FORMULATION OF A REPELLENT LOTION FROM ESSENTIAL OILS OF *ALPINIA GALANGA, CITRUS GRANDIS* AND *CITRUS AURANTIFOLIA* AGAINST MOSQUITOES *AEDES AEGYPTI* AND *CULEX QUINQUEFASCIATUS*

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2018

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THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICNE
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ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Norashiqin Binti Misni

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Field of Study: Parasitology

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FORMULATION OF A REPELLENT LOTION FROM ESSENTIAL OILS OF *ALPINIA GALANGA*, *CITRUS GRANDIS* AND *CITRUS AURANTIFOLIA* AGAINST MOSQUITOES *AEDES AEGYPTI* AND *CULEX QUINQUEFASCIATUS*

ABSTRACT

Mosquitoes are one of the deadliest insects in the world. Their ability to carry and spread disease to humans causes millions of deaths every year. The use of mosquito repellent to protect oneself against mosquito bites is now common with plenty of repellent products available in the market. Plant-based repellent products gained much attention among users as they are believed to be safer compared to the synthetic repellent. Unfortunately, most of the plant-based repellent in the market were found to have short protection time and thus most likely to be unreliable to be considered as vector control tools. The development of new repellent formulation that offer longer duration of protection is therefore essential. In this study, in order to obtain the information regarding local plant species that are traditionally used for protection against mosquito bites, 350 questionnaires were distributed among the community in Kota Tinggi District, Johor Malaysia. From the information attained, three plant species were selected to be developed into repellent formulation. The essential oils (EOs) extracted from each plant species were then exposed to a method known to help improve the protection time/repellent effect of the formulation called microencapsulation via interfacial precipitation chemistry technique. Diethyl-m-toluamide (DEET), the synthetic repellent was also exposed to the similar method and used for comparison purpose. Encapsulated EOs and DEET were then formulated into lotion form in order to produce microencapsulated (ME) formulation. The pure active ingredients of EOs and DEET were also formulated using similar procedures to produce

non-encapsulated (NE) formulation. Both types of formulations were then evaluated for their efficacy against mosquito bites in the laboratory and field conditions. The physical stability of the formulations stored for 12 months at 25^0C and 40^0C storage conditions was also assessed. The formulations were further analyzed to determine their preservative capacity against microbe followed by skin irritation and skin sensitization study using rabbit and guinea pig as animal model. Based on the ethnobotanical survey, ten plants species were identified by respondents as being used as mosquito repellent. Three plants species selected for further investigation were *C. aurantifolia* (leaves), *A. galanga* (rhizome) and *C. grandis* (fruit peel). The EOs extracted from these plants was successfully encapsulated with more than 95% encapsulation efficiency were observed for all the microcapsules. ME and NE of EOs and DEET were successfully formulated into lotion form. Efficacy study of the formulation demonstrated that ME formulation of EOs had a remarkable potency as repellent by providing longer duration of protection compared to NE formulations and shown to have comparable effect to repellent available in the market, Citriodiol®-based repellent. This formulation also appeared to demonstrate excellent ability in retaining its physical stability and efficacy over 6 months of storage compared with NE formulations. All formulations presented good preservative capacity against bacteria and fungus. All formulations also indicated absence of skin irritation and sensitization effect upon application on the skin. In conclusion, microencapsulation method used in the development of repellent formulation adds an important dimension in the development of long-lasting protection plant-based repellent formulation against mosquito bites.

Keywords: essential oil, microencapsulation, repellent, formulation, *Aedes aegypti*

FORMULASI LOSYEN REPELAN DARIPADA MINYAK PATI *ALPINIA GALANGA, CITRUS GRANDIS* DAN *CITRUS AURANTIFOLIA* TERHADAP NYAMUK *AEDES AEGYPTI* DAN *CULEX QUINQUEFASCIATUS*

ABSTRAK

Nyamuk adalah salah satu serangga paling berbahaya di dunia. Kemampuannya membawa dan menyebarkan penyakit kepada manusia menyebabkan jutaan kematian setiap tahun. Kini, penggunaan repelan nyamuk untuk melindungi diri daripada gigitan nyamuk adalah lazim dengan kehadiran banyak produk repelan di pasaran. Produk repelan berasaskan tumbuhan mendapat perhatian dikalangan pengguna kerana dipercayai ia adalah selamat berbanding repelan sintetik. Malangnya, kebanyakkan repelan berasaskan tumbuhan di pasaran mempunyai masa perlindungan/kesan repelan yang pendek dan dengan itu dianggap sebagai alat kawalan vektor yang kurang sesuai. Oleh itu, pembangunan formulasi repelan yang baru dan mampu memberikan tempoh perlindungan yang panjang adalah penting. Dalam kajian ini, untuk memperolehi maklumat berkenaan dengan spesies tumbuhan yang digunakan secara tradisi untuk melindungi diri daripada gigitan nyamuk, 350 soal selidik telah diedarkan kepada komuniti di daerah Kota Tinggi, Johor Malaysia. Daripada maklumat yang diperolehi, tiga spesies tumbuhan dipilih untuk dibangunkan kepada formulasi repelan. Minyak pati (EOs) yang diekstrak daripada setiap spesies tumbuhan kemudiannya didedahkan kepada kaedah formulasi yang boleh meningkatkan masa perlindungan/kesan repelan iaitu mikroenkapsulasi melalui teknik *interfacial precipitation chemistry*. Diethyl-m-toluamide (DEET), yang merupakan repelan sintetik juga didedahkan kepada tatacara yang sama dan digunakan sebagai perbandingan. EOs dan DEET terenkapsulasi kemudiannya diformulasi dalam bentuk losyen bagi menghasilkan formulasi terenkapsulasi (ME). Bahan aktif asli EOs dan DEET tanpa dienkapsulasi juga

diformulasi menggunakan tatacara yang sama bagi menghasilkan formulasi tidak-terenkapsulasi (NE). Kedua-dua jenis formulasi ini (ME dan NE) kemudiannya dinilai keberkesanannya terhadap gigitan nyamuk di makmal dan di lapangan. Kajian kestabilan fizikal bagi formulasi sepanjang 12 bulan penyimpanan pada suhu 25^0C dan 40^0C juga diuji. Formulasi kemudiannya dianalisis untuk menentukan keupayaan pengawetannya terhadap mikrob sebelum kajian iritasi dan sensitisasi kulit dijalankan menggunakan model haiwan. Melalui kajian tinjauan etnobotanikal, sepuluh spesies tumbuhan yang digunakan sebagai repelan nyamuk telah dikenalpasti oleh responden. Tiga spesies tumbuhan telah dipilih untuk kajian selanjutnya yang terdiri daripada *Citrus aurantifolia* (daun), *Alpinia galanga* (rizom) and *Citrus grandis* (kulit buah). EOs yang telah diektrak daripada tumbuhan ini telah berjaya dienkapsulasi dengan lebih daripada 95% keupayaan mengenkapsulasi telah diperhatikan untuk semua mikrokapsul. ME dan NE EOs dan DEET telah berjaya diformulasi dalam bentuk losyen. Kajian keberkesanan formulasi menunjukkan formulasi ME bagi EOs mempunyai potensi yang luar biasa sebagai repelan dengan menyediakan tempoh perlindungan yang lebih lama berbanding formulasi NE selain daripada menunjukkan keberkesanan yang sama dengan repelan berasaskan Citriodiol®. Formulasi ME juga menunjukkan keupayaan yang bagus dalam mengekalkan kestabilan fizikal dan keberkesanannya selama 6 bulan penyimpanan berbanding formulasi NE. Kesemua formulasi menunjukkan keupayaan pengawetan yang baik terhadap bakteria dan fungus. Kesemua formulasi juga menunjukkan tiada kesan iritasi dan sensitisasi apabila digunakan pada kulit. Sebagai kesimpulan, teknik mikroenkapsulasi yang digunakan untuk membangunkan formulasi repelan menambah dimensi yang penting dalam pembangunan repelan berasaskan tumbuhan yang mempunyai kesan perlindungan yang panjang terhadap gigitan nyamuk.

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LIST OF ABBREVIATIONS

ACT	Artemisinins-combination therapy
AGRO	*Alpinia galanga* rhizome oil
ANOVA	Analysis of variance
ANSM	Agence Nationale de Sécurité du Médicament
BKC	Benzalkonium chloride
Bs	*Bacillus sphaericus*
BTH-14	*Bacillus thuringiensis* serotip H-14
CALO	*Citrus aurantifolia* leaf oil
CDC	Control Disease Center
CGPO	*Citrus grandis* peel oil
CIR	Cosmetic Ingredient Review
CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide
DEC	Diethylcarbamazine
DEET	N,N-diethyl-3-methylbenzamide
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue Hemorrhagic Fever
EE	Encapsulation efficacy
EO	Essential oil
EPA	Environmental Protection Agency
FASST	Fast automated SIM/Scan Type
FDA	Food and Drug Administration
FRIM	Forest Research Institute of Malaysia
FTIR	Fourier Transform Infrared spectrometer
GC/MS	Gas chromatography/mass spectroscopy
GRAS	Generally Recognized as Safe
GPELF	Global Programme to Eliminate Lymphatic Filariasis
HLB	Hydrophile-Lipophile Balance
IGRs	Insect growth regulators
IFP	Interfacial polymerization
IMR	Institute for Medical Research of Malaysia
JE	Japanese Encephalities
ME	Microencapsulated
MF	Melamine formaldehyde
MHA	Muller Hilton Agar
MSD	Malaysian Standard Method
NE	Non-encapsulated
NIST	National Institute of Standards and Technology
OR	Odorant receptor
OECD	Organisation for Economic Co-operation and Development

OLE	Oil of lemon eucalyptus
ORNs	Olfactory response neurons
O/W	Oil in water
SD	Standard deviation
SDA	Sabouround agar
SDP	Size distribution processor
SEM	Standard error mean
SPANOVA	Split-plot ANOVA
SPSS	Statistical Product and Service Solution
TGA	Themogravity analysis
PEG	Polyethylene glycol
PMD	para-methane 3,8 diol
PVA	Polyvinyl alcohol
UF	Urea formaldehyde
ULV	Ultra-low volume
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

1.1.1 Mosquito

Mosquitoes are of great concern throughout the world, not only they pose as nuisance, but more importantly they cause a direct health threat as they transmit diseases through their bites (Linthicum *et al.*, 2006). Few examples of the diseases transmitted by mosquito are dengue, dengue hemorrhagic fever, malaria, filariasis, and Japanese Encephalities (JE) (McHugh, 1994). Figure 1.1 shows the global map of mortality due to the vector-borne diseases that also impose Southeast Asian region with estimates mortality about 50 -200 million cases in year 2002 (WHO, 2018).

The World Health Organization (WHO) estimated around 390 million dengue infections worldwide every year (WHO, 2015a). As for malaria WHO reported that almost half of the world's population are at risk with young children, pregnant women and non-immune travelers from malaria-free areas are particularly vulnerable to the disease when infected (WHO, 2015b). In the case of filariasis, it was estimated to infect more than 120 million people worldwide. One third of the people affected with this disease live in India, another one third in Africa and the most of the remainder were in South Asia, The Pacific and the Americas (WHO, 2015c). JE which is the most important cause of viral encephalitis in Asia, was estimated to have caused 68,000 clinical cases globally each year, and up to 20,400 death (WHO, 2015d). These data clearly demonstrated the severity of vector borne diseases as far as global health is

concern. Therefore, for any effort to be effective, successful and sustainable in combating these diseases, without a doubt it must include the control of vector.

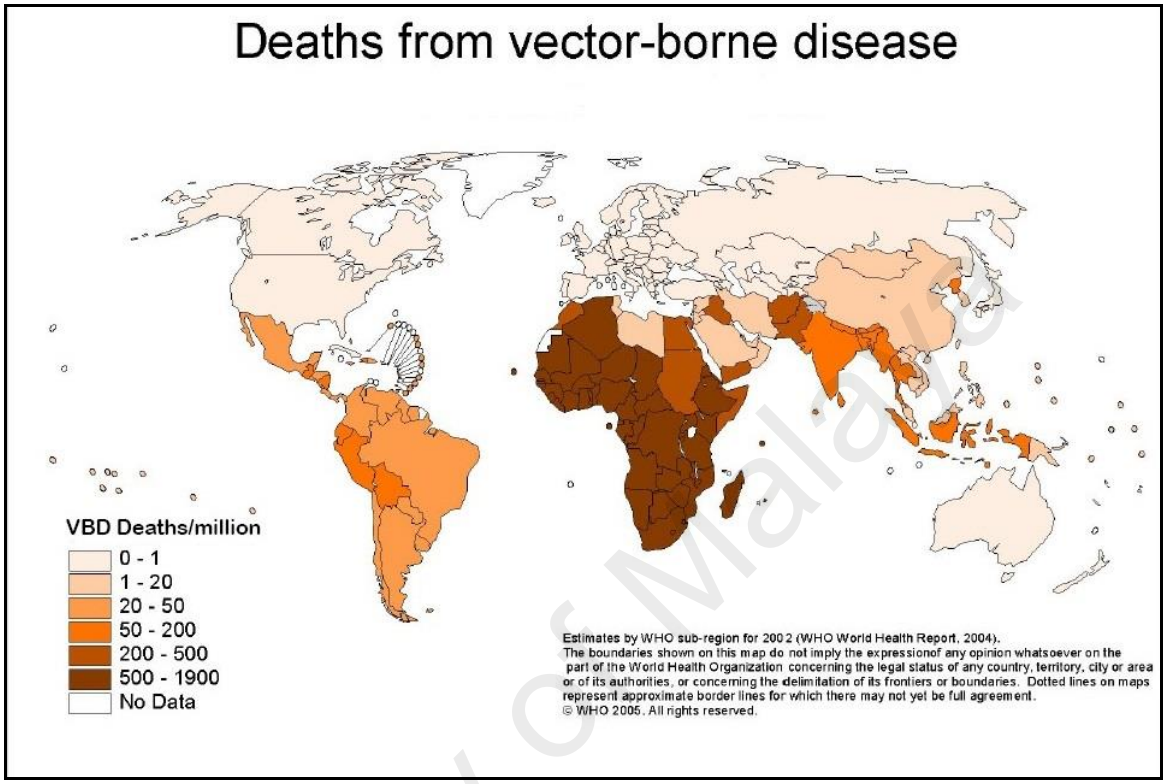


Figure 1.1: Global mortality distribution due vector borne disease. (Source: WHO, 2018).

a) Mosquito control: community level

Mosquito control can be divided into three categories: (i) chemical control, (ii) biological control and (iii) environmental management. Chemical control involves the use of organochlorine, organophosphate, carbamate or synthetic pyrethroids as larvacides or adulticides. This method although found to be effective in killing the adult and larvae of mosquito, when used frequently they have shown to cause pollution to the environment, toxicity effect to human, non-target animal and insects (Service, 2000).

More importantly it can cause mosquito resistance towards these insecticides (Service, 2000).

Biological control on the other hand is intervention based on the use of other organisms that prey upon, parasitize and compete with mosquitoes. For example, predator fish (*Gambusia affinis* Holbrooki, *Fundulus spp.* and *Rivulus spp.*) or bacteria (*Bacillus thuringiensis* and *Bacillus sphaericus*) were used to kill mosquito larvae. This type of control measure is considered better as it is environment friendly and specific. Its usage unfortunately, is somewhat limited due to high expenses in growing/producing these organisms.

Environmental management refers to changes made to the environment in order to prevent or minimize vector propagation and human contact with the vector-pathogen by destroying, altering, removing or recycling non-essential containers that provide mosquito habitats. There are three types of environmental management: (i) environmental modification, (ii) environmental manipulation and (iii) changes to human habitation or behavior. Environmental modification involved the long-lasting physical transformations to reduce vector larval habitats, such as installation of a reliable piped water supply to communities, including household connections. While environmental manipulation is the temporary changes to vector habitats involving the management of "essential" containers, such as frequent emptying and cleaning by scrubbing of water-storage vessels, flower vases and desert room coolers; cleaning of gutters; recycling or proper disposal of discarded containers and tires. Changes to human habitation or behaviour involved actions that can reduce human–vector contact, such as installing mosquito screening on windows, doors and other entry points, and using mosquito nets

(Service, 2000). All these mosquito control measures are conducted usually at large scale which involved community level.

(b) Mosquito control: individual level

At the smaller scale or individual level, personal protection using insect repellent is a popular method that helped avoid human-mosquito contact. Personal protection with insect repellent can be considered as the first line of defense against mosquito bites. It is usually applied in combination with behavioral practices (i.e., destroy the mosquito habitats and avoidance of mosquito peak biting times) and physical barriers (i.e., use of protective clothing and bed-nets) (Freedman, 2008; Maguranyi *et al.*, 2009). It plays an important role in preventing man-mosquito contact and therefore help minimizes the possibility of getting infections.

Commonly use repellent product can be divided into two types: (i) chemical-based repellent and (ii) plant-based repellent. There are two active ingredients that can be found in chemical-based repellent; N,N-diethyl-3-methylbenzamide (DEET) and picaridin. Their effectiveness is very excellent as they shown to provide up to 8 hours of protection from mosquito bite. Unfortunately, for some people these repellents have shown to cause irritation, rashes on the skin, dizziness, headaches and nausea (Corazza *et al.*, 2004; Potera, 2008). As a result, plant-based repellent products have found favor with consumers and the demands for formulation containing plant products are increasing. Citronella oil, neem oil, eucalyptus oil and para-methane 3,8 diol (PMD) are among the plant ingredient commonly found in plant-based repellent. Although most of them shown of having low repellent effect compared to chemical-based repellents,

they are believed to be safer (does not cause irritation, rashes on the skin, dizziness, headaches and nausea) (Maia & Moore, 2011)

1.1.2 Plant-based product as protection against mosquito

In recent years, environmental friendly and biodegradable natural insecticides of plant origin have been receiving great attention which demands more scientific survey to look for new repellent or insecticidal plants (Karunamoorthi *et al.*, 2009a). Several plants have been studied and many shown to possess such effect. Unfortunately only a few of them namely citronella, lemon and eucalyptus oil have been qualified for registration by US Environmental Protection Agency (US EPA) due to their safety and acceptable effectiveness (Katz *et al.*, 2008).

It is widely known that prior to advance use of synthetic repellents such as DEET, humans have used plants to protect themselves against blood sucking insect (Curtis *et al.*, 1991). They either planted or burned local aromatic plants outside their house to avoid themselves from mosquito bite (Karunamoorthi & Husen, 2012). The knowledge and usage custom of these plants is very important in plant-based products development. In reality this information usually passed down to many generations mostly through word of mouth or informal education (Karunamoorthi & Husen, 2012). This mode of information passage more often than not may result in falsification or loss of indigenous knowledge and usage custom of repellent plant. Hence, it is crucial to protect this information through documentation (Karunamoorthi *et al.*, 2009a). Ethnobotanical survey is an important tool that can be used to gather such information.

The documentation of such information will assist in generating data-base on plants traditionally used as insect/mosquito repellent (Karunamoorthi & Husen, 2012).

Many plant species that produce essential oil (aromatic oil) have been reported as natural sources of insect repellent (Hay & Waterman, 1993). Due to their high volatility and biodegradability characteristics however they tend to present low repellent effect that last for a short period of time only (Trongtokit *et al.,* 2005a; Soonwera & Phasomkusolsil, 2015). There were also claims that topical plant-based repellent usually need to be reapplied frequently, sometimes several times in an hour, to provide sufficient protection from mosquito bites (Revay *et al.,* 2013).

It is now known that by controlling the release rates of the essential oil can help increase the protection time of plant based repellent. The study on plant-based repellent of this sort is still considered as an active research area and being studied all over the world (Beestman, 2003; Nerio *et al.,* 2010). One of the methods used to prepare such plant-based repellent is known as microencapsulation. Microencapsulation is the method that encapsulates the volatile oil of a plant studied with wall material that protects it from the external atmosphere (Alikhani & Garmakhany, 2012). The wall materials can be made up from natural resources such as gelatin, gum Arabic and whey protein or synthetic polymer such as polyvinyl alcohol and carboxymethylcellulose (Jo *et al.,* 2015). Encapsulation can be done either through chemical, physicochemical or physical methods where micron size capsule will be produced. The wall that surrounds the core material (essential oil) is the part that will help extend the release of the essential oil to achieve the intended protection time. The application of microencapsulation technologies on essential oils also affords several advantages

including protecting unstable core materials and preventing the core materials from poor environments (Jo *et al.*, 2015).

Apart from having longer protection time, it is also important to ensure the plant-based repellent prepared to possess the ability to maintain its physical and chemical characteristics during storage. The chemical interactions that may arise between the chemical substances inside the plant-based repellent prepared can ruin its stability. For example, an unsuitable temperature during storage will change the physical stability of the plant-based repellent prepared and lead to destabilization effect that will have a direct effect on the quality, efficacy and safety of the plant-based repellent prepared (Bilia *et al.*, 2001). Hence, it is necessary to determine its stability status especially when involving long storage time. In the case where the plant-based repellent prepared has potential to be commercialized, it is a requirement that it has an acceptable safety level to be qualified for registration and only then it can be commercialized (Tripathi *et al.*, 2009).

In this study, an ethnobotanical survey have been performed on a population in a district of Kota Tinggi, Johor in the attempt to gather information regarding traditionally used plant among the Malay ethnic. Based on the information obtained, several plants were selected, extracted and formulated using microencapsulation method to produce plant-based formulation in lotion form. They were then tested for repellent effect on mosquito by comparing them with commercial repellents and the standard chemical, DEET in the laboratory and in the field conditions. The formulation also tested for their stability under the influence of different temperature and time. The toxicity/safety assessments were also conducted.

1.2 RESEARCH OBJECTIVES

1.2.1 Main objective

The main objective of this study is to produce formulation for the development of plant-based repellent against *Aedes aegypti* and *Culex quinquefasciatus* that provides longer protection time using microencapsulation method.

1.2.2 Specific objectives

The specific objectives of the study were:

a) To formulate an effective repellent lotion using microencapsulation method from the essential oil of *Alpinia galanga, Citrus grandis* and *Citrus aurantifolia*.
b) To evaluate the effectiveness of the repellent effect of the formulation in the laboratory and in the field settings.
c) To examine the physical stability and effectiveness of the formulation under the influence of different temperature with time.
d) To determine the toxicity effect of the formulation on skin using animal model.

1.3 Problem statement

#Problem 1: Selection of plant and type of botanical insecticides

It is nearly impossible and impractical to choose plants species via random selections in search of active ingredient that can provide protection against mosquito bite effectively. A practical tool is certainly required not only cost and time friendly but able to provide exact information regarding the potential plant species (Gurib-Fakim,

2006). One of the research tools that are commonly used for such quest is the ethnobotanical study. The main goal of ethnobotanical has been to discover plants extracts or novel compounds derived from plants used in indigenous medical systems, and which can be utilized in the development of new pharmaceuticals (Verpoorte *et al.*, 2005). For example, in the treatment of malaria, two major antimalarial drugs widely used to date were originally came from indigenous medical systems, quinine and artemisinin from Peruvian and Chinese ancestral treatments, respectively (Soh & Benoit-Vical, 2007) while neem oil from *Azadirachta indica* spp, used as active ingredient in various insecticide products, originally came from traditional Indian medicine (Kumar & Navaratnam, 2013). In addition citronella oil and eucalyptus oil, are ingredients that mostly can be found in repellent originally came from Javanese and Australian Aboriginals ancestral treatments (Barr *et al.*, 1988). Based on these discoveries, ethnobotanical study definitely can be recognized as one of the useful tools in which new therapies or insecticides may possibly be discovered.

To date, essential oil-based products are the most diverse botanical insecticides in the market as compared to other plant-based products such as pyrethrum and neem. The wide use of essential oil as active ingredient in plant-based product might be due to the complex mixture of metabolites in essential oil that exhibit internal interactions in the form of synergy, which can contributed to the overall toxicity of the mixture (Miresmailli *et al.*, 2006). In addition, essential oil by its nature also plays as defensive role against fungi, pathogenic microorganism, herbivores and insects. Owing to these characteristics, they have been used as variety of products, from contact toxicants to fumigants and even in behavior–modifier products, such as attractants and repellents (Miresmailli & Isman, 2014).

In this study, ethnobotanical study was conducted in order to gather information regarding traditionally used plants as repellent in the study area.

#Problem 2: Duration of the protection time of plant-based repellent

Essential oil act as repellent when its chemical compounds are release to the environment and this drive away the mosquito. However, essential oil tends to have rapid release rate that contribute to poor longevity of its repellent effect (Maia & Moore, 2011). In order to improve the longevity of the repellent effect, many formulation of plant-based repellent prepared was added with fixative materials such as liquid paraffin (Oyedele *et al.,* 2002), vanillin (Tawatsin *et al.,* 2001), salicyluric acid (Blackwell *et al.,* 2003), mustard and coconut oils (Das *et al.,* 1999). In some of these preparations carrier oil such as coconut oil, palm nut oil and andiroba oil and emulsifier (such as polyethelene glycol, sodium laurate sulfate) were also added. Fixative materials and carrier oil helped to improve the repellence activity by slowing down the evaporation of volatile repellent molecules (Reifenrath *et al.,* 1989; Campbell & Gries, 2010). Some studies done on the formulations prepared this way unfortunately showed that prolong protection somehow is not guaranteed (Thavara *et al.,* 2002; Trongtokit *et al.*, 2005a).

It is now known that for optimum protection, the release rate of the essential oil has to be controlled so that the release of the chemical compounds is sufficient for a longer period. Controlled release formulation is the most recent type of formulation produced in repellent product development. A method that is actively being studied nowadays for that purpose is microencapsulation. Previous research by Solomon *et al.* (2011) successfully encapsulated citronella oil by using simple coacervation technique

and study showed that microencapsulated citronella oil released only 70% of oil content for 10 hours. Study by Maji *et al.* (2007) successfully encapsulated *Zanthoxylum limonella* oil using similar technique with added glutaraldehyde crosslinked gelatin resulted in microencapsulated oil releases 40% oil for 25 hours. Previous study by Karr *et al.* (2012) successfully microencapsulated DEET that provides more than 48 hours release rate. Although there were only few studies regarding microencapsulation technique in the production of repellent product and most of them are ongoing research, these findings were quite promising with regards to controlling the release of essential oil therefore worthy of further investigation.

#Problem 3: Stability of the formulation

Another important issue to be addressed in the process of producing formulation for developing repellent product is the stability of the formulation. It is necessary to determine the suitable storage condition in which the stability of the product can be preserved. It is a fact that unsuitable temperature will change the physical stability of the formulation and lead to the destabilization effect and that will have a direct effect on the quality, efficacy and safety of the product (Bilia *et al.*, 2001).

#Problem 4: Safety/toxicity of the formulation

Several essential oils have been studied for the repellent effect and most of them shown to have the potential to be developed further. For example, Citronella, lemon and eucalyptus oil and Citriodiol® (PMD) are among ingredients of plant repellent products that have been approved by EPA due to their low toxicity and acceptable

efficacy (Nerio *et al.,* 2010). Recent study on skin toxicity assessment showed that oil of lemon eucalyptus do have the potential to cause skin irritation while citronella oil has the potential to cause not only skin irritation but allergies as well (Diaz, 2016). Therefore, the safety evaluation of the formulation is an important component in plant-based product development to be examined to ensure safety of the product.

1.4 Expected output

a) Documentation of plant species traditionally used as mosquito repellent

b) Encapsulation of the essential oil extracted from selected plant species by interfacial precipitation chemistry technique.

c) The formulation in the lotion forms provides prolong protection against mosquito bites in the laboratory and field conditions.

d) The formulation presented stability under different temperature with time.

e) The formulation demonstrated no irritation and sensitization effect on skin.

CHAPTER 2

LITERITURE REVIEW

2.1 BIOLOGY OF THE MOSQUITO

There are more than 3,000 species of mosquitoes, but the members of four genera; *Aedes, Anopheles, Culex* and *Mansonia* bear primary responsibility for the spread of human diseases (Norris, 2004). *Aedes* mosquitoes, of which the voracious Asian tiger is a member, carry dengue fever, zika, yellow fever and encephalitis. *Anopheles* mosquitoes are the only species known to carry malaria and some of the species also vectors for brugian and bancroftian filariasis. Meanwhile certain *Culex* mosquitoes can transmit bancroftian filariasis, encephalitis and the West Nile virus (Service, 2000). Whereas *Mansonia spp.* can transmit brugian filariasis and a few arbovirus diseases.

2.1.1 Life cycle

Most of the mosquitoes mate shortly after emergence from pupa. Spermatozoa, passed by the male into the spermatheca of the female, usually serve to fertilize all eggs laid during her lifetime; thus only one mating and insemination per female is required. A female mosquito must bite a host and take a blood-meal to obtain nutrient for the development of the eggs in the ovaries. This process is referred as anautagenous development. Full development of egg will occur after 2-3 days of blood-meal and this gravid mosquito will search for suitable larval habitats in which to lay her eggs. After oviposition (lay eggs), the female mosquito will take another blood feeding and a further batch of eggs is matured. The process of blood-feeding, eggs maturation,

followed by oviposition, is repeated several times throughout the female's life and the whole process is referred as the gonotrophic cycle (Figure 2.1). Only the female mosquito will feed on blood while male mosquito feed on sugar or nectar of fruits and flowers. Male are consequently unable to transmit any diseases (Service, 2000).

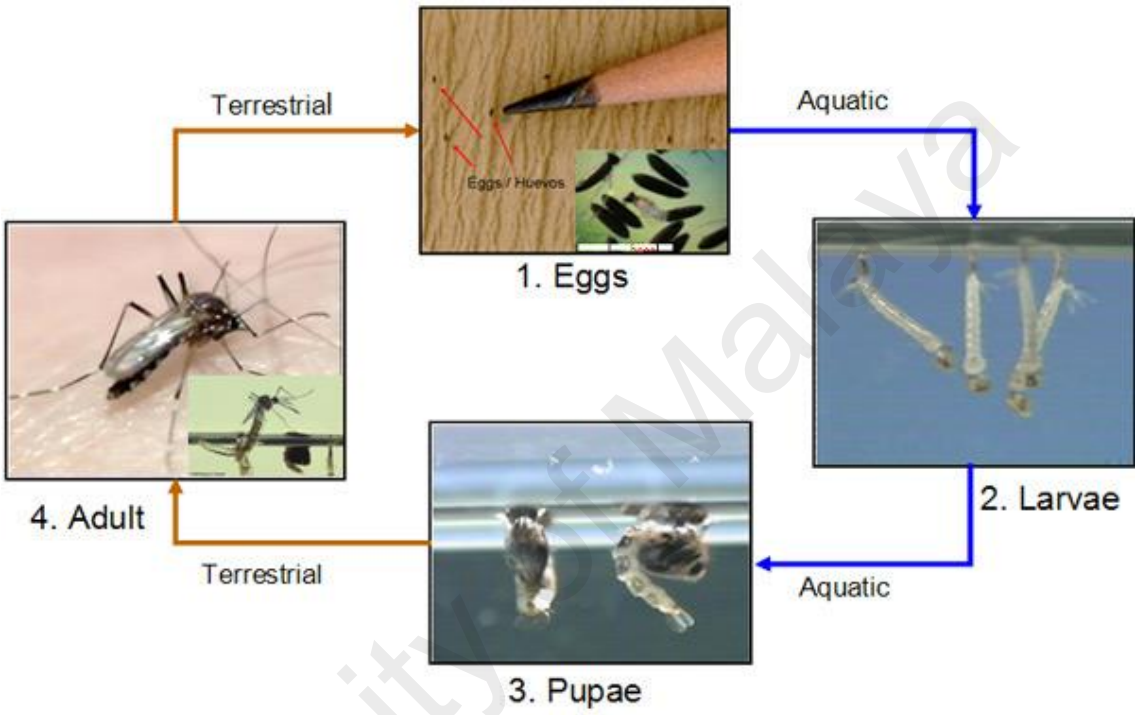


Figure 2.1: General life cycle of the mosquito (Source: CDC, 2012)

Figure 2.1 shows the general life cycle of the mosquito and depending on the species, the female mosquitoes lay about 30-300 eggs at one oviposition on the water surface. In tropics they will hatch into larvae stage within 2-3 days, but in the cooler temperate countries they may hatch after 7-14 days, or longer. Mosquito larvae feed on yeasts, bacteria, protozoans, animal and plants microorganism that are found in the water. There are four larval instars and in the tropical countries larval development from egg hatching to pupation will take about 5-7 days. However, in the temperate areas the larval period may last several weeks or months (Service, 2000).

Pupae do not feed but spend most of their time at the water surface taking in air through the respiratory trumpets. In the tropical countries, the development from pupae to adult will take about 2-3 days but in the colder climates pupal development may extend over 9-12 days, or even longer. At the end of pupal life, the skin on the dorsal surface of the cephalothorax splits, and the adult mosquito struggles out (Service, 2000).

2.1.2 Morphology of the mosquito

2.1.2.1 Egg

The morphology of mosquito eggs is different between genera but generally have brown or blackish in color, having size 1 mm or less. For *Aedes*, the eggs are elongated or approximately ovoid in shape and laid singly on water substrate (Figure 2.2a). For *Culex* mosquito the egg also have ovoid shape but laid vertically in several rows held together by surface tension to form an egg raft which float on the water (Figure 2.2b). As for *Mansonia*, the egg have ovoid shape and drawn out into a terminal filament. They lay their eggs in a sticky mass that is glued to the underside of floating plants (Figure 2.2c). Meanwhile for *Anopheles* mosquito they are usually boat-shaped, laid singly and float on the water (Figure 2.2d).

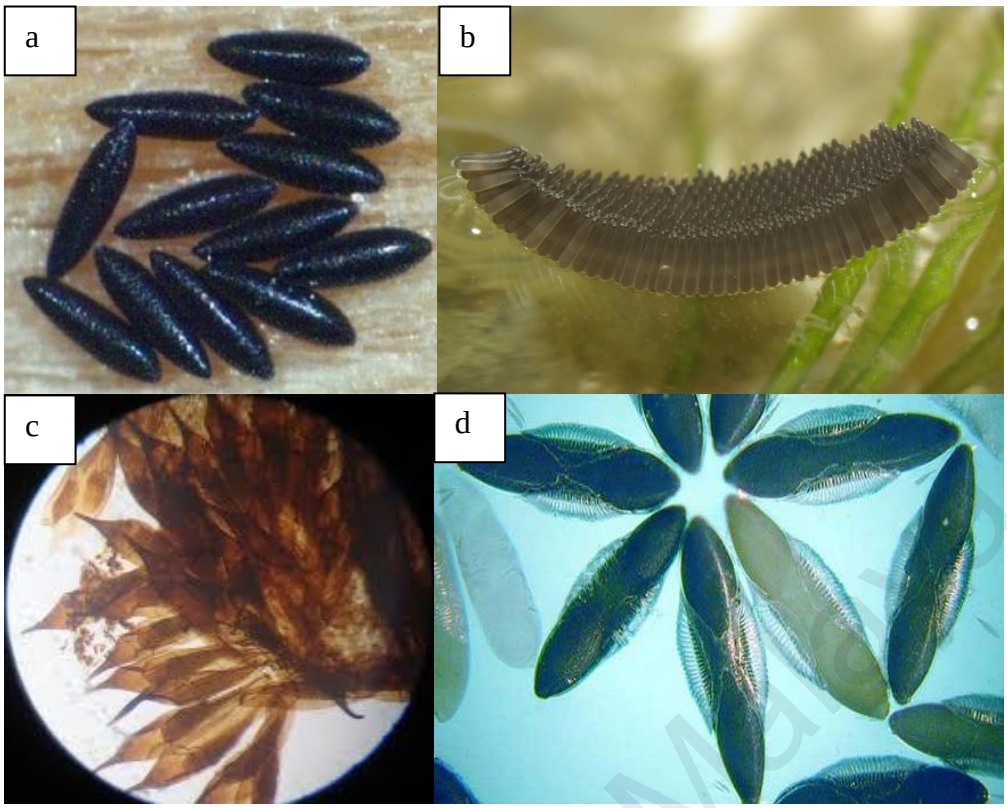


Figure 2.2: Morphology of mosquito egg a) *Aedes spp.* b) *Culex spp.* c) *Mansonia spp.* and d) *Anopheles spp.*

2.1.2.2 Larvae

Mosquito larvae have a well-developed head, bearing a pair of antennae and a pair of compounds eyes. They also have mouthbrushes to sweep water containing food particles into the mouth. All mosquito larvae have 10 segmented abdomens but only nine segments are visible. The last segment has two paired groups of long hairs forming caudal setae, and a larger fan-like group comprising the ventral brush, and at the end it has transparent sausage-shape anal papilla. Anal papilla also known as gills are not concerned with respiration but important for osmoregulation function. The thorax is located between the head and abdomen of the larvae. The morphology of the mosquito larva according to genera are shown in Figure 2.3. *Aedes spp.* larvae were identified by their short siphon (Figure 2.3a), while *Culex spp.* were identified by their long siphon

(Figure 2.3b). *Anopheles spp.* has no siphon but they have spiracle on their dorsal part of abdomen and in the water they rest parallel to the water surface (Figure 2.3c). Meanwhile for *Mansonia spp.*, the siphon was modified into trumpet like structure use to pierce water plants (Figure 2.3d).

Figure 2.3: Morphology of mosquito larvae a) *Aedes spp.* b) *Culex spp.* c) *Anopheles spp.* and d) *Mansonia spp.*

2.1.2.3 Pupae

All mosquito pupae have coma-shaped body with their head and thorax are combined to form the cephalothorax, which has a pair of respiratory trumpets dorsally. The abdomen is 10-segmented although only eight segments are visible. Each segment has numerous short hairs called palmate hair and the last segment there is a flattened structures termed paddles (Service, 2000).

2.1.2.4 Adult

Adult mosquitoes are measuring about 3-6 mm in length. The body is divided into three part; head, thorax and abdomen. The head has a pair of kidney-shaped compound eyes and between the compound eyes ascends a pair of antenna. For female mosquitoes the antenna is equipped with whorls short hairs or known as pilose antenna, while in male mosquitoes the antenna have long hairs giving them feathery appearance. Thus, sexes of the mosquitoes can be differentiating by their antenna. Just below the antenna there is a pair of palps which may be long or short and dilated or pointed at their tips, depending on the sex and genus of the mosquitoes. Between the palps, a single elongated proboscis arises, which contains the piercing mouthparts of the mosquito. The thorax is covered, dorsally and laterally, with scales whether dull or shiny, white, black or brown, which depends on the species. For some species, the color and the pattern of the scales are important for the species identification. Figure 2.4, 2.5 and 2.6 show the general characteristic of four genera of mosquito known to transmit diseases.

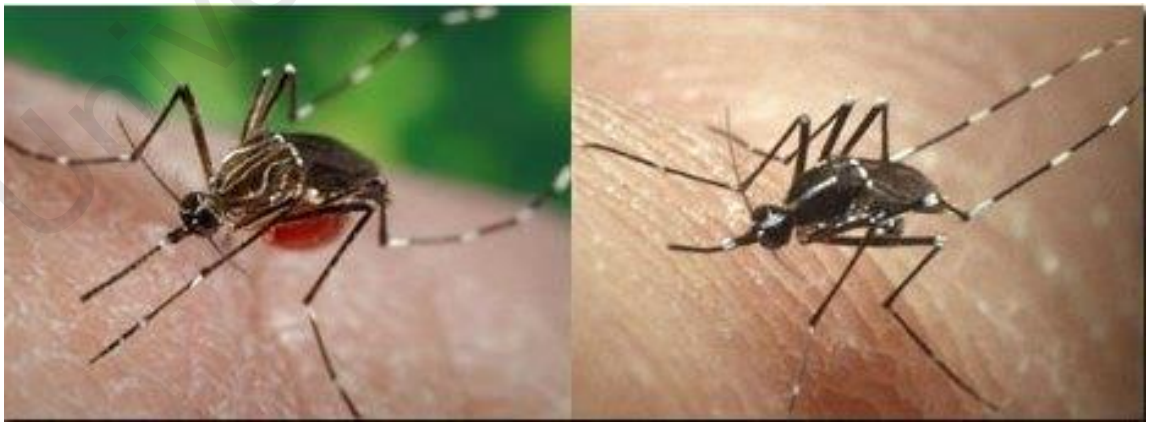


Figure 2.4: Adult mosquito *Aedes aegypti* (left) and *Aedes albopictus* (right) transmit dengue fever and dengue hemorrhagic fever. *Aedes* mosquito is characterized by dark in color with distinct white stripes at their legs. (CDC, 2015a).

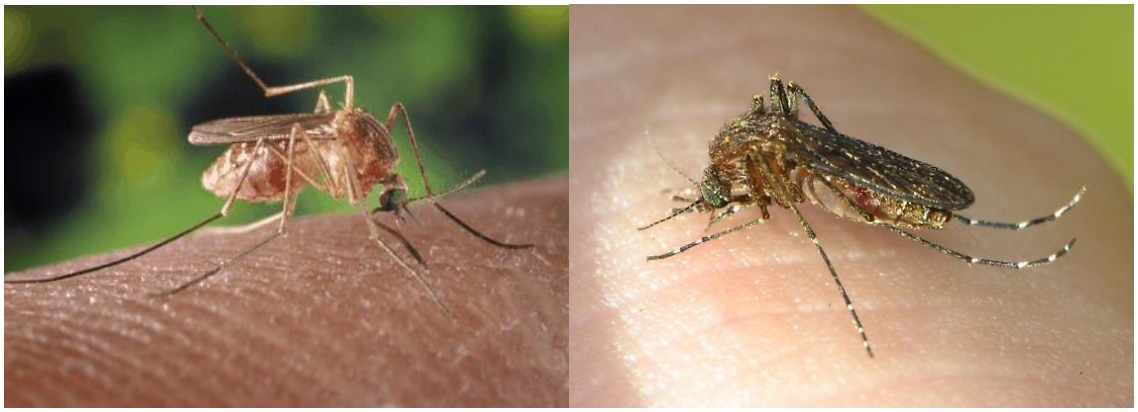


Figure 2.5: **Adult mosquito *Culex spp.* (left) vector for Japanese encephalitis and lymphatic filariasis. Adult characterized by yellowish to brownish in color. *Mansonia spp.* (right) vector for lymphatic filariasis can be recognized by their dusty appearance like salt and pepper. (CDC, 2015c).**

Figure 2.6: **Adult *Anopheles spp.* (left) vector for malaria which characterized by spotted wing that is the dark and pale scales are arranged in small blocks or areas on the veins. *Amigeres spp.*, (right) not a vector for any diseases but their bites can cause irritation and sensitization effect in certain individual (CDC, 2015b).**

Each mosquito has a pair of wings that long, narrow and covered with scales which are usually brown, black, white or creamy yellow depends on the species. The shape of scales and pattern differ between both genera and species of the mosquitoes. Mosquito have three pairs of leg situated at both lateral of the thorax. The abdomen is composed of 10 segments but only the first seven or eight are visible. The last segment of the abdomen of the female mosquito terminates in a pair of small finger-like cerci,

whereas in the males pair of prominent claspers, comprising part of the male external genitalia, is present.

2.1.3 Behavior of mosquito

Female mosquitoes are attracted to the hosts by various stimuli, which are actually by product from respiration and perspiration process that emanating from them such as body odors, carbon dioxide and heat. The vision usually plays only a minor role in host orientation although some species are attracted by the movement of their hosts. The antenna and palp also play an important role in localizing their host and detect any changes in the environment (Service, 2000).

Generally, many species of female mosquitoes will feed on humans to obtain their blood meals and few prefer feeding on non-human host. Some species never bites human. Anthropophagic is the term refer to the species that usually feed on humans, whereas those that feed mainly on other animals are called zoophagic. Sometimes, mosquitoes that feed on birds are called as ornithophagic instead of zoophagic (Service, 2000).

Some of the mosquito species are endophagic and some of them are exophagic in their feeding habits. Endophagic refers to the mosquitoes that enter houses for blood meal while exophagic refers to the mosquitoes that bite their host outdoor. After blood feeding, mosquitoes are usually seeking resting places in which to shelter during digestion of their blood meals and for the maturation of the eggs. Mosquitoes that rest indoor or inside house are called endophilic, while mosquitoes that rest outdoors are termed exophilic. The biting behavior and resting behavior of each mosquito species are

very important in the epidemiology of diseases transmission and control measures planning.

Some of the mosquitoes bite predominantly within forest and wooded area. As a consequent, people will only get bitten when they enter these places. Hence, the behavior of both humans and mosquitoes may be relevant in diseases transmission. The biting behavior of the mosquito also play a role in control measure such as in the use of impregnated bed-net which effective in killing / repelling mosquitoes that come to feed indoors during night hours. In addition, understanding the resting behavior of adults mosquitoes also help in planning the control measure for examples in malaria control, the interior surfaces of houses, such as walls and ceilings, are sprayed with residual insecticides to kill adult mosquitoes that resting on them (EPA, 2016).

2.1.4 Factor influence the host orientation behavior

Many biting insects, including anautogenous mosquitoes, require a blood meal before oogenesis where serves as a primary source of energy for egg development. In order to have a blood meal, the female mosquito must locate a host for feeding known as host orientation behaviors. This behavior is controlled by signals released by the host, including heat, moisture, sounds, and odor as well as visual and olfactory cues (Takken, 1991).

Carbon dioxide (CO_2) is one of the sensory cues that involved in this host-seeking behavior. CO_2 is known as a primary byproduct of cellular respiration, which is released in large quantities by all potential hosts (Takken, 1991). Besides CO_2, other compound such as lactic acid also appears to be important stimuli for mosquito

orientation. Lactic acid is the chemical substances released from our respiration along with octenol, uric acid and fatty acids to form a unique carbon dioxide cocktail (Gillies, 1980). This combination of chemical substances produced a scent which can attract the mosquito. The scent and amount of CO_2 is distinctive to each individual and genetic is one of the factors involved in this variation. For examples, larger people exhale more CO_2, which is why adults are more likely to be bitten than children. Pregnant women also exhale above average amounts and are therefore more attractive to mosquitoes (Brown, 1966).

Other than CO_2 and lactic acids, body odor also is an attractant agent released by host that can attract the mosquito. Bacteria colonies on the skin in combination with sweat also produced attractive scents for mosquito. Besides skin microbiome, genetic factors, diet, immunity, blood metabolites are among of the physiological factors that contribute to odors emanating from human skin, even though their mechanism is still unclear. Differences in skin odor alone can modulate mosquito attraction, when temperature and CO_2 are held constant. The presence of lactic acid in human sweat also contributed in the host orientation behavior in mosquito (Brown, 1966).

Female mosquitoes, as well as many other biting insects, are well equipped with an array of sensors that are highly sensitive and specifically tuned in respond to CO_2 and lactic acid (Grant & O' Connell, 1996; Grant & Kline, 2003). Chemoreceptor neurons in sensilla located on the maxillary palps together with those on the antenna, play an important role in the detection and processing of the chemical stimuli implicated in initiating and modulating host location and feeding behaviors in insects (Kline *et al.,* 1991). For examples CO_2 –sensitive receptor neuron, which is located on maxillary

palps have been identified as sensory structure in responds to CO_2 (Grant & O'Connell, 1996), while antenna that contain a highly sensitive L-lactic acid receptor neuron in responds to lactic acid (Takken & Knols, 2010).

The understanding regarding host orientation in seeking blood play vital role in repellent product development. For examples, DEET the most effective repellent to date, modify or block the responses of olfactory response neurons (ORNs) that normally sensitive to the attractants (Davis, 1988). DEET decreased the sensitivity of lactic acid sensitive ORNs to lactic acid, a component in human sweat and breathe (Davis & Sokolove, 1976). Study by Syed and Lael (2008) reported that DEET also reduced the response of octenol ORNs in mosquitoes.

2.2 MOSQUITO AND THE DISEASES IT TRANSMITS

2.2.1 Dengue Fever (DF) and Dengue Hemorrhagic Fever (DHF)

The etiology agent of dengue virus (DENV) belongs to flavivirus genus, family flaviviridae. There are four serotypes of dengue namely DEN-1, DEN-2, DEN-3 and DEN-4. Each serotype is classified based on their antigenic complex (Calisher *et al.,* 1989). *Ae. aegypti* is the main vector for these viruses followed by *Ae. albopictus* as secondary vector. Under natural conditions, a susceptible mosquito can only acquire a DENV infection after it has taken a blood meal from a viremic host. However, they can also get infection from transovarial transmission in which the DENV from the maternal body transmit to eggs within the ovaries (Gunther *et al.,* 2007; Angel & Joshi, 2008). Humans are the primary host of the virus, (Gould & Solomon, 2008; WHO, 2009a) but it also circulates in nonhuman primates (WHO, 2011). Dengue is the disease that can be acquired via a single bite (CDC, 2010).

The extrinsic cycle of this virus occur in the mosquito and its can take 8-12 days to complete the cycle, depending on temperature, types of DENV and mosquito species. DENV first infects and replicates in the mosquito midgut epithelium. It subsequently spreads through the hemolymph to replicate in other organs such as the fat body and trachea, finally infecting the salivary gland at approximately 10–14 days post-blood meal (Salazar *et al.*, 2007). Once in the saliva, DENV can be inoculated into a human host when the mosquito acquires a blood meal, thus spreading the disease (Sim *et al.*, 2012). Figure 2.7 demonstrated the life cycle of the DENV in the mosquito and how they transmit to human and spread the diseases.

The intrinsic incubation period of DENV in human is about four to seven days. The principle symptoms of dengue fever consisting high fever, severe headache, myalgia, arthralgia, retro-orbital pain and maculopapular rash. Meanwhile, DHF is characterized by increases in blood vessel permeability, hypovolemic and abnormality in blood clotting mechanism (WHO, 2005). Control and prevention of dengue is not only focus on the treatment and vaccine development, the involvement in vector control also plays an important role in controlling the endemic of dengue virus. The dependence on vaccine solely and disregard the vector control programs will increase the vector population and spreading of dengue virus. For the time being, the only methods for preventing and controlling DF/DHF are to control the mosquito vectors and to reduce human-vector contact.

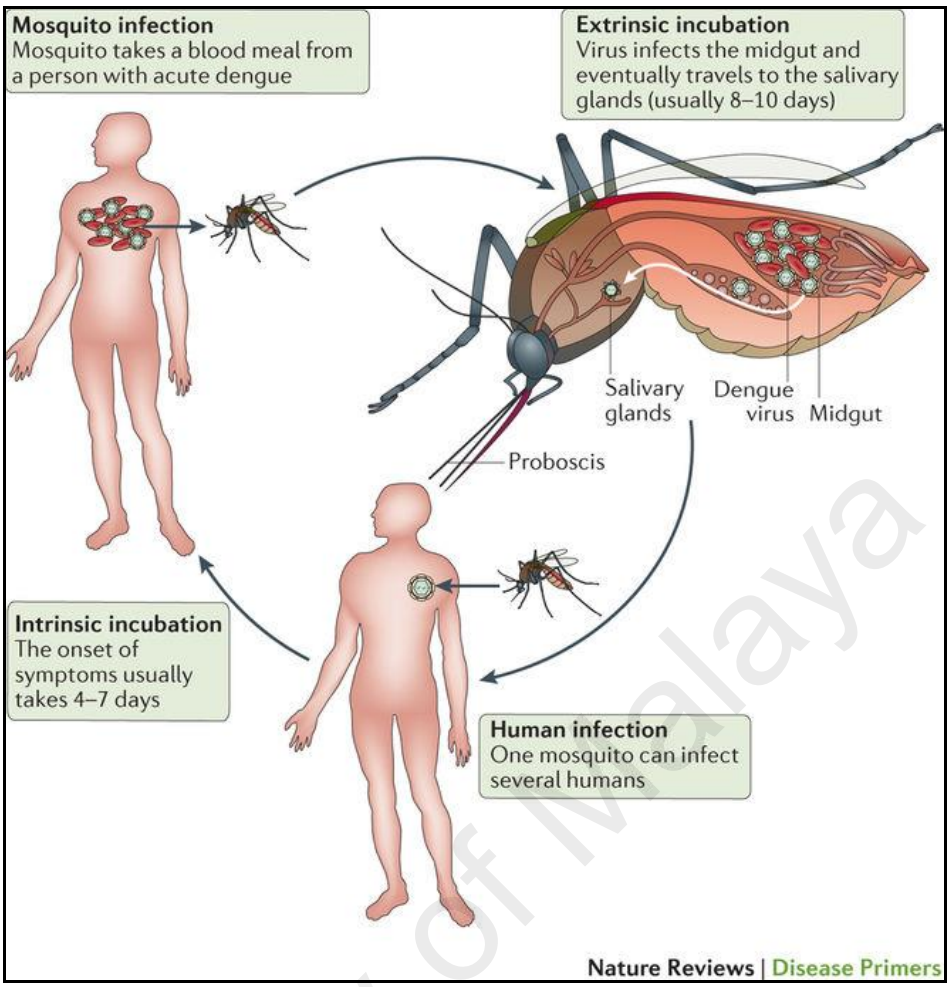


Figure 2.7: Life cycle of DENV in mosquito and the transmission of the disease. (Source: Guzman *et al.*, 2016)

2.2.2 Malaria

Malaria is a mosquito-borne infectious disease of human and other animals caused by parasitic protozoan belonging to the genus *Plasmodium*. The parasites are transmitted from person to person by *Anopheline* mosquitoes. There are five species of malaria parasites that infect humans: (1) *Plasmodium falciparum* occurs throughout tropical Africa and in parts of Asia, the Western Pacific, South and Central America, Haiti and Dominican Republic. (2) *Plasmodium vivax* is the predominant malaria parasite in Asia and South and Central America. (3) *Plasmodium malariae* that can be found worldwide but has very patchy distribution. (4) *Plasmodium ovale* which occurs mainly in tropical West Africa and rarely in the Western Pacific. (5) *P. knowlesi* a

species that naturally occurs in long-tailed and pig-tailed macaques that inhabit forested areas in Southeast Asia. It can be transmitted from monkeys to humans by the bite of an infected mosquito, but infection with *P. knowlesi* was traditionally regarded as a rare disease, occurring only sporadically in humans (Hellemond *et al.,* 2009). A study conducted in Malaysia and Thailand reported that Anopheles mosquitoes are tend to feed outdoor rather than indoors as reported by Hassan *et al.* (2001). In their study, *An. maculatus*, *An. barbirostris* and *An. sinensis* biting were observed throughout the night when peak outdoor biting was higher after midnight (2130 h). Meanwhile, their indoor biting activities were observed before midnight hour. Some of the species such as *An. kochi* and *An. philippinensis* were reported to be more active after dusk and steadily declined after 2130 h.

Malaria parasites enter the human body via the bite of an infected female *Anopheles* mosquito which introduces the parasites from the mosquito's saliva into a person's blood (WHO, 2014). The parasites invade the liver via the bloodstream and multiply. After about nine days or longer (depending on the species), the parasites (called merozoits) enter the bloodstream, invade the red blood cells and again multiply. A few days after the appearance of the first symptoms some merozoits develop into trophozoites and some will develop into gametocytes (Figure 2.8), the sexual stage in the life cycle. Male and female malaria gametocytes are ingested by female mosquitoes during blood-feeding and pass to the mosquito's stomach where they undergo another phase of reproduction in the insects. At the end of this process a new generation of malaria parasites, called sporozoites, migrates to the salivary glands of the mosquito where they remain until the mosquito bites a person and injects the sporozoites into a new human host (Service, 2000; WHO, 2014).

The signs and symptoms of malaria typically begin 8-25 days following infection. The initial manifestations of the disease are common to all malaria species which are similar to flu-like symptoms (Bartoloni & Zammarchi, 2012). The classic symptom of malaria is paroxysm which is a cyclical occurrence of fever, shaking chills, drenching sweats and headaches (Ferri, 2009). The frequency and severity of the fever depends on the malaria species involved but it usually lasts 2-3 days. The attacks of fever coincide with waves of parasite multiplication and the destruction of red blood cell (WHO, 2014). Malaria caused by *P. falciparum* does not always show this cyclic pattern. It is the most severe type of malaria and if untreated, may progress to shock, kidney and liver failure, coma and death. If blood vessels of the brain are affected, the condition is called cerebral malaria (WHO, 2014).

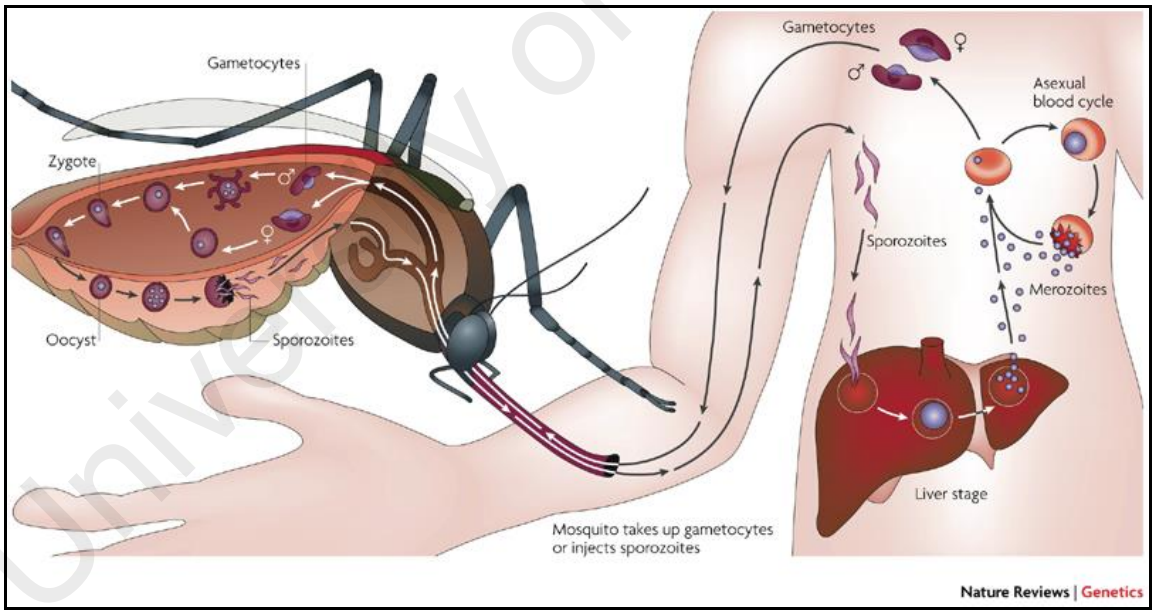


Figure 2.8: Life cycle of malaria parasite in human and mosquito (Su *et al.*, 2007).

Malaria can be prevented if measures are taken to avoid being bitten by *Anopheles* mosquitoes. Wearing protective clothing, use repellents on exposed skin, mosquito coils and other insecticide vaporizers, using bed net and aerosol spray are

among of the protective measure. Travellers or visitors to malaria endemic areas should take prophylactic drugs to prevent the development of the disease in the event of receiving an infective bite (WHO, 2014). Malaria can be treated with antimalarial medication depends on the type and severity of the disease (Meremikwu *et al.*, 2012). *P. falciparum* infection usually treated with artemisinins in combination with other antimalarial drugs (known as artemisinins-combination therapy, or ACT). Other drugs include amodiaquine, lumefantrine, mefloquine or sulfadoxine. While for *P. vivax, P. ovale, P. malariae* and *P. knowlesi* chloroquine or ACT and primaquine are the drugs use for their treatment (WHO, 2010).

2.2.3 Lymphatic Filariasis

Lymphtic filariasis, commonly known as elephantiasis, is a parasitic disease caused by an infection of filarial worms namely *Wuchereria bancrofti, Brugia malayi* and *B. timori* . These parasites are transmitted to humans through the bite of an infected mosquito. Filariasis caused by *W. bancrofti* occurs throughout much of the tropic (Asia, Africa, Pacific, Central and South America) and subtropical countries in the Middle East, while *B. malayi* occurs in West Malaysia, Indonesia, Thailand and Philippines (WHO, 2015c). *Brugia timori* has only been found in small Indonesian islands of Timor, Flores, Alor and Roti (Service, 2000). Lymphatic filariasis is transmitted by different species of mosquitoes; for example the *Cx. quinquefasciatus* that is widespread across urban and semi-urban area is the vector for bancroftian filariasis. The *Anopheles* and *Mansonia* mosquito which are mainly found in rural areas are responsible for brugian filariasis, while *Aedes spp.*, are the important vector for diurnal subperiodic bancroftian filariasis in Pacific Islands.

Filarial worm enter human body via the bites of infected female mosquitoes. The infective stage of larvae rupture the skin of labella of the labium and crawl onto the surface of the host's skin (Service, 2000). They will enter the skin through small lesion caused by mosquito's bite, and pass to the lymphatic system where they develop into adults' worms. Adult worms lodge in the lymphatic system and disrupt the immune system. The worms can live for an average of 6-8 years and during their life time; they produce millions of microfilariae (immature larvae) that circulate in the blood (WHO, 2015c). These circulating microfilariae can be taken up with a blood meal by the mosquito and pass into the stomach. Within a few minutes they exsheath, penetrate the stomach wall and pass into the haemoceal, from where they migrate to the thoracic muscle of the mosquito. In the thorax, they developed into third stage larvae (infective stage), leave the muscle and migrate through the head and down the fleshy labium of the proboscis. This infective larva will enter the host's blood stream during blood meal and continue the cycle (Service, 2000). Figure 2.9 demonstrated the life cycle of microfilariae in mosquito.

Lymphatic filariasis infection involves asymptomatic, acute, and chronic conditions. The majority of the infections are asymptomatic, showing no external signs of infection. However, these asymptomatic infections still cause damage to the lymphatic system and the kidneys as well as the body's immune system (WHO, 2015c). Acute symptoms involve the inflammation around the skin, lymph nodes and lymphatic vessels and often accompany the chronic lymphedema or elephantiasis. When lymphatic filariasis develops into chronic conditions, it leads to lymphedema (tissue swelling) or elephantiasis (skin/tissue thickening) of limbs and hydrocele (scrotal swelling).

Control of the lymphatic filariasis consists of treating the human host with microfilaricidal drugs (e.g. DEC, ivermectin) and concurrently reducing vector populations. Vector control involves the application of larvicides such as microbial insecticides (*B. thuringiensis*), use of expended polystyrene beads in pit latrines and septic tanks, use of impregnated bed nets, residual house spraying and fogging (Service, 2000).

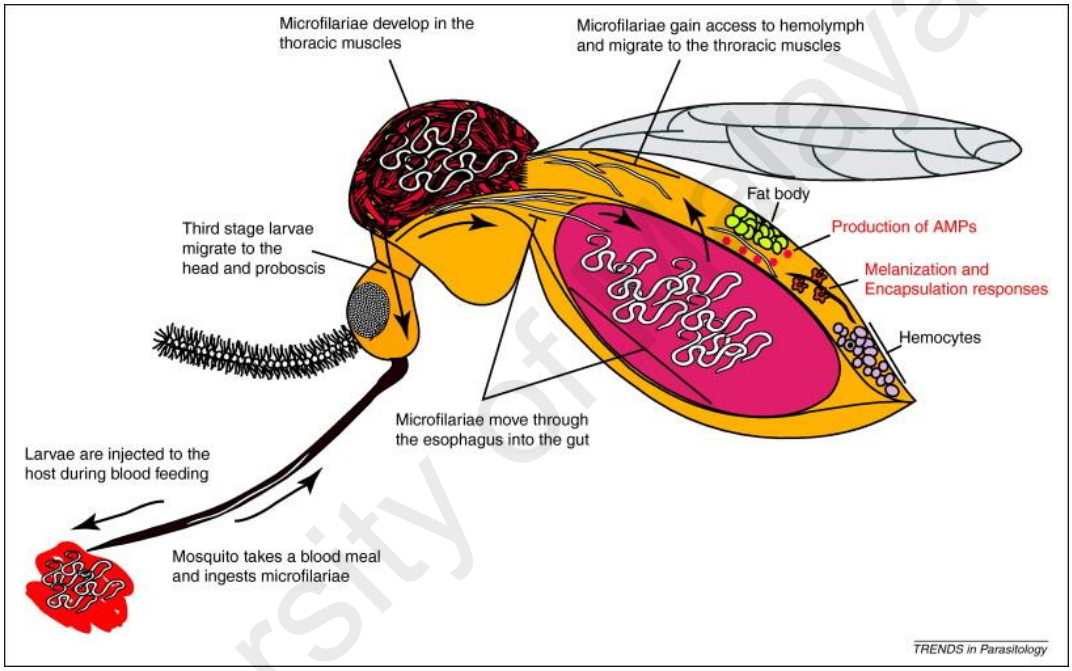


Figure 2.9: Life cycle of filariasis microfilaria in mosquito (Castillo *et al.*, 2011).

2.2.4 Japanese Encephalitis (JE)

Japanese encephalitis (JE) virus is a single-stranded RNA virus that belongs to the genus *Flavivirus* and is closely related to West Nile and Saint Louis encephalitis viruses. Japanese Encephalitis (JE) is mainly occurs in Japan, China, Malaysia, Korea and other areas of Southeast Asia and India (Service, 2000; CDC, 2015d). Figure 2.10 shows the life cycle of the JE virus. JE virus is transmitted to humans through the bite of an infected mosquito, primarily *Culex* species, mainly *Cx. tritaeniorhynchus*, *Cx. gelidus* and *Cx. vishnui*. The virus is maintained in an enzootic cycle between

mosquitoes and amplifying vertebrate hosts, primarily pigs and wading birds. Humans are incidental or dead-end hosts, because they usually do not develop a level or duration of viremia sufficient to infect mosquitoes (Gould & Solomon, 2008). Most human infections are asymptomatic or result in only mild symptoms. However, a small percentage of infected persons develop inflammation of the brain (encephalitis), with symptoms including sudden onset of headache, high fever, disorientation, coma, tremors and convulsions. About 1 in 4 cases are fatal (Service, 2000; CDC, 2015d).

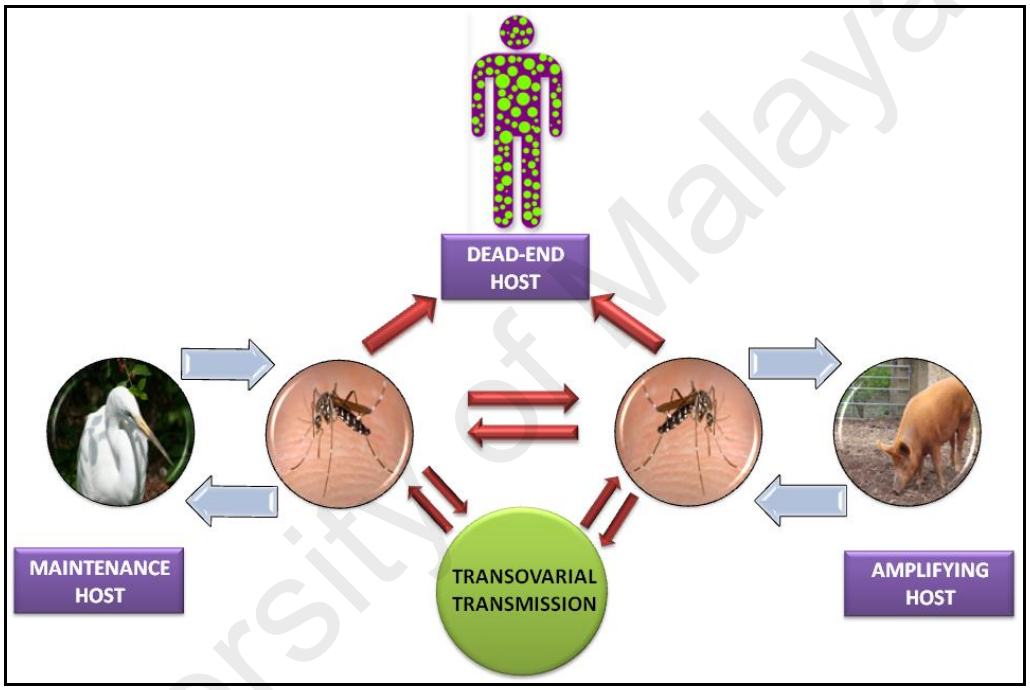


Figure 2.10: Transmission cycle of Japanese encephalitis virus. Infected *Culex* mosquitoes (vectors) play the role in the spread of JEV. Pigs are the amplifying hosts and birds (egrets) are the maintenance hosts while humans are the dead-end hosts. (Saxena *et al.*, 2014).

2.3 MOSQUITO CONTROL

The menace posed by mosquito bites have surged so much that mosquito control measurements are almost crucial to keep oneself safe from contracting serious illness as mention above. In Malaysia, the Vector Borne Disease Control Program was established in 1983 by Ministry of Health. This program is responsible to control seven vector

borne diseases including dengue, malaria, filariasis, JE, Plague, Scrub Typhus and Yellow Fever. Generally, mosquito control can be divided into three categories; which are chemical control, biological control and environmental management.

2.3.1 Chemical control

Chemical approached consist uses of organochlorine, organophosphate and carbamate as adulticide or larvacide (Yeager & Munson, 1945). In 1940s, these insecticides are the most potent insecticides in vector control. However, they gave persistence side effects to the environment, gave toxicity effect to human and other non-target animal or insect. It was also found to increased mosquito resistance towards insecticides used to the point that these insecticides was no longer effective and cannot be used anymore (Curtis, 1994).

It was in the late 1940s that pyrethrum and pyrethrin extracted from plant species named *Chrysanthemum* were introduced before the production of first generation of synthetic pyrethrin which have led to the development of permethrin, cypermethrin, deltamethrin and lambdacyhalothrin which are more stable to light (WHO, 2009a). To date, these pyrethroids have been known as the most ideal insecticide due to their effectiveness, low toxicity effect and environmentally friendly.

2.3.1.1 Adulticides

Mosquito adulticides are applied as ultra-low volume (ULV) sprays. ULV sprayers dispense very fine aerosol droplets that stay aloft and kill flying mosquitoes on contact (EPA, 2016). In Malaysia, chemical insecticides that have been used in ULV

spraying are of pyrethroid class such as deltamethrin and lambda-cyhalothrin (Chua *et al.,* 2005). Besides using as ULV spraying, pyrethroid also were the only chemical recommended for the treatment of bet nets, the main tool for preventing malaria (Liu *et al.,* 2006). Impregnated bed nets not only able to repel from mosquito bites, they also able to kill the mosquito when it makes contact with the insecticides on their surface. The use of pyrethroid insecticide for public health mosquito control programs is safe without posing risks of concern to the general population or to the environment when applied according to the pesticide label. However, there were several studies reported that pyrethroid resistance in the mosquito vectors (Philips, 2001; Liu *et al.,* 2006). Thus, mosquito control program that depend solely on the ULV spraying and impregnated bed net could be no longer sufficient or effective (Philips, 2001). In such situation, the application of larvacidal treatment is necessary to prevent the emergence of adult mosquitoes.

2.3.1.2 Larvicides

Application of larvicidal is usually limited to the containers maintained for domestic use that cannot be eliminated. This is the best method that can be used in the situation where the disease and vector surveillance indicated high risk that they may result in disease outbreaks (WHO, 1995). There are three types of insecticide that can be used to treat containers that hold drinking-water: i) temephos 1% (sand granule), ii) insect growth regulators (IGRs), and iii) *Bacillus thuringiensis* H-14. All this larvicides have extremely low mammalian toxicity, and properly treated the drinking-water for safe human consumption (WHO, 1995). Unfortunately, research has shown that resistance towards temephos has occurred in Caribbean and their neighborhoods countries (Rawlins, 1998). Although, resistance to temephos in *Ae.aegypti* and *Ae.*

albopictus populations has not been reported in the South-East Asian region, the susceptibility level of *Aedes spp.* should be monitor regularly to ensure the effective use of the insecticides (WHO, 1995).

2.3.2 Biological control

Interventions based on the introduction of organisms that prey upon, parasitize, compete with larvae are known as biological control (WHO, 1995). The advantages of biological control measures include no chemical contamination of the environmental and specificity against targets organism (WHO, 1995). *Gambusia affinis* Holbrooki and *Poecillia reticulate* Peters are among the larvivorus fish that can be used to control the larvae of mosquitoes in large water bodies or water container (WHO, 1995). Meanwhile, *Fundulus spp.* and *Rivulus spp.* are among of the species that able to control the larvae population especially in the open swamp area (Rose, 2001). Other than using predator fish, bacteria such as *Bacillus thuringiensis* serotip H-14 (BTH-14) and *Bacillus sphaericus* (Bs) have also been used in vector control. These bacteria produce protein which is toxic to mosquito larvae they ingested which can cause lysis and death (Rodcharoen & Mulla, 1994). This control measure is considered better than chemical control as it is environment friendly and target specific. Its usage unfortunately, is somewhat limited due to high expenses in growing/producing these organisms, difficulty in their application and production as well as they only effective against the immature stages of mosquitoes.

2.3.3 Environmental management

Environment management for vector control refers to changes made to the environment in order to prevent or minimize vector propagation and human contact with

the vector-pathogen by destroying, altering, removing or recycling non-essential containers that provide mosquito habitats. WHO Expert Committee on Vector Biology and Control had categorized environmental management into three types (WHO, 1980) which includes (i) environmental modification which is long-lasting physical transformations of vector habitats, (ii) environmental manipulation which is temporary changes to vector habitats as a result of planned activity to produce conditions unsuitable for vector breeding, and (iii) changes to human habitation or behavior which reduce human-vector-pathogen contact.

Environmental modification involved the long-lasting physical transformations to reduce vector larval habitats, such as installation of a reliable piped water supply to communities, including household connections. Improvement of water supply and storage is one of the methods applied to control urban *Aedes* vectors, particularly, *Ae. aegypti* since it helps improve the delivery of portable water to neighborhoods or individual homes and reduce the use of water storage containers that play a dominant role in *Ae. aegypti* breeding in many urban areas. Insufficient funding and lacking of inter-sector collaboration however are the major challenges for this type of control programs (EPA, 2009).

Environmental manipulation is the temporary changes to vector habitats involving the management of "essential" containers, such as frequent emptying and cleaning by scrubbing of water-storage vessels, flower vases and desert room coolers; cleaning of gutters; recycling or proper disposal of discarded containers and tires. Environmental manipulation methods for different diseases such as malaria, filariasis and JE depend on their vectors and habitats. For example removing or killing of aquatic

plants in swamps areas may be helpful in controlling *Mansonia spp.* the vector for filariasis and may be the most affordable control measures that can be done. Lack of community awareness and involvement in such activities however will limit the successful of this method of control program. In addition, removing of these aquatic plants will alter and indirectly promoting ecological changes of the areas (EPA, 2009).

Improving drainage system, sewage management, and hygiene is effective in controlling *Culex* mosquitoes, vector for filariais and JE since *Culex* mosquitoes prefer to breed in polluted water with high organic matters (Service, 2000). As for *Anopheles* mosquitoes, the vector for malaria, the environmental manipulation found effective for its control include drainage, grading, filling, marsh alteration, vegetative planting, water management activities such as changing water levels in reservoirs, flushing streams or canals, providing intermittent irrigation to agricultural fields (particularly rice), flooding or temporarily dewatering man-made or (where feasible) natural wetlands, and changing water salinity. Manipulation of vegetation may also be useful for example planting water-intensive tree species, such as *Eucalyptus robusta* which can reduce standing water in marshy areas. Planting shade trees near potential larval habitats may also help reduce the abundance of vectors, such as *An. gambiae, An. funestus, An. minimus* and *An. sundaicus* that prefer sunny conditions for larval development (Service, 2000).

In addition, an effective environmental friendly vector control for solid waste management should promote the basic rule of "reduce, reuse, and recycle". For example, plastic container that may serve as potential larval habitats should be recycled. Used tires are another form of solid waste that can serve as breeding site of larvae; thus

should also be recycled or disposed by proper incineration in waste transformation facilities (e.g. incinerator, energy-production plant) (EPA, 2009).

Changes to human habitation or behavior which reduce human-vector-pathogen contact refer to common-sense approaches that help to reduce the potential habitat for *Ae. aegypti* mosquitoes to breed (for examples flower pots, flower vases and ant traps) in and around human surrounding (WHO, 1995). Ant traps to protect food storage cabinets can be treated with common salt or oil (EPA, 2009). Floor drains should be cleaned and kept covered. Roof gutters, outdoor sink, laundry basins and similar items that can retain water and serve as larval habitats should be drained and kept free of debris (WHO, 1995). These measures and others will help in reducing or preventing the breeding of mosquitoes near humans, and thereby reduce the risk of dengue fever.

2.4 REPELLENT AS PERSONAL PROTECTION AGAINST MOSQUITO BITES

Apart from control programs that are implemented at the community level, control measure at individual level is also considered vital as far as disease transmission is concerned. The use of repellent such as topical mosquito repellent, protective clothing and mosquito net are measures that can prevent mosquito-individual contact. To date, many types of repellents, such as mats, coils, lotions and vaporizers are available in the market. Allethrin, bioallethrin, d-allethrin, d-transallethrin and prallethrin are the pyrethroid insecticides that usually used as active ingredient in these mats, coils and vaporizers. The use of repellent not only help protecting individuals against mosquito bites, it indirectly reduce the mosquito population where essential

nourishment for eggs was denied when mosquito fail to feed blood from protective individuals.

Repellent is an English word derived from Latin verb repellere, meaning “to drive back”, and the movement away being repulsion. Dethier defined repellents as “any stimulus which elicits an avoiding reaction” and made a further distinction, in terms of the physical state of the chemical, by recognizing contact repellent and vapor repellents, i.e., those that have to be touched by the insect or detected in the air (Dethier, 1976). Repellent acts by inhibiting the receptor on mosquito’s antenna from detecting the human body temperature, moisture, and carbon dioxide, which then cause misperception and target-direction’s incapability.

There were various types of repellent on the market. Repellent that applied on the skin or clothes known as topical repellents due to the topical treatment. Some devices such as mosquito coils and repellent vaporizers are designed to protect an area or space outdoors or indoors by releasing vapors as long as the device operates.

2.4.1 Type of repellent

2.4.1.1. Chemical-based repellent

a. DEET

N,N-diethyl-3-methylbenzamide (DEET) is the repellent that had been used for more than 50 years, by approximately 200 million people worldwide (Moore & Debboun, 2007). Since 1950s, DEET has been considered as the most broad-spectrum and efficacious repellent against mosquitoes, ticks and other arthropods. In 1997, Brown and Hebert reported that the mechanism of action of DEET, along with certain other

insect repellents, is to provide a vapor barrier that deters the insect with an offensive odor and a bad taste. DEET also had been reported to have ability to modify the physiology response of lactic-acid-sensitive olfactory receptor neuron (ORNs) on the adult female mosquito's antenna (Davis and Sokolove, 1976) that prevent the response of olfactory system from the chemical signal's attraction (Davis, 1985).

To date, there were various repellent products available in the market containing DEET as active ingredient such as Off (SC Johnson, Racine, Wis), Cutter (Spectrum Brands, Atlanta, Ga), Outdoorsman (Spectrum Brands), Skedaddle (Minnetonka Brand INC, Chaska, Minn) and Ultrathon (3M, St. Paul, Minn). Although DEET has been shown of having excellent protection effect, there were several reported cases and research documented the presence of toxicity effect. The toxicity effect most commonly seen is local skin irritation, including erythema and pruritis, at the site of application. A case of anaphylaxis after brief exposure to DEET also has been reported. Many people, including military and forest service personnel, apply high concentrations of DEET on a daily basis developed more severe adverse effects due to chronic exposure that include insomnia, muscle cramps, mood disturbances and rashes (Osimitz & Murphy, 1997). Furthermore, the use of DEET-containing repellent and sunscreen concurrently can increase the systemic absorption of DEET six times faster (Katz *et al.*, 2008). Due to these limitations, DEET free-based repellent products received great attention among the consumers

b) Permethrin

Permethrin is the synthetic pyrethroid that can act as a repellent and insecticides against ticks, mosquitoes and other arthropods. This compound may be used on clothes,

shoes, bed nets, and camping gear, and usually requires reapplication after every five washings. Once in contact it interfere the ionic conductance of nerve membranes of the insects by prolonging the sodium current. This will stimulate nerves to discharge repeatedly causing hyper-excitability, tremors, and paralysis in poisoned insects. However, permethrin is not suitable to use as topical application (CDC, 2006). This might be due to the mechanism of action of permethrin that requires direct contact with the insects, thus making this compound poorly suited for skin application. When exposed to human especially at high concentration, permethrin is known to give neurotoxicity effect, such as tremors, hyperactivity, paralysis, and increase body temperature. Other side effects are irritation on the skin and eyes, reproductive system, mutagenicity, and immune system (Alonso, 1991).

c) Picaridin

Picaridin, 1-piperidine carboxylic acid-2(2-hydroxxyethyl)-1- methpropylester was developed by Bayer in the 1980s as synthetic molecule chemically based on the active agent in pepper (*Piper spp.*). Picaridin was first registered with the US Environmental Protection Agency (EPA) in 2001 and entered the market in 2005 (Katz *et al.,* 2008). Similar to DEET, picaridin's mechanism of action is to provide a vapor barrier that works to deter the insects from biting. Previous research indicated that picaridin may be detected by insects via gustatory receptors neuron (contact chemoreceptors) on the labella and function as feeding deterrent (something that inhibits feeding) (Dickens & Bohbot, 2013). Unlike DEET, picaridin has many characteristics of an ideal insect repellent as it is odorless, does not feel sticky or greasy on application, is less likely to irritate the skin, and will not damage plastic and fabrics.

Unfortunately the allergic contact dermatitis cases due to continues use of Picaridin is increasing (Corazza *et al.*, 2004).

2.4.1.2 Plant-based repellent

The potential of plant as repellent had been discovered prior the development of chemical repellent. In 1901, citronella oil from *Cymbopogon spp.* was discovered and since then it has been widely used as active ingredient in repellent and insecticides product. In 1940s however, the use of plant as repellent was overshadowed by the emergence of synthetic chemical, DEET in 1946. Since then onwards, many people hinge on DEET product for protection against mosquito bites as it was found to be more effective than citronella oil. At present, there are several repellents based on plant available in the market. Most of these plant-based repellents consist of citronella oil, neem oil, and eucalyptus (Maia & Moore, 2011).

a. Citronella oil:

Citronella oil is an essential oil extracted from plants of citronella of genus Poaceae. It is commonly used as the ingredient of plant-based repellent in commercial preparations. It was registered for commercial use in the USA in 1948 and to date citronella is still one of the most widely used natural repellents in the market (Maia & Moore, 2011). Treo, Avon Skin So Soft, Natrapel and Buzzaway are among the commercially available repellents, which contained citronella oil. In general, most of the citronella-based repellent shown to have protection time of about two hours (Trongtokit *et al.*, 2005a; Trongtokit *et al.*, 2005b).

b. Neem oil:

Neem oil is an essential oil extracted from plants of genus Azadirachta especially *Azadirachta indica*. Neem is widely advertised as the alternative to DEET (Ava, 2009) and has been reported to have repellent effect against a wide range of medically important arthropods. Several studies demonstrated that neem oil exhibit better repellency compared to citronella oil (Moore *et al.,* 2002; Barnard & Xue, 2004). Although it presented low dermal toxicity, it can cause skin irritation, such as dermatitis particularly when used undiluted (Reutemann & Ehlich, 2008). It is for this reason that the EPA has not approved neem for use as a topical insect repellent. Neem oil is not recommended for use by travelers in disease endemic areas due to low repellency effect (Goodyer *et al.,* 2010).

c. Lemon eucalyptus:

Scientifically known as *Corymbia citriodora,* lemon eucalyptus is a potent natural repellent extract that was discovered in the 1960s. The main compound (around 85%) of eucalyptus oils is citronellal. This is the compound that gives fresh smell to this oil and therefore widely used in cosmetic industries (Veira, 2004). Barasa *et al.* (2002) showed that eucalyptus oil contained para-methane 3,8 diol (PMD) which is one of the monoterpene compound reported to cause lower vapor pressure compared to other compounds. Another study reported that PMD provide high protection over several hours (Carroll & Loye, 2006).

In United States PMD had been isolated and known as oil of lemon eucalyptus (OLE) or by its trade name Citriodiol (Pichersky & Gerhenzon, 2002). Several PMD-based insect repellents developed had been approved by USEPA for protection against mosquitoes (Emily Zielinski-Gutierez & Roger, 2010; Maia & Moore, 2011). Quwenling is one of the eucalyptus-based products that have been successfully marketed as an insect-repellent in China. It provides protection against *Anopheles* mosquitoes parallel to DEET. Mosiguard Natural is another eucalyptus oil-based repellent that can be found in the market and it contains 50% of eucalyptus oil. MyggA1 Natural is another mosquito repellent product based on PMD and is shown to repel ticks (Jaenson *et al.*, 2006).

PMD is the only plant-based repellent that has been advocated for use in disease endemic areas by the CDC (Centres for Disease Control) (Emily Zielinski-Gutierez & Roger, 2010), due to its proven clinical efficacy in preventing malaria (Hill *et al.*, 2007) and pose no risk to human health (EPA, 2000).

d. Other essential oils:

Other essential oils such as pennyroyal, clove, nutmeg, juniper berries, wintergreen, and pine were also in demand but more as synergistic ingredients to be used in combination with citronella, eucalyptus and neem oil. For example Green Ban, is a repellent that combines citronella oil, cajuput grass, sassafras, peppermint, and myrrh oil. Such combination however has made Green Ban not only more effective but expensive as well. Other essential oil such as coconut oil, palm nut oils (Konan *et al.*, 2003) and andiroba oil (Miot *et al.*, 2004) also shown to possess not only repellent

effect but they are useful as carriers for other repellent actives. They are also cheap and contain unsaturated fatty acids and emulsifiers that improve repellent coverage and slow evaporation of volatile repellent molecules (Reifenrath *et al.*, 1989; Campbell & Gries, 2010).

2.5 DEVELOPMENT OF PLANT-BASED REPELLENT

There are many stages involved from development to commercialization of plant-based repellent product. These include selection of plants, extraction, isolation and chemical characterization of the active compounds. All these processes can be very tedious and time consuming. Therefore, it is necessary to develop a research frame which focused on methodology standardization (the extraction methodology, appropriate and efficacious formulation and bioassay technique), the cost and the quality such as safety evaluation, stability and shelf life as well as proper packaging and labeling of the plant product (Mann & Kaufman, 2012).

2.5.1 Plant selection: Ethnobotanical study

The tropical forests which provide about half of the world's plants species (125,000) continue to sustain a vast reservoir of potential drugs species and remain to provide invaluable compounds for the development of new drugs (Gurib-Fakim, 2006). Since ancient times, human relied so much on nature for their basic needs including foods, shelters, clothing, transportation, fragrances, medicine, as well as for controlling of pest. Plants have become the main sources for traditional medicine system for thousands of years and continue to provide mankind with new remedies. Although about 80% of the world populations primarily those from developing countries depend on plants for their healthcare (Gurib-Fakim, 2006) it is only 1% of the tropical species

have been studied for their pharmaceutical potential. Clearly, there are vast research opportunities out there with regards to plants and its relation to medicine and health.

Apart from medicinal purpose, human also used plants as pest control or repellent to protect themselves against blood sucking insects (Curtis *et al.*, 1991). They either planted or burned the local aromatic plants outside their house to avoid mosquito bites (Karunamoorthi & Husen, 2012). Figure 2.11 showed Maghull painting illustrating a man burning neem leaves near a river where biting insects most likely present. Numerous plants that traditionally used as repellent or pest control had been documented through many studies. An ethnobotanical study that carried out by Samuel *et al.* (2010) among orang asli community in Kampung Bawang, Perak of West Malaysia reported that the use of *Lantana camara* Linn. to repel insects. Previous research by Karunamoorthi *et al.* (2009b), documented that people in Ethiopia used *Boswellia papyrifera* (Del.) Hochst, *Echinops* species, *Croton macrostachyus* Del. *Melia azedarach* L., *Eucalyptus globulus* Labill and *Cymbopogon citratus* (DC.ex.Nees) Stapf to drive away blood sucking insects. Another study done by Waka *et al.* (2004) reported that people in Eritea used *Ocimum forskolei* and *Nicotiana glauca* against mosquitoes, *Salvia shimperi* and *Otostegia integrifolia* against fleas and *Neorautanenia mitis* and *Calpurnea aurea* against animal lice. Some of these plant species have already been developed as insecticides or repellent, many others however have not been developed yet.

The knowledge and usage custom of traditional medicinal plants usually passed down to many generations mostly through word of mouth or informal education (Karunamoorthi & Husen, 2012). This mode of information passage may result in

falsification or loss of indigenous knowledge and usage custom of medicinal plant. Hence, it is crucial to protect this information through documentation (Karunamoorthi *et al.,* 2009a). The documentation of such information will assist in generating national and international data-base on plants traditionally used as medicines (Karunamoorthi & Husen, 2012).

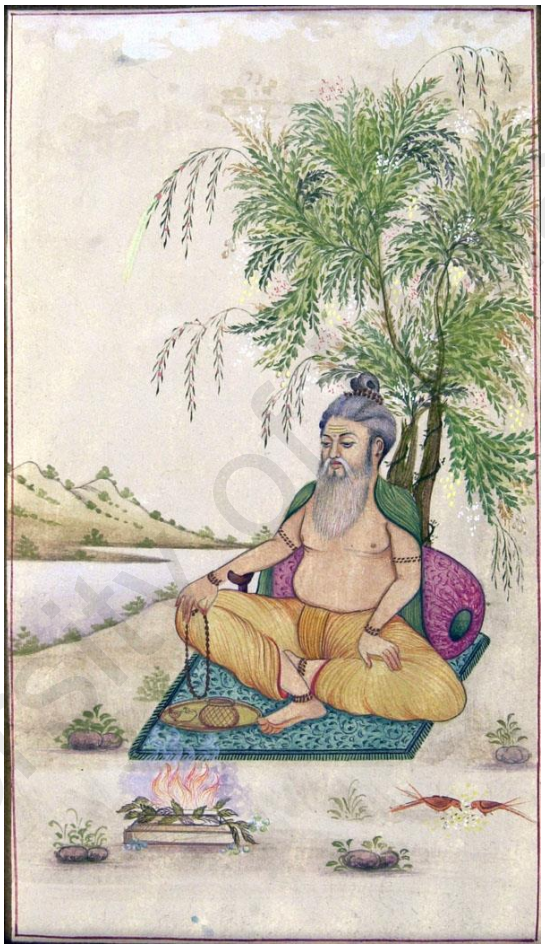


Figure 2.11: Moghul painting illustrating a man burning neem leaves near a river where biting insects most likely present (Maia & Moore, 2011).

It is nearly impossible and impractical however to choose the plants species for research purpose via random selections. Using practical tools not only will save money and time but will also provide exact information regarding the potential plant species. One of the research tools commonly used is ethnobotanical study. Ethnobotanical study

is the study of relationships that exist between people and plants. There are two purposes of this study: (i) to find a new compound and (ii) to document the traditional uses of plants (Waka *et al.*, 2004).

2.5.2 Plant phytochemicals and its secondary metabolites

Plants produce a wide spectrum of chemicals in various tissues above and below ground that are used not only to communicate with other plants (Kegge & Pierik, 2009) and organisms (Dicke & Baldwin, 2010), but also to defend themselves against abiotic stressors (Holopainen & Gershenzon, 2010; Loreto & Schnitzler, 2010). Extraction of these tissues produces many **phytochemicals** that have various biological roles that are significant to human health. For decades, researchers have been studying the used of phytochemicals in medicine, food, textile, fragrance, hygiene products, and pest and disease management tools (Miresmailli & Isman, 2014). Some of these phytochemical such as nucleic acids, proteins and carbohydrates involved in primary metabolism that crucial in growth and development of plants. **Secondary metabolites** on the other hand are produced to facilitates these functions and keeps the plants' system working properly. A common role of secondary metabolites in plants is defensive mechanism. These metabolites are classified as nitrogen-containing compounds, phenolics, polyacetates, and terpenoids (Miresmailli & Isman, 2014).

A secondary metabolite that has been marketed for more than 20 years is terpenoid azadirachtin. It is found in the seed of Indian neem tree, *Azadirachta indica* which are used as contact fumigants, attractants, and repellents to control agriculture pests (i.e green peach aphid and greenhouse whitefly), urban pests (i.e housefly,

bedbug, cockroaches, and ants) and medical pests (i.e mosquitoes, ticks, and lice). Individual metabolite may or may not give the toxicity effect required however complex mixtures of several, often closely related secondary metabolites known as botanical insecticides shown to exhibit internal interactions in the form of synergy or antagonism, which can affect the overall toxicity of the mixture (Miresmailli *et al.*, 2006). Pesticide compounds exist within almost all classes of secondary metabolites. For example, the alkaloids nicotine which is found in the nightshade, Solanaceae family and strychynine which is found in the seeds of *Strychnos* spp have been historically used as pesticides (Metcalf, 1955; Miresmailli & Isman, 2014).

To date, the most important botanical insecticides available on the market are pyrethrum, neem, and essential oil-based products. They are complex mixtures of low-molecular-weight, highly volatile secondary metabolites. Owing to their versatile nature, they have been used in a variety of products, from contact toxicants to fumigants and even in behavior –modifier products, such as attractants and repellents (Miresmailli & Isman, 2014). Among the three, essential oil-based products are the most diverse botanical insecticide.

2.5.3 Essential oil

Essential oils are biosynthesized, accumulated and stored in specialized histological structure named secretory glandules (Bouwmeester *et al.*, 1995). There are two types of secretory glandules; those located on the plant surfaces with exogenous secretion and those located inside the plant in the internal organ with endogenous secretion (Svoboda & Greenaway, 2003). It is available at different plant parts such as

leaf, stem, flower, root, and rhizome depending on the plant species. Essential oil can be obtained from most of the aromatic plants such as family Rutaceae, Umbelliferae, Myrtaceae, Zingiberaceae, Apiaceae, Labiatae, Poaceae, and Piperaceae (Shaaya & Rafaeli, 2007).

Biological roles of essential oils in plants remain hypothetical but it seems that they play a role in plant-plant interactions (inhibition of germination and growth of other plants), plant-animal interaction (attractors of pollinators and pest repellents), defensive role against fungi and pathogenic microorganism, against herbivores and insects. It also produced chemical signals that allow the plant to control and to regulate their environment (Bruneton, 2009). For example, resistance in conifer *Pinus sylvestris* L. against *Callidium violaceum* L. is due to the high essential oil composition in its barks (Smelyanets & Khursin, 1973; Karasev, 1974).

In general, essential oil represents a small fraction of plant composition (less than 5% of the vegetal dry matter) and comprises mainly hydrocarbon terpenes and terpenoids. The main compounds are monoterpenes and it represents more than 80% of the essential oils composition. Terpenes have various functions as potent drugs including anticancer (Ebada *et al.,* 2010), heart disease (Liebgott *et al.,* 2000) as well as insecticidal properties (Rossi *et al.,* 2012). Monoterpenoids such as α-pinene, cineole, eugenol, limonene, terpinolene, citronellal, camphor and thymol are among the common constituents found in the essential oil responsible in repellent activity (Yang *et al.,* 2004; Park *et al.,* 2005; Jaenson *et al.,* 2006). Gilllij *et al.,* (2008) reported that sesquiterpenes found in essential oil such as β-caryophyllene also possess strong repellent effect against mosquito. Other than monoterpenes and sesquiterpenes, phytol

also has high repellent activity against *Anopheles* spp (Odalo *et al.,* 2005). Although repellent activity of essential oils is generally attributed to some particular compounds, a synergistic phenomenon among these metabolites may results in a higher bioactivity compared to the isolated compounds (Hummelbrunner & Isman, 2001; Gillij *et al.,* 2008; Nerio *et al.,* 2010). Previous research by Omolo *et al.* (2004) reported that repellent activity of major compound was much smaller than those of the corresponding essential oil thus indicate that minor constituents also contribute to the repellent activity and reflect the importance of compositional complexity in conferring bioactivity to natural terpenoid mixtures (Nerio *et al.,* 2010).

Vast studies have revealed that essential oil from plants of having the potential as bioactive agent against mosquito vector. Amongst them Basil oil from *Ocimum basilicum* L., cinnamon (*Cinnamomum cassia* Blume), citronella (*Cymbopogon nardus* L.), and thymus oil (*Thymus vulgaris* L.) which act as mosquito larvicidal (Mansour *et al.,* 2000; Cavalcanti *et al.,* 2004). Meanwhile *Lantana camara* leaves Linn essential oil (Dua *et al.,* 2010), celery (*Apium graveolens*), caraway (*Carum carvi*), zedoary (*Curcuma zedoaria*), long pepper (*Piper longum*), and Chinese star anise (*Illicium verum*) are found to be effective mosquitos' adulticide (Chaiyasit *et al.,* 2006). Others such as *Cananga odorata, Cymbopogon citratus, Cymbopogon nardus, Eucalyptus citriodora, Ocimum basilicum* and *Syzygium aromaticum* have been shown to possess oviposition-deterrent and ovicidal effect on mosquitoes (Chaiyasit *et al.,* 2006). Over the last 50 years, thousands of plants have been screened as potential sources of repellents and insecticides (Sukumar *et al.,* 1991). Based on these screenings the repellent activity possessed by these potential plants is said to be related to the evaporation property of the essential oil. It is said to have caused irritation on the

insects' leg once contact with the repellent/insecticide took place and eventually causing them to fly away from the treated area (Peterson & Coats, 2001). Nevertheless, mechanism of action of essential oil as repellent is still a controversial issue (Tripathi *et al*., 2009) although Brown and Hebert (1997) reported that the way the essential oil works is by providing a vapor barrier that deter the arthropod from coming into contact with the surface. While in another study, it was reported that the lipophilic properties of the essential oil caused alteration of the olfactory receptor in the mosquito thus reducing short range of the attractive cues (Wang *et al*., 2008).

Insects detect odor when that volatile odor binds to odorant receptor (OR) proteins displayed on ciliated dendrites of specialized odor receptor neurons (ORNs) that are exposed to the external environment, often on the antennae and maxillary palps of the insect. Ditzen *et al.,* (2008) reported that DEET-based repellent (synthetic repellent) blocked OR83b which is the important ORN in olfaction. It is also the same odor receptors that respond to thujone eucalyptol and linalool (compounds in eucalyptus oil) in *Culex quinquefasciatus* (Syed & Lael, 2008). Several other authors have shown strong responses of mosquito odor receptors (ORN) to volatiles compounds of essential oil including geranyl acetate and citronellal (Carey *et al.,* 2010), 6-methyl-5- hepten-2-one and geranylacetone (Logan *et al.,* 2010) suggesting that essential oil block the odorant receptor of insect thus deter the insect away from the host.

2.5.4 Improvement of essential oil repellent efficiency

There have been many studies that described the effects of extracts and essential oils of plants on insect. These botanical insecticides however come with poor physicochemical stability, high volatility and thermal decomposition criteria that limit

the effectiveness of extracts and essential oils compared to synthetic insecticides which are known to be more stable and effective. Botanical insecticide therefore must be rigorously standardized to a degree that can bring them to a certain level of effectiveness in their final use, if it is to compete with the synthetic insecticides (Isman, 2006; Tramon, 2014). Thus, any production of new formulation needs to consider improvement in potency and stability of the essential oil when use as the active ingredient in repellent product.

As mentioned earlier, the repellent activity of a product is highly dependent on the composition of the active ingredient used. Botanical extract and essential oils often comprise of lipophilic and highly volatile constituents. These compositions however known to be susceptible to conversion and degradation reactions, such as oxidative processes which can result in loss of quality and certain properties (Sell, 2010). The stability of these substances is affected when exposed to elements such as air, lights and elevated temperature. Compared to the synthetic insecticides, botanical insecticides are relatively unstable and breakdown significantly faster when exposed to these elements (Turek & Stintzing, 2013). Once plant chemicals have been removed from their protective compartments as a result of destructive extraction methods, their constituents are prone to oxidative damage, chemical transformation damage and polymerization reactions. Furthermore, as plants extract age, their quality declines further. Over the times, they might lose some of their traits such as odors, flavors, color and consistency (Pokorny *et al.,* 1998; Grassmann & Elstner, 2003).

Some fixative materials such as liquid paraffin (Oyedele *et al.,* 2002), vanillin (Tawatsin *et al.,* 2001), salicyluric acid (Blackwell *et al.,* 2003), mustard and coconut oils (Das *et al.,* 1999) have been used to help improve the repellent activity of essential oils. Amongst all, vanillin is the compound that mostly used by mixing it with essential

oil (Kamsuk *et al.*, 2006; Choochote *et al.*, 2007). This fixative material has been reported to improve the protection time of essential oil against various species of mosquito (Khan *et al.*, 1975; Yang and Ma, 2005; Kamsuk *et al.*, 2006). In addition, the use of carrier oil such as coconut oil, palm nut oil and andiroba oil and emulsifier (such as polyethelene glycol, sodium laurate sulfate) in the formulation also aid in improving the repellent activity. It helps to slow down the evaporation process of volatile repellent molecules (Reifenrath *et al.*, 1989; Campbell & Gries, 2010).

Apart from stability of the oils low skin penetration and water resistance of the formulation to be developed also play an important roles in providing optimum protection against mosquito bites. These can be achieved by addition of polymer into the formulation prepared (Calton *et al.*, 2001). Other components including emollients, fragrances, preservatives, vitamins, humectants and antioxidants are commonly included although not compulsory, to enhance the feel of the material upon application, to moist the skin, and/or to protect the formulation against microbial contamination. Most of the plant-based repellent products available in the market were prepared following such techniques. Unfortunately, there were several studies that demonstrated repellent products available in the market having protection time less than two hours and some required reapplication after one or two hours to get the protection required (Thavara *et al.*, 2002; Trongtokit *et al.*, 2005a). For examples, a study in Thailand, reported that BuzzAway® (containing citronella, cedar wood, eucalyptus, lemongrass, alcohol, and water) and Green Ban® (containing citronella, cajuput, lavender, safrole-free sassafras, peppermint, bergaptene-free bergamot, calendula, soya, and tea tree oils) exhibited a repellent effect of two hours against mosquito bites (Trongtokit *et al.*, 2005b). Another study demonstrated that a citronella-based repellent providing complete protection for only half an hour (Tuetun *et al.*, 2008). Obviously, more

research are required if these problems are to be addressed through development of new technique or formulations.

2.5.5 Safety issues of repellent product

Thousands of plants have been screened as potential sources of repellents and insecticides but only a few of them have been registered by US Environmental Protection Agency (US EPA) (Sukumar *et al.,* 1991; Katz *et al.,* 2008). Citronella, lemon and eucalyptus oil are among the natural ingredients already obtained approval due to their relative low toxicity and acceptable efficacy and therefore often selected by many manufactures as the ingredients in their development of plant-based repellent (Nerio *et al.,* 2010). Although the majority of the essential oils are classified as Generally Recognized as Safe (GRAS) by US Food and Drug Administration (FDA), nevertheless it is important to realize that plant products are not necessarily safer than synthetic ones. For example, their use as topical application often limited due to the irritation and sensitization effect that may arise from the preparation and formulation method (Tripathi *et al.,* 2009).

It is frequently found that although essential oils may have promising properties as insect repellent, like those isolated from the Australian species *Dacrydium franklini* and *Melaleuca bracteata*, they could be unsuitable for use because they were found to cause side effect such as skin irritation (Jacobson, 1966). In addition, even though it had been listed in US EPA's and GRAS list, d-limonene which is the compound that commonly found in citrus plants like orange, lemon, mandarin and grapefruit was found to cause dermatitis in certain individual (Nilsson *et al.,* 1999; Tripathi *et al.,* 2009).

Plants produce a wide variety of insect toxins, many of which are dangerous to mammals (D'Mello, 1997). Thus, simply assuming that natural is safe can be quite dangerous. Another example is essential oil of *Mentha pulegium*. It is widely used as

insect control agent. It contains an active ingredient, pulegone. Upon ingestion pulegone is oxidized by cytochrome P-450 system into toxic metabolite including methofuran (Nelson *et al.,* 1992; Tripathi *et al.,* 2009). These metabolites bind to proteins (Thomassen *et al.,* 1992) causing loss of organ function, seizures, acute poisoning and death (Burkhard *et al.,* 1999). Therefore, the use of such ingredient is strictly not advisable. The toxicity effect of the plant product is not only due to it active ingredient alone. Other factors such as chemical interaction between active ingredient and the other component in the formulation, interaction with the container, as well as photo degradation could also contribute toward the toxicity effect. For example, tea tree oil (*Melaleuca alternifolia*) which is known to have insecticide activity, known to cause contact dermatitis upon application on skin. Study by Hausen *et al.* (1999) revealed that the dermatitis it caused was due to the formation of degradation products (epoxides, peroxides and endoperoxides) resulted from photodegradation mechanism in the containers.

It is therefore important to run the toxicity evaluation after the formulation to determine the toxicity level of the final products for safe usage. The stability assessment also will help determine the safety status of the formulation by looking at the changes in their pH and organoleptic characteristics during the storage time and condition.

2.6 RECENT TECHNOLOGY IN PLANT-BASED TOPICAL REPELLENT PRODUCTION

Essential oil acts as repellent when it is in the vapor phase by releasing its chemical compounds. The speed of chemical compounds released however tend to influence the longevity of the repellent effect. The faster it is released the shorter the

longevity of the repellent effect become thus left users unprotected quicker (Maia & Moore, 2011). In order to provide optimum protection/longer repellent effect, the release rate of chemical compounds needs to be controlled so that it is sustained for a longer period of time. As previously mentioned, many studies used fixative materials, emulsifier and carrier oil to try and improve the longevity of the repellent effect. They were however proven to be not very successful. For that reason, new techniques have been developed to achieve the same goal which is to control the release rate of chemical compounds to enhance the performance of plant repellent by providing long-lasting repellents effect.

Development of "controlled release formulation" is the most recent formulation produced in the repellent product development. It allows smaller quantities of insecticides to be used with high protection effect over a given period of time. Nanotechnology is one of the recent technologies used in the production of controlled release formulation. Study by Sakulku *et al.* (2009) reported that a mixture of citronella oil in nanoemulsion complex increased the repellency effect with 95% protection that lasted for 4.7 hours post application. Another study by Nuchuchua *et al*. (2009) showed that encapsulated citronella oil nanoemulsion achieved 95% protection against *Ae. aegypti* for 2.8 hours.

2.6.1 Nanoemulsion technique

Nanoemulsion technique creates stable droplets that increase the retention of the oil and slow down the release, thus prolonging the protection effect. Nanotechnology application for repellent product development is still ongoing research but results

obtained thus far have shown that nano size oil droplets produced were having size of less than 1 µm and shown to have high absorption rate through skin (Karr *et al.*, 2012).

2.6.2 Encapsulation technique

Another technology used in producing controlled release formulation is encapsulation technique. Microencapsulation produces micron size of oil droplets that help decrease the absorption rate of essential oil through skin (Karr *et al.*, 2012). This technique also reduces loss of active agent leading to high loaded particles that offer protection against environmental agents and also offer the possibility of controlled active agent release (Moretti *et al.*, 2002; Tripathi *et al.*, 2009). Solomon *et al.* (2011) who developed a method to encapsulate citronella oil by using simple coacervation technique that controls the release rate of oil, demonstrated that microencapsulated citronella oil releases 70% of the oil content for 10 hours. Maji *et al.* (2007), successfully encapsulated *Zanthoxylum limonella* oil using coacervation technique by using glutaraldehyde crosslinked gelatin. Study by Asnawi *et al.* (2008) also successfully produced geranium oil loaded solid lipid nanoparticles by using ultrasonic-solvent emulsification technique for mosquito repellent application. All these studies reported that microencapsulation proven to have slowed down the release rate of the essential oil thus help to improve the duration of the repellent effect. Although there were only handful studies that employed microencapsulation in plant-based repellent production and most of them are ongoing research, the findings were quite promising with regards to controlling of essential oil and worthy to be explored further.

2.7 MICROENCAPSULATION APPLICATION IN REPELLENT PRODUCT

2.7.1 Definition and characteristics

Microcapsulation is a technology in which a "core" material is coated with or entrapped within a "shell" or "wall" material (Figure 2.12). The microparticles formed by this process are defined as solid and spherical systems with a diameter ranging between 1 and 1000 µm. This microparticle can be classified as microcapsule or microspheres (Schaffazick *et al.*, 2003). The core material can be solid particles, liquid droplets or gas bubbles while the shell materials are consisting of natural or synthetic polymer. Natural polymer that commonly used as shell material are gelatin, gum Arabic, agarose agar, alginate, carrageenan, insect wax, paraffin, beeswax, starch, dextrin, oligosaccharide and chitin. Whereas polyurethane, polyester, polyacrylate, polyuria, polyvinyl alcohol, polyamide, epoxy resin, polyether, phenol-polyamide resin and melamin resin are among of the synthetic polymer use for shell material (Wang *et al.*, 2015). In term of morphology, microcapsule can be divided into three types namely matrix, mononuclear or polynuclear microcapsule which depend on the deposition process of the shell as well as on the core material used as shown in Figure 2.13. While in term of application microcapsule can be divided into three classes slow-release, pressure-sensitive type, thermal type and intumescent photosensitive microcapsule type (Wang *et al.*, 2015).

2.7.2 History and its application

Microencapsulation technology was invented in 1950s when Green and Schleiches (Green & Schleicher, 1957; Green, 1957) produced microencapsulated dyes by complex coacervation of gelatin and gum Arabic for the manufacture of carbonless

copying paper. Since then the encapsulation technology has developed significantly and has been widely used in the pharmaceutical, food, pesticides, paint, cosmetics,

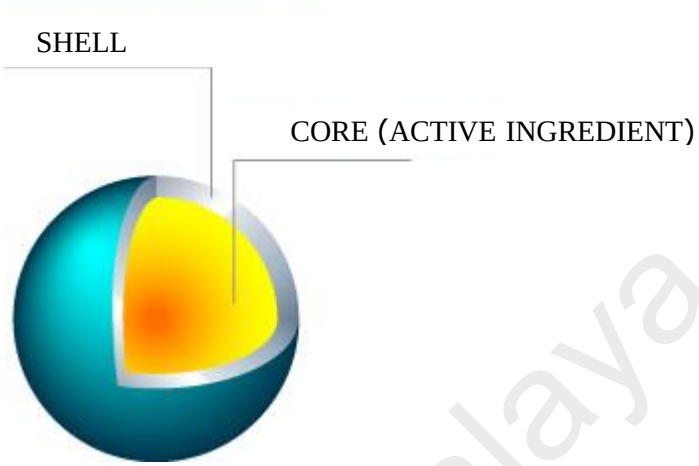


Figure 2.12: Microcapsule that is divided into two compartments; the outer layer known as shell containing wall materials such as polymer and the inner layer known as core which is containing the active ingredient. (Source: Jyothi *et al.*, (2012).

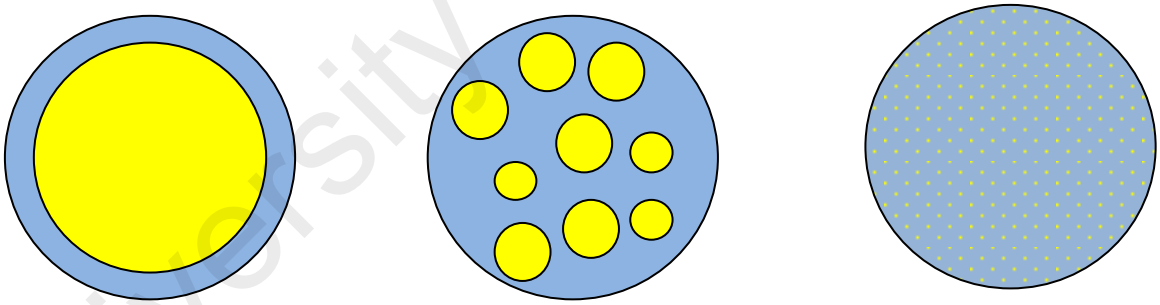


Figure 2.13: Morphology of the microcapsule; mononuclear (microcapsules contain the shell around the core, polynuclear (capsules have many cores enclosed within the shell), matrix (core material is distributed homogeneously into the shell material). Source: Jyothi *et al.*, (2012).

detergents, photographic material, textile and other industries (Jyothi *et al.*, 2012). Various bioactive materials like drug (Bouchemal *et al.*, 2004), bioactive protein (Kwak *et al.*, 2001), pesticides (Scher *et al.*, 1998), probiotics, phase change materials (Mondal,

2008), antimicrobial fabrics (Saraswathi *et al.,* 2010), insect repellent (Specos *et al.,* 2010), long lasting perfume and skin softener (Rodrigues *et al.,* 2009) have been successfully encapsulated into microscopic size materials (Chatterjee *et al.,* 2012).

In plant insecticides development, there was few research that employed this technology. Maji *et al.,* (2007) produced microcapsules that repelled mosquitos using *Zanthoxylum limonella* oil as an insect repellent and gelatin as wall material. Jo *et al.* (2015) developed an anti-insect PVA Polymer strip embedding co-droplets that efficiently repelled *P. interpunctella* larvae. In year 2016, a study conducted by Aziz and co-investigators reported that they had successfully encapsulated citronella oil by using complex coacervation using chitosan-gelatin for repellent application (Aziz *et al.,* 2016). Another study that had successfully encapsulated citronella oil by using different technique which was complex coacervation and applied to cotton textiles for development of repellent textile (Specos *et al.,* 2010). The most current study reported by Pujiastuti *et al.* (2017) also reported the successful of encapsulation of Citronellal by using β-Cyclodextrin for repellent application.

2.7.3 Advantages of microencapsulation

The advantages of microencapsulation are described as the controlled release of encapsulated bioactive material from oxidation and imparting stability to environmental stress (Bansode *et al.,* 2010). In botanical insecticide, this technology can extend the efficacy of botanical insecticides over longer periods of time by enhancing their target specificity, optimizing action of active ingredients and minimizing its residual impacts (Risch & Reineccius, 1995). The encapsulation of essential oils allows optimization of its functionality, a factor that enables a more prolonged action of its active ingredient, since essential oils are characterized by their high volatility. The natural or synthetic polymer that surrounds the core material provides continues film upon skin application

as well as water resistance. This thin-film polymer also offers a low skin penetration and high residual action to the formulation (Calton, 2001; Karr *et al.,* 2012). Obviously repellent with low skin penetration and high residual action is expected to have increased repellent effect. By encapsulating the essential oil, it also prevented the evaporation of volatile compounds and controlled the release of the essential oil. Furthermore, micro-encapsulation also protects the essential oil from oxidation caused by heat, moisture, and contact with other substances over a long shelf life, thus improving the efficacy of essential oil in the repellent product (Lumsdon *et al.,* 2005; Ghosh, 2006; Soest, 2007).

2.7.4 Method of microencapsulation

Microcapsule formation involves different technique and can be divided into three; chemical, physicochemical and physical methods. Briefly, for the **chemical method**, it involves the use of biocide that is chemically attached to a polymer either as pendent side groups or as part of the main backbone. Obviously, only those biocides that contain structural moiety with one functional group suitable for use as a link to a functional polymer can be used in this method. The wall material is formed *in situ* by polymerization between monomers at the core material's surface. The chemical combination can be prepared via three techniques; interfacial polymerization, *in situ* polymerization, and interfacial precipitation chemistry. **Physicochemical method** on the other hand is based on phase separation in colloidal system or a soluble shell material aggregates around the core material to form a solid wall. Coacervation, solvent evaporation and liposome technique are among the techniques under this method (Dong *et al.,* 2011). As for **physical method**, the wall material is mechanically applied or condensed around the core material through either space drying, spray chilling,

extrusion, fluidized bed coating and co-crystallization (Fang & Bhandari, 2010; Laohasongkram *et al.*, 2011).

2.7.4.1. Chemical method

a) Interfacial polymerization (IFP) technique

Interfacial polymerization (IFP) technique also known as condensation polymerization is the technique of condensation reaction between a diamine/diol and a diacid. The water phase contains the diamine/diol (water soluble monomer) and usually an inorganic base to activate any diol and neutralize the by-product acid. The other phase consists of the diacid chloride and an organic liquid (water insoluble monomer) such as dichloromethane, toluene, or hexane. Polymer formation takes place at or near the liquid–liquid interface when the two solutions are brought in contact or stirred together. Through this process and its variations, thousands of polymers have been synthesized (Morgan, 2011) (Figure 2.14).

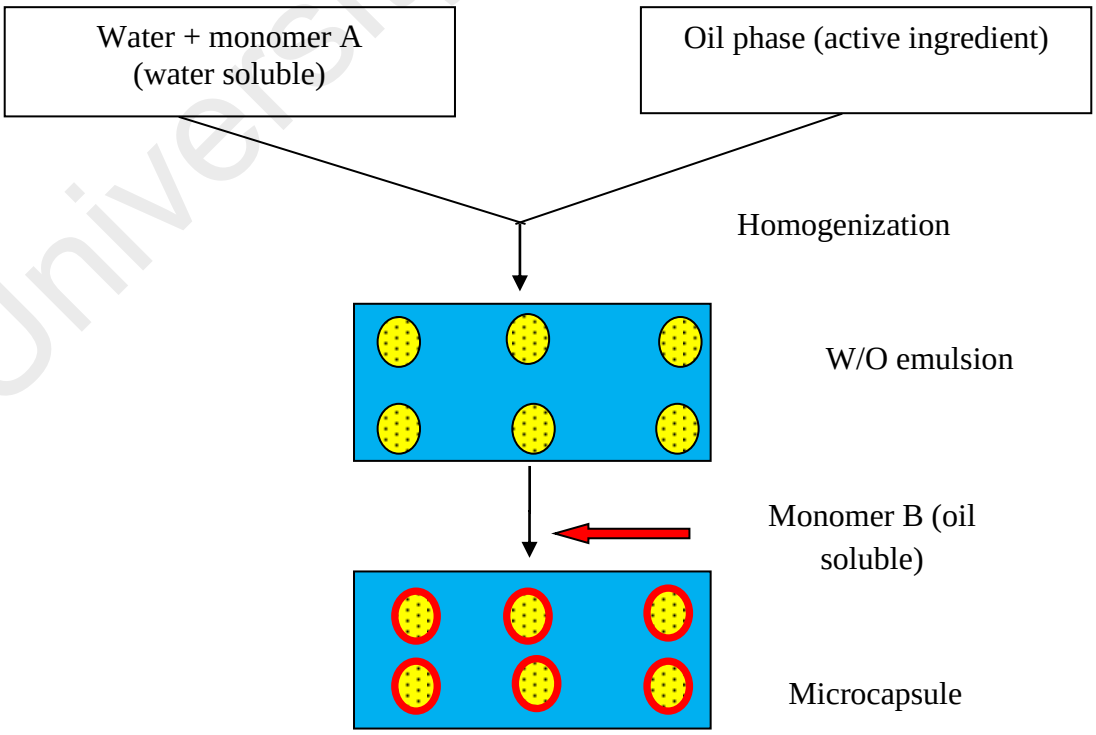


Figure 2.14: Interfacial polymerization technique

b) *In situ* polymerization technique

In situ polymerization technique is a chemical encapsulation technique very similar to interfacial polymerization. The distinguishing characteristic of *in situ* polymerization is that no reactants are included in the core material. All polymerization occurs in the continuous phase, rather than on both sides of the interface between the continuous phase and the core material, as in IFP.

c) Interfacial precipitation chemistry technique

Interfacial precipitation chemistry technique involved formation of multiple phases. First phase is formation of an emulsion including droplets of a water-immiscible core material in a first wall-forming reactant solution that preferentially accumulates at the surface of the droplets. A water-immiscible core material is encapsulated by a process in which the core material is emulsified in an aqueous solution of a first wall-forming reactant to form dispersed droplets of the core materials in the solution. The reactant in the 1st mixture must be amphiphilic (having both hydrophilic and lipophilic moieties) and one member of Lewis acid - Lewis base reactant pair. Forces of polar solvent interaction drive the lipophilic end solvate into the less-polar interior of the droplet, leaving the hydrophilic moieties solvated in the aqueous phase and thus causing the reactant to preferentially accumulate at the droplet surface. Thus it is believed that the reactant in the 1st mixture tends to rapidly collect at the droplets-continues phase interface as the emulsion is formed, stabilizing the emulsion and providing a reaction for the second reactant (Speaker *et al.*, 2011).

The emulsion may be further stabilized by the use of an emulsifying, wetting or surfactant agent such as sodium lauryl sulfate or other material such as polyethylene

glycol, ester, sorbital esters or similar emulsion-promoting materials either in the continuous phase or in the core material prior to dispersion. The emulsion is then combined with the second mixture containing the second, complementary Lewis acid or Lewis base reactant of wall-forming reaction pair, which may be but not necessarily an amphiphilic. Upon mixing of the emulsion and the second mixture, the 2^{nd} reactant reacts with the 1^{st} to from an encapsulating wall surrounding the core material of each emulsion droplets. The resulting reaction products, a salt of Lewis acid-Lewis base reactants, precipitates at the droplet interface to form a stabilized wall and thereby microencapsulates the emulsified droplet containing the water-immiscible core material. The precipitation reaction is spontaneously and essentially instantaneously upon mixing at 25^0C and requires no additional heating step. Figure 2.15 illustrate the methodology in chemistry precipitation technique which involved reaction between 1^{st} wall reactant (amphiphilic) and 2^{nd} wall reactant which is complementary to the 1^{st} wall reactant to form wall surrounding the droplet of core material.

Lewis acid reactant may be contributed by any one or combination of acacia, agar, Arabic acid, carboxymethylcellulose, ghatti gum, polyacrylic acid, sodium alginate, sodium carboxymethylcellulose or acidic gum. If the Lewis acid reactant is used as the 1^{st} reactant introduced to the dispersed core material, it must be amphiphilic so it can properly associate with both aqueous phase and the core material. Source of the complementary wall-forming Lewis base reactant include benzalkonium chloride, cetylpyridinium chloride, hexanediamine or other similar amine that capable of precipitating the Lewis acid (Speaker *et al*., 2011)

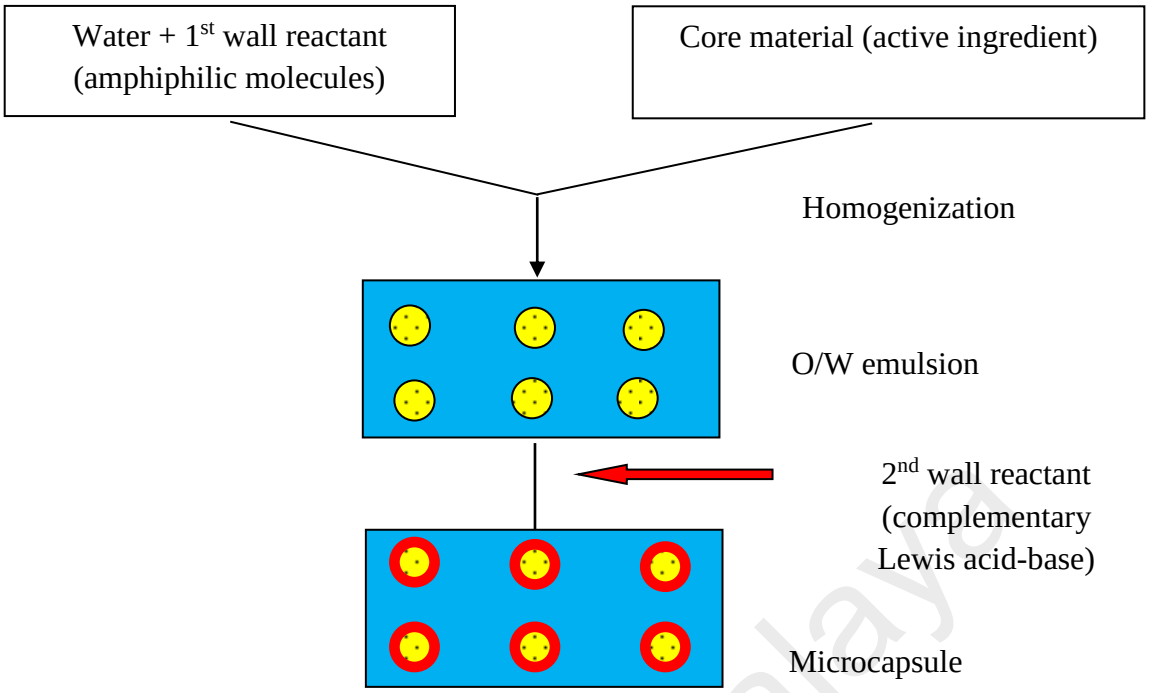


Figure 2.15: Interfacial precipitation chemistry technique.

2.7.4.2 Physicochemical method

a. Coacervation technique

Coacervation technique is generally described as the phase separation of two liquid phases in a colloidal solution or suspension. One phase is rich in polymer and called coacervate phase and the other polymer free phase is called equilibrium solution. There are two types of coacervation technique; simple and complex coacervation. In the case of simple coacervation there is only one polymer involves whereas complex coacervation involves the interaction of two oppositely charged polymers (Kaushik *et al.*, 2015). Simple coacervation is based on the addition of a poor solvent to a hydrophilic solution which results in the formation of two phases; one is rich of colloid molecules (coacervate) and the other is almost coacervate free. For example when sodium sulfate solution, acetone or alcohol were gradually added to a gelatin solution under stirring, a coacervate is formed (Shimokawa *et al.*, 2013). Complex coacervation

is the spontaneous phenomenon that occurs between two oppositely charged polymers. The neutralization of these charges induces a phase separation (polymer rich phase versus aqueous phase). Gelatin and Arabic gum are the commonly used wall material for complex coacervation (Lemetter *at al.*, 2009). Figure 2.16 shows the general possessing of microencapsulation method using complex coacervation technique as wall material.

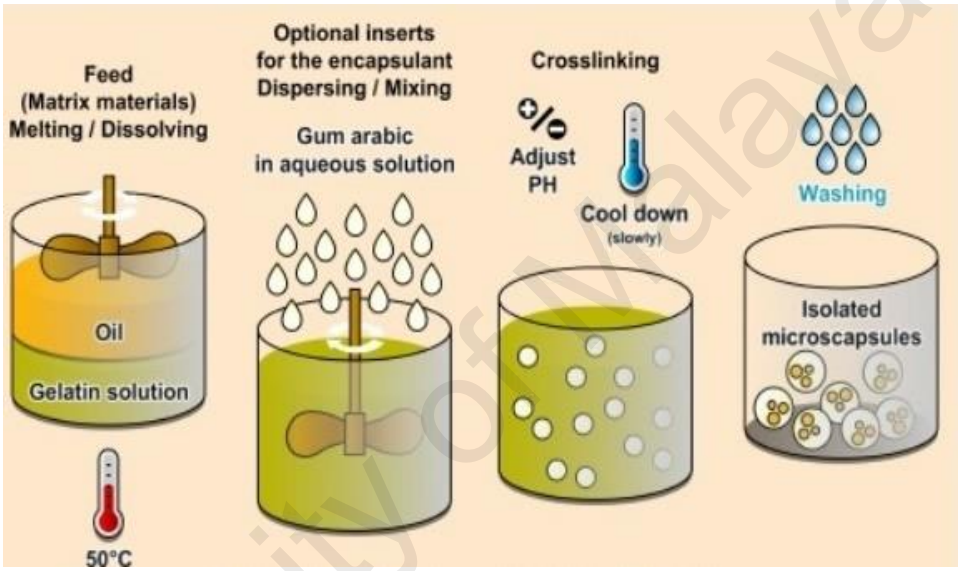


Figure 2.16: General possessing schemes for microcapsule preparation by complex coacervation using gelatin and gum Arabic as core material. (Source: Zuidam & Shimoni, 2010).

b) Solvent evaporation technique

Solvent evaporation technique involves four major steps which include incorporation of pharmaceuticals, droplet formation, solvent removal, and drying. Figure 2.17 shows the process of microencapsulation preparation by using solvent evaporation technique. The polymer is dissolved in a suitable water immiscible solvent, and the core material is directly added into the solution of polymeric-matrix materials by dissolution/dispersion in suitable solvents, or emulsification of aqueous solution of

the core materials immiscible with the matrix-material solution. For the preparation of solution or dispersion of core material, impeller or static mixing, high speed-stator mixing or microfluidization techniques are generally used. Agitation of the system is continued until the solvent partitions into the aqueous phase, removed by evaporation and later results in hardened microsphere which contains the active moiety (Poshadri & Aparna, 2010).

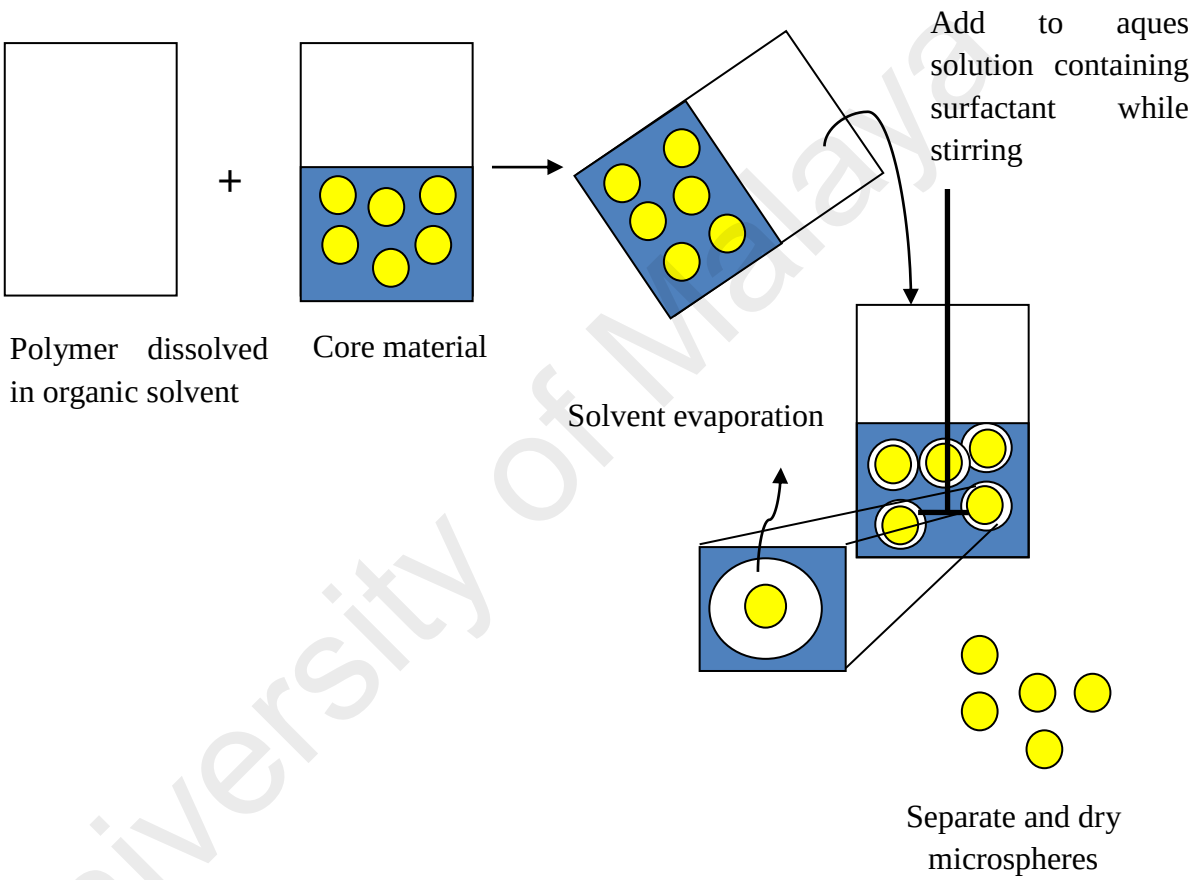


Figure 2.17: Solvent evaporation technique (Chanana *et al.*, 2016)

c) Liposome technique

Liposome is the system formed by one or several phospholipids bilayer defining one or several aqueous compartment (Figure 2.18). Phospholipids are amphiphile

molecules that are able to self-organize spontaneously in aqueous media. They have been used for delivery of vaccines, hormones, enzymes and vitamins into the body. They consist of one or more layers of lipids and are nontoxic and acceptable for foods. Permeability, stability, surface activity and affinity can be varied through size and lipid composition variations. They can range from 25 nm to several microns in diameter, are easy to make, and can be stored by freeze-drying (Poshadri & Aparna, 2010).

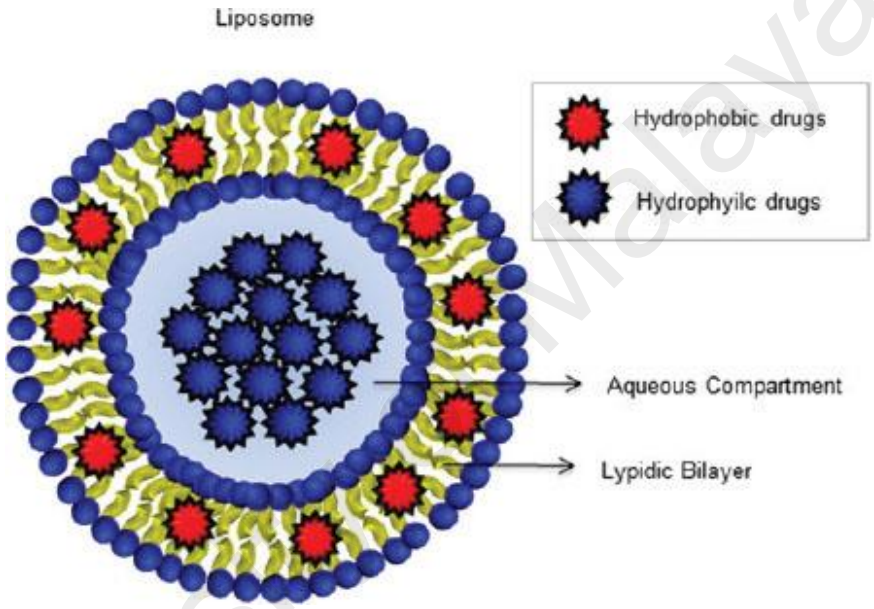


Figure 2.18: Liposome drug delivery system that consist phospholipid bilayer as wall material and aqueous compartment as core material. Source: Cordon *et al.*, (2013).

2.7.4.3 Physical method

a) Space drying technique

Space drying technique is the popular technique for forming microparticles because it is easy to perform on an industrial level and allows continues production (Wu *et al.,* 2014). It consists of liquid atomization into small droplets, a drying step is carried out using a warmed gas and collection of the solid particles (Wu *et al.,* 2014)

b) Extrusion technique

Extrusion technique has been used almost exclusively for the encapsulation of volatile and unstable flavors in glassy carbohydrate matrices. The main advantage of this process is the very long shelf life imparted to normally oxidation-prone flavor compounds, such as citrus oils, because atmospheric gases diffuse very slowly through the hydrophilic glassy matrix, thus providing an almost impermeable barrier against oxygen. Shelf lives of up to 5 years have been reported for extruded flavor oils, compared to typically 1 year for spray dried flavors and a few months for nonencapsulated citrus oils (Poshadri & Aparna 2010).

c) Fluidized bed coating technique

This is a very efficient way to apply a uniform layer of shell material onto solid particles. Interestingly, this technique is one of the few advanced technologies capable of coating particles with any kind of shell material like polysaccharides, proteins, emulsifiers, fats, complex formulations, enteric coating, powder coatings, yeast cell extract, etc. Therefore, the controlled release possibilities are considerably more versatile with any other technologies (Poshadri & Aparna, 2010).

d) Cocrystallization technique

This technique utilizing sucrose as a matrix for the incorporation of core materials. The sucrose syrup is concentrated to the supersaturated state and maintained at a temperature high enough to prevent crystallization. A predetermined amount of core

material is then added to the concentrated syrup with vigorous mechanical agitation, thus providing nucleation for the sucrose/ingredient mixture to crystallize. As the syrup reaches the temperature at which transformation and crystallization begin, a substantial amount of heat is emitted. Agitation is continued in order to promote and extend transformation/ crystallization until the agglomerates are discharged from the vessel. The encapsulated products are then dried to the desired moisture if necessary and screened to a uniform size (Poshadri & Aparna, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

This chapter described all methodology applied in this study including the materials and apparatus.

3.2 MATERIALS

3.2.1

Chemical

The chemicals used in this study are listed in accordance to the flow of works. During essential oils preparation, distilled water was used for heating and condensation process. In order to separate water and oil, drying agent sodium sulphate anhydrous powder purchased from BDH, England was used while the water content in plants was determined using Toluene solvent (Ajax, Chemical, Australia). In the preparation of essential oil-based formulation for topical application, acetyl alcohol, stearic acid, vanillin, span 80, tween 80, sodium carboxymethyl cellulose (CMC), polyethelene glycol (PEG) 3350, benzalkonium chloride (BKC) and diethyl-m-toluamide (DEET) used were purchased from Sigma-Aldrich, USA while Dow corning 200 was purchased from Dow corning, USA. Jojoba oil, sweet almond oil, coconut oil, emulsifying wax, shear butter and cocoa butter were purchased from BF1 Malaysia.

For the efficacy study, all marketed plant-based repellent (KAPS®, MozAway®, BioZ Natural® and Mosiguard®) were purchased from local pharmacy stores. For

microencapsulation and stability studies, deionized water was used to dilute the formulated product prior the droplet size, zeta potential and pH measurements. Muller-Hinton Agar (MHA) and Sabouroud Agar (SDA) used in the microbiological study were purchased from Merck, Germany while anesthethic drug (zoletil and xylaxine) and Euthanasia drug (dolethal) used in skin irritation and sensitization studies were purchased from Per Arcade Veterinary Clinic, Petaling Jaya, Selangor.

3.2.2 Apparatus

Clevenger apparatus (Favorit®), 1000 ml round flask (Favorit®), heating mantle (Toshnival, India), and cooler CBN8-30 (Heto®, Denmark) were apparatus used for essential oil extraction. For plants water content measurement, dean stark apparatus (Favorit®) was used. For formulation preparations, the mixture was stirred using digital stirrer RW and Hot plate C-MAG HS7 (IKA® United State). The mosquito cages used in the efficacy study in the laboratory setting were purchased from Saujana Scientific, Malaysia.

Several apparatus required for other studies such as microbiology, skin irritation, sensitization and stability testing were made available by using those located in laboratories at two different universities. Optical microscope (Leica, Germany) used was the one available at University Putra Malaysia (UPM), while Zetasizer Nano (Malvern Instrument Ltd. United Kingdom), Fourier Transform Infrared spectrometer model Spectrum 400 (PerkinElmer, MA, USA) (FTIR) and themogravity analysis (TGA) model 4000 (PerkinElmer, MA, USA) were located at Chemistry Department, University of Malaya (UM).

Other apparatus such as centrifuge (Hittich, Germany) and pH meter (Mettler Toledo, Switzerland) used in this study were those that belong to Medical Microbiology and Parasitology Department, UPM while viscometer used in stability testing was owned by Chemistry Department, UM. All equipment used in the microbiology study including autoclave and biosafety cabinet were located at the Medical Microbiology and Parasitology Department, UPM. For skin irritation and sensitization study, the cages, drinking bottle and study area were provided by Animal Laboratory Unit, Faculty Medicine University of Malaya.

3.3 ETHNOBOTANICAL STUDY

An ethnobotanical study was conducted in order to obtain and document the knowledge and usage of plants traditionally used as insect or mosquito repellent among the Malay ethnic group of Kota Tinggi district, Johor. The knowledge of the local people on diseases transmitted through mosquito bites and their concerns regarding the risk of mosquito bites and protection measures against mosquito bites were evaluated.

3.3.1 Study area

The survey was conducted in Kota Tinggi District, Johor, Malaysia. Three villages selected for this study were Kampung Semangar Dalam, Perkampungan Felda Simpang Waha and Perkampungan Felda Lok Heng Timur. Figure 3.1 shows the map of Kota Tinggi district where the study areas where located. They were located approximately 30 km away from the nearest town, Kota Tinggi, and 100 km from the capital, Johor Bahru. There are two primary, two secondary schools and one health

center located in each village. For common illnesses, most of the people here will approach the local health center and will be referred to the nearby hospital in Kota Tinggi town for any unmanageable cases. Most of the houses in these study areas were built out of concrete with zinc roofs.

Majority of villagers in Kampung Semangar Dalam are rubber tappers whereas those who living in Perkampungan Felda Simpang Waha and Perkampungan Felda Lok Heng Timur work as settlers. These study areas are surrounded with palm oil and rubber tree estates. Some local people participated in agriculture activity such as fish farming, rearing of cow, goat and chicken as well as growing fruit and vegetable. The man of each household will leave for work early in the morning therefore exposing themselves to mosquitoes.

3.3.2 Study design

A cross-sectional descriptive study was carried out in obtaining data on knowledge and usage customs of insect and mosquito repellent plants among the Malay ethnic group in Kota Tinggi, District, Johor, Malaysia. This study was carried out between the months of October to December 2013. Data was collected using a standardized and pre-test structured questionnaire. A random sampling was used for the selection of 350 households out of a total of 2291 households.

Figure 3.1: Map of Kota Tinggi District, Johor, Malaysia that showing the location of the study areas.

3.3.3 Interview

The questionnaire was prepared in English and translated into the local language, Bahasa Malaysia, for the respondents' easy understanding to avoid bias information and other variables. In order to improve the quality of the data, pretesting of the questionnaire was carried out prior to the actual survey. Respondents were adults including males and females. The questionnaire focused on the socio-demographic of respondents, their knowledge, attitude and practice in relation to mosquito transmitted diseases and their knowledge with regard to plants traditionally used as insect repellent.

3.3.4 Collection of plants

Based on description by respondents, plants were collected and voucher specimens were prepared for identification by a plant taxonomist at the Herbarium of University Malaya, Kuala Lumpur Malaysia.

3.4 PREPARATION OF PLANT EXTRACT

From ethnobotanical survey, plants were frequently named but has not been marketed were selected for study. *Citrus grandis* (the fruit mainly its peel), *C. aurantifolia* (the leaf), and *Alpinia galanga* (the rhizome) were chosen. The fruits of *C. grandis* and rhizome of *A. galanga* were purchased from Pasar Borong Selangor, Seri Kembangan. While leaves of *C. aurantifolia* were obtained from Felda settlement Cluster, Kota Tinggi Johor, Malaysia.

3.4.1 Essential oil extraction

Plant essential oils (EOs) were produced by steam distillation method according to Forest Research Institute of Malaysia (FRIM) procedure (FRIM, 2000) using extraction apparatus that consist of clevenger, heating mantle, cooler and round flask (Figure 3.2). The clevenger is divided into three parts: receiving duct, condenser duct and collecting duct (Figure 3.3a). All plants part to be extracted were cut into small pieces and clean with water prior subjected into round flask. The weights of these parts were measured by using electronic balance (Sartorius, Jerman) and recorded before the extraction process.

The cooler machine was set to 10^0C before the heating mantle was switched on to boil the round flask (5 L) containing 4 L of plants and distilled water. The heating temperature was set up around 70-80^0C. Vapors released from boiling plants mixture and distilled water was eventually filled the receiving duct and later flowed to the condenser duct where condensation took place. The condensation process produced oil, which was collected in the collector duct of the clevenger. After 6-8 hours of collecting, the extracted oil collected was then transferred in a vial. Sodium sulphate anhydrous powder was added into vial to remove water from the oil. The pure oil was then transferred into fresh vial, where the volume was recorded and then stored in the fridge (4^0C) until used.

3.4.2 Water content measurement

The percentages of water in plants were determined by using Dean-Stark apparatus (Figure 3.2b) based on FRIM procedure (FRIM, 1998) that apply the used of organic solvent toluene and water system. Ten gram of plant were placed in the round flask (250 ml) and 100 ml of toluene were added. The flask was then connected to the Dean-Stark apparatus for extraction process. The plant was heated for 2-3 hours using heating mantle. Vapors released from boiling mixture of plants and toluene would filled the receiving duct and later to the condenser duct for condensation process. The water was separated by condensation process and collected in the collecting duct while toluene solvent was channeled back into flask.

Water content was calculated based on this formula:

$$M = W/S \times 100$$

M is water content in plant part (%)

W is the volume of water collected from Dean-stark method (ml)

S is the dry weight of plant part used (g)

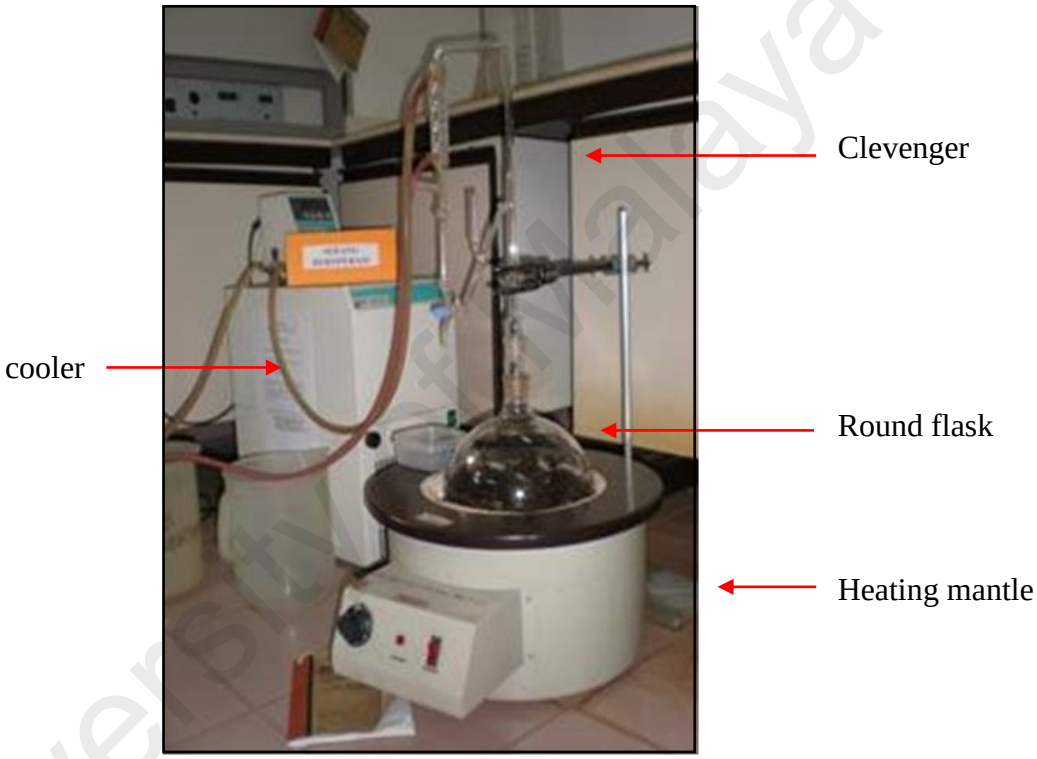


Figure 3.2: The extraction apparatus consist of clevenger, heating mantle, cooler and round flask

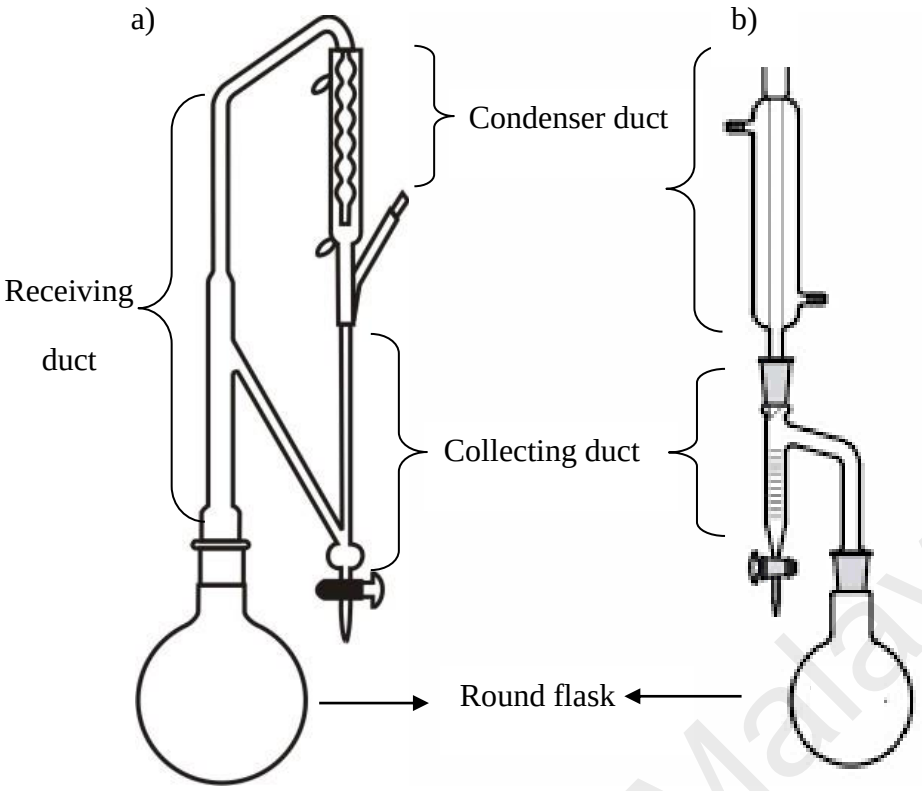


Figure 3.3: a) Clevenger apparatus for essential oil extraction
b) Dean-stark apparatus for water content measurement

3.4.3 Identification of the essential oils compounds

Analysis of EOs compounds was performed by gas chromatography/mass spectroscopy GC/MS-QP2010 Plus (Shimadzu, Japan) equipped with FASST (Fast automated SIM/Scan Type) Software. A fuse silica column 5% phenyl-poly-dimethyl-siloxane (TG-5MS VB-5 BPX 5 30m x 0.25 mm i.d and 0.25 µm film thickness, ThermoScientific) were used. Helium was used as carrier gas. Significant MS operating parameters include: ionization voltage, 70 eV; ion source temperature at 230°C; and mass range of 50–600 u. The identification of compounds was performed by comparing their mass spectra with data from Adams, US National Institute of Standards and Technology (NIST, USA), and confirmed by comparison of their retention indices with those of authentic compounds or with data in the literature.

3.5 MICROENCAPSULATION PROCESS

The microencapsulation process was conducted based on the work of Kasting *et al.* (2008) and Karr *et al.* (2012) with modifications. The encapsulation process utilizes interfacial precipitation chemistry technique that formed a polysaccharide film around the dispersal droplet (Speaker, 2011). Briefly, microcapsule walls were formed through two steps process involving reacting the amphiphilic macromolecule sodium carboxymethylcellulose (CMC) with a complementary reactant benzalkonium chloride (BKC). The first step involved the formation of emulsions containing droplets of water-immiscible core material (the essential oils) in a first wall-forming reactant solution (CMC) that preferentially accumulated at the droplet surface by polar solvent forces. This step is then followed by the second step where the second wall forming reactant (BKC) was added to the system that spontaneously precipitating the CMC to form a membrane-like wall that surrounded each droplet.

In this study, microencapsulation of each EO and DEET (used as control) involved several phases as follow: **Phase A** where active ingredient (40% w/v EOs or DEET) was mixed with an adjuvant to form a core phase consisting 60% active ingredient and 40% adjuvant. Adjuvant was prepared by mixing Dow Corning 200 (silicon oil) and vanillin and then blended in a 200 ml beaker using a magnetic stirrer bar set to rotate at a minimal speed of 200 rpm at 60°C. The solution was allowed to cool down to 45°C before the active ingredient was subsequently added to the mixture and stirring was continued to complete the process.

Phase B is where ingredients such as cetyl alcohol, PEG 3350, SPAN 80, and TWEEN 60 were heated (60^0C) in a beaker to melting and stirred to mix them completely. Phase A mixture was then slowly added into phase B mixture and stirred continuously to form an emulsion referred as a core emulsion mixture. **Phase C** is the aqueous solution of the first wall forming reactant (CMC) in distilled water to make a 1% solution by mixing it using a 40 mm diameter four-bladed propeller at 600 rpm and 45^0C until all the CMC dissolved in water. Once ready, the core emulsion mixture prepared earlier was then dispersed into the Phase C solution at the same rotation speed and temperature. Stirring was maintained for one hour in order to produce uniform oil-in-water dispersion referred as **Phase E.**

Phase D is a mixture of the second wall-forming reactant (BKC) in distilled water. This solution was gradually added to Phase E, while slowly increasing the stirrer speed to 800 rpm in order to accomplish the formation of a microcapsule wall. This mixture was then removed from heating after 120 sec and allowed to cool to ambient temperature with stirring maintained until reaction was completed indicated by formation of semisolid microcapsules within the aqueous solution. The resultant mixture was transferred into a screw cap container after ambient temperature was achieved. Table 3.1 below shows the amount of active ingredients and other ingredients used in this encapsulation technique. An additional amount of distilled water was added at the conclusion of the preparation in order to account for aqueous water losses during encapsulation process.

Table 3.1: Essential oil and DEET microcapsule, formulation composition (Total weight: ~200 g)

Ingredients	Weight (g)
Phase A	
EO/DEET	60
Dow Corning 200	20
Vanillin	20
Phase B	
Cetyl alcohol	30
PEG 3350	10
Span 80	5
Tween 60	5
Phase C	
1% CMC solution	20
Distilled water	~100
Phase D	
1% BKC solution	10

3.6 THE CHARACTERISTICS OF MICROCAPSULES

3.6.1 Encapsulation efficiency

The encapsulation efficiency was conducted to determine the percentage of EOs and DEET that successfully encapsulated. This was determined by using UV-visible spectrophotometer. An analytical curve was produced from 5 ml solution of each EOs and DEET in dichloromethane ranging between 0.2 mg/ml – 5 mg/ml. The maximum absorbance of EOs and DEET was scanned at 200 – 500 nm by the spectrophotometer and presented maximum absorbance at 284 nm (EOs) and 320 nm (DEET). 1 g of microcapsules dissolved in dichloromethane to a final volume of 1 ml. The suspension were mixed gently and kept for 20 minutes before it was sonicated for 10 minutes. The microcapsules reside were filtered through nylon syringe filter with 0.22 ųm pore size. The absorbance of EOs/DEET in microcapsule was calculated using the calibration curve.

Actual amount of EOs/DEET (g) = Total amount of EOs/DEET (g) – free EOs/DEET amount by spectrophotometer measurement (g)

Encapsulation efficiency (%) = $\frac{\text{Actual amount of EOs/DEET (g)}}{\text{Total amount of EOs/DEET (g)}} \times 100$

For the following microcapsule characteristic assays, the EOs and DEET microcapsules prepared in 3.5 were centrifuged at 3500rpm for 15 min. The microcapsules were then washed three times with distilled water. After each wash the samples were centrifuged and the wastewater was discarded. The microcapsules were dried in the petri dish at room temperature overnight before further used.

3.6.2 Morphology of the microcapsule

Small amount of microcapsules were placed on the glass slide prior observation under optical microscope (Leica, Germany). The morphology of microcapsule were captured by using Nikon DS-Fi1 camera and NIS-Elements imaging software that combined with microscope.

3.6.3 Microcapsule size, microcapsule size distribution and zeta potential value

Twenty mg microcapsules were dispersed in 50 ml deionized water and sonicated at 25^0C for 15 minutes. The solution was then transferred into folded capillary cell (polycarbonate with gold plated electrodes) to test for microcapsules size, microcapsules size distribution and zeta potential using Zetasizer Nano (Malvern Instrument Ltd. United Kingdom) at 25^0C. Zeta potential was measured to obtain the

value of charges around microcapsules that determine the stability of the microcapsules. The mean particle diameter was calculated using differential size distribution processor (SDP) intensity analysis program. Figure 3.4 shows the Zetasizer equipment connected with the SDP intensity analysis program. The measurements were done in triplicate and the average results for the measurement were recorded.

Figure 3.4: Zetasizer equipment connected with the SDP intensity analysis program that used for droplet size, particle size distribution and zeta potential value

3.6.4 Fourier Transform Infrared spectrometer (FTIR) analysis

Fourier Transform Infrared spectrometer (FTIR) analysis was done to detect any chemical interaction between EOs and DEET with wall materials by using FTIR model Spectrum 400 (PerkinElmer, MA, USA) (Figure 3.5). The FTIR measurements were recorded as wave number region with expected value ranged between 400 - 4000 cm^{-1} at 4 cm^{-1} resolution.

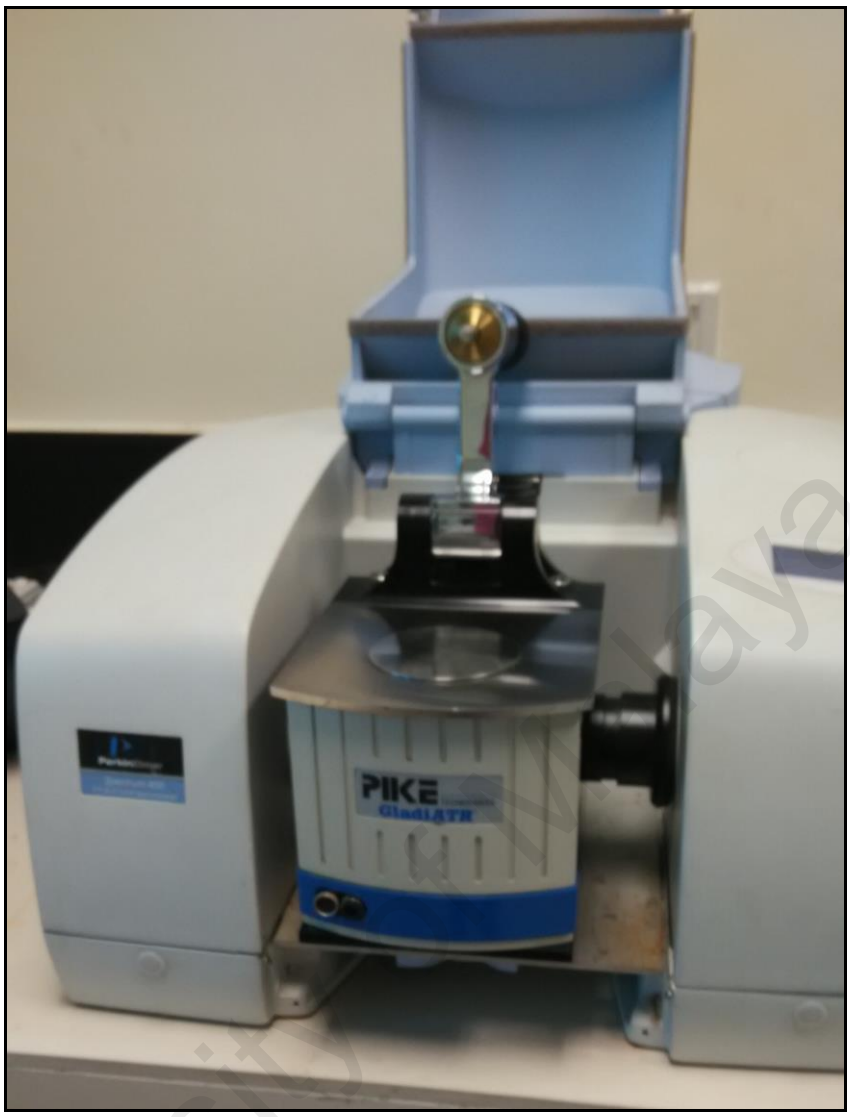


Figure 3.5: FTIR equipment model Spectrum 400 (PerkinElmer, MA, USA).

3.6.5 Thermogravimetric analysis (TGA)

Thermal degradation of the microcapsule was carried out via TGA model 4000 (PerkinElmer, MA, USA) (Figure 3.6) in nitrogen atmosphere applying a heating rate of 10^0C min^{-1}, from 25 to 400^0C. The thermal profiles were recorded in the form of graphs and the TGA curves were then derived.

Figure 3.6: TGA model 4000, PerkinElmar

3.7 INCORPORATION OF MICROCAPSULE INTO LOTION FORM

WHO guidelines had recommended that the maximum concentration of DEET (standard gold repellent) to be used in formulations was 20% (WHO, 2009b). Therefore, all formulations prepared were made to contain 20% active ingredients for better comparison. After confirming that all EOs and DEET were successfully encapsulated, each microencapsulated (ME) active ingredients was then formulated into a lotion that will work against the mosquito and at the same time moisturizes skin. For this purpose, emulsifying wax (5 g), stearic acid (2.5 g), shear butter (10 g), cocoa butter (5 g), coconut oil (2.5 g), sweet almond oil (10 g), and jojoba oil (5 g) were mixed together using a propeller at 400 rpm and heat at 70^0C. The mixture was then allowed to cool down to 45^0C before glycerin (5 g), aloe vera gel (5 g), and a ME active ingredient (100 g) were added with stirring maintained at the same speed, until uniform mixture/lotion

was produced (150 g ME formulation). For comparison purpose, another formulation was prepared employing similar procedure but 20% of non-encapsulated (NE) active ingredient (dilute in coconut oil) was added into the mixture to replace the microencapsulated EOs and DEET.

3.8 CHARACTERISTICS OF THE FORMULATIONS

After all the EOs and DEET were formulated into lotion form, the organoleptic and physicochemical characteristics of the formulations were studied.

3.8.1 Organoleptic characteristics

The color and homogeneity of the formulations was recorded visually while the odor was determined by smelling. The texture of the formulations was tested by pressing a small quantity of the formulation between the thumb and index finger. The consistency of the formulations and the presence of coarse particles were used to evaluate the texture of formulations. Immediate skin feel including the softness and greasiness was also evaluated.

3.8.2 Physicochemical characteristics

The physicochemical characteristics which include the particle size, particle size distribution, zeta potential value, viscosity and pH of the formulations were measured.

3.8.2.1 Particle size, particle size distribution and zeta potential

Twenty mg of formulation was dispersed in 50 ml deionized water and sonicated at 25^0C° for 15 minutes. The solution was then transferred into a folded capillary cell (polycarbonate with gold-plated electrodes) to test for particle size and zeta potential using the Zetasizer Nano (Malvern Instrument Ltd. United Kingdom) at 25^0C. The mean particle diameter was calculated using the differential size distribution processor (SDP) intensity analysis program.

3.8.2.2 pH measurement

One gram of formulation was diluted in distilled water of up to 10 ml, homogenized and then the pH measurement was carried out by using a pH meter (Mettler Toledo, Switzerland) (Marquele-Oliveira *et al.,* 2007).

3.8.2.3 Viscosity measurement

The viscosity of the formulations was measured by using the Brookfield digital viscometer, using the spindle no.7. The test was done at 25^0C and the rotation speed of the spindle was fixed at 20 rpm. Formulation to be tested was left at an ambient temperature for three days before testing. The data was expressed in cp (centipoise) (Marquele-Oliveira *et al.,* 2007).

3.9 EFFICACY STUDY

The repellent effect of each formulation (ME and NE formulations) was evaluated against mosquito in the laboratory and field conditions. For laboratory evaluation, two species of mosquito were used; *Aedes aegypti* (susceptible strain) and *Culex quinquefasciatus* (susceptible strain). For field evaluation, human volunteers were used to determine the effectiveness of the formulations against wild mosquito species in field settings. For comparison purposes, four marketed plant-based repellent products brands KAPS®, MozAway®, BioZ Natural® (citronella-based repellent) and Mosiguard® (citriodiol®-based repellent) were also included in this study.

3.9.1 Laboratory evaluation

Repellency study was conducted based on Malaysian Standard Method (MSD) (2000) for repellent MS 1497:2000 (modified from WHO 1996) (Malaysian Standard, 2000; WHO, 1996). Mosquitoes used for the repellent efficacy study in laboratory were provided by the Institute for Medical Research of Malaysia (IMR). Susceptible strain and nulliparous three- to seven- day old *Ae. aegypti* and *Cx. quinquefasciatus* were used as test species. All the mosquitoes were unfed for overnight prior the testing. The test were conducted at 0800 h to 1600 h in the laboratory that set to have room temperature 25^0C until 30^0C and relative humidity at 60% until 80%.

Bioassays were conducted using a 60 x 60 x 60 cm cage with two 15 cm diameter circular openings fitted with cloth sleeves. The cage had two compartments divided by a Perspex partition in the middle (Figure 3.7). Twenty five females fresh batch of mosquito was introduced into each compartment through the circular opening.

Two square areas of 25 cm^2 were drawn on the back of the hands of the human volunteers. One of these areas was left untreated (act as control); while the others were pre-treated with 0.4 g of formulations (treatment).

To test for any inherent repellency characteristics of the blank formulation (formulation without active ingredient), a comparison of mean landing rates per minutes of mosquitoes were conducted. Five human volunteers applied with 0.4 g of lotion base to the treatment area, leaving their second area as untreated control. Prior the treatment, both hands were covered with rubber gloves with a 25 cm^2 opening up to the wrist to confine mosquito bites to only the exposed areas, and the hands were inserted through the circular opening into the cage containing the mosquitoes. Both hands were exposed simultaneously for a period of three minutes, and the numbers of mosquito that lands and/or bites were recorded. The procedure was repeated 5 times with 5 min interval between each exposure.

For testing the repellent formulations, 0.4 g formulations was applied evenly to the treatment area of each volunteer. The untreated area was used as a control. Gloves were worn to ensure the biting was focused on the exposed area. The test began by introducing both hands into the cage containing mosquitoes for three minutes and the number of mosquito that lands and/or bites were recorded. The assessment periods were one, two, four, six, and eight hours post application. Each formulation was tested on five human volunteers for three iterations. The effectiveness of the formulations were determined by the percentage protection reduction based on the reduction in mosquito that lands and/or bites on the treated arm as compared to the untreated/control arm using the following formula:

% protection reduction = [(C-T)/C] X 100

where *C* is the total number of mosquito that lands and/or bites on the control and *T* is the total number of mosquito that lands and/or bites on the treated arm.

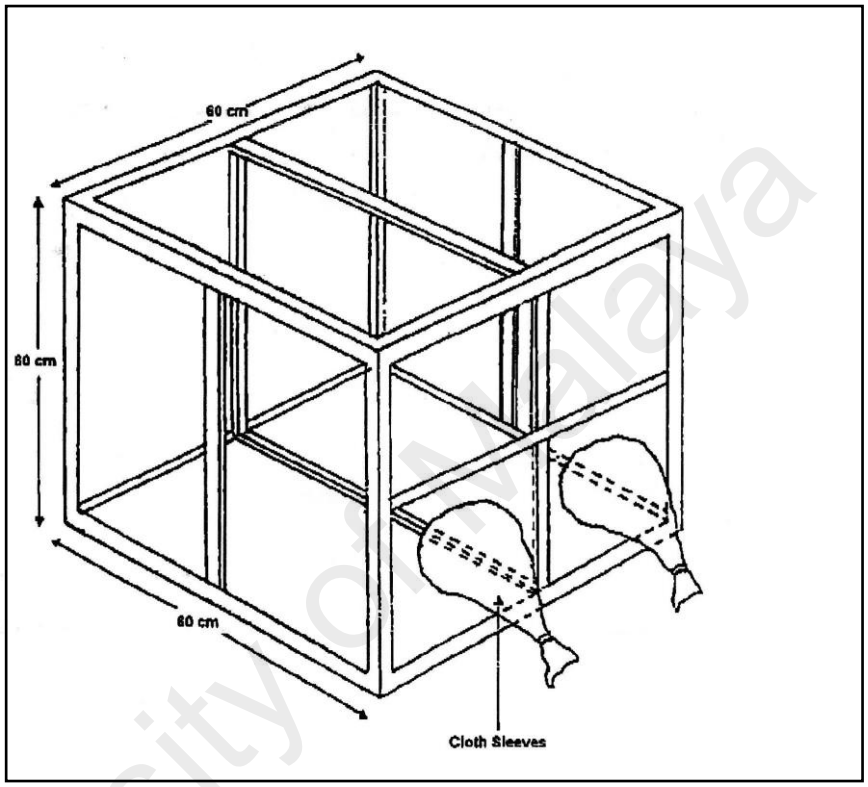


Figure 3.7: Screen cage used for the evaluation of mosquito repellent efficacy

3.9.2 Field evaluation

Study sites:

The field evaluation was conducted in various areas in Peninsular Malaysia having zero or minimal risk of mosquito transmitted diseases (no or only few cases of mosquito borne diseases were reported). The information regarding these locations was obtained from Ministry of Health, Malaysia. Three locations were selected and preliminary studies using human-bait landing catches were done to determine the mosquito population density with broad spectrum of mosquito species in the selected

areas. Based on this preliminary study, Kampung Paya Rumput Jaya, Sungai Udang, Melaka; Kampung Pokok Asam, Sungai Petani, Kedah and Felda Purun, Bera, Pahang were selected for this study. Figure 3.8 shows the three locations selected for the field evaluation study which were situated in three different states in Peninsular Malaysia.

Kampung Paya Rumput Jaya, Sg Udang (2°16'22"N 102°9'7"E) is located in Melaka approximately 21 km away from Melaka Capital. People in this village mostly involve in agriculture and farming activity. The study sites for field evaluation were surrounded mainly by fruit plantation. Kampung Pokok Asam (5°34'49"N 100°28'16"E) is situated in Sungai Petani, Kedah approximately 8 km away from Sungai Petani Town and 74 km from the capital, Alor Setar. People in this village mostly involve in agriculture mainly paddy farming and the study sites selected for field evaluation were located nearby paddy field. Felda Purun (3°22'32"N 102°37'51"E) is one of the FELDA settlements situated in Bera, Pahang. It is approximately 32 km away from the nearest town, Bera New Town and 161 km from the capital, Kuantan. Most of the people in this settlement involve in palm oil industry and the study site selected for field evaluation was surrounded with palm oil and swamp area.

Test procedure:

The field evaluation was conducted based on WHO methodology (WHO, 2009b). For every study site, 13 volunteers took part in the study. Each one was positioned at 13 different spots and each of them was tested with different treatments while one volunteer was not treated (control). One gram of formulation was applied evenly from knee to ankle of each leg. Shorts and shoes were used to standardize the

exposure area. Other parts of the body were covered to avoid mosquitos attack using jacket with hood, gloves, or plastic sheet.

Figure 3.8: Peninsular Malaysia maps that showing the study sites for field evaluation.
★ Symbol represents the three locations for field evaluation.

Care is taken to minimize contact of the treated legs with clothing between tests. All volunteers were seated on chairs; at least 10 meters apart and both legs were exposed for a period of 40 minutes. With the help of flashlight, mosquito that lands

and/or bites were captured using vial and then transferred into fresh cups to be counted and identified later. Each exposure period is followed by a 20-minute break before the next mosquito collection was carried out. After each 40-minute period, the volunteers moved to a new site at least 10 m from the last spot. The test started at 1630 and continued until 2130 hours and was performed for 13 nights. Each volunteer received different treatment each night and sat at a different spot nightly.

Volunteers were instructed to avoid mosquito from feeding on them during the testing. In order to protect them from mosquito borne diseases, prophylaxis treatment were given to each subject prior the study. They were also instructed to avoid applying any cosmetic products including perfume, cologne or lotion on the day of trial. All volunteers were required to wash their legs with soap after testing, and again the following morning. Washing and use of soap or deodorant after midday are prohibited. Skin irritation is observed during the testing period. The captured mosquitos were then transported to the laboratory and identified to species under a stereo microscope. The effectiveness of the formulations and repellent products tested were calculated based on the protection reduction percentage of mosquito that lands and/or bites on the treated leg compared with the untreated leg (control) using the following formula:

$$\% \text{ protection reduction} = [(C-T)/C] \times 100$$

Where C is the total number of mosquito lands and/bites on the control leg and T is the total number of mosquito lands and/or bites on the treated leg.

3.10 STABILITY STUDY

Stability testing was carried out in order to evaluate the stability of the formulation under the influence of various temperatures and duration of storage. The

samples to be tested (microencapsulated formulations and non-encapsulated formulations) were packaged in impermeable polypropylene containers and stored in different conditions (temperature and humidity) for different period of time. Room 1 (25 ± 2^0C/60% ± 5% RH) and Room 2 (40 ± 2^0C/75 ± 5% RH). These formulations were evaluated for their stability after 7 days, 1 month, 6 months and 1 year of storage period. The changes in their organoleptic and physicochemical characteristics were observed during these periods. In addition, the changes in their efficacy level also were examined in this study.

3.10.1 Centrifugation assay

The centrifugation assay was conducted to determine the presence/absence of phase separation during storage. This test was carried out by weighting 2 g of each formulations followed by a centrifugation at 3000 rpm for 30 minutes for a phase separation (Marquele-Oliveira *et al.,* 2007). The present or absent of phase separation were observed and recorded.

3.10.2 Organoleptic assay-physical appearance

The organoleptic features of the formulations were examined by observing changes in color, smell, texture, sediment, phase separation, crystallization, absent/present of residue when applied on the skin, as well as the hardness of the lotions (Marquele-Oliveira *et al.,* 2007).

3.10.3 Particle size and zeta potential analyses

Twenty mg formulations were dispersed in 50 ml deionized water and sonicated at 25^0C° for 15 minutes. The solutions was then transferred into a folded capillary cell (polycarbonate with gold-plated electrodes) to measure the particle size and zeta potential using the Zetasizer Nano (Malvern Instrument Ltd. United Kingdom) at 25^0C. The mean particle diameter was calculated using the differential size distribution processor (SDP) intensity analysis program.

3.10.4 pH measurement

One gram of formulation was diluted in distilled water of up to 10 ml, homogenized and then the pH measurement was carried out by using a pH meter (Mettler Toledo, Switzerland) (Marquele-Oliveira *et al.*, 2007).

3.10.5 Viscosity measurement

The viscosity of the formulations was measured by using the Brookfield digital viscometer, using the spindle no.7. The test was done at 25^0C and the rotation speed of the spindle was fixed at 20 rpm. Sample to be tested was left at an ambient temperature for three days before testing. The data was expressed in cp (centipoise) (Marquele-Oliveira *et al.*, 2007).

3.10.6 Efficacy study during storage

In order to determine the efficacy level of formulations during 12 months of storage, the evaluation of their repellent effect was conducted right after the preparation and every 30 days for 12 months period. The procedure was similar as mentioned in point 3.9.1.

3.11 MICROBIOLOGY STUDY

For microbiology testing, two types of methodology were employed. First test was the basic microbiology testing to determine the good laboratory practice and hygiene during the production of repellent formulations and the second test was the challenge testing to evaluate the preservative capacity of the products. Both tests were done according to Hugbo *et al.* (2003) methodology.

3.11.1 Basic microbiology testing

A loopful formulation was spread thinly on Muller Hilton agar (MHA) and Sabouround agar (SDA) plates. The plates were then incubated for 24-48h at 37^0C and 5 days at 30^0C respectively for bacterial and mold growth. For any growth appeared, it was isolated and its species identified using standard procedures. Each formulation was tested in triplicate.

3.11.2 Preservative capacity evaluation

To ensure the products continue to have lasting preservative effect, challenge testing was done against multiples inoculates via disc diffusion method. *Staphylococcus aureus, Escherichia coli, Candida albicans* and *Aspergillus fumigatus* were employed as the challenge inoculates as these were among bacteria/fungus strain that commonly contaminate cosmetic products. Microorganism was cultured at 37^0C for 4 hours and prepared to turbidity equivalent to 0.5 McFarland Standard. 100 µl of suspension of tested microorganism, containing 10^8 cfu/ml of bacteria cell and 10^4 cfu/ml spores of

fungal strain were spread on MHA and SDA, respectively. The filter disc (6 mm) were individually soaked with 15 ml of formulation and placed on the agar plates. Disc without formulation were used as –ve control. Ampicilin (10µg/disc), Streptomycin (10µg/disc), and Flumequine (30µg/disc) were used as positive control for bacteria gram positive, gram negative and fungus, respectively. The petri dishes were kept at 4^0C for 2 hours and then incubated at 37^0C for 24 hours for bacteria and at 30^0C for 48 hours for fungus. The inhibition zone diameters were measured and each product was tested in triplicate and the mean inhibition zone diameter was recorded.

3.12 SKIN TOXICITY/SAFETY STUDY

3.12.1 Acute dermal irritation/corrosive

Only rabbit of 17 weeks of age in the range of 2.7 kg-3.6 kg of body weight with healthy, intact skin were used. All rabbit were housed individually in cages to avoid the animal from scratching each other and disturbed the test area/patches. All rabbits were acclimatized to the laboratory condition 14 days prior the experiment. For feeding, conventional laboratory diets were used with an unlimited supply of drinking water and maintained in an air-conditioned room at 19-25^0C with relative humidity of 40-70%, 12-hour light (7:00 -19:00)/dark (19:00-7:00) cycle.

For this study four groups of animals were tested: (i) with AGRO ME formulation, (ii) with CGPO ME formulation, (iii) with CALO ME formulation and (iv) with DEET ME formulation. Up to three patches were applied sequentially to each animal. An area of 6 cm^2 (treatment area) on the back of each (posterior flank) rabbit were made free of fur using an electric shaver 24 hours prior the experiment. Animal were anesthetized before shaving to avoid injury during the process. The posterior flank

of animal was chosen for test area in order to avoid animal from scratching/disturbing the area.

Another 6 cm^2 at adjacent area was made available as control area (no treatment). 0.5 g of formulations were spread on the test area which then covered with gauze patch which held in place with non-irritating elastic bandage. The patch was hold in place with bandage for one minute, after which the patch was removed and the skin was cleaned from residual formulation by washing with water. If no serious skin reaction (no corrosive effect) observed, a second patch was applied at a different site and removed after one hour. If the observation at this stage indicated no serious skin reaction (no corrosive effect), a third patch was applied and removed after four hours, and the response was graded as before.

In the case where corrosive effect was observed after any of the three sequential exposures, the test was immediately terminated by sacrificing the animal. If corrosive effect was not observed after the removal of the last patch, observation was continued for 14 days. Within that 14 days if corrosive effect was seen developed the test was immediately terminated.

Skin irritation effects were assessed at approximately 1, 24, 48 and 72 hours after the removal of the dressings according to the OECD guideline (2002). Erythema and edema were scored on a scale of 0 - 4 with 0 showing no effect and 4 representing severe erythema or edema. For each animal, dermal response scores (sum of the scores of erythema, eschar and edema formation) at 1, 24, 48, and 72 hours after removal of the patches were summed and divided by three to obtain a mean irritation score per time

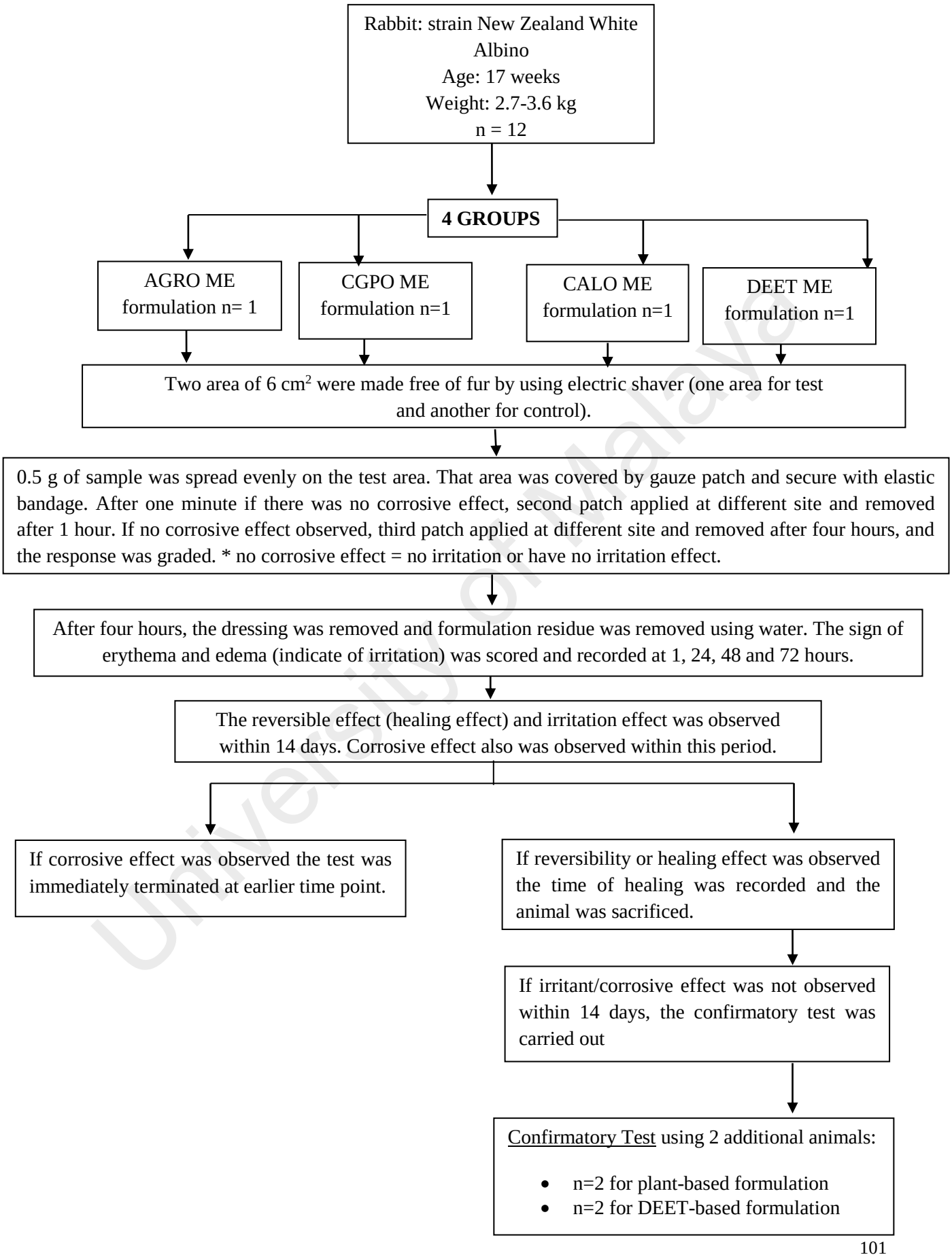
point. The mean scores at 24, 48, and 72hours were summed and averaged to obtain the primary irritation index.

$$\text{Primary irritation index} = \frac{\text{Mean score at 24 h + mean score at 48 h + mean score at 72}}{3}$$

If a corrosive effect was not observed in the initial test, the irritant or negative response was confirmed using up to two additional animals, each with one patch, for an exposure period of four hours. If there was no corrosive effect or irritation effect in the initial test, it is not required to do three sequential patches on the confirmatory animals. If both animals exhibit the same response (no irritation), no further testing is needed.

The duration of the observation period should be sufficient (14 days) to evaluate fully reversibility of the effects observed (i.e healing). However, the experiment should be terminated at any time when animal shows continuing signs of severe pain or distress. Animals were observed up to 14 days after removal of the patches for reversibility effects (healing). If healing is seen within 14 days, the experiment was terminated immediately.

ACUTE DERMAL IRRITATION/CORROSION STUDY



3.12.2 Skin sensitization

The skin sensitization experiment was carried out in accordance to OECD Guideline 406 "skin sensitization" (OECD, 1992). Guinea pigs aged 5-6 weeks with body weight of 0.33-0.41 kg were used in the study. This study involved two phases: (i) induction exposure phase and (ii) challenge exposure phase. Induction exposure referred to exposure of test substance onto a subject with the intention to induce hypersensitive state. While challenge exposure referred to exposure of similar test substance onto previously treated subject (induction period) to determine if the subject reacts in a hypersensitive manner.

Twenty four hours prior the first induction, the guinea pigs were assigned to six groups to be treated/tested with (i) AGRO ME formulation (n=10), (ii) CGPO ME formulation (n=10), (iii) CALO ME formulation (n=10), (iv) DEET ME formulation (n=10), (v) 10% formaldehyde (positive control which have mild sensitization effect) n=5, and (vi) Saline water (negative control) n=5.

(i) Induction exposure

Day 0 – Test group and positive control group

4 cm^2 area (on posterior flank) was cleared of hair by using electric shaver. The posterior flank of animal was chosen for test area in order to avoid animal from scratching the test area. Before shaving, the animals were anesthetized to avoid injury during the process. The test patch (gauze patch which was held in place with non-irritating elastic bandage) was applied with test substance (0.4 g). The test patch was

applied to the test area and held in contact with the skin for 6 hours. After 6 hours the dressing was removed and residual of sample was removed using water. The sign of sensitization on the skin was graded before all animal were led to rest for a week (without treatment).

Day 0 - Control negative group

The same procedures to that performed on test group was carried out for the negative control group. Test patch however, were with saline water.

Days 7 and 14 – All group

The same application as conducted on day 0 was carried out on the same test area on day 7^{th}, and again on day 14^{th}.

ii. Challenge exposure

Day 29^{th} – All group

The untested flank of treated and control animals was cleared of hair. Before shaving the animals were anesthetized to avoid injury during the process. A patch containing test substance (0.4 g) was applied to the posterior untested flank of test and control animals. The patch was held in contact by a suitable dressing for 6 hours.

Skin irritation effects were assessed at approximately 1, 24, 48 and 72 hours after the removal of the patch according to the OECD guideline (2002). Erythema and edema if detected were scored on a scale of 0 - 4 with 0 showing no effect and 4 representing severe erythema or edema. After 72 hours the animals were sacrificed.

GRADING OF SKIN REACTIONS:

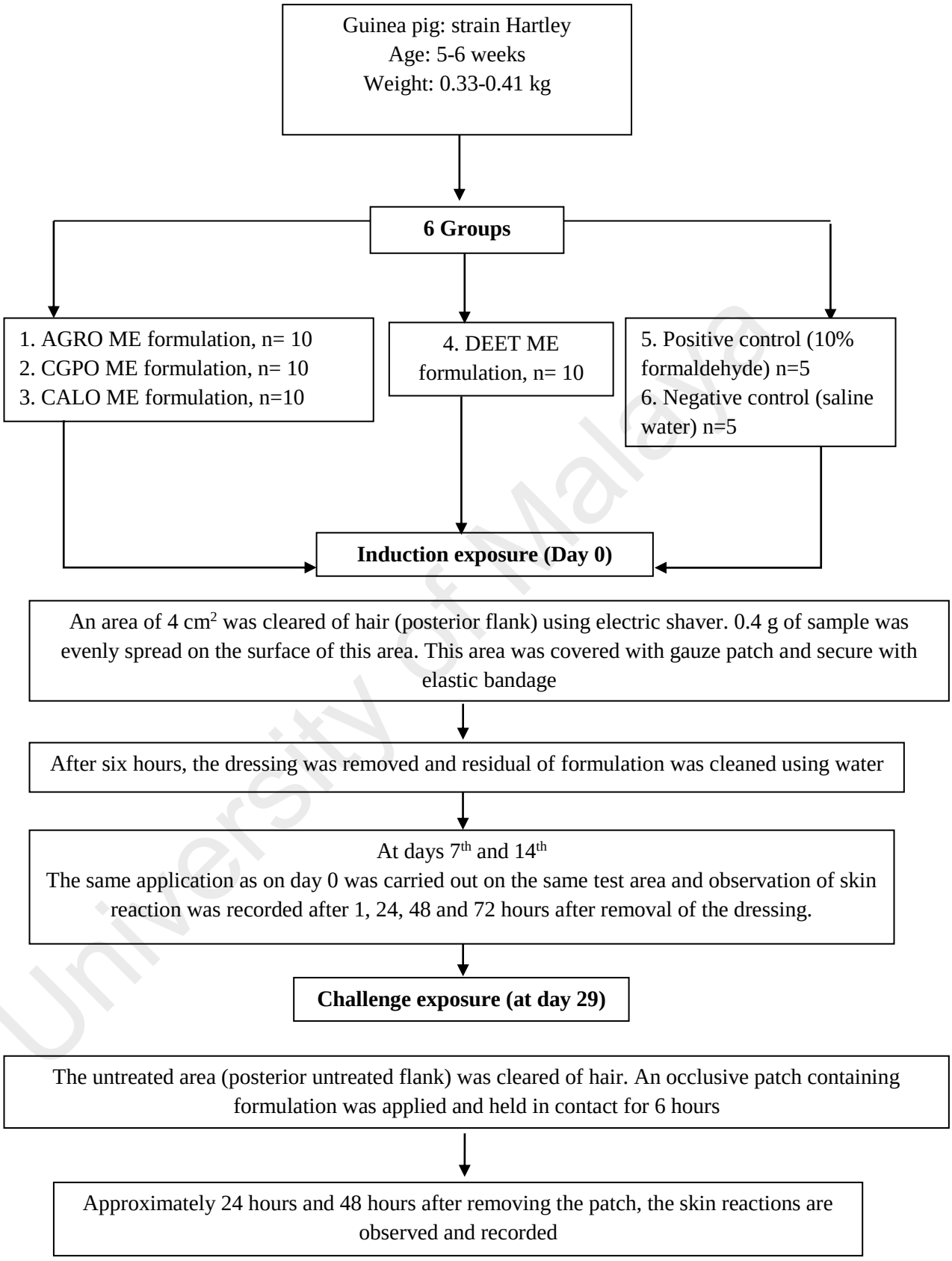
Erythema and eschar formation:

No erythema……	0
Very slight erythema (barely perceptible)……..	1
Well defined erythema…….	2
Moderate to severe erythema……..	3
Severe erythema (beef redness) to eschar formation preventing grading of erythema.	4

Edema formation:

No edema……	0
Very slight edema (barely perceptible)……...	1
Slight edema……	2
Moderate edema……..	3
Severe edema (raised more than 1 mm and extending beyond area of exposure…..	4

SKIN SENSITIZATION STUDY



3.13 ETHICAL APPROVAL

For repellency testing using human volunteer: Full ethics approval was granted by the Faculty of Medicine of University Malaya Ethic Committee (Ethic No: 988.11). For animal study, full ethic approval was granted by Animal ethic committee Faculty of Medicine of University Malaya (Ethic No: 2014-04-01/PARA/R/NM)

3.14 STATISTICAL ANALYSIS

All the data were analyzed by using IBM SPSS version 20 software. The descriptive analyses to determine the mean, median and average were conducted to every single data. The Levene test was conducted to determine the normality of the data. For ethnobotanical study, the data were analyzed by using chi-square analysis to determine the association between the respondent's knowledge and usage custom of insect repellent plants with the demographic parameter. For microencapsulation characteristics, the encapsulation efficacy, particle size, particle size distribution and zeta potential were analyzed by using one-way ANOVA. For repellency testing of formulations, the percentage of repellency was analyzed by split-plot ANOVA (SPANOVA) to identify the mean differences between each formulation over time. Prior to this analysis, data had to be transformed using arcsine-square root transformation to better reach the parametric assumptions. For stability and microbiology study, the data were also analyzed by using SPANOVA analysis as well as t-paired independent analysis depends on the requirement. A value of $p<0.05$; 95% confidence interval was considered statistically significant.

CHAPTER 4

RESULTS

4.1 INTRODUCTION

This chapter presents results of experiments conducted comprising ethnobotanical study, plant selection and extraction, chemical compounds of the essential oil, microencapsulation: characteristic of the microcapsules, characteristic of the formulations, efficacy of the formulations, physical stability of the formulations, microbiology test and skin toxicity or safety studies.

4.2 ETHNOBOTANICAL STUDY

4.2.1 Socio-demographic characteristics of respondents

Out of 350 respondents, female respondent's constituted 64.6% while males were 35.4%. Age of respondents ranged between 35 to 67 years old and the mean of age was 53.8 years old. About 45.1% of respondent had an education background of secondary level, 39.4% had only primary education followed by 12% received tertiary education and some 3.4% were found illiterate (without formal education). The main occupation in the area is settler. Large numbers of the respondents (43.7%) were in the unemployed group which includes retirees and housewives. For those who are in the working group, nearly half of them (44.6%) earned more than MYR1500. The socio-demographic characteristics of the respondents are shown in Table 4.1.

Table 4.1: Socio-demographic characteristics of respondents.

Variables	Frequency	%
Gender		
Male	124	35.4
Female	226	64.6
Age of respondents		
35-40 years	17	4.9
41-46 years	40	11.4
47-52 years	101	28.9
53-58 years	93	26.6
59-64 years	80	22.9
≥ 65 years	19	5.4
Educational level		
Primary	138	39.4
Secondary	158	45.1
Tertiary	42	12.0
Illiterate	12	3.4
Monthly income (MYR)		
≤ 1500	156	44.6
1600-2000	97	27.7
2100-2500	37	10.6
2600-3000	42	12.0
3100-3500	13	3.7
≥ 3600	5	1.4
Occupation		
Settler	109	31.1
Business sector	15	4.3
Agriculture sector	21	6.0
Civil servant	39	11.1
Other	13	3.7
Unemployed	153	43.7

4.2.2 Knowledge, attitude and practice with regards to mosquito transmitted diseases

All respondents agreed that mosquitoes transmit dengue but only half of them are aware that mosquitoes can also transmit malaria. 65.7% of respondents agreed that mosquitoes transmit filariasis but only a small number (8.3%) seemed to know that

Japanese encephalitis (JE) is transmitted by mosquitoes. In general, majority of them knew that mosquito bites pose threat to human but some 2% admitted that they did not know about this. All respondents practice closing of doors and windows from dawn to dusk as a way to prevent contact with mosquito. 61.4% of respondents used aerosol spray, 38.3% use bed nets while the other 20.3% said they use repellents to avoid mosquitoes bite. 44% of respondents admitted they had knowledge about plant that can repel mosquito and some of them use these plats for that purpose. The knowledge, attitude and practice of respondents with regards to mosquito transmitted diseases are presented in Table 4.2.

4.2.3 Knowledge on plants traditionally used as insect repellent

Table 4.3 presented the list of plants commonly used as repellent obtained from the survey. All together there are 16 species of plants known to be used by the local community to repel insects and mosquitoes. Ten of them were already reported to have repellent effect against mosquitoes. These plant species were found belonging to 15 genera and 14 families. 13.7% of the respondents mentioned that *Cymbopogon nardus* (L.) Rendle was the plant commonly used to drive away or avoid mosquitoes and flies followed by *Allium sativum* L. (9.1%), *Cinnamomum zeylicum* Blume (7.7%) while *Illicium verum* Hook. F (7.4%) was used against ants and cockroaches. Others (5.7%) mentioned *Pelargonium graveolens* (Cav.) L'Herit, *Citrus aurantifolia* (Christm.) (4.6%), *Alpinia galanga* (L.) Willd (4.3%), *Lantana camara* L. (3.4%) and *Citrus grandis* (L.) Osbeck (3.1%) as plants used specifically against mosquito bites. The leaf was the most common part used for this purpose. Other parts sometimes used as mosquito repellent were the bark, rhizome, flower and fruit peel.

Table 4.2: Respondents knowledge on diseases transmitted by mosquitoes, preventive measures, and knowledge and usage of insect or mosquito repellent plants.

Variables	Frequency	%
Are mosquito bites dangerous to you and your family?		
Yes	343	98
No	7	2
Disease transmitted by mosquito bites*		
Dengue / Dengue Hemorrhagic fever	350	100
Malaria	175	50
Filariasis	230	65.7
JE	29	8.3
Prevention measures*		
Using bed nets	134	38.3
Using aerosol spray	215	61.4
Using repellents	71	20.3
Close windows and doors during dusk to dawn	350	100
Burning plant material to make smoke	270	77.1
Knowledge and usage of insect / mosquito repellent plants		
Yes	154	44.0
No	196	56.0

* Note: multiple answers is allowed and hence the score is >100% statistic

Table 4.3: List of information on repellent plants obtained from the ethnobotanical survey.

Family name	Plant scientific name	Vernacular name	Voucher number	n=350	%	Part used	Method of application	Type of insect repelled
1.Graminae	*Cymbopogon nardus* (L.) Rendle	Serai wangi	KLU 48235	48	13.7	Leaves	Growing plant nearby house / Laying the part of plant	Mosquitoes and flies
2.Rutaceae	*Citrus aurantifolia* Christm	Limau nipis	KLU 48243	16	4.6	Leaves	Spray the crushed leaf suspension / Laying the part of plant	Mosquitoes
3.Rutaceae	*Citrus grandis* Osbeck	Limau bali/limau tambun	KLU 48245	11	3.1	Fruit peel	Spray the crushed fruit peel suspension	Mosquitoes
4.Zingiberaceae	*Alpinia galanga* (L.) Willd	Lengkuas	KLU 48244	15	4.3	Rhizome	Spray the crushed rhizome suspension	Mosquitoes
5.Geraniaceae	*Pelargonium graveolens* (Cav.) L'Hérit	Jeremin	KLU 48236	20	5.7	Leaves	Growing the plant nearby house	Mosquitoes
6.Verbenaceae	*Lantana camara* L.	Bunga tahi ayam	KLU 48238	12	3.4	Flower	Growing the plant nearby house	Mosquitoes
7.Alliaceae	*Allium sativum* L.	Bawang putih	NA	32	9.1	Rhizome	Laying the part of plant	Ants and cockroaches
8.Lauraceae	*Cinnamomum zeylanicum* Blume	Kayu manis	NA	27	7.7	Bark	Laying the part of plant	Ants and cockroaches
9.Pandanaceae	*Pandanus amaryllifolius* Roxb.	Daun pandan	KLU 48241	18	5.1	Leaves	Laying the part of plant	Cockroaches

NA = Not applicable

Table 4.3, continued

Family name	Plant scientific name	Vernacular name	Voucher number	n=350	%	Part used	Method of application	Type of insect repelled
10.Illiciaceae	*Illicium verum* Hooker fil.	Bunga lawang/star anise	NA	26	7.4	Flower	Laying the part of plant	Ants and cockroaches
11.Annonaceae	*Annona muricata* L.	Durian belanda	KLU 48242	9	2.6	Leaves	Laying the part of plant	Cockroaches and fleas
12.Rutaceae	*Murraya koenigii* (L.) Spreng	Daun kari	KLU 48240	3	0.9	Leaves	Laying the part of plant	Flies
13.Zingiberaceae	*Etlingera elatior* (Jack) R.M.Sm	Bunga kantan	KLU 48246	4	1.1	Flower	Growing the plant nearby house / Laying the part of plant	Mosquitoes
14.Labiatae/ Lamiacea	*Pogostemon cablin* (Blanco) Benth	Nilam	KLU 48237	3	0.9	Leaves	Growing the plant nearby house	Mosquitoes
15.Asteraceae	*Tagetes erecta* L.	Marigold	KLU 48239	1	0.3	Flower	Growing the plant nearby house	Mosquitoes
16.Fabaceae	*Sesbania grandiflora* (L.) Pers	Geti/turi	KLU 48247	3	0.9	Leaves	Spray the crushed leaf suspension	Mosquitoes

NA = Not applicable

Based on this survey, about 89.9% of the local community who had knowledge and used repellent plants perceived that insect repellent plants as easily accessible, around 80.7% felt that these plants are pleasant to use and 85.4% believed that they are effective (Figure 4.1). Various methods in using plants were adopted by the local community to repel mosquitoes and other insects. For example, laying plant part on the flat surface was the most common practice among respondents, which indicated by 51.1% respondent, followed by those who grow the plant nearby their house (25.1%). Spraying the plant extracts after crushing and grinding the plant parts was also quite a common method employed (Figure 4.2). According to the respondents, such preparation was usually applied before they leave for work either in the estate, farm or in the jungle (Figure 4.3).

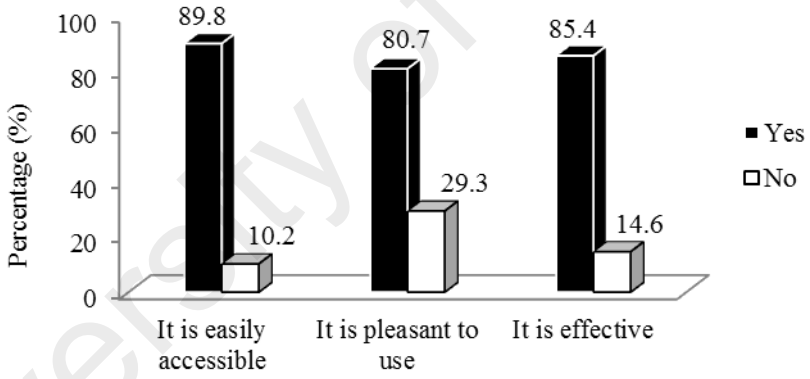


Figure 4.1: Perception of the respondents regarding the insect repellent plant accessibility, pleasantness and effectiveness.

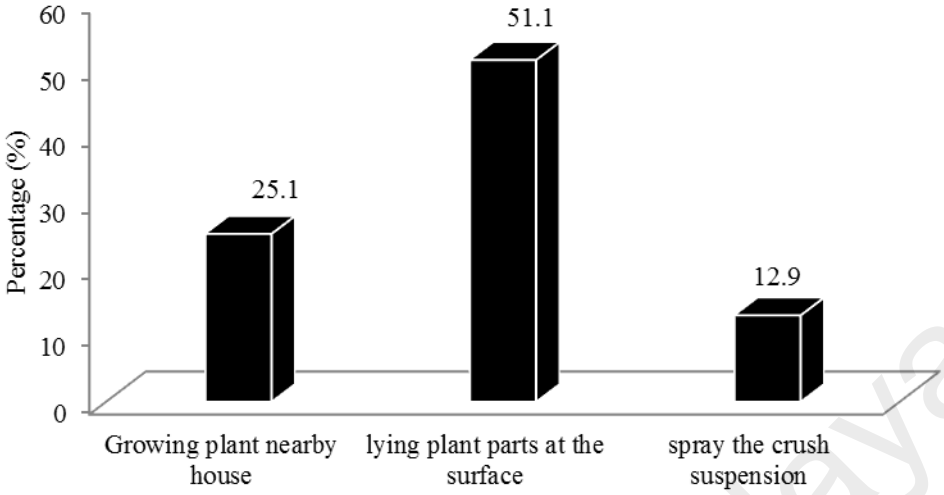


Figure 4.2: Usage customs of respondents regarding method of applications of insect repellent plants. * Note: percentage does not add up to 100, due to multiple responses.

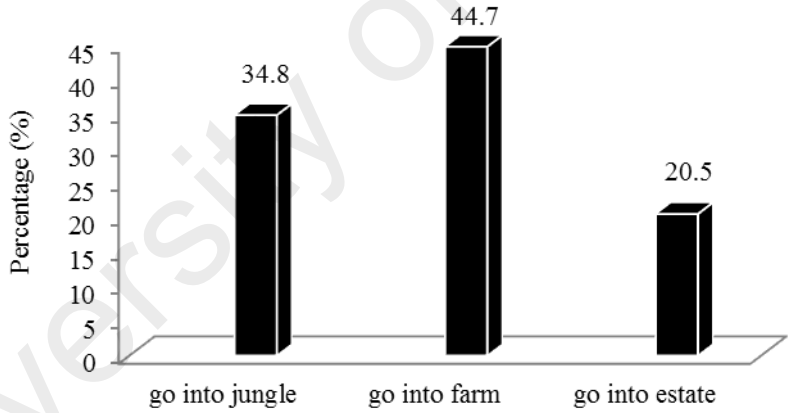


Figure 4.3: Practice and usage custom of respondents regarding the purpose of repellent plant applications

The association between respondent's knowledge and usage customs of insect repellent plants with their gender, educational status, age and monthly income is demonstrated in Table 4.4. The analyses revealed that there was evidence of a significant relationship between the knowledge with education status (p=0.003). However, there were

no significant relationship between the knowledge on repellent plants with the gender, age and monthly income of the respondents ($p>0.05$). This present study detected a significant relationship between education status and age with the usage customs of repellent plants ($p<0.05$). Significant relationship however was not demonstrated between gender and monthly income with the usage customs of repellent plants ($p>0.05$).

Table 4.4: Association between knowledge and usage custom of insect / mosquito repellent plants in relation with gender, educational status, age and monthly income.

Variable	Total no of respondents	Knowledge towards insect / mosquito repellent usage		p-value	Usage custom of insect / mosquito repellent		p-value
		Yes	No		Yes	No	
Gender							
Male	124	61	63	χ^2=2.2,df=1,	50	74	χ^2=2.8,df=1,
Female	226	93	133	p=0.147	71	155	p=0.09
Educational status							
Illiterate	12	10	2	χ^2=13.7,df=3,	10	2	χ^2=17.7,df=3,
Primary school	138	58	80	p=0.003	43	95	p=0.001
Secondary school	158	61	97		48	110	
Higher education	42	25	17		20	22	
Age of respondents							
35-40 years	17	10	7	χ^2=5.4,df=5,	9	8	χ^2=14.7,df=5,
41-46 years	40	20	20	p=0.369	18	22	p=0.01
47-52 years	101	42	59		27	74	
53-58 years	93	34	59		24	69	
59-64 years	80	38	42		33	47	
≥ 65 years	19	10	9		10	9	
Monthly income (MYR)							
≤ 1500	156	67	89	χ^2=2.5,df=5,	52	104	χ^2=4.1,df=5,
1600-2000	97	41	56	p=0.771	29	68	p=0.529
2100-2500	37	17	20		15	22	
2600-3000	42	21	21		18	24	
3100-3500	13	7	6		6	7	
≥ 3600	5	1	4		1	4	

4.3 PLANTS SELECTION AND EXTRACTION

Based on the data obtained from ethnobotanical study, three plants species were chosen for this study. They are *Alpinia galanga* (the rhizome) (AGRO), *Citrus grandis* (the fruit peel) (CGPO) and *C. aurantifolia* (the leaf) (CALO). The selection was based on the frequency being named during the survey, and have not been commercialized, and their availability. The essential oils (EOs) from these plants were extracted by using hydrodistillation method. The amount of EO collected was presented in percentage calculated from the dry weight of the samples (Table 4.5). Table 4.5 also recorded the water content in each plant part obtained using Dean-stark apparatus. The percentage for AGRO was 0.3% with 8% water content while the percentage for CGPO was 0.6% of oil and 9% water content. The percentage for CALO was 0.5% oil and 7% of water.

Table 4.5: The amount (%) of essential oils obtained from AGRO/CGPO/CALO

	AGRO	CGPO	CALO
Dry weight (g)	100	100	100
Essential oil weight (g)	0.3	0.6	0.5
Amount of essential oil (%)	0.3	0.6	0.5
Amount of water content (%)	8	9	7

4.4 CHEMICAL COMPOUNDS OF THE ESSENTIAL OIL

4.4.1 Chemical compounds of AGRO

The EO extracted from *A. galanga* presented itself as a liquid that is bright yellow in colour with camphoraceous, floral and spicy smell. The yield obtained was 0.3%. In order to determine its compounds, the EO was analysed by GC-MS (Figure 4.4). List of its compounds determined is shown in Table 4.6 along with the retention indices of the identified compounds. Thirty-two compounds were identified representing 100% of the EO. The major compound is tetradecyl ester which contributed 15.07% of the total EO, followed by the 1-undecene (13.59%), hexadecyl ester (11.38%), 3-methylpentane (6.60%) and α- limonene (5.27%). About 64.94% of the AGRO mainly compose of oxygenated compounds such as ester (43%), carboxylic acid (12.38%) and benzoates (9.56%). The remaining compounds (35.06%) found in AGRO were terpenes such as 1-undecene, 3-methyl pentane, α-thujene, α-phellandrene, p-cymene, β-pinene, and α-limonene.

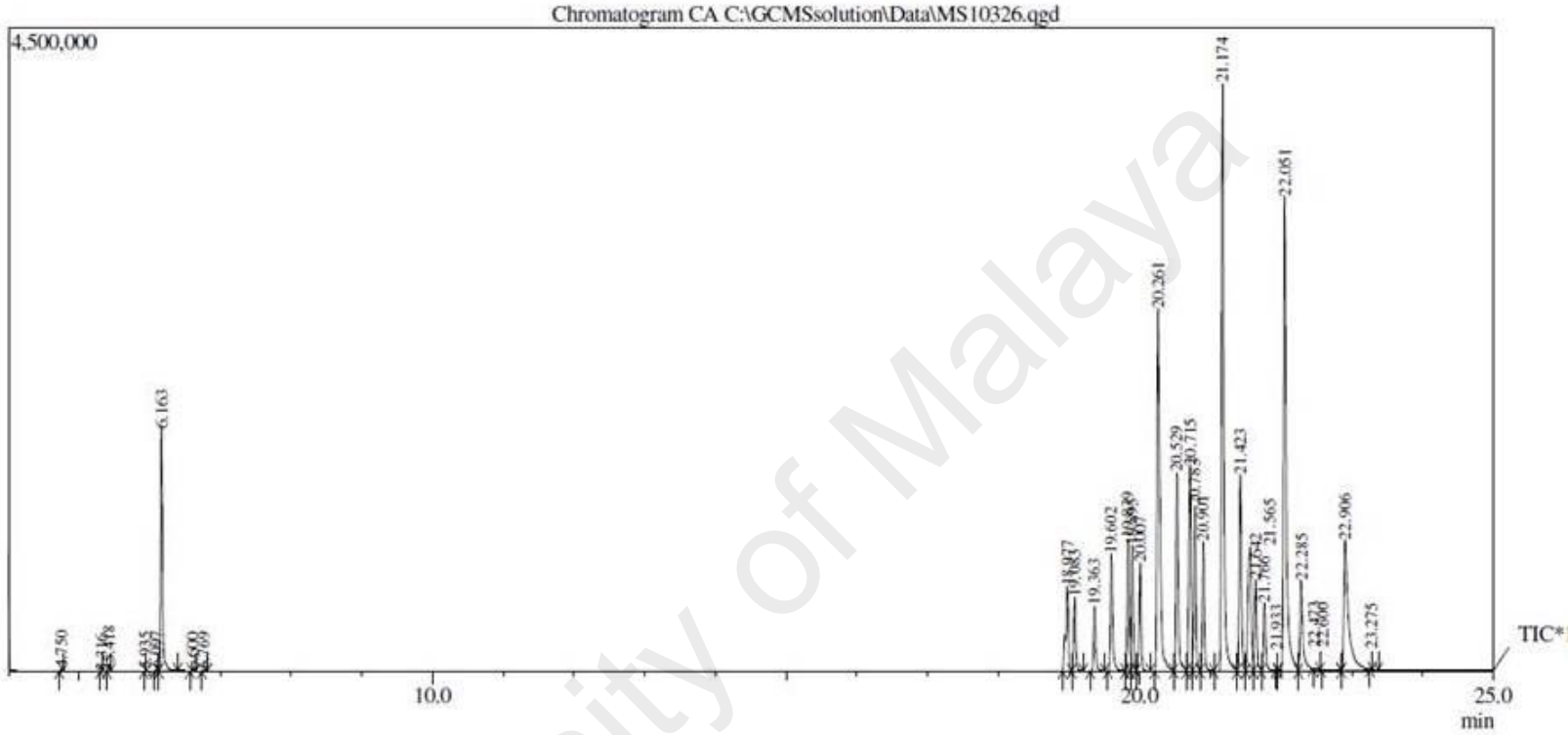


Figure 4.4: GC-MS chromatogram of AGRO

Table 4.6: List of chemical compounds and retention indices of AGRO

Peak number	Retention indices (min)	Compounds	Composition of total oil (%)
1	4.75	α-thujene	0.04
2	5.316	2-cyanofuron	0.001
3	5.418	β-pinene	0.12
4	5.935	2-propenamide	0.01
5	6.097	p-cymene	0.05
6	6.163	α-limonene	5.27
7	6.600	Phenylamine	0.01
8	6.769	2-pentanol	0.02
9	18.977	Benzoic acid	2.40
10	19.083	Undecyl benzoate	1.55
11	19.363	Tetradecyl ester	1.50
12	19.602	2-octyl benzoate	2.86
13	19.839	4-chlorobutyric acid	2.65
14	19.895	Malonic acid	2.65
15	20.007	2-methylpentyl ester	2.33
16	20.261	Hexadecyl ester	11.38
17	20.529	Pentadecyl ester	4.78
18	20.715	4-cholobutyric acid	5.26
19	20.783	Pentadecyl ester	3.40
20	20.901	2-octyl benzoate	2.75

Table 4.6, Continued

Peak number	Retention indices (min)	Compounds	Composition of total oil (%)
21	21.174	Tetradecyl ester	15.07
22	21.423	Tridecyl ester	4.54
23	21.565	Carbonic acid	4.47
24	21.642	Eicosyl ester	2.05
25	21.766	1-propanol	1.60
26	21.933	Quinoalamine	0.001
27	22.051	1-undecene	13.59
28	22.285	4-chlorobutyric acid	3.00
29	22.473	1-heptene	0.02
30	22.600	Neopentanol	0.001
31	22.906	3-methylpentane	6.60
32	23.275	Vinyl crotonate	0.001

4.4.2 Chemical compounds of CGPO

The EO extracted from CGPO presented itself as a liquid that was bright yellow in colour with citrus-like odour. The yield was 0.6%. The EO was analysed by GC-MS (Figure 4.5) and the compounds of EO is listed in Table 4.7 along with the retention indices of the identified compounds. The major compound detected was D-limonene which contributed 91.89% of the EO. Other compounds detected showed very small percentage such as β-myrcene (2.24%), β-pinene (1.40%), α-pinene (1.22%) and few more that detected as <1% (β-cymene, L-limonene, β-terpinene, α-zingiberene, caryophyllene,

napthalene, 1-6, cyclodecadiene, α-cubebene , β-ocimene, α-thujene, α-terpineol, δ-cadinene and α-phellandrene). Almost 100% (99.97%) of the CGPO was composed mainly by the hydrocarbon terpenes groups (D-limonene, β-myrcene, β-pinene, β-cymene, α-terpinene, caryophyllene, β-ocimene, α-phellandrene, α-zingiberene, α-thujene, α-cubebene) and very small amount was component of oxygenated compound (monoterpene alcohol) which is α-terpineol (0.03%).

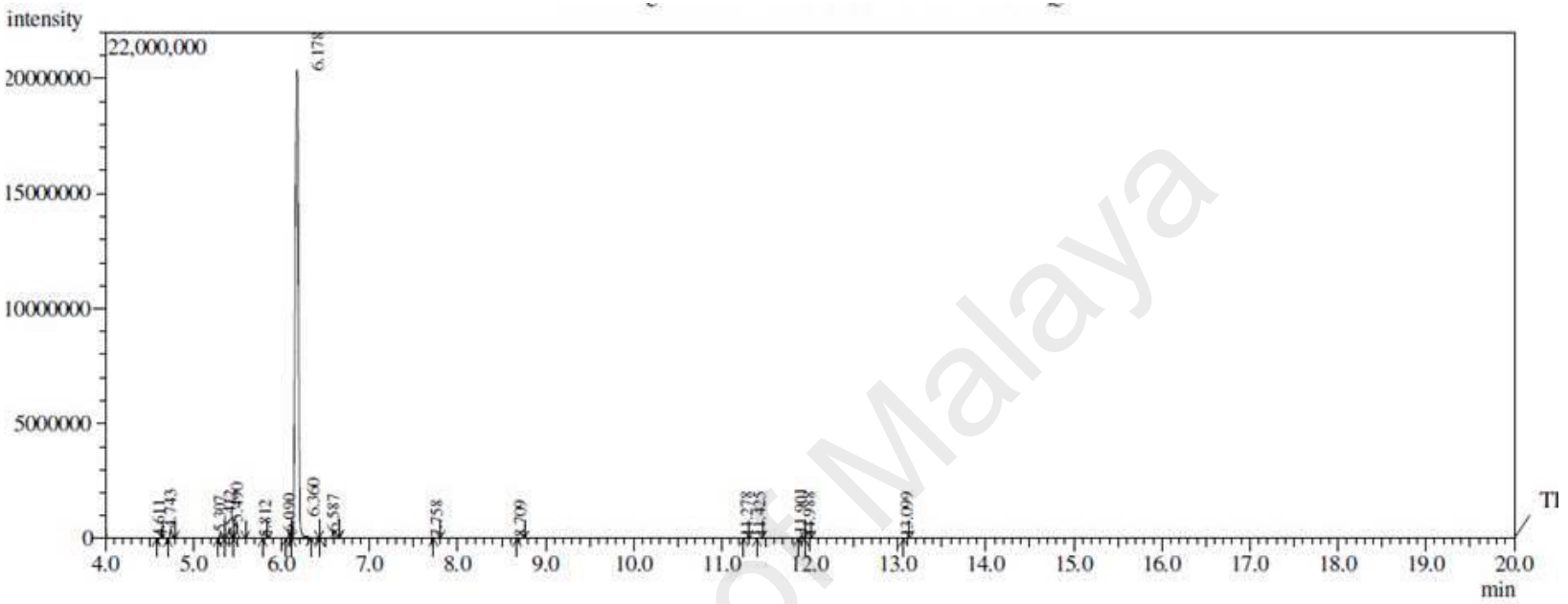


Figure 4.5: GC-MS chromatogram of CGPO

Table 4.7: List of chemical compounds and retention indices of CGPO

Peak number	Retention indices (min)	Compounds	Composition of total oil (%)
1	4.611	α-thujene	0.05
2	4.743	α-pinene	1.22
3	5.307	β-terpinene	0.66
4	5.412	β-pinene	1.40
5	5.490	β-myrcene	2.24
6	5.812	α-phellandrene	0.02
7	6.090	β-cymene	0.80
8	6.178	D-limonene	91.89
9	6.360	β-ocimene	0.07
10	6.587	L-limonene	0.73
11	7.758	α-terpineol	0.03
12	8.709	α-cubebene	0.09
13	11.278	Naphthalene	0.13
14	11.425	Caryophyllene	0.13
15	11.901	α-zingiberene	0.41
16	11.998	δ-cadinene	0.03
17	13.099	1,6-cyclodecadiene	0.10

4.4.3 Chemical compounds of CALO

The EO extracted from CALO presented itself as a liquid that was dark green in colour and having citrus smell. The yield for CALO was 0.5%. Similarly, CALO was analysed by GC-MS (Figure 4.6) to determine its compounds. The list of its compounds is presented in Table 4.8 along with the retention indices of the identified compounds. Seven compounds were identified representing 100% of the oil. The major compounds is D-limonene which contributed 96.09% of the total EO. The other six compounds showed very low percentage with cyclohexene contributed 1.81%, while others contibuted < 1% (crodamol 0.65%, linalool 0.21%, β-pinene 0.15% and α-phellandrene 0.07%).

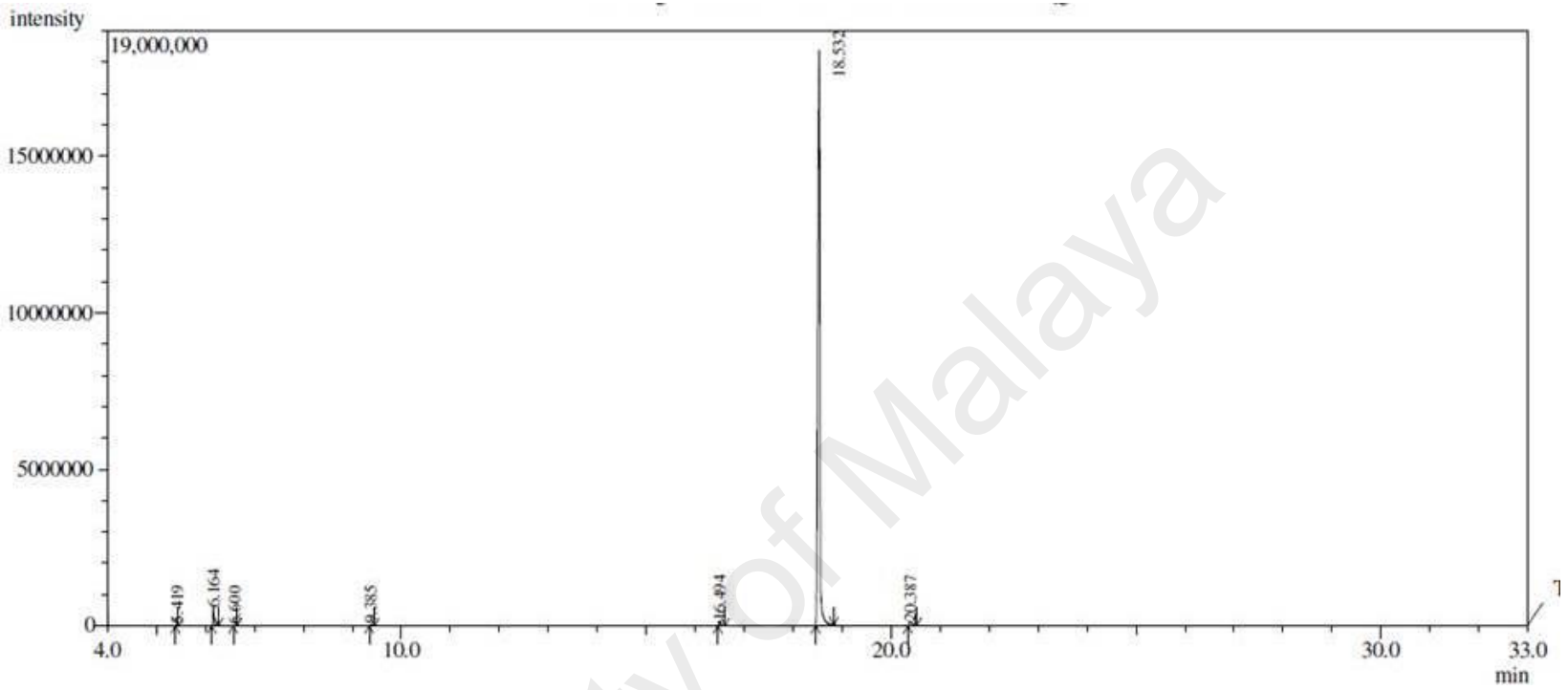


Figure 4.6: GC-MS chromatogram of CALO.

Table 4.8: List of chemical compounds and retention indices of CALO

Peak number	Retention indices (min)	Compounds	Composition of total oil (%)
1	5.419	β-pinene	0.15
2	6.164	Cyclohexene	1.81
3	6.600	α-phellandrene	0.07
4	9.350	Linalool	0.21
5	16.494	Crodamol	0.65
6	18.532	D-limonene	96.09
7	20.387	Octadecanoic acid	1.02

4.5 MICROENCAPSULATION: CHARACTERISTICS OF THE MICROCAPSULES

4.5.1 Morphology of microcapsule

Microencapsulation process produced suspensions of semisolid microcapsule of EOs (AGRO/CGPO/CALO) and DEET as milky white liquid (Figure 4.7). There were no EOs and DEET observed either on the surface of the suspensions or after centrifugation of the discarded aqueous supernatant indicating very high encapsulation efficacy taken placed. No coalescence and/or other appearance of emulsion failure were observed indicating well homogenized suspension obtained.

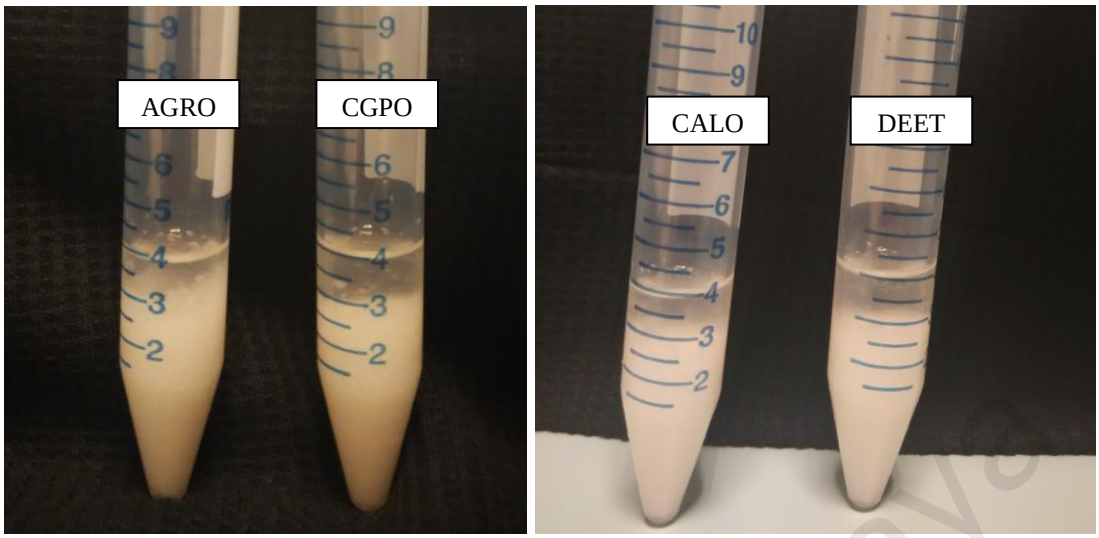


Figure 4.7: Suspensions of semisolid microcapsule of EOs (AGRO/CGPO/CALO) and DEET

Figure 4.8 and 4.9 show the optical micrograph of EOs and DEET microcapsules. Figure 4.8 shows a single microcapsule of EOs: (a) AGRO, (b) CGPO, (c) CALO and (d) DEET all having thick wall surrounding the core. Figure 4.9 shows several microcapsules of EOs: (a) AGRO, (b) CGPO, (c) CALO and (d) DEET. All EOs and DEET microcapsules appeared as spherical and oval in shape. The microcapsules were shown distributed individually without excessive agglomeration. The microcapsules diameter indicated that it varies in the range of 2 to 8 µm.

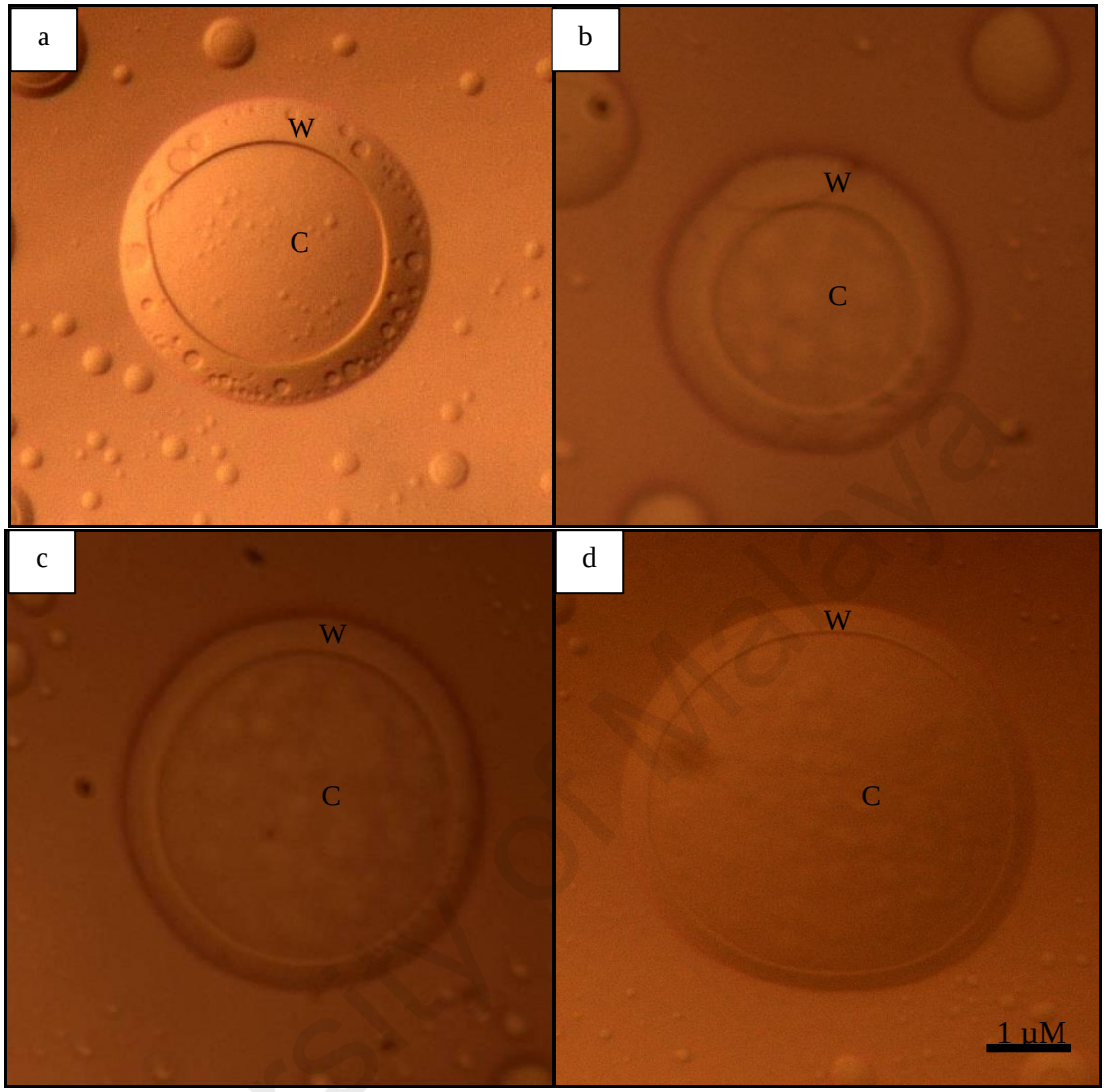


Figure 4.8: **Optical micrographs showing single microcapsule of (a) AGRO (b) CGPO (c) CALO and (d) DEET (10x 40 magnifications). Bar represents 1 µm. w = wall and c = core.**

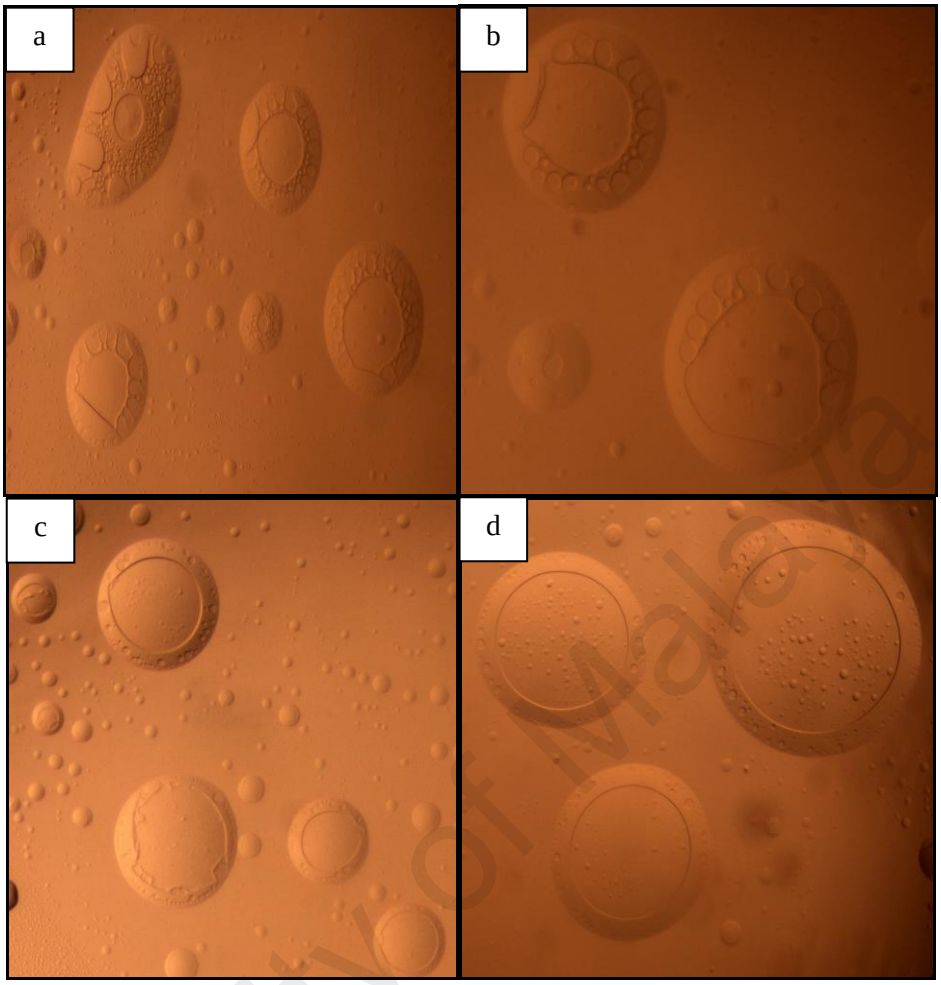


Figure 4.9: Optical micrographs of several microcapsules (a) AGRO (b) CGPO and (c) CALO and (d) DEET (10 x 10 magnifications).

4.5.2 Microcapsule diameter size, microcapsule size distribution, encapsulation efficiency (EE) and zeta potential values

Table 4.9 shows the microcapsule diameter size, microcapsule size distribution, percentage of EE and zeta potential values of EOs and DEET microcapsules. The measurement of microcapsule demonstrated that DEET microcapsule possessed significantly larger size (6.5 µm) compared to the EOs microcapsules. Among the EOs, CALO presented larger size of microcapsule (4.0 µm) followed by AGRO (3.3 µm) and

CGPO (2.7 µm). Statistically, all EOs microcapsules demonstrated significantly different in particle sizes ($p<0.05$).

The size distribution of the microcapsules was characterized by PDI value. The lower the index value the narrower the size distribution of the microcapsules. The PDI was measured between 0 and 1. Values close to 0 indicate a monodisperse, while values close to 1 indicated polydisperse in particle size distribution. Results obtained showed that PDI values were 0.6 for both CGPO and AGRO microcapsules, 0.5 for CALO microcapsules and 0.4 for DEET microcapsules. This data indicated that DEET microcapsules of having had moderate polydisperse type of size of distribution while EOs had broad polydisperse type.

These results were confirmed by the percentage of size distribution histograms shown in Figure 4.10. AGRO and CGPO microcapsules had almost similar particle size distribution ranging from 0.4 µm to 9.6 µm (Figure 4.10a) and 0.4 µm to 8.3 µm (Figure 4.10b), respectively. CALO microcapsule had a wider distribution ranging from 0.24 µm to 9.6 µm (Figure 4.10c) while DEET microcapsule showed a narrow distribution ranging from 2.2 µm to 6.2 µm (Figure 4.10d).

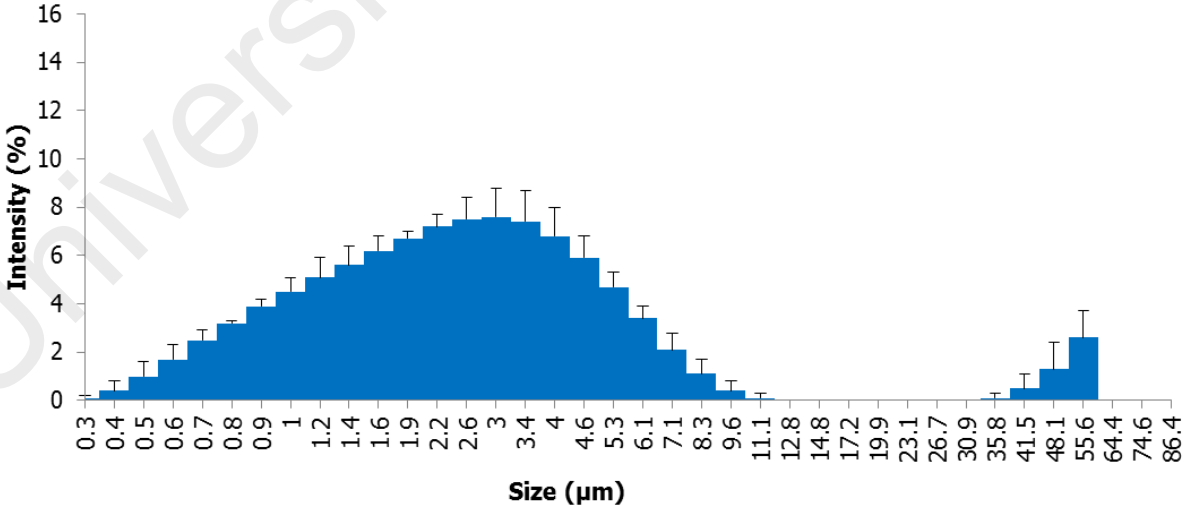
The EE results showed that more than 96% and 98% of EOs and DEET were successfully encapsulated, respectively. All EOs shown to have comparable EE as no significant different were detected ($p>0.05$). However DEET possess significantly higher EE compared with EOs ($p<0.05$). Based on the zeta potential values recorded CGPO microcapsules demonstrated the strongest charges (-47.9 mV), followed by CALO (-46.1

mV), AGRO (-45.0 mV) and lastly DEET (-43.0 mV). Statistically all of these values were significantly different when compared between one another.

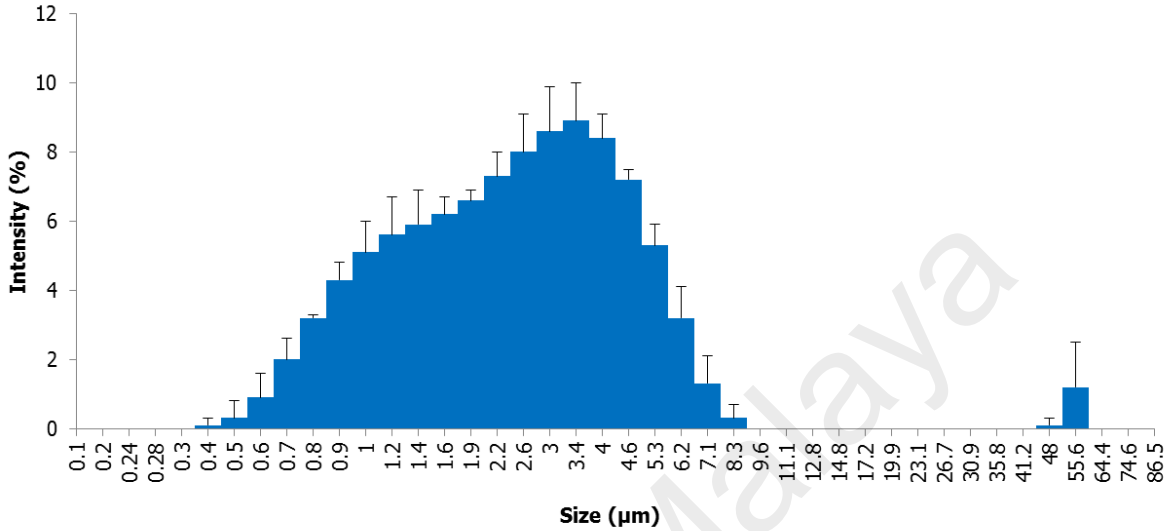
Table 4.9: Diameter size, microcapsule size distribution, encapsulation efficiency (EE) and zeta potential value of microcapsules.

Microcapsule	Diameter size (µm ± SD)	PDI	EE (%)	Zeta potential (mV ± SD)
AGRO	3.3 ± 1.6	0.6	96.81 ± 0.3	-45.0 ± 3.5
CGPO	2.7 ± 1.3	0.6	96.42 ± 0.5	-47.9 ± 5.6
CALO	4.0 ± 1.4	0.5	97.22 ± 0.4	-46.1 ± 3.9
DEET	6.5 ± 2.4	0.4	98.36 ± 0.3	-43.0 ± 4.5

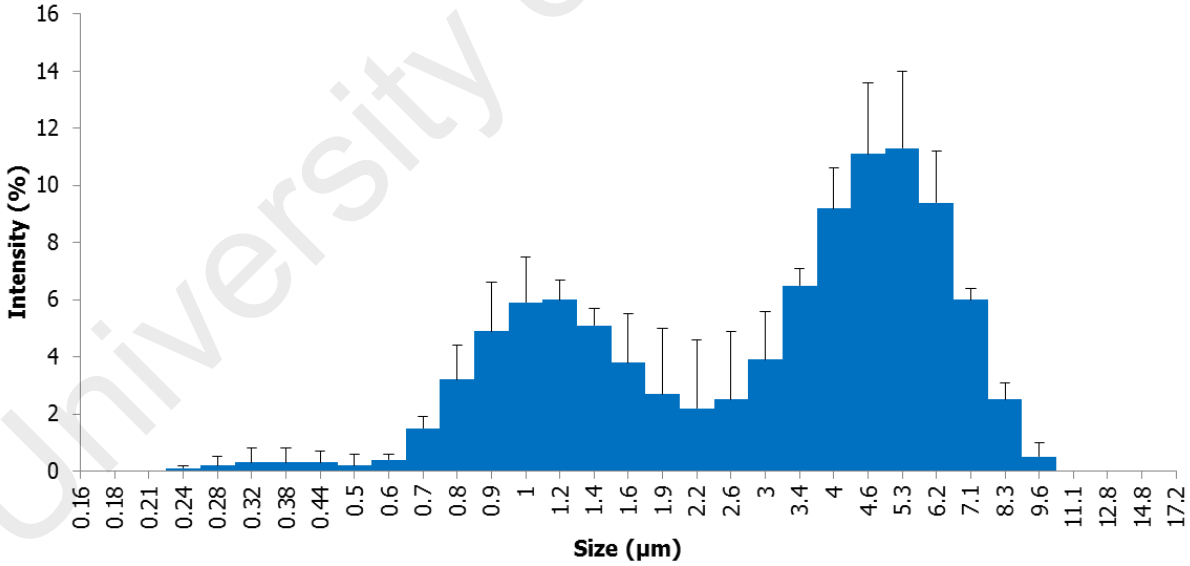
a)



b)



c)



d)

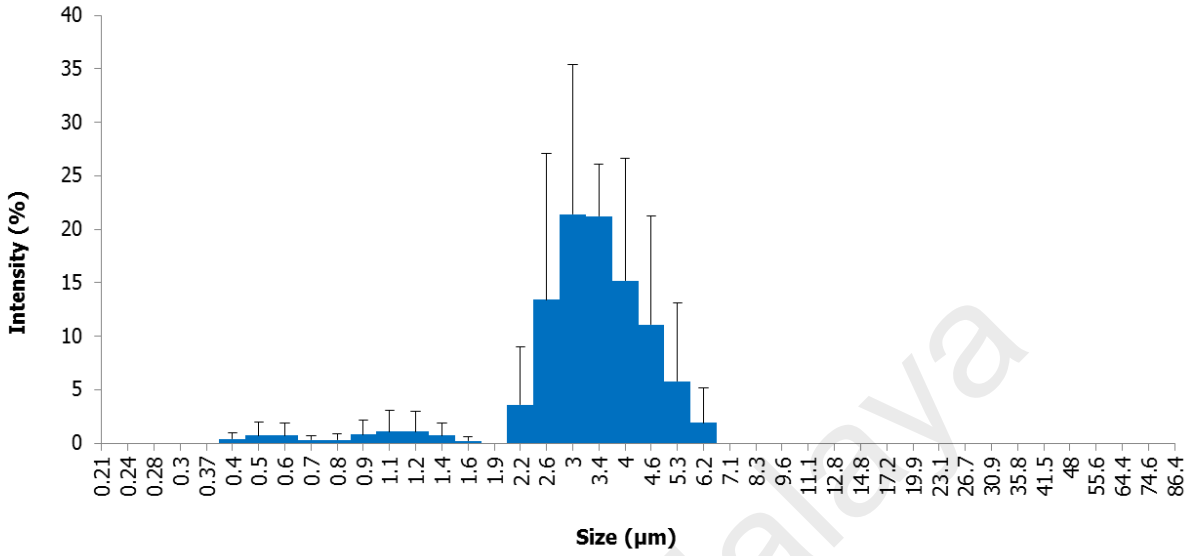


Figure 4.10: Size distribution histograms of (a) AGRO microcapsule and (b) CGPO microcapsule (c) CALO microcapsule and (d) DEET microcapsule.

4.5.3 Fourier transforms infrared spectroscopy (FTIR) analysis

FTIR analyses were performed in order to examine the interaction between the wall materials (BKC and CMC) and the success of the entrapment of the EOs in the microcapsules. The FTIR spectra of BKC, CMC, pure EOs, EOs microcapsules, pure DEET and DEET microcapsules are shown in Figure 4.11- 4.14. The absorption peaks obtained were then compared with the previous literature for analysis purposes. The FTIR spectrum analysis of the wall materials (BKC and CMC) and pure EOs and DEET were found to demonstrate similar absorption peaks when compared with previous literature. The results of the analysis are summarized in Table 4.10. The FTIR spectrum analysis of all EOs and DEET microcapsules showed two new peaks that associated with the presence of interaction between wall materials and may indicate successful encapsulation has occurred.

The presence of absorption peaks pure EOs and pure DEET in the EOs microcapsule and DEET microcapsule spectrum may also indicate the successful encapsulation of pure EOs and DEET (Table 4.10).

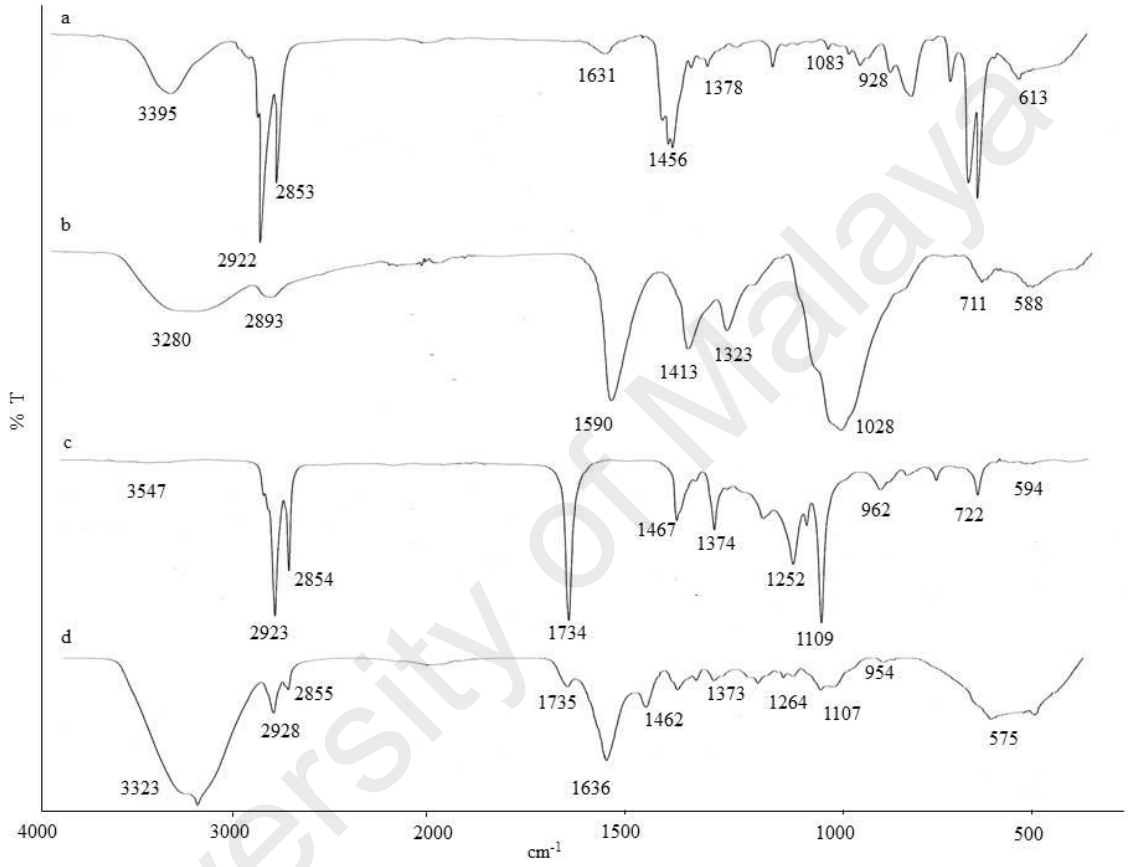


Figure 4.11: FTIR spectrums of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure AGRO and (d) AGRO microcapsule.

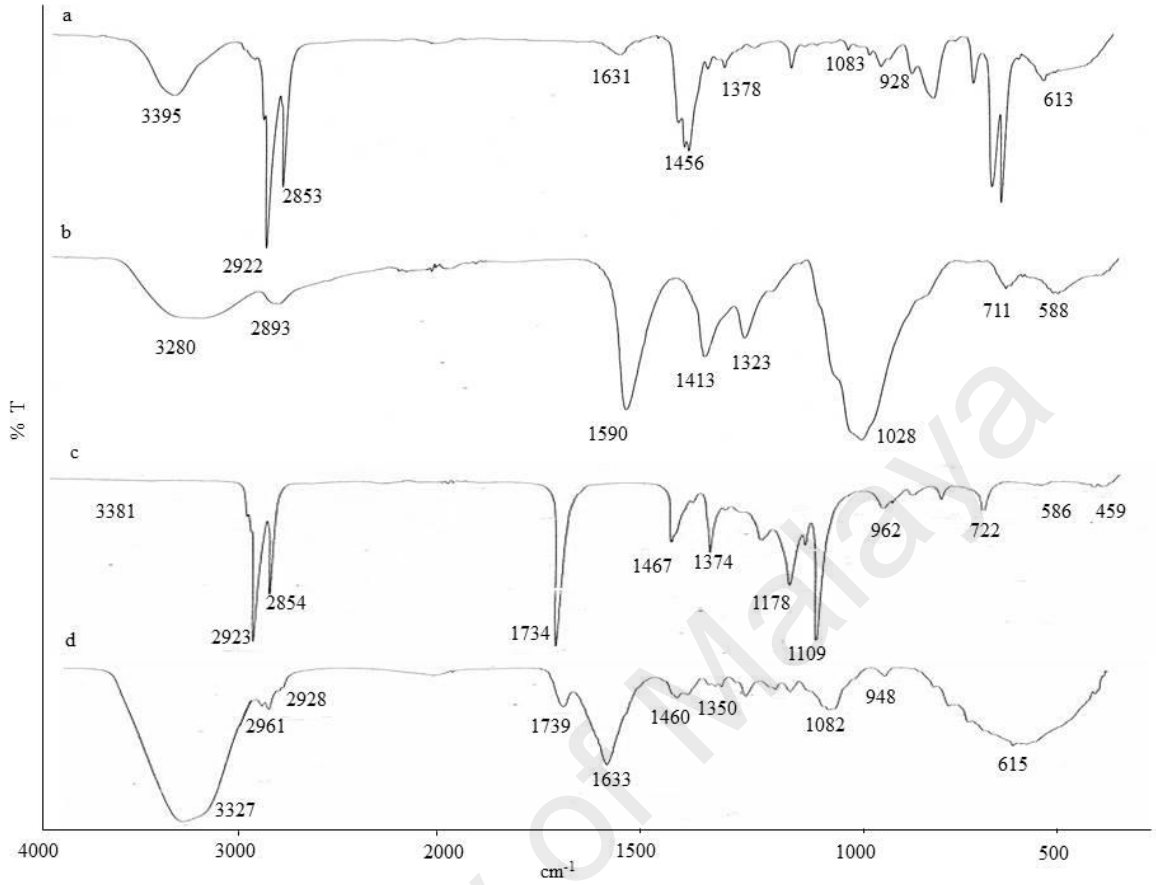


Figure 4.12: FTIR spectrums of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure CGPO and (d) CGPO microcapsule.

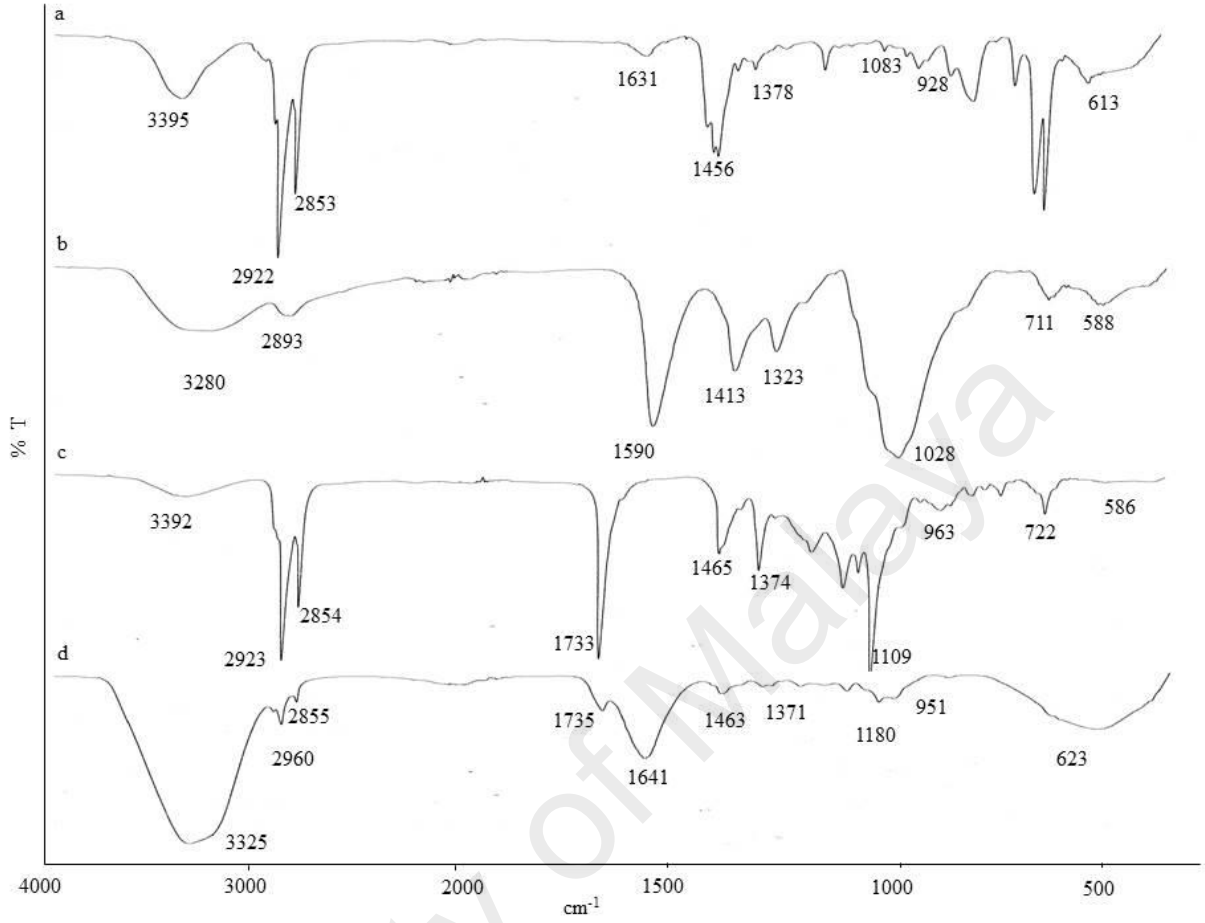


Figure 4.13: FTIR spectrums of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure CALO and (d) CALO microcapsule.

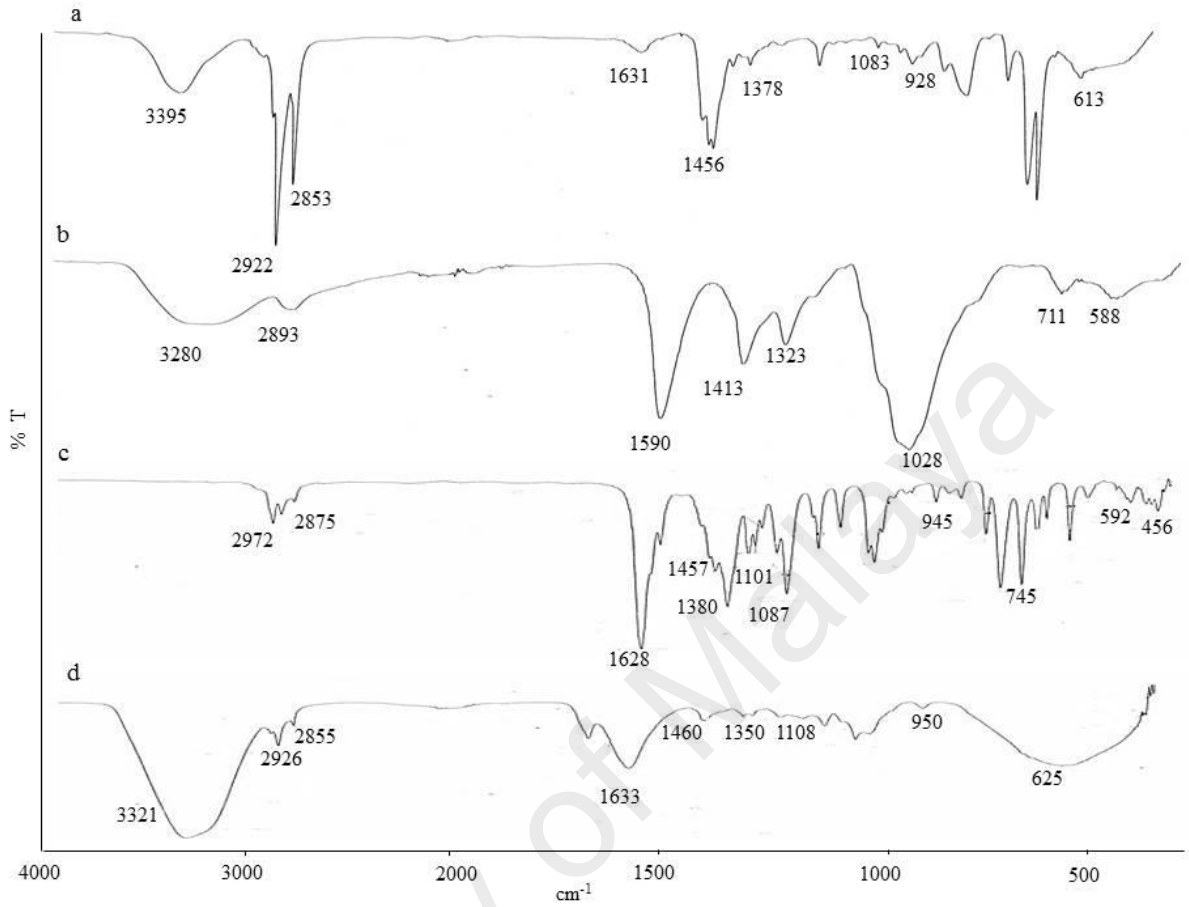


Figure 4.14: FTIR spectrums of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure DEET and (d) DEET microcapsule.

Table 4.10: The analysis of FTIR spectrum of wall materials (BKC and CMC), pure EOs, pure DEET, EOs microcapsule and DEET microcapsule.

Component	Absorption peak (cm^{-1})	Assignation	References
BKC	3395	-NH group stretching	Theron *et al*., 2012
	2922, 2853, 1456	-CH stretching vibration of the surfactant tail and –CH bonding in methyl and methylene group, respectively.	Farias *et al.,* 2011
CMC	3280	-OH group stretching	Bozaci *et al.,* 2015; Koupantsis *et al.*, 2016
	1590, 1413	Asymmetrical and symmetrical stretching vibration of the –COO group, respectively.	Bordallo *et al.,* 2015; Yadav *et al.*, 2014.
	2893, 1323	-CH_2 and –OH group stretching vibration, respectively.	Bozaci *et al.,* 2015; Adebejo & Frost, 2004.
	1028	C-O stretching vibration cellulose backbone of the CMC	Bozaci *et al.,* 2015; Adebejo & Frost, 2004.
Pure EO	3547 (AGRO), 3381 (CGPO), 3392 (CALO)	Asymmetric C-H stretching vibrations in =CH_2	Senhorini *et al.,* 2012
	2923 and 2854 (all EOs)	Aliphatic C-H stretching vibrations in –CH_3 and –CH_2, respectively.	Sutaphanit & Chitprasert, 2014
	1734 (AGRO and CGPO) and 1733 (CALO)	C=O of saturated aliphatic ester.	Senhorini *et al.,* 2012

Table 4.10, Continued

	1467 (AGRO and CGPO), 1465 (CALO)	Scissor C-H bending vibrations in $=CH_2$.	Sutaphanit & Chitprasert, 2014
	1374 (all EOs)	Symmetric C-H bending vibration in $–CH_3$.	Senhorini *et al.*, 2012
	962 cm^{-1} and 594 cm^{-1} in AGRO, 962 cm^{-1} and 586 cm^{-1} in CGPO and 963 cm^{-1} and 586 cm-1 in CALO.	C-H bending of the alkene or aromatic groups	Sajomsang *et al.*, 2007.
Pure DEET	2972, 2875	Aliphatic C-H stretching vibration	NIST, 2016
	1628	-C=C stretching of aromatic group	
	1500 – 1000	Vibration of the C-C and C-H groups.	
	945 -456	'oop' =C-H stretch bending	
EOs and DEET microcapsules	3323 (AGRO), 3327 (CGPO), 3325 (CALO) and 3321 (DEET)	Presence of –OH group and -NH group from CMC and BKC. H bonding interaction between CMC and BKC which confirmed the formation of membrane around the microcapsules. Shifting of –OH (3280 cm^{-1}) in CMC and -NH group (3395 cm^{-1}) in BKC.	Lawrie *et al.*, 2007; Natrajan *et al.*, 2015
	1636 (AGRO), 1633 (CGPO), 1641 (CALO), and 1633 (DEET)	Associated with the formation of –CONH group which confirmed the formation of membrane around the microcapsules due to	Theron *et al.*, 2012; Gite *et al.*, 2015.

	the electrostatic interaction between –COOH group of CMC and –NH_2 group of BKC.	
	Most of the characteristic peaks of EOs and DEET could be observed in the spectrum of microcapsule with minor differences in frequencies which confirmed the successfully encapsulated EOs and DEET.	

4.5.4 Thermogravimetric Analysis (TGA)

TGA is done in order to examine stability and weight changes with temperature. The thermal analysis of BKC, CMC, pure EOs, pure DEET, EOs and DEET microcapsules is shown in Figure 4.15 – 4.18. The TGA curves obtained were compared with the previous literature for analysis purposes. The results of the analysis were presented in Table 4.11. The TGA curve analysis of the wall materials BKC, CMC, pure EOs and DEET indicated the specific weight loss step for each curve when compared with previous literature. Based on the TGA curves of EOs and DEET microcapsules almost similar pattern of weight loss step curves (three weight loss steps) were observed with minimal differences in temperature.

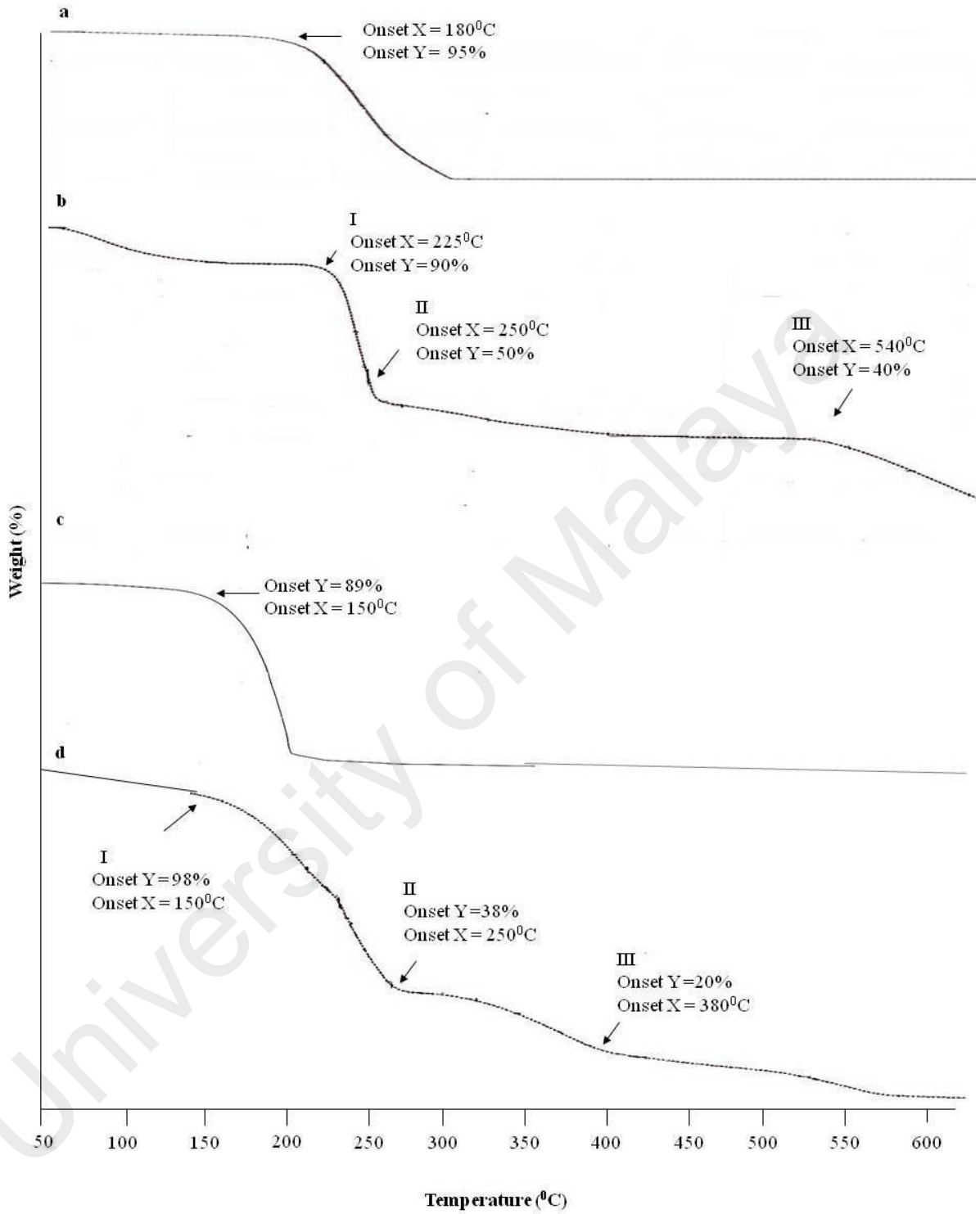


Figure 4.15: TGA curves of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure AGRO and d) AGRO microcapsule.

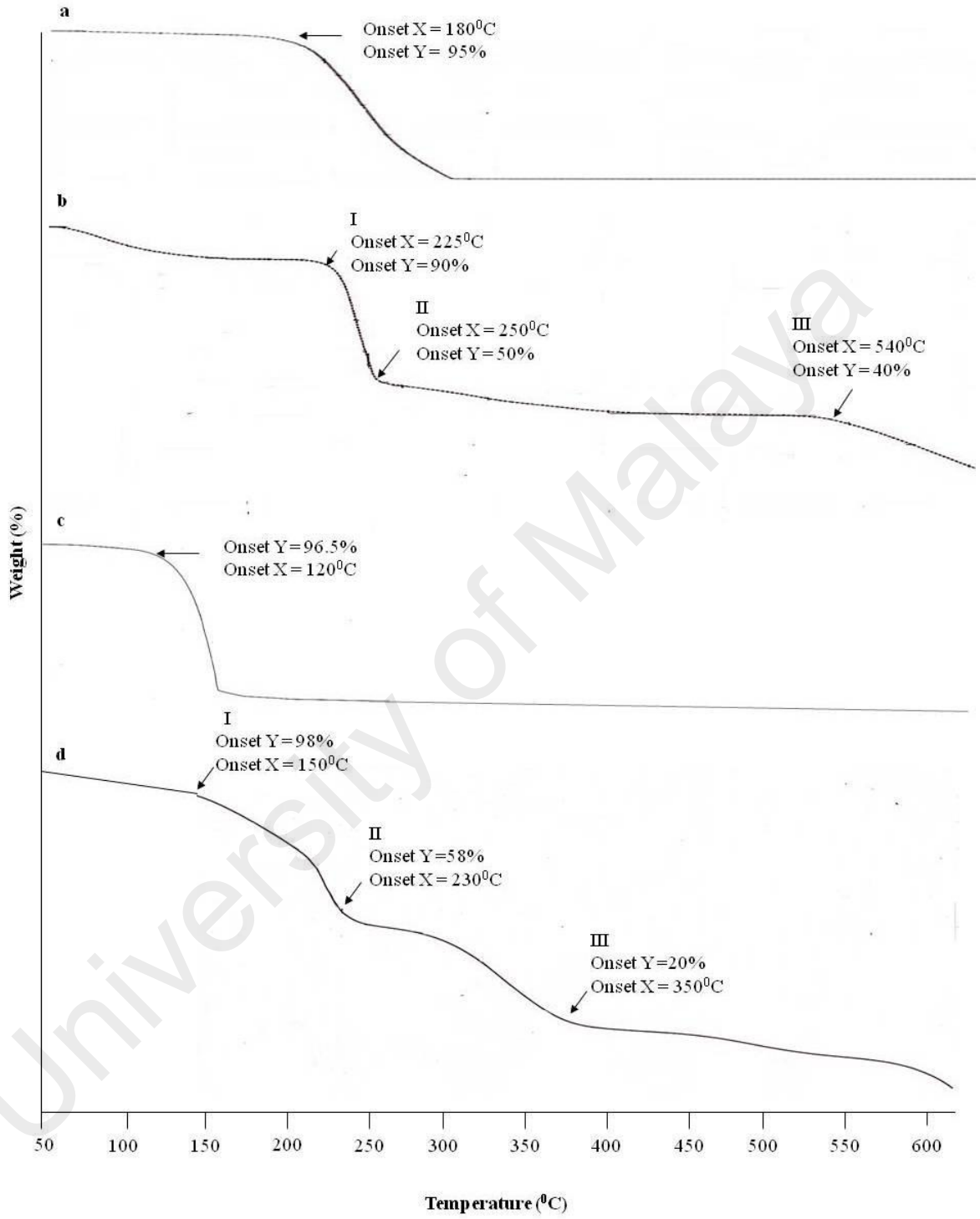


Figure 4.16: TGA curves of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure CGPO and d) CGPO microcapsule.

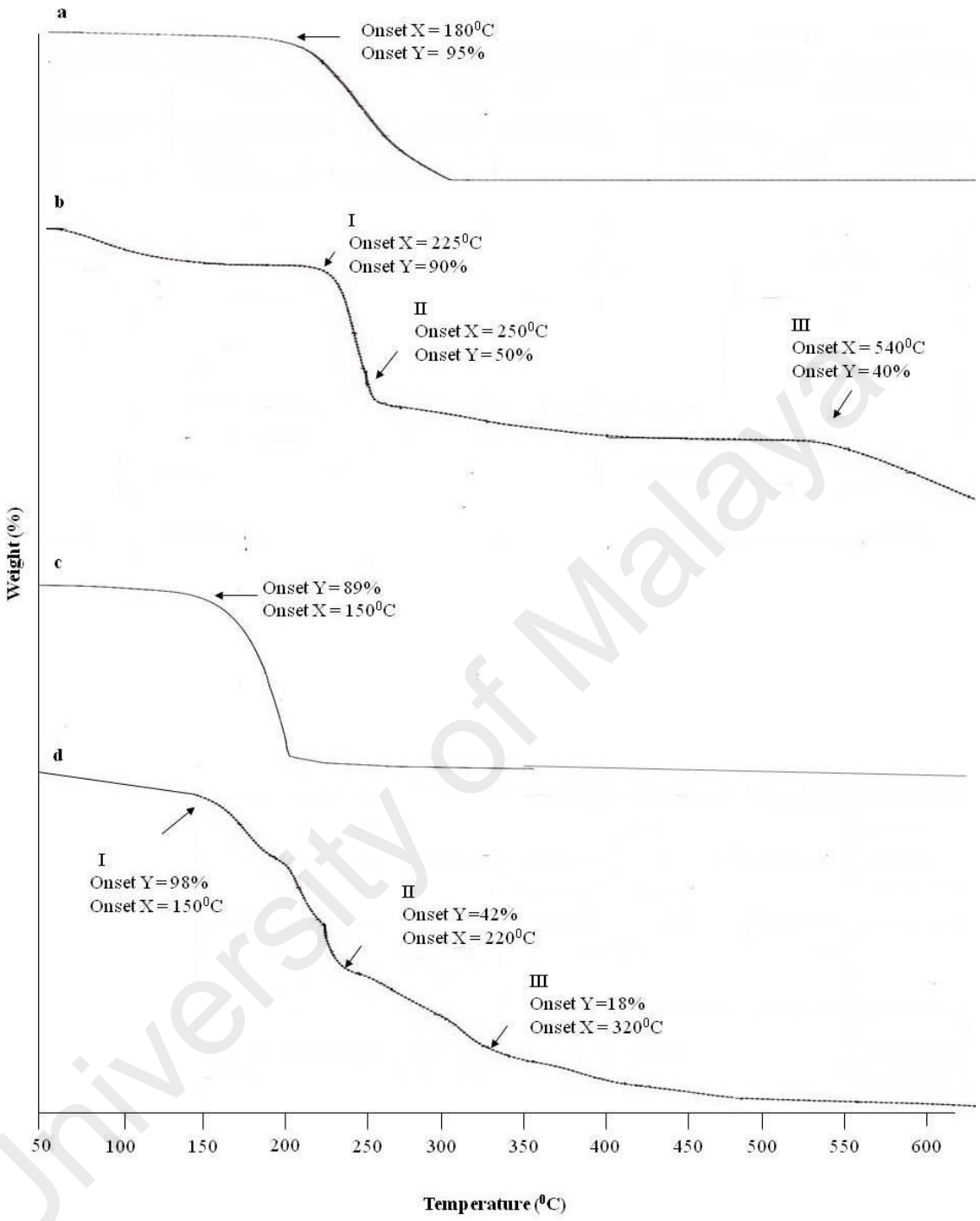


Figure 4.17: TGA curves of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure CALO and d) CALO microcapsule.

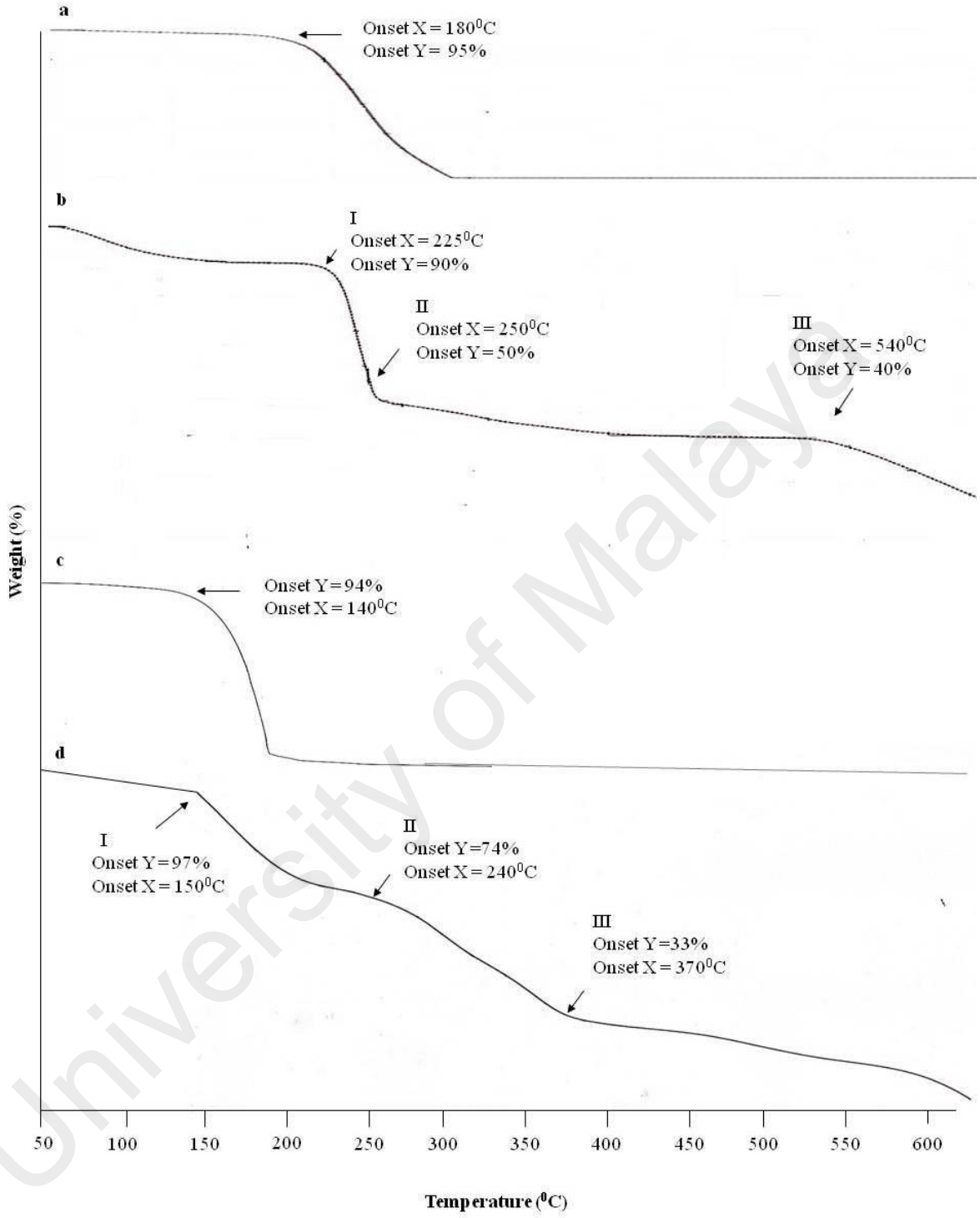


Figure 4.18: TGA curves of (a) Benzalkonium chloride (BKC), (b) Carboxymethylcellulose (CMC), (c) pure DEET and d) DEET microcapsule.

Table 4.11: TGA curve analysis of wall materials (BKC and CMC), pure EOs and DEET and EOs and DEET microcapsules

Component	Weight loss step	Assignation	References
BKC	One weight loss step starting at 180°C and completed at 300°C		
CMC	Three weight loss step: I: 10% weight loss starting at 50 to 225°C. II: 50% weight loss when temperature increased from 225 – 250°C. III: 40% weight loss when temperature increased from 250 – 540°C.	 Evaporation of water Decarboxylation process Main chain decomposition of the cellulose. At 540°C the CMC started to decompose.	Ünlü 2013
Pure AGRO	One weight loss step: Completed weight loss for AGRO when temperature reached 200°C.		Senhorini *et al.*, 2012
Pure CGPO	One weight loss step: Completed weight loss when the temperature reached 150°C.		
Pure CALO	One weight loss step: Completed weight loss when the temperature reached 200°C		

Table 4.11, Continued

Pure DEET	One weight loss step: Completed weight loss was observed when temperature reached 180°		Drapeau *et al.*, 2011
EOs/DEET microcapsules	Three weight loss steps: I: At around 150°C there was approximately 2% weight loss.	The evaporation of water remaining entrapped in the capsules after the encapsulation process.	Gite *et al.*, 2015; Wu *et al.*, 2014; Fei *et al*., 2015
	After 150^0C until around 250^0C resulted in 60% (AGRO), 40% (CGPO), 56% (CALO) and 23% (DEET) weight loss.	The EOs/DEET reached the boiling point and the microcapsule wall broke. Part of the core material was released from the microcapsules. Compared with pure EOs/DEET, the microcapsule had slower weight loss rate under temperature range.	
	II: 18% (AGRO), 38% (CGPO), 24% (CALO) and 41% (DEET) weight loss at temperature ranging from 250°C and 380°C.	The degradation of microcapsule wall and the temperature range was consistent with that of the third weight loss stage and complete degradation for the CMC and BKC, respectively.	
	III: weight loss observed above 320°C	Residual degradation of the microcapsule wall	

4.6 CHARACTERISTICS OF THE FORMULATIONS

Right after the preparations, all formulations were observed for their physical appearance which also known as organoleptic characteristics. Observation on all ME and NE formulations showed that they presented milky white color appearance, smooth texture and no phase separation (well homogenized). All of them emitted very pleasant aroma where AGRO formulations presented fresh, spicy, camphorous aroma, CGPO and CALO have citrus aroma while DEET have shea butter-like odour. Upon application on the skin, all the formulations presented non-greasy feeling and have good spreadreadily (easily spreadable). ME and NE formulations presented different softness upon application on skin with NE formulations was found much softer than ME formulation. It was also found that the formulations do not left residue on the skin surface after application.

The physicochemical characteristics of the formulations were also observed right after the preparation and the results are presented in Table 4.12. ME formulation demonstrated higher viscose ranged between 6842 cp and 7724 cp compared to NE formulations which ranged between 6234 cp and 6778 cp. ME formulations indicated micron in particle sizes (more than 1 µm) ranged between 3.29 and 6.5 µm, while NE formulations presented nano sized (less than 1 µm) ranged between 0.21 and 0.53 µm. The PDI values of ME formulations showed broader particle size distribution (range from 0.5 to 0.6) compared with NE formulations which PDI values range from 0.3 to 0.4. ME formulations presented higher zeta potential values (range between -43 mV to -46 mV) compared with NE formulations that presented values range between -30 mV to -35 mV. All formulations were found to have pH values ranged from 5.0 to 5.5.

Table 4.12: Physicochemical characteristics of the formulations.

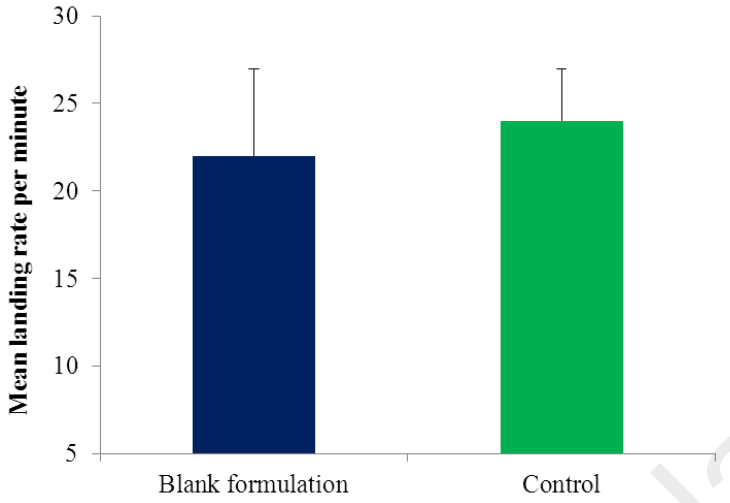
	Formulation	AGRO	CGPO	CALO	DEET
Viscosity (cp)	ME	7575	7581	6852	7724
	NE	6645	6778	6234	6432
Particle size (µm)	ME	3.3	3.29	4.0	6.5
	NE	0.31	0.21	0.34	0.53
Polydispersity	ME	0.6	0.6	0.6	0.5
index (PDI)	NE	0.3	0.4	0.4	0.3
Zeta potential	ME	-45	-47.9	-46.1	-43
(mV)	NE	-30	-32.7	-31.9	-34.1
pH	ME	5.54	5.56	5.50	5.53
	NE	5.55	5.54	5.42	5.5

4.7 EFFICACY OF THE FORMULATIONS

4.7.1 Laboratory evaluation

Statistically, no significant difference was detected with regards to the mean landing rate per minute on area tested with blank formulation and untested area ($p>0.05$) for both species *Aedes aegypti* and *Cx quinquefasciatus* (Figure 4.19). This indicated that the blank formulation did not possess inherent repellent properties and therefore did not affect the repellency response of the essential oils and DEET.

a)



b)

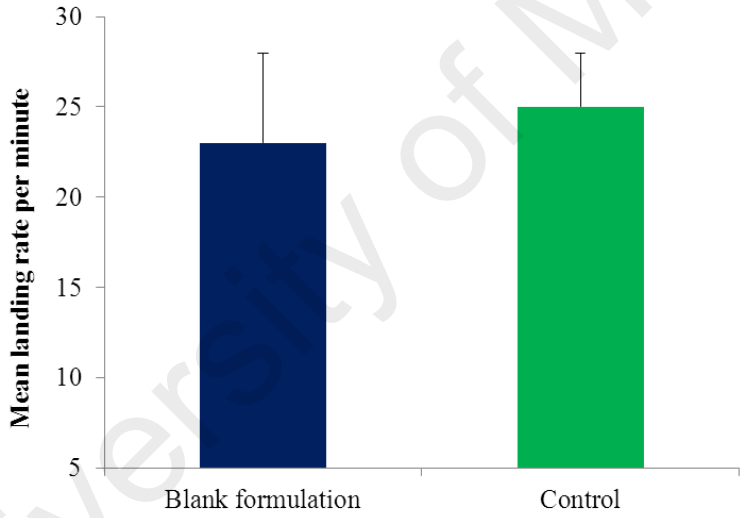


Figure 4.19: Mean landing rate of *Aedes aegypti* (a) and *Cx. quinquefasciatus* (b) on area tested with blank formulation and untested area (control). No significant difference was detected between blank formulation and control. Error bars indicate SEM.

Table 4.13 shows the mean percentage reduction of *Aedes aegypti* bites after application of ME and NE EOs and DEET formulations. Commercial plant-based repellent containing citronella and Citriodiol® were also tested as comparison. Based on the data obtained, all the ME and NE formulations of AGRO and CGPO presented similar efficacy level where complete protection was detected up to 2 hours post application. After 4 hours post application however, only ME formulation maintained the significantly higher protection than the NE formulation ($p<0.05$). At the end of observation time, >80% and >70% of protection were observed in ME formulation of AGRO and CGPO, respectively while NE formulation demonstrated protection of around 60%.

As for CALO, both ME and NE formulations presented no significant difference in term of protection during the first hour of application ($p>0.05$). After 2 hours post application however, ME formulation presented protection which is significantly higher than the NE formulation. At 8 hours post application, CALO ME formulation demonstrated 75.86% of protection while NE formulation presented only 41.89% of protection. As for DEET, both formulation presented no significant different in protection provided ($p>0.05$).

When comparison were made between EOs formulations with DEET formulation, EOs formulations presented a significantly lower repellency effect ($p<0.05$). Comparison made between EOs formulations either ME or NE with commercial citronella-based repellent demonstrated that EOs formulations of having significantly better repellent effect ($p<0.05$). When compared with Citrodiol®- based repellent, ME formulations of AGRO and CGPO showed similar repellent effect ($p>0.05$).

Table 4.13: Mean percentage reduction of *Aedes aegypti* bites on volunteers after application of ME and NE EOs and DEET formulations.

		Percentage reduction of repellency (%)					
Treatment	**Formulation**	**0 h**	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**
AGRO	ME	100	100	100	98.91	88.88[a]	80.02[a]
	NE	100	100	100	92.70[a,b]	82.90[a,b]	64.13[a,b]
CGPO	ME	100	100	100	98.82[a]	88.40[a]	75.86[a]
	NE	100	100	100	91.94[a,b]	80.39[a,b]	62.07[a,b]
CALO	ME	100	100	100	90.82[a]	83.59[a]	75.86[a]
	NE	100	98.97	83.21[a,b]	70.34[a,b]	54.26[a,b]	41.89[a,b]
DEET	ME	100	100	100	100	94.64	85.68
	NE	100	100	100	100	93.79	82.85
Commercial plant-based repellent							
KAPS®	Citronella oil	100	100	95.13	82.95	74.11	63.01
MozAway®	Citronella oil	100	100	96.66	82.21	68.78	58.15
BioZ Natural®	Citronella oil	100	100	92.39	80.39	70.61	57.44
Mosiguard®	Citriodiol®	100	100	100	96.58	89.79	83.25

a denotes significantly different compared with DEET at similar time of exposure ($P<0.05$)
b denotes significantly different compared with ME formulation of similar EOs and time of exposure ($p<0.05$)

The laboratory trial of ME and NE formulation of EOs and DEET against *Culex quinquefasciatus* is shown in Table 4.14. Commercial plant-based repellent containing citronella and Citriodiol® were also tested as comparison. All EOs ME formulation presented similar repellent effect where complete protection was observed up to 2 hours against *Cx. quinquefasciatus*. At 6 hours post application the protection was maintained at >80%. AGRO ME formulation even maintained this efficacy up to 8 hours post application while CGPO and CALO ME formulations demonstrated slightly less protection (around 70%). No significant difference in protection time were detected between EOs ME formulations and DEET ME formulation ($p>0.05$) up to 4 hours post application. After 6

hours application however, DEET ME formulation demonstrated significantly better repellent effect ($p<0.05$) than EOs ME formulations. All ME formulations shown to have significantly better repellent effect when compared with NE formulations ($p<0.05$). At the end of observation, ME formulations were found having significantly higher repellent effect: >80% protection for AGRO and >70% for CGPO and CALO; while NE formulation demonstrated protection around 60% of ($p<0.05$).

Comparison made between EOs formulations either ME or NE with commercial citronella-based repellent shown EOs formulations having significantly better repellent effect ($p<0.05$). When EOs formulations were compared with Citrodiol®- based repellent, AGRO and CGPO ME formulations were found to have similar repellent effect ($p>0.05$) to Citrodiol®- based repellent.

Table 4.14: Mean percentage of reduction of *Culex quinquefasciatus* bites on volunteers after application of ME and NE EOs and DEET formulations.

		Percentage reduction of repellency (%)					
Treatment	**Formulation**	**0 h**	**1 h**	**2 h**	**4 h**	**6 h**	**8 h**
AGRO	ME	100	100	100	99.03	87.85[a]	81.04[a]
	NE	100	100	98.13[a,b]	81.19[a,b]	73.43[a,b]	65.72[a,b]
CGPO	ME	100	100	100	98.96	86.95[a]	75.86[a]
	NE	100	100	98.09[a,b]	80.97[a,b]	72.98[a,b]	63.12[a,b]
CALO	ME	100	100	100	98.95	85.13[a]	74.97[a]
	NE	100	100	98.11[a,b]	81.23[a,b]	71.19[a,b]	61.41[a,b]
DEET	ME	100	100	100	100	97.81	90.92
	NE	100	100	100	98.42[b]	91.05[b]	81.03[b]
Commercial plant-based repellent							
KAPS®	Citronella oil	100	100	96.16[a]	84.96[a]	75.32[a]	62.11[a]
MozAway®	Citronella oil	100	100	97.13[a]	84.15[a]	61.47[a]	54.33[a]
BioZ Natural®	Citronella oil	100	100	94.14[a]	81.33[a]	71.98[a]	53.22[a]
Mosiguard®	Citriodiol®	100	100	100	98.90	88.11[a]	79.86[a]

a denotes significantly different compared with DEET at similar time of exposure (P<0.05)
b denotes significantly different compared with ME formulation of similar EOs and time of exposure (p<0.05)

4.7.2 Field evaluation

The number of mosquito collected on the untested volunteers at three study sites is presented in Table 4.15. Four genera of mosquitoes namely *Amigeres*, *Aedes*, *Culex* and *Mansonia* were collected. *Ae. albopictus* were the predominant mosquito captured followed by *A. subalbatus*, *Culex spp* and *Mansonia spp*. Among the three areas involved Felda Purun, Pahang demonstrated the highest mosquito number (2,137) while the other two sites presented about the same number of mosquito and lesser than that of Felda, Purun.

The percentage of repellency of ME and NE formulation of EOs and DEET are shown in Table 4.16 – 4.18. Based on results obtained from all study sites, all formulations shown to present almost similar repellent effect where for all ME EOs and DEET formulations complete protection (100%) was presented up to 2 hours post application. ME AGRO and DEET formulations even maintained complete protection up to three hours and four hours post applications, respectively.

For NE EOs formulation, complete protection was observed during first hour post application and the protection decreased to around 50% at five hours post application. NE DEET formulation however presented complete protection up to three hours post application and the protection decreased to around 80% at five hours post application. It can be observed that ME EOs formulation presented significantly better repellent effect when compared with citronella-based repellents (KAPS®, MozAway® and BioZ Natural®) for all study sites ($p<0.05$). They presented complete protection against mosquito bites during first hour post application but lost 50% of its protection at five hours post application. Similar protection pattern was observed for NE formulations, thus indicated the equal repellent effect between ME and NE formulations. When comparison were made with Citriodiol®-based repellent (Mosiguard®), ME EOs formulations shown to present comparable effect ($p>0.05$).

Table 4.15: Number of captured mosquito, biting rate and mosquito species collected on untested volunteers at various study sites in Malaysia

Study site	Total mosquito	Mosquito biting rate (mean ± SEM)	Mosquito species	Percent of mosquito
Kampung Paya	1,725	287.5 ± 55.3	*Amigeres subalbatus*	47.1%
Rumput Jaya, Sungai			*Ae. Albopictus*	48.9%
Udang			*Cx. quinquefasciatus*	2.26%
			Cx. tritaenarhynchus	1.74%
Kampung Pokok	1,772	295.4 ± 32.9	*Amigeres subalbatus*	45.8%
Asam, Sungai petani			*Ae. Albopictus*	47.6%
			Ae. Aegypti	4.33%
			Cx. quinquefasciatus	2.27%
Felda Purun, Bera	2,137	356.2 ± 33.2	*Amigeres subalbatus*	47.4%
			Ae. Albopictus	48.6%
			Cx. gelidus	2.07%
			Cx. tritaenarhynchus	1.57%
			M. annulifera	0.36%

Table 4.16: Average mean (± SEM) number of mosquito bites on control and mean percentage of repellency of ME and NE formulations of EOs and DEET and the marketed topical plant-based repellent against mosquito in Kampung Paya Rumput Jaya, Sungai Udang, Melaka.

Active ingredient	Formulation	Percentage of repellency (%)					
		0	1	2	3	4	5
Alpinia galanga	ME	100	100	100	100	97.5	89.9
	NE	100	100	97.1	82.6	65.2	53.9
Citrus grandis	ME	100	100	100	98.3	95.6	88.6
	NE	100	100	96.9	80.1	61.9	52.2
Citrus aurantifolia	ME	100	100	100	97.9	94.5	88.4
	NE	100	100	95.3	81.4	62.3	50.8
DEET	ME	100	100	100	100	100	95.7
	NE	100	100	100	100	95.3	87.9
Citronella	KAPS®	100	100	94.7	84.9	79.3	53.8
	MozAway®	100	100	95.5	83.2	70.6	54.9
	BioZ Natural®	100	100	94.1	66.4	61.7	51.1
Citriodiol®	Mosiguard®	100	100	100	100	97.3	88.5
Control		56.8 ± 12.8	97.4 ± 23.9	141.3 ± 46.1	212.5 ± 33.9	250.4 ± 41.7	287.5 ± 37.6

Table 4.17: Average mean (± SEM) number of mosquito bites on control and mean percentage of repellency of ME and NE formulations of EOs and DEET and the marketed topical plant-based repellent against mosquito in Kampung Pokok Asam, Sungai Petani, Kedah.

Active ingredient	Formulation	Percentage of repellency (%)					
		0	**1**	**2**	**3**	**4**	**5**
Alpinia galanga	ME	100	100	100	100	96.8	87.9
	NE	100	100	98.7	81.7	63.4	54.8
Citrus grandis	ME	100	100	100	98.6	94.0	85.1
	NE	100	100	97.7	80.9	62.6	52.5
Citrus aurantifolia	ME	100	100	100	97.3	90.9	84.7
	NE	100	100	95.1	79.4	60.7	51.9
DEET	ME	100	100	100	100	100	95.1
	NE	100	100	100	100	94.8	85.9
Citronella	KAPS®	100	100	95.5	86.4	77.5	52.9
	MozAway®	100	100	94.3	78.5	62.5	49.3
	BioZ Natural®	100	100	94.6	75.9	59.0	48.2
Citriodiol®	Mosiguard®	100	100	100	100	96.9	85.4
Control		77.7 ± 17.2	114.0 ± 31.5	160.6 ± 39.6	247.8 ± 27.9	252.9 ± 44.2	295.4 ± 40.2

Table 4.18: Average mean (± SEM) number of mosquito bites on control and mean percentage of repellency of ME and NE formulations of EOs and DEET and the marketed topical plant-based repellent against mosquito in Felda Purun, Bera, Pahang.

Active ingredient	Formulation	Percentage of repellency (%)					
		0	**1**	**2**	**3**	**4**	**5**
Alpinia galanga	ME	100	100	100	100	96.6	89.7
	NE	100	100	97.3	82.9	64.7	52.9
Citrus grandis	ME	100	100	100	98.0	92.2	86.2
	NE	100	100	96.3	80.7	62.6	49.7
Citrus aurantifolia	ME	100	100	100	97.7	90.6	83.6
	NE	100	100	95.2	79.6	60.4	48.9
DEET	ME	100	100	100	100	100	96.1
	NE	100	100	100	100	96.8	84.7
Citronella	KAPS®	100	100	96.5	81.8	68.9	55.2
	MozAway®	100	100	96	79.8	56.3	47.8
	BioZ Natural®	100	100	95.6	76.5	59.7	50.1
Citriodiol®	Mosiguard®	100	100	100	100	97.9	88.9
Control		69.9 ± 13.9	110.0 ± 39.2	166.2 ± 40.5	216.2 ± 34.4	294.8 ± 47.8	356.2 ± 41.1

4.8 PHYSICAL STABILITY OF THE FORMULATIONS

4.8.1 Centrifugation test

Centrifugation test performed on all the ME and NE EOs and DEET formulations stored at stated duration at 25^0C storage condition demonstrated absence of phase separation (Figure 4.20 and 4.21). At 40^0C storage condition however, all formulations presented phase separation after 6 and 12 months of storage (Figure 4.22 and 4.23).

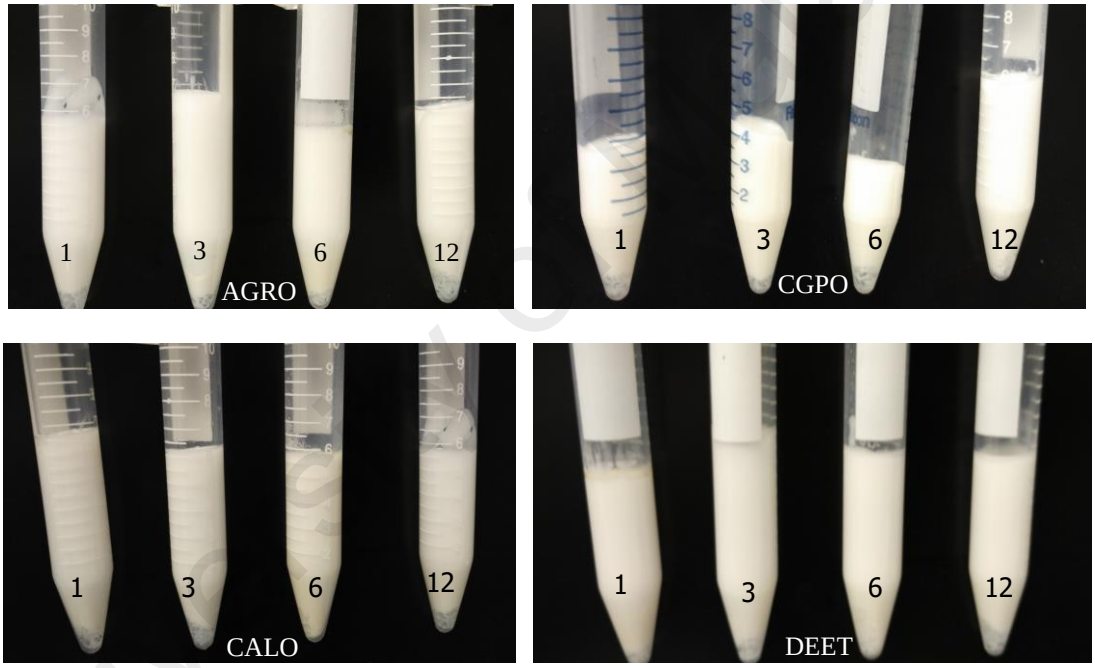


Figure 4.20: ME EOs and DEET formulation stored in 25^0C storage condition after 1, 3, 6 and 12 months showed absence of phase separation.

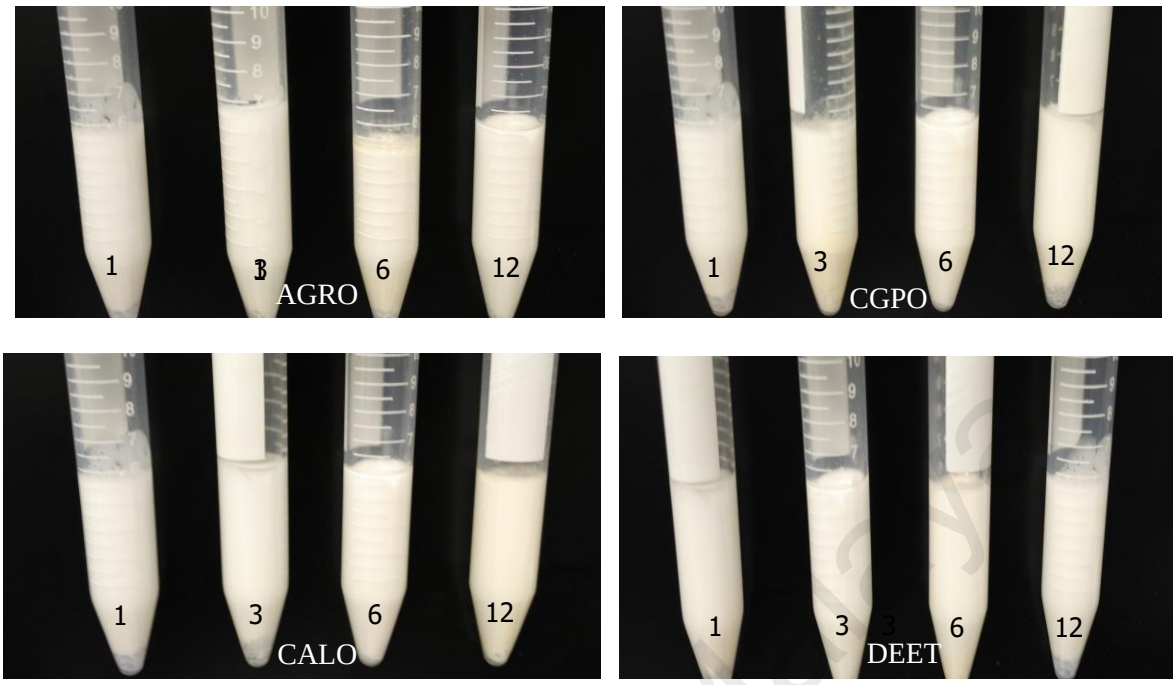


Figure 4.21: NE EOs and DEET formulations stored in 25^{0}C storage condition after 1, 3, 6 and 12 months showed absence of phase separation.

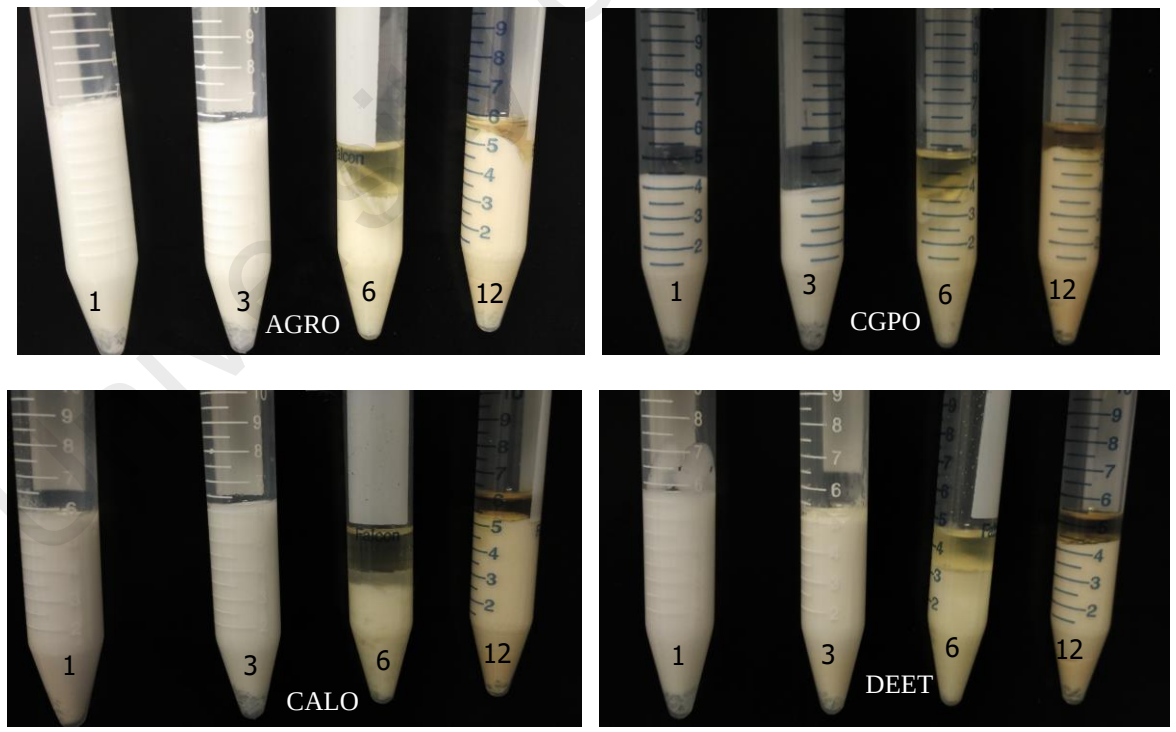


Figure 4.22: ME EOs and DEET formulation stored in 40^{0}C storage condition after 6 and 12 months showed presence of phase separation.

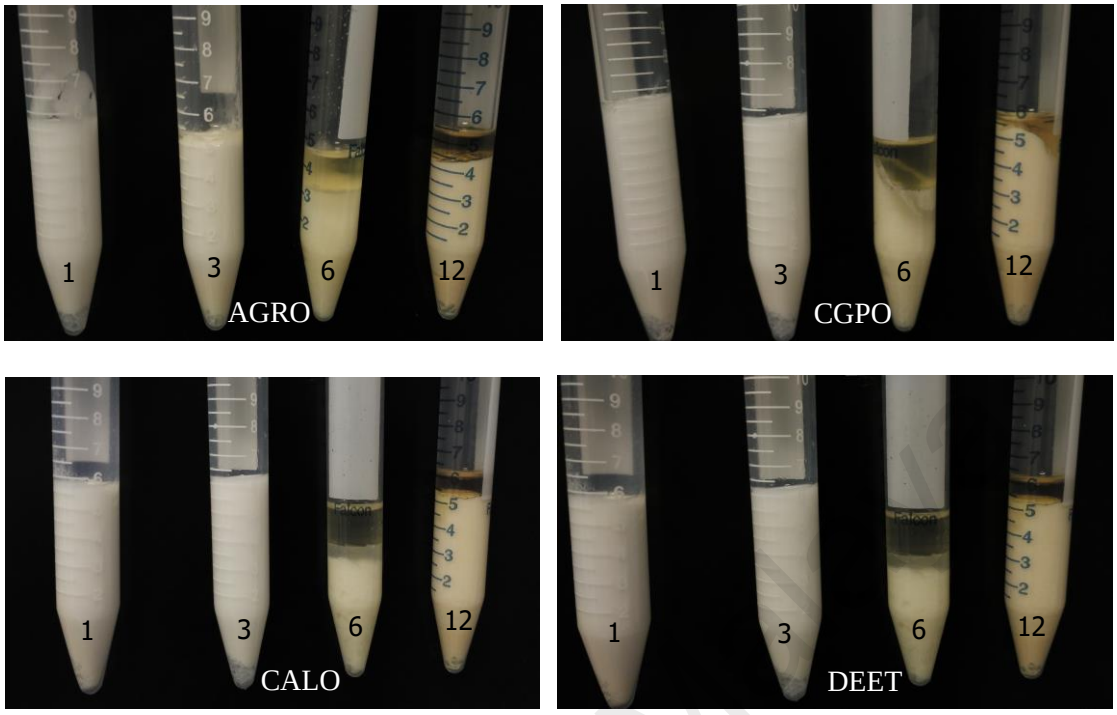


Figure 4.23: NE EOs and DEET formulation stored in 40^{0}C storage condition after 6 and 12 months showed presence of phase separation.

4.8.2 Organoleptic test

All ME formulations maintained their white color and intensity of color for 12 months of storage when stored at 25^{0}C storage conditions. Similarly, the physical appearance, smell, homogeneity, spread ability, and texture of all formulations remained the same until the end of the storage period. None of the formulations showed signs of physical or chemical instability in any of the containers (Figure 4.24). ME formulations stored at 40^{0}C storage condition however, showed slight changes in their color (turned yellowish), produced strong odor, presented phase separation and became very light in their texture after 6 months of storage. Twelve months of storage caused both formulations to turn slightly darker in color and released an irritating odor (Figure 4.25). Phase separation

was also observed, change in spread ability (very light) was noted, and an oily feeling upon application on the skin was also detected.

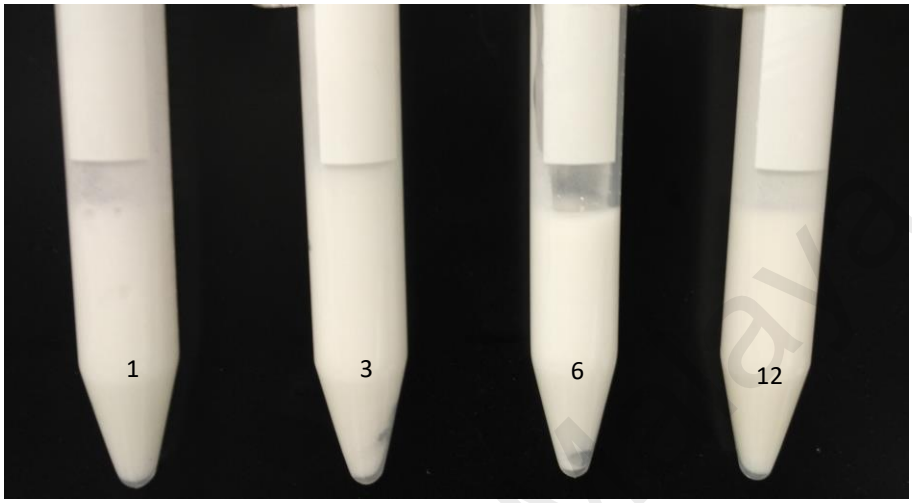


Figure 4.24: ME AGRO formulations stored at 25^{0}C storage conditions showed absence of change in appearance and phase separation after 1, 3, 6 and 12 months. Other ME EOs formulations also presented similar characteristics as AGRO.

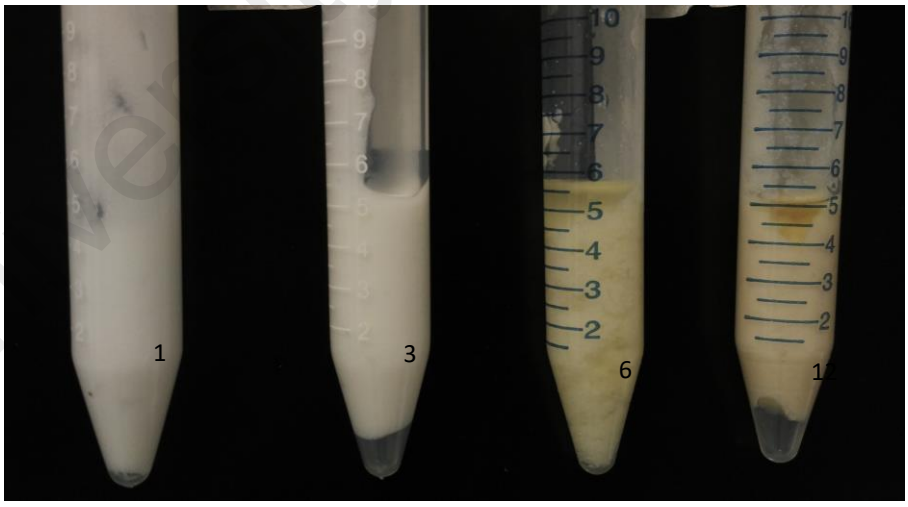


Figure 4.25: ME AGRO formulation stored at 40^{0}C storage conditions showed presence of change in appearance and phase separation after 6 and 12 months. Other ME EOs formulations also presented similar characteristics as AGRO.

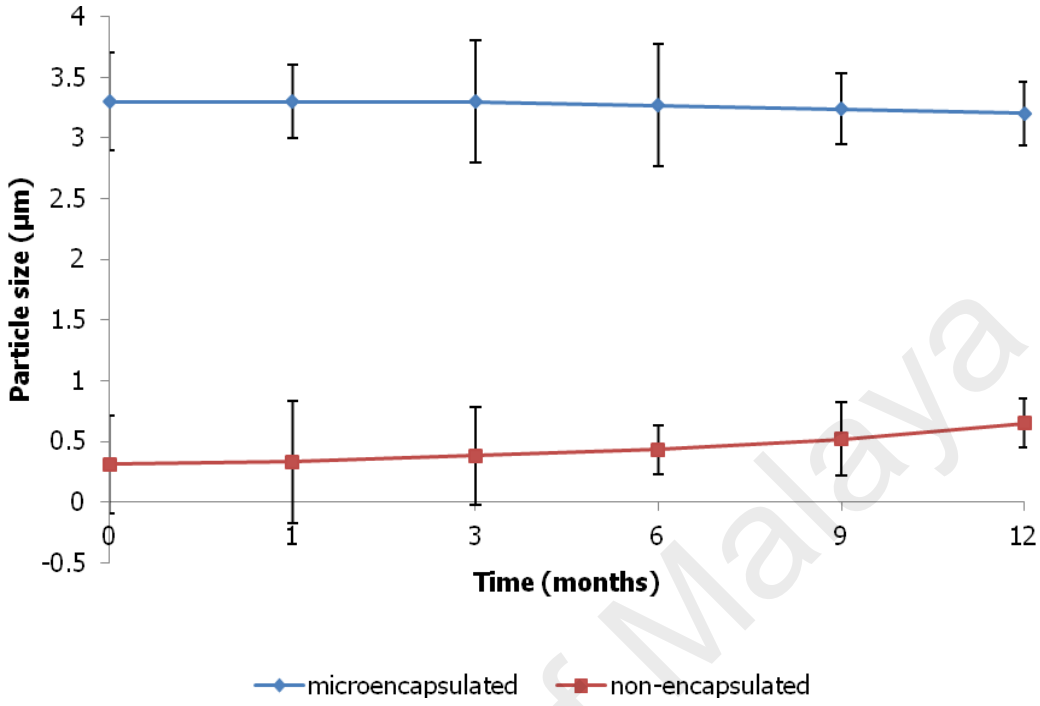
4.8.3 Particle size

The changes of particle sizes for ME and NE EOs and DEET formulations during 12 months of storage at different storage conditions ($25^0C \pm 2^0C/60\% \pm 5\%$ RH and $40^0C \pm 2^0C/70\% \pm 5\%$ RH) were shown in Figure 4.26- 4.29. The initial (right after the preparation) average particle size of ME formulations were 3.3 µm for AGRO, 3.29 µm for CGPO, 4.0 µm for CALO and 6.5 µm for DEET while for NE formulations, AGRO presented particle size of 0.31 µm, CGPO presented particle size of 0.21 µm, CALO presented particle size of 0.34 µm and DEET presented particle size of 0.53 µm.

At 25^0C storage condition, the particle sizes for ME formulations did not change much. In fact during the 1st three months of storage no change in particle size were observed. After three months, even though reduction in particle size were observed with AGRO showed 3%, CGPO 17.9%, CALO showed 7.5% and DEET showed 13.8% reduction respectively, statistically the difference is not significant ($p>0.05$). On the contrary, the average particle size for NE formulations showed an opposite trend. The particle size significantly increased in size as much as 52.3% for AGRO, 72% for CGPO, 52.1% for CALO and 33.8% for DEET for 12 months ($p<0.05$).

At 40^0C storage condition shown to cause significant reduction in particle size of ME formulations as much as 66.9% for AGRO, 68.7% for CGPO, 88.9% for CALO and 64.9% for DEET for 12 months of storage ($p<0.05$). Meanwhile, NE formulations presented significant enlargement in particle size with AGRO demonstrated an increase up to 89.5%, CGPO to 92.7%, CALO to 88.9% and DEET to 82.2% for 12 months ($p<0.05$).

a)



b)

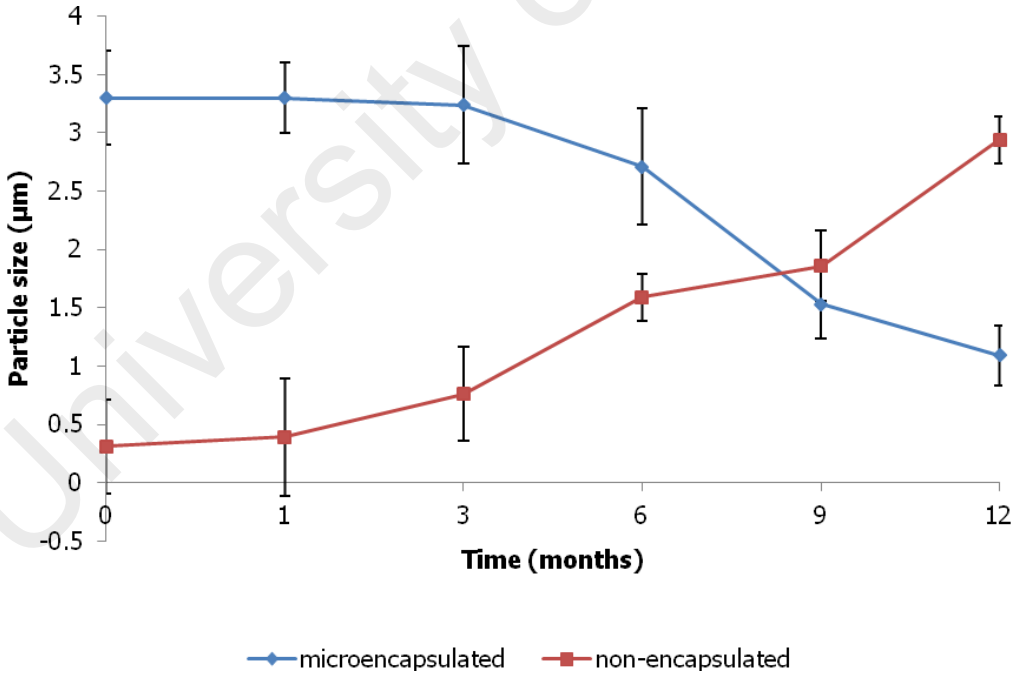
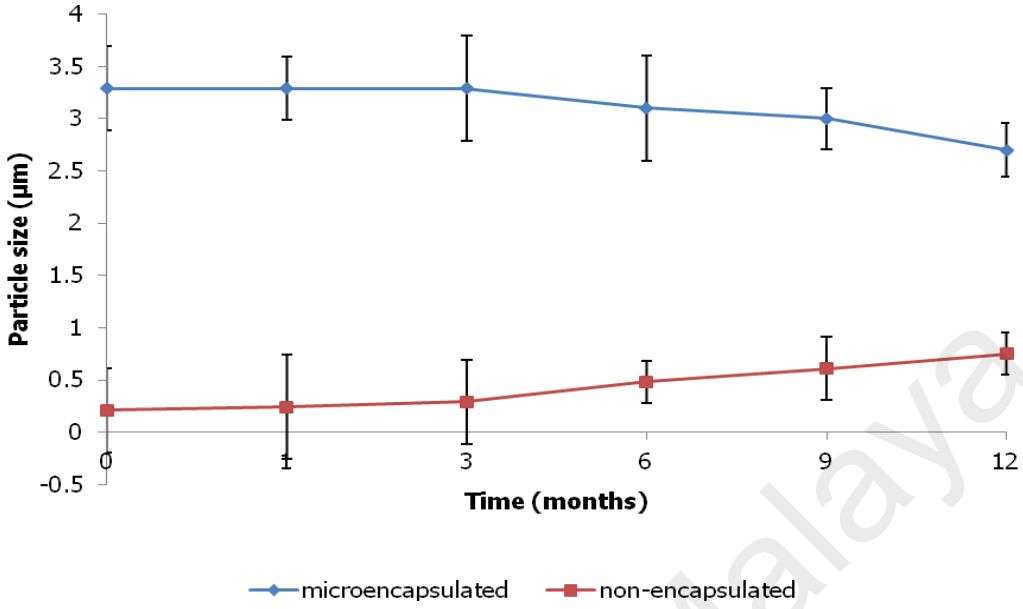


Figure 4.26: Particle size of ME and NE AGRO formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

a)



b)

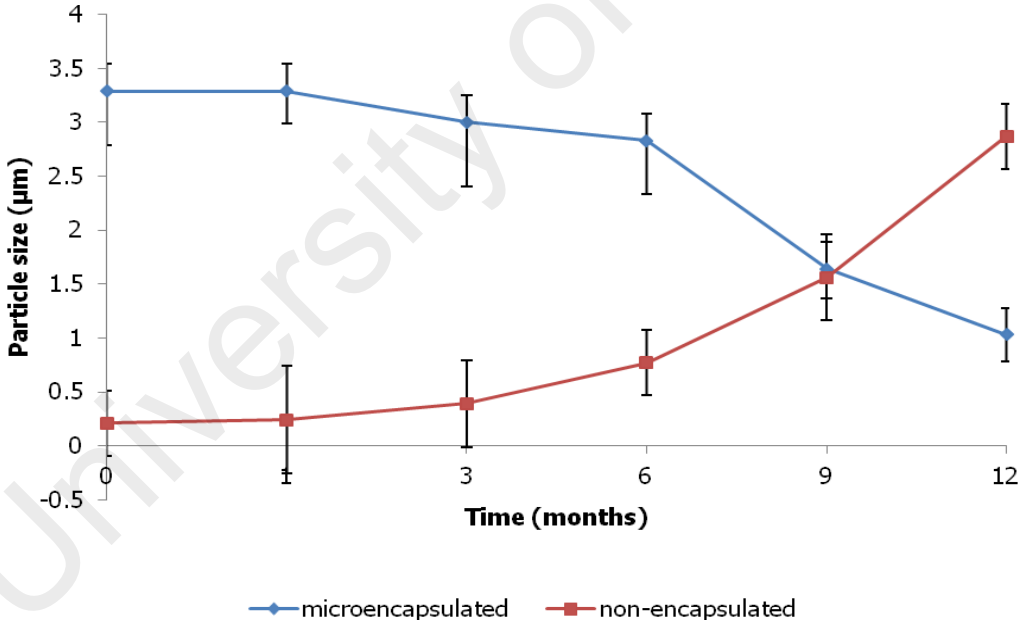
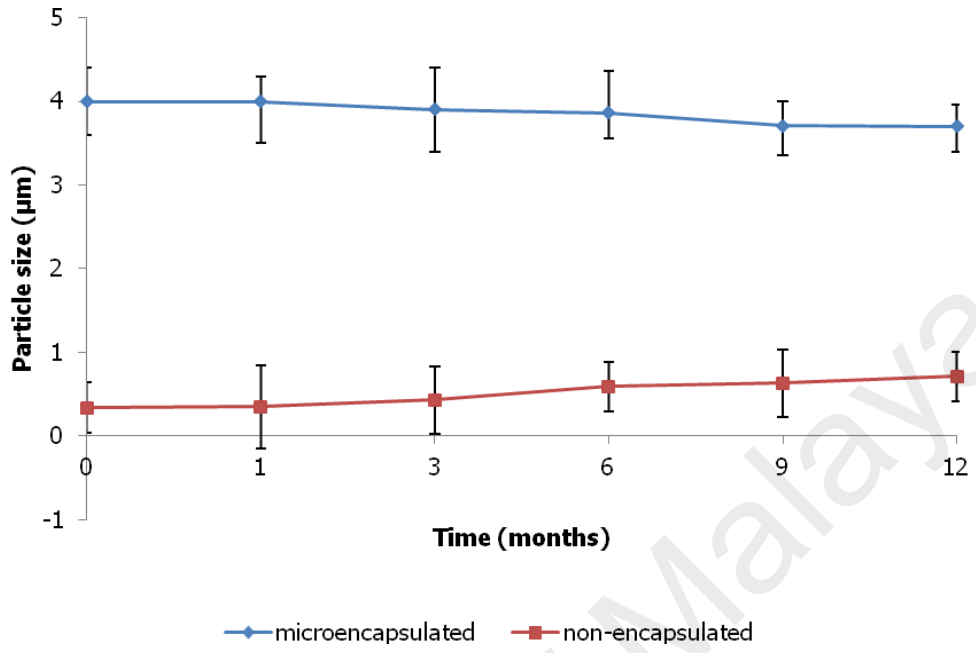


Figure 4.27: Particle size of ME and NE CGPO formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

a)



b)

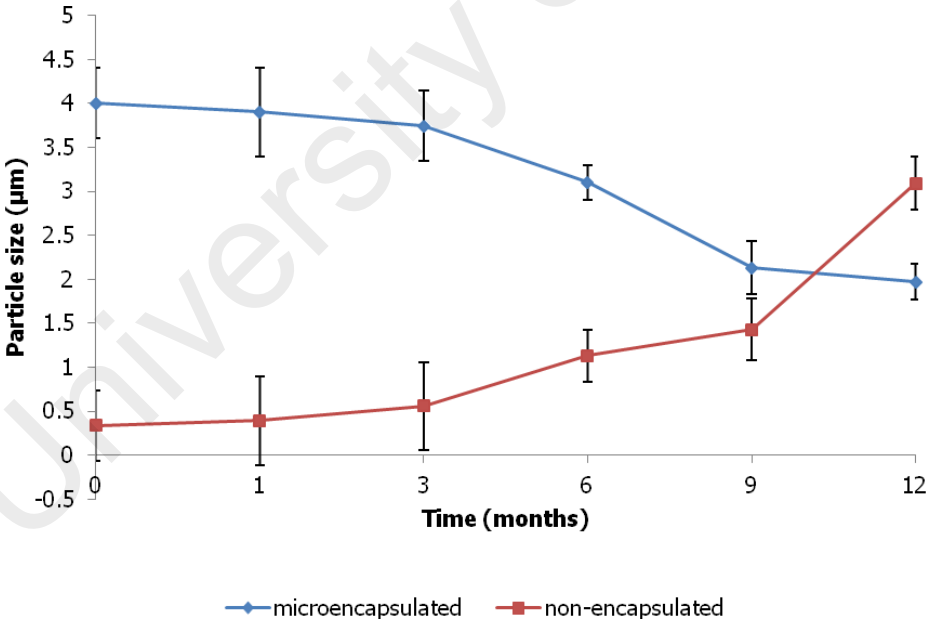
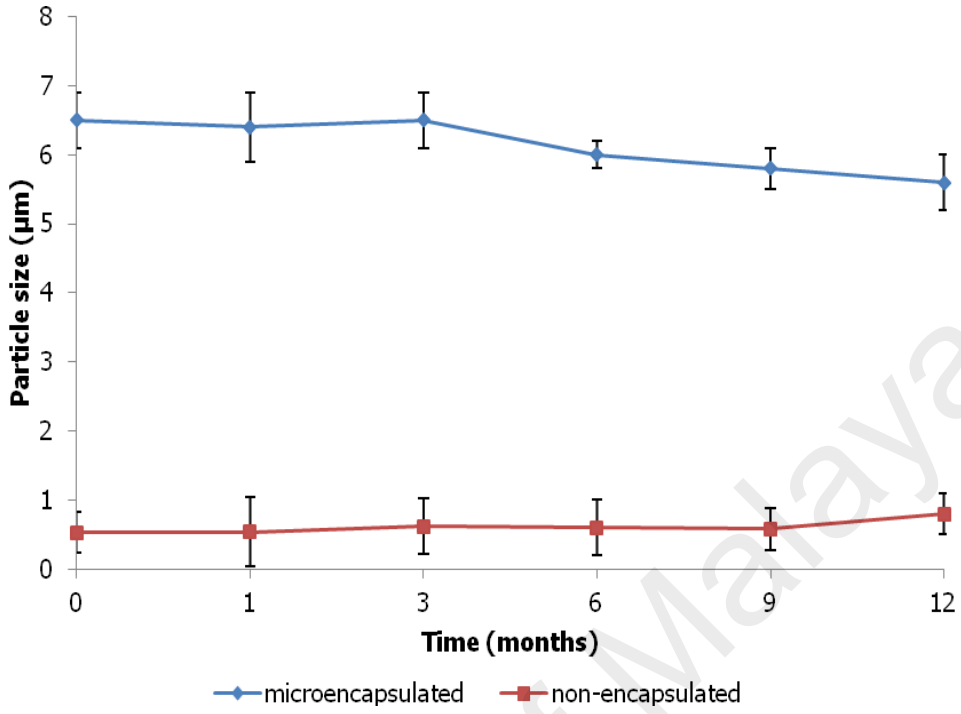


Figure 4.28: Particle size of ME and NE CALO formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

a)



b)

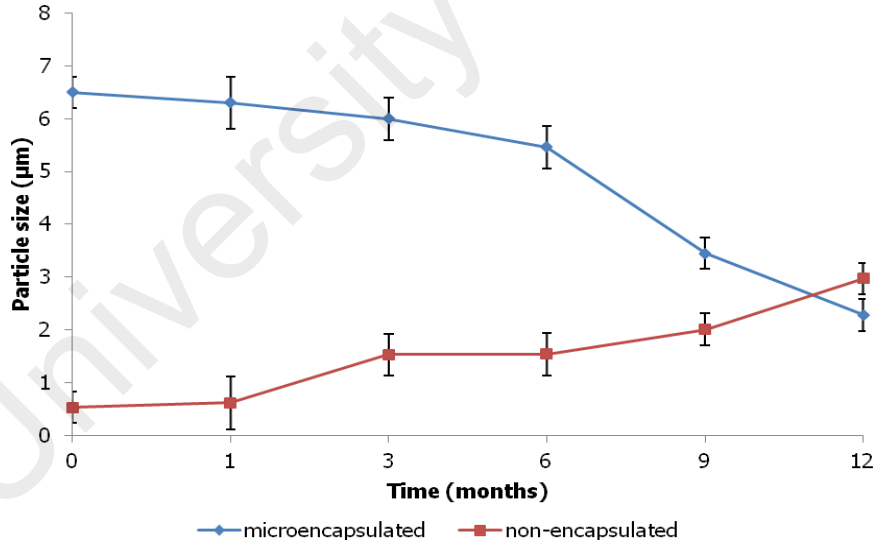


Figure 4.29: Particle size of ME and NE DEET formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

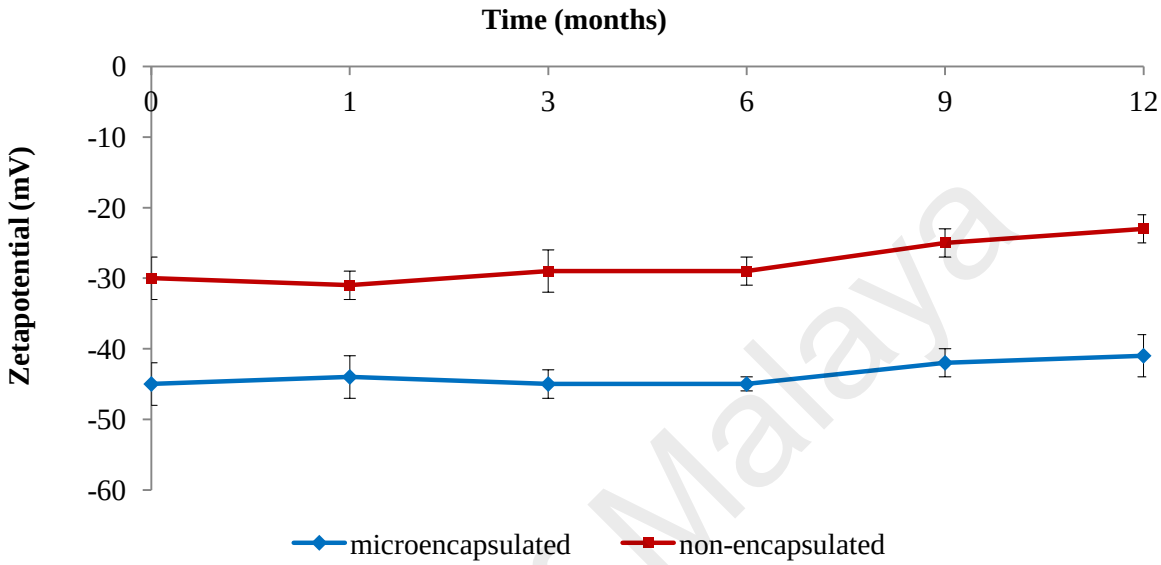
4.8.4 Zeta potential value

The changes in zeta potential values of ME and NE EOs and DEET formulations during 12 months of storage at $25^0C \pm 2^0C/60\% \pm 5\%$ RH and $40^0C \pm 2^0C/70\% \pm 5\%$ RH were shown in Figure 4.30 – 4.33. In general, almost similar pattern were observed for all the formulations with regards to zeta potential values. At 25^0C storage condition, the zeta potential values for ME formulations measured right after the preparation demonstrated high stability in AGRO (-45 mV), CGPO (-47.9 mV), CALO (-46.1 mV) and DEET (-43 mV) while for NE formulations, they presented moderate stability with AGRO showed -30 mV, CGPO -32.7 mV, CALO -31.9 mV and DEET -34.1 mV.

All formulations showed no significant changes in zeta potential values within 6 months of storage ($p>0.05$). After 6 months of storage ME formulations continue to present insignificant reduction in which AGRO presented only 9% reduction, 5.4% reduction for CGPO, 4.8% and 3.1% reduction in CALO and DEET, respectively. As for NE formulations however, significant reduction in zeta potential values was observed with AGRO showed 20% reduction, CGPO showed18.7% reduction, CALO showed 26.7% reduction and DEET showed 20.6% reduction. At the end of observation periods, ME formulations were seen to maintained high stability ranged between -40.7 mV and -44.1 mV while the NE formulations demonstrated slight dispersion ranged between -21.7 mV and -25.8 mV. At 40^0C storage condition the zeta potential values of both formulations shown to decreased significant within a month of storage ($p<0.05$) and continued to decrease with time where ME formulations presented percentage of reduction ranged

between 66.7% and 75.8% while NE formulations presented percentage reduction that ranged between 72.8% and 83.3% from the 0 to 12 months of storage time.

a)



b)

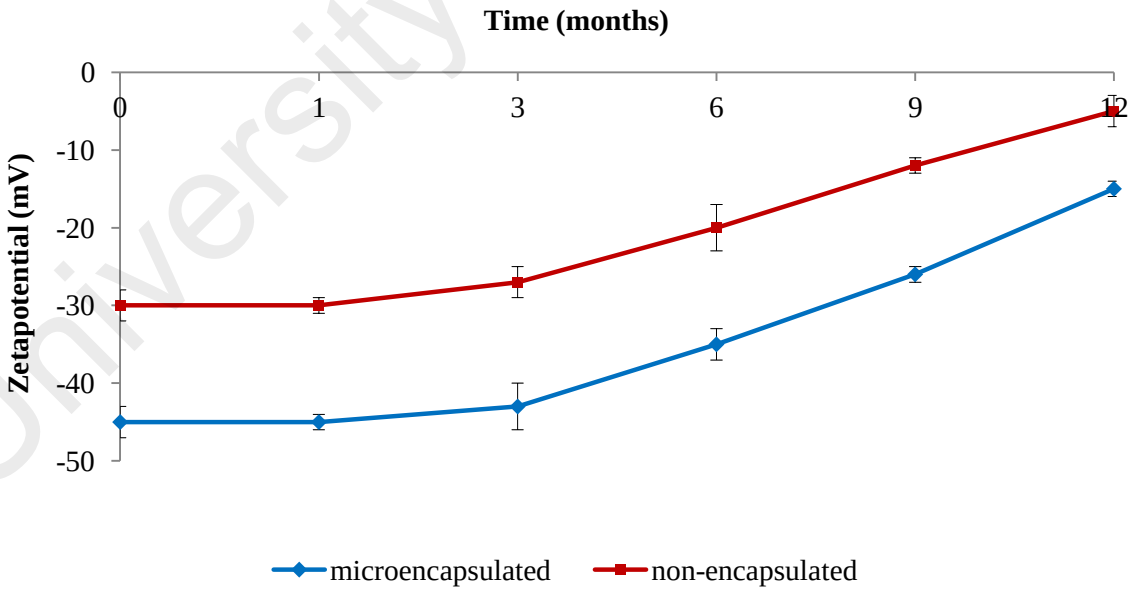
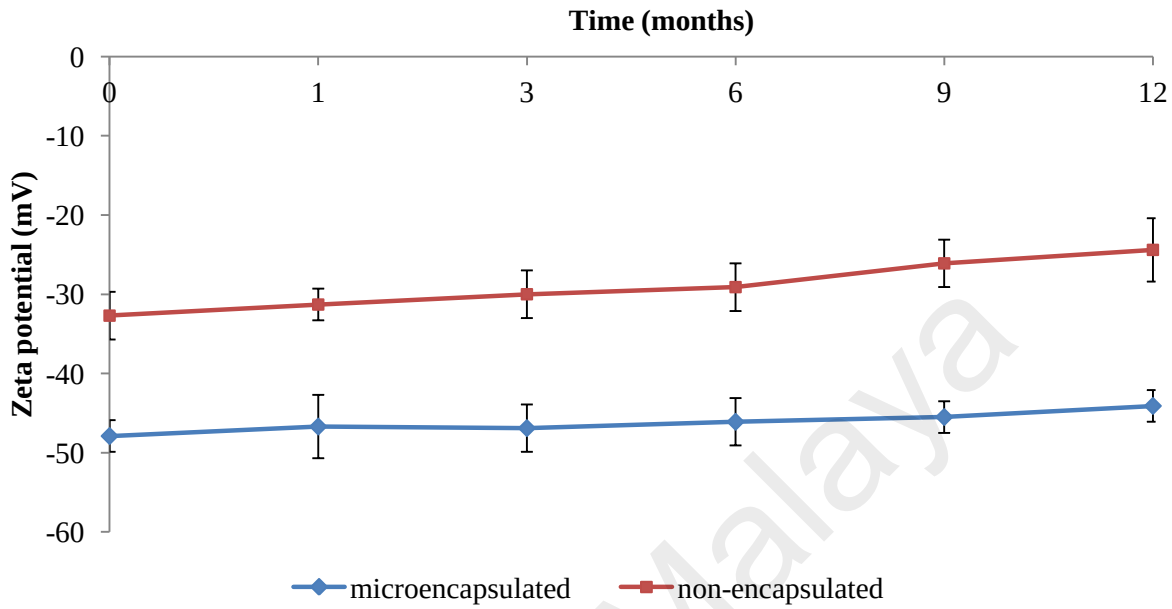


Figure 4.30: Zeta potential value of ME and NE AGRO formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

a)



b)

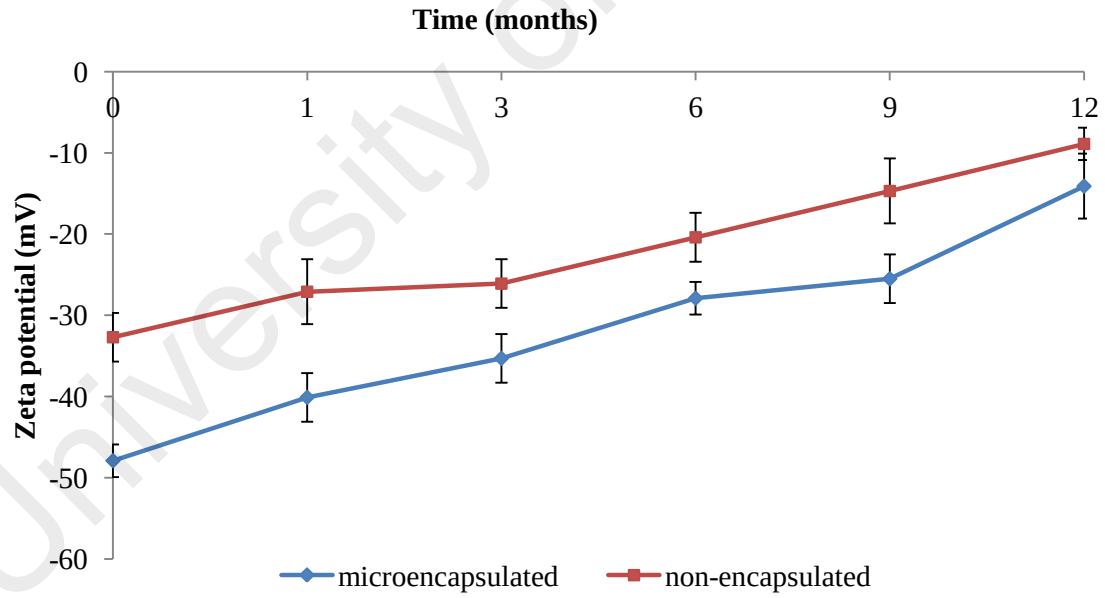
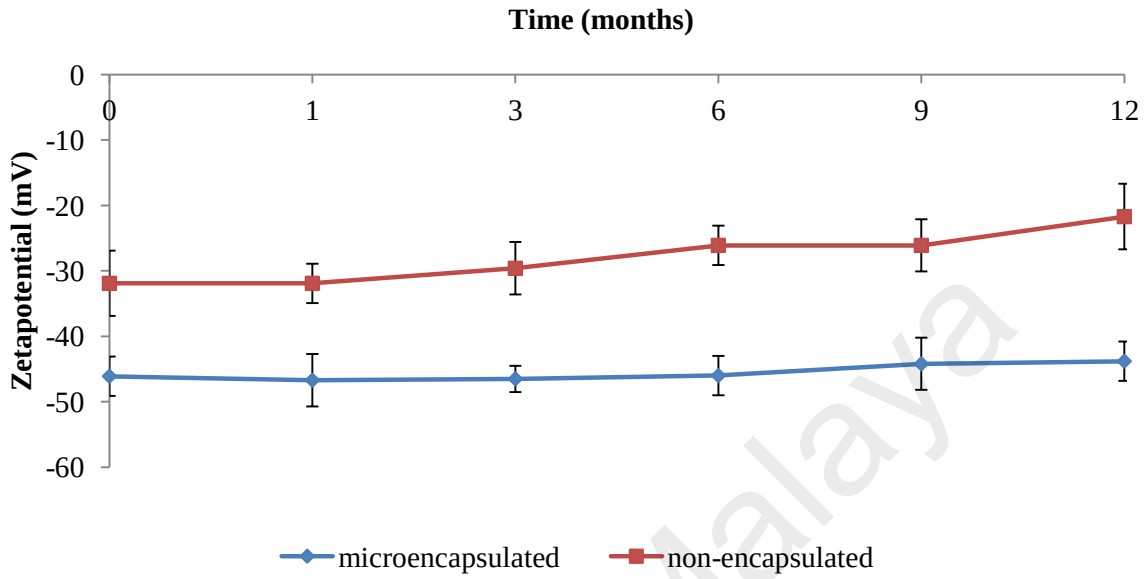


Figure 4.31: Zeta potential value of ME and NE CGPO formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

a)



b)

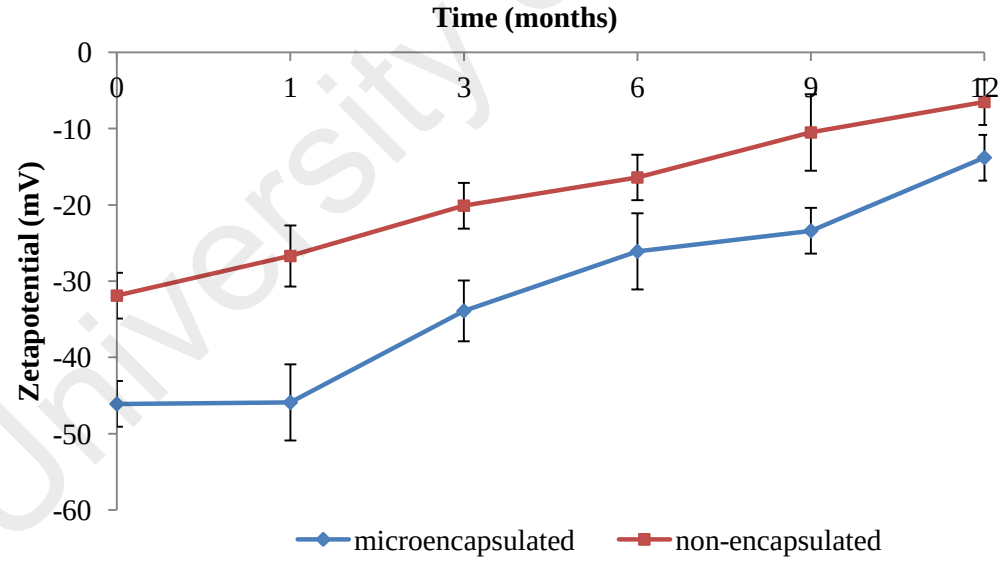
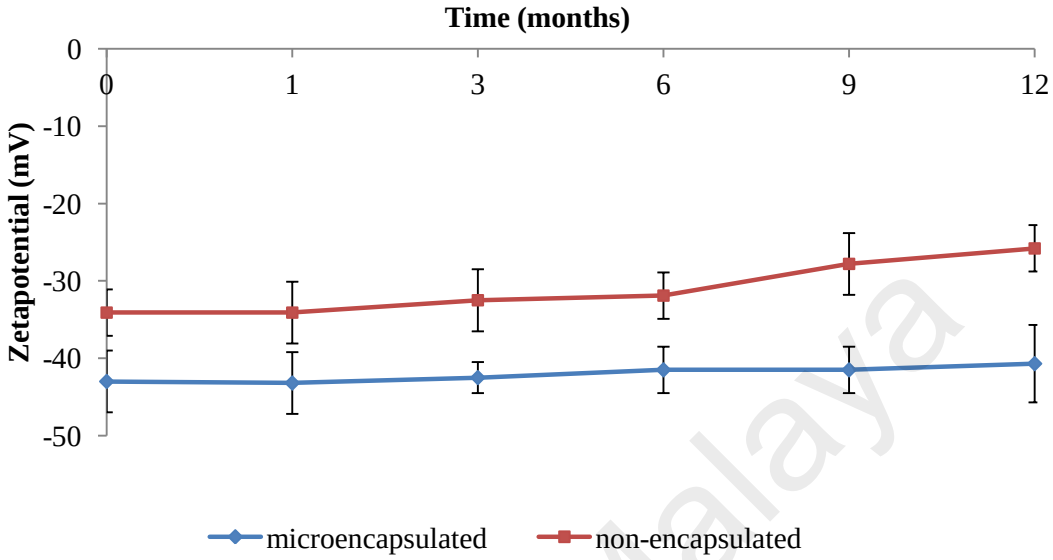


Figure 4.32: Zeta potential value of ME and NE CALO formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

a)



b)

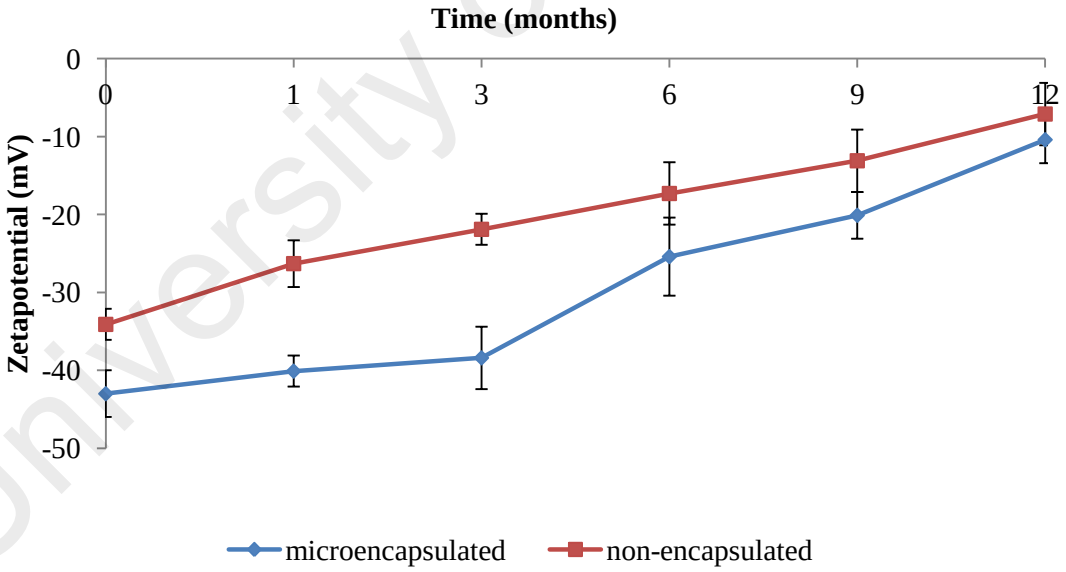


Figure 4.33: Zeta potential value of ME and NE DEET formulations stored at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) within 12 months of storage.

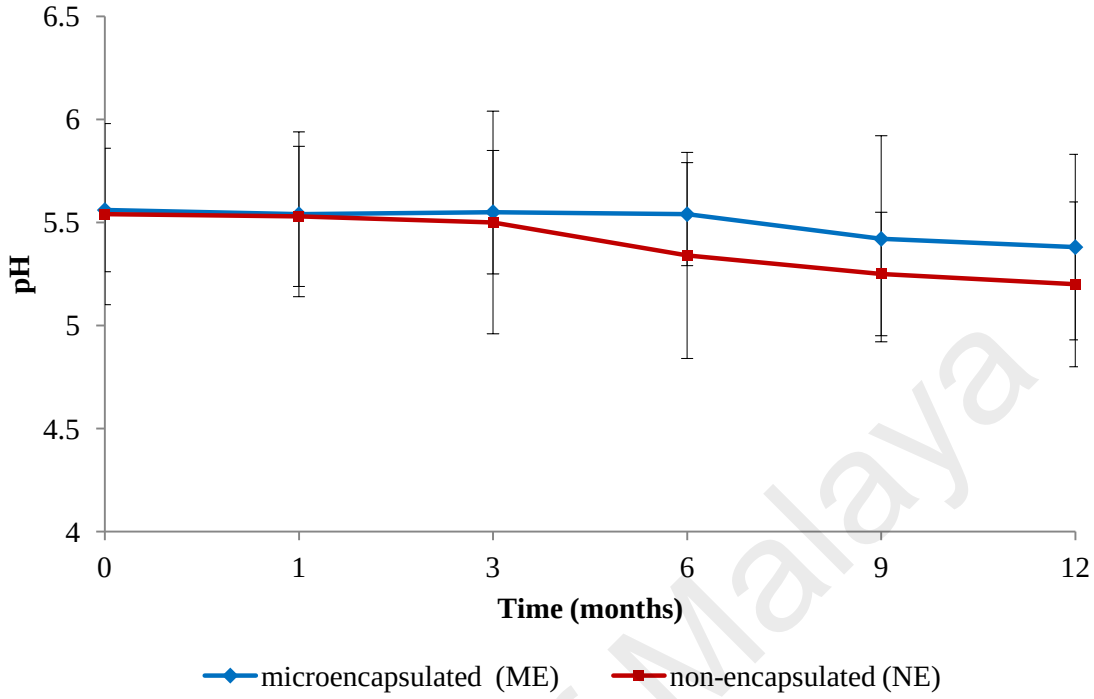
4.8.5 pH value

Figure 4.34 to 4.37 showed the changes in pH values of ME and NE EOs and DEET formulations over 12 months of storage at 25^0C ± 2^0C/60% ± 5% RH and 40^0C ± 2^0C/70% ± 5% RH storage conditions. Right after the preparations, pH values for ME formulations of AGRO were recorded as 5.54, 5.56 for CGPO, 5.5 for CALO and 5.53 for DEET while pH values for NE formulations was 5.55 for AGRO, 5.54 for CGPO, 5.42 for CALO and 5.50 for DEET.

Based on all figures, almost similar pattern were observed with regards to pH value for allformulations kept at 25^0C storage condition. Storage duration of more than 6 months shown to cause slight changes (insignificant) in pH values for ME formulations ($p>0.05$). ME formulations of AGRO and CGPO showed 2.8% reduction, CALO presented 1.3% reduction and DEET presented 3% reduction. As for NE formulations, the reduction in pH value shown to occur faster (after 3 months of storage) and slightly more than percentage shown by ME formulations (AGRO presented 5% reduction, CGPO showed 4.5% reduction, CALO with 2.7% reduction and DEET presented 5.3% reduction). At the end of observation period, all the ME and NE formulations demonstrated pH values that range between 5.0-5.5 which was still suitable for topical application.

At 40^0C storage condition, a significant reduction in pH values occurred right after a month of storage for both ME and NE formulations ($p<0.05$). At the end of observation periods, all the formulations demonstrated pH values which were less than 5.00.

a)



b)

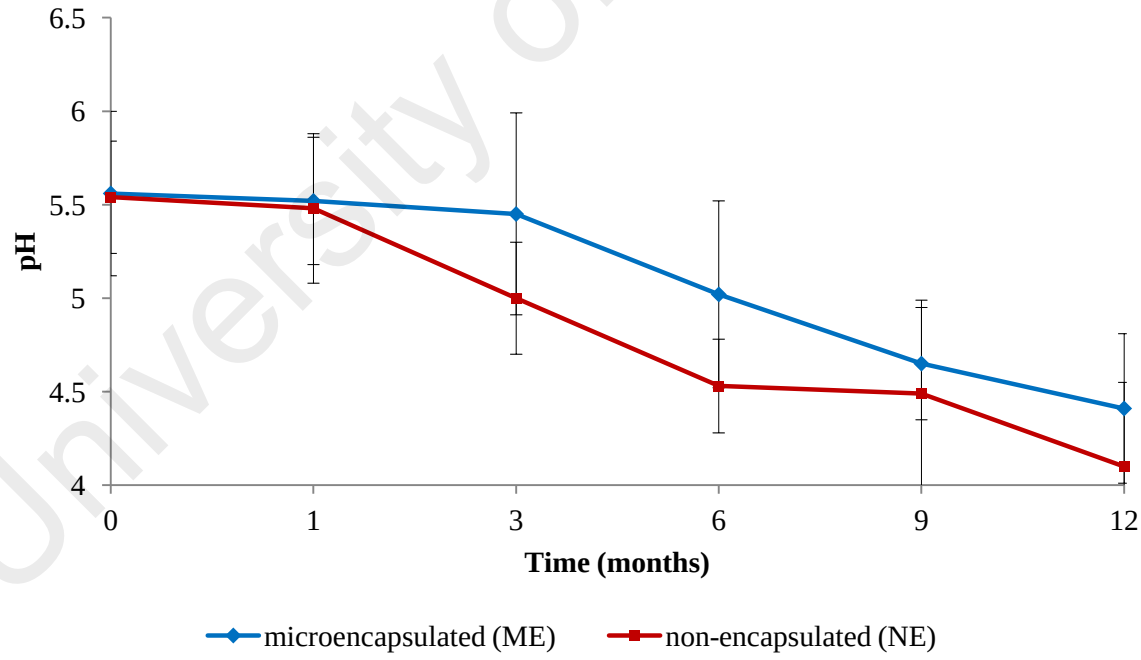
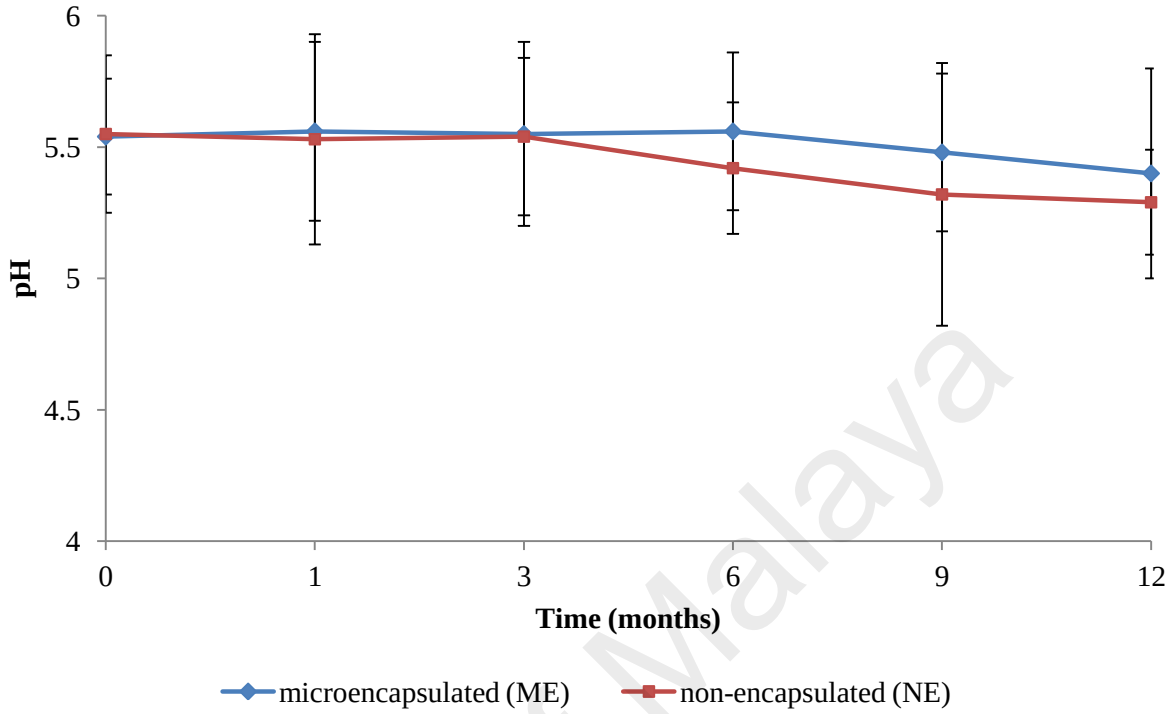


Figure 4.34: pH values of ME and NE AGRO formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

a)



b)

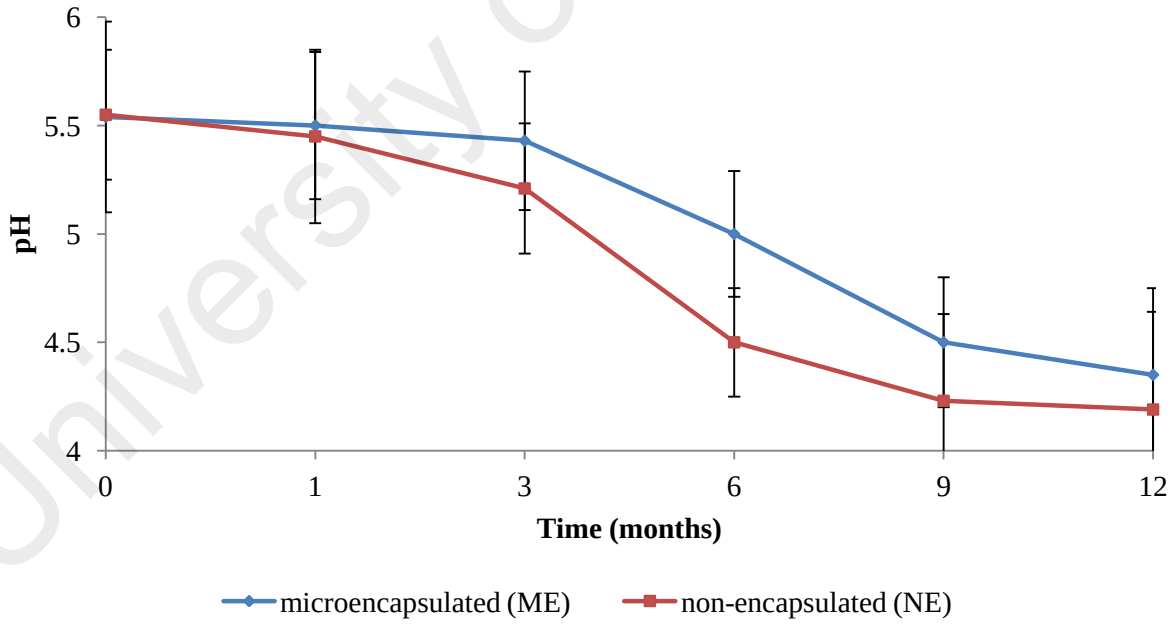
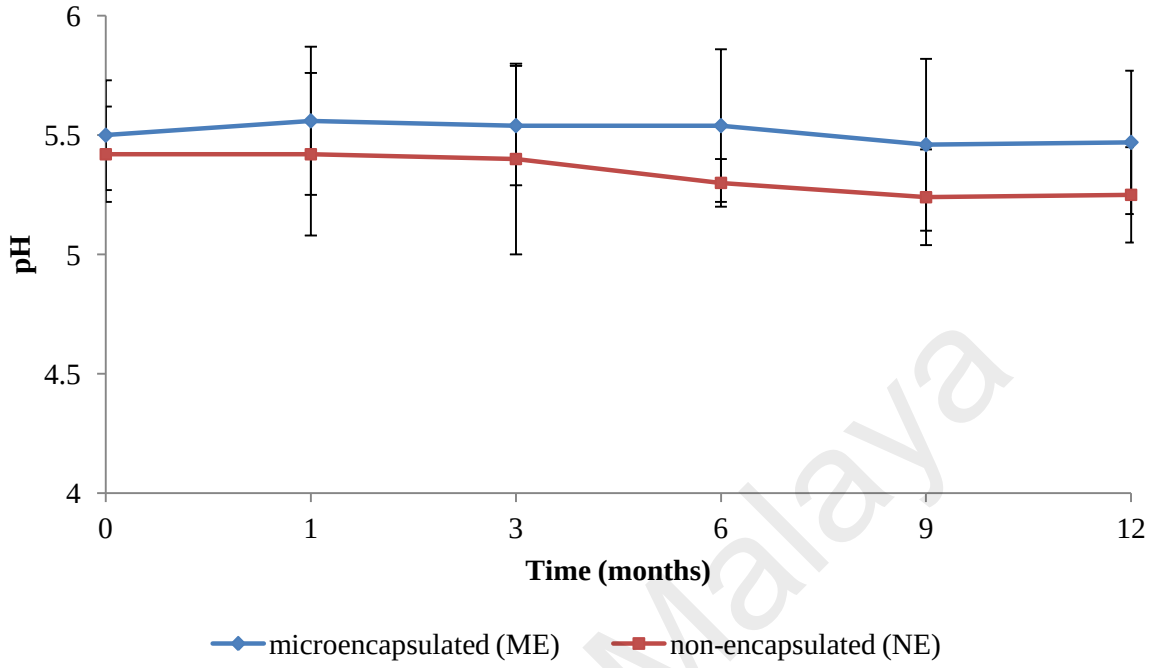


Figure 4.35: pH values of ME and NE CGPO formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

a)



b)

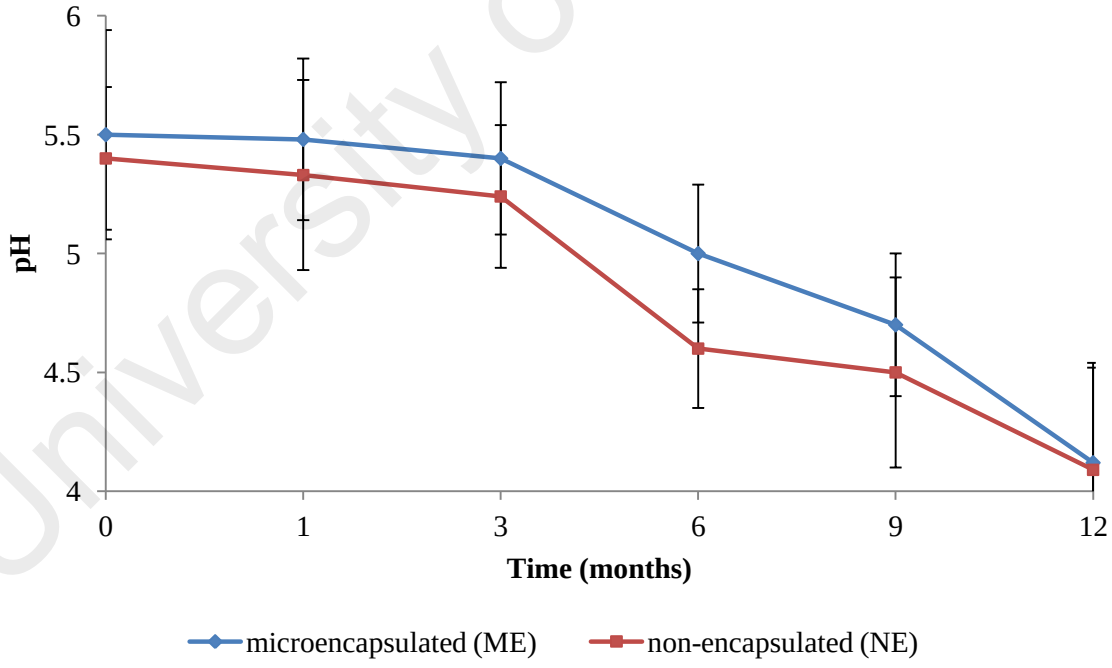
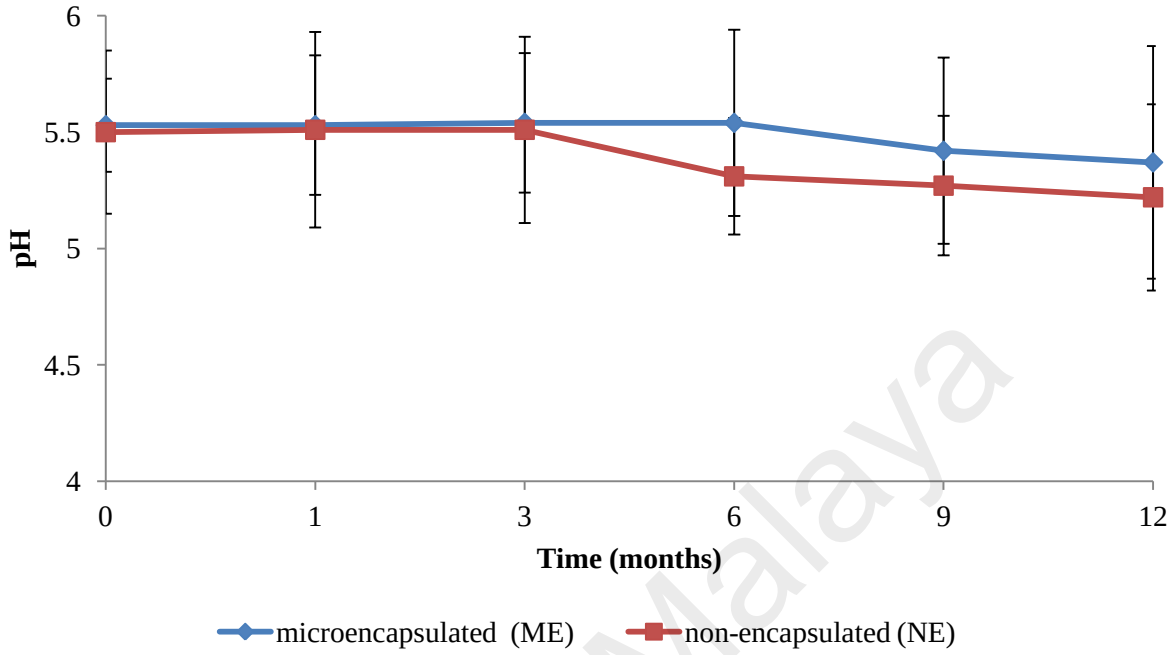


Figure 4.36: pH values of ME and NE CALO formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

a)



b)

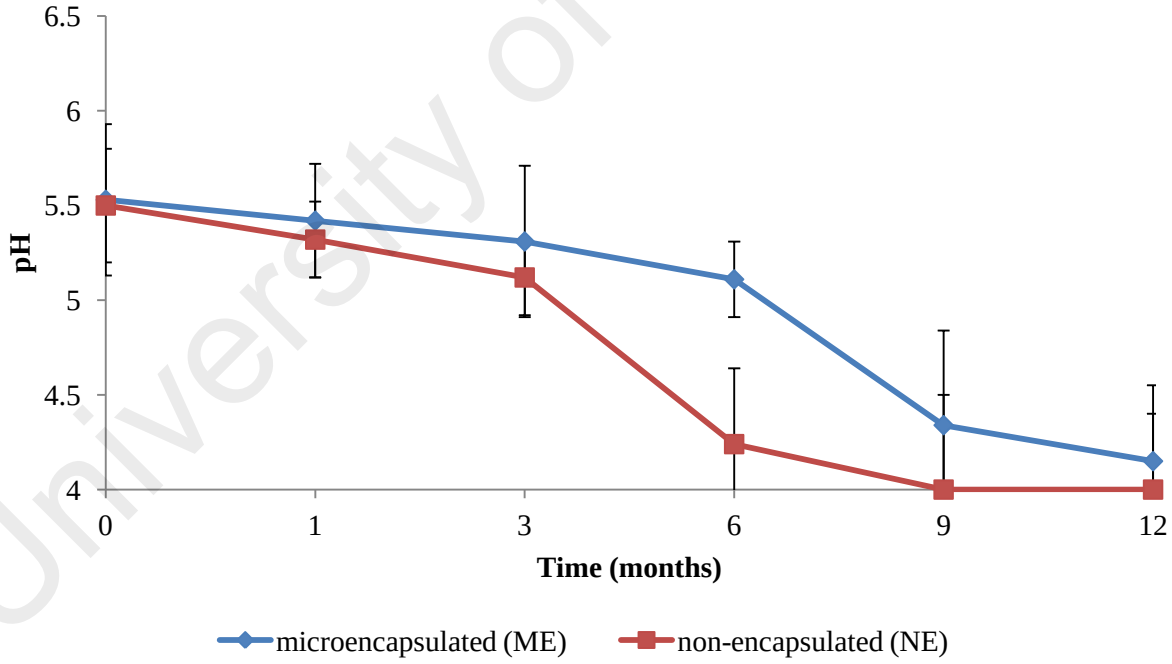


Figure 4.37: pH values of ME and NE DEET formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

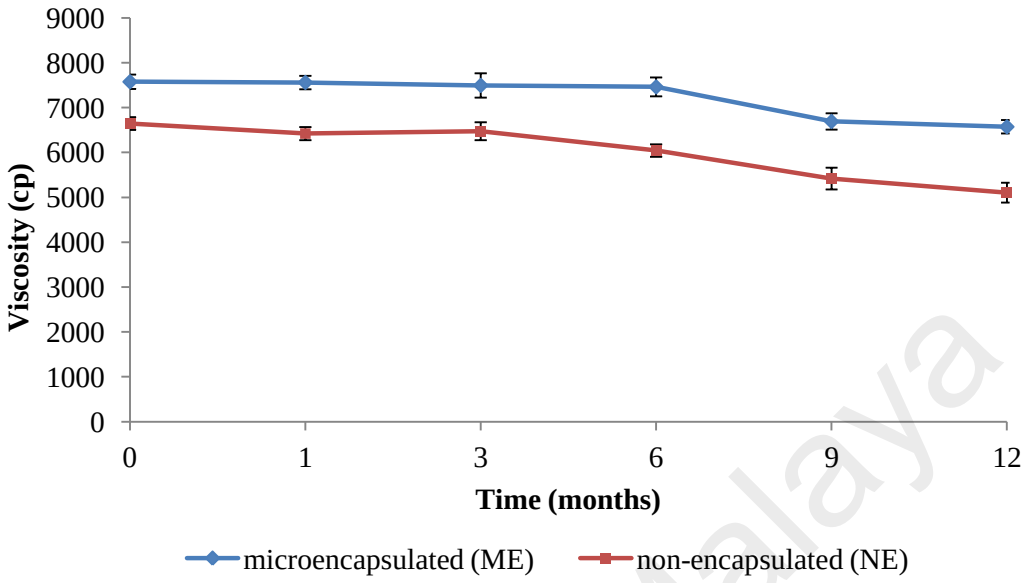
4.8.6 Viscosity

Figure 4.38 to 4.41 showed the changes in viscosity of ME and NE EOs and DEET formulations over 12 months of storage at 25^0C ± 2^0C/60% ± 5% RH and 40^0C ± 2^0C/70% ± 5% RH storage conditions. Right after the preparation, ME formulations demonstrated viscosity values of 7575 cp for AGRO, 7581 cp for CGPO, 6852 cp for CALO and 7724 cp for DEET. As for NE formulations, lower of viscosity values were presented slightly with 6645 cp for AGRO, 6778 cp for CGPO, 6234 cp for CALO and 6432 cp for DEET.

Based on all figures, almost similar trends were observed with regards to viscosity for all formulations. At 25^0C storage condition, both ME and NE formulations presented no significant changes within 6 months of storage time ($p>0.05$). After 6 months of storage, ME formulation continued to present insignificant reduction in viscosity where AGRO showed 11.9% reduction, CGPO showed 8.5% reduction, CALO presented 10.9% reduction and DEET presented 4.4% reduction ($p>0.05$). In contrast NE formulation presented larger reduction in viscosity values with AGRO showed 15.5%, CGPO with 21.8% reduction, CALO showed 18.9% reduction and DEET having 5.1% reduction.

At 40^0C storage condition, a significant reduction in viscosity occurred right after a month of storage for both formulations. ME formulations presented reduction between 64% to 71.2% while NE formulations presented reduction in viscosity between 70.5% to 83.2%.

a)



b)

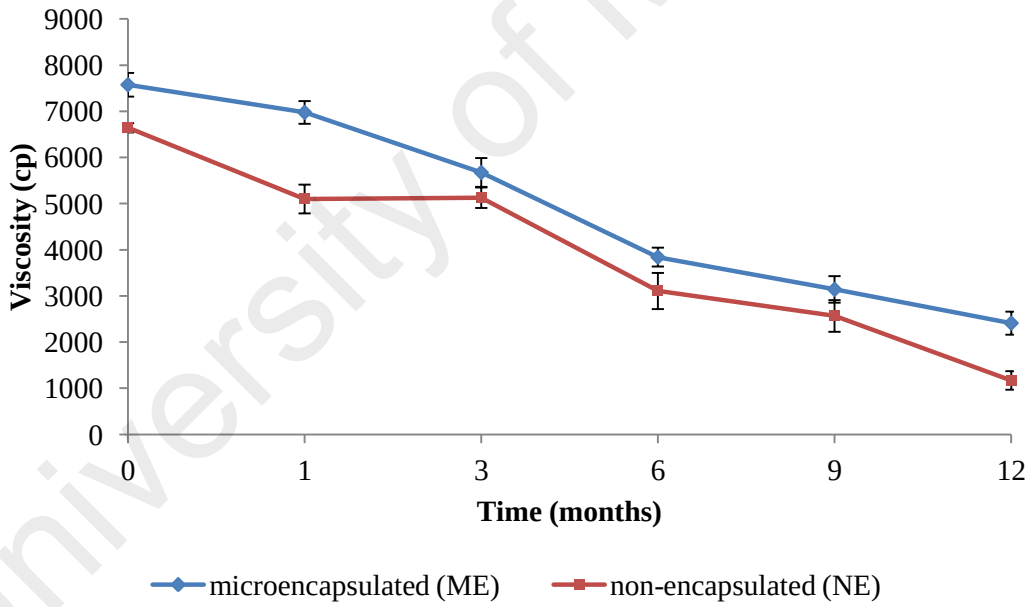
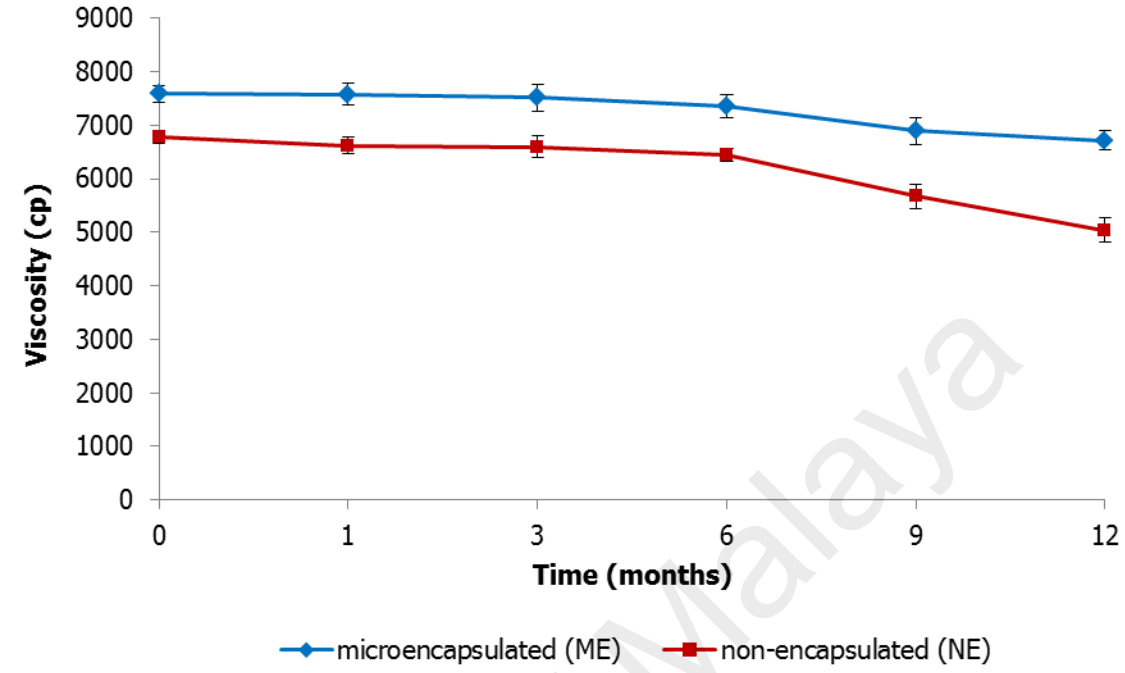


Figure 4.38: Viscosity of ME and NE AGRO formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

a)



b)

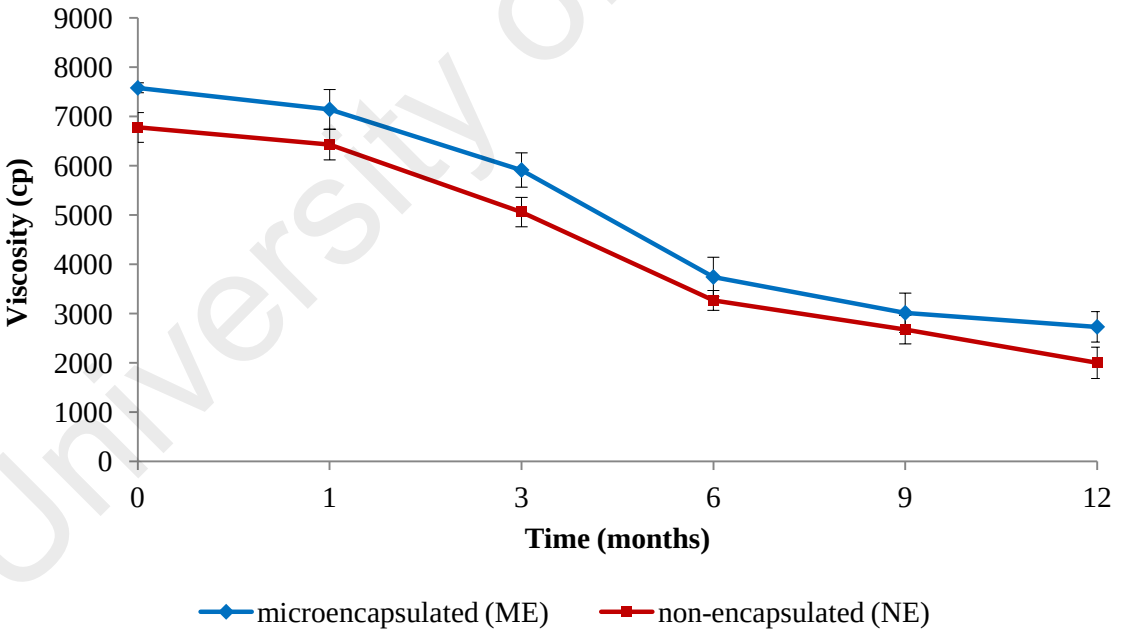
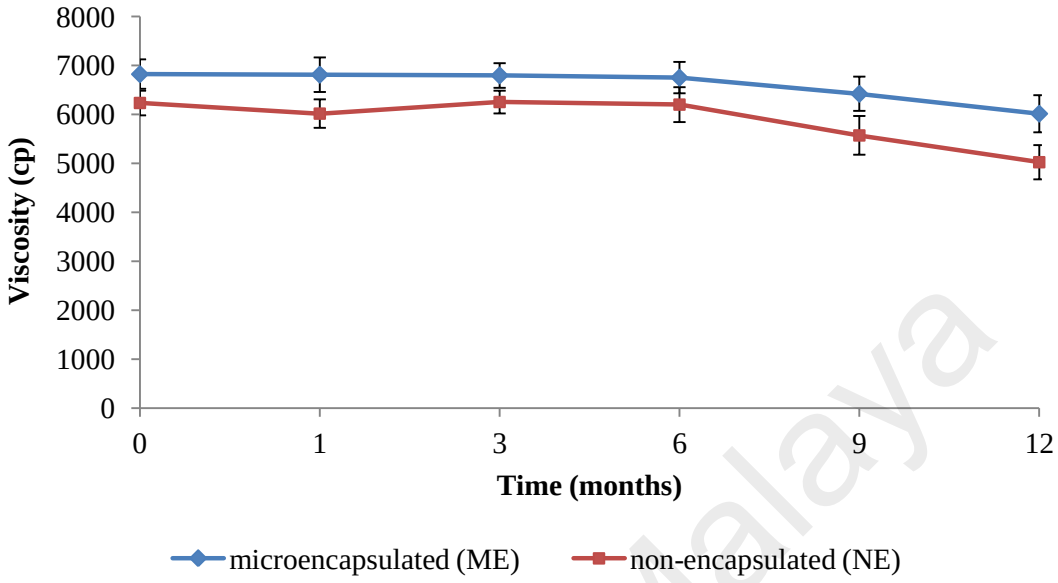


Figure 4.39: Viscosity of ME and NE CGPO formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

a)



b)

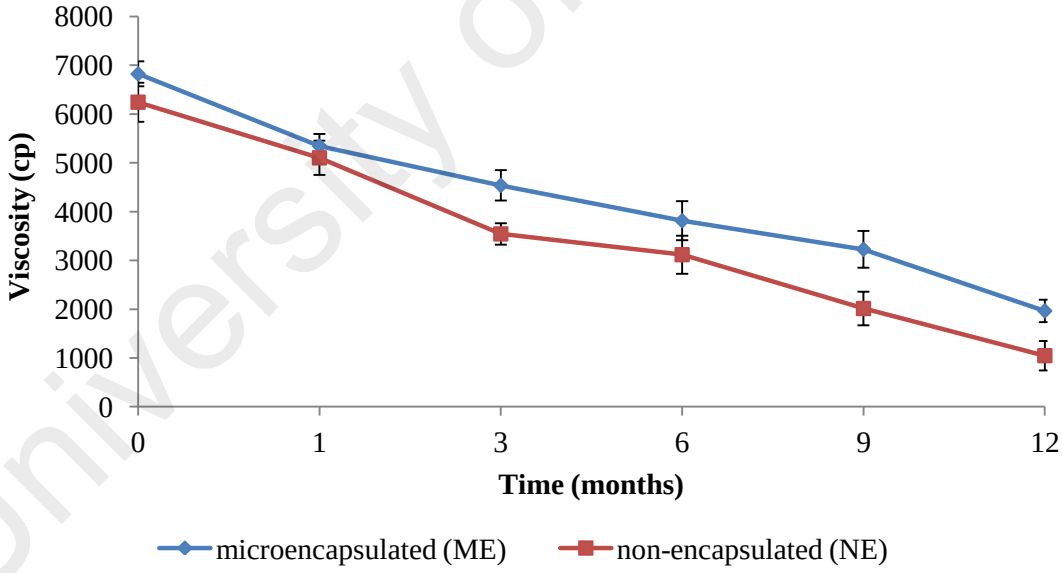
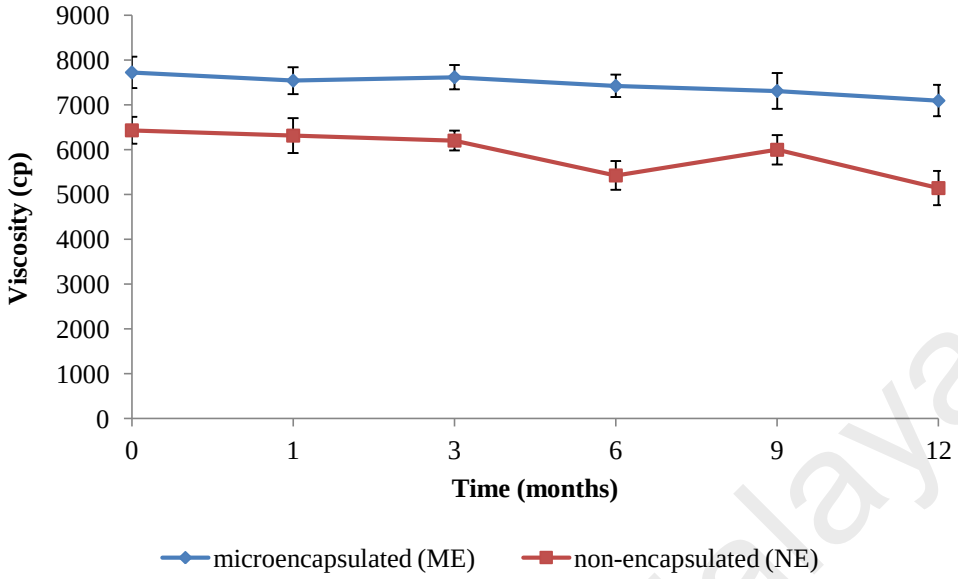


Figure 4.40: Viscosity of CALO ME and NE formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

a)



b)

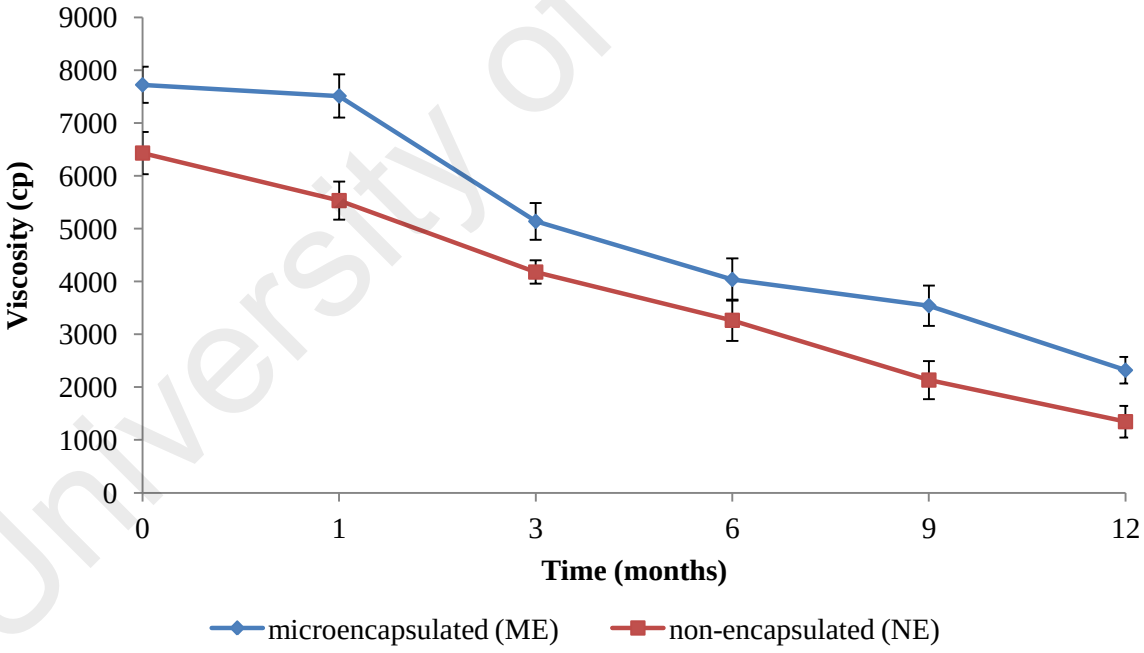


Figure 4.41: Viscosity of ME and NE DEET formulations over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) and 40^{0}C ± 2^{0}C/70% ± 5% RH (b) storage conditions.

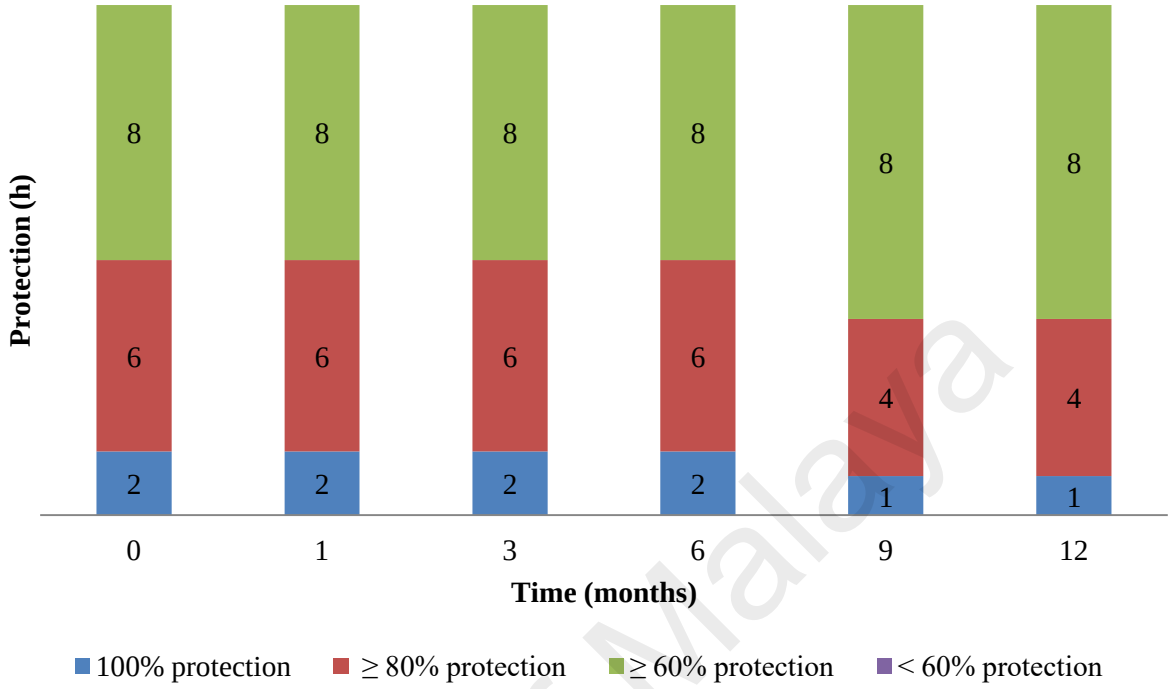
4.8.7 Efficacy during storage

Based on results of the stability study, it is quite obvious that the 25^0C storage condition provided the suitable condition where it allows stability of the formulations to be maintained for a certain period of time (up to 6 months). Thus, formulations stored in this condition were then tested for their repellent effects to ensure that effectiveness of the formulation is also maintained during storage. Figure 4.42 – 4.44 show the changes in the protection time of ME and NE EOs formulations over 12 months of storage. Based on all figures, all the formulations presented almost similar pattern with regards to the efficacy level when stored over 12 months of storage time.

All the ME and NE EOs formulations presented similar efficacy level when tested right after the preparation by demonstrating complete protection (100%) up to 2 hours, ≥80% protection at 6 hours and ≥60% protection at 8 hours post application. This efficacy level was maintained by all ME EOs formulation stored up to 6 months of storage time. As for NE formulations, reduction in protection time was observed by formulation stored up to 3 months of storage time where complete protection time was reduced to 1 hour, ≥80% protection only at 2 hours and <60% protection at 8 hours post-application. For ME formulation stored up to 9 months of storage time, the complete protection was shown reduced to 1 hour post application while complete protection was not observed for the NE formulation stored at ≥ 6 months of storage time. As for ME and NE DEET formulations, both formulations showed similar efficacy level right after the preparation by demonstrating complete protection (100%) up to 4 hours and ≥80% at 8 hours post-application (Figure 4.45). The ME formulation shown to maintained this efficacy level

when kept up to 12 months of storage time while NE formulation only maintained the same level of efficacy up to 3 months of storage time.

a)



b)

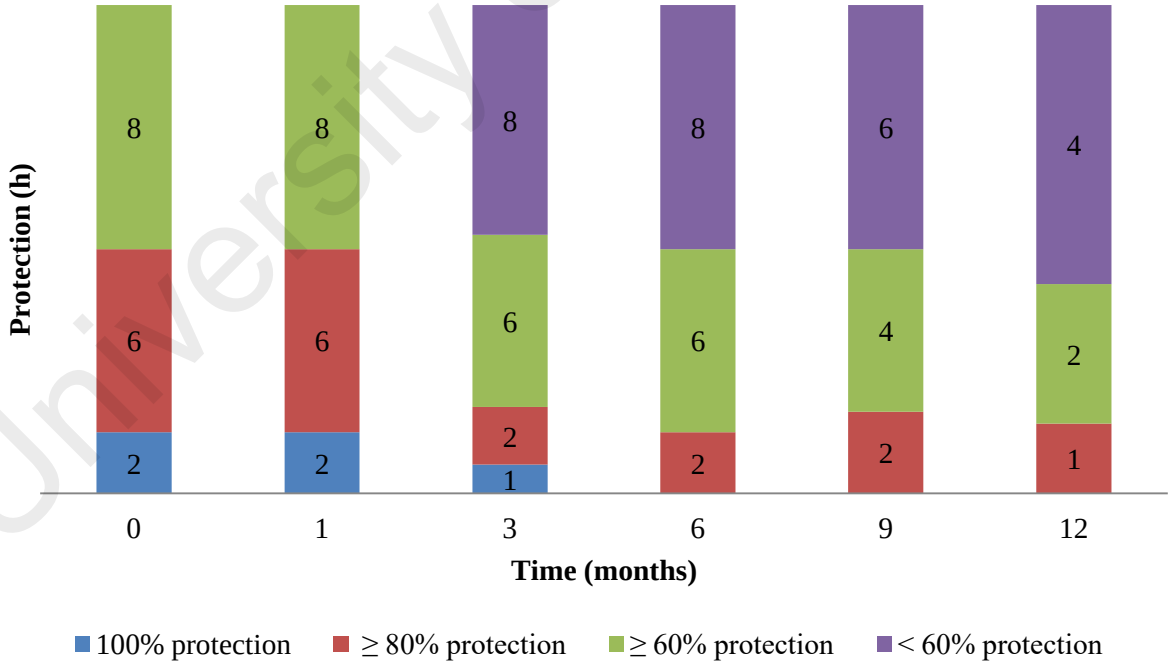
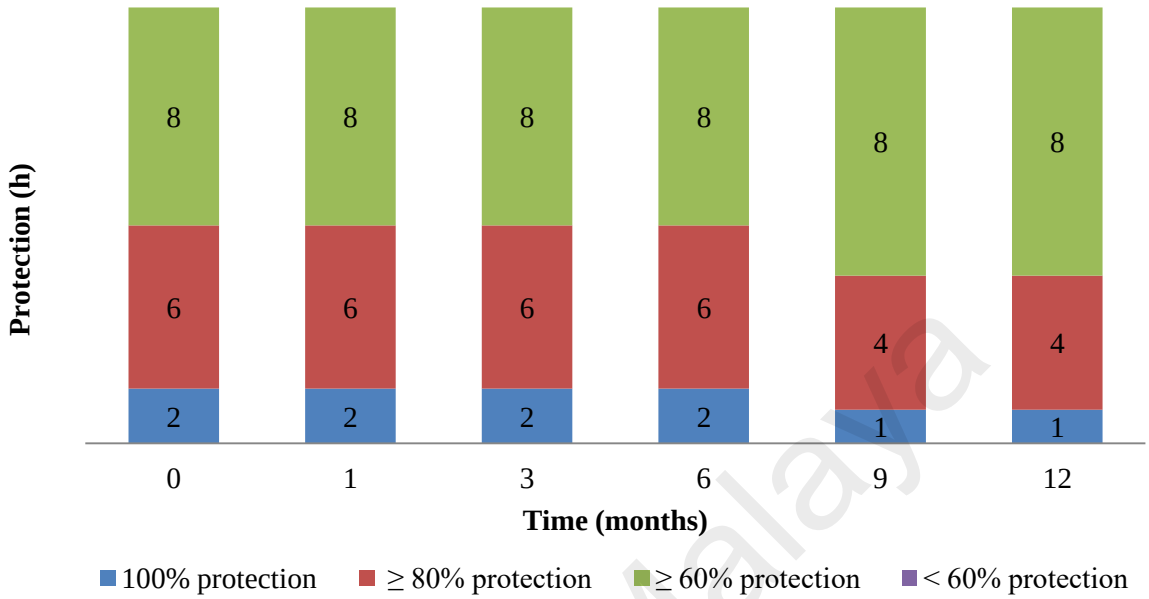


Figure 4.42: Repellent effect of the ME AGRO (a) and NE AGRO formulations (b) over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH (a) storage condition.

a)



b)

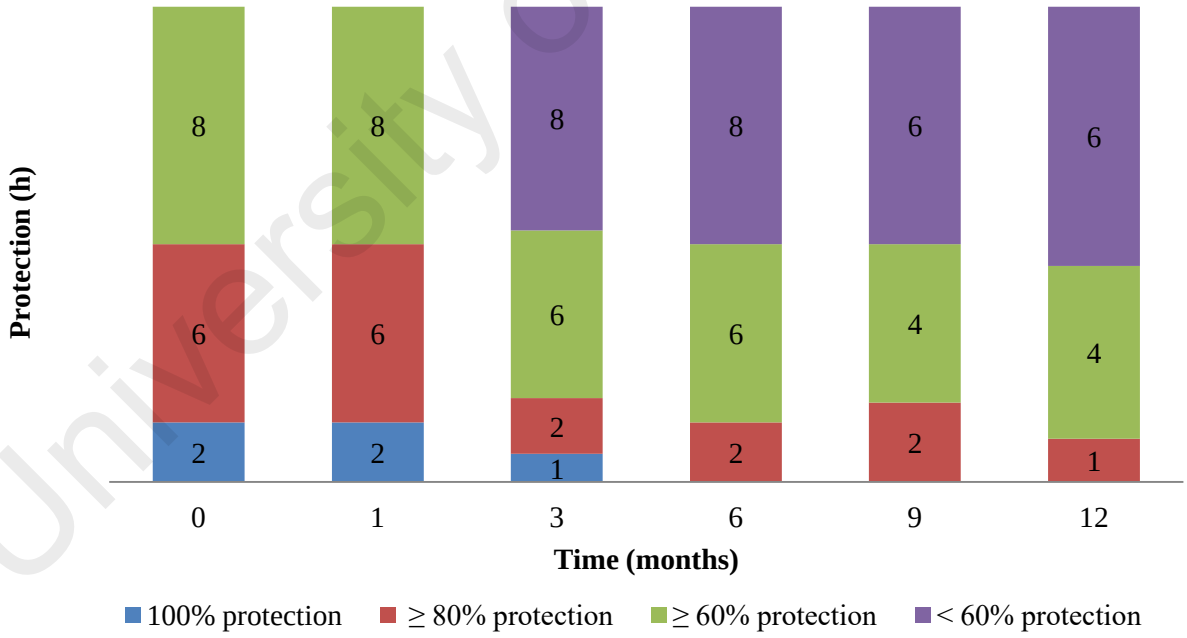
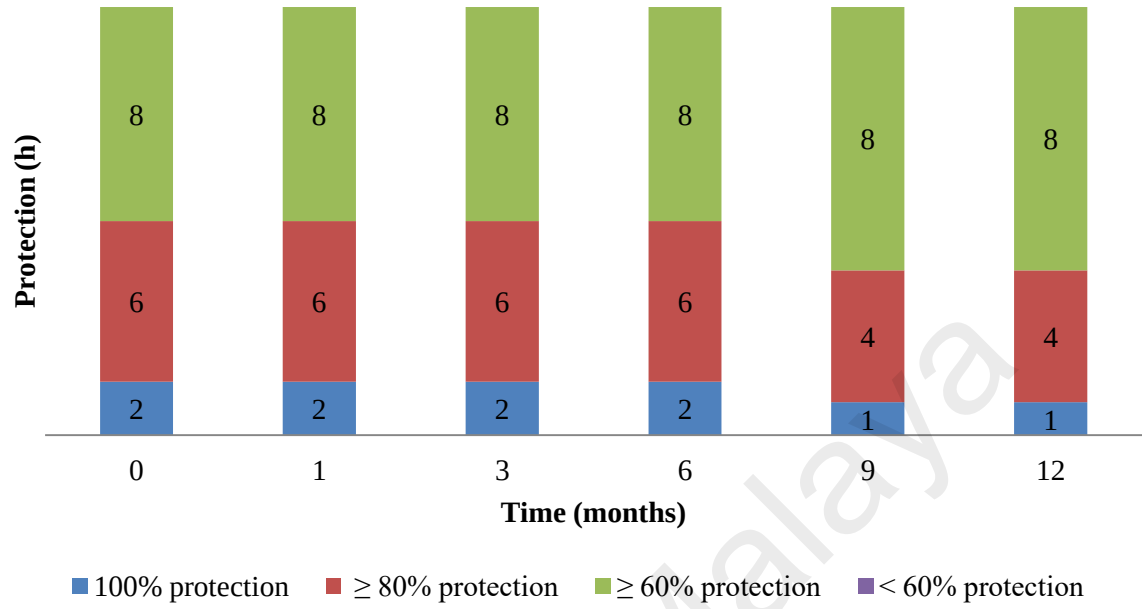


Figure 4.43: Repellent effect of the ME CGPO (a) and NE CGPO formulations (b) over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH storage condition.

a)



b)

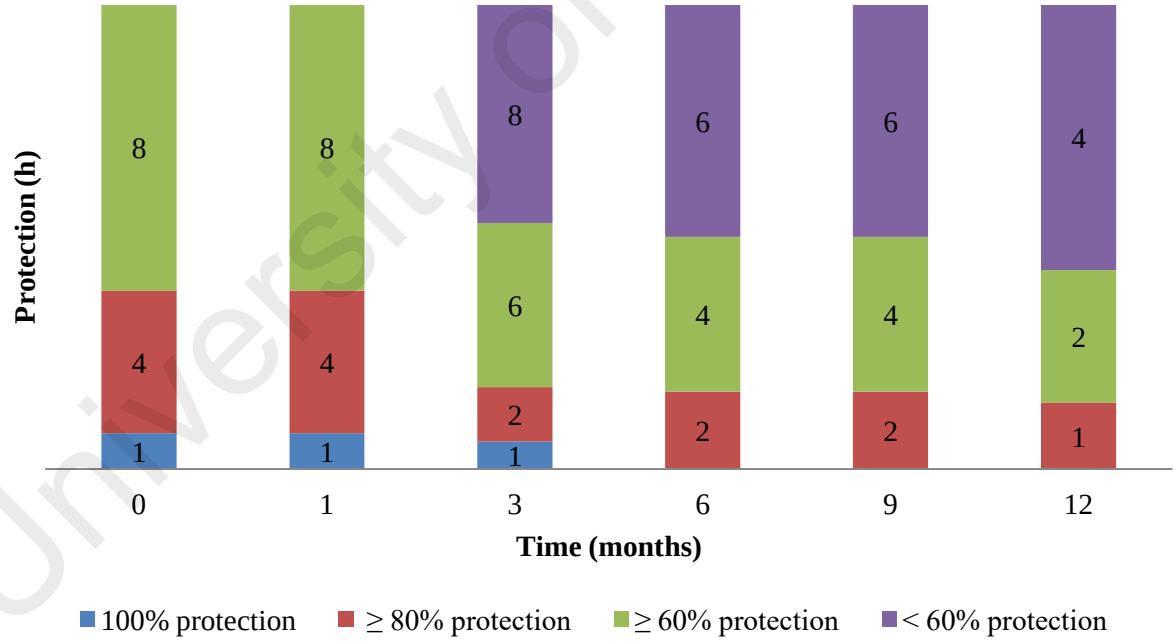
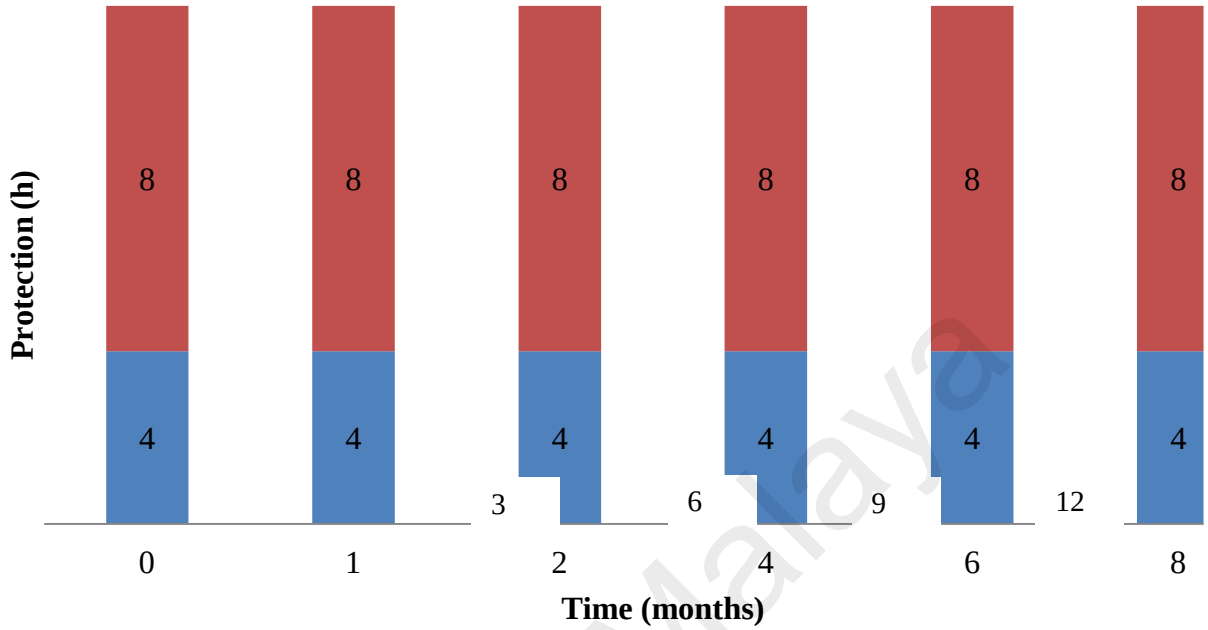


Figure 4.44: Repellent effect of the ME CALO (a) and NE CALO formulations (b) over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH storage condition.

a)



b)

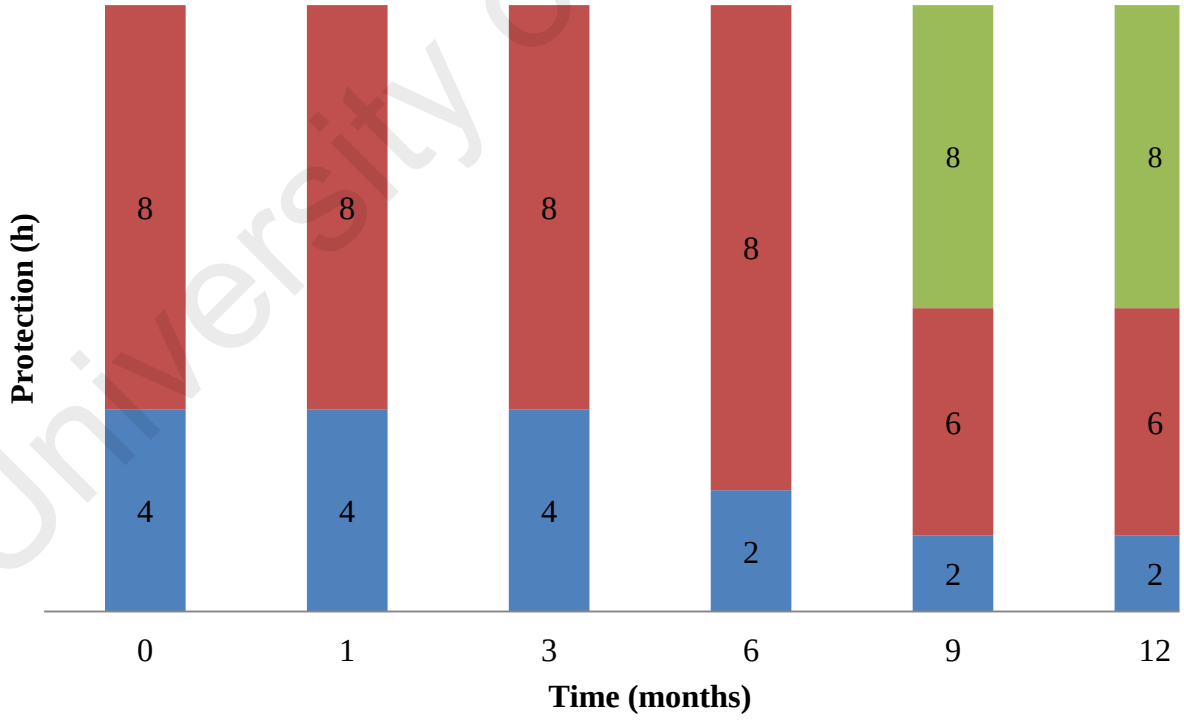


Figure 4.45: Repellent effect of the ME DEET (a) and NE DEET formulations (b) over 12 months of storage at 25^{0}C ± 2^{0}C/60% ± 5% RH storage condition.

4.9 MICROBIOLOGY TEST

4.9.1 Basic microbiology test

Figure 4.46 shows the absence of bacteria and fungi colony on the agar plates exposed to ME EOs and DEET formulations right after the preparation. Performing similar test on the same formulations after they were stored for 1, 3, 6 and 12 months also demonstrated absence of bacteria and fungi colonies. Figure 4.47 shows result obtained when test was performed on NE EOs and DEET formulations. Similar results to that of ME EOs and DEET formulations were obtained.

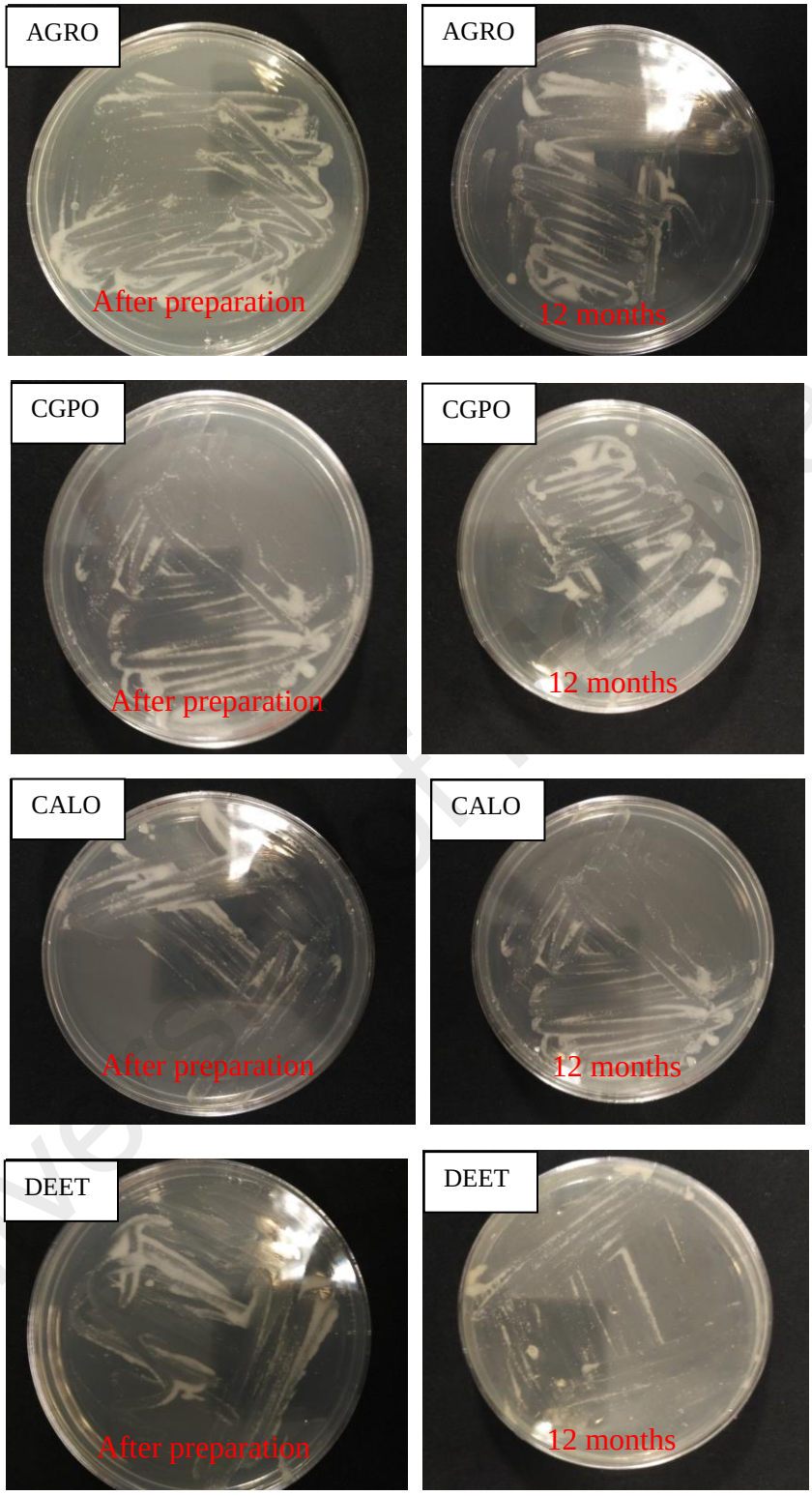


Figure 4.46: Agar exposed to ME EOs and DEET formulations showed absence of colony of bacteria or fungus right after the preparation and after 12 months of storage.

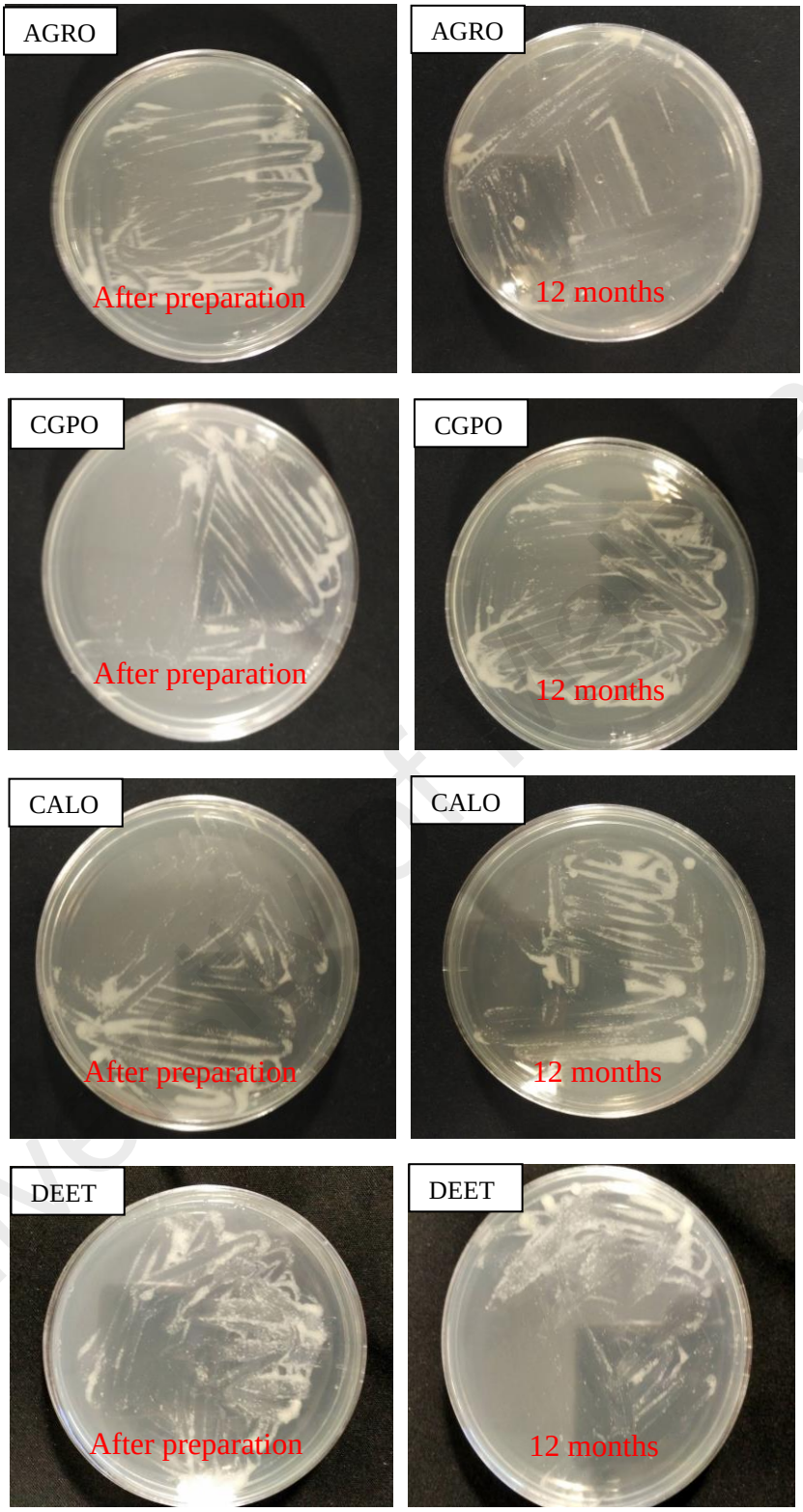


Figure 4.47 **Agar exposed to NE EOs and DEET formulations showed absence of colony of bacteria or fungus right after the preparation and after 12 months of storage.**

4.9.2 Preservative efficacy evaluation

Table 4.19 presented the results on preservative efficacy of the formulations right after their production indicating that all the formulations displayed variable degree of antimicrobial activity against the different strains tested. The ME AGRO formulation showed the highest antimicrobial activity when tested against *S. aureus, E. coli, C albicans and A. fumigatus*, followed by ME DEET formulations, ME CGPO formulations and ME CALO formulation. It was noted that although most of the ME formulations presented greater antimicrobial activity compared to the NE formulations, the different was statistically insignificant ($p>0.05$).

When comparison was made between formulations and gram positive standard drugs (Ampicillin), antimicrobial activity of all formulations demonstrated weaker antimicrobial activity against *S. aureaus*. Similar results were observed when comparison was made between all formulations and antifungus standard drug (Flumequine) against *C. albicans* and *A. fumigatus*. In contrast, when comparison were made with gram negative standard drugs (Streptomycin), AGRO and DEET formulations presented stronger antimicrobial activity against *E.coli*.

After 6 months of storage, all formulations presented no significant change in their degree of antimicrobial activity ($p>0.05$) (Table 4.20). After 9 months and 12 months storage duration however, some reduction in antimicrobial activity were detected but statistically not significant ($p>0.05$) (Table 4.21 and Table 4.22).

Table 4.19: Mean diameter inhibition zone (mm) of all formulations against *Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus fumigatus* right after the production.

	Mean diameter inhibition zone (mm) ± SEM			
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
AGRO-MEF	20.3 ± 1.4	28.5 ± 1.6	21.7 ± 0.6	18.4 ± 1.2
CGPO-MEF	15.2 ± 1.3	12.2 ± 1.0	11.4 ± 0.8	14.8 ± 1.1
CALO-MEF	14.3 ± 1.2	10.1 ± 1.1	9.6 ± 1.1	12.3 ± 0.7
DEET-MEF	16.5 ± 1.2	20.1 ± 1.3	22.1 ± 1.3	16.9 ± 0.9
AGRO-NEF	18.8 ± 1.1	26.2 ± 1.2	20.3 ± 1.2	19.2 ± 0.4
CGPO-NEF	13.3 ± 1.0	12.1 ± 1.2	8.2 ± 0.9	10.2 ± 1.1
CALO-NEF	12.9 ± 1.3	10.3 ± 1.0	8.8 ± 1.0	11.4 ± 0.6
DEET-NEF	14.2 ± 1.0	20.1 ± 1.3	15.2 ± 0.7	17.5 ± 1.0
Ampicilin (10 µg)	22.5 ± 1.8	-	-	-
Streptomicin (10 µg)	-	15. 2 ± 1.4	-	-
Flumequine (30 µg)	-	-	28.2 ± 0.6	26. 4 ± 0.5
Control negative	No inhibition zone	No inhibition zone	No inhibition zone	No inhibition zone

Table 4.20: Mean diameter inhibition zone (mm) of all formulations against *Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus fumigatus* right after 6 months of storage time.

	Mean diameter inhibition zone (mm) ± SEM			
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
AGRO-MEF	21.5 ± 0.7	28.1 ± 0.5	20.6 ± 1.4	17.8 ± 1.0
CGPO-MEF	15.0 ± 1.1	11.4 ± 1.3	12.0 ± 0.2	14.2 ± 1.2
CALO-MEF	13.9 ± 1.0	11.2 ± 1.0	10.1 ± 0.9	11.9 ± 0.6
DEET-MEF	17.2 ± 1.5	20.1 ± 1.4	21.6 ± 1.1	16.2 ± 0.7
AGRO-NEF	18.9 ± 0.9	25.8 ± 1.1	19.9 ± 1.0	18.8 ± 1.4
CGPO-NEF	14.1 ± 1.1	11.2 ± 0.2	9.1 ± 0.7	11.0 ± 1.2
CALO-NEF	12.7 ± 1.2	10.0 ± 1.1	8.6 ± 1.3	10.9 ± 0.8
DEET-NEF	14.9 ± 0.8	19.8 ± 0.9	14.6 ± 1.6	18.1 ± 1.3
Ampicilin (10 µg)	24.1 ± 1.2	-	-	-
Streptomicin (10 µg)	-	16.1 ± 1.2	-	-
Flumequine (30 µg)	-	-	26.8 ± 1.5	25.3 ± 0.8
Control negative	No inhibition zone	No inhibition zone	No inhibition zone	No inhibition zone

Table 4.21: Mean diameter inhibition zone (mm) of all formulations against *Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus fumigatus* right after 9 months of the storage time.

	Mean diameter inhibition zone (mm) ± SEM			
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
AGRO-MEF	19.8 ± 0.4	27.3 ± 0.4	20.0 ± 1.2	17.2 ± 1.3
CGPO-MEF	14.4 ± 1.2	11.0 ± 1.2	10.8 ± 0.3	13.8 ± 0.8
CALO-MEF	13.5 ± 0.9	10.9 ± 1.1	9.7 ± 0.8	11.2 ± 0.5
DEET-MEF	16.6 ± 1.3	19.3 ± 1.2	20.6 ± 1.3	15.2 ± 1.3
AGRO-NEF	18.2 ± 0.5	25.2 ± 1.2	19.1 ± 1.1	17.9 ± 1.2
CGPO-NEF	13.3 ± 1.0	10.5 ± 0.5	8.0 ± 0.6	10.1 ± 1.2
CALO-NEF	12.6 ± 1.2	9.4 ± 1.3	8.2 ± 1.1	10.7 ± 1.4
DEET-NEF	14.0 ± 0.3	18.3 ± 1.1	14.0 ± 1.3	17.9 ± 1.5
Ampicilin (10 µg)	24.2 ± 1.2	-	-	-
Streptomicin (10 µg)	-	16.0 ± 1.0	-	-
Flumequine (30 µg)	-	-	26.4 ± 1.3	24.0 ± 0.9
Control negative	No inhibition zone	No inhibition zone	No inhibition zone	No inhibition zone

Table 4.22: Mean diameter inhibition zone (mm) of all formulations against *Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus fumigatus* after 12 months of storage.

	Mean diameter inhibition zone (mm) ± SEM			
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*	*Aspergillus fumigatus*
AGRO-MEF	19.4 ± 1.1	27.2 ± 0.8	19.8 ± 0.4	17.3 ± 1.5
CGPO-MEF	14.9 ± 1.2	11.2 ± 1.0	9.9 ± 0.6	13.5 ± 1.3
CALO-MEF	13.2 ± 1.4	10.0 ± 1.2	9.2 ± 1.7	10.9 ± 0.5
DEET-MEF	16.2 ± 1.3	19.1 ± 1.3	20.2 ± 1.4	15.8 ± 0.6
AGRO-NEF	18.3 ± 1.4	24.5 ± 1.2	19.3 ± 1.1	17.0 ± 0.4
CGPO-NEF	13.0 ± 1.1	9.8 ± 1.0	8.0 ± 0.3	9.3 ± 1.0
CALO-NEF	12.1 ± 1.2	8.9 ± 0.6	8.4 ± 1.2	9.4 ± 0.3
DEET-NEF	14.7 ± 1.0	18.0 ± 1.2	13.9 ± 1.0	17.1 ± 1.5
Ampicilin (10 µg)	21.6 ± 1.3	-	-	-
Streptomicin (10 µg)	-	15.4 ± 1.3	-	-
Flumequine (30 µg)	-	-	24.3 ± 0.2	25. 3 ± 0.7
Control negative	No inhibition zone	No inhibition zone	No inhibition zone	No inhibition zone

4.10 SKIN TOXICITY/SAFETY STUDIES

4.10.1 Acute dermal irritation/corrosive

The results of skin irritation studies of all the formulations are summarized in Table 4.23. Data obtained indicate absence of dermal responses (including edema, erythema or eschar) in rabbits tested with ME EOs and DEET formulations. The primary irritation index (PII) was calculated was = 0 in these groups. Examination done after Day 14, also indicated absence of irritation effect, normal hair growth of all animals and no changes in body weight on animal tested with all formulations (Figure 4.48). Confirmatory test using two additional rabbits indicated no sign of irritation effect for all formulations tested. Figure 4.49 shows no significant changes in body weight caused by the treatment.

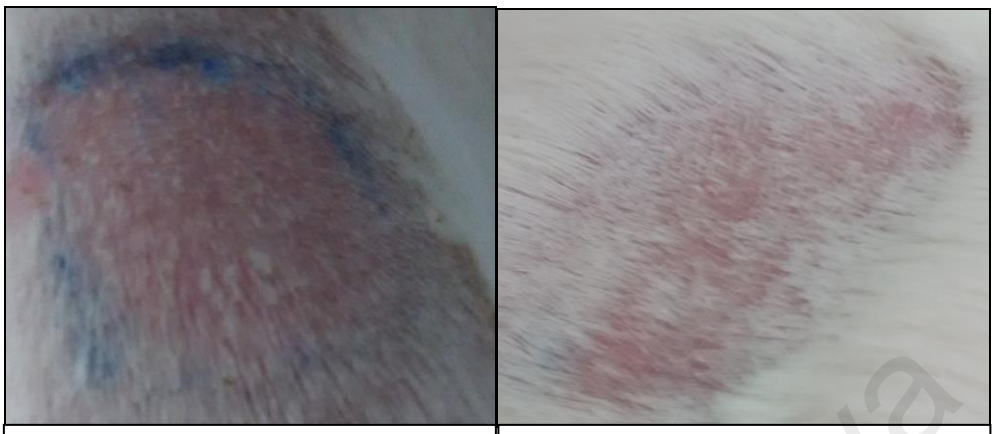
Table 4.23: Skin irritation study of ME EOs and DEET formulations in rabbits.

	ME formulation				
	AGRO	**CGPO**	**CALO**	**DEET**	**Control**
Dermal responses (mean score)[a]					
1 h after removal of patches	0	0	0	0	0
24 h after removal of patches	0	0	0	0	0
48 h after removal of patches	0	0	0	0	0
72 h after removal of patches	0	0	0	0	0
Primary Irritation Index (PII)[b]	0	0	0	0	0

[a] Dermal responses were scored according to OECD 404 (2002). Mean score of dermal responses = (total score of erythema and eschar formation + total score of edema formation)/3.

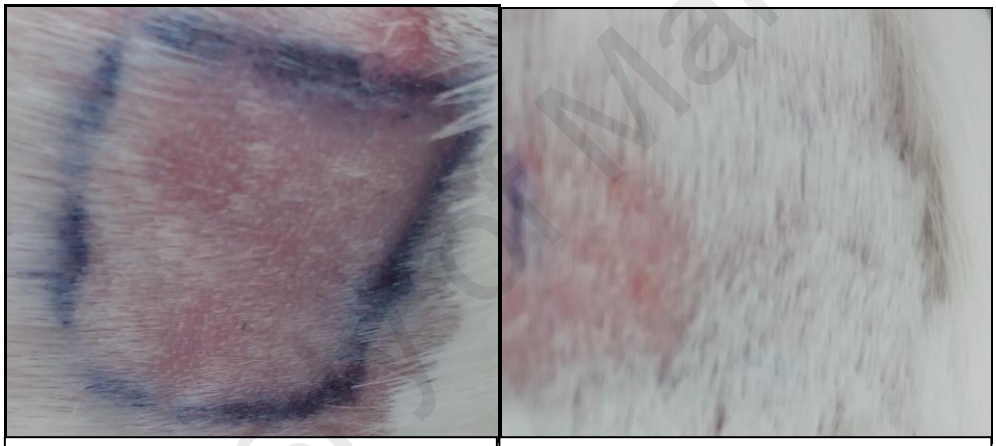
[b] Primary Irritation Index (PII) = (Mean score at 24 h + mean score at 48 h + mean score at 72 h)/3

a) ME AGRO formulation



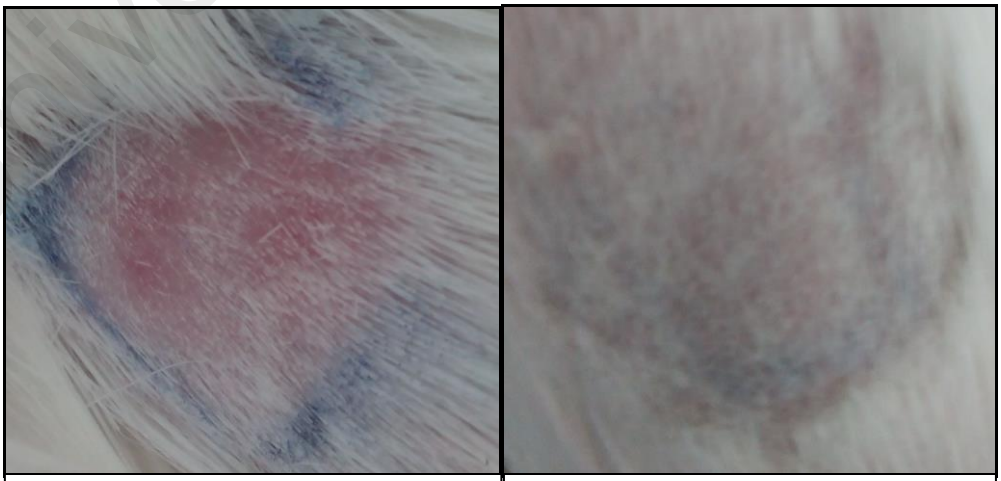
Day 1	Day 14

b) ME CGPO formulation



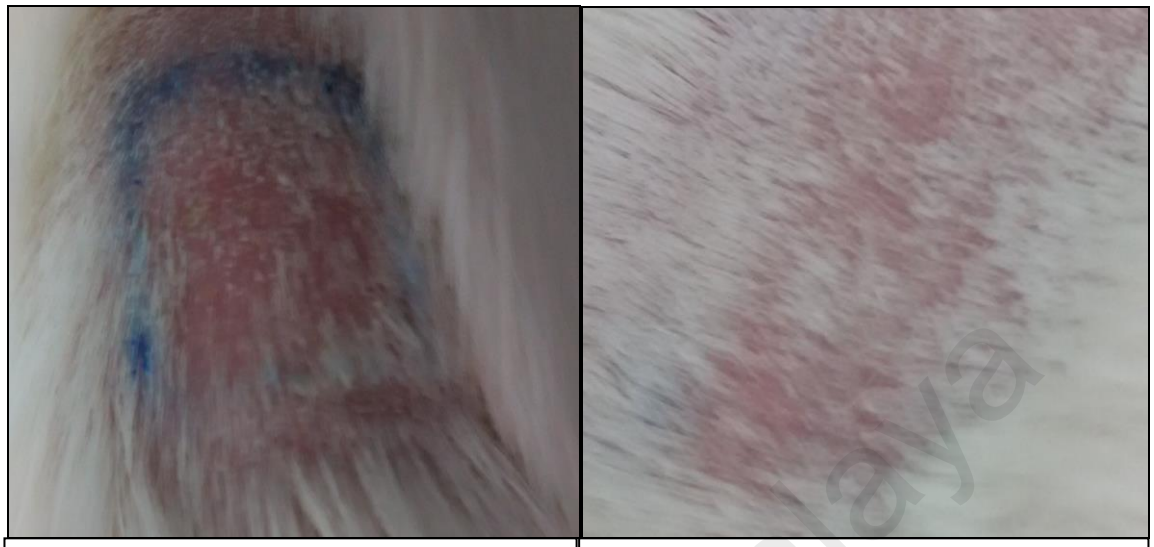
Day 1	Day 14

c) ME CALO formulation



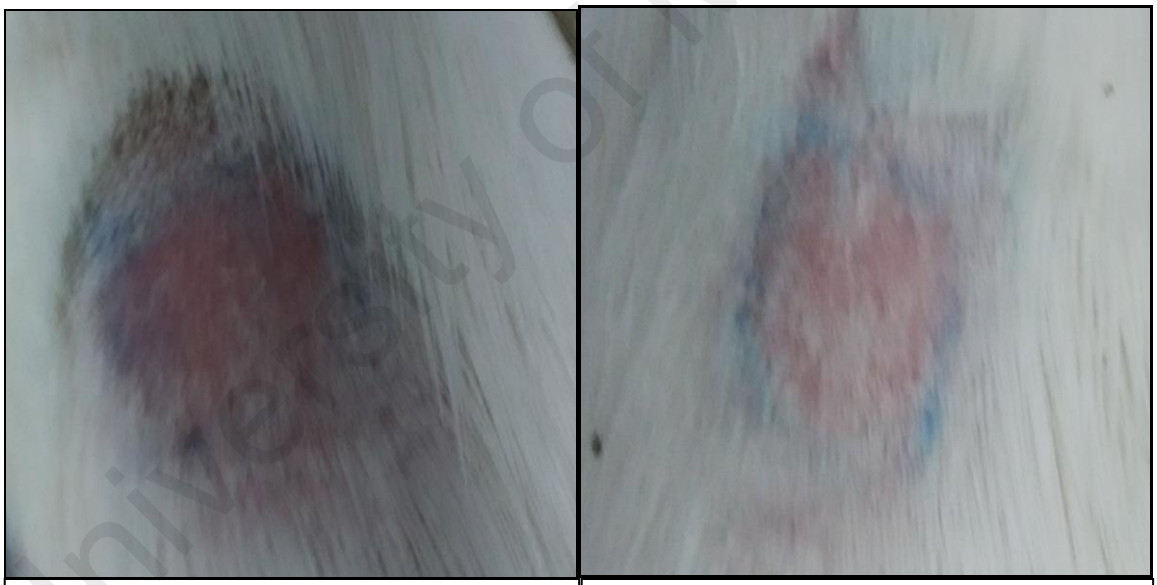
Day 1	Day 14

d) ME DEET formulation



Day 1	Day 14

e) Saline water



Day 1	Day 14

Figure 4.48: Skin irritation study: No sign of irritation were observed on rabbits that dermally exposed to ME EOs and DEET formulations of after Day 1 and Day 14 of exposure.

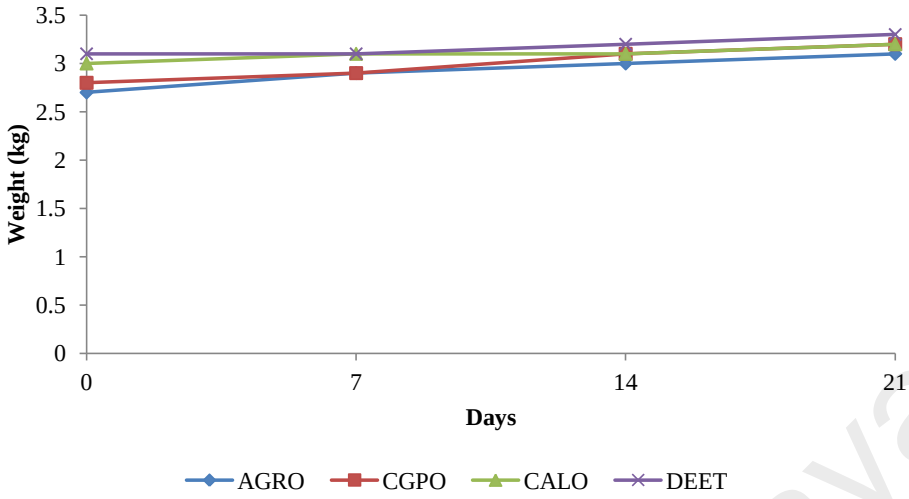


Figure 4.49 Changes in body weight of rabbits dermally tested with the formulations.

4.10.2 Skin sensitization

The results of skin sensitization studies of EOs and DEET ME formulation are summarized in Table 4.24 - Table 4.28. Table 4.24 - 4.26 presented the sensitization effect of all formulations during induction phase at day 0, day 7 and day 14 of the study. At day 0, day 7 and day 14, all the guinea pigs treated with EOs and DEET ME formulations presented no erythema and edema effect even after 72 hours of treatment. The negative control group which was tested with saline water also presented similar effect. However, for positive control group that was tested with 10% formaldehyde presented very slight erythema (Table 4.24). At day 7 and 14, all the guinea pig of the positive control group presented moderate erythema and edema (Table 4.25 and Table 4.26). Table 4.27 and Table 4.28 presented the sensitization effect of all formulations in tested and control group during the challenge phase at day 29 of the study, respectively. No erythema and edema

was observed after the challenge phase with all the formulations in tested group (Table 4.27) and negative control group (Table 4.28). Statistical analysis on the body weight of guinea pigs did not reveal significant different between tested and negative control groups ($p>0.05$). However all animal showed significant changes in their body weight when compared to positive control group ($p<0.05$) (Figure 4.50).

Table 4.24: Dermal responses in tested and control groups of guinea pig at day 0.

Groups	No of guinea pig	After 6 hours of treatment	Mean score[a]	Positive ratio[b]
AGRO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
CGPO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
CALO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
DEET	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
Negative control (saline water)	5	1	0	0/5
		24	0	0/5
		48	0	0/5
		72	0	0/5
Positive control (10% Formaldehyde)	5	1	1	5/0
		24	1	5/0
		48	1	5/0
		72	1	5/0

[a] dermal responses were scored according to OECD (2002)
[b] Positive ratio = no. of guinea pigs with positive response/no. of guinea pigs with negative response.

Table 4.25: Dermal responses in tested and control groups of guinea pig at day 7th.

Groups	No of guinea pig	After 6 hours of treatment	Mean score[a]	Positive ratio[b]
AGRO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
CGPO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
CALO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
DEET	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
Negative control (saline water)	5	1	0	0/5
		24	0	0/5
		48	0	0/5
		72	0	0/5
Positive control (10% Formaldehyde)	5	1	1	5/0
		24	2	5/0
		48	2	5/0
		72	3	5/0

[a] dermal responses were scored according to OECD (2002)
[b] Positive ratio = no. of guinea pigs with positive response/no. of guinea pigs with negative response.

Table 4.26: Dermal responses in tested and control groups of guinea pig at day 14th.

Groups	No of guinea pig	After 6 hours of treatment	Mean score[a]	Positive ratio[b]
AGRO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
CGPO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
CALO	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
DEET	10	1	0	0/10
		24	0	0/10
		48	0	0/10
		72	0	0/10
Negative control (saline water)	5	1	0	0/5
		24	0	0/5
		48	0	0/5
		72	0	0/5
Positive control (10% Formaldehyde)	5	1	2	5/0
		24	2	5/0
		48	3	5/0
		72	3	5/0

[a] dermal responses were scored according to OECD (2002)
[b] Positive ratio = no. of guinea pigs with positive response/no. of guinea pigs with negative response.

Table 4.27: Dermal responses in tested group of guinea pig after challenge with ME EOs and DEET formulations (Day 29th).

Groups	Substance for induction	Substance for challenge	No of guinea pig	Hour after the end of challenge	Mean score[a]	Positive ratio[b]
AGRO	AGRO	AGRO	10	1	0	0/10
				24	0	0/10
				48	0	0/10
				72	0	0/10
CGPO	CGPO	CGPO	10	1	0	0/10
				24	0	0/10
				48	0	0/10
				72	0	0/10
CALO	CALO	CALO	10	1	0	0/10
				24	0	0/10
				48	0	0/10
				72	0	0/10
DEET	DEET	DEET	10	1	0	0/10
				24	0	0/10
				48	0	0/10
				72	0	0/10

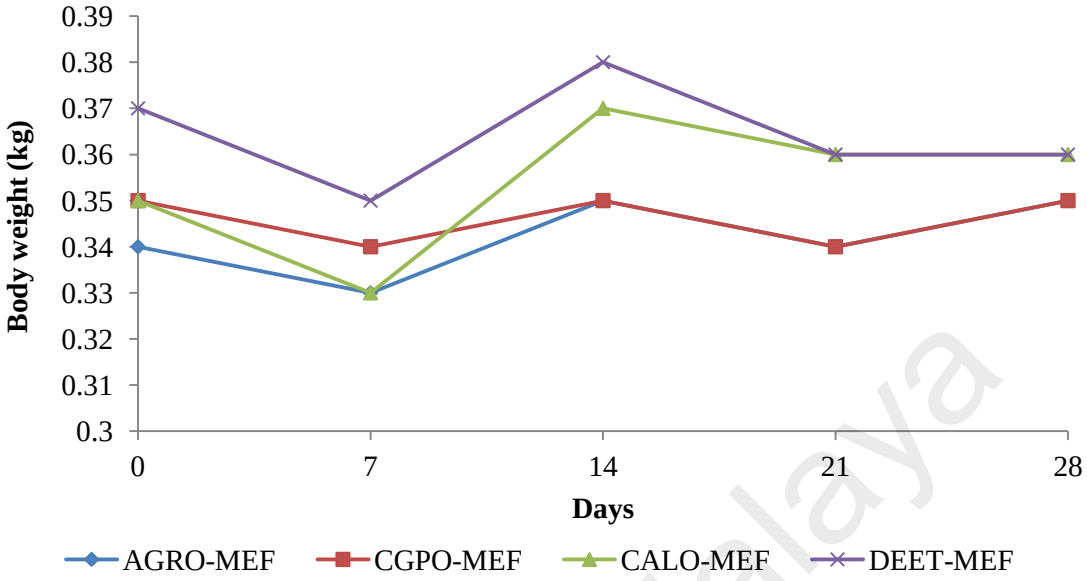
[a] dermal responses were scored according to OECD (2002)
[b] Positive ratio = no. of guinea pigs with positive response/no. of guinea pigs with negative response.

Table 4.28: Dermal responses in control negative group of guinea pig after challenge with ME EOs and DEET formulations (Day 29th).

Groups	Substance for induction	Substance for challenge	No of guinea pig	Hour after the end of challenge	Mean score[a]	Positive ratio[b]
Negative Control	Saline water	AGRO	5	1	0	0/5
				24	0	0/5
				48	0	0/5
				72	0	0/5
	Saline water	CGPO	5	1	0	0/5
				24	0	0/5
				48	0	0/5
				72	0	0/5
	Saline water	CALO	5	1	0	0/5
				24	0	0/5
				48	0	0/5
				72	0	0/5
	Saline water	DEET	5	1	0	0/5
				24	0	0/5
				48	0	0/5
				72	0	0/5
Positive Control	10% Formaldehyde	10% Formaldehyde	5	1	2	5/0
				24	2	5/0
				48	3	5/0
				72	3	5/0

[a] dermal responses were scored according to OECD (2002).
[b] Positive ratio = no. of guinea pigs with positive response/no. of guinea pigs with negative response.

a)



b)

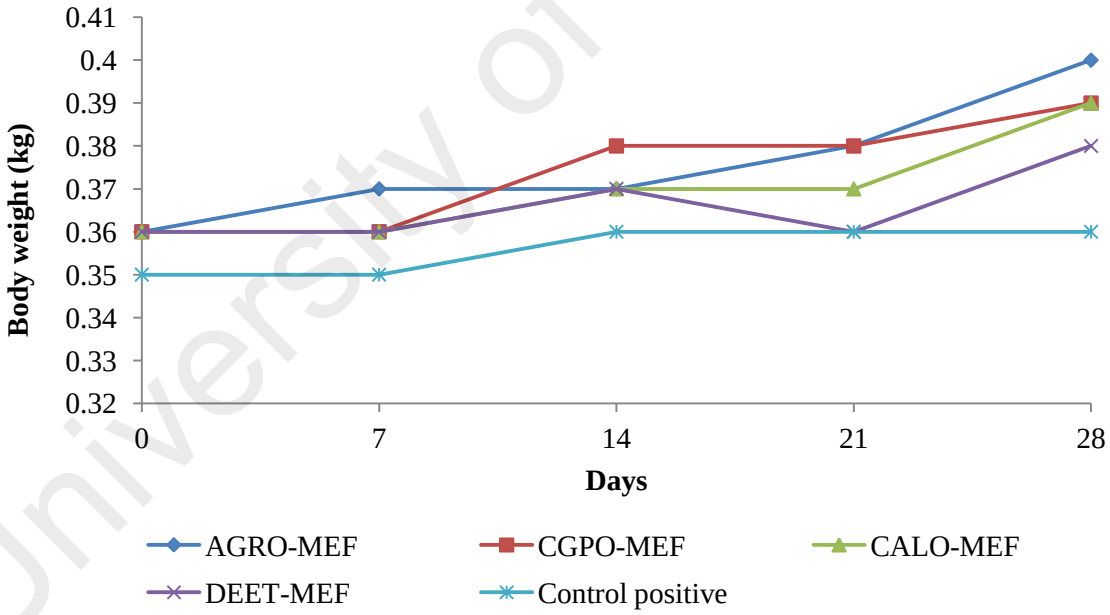


Figure 4.50: Changes in body weight of (a) tested and (b) control group of guinea pigs after challenge with ME formulation.

CHAPTER 5

DISCUSSION

5.1 Ethnobotanical study on repellent plants

Prior the development of synthetic repellent, insect repellents were very much based on plants. Therefore in this study, an attempt was made to record and document plants that are used traditionally as insect repellent in Malaysia. To our best knowledge, this study is the first study in Malaysia that records the knowledge on repellent plants and the methods on how these plants were used (usage customs), based on survey, in order to generate preliminary base-line data (reference document) for such plants.

Most of the studies on knowledge and usage customs of repellent plants came from rural areas of third world countries such as Ethiopia, Kenya and Eritrea (Palsson & Jaenson, 1999; Odalo *et al.,* 2005; Ntonifor *et al.,* 2006; Karunamoorthi *et al.,* 2009a; Kidane *et al.,* 2013). Their studies showed that not only the population in these countries had some knowledge about plants having the ability to repel insects / mosquitoes; they also demonstrated awareness on the importance of mosquitoes in relation to diseases. Therefore, efforts to avoid contact with mosquitoes were widely applied. In those studies, more than half of the respondents choose to use plants as a material to repel mosquitoes since synthetic commercial mosquito repellents and insecticides were considered costly, if available.

In this current study, the level of knowledge and usage customs of repellent plants was found lower (44%) than studies conducted, for example, in Western Hararghe zone, Ethiopia; where 92.1% of the respondents demonstrated adequate awareness on repellent plants (Karunamoorthi & Husen, 2012). Similarly, a study among the Oromo ethnic group in Ethiopia, presented 83.6% of awareness (Karunamoorthi *et al.,* 2009a) and 64.8% observation was recorded among respondents in Bolifomba village, Cameroon (Ntonifor *et al.,* 2006). Lower percentage observed in this study could be due to the fact that many Malaysian can afford to buy commercial repellent or insecticides and consequently influence their knowledge regarding repellent plants. It is consistent with the result about 60% of the respondents use aerosol spray to protect themselves against mosquito bites.

This survey demonstrated that 89.8% of the respondents, who had knowledge and used repellent plants, mentioned that they had easy access to these plants. All study areas were situated adjacent to the jungle and most of the villagers owned the plantation estates where some of these repellent plants grew freely and therefore are available for use almost all year round. Moreover, most of the respondents mentioned that the plants they use were effective in repelling mosquitoes and had a pleasant smell when used. These could be the reasons why repellent plants were preferred by some respondents compared to synthetic chemical repellents in these areas. The fact that more than half of the respondents use aerosol spray as one of the methods to repel mosquitoes suggested that cost may not be a major issue in deciding the types of preventive measures used here. In addition, this study also showed that those who used the repellent plants mostly applied them before they leave home for work in the plantation estate, jungle and farms. Obviously, this is because they are more exposed to mosquitoes while working compared to when they are at home.

Sixteen plant species were mentioned by the respondents as effective repellent plants against insects / mosquitoes. The most common plant reported was *Cymbopogon nardus* (family Poeceae) (13.7%). Similar findings have been reported in Ethiopia and Cameroon where one of the repellent plants that is traditionally used by the community in those areas was also from Poaceae family, *Cymbopogon citratus* (Ntonifor *et al.,* 2006; Karunamoorthi & Husen, 2012). Our findings also indicated that 3.4% of respondents used *Lantana camara*, the same plant used in North-eastern Tanzania for the same purpose (Kweka *et al.,* 2008). Other plants mentioned were two species belonging to the Rutaceae family, *Citrus aurantifolia* (4.6%) and *C. grandis* (3.1%), which were in agreement with studies done in Cameroon that reported 7% of the community in their study used *Citrus spp.* as mosquito repellent (Ntonifor *et al.,* 2006). Thus, *Cymbopogon spp., L. camara* and *Citrus spp.* were among the plants known to be used as mosquito repellent by several communities in different parts of the world.

Half of the respondents stated that the leaf was the main part of the plant conventionally used for repellent purposes followed by the flower (25%), rhizome (12.5%), bark (6.25%), and fruit peel (6.25%). These results were comparable with a previous study reported by Kweka *et al.* (2008) that the plant part mostly used as repellent was leaf (70%), followed by bark (10%), mixed plant parts (13%) and root (7%). A study conducted in Ethiopia and South Africa also demonstrated that the leaf was the part mostly used as repellent (Karunamoorthi *et al.,* 2009a; Karunamoorthi *et al.,* 2009b; Mavundza *et al.,* 2011). Community in this area used the leaves of *C. nardus*, *C. aurantifolia* and the flower of *Etlingera eliator* to repel mosquitoes by laying them on the surface of cupboards and drawers. This was almost similar to a practice performed by people in Western Hararghe

zone, Ethiopia, where leaves of *C. citratus* were placed all over the floor of the house (Karunamoorthi & Husen, 2012). In South Africa, it was reported that when leaves of *Clausena anisata* were used to drive away mosquitoes, they tend to hang these leaves inside their house (Mavundza *et al.,* 2011). Community of Lua in East Africa who used branches of *Ocimum basilicum* (Labiatae) to repel mosquitoes laid the branches inside their houses (Kokwaro, 1976).

Another method used was burning or smouldering of the dried plant materials on a traditional charcoal stove or outside houses. This method was commonly used in Ethiopia (Karunamoorthi *et al.,* 2009a; Karunamoorthi & Husen, 2012), Eritea (Waka *et al.,* 2004) and Guatemala (Klien *et al.,* 1995). These findings suggested that although similar or closely related plants were selected, methods applied to repel mosquitoes differ from one community to another.

Apart from laying leaves on surfaces, this present study showed that 12.9% of respondent sprayed liquid obtained from crushed leaves of *Citrus aurantifolia* and *Sesbania grandiflora*, crushed fruit peel of *C. grandis,* and crushed rhizome of *Alpinia galanga* on their bodies to repel mosquitoes. This practice was also found employed by the community in Ethiopia, where they use the crushed leaves of *Echinops spp.*, *Silene macroserene* L., and *Otostegia integrifolia* L. The difference between these communities was that, in Ethiopia, spraying was done not on the body but on the house floor instead (Karunamoorthi & Husen, 2012). From these findings, it can be concluded that protection of this sort allows protection to be done at home by spraying the house (floor) and while away from home it can be done by spraying it on the body.

It was interesting to note that the community in Kota Tinggi, Johor believed that growing plants such as *C. nardus, P. graveolens, L. camara, E. eliator* and *P. cablin* close to their houses helped drive away mosquitoes. Such practice was found to be mentioned for the first time with regard to plants as a mosquito repellent.

The statistical analysis shows that there was no significant association between knowledge on repellent plants with gender, age and monthly income. This result could suggest that the plants are well known by majority members in the community regardless of their gender, age and income (Klien *et al.,* 1995). It is also quite comparable with the earlier studies conducted in Ethiopia, except for the age, where their study showed a significant association between knowledge on repellent plants with age (Ntonifor *et al.,* 2006; Karunamoorthi & Husen, 2012). Nevertheless, our study did show a significant association between knowledge on repellent plants with educational status. It could be that knowledge with regard to repellent plants was obtained via informal education passed on from a generation to another since this was not taught at school. This kind of information passage however can lead to the loss of indigenous knowledge when it comes to the next generation. Therefore, the educated respondents, which consist of the younger generations, could be those who knew less about such plants compared to those who are older and uneducated.

Analysis also showed that the usage of repellent plants is significantly associated with age and educational status. It could be that the elderly people tend to use repellent plants more than the younger individuals as a result of common practice introduced to them

by earlier generations, whereas the younger people would choose commercialized repellent as they were widely known through mass media and available in the market for easy use. Correspondingly, the older generation may not have enough knowledge regarding recent personal protective devices such as aerosol spray and other commercialized repellent products which lead them to stick to application of insect repellent plants. This finding is consistent with another study done in Ethiopia where a significant association between knowledge on repellent plants with educational status was observed (Karunamoorthi & Husen, 2012).

Our study revealed that gender and monthly income showed no significant association with the usage of repellent plants, suggesting that men or women used repellent plants equally whether they were at home or out to work. The finding is contrary with the study conducted in Ethiopia where it was reported that there was a significant association between gender and the usage customs of insect repellent plants. In that study, women tend to use insect repellent plants more compared to men (Karunamoorthi & Husen, 2012).

The significant association of knowledge/usage with age and educational status might be due to the *shifting baseline syndrome* in the Traditional Ecological Knowledge (TEK) among the community. According to Hanazaki *et al.*, (2013), TEK is the knowledge that developed by local communities through adaptive experiences with natural resources. It is dynamic and continuously modified as adaptive responses to new environmental, social, and economic conditions (Gómez-Baggethun & Reyes-García, 2013). Such changes can also be related to a "loss of knowledge", especially when the social reproduction of people holding TEK is at risk, resulting in the loss of local knowledge systems. The loss of

local knowledge can result in a diminished ability to cope with environmental alterations, and also can be related to a changing baseline in the perception of natural resources or known as *shifting baseline syndrome* (Pauly, 1995).

In general this study demonstrated that the local people in Kota Tinggi district had knowledge and usage customs with regard to repellent plants. They also had knowledge and awareness about mosquitoes and its importance in relation to diseases. Most of them possessed basic good practice in terms of preventive measures. Although 16 plants were recorded in this study as having repellent effects, the efficacy of these so called "repellent plants" is however still uncertain.

5.2 CHEMICAL COMPOUNDS OF THE ESSENTIAL OIL

Based on the ethnobotanical survey conducted, three species of plants were selected; *Alpinia galanga, Citrus grandis and C. aurantifolia*. These plants were reported to have repellent effect against mosquito as documented during the survey. This information is supported by previous studies that demonstrated essential oil of these plants shown to have mortality and repellent effect against mosquitoes. For example, Tawatsin *et al.* (2002) demonstrated that *A. galanga* extract had knockdown effect against mosquito when used as mosquito coil and *A. galanga* essential oil having repellent effect against *Ae. aegypti* (Tawatsin *et al.,* 2006). *Citrus spp.* was shown to have mortality and repellent effect against mosquito, cockroach and houseflies (Ezeonu *et al.*, 2001). Based on these findings, essential oil of *A. galanga* and *Citrus spp.* are proven to have repellent effect

against various species of insects and thus have the potential to be developed as repellent products having broader spectrum.

Essential oils are complex mixtures of various chemical compounds. Insecticidal activity of the *A. galanga* and *Citrus spp*. may be related to the chemical compounds in the essential oils. The biological effects of essential oil might be either the result of a synergism of all compounds or could reflect only those of the main compounds. For examples, study conducted by Rohani *et al.* (1997) reported that essential oils of *Litsea elliptica* Blume having an adulticide effect against Aedes aegypti and another study conducted by Sulaiman *et al.* (2001) also reported that essential oils of *Melaleuca cajuputi* and *Cymbopogon nardus* have an adulticide effect against Aedes mosquito. In that sense, for biological purposes, it could be more informative to study the entire oil rather than some of its component because the concept of synergism seems to be important (Zoubiri & Baaliouamer, 2014).

Analysis using GC-MS demonstrated that the main compound of AGRO were tetradecyl ester which contributed 15.07% of the essential oil, followed by the 1-undecene (13.59%), hexadecyl ester (11.38%), 3-methyl pentene (6.60%) and α- limonene (5.27%). About 64.94% of the AGRO was mainly compose of oxygenated compounds such as ester (43%), carboxylic acid (12.38%) and benzoates (9.56%). Esters are the common compound found in the aromatic plants and derived from the interaction between carboxylic acid and alcohol. Previous study demonstrated that ester in essential oil shown to have antimicrobial activity (Inouye *et al.*, 2001). Although no specific study on the repellent effect or mosquitocidal of ester compounds in essential oil were conducted, plant

species that contain esters as one of the chemical compounds shown to provide repellent and mosquitocidal effect (Nerio *et al.,* 2010; Seebaluck *et al.,* 2015). Therefore, esters detected as the main compound of AGRO may contribute in its repellent activity.

The remaining compounds found in AGRO were terpenes such as 1-undecene, 3-methyl pentane, α-thujene, α-phellandrene, p-cymene, β-pinene, and α-limonene. Among those terpenes, 1-undecene contributed the highest percentage. Undecene was among the common compounds that can be found in plant essential oil including rhizome oil of *Kaempferia galanga* (Bhuiyan *et al.,* 2008) and essential oils from *Porophyllum ruderale* (10%) (Souza *et al.,* 2003). Previous study on 1-undecene demonstrated that this compound has antifeedant activity against pest *Ambrostoma quadriimpressum* (Coleoptera: Chrysomelidae) (Wang *et al.,* 2016) and insecticidal effect against fruit fly *Dacus dorsalis* Hendel (Marr & Tang, 1992).

Previous studies also demonstrated that all remaining terpenes such as α-thujene, α-phellandrene, p-cymene, β-pinene, and α-limonene were involved in repellent and insecticides activity (Nerio *et al.,* 2010). For instance, it was demonstrated that the insecticidal activities of leaf essential oils of *Lantana camara, Tagetes minuta, Satureja khuzestanica* and *Ligusticum hultenii* were linked with separate and synergistic actions of monoterpenoids in the oils (Meepagala *et al.,* 2006; Koul *et al.,* 2008). Therefore, these minor components might have a critical part for action in repellent activity, possibly by producing a synergistic effect between other compounds.

It is interesting to note that the composition of AGRO determined in this study differs from that described by other authors. De Pooter *et al.* (1985) reported that galanga

oil from Malaysia contain terpenes as major components such as (E)- farnasene (18.2%), β-bisabolene (16.2%), α-bergamontene (10.7%) and α-pinene (10.2%). In India, Jirovetz *et al.* (2003) demonstrated that rhizome oil of galangal contained mainly 1,8-cineole (28.4%), α-fenchyl acetate (18.4%), camphor (7.7%), (E)-methyl cinnamate (4.2%) and guaiol (3.3%). Another study also reported that the main compounds of galanga oil consist of 1,8-cineole, β-caryophyllene, β-selinene and 1,2-benzenedicarboxylic acid (Mayachiew & Devahastin, 2008). In year 2016, Abdullah and co-researcher reported that the rhizome of *A. galanga* from Malaysia having 1,8- cineol as the main compound (Abdullah *et al.*, 2016). The dissimilarity observed might be due to several factors such as planting, climate seasonal and experimental condition that can influence the composition of plant essential oil (Mayachiew & Devahastin 2008).

The main compound for CGPO and CALO is D-limonene which contributed 91.89% and 96.09% of the essential oil of CGPO and CALO, respectively. The remaining compounds for CGPO are β-myrcene, β-pinene, β-cymene, α-terpinene, caryophyllene, β-ocimene, α-phellandrene, α-Zingiberene, α-thujene and α-cubebene while cyclohexene, crodamol, linalool, β-pinene and α-phellandrene are the remaining compounds for CALO. Similar compound have been reported in several other studies. Tu *et al.* (2002) found that Vietnamese pummelo peel oil consist mainly of limonene which represent more than 90% of the peel oil. In Tunisia, Hosni *et al.* (2010) also reported that most of the component in peel oil is D-limonene (95.4%) followed by β-pinene, β-ocimene, sabine and α-pinene. D-limonene is cyclohexanoid monoterpene found in a variety of plants, particularly in oils of lemon, orange and bergamot. As previously reported, limonene not only has antitumor,

antibiotic and antiprotozoa properties, but it also shown to be effective as insecticides and repellent agent (Raphael & Kuttan, 2003; Arruda *et al.*, 2009).

5.3 MICROENCAPSULATION METHODS

In the attempt to produce formulation that possesses longer protection time/repellent effect to then use in the development of repellent product, microencapsulation method was employed. Several studies have proposed different techniques with regards to encapsulation of essential oil for the purpose of repellent product development (Maji *et al.*, 2007; Kasting *et al.*, 2008; Karr *et al.*, 2012). Basically, encapsulation methods are categorized into three categories namely physical, chemical and physicochemical methods. **Physical method** include techniques such as extrusion, fluidized bed, pan coating and atomization (spinning disk, spray drying and spray chilling) while **chemical method** include techniques such as *in situ* polymerization, interfacial polymerization and interfacial precipitation. Techniques such as coacervation, solvent evaporation, liposome and sol-gel were techniques under the **physicochemical method**. The selection for the most appropriate technique to be used depend on many parameters such as the particle size requirement, thermal and chemical stability of the active ingredient, and several other variables such as requirement for specialized equipment and cost (Mishra, 2015).

As far as particle size is concerned, physical method is known to produce particle size that range between 10 µm to 10,000 µm. Chemical method on the other hand is known to produce smaller range of particle sizes that range between 0.1 µm to 500 µm. Meanwhile some techniques under the physicochemical method (i.e sol-gel and liposome)

are known to produce nano size particle that range between 10 to 100 nm (Mishra, 2015). Based on previous study in microencapsulation application for repellent development, the particle size required was in the range between 2.8 to 30 µm, indicating that chemical method is more likely to be the suitable method to be employed for encapsulation of essential oil for repellent application. Particle having size of this range have shown not only produce better controlled release of EOs and DEET but able to decrease its permeation through skin (Maji *et al.*, 2007; Kasting *et al.*, 2008; Karr *et al.*, 2012).

Apart from choosing the right size of the particle, the types of core materials used in the encapsulation process also play an important role in technique selection. Some of the techniques such as extrusion and spray drying techniques are applied when gas and solids core materials are used. While others, such as spinning disk technique is found most suitable when high viscosity liquids and high solids core materials is used (Mishra, 2015). Most of the chemical and physicochemical methods are usually used to encapsulate oil-in water emulsions, water- in oil emulsion to produce core-shell capsule with stable dispersion. In this current study, the core materials were oil-in water emulsion therefore chemical and physicochemical methods were obviously the methods most suitable to be applied. Furthermore, in some other techniques such as spray chilling or spray coating under the physical method require the core materials to be exposed to elevated temperature for longer period of time a condition that is unsuitable for encapsulation of essential oil as it can cause physical and chemical instability compared to chemical and physicochemical methods that can be carried out at room temperature (Mishra, 2015).

The availability of specialized equipment also plays an important role in the selection of the encapsulation technique. It can be difficult sometimes when specialized equipment is required to perform a technique. For example the need to have the spray drying equipment when performing atomization technique or the need to have fluid bed coating and coextrusion equipment in order to perform coextrusion technique. These equipments could be costly to have and maintain. It is always easier when the suitable technique to be used require no specialized equipment and involve only small scale, simple laboratory glassware, mixing, homogenization, temperature control, and pH monitoring as the general required components (Mishra, 2015). After considering all the above factors, for this current study the interfacial precipitation technique under the chemical method was selected to encapsulate EOs of the selected plants and DEET.

5.4 CHARACTERISTICS OF THE MICROCAPSULES

Microencapsulation performed on EOs and DEET produced semisolid suspensions presented as a milky white liquid without any of the EOs and DEET being detected either on the surface of the suspensions or on the discarded aqueous supernatant after centrifugation. In addition there was no coalescence and/or other appearance of emulsion failure detected indicating possibility of very high encapsulation efficiency has taken placed (Kasting *et al.,* 2008).

Morphologically, microencapsulation has produced mononuclear type of microcapsule for EOs and DEET in which the cores enclosed within the shell and concentrated mainly in the center of microcapsules with wall membrane surrounding

around the center. Dong *et al.* (2011) reported that this type of microcapsules own excellent controlled-release characteristics suitable for repellent application.

These particles were having size ranged between 2-8 µm bearing round and oval shape with no irregular microcapsules and no agglomeration observed. This result is in line with findings to that obtained by other studies using similar technique (Kasting *et al.,* 2008; Karr *et al.,* 2012). Microcapsules of this size are suitable for controlled release applications in repellent product. Nevertheless, our results were also found in contrast with other studies. These studies however used different technique of microencapsulation. For examples work by Sakulku *et al.* (2009) found that Citronella oil encapsulated by using high pressure homogenization technique produced particle sizes that range between 100 to 200 nm and work by Maji *et al.* (2007) that demonstrated that *Zanthoxylum limonella* oil encapsulated by using coacervation technique produced particle size of 20 µm thus proven that different technique of microencapsulation produced different particle sizes (Mishra, 2015).

The particle size distribution of the microcapsules was characterized by the polydispersity index (PDI). Dispersity is a measure of the heterogeneity of sizes of microcapsules in a system. A system is called uniform (monodisperse) if the microcapsules have same size, shape or mass. In contrast, a system that has an inconsistent size, shape and mass distribution is called non-uniform (polydisperse). Results obtained show that the PDI values for both EOs and DEET microcapsules were between 0.5-0.6 and 0.4, respectively. Indicating polydisperse type of particle size distribution with DEET microcapsules had moderate polydisperse type while EOs microcapsules had broad polydisperse type

(Malvern Technical Note, 2011). There is no general limit for acceptable polydispersity index; however values smaller than 0.05 are rarely seen other than with highly monodisperse standard. Values greater than 0.7 indicate the system has very broad size of distribution. The fact that PDI value obtained was range between 0.05 - 0.6 indicated that the technique selected is suitable.

Generally, the particle size and the particle size distribution are influence by several factors applied in the encapsulation technique. Those factors include agitation rate, surfactant concentration, viscosity of the reactant media, and temperature (Podshivalov *et al.*, 2013). According to Podshivalov *et al.* (2013), high stirring rate or homogenization rate will produced smaller particle compared to low stirring rate as it increases the break-up of oil droplets. The stirring rate therefore enables the mean particle size of microcapsules to be controlled (Touitou, 1998). Narrower particle size distribution and high loading capacity also can be obtained by increasing the stirring rate, therefore more effective use of shell materials for encapsulation for larger amount of oil. In contrast, at lower agitation rate, the tendency of the droplets to coalesce and aggregate is higher and the outcome is larger mean particle size and a wider size distribution (Brown *et al.*, 2003). In order to produce a certain preferred range of particle size and particle size distribution, stirring rate has to be optimized. In this study, the stirring rate applied already been optimized in previous study by Karr *et al.* (2012) and Kasting *et al.* (2008), hence the particle sizes and the particle size distribution obtained were assumed and found to be similar to these works.

Surfactant plays an important role in the synthesis of individual spherical microcapsules through a combination of interfacial tension reduction and increasing the

repulsive between the droplets when they collide (Yuan *et al.,* 2009). The choice and concentrations of the surfactant used determine the microcapsules size produced. Several works have shown the effect of surfactant type and concentration on the size of microcapsules. Thakare *et al.* (2013) for example used Tween 20, Tween 60, and Tween 85 to study the effect of surfactant type on the ethylcellulose microcapsule of theophylline and found that the mean particle sizes increased with the decreased in the Hydrophile-Lipophile Balance (HLB) value of the surfactant used. Beyger and Nairn (2012) used Span 80 to produce cellulose acetate phthalate microcapsule and found that the particle size decreased as the surfactant concentration increased.

A viscous dispersed phase retard the emulsification rate which leads to formation of a relatively coarse emulsion with larger particle size. The probability of the collision between the droplets is increased in high viscous phase since the density of dispersed droplets in the fixed vessel is higher. As a result, the coalescence rate increases and the size of microcapsules increase. The viscosity of polymer or wall materials also involved in the viscosity of dispersed phase. Both the molecular weight and concentration of the polymer affect the viscosity of the polymer solution. Either increasing polymer concentration or using a polymer with higher molecular will results in larger microcapsules. It was demonstrated in this study, various viscosity of dispersed phase of EOs and DEET may involve in the different particle sizes obtained. DEET which is more viscous (~80 cp) compared to EOs (~50 cp), has resulted in DEET microcapsules showing larger size compared to EOs microcapsules.

By performing microencapsulation encapsulated AGRO, CGPO, CALO and DEET demonstrated encapsulation efficacy (EE) of more than 95%. EE reflects the presence of oil inside the wall material of encapsulated particles (Lewandowski *et al.,* 2012). The sequence of EE in this study was as follows: DEET>CALO>AGRO>CGPO and found corresponded with the results of the particle size measured earlier. According to Jyothi *et al.* (2012) higher EE usually associated with larger particle size and it was shown in this study and study by Karr *et al.* (2012) where DEET encapsulated using similar technique demonstrated very high EE (98%).

Studies have shown that EE can be influenced by several factors such as stirring rate, ratio between core and wall materials and concentration of wall material (Maji & Hussain, 2008; Solomon *et al.*, 2011; Jyothi *et al.*, 2012). By optimizing these factors during encapsulation process it allows high EE to be obtained. Work by Solomon *et al.* (2011) reported that when the stirring rate is increased from 500 rpm to 720 rpm the EE shown to increase from 30% to 60%. The EE increased from 34% to 60% when the ratio of core material (*Zanthoxylum limonella*) and wall materials (chitosan: gelatin) were decreased from 4:0.25:1 to 4:1:1 (Maji & Hussain, 2009). The EE also increased with the increasing polymer concentration (Li *et al.,* 1999; Jyothi *et al.,* 2012). For examples, EE increased from 53.1 to 70.9% when concentration of the polymer increased from 20.0 to 32.5% (Mehta *et al.,* 1996). According to Rafati *et al.* (1997) highly concentrated polymer precipitates faster on the surface of the core materials and prevented the core material diffusion across the phase boundary. Based on the results it was believed that the EE of EOs and DEET have been improved due to the microencapsulation. Nevertheless, there were several studies that applied encapsulation technique for repellent application that

demonstrated EE which was not as high. For example, study conducted by Solomon *et al.* (2011) and Maji and Hussain (2009) demonstrated that encapsulation of citronella oil and *Zanthoxylum limonella* oil respectively using coacervation technique produced EE of 60% only. It looks like different encapsulation technique may contribute in the EE achieved. The fact that the EE obtained in this study were high (> 96% for all EOs) indicated that the technique selected was suitable to be used to encapsulate EOs.

The measurement of zeta potential was done in order to measure the charge on a particle surface used as indicator that determines the stability of an emulsion. High absolute values, whether negative or positive lead to repulsive forces between particles which improve the repellency between particles, thus inhibiting aggregations (Müller, 1996). Low potential values on the contrary, indicate the absence of repulsive interaction resulted in agglomeration and the emulsion become unstable and precipitate (Morrison & Ross, 2002). According to Greenwood and Kendall (1999), zeta potential that value from ±40 to ± 60 indicate good stability. The zeta potential of EOs and DEET microcapsules in this study demonstrated strong negative charge that range between -43 to -47.9 mV indicating that the particles in the suspension are stable and less tendency to build up flocculation.

The negative charges of microcapsules are due to the carboxylate ion ($-COO^-$) and chloride ion (Cl^-) in the emulsion system. The reaction between CMC (1st wall reactant) and BKC (2nd wall reactant) produces two pairs of oppositely charged ions namely benzalkonium methylcellulose carboxylate and sodium chloride. Benzalkonium methylcellulose carboxylate is poorly soluble at the phase interface and precipitates to form

the wall of the microcapsule while sodium chloride is typically fairly water soluble and readily dissolves in the aqueous medium (Speaker *et al.,* 1989).

FTIR analyses on EOs and DEET microcapsules indicated that both of these active ingredients were successfully encapsulated and presence of interaction between the two wall reactant, BKC and CMC. The presence of CMC and BKC in the microcapsules was verified by the presence of peak at 3323 for AGRO microcapsule, 3327 for CGPO microcapsule, 3325 for CALO microcapsule, and 3321 cm^{-1} for DEET microcapsule. These peaks were referred to the–OH group and N-H group from CMC and BKC, respectively (Lawrie *et al.,* 2007; Natrajan *et al.,* 2015). The chemical interaction between CMC and BKC were confirmed by the presence of stretching frequency of –OH (3280 cm^{-1}) in CMC and N-H group (3395 cm^{-1}) in BKC shifted to 3323 cm^{-1} for AGRO microcapsule, to 3327 cm^{-1} for CGPO microcapsule, to 3325 cm^{-1} for CALO microcapsule and to 3321 cm^{-1} for DEET microcapsule. As reported by Yadav *et al.* (2014) and Natrajan *et al.* (2015) this shifting indicated the presence of –H bonding type interaction between CMC and BKC. In addition, the presence of new peak at 1636 for AGRO microcapsule, 1633 for CGPO microcapsule, 1641 for CALO microcapsule, and 1642 for DEET microcapsule associated with the formation of –CONH- group which confirmed the formation of membrane around the microcapsule due to the electrostatic interaction between –COOH group of CMC and $–NH_2$ group of BKC (Theron *et al.*, 2012; Gite *et al.,* 2015). Most of the characteristic peaks of EOs and DEET could be observed in the spectrum of their microcapsule with minor differences in frequencies indicating successful incorporation of EOs or DEET into the microcapsule and chemical stability after encapsulation (Senhorini *et al.,* 2012).

Encapsulation also significantly improved the thermal stability of EOs and DEET shown by the TGA curve where the weight loss rate of the microcapsules was found slower that their pure compounds. Based on studies by Gite *et al.* (2015) and Fei *et al.* (2015), such results indicated that the wall materials, CMC and BKC have encapsulated these compounds appropriately. In addition, due to the compact encapsulation of the EOs and DEET microcapsules, the thermal stability curve of the microcapsule were found more complicated than their pure compounds thus suggesting that the EOs and DEET were most likely being successfully encapsulated (Wu *et al.,* 2014).

5.5 CHARACTERISTICS OF THE FORMULATION

To date, there are many topical mosquito repellents in the market and made available in many forms: spray, creams, lotions, aerosols, oils, evaporators, patch and canister. Despite of the remarkable advancement in the insect repellent research, the menace of vector-borne diseases is yet to be controlled effectively and the main reason behind this failure as far as repellent application is concerned is the poor user compliance. For example, topical repellents need to be reapplied frequently, which certainly results in inconsistent amenability and inaccurate application and thus are considered to be unreliable vector control tools (Moore, 2016; Islam *et al.*, 2017). Thus one approach that can be explored to overcome this problem is to design formulation which could enhance user compliance and acceptability to be used in the development of topical repellent.

With the intention of developing new effective insect repellent using formulation with improved efficacy; it is important to investigate the manufacturing conditions and parameters that can influence the efficacy as well as the safety of the formulation prepared (Islam *et al.*, 2017). An understanding of the relationships between product performance, material properties and structural attributes enables the designer to select the proper ingredients and design the manufacturing process to obtain a product with the desired performance. For example, the used of microencapsulation technique that produce controlled release formulation has shown to improve the effectiveness of the repellent. In addition it also helped the formulation to demonstrate better physical characteristics (such as their spreadreadily, emolliency, aroma, pleasant feeling and non-greasy feel) upon application on skin that can influence the consumer acceptance (Cheng *et al*., 2009).

In this present study, the lotion form was chosen as the ideal repellent formulation. Lotion form formulation was found to be less viscous and have better spreadreadily property compared to cream form formulation therefore qualify as more suitable for outdoor use and right for a larger area application such as leg and body (Cheng *et al.,* 2009). Unlike cream form formulation is more for application on smaller area, therefore unsuitable to be used for outdoor purpose.

The fact that the spreadreadily property is influence by the viscosity of the formulation it has to be adjusted by optimizing the amount of thickener agent (emulsifying wax) used during formulation process (Cheng *et al.,* 2009). According to Cheng *et al.* (2009), viscosity between 6000 and 50,000 cp would give a spreadreadily effect to the formulation and pleasant feel (non-greasy) upon application on the large area of skin

(Cheng *et al.,* 2009). In this study the viscosity of the formulations obtained right after the preparation were between 6200 to 7700 cp and this was consistence with the results obtained from organoleptic assay where all the formulations prepared have spreadreadily and non-greasy feel when applied on the skin.

Cheng *et al.* (2009) in their study reported that most consumers expressed their dislike for product which is not smooth and having greasy feeling. Therefore these are the sensorial quality factors that need to be considered to fulfill the user compliance. Since EOs and DEET are oily in nature, step that can hinder the greasy feel of these active ingredients clearly needs to be addressed. For this purpose, a humectant, an agent that can give moisturizing effect such as glycerol and propylene glycol and emollient agent that can give softening effect such as sweet almond oil and coconut oil can be added to the formulation. In this study, the types and amount of each humectant and emollient agents used were based on the ingredient mostly used and with suggested concentration by previous researcher. Cheng *et al.* (2009) provided list of all ingredients and the suggested concentrations commonly used in cosmetic products.

It can be observed from this study that there was no phase separation shown by the formulation right after its preparation indicating that aqueous and oil phase were well homogenized and the emulsification was successful (Cheng *et al.,* 2009). In fact while preparing the formulation at the point where oil phase was added to water phase the emulsification shown to occur spontaneously indicating that the type and amount of emulsifier used were suitable and sufficient to produce well homogenized formulation.

According to Cheng *et al.* (2009), the types and concentrations of emulsifier used in the formulation were the factors that determine the effective homogenization.

As expected, the particle sizes measurement on the both formulations indicated that ME formulation of having micron size particle range between 3.29 to 6.50 µm, while NE formulation presented nano size particle range between 210 to 530 nm. The larger sizes of particle size in ME formulation is due to the presence of microcapsules of EOs/DEET as their active ingredients. The particle size is the factor involved in producing the softness feeling of the formulations. According to Cheng *et al*. (2009), the formulations with particle size range from 1 to 10 µm should have an acceptable softness. This study however demonstrated that formulation having nano size particles presented softer texture compared to formulation based on particles of micron size.

The zeta potential values of both formulations also shown to follow the zeta potential values of the particles used to prepare the formulation. As explained earlier, for particles with high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential value is small, attractive forces may exceed this repulsion and the dispersion may break and flocculate (Hanaor *et al.*, 2012). According to Greenwood and Kendall (1999); zeta potential value from ±30 to ± 60 indicate good stability and the particles in the suspension are stable and having less tendency to build up flocculation. Therefore, it can be concluded that all the formulations prepared in this study either ME or NE formulations have obtained good stability.

All the formulations prepared in this study presented pH that range from 5.40 to 5.50. This range of pH value was found suitable for product to be applied on the skin (Flynn *et al*., 2001). The pH range from 5.0 to 5.5 was recommended for the entire topical product since too high and to low pH value will lead to skin irritation and sensitization (Cheng *et al*., 2009). In a normal condition, the pH of the skin is between 5 and 6; however, an application of any topical products including repellent can change this value (Flynn *et al.*, 2001). Hence, it is a requirement to prepare a formulation that has a pH which is in accordance with the skin's pH to ensure tolerance. Therefore, in this study both formulations were prepared for pH between the ranged of 5.0 to 5.5 by adding triethanolamine (TEA) gradually into the formulation until the desired pH were achieved.

Based on the results obtained it can be concluded that all formulations showed good physical characteristic: well homogenized, stable and soft upon application on the skin. Additionally, the great aroma from the essential oils itself was pleasant and the smell of DEET was hindered by the aroma that came from sweet almond oil and coconut oil in the formulations. The moisturizing effect that come from the glycerol, butter and oil also gave extra benefit to the formulation.

5.6 EFFECTIVENESS OF THE FORMULATION

Plant essential oils are rich sources of many compounds, such as hydrocarbon terpenes and terpenoids which can be utilized in the development of alternative repellents to synthetic repellents. Studies however have shown that repellents based on essential oils generally do not provide sufficient repellent effect against mosquito bites (in terms of 100%

repellent effect maintained for a long duration) compared to synthetic repellents where complete protection (100%) can be maintained between 6–8 hours. It is well understood now that such limitation is caused by the high volatility nature of the essential oils (Trongtokit *et al.,* 2005a).

In order to overcome this limitation, some of the techniques applied in the production of plant-based repellents are making use of fixative materials such as vanillin, polymers, and adjuvant to control the volatility of the essential oils (Nerio *et al.,* 2010). Although such fixative materials were shown to have helped increase the repellent effect the duration of the repellent effect maintained is yet to match the duration of the synthetic repellent. Most of the plant-based repellent products in the market have less than two hours of 100% repellent effect. Several formulation techniques have been developed to help improve previous techniques in addressing this limitation and one of techniques is microencapsulation method (Nerio *et al.,* 2010).

This present study showed that the microencapsulation method helped to extend the duration of the repellent effect of the formulations prepared. Evaluation performed in the laboratory and field conditions, demonstrated that the ME formulation possessed repellent effect of having duration between 1–2 hours compared with the NE formulation for all the essential oils. In line with this finding, other researchers also demonstrated ME formulation presented better repellent effect compared with NE formulation. For example study by Sakulku *et al.* (2009) showed citronella oil encapsulated by using high pressure homogenization technique demonstrated 95% protection against *Ae. aegypti* bites for 2.8 hours. Study by Yuvasri *et al.* (2016) on encapsulated citronella oil using complex

coacervation technique and treated on cotton fabric demonstrated better repellency effect (90%) compared to direct oil-treated fabric for 9 days.

The presence of the wall surrounding the essential oil droplets produced by microencapsulation is shown to have provided several advantages to the ME formulations. The use of this method not only created better controlled release of the essential oil, the larger droplet size of the microcapsules formed tend to inhibit skin penetration, therefore decreasing the amount of metabolites of the essential oil that may penetrate the skin. In addition, the polymer added in the formulation provides skin resistance effect to the formulation by reducing the wash out effect from perspiration. Both characteristics allow more essential oil to be retained on the skin for action against mosquito bites, hence extending the required repellent effect (Calton *et al.,* 2001: Karr *et al.,* 2012).

This present study demonstrated that DEET- based formulation of having significantly better repellent effect compared to EOs- based formulation in both laboratory and field conditions. DEET being accepted widely as the synthetic gold standard for repellent able to provide 8 hours of protection against mosquito bites in both conditions (Tawatsin *et al.,* 2001; Thavara *et al.,* 2002). DEET was designed to be relatively non-volatile having boiling point at 280°C (McCabe *et al,.* 1954) to confers lower evaporation rate and, consequently, a long protection time is achieved, which is one of the essential properties a repellent must have (Leal, 2014). In addition, factors such as environmental conditions (temperature and humidity) and the volunteers' perspiration are believed to have exaggerated the poor physical-chemistry properties possessed by the essential oils compared to DEET.

Interestingly, although the ME formulation of EOs did not perform as excellent as the ME formulation of DEET the ME formulation of EOs showed comparable effect compared to Citriodiol®-based repellent (Mosiguard®) by providing >80% protection for 6 hours. Citriodiol® is the trade name of p-menthane-3,8-diol (PMD) and also known as oil of lemon eucalyptus (OLE). PMD-based insect repellents had been approved by USEPA for protection against mosquitoes and were the only plant-based repellent that has been promoted for use in disease endemic areas by the Centres for Disease Control (CDC) (Emily Zielinski-Gutierez &Roger, 2010; Maia & Moore, 2011).

In the laboratory and field conditions, all ME formulations of EOs demonstrated repellent effect that was better than several citronella-based repellents (KAPS®, MozAway®, and BioZ Natural®). In agreement with this finding was a study done by Tuetun *et al.* (2008) who reported that most of the commercial citronella-based repellents tested were found to show 100% repellent effect for only half an hour. Surprisingly some of these citronella-based repellents were even found to have no repellent effect against mosquitoes namely Buzz Away® (containing citronella, cedar wood, eucalyptus, lemongrass, alcohol, and water) and Green Ban® (containing citronella, cajuput, lavender, safrole-free sassafras, peppermint, bergaptene-free bergamot, calendula, soya, and tea tree oils) (Trongtokit *et al.*, 2004). To the best of our knowledge, all these repellents were prepared as lotion and spray forms without undergoing the microencapsulation method. Therefore, performing microencapsulation on EOs before preparation of formulation proved help prolong the duration of repellent effect.

This present study demonstrated that there was no significant different in the efficacy level for all formulations against both species, *Ae. aegypti* and *Cx. quinquefasciatus* when tested in laboratory condition ($p>0.05$). Several studies that conducted repellent study against different species/genus of mosquito also showed similar findings. For examples, study conducted by Soonwera and Phasomkusolsil (2015) demonstrated that ylang-ylang oil and lemongrass oil repellent formulations showed no significant different in their repellent effect against both species, *Ae. aegypti* and *Cx. quinquefasciatus.* Another study conducted by Gokulakrishnan *et al.* (2013) also demonstrated that five major compounds in *Pogostemon cablin* EO provided no significant different in their repellent effect against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus*. In contrast, study by Sritabutra *et al.* (2011) revealed that the combination of eucalyptus oil and sweet basil oil presented significant different in their repellent effect against *Ae. aegypti* and *An. dirus*. Study by Govindarajan (2011) also reported that *Zingiber officinale* offered significant difference in their repellent effect against *Cx. quinquefasciatus* and *An. subpictus*. Both studies suggested that the different in the effectiveness of repellent formulation/active ingredient seen on different species of mosquito could be due to the mosquito species sensitivity towards repellent formulation/active ingredient tested. Some mosquitoes were believed to be more sensitive to the repellent formulation/active ingredient than others (Sritabutra *et al.*, 2011). However, it will be good if the repellent formulation provide a fair repellent effect without counting the different between mosquito species.

Among the EOs AGRO formulation presented better repellent effect compared to CGPO and CALO formulation in both laboratory and field condition. AGRO was already

known for having repellent effect against mosquito bites as reported in several studies. Tawatsin *et al.* (2006) reported that 10% AGRO lotion formulation provided 0.6 and 7.8 hours of protection against *Ae. aegypti* and *Ae. albopictus*, respectively. AGRO formulated in mosquito coil also demonstrated high knockdown effect (62%) against mosquitoes in the field condition (Tawatsin *et al.*, 2002). Interestingly, in the study by Sinthusiri and Soonwera (2013), AGRO was also shown to have a knockdown effect against houseflies *Musca domestica* L. indicating the possibility of AGRO to be employed as active ingredient in repellent and in insecticide products.

As for *Citrus*-based formulation study done by Reegan *et al.* (2014) recorded that essential oil-based cream formulation containing peel oil of fruit *C. aurantifolia* and *C sinensis* provided 3 hours and 4 hours of protection (90%) against *Ae. aegypti* and *Cx. quinquefasciatus*, respectively. Another study by Tawatsin *et al.* (2001) reported that formulation of 25% *C. hystrix* essential oil with 5% vanillin provided 3 hours of protection (90%) against *Ae. Aegypti* and formulation of *C. sinensis* essential oil with eucalyptus oil and citronella oil in ethyl alcohol demonstrated 2.5 hours of protection (90%) against *Cx. quinquefasciatus* (Phasomkusolsil & Soonwera, 2010).

The difference seen in the efficacy level between EOs might have something to do with the chemical compounds possessed by individual EOs. Result from GC-MS indicated that more than 90% of AGRO contained aromatic compounds such as benzoic acid, carboxylic acid and ester. Previous study by Bernard *et al.* (1995) reported that benzoic acid derivatives from Piperaceae plants had insecticidal effect against Lepidopteran, *Ostrinia nubilalis*. The repellent activity of carboxylic acid had been widely studied since

1970s. In 1973, Johnson and co-investigators reported that carboxylic acid derivatives shown to have repellent effect *Ae. aegypti*. The repellent activity of ester also had been reported since 1980, when McGovern and Schreck (1988) demonstrated that it provided 21 days of protection against mosquito when impregnated on cloth. In 2013, Seenivasagan *et al.* demonstrated that 14 from 16 esters that had been studied shown to provide highly significant repellency (67-96%) against *Culex quinquefasciatus*. Based on these findings, in can be concluded that all chemical compounds of AGRO possess excellent repellent effect against mosquito and their synergistic effect may also contributed to high repellent effect of AGRO.

As for CGPO and CALO D-limonene was the main compounds that contributed around 90% of the whole EOs. D-limonene was categorized under oxygenated monoterpene hydrocarbons (Parthasarathy & Zachariah, 2008) and this compound also had been known to have repellent effect against mosquito (Jaenson *et al.*, 2006). Most of the research however, demonstrated that limonene have low repellent effect and generally lasted less than 1 hour (Thorsell *et al.*, 1998). The high volatility of the D-limonene was suggested contributed to the low repellent effect of CGPO and CALO (Thorsell *et al.*, 1998). Although the pure compounds of CGPO and CALO presented low repellent effect, once exposed to microencapsulation method the duration of repellent effect were found extended. This present study demonstrated that ME CGPO and CALO formulations presented >90% protection for 4 hours.

5.7 PHYSICAL STABILITY OF FORMULATIONS

During the development of the repellent product, it is necessary to determine the suitable storage condition in which the stability of the repellent can be preserved. For example, unsuitable storage temperature can change the physical stability of the formulation and lead to the destabilization effect and that will have a direct effect on the quality, efficacy and safety of the product (Bilia *et al.*, 2001).

The centrifugation test provided fast information regarding the stability properties of the emulsion system in the formulation. Instability of the emulsion is indicated when phase separation occurred after centrifugation (Marquele-Oliveira *et al.*, 2007). In this present study it was demonstrated that during the 12 months of storage, the ME and NE formulations presented no phase separation when stored at 25^0C storage condition compared to 40^0C storage condition indicating that 25^0C storage condition allows stability of the formulations to be maintained.

The organoleptic assays also demonstrated that at 25^0C storage condition both formulations presented no changes in their physical appearance over 12 months of storage. Compared to 40^0C storage condition both formulations presented changes in physical appearance after six months of storage which was consistent with the centrifugation study, where the physical instability of the emulsion system in the formulations was observed after six months of storage when stored at 40^0C storage condition. Idson (1993) in his article stated that the physical instability of the emulsion system will not only cause phase

separation, it can also leads to the changes in appearance, consistency, spreadability and performance.

This present study also demonstrated that at 25^0C storage condition, ME formulation presented no significant reduction in the particle sizes over 12 months of storage ($p>0.05$). NE formulations however presented significant upsurge in particle size after three months of storage ($p<0.05$) suggesting encapsulation help to retain the particle size when stored at 25^0C storage condition. This study also revealed that both types of formulations presented contrary trend in particle sizes during the 12 months period. This finding was parallel to the previous study by Karr *et al.* (2012) who reported that the particle size of DEET microcapsules showed decline trend after three months of storage. This reduction was believed caused by the release of the essential oil within the microcapsule into continues phase, thus decreasing the particle size during storage. Increase in particle size of the NE formulation during storage indicated coalescence between the particles due to coalescence phenomena as suggested by several other studies (Marquele-Oliveira *et al.*, 2007).

In emulsion system, generally all particles are constantly moving in all directions known as Brownian motion. High temperature will increase the Brownian motion between the particles and therefore causing frequent collisions between them (Tadros, 2013). Some of the particles will get ruptured and some will coalesce to form a large droplet. Exposure to higher temperature could cause exaggeration of these mechanisms in which the release rate of the essential oil to the microcapsule formulation and increase the coalescence to the pure formulation. If this process continues, the dispersed phase will separate from the

continues phase and the emulsion will break (Tadros, 2013). This was consistence with the findings based on centrifugation test where both formulations showed phase separation after 12 months when stored particularly at 40^0C condition.

During the storage at 25^OC condition, it can be observed that ME formulations showed no significant changes in zeta potential value over 12 months of storage while NE formulation presented significant reduction in zeta potential value after six months of storage. According to Morrison and Ross (2002), zeta potential values are divided into several grades. Zeta potential value from ±60 to ±40 mV indicates high stability of the emulsion system, ±40 to 30 mV indicate moderate stability, ±30 to ±15 mV indicate light dispersion and ±15 to ±10 mV indicate agglomeration. Based on these grades, ME EOs and DEET formulations demonstrated high stability while NE formulations presented moderate stability.

These results were found in line with the theory regarding zeta potential value. In the case of ME formulations, high zeta potential values provide strong repulsive forces between particles, so aggregations are prevented and the emulsion system becomes stable. On the contrary NE formulations with low zeta potential values caused low repulsive forces and leads to particle aggregation and instability of the emulsion system (Morrison & Ross, 2002).

At 40^0C storage condition six months and 12 months storage resulted in the instability for both formulations as ME formulation demonstrated agglomeration and NE formulation indicated precipitation and phase separation. Temperature is one of the factors

that can changes of zeta potential values. Exposure to high temperature will decrease the repulsive forces between the particles which then encourage aggregation and caused the emulsion system to become unstable (Gaonkar, 1991; Tadrus, 2013).

Since alteration in pH value can cause formulation to become incompatible with the skin and may cause skin irritation (Marquele-Oliveira *et al.,* 2007), it is important to detect pH alterations of the formulations during storage. Results based on stability study indicated that although the pH was shown to reduce for both formulations over 12 months storage at 25^0C storage condition; the change was within the range of pH 5.0 to 5.5 which is still in the range that is suitable for topical application. The decreasing of pH values in both formulations might be due to the presence of free essential oil in the emulsion system due to the release of essential oil from the microcapsule for ME formulation or due to breakdown of the particles for the NE formulation. At 40^0C storage condition, the pH value went below 5.0 (acidic state) and likely unsuitable for topical application.

In this present study, both types of formulations presented significant reduction in viscosity at 25^oC condition after six months of storage with NE formulations presented larger reduction (5.1 - 22%) compared to ME formulations (4 - 12 %). These results indicated that ME formulation possessed better stability in viscosity compared with NE formulation during the storage in 25^OC condition over the period of study. The higher viscosity level in ME formulations contributed in the better stability of ME formulations. Based on the theory of Stoke-Einstein, the diffusion coefficient was lowered in high viscosity of emulsion system and therefore lowered the coalescence between particles and improved the stability of the system (Sabliov *et al.,* 2015). When exposed to the high

temperature (40^0C), both formulations showed instability in viscosity over 12 months of storage. This was believed due to reduction in the viscosity of surfactant layers when exposed to high temperature and later promoted destabilization in the emulsion system (Mitri *et al.*, 2011).

Data regarding formulation repellent activity showed that both of the formulations demonstrated reduction in repellent activity throughout the study period however ME formulations was considered to better than the NE formulations as it possesses repellent activity that was maintained up to six months of storage compared to NE formulation (up to three months of storage). The superior repellent activity in the ME formulation was possibly due to the wall surrounding the particle that provided the slow release of the essential oil into the environment upon application to the skin. In addition microencapsulation gave protection to EOs from oxidation when exposed to the environment and chemical interaction with other chemicals inside the formulation, therefore improving their functional activity as a repellent.

This present study demonstrated that ME DEET formulation able to provide complete protection for four hours even after 12 months of storage which is far better compared to ME EOs formulations that only able to maintain their efficacy level (complete protection for 2 hours) for only six month of storage. Although the physical stability study demonstrated that both of them have similar pattern; the changes in the physical stability of ME DEET formulation did not affect its effectiveness as it affected the ME EOs formulations.

Overall, it can be concluded that all ME formulations presented better physical characteristics stability compared with NE formulations when stored at 25^0C storage condition for over 12 months of storage. Although there were no significant changes in centrifugation assay and organoleptic characteristics observed in NE formulations which indicated the emulsion system in NE formulations were stable; however the particle sizes, zeta potential values and pH values demonstrated significant changes right after six months of storage. Efficacy study also demonstrated that ME formulations able to maintain their efficacy level longer than NE formulations. This study therefore revealed that by doing microencapsulation, not only the physical stability of the formulations could be preserved but their effectiveness could also be maintained over 12 months of storage.

Since the formulation produced in this study is based mainly on mixture of oil and water therefore it is also known as emulsion. Study by Pather demonstrated that emulsion shown to have an unstable system and can caused phases in the system which ultimately separate completely (Pather *et al.*, 1995). This would leads to breakdown of the emulsion which may occur during storage by many processes including flocculation, coalescence, creaming and Ostwald ripening (Pather *et al.,* 1995). This phenomenon was believed associated with the changes in physical characteristic in both formulations during storage. However, this study revealed that by performing encapsulation these processes were made slower and allow stability to be maintained for a longer period of time.

5.8 MICROBIOLOGY TEST OF THE FORMULATION

Results of basic microbiology test showed absence of bacterial and fungus contaminations during preparations up to 12 months of storage indicating good laboratory practice was achieved during preparation and the formulations produced were able to inhibit bacterial and fungus growth during storage. According to Steinberg (2006), topical products are not necessarily to be sterile microbiologically but they have to be quality proper with regards to consumer health. Studies have shown that such products can be contaminated with microorganisms present in the production environment or in raw materials, especially water, and that the contamination can occur after production due to unhealthy storage conditions or during consumer use (Underwood, 1998; Yorgancioglu & Bayramoglu, 2013).

Microorganism contamination of topical products can cause unwanted changes to the product such as the smell, color, viscosity, and performance. Disintegrated skin may become infected while the endotoxins and metabolites produced by microorganism may cause abrasion, irritation and allergies on the skin (Yorgancioglu & Bayramoglu, 2013). Therefore, in order to avoid possibility of microorganism contamination, it is necessary to add preservative to the topical pharmaceutical product (Sasseville, 2004).

In this present study no preservative was added and yet bacterial and fungus contaminations were not detected during preparations up to 12 months of storage. According to Inouye *et al.* (2003) essential oil can be more efficient as preservative than various artificial antimicrobial agents. In fact natural food preservatives has been widely

used and accepted by the consumers, who prefer natural and healthy products with low synthetic additives (Militello *et al.,* 2011). Therefore it can be concluded that essential oils in the formulation play an important role as preservative agent as well.

Preservative capacity study demonstrated that all the formulations showed microbiology activity against all microorganisms tested by presenting inhibitory zone of more than 8 mm. All EOs used in this study had been reported to have microbiology activity by previous studies (Settanni *et al.,* 2012). As reported by Trakranrungsie *et al.* (2008) AGRO had shown to have microbiology effect against *Candida albicans* and *Trichophyton mentagrophyte*. Study conducted by Settanni *et al.* (2012) demonstrated that EOs extracted from pummelo fruit peel having antibacterial effect against *S. aureus* and *S. enterica* and study conducted by Fekrazad *et al.* (2014) demontrated that *Citrus aurantifolia* leaf oil of having antifungal effect against *C. albicans* and *Tricophyton rubrum*.

As mentioned earlier, the main compound of AGRO is benzoic acid. Since 1875, it was discovered that benzoic acid is having antifungal abilities and has been used for a long time as preservative agent (Salkowski, 1875). Benzoic acid was also found to inhibit mold and bacteria growth and widely use as food preservative represented by the E-numbers E210, E211, E212 and E213. This study therefore not only proves that AGRO formulation provide excellent repellent effect against mosquito but inhibitory effect against microorganisms.

Microencapsulation technology of essential oil for antimicrobial or food preservative had been widely studied. Study by Peng *et al*. (2014) demonstrated that encapsulation of mustard seed oil provided better antimicrobial compared with non-encapsulated oil. Another study by Khalili *et al.* (2015) also reported that encapsulated thyme essential oil provided better antimicrobial activity against *Aspergillus flavus* compared to non-encapsulated thyme oil. When antimicrobial activity of the ME formulations were monitored every three months during the 12 months of storage, all formulations presented no significant reduction in their activity indicating that they have good preservative capacity against microorganism namely *S. aureus, E.coli, C albicans and A. fumigatus*. NE formulation also presented no significant reduction in their antimicrobial activity ($p>0.05$) indicating microencapsulated or not these formulations possessed protection against contamination.

Many studies have reported that by adding natural essential oil in a product promotes excellent preservative capacity. This antimicrobial activity of essential oils is known to provide protection to the products (Bayramoglu *et al*., 2009; Bayramoglu, 2010; Sekeroglu *et al.*, 2011). Even as low as 2% concentrations, essential oils able to promote excellent preservative capacity in formulation (Yorgancioglu & Bayramoglu, 2013). One of the most widely used synthetic preservative agents is paraben. The use of paraben however has been shown to cause contact allergies (Charnock & Finsrud, 2007), disruption to estrogen hormone (Nagel *et al.*, 1977) and was linked to breast cancer (Darbre, 2003). Due to these reasons many manufactures in food, cosmetic and pharmaceutical industry prefer to use EOs as alternative to synthetic preservatives to be incorporated into their products.

5.8 SAFETY OF THE FORMULATION

The properties of ideal insect repellents are not only that they have to be effective and pleasant to apply on skin but more importantly it is safe upon application on skin (Katz *et al.*, 2008). Essential oil is one of the substances that generally recognized as safe (GRAS) by United States of Food and Drugs Administration (FDA, 2016). EOs contains chemicals such as cineole, camphor or menthol, exerts a pharmacological action by penetrating the body through different routes, i.e. by oral intake, inhalation or by dermal route after transcutaneous penetration (ANSM, 2008). Although EOs have been used in the treatment of disease according to the knowledge accumulated over centuries, it is also known that many plant synthesizes toxic substances, which in nature act as defense against infections, insects and herbivores. Previous studies have shown that some compounds present in EOs are potentially toxic (de sa Ferreira & Vargas, 1999). The dermal application of these substances may exert local toxicity after topical exposure, e.g. irritation, corrosion, burn and contact sensitization (Posadzki *et al.*, 2012). Therefore although EOs was coded as GRAS, they actually cause toxicity upon dermal application.

EPA in 1997 had designated all product containing citronella oil as slight toxic which is Toxicity Category III (EPA, 1997). Citronella oil derived from "Ceylon type" (*Cymbopogon nardus*) oil as a week dermal sensitizer while citronella oil derived from "Java type" (*Cymbopogon winterianus*) as non-sensitizer (EPA, 1997). Dermal irritation study on product containing 30% Citriodiol® (PMD) indicated Toxicity Category III (slight toxic) with all the animals showed grade 1 erythema and edema. Meanwhile for skin sensitization study, demonstrated that this product presented no positive response (Toxicity

Category Non-sensitizer) (EPA, 2000a). Contrary with this study, ME formulation of EOs and DEET presented no sign of skin irritation and sensitization and can be categorized as non- Irritant and non- sensitization (Toxicity category IV) as per EPA guidelines. Hence, these formulations can be regarded as quite safe and may be suitable for use in topical application of repellent.

This present study also demonstrated that at 20% concentration of DEET in formulation presented no irritation and sensitization effects. This was supported by previous research that also reported no irritation and sensitization signs were observed in rabbit and guinea pig when used in similar concentration, respectively. Research on formulation containing 40% of DEET presented moderate irritation in rabbit (Busch, 1985). On the other hand, no dermal irritation was observed with a formulation containing 25% DEET and another formulation containing 9% DEET and 0.09% fenvalerate (Thompson, 1980; Rothstein, 1986). EPA in 1998 has been placed DEET in Toxicity Category III (slight toxic) and non-sensitizer when applied dermally (EPA, 2000b).

The method selected to produce formulation that provides longer protection time /repellent effect to be used in the development of repellent product might also involve in the determination of the safety profile. It is well understood that immune system will be induced when the active ingredients/chemicals penetrated the skin and react with the endogenous protein which then promote the inflammatory response and lead to dermal injury (Goebel *et al.,* 2012). The microencapsulation method chosen for this study has been shown to help produced micron size particle which is larger compared to other techniques that are known to produce smaller particles (nano size). Skin penetration is avoided when

active ingredient used having particle of larger size causing absence of inflammatory response and dermal injury as shown by several studies (Kasting *et al.*, 2008; Karr *et al.*, 2012). Although these studies did not conduct safety assessment on their formulations, the results from their skin permeation study indicated that the used of microencapsulated DEET in their formulation inhibited skin absorption.

Other than active ingredients, the other substances added in the formulation may also contribute in the irritation and sensitization effect of the formulation (Dornic *et al.*, 2016). The used of surfactants and solvents in most of formulations preparation could also enhance skin penetration. For example, acetyl alcohol used as solvent and emollient had been reported to cause minimal to slight irritation to the skin of albino white rabbit when used at 4% concentration but absence in sensitization effect on guinea pig. However, clinical study using human volunteer indicated no irritation and sensitization when used at 100% pure acetyl alcohol and 8.4% concentrations in lotion formulation (CIR, 1988). In this present study no irritation and sensitization effect were detected when 3% acetyl alcohol is used of in all formulations.

Other surfactants used in this study were Tween 60 and Span 80, but both surfactants had already been verified by Cosmetic Ingredient Review (CIR) Expert Panel as safe to be used (at 1.0 to 8.4%) in a formulation for topical application (CIR, 2015). In 2013, European Union restricted the use wall reagent, BKC more than 5% concentration as it was found to cause dermal irritation (Cameron *et al.*, 2013). At lower concentrations (3%), BKC were not sensitizer in guinea pig (CIR, 2008). However another wall reagent, CMC was regarded as non-toxic and non-irritant materials and had been used widely as

polymer in food, pharmaceutical and cosmetic products. The concentration between 1-3% of cellulose and its derivatives in product formulation demonstrated no irritation effect upon application on the skin of rabbit (CIR, 2009).

Most of topical formulations that include PEGs and their derivatives as surfactant demonstrated skin irritation (CIR, 2001). Their dermal toxicity effect however depends on the molecular weight of PEG used. Low molecular weight PEG (PEG-2, 4, and 6) can be absorbed through the intact skin and may exert immunology reaction. CIR (2001) demonstrated that PEG-25 at 2% in antiperspiration product can still cause a minimal irritation effect in rabbit. Several studies showed PEGs with higher molecular (PEG-40 or greater) were unable to penetrate skin (Barany *et al.*, 2000). A formulation containing high molecular PEG was shown to effectively reduce skin penetration of organophosphate compounds (Oison *et al.,* 1991) as well as skin permeation of the insect repellent DEET (Qiu *et al.,* 1998). In this present study, high molecular weight PEG (PEG-3350) was used as barriers to dermal penetration.

CHAPTER 6

CONCLUSION

6.1 Main findings

From ethnobotanical study, ten plants species which include *Cymbopogon nardus* (L.) Rendle, *Pelargonium graveolens* L'He´r, *Citrus aurantifolia* (Christm.) Swingle, *Alpinia galanga* (L.) Willd, *Lantana camara* (L.), *Citrus grandis* Osbeck, *Etlingera elatior* (Jack) R.M. Sm, *Pogostemon cablin* (Blanco) Benth, *Tagetes erecta* (L.) and *Sesbania grandiflora* (L.) Pers were identified by the local community as repellent plant. Based on the results obtained from ethnobotanical study, three plants species were selected for this study namely *Alpinia galanga* (rhizome), *Citrus grandis* (peel) and *C. aurantifolia* (leaf). All the EOs were successfully encapsulated using interfacial precipitation technique with more than 95% of encapsulation efficiency were observed in all the microcapsules. All the microcapsules produced also were successfully incorporated into lotion formulation with no phase separation observed indicate very well homogenized. Results on efficacy study showed that all the microencapsulated formulations of essential oils possessed long lasting repellent effect (1- 2 hours more) compared with non-encapsulated formulations. Even though they presented significantly low in repellency effect when compared with synthetic repellent, DEET, when compared with Citriodiol®-based repellent (standard botanical repellent) they presented comparable effect. The formulations also demonstrate other characteristics that should make them suitable enough to be used in the development of repellent product. These characteristics include (i) efficacy level that can be maintained up to 6 months (ii) physical stability that can be maintained up to 12 months (iii) good

preservative capacity that can be maintained up to 12 months (iv) pH values that is safe for topical application (v) absence of irritation and sensitization effect upon application on the skin (vi) antimicrobial activity (vii) cosmetically appealing appearance such as smooth texture, good in spread ability, and homogenous.

In conclusion, this study has shown that the use of microencapsulation technique in the production of formulation has helped improve the characteristics of the formulation and have it used in the development of repellent will most likely help to produce new repellent that has a better effect than the existing ones.

6.2 Limitation of this study

There were several limitations encountered throughout the duration of this study.

1. The process of extracting the essential oils and the study on physical characteristic of microcapsules took several months to complete due to lack of equipment facilities and sharing basic usage.

2. The morphology study of microcapsules based on SEM and TEM were canceled due to lack of financial support.

3. Efficacy study of the formulation against *Anopheles* mosquito was not performed due to lack of mosquito supply

4. The numbers of animals used for skin sensitization study was not in accordance to that recommended by the guidelines due to financial constrain

6.3 Future studies

1. Identification and isolation of the bioactive compounds of each EO.

   The synergistic effect of compounds in EOs is undeniable; however there are also possibility that individual compound may have outstanding repellent effect.

2. The morphology study based on SEM and TEM

   By performing this study shape, surface structure and the thickness of the wall can be measured. Wall thickness is known to affect the release of the active ingredient. By knowing the thickness of the wall adjustment can be made by the optimization during microencapsulation process so that the suitable thickness is obtained.

3. The assessment of the release rate of EOs and DEET from microcapsules

   This study will determine the amount and time of EOs and DEET released from microcapsule. By knowing the amount and time of EOs and DEET released they can be optimized during the microencapsulation process, so that the optimum release rate can be achieved.

4. Evaluation on penetration rate of the microcapsules on the skin

   High penetration rate will lead to low efficacy level and will have effect on the safety of the formulation when applied on the skin.

5. Evaluation of the efficacy level in laboratory setting

To be conducted against other species of mosquito such as *Aedes albopictus* and *Anopheles spp.* because the effectiveness of the formulation could be different against these species.

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