CHAPTER 4

DISCUSSION

Repellents of natural origin, particularly plant products have nowadays received renewed attention as an ideal way for protection against nuisance and vector arthropods leading to reduce annoyance as well as risk of infection and spread of disease. Although herbal repellents are registered as commercially available and demand is gradually increasing, only few plant substances showed sufficient and long-lasting repellency (Amer and Mehlhorn, 2006; Maia and Moore, 2011; Patel et al., 2012). Furthermore, disadvantages such as expense and need more frequent re-application to maintain full protection make the plant-based materials not be used practically. Therefore, the search for additional bioactive compounds that are expected to improve efficiency, economical feasibility, and user friendliness continues in earnest.

In the present study, essential oils as well as ethanolic and hexane extracts derived from fifteen plant species were screened for repellent activity against *Ae. aegypti* under laboratory conditions. The selection of plants used in repellency screening was focused principally on plants that are abundant and in the similar family of those reported to have the repellent potential against mosquitoes. Preparations of plant products were performed by 2 procedures; steam distillation that generated volatile oils and solvent maceration, which yielded ethanolic and hexane extracts. Steam distillation, a common and economical technique used for separating volatile compounds from nonvolatile contaminants, has been employed extensively in the isolation of natural products. From 17 plant samples, only two plant materials, *S. lappa* root and *A. marmelos* leaf, provided essential oils with yields of 0.32 and 1.50% (v/w), respectively. These are quite large yields when compared to those obtained by other essential oil extractions, which ranged from 0.01-1.0% (v/w) on a fresh/dry weight basis. In earlier studies, the oil yields obtained from hydro-distillation for 3.0-6.0 hr using Clevenger apparatus of *S. lappa* root and *A. marmelos* leaf derived from different locations varied considerably. While the yields of *S. lappa* root oil varied from 0.02% to 0.89% (v/w) (Liu et al., 2012; Gwari et al., 2012; Negi et al., 2013), those of *A. marmelos* leaf oil were 0.30% to 1.50% (v/w) (Kumar et al., 2008; Satyal et al., 2012; Verma et al., 2013). The remaining plant samples, such as *O. basilicum* leaf, *O. americanum* seed, and *L. camara* flower were reported previously with yields of essential oils ranging from 0.05-0.3, when extract by hydro-distillation in conventional Clevenger-type apparatus (Khan et al., 2002; Shadia et al., 2007; al-Maskri et al., 2011). However, in current extraction steps they provide no essential oil when extracted by steam distillation. In addition to plant species and extraction procedures, parts of plant also affected the yield of extracted oils. Examples of these were afforded by the fact that while the leaf of *A. marmelos* provided light yellow oil with an aromatic odor, its fruit yielded no oil. Although extraction by the similar method, steam distillation, the oil yield of *H. cordata* flower previously reported was 0.20% (Tawatsin, 2006) whereas that of its leaf present in this study was zero.

Most plants, except *O. americanum*, were found to provide higher yields when extracted by ethanol (5.12-65.0%, w/w) than those obtained by hexane extraction (0.66-15.98%, w/w). Why ethanolic extraction generates greater yields could be attributed to its intermediate polarity, which leads to a large number of chemical constituents, both polar and non-polar compounds, being extracted (Harborne, 1984). In addition to this advantage, ethanol is often chosen as a first choice use for phytochemical extraction because it is non-toxic, economical, and easy to evaporate at low temperature (Mehta, 2002). Quantity of the resulting products is not the only key to successful extraction, but quality also is, and this should be taken into consideration. Therefore, this study used hexane as extracting solvent, due to reports of its products with strong repellent against many species of mosquito vectors (Choochote et al., 1999; Tuetun et al., 2004; Tuetun et al 2009; Panneerselvam and Muragun, 2013; Singh and Mittal, 2013), and apparent findings supported this information.

In repellent screening of plant samples, the effectiveness of different plant products, including essential oils as well as ethanolic and hexane extracts, for protection against *Ae. aegypti* was documented at varying degrees. Most hexane extracts, including

S. lappa root, *B. orellana* seed, *C. ternatea* seed, *V. zizanioides* (rhizome & root), *A. marmelos* leaf, *H. cordata* leaf, *L. sinense* rhizome, and *Z. zerumbet* rhizome offered higher repellent activity than the other products of the same plant. These findings indicate that the active compounds are more soluble in hexane. The chemical ingredients of hexane-extracted products, which demonstrate greater repellency, are principally non-polar substances, due to hexane being a non-polar solvent that usually dissolves non-polar molecules (Mehta, 2002). Various products derived from the same plant species, which were extracted by distinct chemicals and processes demonstrated differences in repellency. Therefore, the natures of the solvent and extraction technique are critical factors, which affect the chemical principles that influence the bioactivity of plant products (Mehta, 2002; Wandscheer et al., 2004). The initial success of this study is the protection time of up the 2.0 hr from three plant hexane extracts, including *S. lappa* (2.0 hr), *A. marmelos* (2.25 hr), and *L. sinense* (6.5 hr). This meets the requirement of the Food and Drug Administration (FDA), which needs a minimum protection time of 2 hr before allowing sales of repellents in Thailand. These plant products are considered as satisfactory potential candidates for developing new and more effective natural repellents.

L. sinense hexane extract (LHE), with the longest lasting complete protection time of 6.5 (5.0-8.0) hr, which was comparable to that of DEET (6.25, 5.0-6.5 hr) was indicated as the most effective repellent. Therefore, LHE was selected as a candidate to evaluate for repellency in comparison to DEET against two target mosquitoes, *Ae. aegypti* and *An. minimus*. In this step, LHE was formulated with 5% vanillin to ensure more lasting repellency. It is generally known that some plant products, particularly essential oils are highly volatile, which results in reduced repellent activity and residual time. This problem can be solved by formulating with appropriate fixatives that can enhance the residual activity by lowering the evaporation rate and/or altering the skin persistence of repellent (Tuetun et al., 2005; Rehman et al., 2014). Some fixatives such as vanillin (Tawatsin et al., 2001), liquid paraffin (Oyedele et al., 2002), salicyluric acid (Blackwell et al., 2003), coconut, and mustard oils (Das et al., 1999) have been formulated with plant products in order to improve the repellent efficiency. Due to its synergist effect, vanillin is widely used to incorporate with plant-based repellents such as essential oils and solvent extracts to prolong efficacy. Many of the studies have revealed under laboratory conditions that herbal substances and their formulations combined with vanillin showed strong and durable repellency to various mosquito species. Tawatsin et al., (2001) demonstrated that essential oils from tumeric (*Curcuma longa*), citronella grass (*Cymbopogon winterianus*), and hairy basil (*Ocimum americanum*), especially with the addition of 5% vanillin, were effective in repelling *Ae. aegypti*, *An. dirus*, and *Cx. quinquefasciatus* under cage conditions for up to 8 hr. Eucalyptus oil showed improved repellency against *Ae. albopictus* with protection times increased from 3 to 5 hr after adding with 5% vanillin (Yang and Ma, 2005). A study of Choochote et al., (2007) also supported the benefit of incorporating 10% vanillin in essential oils such as *Zanthoxylum piperitum*, *Anethum graveolens*, and *Kaempferia galanga* oils, and their combinations, which provided improved protective effect against *Ae. aegypti*. Addition of 5% vanillin to essential oils such as cassia, rosemary, lemongrass, and xanthoxylum oils, and mixtures of them significantly increased the repellent efficacy against *Ae. aegypti* (Kim et al., 2012). Furthermore, repellent studies of many researchers revealed that vanillin enhanced efficacy not only in plant products, but also in DEET (Tawatsin et al., 2001; Tuetun et al., 2005; Amer and Mehlhorn, 2006; Kamsuk et al., 2007). These findings were corresponded to that of the current study, which demonstrated that vanillin extended the protection times of 25% LHEv and 25% DEETv against *Ae. aegypti* from 6.5 (5.5-9.5) hr to 11.0 (7.0-13.5) hr and 8.0 (5.0-9.5) hr to 8.75 (7.5-11.0) hr, respectively; and against *An. minimus* from 11.5 (9.0-14.0) hr to 12.5 (9.0-16.0) hr and 11.5 (10.5-15.0) hr to 14.25 (11.0-18.0) hr, respectively. Regarding to the present results and those of earlier studies, it was suggested that one promising way to improve repellent efficacy is by improving the plant preparations such as formulation with vanillin.

Repellent evaluation of plant products has been carried out against various mosquitoes both diurnal and nocturnal species under laboratory and field conditions (Patel et al., 2012; Rehman et al., 2014). In this study, mosquitoes of medical importance, *Ae. aegypti* and *An. minimus*, were used as targets for repellent investigations under laboratory situations. In addition to the importance as disease vector, *Ae. aegypti* is generally accepted as a mosquito model used worldwide for repellent screening because of its avidity, easy collection, and conveniences as a laboratory strain; and its sensitivity to test materials can be an indicator of repellent activity (Badolo et al., 2004). Furthermore, results obtained from the study of Amer and Mehlhorn (2006) indicated that *Ae. aegypti* was the most aggressive species and considerably less long repelled by plant extracts as well as synthetic repellents such as DEET and Icaridin/Saltidin, when compared to *An. stephensi* and *Cx. quinquefasciatus*. Although sensitivity to tested substances of *Ae. aegypti* observed from this study can be an initial indicator of repellent activity, the protective effect of repellents against this mosquito species may not ensure success against other species under similar or different circumstances. Therefore, another type of mosquitoes, *An. minimus*, an important vector of malaria was added in order to observe repellent activity of test plant substances in different mosquito species. The results obtained also corroborated that the responses of two mosquito species were relatively different. *Ae. aegypti* was proved to be more tolerant than *An. minimus* for both LHE and DEET. The median complete-protection times of all repellent samples, with and without 5% vanillin, including LHE, DEET, LHEv, DEETv, against *Ae. aegypti* were shorter than those of *An. minimus*. For practical use of the plant product, repellent testing in the field against a wide range of mosquito species also is necessary; therefore, the most effective plant sample (LHEv) was subsequently investigated for repellency comparing to DEETv under field conditions with ambient environments; temperature, humidity, and wind speed.

Results obtained from the field repellent study corresponded to that of the preliminary trials that there were large and mixed mosquito populations, of which some species comprising *Armigeres*, *Aedes*, *Anopheles*, *Culex*, and *Mansonia* were abundant and available for repellency evaluation. It appeared that 25% LHEv exhibited remarkable repellency that was comparable to that of 25% DEETv. No mosquito bite was observed on the volunteers treated with 25% LHEv and 25% DEETv throughout the field study. Therefore, it should be calculated that 25% LHEv and 25% DEETv exerted similarly strong repellent activities by reducing bites with 100% protection. During the field experiment, sunset at the testing site occurred at ≈ 19.30 hr local time and the crowded mosquitoes were observed for at least 3 hr between 18.00-21.30 hr. Varying number and species of mosquitoes were collected from control volunteers in different periods. The maximum mean collecting rates of the predominant, *Ar. subalbatus* (42.76%), *Cx. quinquefasciatus* (41.27%), and *Cx. vishnui* (9.69%) also were observed in different times. While the former crowded in the evening before sunset and gradually decreased after sunset, the two latter mosquitoes were fewer before sunset and increased after the sun set. It was apparent that *Ar. subalbatus* was active in the hours before sunset with the activity peak between 18.44-19.04 hr. Conversely, *Culex* species responded to negative light change i.e. decreasing light intensity and active in the hours after sunset with the activity peak between 19.50-20.10 hr. Similar patterns in feeding behaviors of these mosquitoes that commonly found close to human habitations particularly in suburban areas with poor sanitation containing polluted water were observed everyday of field collections. It is interesting to note that, although *Ar. subalbatus* and *Cx. quinquefasciatus* have been incriminated as the important vectors of the dog heartworm *Dirofilaria immitis* (Siriyasatien et al., 2005) and Japanese encephalitis (Nitatpattana et al., 2008; Changbunjong et al., 2013), respectively, no risk from mosquito-borne diseases had not been previously reported in this field location. The relevance between diseases and their vectors in this area is not known and warrants a more extensive study such as dissecting collected mosquitoes for the presence of parasite infection.

The complete protection of 25% LHEv against the predominant *Ar. subalbatus*, *Cx. quinquefasciatus*, *Cx. vishnui*, and other mosquito species; *Ae. vexans*, *Ae. albopictus*, *An. barbirostris*, *Cx. tritaeniorhynchus*, *Cx. gelidus*, *Ma. indiana*, *Ma. annulifera*, and *Ma. uniformis* was considered as significantly promising potential. However, number of the remaining collected mosquitoes, *Ae. aegypti* (4) and *Ae. lineatopennis* (8) were too small to allow a valid estimation of the protective level against them. Although 25% LHEv possibly protect against *Ae. aegypti* as proven in the laboratory testing, insufficient mosquito number collected in this field experiment could not help to confirm its repellency against this mosquito. Due to no local skin reaction such as rash, swelling, irritation, or other allergic responses was observed during both laboratory and field study periods, this plant product was considered relatively safe to be applied on the skin.

Physical and biological stability of LHE samples determined after storage under different conditions that varied in temperature and time demonstrated slight differences. Some changes in physical characteristics and varying degrees of repellency were recorded among the stored samples of LHE. For physical observation, appearance and odor of all stored LHE samples were similar to that of the fresh sample, showing viscous and pleasant aromatic odor; whereas color of samples kept at ambient temperature (21-35 °C) and 45 °C for 1, 2, and 3 months changed from light- to dark-brown. This finding suggested the relatively changeable property depending on the storing circumstances of this product. It was possibly that higher temperature induced some reactivity leading to color changes. Turek and Stintzing (2012) also reported that the temperature crucially influenced essential oil stability in several respects through oxidative and polymerization processes that resulted in a loss of quality and pharmacological properties. The effect of temperature in reducing repellency against mosquitoes of plant-based products such as essential oils and solvent extracts had been reported by many researchers (Choochote et al., 2007; Tuetun et al., 2004). However, at present higher temperature may slightly affect biological activity of stored LHE because their repellency against *Ae. aegypti* was presented for a period of at least 3 months with insignificantly varied efficacy. Furthermore, LHE samples kept at 4 °C, ambient temperature, and 45 °C for 1 month offered the median complete-protection times of 7.5 (5.0-9.0) hr, 7.25 (5.0-10.5) hr, and 8.0 (4.5-8.5) hr, respectively, which were slightly greater than that of the fresh sample (6.5, 5.0-8.0 hr). In contrast, most samples stored at each temperature for 2 and 3 months exhibited slightly lower repellency than the fresh sample and those kept for 1 month. Nevertheless, repellent activities offered from the stored samples of LHE still produced satisfactory protection times of more than 2 hr (3.5-8.0 hr), which meet the requirement of FDA for sale in Thailand. To determine the feasibility of such results, analysis of chemical constituents and their alterations in the stored and fresh samples is important to indicate any bioactive substances that responsible for repellent efficiency. Among the stored samples, LHE kept at ambient temperature for various durations afforded the greatest repellency, which was comparable to that of the fresh sample. This suggests that this product can be placed under general environment, which makes it convenient and practical to use and maintain.

Chemical analysis by GC/MS presented that the most abundant components derived from the non-polar constituents of LHE were 3-n-butylphthalide (31.46%), 2, 5-lutidin (21.94%), and linoleic acid (16.41%), constituting 69.81% of all the volatile constituents. The minor constituents of LHE were 4-hydroxyindole (7.05%), butylidene phthalide (6.25%), bis (2-ethylhexyl) phthalate (4.84%), and *β*-selinene (2.41%). Many studies previously reported that phthalides are the main biological components found in rhizome of *L. sinense*, besides of phenolic acids and polysaccharide (Luo, 1998; Luo et al., 1994; Zhu and Luo, 1997; Wang et al., 2011a; Wei et al., 2014). However, there have been no publications on chemical constituents of the hexane-extracted *L. sinense* rhizome. Chemical characterization by similar technique, GC/MS, of other products from *L. sinense* such as essential oils demonstrated slight variations in type and amount of compound substances. The principal constituents of *L. sinense* oil studied by Huang and Pu (1988) were ligustilide (58.00%), 3-butyl phthalide (5.29%), and sabinene (6.08%) whereas that reported by Wang et al (2011b) were 5-Oxo-*δ*-4-decahydrobenzindene (50.1%), ligustilide (16.4%), *β*-phellandrene (7.8%), myristicine (5.5%), and spathulenol (3.3%). It is noted that chemical composition of *L. sinense* rhizome reported in the current and previous studies varied qualitatively and quantitatively. Variable factors such as type of plant products, method of extraction, and other plant-related factors including rearing condition (climate and geography), maturation of the harvested plant, plant storage or preservation, and plant preparation possibly affect the production and alteration of plant components (Vieira and Simon, 2000; Tawatsin et al., 2001; Wandscheer et al., 2004; Nurzyńska-wierdak et al., 2013). The phthalides such as 3-n-butylphthalide accounted in both hexane extract and essential oil of *L. sinense* rhizome were also reported as the major constituents of *Apium graveolens* hexane extract (AHE) that offered remarkable repellency against *Ae. aegypti* (Tuetun et al., 2008). According to these findings, it is therefore reasonable to assume that the observed repellent activity of LHE and AHE was partly attributable to the presence of phthalides. However, determination on repellency of this compound needs to be carried out in order to clarify its potential against mosquitoes.

Regarding the results obtained from this study, LHE with proven repellent efficacy, rather physical and biological stable, and no irritant side effect are potential candidates for the development of a new natural alternative to DEET, or an additional weapon used together with other chemicals/measures for integrated vector control.