CHAPTER 4

Discussion

Phytochemicals have received renewed interest as current and future insecticide alternatives because of their advantages over synthetic chemicals, including less toxicity, less chance of vector resistance, easier degradability in the environment, and ready availability worldwide (Shaalan et al., 2005; Ghosh et al., 2012; Tong and Bloomquist, 2013). A large number of plant species have been surveyed and studied for antimosquito potential, with the goal of discovering and developing a viable alternative for controlling mosquitoes. Although certain compounds of plant origin were proven as being potential candidates for insecticide, further scientific research and development have not been performed frequently. Therefore, few commercial bioinsecticides such as pyrethrum, nicotine, and azadirachtin, account for less than 5% of global pesticides in the marketplace (Mann and Kaufman, 2012; Web bio, 2015). Due to the current lack of choice, the major tool in mosquito monitoring is still application of synthetic insecticides, with limited success because of increasing vector resistance and refusal of community due to concerns about the long-term impacts on health and the environment (Ghosh et al., 2012; Chareonviriyaphap et al., 2013). Encouraging more systematic study of plant-derived products as potential candidates for use in controlling mosquito vectors also is challenging. This study therefore aimed to identify bioactive substances that could be developed further for commercial production of novel natural insecticides, which would possibly meet desired demands. Seventeen plant species were subjected to extraction by two different methods; steam distillation and ethanolic solvent extraction, with 9 essential oils (EOs) and 17 ethanolic extracts (EEs) being obtained in varying yields and different physical characteristics. Although steam distillation provided fewer herbal extracts than ethanolic extraction, almost all EOs obtained from the former exerted significant larvicidal activity against MCM-S *Ae. aegypti*. The preliminary trials for larvicidal screening, at a discriminating dosage of 100 ppm, showed that six of nine EOs, including

Petroselinum crispum, *Foeniculum vulgare*, *Myristica fragrans*, *Limnophila aromatica*, *Piper sarmentosum*, and *Curcuma longa* produced 100% larval mortality. However, of the 17 EEs screened, only *M. fragans* provided 90% larval mortality, whereas the remainder offered no or low larval mortality, ranging from 0 to 36%. Consequently, it is evident that all the effective oils exhibited greater larvicidal potential than the EEs. Also, the efficacy of extracted products, EOs and EEs, which derived from identical plant species, did not correlate with each other consistently. Variation in the larvicidal efficacy of products extracted from the same plant material could be attributed to using different chemicals and techniques, which lead to the difference in insecticidal ingredients presented, qualitatively and/or quantitatively. It has been established that the nature of solvent and extraction procedure are essential factors which affect the chemical principles responsible for the bioefficacy of plant products (Mehta, 2002; Wandscheer et al., 2004).

In the dose-response from larvicidal bioassays, all selected plant products elicited notable activity against MCM-S *Ae. aegypti*, in varied degrees and with different dose dependence. *P. crispum* oil proved to have the highest larvicidal efficacy with the lowest LC_{50} value (43.22 ppm), followed by the oils of *F. vulgare*, *M. fragrans*, *L. aromatica*, *P. sarmentosum*, *C. longa*, and *M. fragrans* ethanolic extract (LC_{50} values of 44.84, 47.42, 47.94, 49.19, 65.51, and 75.45 ppm, respectively). Some of these plant oils were investigated previously and reported to have larvicidal, adulticidal and/or repellent activity against various mosquito species (Choochote et al., 2006; Pitasawat et al., 2007; Knio et al., 2008; Intirach et al., 2012; Zoubiri and Baaliouamer, 2014; Champakaew et al., 2015). *P. crispum* oil exhibiting the highest larvicidal efficacy was, therefore, selected herein as a candidate for further studies, including chemical analysis, antimosquito assessment, and determination of mosquitocidal actions against both larval and adult stages of MCM-S, PMD-R, and UPK-R strains of *Ae. aegypti*.

In antimosquito assessment, *P. crispum* oil and all synthetic chemicals demonstrated efficacy with dosage dependence and different levels of toxicity against the pyrethroid susceptible (MCM-S) and resistant (PMD-R and UPK-R) strains of *Ae. aegypti*. Larvicidal investigations revealed that commercial insecticides showed drastically different levels of larvicidal activity against MCM-S, PMD-R, and UPK-R, with LC_{50} values of 3.54, 6.62, and 6.89 ppb, respectively, for temephos; 1.46, 5.97, and

417.59 ppb, respectively, for permethrin; and 0.16, 1.50, and 9.37 ppb, respectively, for deltamethrin. On the contrary, *P. crispum* oil possessed relatively equal efficacy against MCM-S, PMD-R, and UPK-R, with LC_{50} values of 43.22, 44.50, and 44.03 ppm, respectively. Based on these larvicide-LC_{50} values, it can be stated that the potency of temephos, permethrin, and deltamethrin was incomparably higher than that of *P. crispum* oil. These consequences also were observed in adulticidal evaluations, demonstrating a similar level of susceptibility to *P. crispum* in MCM-S, PMD-R, and UPK-R, with LD_{50} values of 6.01, 6.15, and 6.12 µg/mg female, respectively. Whereas, adulticidal activities of insecticides against MCM-S, PMD-R, and UPK-R also were significantly different, with LD_{50} values of 0.47, 3.81, and 29.36 ng/mg female, respectively, for permethrin as well as 0.06, 3.66, and 7.10 ng/mg female, respectively, for deltamethrin. The effectiveness of each treatment is generally influenced not only by the different susceptibilities of mosquito strains, but also toxicity, depending on the quality and quantity of the insecticidal ingredients among the test samples. The considerably higher efficacy of commercial synthetic insecticides is, therefore, possibly due to their products being prepared specifically with defined contents of active ingredients that affect target species at very low concentrations. Whereas, the active ingredient(s) in crude plant extracts contain several phytochemicals with different bioinsecticidal activity that usually present in very little content when compared to those of traditional synthetics. Furthermore, the purity of plant products is highly variable, depending on either the extraction method or plant-related factors such as age, part used, harvest and storage times as well as the geographical origin of the plant (Sukumar, 1991; Mann and Kaufman, 2012; Mansour et al., 2012). Such a difference has to be taken into consideration when comparing biocidal activity of herbal products with that of synthetic chemicals.

However, unlike conventional insecticides, which are based generally on a single active ingredient, insecticides of plant origin, such as EOs, comprise botanical blends of chemical components that act concertedly on both behavioral and physiological processes, leading to their broad-spectrum of activity and decreasing chance of developing vector resistance (Ghosh et al., 2012; Mansour et al., 2012). In addition, based on LC_{50} values obtained from larvicidal testing, it is noticeable that low levels of susceptibility toward temephos, permethrin, and deltamethrin were found in populations of PMD-R and UPK-R, by 1.9- and 1.9-fold; 4.1- and 286.0-fold; and 9.4- and 58.6-fold,

respectively, over levels in MCM-S. These findings corresponded to those of adulticidal investigation, exhibiting low susceptibility of PMD-R and UPK-R to permethrin and deltamethrin, with LD_{50} values of 8.1- and 62.5-fold and 61.0- and 118.3-fold, respectively, over those of MCM-S. Regarding this, the pyrethroid resistance of both PMD-R and UPK-R *Ae. aegypti* strains can be indicated and confirmed as significantly resistant to all tested insecticides (temephos, permethrin, and deltamethrin) in either the larval or adult stage. Interestingly, the high susceptibility to *P. crispum* oil observed in the larvae and adults of pyrethroid susceptible MCM-S *Ae. aegypti* was comparable to that of PMD-R and UPK-R. These results suggested the promising role of *P. crispum* oil as an alternative in eradicating mosquito vectors, even those resistant strains to insecticides such as organophosphates and pyrethroids. Unfortunately, although some EOs are extracted directly from plants that have been developed and distributed as commercial insecticides for decades, their use as antimosquito products is limited, due to their lower and short-lived effectiveness when compared to conventional insecticides (Fradin and Day, 2002; Kweka et al., 2011). However, there are only a few comparative studies demonstrating the advantage of EOs over synthetic chemicals, by way of affecting both insecticide susceptible and resistant vectors. Despite *P. crispum* oil producing less potential than synthetic compounds in this study, its comparative efficacy on pyrethroid susceptible and resistant *Ae. aegypti* makes it a promising alternative bioinsecticide or probable candidate for further research on the management of insecticide resistant mosquitoes. The application of EOs as a synergist or supplementary agent in synthetic chemicals, which results in reducing vector resistance, cost, and risk of increasing insecticides, could be of great efficacious, economic, and ecological benefit. Investigation on the possibility of using *P. crispum* oil in combination with other insecticides, either natural or synthetic substances, is therefore an important next step from this study.

Determination of mosquitocidal actions by observations on behavioral changes revealed that the toxic symptoms observed in larvae treated with *P. crispum* oil were initiated from the abnormal movement, followed by excitation, restlessness, tremor, convulsion, paralysis, and death within 6 h. Some symptoms such as excitation, convulsion, paralysis, and death were similar to those caused by nerve poison. However, the apparent results observed in the parsley oil-treated larvae that died within a long period of time (6 h) indicated a delayed type of larval killing. Our findings corresponded to those

of the previous studies, which investigated the larvicidal potential against various mosquito species of products derived from plants such as *Kaempferia galanga*, *Tagetes patula*, *Apium graveolens*, *Curcuma aromatica*, *Pinus longifolia*, *Cinnamomum zeylanicum*, and *Piper* species (Insun et al., 1999; Choochote et al., 2004, 2005; Dharmagadda et al., 2005; Chaithong et al., 2006; Warikoo et al., 2011). Although the symptoms in *P. crispum* oil-treated larvae seem to be similar to those observed in these studies, the times generating toxic symptoms caused by these plants were relatively different. Similarity in the behaviour and symptoms of larvae exposed with the parsley oil to those of nerve poisons, suggested that this plant oil probably had a toxic effect on the neuromuscular system (Sakthivadivel and Thilagavathy, 2003; Choochote et al., 2004; Warikoo et al., 2011). However, the larval symptoms caused by nerve poisons occurred more rapidly than those observed in the plant-treated ones.

Observations on physical changes of the parsley oil-treated larvae under LM and SEM demonstrated that while most organs of MCM-S, PMD-R, and UPK-R *Ae. aegypti* had a normal appearance, the anal gills showed remarkable shrinkage with extensive damage. These results therefore pointed out that the toxic effect of parsley oil is mainly on the anal gills, causing the structural deformation. These findings were in agreement with that of Insun et al. (1999) who reported the morphological disruption of anal papillae, with a destroyed surface and shrunken border of cuticle in *K. galanga*-treated *Culex quinquefasciatus* larvae under LM and SEM. Chaithong et al. (2006) also reported the morphological deformation of anal papillae in pepper-treated *Ae. aegypti* larvae under LM and SEM. The shrinkage of internal membrane of the anal papillae was also observed in *Ae. aegypti* larvae treated with cinnamon and pine oils (Warikoo et al., 2011). Determining the toxic effect of pellitorine, an isobutylamide alkaloid obtained from *Asarum heterotropoides* root, on *Ae. aegypti* larvae revealed the morphological destructions in both external and internal bodies (Perumalsamy et al., 2013). The whole body of the pellitorine-treated larvae became dark in color, particularly damaged thorax and abdominal regions. The main targets of pellitorine in the internal body were midgut epithelium and anal gills. The anterior and posterior midgut was found to be entirely necrosed, bearing only gut lumen residues inside the peritrophic membranes. Comprehensive damage of anal gill cells and branches of tracheole and debris was found in hemolymph of the anal gills. In the fresh-water mosquito larvae, uptake and elimination

of most ions occur through the anal gills, while the ion conservation is mainly processed in the alimentary canal (Garrett and Bradley, 1984; Clements, 1992). The ability to take up ions i.e., sodium, potassium, chloride, and phosphate from the medium was evidently decreased or lost in anal gill-less larvae (Koch, 1938; Hassett and Jenkins, 1951; Ramsay, 1953). This indicated that structural destructions of the midgut and anal gills causing their dysfunction may lead to an interruption of ionic regulation and subsequent ionic imbalance, which is intrinsically associated with the death of mosquito larvae.

Observations on behavioral changes in the parsley oil-treated adult females revealed the similarly abnormal appearance in MCM-S, PMD-R, and UPK-R *Ae. aegypti*. After treatment with the plant oil, the adult mosquitoes could not rest on the wall of the cup, lay down on the bottom with inability to move their legs and wings, and then died immediately. However, no morphological change was observed in the females under LM and SEM after treating with the oil for 12, 24, and 36 h. Normality in physical appearance found in the dead females may be due to their exoskeleton, a hard outer layer made mostly of chitin that makes difficult to observe any changes, particularly those in the internal body. Further studies on histopathological and ultrastructural characteristics by using TEM or other effective tools are, therefore, needed to verify the target and mechanism of adulticidal action of this oil. From the results obtained herein, it is insufficient to justify the cause of mosquito death. However, the study of Waliwitiya et al. (2009) investigating the *in vivo* actions of the neuroactive compounds derived from plants in the adults of blowfly *Phaenicia sericata* demonstrated that thymol, the main component identified in *P. crispum* oil tested in this study, penetrated the cuticle and interfered with flight muscle and central nervous function. Due to the similarity of the action of thymol and GABA, the authors suggested that this terpenoid acts centrally in adult blowflies by mimicking or facilitating GABA action. This is supported by the research of Jankowska et al. (2018) who reviewed about molecular targets for components of EOs in the insect nervous system, which indicated that thymol might interfere the GABA receptors and/or central nervous system. Although there are a lot of studies showing neurotoxic actions of essential oil components, the toxic action of the oil on the GABA receptors leading to insect's death needs further studies.

Major mechanisms of insecticide resistance involve in either mutation within the target site of the insecticide and/or insecticide detoxification enzymes, including glutathione-S-transferases (GSTs), esterases (α and β), mixed-function oxidases (MFO), acid (ACP) and alkaline (ALK) phosphatases, and acetylcholinesterase (AChE). Qualitative and/or quantitative changes of these enzymes may be the important processes in the resistance mechanism (Hemingway and Karunaratne, 1998; Chareonviriyaphap et al., 2013). Three mosquito strains, including MCM-S, PMD-R, and UPK-R have previously been studied for mechanism and status of insecticide resistance (Prapanthadara et al., 2002; Somwang et al., 2011; Lumjuan et al., 2014; Plernsub et al., 2016b). Based on LC_{50} and LD_{50} values obtained from larvicidal and adulticidal bioassays, respectively, levels of susceptibility toward temephos, permethrin, or deltamethrin of PMD-R and UPK-R were lower than that of MCM-S. Resistance of PMD-R and UPK-R strains to temephos, permethrin, and deltamethrin may be due partly to the increased level of activity of detoxifying enzymes.

GSTs are enzymes involved in the detoxification of endogenous and exogenous compounds by catalyzing the conjugation of glutathione with electrophilic compounds. In addition to catalytic function, GSTs also act as ligand-binding proteins leading to the sequestration of xenobiotics (Hayes and Wolf, 1988; Mannervik and Danielson, 1988; Pickett and Lu, 1989; Yang et al., 2001). In this study, the highest levels of GSTs activity at 0 h-time point were detected in both of larvae and adults of UPK-R, followed by those of PMD-R and MCM-S, respectively. The GSTs activity in UPK-R larvae was significantly higher than those in PMD-R and MCM-S larvae by 1.29- and 2.46-fold, respectively. Also, the high level of GSTs activity was detected in UPK-R adults, by 1.06- and 1.37-fold, respectively, over levels in those of PMD-R and MCM-S. These findings were in agreement with those obtained from the study of Choovattanapakorn et al. (2017), who reported that the GSTs activities in UPK-R larvae and adults were higher than those in larvae (~1.3-fold) and adults (~1.2-fold) of PMD, which is susceptible to pyrethroid insecticides.

Many studies revealed that insecticide-resistant insects have elevated levels of GSTs activity in crude homogenates, which suggests a role for GSTs in resistance (Grant 1991; Grant et al., 1991). A review on the resistance in insect vectors of diseases by

Hemingway and Ranson (2000) indicated that resistance mechanism to DDT is based on GSTs, which are capable of metabolizing DDT to nontoxic products. GST-based DDT resistance is common in a number of anopheline species, of which a large number of different GSTs are elevated. The GSTs in resistant *An. gambiae* and *Ae. aegypti* are constitutively over-expressed. Likewise, the study of Prapanthadara et al. (2002, 2005) have indicated that elevation of GSTs activity confer resistance to DDT in PMD and PMD-R strains of *Ae. aegypti* by increasing of DDTase activity. Although the PMD-R and UPK-R strains used in this study are the DDT-resistant mosquitoes, the role of DDTase activity conferring resistance to DDT in these mosquitoes has not been investigated herein.

In this study, GSTs activity in the *P. crispum* oil-treated larvae of MCM-S, PMD-R, and UPK-R as compared to the controls were increased by 87.5, 82.6, and 86.07%, respectively. These findings corresponded to those of many studies demonstrating that plant product-treated insects have increased activity of GSTs comparing to the controls (Sarita et al., 2010; Zibaee and Bandani, 2010). The significant increase of GSTs activity was detected in *Ae. aegypti* larvae after treatment with methanolic extract of *Alangium salvifolium* (Thanigaivel et al., 2017). Similar results on increased activities of GSTs at all-time intervals were reported in the rice striped stem borer (*Chilo suppressalis*) larvae exposed to different concentrations of pyriproxyfen (Mirhaghparast et al., 2016). The authors suggested that these enzymes possible involved in the degradation of pyriproxyfen in hemolymph. The cotton leafworm (*Spodoptera littoralis*) larvae showed an increased GSTs activity after treating with indoxacarb, with the elevated levels by 26 and 22.38% in 2nd and 4th instar larvae, respectively (Gamil et al., 2011). Wang et al. (2010) revealed that GSTs activity was increased by > 50% in the *Helicoverpa assulta* larvae fed with the artificial diet and tobacco. They concluded that the high activities of GSTs lead to *H. assulta* become more susceptible to the tested substances. As GSTs are dimeric multifunctional enzymes that play a role in detoxification of a large range of xenobiotics (Prapanthadara et al., 1996), the elevated levels of GSTs activity in these plant product-treated larvae probably associate with the detoxification mechanisms of test substances. However, the unchanged level of GSTs activity in *P. crispum* oil-treated adults of MCM-S, PMD-R, and UPK-R at all-time points may suggest that these enzymes play little or no role in detoxification of this oil in the adult stage.

Esterases are classified as hydrolase enzymes, a large and diverse group that catalyze ester bonds, which are found in a wide range of insecticides including organophosphates, carbamates, and pyrethroids (Dauterman, 1985). Carboxylesterase also play an important role in allelochemical metabolism and tolerance, although the role in a few cases was validated at only the biochemical level (Li et al., 2007). As elevated esterase activities are commonly associated with resistance to organophosphate, carbamate, and pyrethroid insecticides in various pests, the involvement of α- and β-carboxylesterase activity in pest resistance has been extensively studied over the years. In this study, the highest activities of α- and β-esterases at 0-h time point were observed in both larvae and adults of UPK-R, followed by PMD-R and MCM-S in larvae; and followed by MCM-S and PMD-R in adults. The α- and β-esterase activity in UPK-R larvae was significantly higher than those in larvae of PMD-R (1.08- and 1.24-fold, respectively) and MCM-S (1.48- and 1.60-fold, respectively). Accordingly, the high level of α- and β-esterase activity was detected in UPK-R adults, by 1.27- and 1.22-fold, respectively as well as by 1.36- and 1.34-fold, respectively, over levels in those of MCM-S as well as PMD-R, respectively. Elevation of esterase activity has been reported in the field populations of organophosphate- and pyrethtoid-resistant mosquitoes (Macoris et al., 2003; Yaicharoen et al., 2005; Ahmad et al., 2007; Pimsaman et al., 2009). Also, the elevation of α- and β- esterases was reported in deltamethrin-resistant strain of *Ae. aegypti* (Jagadeshwaran and Vijayan, 2009). The elevated levels of esterase activity in these insecticide-resistant mosquitoes indicated an essential role for esterase in resistance mechanism of organophosphate and pyrethroid substances.

The present study demonstrated significantly increased activity of α- and β-carboxylesterases in *P. crispum* oil-treated *Ae. aegypti* larvae of MCM-S (60.57 and 62.31%, respectively), PMD-R (25.69 and 17.67%, respectively), and UPK-R (11.28 and 25.00%, respectively) as compared with those of the controls. These results were in agreement with those of Huron et al. (2016) who studied the toxicity of four crude plant extracts as natural insecticides against the Cowpea aphid (*Aphis carricivora*). It was revealed that treatment of *A. carricivora* with LC_{50} of lupine and lemon grass extracts activated defensive enzymes by increasing the level of α- and β-esterases at 24 and 72 h post treatment. For the adult stage, treatment of *P. crispum* oil for 24 h showed insignificant increase of α-esterase activity in all strains of *Ae. aegypti*, whereas the

activity level of β- esterase was significantly increased after exposure with *P. crispum* oil for 24 h in MCM-S and UPK-R, and 48 h in PMD-R, as compared to the controls. The increase of α- and β- esterase activities have been reported in several insects including mosquitoes after exposure to insecticides and plant-derived extracts (Brengues et al., 2003; Ahmad et al., 2007; Wang et al., 2010; Gamil et al., 2011; Huron et al., 2016). These findings supported the potential role of the esterase enzymes in detoxification of the tested compounds.

Monooxygenases or MFO enzyme plays an important role in the metabolism of pyrethroid and organophosphorus insecticides in both activation and/or detoxification (Hemingway et al., 2004; Yaicharoen et al., 2005). In the current study, the highest levels of MFO activity at 0-h time point were detected in both larvae and adults of UPK-R, followed by those of PMD-R and MCM-S, respectively. The MFO activity in UPK-R larvae was significantly higher than those in PMD-R and MCM-S larvae by 1.36- and 1.42-fold, respectively. Correspondingly, the high level of MFO activity was detected in UPK-R adults, by 1.35- and 1.90-fold, over levels in those of PMD-R and MCM-S, respectively. These were similar to those of Choovattanapakorn et al. (2017), who reported that the MFO activities in the UPK-R were relatively higher than those in the susceptible PMD. Elevated levels of MFO activity have also been observed in mosquitoes resistant to permethrin and deltamethrin (Amin, 1989) and permethrin/organophosphate cross-resistant mosquitoes (Putra et al., 2016).

As comparing to the controls, the decreased MFO activity was observed in *P. crispum* oil-treated larvae of UPK-R, with significant difference at 24-h time point. However, the decreases/increases of MFO activity were detected in *P. crispum* oil-treated larvae of MCM-S and PMD-R, with insignificant difference from those of the controls at all-time points. It can be noted that this enzyme play a minimal or no role in detoxification of the tested oil. Joffe et al. (2011) reported that the parsley seed oil combined with dillapiole oil inhibited cytochrome P450s (CYP450) activity, but not esterase activity, providing a synergistic lethal effect for pyrethrum on *Musca domestica* adults. Earlier studies revealed that several groups of plant products such as plant flavonoids (Mclaughlin et al., 2008); thymol and eugenol (Waliwitiya et al., 2012); EOs from Columbian plants that possess repellent activity (Ramirez et al., 2012); and six

commercial EOs displaying synergistic larvicidal activity against *Ae. aegypti* (Tong and Bloomquist, 2013) acted as inhibitors of CYP450. In contrast to the results of larvae, the *P. crispum* oil-treated adults of MCM-S (18.6 and 8.7% at 24- and 48-h time points, respectively) and PMD-R (7.1% at 24 h-time point) showed significantly increased MFO activity as compared to the controls. However, the insignificantly increased levels of MFO activity were observed in *P. crispum* oil-UPK-R adults at all-time points. These were similar to those of Vasantha-Srinivasan et al. (2017), who found the upregulated levels of CYP450 activity in the field collected wild strain and a susceptible laboratory strain of *Ae. aegypti* after treatment with crude volatile oil of *Piper betle*. These findings suggested the possible involvement of this enzyme in detoxifying the tested oil.

Phosphatases play an important role in diverse physiological processes and act as hydrolytic enzymes that catalyze the removal of phosphate groups by hydrolysis of phosphate ester bonds (Callaghan, 1991; Majerus et al., 1999). These enzymes are able to breakdown phosphotriester bonds in organophosphate insecticides. In this study, the highest levels of acid phosphatase activity at 0-h time point were detected in larvae of PMD-R, followed by those of UPK-R and MCM-S larvae, respectively; whereas UPK-R larvae showed the highest activity of ALK, followed by PMD-R and MCM-S. The ACP activity in PMD-R larvae was slightly higher than those in UPK-R and MCM-S larvae by 1.1- and 1.3-fold, respectively. The activity of ALK in the UPK-R strain was significantly higher than those in PMD-R (1.7-fold) and MCM-S (1.6-fold). Conversely, the highest activities of ACP and ALK in the adults at 0-h time point were observed in UPK-R and PMD-R, respectively. It was found that the high levels of ACP and ALK were detected in either larvae or adults of PMD-R and UPK-R that significantly resistant to temephos, permethrin, and deltamethrin (Intirach et al. 2016), whereas the susceptible MCM-S showed the lowest phosphatase activities in both larval and adult stages. The increased activity of phosphatases in the insecticide-resistant strains indicated their possible role in resistance mechanism to organophosphates. Correspondingly, several studies have suggested that the elevated levels of phosphatase activity is correlated with resistance to organophosphates, such as chlorpyrifos (Emtithal and Thanaa, 2012).

The present study showed significantly increased activity of ACP and ALK in all strains of *Ae. aegypti* larvae after exposure to *P. crispum* oil for 24 h. The levels of ACP

and ALK activity in the *P. crispum* oil-treated larvae of MCM-S (16 and 112%, respectively), PMD-R (34 and 89%, respectively), and UPK-R (6 and 63%, respectively) were increased as compared to the controls. Although the *P. crispum* oil-treated adults of MCM-S (28% at 48-h time point), PMD-R (32% at 24-h time point), and UPK-R (25% at 24-h time point) showed significantly increased ALK activity, the levels of ACP activity in these mosquitoes were not significantly different from those of the controls. The significant increase of ALK activity in all strains of *Ae. aegypti* larvae and adults demonstrated herein indicated the possible involvement of this enzyme in detoxifying toxicants contained in the *P. crispum* oil. Koodalingam et al. (2012) reported that *Ae. aegypti* larvae treated with Bt-based product (Vectobar) showed a 40% increase in ACP activity, but a declined level of ALK activity. They suggested that the imbalance levels of ACP and ALK that produced from the Vectobar led to different impact and deterioration of various biochemical functions, i.e., nucleotide metabolism, energy transfer through ATP, tissue growth and differentiation of the target vector mosquito. Several studies reported the enhancing effects of some botanicals on phosphatase enzymes in various insects, such as *Schistocerca gregaria* (Shekari et al., 2008; Ghoneim et al., 2014), *Culex pipiens* (El-Bassal, 1993), *Helicoverpa armigra* (Babu et al., 1996), *Agrotis ipsilon* (El-Sheikh, 2002), *Rhynchophorus ferrugineus* (Bream, 2003), and *Ae. aegypti* (Koodalingam et al., 2012). The induction of acid phosphatase activity may be attributed to increasing number of lysosomes since ecdysone (molting hormone) is responsible for an increase of lysosome number as a lysosomal acid phosphatase enzyme (Redford and Misch, 1971; van Pelt-Verkuil, 1979; Bassal and Ismail, 1985). These enzymes could affect physiological mechanisms of insect's midgut by interfering the digestion, absorption, and transportation of nutrient (Smirle et al., 1996; Senthil-Nathan et al., 2004).

AChE is primarily a hydrolytic enzyme, metabolizing the neurotransmitter acetylcholine (Ach), thus stopping transmission of nerve impulses at cholinergic synapses in the central and peripheral nervous systems of vertebrates and invertebrates (Grundy and Still, 1985; Wang et al., 2004; Zibaee, 2011). Consequently, inhibition of AChE results in the over accumulation of Ach at the synaptic cleft, leading to the overstimulation of the cholinergic receptors, and ultimately paralysis, secretions, seizures, and death. In insects, AChE is the primary target of organophosphorus and carbamate compounds,

which remain widely used insecticides (Harel et al., 2000). In this study, the level of AChE activity at 0 h-time point in larvae was not significantly different among MCM-S, PMD-R, and UPK-R strains of *Ae. aegypti*.

After exposure to *P. crispum* oil, larvae (24-h time point) and adults (48-h time point) of MCM-S (33 and 14%, respectively), PMD-R (27 and 18%, respectively), and UPK-R (15 and 15%, respectively) showed significantly decreased AChE activity, as comparing to the controls. The lethal effect of *P. crispum* oil may be attributed to its ability to inhibit the activity of AChE, which catalyzes the hydrolysis of the neurotransmitter Ach at nerve synapses and neuromuscular junction leading to paralysis and death. These findings were in agreement with those of many research studies demonstrating botanical effects on AChE activity in various insects. Exposure of *Ae. aegypti* larvae to soapnut kernel extract (Koodalingam et al., 2011) and Vectobar (Koodalingam et al., 2012) significantly decreased the level of AChE activity. Seo et al. (2015) reported the larvicidal and AChE inhibitory activities of Apiaceae EOs and their constituents against *Ae. albopictus*. Of the constituents identified, the highest AChE inhibitory activity in *Ae. albopictus* larvae was established from carvacrol, followed by α-pinene, and β-pinene with IC_{50} values of 0.057, 0.062, and 0.190 mg/ml, respectively. Investigations of fumigant toxicity and effect on AChE activity of EOs against rice weevil, *Sitophilus oryzae*, discovered that reduction in AChE activity was 50.0 and 53.57% after 24 h of fumigation with 80% of 24-h LC_{50} of *Cumunum cyminum* and *Piper nigrum* EOs, respectively (Chaubey, 2011). It can be concluded that these EOs probably induced toxicity in insects by inhibiting AChE activity. Toxicity evaluation of two bio-insecticides (Spinotoram and Vertemic) and Methomyl (Lannete 90% SP) by El-Kady et al. (2008) showed the decreased AChE activity in treated mosquitoes, *Cx. pipiens* and *Anopheles multicolor*. The authors suggested that inhibition of AChE by these pesticides caused a desensitization of the ACh receptor and led to eventual death of the organism.

Regarding the results obtained from the present and previous studies, the higher levels of some detoxifying enzymes, including GSTs, α- and β- carboxylesterases, and ACP and ALK in the resistant UPK-R and PMD-R than the susceptible MCM-S could be due to their associations with resistance to insecticides such as organophosphates and pyrethroids in these mosquitoes. However, as AChE is normally responsible for

inactivation of the neurotransmitter ACh at synaptic and neuroeffector, the levels of AChE activity were not significantly different between the resistant (UPK-R and PMD-R) and susceptible (MCM-S) strains of *Ae. aegypti* larvae. In this study, *P. crispum* oil exhibited the strong larvicidal and adulticidal activities against both pyrethroid-susceptible and resistant *Ae. aegypti*. The significantly increased levels of the enzyme activity in *P. crispum* oil-treated mosquitoes indicated their possible role in detoxification of the oil components. It is hypothesized that some oil constituents that entered the insect's body could be metabolized directly by the detoxifying enzymes, while the rest of these enzymes binding to receptors could induce more production of enzymes (Ahmad et al., 2007). In the other hand, the decrease of AChE activity level in all strains of *P. crispum* oil-treated mosquitoes indicated that this enzyme is the possible target of the oil components.

In this study, GC/MS technique was carried out in order to show the profile of constituents and characterize the main active substances in *P. crispum* oil, the most effective product. A total of 19 compounds were characterized from the oil of *P. crispum*, which represented 98.25% of the whole oil. The main components of this fruit oil were thymol (42.41%), *p*-cymene (27.71%), and *γ*-terpinene (20.98%), followed by a minor amount of *β*-pinene (2.54%). In accordance with the findings of this study, Knio et al. (2008) identified thymol (49.7%), *p*-cymene (28.7%), and *γ*-terpinene (15.0%) as dominant compounds, with trace amounts of trans-anethole (1.6%) and *β*-pinene (1.0%) in the seed oil of *P. crispum* purchased from a local market in Beirut, Lebanon. The oil yields from *P. crispum* fruit and seed, which derived from different localities and were extracted by different techniques, also bore close similarity. While the fruit oil extracted herein yielded 1.74% (v/w) by steam distillation, seed oil extracted by hydrodistillation gave 2.1% (v/w) (Knio et al., 2008). Additionally, the study of Knio et al. (2008) observed that *P. crispum* seed oil and its major compound, thymol, demonstrated larvicidal potential against the seaside mosquito, *Ochlerotatus caspius*, with LC_{50} values of 34.3 and 33.7 µg/ml (ppm), respectively. Thymol is an alkylated phenol derivative, which also has been identified as the active principle responsible for the insecticidal and repellent actions of various plant oils such as *Lippia sidoides*, *Trachyspermum ammi*, *Origanum syriacum*, and *Thymus vulgaris* against many mosquito species, for example, *Ae. aegypti*, *Anopheles stephensi*, *Culex pipiens molestus*, and *Culex pipiens pallens* (Traboulsi et al.,

2002; Choi et al., 2002; Carvalho et al., 2003; Park et al., 2005; Pandey et al., 2009). Therefore, it is reasonable to note that the antimosquito performance of *P. crispum* oils, revealed herein and in other studies, was possibly influenced by the presence of a principal constituent such as thymol. However, the interaction and synergistic effect between the active constituent, thymol, and other components that are either major or minor compounds, such as *p*-cymene, *γ*-terpinene, trans-anethole, and *β*-pinene, could be influential cofactors. These compounds were found to present also in a wide range of plants, with antimosquito properties against a variety of vector populations (Choi et al., 2002; Knio et al., 2008; Vongsombath et al., 2012; El-Akhal et al., 2014; Zoubiri and Baaliouamer, 2014).

The present study established the promising mosquitocidal efficacy of plant-derived products against the pyrethroid-susceptible and resistant strains of *Ae. aegypti*. The successful results demonstrated herein may reflect the potential of EOs, specifically *P. crispum* oil, as an alternative to further development of larvicides and adulticides used for management of mosquito vectors, particularly insecticide resistant populations.