FORMULATION, CHARACTERISATION AND *IN VITRO* SKIN IRRITATION STUDIES OF *JASMINUM OFFICINALE* AND *ANTHEMIS NOBILIS* ESSENTIAL OIL NANOEMULSION FOR *AEDES AEGYPTI* REPELLENT ACTIVITY

NITHYA NIRANJINI MUTTIAH

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by

NITHYA NIRANJINI MUTTIAH

Thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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Specially dedicated

To,

My beloved parents Mr. Muttiah Sinnathamby
and Mrs. Krishnaveni Ramasami

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LIST OF ABBREVIATIONS AND SYMBOLS

µL	Microliter
µm	Micrometer
3Rs	Replacement, refinement and reduction
amu	Atomic mass unit
ANOVA	Analysis of variance
BA	Benzoic acid
BC	Benzalkonium chloride
cm	Centimeter
CO_2	Carbon dioxide
DEET	*N*,*N*-diethyl-3-methylbenzamide
DLS	Dynamic light scattering
DPBS	Dulbecco's phosphate-buffered saline
ECVAM	European Centre for the Validation of Alternative Methods
ELISA	Enzyme-linked immunosorbent assay
eV	Electron volt
FCO	Fractionated coconut oil
FDA	Food and Drug Administration
GC-MS	Gas chromatography-mass spectrometry
GHS	Globally Harmonised System of Classification and Labelling of Chemicals
h	Hour (s)
ICH	International Conference on Harmonisation
IFN-ɣ	Interferon gamma
IS	Internal standard
LC	*Litsea cubeba*
LOD	Limit of detection
LOQ	Limit of quantification
LS	*Litsea salicifolia*
m	Meter
m/z	Ratio of mass per charge

MFI	Median fluorescence intensity
mg/cm^2	Milligram per square centimeter
mg/kg	Milligram per kilogram
mg/mL	Milligram per milliliter
min	Minute (s)
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
MTT	(3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyltetrazolium bromide)
mV	Millivolt
Na_2SO_4	Sodium sulfate
NC	Negative control
NIST	National Institute of Standards and Technology
nm	Nanometer
ºC	Degrees Celsius
ºC/min	Degrees Celcius per minute
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
PC	Positive control
PDI	Polydispersity index
pg/mL	Picogram per milliliter
ppb	Parts per billion
ppm	Parts per million
QC	Quality control
RE	Relative error
RH	Relative humidity
RI	Retention index
rpm	Revolutions per minute
RSD	Relative standard deviation
RT	Retention time
SCC	Standard culture conditions
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
SIM	Selective ion monitoring
SLS	Sodium lauryl sulfate
spp.	Species
TEM	Transmission electron microscopy
TG	Test Guideline
TIC	Total ion chromatogram
TNF-α	Tumour necrosis factor alpha
TS	Test substance
UNECE	United Nations Economic Commission for Europe
USEPA	United States Environmental Protection Agency
v/v	Volume to volume
WHO	World Health Organisation
WHOPES	World Health Organisation Pesticide Evaluation Scheme

KAJIAN FORMULASI, PENCIRIAN DAN IRITASI KULIT *IN VITRO* KE ATAS NANOEMULSI MINYAK PATI *JASMINUM OFFICINALE* DAN *ANTHEMIS NOBILIS* UNTUK AKTIVITI PENGHALAU *AEDES AEGYPTI*

ABSTRAK

Nyamuk merupakan vektor penting yang bertanggungjawab dalam penyebaran pelbagai patogen yang menyebabkan morbiditi dan kematian utama manusia. *Aedes aegypti* ialah spesies utama yang terlibat dalam penyebaran demam denggi. Penghalau nyamuk semulajadi seperti minyak pati boleh memberi perlindungan daripada gigitan nyamuk, yang lebih selamat dan lebih selesa untuk digunakan. Walau bagaimanapun, keberkesanan minyak pati didapati berkurang dengan agak cepat dari masa ke masa disebabkan oleh kemeruapan yang tinggi. Formulasi nanoemulsi boleh mengawal kemeruapan minyak pati dan dengan itu dapat memanjangkan tempoh kadar penghalau. Oleh itu, nanoemulsi minyak dalam air yang mengandungi campuran minyak pati *Jasminum officinale* dan *Anthemis nobilis* telah diformulasi melalui kaedah ultrasonikasi, dan dicirikan berdasarkan saiz titisan, indeks poliserakan (PDI), potensi zeta dan kestabilan penyimpanan. Analisis kromatografi gas-spektrometri jisim (GC-MS) menunjukkan bahawa kandungan monoterpena dan diterpena oksigen dalam minyak pati *J. officinale* masing-masing adalah sebanyak 31.14% dan 21.20%. Kandungan monoterpena beroksigen dalam minyak pati *A. nobilis* menyumbang kepada 84.79% sebatian yang dikenalpasti. Gabungan *J. officinale* dan *A. nobilis* dalam nisbah 1:1 menunjukkan kadar penghalau yang jauh lebih tinggi terhadap *Ae. aegypti* menggunakan model tikus untuk selama 120 minit daripada kadar penghalau minyak pati secara individu. Saiz titisan nanoemulsi; nJC1, nJC2 dan nJC3 adalah 264.23, 291.43 dan 351.37 nm, masing-masing. Potensi zeta dan PDI nanoemulsi adalah -32.77 hingga -46.93 mV dan 0.232 hingga 0.264, masing-masing. Imej mikroskop transmisi electron mengesahkan saiz nano titisan minyak yang kelihatan dalam bentuk sfera. nJC1 menunjukkan kecekapan pemerangkapan minyak pati yang paling tinggi dengan kapasiti muatan sebatian penanda, isophytol dan 1R-α-Pinene yang mencukupi. Saiz titisan nanoemulsi didapati meningkat dalam jangkamasa 60 hari penyimpanan pada suhu bilik dan suhu penyejukan, walau bagaimanapun PDI yang rendah dan peningkatan nilai potensi negatif zeta menunjukkan sistem koloidal yang stabil. Semua formulasi menunjukkan 100% kadar penghalau terhadap *Ae. aegypti* untuk 3 jam yang pertama, dan kesan ini mula berkurangan pada jam berikutnya. Menariknya, nJC1 menunjukkan kadar penghalau yang jauh lebih tinggi iaitu 81.02% berbanding dengan 10% DEET, 62.10% pada 8 jam selepas rawatan, Kajian pelepasan *in vitro* menunjukkan pelepasan minyak pati yang berterusan daripada nanoemulsi melaui mekanisma pelepasan secara kawalan penyebaran. Selain itu, nanoemulsi telah terbukti bebas-irritasi dalam ujian iritasi kulit EpiDerm™, dan profil pelepasan IL-1α dan IL-8 selanjutnya mengesahkan penemuan tersebut. Hasil kajian ini menunjukkan bahawa formulasi nanoemulsi meningkatkan kadar penghalau minyak pati *J. officinale* dan *A. nobilis* dengan ketara dengan tempoh perlindungan yang lebih panjang, mencadangkan potensinya untuk digunakan sebagai satu alternatif penghalau nyamuk berasaskan tumbuh-tumbuhan.

FORMULATION, CHARACTERISATION AND *IN VITRO* SKIN IRRITATION STUDIES OF *JASMINUM OFFICINALE* AND *ANTHEMIS NOBILIS* ESSENTIAL OIL NANOEMULSION FOR *AEDES AEGYPTI* REPELLENT ACTIVITY

ABSTRACT

Mosquitoes are important vectors responsible for the transmission of many pathogens that cause major human morbidity and mortality. *Aedes aegypti* is the main species engaged in the transmission of dengue fever. Natural repellents such as essential oils may provide a means of protection from mosquito bites that are safer and more pleasant to use. However, their effectiveness decreases relatively fast over time due to high volatility. Nanoemulsion formulation enables to control the volatility of essential oil and thereby extends the duration of repellency. Therefore, oil-in-water nanoemulsion containing the mixture of *Jasminum officinale* and *Anthemis nobilis* essential oils were formulated *via* ultrasonication, and characterised with respect to droplet size, polydispersity index (PDI), zeta potential and storage stability. Gas chromatography-mass spectrometry (GC-MS) analysis revealed that oxygenated monoterpenes and diterpenes constituted 31.14% and 21.20% of *J. officinale*, respectively. For *A. nobilis*, oxygenated monoterpenes accounted for 84.79% of the compounds identified. The combination of *J. officinale* and *A. nobilis* in a 1:1 ratio exhibited significantly ($p < 0.05$) higher repellency against *Ae. aegypti* using a rat model for 120 min than the individual oils. The droplet size of nanoemulsions; nJC1, nJC2 and nJC3 were 264.23, 291.43 and 351.37 nm, respectively. The zeta potential and PDI of the nanoemulsions were -32.77 to -46.93 mV and 0.232 to 0.264, respectively. Transmission electron microscopy (TEM) images verified the nanosize of oil droplets which appeared spherical in shape. nJC1 showed the highest entrapment efficiency with an adequate loading capacity of the marker compounds, isophytol and 1R-*α*-Pinene. The nanoemulsions increased in droplet size over 60 days storage at room temperature and refrigeration, however the low PDI and increasing negative potential zeta values indicated a stable colloidal system. All the formulations showed 100% repellency against *Ae. aegypti* for the first 3 h, and this effect starts to decrease in subsequent hours. Interestingly, nJC1 exhibited significantly ($p < 0.05$) higher repellency, 81.02% than 10% DEET, 62.10% at 8 h post-treatment. The *in vitro* release study demonstrated a sustained release of the essential oils from the nanoemulsion *via* a diffusion-controlled release mechanism. In addition, the nanoemulsions were proven to be non-irritant in the EpiDerm™ skin irritation test, and IL-1α and IL-8 release profiles further confirmed the outcome. The findings of this study demonstrated that nanoemulsion formulation significantly increases the repellency of *J. officinale* and *A. nobilis* essential oils with prolonged protection period, suggesting its potential to be utilised as an alternative plant-based mosquito repellent.

CHAPTER 1

INTRODUCTION

1.1 Problem statement and justification of the study

Mosquitoes are important vectors responsible for transmission of many pathogens that cause major human morbidity and mortality. Dengue fever is an arboviral infection spread by *Aedes aegypti* or *Aedes albopictus* mosquitoes *via* a flavivirus transmission (Pinheiro and Corber, 1997). It is by far the most rapidly developing vector-borne disease with an estimated 390 million infections occurring every year (Bhatt *et al*., 2013). Personal protection plays a crucial role in averting mosquito bites and thus minimize the risk of infection.

Essential oils are complex mixtures of volatile organic compounds that contribute to the flavour and fragrance of a plant. They also act as a repellent/deterrent against phytophagous insects (Nasr *et al*., 2017; Gershenzon and Dudareva, 2007; Lee *et al*., 2001). Therefore, plants have been used for protection against insects or other pests. For instance, people burn or bruise plant materials and also apply the oil or oil mixtures to the skin to protect themselves from biting insects, particularly mosquitoes (Seyoum *et al*., 2002; Moore *et al*., 2007; Innocent *et al*., 2010). Plant-based insect repellents have acquired increasing acceptance among customers as they may provide a means of protection from mosquito bites that are safer and more pleasing to use than synthetic repellents. DEET (*N*,*N*-diethyl-3-methylbenzamide)-based insect repellents have been used globally for more than 60 years. However, DEET has an unpleasant odour and causes inconvenience when applied constantly to the skin at high concentrations (Kain *et al*., 2013; Leal, 2014). DEET also blocks sodium and potassium ion channels of mammals, which may contribute to lip numbness (Swale *et al*., 2014). Even though essential oils are effective when freshly applied, their efficacy usually decreases relatively fast over time. This problem is most likely related to their high volatility. Thus, the most important consideration in developing plant-based repellents is to increase their longevity. One of the ways of improving the longevity and efficiency of essential oils is through the formulation of nanoemulsion that would retain the active ingredients onto the skin for a longer duration. The unique properties of nanoemulsion such as large surface area, transparent appearance, robust stability and adjustable rheology have gained a vast interest among researchers over the past decade (Gupta *et al*., 2016).

In this study, two plants were brought to our attention, jasmine (*Jasminum officinale* L.) and Roman chamomile (*Anthemis nobilis* L.). The flower buds of jasmine are used for the treatment of ulcers, boils, skin ailments and eye diseases (Patil *et al*., 2012). In south China, jasmine was traditionally used to treat hepatitis and duodenitis (Nanjing, 2006). Chamomile is commonly used in herbal teas to relieve spasms or inflammatory disorders related to the gastrointestinal tract (Srivastava and Gupta, 2010), and it also is used to relieve diarrhoea, sleeping disorders, mucositis, colic, eczema and injuries (Petronilho *et al*., 2012; McKay and Blumberg, 2006). The mosquito repellent studies of jasmine were reported concerning the essential oil derived from *Jasminum grandiflorum* L. (Uniyal *et al*., 2016; Uniyal *et al*., 2014; Amer and Mehlhorn, 2006). The chemical composition of Roman chamomile essential oil has been determined previously (Omidbaigi *et al*., 2004;

Farkas *et al*., 2003; Omidbaigi *et al*., 2003), and only three studies have evaluated its mosquito repellent activity to date (Uniyal *et al*., 2016; Uniyal *et al*., 2014; Amer and Mehlhorn, 2006). To our knowledge, this study reports for the first time the chemical composition and mosquito repellent activity of *Jasminum officinale*. Moreover, it is interesting to evaluate the repellent efficacy of *J. officinale* in combination with chamomile, the plant renowned for its enormous medicinal properties (Srivastava *et al*., 2010). Furthermore, chemical compounds such as champene, *α*-pinene, *β*-pinene and 1,8-cineole found in *A. nobilis* have been shown to repel mosquitoes (Omolo *et al*., 2004; Traboulsi *et al*., 2005; Hwang *et al*., 1985). Thus, *A. nobilis* can be a good candidate for mosquito repellent study.

Skin irritation testing is required as part of the safety evaluation as the final product of the repellent is aimed for topical application to human skin. *In vivo* acute dermal irritation test in rabbits has typically been used for the irritation testing of test chemicals (Draize *et al*., 1944; OECD, 2002). However, the *in vivo* rabbit studies were found to poorly reflect human exposure scenarios in which mostly shown to overpredict human responses for the examined chemicals (Basketter *et al*., 2012). Moreover, the legislation and a total ban of animal testing for cosmetics following European Cosmetics Directive (76/768/EEC) starting in 2013 have heightened the need for non-animal alternative test methods (Gura, 2008; Adler *et al*., 2011). In addition, the 3Rs principles (replacement, refinement and reduction) of the use of animals in research are an important ethical concept in providing a framework in minimising animal pain and suffering (Robinson, 2005). Hence, the present study is initiated to support the 3Rs thus promoting the use of alternative test method for skin irritation evaluation employing the reconstructed human epidermis model, EpiDerm™ which mimic skin barrier *in vivo*. European Centre for the Validation of Alternative Methods (ECVAM) approved and validated the EpiDerm™ skin irritation testing (OECD TG 439) in 2008 to fully replace the regulatory-accepted rabbit skin irritation test (Kandarova *et al*., 2009a; Kandarova *et al*., 2009b). Following EpiDerm™ tissue viability determination by MTT assay, the extracellular secretion of prominent pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1β, TNF-α, IL-8 and IL-6 which are elicited upon irritation responses in the epidermis, will be quantified *via* multiplexed fluorescent bead-based immunoassay. It is relatively a new technique which incorporates the antibody-coated fluorescence beads to efficiently capture the cytokines (Kellar *et al*., 2001; Yigitbasi, 2012).

1.2 Hypothesis

Previous studies have shown that formulation of essential oils by different techniques resulted in improvement of their repellent activities (Nuchuchua *et al*., 2009; Misni *et al*., 2017). Therefore, it is hypothesised that *'The use of nanoemulsion technology in formulating the essential oils of J. officinale and A. nobilis may enhance their mosquito repellent efficacy and stability'*.

1.3 Aims and objectives

Overall, this study aims at the formulation and characterisation of nanoemulsion containing *J. officinale* and *A. nobilis* essential oil mixture for *Ae. aegypti* repellent activity, followed by testing for skin irritation.

The sub-objectives are as follows:

1. To identify the chemical composition of *J. officinale* and *A. nobilis* essential oils *via* GC-MS, and to evaluate their repellent activities against *Ae. aegypti* individually and in combinations using a rat model.
2. To formulate and characterise the nanoemulsion containing *J. officinale* and *A. nobilis* essential oil mixture.
3. To evaluate the mosquito repellent efficacy of the newly synthesised nanoemulsions.
4. To assess the release kinetics of essential oils from nanoemulsion, and to validate the GC-MS method used for analysis of drug release test samples.
5. To evaluate the skin irritation potential of nanoemulsions using reconstructed human epidermis model and to determine the association between the irritation response and cytokines release.

CHAPTER 2

LITERATURE REVIEW

2.1 Insect repellents

2.1.1 Synthetic repellents

The use of synthetic repellents increased when they were utilised by the militaries for protection from vector-borne diseases (Strickman, 2007). DEET product was registered and introduced for public use in 1957. Since then, it remained the most popular insect repellent, and eventually turned out as the gold standard of arthropod repellents (Strickman, 2007). DEET is known to repel several insects and arthropods.

The mode of action of DEET were studied primarily involving the insect olfactory and gustatory receptors. Two hypotheses have been proposed pertaining to its mode of action which includes activation of ionotropic receptor *Ir40a* and activation of odourant receptor(s) (DeGennaro *et al*., 2013; Xu *et al*., 2014). Firstly, it is a repellent sensu stricto (Frances and Wirtz, 2005; Leal, 2014), an odourant that causes mosquitoes to steer away from the source of stimulus. It acts at a distance, but not too far away from the source (Xu *et al*., 2014). This hypothesis was supported by recent studies where highly conserved *Ir40a* receptor pathway shown to mediate non-contact DEET repellency (Kain *et al*., 2013; Ray and Boyle, 2015), yet the findings were debatable (Silbering *et al*., 2016). Nevertheless, the involvement of odourant receptors in mosquitoes has been highlighted in another study which suggests that DEET detection in *Culex quinquifasciatus* depends solely on the *CquiOr136* receptor (Xu *et al*., 2014) and not the *Ir40a* pathway (Silbering *et al*., 2016). In addition, DEET acts as both a repellent and an antifeedant on contact with insects (Deletre *et al*., 2016; DeGennaro, 2015; Lee *et al*., 2010). Yet, whether the same receptors mediate these behaviours remains unclear. The external uniporous gustatory sensilla (olfactory sensilla are multiporous) of taste organs enable the insect to sense potential food sources without consuming them (Altner and Prillinger, 1980; Montell, 2009). DEET can also disrupt insect movement towards the skin by its extensive neurological effects on ion channels and acetylcholinesterase (Antwi *et al*., 2008; Corbel *et al*., 2009; Swale *et al*., 2014).

Despite the wide usage of DEET-based insect repellents for protection against mosquito bites and its proven effectiveness, DEET comes with several drawbacks. For example, it emits an unpleasant odour and it was found to harm synthetic textiles, plastics and painted exteriors which prevents its use in bed nets and in many urban locations (Krajick, 2006). Additionally, instances of DEET resistance have been shown in *Ae. aegypti* mosquitoes (Klun *et al*., 2004; Stanczyk *et al*., 2010), as well as in flies (Reeder *et al*., 2001). DEET have also been reported to cause sensory problems and affect motor ability, memory, and learning capability (Abdel-Rahman *et al*., 2004; Abou-Donia *et al*., 2001, Briassoulis *et al*., 2001). Besides that, high concentrations of DEET can cause encephalopathy and other side effects and therefore DEET is not suggested for children (Abdel-Rahman *et al*., 2001; Clem *et al*., 1993). Therefore, extreme precautions must be practiced while applying DEET containing products.

Permethrin is an odourless, biodegradable synthetic pyrethroid insecticide derived from the plant *Chrysanthemum cinerariifolium* (Davies *et al*., 2007; Katsuda, 2012). It was registered in the US in 1979 as a repellent. In the present days, it is used as an insecticide on fabrics such as clothing, bed nets, etc. and equally as an insect repellent (Banks *et al*., 2014). Permethrin mainly acts by blocking the movement of sodium ions into the nerve cells of insects *via* inhibition of acetylcholinesterase, adenosine triphosphatase, and the gamma aminobutyric acid-A receptor, eventually leading to paralysis (Cox, 1998; DeLorenzo *et al*., 2006). Permethrin requires direct contact with arthropods, making this compound poorly suited for topical application.

It provides protection against mosquitoes, ticks, sand flies, tsetse flies, chigger mites, fleas, lice, and kissing bugs (Goodyer *et al*., 2010). However, the level of protection varies upon several factors such as insect to be tested, methods of treating fabrics, etc. For instance, permethrin impregnated clothing provides better protection against *Aedes* and *Anopheles* spp., while a low level of protection against *Culex* spp. (Banks *et al*., 2014). Techniques by which permethrin is infused to clothing may affect the efficacy, protection period and determines the cost. Individually treated or pre-treated permethrin impregnated clothing are generally used in endemic areas depending on the severity of vector-borne diseases. Permethrin may be impregnated individually either by spraying or dipping and it is considered as an affordable and cost-effective method, but it offers less consistent protection and does not last as long as pre-treated clothing (Banks *et al*., 2014). Therefore, reapplication of permethrin is recommended after every five washes. Toxicity concern is minimal when it is applied appropriately (Moore *et al*., 2012).

Apart from that, permethrin-treated mosquito nets have been widely tested in tropical nations where vector-borne diseases are prevalent. These nets were shown to reduce morbidity and mortality particularly related with malaria and other mosquito-borne illnesses (Gershman and Staples, 2016). In addition, World Health Organisation (WHO) recommended the use of treated bed nets against malaria vectors, especially endophagic species such as *Anopheles gambiae* and *Anopheles funestus* which are responsible for indoor transmission (Flaxman *et al*., 2010; Lines *et al*., 2003; WHO, 2002; Wilson *et al*., 2014). The protection displayed against various arthropod species, and negligible safety concerns, make permethrin-treated clothing an important arthropod-protection strategy when used together with other protection methods, including topical insect repellents. The reported toxicity caused by permethrin are uncommon, yet inappropriate application at elevated doses, initiate neurotoxic effects such as tremors, loss of coordination, hyperactivity, paralysis and an increase in body temperature. Skin and eye irritation, reproductive effects, mutagenicity, and changes in the immune system, etc. were other side effects reported (Alonso *et al*., 1991).

Picaridin (1-piperidinecarboxylic acid 2-(2-hydroxyethyl)-1-methylpro-pylester), is a colourless and relatively scentless piperidine analog invented by Bayer in the1980s *via* molecular modelling (Moore and Debboun, 2007; Swale *et al*., 2014). It was first sold in Europe in 1990s and later in the US in 2005 (Boeckh *et al*., 1996; Dé *et al*., 2014). It is also called as KBR 3023, Icaridin, and Bayrepel™ (Moore and Debboun, 2007). The exact mode of action of picaridin is yet unidentified in *Aedes, Anopheles* and other arthropod vectors, however, similar odourant receptor, *CquiOR136* as of DEET in *C.*

quinquefasciatus is thought to be involved which discourages biting insects (Xu *et al*., 2014). The effectiveness of picaridin is equivalent to DEET and particularly 20% picaridin spray gave protection against three mosquito vectors, *Aedes*, *Anopheles* and *Culex* for about 5 h with better efficacy compared to DEET. Though, reapplication is required after every 4 to 6 h (Alpern *et al*., 2016).

DEPA (*N*,*N*-diethyl-2-phenyl-acetamide) is a multi-insect repellent which regained interest recently and could prove to be an important repellent in developing countries because of its low cost in comparison to DEET (Moore and Debboun, 2007). In India, DEPA is used as a repellent due to the unavailability of 3-methylbenzoic acid, a compound required to produce DEET (Kumar *et al*., 1992). DEPA exhibited repellency almost comparable to DEET against four mosquito vectors, *Ae*. *aegypti*, *Ae*. *albopictus*, *A*. *stephensi* and *C*. *quinquefasciatus* (Kumar *et al*., 1984; Tikar *et al*., 2014). Moreover, it exhibits moderate oral toxicity (mouse oral LD_{50} 900 mg/kg) and low to moderate dermal toxicity (rabbit and female mouse LD_{50} of 3500 and 2200 mg/kg, respectively) (Rao *et al*., 1993).

2.1.2 Natural repellents

PMD (*p*-menthane-3,8-diol) is a major constituent of the by-product derived from the distillation of lemon-scented gum tree leaves, *Corymbia citriodora* from Myrtaceae family (previously known as *Eucalyptus maculata citriodora*). PMD turns out to be an important active ingredient of commercial repellents listed by the United States Environmental Protection Agency (USEPA) and Canadian Pest Management Regulatory

Agency in 2000 and 2002, respectively (Strickman, 2007). It is effective against some species of mosquitoes and ticks (Bissinger and Roe, 2010). The essential oil from *C. citriodora* also contains citronella, citronellol, geraniol, isopulegol, and delta pinene (Moore and Debboun, 2007). These active constituents work independently or in combination in repelling mosquitoes and ticks (Pohlit *et al*., 2011). Protection against *Anopheles* by PMD was found to be comparable to that of DEET. Therefore, its use is advocated by the Centers for Disease Control (CDC) for use in malaria endemic areas (EPA, 2000; Alpern *et al*., 2016). PMD gives complete protection for 4 to 6 h against *Aedes*, *Anopheles*, and *Culex* spp., and also ticks at a concentration of 30% (Lupi *et al*., 2013; Stanczyk *et al*., 2015). The repellent mechanism of PMD in mosquitoes (*Aedes* and *Anopheles*) and ticks is yet to be discovered. However, an odourant receptor, *CquiOR136* is believed to be involved in *C. quinquefasciatus* (Xu *et al*., 2014).

Insect repellent 3535 (Ethyl 3-[acetyl(butyl)amino]-propanoate) is a synthetic repellent invented by Merck in 1970 and therefore named IR 3535 or Merck 3535. Since its commencement, it has been accessible in Europe while it was unavailable in the US until 1999 (Puccetti, 2007). It has structural similarity with naturally occurring beta-alanine and as of now it has been enlisted by the USEPA as a biopesticide (Carroll, 2008). IR 3535 was found to repel mosquitoes and ticks, however its exact mode of repellency is yet to be investigated in a number of arthropod vectors. Nonetheless, a research group hypothesizes that it behaves in the similar manner as DEET *via* an odourant receptor, *CquiOR136* in southern house mosquito, *C. quinquefasciatus* (Xu *et al*., 2014). IR 3535 at 20% gives complete protection against *Aedes* and *Culex* mosquitoes for 7 to 10 h but provides lesser protection against *Anopheles* which is around 3.8 h, thus preventing its use in malaria endemic regions (Lupi *et al*., 2013; Stanczyk *et al*., 2015). The efficacy of IR 3535 perhaps similar to DEET, however it needs to be reapplied every 6 to 8 h.

2.2 Essential oils as mosquito repellent

Essential oils are volatile blends of hydrocarbons with a variety of functional groups produced in plants. Their repellent property has been related to the existence of monoterpenes and sesquiterpenes. However, in certain some instances, these compounds can work synergistically which improves their efficacy. Essential oils were recognised as a vital natural source of insecticides (Prabakaran *et al*., 2017; Duarte *et al*., 2015; Sugumar *et al*., 2014a) and have the potential of being acute ovicidal, fumigant and insect growth regulator of different insect species (Govindarajan *et al*., 2008; Papachristos and Stamopoulos, 2004; Osman *et al*., 2016). The repellency of essential oils against mosquitoes is usually attributed to its main compounds (Gurunathan *et al*., 2016; Cantrell *et al*., 2018; Trongtokit *et al*., 2005). Nevertheless, the diverse constituents of essential oils may result in a greater repellency than its individual or blends of compounds (Hori, 2003; Müller *et al*., 2009).

2.2.1 Chemical constituents responsible for mosquito repellent activity

Monoterpenes and their oxygenated derivatives such as α-pinene, cineole, limonene, terpinolene, citronellol, citronellal, camphor and thymol of various essential oils were shown to exhibit mosquito repellent activity (Jaenson *et al*., 2006; Park *et al*., 2005; Yang *et al*., 2004). β-caryophyllene, a sesquiterpene is indicated as a strong repellent against

Ae. aegypti (Gillij *et al*., 2008). Repellent properties of several essential oils have been frequently related to the existence of monoterpenoids and sesquiterpenes (Kiran and Devi, 2007; Jaenson *et al*., 2006; Sukumar *et al*., 1991). Interestingly, Odalo *et al*. (2005) revealed that phytol, a linear diterpene alcohol possess high repellency against *A. gambiae*. The repellent activities of pure compounds extracted from different Kenyan plants have been evaluated against *A. gambiae* by Omolo *et al*. (2004) and Odalo *et al*. (2005). Citronellal, perillyl alcohol, *cis*-verbenol, *cis*-carveol, geraniol, carvacrol, thymol, 3-carene, myrcene, caryophyllene oxide and perillaldehyde were identified as effective repellents. These compounds belong to different groups including monoterpenoid, diterpenoid and sesquiterpenoid. It is noted that most of the repellent compounds which have been mentioned earlier are oxygenated, having the hydroxyl group connected to a primary, secondary or aromatic carbon.

Wang *et al*. (2008) showed that terpenoids with two functional groups were biologically active as mosquito repellents. Terpenoids with two functional groups (one negatively charged end possessing either ester/ether bonds or an ethanol hydroxyl group, and one positively charged end having alkane groups) were synthesised and evaluated for their repellent activities. The positive end was found to be more favourable for receptor interactions as discovered by a computational model (Wang *et al*., 2008). The extent of the positive charge depicts the electrophilic nature of the group (Ma *et al*., 1999) and thus the interaction between the repellent and receptor are most probably linked to electrophilicity. Apart from that, the model created by Wang *et al*. (2008) displayed that molecular descriptors such as dipole moment and boiling point are closely associated with repellent action. Lipophilicity or specific electrostatic relations with the receptor could be connected to the dipole moment, while the period of contact time with the olfactory chemosensilla of mosquitoes might be decided by the boiling point/vapour pressure (Wang *et al*., 2008).

2.2.2 Synergism associated with repellent activity

Even though repellent activity of essential oils is usually credited to some specific compounds, a synergistic interaction among these compounds may result in a higher bioactivity compared to the individual constituents (Hummelbrunner and Isman, 2001; Gillij *et al*., 2008). Wathoni *et al*. (2018) demonstrated that the blend of ylang-ylang and citronella essential oils on corn starch-based thixogel significantly enhanced repellent activity against *Ae*. *aegypti* compared to the oil independently. Das *et al*. (2015) evaluated the mosquito repellent activities of *Curcuma longa*, *Pogostemon heyneanus*, and *Zanthoxylum limonella* essential oils separately and in combination. The individual oils showed much lower repellent activity compared to the oil mixtures. The 1:1:2 mixture of essential oils provided 329.4 and 391.0 min of complete protection time in the laboratory and under field conditions, respectively.

Moreover, synergist actions were claimed to be functioning in 8% of essential oil containing patents. For instance, essential oil of lippia in combination with geranium, lemon eucalyptus or basil essential oils formulated into a gradually vapourising hydrocarbon soluble composition was thought to alter neuronal action in adult mosquitoes and exhibit repellent activity comparable to commercial pyrethroids (Singh *et al*., 2010).

Another example of a synergist formulation included almost equal quantities of eucalyptus and cassia essential oils together with an emulsifier and butyl acetate solvent which was intended to be used in incense preparations (Wang *et al*., 2009). Furthermore, a synergist repellent meant for use with pyrethroids utilised essential oils of wintergreen, *Gaultheria procumbens* L. and camphor, *Cinnamomum camphora* (L.) J. Presl, emulsifier and solvent (He *et al*., 2009).

2.3 The plants *Jasminum officinale* and *Anthemis nobilis*

2.3.1 Description of the plants

J. *officinale* is recognised as common jasmine or simply jasmine, is a flowering plant native to India, Caucasus, Nepal, northern Iran, Pakistan, the Himalayas and western China (Brickell *et al*., 2004). *Jasminum* is a genus belonging to the family of Oleaceae (olive family) comprising of about 600 species of small trees and vines. These vigorous twining shrubs with pinnate leaves are usually cultivated in gardens and they are usually found to be growing in the forests throughout tropical Asia and warm temperate regions in Europe and Africa (Kunhachan *et al*., 2012). Jasmine bears intensely aromatic, white star-shaped flowers from early to late summer. This plant is best grown in full sun for the strongest fragrance, but it does grow well in partial shade. It needs support to climb up to its maximum height of 12 ft (Figure 2.1).

The Roman chamomile (*A. nobilis* L. syn: *Chamaemelum nobile* L.) is a lasting, herbaceous plant from the Asteraceae family (daisy family). It is originated from

Mediterranean, native primarily to Portugal, France and Algeria. Only the variety with full flowers and comprising huge amount of essential oil is cultivated (var. flora plena syn: var. ligulosa), which does not grow wildly (Bernath, 2002; Hornok, 1992). The full-flowered variety only occasionally produce seeds which are fit for germination, and it is propagated vegetatively. The plant can reach 16 to 32 cm long, trailing partitioned stems. The flowers are large and white, and they blossom from mid-summer until killed frost late in the fall or early winter (Figure 2.2). The flowers have a strong pleasant aroma (Hornok, 1992; Shaath *et al*., 1990). Pubescent glands containing essential oil can be found on any parts of the flowers, with the dry flowers consist of 0.3-1.5% essential oil (Bernath, 2002; Hornok, 1992; Shaath *et al*., 1990).

Figure 2.1: *Jasminum officinale*. The image was taken from https://futureforests.ie

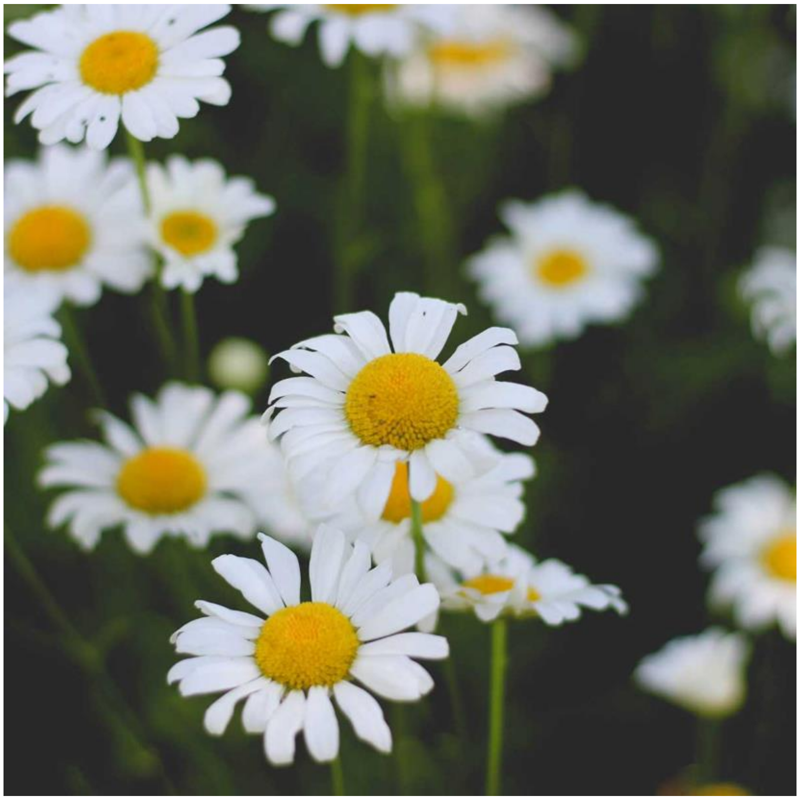

Figure 2.2: *Anthemis nobilis*. The image was taken from https://www.rockymountainoils.com

2.3.2 Chemical constituents of the plants

The technique used for the extraction of volatile components from the jasmine flowers has a significant impact on the composition as well as the olfactory property of the volatiles (Kapoor, 1991; Shaath and Azzo, 1992). Rout *et al*. (2010) have reported the chemical composition of *Jasminum sambac* absolutes obtained from three different locations, Delhi, Bhubaneswar and Saluru. The major components identified were (Z)-3-hexenyl benzoate, linalool, benzyl acetate, (Z)-3-hexenyl acetate, methyl anthranilate, methyl stearate, (E,E)-α-farnesene, tetracosane, oleic acid and 5-methyltricosane. The chemical components varied in composition from one location to another. The chemical composition of *Jasminum grandiflorum* L. essential oil was reported by Wei *et al*. (2015). The main constituents were phytol (25.77%), 3,7,11-trimethyldodeca-1,6,10-trien-3-ol (12.54%), 3,7,11,15-tetramethyl-1-Hexadecen-3-ol (12.42%) and hexadecanoic acid (9.16%).

The chemical compositions of chamomile essential oils may differ largely between samples depending upon different geographical sites, cultivars, harvesting time, and origin. Besides that, the chemical fingerprints particularly for volatile oil constituents can be affected by sample collection and handling techniques (Wang *et al*., 2014). The Roman chamomile oil was reported to mainly contain angelate, tiglate and butyrate esters. Besides that, monoterpene and sesquiterepene derivatives are commonly present in Roman chamomile oils (Wang *et al*., 2014). Omidbaigi *et al*. (2003) analysed the diferrence between chemical composition of *Anthemis nobilis* essential oil extracted by hydro-distillation and supercritical fluid extraction (SFE). The main constituents extracted by hydro-distillation were iso-butyl angelate (25.85%), 2-methy butyl angelate (13.02%), propyl tiglate (12.00%), iso-amyl angelate (6.63%), 3-methyl butyl iso-butyrate (5.20%) and iso-amyl-2-methylbutyrate (4.08%). As for SFE method, 2-methyl-butyl angelate (8.59%) iso-amyl-angelate (4.21%), iso-amyl-2-methyl butyrate (2.77%) and propyl tiglate (2.34%) were mainly present.

The essential oil derived from German chamomile flowers primarily comprised of α-bisabolol and its oxides, azulenes including chamazulene and acetylene derivatives

(Adams *et al*., 2009; Bucko and Salamon, 2007; McKay and Blumberg, 2006; Orav *et al*., 2010). Stanojevic *et al*. (2016) have reported the chemical composition of German chamomile (*Matricaria chamomilla* L.) essential oil obtained by hydro-distillation method. The major components were (E)-β-farnesene (29.8%), (E,E)-α-farnesene (9.3%), α-bisabolol oxide A (7%), chamazulene (6.4%), α-bisabolol oxide B (6.3%), germacrene D (6.2%), (Z)-spiroether (5.1%), α-bisabolone oxide A (3%) and α-bisabolol (2.4%).

2.3.3 Pharmacological properties of the plants

The flowers and leaves of jasmine are well-known for their multiuse. For instance, the flowers were used conventionally in Asia for the treatment of various ailments such as fever, diarrhea, abdominal pain, conjunctivitis, dermatitis, abscess, asthma, toothache and uterine bleeding and (Kunhachan *et al*., 2012). The leaves were used to treat quadriplegia gall, dysentery, and stomach pain in China (Kunhachan *et al*., 2012). The plant is cultivated in many locations of the world, however the essential oil of jasmine (jasmine absolute) is largely produced in Egypt from the delicate flowers (Lis-Balchin *et al*., 2002). It is used as a comprehensive medication for lethargy, fear, anxiety, hyposensitivity, panic, uterine ailments, labour pain, skin care, frigidity, coughs, hoarseness and muscular contraction, in aromatherapy (Tisserand, 1985; Lawless, 1995). Lis-Balchin *et al*. (1996) reported the spasmolytic activity of jasmine absolute on guinea pig ileum *in vitro*. The relaxant effect of jasmine was correlated with spasmolysis of the smooth muscle of the ileum *in vitro* (Lis-Balchin and Hart, 1997).

Chamomile is one of the most broadly used and well recognised therapeutic plants on the planet and it has been recommended for a range of healing purposes (Astin *et al*., 2000).

Essential oils derived from chamomile are used widely in cosmetics and aromatherapy. For example, chamomile tea and essential oil aromatherapy have been used traditionally to treat sleeplessness and to incite sedation effects. Besides that, inhalation of the vaporised chamomile oil was shown to reduce a stress-induced increase of adrenocorticotropic hormone levels in plasma (Paladini *et al*., 1999). In another study, the potential of topical use of chamomile to improve wound healing was assessed using a double-blind test on fourteen patients who underwent dermabrasion of tattoos. Chamomile was shown to be significantly effective in creating wound drying and in speeding up epithelialisation (Glowania *et al*., 1987).

In addition, topical applications of chamomile have been indicated to be moderately effective in treating atopic eczema. A partially double-blind, randomised study was carried out for a duration of two weeks to compare Kamillosan (R) cream containing chamomile extract with 0.5% hydrocortisone cream and the vehicle cream (placebo), in patients suffering from medium-degree atopic eczema (Patzelt-Wenczler *et al*., 2000). The findings showed that Kamillosan (R) cream exhibited a mild betterment than 0.5% hydrocortisone and a marginal difference compared to placebo. Apart from that, chamomile was proposed to inhibit *Helicobacter pylori*, the bacteria that can lead to stomach ulcers, based on studies using preclinical models (Weseler *et al*., 2005).

2.4 Dengue vector *Aedes*

In recent decades, dengue incidence has increased significantly around the world. The WHO reported the global occurrence of dengue has expanded over the last 50 years and estimated about 50 to 100 million new infections happen every year, with around 20,000 deaths (WHO, 2012). In addition, a more recent study has revealed an estimation of 390 million dengue infections to occur each year using cartographic approaches (Bhatt *et al*., 2013). In Malaysia, a total of 101,357 dengue cases were diagnosed with 237 deaths were reported in 2016 (CPRC, 2016).

Ae. *aegypti* is regarded as the main species responsible for the transmission of dengue fever (CPRC, 2016; Lim *et al*., 2010). Its capacity as an effective vector of dengue infection is described by its aptitude to adjust to the diverse environmental conditions; its inclination for human habitats and skip oviposition manners (Ng *et al*., 2009; Severson *et al*., 2004). Female *Ae*. *aegypti* feed primarily on human blood which results in regular contacts of the vector with human. This anthropophilic nature is hypothesised to be a factor that cause *Ae*. *aegypti* to be more capable in transmitting the dengue virus compared to *Ae*. *albopictus* that feeds on both human and animal blood (CPRC, 2016; Ng *et al*., 2009). *Ae*. *albopictus* is a secondary vector of dengue infection in Southeast Asia but it has also been recognised as the sole vector during some outbreaks where *Ae*. *aegypti* was not present (Kyle and Harris, 2008).

2.4.1 Biology and ecology of mosquitoes

Mosquitoes belong to the Culicidae family of the Diptera order (true flies). Adult female mosquitoes have one sets of scale-enclosed wings and one sets of vestigial hindwings (halteres), long legs and a long thin midriff. The mouthparts are altered for penetrating and sucking with the proboscis comprising of the six puncturing stylets which include labrum-epipharynx, two mandibles, two maxillae, and the hypopharynx, all ensheathed in the extended labium. Except for Antarctica, mosquitoes exist on each landmass of the globe and adjusted to nearly every habitat. As per larval habitat, species are gathered into holder/tree-hole breeding and pool breeding (Chase and Knight 2003; Juliano, 2009). The earlier incorporates most *Aedes* spp., which store eggs separately along the inner walls of artificial containers at the waterline. The eggs of these species go through a period of desiccation and hatch when immersed in water.

The general fitness and survival of mosquitoes results from mostly genetically determined behaviour in response to various internal and external stimuli, the latter of which are principally olfactory. Olfactory receptors on the antennae and maxillary palps detect semiochemicals for sugar feeding, host-seeking, oviposition, and to a lesser extent mate finding. Species-specific contact pheromones have been described, but their functional roles are not completely understood. Mating behaviour, which normally occurs 3 to 5 days post-emergence is believed to be mediated primarily by acoustic signals. Conspecific males recognise the slightly lower wing-beat frequency of females (Göpfert *et al*. 1999).

Nectar is the sole food of male mosquitoes. Females of many species consume nectar prior to host seeking, presumably as a source of energy for the host-seeking flights. Mosquitoes locate host plants by way of volatile semiochemicals. For example, volatiles of the oxeye daisy, *Leucanthemum vulgare*, are attractive to *Ae. aegypti* (Jepson and Healy, 1988). The nectar sugars supplement energy reserves thus enabling females to fly for extended distances in search of a blood host, a behaviour that is mediated heavily by olfaction. Host-seeking mosquitoes detect host-derived chemical cues by the two major sensory organs. The maxillary palps detect and assess carbon dioxide (CO_2) levels which activate and alert mosquitoes to other host-related odours that are received primarily by the olfactory receptors of the antennae and some receptors on the palps (Zwiebel and Takken, 2004; Dekker *et al*., 2005). Approximately 350 different chemical compounds are associated with human skin, of which L-lactic acid, ammonia, acetone, and carboxylic acids are integral kairomones during host seeking behaviour (Bernier *et al*., 2000; Steib *et al*., 2001; Bernier *et al*., 2003; Smallegange *et al*., 2005).

The saliva of mosquitoes contains various compounds that prevent haemostasis and the inflammatory response of the host (Rodriguez and Hernández-Hernández, 2004). Antigenic compounds of the saliva cause an initial histamine-mediated immunological response resulting in the classic itchy bite, which disappears after a few hours, followed by a delayed cell-mediated response appearing 1-2 days later as the signature hive (Hill and van Haselen, 1996). Not only are the bites of mosquitoes a nuisance, the exchange of fluids between the mosquito and the host creates the opportunity for the transfer of pathogens that may cause disease. Mosquitoes are vectors of some of the most important disease-causing pathogens, including the protozoan parasite *Plasmodium* spp. (the causal agent of malaria), Western Equine Encephalitis virus, West Nile virus, Dengue Fever and Yellow Fever flaviviruses, and many more pathogens responsible for significant morbidity and mortality. As such, the mosquito has received the distinct notoriety as the most dangerous animal in the world.

2.4.2 Life cycle of *Aedes aegypti*

Ae. *aegypti* is a container or tree-hole breeding mosquito (Roberts and Janovy, 2000). As an outstandingly resilient species, *Ae*. *aegypti* breeds in almost any small container of water, while their eggs can survive extensive durations of dehydration (Malavige *et al*., 2004). Adults typically rest indoors like living rooms and bedrooms expanding their vector capacity through increased biting opportunity (Mackenzie *et al*., 2004; Malavige *et al*., 2004). Female *Ae*. *aegypti* lay eggs individually on the inner walls of containers at the waterline and following a period of desiccation, eggs hatch when submerged. Larvae are aquatic and develop through four larval stages prior to metamorphosing into active non-feeding pupae. Pupation is relatively short with completely developed adults emerging through a split in the integument of the thorax after around 2-3 days (Roberts and Janovy, 2000). The life cycle process is illustrated in Figure 2.3. Adult mosquitoes live for about three weeks (CDC, 2012). Both males and females feed on nectaries and extra-floral nectaries of plants as a source of carbohydrates for energy, but only females feed on the blood of humans to supplement their diet with protein for the growth of eggs. Several gonotropic cycles occur in a female's life and consequently blood feeding occurs