CHAPTER 4

Discussion

In the present study, botanical products with different yields and physical characteristics were obtained from 33 plant species by using conventional extraction methods, including steam distillation and ethanolic solvent extraction. The extraction procedures employed in this study were simple and economical and used extensively for separating naturally bioactive compounds. In essential oil steam distillation, only nine plant samples provided liquids oils, with low yields (v/w) ranging from 0.02% for *Angelica sinensis* to 1.75% for *Zingiber cassumunar*. Although there are several processes for extracting essential oil from plants, the majority of natural oils are obtained by steam distillation, which is the most common, gentle, and productive method for this purpose. Apart from the benefit of low cost and no need for special solvents, the distinct advantage of this technique is preserving integrity of the plant oils, because the volatile components can be extracted at temperatures substantially lower than boiling point of the individual constituents, thus resulting in reduced risk of thermal degradation (FAO 1995; Tripathi et al. 2009; Newman 2013). However, steam distillation has been deemed an inappropriate means of isolating certain essential oils such as lemon and orange oils as well as those derived from flower petals, which would be denatured significantly by even moderate heat from steam. Therefore, these essential oils are obtained usually by a suitable mechanical process such as expression, which does not need heating (Miguel 2010; Newman 2013). Due to the low productivity in both type and amount of oils recovered from steam distillation herein, optimization of the current extraction process, or finding new methods, is needed to improve the production of volatiles. However, the promising repellency offered from all the oils obtained supports the advantage of steam distillation, which is capable of producing oil with acceptable quality.

Solvent extractions by macerating plant materials in 95% ethanol provided 33 crude ethanolic extracts, with varied yields that ranged in dry weight from 2.52% (w/w) for *Tamarindus indica* to 33.71% (w/w) for *Murraya paniculata*. In addition to being nontoxic, inexpensive and easily evaporated at low temperature, 95% ethanol was used in this study as extracting solvent because of its intermediate polarity that resulted in isolating a large number of chemical components, which were both polar and nonpolar molecules (Harborne 1984; Mehta 2002). This provided not only good percentage yields, but also a variety of constituents with expected sufficiency for screening bioactive potential in plant products.

In repellent screening of plant samples, the effectiveness of different plant products, including essential oils as well as ethanolic extracts, for protection against *Ae. aegypti* was documented at varying degrees. While nine essential oils possessed promising repellent potential against *Ae. aegypti* (0.5 to 7.0 h), only four of 33 ethanolic extracts demonstrated moderate repellency, with the median complete protection times (MPTs) ranging from 0.5 to 2.5 h. This might be due primarily to the aromatic nature and strong odor of the essential oils, from which vaporized oils gained easy access through the insects' tracheal system, thus causing choking and irritation simultaneously, and compelling them to leave the treated sites (Mehmood et al. 2012). All ethanolic extracts exhibited lower repellent activity than the essential oils of the same plants such as *Angelica sinensis* and *Curcuma zedoaria*. Furthermore, while the oils of *Acorus calamus*, *Cinnamomum verum*, *Homalomena aromatica*, *Limnophila aromatica*, *Petroselinum crispum*, *M. paniculata*, and *Z. cassumunar* were effective in repelling *Ae. aegypti* with MPTs of 2.0, 2.0, 1.5, 1.0, 0.5, 0.5, and 0.5 h, respectively; no repellency was observed from the ethanolic extracts of these plants. Variation in repellency from products extracted from the same plant sample by different procedures may be attributed to differences in the active principles presented, qualitatively and/or quantitatively. In addition to the strong aromatic odor of the essential oils from each plant, the greater repellency of essential oils is possibly due to bioactive components, which presented more than those of ethanolic extracts. These findings indicated that the extraction technique and nature of solvent are essential factors facilitating the active chemicals responsible for bioactivity (Mehta 2002; Wandscheer et al. 2004). However, this aspect needs further investigation in order to determine the appropriate extraction method for yielding bioactive compounds as much as possible.

In this study, up to 33 plant species belonging to 23 families were subjected to extractions yielding 42 plant products, but only 13 extracts from 11 plants in 8 families proved to have repellent activity. This outcome was not surprising, due to the selection of plants being based only on botanical and pharmacological data, without support from antimosquito information because of the extensive search required for new repellents from various plants. However, *Sesamum orientale* and *L. aromatica* were recorded herein for the first time as repellents against *Ae. aegypti*, with complete protection times of 0.5 and 1.0 h, respectively, while the other effective products were reported previously as having repellency against mosquitoes and other arthropods (Kamalakannan et al. 2011; Evergetis et al. 2012; Hazarika et al. 2012; Mehmood et al. 2012; Rehman et al. 2014). Furthermore, the highest repellency was established from both *A. sinensis* products that belong to the family, Umbelliferae (Apiaceae), of which members such as *Apium graveolens*, *Kaempferia galanga*, and *Ligusticum sinense* are endowed with repellent properties (Choochote et al. 2007; Tuetun et al. 2009; Evergetis et al. 2012; Sanghong et al. 2014). These findings suggest that the more the research effort, the greater the chance of finding an efficient alternative. However, to have the ability to produce practical and proficient repellents that serve as potential candidates for commercial production and exploitation still requires many working procedures.

There was no detectable local reaction such as rash, irritation, or swelling in most repellent-applied subjects during the study period; however, a little itching was described as an irritant in volunteers treated with oil of *A. sinensis*. Such dermal or skin sensitization also was reported from some essential oils, including lemon eucalyptus, lemongrass, and neem oils, which generally act as insect repellents (Reutemann and Ehrlich 2008; Goodyer et al. 2010; Maia and Moore 2011). This drawback resulted in restricted use as a topical repellent, due to it being a possible skin irritant at a specifically pure or high concentration. It is essential to keep in mind that natural products are not always safer than synthetic ones, and their utilization has been limited in many cases, due to their defective and adverse effects (Trumble 2002; Foster et al. 2005; Strickman et al. 2009). In the present study, *A. sinensis* oil (AEO) was the strongest repellent observed in the screening experiment, but it was considered an unsuitably active ingredient for producing repellents, because of its low yield (0.02%), pungent smell, and cause of itchy skin. However, significant pronounced repelleny against *Ae. aegypti* of *A. sinensis* products both essential oil and ethanolic extract highlighted its qualified promise of being developed as a potential natural alternative for protection against mosquitoes. Therefore, *A. sinensis* was subjected to extracting with different organic solvents of increasing polarity (hexane, acetone, methanol, and absolute ethanol), in order to isolate the bioactive components with the highest repellency as much as possible. The solvent extracts of *A. sinensis* thus obtained were evaluated in comparison to the standard compound, DEET, for repelleny against *Ae. aegypti*. Among four extractants of *A. sinensis*, including hexane extract (AHE), acetone extract (AAE), methanol extract (AME), and ethanol extract (AEE), AHE was considered as the most effective product, with the longest lasting complete protection time of 7.5 h (6.5-8.5), which compared favorably to that of its essential oil (AEO: 7.0, 6.0-7.5 h) and DEET (6.25, 5.0-6.5 h). These findings corroborated with those of many works, thus indicating that hexane-extracted plant products are one of the best repellents against various mosquito species (Choochote et al. 1999; Tuetun et al. 2009; Panneerselvam and Murugan 2013; Singh and Mittal 2013). In general, productivity obtained from extraction with different types of solvents depends on the relative polarity of phytochemicals and extracting solvents (Harborne 1984; Mehta 2002). While moderately polar solvents such as acetone, methanol, and ethanol yield products of mixed polarity, hexane, which is a nonpolar solvent, usually generates nonpolar components. According to the highest repellency observed in AHE, it is note worthy that active principles possessing pronounced repellency are nonpolar extractables. However, the deeper investigation is needed in order to identify effective substances that are accountable for repellent activity, and a possible pilot study would be determination of chemical compositions in each plant product.

In this study, GC/MS was the technique chosen for chemical analysis. It was used to show the profile of constituents and characterize the main active substances that were possibly responsible for the repellent potential of extracted products derived from *A. sinensis*. Chemical compositions in solvent extracts of *A. sinensis*, including AHE, AEO, AME, AEE, and AAE were almost similar because phthalides or phthalates were principal constituents. Phthalides such as 3-*N*-butylphthalide were found as the principal constituents of AHE (70.14%), AEO (50.71%), AME (35.25%), and AEE (28.46%), whereas a dominant constituent in AAE was phthalates such as Di-iso-octyl phthalate. The other phthalides, such as butylidenephthalide and ligustilide, were present in all the extracts with a minor or trace amount. *Cis, cis*-linoleic acid, and/or linoleic acid methyl ester also were recorded as minor constituents in AAE, AME, and AEE. The presence of these phytochemicals, individually or in combinations, is expected to be involved and result in biological activity such as repellency in these plant products. Analysis of the chemical components and repellent activity of each product is needed in order to elucidate on the main active constituents and their probable role in repellent activity. According to repellent evaluation and chemical analysis, it is logical to postulate that the high level of repellent potential, which derived from AHE (7.5 h) and AEO (7.0 h), is possibly associated with the higher percentage of phthalides, particularly 3-*N*-butylphthalide. This phthalide was present in both extracts as a principal constituent in more than half of the total extractable content. These findings are in agreement with the remarkable repellency established from the hexane extract of *A. graveolens* (celery) and *L. sinense* (*Ligusticum chuanxiong*, synonym) essential oil, which contain phthalides such as 3-*N*-butylphthalide, butylidenephthalide, and ligustilide as main biological constituents (Tuetun et al. 2004; Tang et al. 2010; Sanghong et al. 2014, 2015). A large number of phthalides, which are a characteristic type of compound, have proven potential of being insecticidal, herbicidal, nematocidal, acaricidal, antimicrobial, and antifungal agents (Tsukamoto et al. 2005; Beck and Chou 2007). Since phthalides and their derivatives have a documented history of insecticidal activity, investigation of (*Z*)-ligustilide, which is a dominant compound determined as a chemical marker for *A. sinensis* root oil, revealed that (*Z*)-ligustilide-treated cloth deterred the biting of both *Ae. aegypti* and *Anopheles stephensi* more effectively than DEET (Wedge et al. 2009). However, this study showed AME with the lowest repellency, despite it having a higher content of phthalides than AAE and AEE, thus suggesting that other substances are influential, particularly phthalates such as di-iso-octyl phthalate and mono (2-ethylhexyl) phthalate, which had a high percentage of 56.47 and 10.40%, respectively, as found in AAE and AEE. Some phthalates such as synthetic diethyl phthalate and dimethyl phthalate were used as active ingredients in insect repellents. However, their widespread public use is prevented by limitations in effectiveness and possible health risks associated with exposure through direct skin contact (USEPA 2000; WHO 2003; Labunska and Santillo 2004). Some plant fatty acids and their esters such as linoleic acid and linoleic acid methyl ester have been reported to possess not only repellent potential against *Protaphorura armata*, a pest to sugar beet seeds (Nilsson and Bengtsson 2004), but also insecticidal and growth inhibition activity against the cotton leafworm, *Spodoptera littoralis* (Farag et al. 2011; Heba et al. 2013). These findings reflect the importance of compositional complexity in offering and enhancing bioefficacy of natural plant products. Although repellent activity is generally attributed to particular compounds, a synergistic phenomenon among these substances may lead to a potentially promising repellency through a variety of mechanisms (Berenbaum 1985; Hummelbrunner and Isman 2001; Gillij et al. 2008; Nerio et al. 2010). This is a possible reason why crude plant extracts, which comprise complex mixtures of active compounds, frequently demonstrate greater overall bioactivity and provide higher effectiveness when compared to individual constituents. However, comparative chemical and biological analyses are needed, particularly for isolation, identification, and quantification of chemical components. Bioefficacy characterization of isolated compounds, which derive from plants individually and in combinations, also is required for specific elucidation of the active substances responsible for repellent properties.

AHE-based products, including AHE ethanolic solutions (AHE-ES) and AHE nanoemulsions (AHE-NE), with and without 5% vanillin supplementation, demonstrated improved repellency in a dose dependent manner against *Ae. aegypti*. The addition of 5% vanillin increased repellent activity of 5-25% AHE-ES and 5-25% AHE-NE, with prolonged MPTs from 0.5-4.0 h to 2.5-6.75 h and 3.25-5.75 h to 4.0-7.75 h, respectively. Correspondingly, vanillin also expanded the protection times against *Ae. aegypti* of 5-25% DEET ethanolic solutions (DEET-ES) from 2.25-7.25 to 4.25-8.25 h. Vanillin was selected in this study as an added fixative to improve the repellency of AHE, due to its noted ability in optimizing lasting quality in not only plant-based products, but also synthetic substances such as DEET (Tawatsin et al. 2001; Tuetun et al. 2005; Choochote et al. 2007). Among a variety of commercial products are two categories of fixatives based on their sources: natural and synthetic materials (Sturm and Peters 2005; Songkro et al. 2012). Natural fixatives can be derived from herbal constituents (vanillin, benzoin, myrrh, tolu balsam, etc.) and animal secretions (civet, castoreum, musk, ambergris, etc.). Vanillin (4-hydroxy-3-methoxybenzaldehyde) also can be derived from bioconversion of related natural products or synthesis. The synthetic fixatives used in repellent formations include glucam P-20, fixolide, and 2,2,4-trimethyl-1,3-pentane diol. Fixative substances such as vanillin (Khan et al. 1975; Tawatsin et al. 2001; Tuetun et al. 2005; Choochote et al. 2007; Songkro et al. 2012), mustard and coconut oils (Das et al. 1999), liquid paraffin (Oyedele et al. 2002), salicyluric acid (Blackwell et al. 2003), and glucam P-20 and fixolide (Songkro et al. 2012), improve repellent efficacy and are considered the simplest method when compared to other formulation techniques, such as microcapsule or nanoemulsion applications. Among fixative materials, vanillin has been preferable and selected widely as a synergistic additive in various mosquito repellents, with encouragingly improved efficacy. Songkro et al. (2012) reported vanillin, a naturally fragrant fixative, as being the most effective in reducing the evaporation rate of citronella oil at 120 °C, when compared to synthetic compounds, such as glucam P-20 and fixolide. Consequently, vanillin was seen as the best fixative of citronella oil for effectively increasing the protective effect against *Ae. aegypti*. However, they suggested that besides the type and concentration of fixatives, the formula composition, such as ingredients incorporated into the preparations, also had some influence and played an important role in controlling repellent property. Correspondingly, Amer and Mehlhorn 2006 stated that vanillin was not good enough to induce the same effect as a complex formulation (e.g., M10 containing 5% of the five best oils at 1% of each in ethanol and vanillin). Therefore, in the next step of developing AHE for exploitable commercial production, other fragrant fixatives, herbal active ingredients and additive materials would be incorporated. Methods of formulation used for amplifying repellency, particularly sustained-release technology that offers extended mosquito protection such as liposomes, microcapsules, nanoemulsions and nanosuspensions, also should be included. However, the formulation technique is important for not only increasing the effectiveness of a product, but also considering other factors such as health and economical aspects (Amer and Mehlhorn 2006; Nerio et al. 2010). These techniques appear to be valuable in producing AHE-based repellents with the concept of customer acceptability of a safe, cheap, convenient, practical and effective repellent.

Generally, botanical-based products that qualify for registering as a commercial material should have a comparable efficacy to conventional repellents. However, it has been reported that certain plant-based repellents provide less effectiveness and short-lived efficacy compared with synthetic chemicals. A possible reason for this could be the uncontrolled release of active substances in herbal products. Many researchers have, therefore, studied how to control the release of activity for enhancing the repellency of plant products. Development of controlled-release formulations to fix and control the release of the active ingredients that result in an increase of repellency duration or sustained mosquito protection time, should help to achieve this goal. In this study, nanoemulsion was a sustained-release technology chosen for preparation of AHE-based repellent products. AHE were prepared into nanoemulsions that have droplets which are very small with size ranging from 20 to 200 nm. AHE nanoemulsions were formulated in various raitos comprising 5-25% AHE (active ingredient), 5% vanillin (fixative), tween 20 (surfactant), glycerine (co-surfactant), dork butterfly pea (fragrance), and deionized water by ultrasonication. Evaluation against *Ae. aegypti* revealed the remarkable repellecny of AHEv-NE (3.5-8.0 h), which were significantly higer than those of AHE-NE (3.0-6.0 h). Due to the impressive repellency against *Ae. aegypti* of AHEv-NE, this formulation were subjected to further preparation as nanoemulsion gel, 10% AHEv-NEG, which was composed of AHEv-NE, Carbopol®940, glycerine, EDTA, SMS, preservative, TEA, and deionized water. The results obtained from repellent investigations demonstrated the improved efficacy of 10% AHEv-NEG, with MPTs of 4.5 (4.0-6.0) h, 7.75 (6.5-11.5) h, and 11.0 (9.5-12.0) h against laboratory-reared *Ae. aegypti*, *Culex quinquefasciatus*, and *Anopheles minimus*, respectively. Therefore, 10% AHEv-NEG was considered as the most effective AHE-based repellent products. The better protection of formulated nanoemulsion was presumably due to the small AHE-loaded nanoemulsion droplet sizes, which increased surface area of the droplets, thereby, increasing the repellent activity. These findings were rather in accordance with those of Nuchuchua et al. (2009), who reported that small nanoscale of essential-oil-loaded nanoemulsion prepared by high-pressure homogenization would play an important role on their repellent efficacy. The nanodroplets could be well-formed and spread on skin surface as thin film, which resulted to increasing the vaporization of essential oils and subsequently prolonging the mosquito repellent activity. The improved repellency obtained from 10% AHEv-NEG, which was prepared in gel formulation may be attributed to the effect of a gelling agent, Carbopol®940. This gelling agent may conserve persistence of the active ingredient by making the gel-film a tightly coated skin surface. These findings corresponded to the study of Tuetun et al. (2009) who revealed that repellency against *Ae. aegypti* afforded by the formulated gels (2-4.5 h) of *A. graveolens* mostly appeared to have higher repellency than the formulated solutions (0-3.5 h). The best formulae, with the longest-lasting protection of 4.5 (4.5-5.0) h was G10 gel-formulation, which comprised 5% AHE, orange oil, eucalyptus oil, vanillin, Carbopol® Ultrez 21, propylene glycol, preservative solution, deionized water, D-ethanol, 50% neutral TE and PEG-RH 40. In this study, other substances formulated in 10% AHEv-NEG such as TEA, glycerine, methyl and propylparaben, EDTA, and SMS; which commonly used as a neutralizer, humectant, preservative, chelating agent, and antioxidant, respectively, might be influential in enhancement of effectiveness in repelling mosquitoes, presumably by synergistic and/or other properties. However, this aspect needs further investigations in order to identify their probable roles.

For viable application of new repellent products, field evaluation against natural mosquito populations, which generally have different behavioral responses from laboratory-reared mosquitoes is of practical importance. Although sensitivity to active compounds of laboratory strains such as *Ae. aegypti*, *An. minimus*, and *Cx. quinquefasciatus* can be an indicator of repellent activity, this may not ensure success against these species or others under similar or different circumstances. Field repellent investigations of AHE-based products were then performed twice by human-baited techniques; once each in Field I and Field II, at the same location in Chiang Mai province during the hot and rainy seasons of 2013 and 2016, respectively. In Field I, either 25% AHEv-ES or 25% DEETv-ES afforded encouragingly good personal protection by reducing bites with 100% protective effect against a wide range of local mosquito populations. The predominant mosquitoes collected were *Cx.*

quinquefasciatus, *Armigeres subalbatus* and *Culex vishnui*, which made up 41.47%, 41.13%, and 10.53%, respectively. These results corresponded to those of pre-liminary surveys, which presented *Culex* and *Armigeres* as the principal mosquito genera collected. However, these findings did not coincide with those obtained from an earlier study conducted at the same place by Tuetun et al. 2004, at that time the dominant mosquito species collected were *Aedes gardnerii* (35.1%), *Culex tritaeniorhynchus* (29.2%), *Cx. vishnui* (19.4%), *Aedes lineatopennis* (5.1%), *Ar. subalbatus* (3.8%), and *Mansonia uniformis* (2.3%). The difference in mosquito populations collected almost a decade apart is likely a major consequence of environmental changes due to the extension of cities and towns. Noticeable changes in the site and surrounding areas of this study are a reduction of rice fields and gardens, with increasing sources of polluted water. It is generally known that *Cx. quinquefasciatus* and *Ar. subalbatus* breed profusely in sewage or polluted water deposits (Rajavel 1992; Weinstein et al. 1997), whereas *Ae. gardnerii* is found usually in tree holes, log holes, bamboo stumps and bamboo cups and *Cx. tritaeniorhynchus* is commonly seen in irrigated rice fields and ditches, as they prefer cleaner water (Huang 1977; Rozendaal 1997). It becomes evident from the outcome of this study that *Ae. gardnerii* and *Cx. tritaeniorhynchus* populations have been replaced by *Cx. quinquefasciatus* and *Ar. subalbatus* in this location. In this study, the maximum mean collecting rates of the dominant mosquito species, *Culex* and *Armigeres*, were observed in different periods. *Ar. subalbatus* gathered in the evening before sunset with an activity peak at between 18:44 and 19:04 h before decreasing continually after sunset. By contrast, fewer *Culex* species were seen before sunset, but increased consecutively after it, with a biting peak between 20:34 and 20:54 h. A varied pattern in biting behavior of these mosquitoes was observed each day of the field collections. This possibly related to differences in feeding or biting behavior of each mosquito species. *Armigeres subalbatus* is a vicious crepuscular biter that frequently feeds at dusk and dawn, whereas *Culex* spp. respond negatively to light intensity and become active after sunset, when they mostly feed at night (Pandian and Chandrashekaran 1980; Rozendaal 1997). These mosquitoes are associated closely with human habitations because of their anthropophilic and breeding areas (Rajavel 1992; Forattini et al. 1993). Despite there being no evidence of *Ar. subalbatus* transmitting pathogens to humans in Thailand, it has been shown as an efficient vector of the dog heartworm, *Dirofilaria immitis* (Siriyasatien et al. 2005). *Culex quinquefasciatus* is presently regarded as an important vector of filariasis and Japanese encephalitis in the tropical and subtropical regions (Julvez et al. 1998; Nitatpattana et al. 2008; Changbunjong et al. 2013). However, the risk from mosquito-borne diseases has not been reported in the area of the present study. One reason for this is probably that almost all inhabitants lived in houses with protection from insect bites such as screened doors and windows. In general, the feeding behavior of mosquitoes is a significant factor that determines whether they are important as nuisance insects or vectors of diseases, which governs the selection of control methods (Rozendaal 1997). Species that prefer to feed on animals are inefficient at transmitting diseases from human to human. Those that feed in the early evening may be more difficult to avoid than those that feed at night. However, information on epidemiology and disease-vector relationships in the locality of this study is not available and warrants more extensive research such as surveys for larval habitats, mosquito collections and evaluation of local mosquito populations for the presence of pathogenic infections. The complete protection of 25% AHEv-ES against the most abundant mosquitoes, *Cx. quinquefasciatus*, *Cx. vishnui*, and *Ar. subalbatus* as well as other mosquito species, such as *Aedes vexans*, *Aedes albopictus*, *Ae. lineatopennis*, *Anopheles wejchoochotei* (formerly *Anopheles campestis*-like), *Culex gelidus*, *Cx. tritaeniorhynchus*, *Mansonia indiana*, *Mn. uniformis*, and *Mansonia annulifera*, was considered to have significantly promising potential. However, the number of remaining *Ae. aegypti* (4; 0.07%) collected was too small to allow a valid estimate of the protective level against this species. The low number of *Ae. aegypti* collected was possibly because either the testing period (between 18:00 and 21:30 h) was not concurrent with its prime biting time or the study site was not a suitable location for finding this mosquito species. Although 25 % AHEv-ES presumably protects against *Ae. aegypti*, as proven in the laboratory, the insufficient number collected in this field experiment could not confirm repellency against the natural population. As *Ae. aegypti* is the most important vector of dengue fever in urban areas of Thailand (Hammon et al. 1960; Scanlon 1966), further field studies should survey the optimal location and time period for repellent testing against its natural populations. Field studies using local mosquito populations not only provide and confirm an accurate potential of repellent against known mosquito pests and disease vectors, but also the results obtained are important when recommending repellent use to the public (Webb 2013; Afify et al. 2014).

In Field II, two nanoemulsion gel products; 10% AHEv-NEG and 10% DEETv-NEG were investigated for repellency, and the former was slightly stronger than the latter (99.9% protection) by reducing bites with 100% against a wide range of natural mosquito populations. The complete protection of 10% AHEv-NEG against the predominant *Cx. quinquefasciatus*, *Cx. vishnui*, *Ae. vexans*, *Cx. gelidus*, and *Ar. subalbatus* as well as other mosquito species comprising *Ae. aegypti*, *Aedes vittatus*, *Ae. gardnerii*, *Ae. albopictus*, *Ae. lineatopennis*, *Anopheles tessellatus*, *An. wejchoochotei*, *Cx. tritaeniorhynchus*, *Mn. indiana*, *Mn. uniformis*, and *Mn. annulifera*, was considered to have significantly promising potential. Furthermore, this AHE product probably protected against the remaining mosquitoes collected, including *Culex fuscocephala*, *Culex bitaeniorhynchus*, and *Lutzia fuscana*. However, the number of these mosquitoes was too small and insufficient to allow a valid estimation of the level of protection against them. During the study period of Field II, no mosquito bite was observed on 10% AHEv-NEG-treated volunteers, whereas 1 *Ar. subalbatus* came to bite on a volunteer treated with 10% DEETv-NEG. These findings corresponded to those derived from a previous field trial of Tuetun et al. (2009), which revealed that 25% DEET was effective in minimizing bites with 99.68% protection against natural mosquitoes. The only two species landing on 25% DEET-treated volunteers were *Anopheles barbirostris* (5 mosquitoes) and *Ar. subalbatus* (2 mosquitoes), which were the most prominent mosquitoes collected in that time. It is therefore reasonable to assume that these two mosquito species, *An. barbirostris* and *Ar. subalbatus* were slightly tolerant to 25% DEET. Although insignificantly slight differences between the percentage protection derived from 10% AHEv-NEG and 10% DEETv-NEG were observed, and DEET is extensively accepted as an effective broad-spectrum repellent, the better repellency against a wide range of natural mosquitoes obtained from 10% AHEv-NEG was certainly non-negligible. Furthermore, the complaints about unpleasant smell, uncomfortable oily feeling, and undesirable effects such as dizziness or nausea from some of the volunteers treated with 10% DEETv-NEG were recorded. The principal mosquito genera collected in Field II were *Culex*, *Aedes*, and *Armigeres*, which also were the predominant mosquitoes collected in the preliminary surveys. Except for the increase of captured *Aedes* species, these results were consistent with those of Field I, which presented *Culex* and *Armigeres* as the main mosquito genera collected. The relative similarity in mosquito populations collected in Field I and Field II, which conducted in different seasons and 3 years apart was possibly due to minor changes in local populations of mosquitoes at the study site and surrounding areas.

The skin irritant potential of *A. sinensis* has not been previously investigated. In this study, evaluation of skin irritation revealed that all volunteers treated with 25% AHE-ES or 10% AHEv-NEG exhibited no irritant whereas 21 of 30 human volunteers showed a skin irritant to 20% sodium lauryl sulfate (20% SLS), a widely used cosmetic ingredient that was applied as a positive control herein. The foregoing results thus suggest that a single and short (4 h) topical application of AHE-based repellent products were not an irritant to human skin. Although this study may not adequately reflect the effects of long-term and routine use of AHE-based repellents as topical materials, the result obtained was important supportive evidence to establish the safety of AHE for its proposed applications to human volunteers in a mosquito repellent study. Moreover, no local skin reactions such as rash, swelling, irritation, hot sensation, or other allergic responses were observed in the subject volunteers during both laboratory and field study periods. The dermal toxicity of AHE has not been evaluated previously in either humans or animals. However, *A. sinensis* has been reported as herbal medicine formulated clinically to treat various forms of skin trauma and wounds (Huang et al. 2004; Hsiao et al. 2012). Evaluation on the pharmacological effects revealed that the ethanolic extracts of this plant contributed in the process of wound healing by effectively promoting skin fibroblast proliferation with low levels of cytotoxicity even at high concentrations (Hsiao et al. 2012). Furthermore, this herbal extract was proven to have the therapeutic property on atopic dermatitis by inhibition of allergic and inflammatory mediators (Choi et al. 2016). These findings supported the relatively safe application of this plant product on skin.

Determination of physical and biological stability of AHE-based repellent products, including 25% AHE-ES and 10% AHEv-NEG, was performed after they were kept under conditions of a varying temperature and time storage and/or a heating/cooling cycle. For AHE investigation, it was found that all stored AHE samples exhibited similar characteristics, liquid phases with aromatic odor, to those of the fresh preparation; whereas color of samples kept at either ambient temperature for 6 months or at 45 °C for 3 and 6 months changed from dark brown to very dark brown. These findings indicate relatively changeable appearance depending on the storing conditions of this product. However, the results obtained from testing these stored AHE samples against *Ae. aegypti* demonstrated that their repellent activity was present for a period of at least six months, with varied efficacy. Apart from the AHE samples kept at 4 °C for one month, most of the others stored in each condition for one, three, and six months provided relatively weaker repellency than the fresh sample. Furthermore, a lower repellency was determined from AHE samples with longer storage time. It is plausible that extended storage times as well as fluctuating ambient temperature ranging from 21 to 35 °C, and high temperature of 45 °C, partially influenced either physical or biological stability of AHE materials. These findings corresponded to those of Turek and Stintzing 2013, who suggested that monitoring volatile plant extracts and essential oil composition generally revealed forfeited stability from prolonged storage time as well as rises in temperature. These authors also reported that extrinsic parameters, particularly temperature, light and oxygen availability, affected stability of herbal products such as essential oils through oxidative and polymerization processes, with a loss of quality and pharmacological properties. Surprisingly, AHE samples kept at 4 °C for one month afforded MPTs of 10.0 (8.0-11.0) h, which was extremely longer than those of the fresh sample (6.50, 6.0-8.0 h). Although these outcomes cannot be explained herein, due to the absence of supportive experimental evidence, whether or not they resulted from low storage temperature is of interest. Turek and Stintzing 2012 reported the strong stability of rosemary oil when kept at low temperatures, such as in the refrigerator, which could prevent oxidative reactions during three months of storage experiments. However, primary oxidation occurred in pine oil at only 5 °C, despite being promoted at 23 °C. Conversely, this reaction developed especially in lavender oil stored at 5 °C, when compared to that in room temperature, while it had almost degraded completely in both oils of pine and lavender at 38 °C (Turek and Stintzing 2012). Consequently, Turek and Stintzing 2013 concluded that, based on their work and literature review, essential oils vary in susceptibility to autoxidation at different storage temperatures. They also suggested that analytical methods should be evaluated to assess both original and altered essential oil profiles with respect to their suitability for tracking chemical alterations. Analyzing chemical constituents in stored and fresh samples of AHE was, therefore, useful in manifesting not only bioactive substances responsible for repellency, but also chemical alterations affecting stability. For 10% AHEv-NEG investigation, it was found that the stored samples of this product exhibited some changes in appearance and differing degrees of repellency, with MPTs ranging between 3.5-5.25 h, after keeping at 4 °C, ambient temperature (16 to 30 °C), and 45°C for 1, 2, 3, and 6 months. However, 10% AHEv-NEG samples demonstrated similarity in appearance and physical property between those kept for 2 and 4 heating/cooling cycles, which provided MPTs of 4.50 (4.0-4.5) and 3.75 (3.5-4.5) h, respectively, when compared to that of 5.50 (5.0-6.0) h in fresh preparation. Results obtained from the stability testing suggested that the stored AHE-based repellents either 25% AHE-ES or 10% AHEv-NEG achieved adequate protection times (3.5-10.0 h), which exceeded the minimum requirement (2 h) of the Food and Drug Administration (FDA) for sale in Thailand. Furthermore, AHE-based repellent samples kept at ambient temperature for all durations serve as sufficient repellency that is relatively close to that of the fresh sample. It is likely that AHE-based repellent products can be kept in an ambient environment, which makes them convenient and practical in use and maintenance. However, the optimal storage conditions of this material are still low temperatures, such as those in a refrigerator.

In the light of the apparent results, it appears that AHE-based repellent products with proven mosquito repellency under both laboratory and field conditions, no side effects on the skin, and relatively stable physical and biological performance could qualify for developing and registering a new natural alternative to DEET.