การควบคุมยุงลายนำไข้เลือดออก (*AEDES AEGYPTI* L.) โดยชีววิธี ด้วยสารสกัดเปลือกมะกรูด (*CITRUS HYSTRIX* DC.) และ เมล็ดมะละกอ (*CARICA PAPAYA* L.)

นางสาวสุพรรณี โชคคุณ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2554

BIOLOGICAL CONTROL OF DENGUE HEMORRHAGIC FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY KAFFIR LIME (*CITRUS HYSTRIX* DC.) PEEL AND PAPAYA (*CARICA PAPAYA* L.) SEED EXTRACTS

Suphunnee Chokkhun

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master in Environmental Biology

Suranaree University of Technology

Academic Year 2011

BIOLOGICAL CONTROL OF DENGUE HEMORHAGIC FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY KAFFIR LIME (*CITRUS HYSTRIX* DC.) PEEL AND PAPAYA (*CARICA PAPAYA* L.) SEED EXTRACTS

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

Thesis Examining Committee

(Asst. Prof. Dr. Griangsak Eumkeb)
Chairperson

(Assoc. Prof. Dr. Korakod Indrapichate)
Member (Thesis Advisor)

(Dr. Pongrit Krubprachaya)
Member

(Assoc. Prof. Dr. Kingkaew Wattanasirmkit)
Member

(Asst. Prof. Dr. Nathawut Thanee)
Member

(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs

(Assoc. Prof. Dr. Prapun Manyum)
Dean of Institute of Science

สุพรรณี โชคคุณ : การควบคุมยุงลายนำไข้เลือดออก โดยชีววิธีด้วยสารสกัดจากเปลือกมะกรูดและเมล็ดมะละกอ (BIOLOGICAL CONTROL OF DENGUE HEMORRHAGIC FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY KAFFIR LIME (*CITRUS HYSTRIX* DC.) PEEL AND PAPAYA (*CARICA PAPAYA* L.) SEED EXTRACTS).
อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.กรกช อินทราพิเชฐ, 116 หน้า.

การควบคุมโดยชีววิธียุงลายพาหะนำโรคไข้เลือดออกโดยชีววิธีด้วยสารสกัดจาก เปลือกมะกรูดและเมล็ดมะละกอสุก โดยทำการสกัดสารด้วยน้ำและเอทานอล ศึกษาพฤกษเคมีหลักของสารสกัดด้วยวิธีทินเลเยอร์โครมาโทกราฟี และวิเคราะห์ความเป็นพิษต่อเซลล์ของสารสกัดในกุ้งฝอยอาร์ทิเมียร์ ศึกษาประสิทธิผลการฟักไข่ยุง การตายของลูกน้ำระยะที่สอง และตัวโม่ง และศึกษาฤทธิ์ต่อการไล่ยุงตัวเต็มวัยด้วยสารสกัดชนิดเดียวและสารสกัดผสมครั้งละสองสารสกัด ผลการศึกษาพบว่าสารสกัดของเปลือกมะกรูดมีปริมาณสารประกอบฟีนอลิกสูงกว่าสารสกัดของเมล็ดมะละกอ การตรวจสอบสารประกอบทางพฤกษเคมีด้วยวิธีทินเลเยอร์โครมาโทกราฟี และตรวจหาสารเฉพาะด้วยกรดวานิลีนซัลฟุริก บ่งชี้ว่ามีสารกลุ่มเทอร์พีน (Terpenes) ความเป็นพิษของสารสกัดต่ออาร์ทิเมียร์มีความแตกต่างกันมากระหว่างสารสกัดด้วยน้ำและสกัดด้วยเอทานอล สารสกัดด้วยน้ำของเปลือกมะกรูด และสารสกัดด้วยเอทานอลของเมล็ดมะละกอสุก มีพิษสูงต่ออาร์ทิเมียร์ มีค่า LC_{50} ที่ 24 ชั่วโมง เท่ากับ 68.81 ± 0.21 และ 68.93 ± 0.22 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สารสกัดด้วยเอทานอลของเมล็ดมะละกอสุกมีประสิทธิภาพสูงสุดในการยับยั้งการฟักไข่ยุงคือ มีค่า EC_{50} ที่ 24 ชั่วโมง เท่ากับ 1.71 ± 0.02 มิลลิกรัมต่อมิลลิลิตร สารสกัดทั้งหมดมีฤทธิ์กำจัดลูกน้ำของสารสกัดได้ตามลำดับดังนี้ สารสกัดด้วยเอทานอลของเมล็ดมะละกอสุก สารสกัดด้วยเอทานอลของเปลือกมะกรูด สารสกัดด้วยน้ำของเมล็ดมะละกอสุก และสารสกัดด้วยน้ำของเปลือกมะกรูด โดยสารสกัดด้วยเอทานอลของเมล็ดมะละกอสุกซึ่งมีประสิทธิผลสูงสุดมีค่า LC_{50} เท่ากับ 0.48 ± 0.12 มิลลิกรัมต่อมิลลิลิตร ซึ่งสูงมากกว่าสารสกัดด้วยน้ำของเมล็ดมะละกอสุกประมาณ 18 เท่า สารสกัดด้วยเอทานอลมีฤทธิ์กำจัดตัวโม่งได้ดี ประสิทธิภาพต่อการตายของตัวโม่งเรียงลำดับดังนี้ สารสกัดด้วยเอทานอลของเมล็ดมะละกอสุก สารสกัดด้วยเอทานอลของเปลือกมะกรูด สารสกัดด้วยน้ำของเมล็ดมะละกอสุก และสารสกัดด้วยน้ำของเปลือกมะกรูด สารสกัดด้วยเอทานอลของเมล็ดมะละกอสุก มีค่า LC_{50} ที่ 24 ชั่วโมง เท่ากับ 1.48 ± 0.94 มิลลิกรัมต่อมิลลิลิตร ซึ่งสูงกว่าสารสกัดด้วยเอทานอลของเปลือกมะกรูด (LC_{50}, 40.58 ± 0.00 มิลลิกรัมต่อมิลลิลิตร) ประมาณ 27 เท่า ประสิทธิภาพในการขับไล่ตัวเต็มวัยของสารสกัดชนิดเดียวโดยวิธีทาบนหนังหนู พบว่าสารสกัดทั้งหมดสามารถไล่ยุงได้ดีมากระหว่าง 30 นาทีแรก และประสิทธิภาพคงอยู่นานถึง

3 ชั่วโมง สารสกัดผสมที่มี สารสกัดด้วยน้ำของเปลือกมะกรูด หรือสารสกัดด้วยเอทานอลของเมล็ดมะละกอสุก ประกอบในคู่ผสมช่วยเสริมฤทธิ์สารสกัดผสม

การศึกษานี้สามารถสรุปได้ว่า สารสกัดเปลือกมะกรูดและเมล็ดมะละกอสุกมีคุณสมบัติเป็นสารกำจัดแมลงที่ดีต่อการกำจัดยุงได้ทุกระยะของการเจริญพัฒนาการ และต่อการไล่ยุงตัวเต็มวัยของยุงสายพันธุ์ *Aedes aegypti* ควรมีการศึกษาความเป็นพิษของพืชทั้งสองนี้ต่อไปเพื่อสนับสนุนการประยุกต์ใช้ในด้านอื่นๆนอกจากใช้ควบคุมโดยชีวภาพของยุง

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2554 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

SUPHUNNEE CHOKKHUN : BIOLOGICAL CONTROL OF DENGUE HEMORRHAGIC FEVER MOSQUITOES (*AEDES AEGYPTI* L.) BY KAFFIR LIME (*CITRUS HYSTRIX* DC.) PEEL AND PAPAYA (*CARICA PAPAYA* L.) SEED EXTRACTS. THESIS ADVISOR : ASSOC. PROF. KORAKOD INDRAPICHATE, Ph.D. 116 PP.

BIOLOGICAL CONTROL/ *AEDES AEGYPTI* L./ *CITRUS HYSTRIX* DC./ *CARICA PAPAYA* L./ MORTALITY

Biological control of *Aedes aegypti*, mosquito vector of dengue fever and dengue hemorrhagic fever using the extracts of kaffir lime (*Citrus hyptrix* DC.) peel and ripe papaya (*Carica papaya* L.) ripe seeds was investigated. The plant products were obtained by water and ethanolic extraction. A selected, major phytochemical group of the extracts was determined by thin layer chromatography (TLC). Their cytoxicity was evaluated by brine shrimp lethality assay (BSLA). Bioefficacy of the extracts on mosquito mortality was investigated on the egg hatching, the second instar larvae, and the pupae of *Ae. aegypti*. Repellent activity of the extracts against the mosquito adults was assessed using individual and paired combination of the extracts by topical application on naked rat skin. The kaffir lime peel ethanolic extract contained highest total phenolic compounds among the extracts. Thin layer chromatographic separation and vanillin-sulphuric detection indicated the presence of terpenes group. The cytotoxicity on brine shrimps, *Artemia salina*, was different in a wide range between the water and ethanolic extracts. The kaffir lime peel water extract and the ripe papaya seed ethanolic extract showed high toxic to *A. salina* with LC_{50} value at 24 h of 68.81 ± 0.00 and 68.92 ± 0.22 µg/mL, respectively. The ripe papaya seed ethanolic extract was most effective on inhibiting the egg hatching of *Ae. aegypti* eggs in to larvae with EC_{50}, 24 h, of 1.72 ± 0.00 mg/mL.

The ripe papaya seed ethanolic extract was most effective on inhibiting the egg hatching of *Ae*. *aeegypti* eggs in to larvae with EC_{50}, 24 h, of 1.72 ± 0.00 mg/mL. The larvicidal efficacy of all extracts on the second instar larvae ranged as ripe papaya seed ethanolic extract, kaffir lime peel ethanolic extract, ripe papaya seed water extract and kaffir lime peel water extract. The ripe papaya seed ethanolic extract with the highest LC_{50} of 0.48 ± 0.12 mg/mL was approximately 18 fold of PSE/w (8.62 ± 0.62 mg/mL). The pupal mortality caused by ethanolic extracts were more potent than the water extracts. The efficacy on pupal mortality of all extracts ranged as ripe papaya seed ethanolic extract, kaffir lime peel ethanolic extract, ripe papaya seed water extract and kaffir lime peel water extract. The highest efficacy was of ripe papaya seed ethanolic extract with LC_{50}, 24 h. 1.47 ± 0.94 mg/mL. It was approximately 27 fold of kaffir lime peel ethanolic extract 40.58 ± 0.00 mg/mL. The repellent activity of all individual extracts on the adult mosquitoes was very potent during 30 minutes after application and considerably potent up to 3 h. The combinations in pairwise of the extracts could protect against the mosquitoes were also very potent and last long up to 3 h. The combination containing kaffir lime peel water extract or ripe papaya seed ethanolic extract was likely to enhance the repellent activity.

It could be concluded that kaffir lime peel and papaya seed extracts possessed insecticidal property that was good for controlling mosquitoes at all developmental stages and repelling the adult of *Aedes aegypti*. Further study of theses plant products showed be encouragesd for toxicity other applications other than mosquito biological control.

School of Biology Student's Signature_____

Academic Year 2011 Advisor's Signature_____

Co-advisor's Signature_____

ACKNOWLEDGEMENTS

During my graduate studies I have been fortunate enough to receive help and encouragement from several people. I would like to recognize and sincerely thank them who have made my studies at the Suranaree University of Technology a pleasant one and who made a completion of this degree possible:

Assoc. Prof. Dr. Korakod Indrapichate, my major advisor, for her excellent guidance, follow up and regular supervision during the study. Dr. Pongrit Krobprachaya my Thesis co-advisors, Asst. Prof. Dr. Griangsak Eumkeb and Asst. Prof. Dr. Nathawut Thanee for their kind help in giving me some technical advices regarding the methodologies I have followed. Assoc. Prof. Dr. Kingkaew Wattanasirmkit thesis committee members, for her guidance.

I sincerely also like to thank the Suranaree University of Technology thesis grant and I gratefully thank Infective Disease Insect Control Office at Saraburi province, the Department of Communicable Disease Control, Ministry of Public Health in Nonthaburi province, and Communicable Disease Control Centre Zone 6, Ministry of Public Health in Khon Kaen province for *Aedes aegypti* L. eggs used in this study.

Thanks to my family, friends and to all who in one way or another help in the successful completion of this study and the continuous encouragement, endless love, warmth and care.

Suphunnee Chokkhun

CONTENTS

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER I

INTRODUCTION

1.1 Background

Dengue fever is a mosquito-borne viral disease in humans (Lee, Lee, Liu and Yang, 2010). It is caused by four closely related arboviruses (designated DENV-1, DENV-2, DENV-3, and DENV-4) which belong to the genus *Flavivirus*, family Flaviviridae (Guzman and Istúriz, 2010). Viruses are overwhelmingly transmitted which is between human beings after a bite from an infected female *Aedes aegyti* L. (*Ae*. *aegyti*) mosquito, which is the most important vector (Meltzer and Schwartz, 2009). The severity of the disease varies from asymptomatic infections, to a febrile fever, or potentially life-threatening dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (Murrell, Wu and Butler, 2011). The World Health Organization (WHO) estimates that 50 million cases of dengue occur per year worldwide, with more than 2.5 billion people living at risk (de-Freitas, Koella and de-Oliveira, 2011). It is estimated that there are around 22,000 deaths from half a million cases per year, mainly children (Ashley, 2011). Dengue is endemic in tropical and subtropical countries, which includes more than 110 countries, and is the arboviral disease that has spread very rapidly. In Thailand, DHF continues to become one of the highest numbers of dengue cases as well as Vietnam and the Philippines among Asian countries (Guzman and Istúriz, 2010). DHF has been increasing with the cyclic outbreaks occurring every 2-3 years. Recently, in Thailand, it has been

reported that approximately 127 cases of DHF per 100,000 populations were found in 2010 (Department of Disease Control, Ministry of Public Health, Online, 2010). Therefore, if prevention approaches of the disease could be seriously concerned, the disease incidence and distribution will be declined. Dengue control can be conducted by three main strategies; source reduction by localization location and destruction of mosquitoes breeding sites, use of larvicides, and use of ultralow-volume aerosolised adulticides (Massad and Coutinho, 2011). Unfortunately, control strategies aimed at elimination of preferred vector larval habitat through source reduction or periodic application of insecticides to water sources has often failed to sufficiently control of *Ae. aegyti*. Furthermore, control of adult mosquitoes using a variety of chemical means is fraught with complications including high cost, slow operational response, ineffective timing of application, low efficacy, and evolution of resistance to insecticides. However, the reduction of vector density alone has often proven insufficient. A better understanding of the complex interplay of diverse factors, including site-specific social determinants, is critical for promoting a broader public health response leading to successful vector control, for instance management practice using routine larviciding and focal space spraying. Nevertheless, insecticides continue to play a crucial role, if not indispensable, in helping to reduce the risk of dengue transmission by complementary reduction of immature and adult vector mosquitoes (Chuaycharoensuk et al., 2011). Moreover, complex problems were created when using synthetic insecticides, such as the development of mosquito resistance, environmental pollution, and undesirable effects on humans, mammals, and other non-target organisms (Chaithong et al., 2006).

Concerning the quality and safety of life and the environment, the emphasis on controlling mosquito vectors has gradually shifted from the use of synthetic chemicals to alternative insecticides of botanical origin which are target-specific, biodegradable, and environmentally safe. Although some plant chemicals and their derivatives have been used for controlling and eradicating mosquitoes and other domestic pests, but only few insecticides of plant origin have been commercially available (Chaiyasit et al., 2006).

Numerous plants and their derived products, particularly essential oils, have been investigated and described as potentially natural sources of insect repellent. Most plant-based insect repellents are currently on markets containing essential oils from one or more of the following plants; citronella (*Cymbopogon nardus*), cedar (*Juniper virginiana*), eucalyptus (*Eucalyptus maculata*), geranium (*Pelargonium reniforme*), lemon-grass (*Cymbopogon excavatus*), peppermint (*Mentha piperita*), soybean (*Neonotonia wightii*) (Choochote et al., 2007), neem (*Azadirachta indica*) (Murugan et al., 2011). Prajapati, Tripathi, Aggarwal and Khanuja (2005) have revealed the oviposition deterrent, ovicidal, and repellent activities of the essential oils of *Cinnamomum zeylanicum*, *Zingiber officinale*, and *Rosemarinus officinalis* against *Aedes aegypti*. Plant-derived bioproducts, however, shown promissing results in controling of mosquito vectors, if they are adequately effective and harmless to beneficial nontarget organisms and the environment. Furthermore, the insect resistance to plant-based mosquitocides has not been documented (Chaiyasit et al., 2006). Therefore, biologically active plant materials are attractively considerable of interest in mosquito control programmes recently (Nath, Bhuyan, and Goswami, 2006).

1.2 Research objectives

1.2.1 To control *Ae. aegypti* L. mosquitoes using kaffir lime (*C. hystrix*) peels and papaya (*C. papaya*) seeds.

1.2.2 To compare the effects of crude extracts of kaffir lime (*C. hystrix*) and ripe papaya (*C. papaya*) ripe seeds on *Ae. aegypti* L.control.

1.3 Scope and limitation of the study

1.3.1 Plants were collected on Suranaree University of Technology campus.

1.3.2 Plants were collected at one time through the study.

1.3.3 Second instar larvae were collected, and the only female adults of *Ae. aegypti* L. were used in all experiments.

1.4 Research hypothesis

C. hystrix peels and *C. papaya* seeds extracts can control *Ae aegypti* L. eggs, larvae, pupae and adults.

มหาวิทยาลัยเทคโนโลยีสุรนารี

1.5 References

Ashley, E.A., (2011). Dengue fever. **Trends in Anaesthesia and Critical Care**. 1: 39 - 41.

Chaithong, U., Choochote,W., Kamsuk, K., Jitpakdi, A., Tippawangkosol, P., Chaiyasit, D., Champakaew, D., Tuetun, B. and Pitasawatet, B. (2006). Larvicidal effect of pepper plants on *Aedes aegypti* (L.) (Diptera: Culicidae). **Journal of Vector Ecology**. 31: 138-144.

Chaiyasit, D., Choochte, W., Rattanachanpichai, E., Chaithong, U., Chaiwong, P., Jitpakdi, A., Tippawangkosol, P., Riyong, D. and Pitasawatet, B. (2006). Essential oils as potential adulticides against two populations of *Aedes aegypti*, the laboratory and natural field strains, in Chiang Mai province, northern Thailand. **Parasitology Research**. 99(6): 715-721.

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

Chuaycharoensuk, T., Juntarajumnong, W., Boonyuan, W., Bangs, M.J. Akratanakul, P., Thammapalo, S., Jirakanjanakit, N., Tanasinchayakul, S. and Chareonviriyaphap, T. (2011). Frequency of pyrethroid resistance in *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Thailand. **Journal of Vector Ecology**. 36: 204-212.

de-Freitas, R.M., Koella, Koella., J.C. and de-Oliveira., R.L. (2011). Lower survivalrate, longevity and fecundity of *Aedes aegypti* (Diptera: Culicidae) females orally challenged with dengue virus serotype 2. **Transactions of the Royal Society of Tropical Medicine and Hygiene**. 105: 452-458.

Department of Disease Control Ministry of Public Health, Bureau of Epidemiology. (2010). **Situation of dengue infection in Thailand**. [On-line]. Available: http://www.boe.moph.go.th/files/report/20101111_85282432.pdf.

Guzman, A. and Istúriz, E.I. (2010). Update on the global spread of dengue. **Journal of Antimicrobial Agents**. 36: 40-42.

Lee, I.K., Lee, W.H., Liu, J.W. and Yang., K.D. (2010). Acute myocarditis in dengue hemorrhagic fever: A case report and review of cardiac complications in

dengue-affected patients. **Journal of Infectious Diseases**. 14: 919-922.

Massad, E. and Coutinho, F.A.B. (2011). The cost of dengue control. **The Lancet**. 377: 1630-1631.

Meltzer, E. and Schwartz, E. (2009). A travel medicine view of dengue and dengue hemorrhagic fever. **Travel Medicine and Infectious Disease**. 7: 278-283.

Murrell, S., Wu., S.C. and Butler, M. (2011). Review of dengue virus and the development of a vaccine. **Biotechnology Advances**. 29: 239-247.

Murugan, K., Hwang, J.S., Kovendan, K., Kumar, K.P., Vasugi, C. and Kumar, A.N. (2011). Use of plant products and copepods for control of the dengue vector, *Aedes aegypti*. **Hydrobiologia**. 666: 331-338.

Nath, D.R., Bhuyan, M. and Goswami, S. (2006). Botanicals as mosquito larvicides. **Defence Science Journal**. 56: 507-511.

Prajapati, V., Tripathi, A.K., Aggarwal, K.K. and Khanuja, S.P.S. (2005). Insecticidal, repellent and oviposition-deterrent activity of selected essential oils against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. **Bioresource Technology**. 96: 1749-1757.

CHAPTER II

LITERATURE REVIEW

2.1 *Aedes aegypti* L.

Aedes aegypti L. is a vector of dengue fever and dengue hemorrhagic fever. These fevers are the most important arthropod-borne viral diseases of humans that infect millions of people in tropical and subtropical countries annually (Ponlawat and Harrington, 2009). The countries with the highest disease burden are in the Asia Pacific region, South and Central America and the Caribbean. The dengue virus is an RNA flavivirus which has 4 serotypes numbered DENV1, DENV2, DENV3 and DENV4. The *Ae. aegypti* mosquito is the most important vector. Worldwide, children are most affected reflecting the age of acquisition of infection rather than increased susceptibility (Ashley, 2011).

The countries of the affected region have been divided into four distinct climatic zones with different dengue transmission potential. Epidemic dengue is a major public health problem in Indonesia, Myanmar, Sri Lanka, Thailand and Timor-Leste which are in the tropical monsoon and equatorial zone where *Ae. aegypti* is widespread in both urban and rural areas. Multiple virus serotypes are circulating, and dengue is a leading cause of hospitalization and death in children (World Health Organization (WHO) (WHO, Online, 2009). In Thailand, dengue is reported from all four regions: Northern, Central, North-Eastern and Southern. In June 2007, the outbreaks were reported from Trat, Bangkok, Chiang Rai, Phetchabun, Phitsanulok,

Kamphaeng Phet, Nakhon Sawan and Phichit. A total of 58,836 cases were reported from January to November 2007. The case-fatality rate in Thailand is below 0.2% (WHO, Online, 2009). The symptoms of dengue fever are high fever, rash, a severe headache (dengue triad), severe joint and muscular pain (breakbone fever) (Ahmad et al., 2011).

There are more than 3,000 mosquito species in the world and at least 150 species are found in Thailand (Muizebelt, 2005). The most dangerous diseases are caused by several species of *Culex* (lymphatic filariasis), *Anopheles* (malaria), and *Aedes* genera. Two *Aedes* mosquitoes are *Ae. albopictus* which transmits dengue fever and *Ae. aegypti* which transmits dengue and yellow fevers (Awoke and Kassa, Online, 2006).

2.1.1 Classification of *Aedes aegypti* L.

Kingdom: Animalia

Phylum: Arthropoda

Class: Insecta

Order: Diptera

Family: Culicidae

Genus: *Aedes*

Species: *Aedes aegypti* L.

Ae. aegypti is unknown when first colonized Southeast Asia (Bosio et al., 2005). It is a predominately human-biting mosquito, is the primary vector worldwide and a common species throughout Thailand (Sukonthabhirom et al, 2009).

A hierarchical population genetic study was conducted among *Ae. aegypti* populations in Thailand from Chiang Mai in the North to Songkhla in the South (Bosio et al., 2005). The analysis based on the single-strand conformation of polymorphism of the NADH dehydrogenase subunit 4 (ND4) mitochondrial DNA gene showed that populations from urban areas were panmictic while suburban/rural sites were more differentiated. In urban settings, the availability of numerous breeding sites for oviposition can favor dispersal within this suitable habitat (Urdaneta-Marquez and Failloux, 2011). Based on allozymes, Sukonthabhirom et al. (2009) found only a low genetic differentiation among populations collected in Bangkok. Conversely, in suburban or rural areas such as in Chiang Mai, breeding sites were more likely separated by open space or agricultural areas leading to limit dispersal of *Ae. aegypti*. The high genetic differentiation found among *Ae. aegypti* samples collected in Chiang Mai has also been demonstrated using allozymes (Mousson et al., 2002). Vector competence assessed by oral infections of mosquito females with DENV-2 gave highly variable disseminated infection rates ranging from 52.9% to 100% (Urdaneta-Marquez and Failloux, 2011). From mark-release-recapture experiments, Harrington et al. (2005) confirmed that, *Ae. Aegypti* in Thailand disperse over relatively short distances suggesting that people rather than mosquitoes are the primary mode of dengue dissemination among communities.

2.1.2 Life cycle of *Aedes aegypti* L.

Ae. aegypti and other mosquitoes have a complex life-cycle with dramatic changes in shapes, functions, and habitats. The life cycle of a mosquito presents four distinct stages: egg, larva, pupa and adult. The first three stages take

place in or near water, while air is the medium for the adult stage (Otero, Solari and Schweigmann, 2006).

Female mosquitoes lay their eggs on the wet inner walls of containers with water such as cans, buckets, flower pots, bottles, jars, urns and rain-water containers. Larvae are hatched when water inundates the eggs as a result of rains or the addition of water by people. Used car tires provide an ideal larval habitat. In tropical climates, larvae can also be found in some natural cavities such as tree holes. In the following days, the larvae will feed on microorganisms and particulate organic matter, shedding their skins three times to be able to grow from first to fourth instars. When the larva has acquired enough energy and size and is in the fourth instars, metamorphosis is triggered, changing the larva into a pupa. Pupae do not feed; they just change in form until the body of the adult, flying mosquito is formed. Then, the newly formed adult emerges from the water after breaking the pupal skin. The entire life cycle lasts 8-10 days at room temperature, depending on the level of feeding. Thus, there is an aquatic phase (larvae, pupae) and a terrestrial phase (eggs, adults) in the *Ae. aegypti* life-cycle. This Complex life-cycle makes it rather difficult to understand where the mosquitoes come from. Similar complex life-cycles with aquatic and terrestrial forms are observed in amphibians. For educational and training purposes, it is rather useful to make life-cycle kits, so people have an opportunity to watch how the aquatic stages turn into terrestrial ones.

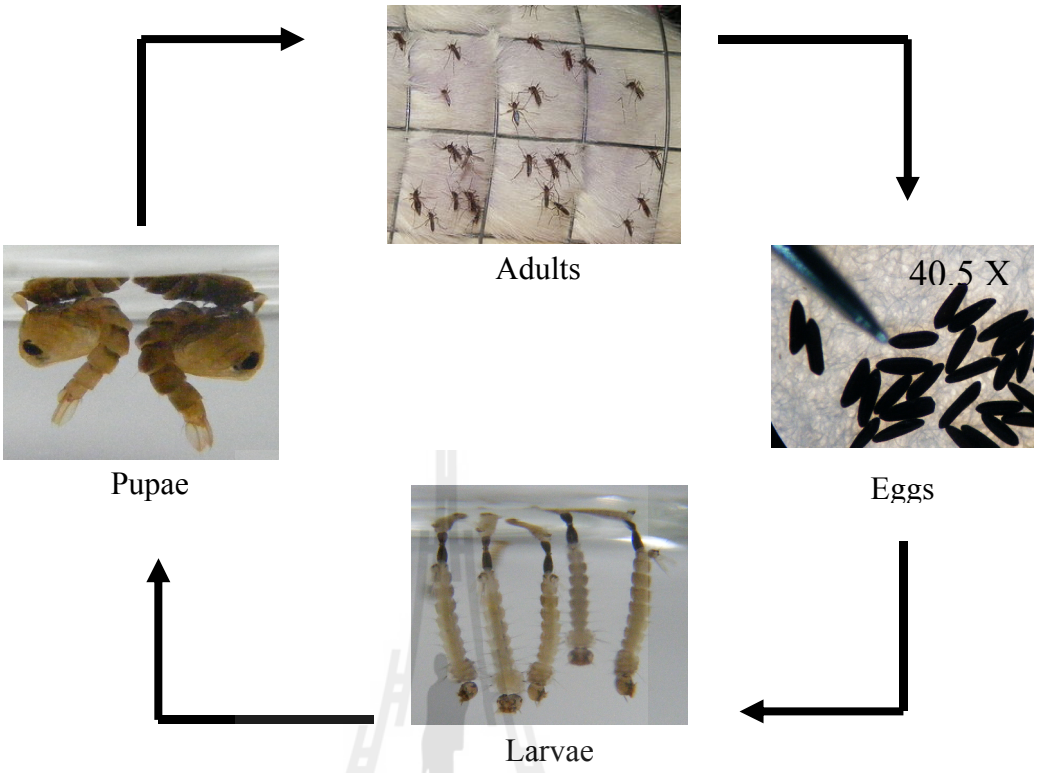

Figure 2.1 *Aedes aegypti* life cycle.

There is a very important adaptation of *Ae. aegypti* and other dengue vectors that makes controlling their populations a difficult task. Their eggs can withstand desiccation for several months, which means that even if all larvae, pupae, and adults were eliminated at some point in time, repopulation will occur as soon as the eggs in the containers are flooded with water. Unfortunately, there is no effective way to control the eggs in containers.

2.1.3 Distribution of *Ae. aegypti* L. and dengue

Ae. aegypti is the principal vector to humans of the four dengue viruses (DENV1- 4) which cause each year millions cases of dengue fever (DF) and dengue

hemorrhagic fever (DHF) around the world (Gubler, 2002). As long as a dengue vaccine and antiviral therapy are not available, dengue prevention will rely on the control of the mosquito vector.

Humans are the main vertebrate reservoir of DENV although there may be a relic monkey–mosquito cycle in some areas (Diallo et al., 2003). *Ae. aegypti formosus* native to Africa and forest dwelling form, may have spread from tropical African forests to urban environments in North Africa or in the near-East resulting isolation of the *Ae. aegypti* domestic populations known as *Ae. aegypti*. Human trading activities later introduced *Ae. aegypti* throughout the tropical and subtropical world. *Ae. aegypti* was probably imported into the New World from West Africa via the African slave trade in the 16–19th centuries. The following decades experienced a dramatic increase of DF epidemics in Southeast Asia with the first occurrence of DHF in the 1950s. Dengue outbreaks are widely endemic in South-East Asia and South America (Urdaneta-Marquez and Failloux, 2011).

Dengue is the most rapidly spreading mosquito-borne viral disease in the world. In the last 50 years, incidence has increased 30-fold with increasing geographic expansion to new countries and, in the present decade, from urban to rural settings (Figure 1.2) (WHO, Online, 2009).

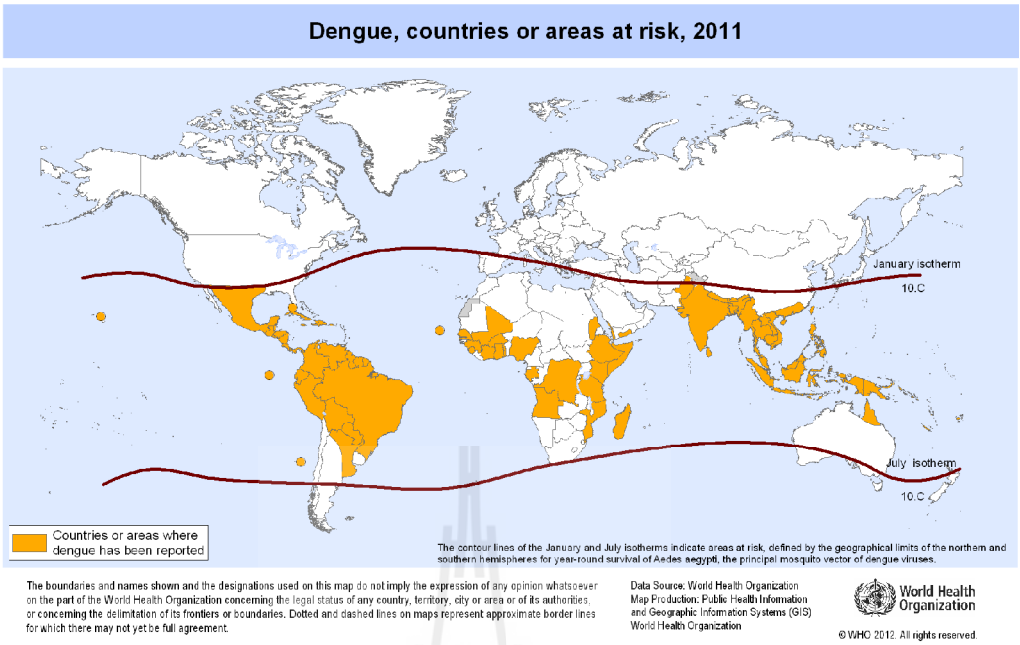

Figure 2.2 Countries or areas at risk of dengue fever throughout the world in 2011 (WHO, Online, 2012).

Ae. aegypti currently has a wide distribution in most tropical and subtropical areas, the present distribution does not reflect the maximum range of its potential distribution as defined by historical records. This is particularly evident in parts of Europe, in North America and in Australia where the species has previously displayed a much larger geographical distribution. Overall, the geographical distribution of *Ae. aegypti* is not static, and appears to have undergone significant changes in a number of continents over time. This mosquito which breeds mainly in domestic containers (Kittayapong and Strickman, 1993) needs several blood-meals before oviposition (Scott, Naksathit, Day, Kittayapong and Edman, 1997). *Ae. aegypti* distributes its eggs among several breeding sites (Apostol, Black, Reiter and Miller, 1994). The adult mosquito usually has a short flight range and disperses, actively in search of oviposition sites (Edman et al., 1998) or passively using human

transportations (Huber, Eisenreich, Hecht and Wächtershäuser, 2003). The direct estimate of dispersal based on the mark–release–recapture technique has shown that, in a dengue endemic area, *Ae. aegypti* females can fly at least 800 meter in one week (Reiter, Amador, Anderson and Clark, 1995; Hono´rio et al., 2003). Dispersal rate is inversely correlated with the abundance of breeding sites (Edman et al., 1998).

Human transport of eggs, immature or adult mosquitoes also contributes to extend the distribution of the species. The current global pandemic of DF/DHF began in Southeast Asia after World War II. Afterwards, displacements of civilians and soldiers who were susceptible to dengue infection, have provided ideal conditions for dissemination of DF and emergence of DHF in the region (Urdaneta-Marquez and Failloux, 2011). Dengue has become one of the leading causes of hospitalization among children. Dramatic ecologic disturbances and demographic changes have resulted in important increases of dengue transmission. Ecological changes have expanded the geographic distribution and increased the densities of *Ae. aegypti* which was introduced into Asia at the end of the 19th century. In 2003 only 8 countries in South East Asia Region reported dengue cases. As of 2006, ten out of the eleven countries in the Region (Bangladesh, Bhutan, India, Indonesia, Maldives, Myanmar, Nepal, Sri Lanka, Thailand and Timor-Leste) reported dengue cases. Bhutan reported the first dengue outbreak in 2004. An outbreak, with a high case fatality rate (3.55%) was first reported in Timor-Leste in 2005. Nepal reported dengue cases for the first time in November 2006. The Democratic Peoples' Republic of Korea is the only country in this Region of WHO that has no report of indigenous transmission of DF/DHF. Of the total world population of 6.2 billion, countries of the South-East Asia Region (SEAR) account for 1.5 billion (24%). On that scale, of the

2.5 billion people (living in the tropics and sub-tropics) at risk of DF/DHF, 52%, i.e. 1.3 billion population, live in SEAR (WHO, Online, 2007a).

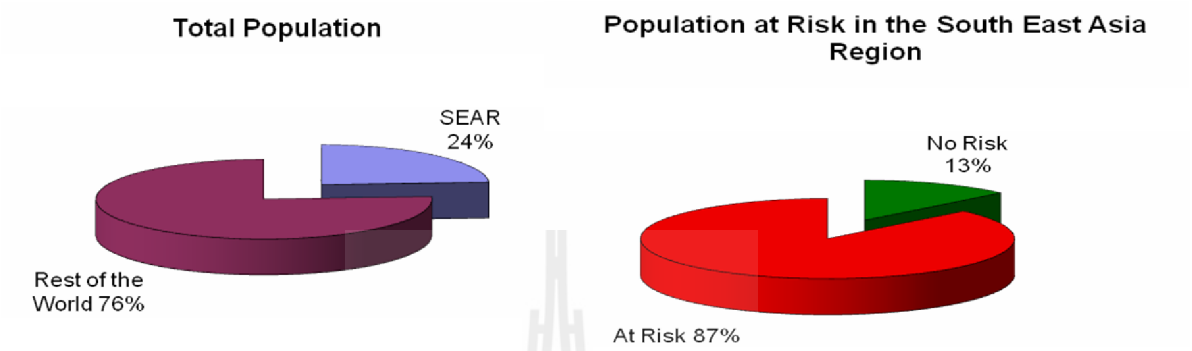

Figure 2.3 Population are at risk of DF/DHF in the Southeast Asia region (WHO, Online, 2007a).

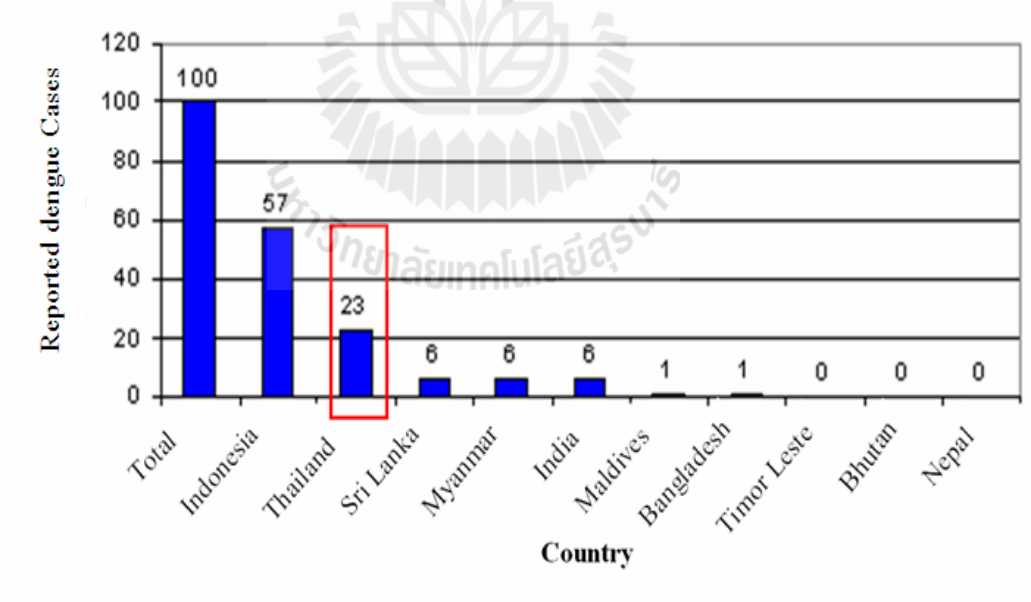

Figure 2.4 Percentage of dengue cases reported by ten countries in Southeast Asia region of WHO in 2006 (WHO, Online, 2007b).

2.2 Biological control of *Aedes aegypti*

Biological control is based on the introduction of organisms that prey upon, parasitize, compete with or otherwise reduce populations of the target species. Biological control, using the natural enemies of *Ae. aegypti*, appears to be an alternative approach to the systematic failure of use of insecticides (Lardeux, Riviere, Séchan and Loncke, 2002).

The application of biological control agents, which are directed against the larval stages of the mosquitoes, in Southeast Asia has been restricted to specific container habitats in small-scale field operations (WHO, Online, 2011). While biological control avoids chemical contamination to the environment. There may be operational limitations such as expense and task of rearing the organisms on a large scale. The difficulty in applying agents and their limited utility in aquatic sites where temperature, pH and organic pollution may exceed the narrow requirements of the organism. Importantly, the biological control of organisms are not resistant to desiccation, hence their utility is mainly restricted to container habitats that are seldom emptied or cleaned, such as large water-storage containers or wells. However, the willingness of communities to accept the introduction of organisms into water containers is essential. Community involvement is also desirable in distributing the agents, monitoring and restocking containers, as necessary. There is a vast array of agents used in the biological control of mosquitoes, including copepods (Schaper and Hernández-Chavarría, 2006). Larvivorus fish (*Gambusia affinis* and *Poecilia reticulata*) have been extensively used for the control of *An. stephensi* and/or *Ae. aegypti* in large waterbodies or large water containers in many countries in Southeast

Asia. For example, the community-based use of larvivorous fish *Poecilia reticulata* to control the dengue vector *Ae. aegypti* in domestic water-storage containers in rural Cambodia (Seng et al., 2008). The applicability and efficiency of this control depends on the type of containers used (WHO, Online, 2011). The predatory role of copepod crustaceans was documented between 1930 and 1950. However, scientific evaluation was carried out only in 1980 in Tahiti, French Polynesia, where it was found that *Mesocyclops aspericornis* could effect a 99.3% mortality rate on *Ae. aegypti* larvae and 9.7% and 1.9%, on *Cx. quinquefasciatus* and *Toxorhynchities amboinensis* larvae respectively (Kittayapong and Strickman, 1993). Trials in crab burrows against *Ae. polynesiensis* and in water tanks, drums and covered wells met with mixed results. In Queensland, Australia, of seven species evaluated in the laboratory all but *Micropterus notius* were found to be effective predators of both *Ae. aegypti* and *An. farauti* but not against *Cx. quinquifasciatus*. Field releases in both northern and southern Queensland, however, showed mixed results. In Thailand too, the results were mixed; but in Vietnam the results were more successful, contributing to the eradication of *Ae. aegypti* from one village. Although the lack of nutrients and frequent cleaning of some containers can prevent the sustainability of copepods, they could be suitable for large containers that need not to be cleaned regularly, such as wells, concrete tanks and tyres (Lardeux, 1992). They can also be used in conjunction with *Bacillus thuringiensis H-14.* Copepods have a role in dengue vector control, but more research is required on the feasibility of operational use.

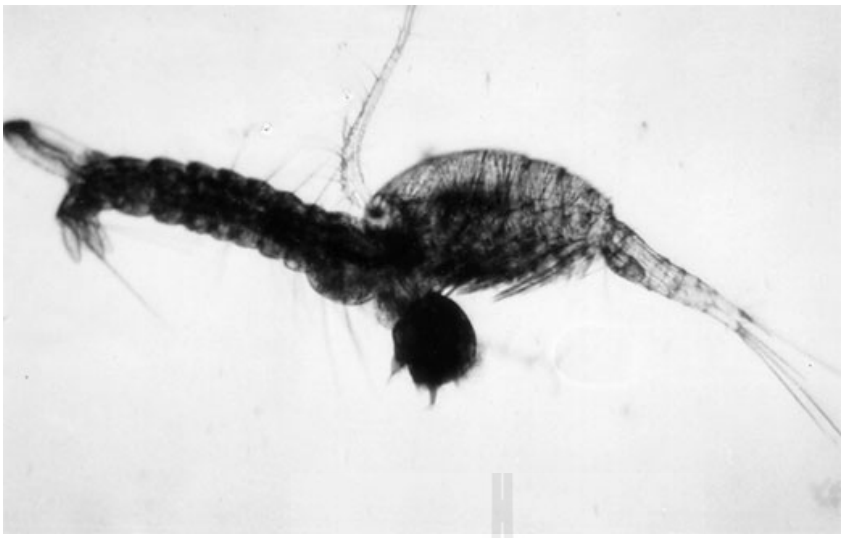

Figure 2.5 *Mesocyclops aspericornis* seizing an *Ae. aegypti* Lavar (Marten, Online, 2001).

Research on insect repellents has explored, among others, the effect of botanical products, promoting the development of "natural" plant-based insect repellents. Essential oils from plants such as andiroba (*Carapa* spp.), basil (*Ocimum* spp.), catnip (*Nepeta* spp.), cedar (*Cedrus* spp.), citronella grass (*Cymbopogon* spp.), clove (*Syzygium* spp.), lemon eucalyptus (*Eucalyptus* spp.), geranium (*Pelargonium* spp.), neem (*Azadirachta* spp.), rosemary (*Rosmarinus* spp.), and thyme (*Thymus* spp.) have all been shown be effective on repelling mosquitoes (Thorsell, Mikiver, Malander and Tunon, 1998; Barnard, 1999; Tawatsin, Wratten, Scott, Thavara and Techadamrongsin 2001). The chemical composition of essential oils is responsible for their repellence (Rota, Herrera, Martinez, Sotomayor and Jordan, 2008). Many constituents have a pleasant fragrance, relatively low mammalian toxicity, and a vapour pressure suitable for action as a volatile spatial repellent or attraction antagonist (Campbell, Gries and Gries, 2010).

2.3 Selected medicinal plants

2.3.1 Kaffir lime (*Citrus hystrix* DC.)

Kaffir lime (*Citrus hystrix*, DC.) is a member of citrus family, and is also known as kieffer lime, Thai bergamot, limau purut or ma-grood.

Kaffir lime (*Citrus hystrix*, DC.) is classified as follows ;

Kingdom: Plantae

Subkingdom: Viridaeplantae

Infrakingdom: Streptophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Infradivision: Angiospermae

Class: Magnoliopsida

Superorder: Rosanae

Order: Sapindales

Family: Rutaceae

Genus: *Citrus* L.

Species: *Citrus hystrix* DC.

Figure 2.6 Kaffir lime (*Citrus hystrix* DC.).

The leaves of kaffir lime trees are a dark green color with a glossy sheen, and are composed of two leaflets. The top leaflet is lightly pointed at its tip and is attached to another leaflet beneath that is broader on its upper edge. Kaffir lime leaves are an important ingredient in many Thai dishes, from soups and salads to curries and stirred fries. Kaffir lime is native to Indonesia, Thailand, and Malaysia, and is commonly used in Asian cuisine and folk medicine. The major compounds in kaffir lime peel are β-pinene, limonene, α-pinene β-pinene α-terpiene α-phellandrene limonene α-terpinolene, cis-linaloloxide, isopulegol, α-terpineol and caryophyllene (Laohakunjit, Kerdchoechuen, Singkhornart, and Chatpaisarn, 2009). Kaffir lime leaves have various medical and culinary uses in Southeast Asia. Fresh peels and dried fruits are used to relieve nausea, dispel gas, and control normal menstruation. The fruits are used for cough suppression, and as a shampoo. Kaffir lime leaves and fruit extracts have antioxidant activity (Siripongvutikorna, Thummaratwasik and Huang, 2005), free radical scavenging ability (Hutadilok-Towatana, Chaiyamutti, Panthong, Mahabusarakam and Rukachaisirikul, 2006),

antimicrobial activity (Siripongvutikorn et al., 2005), and anti-inflammatory activity (Lertsatitthanakorn, Taweechaisupapong, Aromdee and Khunkitti, 2006). In regard to cancer research, kaffir lime extracts have been shown to have anti-proliferative activity on KB (cervical cancer) and P388 (mouse leukemia) cell lines (Manosroi, Dhumtanom and Manosroi, 2006). Glyceroglycolipids in kaffir lime leaves could inhibit 12-Otetradecanoylphorbol 13-acetate (TPA) and skin carcinogen activities in mice (Murakami, Nakamura, Koshimizu and Ohigashi, 1995). Tawatsin et al. (2001) found that kaffir lime oil provided protection against the three mosquito species, *Ae aegypti* ; *Anopheles dirus* and *Culex quinquefasciatus*. It could be formulated with vanillin as mosquito repellant.

2.3.2 Papaya (*Carica papaya* L.)

Papaya (*Carica papaya* L.) is classified as follows ;

Kingdom: Plantae

Subkingdom: Viridaeplantae

Infrakingdom: Streptophyta

Division: Tracheophyta

Subdivision: Spermatophytina

Infradivision: Angiospermae

Class: Magnoliopsida

Superorder: Rosanae

Order: Brassicales

Family: Caricaceae

Genus: *Carica* L.

Species *papaya*

Papaya is native to the tropics of the Americas, and was cultivated in Mexico several centuries before the emergence of the Mesoamerican classic cultures. Papaya (*Carica papaya* L.) is a popular and economically important fruit tree of tropical and subtropical countries. The fruit is consumed world-wide as fresh fruit and as a vegetable or used as processed products (Teixeira da Silva et al., 2007).

Figure 2.7 *Carica papaya* fruit and its seeds.

Papaya is a fast-growing, semi-woody tropical herb. The stem is single, straight and hollow and contains prominent leaf scars. Papaya exhibits strong apical dominance rarely branching unless the apical meristem is removed, or damaged. Palmately-lobed leaves, usually large, are arranged spirally and clustered at the crown, although some differences in the structure and arrangement of leaves have been reported on Malaysian cultivars (Chan and Teo, 2002). Generally, papaya cultivars are differentiated by the number of main leaf veins, the number of lobes at the leaf margins, leaf shape, stomata type, and wax structures on the leaf surface, as well as the colour of the leaf petiole. The fruit is melon-like, oval to nearly round,

somewhat pyriform, or elongated club-shaped, 15-50 cm long and 10-20 cm thick and weighing up to 9 kg (Morton, 1987). Semiwild (naturalized) plants bear small fruits 2.5-15 cm in length. The skin is waxy and thin but fairly tough. When the fruit is immature, it is rich in white latex and the skin is green and hard. As ripening progresses, papaya fruits develop a light- or deep- yellow-orange coloured skin while the thick wall of succulent flesh becomes aromatic, yellow-orange or various shades of salmon or red. It is then juicy, sweetish and somewhat like a cantaloupe in flavor but some types are quite musky (Morton, 1987). Mature fruits contain numerous grey-black ovoid seeds attached lightly to the flesh by soft, white, fibrous tissue.

C. papaya contains many biologically active compounds. Two important compounds are chymopapain and papain which are widely known as being useful for digestive disorders and disturbances of the gastrointestinal tract. Huet et al. (2006) showed that papaya-derived papain, caricain, chymopapain, and glycine endopeptidase can survive acidic pH conditions and pepsin degradation. However, at low pH, a conformational transition that instantaneously converts their native forms into molten globules that are quite unstable and rapidly degraded by pepsin. Thus, they may need to be protected against both acid denaturation and proteolysis for them to be effective in the gut after oral administration for the control of gastrointestinal nematodes. Apart from papain and chymopapain, *C. papaya* contains many biologically active compounds. *C. papaya* lipase, or CPL, a hydrolase, is tightly bonded to the water-insoluble fraction of crude papain and is thus considered as a "naturally immobilized" biocatalyst. Domínguez de María, Sinisterra, Tsai and Alcántara (2006) reviewed several applications of CPL: (i) fats and oils modification, derived from the sn-3 selectivity of CPL as well as from its preference for short-chain

fatty acids; (ii) esterification and inter-esterification reactions in organic media, accepting a wide range of acids and alcohols as substrates; and (iii) more recently, the asymmetric resolution of different non-steroidal anti-inflammatory drugs (NSAIDs), 2-(chlorophenoxy)propionic acids, and nonnatural amino acids. The papaya Kunitz-type trypsin inhibitor, a 24-kDa glycoprotein, when purified, stoichiometrically inhibits bovine trypsin in a 1:1 molar ratio (Azarkan, Dibiani, Goormaghtigh,

Raussens and Baeyens-Volant, 2006). A novel α-amylase inhibitor from *C. papaya* seeds was recently shown to be effective against cowpea weevil (Callosobruchus maculatus) (Farias et al., 2007). A comprehensive list of the compounds found in various parts of the papaya plant can be accessed from the USDA Phytochemical and Ethnobotanical Databases. Of note, levels of the compounds vary in the fruit, latex, leaves, and roots. Furthermore, plant parts from male and female trees have been found to differ in the amounts of the compounds produced. For example, phenolic compounds tend to be higher in male plants than female plants. Cultivars also vary in the quantity of the compounds.

The seeds of *Carica papaya* L. (Caricaceae) have been used for decades in parts of Asia and South America as vermifugicidal agent. In India, the seeds are administered with honey for expelling roundworms (Krishnakumari and Majumder, 1960; Adebiyi, Adaikan, and Prasad, 2003).

In Panama, powdered papaya seeds mixed with honey and castor oil are orally taken by (as laxative) to get rid of intestinal worms (Gupta, Arias, Correa and Lamba, 1979; Adebiyi, Adaikan and Prasad, 2003). There are some studies showed that papaya seeds extracts are capable of killing worms such as *Toxascaris transfuga,*

Ascaris lumbricoides, Pheretima sp. and *Caenorhabditis elegans in vitro* and also deworm infected animals (Adebiyi, Adaikan and Prasad, 2003). Apart from their use as vermifugicidal agents, papaya seed preparations are reported to use in folk medicine to facilitate good menstrual flow and are thought to have abortifacient properties. Benzyl isothiocyanate (BITC), a chemopreventive phytochemical found in cruciferous vegetables has also been shown to present in different extracts of papaya seeds (Adebiyi et al., 2003). Fruit and seed extracts have profound bactericidal activity against *Staphylococcus aureus Bacillus cereus Esherischia coli, Pseudomanas aeruginosa* and *Shigella flexneri*. (Emeruwa, 1982 quoted in Oloyede, 2005) The juice of fruit is used for curing warts, cancer, tumors and indurations of the skin. Leaves have been poulticed onto nervous pains, elephantoid growths. They were smoked for asthma relief in various remote areas. In addition, the hypoglycemic effect of papaya has been reported (Reed, 1976 quoted in Oloyede, 2005).

Ahmad et al. (2011) investigated the potential of *C. papaya* leaves extracts against dengue fever in 45 year old patient bitten by carrier mosquitoes. For the treatment of dengue fever the extract prepared in water. Twenty five milliliters of aqueous extract of *C. papaya* leave was administered to patient infected with dengue fever twice daily, i.e. morning and evening for five consecutive days. Before the extract administration the blood samples from patient were analyzed. Platelets count (PLT), white blood cells (WBC) and neutrophils (NEUT) decreased. Subsequently, It after admin is traction of the extract was found that the PLT count, WBC and NEUT increased. It demonstrated that *C. papaya* leaves aqueous extract exhibited potential activity against Dengue fever. Kovendan, Murugan, Kumar, Vincent and Hwang (2012) carried out to establish the properties of *C. papaya* leaf extract against *Ae.*

aegypti. This is extract showed larvicidal and pupicidal effects after 24 h of exposure; however, the highest larval and pupal mortalitie were found in the methanolic leaf extract of *C. papaya* against the first-to fourth-instar larvae and pupae with LC_{50} of 51.76 ppm, 61.87 ppm, 74.07 ppm, 82.18 ppm, and 440.65 ppm, respectively. It suggest that the leaves extract of *C. papaya* is promising as good larvicidal and pupicidal properties of against *A. aegypti*.

2.4 References

Adebiyi, A., Adaikan, P.G. and Prasad, R.N.V. (2003). Tocolytic and toxic activity of papaya seed extract on isolated rat uterus. **Life Sciences**. 74: 581-592.

Ahmad, N., Fazal, H., Ayaz, M., Abbasi, B.H., Mohammad, I. and Fazal, L. (2011). Dengue fever treatment with *Carica papaya* leaves extracts. **Asian Pacific Journal of Tropical Biomedicine**. 1: 330-333.

Apostol, B.L., Black. W.C., Reiter, P. and Miller, B.R. (1994). Use of randomly amplified polymorphic DNA amplified by polymerase chain reaction markers to estimate the number of *Aedes aegypti* families at oviposition sites in San Juan, Puerto Rico. **American Journal of Tropical Medicine and Hygiene**. 51: 89-97.

Ashley, E.A. (2011). Review Dengue fever. **Trends in Anaesthesia and Critical Care**. 1(1): 39-41.

Awoke, A. and Kassa L. (2006). **Vector and Rodent Control** [On-line]. Available: http://www.cartercenter.org/ resources/pdfs/health/ephti/library/lecture_notes/en

v_health_science_students/VectorRodent.pdf.

Azarkan, M., Dibiani, R., Goormaghtigh, E., Raussens, V. and Baeyens-Volant, D. (2006). The papaya Kunitz-type trypsin inhibitor is a highly stable beta-sheet glycoprotein. **Biochimica et Biophysica Acta**. 1764(6): 1063-1072.

Barnard, D.R. (1999). Repellency of essential oils to mosquitoes (Diptera: Culicidae). **Journal of Medical Entomology**. 36: 625-629.

Bosio, C.F., Harrington, L.C., Jones, J.W., Sithiprasasna, R. Norris, D.E. and Scott, T.W. (2005). Genetic structure of *Aedes aegypti* populations in Thailand using mitochondrial DNA. **American Journal of Tropical Medicine and Hygiene**. 72(4): 434-442.

Campbell, C., Gries, R. and Gries, G. (2010). Forty-two compounds in eleven essential oils elicit antennal responses from *Aedes aegypti*. **Entomologia Experimentalis et Applicata**. 138(1): 21-32.

Chan, L.-K. and Teo, C.-K.H. (2002). Micropropagation of Eksotika, a Malaysia papaya cultivar, and the field performance of the tissue culture derived clones. **Acta Horticulturae**. 575: 99-105.

Diallo, M., Ba, Y., Sall, A.A., Diop, O.M., Ndione, J.A., Mondo, M., Girault, L. and Mathiot, C. (2003). Amplification of the sylvatic cycle of dengue virus type 2, Senegal, 1999-2000: Entomologic findings and epidemiologic considerations. **Emerging Infectious Disease**. 9(3): 362-367.

Domínguez de María, P., Sinisterra, J.V., Tsai, S.-W. and Alcántara, A.R. (2006). *Carica papaya* lipase (CPL): An emerging and versatile biocatalyst. **Biotechnology Advances**. 24: 493-499.

Edman, J.D., Scott, T.W., Costero, A., Morrison, A.C., Harrington, L.C. and Clark, G.G. (1998). *Aedes aegypti* (Diptera: Culicidae) movement influenced by availability of oviposition sites. **Journal of Medical Entomology**. 35: 578-583.

Emeruwa, A.C. (1982). Antibacterial substance from *Carica papaya* fruit extract. **Journal of Natural Products**. 45: 123-127.

Farias, L.R., Costa, F.T., Souza, L.A., Pelegrini, P.B., Grossi-de-Sá, M.F., Neto Jr, S.M., Bloch, C., Laumann, R.A., Noronha, E.F. and Franco, O.L. (2007). Isolation of a novel *Carica papaya* α-amylase inhibitor with deleterious activity toward *Callosobruchus maculatus*. **Pesticide Biochemistry and Physiology**. 87: 255-260.

Gubler, D.J. (2002). How effectively is epidemiological surveillance used for dengue programme planning and epidemic response. **Dengue Bulletin**. 26: 96-106.

Gupta, M.P., Arias, T.D., Correa, M., and Lamba, S.S. (1979). Ethnopharmacognostic observations on Panamanian medicinal plants (Part 1). **Quarterly Journal of Crude Drug Research**. 17(3-4): 115-130.

Harrington, L.C., Scott, T.W., Lerdthusnee, K., Coleman, R.C., Costero, A., Clark, G.G., Jones, J.J., Kitthawee, S., Kittayapong, P., Sithiprasaana, R. and Edman, J. D. (2005). Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. **American Society of Tropical Medicine and Hygiene**. 72(2): 209-220.

Honório, N.A., Silva Wda, C., Leite, P.J., Goncalves, J.M., Lounibos, L.P. and Lourenco-de-Oliveira, R. (2003). Dispersal of *Aedes aegypti* and *Aedes*

albopictus (Diptera: Culicidae) in an urban endemic dengue area in the State of Rio de Janeiro, Brazil. **Memórias do Instituto Oswaldo Cruz**. 98: 191-198.

Huber, C., Eisenreich, W., Hecht, S. and Wächtershäuser, G. (2003). A possible primordial peptide cycle. **Science**. 301: 938-940.

Huet, J., Looze, Y., Bartik, K., Raussens, V., Wintjens, R. and Boussard, P. (2006). tructural characterization of the papaya cysteine proteinases at low pH. **Biochemical and Biophysical Research Communications**. 341: 620-626.

Hutadilok-Towatana, N., Chaiyamutti, P., Panthong, K., Mahabusarakam, W. and Rukachaisirikul, V. (2006). Antioxidative and free radical scavenging activities of some plants used in Thai folk medicine. **Pharmaceutical Biology**. 44(3): 221-228.

Kittayapong, P. and Strickman, D. (1993). Three simple devices for preventing development of *Aedes aegypti* larvae in water jars. **American Journal of Tropical Medicine and Hygiene**. 49(2): 158-165.

Kovendan, K., Murugan, K., Kumar, A.N., Vincent, S. and Hwang, J.-H. (2012). Bioefficacy of larvicdial and pupicidal properties of *Carica papaya* (Caricaceae) leaf extract and bacterial insecticide, spinosad, against chikungunya vector, *Aedes aegypti* (Diptera: Culicidae). **Parasitology Research**. 110(2): 669-678.

Krishnakumari, M.K. and Majumder, S.K. (1960). Studies on the anthelmintic activities of seeds of *Carica papaya* Linn. **Annals of Biochemistry and Experimental Medicine** 20: 551-556.

Laohakunjit, N., Kerdchoechuen, O., Singkhornart, S. and Chatpaisarn, A. (2009).

Simultaneous distillation extraction for essential oil from Rutaceae. **Agricultural Science Journal**. 40: 1 (Suppl.): 79-82.

Lardeux, F.R. (1992). Biological control of culicidae with the copepod mesocyclops aspericornis and larvivorus fish (Poeciliidae) in a village of French Polynesia. **Medical and Veterinary Entomology**. 6: 9-15.

Lardeux, F., Riviere, F., Séchan, Y. and Loncke, S. (2002). Control of the *Aedes* vector of the dengue viruses and *Wuchereria bancrofti*: The French Polynesian experience. **Annuals of Tropical Medicine and Parasitology**. 96: S105-S116.

Lertsatitthanakorn, P., Taweechaisupapong, S., Aromdee, C. and Khunkitti, W. (2006). *In vitro* bioactivities of essential oils used for acne control. **International Journal of Aromatherapy**. 16(1): 43-49.

Manosroi, J., Dhumtanom, P. and Manosroi, A. (2006). Anti-proliferative activity of essential oil extracted from Thai medicinal plants on KB and P388 cell lines. **Cancer Letters**. 235: 114-120.

Marten, G. (2001). **Vietnam-dengue hemorrhagic fever, copepods, and biological control of mosquitoes** [On-line]. Available: http://www.ecotippingpoints. org/our-stories/ indepth/ vietnam-dengue-mosquito-copepod-biological-control. html.

Morton, J.F. (1987). Papaya. In: J.F. Morton (ed). **Fruits of warm climates** (pp 336-346). Florida: Creative resource systems.

Mousson, L., Vazeille, M., Chawprom, S., Prajakwong, S., Rodhain, F. and Failloux, A-B. (2002). Genetic structure of *Aedes aegypti* populations in Chiang Mai

(Thailand) and relation with dengue transmission. **Tropical Medicine and International Health**. 7: 865-872.

Muizebelt, M. (2005). **Mosquito bite/blood**. [On-line]. Available: http://www.treknature.com/ gallery/ Asia/ Thailand/ photo36161.htm.

Murakami, A., Nakamura, Y., Koshimizu, K. and Ohigashi, H. (1995). Glyceroglycolipids from *Citrus hystrix*, a traditional herb in Thailand, potently inhibit the tumor-promoting activity of 12-O-Tetradecanoylphorbol 13-Acetate in mouse skin. **Journal of Agricultural and Food Chemistry**. 43(10): 2779-2783.

Oloyede, O.I. (2005). Chemical Profile of Unripe Pulp of *Carica papaya*. **Pakistan Journal of Nutrition**. 4(6): 379-381.

Otero, M., Solari, H.G., and Schweigmann, N. (2006). A stochastic population dynamics model for *Aedes aegypti*: Formulation and application to a city with temperate climate. Bulletin of Mathematical Biology. 68: 1945-1974.

Ponlawat, A. and Harrington, L.C. (2009). Factors associated with male mating success of the dengue vector mosquito, *Aedes aegypti*. **American Journal of Tropical Medicine and Hygiene**. 80(3): 395-400. Reed, C.F. (1976). Information summaries on 1000 economic plants. Typescripts submitted to the USDA.

Reiter, P., Amador, M.A., Anderson, R.A. and Clark, G.G. (1995). Short report: dispersal of *Aedes aegypti* in an urban area after blood feeding as demonstrated by rubidium-marked eggs. **American Journal of Tropical Medicine and Hygiene**. 52: 177-179.

Rota, M.C., Herrera, A., Martinez, R.M., Sotomayor, J.A. and Jordan, M.J. (2008). Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. **Food Control**. 19: 681-687.

Schaper, S. and Hernández-Chavarría. F. (2006). Scanning electron microscopy of damage caused by *Mesocyclops thermocyclopoides* (Copepoda: Cyclopoidea) on larvae of the dengue fever vector *Aedes aegypti* (Diptera: Culicidae). **Revista de Biologia Tropical**. 54(3): 843-846.

Scott, T.W., Naksathit, A., Day, J.F., Kittayapong, P. and Edman, J.D. (1997). Fitness advantage for *Aedes aegypti* and the viruses it transmits when females feed only on human blood. **American Journal of Tropical Medicine and Hygiene**. 57: 235-239.

Seng, C.M., Setha, T., Nealon, J., Socheat, D., Chantha, N. and Nathan, M.B. (2008). Community-based use of the larvivorous fish *Poecilia reticulata* to control the dengue vector *Aedes aegypti* in domestic water storage containers in rural Cambodia. **Journal of Vector Ecology**. 33(1): 139-144.

Siripongvutikorn, S., Thummaratwasik, P. and Huang, Y. (2005). Antimicrobial and antioxidation effects of Thai seasoning, Tom-Yum. **LTW-Food Science and Technology**. 38(4): 347-352.

Sukonthabhirom, S., Saengtharatip, S., Jirakanchanakit, N., Rongnoparut, P., Yoksan, S., Daorai, A. and Chareonviriyaphap, T. (2009). Genetic structure among Thai populations of *Aedes aegypti* mosquitoes. **Journal of Vector Ecology**. 34(1): 43-49.

Tawatsin, A., Wratten, S.D., Scott, R.R., Thavara, U. and Techadamrongsin, Y. (2001). Repellency of volatile oils from plants against three mosquito vectors. **Journal of Vector Ecology**. 26(1): 76-82.

Teixeira da Silva, J.A., Rashid, Z., Nhut, D.T., Sivakumar, D., Gera, A., Teixeira Souza Jr, M. and Tennant, P.F. (2007). Papaya (*Carica papaya* L.) Biology and biotechnology. **Tree and Forestry Science and Biotechnology**. 1(1): 47-73.

Thorsell, W., Mikiver, A., Malander, I. and Tunon, H. (1998). Efficacy of plant extracts and oils as mosquito repellents. **Phytomedicine**. 5(4): 311-323.

Urdaneta-Marquez, L. and Failloux, A-B. (2011). Population genetic structure of *Aedes aegypti*, the principal vector of dengue viruses. **Infection, Genetics and Evolution**. 11: 253-261.

World Health Organization (WHO). (2007a). **Dengue/DHF: Situation of dengue/dengue haemorrhagic fever in South-East Asia region** [On-line]. Available: http://www.searo. who. int/ en/ Section 10/ Section 332_1098.htm.

World Health Organization (WHO). (2007b). **Dengue/ DHF reported cases of DF/DHF in selected countries in SEA region (1985-2005)** [On-line]. Available: http://www.searo.who.int/en/Section10/Section332_1101.htm.

World Health Organization (WHO). (2009). **Treatment, prevention and control**. [On-line]. Available: http://whqlibdoc.who.int/ publications/2009/9789241547871_ eng.pdf.

World Health Organization (WHO). (2011). **Dengue/DHF**. [On-line]. Available: http://www.searo.who. int/ en / Section10 / Section 332/ Section 554_2569.htm.

World Health Organization (WHO). (2012). **Global dengue transmission world** [On-line]. Available: http:// gamapserver. who. Int / mapLibrary / Files/ Maps/ Global_DengueTransmission_ITHRiskMap.png.

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER III

SOME PHYTOCHEMICALS AND CYTOTOXICITY OF KAFFIR LIME (*CITRUS HYSTRIX* DC.) PEEL AND PAPAYA (*CARICA PAPAYA* L.) SEED EXTRACTS

3.1 Abstract

Kaffir lime (*Citrus hyptrix* DC.) peel and ripe papaya (*Carica papaya* L.) seeds were extracted in 70% ethanol and water, kaffir lime peel ethanolic extract (KPE/e), kaffir lime peel water extract (KPE/w), ripe papaya seed ethanolic extract (PSE/e), and ripe papaya seed water extract (PSE/w) respectively. Total phenolic compounds (TPC) of the extracts were quantified by the Folin-Ciocalteau assay. The TPCs of the ethanolic and water extracts of kaffir lime peel and papaya seeds were 51.26 ± 0.05, 35.80 ± 0.07, 27.62 ± 0.01 and 31.11 ± 0.31 mg GAE/g, respectively. TLC analysis and vanillin sulphuric reagent detection indicated that the presence of a terpenes group as the major compound constituent in the extracts. The cytotoxicity of the extracts was determined by Shrimp Lethality Assay (BSLA). The cytotoxic efficacy of KPE/w and PSE/e were the most with LC_{50} value at 24 h of 68.81 ± 0.00 and 68.92 ± 0.00 µg/mL. KPE/e cytotoxic efficacy was moderate with LC_{50} of 125.32 ± 0.56 µg/mL. The cytotoxic efficacy of PSE/w was least at LC_{50} of 341.44 ± 0.29 µg/mL.

The cytotoxicity of all extracts was likely to depend on the terpenes, but was opposite to the amount of TPC.

3.2 Introduction

The lime is a tropical fruit, yet it is ubiquitous and used for everything from food to cosmetics and cleaning products. There are many varieties of this citrus fruit and they are extremely nutritious. Kaffir lime *Citrus hystrix* CD. (family: Rutaceae) is native to Southeast Asia. It is a small tree, about 3 - 5 meters tall. Kaffir lime fruits are pear-shaped with rough warty skin (Koh and Ong, 1999). Kaffir lime is known for their beautiful aroma and strong citrus flavor. Due to its culinary and medicinal uses. Kaffir lime fruits are bright green with rough bumpy skin.

Figure 3.1 Kaffir lime peel.

Kaffir lime fruits have a stronger astringent, more intense flavor that blends well with the complex flavors food in Thai, Vietnamese and Cambodian cuisine. The oil from the rind/peel has strong insecticidal properties. The juice is generally regarded as too acidic to use in food preparation, but finds use as a cleanser for

clothing and hair in Thailand. Kaffir lime leaves and fruits have pleasant smell and are referred to as medicinal lime (Yaacob and Subhadrabandhu, 1995). The kaffir lime juice was deliberately applied as a treatment for insect bites and as an insect repellent (Koh and Ong, 1999). The leaf oil of kaffir lime yielded high number of oxygenated monoterpenes. The identified oxygenated compounds contained approximately over 85% of total oil. The major components characterized from the kaffir lime leaf oil were β-citronellal, a monoterpenoid, representing 66.85% of total oil. This was followed by β-citronellol (6.59%), linalool (3.90%) and citronellol (1.76%). Others components were never exceeded 2% of the oil composition. Kaffir lime essential oil showed good repellence properties against cluster caterpillar *Spodoptera litura* larvae. (Loh, Awang, Omar and Rahmani, 2011).

Papaya (*Carica papaya* L.) is a tropical fruit tree with a single stem growing from 5 to 10 meters and commonly well known for its food and nutritional values throughout the world (Afolabi and Ofobrukweta, 2011). The fruit is ripe when it feels soft and its skin has attained amber to orange.

มหาวิทยาลัยเทคโนโลยีสุรนารี

Figure 3.2 Papaya (*Carica papaya* L.) seeds.

Papaya is a plant that is widely cultivated in many parts of the tropics. Papaya can be used as a food, a cooking aid, and in traditional medicine. The stem and bark may be used in rope production. Its fruits and it is favored by the people of the tropics, as breakfast, as ingredients in jellies, preserves, or cooked in various ways. Papaya fruit is a rich source of nutrients such as provitamin A carotenoids , vitamin C, B vitamins, dietary minerals and dietay fiber. Papaya skin, pulp and seeds also contain a variety of phytochemicals, including natural phenols. Danielone is a phytoalexin found in the papaya fruit. This compound showed high antifungal activity against *Colletrichum gloesporioides*, a pathogenic fungus of papaya (Echeverri et al., 1997). The unripe green fruit can be eaten cooked, usually in curries, salads, and stews. Green papaya is used in Southeast Asian cooking, both raw and cooked. In Thai cuisine, papaya is used to make som tam and kaeng som when still not fully ripe. The papaya fruit, seeds, latex, and leaves also contains carpaine, an anthelmintic alkaloid, a drug that removes parasitic worms from the body, which can

be dangerous in high doses. The juice makes a popular beverage; young leaves, shoots and fruits cooked as vegetable. The juice is used for curing warts, cancer, tumors and indurations of the skin (Oloyede, 2005). The ripe seeds, black seeds, of the papaya are edible and have a sharp, spicy taste. Papaya seed extract was reported that the major fatty acids in the extracted oil were oleic, followed by palmitic, stearic, and linoleic (Puangsri, Abdulkarim and Ghazali, 2005). The seeds of papaya have antimicrobial activity against *Trichomonas vaginalis* trophozoites. They could also be used in urinogenital disorder like trichomoniasis with care to avoid toxicity (Calzada Mulia abd Contreras, 2007). The papaya seeds, irrespective of its fruit maturity stages had bacteriostatic activity on gram positive and negative organisms, which could be useful in treating chronic skin ulcer (Afolabi, Marcus, Olanrewaju and Chizea, 2011). They have been shown to be a good source of oil (25.6%) that may be useful for medicinal, biofuel, and industrial purposes (Afolabi et al., 2011). The physicochemical properties of the papaya seed oils were qualitatively determined that they are suitable for consumption (Fokou et al., 2009). Benzylisothiocynate presenting in seeds is the chief or sole antihelminthic (Kermanshai et al., 2001).

Thin layer chromatography (TLC) is a chromatographic technique that is useful for separating organic compounds (Brian, Online, 2000). TLC separation can also be used to select column chromatography conditions. TLC conditions that give a useful R_f value indicate compound separates from the majority of other components without staying at the origin or with the solvent front, can be approximately transferred to column chromatography for further purification. Identification of the target compounds or the TLC plate can be carried out by comparison with a standard, by chemical staining, or by an overlay assay carried out on top of the developed plate in

the case of an unknown biologically active components, or by scraping off, extracting, and assaying potions of the adsorbent (Cannell, 1998).

The brine shrimp lethality assay is very useful tool for the isolation of bioactive compounds from plant extracts (Sam, 1993). The method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the microwell scale (Krishnarajua, Raoa, Sundararajua and Vanisreeb, 2005).

This study intended to evaluate some phytochemicals and cytotoxicity of the kaffir lime peel and papaya seed extracts, which would support the use of them in the biological control of mosquitoes.

3.3 Materials and methods

3.3.1 Plant collection and extraction

Citrus hystrix peel and *Carica papaya* ripe seeds were collected on SUT campus and then dried by sun light for 2-3 days before extraction. The plant samples of 10 g were extracted in Soxhlet extraction apparatus in 1 L of water or 70% ethanol. The extracts were evaporated, dried by lyophilizer (Labconco Corp., Kansas City, MO, USA), and stored at -20°C for further use. The dried extracts were dissolved in its original solvent and stored at 4°C during study.

3.3.2 Determination of total phenolic compounds

The amount of total polyphenolic compounds was quantified by the Folin-Ciocalteau assay (Matthaus, 2002). In brief, 5 mg of the extract was dissolved in 5 mL of methanol, and 2 mL of Folin-Ciocalteau reagent were purchased from Carlo Erba Reagents (Strada Rivoltana, Spain). A 100 μL aliquot of this mixture was

added to 2 mL of 2% Na_2CO_3. After 2 minutes of incubation, 100 µL of Folin-Ciocalteau reagent diluted with methanol 1:1 was added. After 30 minutes, the absorbance was measured at 750 nm. The concentration was calculated using gallic acid as a standard. The results were expressed as milligrams gallic acid equivalents (GAE) per gram extract.

3.3.3 Thin layer chromatography (TLC)

Thin Layer Chromatography (TLC) was conducted to partially separate the crude extracts of *C. hystrix* peel and *C. papaya* seeds. The extract of 0.01 g. was diluted in solvents, spotted on a TLC plate, silica gel 60 F_{254} (2 x 7 cm^2) (Carlo Erba Reagents, Strada Rivoltana, Spain). The TLC system for *C. hystrix* peel extraction was the mobile phase systems of toluene : chloroform : ethanol (5:4:1). The TLC system for *C. papaya* seed extraction was the mobile phase systems of diethyl ether : diethyl acetate (8:2). The TLC plate was air dried and developed with different solvents. The plate was visualized under UV lamp at 254 nm. The relative migration, R_f, of TLC bands was measured. The TLC gels were sprayed with vanillin-sulphuric reagent for detection of essential oils, steroids, terpenoids, and phenols. The vanillin-sulphuric acid reagent produced pink-red and blue spots. The TLC plates then were heated at 100ºC until color appeared (Cannell, 1998).

$$Rf = \frac{\text{Distance traveled by the center of substance spot from the origin (cm)}}{\text{Distance traveled by the solvent from the origin (cm)}}$$

where : R_f stands for ratio of front and its characteristics of any given compounds on each stationary phase using the appropriate mobile phase for the development of the plate.

3.3.4 Cytotoxicity test by brine shrimp lethality assay (BSLA).

Cytotoxicity of the extracts was performed by brine shrimp lethality assay (BSLA) (Solis, Wright, Anderson, Gupta and Philhipso, 1992). The brine shrimp (*Artemia salina* L.) eggs were obtained from a local aquatic shop in Nakhon Ratchasima. The eggs were allowed to hatch in artificial sea water, 3.8% NaCl (w/v) in a small chamber of a 2-unequal chamber plastic container with a multi-hole hole divider. The egg hating chamber was completely covered with a lid, while the larger chamber for brine shrimp larvae was illuminated (Figure 3.3). The eggs were incubated at room temperature (21-22°C) and allowed to develop into nauplii larvae.

Figure 3.3 The two-chamber container with a perforate divider and light for rearing brine shrimps.

The smaller chamber was for brine shrimp egg hatching. The larger chamber was for the nauplii, migrated toward the light. Ten nauplii were transferred into a 24-well plate. The extractions of various concentrations, ranging of 10, 50, 100, 500 and 1,000 µg/mL were added to the well and incubated from 24 h. The numbers of dead and living larvae were counted under a stereomicroscope. The median lethal concentration, $LC_{50,}$ was calculated (Solis, Wright, Anderson, Gupta, and Phillipson, 1993).

3.3.5 Data analysis

Data from the experiments of TPC was analyzed by one way ANOVA, followed by Schffe's test with significance levels of 0.01 ($P \leq 0.01$). the data from the BSLA was analyzed by Probit analysis. All statistical analysis were performed using Statistical Package for the Social Sciences (SPSS) program for Windows v.11.5.

3.4 Results and discussion

3.4.1 Total phenolic compounds (TPC)

Total phenolic compounds (TPC) of the extracts were quantified by the Folin-Ciocalteau assay. TPCs of kaffir lime peel ethanolic (KPE/e) and water extracts (KPE/w) were 51.26 ± 0.05 and 35.80 ± 0.07 mg GAE/g respectively (Table 3.1). TPCs of papaya seed ethanolic (PSE/e) and water extracts (PSE/w) were 27.62 ± 0.10 and 31.11 ± 0.31 mg GAE/g respectively.

Table 3.1 The results of total phenolic compounds (TPC) of the *C. hystrix* peel and *C. papaya* seed extracts.

Plant	Extracts	Total Phenolic Compounds mg GAE/g ± S.D., n = 4
Citrus hyptrix	KPE/e	51.26 ± 0.05^{a}
	KPE/w	35.80 ± 0.07^{b}
Carica papaya	PSE/e	27.62 ± 0.01^{d}
	PSE/w	31.11 ± 0.31^{c}

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). KPE/e, kaffir lime peel ethanolic extract; KPE/w, kaffir lime peel water extract; PSE/e, papaya seed ethanolic extract; PSE/w, papaya seed water extract

Similarly, Ho and Lin (2008) reported that ponkan mandarins *Citrus reticulata* peel extracts contained TPCs ranged from 40.8 ± 0.9 to 54.1 ± 1.8 mg GAE/g. The optimisation of extraction conditions of limau purut *Citrus hystrix* peels was conducted and the amount of TPCs was found higher with higher concentration of organic solvents (Chan, Lee, Yap, Wan Aida and Ho, 2009; Zhou, Wang, Mei, Li, Luo and Dai, 2011). TPCs in edible, papaya seed oils were reported low (Sammarphet, 2008).

3.4.2 Thin layer chromatography (TLC) analysis

Some major phytochemicals of kaffir lime peel extract and ripe papaya seed extract extracts were investigated by TLC analysis. The TLC profiles of KPE/w, KPE/e, PSE/w, and PSE/e were shown in Figures 3.4-3.7, respectively. The relative migration, R_f, would roughly indicate phytochemical group in these crude extracts. There are two R_f in KPE/w, three R_f in KPE/e, one R_f in PSE/w, and six R_f in PSE/e. The R_f profiles were correspond to the staining of vanillin-sulphuric acid reagent indicating that the major compounds constituted in the extracts were terpenes.

มหาวิทยาลัยเทคโนโลยีสุรนารี

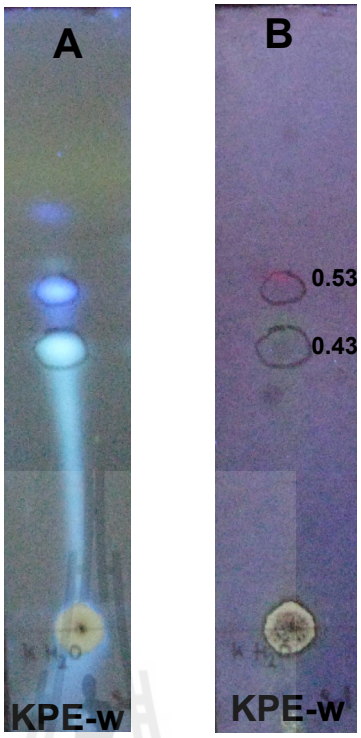

Figure 3.4 TLC chromatographic profiles of the peel water extract of kaffir lime (*C. hytrix*) using a mobile phase of toluene: chloroform: ethyl alcohol in the ratio of 5:4:1.

A: before spraying with vanillin-sulphuric acid reagent.

B: after spraying with vanillin-sulphuric acid reagent.

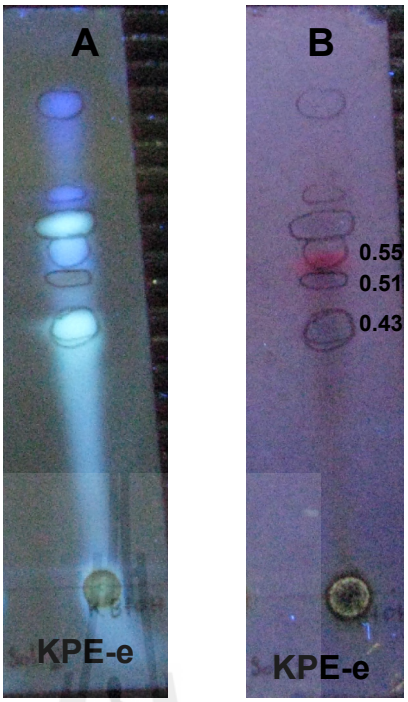

Figure 3.5 TLC chromatographic profiles of the peel ethanol extract of kaffir (*C. hytrix*) using a mobile phase of toluene: chloroform: ethyl alcohol in the ratio of 5:4:1.

A: before spraying with vanillin-sulphuric acid reagent.

B: after spraying with vanillin-sulphuric acid reagent.

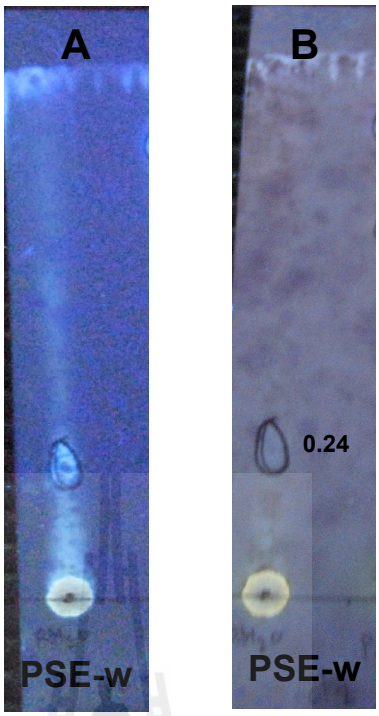

Figure 3.6 TLC chromatographic profiles of the seed water extract of papaya (*C. papaya*) using a mobile phase of diethyl ether : ethyl acetate in the ratio of 8:2.

A: before spraying with vanillin-sulphuric acid reagent.

B: after spraying with vanillin-sulphuric acid reagent.

มหาวิทยาลัยเทคโนโลยีสุรนารี

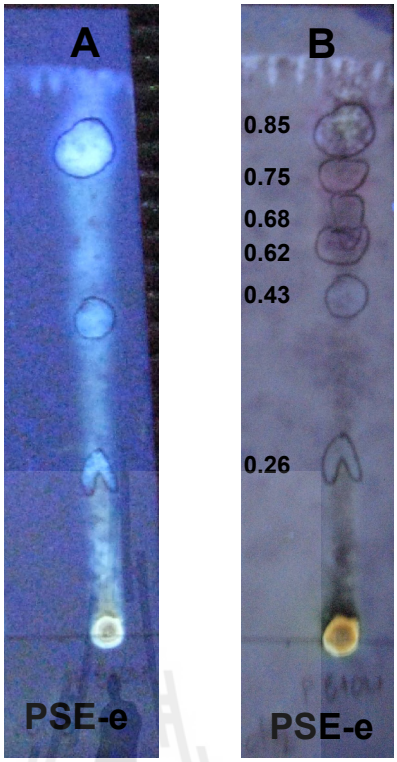

Figure 3.7 TLC chromatographic profiles of the seed ethanol extract of papaya (*C. papaya*) using a mobile phase of diethyl ether : ethyl acetate in the ratio of 8:2.

A: before spraying with vanillin-sulphuric acid reagent.

B: after spraying with vanillin-sulphuric acid reagent.

Kaffir lime essential oil from fresh peel was reported that it mainly consisted of monoterpene hydrocarbons, with limonene and β-pinene as the main components. Other minor components were terpinene-4-ol, α-terpineol, γ-terpinene, α-terpinene and terpinolene (Hongratanaworakit and Buchbauer, 2007). Limonene, citronellal and β-pinene were reported as the major components of the ethyl acetate extract from kaffir lime peel extract and β-pinene, sabinene and citronellal were appeared as the major compounds of kaffir lime peel essential oil (Chanthaphon,

Chanthachum and Hongpattarakere, 2008). Similarly, kaffir lime peel oil was reported to contain monoterpene hydrocarbon, monoterpene oxygenated, terpenes, sesquiterpenes, and other aliphatic hydrocarbons (Shahidi and Zhong, 2005). In addition, essential oils of kaffir lime leaf were rich in monoterpenes, with *b*-pinene as major component, and poor in limonene (Waikedre et al., 2010). These findings were in agreement with my study on kaffir lime extracts.

Ethanol and aqueous extracts of seeds of ripe papaya showed the presence of alkaloids, flavonoids, tannins, saponins (Okoye, 2011), and additionally glycosides and anthraquinones, (Imaga et al., 2010). The pentane extract of papaya seeds were reported to possess benzyl isothiocyanate (BITC) (Wilson, Kwan, Kwan and Sorger, 2002; Adebiyi, Adaikan and Prasad, 2003 and 2004; Nakamur et al., 2007). All fruit development and ripening of papaya fruits, high amounts of benzylglucosinolates (BG) and benzylisothiocyanates (BITC) were detected (Rossetto et al., 2008).

3.4.4 Determination of cytotoxicity of plant extracts by brine shrimp lethality assay (BSLA).

The cytotoxicity of the extracts on *Artemia salina* was determined by Brine Shrimp Lethality Assay (BSLA). The efficacy, the median lethal concentration was evaluated at 24 h of treatment. The LC_{50} values of toxicity to brine shrimp at 24 h of the ethanolic and water extracts of kaffir lime peel (KPE/e, KPE/w) and papaya seeds (PSEL/e, PSE/w) were 125.32 ± 0.56, 68.81 ± 0.00, 68.92 ± 0.00 and 341.44 ± 0.29 ug/mL, respectively (Table 3.2). It was noticed that the most cytotoxicity of the extracts was KPE/w and PSE/e, which was merely equal.

Table 3.2 Cytotoxicity, LC_{50} at 24 h, of the ethanolic and water peel extracts of kaffir lime *Citrus hystrix* and *Carica papaya* perfomed by brine shrimp lethality assay (BSLA).

Plant Extracts	**Extracts**	**LC_{50} of cytotoxicity µg/mL ± S.D., 24 h, n=6**
kaffir lime (*Citrus hyptrix*)	KPE/e	125.32 ± 0.56^{b}
	KPE/w	68.81 ± 0.00^{c}
Papaya (*Carica papaya*)	PSE/e	68.92 ± 0.00^{c}
	PSE/w	341.44 ± 0.29^{a}

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). LC_{50}, median lethal concentration KPE/e, kaffir lime peel ethanolic extract; KPE/w, kaffir lime peel water extract; PSE/e, papaya seed ethanolic extract; PSE/w, papaya seed water extract

The brine shrimp lethality assay was applied for preliminary assessment of toxicity of the plant extracts. Kaffir lime peel and leaf oils were found to have very high cytotoxicity using BSLA (Veeraphant, Mahakittikun and Soonthornchareonnon, 2011). The cytotoxicity of papaya fruit extracted with water / hydro-alcohol / alcohol (Krishnaraju et al., 2005), of the fresh leaves (Awolola, Oluwaniyi, Solanke, Dosumu and Shuiab, 2010) and leaves, stem and roots (Olawale, Oladimeji, Nia, Ndukwe and Attih, 2007) were similar to this study.

3.5 Conclusion

The ethanolic extracts of kaffir lime peels had significant higher TPC than the water extracts, whereas the water extracts of ripe papaya seeds had higher TPC than the ethanolic extracts. TLC fingerprinting revealed that the phytochemicals found in kaffir lime peels and ripe papaya seeds were highly soluble in ethanol. The TLC profiles and color detection by vanillin-sulphuric acid reagent demonstrated terpenes were likely to be the major compounds constituted in the extracts. The cytotoxicity, LC_{50} values, of kaffir lime peel water extract (KPE/w) and ethanolic of papaya seeds (PSE/e) showed the highest cytotoxicity was equal.

3.6 References

Adebiyi, A., Adaikan, P.G. and Prasad., R.N.V. (2003). Tocolytic and toxic activity of papaya seed extract on isolated rat uterus. **Life Sciences.** 74(5): 581-592.

Adebiyi, A.P., Adaikan, G. and Prasad, R.N.V. (2004). Effect of benzyl isothiocyanate on spontaneous and induced force of rat uterine contraction **Pharmacological Research**. 49: 415-422.

Afolabi, I.S., Marcus, G.D., Olanrewaju, T.O. and Chizea, V. (2011). Biochemical effect of some food processing methods on the health promoting properties of under-utilized *Carica papaya* seed. **Journal of Natural Products**. 4: 17-24.

Afolabi, I.S. and Ofobrukweta, K. (2011). Physicochemical and nutritional qualities of *Carica papaya* seed products. **Journal of Medicinal Plants Research**. 5(14): 3113-3117.

Awolola, G.V., Oluwaniyi, O.O., Solanke, A., Dosumu, O.O. and Shuiab, A.O. (2010). Toxicity assessment of natural and chemical coagulants using brine

shrimp (*Artemia salina*) bioassay. **International Journal of Biological and Chemical Sciences**. 4(3): 633-641.

Brian, M. (2000). **Thin-Layer chromatography**. **(TLC)** [On-line]. **Available**: http://www.files.chem.vt.edu/chem-ed/sep/tlc/tlc.html.

Calzada F, Yepez, M.I and Tapia, C.A. (2007). Effect of Mexican medicinal plant used to treat trichomoniasis on *Trochomonas vaginalis* trophozoites. **Journal of Ethnopharmacology**. 113(2): 248-251.

Cannell, R.J.P. (1998). **Natural Products Isolation**. New Jersey, The United States of America: Humana Press.

Chan, S.W., Lee, C.Y., Yap, C.F., Wan, A., W.M. and Ho, C.W. (2009). Optimisation of extraction conditions for phenolic compounds from limau purut (*Citrus hystrix*) peel. **International Food Research Journal**. 16: 203-213.

Chanthaphon1, S., Chanthachums, S. and Hongpattarakere1, T. (2008). Antimicrobial activities of essential oils and crude extracts from tropical *Citrus* spp. against food-related microorganisms. **Songklanakarin Journal of Science and Technology**. 30(Suppl. 1): 125-131.

Echeverri, F., Torres, F., Quiñones, W., Cardona, G., Archbold, R., Roldan, J., Brito, I., Luis., J.G. and Lahlou, E.H. (1997). Danielone, aphytoalexin from papaya fruit. **Phytochemistry**. 44(2): 255-256.

Fokou, E., Achu, M.B., Kansci, G., Ponka. R., Fotso, M., Tchiégang, C. and Tchouanguep, F.M. (2009). Chemical properties of some cucurbitaceae oils from Cameroon. **Pakistan Journal of Nutrition**. 8(9): 1325-1334.

Ho, S.C. and Lin, C.C. (2008). Investigation of Heat Treating Conditions for Enhancing the Anti-Inflammatory Activity of Citrus Fruit (*Citrus reticulata*) Peels. **Journal of Agricultural and Food Chemistry**. 56(17): 7976-7982.

Hongratanaworakit, T. and Buchbauer, G. (2007). Chemical composition and stimulating effect of *Citrus hystrix* oil on humans. **Flavour and Fragance Journal**. 22: 443-449.

Imaga, N.A., Gbenle, G.O., Okochi, V.I., Adenekan, S., Emmanuel, B.D., Oyeniyi, B., Dokai, P.N., Oyenuga, M., Otumara, A. and Ekeh, F. (2010). Phytochemical and antioxidant nutrient constituents of *Carica papaya* and *Parquetina nigrescens* extracts. **Scientific Research and Essays**. 5(16): 2201-2205.

Kermanshai, R., McCarry, B.E., Rosenfeld, J., Summers, P.S., Weretilny, EA., and Sorger, G.J. (2001). Benzyl isothiocynate is the sole or chief anthelmintic in papaya seed extracts. **Phytochemistry**. 57(3): 427-435.

Koh, D. and Ong, C.N. (1999). Phytophotodermatitis due to the application of *Citrus hystrix* as a folk remedy. **British Journal of Dermatology**. 140(4): 737-738. Krishnaraju, A.V., Rao, T.V.N., Sundararaju, D., Vanisree, M., Tsay, H.S. and Subbaraju, G.V. (2003). Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. **International Journal of Applied Science and Engineering**. 3(2): 125-134.

Loh1, F.S. and Awang1, R.M. (2011). Dzolkhifli Omar1 and Mawardi Rahmani2. Insecticidal properties of *Citrus hystrix* DC leaves essential oil against *Spodoptera litura* fabricius. **Journal of Medicinal Plants Research**. 5(16): 3739-3744.

Matthus, B. (2002). Antioxidant activity of extracts obtained from residues of different oilseeds. **Journal of Agricultural and Food Chemistry**. 50(12): 3444-3452.

Nakamura, Y., Yoshimoto, M., Murata, Y., Shimoishi, Y., Asai, Y., Park, E.Y., Sato, K. and Nakamura, Y. (2007). Papaya seed represents a rich source of biologically active isothiocyanate. **Journal of Agricultural and Food Chemistry**. 55(11): 4407-4413.

Okoye, E.I. (2011). Preliminry phytochemical analysis and antimicrobial activity of seeds of *Carica papaya*. **Journal of Basic Physical Research**. 2(1): 66-69.

Olawale, H., Oladimeji, Nia, R., Ndukwe, K. and Attih, E. (2007). *In vitro* biological activities of *Carica papaya*. **Research Journal of Medicinal Plant**. 1(3): 92-99.

Oloyede, O.I. (2005). Chemical profile of unripe pulp of *Carica papaya*. **Pakistan Journal of Nutrition**. 4(6): 379-381.

Puangsri, T., Abdulkarim, S.M. and Ghazali, H.M. (2005). Properties of *Carica papaya* L. (papaya) seed oil following extraction using solvent and aqueous enzymatic methods. **Journal of Food Lipids**. 12(1): 62-76.

Rossetto, M.R., Oliveira de Nascimento J.R., Purgatto, E., Fabi, J.P., Lajolo, F.M. and Cordenunsi, B.R. (2008). Benzylglucosinolate, benzylisothiocyanate, and myrosinase activity in papaya fruit during development and ripening. **Journal of Agricultural and Food Chemistry**. 56(20): 9592-9599.

Sam, T.W. (1993). **Toxicity testing using the brine shrimp: *Artemia salina***. In: Colegate, S.M. and Molyneux, R.J. (Eds.), Bioactive Natural Products 442-

456 Detection, Isolation and Structural Determination. CRC Press, Boca Raton, FL: 442-456.

Sammarphet, P. (2008). **Investigation of the papaya seed oil properties for development into edible oil**. Master of Science Thesis, Mahidol University.

Shahidi, F. and Zhong, Y. (2005). **Antioxidants: Regulation Status.** In F. Shahidi (ed). Bailey's Industrial Oil and Fat Products (pp. 491-512). USA: Wiley-Interscience.

Solis, P.N., Wright1,C.W., Anderson M.M., Gupta, M.P. and Philhipson, J.D. (1993). A microwell cytotoxicity assay using *Artemia salina* (Brine Shrimp). **Planta Medica**. 59: 250-252.

Veeraphantl, C., Mahakittikun, V. and Soonthornchareonnon1, N. (2011). Acaricidal Effects of Thai herbal essential oils against *Dermatophagoides pteronyssinus*. **Mahidol University Journal of Pharmaceutical Science**. 38(3-4): 1-12.

Waikedre, J., Dugayb, A., Barrachinac, I., Herrenknechtc, C., Cabaliona, P. and Fournet, A. (2010). Chemical composition and antimicrobial activity of the essential oils from New Caledonian *Citrus macroptera* and *Citrus hystrix*. **Chemistry and Biodiversity**. 7: 871-877.

Wilson, R.K., Kwan, T.K., Kwan, C.Y. and Sorger, G.J. (2002). Effects of papaya seed extract and benzyl isothiocyanate on vascular contraction. **Life Sciences** 71: 497-507.

Yaacob, O. and Subhadrabandhu, S. (1995). **The Production of Economic Fruits in South East Asia. Kuala Lumpur**. Oxford University Press.

Zhou, K., Wang, H., Mei, W., Li, X., Luo, Y. and Dai, H. (2011). Antioxidant activity of papaya seed extracts. **Molecules**. 16(8): 6179-6192.

CHAPTER IV

THE EFFECTS OF KAFFIR LIME (*CITRUS HYSTRIX* DC.) PEEL AND PAPAYA (*CARICA PAPAYA* L.) SEED EXTRACTS ON EGG HATCHING AND LARVAL AND PUPAL MORTALITIES OF *AEDES AEGYPTI* L.

4.1 Abstract

The ethanolic and water extracts of the kaffir lime peel and the papaya seeds were studied for biological control of *Aedes aegypti*. The egg hatching and larval and pupal mortality were observed after 24 h treatment. In all aspects, the ethanolic extracts were more potent than the water extracts. The efficacy of egg hatching inhibition and the mortality of larvae and pupae ranged as ripe papaya seed ethanolic extract (PSE/e), kaffir lime peel ethanolic extract (KPE/e), ripe papaya seed water extract (PSE/w) and kaffir lime peel water extract (KPE/w). The EC_{50} of egg hatching of KPE/w, KPE/e, PSE/w and PSE/e was 16.91 ± 0.01, 4.46 ± 0.01, 4.71 ± 0.01 and 1.72 ± 0.02 mg/mL respectively; the LC_{50} of second instar larval mortality was 16.55 ± 0.59 mg/mL, 3.10 ± 0.56, 8.62 ± 0.62 and 0.48±0.12 mg/mL respectively; and the LC_{50} of pupal mortality was 180.98 ± 0.00, 40.58 ± 0.00, 131.84 ± 0.00 and 1.48 ± 0.94 mg/mL, respectively. This study demonstrated that kaffir lime peel and papaya seeds were potent in biological control of *Aedes aegypti*, the dengue fever vector.

4.2 Introduction

Mosquito control is a difficult task and becoming even more, due to a variety of factors including the development of insecticide resistance, the concern over environmental pollution, and the immunological suppression (Srivastava and Sharma, 2000). Failure and ineffectiveness of conventional insecticides eventually produce resistance in the mosquito vectors (Vincent, 2000). Recently, this failure became acute and caused death of millions of people each year and loss of socio-economic wealth in many countries (Venkatachalam and Jebanesan, 2001). As the result the insect resistance and environmental imbalance, the chemical control of mosquitoes was not presently flavored. Therefore, alternative means of control of mosquitoes was needed (Villanueva, Gaona and Perez, 2008), in particularly by plant products. Some phytochemicals were toxic to mosquito larvae (Villanueva, Gaona and Perez, 2008). The essential oils from *Citrus* species consist of volatile compounds such as limonene, pinene, terpinene, and cymene. Limonene, a monoterpene hydrocarbon compound were used for antisepsis for bacteria, metabolism stimulating. In northern Thailand, various *Citrus* species, especially citron (*Citrus medica* L.var *medica*) are grown mostly (Kamkuan, Suteerapataranon and Tovaranonte, 2005). Citrus essential oils are a mixture of volatile compounds and mainly consisted of monoterpene hydrocarbons (Sawamura et al., 2004). Citrus oils are mixtures of over a hundred compounds that can be approximated into three fractions: terpene hydrocarbons, oxygenated compounds and non-volatile compounds. The terpene fraction constituted from 50 to more than 95% of the oil. However, it made little contribution to the flavor and fragrance of the oil (Chanthaphon, Chanthachum and Hongpattaraker, 2008). *Carica papaya* L. leaves and seeds were known to contain proteolytic

enzymes (papain, chymopapain), alkaloids (carpain, carpasemine), sulfurous compounds (benzyl isothiocyanate), flavonoids, triterpenes, organic acids and oils (Cowan, 1999; Osuna, Tapia and Aguilar, 2005; Quintal, Flores, Buenfil and Tintore, 2011). Extracts from different papaya tissues have been shown to be bioactive. Aqueous extracts of leaves and seeds had antifungal activity against *Colletotrichum gloeosporioides* (Bautista, Barrera, Bravo and Bermudes, 2002; Quintal et al., 2011), and aqueous and organic extracts of seeds had antihelminthic activity against *Caenorhabditis elegans* (Kermanshai et al., 2001; Adebiyi and Adaikan, 2005; Quintal et al., 2011). Alcoholic extracts of the epicarp, endocarp, roots and seeds from ripe and unripe papaya fruit have antidiarrheic, antidysenteric and antibacterial properties (Emeruwa, 1982; Osuna, 2005; Doughari, Elmahmood and Manzara, 2007; Quintal et al., 2011), and aqueous extracts of seeds had contraceptive effects on male albino rats (Lohiya, 2005; Quintal et al., 2011). Two compounds recently isolated from the defensive gland of *Necrodes surinamensis*, α-and β-necrodol, first representatives of a new category of monoterpenes (the necrodanes), was shown to be repellent to ants and other insects and irritating to cockroaches and flies (Eisner, Deyrup, Jacobs and Meinwald, 1985). Terpenes have been known for several centuries as components of the fragrant oils obtained from leaves, flowers and fruits. Monoterpenes, with sesquiterpenes, are the main constituents of essential oils (Leray, Online, 2011).

4.3 Materials and methods

4.3.1 Preparation for extracts

The extracts of ripe papaya seeds and kaffir lime peel were prepared as in chapter III.

4.3.2 Mosquito collection and culture

Aedes aegypti eggs were a gift from the Office of Vector Borne Disease Control Region 1, Prabuddhabat, Saraburi. *Ae. aegypti* was reared at Building F9, the Center for Scientific and Technological Equipment, SUT by rearing standard techniques of Sutthanont et al. (2010). The eggs on filter paper were placed on a water filled tray for allowing hatching (Figure 4.1). The mosquito culture was conducted at 27 ± 2°C, 75% relative humidity. The larvae of *Ae. aegypti* mosquitoes were bred in chlorine-free tap water. The larvae were fed with rat feed. The pupae were transferred to a cup containing dechlorinated tap water and placed in mosquito-net cages (Figures 4.1 and 4.2). Adults of *Ae. aegypti* were reared in 1x1x1 m net cages. The adult males were fed with vitamin syrup in jars with cotton wick. The adult females were fed with rat blood (Nathan, 2007).

A B

Figure 4.1 *Aedes aegypti* female laid eggs on a filter paper (A) and the egg were placde in a tray filled with water for hatching (B).

A B

Figure 4.2 *Aedes aegypti* rearing in laboratory.

A: Larvae in enamel trays **B**: Adult females were fed with rat blood

4.3.3 Eggs hatching test

Female mosquitos were allowed to lay eggs in a 30-ml wide plastic chamber filled with 5 ml water. Afer, the mosquito laid eggs, the remaining water was allowed to dried. The eggs were counted under stereomicroscope to get one hundred eggs per chamber. Ten milliliters of various concentration of extract, which dissolved in water or 0.1% was added into each chamber. Water and 0.1% DMSO were used as controls. The number of hatched larvae was counted at 24 h. The experiment was performed in triplicate and repeated four time in each experiment. The median effective concentration, EC_{50} was calculated by Probit analysis using Statistical Package for the Social Sciences (SPSS) program for Windows v.11.5.

A **B**

Figure 4.3 Preparation for testing eggs hatching.

A: Stereo microscope OLYMPUS SZ61 modal

B: Eggs of *Ae. Aegypti* in a wide plastic chamber

4.3.4 Larval mortality test

The extract powder was dissolved in water or 0.1% DMSO v/v. Thirty 2nd instar larvae (Figure 4.4) were gently transferred into 20 mL in vial. Remove the water, various concentration of extracts into 20 mL were added (Figure 4.5). Water and 0.1% DMSO were used as controls. The number of larval mortality was counted at 24 h. The experiment was performed in triplicate and repeated four times in each experiment. The median lethal concentration, LC_{50} was calculated by Probit analysis using Statistical Package for the Social Sciences (SPSS) program for Windows v.11.5.

Figure 4.4 Second instar larve of *Ae. aegypti* in big vial before exposure.

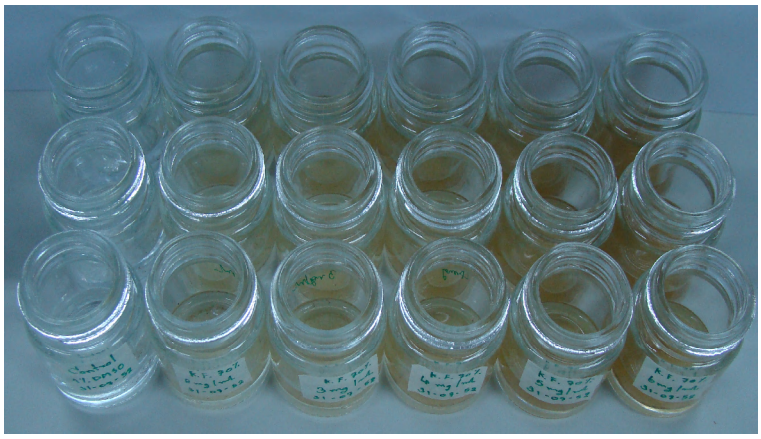

Figure 4.5 Larvicidial mortality tests using various concentrations of extracts.

4.3.5 Pupal mortality test

The extract powder was dissolved in water or 0.1% DMSO v/v. Thirty pupae were gently transferred into 20 ml in vial. Remove the water, various concentration of extracts into 20 mL were added (Figure 4.6). The dead pupae were counted at 24 hr. Water and 0.1% DMSO were used as controls. The experiment was performed in triplicate and repeated four times in each experiment. The LC_{50} was calculated by Probit analysis using Statistical Package for the Social Sciences (SPSS) program for Windows v.11.5.

A B

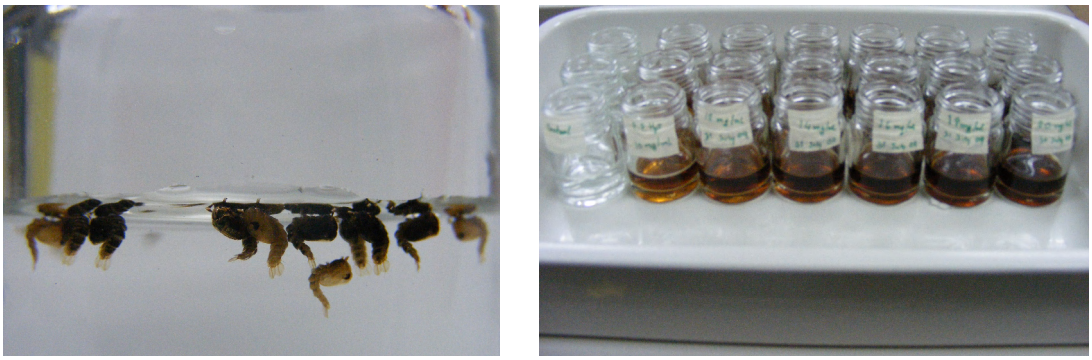

Figure 4.6 *Ae. aegypti* pupae in a big vial before exposure (A) and plant extract for pupicidal mortality test (B).

4.3.6 Data analysis

All data were analyzed using analysis of variance (ANOVA) and in completely randomized design (CRD) using Statistical Package for the Social Sciences (SPSS) program for Windows v.11.5. The means were compared using Scheffe's test. The percentage of hatching (Govindarajan, Mathivanan, Elumalai, Krishnappa and Anandan, 2011) was calculated as follwing;

$$\% \text{ Hatching} = \frac{\text{Number of hatched larvae}}{\text{Total number of eggs}} \times 100.$$

The percentage of mortality was corrected by Abbott's formula (Sharma and Ansari, 1994; Yap et al., 1998; Tawatsin,Wratten, Scott, Thavara and Techadamrongsin, 2001) as following;

$$\% \text{ Mortality} = \frac{\% \text{ Test mortality} - \% \text{ Control mortality}}{100 - \% \text{ Control mortality}} \times 100.$$

4.4 Results and Discussion

4.4.1 Effects of the extracts on *Ae. aegypti* eggs hatching

Ae. aegypti egg hatching was affected by kaffir lime peel and ripe papaya seed extracts. The ripe papaya seed ethanolic extract, PSE/e, produced highest inhibitory effect on the mosquito egg hatching with EC_{50} value of 1.72 ± 0.02 mg/mL (Table 4.2). The inhibitory egg hatching EC_{50} of the kaffir lime peel ethanolic extract, KPE/e, was nearly equal to that of the papaya seed water extract, PSE/w which were 4.46 ± 0.01 and 4.71 ± 0.01 mg/mL, respectively. The kaffir lime peel water extract, KPE/w was least effect on the egg hatching with EC_{50} value of 16.91 ± 0.01 mg/mL. Thus, the effects of all extracts on *Ae. aegypti* egg hatching ranged as PSE/e > KPE/e, PSE/w > KPE/w.

A few plants, *Mammea siamensis*, *Anethum graveolens* and *Annona muricata*, were repeated that their ethanolic extracts were able to reduce egg laying and hatching of *Ae. aegypti* (Promsiri, Naksathit, Kruatrachue and Thavara, 2006). *M. siamensis* possessed mammea coumarin (Mahidol, Kaweetripob, Prawat and Ruchirawat, 2002) which was likely to inhibit ecdysteriod and juvenile hormones affecting egg development with poor quality and hatchability (Promsiri et al., 2006). There was also a repot demonstrated that the egg hatching of *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* was highly decreased by *Abutilon indicum* flower extract, extracted with water, hexane, diethyl ether, dicholoromethane

and ethyl acetate (Arivoli and Tennyson, 2011). These demonstrated that plant extracts can be used to control on mosquito egg hatching. However, there is no evidence of the effects of kaffir lime and papaya extracts on the egg hatching of *Ae. aegypti*. Thus, this study was the first observation demonstrating these two plant products effectively inhibited *Ae. aegypti* egg hatching. However, essential oil of lemongrass was repeated to inhibit filarial mosquito *Culex quinquefasciatus* (Pushpanathan, Jebanesan and Govindarajan, 2006). Egg hatchability of *Ae. aegypti*, *An. stephensi* and *C. quinquefasciatus* mosquitoes was effectively inhibited by grape jasmine (Thai: poot) *Ervatamia coronaria* and *Caesalpinia pulcherrima* leaf benzene and ethyl acetate extracts (Govindarajan, Mathivanan, Elumalai, Krishnappa and Anandan, 2011).

มหาวิทยาลัยเทคโนโลยีสุรนารี

Table 4.1 Effects of kaffir lime (*C. hystrix*) peel extracts on *Ae. aegypti* egg hatching.

Plant Extracts	Concentrations mg/mL	% Hatching ± S.D.	EC_{50} ± S.D., n= 4
KPE/w	Control (water)	99.00 ± 1.00[a]	
	0.50	78.00 ± 3.60[b]	
	1.00	67.33 ± 1.52[b,c]	
	5.00	56.33 ± 1.52[c]	16.91 ± 0.01
	25.00	24.33 ± 4.93[d]	
	50.00	14.00 ± 2.00[d]	
KPE/e	Control (water)	99.00 ± 1.00[a]	
	0.1% DMSO	98.33 ± 0.57[a]	
	1.00	98.66 ± 0.57[a]	
	5.00	85.00 ± 1.00[b]	
	10.00	75.00 ± 2.00[c]	4.46 ± 0.01
	15.00	23.33 ± 2.00[d]	
	20.00	0.00 ± 0.00[e]	

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). EC_{50}, median effective concentration. KPE/w, kaffir lime peel water; KPE/e, kaffir lime peel ethanolic extract

Table 4.2 Effects of ripe papaya (*C. papaya*) seed extracts on *Ae. aegypti* egg hatching.

Plant Extracts	**Concentrations mg/mL**	**% Hatching ± S.D.**	**EC_{50} ± S.D., n=4**
PSE/w	Control (water)	99.33 ± 1.15[a]	
	1.00	73.00 ± 5.29[b]	
	2.00	63.33 ± 2.51[b,c]	
	4.00	52.66 ± 3.05[c]	4.71 ± 0.01
	8.00	25.00 ± 1.73[d]	
	16.00	1.66 ± 2.88[e]	
PSE/e	Control (water)	99.00 ± 1.00[a]	
	0.1% DMSO	98.66 ± 1.15[a]	
	0.30	73.66 ± 3.78[b]	
	0.50	67.33 ± 1.52[b,c]	1.72 ± 0.00
	1.00	56.66 ± 2.08[c]	
	3.00	21.33 ± 1.15 [d]	
	5.00	3.66 ± 3.05 [e]	

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). EC_{50}, median effective concentration. PSE/w = ripe papaya seed water extract; PSE/e = papaya seed ethanolic extract

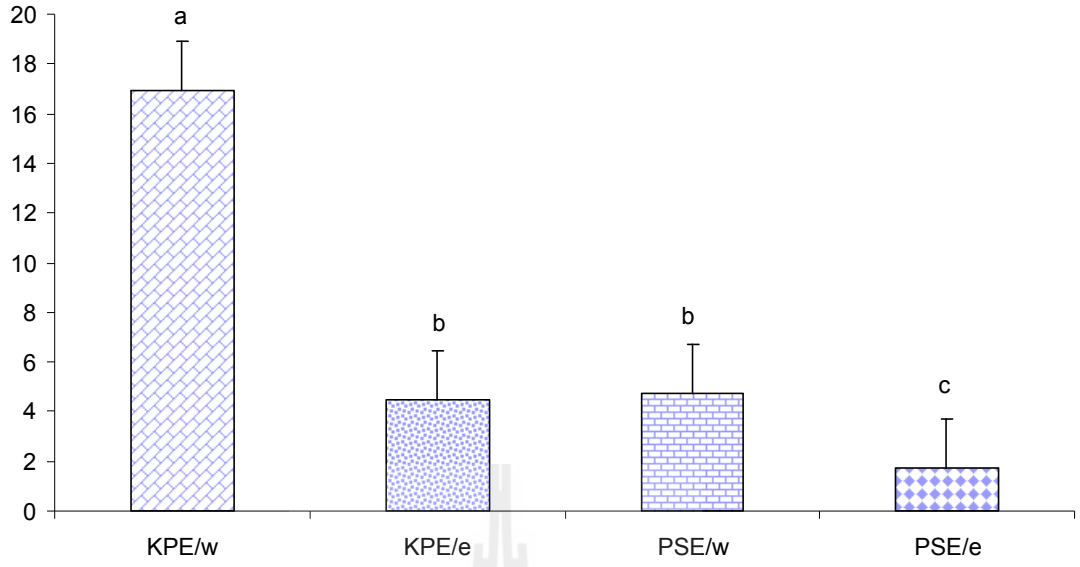

Figure 4.7 Effects, EC_{50}, values of *C. hystrix* peel and *C. papaya* ripe seed extracts on eggs hatching of *Ae. aegypti*.

4.4.2 Effects of the extracts on *Ae. aegypti* larvae

The effects of kaffir lime peel and ripe papaya seed extracts on the control of *Ae. aegypti* larvae were conducted and observed at 24 h of treatment. The ethanolic extracts of them, KPE/e and PSE/e, were more effective on ridding the mosquito 2nd instar larvae than those of the water extracts, KPE/w and PSE/w. The larvicidal efficacy of all extracts ranged as PSE/e > KPE/e > PSE/w > KPE/w (Tables 4.3 and 4.4). PSE/e produced highest efficacy on the control of the mosquito larvae with LC_{50} of 0.48 ± 0.12 mg/mL, which was approximately 18 fold of PSE/w (8.62 ± 0.62 mg/ml). KPE/w efficacy was least with LC_{50} of 16.55 ± 0.59 mg/mL which was approximately 5 fold lower than KPE/e (3.10 ± 0.56).

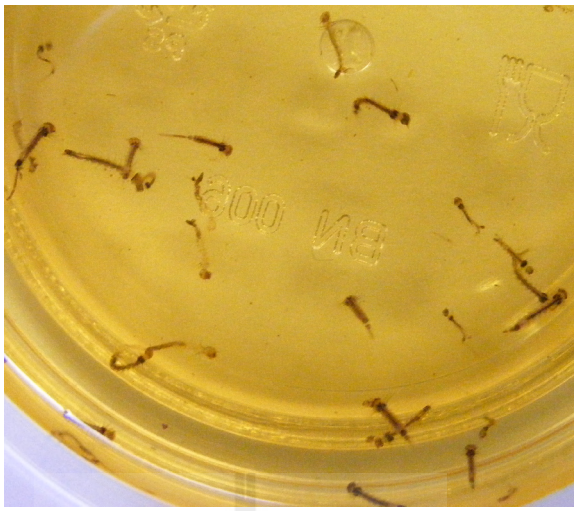

Figure 4.8 Larvae of *Ae. aegypti* in vial after 24 h of exposure.

The efficacy of the extracts of the same plants was compared as following; KPE/e was about 4 fold greater than KPE/w and PSE/e was about 23 fold greater than PSE/w. There was no direct evidence demonstrating the bioactivity of kaffir lime peel and ripe papaya seed extracts on *Ae. aegypti* larvae. However, papaya methanolic leaf extract strongly caused mortality of *Ae. aegypti* at 1st to 4th instars larvae and early 3rd instar larvae (Sakthivadivel and Daniel, 2008). Papaya seed water extract was found to cause high death to filarial vector mosquito *Culex quinquefasciatus* larvae, all instars (Rawani, Haldar, Ghosh and Chandra, 2009). These finding well supported the papaya seed extracts on *Ae. aegypti* of my study. However, the kaffir lime peel extract could be toxic to insect larvae via biological of cytochrome c oxidase as evidence of the aroma of kaffir lime leaf extract on rice weevil larvae (Buatone and Indrapichate, 2011). In addition, mallow *Abutilon indicum* leaf hexane extract arrested the larval development of *Ae. aegypti*, *An. stephensi* and *C. quinquefasciatus* leading to the reduction of the mosquito pupae

(Arivoli and Tennyson, 2011). Cashew, *Anacardium accidentale*, was demonstrated that its seed hexane extract highly caused mortality of larvae, pupae and adults of mosquitos *Anopheles gambiae* (Akinkurolere, Adedire, Odeyemi, Raji and Owoeye, 2011). There is a repeat demonstration that papaya leaf aqueous extract was administrated to patient infected with Dengue fever in order to direct against the fever (Ahmad, Fazal, Ayaz, Abbasi and Moham, 2011). The leaf extracts of giant milkweed (rak in Thai) *Calotropis gigantea* were also effectively control first to fourth-instar larvae and pupae of mosquito vectors including *Ae. aegypti* (Kovendan, Murugan, Kumar, Vincent and Hwang, 2012). There are a number of essential oils, such as camphor *Cinnamonum camphora*, lemon *Citrus limon*, black paper *Piper nigerum* and cinnamon *Cinnamonum zeylanicum* were able to reduce mortality rate of *Ae. aegypti*, *An. stephensi* and *C. quinquefasciatus* larvae (Amer and Mehlhorn, 2006).

มหาวิทยาลัยเทคโนโลยีสุรนารี

Table 4.3 Effects of kaffir lime (*C. hystrix*) peel extracts on *Ae. aegypti* larval mortality.

Plant Extracts	Concentrations mg/mL	% Mortality ± S.D.	LC_{50} ± S.D., n=4
KPE/w	Control (water)	0.00 ± 0.00^{c}	
	5.00	1.81 ± 0.06^{c}	
	10.00	15.55 ± 1.92^{d}	
	15.00	41.40 ± 1.44^{c}	16.55 ± 0.59
	20.00	71.32 ± 0.26^{b}	
	25.00	100.00 ± 0.00^{a}	
KPE/e	Control (water)	0.00 ± 0.00^{f}	
	0.1% DMSO	0.00 ± 0.00^{f}	
	1.00	2.21 ± 0.12^{e}	
	2.00	18.43 ± 0.44^{d}	
	4.00	51.25 ± 0.25^{c}	3.10 ± 0.56
	6.00	87.34 ± 0.59^{b}	
	8.00	100.00 ± 0.00^{a}	

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). LC_{50}, median lethal concentration. KPE/w = Kaffir lime peel water extract; KPE/e = Kaffir lime peel ethanolic extract,

Table 4.4 Effects of ripe papaya (*C. papaya*) seed extracts on *Ae. aegypti* larval mortality.

Plant Extracts	Concentrations mg/mL	% Mortality ± S.D.	LC_{50} ± S.D., n=4
PSE/w	Control (water)	0.00 ± 0.00^{e}	
	1.00	1.79 ± 0.02^{e}	
	5.00	21.45 ± 1.26^{d}	
	10.00	50.67 ± 0.87^{c}	8.62 ± 0.62
	15.00	77.14 ± 0.56^{b}	
	20.00	100.00 ± 0.00^{a}	
PSE/e	Control (water)	0.00 ± 0.00^{d}	
	0.1% DMSO	0.00 ± 0.00^{d}	
	0.10	1.33 ± 1.15^{d}	
	0.20	11.11 ± 1.92^{c}	
	0.40	47.55 ± 0.38^{b}	0.48 ± 0.12
	0.60	98.55 ± 1.38^{a}	
	0.80	100.00 ± 0.00^{a}	

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). LC_{50}, median lethal concentration. PSE/e = ripe papaya seed ethanolic extract; PSE/w = ripe papaya seed water extract

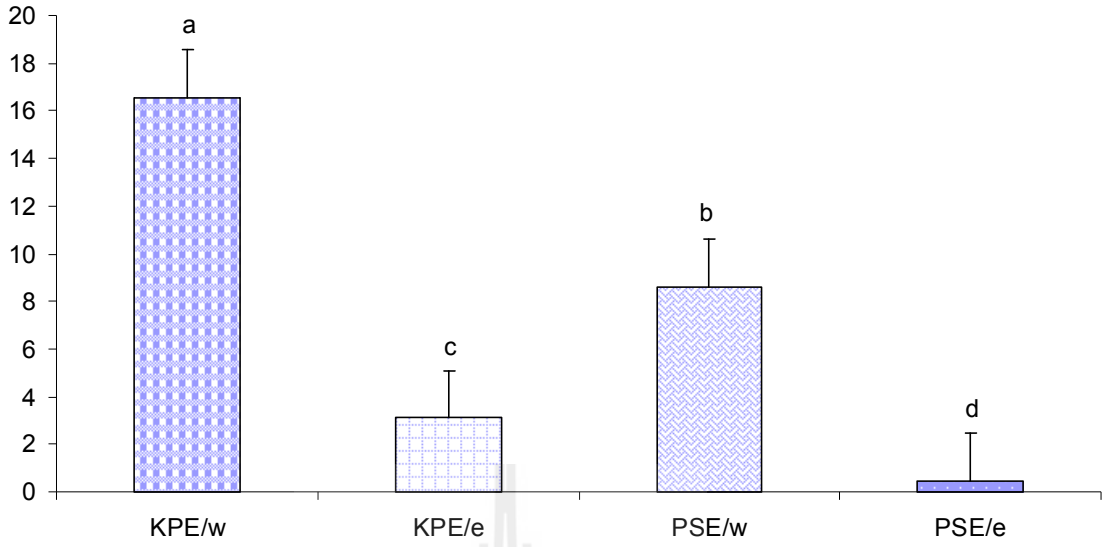

Figure 4.9 Efficacy $LC_{50,}$ *C. hystrix* peel and *C. papaya* ripe seed extracts on 2nd instar larvae of *Ae. aegypti*.

4.4.3 Effects of the extracts on *Ae. aegypti* pupicidal mortality

The biocontrol on *Ae. aegypti* pupae by the kaffir lime peel and the ripe papaya seeds extracts was conducted. The pupal mortality was observed at 24 h of treatment. The effects of the extracts on the mosquito pupae were similar to those on the egg hatching and the larvae. The ethanolic extracts were more potent than the water extracts and their efficacy ranged as PSE/e > KPE/e > PSE/w > KPE/w (Table 4.5 and 4.6). The highest efficacy was PSE/e with LC_{50} of 1.48 ± 0.94 mg/mL. It was approximately 27 fold of KPE/e which it LC_{50} was 40.58 ± 0.00. The lowest efficacy was KPE/w with LC_{50} of 180.98 ± 0.00 mg/mL which was approximately 1.4 fold lesser than of PSE/w, 131.84 ± 0.00 mg/mL. As comparing between the extracts of KPE/e was about 4.5 fold higher than KPE/w, and the efficacy of PSE/e was about 89 fold greater than PSE/w. *Citrus limelta* peel hexane and petroleum ether extracts possessed terpenoids and flavonoids and were assessed toxicity effects against early

fourth instar larvae of *Ae. aegypti* and *An. stephensi* (Kumar, Warikoo, Mishrn, Seth and Wahah, 2012). In addition, essential oil of kaffir lime leaves containing beta-citronellal, beta-citronellol, linalool, and citronellol was effective in controlling second instar larvae of cotton warm *Spodoptera litura* (Loh, Awang, Omar and Rahmani, 2011). Essential oil and crude ethanolic extracts of kaffir lime peel were reported to highly inhibit growth of 21 serotypes of Salmonella and 5 of Enterobacteria spp and greater than those of kaffir lime leaves (Nanasombat and Lohasupthawee, 2005). Kaffir lime peel essential oil also suppressed growth of yeast and mold in Chinese sausage (Kun-Chiang) (Kingchaiyaphum and Rachtanapun, 2012). It could be possible that beta-citronellal, the major composition in kaffir lime, was the crucial chemical against all developmental stages of *Ae. aegypti* mosquitoes and other microorganisms. There is a number of insecticidal plants, including papaya leaf extracts, against all instar larvae and pupae of mosquito vector, including *Ae. aegypti* (Lukwa, Mutambu, Makaza, Molgaard and Fura, 2001; Sakthivadivel and Daniel, 2008; Rawani, Haldar, Grhosh and Chandra, 2009; Rernia and Logaswamy, 2010; Akinkurolere et al., 2011; Kovendan et al., 2012). However, there is no report on the effects of papaya seed extract on the pupae of *Ae. aegypti*. The ethanolic extract of seeds of lueat raet *Knema globularia*, salaeng chai *Stryehnos nux-vomica*, duria belanda *Annona muricata* effectively reduce adult emergence of *Ae aegypti* (Prosiri, Naksathit, Kruatrachue and Thavara, 2006). There were same seed petroleum ether extracts, including *Argemone mexicana*, *Aristalochia bructeolata*, and *Citrullus colocynthis* were potentially against fourth instar larvae of *Ae aegypti* mosquitos (Sakthivadivel and Daniel, 2008), which was likely to against their pupae. The papaya seed were rich in biologically active benzylisothiocyanate and

benzylglucosinolate with potential benefite for controlling agricultural pest (Nakamura et al., 2007). These phytochemical were found to remain stable in papaya seeds during papaya fruit development and repening by ethylene treatment (Rossetto et al., 2008). Therefor, it is likely that benzylisothiocyanate and benzylglucosinolate of papaya seeds are responsible for controlling *Ae. aegypti* mosquito.

Table 4.5 Effects of kaffir lime (*C. hystrix*) peel extracts on *Ae. aegypti* pupal mortality.

Plant Extracts	Concentrations mg/mL	% Mortality ± S.D.	LC_{50} ± S.D., n=6
KPE/w	Control (water)	0.00 ± 0.00	
	90.00	2.06 ± 1.57^{e}	
	120.00	4.13 ± 1.34^{d}	
	150.00	14.35 ± 1.02^{c}	180.98 ± 0.00
	180.00	35.55 ± 2.51^{b}	
	210.00	91.57 ± 2.44^{a}	
KPE/e	Control(water)	0.00 ± 0.00^{e}	
	0.1% DMSO	0.00 ± 0.00^{e}	
	10.00	0.00 ± 0.00^{e}	
	25.00	27.25 ± 1.96^{d}	
	40.00	43.20 ± 3.06^{c}	40.58 ± 0.00
	55.00	78.88 ± 5.57^{b}	
	70.00	97.29 ± 6.91^{a}	

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). LC_{50}, median lethal concentration. KPE/w = Kaffir lime peel water extract, KPE/e = Kaffir lime peel ethanolic extract

Table 4.6 Effects of ripe papaya (*C. papaya*) seed extracts on *Ae. aegypti* pupal mortality.

Plant Extracts	Concentrations mg/mL	% Mortality ± S.D.	LC_{50} ± S.D., n=6
PSE/w	Control (water)	0.00 ± 0.00^{f}	
	75.00	1.03 ± 0.78^{e}	
	90.00	11.81 ± 0.85^{d}	
	105.00	23.10 ± 1.64^{c}	131.84 ± 0.00
	120.00	47.75 ± 3.37^{b}	
	135.00	94.28 ± 2.66^{a}	
PSE/e	Control (water)	0.00 ± 0.00^{e}	
	0.1% DMSO	0.00 ± 0.00^{e}	
	0.10	0.00 ± 0.00^{e}	
	0.50	11.89 ± 2.78^{d}	
	1.00	48.13 ± 3.45^{c}	1.48 ± 0.94
	3.00	92.22 ± 6.52^{b}	
	5.00	100 ± 0.00^{a}	

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.). LC_{50}, median lethal concentration. PSE/w = ripe papaya seed water extract, PSE/e = papaya seed ethanolic extract

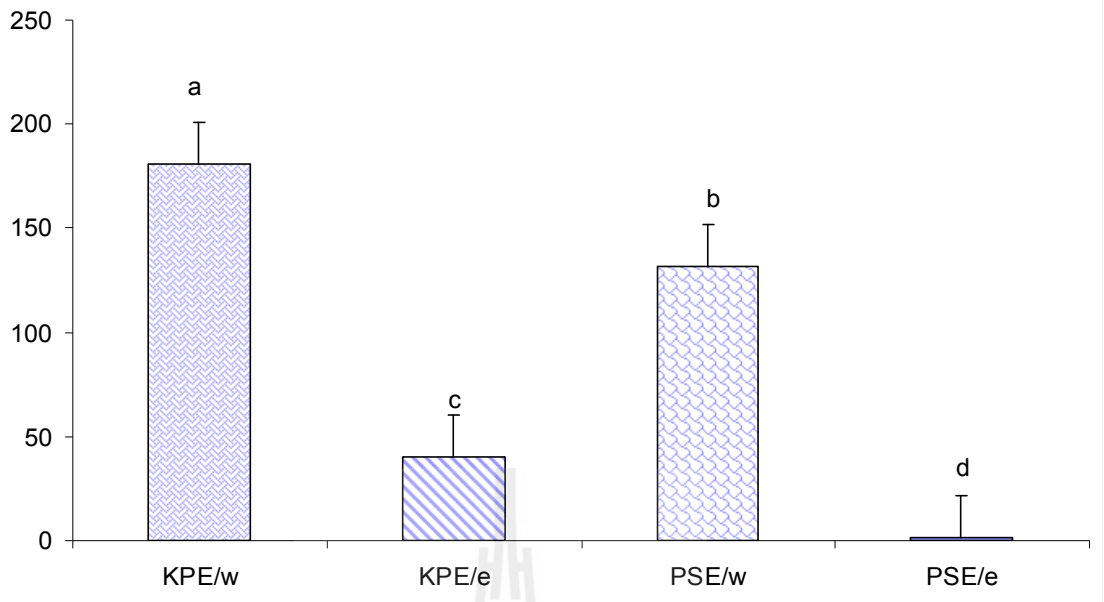

Figure 4.10 Efficacy LC_{50}, *C. hystrix* peel and *C. papaya* ripe seed extracts on pupae of *Ae. aegypti*.

Plant sources possess a wide range of pharmaceutical and insecticidal properties. The insecticides of plant origin did not disturb the environment. Besides reducing the cost-factor, plants exhibit different degrees of toxicity to the different stages and conditions of mosquitoes such as larvicidal, pupicidal, adulticidal, growth and reproduction inhibition, repellent and ovipositional deterrents. The very high activity of the extracts especially *Albizia amara*, *Areca catechu*, *Leucas aspera* and *Ocimum sanctum* against the larvae of *Anopheles stephensi* suggested that the methanol extracts might be used directly as larvicidal agents in small volume aquatic habitats or breeding sites of around human dwellings (Vinayagam**,** Senthilkumar and Umamaheswari**,** 2008).

4.5 Conclusion

This study was the first report demonstrating that kaffir lime peel and ripe papaya seed extracts were effectively control egg hatching, larvae, and pupae of Dengue vector mosquito *Ae. aegypti.* The efficacy of ethanolic extracts was higher than water extracts. The papaya seed ethanolic extract was most potent and the kaffir lime water extract was least potent. All the extracts were good alternative choices for biological control of Dengue fever vector, *Ae. aegypti.* The bioactive control of the kaffir lime peel and the papaya seed could be due to the activities of their phytochemical constituents. Beta-citronellal, beta-citronellol, linalool, and citronellol in the kaffeir lime peel and benzylisothiocyanate and benzylglucosinolate in the papaya seeds.

4.6 References

Adebiyi, A., Adaikan, P.G. and Prasad., R.N.V. (2003). Tocolytic and toxic activity of papaya seed extract on isolated rat uterus. **Life Sciences.** 74(5): 581-592.

Ahmad, N., Fazal, H., Ayaz, M., Abbasi1, B. H., Mohammad, I. and Fazal, L. (2011). Dengue fever treatment with *Carica papaya* leaves extracts. **Asian Pacific Journal of Tropical Biomedicine**. 330-333.

Akinkurolere, R.O., Adedire, C.O., Odeyemi, O.O., Raji, J. and Owoeye, J.A. (2011). Bioefficacy of extracts of some indigenous Nigerian plants on the developmental stages of mosquito (*Anopheles gambiae*). **Jordan Journal of Biological Sciences**. 237-242.

Amer, A. and Mehlhor, H. (2006). Larvicidal effects of various essential oils against *Aedes*, *Anopheles*, and *Culex* larvae (Diptera, Culicidae). **Journal of Parasitology Research**. 99(4): 466-472.

Arivoli, S. and Tennyson, S. (2011). Larvicidal and adult emergence inhibition activity of *Abutilon indicum* (Linn.) (Malvaceae) leaf extracts against vector mosquitoes (Diptera : Culicidae). **Journal of Biopesticides**. 4(1): 27-35.

Buatone, S. and Indrapichate, K. (2011). Protective effects of mintweed, kitchen mint and kaffir lime leaf extract against rice weevils, *Stitopilus oryzae* L., in stored, milled rice. **International Journal of Agriculture Sciences**. 3(3): 133-139.

Chanthaphon, S., Chanthachum, S. and Hongpattaraker, T. (2008). Antimicrobial activities of essential oils and crude extracts from tropical *Citrus* spp. against food-related microorganisms. **Songklanakarin Journal of Science and Technology**. 30(1): 125-131.

Eisner, T., Deyrup, M., Jacobs, R. and Meinwald, J. (1985) Necrodols: Anti-insectan terpenes from defensive secretion of carrion beetle *(Necrodes surinamensis*). **Journal of Chemical Ecology**. 12(6): 1407-1415.

Govindarajan, M., Mathivanan, T., Elumalai, K., Krishnappa, K. and Anandan, A. (2011). Mosquito larvicidal, ovicidal, and repellent properties of botanical extracts against *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* (Diptera: Culicidae). **Journal of Parasitology Research**. 109(2): 353-367.

Kamkuan, W., Suteerapataranon, S. and Tovaranonte, J. (2005). Limonene in volatile oil extracted from some *Citrus* peels. **31st Congress on Science and**

Technology of Thailand. Nakhon Ratchasima: Suranaree University of Technology.

Kingchaiyaphum, W. and Rachtanapun, C. (2012). Antimicrobial and antioxidative activities of essential oils in Chinese sausage (Kun-Chiang). **Asian Journal of Food and Agro-Industry**. 5(2): 156-162.

Kovendan, K., Murugan, K., Kumar, N.A., Vincent, S. and Hwang, J.S. (2012). Bioefficacy of larvicdial and pupicidal properties of *Carica papaya* (Caricaceae) leaf extract and bacterial insecticide, spinosad, against chikungunya vector, *Aedes aegypti* (Diptera: Culicidae). **Journal of Parasitology Research**. 110(2): 669-678.

Kovendan, K., Murugan, K., Kumar, K.P., Panneerselvam, C., Kumar, P.M., Amerasan, D., Subramaniam, J. and Vincent, S. (2012). Mosquitocidal properties of *Calotropis gigantea* (Family:Asclepiadaceae) leaf extract and bacterial insecticide, *Bacillus thuringiensis*, against the mosquito vectors. **Journal of Parasitology Research**. 111(2): 531-544.

Kumar, S., Warikoo, R., Mishrn, M., Seth, A. and Wahah, N. (2012). Larvicidal efficacy of the *Citrus limetta* peel extracts against Indian strains of *Anopheles stephensi* Liston and *Aedes aegypti* L. **Journal of Parasitology Research**. 111(1):173-178.

Leray, C. (2011). **Monoterpenes**. [On-line]. http://www.cyberlipid.org/simple/ simp 00041.htm.

Loh, F.S., Awang, R.M., Omar, D., and Rahmani, M. (2011). Insecticidal properties of *Citrus hystrix* DC leaves essential oil against *Spodoptera litura*

fabricius. **Journal of Medicinal Plants Research**. 5(16): 3739-3744.

Lohiya, N.K., Mishra, P.K., Pathak. N., Manivannan. B., Bhande. S.S., Panneerdoss, S. and Sriram, S. (2005). Efficacy trial on the purified compounds of the seeds of *Carica papaya* for male contraception in albino rat. **Reproductive Toxicology**. 20(1): 135-148.

Mahidol, C., Kaweetripob, W., Prawat, H. and Ruchirawat, S. (2002). Mammea coumarins from the flowers of *Mammea siamensis.* **Journal of Natural Products**. 65(5): 757-760.

Nakamura, Y., Yoshimoto, M., Murata, Y., Shimoishi,Y., Asai, Y., Park, E.Y., Sato, K. and Nakamura, Y. (2007). Papaya seed represents a rich source of biologically active isothiocyanate. **Journal of Agricultural and Food Chemistry**. 55(11): 4407-4413.

Nanasombat, S. and Lohasupthawee, P. (2005). Antibacterial activity of crude ethanolic extracts and essential oils of spices against Salmonellae and other entrobacteria. **KMITL Science and Technology Journal**. 5(3): 527-538.

Nathan, S.S. (2007). The use of *Eucalyptus tereticornis* Sm. (Myrtaceae) oil (leaf extract) as a natural larvicidal agent against the malaria vector *Anopheles stephensi* Liston (Diptera: Culicidae). **Bioresource Technology**. 98(9): 1856-1860.

Promsiri, S., Naksathit, A., Yakruatrachue, M. and Thavara, U. (2006). Evaluations of larvicidal activity of medicinal plant extracts to *Aedes aegypti* (Diptera: Culicidae) and other effects on a non target fish. **Insect Science**. 13: 179-188.

Pushpanathan, T., Jebanesan, A. and Govindarajan, M. (2006). Larvicidal, ovicidal and repellent activities of *Cymbopogon citratus* Stapf (Gramineae) essential oil against the filarial mosquito *Culex quinquefasciatus* (Say) (Diptera: Culicidae). **Tropical Biomedicine**. 23(2): 208-212.

Quintal, P.C., Flores, T.G., Buenfil, I.R. and Tintore, S.G. (2011). Antifungal Activity in ethanolic extracts of *Carica papaya* L. cv. maradol leaves and seeds. **Indian Journal of Microbiology**. 51(1): 54-60.

Rawani, A., Haldar, K.M., Ghosh, A. and Chandra, G. (2009). Larvicidal activities of three plants against filarial vector *Culex quinquefasciatus* Say (Diptera: Culicidae). **Parasitology Research.** 105(5): 1411-1417.

Rossetto, M.R.M., de Nascimento. J.R.O., Purgatto, E., Fabi, J.P., Lajolo, M.F. and Cordenunsi, B. (2008). Benzylglucosinolate, benzylisothiocyanate, and myrosinase activity in papaya fruit during development and ripening. **Journal of Agricultural and Food Chemistry**. 56(20): 9592-9599.

Sakkhivadivel, M. and Daniel, T. (2008). Evaluation of certain insecticidal plants for the control of vector mosquitoes viz. *Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*. **Applied Entomology and Zoology**. 43(1): 57-63.

Sawamura, M., Son, U.S., Choi, H.S., Kim, M.S.L., Phi, N.T.L., Fears, M. and Kumagai, C. (2004). Compositional changes in commercial lemon essential oil for aromatherapy. **International Journal of Aromatherapy**. 4(1): 27-36.

Sharma, V.P. and Ansari, M.A. (1994). Personal protection from mosquito (Diptera: Culicidae) by burning neem oil in kerosene. **Journal of Medical Entomology**. 31(3): 505-507.

Srivastava, H.C. and Sharma, S.K. (2000). Chloroquine resistant *Plasmodium falciparum* in migrant population. **Indian Journal of Malariology**. 37(1-2): 39-42.

Sutthanont, N., Choochote, W., Tuetun, B., Junkum, A., Jitpakdi, A., Chaithong, U., Riyong, D. and Pitasawat, B. (2010). Chemical composition and larvicidal activity of edible plant-derived essential oils against the pyrethroid-susceptible and resistant strains of *Aedes aegypti* (Diptera: Culicidae). **Journal of Vector Ecology**. 35(1): 106-115.

Tawatsin, A., Wratten, S.D., Scott, R.R., Thavara, U. and Techadamrongsin, Y. (2001). Repellency of volatile oils from plants against three mosquito vectors. **Journal of Vector Ecology**. 26(1): 76-82.

Venkatachalam, M.R. and Jebanesan, A. (2001). Repellent activity of *Ferronia elephantum* Corr. (Rutaceae) leaf extract against *Aedes aegypti* (L.). **Bioresource Technology**. 76(3): 287-288.

Villanueva, F.R., Gaona, O.J.G. and Perez, M.A.R. (2008). Larvicidal effect of medicinal plants against *Aedes aegypti* (L.) (Diptera: Culicidae) in Mexico. **Bio Assay**. 3(7): 1-4.

Vincent, I. (2000). Cycling to the finish. **Neurobiology of Aging**. 21(6): 757-760.

Yap, H.H., Jahangir, K., Chong, A.S.C., Adanan, C.R., Chong. N.L., Malik, Y.A. and Rohaizat, B. (1998). Field efficacy of a new repellent, KBR 3023, against

Aedes albopictus (Skuse) and *Culex quinquefasciatus* (Say) in a tropical environment. **Journal of Vector Ecology**. 23(1): 62-68.

CHAPTER V

REPELLENT EFFECTS OF KAFFIR LIME *CITRUS HYSTRIX* CD. PEEL AND PAPAYA *CARICA PAPAYA* L. SEED EXTRACTS ON ADULTS OF *AEDES AEGYPTI* L.

5.1 Abstract

The repellent activity of the water and ethanolic extracts of kaffir lime peel (KPE/w, KPE/e) and papaya seed (PSE/w, PSE/e) against *Aedes aegypti* adult mosquitoes was investigated by topical application on nude rat skin. The individual extracts and paired combination of them were performed. Each individual extract was able to repel the mosquitoes effectively up to 10-30 minutes and could repel up to 3 h with high concentration of 1.70 g/mL KPE/w and KPE/e, 3.00 g/mL PSE/w, and 2.2 g/mL PSE/e. The repellent efficacy, EC_{50} at 3 h, of them ranged as KPE/e > PSE/e > KPE/w > PSE/w (0.38, 0.35, 0.50 and 1.33 g/mL). The combination of KPE/w + KPE/e could fully protect against the mosquitoes up to 3 h which was longer PSE/w + PSE/e (10 min). KPE/w and PSE/e were likely to enhance the other extracts in paired combinations. The monoterpenes detectd in the extracts could be responsible for their repellent activities. Further study for more rough information including specific phytochemicals involved in the repellent property of these extracts is needed, so that it will be beneficial for public health and coating of cloth in fabric industry.

5.2 Introduction

Dengue fevers are found in the tropical and subtropical regions around the world, predominantly in urban and semi-urban areas. The disease is caused by a virus, which belongs to family Flaviviradae. The virus is spreaded by *Aedes* (*Stegomyia*) mosquitoes. There is no specific treatment for dengue, but appropriate medical care frequently saves lives of patients with the more serious dengue hemorrhagic fever. The most effective way to prevent dengue virus transmission is to rid the disease-vector mosquitoes (WHO, 2012). The Dengue attacks starting in late summer and end in early winter. Most patients infected are in the age of 30-45 years old. The Dengue fever repeats every year and causes several deaths (Ahmad et al., 2011). In 2003, Thailand reported the highest number of dengue cases in the region. Since 2004 Indonesia reported the highest number of cases from the region. In 2006 57% of the cases were reported from Indonesia alone (WHO, 2007).

Mosquitoes are important vectors of diseases, besides nuisance pests. Repellence is a means to minimize contact to mosquitoes. Repellents are reagents, commonly used for personal protection against mosquitoes. They are one of the most effective means in prevention and control of mosquito borne diseases or for protection of mosquito bites (Thavara, Tawatsin and Chompoosri, 2002). Synthetic insect repellents such as deet (N, Ndiethyl - 3 - methylbenzamide) and picaridin (2- (2-hydroxyethyl)- 1 -piperidinecarboxylic acid 1-methylpropyl ester) have been shown to be effective at providing protection against mosquitoes biting (Qiu et al., 1998, Barnard and Xue, 2004). As the fact of the increase of resistance to the use of these synthetic chemicals and the adverse health effects perceived by communities (Fradin, 1998), there is a growing demand for alternative natural products, in particular those

derived from botanical materials for insecticides, deterrents and repellents (Fradin and Day 2002; Isman, 2006). Plant-based mosquito repellents are especially useful for people who spend a great deal of time outdoor or opened areas. There are a variety of both wild and cultivated plants that repel mosquitoes. It is important to note that it is compounds found within the plants that do the repelling. These compounds need to be released from the plant to unlock the mosquito-repelling qualities. Depending on the species of plant, they can be released by either crushing, drying, or infusing the plant into an oil or alcohol base that can be applied to skin, clothing, or living spaces. Others are best used as a smudge, which releases the compounds in a smoke. Just standing near living plants that repel mosquitoes is often not effective. Extracts from plants have been suggested as potential repellents for the uses against biting arthropods (Novak and Gerberg, 2005) and are often perceived to be safer than those containing synthetic chemicals such as mentioned above (Osimitz and Grothaus, 1995). The repellent properties of many plant essential oils have been investigated, including clove, peppermint, citronella, turmeric, hairy basil, eucalyptus, lavender, peppermint, and catmint essential oils and found to be effective repellents (Tawatsin et al., 2001) It is well known that citronella grass / tak ky hom *Cymbopogon nardus* is the traditionally most popular cultivated aromatic plant used for repelling mosquitoes in Thailand. Its oil, citronella oil, is the primary ingredient in most natural insect repellents sold in stores. Citronella products applied to the skin are most effective. There is a variety of other aromatic plants were reported effective in mosquito repellence (Tawatsin et al., 2001; Gillij, Gleiser and Pitasawat, 2007; Zygadlo, 2008) and other insecticide (Kumar, Mishra, Malik and Satya, 2011). This study aimed to investigate the repellent effects of kaffir lime *Citrus hystrix* peel and

papaya *Carica papaya* seed extracts on the *Ae. aegypti* adult mosquitoes, in searching for naturally alternative agents to control this Dengue vector.

5.3 Materials and methods

5.3.1 Repellent test of individual extracts by skin topical application

The repellent test was performed as described by Tawatsin, Wratten, Scott, Thavara and Techadamrongsin (2001). A Wistar rat (obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, housed at the Animal Facility of Suranaree University of Technology at 25°C, 50-70% RH and fed *ad libitum*) was thoroughly shaved on its back about 1x3 cm^2. the rat was trapped to retain in place. The individual plant extracts were spreaded on the naked area of the rat skin. The treated rat was moved in to a 30x30x30 cm^3 net cage (Figure 5.1), containing 100 female mosquitoes. The untreated rat was spreaded with 0.1% of DMSO, used as a control. The repellent activity was observed at 5, 10, 30 min, 1, 3, and 6 h. The tests were performed in triplicate and repeated thrice. The median effective concentration, EC_{50}, was calculated by Probit analysis, used SPSS (Statistical Package for the Social Sciences) program for Windows v.11.5.

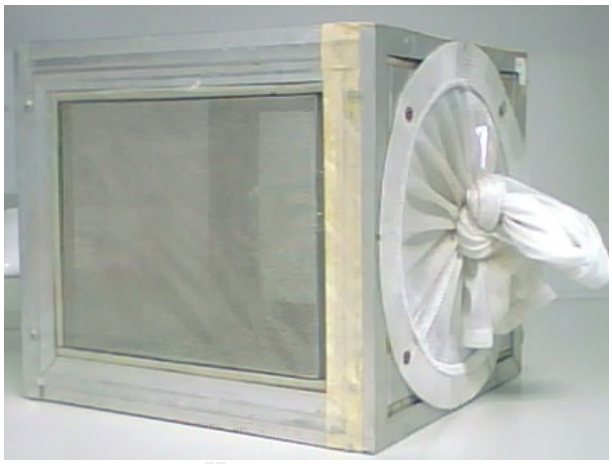

Figure 5.1 Repellent test in a net cage.

A B

Figure 5.2 A rat was shaved on back, trapped (A), and topical applied with the extracts (B).

5.3.2 Repellent test of combined extracts by skin topical application

Repellent test of extract combination was conducted as the methods of Choockote et al. (2007) with modification. A hundred females *Ae. aegypti* mosquitoes were kept in a net cage (30x30x30 cm^3). A back hairless rat, prepared as in 5.3.1, was marked with a permanent marker and then rubbed with various concentrations of combined extracts of kaffir lime peel and papaya seeds at the ratio

of 1:3, 1:1 and 3:1of the same plants and different plants (KPE/w + KPE/e; PSE/w + PSE/e; KPE/e + PSE/e; KPE/w + PSE/e; and KPE/w + PSE/w). DMSO at 0.1% was used as a control. The repellent activity was observed at 5, 10, 30 min, 1, 3 and 6 h. The results were expressed as percentage of repellency as following.

$$\% \text{ Repellent} = \frac{\text{Number collected from control - Number collected from treated}}{\text{Number collected from the treated}} \times 100.$$

The tests were performed in triplicate and repeated twice. The percentage of repellence was corrected by Abbott's formula (Abbott, 1925).

5.3.5 Data analysis

The mortality counts were corrected by Abbott's formula (1925). Data were expressed as mean ± S.D. The statistical analysis was analyzed by one way ANOVA, followed by Schffe's test with significance levels of 0.01 ($P \leq 0.01$). EC_{50} value was estimated by Probit analysis. All statistical analysis were performed using Statistical Package for the Social Sciences (SPSS) program for Windows v.11.5.

5.4 Results and discussion

5.4.1 Repellent effects of individual extracts of kaffir lime peel and papaya seed against *Ae. aegypti* adults by skin topical application

The repellent effect of water peel extract of kaffir lime on the adults of *Ae aegypti* by topical application on a rat nude skin at various concentrations was

investigated (Table 5.1). During 10 min of the beginning, all KPE/w concentrations were able to totally repel the mosquitoes. The lowest concentration that KPE/w fully repel the mosquitoes within 10 min was 0.10 g/mL. While the highest concentration of 1.70 g/mL, KPE/w was able to fully repel the mosquitoes last to 3 h and it could repel 90% up to 6 h. In addition, the repellent efficacy declined with time of exposure, i.e., the EC_{50} increased, as followed, 0.06 ± 0.47 g/mL at 10 min; 0.17 ± 0.26 g/mL at 30 min; 0.38 ± 0.01 g/mL at 1 h; 0.51 ± 0.09 g/mL at 3 h and 0.80 ± 0.06 g/mL at 6 h.

The repellent effect of KPE/e was slightly lower, but later than the KPE/w (Table 5.2). The total repellent activity obtained within 5 min of all concentrations and the lowest one was last only to 10 min at 0.90 g/mL. At 6 h, KPE/e at 1.7 g/mL could repel against the adult mosquitoes over 90%. The repellent efficacy reduced, increased EC_{50}, as followed, 0.07 ± 0.40 g/mL at 10 min; 0.11 ± 0.17 g/mL at 30 min; 0.18 ± 0.09 g/mL at 1 h; 0.23 ± 0.09 g/mL at 3 h and 0.49 ± 0.06 g/mL at 6 h.

The activity of PSE/w showed total repellence in 5 min at a high concentration of 1.50 g/mL (Table 5.3). This concentration was the lowest that could obtain 100% activity of PSE/w. While the high ones were 2.50 and 3.00 g/mL at 30 min. At 6 hour of treatment, PSE/w at 2.50 g/mL could protect against the mosquitoes 77%. The repellent efficacy, EC_{50}, was followed, 0.72 ± 0.09 g/mL at 10 min; 0.75 ± 0.08 g/mL at 30 min; 0.95 ± 0.04 g/mL at 1 h; 1.33 ± 0.04 g/mL at 3 h and 1.57 ± 0.03 g/mL at 6 h.

The repellent activity of PSE/e began to completely repel the mosquitoes at 5 min (Table 5.4). Its effect was slightly higher and last longer than

that of PSE/w, as compared between 100% repellence of PSE/e in 30 min at 1.70 g/mL and of PSE/w in 5 min at 1.50 g/mL (Table 5.3). The EC_{50} of PSE/e repellency was 0.06 ± 0.47 g/mL; 0.26 ± 0.01 g/mL at 30 min; 0.35 ± 0.06 g/mL at 1 h; 0.42 ± 0.05 g/mL at 3 h and 0.67 ± 0.04 g/mL at 6 h.

Table 5.1 Repellent effect of kaffir lime (*Citrus hystrix* DC.) peel water extract, KPE/w, on *Ae. aegypti* adults by skin topical application.

KPE/w Concentration g/ml	% Repellent ± S.D., n=3					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{d,a}$	$0.00 \pm 0.00^{e,a}$	$0.00 \pm 0.00^{e,a}$	$0.00 \pm 0.00^{e,a}$
0.1	$100.00 \pm 0.00^{a,a}$	$99.59 \pm 0.71^{a,a}$	$64.15 \pm 0.05^{c,b}$	$47.65 \pm 1.15^{d,c}$	$34.82 \pm 0.24^{d,d}$	$9.40 \pm 0.80^{c,d}$
0.5	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$89.83 \pm 0.02^{b,a}$	$62.66 \pm 0.06^{c,b}$	$48.40 \pm 0.73^{c,c}$	$44.22 \pm 2.71^{d,c}$
0.9	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$82.32 \pm 0.46^{b,b}$	$79.10 \pm 2.46^{b,b}$	$61.22 \pm 1.07^{c,c}$
1.3	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$96.28 \pm 0.28^{a,a}$	$76.65 \pm 3.32^{b,b}$
1.7	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$90.61 \pm 0.82^{a,b}$
EC_{50} (g/mL)		0.06 ± 0.47^{e}	0.17 ± 0.26^{d}	0.38 ± 0.01^{c}	0.50 ± 0.09^{b}	0.80 ± 0.06^{a}

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

EC_{50}, median effective concentration

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

Table 5.2 Repellent effect of kaffir lime (*Citrus hystrix* DC.) peel ethanolic extract, KPE/e, on *Ae. aegypti* adults by skin topical application.

KPE/e Concentration g/ml	% Repellent ± S.D., n=3					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{d,a}$	$0.00 \pm 0.00^{c,a}$	$0.00 \pm 0.00^{d,a}$	$0.00 \pm 0.00^{f,a}$	$0.00 \pm 0.00^{c,a}$
0.1	$100.00 \pm 0.00^{a,a}$	$94.66 \pm 0.80^{c,ab}$	$87.47 \pm 3.5^{b,b}$	$76.31 \pm 2.14^{c,c}$	$70.84 \pm 0.8^{e,c}$	$55.76 \pm 0.81^{d,d}$
0.5	$100.00 \pm 0.00^{a,a}$	$97.49 \pm 0.45^{b,b}$	$91.35 \pm 0.29^{ab,c}$	$84.31 \pm 0.17^{bc,d}$	$79.58 \pm 0.48^{d,e}$	$53.67 \pm 0.59^{d,f}$
0.9	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$95.65 \pm 2.23^{ab,ab}$	$87.58 \pm 5.71^{abc,ab}$	$83.82 \pm 0.23^{c,bc}$	$72.41 \pm 0.32^{c,c}$
1.3	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$96.55 \pm 1.26^{ab,b}$	$96.22 \pm 0.36^{b,b}$	$80.17 \pm 0.33^{b,c}$
1.7	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$94.65 \pm 0.37^{a,b}$
EC_{50} (g/mL)		0.07 ± 0.40^{a}	0.11 ± 0.17^{a}	0.18 ± 0.09^{a}	0.23 ± 0.09^{a}	0.49 ± 0.06^{a}

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

EC_{50}, median effective concentration

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

Table 5.3 Repellent effect of papaya (*Carica papaya* L.) seed water extracts, PSE/w, on *Ae. aegypti* adults by skin topical application.

PSE/w Concentration g/ml	% Repellent ± S.D., n=3					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{d,a}$	$0.00 \pm 0.00^{e,a}$	$0.00 \pm 0.00^{f,a}$	$0.00 \pm 0.00^{e,a}$
0.1	$96.71 \pm 0.22^{a,a}$	$94.19 \pm 5.04^{b,a}$	$86.77 \pm 0.88^{c,a}$	$73.33 \pm 0.50^{d,b}$	$47.10 \pm 0.73^{e,c}$	$32.53 \pm 0.42^{b,a}$
1.5	$100.00 \pm 0.00^{a,a}$	$96.66 \pm 0.35^{a,ab}$	$92.27 \pm 0.36^{b,b}$	$79.61 \pm 2.61^{c,c}$	$67.69 \pm 0.09^{d,d}$	$66.42 \pm 1.12^{c,d}$
2	$100.00 \pm 0.00^{a,a}$	$97.23 \pm 0.20^{a,b}$	$93.25 \pm 0.20^{b,c}$	$88.12 \pm 0.71^{b,b}$	$75.56 \pm 0.30^{c,e}$	$72.56 \pm 0.30^{b,f}$
2.5	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$91.29 \pm 0.37^{ab,b}$	$87.61 \pm 0.30^{b,c}$	$77.54 \pm 0.26^{a,d}$
3	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$96.62 \pm 0.07^{a,b}$	$83.41 \pm 0.15^{a,c}$	$72.71 \pm 0.29^{b,d}$
EC_{50} (g/mL)		0.72 ± 0.09^{a}	0.75 ± 0.08^{a}	0.95 ± 0.04^{a}	1.33 ± 0.04^{a}	1.57 ± 0.03^{a}

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

EC_{50}, median effective concentration

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

Table 5.4 Repellent effect of papaya (*Carica papaya* L.) seed ethanolic extracts, PSE/e, on *Ae. aegypti* adults by skin topical application.

PSE/e Concentration g/ml	% Repellent ± S.D., n=3					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{c,a}$	$0.00 \pm 0.00^{e,a}$	$0.00 \pm 0.00^{f,a}$	$0.00 \pm 0.00^{f,a}$	$0.00 \pm 0.00^{f,a}$
0.2	$100.00 \pm 0.00^{a,a}$	$96.64 \pm 0.01^{b,b}$	$80.52 \pm 0.47^{d,c}$	$71.59 \pm 0.43^{e,a}$	$67.22 \pm 0.14^{e,e}$	$57.45 \pm 0.57^{e,f}$
0.7	$100.00 \pm 0.00^{a,a}$	$95.45 \pm 0.19^{b,b}$	$82.30 \pm 0.14^{c,c}$	$75.57 \pm 0.40^{d,d}$	$70.94 \pm 0.07^{d,e}$	$63.67 \pm 0.31^{d,f}$
1.2	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$93.47 \pm 0.51^{b,b}$	$89.71 \pm 0.27^{c,c}$	$85.55 \pm 0.10^{c,d}$	$68.36 \pm 0.51^{c,e}$
1.7	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$93.56 \pm 0.72^{b,b}$	$89.45 \pm 0.47^{b,c}$	$79.27 \pm 0.26^{b,d}$
2.2	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$97.40 \pm 0.33^{a,b}$	$96.44 \pm 0.53^{a,b}$	$84.55 \pm 0.46^{a,c}$
EC_{50} (g/mL)		0.06 ± 0.47^{e}	0.26 ± 0.011^{d}	0.35 ± 0.06^{c}	0.42 ± 0.05^{b}	0.67 ± 0.04^{a}

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

EC_{50}, median effective concentration

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

It is summarized that the individual extracts of kaffir lime peel and papaya seed were potent in repelling the adult *Ae aegypti* and their activities could last up to 6 h. the repellent activity of KPE/e was highest, followed by the activity of PSE/e. A number of plants have been traditionally used as repellents against insect pests, including mosquitoes. The essential oils from some aromatic plants such as tagetes, eucalyptus, rosemary (Gillij, Gleiser and Zygadlo, 2008); gallic, clove, orange, and sweet basil (Sritabutra, Soonwera, Waltanachanobon and Poungiai, 2011); and mintweed (Conti et al., 2012) were assessed for the repellent potential against *Aedes* spp. mosquitoes. Peppermint *Mentha piperita* essential oil (Kumer, Wahab and Warikoo, 2011) and sages *Salvia* spp. (Conti et al., 2012) were found effectively repel *Aedes* spp. adults by human-bait technique. Eucalyptus essential oils with strong volatility were highly potential knockdown agents against *Ae. aegypti* in fumigation observation (Lucia, Licastro, Zerba, Audino and Masuh, 2009). The substances those plant products possess repellent activity must provoke antennal responses from the mosquitoes. There were some plants reported that their essential oils elicited the antennal responses from *Aedes aegypti*, for instance, cinnamon, citronella, cumin, eucalyptus, ginger, and peppermint. The most common compounds from plant products found were limonene and camphor (Gillij, Gleiser and Zygadlo, 2008); (b-caryophyllene, linalool, 1,8-cineole, geraniol, and geranial (Campbell, Gries and Gries, 2011); high amount of monoterpenes and oxygenated sesquiterpenes (Park, Chol, Kim, Kim and Lee, 2005; Conti et al., 2012b). However, there is no report on the repellent activity of kaffir lime peel and papaya seed extracts against *Ae. aegypti* adult mosquitoes. This would be the first investigation of these two plant products on repellent activity on Dengue vector mosquito.

5.4.2 Repellent effects of extract combination on *Ae. aegypti* adult mosquitoes by topical skin application

The combination of two types of the kaffir lime and papaya seed extracts was investigated for repellent activity against the *Ae. aegypti* adult mosquitoes. The combination was varied in proportion (v/v) of each extract and then topically applied on rat naked skin.

The combination was set up as following:

(1) KPE/w + KPE/e;

(2) PSE/w + PSE/e;

(3) KPE/w + PSE/w;

(4) KPE/w + PSE/e;

(5) KPE/e + PSE/w;

(6) KPE/e + PSE/e.

The repellent activities of KPE/w + KPE/e combination, 1:3; 1:1; 3:1 (v/v), were similar (Table 5.5). The repellency was 100% potent and last long up to 1 h and slight declined at 3 h. The activity against the mosquitoes was then reduced to approximately 65% at 6 h. It was noticeable that at prolong treatment at 3-6 h the repellence was dependent on the amount of KPE/e.

The combination of PSE/w + PSE/e could repel 100% mosquitoes until only to 10 min (Table 5.6). The repellence as then gradually declined, which the activity was depend on the amount of each extract in the combination. However, it was noticed that the combination with the high proportion of PSE/w produced stronger repellence than those with high PSE/e. the equal amount of them was less

repellence. This indicated that the water extract of papaya seeds was more potent than the ethanolic extract.

The combination of KPE/w + PSE/w could protect against the mosquito attack 100% until 30 min (Table 5.7). The repellent activity declined to 99 and 85% at 1 and 3 h respectively. The repellence at 6 h was dependent on the amount of PSE/w in the mixture.

The repellent activity of KPE/w + PSE/e combination protected against the mosquitoes up to 1 h and slight declined at 3 h (Table 5.8). The mixture was not potent when prolong applied up to 6 h.

The combination of KPE/e + PSE/w could totally repel the mosquitoes up to 1 h and to 3 h when reduced the proportions of PSE/w to at least equal to KPE/e (Table 5.9). Prolong application up to 6 h, the repellent activity was potent with the amount of KPE/e.

The repellent effect of the KPE/e + PSE/e combination on the mosquitoes was up to 3 h (Table 5.10). At 6 h application, the repellent activity was moderate and dependent on PSE/e.

This investigation could conclude that the extracts of kaffir lime peel and papaya seeds were likely to possess the repellent property against *Aedes aegypti* adult mosquitoes. KPE/w seemed to be most potent, followed by PSE/e at a short time of application. PSE/e was most potent at a long time of application. KPE/w and PSE/e were likely to enhance the other extracts in paired combinations. It could be possible that kaffir lime peel and papaya seed contained monoterpenes (Chapter III) which were reported the major source in repelling insect pests (Park et al., 2005; Conti et al.,

2012b) and other phytochemicals, such as limonene and camphor (Gillij, Gleiser and Zygadlo, 2008).

Table 5.5 The repellent effects of combination of kaffir lime (*Citrus hystrix* DC.) peel extracts on adult of *Ae. aegypti* by animal skin rubbing.

KPE/w : KPE/e Concentration g/ml	% Repellent ± S.D., n=3					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{e,a}$	$0.00 \pm 0.00^{e,a}$	$0.00 \pm 0.00^{c,a}$
1: 3	$100.00 \pm 0.00^{a,a}$	$99.59 \pm 0.71^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.44 \pm 0.50^{a,a}$	$90.33 \pm 0.83^{b,a}$	$59.54 \pm 3.85^{b,c}$
1 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$97.59 \pm 0.48^{a,a}$	$74.44 \pm 3.18^{a,b}$
3 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.27 \pm 0.51^{a,a}$	$98.25 \pm 0.21^{a,a}$	$70.08 \pm 1.75^{a,b}$

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

KPE/w, kaffir lime peel water extract, KPE/e, kaffir lime peel ethanolic extrac

Table 5.6 The repellent effects of combination of papaya (*Carica papaya* L.) seed extracts on adult of *Ae. aegypti* by animal skin rubbing.

PSE/w : PSE/e Concentration g/ml	% Repellent ± S.D., n=3					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{c,a}$	$0.00 \pm 0.00^{d,a}$
1: 3	$100.00 \pm 0.00^{a,a}$	$99.14 \pm 0.49^{a,a}$	$88.32 \pm 0.47^{a,b}$	$63.86 \pm 1.52^{a,c}$	$57.46 \pm 7.13^{ab,c}$	$53.43 \pm 3.81^{b,c}$
1 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$89.99 \pm 5.29^{a,a}$	$66.36 \pm 4.00^{a,b}$	$43.47 \pm 7.15^{b,c}$	$36.25 \pm 2.15^{c,c}$
3 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$95.44 \pm 4.44^{a,ab}$	$77.72 \pm 7.13^{a,bc}$	$70.07 \pm 7.02^{a,c}$	$73.61 \pm 6.96^{a,c}$

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

PSE/w = papaya seed water extract, PSE/e = papaya seed ethanolic extract

มหาวิทยาลัยเทคโนโลยีสุรนารี

Table 5.7 The repellent effects of combination of kaffir lime (*Citrus hystrix* CD.) peel water extract and ripe papaya (*Carica papaya* L.) seed water extract on adult of *Ae. aegypti* by animal skin rubbing.

KPE/w : PSE/w Concentration g/ml	% Repellent ± S.D., n=3					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{c,a}$	$0.00 \pm 0.00^{c,a}$
1: 3	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.35 \pm 0.26^{a,a}$	$88.17 \pm 1.02^{a,b}$	$71.60 \pm 5.2^{a,c}$
1 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$97.31 \pm 1.69^{a,a}$	$95.04 \pm 4.00^{a,a}$	$64.77 \pm 3.54^{a,b}$
3 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.71 \pm 0.33^{a,a}$	$85.58 \pm 7.08^{a,a}$	$35.86 \pm 4.88^{b,b}$

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

KPE/w, kaffir lime peel water extract; SPE/w, ripe papaya seed water extract

Table 5.8 The repellent effects of combination of kaffir lime (*Citrus hystrix* DC.) peel water extract and papaya (*Carica papaya* L.) seed ethanolic extracts on adult of *Ae. aegypti* by animal skin rubbing.

KPE/w : PSE/e	**% Repellent ± S.D., n=3**					
Concentration g/ml	**5 min**	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{d,a}$
1 : 3	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$97.71 \pm 1.16^{a,a}$	$87.46 \pm 0.99^{a,a}$	$39.36 \pm 1.23^{b,c}$
1 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.80 \pm 0.33^{a,a}$	$95.42 \pm 4.02^{a,a}$	$45.71 \pm 1.20^{a,b}$
3 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.58 \pm 0.36^{a,a}$	$92.19 \pm 4.12^{a,b}$	$34.37 \pm 0.93^{c,c}$

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

KPE/w, kaffir lime peel water extract; PSE/e, ripe papaya seed ethanolic extract

Table 5.9 The repellent effects of combination of kaffir lime (*Citrus hystrix* CD.) peel ethanolic and ripe papaya (*Carica papaya* L.) seed water extracts on adult of *Ae. aegypti* by animal skin rubbing.

KPE/e : PSE/w	**% Repellent ± S.D., n=3**					
Concentration g/ml	**5 min**	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{c,a}$	$0.00 \pm 0.00^{d,a}$
1: 3	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$86.43 \pm 3.67^{b,b}$	$35.61 \pm 1.55^{c,c}$
1 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$73.84 \pm 3.89^{a,b}$
3 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$58.53 \pm 58.86^{b,b}$

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

KPE/e, kaffir lime peel ethanolic extract; PSE/w, papaya seed water extract

Table 5.10 The repellent effects of combination of kaffir lime (*Citrus hystrix* DC.) peel ethanolic extracts and ripe papaya (*Carica papaya* L.) seed ethanolic extracts on adult of *Ae. aegypti* by animal skin rubbing.

KPE/e : PSE/e Concentration g/ml	**% Repellent ± S.D., n=3**					
	5 min	**10 min**	**30 min**	**1 h**	**3 h**	**6 h**
control	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{b,a}$	$0.00 \pm 0.00^{d,a}$	$0.00 \pm 0.00^{c,a}$
1: 3	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.75 \pm 0.42^{a,a}$	$90.37 \pm 0.91^{c,a}$	$62.70 \pm 6.76^{a,b}$
1 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$97.87 \pm 1.70^{a,a}$	$53.15 \pm 5.88^{a,b}$
3 : 1	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$100.00 \pm 0.00^{a,a}$	$99.69 \pm 0.52^{a,a}$	$98.50 \pm 0.50^{a,a}$	$34.28 \pm 0.88^{b,b}$

Numbers with different letters within the same column are significantly different $P \leq 0.01$ mean± standard deviation (S.D.).

The first superscript (^{a,b}) in the different between time

The second superscript (^{a,b}) in the different between concentration

KPE/e, kaffir lime peel ethanolic extract; PSE/e, papaya seed ethanolic extract

5.5 Conclusion

The kaffir lime peel and papaya seed extracts possessed potent repellent property against *Aedes aegypti* adult mosquitoes. When they were topically applied on the nude rat skin, individuals or combinations. They could protect against the *Aedes aegypti* mosquito attack up to 3 h, roughly. These plant products could contain one or more of those compounds which exhibited potent repellent activity against mosquitoes. Monoterpenes could be the major compound that was responsible for the repellent activity of the extracts. Further study ought to be conducted since these compounds would be used in cloth and fabric industry as well as in public health, including the value added to the plant products.

5.6 References

Abbott, W.S. (1925). A method of computing the effectiveness of an insecticide. **American Mosquito Control Association.** 3(2): 302-303.

Ahmad, N., Fazal, H., Ayaz, M., Abbasi, B.H., Mohammad, I. and Fazal, L. (2011). Dengue fever treatment with *Carica papaya* leaves extracts. **Asian Pacific Journal of Topical Biomedicine**. 330-333.

Barnard, D. and Xue, R.D. (2004). Laboratory evaluaton of mosquito repellent against *Aedes albopitus*, *Culex nigripalus*, and *Ochlerotatus triseriatus* (Diptera: Culicidae). **Journal of Medical Entomology**. 41: 726-730.

Conti, B., Benelli, B., Flamini, G., Cioni, P.L., Profeti, R., Ceccarini, M.M. and Canale, A. (2012a). Larvicidal and repellent activity of *Hyptis suaveolens* (Lamiaceae) essential oil against the mosquito *Aedes albopictus* Skuse (Diptera: Culicidae). **Parasitology Research**. 110: 2013-2021.

Choochote, W., Chaithong, U., Kamsuk, K., Jitpakdi, A., Tippawangkosol, P., Tuetun, B., Champakaew, D. and Pitasawat, B. (2007). Repellent activity of selected essential oils against *Aedes aegypti*. **Fitoterapia**. 78: 359-64.

Campbell, C., Gries, R. and Gries, G. (2011). Forty-two compounds in eleven essential oils elicit antennal responses from *Aedes aegypti*. **Entomologia Experimentalis et Applicata**. 138: 21-32.

Fradin, M.S. (1998). Mosquitoes and mosquito repellents: a clinician's guide. **Annuals of Internal Medicine**. 128: 931-940.

Fradin, M.S. and Day, J.F. (2002). Comparative efficacy of insect repellents against mosquito bites. **New England Journal of Medicine**. 347: 13-18.

Gillij, Y.G., Gleiser, R.M. and Zygadlo, J.A. (2008). Mosquito repellent activity of essential oils of aromatic plants growing in Argentina. **Bioresource Technology**. 99: 2507-2515.

Isman, M.B. (2006). Botanical insecticides, deterrents and repellents in modern agriculture and an increasingly regulated world. **Annual Review of Entomology**. 51: 45-66.

Kumar, P., Mishra, S., Malik, A. and Satya, S. (2011). Insecticidal properties of *Mentha* species: A review. **Industrial Crops and Products**. 34(1): 802-817.

Kumar, S., Wahab, N. and Warikoo, R. (2011). Bioefficacy of *Mentha piperita* essential oil against dengue fever mosquito *Aedes aegypti* L. **Asian Pacific Journal of Tropical Biomedicine**. 85-88.

Lucia, A., Licastro, S., Zerba, E., Paola Gonzalez Audino, P.G. and Masuh, H. (2009). Sensitivity of *Aedes aegypti* adults (Diptera: Culicidae) to the vapors

of eucalyptus essential oils. **Bioresource Technology**. 100: 6083-6087.

Novak, R.J. and Gerberg, E.J. (2005). Natural-based repellent products: efficacy for military and general public uses. **American Mosquito Control Association**. 21(sp1): 7-11.

Osimitz. T.G. and Grothaus, R.H. (1995). The present safety assessment of deet. **American Mosquito Control Association**. 11: 274-278.

Park, B.S., Choi, W.S., Kim, J.H., Kim, K.H. and Lee, S.E. (2005). Monoterpenes from thyme (*Thymus vulgaris*) as potential moaquito repellents. **American Mosquito Control Association**. 21(1): 80-83.

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

Qiu, H., Jun, H. W. and Mc Call, J. W. (1998). Pharmacokinetics, formulation, and safety of insect repellent N, Ndiethyl-3-methylbenzamide (deet): a review. **Journal of the American Mosquito Control Association**. 14:12-27.

Sritabutra, D., Soonwera, M., Waltanachanobon, S. and Poungjai, S. (2011) Evaluation of herbal essential oil as repellents against *Aedes aegypti* (L.) and *Anopheles dirus* Peyton & Harrion. **Asian Pacific Journal of Tropical Biomedicine**. S124-S128.

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

Thavara, U., Tawatsin, A. and Chompoosri, J. (2002)**.** Phytochemicals as repellents against mosquitoes in Thailand**.** Scientific Publications Relating to Insect Vectors from 1995 to 2004**.** National Institute of Health, Department of Medical Sciences, Ministry of Public Health. **In Proceedings International Conference on Biopesticide** 3, April 21-26, 2002, Kuala Lumpur, Malaysia, 244-250.

World Health Organization (WHO) (2012). **Dengue/ DHF**. [On-line]. **Available**: http://www.searo.who.int / LinkFiles / Dengue_DHF_pcg_ introduction.pdf.

World Health Organization (WHO) (2007). **Dengue/ DHF** Reported cases of DF/DHF in Selected Countries in SEA Region (1985 - 2005). [On-line]. **Available:** http://www.searo.who.int/ en/ Section 10/ Section332_1101.htm.

มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER VI

CONCLUSION

Aedes aegypti is a mosquito that can spread the dengue fever, Chikungunya and yellow fever, and other diseases, especially in tropical and subtropical countries of the world. For Thailand, dengue hemorrhagic fever is a major public health problem, because the chemical control of mosquito can cause many environmental problems. Biological control is an alternative way to control the mosquito with human and environmental safety. Therefore, the plant containing mumerous phytochemicals could be used for the mosquito control. In the present study, the kaffir lime (*Citrus hyptrix* DC.) peel and papaya (*Carica papaya* L.) seeds extracts were investigated for the efficiency in biological control of *Ae*. *aegypti* egg hatching inhibition, larval and pupal mortality and adult repellence.

Total phenolic compounds (TPC) of ethanolic extracts and water extract of kaffir lime peel was slightly higher then papaya seeds which were 51.26 ± 0.05 mg and 35.80 ± 0.07 GAE/g respectively. TLC fingerprint with vanillin detection indicated that the major compound constituted in the extracts was terpenes group.

The cytotoxicity of the extracts was determined by brine shrimp lethality assay (BSLA). The toxicity of kaffir lime peel water extract (KPE/w) and ripe papaya seed ethanolic extract (PSE/e) were the most with LC_{50} values of 68.81 ± 0.00 and 68.92 ± 0.00 µg/mL at 24 h kaffir lime peel ethanolic extract (KPE/e) cytotoxic efficacy was moderate at LC_{50} of 125.32 ± 0.56 µg/mL.

The cytotoxicity of ripe papaya seed water extract (PSE/w) was least at LC_{50} of 341.44 ± 0.29 μg/mL.

The efficacy of PSE/e on egg hatching inhibition and the mortality of larvae and pupae was the highest with the EC_{50} value of 1.72 ± 0.00 mg/mL and LC_{50} of 0.48 ± 0.12 and 1.48 ± 0.94 mg/mL, respectively.

The repellent efficacy of plant extract was examined by using animal rubbing test and monitored for 6 h. The results showed that the water extract of kaffir lime and the ethanolic extracts of kaffir lime peel (1.7 g/mL) and papaya seeds (2.2 g/mL) could repell against *Aedes aegypti* for at least 3 h. The combination of the ethanolic peel extract of kaffir lime and the water extract of papaya seeds at the ratios of 1:1 and 3:1 provided protection for at least 3 h. In conclusion, papaya seed ethanolic extract were high toxic to control the mosquito by egg hatching inhibition, second instar larval mortality and pupal mortality. The kaffir lime peel extract was effective in repelling the adults of *Ae. aedgypti*.

มหาวิทยาลัยเทคโนโลยีสุรนารี

CURRICULUM VITAE

FIRST NAME: SUPHUNNEE **LAST NAME:** CHOKKHUN

GENDER: Female **NATIONALITY:** Thai

DATE OF BIRTH: August 31, 1980. **PLACE OF BIRTH:** Nakhon Ratchasima

EDUCATION BACKGROUND:

2002 B.Sc. (Animal Science), Rajamangala University of Technology Lanna, Phitsanulok Campus, Thailand.

WORK EXPERIENCE:

2002-2005 Assistant veterinarians Suwannachart Animal Hospital and Plubplachai Animal Hospital, Bangkok, Thailand.

2005-2007 Research Assistant, School of Biology, Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand.