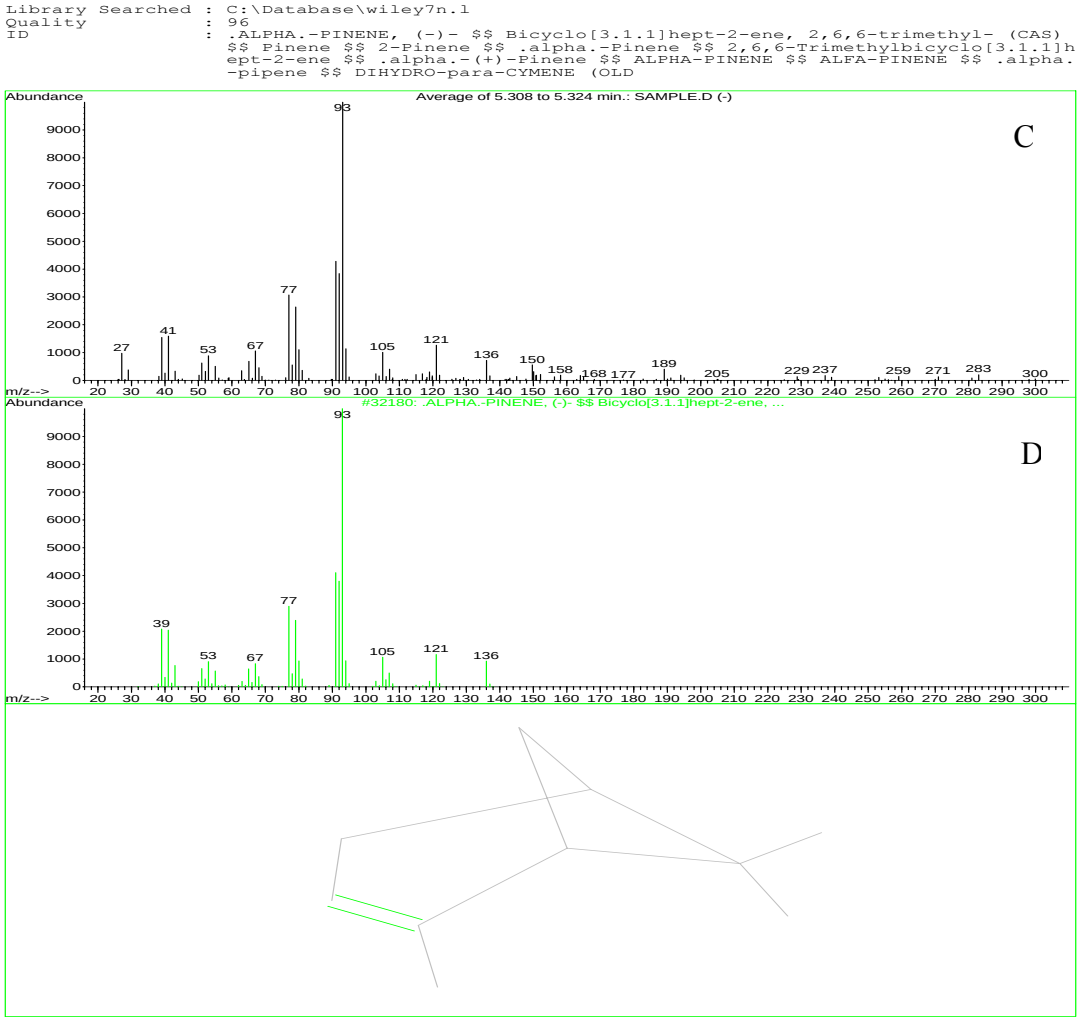


Figure 11 Mass spectra of α-pinene **(C)** and its library reference **(D)**

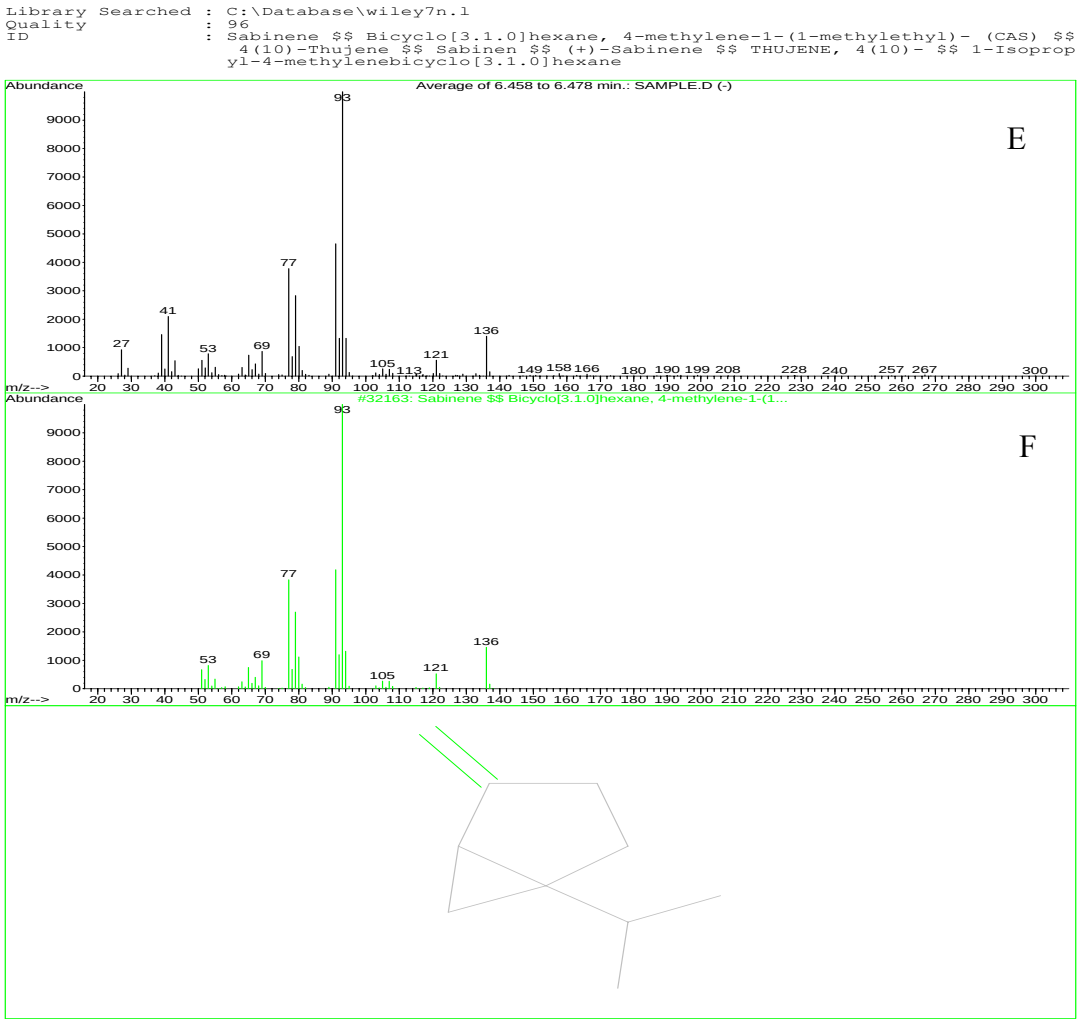


Figure 12 Mass spectra of sabinene **(E)** and its library reference **(F)**

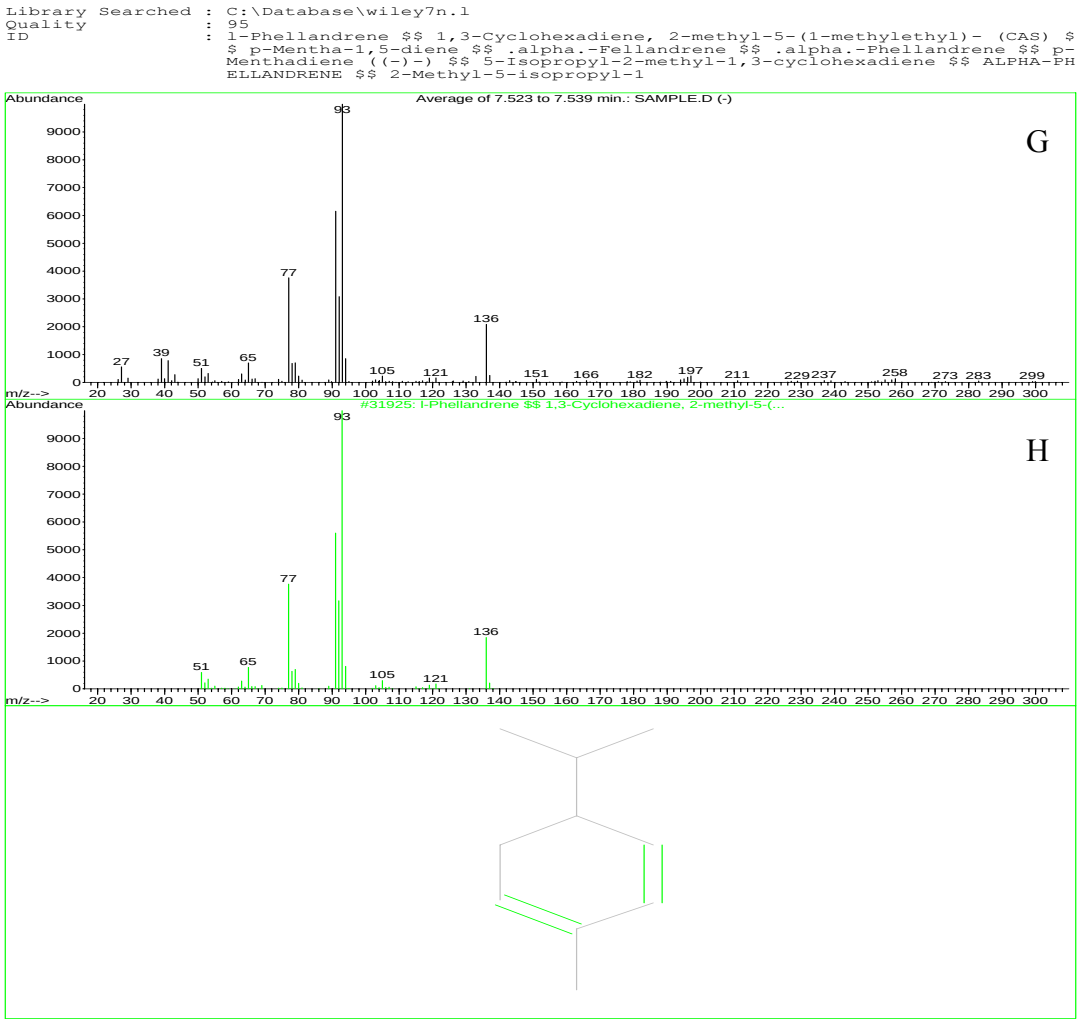


Figure 13 Mass spectra of α-phellandrene **(G)** and its library reference **(H)**

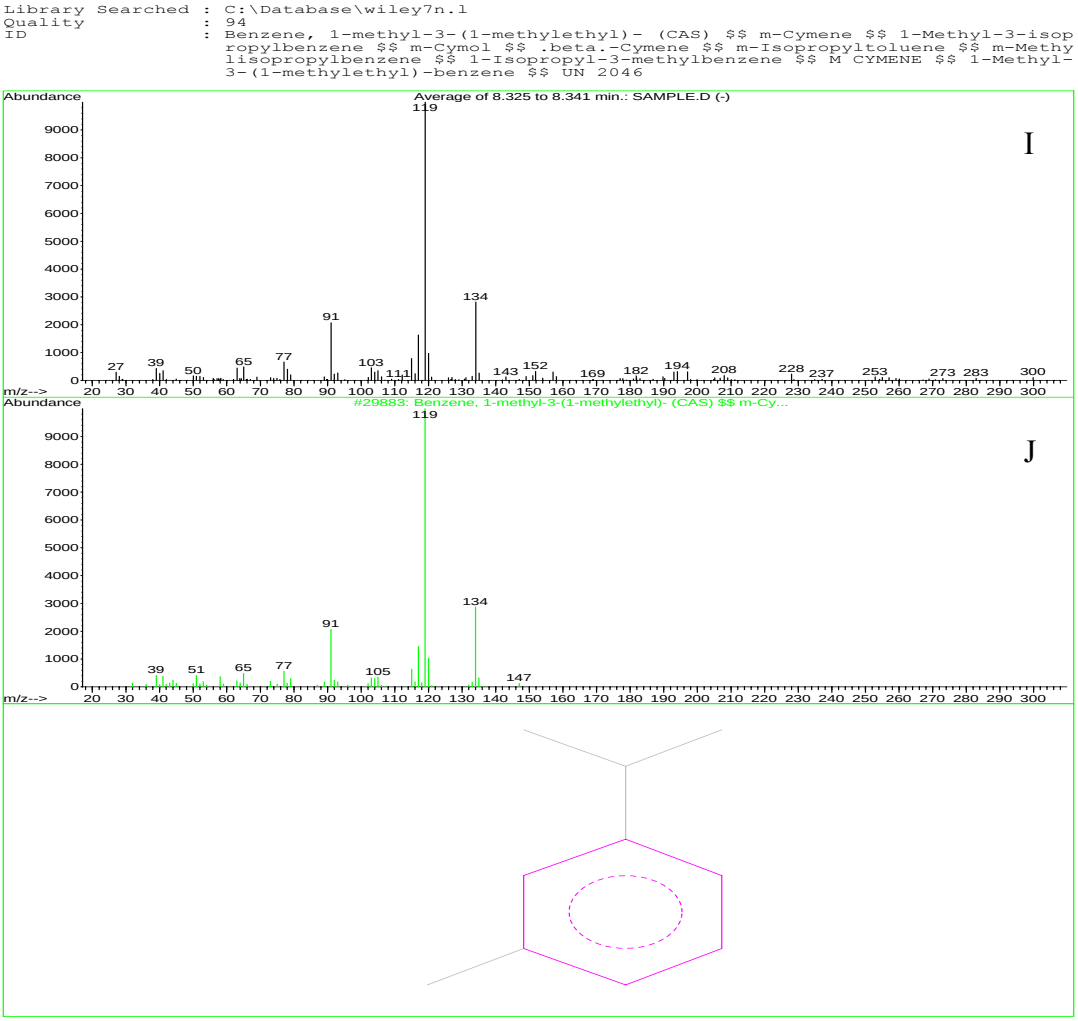


Figure 14 Mass spectra of β-cymene **(I)** and its library reference **(J)**

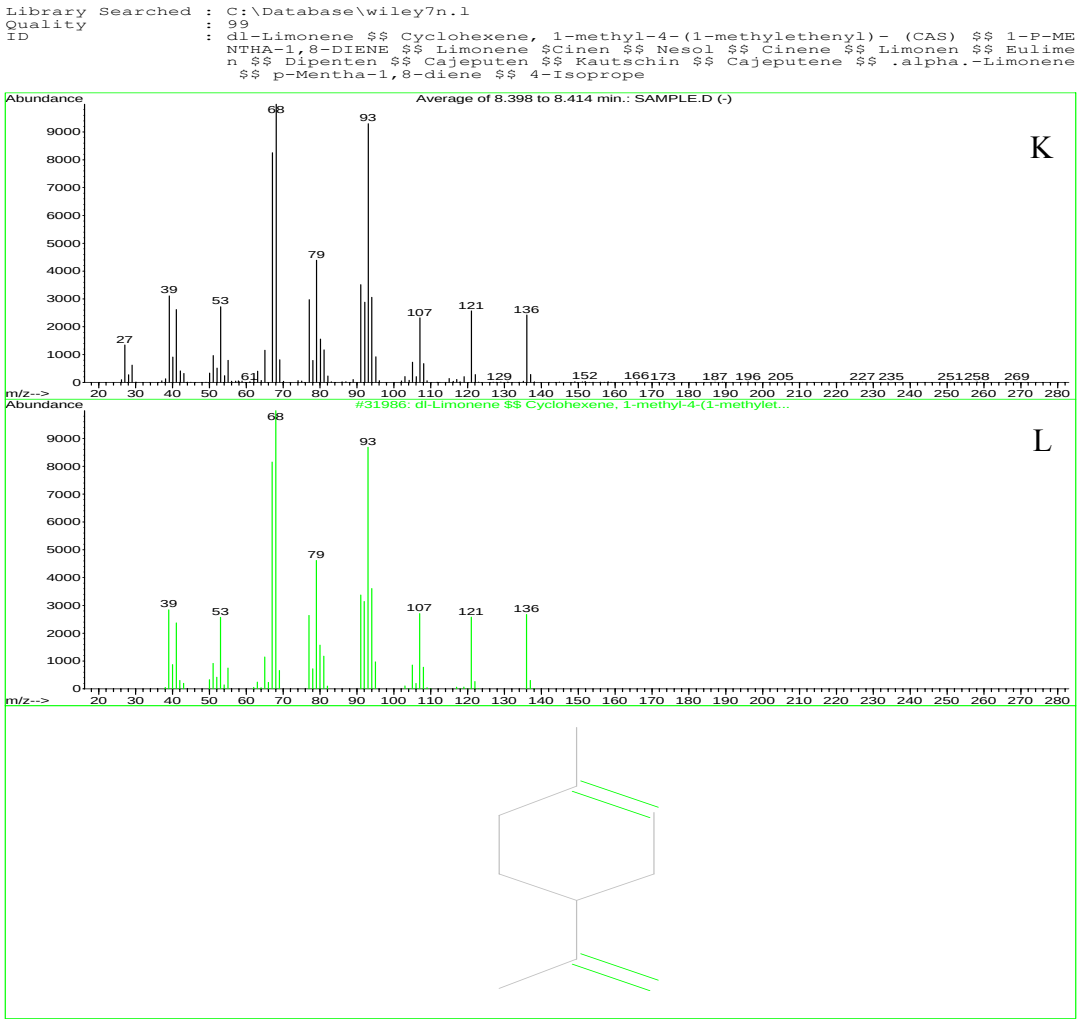


Figure 15 Mass spectra of d-limonene **(K)** and its library reference **(L)**

These results were in agreement with those reported by Itthipanichpong et al. 2002 and Tangjitjaroenkun et al. 2010 which demonstrated that *Zanthoxylum limonella* fruit essential oils composed of 27-33 chemical constituents and the main constituents were limonene (31-39%) and sabinene (9-43%). All these essential oils constituents, α-pinene, sabinene, α-phellandrene, β-cymene and d-limonene, were classified as monoterpene.

3.3 GC analysis of limonene in *Zanthoxylum limonella* essential oils

The GC chromatogram of the standard d-limonene showed in a single peak at a retention time of 4.40 minute (**Figure 16**). A typical calibration curve of d-limonene was shown in **Figure 17**. In a concentration range of 5-16 mg/ml, peak areas were linearly related to d-limonene concentrations with the coefficient of determination, R^2 of 0.999.

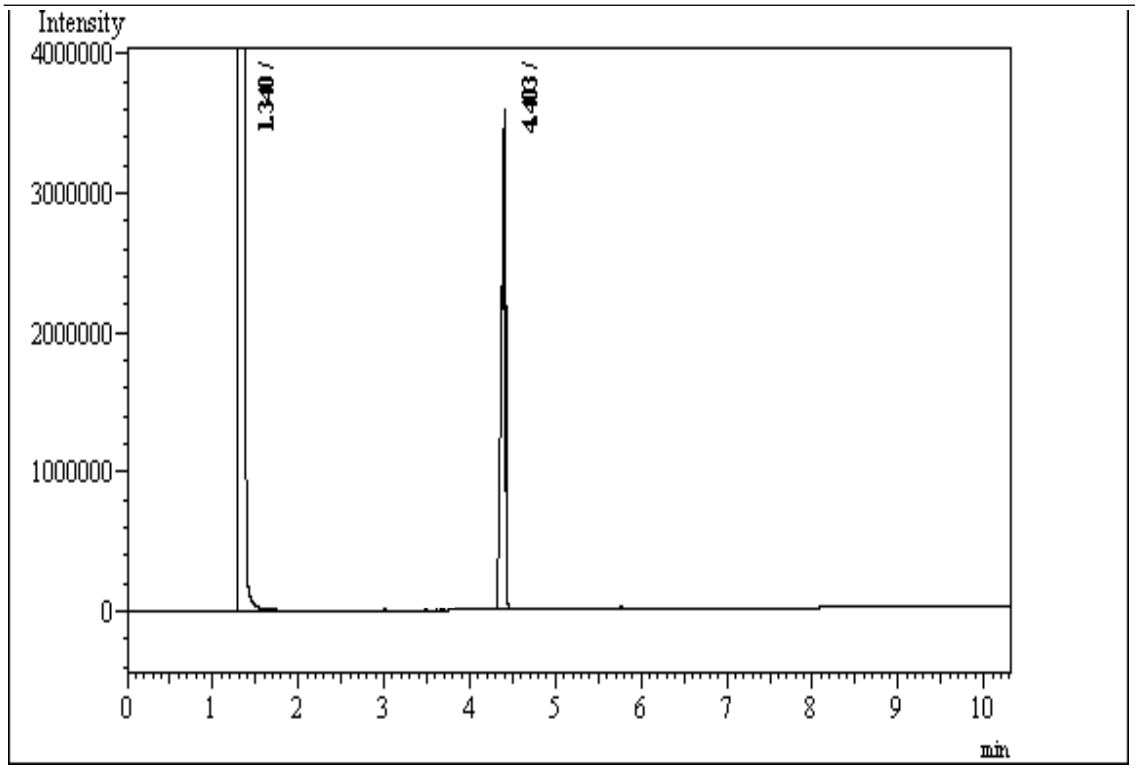


Figure 16 GC chromatogram of standard limonene

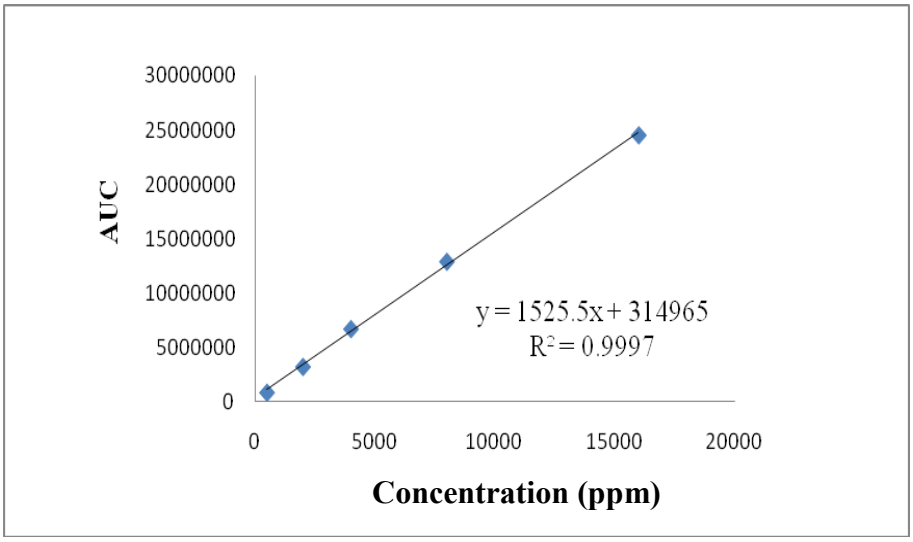


Figure 17 Calibration curve of standard limonene

The GC chromatogram of essential oils showed six major peaks (**Figure 18)**. Peaks eluted at the retention time of 4.45 and 4.49 minutes were considered as the peak of limonene by comparing the retention time of standard limonene. The concentration of d-limonene in was found as 71.07% w/w.

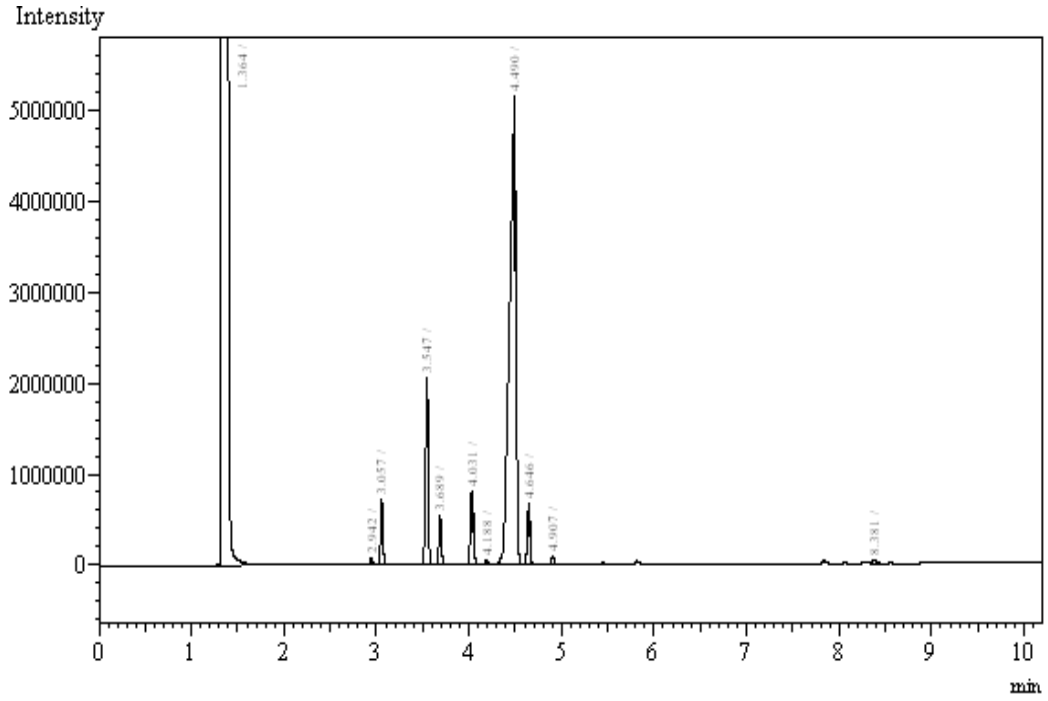


Figure 18 GC chromatograms of *Zanthoxylum limonella* essential oils

3.4 Preparation of microencapsulated *Zanthoxylum limonella* essential oils

The alginate microcapsules containing *Zanthoxylum limonella* essential oils were prepared by the extrusion method using sodium alginate as a polymer and calcium chloride as a cross-linking agent. The extrusion method involved emulsification and cross-linking processes. Factors affecting the formation and physical characteristics such as size, shape and aggregation of microcapsules were investigated by varying one factor at a time.

Various types (Span 80, Span 20, Tween 80 or Tween 20) and concentrations (1-5 %w/w) of emulsifiers were employed in formulations of emulsion containing essential oils. The types of the emulsions, w/o or o/w emulsion, were classified by the dilution test. The emulsion formulations M1-M3 were w/o emulsions while

formulations M4-M12 were o/w emulsions. Formulation M13 gave rise to a gel-like product; therefore, determination of emulsion type using the dilution test could not be performed.

The obtained emulsion was cross-linked with 2% calcium chloride solution to obtain calcium alginate microcapsules containing essential oils. In the formulations M1-M3, Span 80 was a solely emulsifer used for forming the emulsions and corresponding microspheres. The emulsions obtained from M1-M3 were w/o where the essential oils were present in the outer phase and were not entrapped within the microcapsules; therefore, no oil globules dispersed in the microcapsules was observed under a light microscope. Span 80 was replaced with Tween 80 or Tween 20 in formulations M4-M7. However, these formulations provided smooth, non-spherical macrobeads with thick wall and tail. The large beads with tail were more obvious when emulsifier concentration increased to 5% w/w. In formulations, M8-M10, span 20 was applied as an emulsifier. Formulation M8 gave irregular and distorted microspheres. Formulation M9 gave spherical and free-flowing microspheres with observable translucent oil globules in the alginate microspheres (**Figure 19**). The mean droplet size of microcapsules obtained from M9 was $\leq$ 200 μm in diameter. On the other hand, M10 became a viscous gel-like system during cross-linking process when the emulsifier concentration was higher up to 5 %w/w. Using emulsifier blends (span 80 and tween 80 or tween 20) M11-M12 gave large, irregular and aggregated microcapsules.

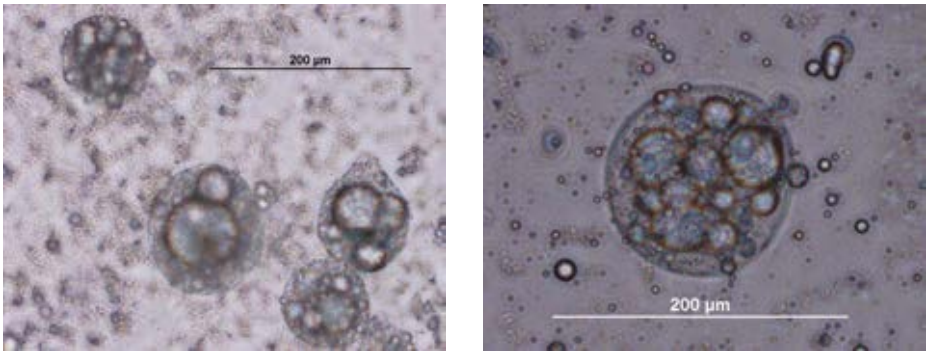


Figure 19 Morphology of calcium alginate microsphere, M9; magnification x 20

The other parameters such as concentration of essential oils, concentration of sodium alginate and stirring rate were studied by comparing of microcapsules obtained from M9. At low concentration of essential oils (5 %w/w) in M13, microspheres were not formed. The beads were considered as spherical with thick wall in M14 and M15 with essential oils 10-15 %w/w. The poor stability of emulsion was occurred with higher oil content (30 %w/w) in M16 and so microspheres were not formed. The alginate concentrations were varied from 0.5-2.5 %w/w. When the alginate concentration was lower than 1.5 %w/w (formulation M17), the microcapsules were not formed. The spherical microspheres containing inner oil core were seen in M9 and M18. The effect of stirring rate during cross-linking process was observed in formulation M9, M19 and M20 where three stirring rates were varied between 500-1000 rpm. When the stirring rate was low (500 rpm in formulation M19), large oval microspheres diameter > 200 µm were obtained. When the stirring rates were 750 rpm and higher than 750 rpm, the obtained microcapsules were similar in both shape and size.

Microcapsule preparations using the extrusion method had a distinct advantage for volatile compounds because the encapsulation process was normally carried out at room temperature without air-flow (Lertsuttiwong et al., 2008). Water-soluble sodium alginate could form insoluble calcium alginate by cross-linking with calcium chloride when divalent calcium ions bound the interchain between the guluronic acid (G) units of alginate polysacccharides.

Surfactant played an important role in the formation of essential oils containing sodium alginate emulsion preparations. Span 80 (HLB-4.3) gave the w/o emulsion while Span 20, Tween 80 or Tween 20, HLB value in a range of 8.6-16.7, gave the o/w emulsion.

The addition of sufficient amount of emulsifier was favorable to from discrete, spherical and non-aggregated microspheres (Yoo et al., 2006; Chuah et al., 2009). The suitable type of emulsifier could reduce the surface tension in coating matrix resulting in spherical and non-aggregate microspheres with inner core (Chang et al., 2000). In this study, when the microcapsules were prepared with different types of emulsifier or emulsifier blends, a marked difference in the characteristics of microcapsules was observed. The influence of the emulsifier on the particle size distribution of the formed microspheres was not obvious with less oil loading emulsions. The appropriate amount of essential oil to surfactant ratio was found as 20:3.

It was suggested that the alginate concentration in a range of 1.5-2.5 %w/w could be formed the spherical microsphere formation (Chan, 2011). The microspheres were not formed when alginate concentration was lower than 1.5 %w/w (formulation

M17). The emulsion droplets containing sodium alginate concentration 0.5 %w/w might not be able to counteract the effect of impact and drag when they were dripped into the calcium chloride solution with constant stirring rate (Chan, 2011). However, 2.5% of sodium alginate concentration was not recommended in this study because microcapsules obtained from M18 gave gritty sensation after application on skin.

The continuous stirring during cross-linking influenced the size and shape of microcapsules formation. The size and shape of microcapsules formed by 500 rpm was markedly large and non-spherical with > 200 μm comparing with that of 750 rpm and 1000 rpm. However, the differences were less evident between 750 rpm and 1000 rpm with approximately ≤ 200 μm in diameter. Formation of uniform and spherical microspheres might attribute to stirring rate to a certain extent. The formulation, M9, prepared with Span 20 (3 %w/w), essential oils (20 %w/w), sodium alginate (1.5 %w/w) and stirring rate (750 rpm) during cross-linking was found to be the suitable formulation in this study.

In this current study, the calcium chloride concentration and cross-linking time were fixed as 2% w/w and 30 minutes because both parameters had no significant effect on the size and morphology of prepared microcapsules (Chang and Dobashi, 2003; Lotfipour et al., 2012). During cross-linking, the rapid reaction was observed at the beginning of the process as calcium ions content in the microcapsule wall rapidly increased at the beginning of the process (Liu et al., 2002). The cross-linking rate was slow down and approached zero within 30 minutes of cross-linking time. Electrostatically interact with high negative charge of alginate at carboxylic groups of guluronic acid resulting in partially neutralizing charge of alginate. Thus the alginate

showed less electrostatic interaction with the next calcium ions. The rapid reaction mainly occurred at the surface of the microsphere and high degree of cross-linking at the interface gives smaller pore size in the external gelation method. The higher calcium ions and alginate concentration at the interface by comparing with the inner core might lead to the formation of vesicles by external cross-linking process (Liu et al., 2002).

Formulation M9 was prepared triplicates and the obtained microcapsules were further vacuum dried. The vacuum dry microcapsules were weighed every hour until the constant weight was obtained. The weight of microcapsules from different batches became constant after 3 hours of drying. Percentage remaining weight after 3 hours of drying was 64.31±0.80 and after 6 hours of drying was 63.87±0.91 respectively as shown in **Figure 20**. After 3 hours of drying, the obtained microcapsules were dispersed in water and seen under the light microscope as shown in **Figure 21**. Microcapsules were found as physically stable with inner oil core. Therefore, 3 hours of drying time was selected for further study on microcapsules preparation.

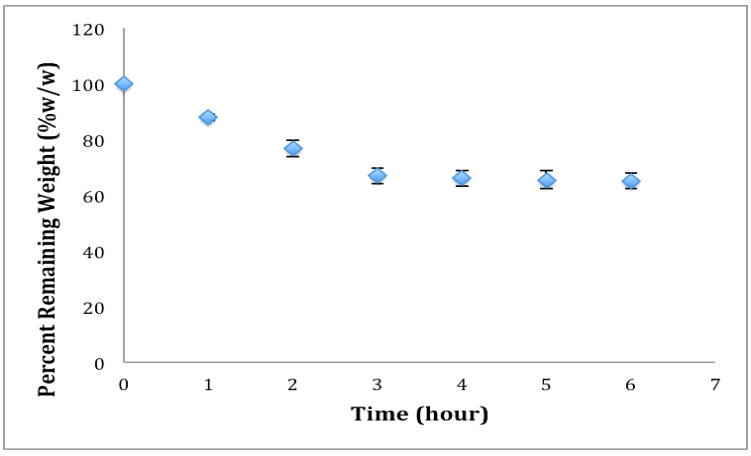


Figure 20 Drying time for microcapules preparation

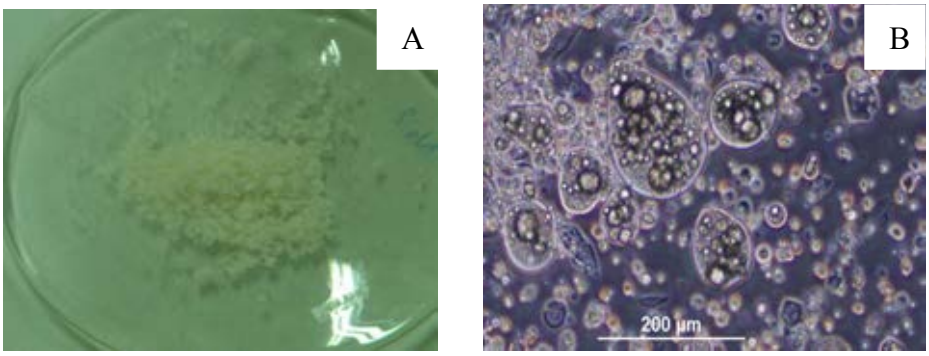


Figure 21 Picture of vacuum dry calcium alginate microsphere (**A**), its morphology magnification x 10 (**B**)

3.4.1 Percentage yield of vacuum dried essential oils containing calcium alginate microcapsules

The percentage yield of microcapsules was determined by preparing formulation M9 in triplicates. The yield of calcium alginate microcapsules was 67.29±2.56 %w/w (n=3).

3.4.2 Percentage entrapment efficiency of limonene in microcapsules

The GC chromatogram of the essential oils extracted from calcium alginate microspheres was shown in **Figure 22**. By comparing their retention times of peaks in **Figure 22** with retention time of standard limonene, the major peak at the retention time 4.49 was limonene. The percentage entrapment efficiency (%EE) of calcium alginate microcapsules was found as 29.96±1.65 %w/w (n=3). In this study, percentage entrapment efficiency was low because of the diameter of the microcapsules was ≤ 200 μm. This finding was contradicted to high percent loading

entrapment efficiency of 90-92% when the alginate capsules were in millimeter scale (Chang and Dobashi, 2003). In order to increase the percentage entrapment efficiency, high concentration of sodium alginate concentration could be employed. However, high concentration of sodium alginate resulted in large size of microcapsule, M18, and gave gritty feeling after skin application. Moreover, a lower percentage entrapment efficiency of the essential oils was due to the nature of the essential oils with high volatility even at room temperature or slightly higher temperature. Each processing step should be strictly controlled in order to prevent the loss of essential oils.

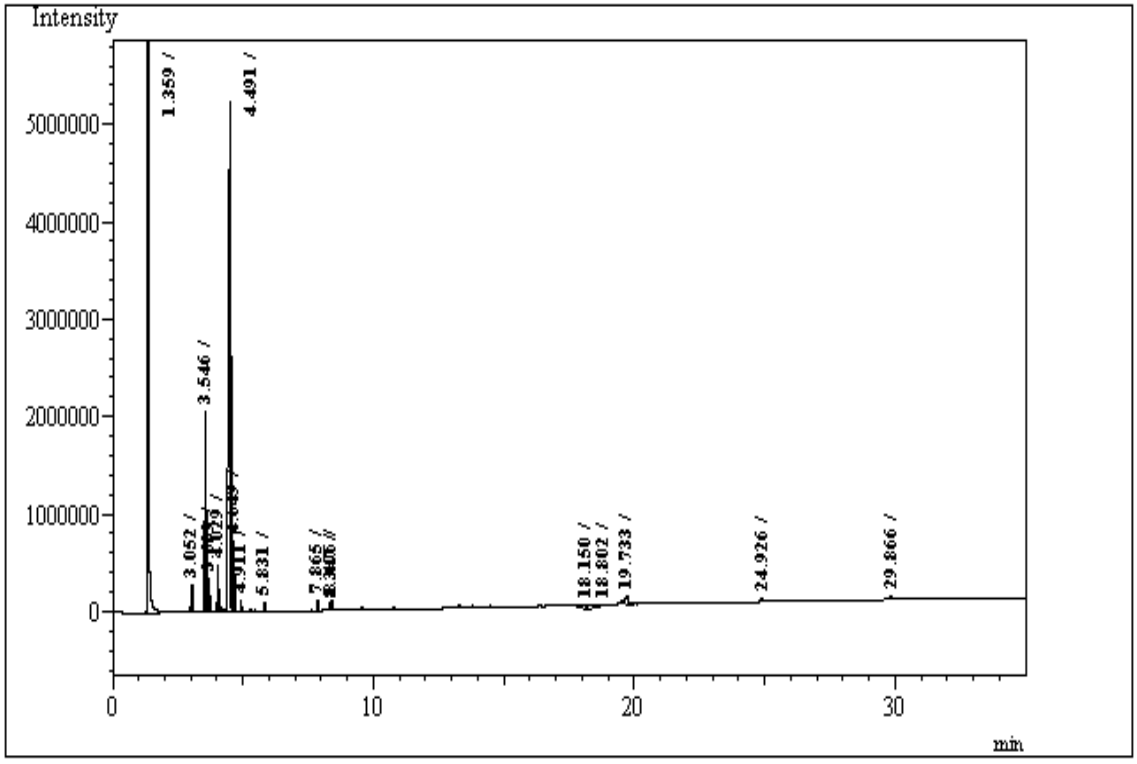


Figure 22 GC chromatogram of essential oils in the calcium alginate microcapsules

3.5 Development of emulgel preparations containing microcapsules of essential oils or essential oils

The obtained microencapsulated essential oils or pure essential oils were separately prepared in emulgel.

3.5.1 Development of emulgel preparation containing standard limonene

The emulgel formulations were developed by two different methods in term of incorporation of gel phase into the formulations. In method A, emulsion formulations E1-E10 were prepared and then mixed with gel phase to obtain emulgel preparations E11-E18. In method B, gel was added to the mixture of oils containing surfactants, proplyglycol and paraben concentrates to obtain emulgel preparations E19-E28.

In the preliminary formulation development steps, the standard limonene was added into the formulations. In the formulation development step, the essential oils were employed. Various types of fixed oils, such as mineral oil, isopropyl myristate, jojoba oil and dimethicone, were selected as oil phase. An emulsifier or emulsifier blends i.e, Tween 80 and Span 80, Brij 721 and Brij 72 or CremophorRH 40, were adjusted to satisfy the HLB value of the oil phase. HPMC or Carbopol 940 was employed as the gelling agent. The effect of emulsifier, types of oil and types of gelling agent on emulgel preparations was evaluated. The emulgel preparations were selected depending on their physical stabilities and feelings after skin application.

3.5.1.1 Method A

The emulsion formulations E1-E4 were prepared with emulsifier blend Brij 721 and Brij 72 or Tween 80 and Span 80 in the present of mineral oil and standard limonene. HLB value of emulsifier blend was adjusted according to that of mineral oil (HLB-10). E1-E2 formulations with Brij 721 and Brij 72 were unstable as creaming

and cracking occurred after storage at room temperature for 24 hours. E3-E4 formulations with Tween 80 and Span 80 were found to be stable. Therefore, emulsion formulations E5-E10 containing isopropyl myristate (HLB 11.5), jojoba oil (HLB 6) or dimethicone (HLB 5) were prepared using tween 80 and span 80 emulsifier blend. Emulsions E3-E10 were physically stable after storage at room temperature for a week. Therefore, stability of emulsions containing the standard limonene did not depend on types of fixed oil but depended on emulsifier or emulsifier blend that satisfy HLB value of the oil phase. The obtained emulsion formulations E3-E4 were mixed with various concentrations of gel phase to obtain emulgel formulations E11-E18. However, the obtained emulgel formulations were physically unstable as creaming occurred after storage for a week at room temperature.

3.5.1.2 Method B

The emulgel formulations E19-E28 were also developed by this method B. The obtained emulgels from formulation E19-E22 were yellowish milky cream. Formulations E19 and E20 employed HPMC as gelling agent and the emulgel contained high concentration of emulsifier blend (10 %w/w in the formulation E20) was physically stable but the formulation E19 with 5 %w/w emulsifier blend showed creaming after 7 days at room temperature. E20 also gave sticky feeling after applying the emulgel on the skin. The sticky feeling was speculated to be due to the presence of HPMC. E21-E22 formulations were employed carbopol 940 and did not give sticky feeling after skin application when compared with HPMC containing emulgels. Both E21 and E22 were physically stable. Therefore, emulgels containing Carbopol 940 as

a gelling agent and emulsifier blend of Span 80 and Tween 80 at the low concentration of 5 %w/w was selected for further development because it showed better physical stability with low concentration of emulsifier which could avoid skin irritation.

The effect of various fixed oils on the emulgel preparations was compared between emulgel preparations (E21, E23-E25). Emulgel containing jojoba oil E25 provided dry smoothness and non-greasy skin feeling comparing with other oil phase. All of the emulgel preparations containing Tween 80 and Span 80 emulsifier blend provided yellowish milky appearance. In order to improve the physical appearance of the emulgel preparation, CremophorRH 40 was employed as an emulsifier and solubilizer for essential oils. The whitish appearance of emulgel preparation was observed in E26.

Furthermore, carbopol concentrations were varied in E26-E28 to prepared emulgel preparation. E26 was found as satisfy preparation as it provided better appearance, better skin feeling and physically stable at room temperature for several weeks.

The formulation obtained from the method B was found as physically stable emulgel than that obtained from method A. It was reported that emulgel containing topical insect repellent, DEET, was successfully prepared by this method B (Qui et al., 1997).

The non-ionic emulsifier or emulsifier blend; i.e. Tween 80 and Span 80 or Brij 721 and Brij 72 or CremophorRH 40, were selected due to their widely used in

topical pharmaceutical formulations and their better chemical stability over a wide pH range. CremophorRH 40 was selected as an emulisifer because it was a well-known solubilizer for vitamins and essential oils. It additionally improved the appearance of the emulgel preparation in this study.

Fixed oil such as mineral oil, isopropyl myristate, jojoba oil and dimethicone were selected for emulgel preparation because they are safe and generally used in pharmaceutical industry. Jojoba oil, a liquid unsaturated wax composed of esters of long carbon chain fatty acids and long carbon chain unsaturated alcohols, is a medium polar oil possessing an appropriate viscosity (35 cPs) and spreading coefficient (19 S). In this study, emulgel containing jojoba oil provided more pleasant feeling on the skin (Shahin et al., 2011).

By employing gelling agent, Carbopol 940, the viscosity of emulgel preparations was evidently increased with the polymer concentrations increased. Carbopol 940 at the concentration of 1% was suitable to get stable emulgel. Hence, carbopol enhanced the entrapment of the active compound in the gelling network system and improved the physical and thermal stability of emulgel preparation. However, it was well known that the gelling capacity of carbopol gel was greatly depended on pH. The carbopol gel at pH 4.5-5 offered the acceptable gel clarity and viscosity. It possessed the optimum rheology property known as pseudoplastic flow behavior that assisted in easier for skin application (Mohamed, 2004; Shahin et al., 2011).

3.5.2 Preparation of emulgel containing *Zanthoxylum limonella* essential oils or microcapsules of essential oils

Loading amount of *Zanthoxylum limonella* essential oils in the emulgel preparations was calculated based on the previous reports (Trongtokitet et al., 2004; Trongtokitet et al., 2005). According to the previous studies, 4.67 %w/w of limonene showed repellency against *Ae. aegypti*. In this study, *Zanthoxylum limonella* essential oils containing 71.07 %w/w of limonene, as mentioned in the section **3**, were employed. In order to obtain 4.67 %w/w of limonene in the preparations, theoretical loading concentration of the essential oils in the preparations should be 6.57 %w/w. Three batches of the microcapsules were prepared and limonene concentration present in each batch was evaluated (**Table B3**). The microcapsules from 3 batches were combined. The amount of limonene in 1 g of microcapsules was calculated to be 0.26±0.01 %w/w. To obtain 4.67 %w/w of limonene in the preparations, theoretical loading concentration of microcapsules containing essential oils should be 17.96 %w/w.

The selected emulgel formulation E26 was prepared with various concentrations of essential oils (7-22 %w/w) and microcapsules containing essential oils (18-28 %w/w) as shown in formulation E29-E38 (**Table 7**). The emulgels containing essential oils or microcapsules of essential oils were white and milky cream with lemon-like odor. However, the emulgels containing microcapsules of essential oils gave weaker smell that the emulgels containing essential oils. **Figure 23** below show the calcium alginate microcapsules dispersed in the emulgel under the light microscope.

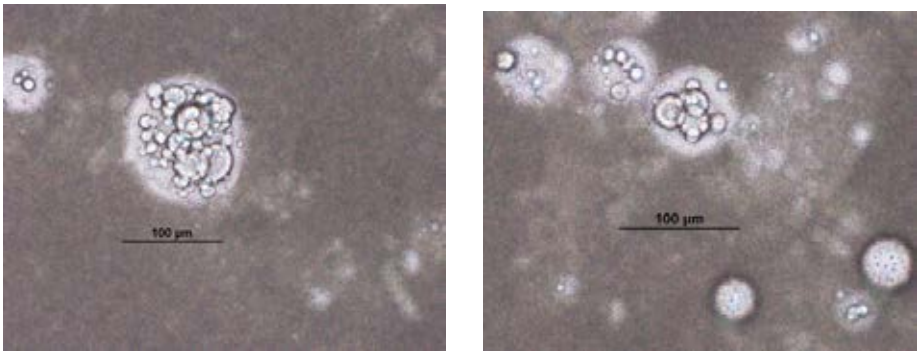


Figure 23 Calcium alginate microcapsules dispersed in the emulgel; magnification x 10

Besides, emulgel formulations E29-E35 were prepared by dissolving BHT in the essential oils to prevent the oxidation of limonene. In emulgel formualtions E36-E38 containing microcapsules of essential oils, BHT concentrated in Arlamol HD was directly added into the emulgel formulations. In addition, emulgel formlations E36 and E37, vanillin, a fixative, was added in order to investigate effects of a fixative on mosquito repellent efficacy.

3.5.2.1 Determination of limonene concentration in emulgel

In this study, concentrations of essential oils or microcapsules of essential oils were varied in a range of 7-22 %w/w (E29-E33, **Table 7**) or 18-28 %w/w (E36-E38, **Table 7**), respectively. Limonene present in each formulation was analyzed, calculated and illustrated as percentage of limonene (**Figure 24, A and B**).

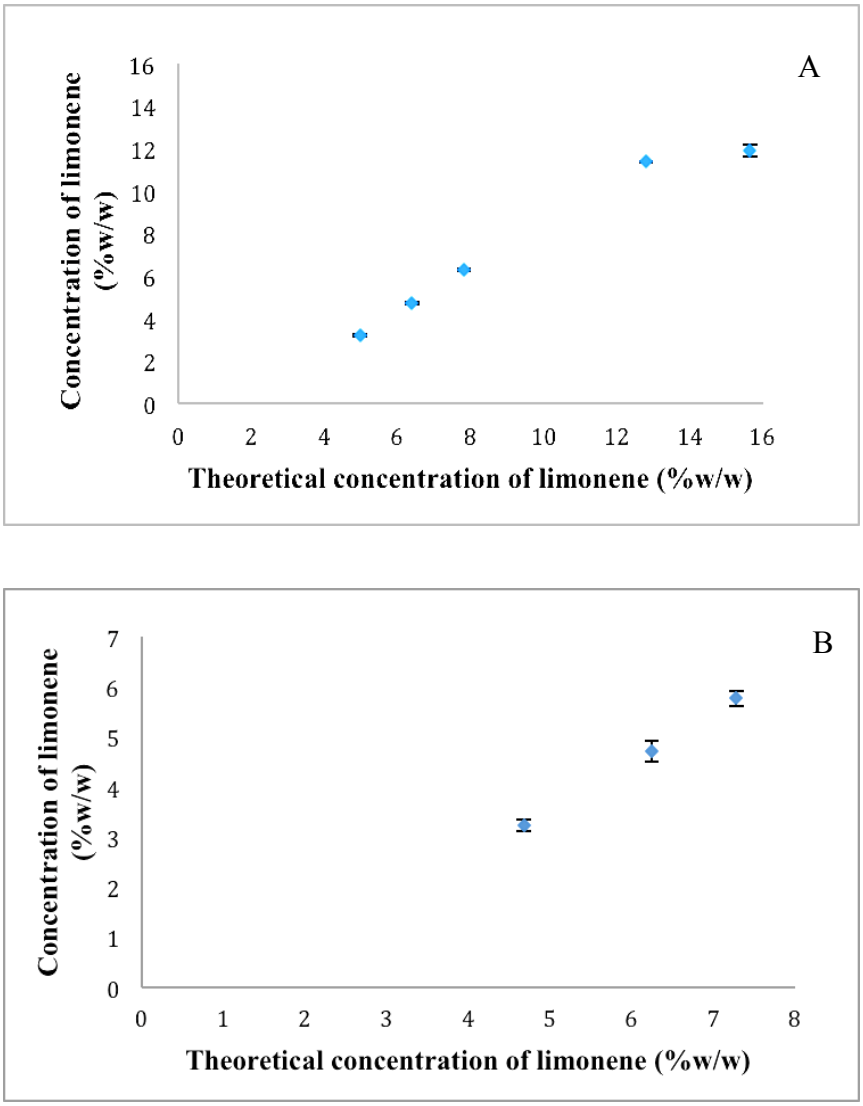


Figure 24 Concentration of limonene found in emulgel preparations containing essential oils (**A**) and microcapsule essential oils (**B**)

Relative limonene content with respect to theoretical amount of limonene (analytical recovery) in emulgel E29-E33 was in a range of 64-88%. The amount of limonene increased when the addition of essential oils was increased and then leveled off at the higher concentration of essential oils (18-22 %w/w) as shown in **Figure 24, A**. Limonene in the emulgel containing microcapsules was in the range of 70-80% of

its theoretical value. The amount of limonene increased when more microcapsules essential oils were added into the emulgel **Figure 24, B**.

Essential oils including limonene were volatile substances; thus, loss of these compounds was obvious even under mild preparation conditions such as at room temperature. For example, formulations E30 and E37 where theoretical concentration of limonene should be 6.40 and 6.24 %w/w, respectively, the limonene concentration found in these two preparations were 4.69 and 4.71 %w/w, respectively. In other words, 26.72 and 24 percent of limonene was lost during the preparation process. Although the percentage loss of limonene during the preparation process was not obvious, this finding suggested that encapsulation of volatile substances could prevent volatility of these compounds.

The emulgel preparation E30, E32, E34, E35 and E37 (**Table 7**) were selected because they contained around 4.69-11.43 %w/w of limonene. These preparations were continued for the physical and chemical stability study and their repellency against *Ae. aegypti*.

3.5.2.2 Physical stability evaluation of emulgels

The selected emulgel preparations E30, E32, E34, E35 and E37 (**Table 7**) were centrifuged at 6000 rpm for 30 minutes for several cycles at room temperature. All the emulgel preparations exhibited a proper resistance to centrifugation until 8 cycles. No creaming and phase separation behavior were observed after each cycle of centrifugation.

Moreover, The selected emulgels were further assessed their stability under heating cooling temperature cycle, at 40˚C for 48 hours and 4˚C for 48 hours, for 6 cycles. All the emulgel preparations were stable because without color and odor changes or phase separation. The pH and viscosity of emulgel preparations, E30, E32, E34, E35 and E37, were constant as shown in **Figure 25 and 26**.

Viscosity values of each preparation were constant throughout 6 heating cooling cycles. The pseudo-plastic flow behavior of emulgel preparations was seen throughout the study (**Figure 27 and 28)**. Steady-state rheological measurements revealed that all the investigated preparations exhibited shear-thinning behavior since the apparent viscosity decreases with an increase in shear rate during heating cooling cycle test. Increasing in shear stress could generally disarrange the molecules of the gelling materials to align themselves in the flow direction. This arrangement reduces the resistance of gelling network to the shear stress.

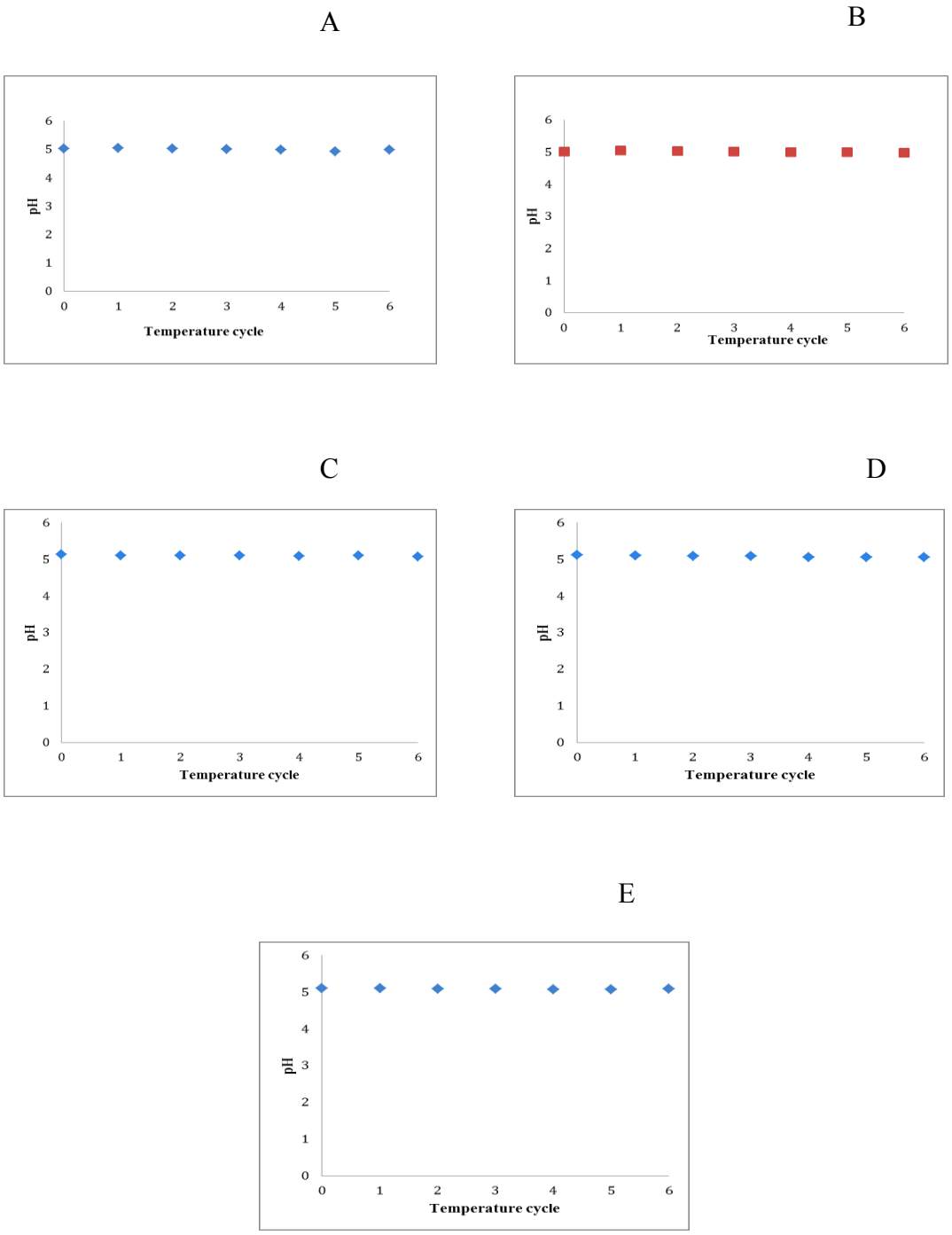


Figure 25 The pH of the formulations; E30 (A), E32 (B), E34 (C), E35 (D) and E37 (E) during heating-cooling cycle

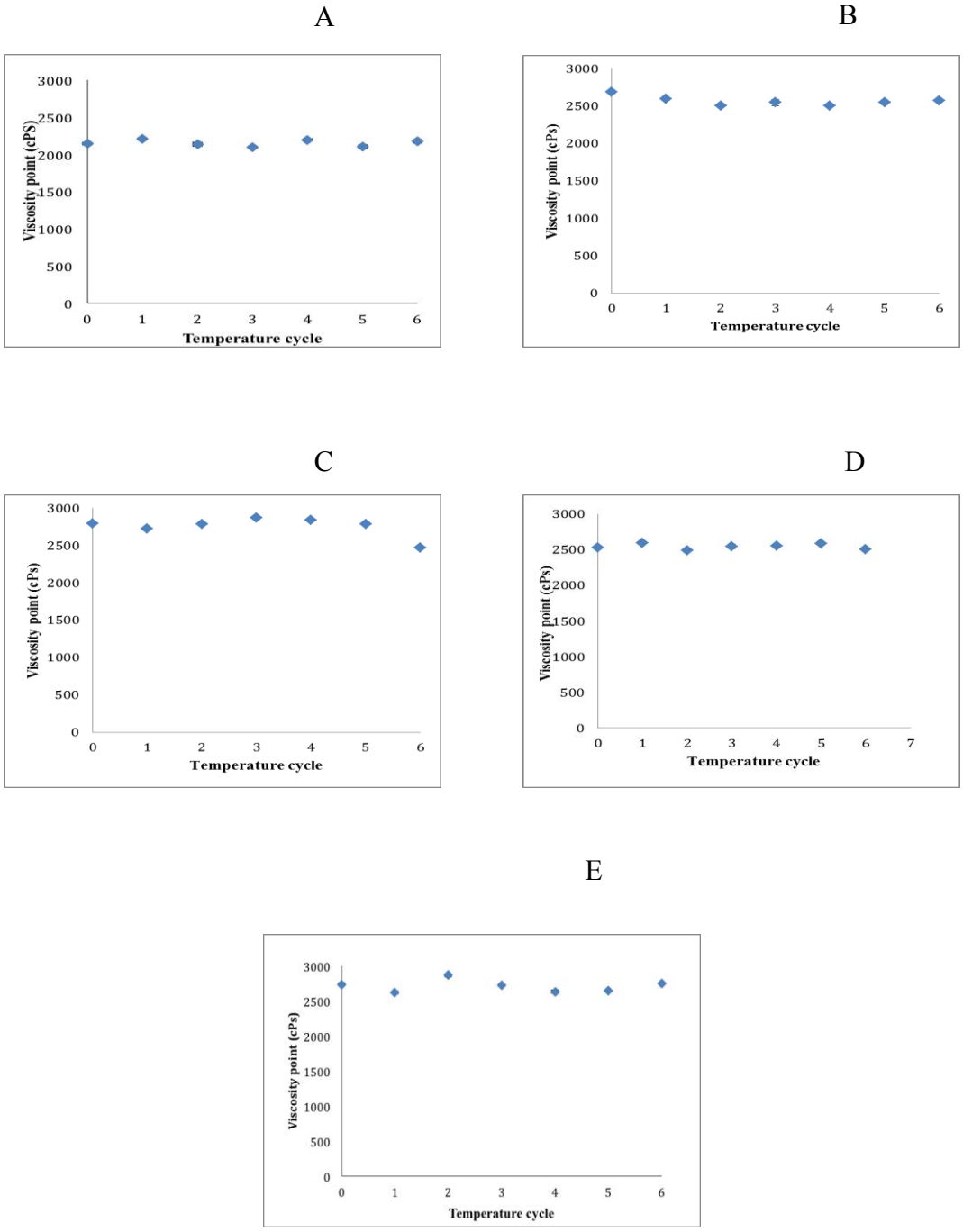
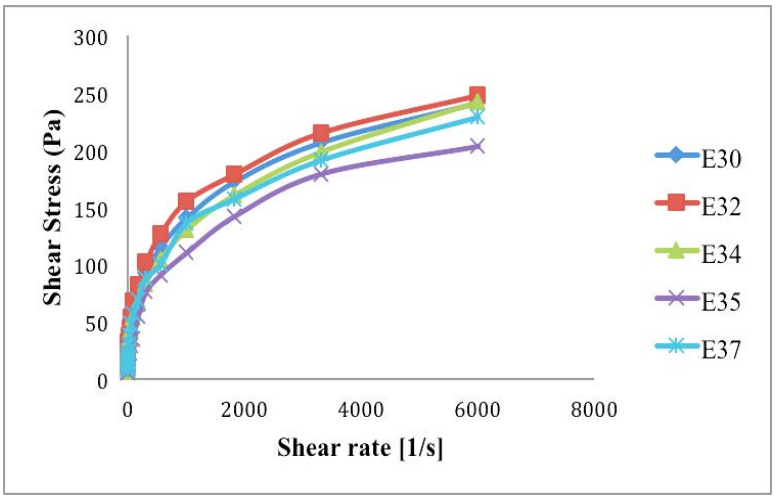


Figure 26 The viscosity of the formulations; E30 (A), E32 (B), E34 (C), E35 (D) and E37 (E) during heating-cooling cycle

A



B

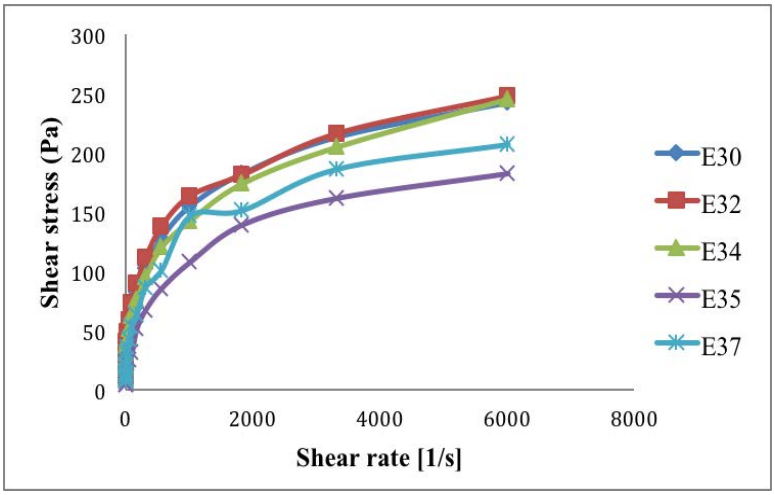


Figure 27 The rheograms of the emulgel formulations at heating cooling cycle 0 (A) and cycle 6 (B)

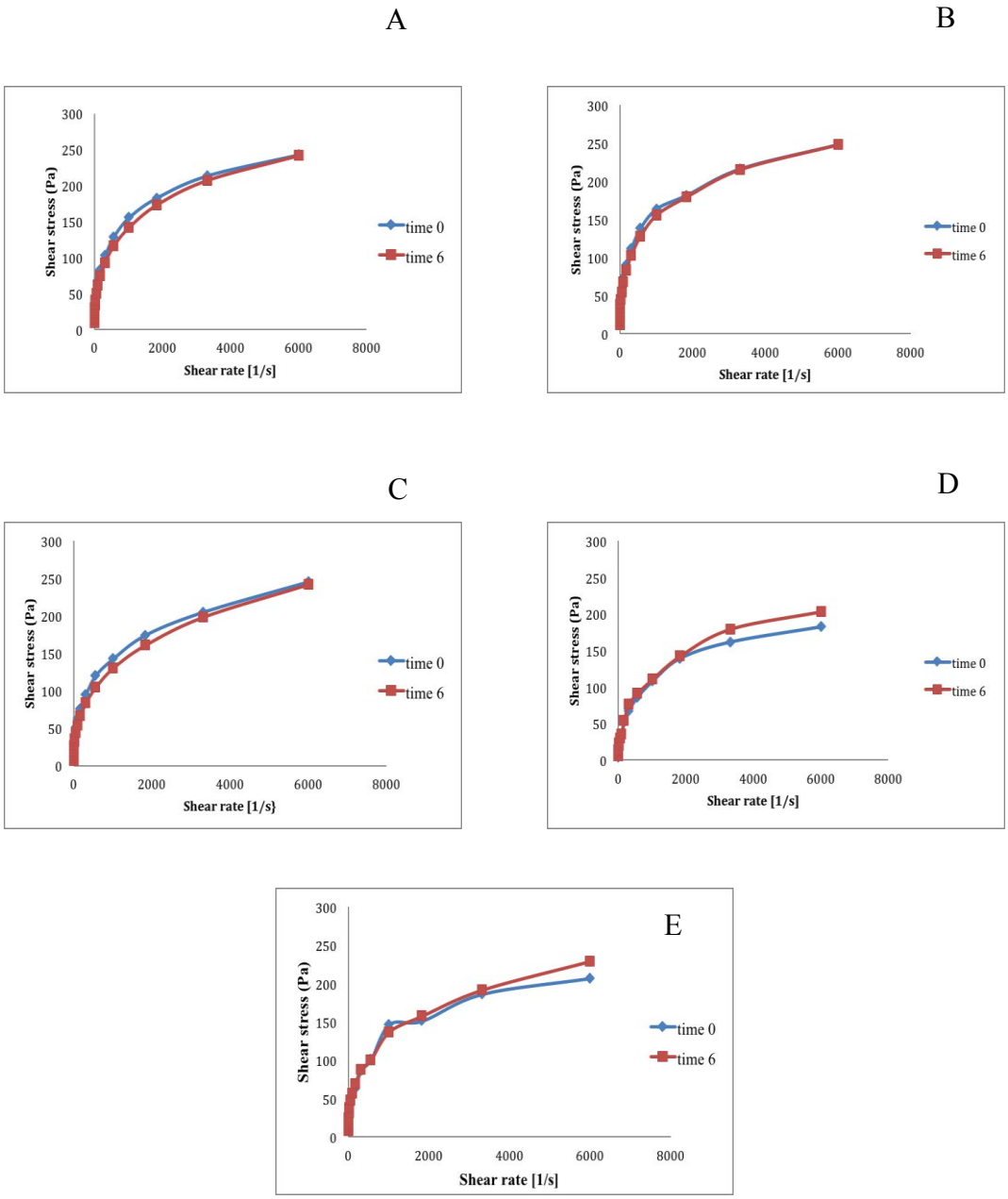


Figure 28 The rheograms of the formulations; E30 (A), E32 (B), E34 (C), E35 (D) and E37 (E) at heating cooling cycle 0 and cycle 6

3.5.2.3 Repellency efficacy test of emulgels containing *Zanthoxylum limonella* essential oils against *Ae. aegypti*

The mosquito repellent efficacy of emulgels containing *Zanthoxylum limonella* essential oils were investigated using the human-bait technique. The volunteers, age in range of 25-61 years, participated in the laboraroty tests where 3-5 volunteers were tested on emulgel products and one volunteer was a standard control using 15% IR3535 in ethanol solution. The emulgel preparations E30, E32, E34, E35 and E37; and emulgel preparation containing standard limonene were prepared for repellency test.

Limonene present in each emulgel preparation was analysed by GC-FID method before testing on mosquito. The percentage of limonene in all submitted emulgel preparation were in the range of 4.68-11.49 %w/w (**Table 10**).

The emulgel preparations were randomly applied to the area on the forearm of each volunteer. The protection time was collected as the time between application of tested emulgel sample and the second mosquito bite. An average protection hour was computed from the data obtained from each volunteer and tabulated (**Table 10**).

According to the test criterion, a qualified mosquito repellent must provide an average protection time of at least 2 hours against *Ae aegypit*. The only E35 was complied with the test criterion with an average protection time around 3.1-3.4 hours. E35, which contained 18 %w/w essential oils and 5 %w/w vanillin, provided the protection hours of 3.1±1.34 hours on the right hand side and 3.4±1.19 hours on the left hand side, respectively.

The results depicted that the average protection time was prolonged with higher concentration of essential oils. It was found out that the average protection time of *Zanthoxylum limonella* essential oils was longer than that of standard limonene where both emulgel preparations contained approximately similar concentration limonene. It might be due to the additive property of other active constituents in the essential oils that can result in longer protection time. The essential oils constituents such as α-pinene, sabinene and β-cymene were the commonly seen chemicals in the essential oils possessing mosquito repellent efficacy (Nerio et al., 2010).

Moreover, the efficacy of the emulgel formulation was imporved by the addition of vanillin which is well-known fixative material in mosquito repellent products (Tawatsin et al., 2001; Choochote et al., 2007). As also shown in this study, the addition of 5% vanillin into emulgel preparation containing 9% and 18% essentail oils greatly improved the protection time.

In the case of emulgel preparation containing microcapsules of essential oils, large amount of microcapsules were prepared in order to reach the loading amount of limonene in the emulgel. Emulgel preparation containing microcapsules of essential oilsdid not show repellency efficacy.

Table 8 Average protection time of emulgel product against *Ae. aegypti* mosquitoes on volunteers.

Formulations	Ingredients (%w/w)	Amount of limonene (%w/w)	Protection time (hours)	Volunteers	Hand side
E30	essential oils (9%)	4.69	0	n = 3	right hand
E32	essential oils (18%)	11.43	1.7±1.03	n = 5	right hand
E34	essential oils (9%) + vanillin (5%)	4.68	0.5	n = 1	right hand
E35	essential oils (18%) + vanillin (5%)	11.44	3.1±1.34	n = 5	right hand
E35	essential oils (18%) + vanillin (5%)	11.44	3.4±1.19	n = 5	left hand
E37	microcapsules essential oils (24%)	4.71	0	n = 3	right hand
Emulgel	Standard limonene	11.49	1.1±0.89	n = 5	right hand
Positive control	15% IR3535	-	7	n = 1	right hand
Positive control	15% IR3535	-	7	n = 1	left hand

3.5.2.4 Chemical stability evaluation of emulgel

In this study, the long-term and accelerated stability tests were carried on the selected emulgel preparation E35 because it was the only preparation showing repellency efficacy complied with the test criterion. The emulgel preparation was filled in the glass bottle and sealed with aluminum cap. Then the preparation was kept at 30˚C, 75 RH and 40˚C, 75 RH for 12 weeks according to Asean Guideline on Stability Study of Drug Product (9th ACCSQ-PPWG Meeting, Philippines, 2005).

The amount of limonene in the emulgel was determined by GC-FID method at the determined time interval. GC chromatograms **Figure 29 and 30** showed that other ingredients in the formulation did not interfere with limonene peak. In addition, peak with a retention time of 14.49 minutes was assigned to be vanillin after comparison with the retention time of standard vanillin under the same analytical conditions. The percentage remaining of limonene was reported as shown in **Figure 31**. The percentage remaining of limonene was in the range of 100 – 95.54 %w/w after 8 weeks of study at both 30˚C and 40˚C. However, it was markedly decreased to 93.17 %w/wat 30˚C and 86.58 w/w at 40˚C after 12 weeks of storage. The percentage remaining of vanillin was also calculated based on the peak area. At 30˚C, the percentage remaining of vanillin was in the range of 100 – 97.05 %w/w after 8 weeks and it decreased to 94.86 w/w after 12 weeks. However, at 40˚C, the percentage remaining of vanillin was in the range of 100 – 74.58 %w/w throughout the studies.

Therefore, emulgel preparation containing essential oils and vanillin was stable for 2 months at 30˚C. At the higher temperature, the loss of limonene and vanillin markedly increased.

The physical characteristic such as color, odor, homogeneity, consistency, and phase separation were inspected at each time point. There were no changes in physical properties of emulgel preparation for 12 weeks. Moreover, pH values and viscosity point were monitored over a period of 12 weeks and also remained unchanged as shown in **Figure 32**.

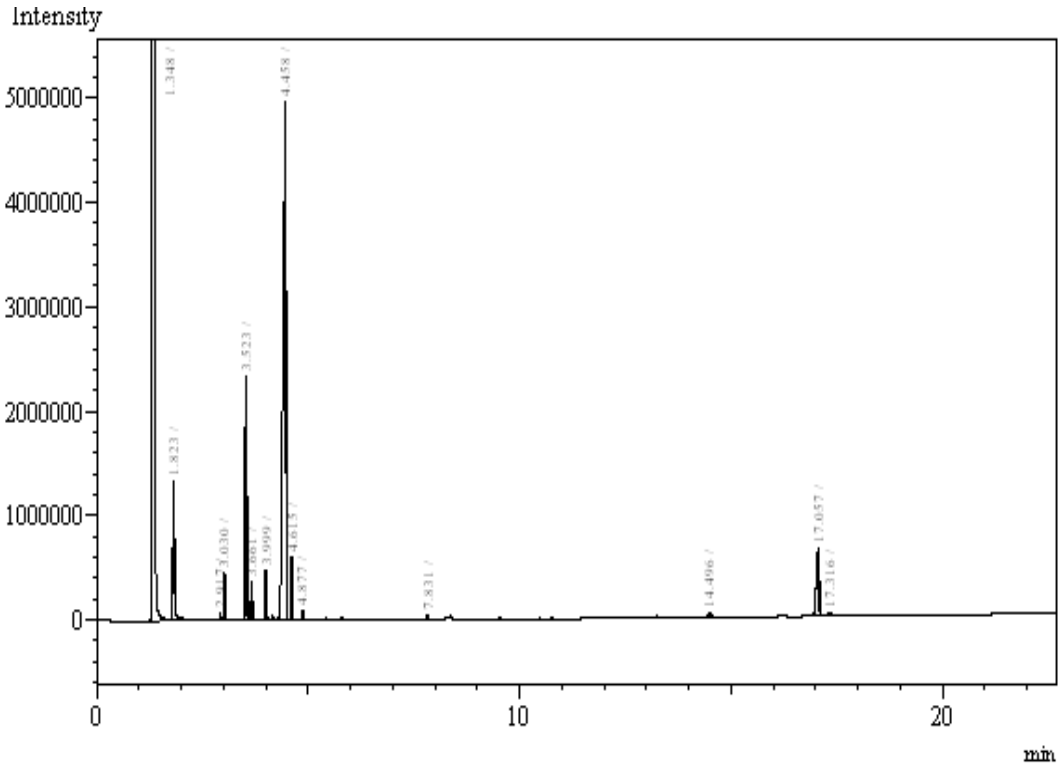


Figure 29 GC chromatogram of emulgel containing essential oils and vanillin

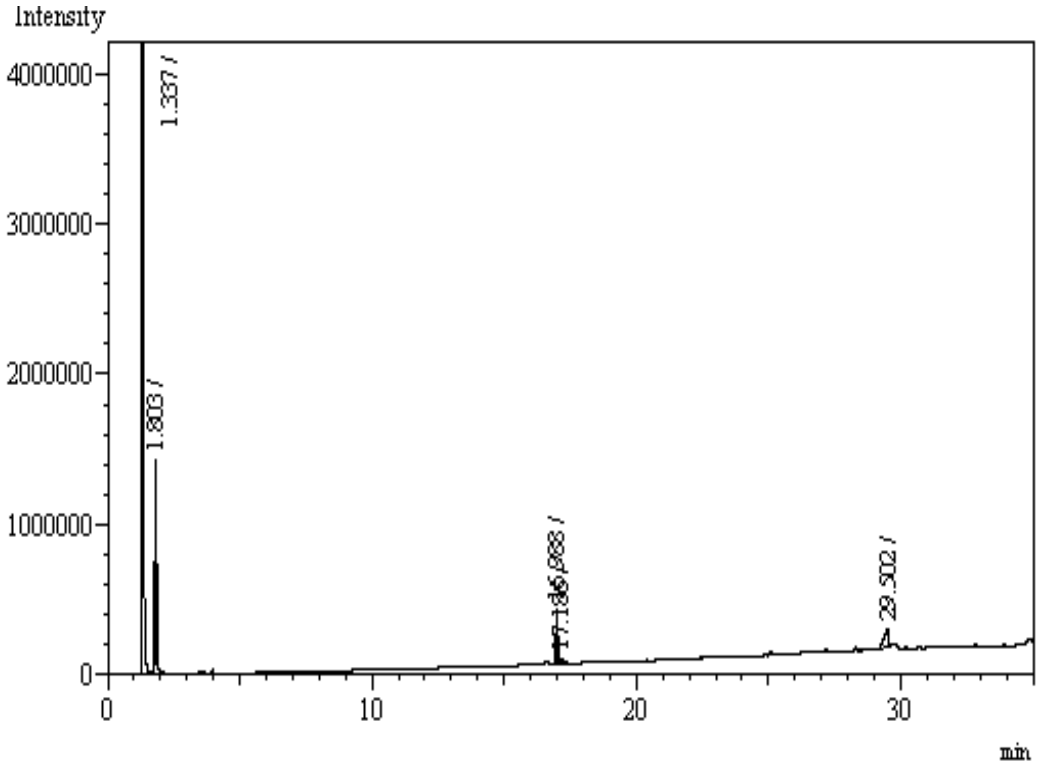


Figure 30 GC chromatogram of blank formulation

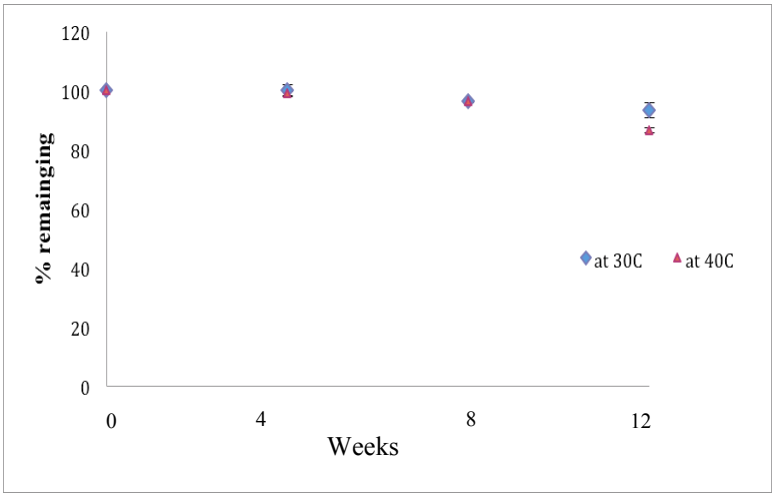
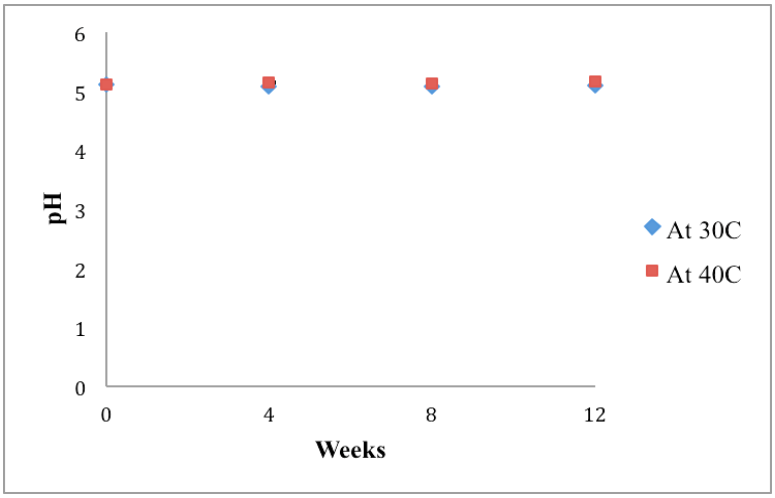
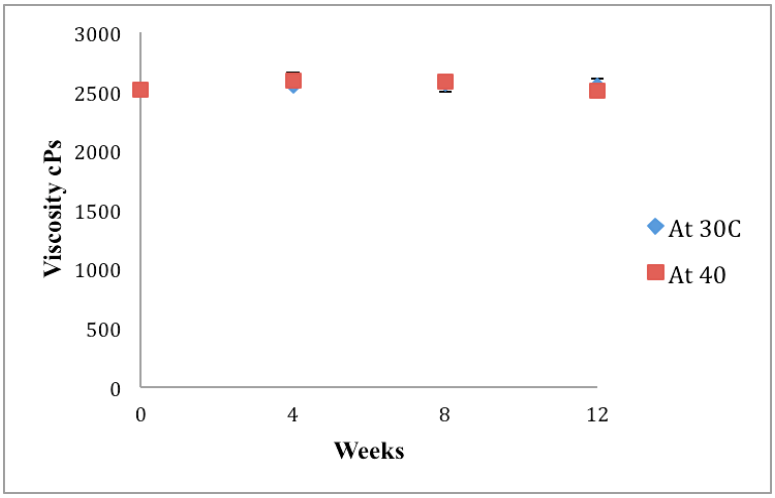


Figure 31 Percentage of remaining limonene in the preparation of E35 over 12 weeks after stage at 30˚C and at 40˚C, 75% RH (n=3)



A



B

Figure 32 pH (**A**) and viscosity (**B**) of the emulgel formulation E35 over 12 weeks after storage at 30˚C and at 40˚C, 75 % RH (n=3)

So far, further experiments are necessary to complement this study. The efficient production method for microcapsules containing essential oils is required in order to imporve stabilities and repellency efficacy of microcapsules containing essential oils. Microcapsules' stabilities may be imporved by combination or changing biopolymers.The stability of limonene in the emulgel preparation may be increased by keeping the emulgel preparation under mild conditions such as at 25˚C or at refrigerated temperature. Tight single used container such as sachet should be

used for this preparation. During repellency testing, essential oils containing emulgel preparations did not show any skin rash or irritation on human volunteers. However, it is still necessary to carried out a proper skin irritation test on human volunteers.

CHAPTER V

CONCLUSION

The extraction of essential oils from *Zanthoxylum limonella* dried fruit clusters was performed by the steam distillation method. The obtained essential oils were characterized for their volatile constituents using GC-MS. The amount of limonene was further quantified by using GC-FID. The essential oils were prepared into microcapsules and emulgels preparation. The emulgel preparations containing microcapsules of essential oils and free essential oils were further investigated for physical stability and repellency efficacy against *Ae. aegypti*. The emulgel preparation showing repellency efficacy which complied with test criterion was subjected to chemical stability test.

Conclusively, *Zanthoxylum limonella* essential oils were successfully extracted. The percentage yield of essential oils from the batch 2 was higher than that of batch 1. GC-MS results showed that the essential oils composed of five main peaks where limonene was found as a major compound. According to the GC-FID results, the concentration of limonene from batch 2 was higher than that from batch 1.The essential oils obtained from batch 2 were prepared as microcapsules. The alginate microcapsules containing essential oils were formed with sodium alginate (1.5 %w/w), Span 20 (3 %w/w) and essential oils (20 %w/w). The microcapsules of essential oils and essential oils were separately added into emulgel preparation. The emulgel preparations containing jojoba oil, CremophorRH 40 and Carbopol 940 provided better appearance, more pleasant skin feeling and physically stable. The

emulgel preparation containing 18% of essential oils and 5% of vanillin, a fixative, showed 3.1-3.4 hours of protection time against *Ae. aegypti*. The emulgel preparation was chemically stable for 8 weeks at 30˚C. The emulgel containing microcapsules of essential oils product has commercially potential, however; the further development on microencapsulation process need to be done in order to improve the product efficacy and stability.

References

Abraham, G. J. and Agshiker, N. V. Anti-inflammatory activity of an essential oil from Zanthoxylum budrunga. Pharmacology 7 (1972) : 109-14.

Agshiker, N. V. and Abraham, G. J. 1972. Pharmacology and acute toxicity of essential oil extracted from Zanthoxylum budrunga. Indian Journal of Medical Research 60 (1972) : 757-62.

Agshiker, N. V., Sehgal, V. N., Rege, V. L. and Abraham, G. J. Topical anti-inflammatory effect of oil from Zanthoxylum budrunga in inflammatorydermatoses, a preliminary report. Indian Practitioner 25 (1972) : 175.

Amer, A. and Mehlhorn, H. Repellency effect of forty-one essential oils against Aedes, Anopheles, and Culex mosquitoes. Parasitology research 99(4) (2006) : 478-490.

Beasley, D. W. C., Barrett, A. D. T., In: Halstead, S. B., (Ed.), Dengue (tropical Medicine; Science and Practice) Imperial College Press, London (5) 2008.

Bunker, C. W and Hirschfelder, A. D. Mosquito repellents From the U.S. Naval Medical School, Washington, D.C., and the Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota (1925) : 359-384.

Chang, C. P. and Dobashi, T. Preparation of alginate complex capsules containing eucalyptus essential oil and its controlled release. Colloids and Surfaces B: Biointerfaces 32 (2003) : 257-262.

Chan, L. W., Lim, L. T., &Heng, P. W. S. Microencapsulation of oils using sodium alginate. Journal of Microencapsulation 17(6) (2000) 757-766.

Charoenying, P., Laosinwattana, C., Phuwiwat, W. and Lomratsiri, J. Biological activities of Zanthoxylum limonella Alston fruit extracts. KMITL Science Journal 8(1) (2008) : 12-15.

Charoenying, P., Teerarak, M. and Laosinwattana, C. An allelopathic substance isolated from *Zanthoxylum limonella* Alston fruit. Scientia Horticulturae 125(3) (2010) : 411-416.

Choochote, W., Chaithong, U., Kamsuk,U., Jitpakdi, A., Tippawangkosol, P., Tuetun, B.,Champakaew, D., and Pitasawat, B. Repellent activity of selected essential oilsagainst *Aedes aegypti*. Fitoterapia 78(5) (2007) : 359-364.

Christensson, J. B., Johansson, S., Hagvall, L., Jonsson, C., Börje, A. and Karlberg, A. T. Limonene hydroperoxide analogues differ in allergenic activity. Contact Dermatitis 59(6) (2008) : 344-352.

Christophers S.R. Mosquito repellent, Being a report of the work of the mosquito repellent.

Chuah, A. M., Kuroiwa, T., Kobayashi, I., Zhang, X. and Nakajima, M. Preparation of uniformly sized alginate microspheres using the novel combined methods of microchannel emulsification and external gelation. Colloids and Surfaces A:Physicochemical and Engineering Aspects 351(1) (2009) : 9-17.

Das, N. G., Baruah, I., Talukdar, P. K. and Das, S. C. Evaluation of botanicals as repellents against mosquitoes. Journal of Vector Borne Diseases 40 (2003) : 49-53.

Davis, E.E. and Sokolove, P.G. Lactic acid-sensitive receptors on the antennae of the mosquito, *Aedes aegypti*. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 105(1) (1976) : 43-54.

Debboun, M., Frances, S. P., Strickman, D., (Ed.), Insect Repellents : Principles, Methods, and Uses. CRS Press, Taylor & Francis Group (1) 2006.

Djordjevic, D., Cercaci, L., Alamed, J., Mcclements, D. J. and Decker, E. A.Chemical and physical stability of protein- and gum arabic- stabilized oil-in-water emulsions containing limoene. Journal of Food Science 73 (2008) : C167-C172.

Handa, S.S., Khanuja, S.P.S., Longo, G. and Rakesh, D.D. Extraction Technologies for Medicinal and Aromatic Plants. Chapter 7 : Distillation Technology for Essential oils (2008)

Hink, W. F. and Fee, B. J. Toxicity of D-limonene, the major component of citrus peel oil, to all life stages of the cat flea, Ctenocephalides felis (Siphonaptera: Pulicidae). Journal of Medical Entomology 23(4) (1986) : 400-404.

Islam, A., Sayeed, A., Bhuiyan, M. S. A., Mosaddik, M. A., Islam, M. A. U. and Astaq Mondal Khan, G. R. M. Antimicrobial activity and cytotoxicity of *Zanthoxylum budrunga*. Fitoterapia 72(4) (2001) : 428-430.

Itthipanichpong, C., Rugangrungsi, N. and Pattanaautsahakit, C. Chemical compostion and pharmacological effect of essential oil from the fruit of Zanthoxylum limonella. Journal of The Medical Association of Thailand 85 (Suppl 1) (2002) : S344-S354.

Karr, L. L. and Coats, J. R. Insecticidal Properties of d-Limonene. Journal of Pesticide Science 13 (1987) : 287-290.

Khullar, R., Kumar, D., Seth, N. and Saini, S. Formulation and Evaluation of Mefenamic Acid Emulgel for Topical Delivery. Saudi Pharmaceutical Journal 20 (2012) : 63-67.

Koul, R., Walia, H. and Dhaliwal, G. S. Essential oils as green pesticides: potential and constraints. Biopestic Int 4(1) (2008) : 63-84.

Kyle, J. L. and Harris, E. Global spread and persistence of dengue. Annu. Rev. Microbiol 62 (2008) : 71-92.

Lertsutthiwong, P., Noomun K., Jongaroonngamsang N., Rojsithisak P. and Nimmannit U. Preparation of alginate nanocapsules containing turmeric Oil. Carbohydrare Polymers 74(2) (2008) : 209-14.

Liu, X. D., Yu, W. Y., Zhang, Y., Xue, W. M., Yu, W. T., Xiong, Y. and Yuan, Q. Characterization of structure and diffusion behaviour of Ca-alginate beads prepared with external or internal calcium sources. Journal of Microencapsulation 19(6) (2002) : 775-782.

Liu, X., Xue, W., Liu, Q., Yu, W., Fu, Y., Xiong, X. and Yuan, Q. Swelling behaviour of alginate–chitosan microcapsules prepared by external gelation or internal gelation technology. Carbohydrate Polymers 56(4) (2004) : 459-464.

Maheswaran, R. and Savarimuthu I. A novel herbal formulation against dengue vector mosquito *Aedes aegypti* and *Aedes albopictus*. Parasitology Research 110(5) (2012) : 1801-1813.

Maji, T. K., Baruah, I., Dube, S. and Hussain, M. R. Microencapsulation of Zanthoxylum limonella oil (ZLO) in glutaraldehyde crosslinked gelatin formosquito repellent application. Bioresource Technology 98 (1998) : 840-844.

Marquardt, D. and Sucker, H. Oil-in-water-emulsion gels: determination and mathematical treatment of flow properties. European Journal of Pharmaceuticsand Biopharmaceutics 46 (1998) : 115-124.

Mohamed, M. I. Optimization Of Chlorphenesin Emulgel Formulaiton. The AAPS Journal 6(3) (2004) : 1-7.

Moore W. Esters as repellents. New York Entomological Society 42(2) (1934) : 185-192 inquiry, Cambridge. Zoological Laboratory, University of Cambridge (1943-5) : 176-232.

Moretti, M., Sanna-Passino, G., Demontis, S. and Bazzoni, E. Essential oilformulations useful as a new tool for insect pest control. AAPS PharmSciTech 3 (2002) : 64-74.

Naucke, T. J., Kropke, R., Benner, G., Schulz, J., Wittern, K.P., Rose, A. and Grunewald, H.W. Field evaluation of the efficacy of proprietary repellent formualtions with IR3535® and Picaridin against *Aedes aegypti*. Parasitology Research 101(1) (2007) : 169-177.

Nayak, K. P. and Dutta, N. K. Role of essential oils and allied drug in experimental cholera of rabbit. Indian Journal of Medical Research 49 (1961) : 51-4.

Nerio, L. S., Olivero-Verbel, J. and Stashenko, E. Repellent activity of essential oils: a review. Bioresource Technology 101(1) (2010) : 372-378.

Nilsson, U., Bergh, M., Shao, L. P. and Karlberg, A. T. Analysis of contact allergenic compounds in oxidized d-limonene. Chromatographia 42(3-4) (1996) : 199-205.

Paluch, G., Bartholomay, L. and Coats, J. Mosquito repellents: a review of chemical structure diversity and olfaction. Pest Management Science 66(9) (2010) : 925-935.

Pitasawat, B., Champakaew, D., Choochote, W., Jitpakdi, A., Chaithong, U., Kanjanapothi, D., Rattanachanpichai, E., Tippawangkosol, P., Riyong, D., Tuetun, B. and Chaiyasit, D. Aromatic plant-derived essential oil: An alternative larvicide for mosquito control. Fitoterapia 78 (2007) : 205-210.

Qiu, H., McCall, J. W. and Won Jun, H. Formulation of topical insect repellent N, N-diethyl-m-toluamide (DEET): vehicle effects on DEET in vitro skin permeation. International Journal of Pharmaceutics 163(1) (1998) 167-176.

Rahman, M. T., Alimuzzaman, M., Ahmad, S. and Chowdhury, A. A. Antinociceptive andantidiarrhoeal activity of Zanthoxylum rhetsa. Fitoterapia 73(4) (2002) : 340-342.

Rahman, M., Islam, M., Khondkar, P. and Gray, A. I. Alkaloids and lignans from *Zanthoxylum budrunga* (Rutaceae).Biochemical systematics and ecology 33 (1) (2005) : 91-96.

Shahin, M., Hady, S. H., Hammad, M. and Mortada, N. Novel Jojoba oil Based Emulsion Gel Formulation For Clotrimezole Delivery. AAPS PharmaSciTech 12 (2011) : 135-147.

Song, J., Wang, Z., Findlater, A., Han, Z., Jiang, Z., Chen, J. and Hyde, S. Terpenoid mosquito repellents: A combined DFT and QSAR study. Bioorganic & medicinal chemistry letters 23(5) (2013) : 1245-1248.

Tawatsin, A., Wratten, S. D., Scott, R. R., Thavara, U. and Techadamrongsin, Y. Repellency of volatile oils from plants against three mosquito vectors. Journal of vector ecology 26 (2001) : 76-82.

Tawatsin, A., Asavadachanukorn, P., Thavara, U., Wongsinkongman, P., Bansidhi, J., Boonruad, T. and Mulla, M. S. Repellency of essential oils extracted from plants in Thailand against four mosquito vectors (Diptera: Culicidae) and oviposition deterrent effects against *Aedes aegypti* (Diptera: Culicidae) 2006.

Tangjitjaroenkun, J., Supabphol, R. and Chavasiri, W. Antioxidant effect of *Zanthoxylum limonella* Alston. Journal of Medicinal Plants Research (2012) : 1407-1414.

Tiwary, M., Naik, S. N., Tewary, D. K., Mittal, P. K. and Yadav, S. Chemical compostion and larvicidal activities of the essential oil of Zanthoxylum armatum DC (Rutaceae) against three mosquito vectors. Journal of Vector Borne Diseases 44 (2007) : 198-204

Tripathi, A. K., Upadhyay, S., Bhuiyan, M. and Bhattacharya, P. R. A review on prospects of essential oils as biopesticide in insect-pest management. Journal of Pharmacognosy and Phytotherapy 1(5) (2009): 052-063.

Trongtokit, Y., Rongsriyam, Y., Komalamisra, N., Krisadaphong, P. and Apiwathnasorn, C. Laboratory and field trial of developing medicinal local Thai plant products against four species of mosquito vectors. Southeast Asian Journal of Tropical Medicine and Public Health 35 (2004) : 325-33.

Trongtokit, Y., Rongsriyam, Y., Komalamisra, N. and Apiwathnasorn, C. Comparative repellency of 38 essential oils against mosquito bites. Phytotherapy Research 19 (4) (2005) : 303-309.

oo, S. H., Song, . B., Chang, P. S. and e e, H. G. Microencapsulation of α-tocopherol using sodium alginate and its controlled release properties. International Journal of Biological Macromolecules 38(1) (2006) : 25-30.

APPENDIX

Appendix A Preparation of standard curve of limonene

Table A1 Peak area of standard limonene at various concentrations

Concentrations	Area	Retention time (min)
16000	24862502	4.50
8000	12698719	4.46
4000	6519823	4.43
2000	3245804	4.40
500	794608	4.38

Appendix B Preparation of microencapsulated *Zanthoxylum limonella* essential oils

Table B1 Weight of vacuum dry microcapsules during drying

Time (hour)	Batch 1 (%w/w)	Batch 2 (%w/w)	Batch 3 (%w/w)	Mean	SD
0	7.512	7.876	7.657	7.682	0.183
1	6.769	6.109	6.946	6.608	0.441
2	6.108	6.101	6.217	6.142	0.065
3	4.925	4.911	4.979	4.938	0.036
4	4.919	4.908	4.977	4.935	0.037
5	4.901	4.898	4.962	4.920	0.036
6	4.879	4.871	4.961	4.904	0.050

Table B2 Percentage yield of calcium alginate microcapsules containing essential oils

	Weight of microcapsules (g)	Weight of ingredients (g)	% Yield
Batch 1	5.09	7.35	69.23
Batch 2	4.73	7.35	64.39
Batch 3	5.02	7.35	68.24
Mean ± SD	4.95±0.19		67.29±2.56

Table B3 Percentage encapsulation efficiency of calcium alginate microcapsules

	Weight of microcapsule (g)	**Amount of limonene (%w/w)**	**Theoretical amount of limonene (%w/w)**	**Amount of limonene in 1 g of microcapsule (%w/w)**	**% EE**
Batch 1	5.09	1.27	4.26	0.25	29.81
Batch 2	4.73	1.21	4.26	0.26	28.40
Batch 3	5.02	1.35	4.26	0.27	31.68
Mean ± SD	4.95±0.19	1.28±0.07	-	0.26±0.01	29.96±1.65

Appendix C Development of emuglel preparations containing microcapsules of essential oils or essential oils

Table C1 Limonene concentration in the emulgels containing essential oils

	E29	**E30**	**E31**	**E32**	**E33**
Batch 1	3.13	4.79	6.27	11.29	11.51
Batch 2	3.29	4.63	6.23	11.47	11.84
Batch 3	3.19	4.64	6.35	11.52	11.62
Mean±SD	3.20±0.08	4.69±0.09	6.28±0.06	11.43±0.12	11.66±0.17

Table C2 Limonene concentration in the emulgels containing microcapsules of essential oils

	E36	**E37**	**E38**
Batch 1	3.11	4.95	5.93
Batch 2	3.29	4.67	5.74
Batch 3	3.31	4.52	5.63
Mean±SD	3.24±0.11	4.71±0.22	5.77±0.15

Appendix D Physical stability evaluation of emulgels

Table D1 pH of the emulgels (n = 3)

	Cycle 0	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
E30	5.01±0.01	5.04±0.01	5.02±0.01	5.01±0.02	4.97±0.01	4.93±0.01	4.98±0.04
E32	5.01±0.01	5.05±0.01	5.02±0.04	5.02±0.01	4.99±0.06	4.99±0.06	4.98±0.04
E34	5.13±0.02	5.10±0.03	5.10±0.04	5.10±0.02	5.08±0.02	5.10±0.01	5.07±0.04
E35	5.11±0.02	5.09±0.01	5.08±0.01	5.08±0.01	5.06±0.03	5.05±0.01	5.05±0.04
E37	5.10±0.02	5.09±0.02	5.08±0.01	5.08±0.01	5.06±0.03	5.07±0.02	5.09±0.01

Table D2 viscosity of the emulgels (n = 3)

	Cycle 0	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
E30	2142± 18.51	2210± 14.29	2132± 23.21	2098± 14.87	2195± 13.25	2101± 24.67	2176± 24.32
E32	2687± 22.61	2594± 22.98	2502± 19.72	2549± 42.72	2508± 12.56	2547± 24.12	2576± 13.45
E34	2797± 11.21	2729± 14.51	2785± 13.21	2871± 21.78	2838± 18.02	2791± 7.06	2472± 18.21
E35	2532± 12.02	2597± 21.02	2490± 18.67	2549± 32.12	2553± 14.24	2587± 17.81	2510± 21.53
E37	2732± 22.31	2619± 18.51	2865± 22.52	2722± 13.24	2631± 21.32	2646± 11.31	2745± 12.92

Appendix E Repellency efficacy test of emulgels containing *Zanthoxylum limonella* essential oils against *Ae. aegypti*

Table E1 Average protection time of emulgel product containing standard limonene against *Ae. aegypti* mosquitoes on right-hand side of volunteers

Volunteers	**1**	**2**	**3**	**4**	**5**
ProtectionTime (hours)	2.5	0.5	0.5	0.5	1.5
Mean±SD	1.1±0.89 hours				

Table E2 Average protection time of emulgel product containing 18% *Zanthoxylum limonella* essential oils against *Ae. aegypti* mosquitoes on right-hand sides on volunteers

Volunteers	**1**	**2**	**3**	**4**	**5**
ProtectionTime (hours)	0.5	1.0	1.5	3.0	2.5
Mean±SD	1.7±1.03 hours				

Table E3 Average protection time of emulgel product containing 18% *Zanthoxylum limonella* essential oils with polyethylene glycol and vanillin against *Ae. aegypti* mosquitoes on right-hand side of volunteers

Volunteers	**1**	**2**	**3**	**4**	**5**
ProtectionTime (hours)	1.5	4.5	2.5	2.5	4.5
Mean±SD	3.1±1.34 hours				

Table E4 Average protection time of emulgel product containing 18% *Zanthoxylum limonella* essential oils with polyethylene glycol and vanillin against *Ae aegypti* mosquitoes on left-hand side of volunteers

Volunteers	**1**	**2**	**3**	**4**	**5**
ProtectionTime (hours)	1.5	4.0	4.5	3.0	4.0
Mean±SD	3.4±1.19 hours				

Appendix F Chemical stability evaluation of emulgels

Table F1 Concentration of limonene in the preparation E35 over 12 weeks after storage at at 30˚C, 75 RH n 3)

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	11.42	11.49	11.01	10.76
Batch 2	11.49	11.33	11.03	10.51
Batch 3	11.42	11.46	10.77	10.71
Mean ± SD	11.44±0.05	11.43±0.08	10.94±0.14	10.66±0.13

Table F2 Concentrations of limonene in the preparation E35 over 12 weeks after storage at at 40˚C, 75 RH n 3)

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	11.41	11.17	10.75	9.75
Batch 2	11.49	11.45	11.09	9.93
Batch 3	11.42	11.33	10.95	10.04
Mean ± SD	11.44±0.05	11.32±0.14	10.93±0.17	9.91±0.15

Table F3 pH of the emulgel formulation E35 over 12 weeks after storage at 30˚C at 75 % RH (n=3)

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	5.12	5.09	5.07	5.1
Batch 2	5.11	5.08	5.09	5.09
Batch 3	5.14	5.1	5.07	5.09
Mean ± SD	5.12±0.02	5.09±0.01	5.08±0.01	5.09±0.01

Table F4 pH of the emulgel formulation E35 over 12 weeks after storage at 40˚C at 75 % RH (n=3)

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	5.12	5.14	5.11	5.17
Batch 2	5.11	5.14	5.13	5.15
Batch 3	5.14	5.17	5.15	5.16
Mean ± SD	5.12±0.02	5.15±0.02	5.13±0.02	5.16±0.01

Table F5 viscosity of the emulgel formulation E35 over 12 weeks after storage at 30˚C at 75 RH n 3)

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	2516	2532	2524	2518
Batch 2	2523	2564	2532	2511
Batch 3	2512	2552	2543	2516
Mean ± SD	2517±5.57	2549±16.17	2566±16.52	2548±18.71

Table F6 Viscosity of the emulgel formulation E35 over 12 weeks after storage at 40˚C, 75 RH n 3)

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	2516	2526	2598	2573
Batch 2	2523	2559	2562	2564
Batch 3	2512	2531	2583	2593
Mean ± SD	2517±5.57	2539±17.79	2581±18.08	2577±14.84

Table F7 Peak area of vanillin in the preparation E35 over 12 weeks after storage at 30˚C, 75 RH

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	224339	224934	217848	210505
Batch 2	232708	221289	223468	221881
Batch 3	230421	239718	225869	219786
Mean ± SD	229156± 4326	228647± 9759	222395± 4117	217390± 6054

Table F8 Peak area of vanillin in the preparation E35 over 12 weeks after storage at at 40˚C, 75 RH

	Week 0	**Week 4**	**Week 8**	**Week 12**
Batch 1	224339	199320	192929	169505
Batch 2	232708	218001	180642	174233
Batch 3	230421	209978	191972	168981
Mean ± SD	229156± 4326	209100± 9371	188514± 6834	170906± 2893

VITA

Ms. Thi Thi San, 5376852633, was born on January 9, 1987 in Yangon, Myanmar. She got her Bachelor's degree in Pharmacy from University of Pharmacy, Yangon, Myanmar, in 2009. She is continuing her master degree at Department of Pharmaceutics and Industrial Pharmacy, majoring in Pharmaceutical Technology (International) Program, Faculty of Pharmaceutical Sciences, Chulalongkorn University.