

**The Activity of Analogs of the Natural Product Dillapiol and  
Sessamol as Detoxification Enzyme Inhibitors  
and Insecticide Synergists**

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## ABSTRACT

In the present thesis, analogs of the plant derived compound, dillapiol, were investigated for their potential as cytochrome P450 inhibitors and insecticide synergists. Dillapiol was chosen as a lead compound because it has a methylenedioxyphenyl (MDP) functionality that serves as a cytochrome P450 inhibitor and reported insecticide synergist activity comparable to the commercially used piperonyl butoxide (PBO).

Initially a set of fifty two dillapiol analogs was investigated for inhibition of cytochrome P450 using cloned Human CYP3A4, a highly standardized preparation that allowed accurate determination of structure activity relationships. A qualitative analysis revealed that analogs with a large acyl group attached via ester bonds had higher *in vitro* CYP3A4 inhibitory activity. However, a Gaussian Quantitative Structure Activity Relationship model also showed the importance of hydrophobic interactions and predicted new structures with higher P450A4 inhibition.

Subsequently selected analogs were investigated as potential pyrethrum synergists in insecticide susceptible (SS-CPB) and resistant (RS-CPB) Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Coleoptera: Chrysomelidae) as well as the European corn borer (ECB, *Ostrinia nubilalis* Hübner). Using discriminating dose and full concentration bioassays, the synergistic activity of selected analogs was studied. Ether analogs demonstrated stronger synergistic activity than ester analogs for both insect species. All tested compounds displayed higher synergistic activity by ingestion than topical administration, and each analog type (ester and ether) had one compound with a synergism ratio greater than 20. Both compounds successfully restored the insecticide susceptibility within RS-CPB resistant strain larvae to pyrethrum.

In greenhouse and field trials, pyrethrum extract combined with dillapiol was effective against SS-CPB, and the pyrethrum + dillapiol formulation demonstrated efficacy at least 10 times higher than that of pyrethrum alone. This suggested the feasibility of dillapiol as a novel PBO replacement for organic farming.

The intrinsic toxicity of the most active analogs compared to dillapiol or PBO were determined by growth inhibition bioassay with ECB larvae which were administered a synergist treated diet. In several growth parameters of ECB evaluated, PBO was found to have the strongest intrinsic toxicity, followed by the ester analog. Dillapiol showed the least toxicity among four tested compounds, while the ether analog, which was the best pyrethrum synergist against both oligophagous CPB and polyphagous ECB, had a similar safety level compared to dillapiol.

To explore the underlying mechanism of the synergists, the impact of selected compounds on three major detoxification enzymes, monooxygenase P450, Glutathione S-transferases (GST) and esterases of both insect species were evaluated. All selected analogs effectively inhibited *in vitro* and *in vivo* P450 monooxygenases activity for both oligophagous CPB and polyphagous ECB. The best pyrethrum synergist also displayed significantly greater P450 inhibitory activity than PBO which was eleven times more effective than PBO as an inhibitor of ECB *in vitro* P450 activity. The inhibition assay with either CPB or ECB GSTs respectively produced the surprising result that the best pyrethrum synergist exhibited a 180-fold or 575-fold lower  $IC_{50}$  than the standard inhibitor of GST. To our knowledge, this is the first report of MDP related compounds showing significant GST inhibitory activity.

A pilot study of insect dysregulation induced by synergists was conducted with Ultra Performance Liquid Chromatography (UPLC)-Quadrupole Time of Flight Mass Spectrometry (QTOF/MS). It was found to be a suitable technique to study the metabolites changes induced by

selected analogs for the two insect species. The application of UPLC-QTOF/MS produced high-resolution metabolites profiles which guarantees the success of the dynamic metabolism research of selected insects in the future.

## RÉSUMÉ

Dans cette thèse, les analogues du composé végétal, dillapiole, ont été étudiés pour leur potentiel comme inhibiteurs du cytochrome P450 et synergiques d'insecticides. Dillapiole a été choisi en tant que composé principal, car il présente une fonctionnalité méthylènedioxyphényl (MDP) qui sert d'inhibiteur du cytochrome P450 et a un effet documenté synergique sur l'activité insecticide qui serait comparable au composé commercial, la butoxyde de pipéronyle (BOP).

Initialement, un ensemble de cinquante-deux analogues de dillapiole a été étudié pour l'inhibition du cytochrome P450 en utilisant le clone humain CYP3A4, une préparation hautement standardisées qui a permis la détermination précise des relations structure-activité. Une analyse qualitative a révélé que les analogues ayant un important groupe acyle lié par des liaisons esters avaient une activité inhibitrice *in vitro* plus élevée du CYP3A4. Cependant, le modèle gaussien de la relation quantitative structure à activité (connu en anglais sous le nom *Quantitative structure-activity relationship* ou QSAR) a également montré l'importance des interactions hydrophobes et prédit de nouvelles structures ayant plus d'inhibition de P4503A4.

Par la suite, des analogues ont été sélectionnés et étudiés comme agents synergiques potentiels du pyrèthre chez les doryphores de la pomme de terre (*Leptinotarsa decemlineata* Say) sensibles (DPT-SS) et résistants (DPT-RS) à l'insecticide ainsi que chez la pyrale du maïs (PDM; *Ostrinia nubilalis* Hübner). L'activité synergique des analogues sélectionnés a été étudiée par des bioessais à dose discriminant et à pleine concentration. Chez les deux espèces d'insectes, les analogues d'éther ont démontré une activité synergique plus importante que les analogues d'ester. Tous les composés testés ont affiché une plus grande activité synergique par ingestion que par administration topique, et chaque type analogique (ester et éther) avait un composé ayant

un rapport de synergie supérieur à 20. Les deux composés ont rétabli avec succès la sensibilité à l'insecticide chez les larves DPT-RS de la souche résistante à pyrèthre.

Dans les serres ainsi que sur le terrain, l'extrait de pyrèthre combiné avec le dillapiole a été efficace contre DPT-SS, et la combinaison pyrèthre / dillapiole a démontré une efficacité au moins 10 fois plus grande que le pyrèthre seul. Cela suggère la possibilité d'utiliser le dillapiole comme un remplacement de BOP pour l'agriculture biologique.

La toxicité intrinsèque des analogues les plus actifs par rapport à dillapiole ou BOP ont été déterminés par un bioessai de l'inhibition de croissance des larves PDM qui ont été administré une diète synergique. Pour plusieurs des paramètres de croissance évaluées chez les PDM, la BOP avait la toxicité intrinsèque la plus forte, suivie de l'analogue d'ester. Le dillapiole a démontré la plus faible toxicité parmi les quatre composés testés, alors que l'analogue d'éther, qui était la meilleure synergie du pyrèthre à la fois contre les DPT oligophages et les PDM polyphages, avait un niveau de sécurité similaire au dillapiole.

Afin d'explorer le mécanisme sous-jacent des agents synergiques, l'impact des composés sélectionnés sur trois enzymes importants de détoxification, le mono-oxygénase P450, les glutathion S-transférases (GST) et les estérases, des deux espèces d'insectes ont été évalués. Tous les analogues sélectionnés peuvent efficacement inhiber l'activité du mono-oxygénase P450 *in vitro* et *in vivo* à la fois chez les DPT oligophages et les PDM polyphages. Le meilleur synergiste du pyrèthre a également démontré significativement une plus grande activité d'inhibition de P450 que le BOP en étant onze fois plus efficace que le BOP comme inhibiteur de l'activité de P450 chez les PDM *in vitro*. Fait surprenant, l'essai d'inhibition avec les GST de DPT ou de PDM a démontré que le meilleur agent synergique d'éther du pyrèthre avait une  $CI_{50}$  de 180 fois ou 575 fois plus faible respectivement que l'inhibiteur courant des GST. À notre connaissance, ce travail

est le premier démontrant une activité inhibitrice significative des GST par des composés reliés au MDP.

Une étude pilote de dysrégulation des insectes induite par des agents synergiques a été menée par la Chromatographie en phase Liquide à Ultra Performance (CLUP)-Spectrométrie de Masse Quantitative à Temps de Vol (QTOF/MS, selon l'acronyme anglais *Quantitative Time of Flight Mass Spectrometry*). Cette technique s'est avérée être adéquate pour étudier les changements de métabolites causés par les analogues sélectionnés chez les deux espèces d'insectes. L'application de CLUP-QTOF/MS a généré un profil à haute résolution des métabolites garantissant ainsi le succès de la recherche sur le métabolisme dynamique des insectes sélectionnés dans le futur.

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Part of this work (Chapter 3) has been presented, and three more manuscripts (Chapter 2, Chapter 4, and Chapter 5) are under preparation for submission:

- 1) **Liu SQ**, Scott IM, Pelletier Y, Kramp K, Durst T, Sims SR, Arnason JT. 2014. Dillapiol: a pyrethrum synergist for control of the Colorado potato beetle. *J. Econ. Entomol.* 107(2):797-805
- 2) Carballo AF, **Liu SQ**, Pineda N, Durst T, Arnason JT, and Compadre CM. Potent CYP3A4 inhibitors derived from dillapiol and sessamol. Ready for submission to *Tetrahedron*.
- 3) **Liu SQ**, Carballo AF, Wang ZL, Scott IM, Durst T, Sims SR, Sánchez-Vindas PE, and Arnason JT. Evaluation of novel dillapiol analogs as insect P450 monooxygenase inhibitors

and insecticide synergists against Colorado potato beetle. In preparation for submission to *Insect Biochemistry and Physiology*

- 4) **Liu SQ**, Carballo AF, Harkin E, Scott IM, Durst T, and Arnason JT. New MDP compound as an effective inhibitor of cytochrome P450 and GSTs of European Corn Borer *Ostrinia nubilalis* Hübner. In preparation for submission to *Pest Management Science*.

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## LIST OF ABBREVIATIONS

C.I.	Confidence interval
CPB	Colorado potato beetle
CYP3A4	Cytochrome P450 3A4
DBF	Dibenzylfluorescein
DEF	S,S,S,-tributylphosphorotrithioate
DEM	Diethyl maleate
ECB	European corn borer
GST	Glutathione S-transferase
IC <sub>50</sub>	Median inhibition concentration
LC <sub>50</sub>	Median lethal concentration
MDP	Methylenedioxyphenyl
Py	Pyrethrum
PBO	Piperonyl butoxide
QSAR	Quantitative Structure-Activity Relationship
RS-	Resistant
S.E.	Standard error
SS-	Susceptible
UPLC- QTOF/MS	Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry

# CHAPTER 1 INTRODUCTION

## 1.1 General Introduction

Resistance to drugs and pesticides is a widespread phenomenon which occurs in both vertebrates and invertebrates, including insect pests. Insect herbivores also become resistant to plant defence mediated by plant secondary metabolites. To restore sensitivity in insect herbivores, many plants produce phytochemicals which inhibit metabolism of defence compounds. Dillapiol, a neolignan produced by dill, wild peppers and other plants is one such phytochemical, and is known to act by inhibition of toxin metabolism. Dillapiol is also an effective insecticide synergist, but its activity could be improved by semi-synthesis of analogs. To develop practical insecticide synergists, dillapiol analogs were first produced by semi-synthesis by medicinal chemistry collaborators. In the present thesis, I then systematically examined the *in vitro* biochemical activity of compounds in both human and insect enzyme assays. Selected compounds were advanced to *in vivo* evaluation as pyrethrin synergists in a polyphagous and an oligophagous insect.

## 1.2 Literature Review

The scientific consensus on best practices to control insect pests is integrated pest management, which includes use of resistant plants, natural enemies, cultural and other emerging practices, as well as insecticides, where necessary. Insecticides remain an important control strategy today but insects have responded by the evolution of insecticide resistant populations.

Resistance to insecticides has been recognized for more than 100 years since the first reported case, which was the resistance of San Jose scale (*Quadraspidiotus perniciosus* Comstock, Hemiptera: Diaspididae) to lime sulfur in the state of Washington in 1908 (Melander, 1914). To date, resistance has been observed in all classes of compounds used for insect pest control,

including insect growth regulators (hormone mimics) (Hemingway, *et al.*, 2004). Resistance can generally be categorized into three major types: behavioral resistance, which involves changes in the behaviors by which insects avoid insecticides; physiological resistance, which is any form of resistance that reduces toxicity through changes in basic physiology; and biochemical resistance which refers to the detoxification of insecticides by one or more insect enzymes before they reach the site of action (Fig.1-1).

### **Behavioral Resistance (BR)**

Behavioral resistance is the evolution of any behavioral change that permits a population to escape from an insecticide-treated area. Behaviors that may be important in the resistance development include movement of immature stages, adult dispersal, oviposition, feeding or any social or non-social interaction in a population. BR can be divided into direct contact excitation or irritancy and non-contact spatial repellency (Roberts *et al.*, 1997). Using a laboratory population of *Plutella xylostella* as the insect model, Head *et al.* (1995) demonstrated increased general larval activity, and a greater tendency of larvae to avoid insecticide pyrethroids in resistant insects.

In other experiments conducted by Jallow and Hoy (2005, 2006, 2007), a lower oviposition rate of *P. xylostella* was observed on cabbage leaf disks and seedlings treated with permethrin, and this oviposition deterrence was correlated with permethrin concentration. In a subsequent study, Jallow and Hoy (2007) discovered that a high proportion of phenotypic variation for adult behavioral response to permethrin was heritable. This adult behavioral response can lower the exposure of larvae to the insecticide, reducing selection pressure for toxicological resistance in larvae. On the other hand, this behavioral response and associated larval survival could help preserve susceptible alleles in the population, which contribute to the success of insect resistance management (Onstad, 2014).

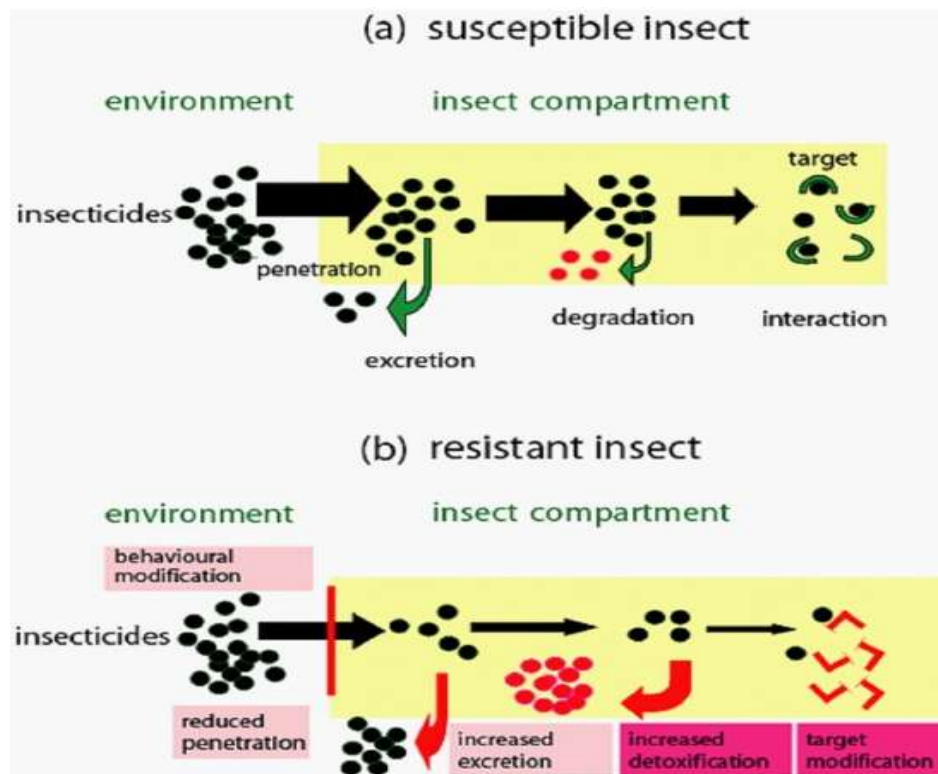


Fig. 1-1 Scheme of potential behavioral, physiological, and biochemical changes associated with insecticide resistance; (a) susceptible insect; (b) resistant insect (source: Lapied *et al*, 2009, with reusing permission)

## **Physiological resistance**

Physiological resistance has been shown to involve two major factors, reduced penetration and target site insensitivity.

### *Penetration Reduction*

Resistance by reduced penetration occurs when insects develop a heritable mechanism that reduces the entry or penetration or decreases the absorption of the toxin or insecticide into the insects by modifying the insect cuticle or digestive tract linings. A study demonstrated that the cuticle of the DDT-resistant strain of tobacco budworm (*Heliothis virescens* Fabricius) contains more protein and lipid than that from the susceptible strain, and an increase of sclerotization of the cuticle of resistant insects was observed (Vinson and Law, 1971). This evidence suggests that the increase in density and hardness of the cuticle decreases its permeability to insecticide molecules. Cuticular resistance was also reported for the domestic fly *Musca domestica* (Plapp, 1984) and house mosquito *Culex quinquefasciatus* (Georghiou, 1983). The use of microarray techniques in *Anopheles* showed that two genes, *cplcg3* and *cplcg4*, which were responsible for encoding cuticular proteins, were upregulated in pyrethroid resistant strains (Djouaka *et al.*, 2008; Vontas *et al.*, 2007; Awolola *et al.*, 2002). The measurement of mean cuticle thickness of *A. funestus* using scanning electron microscopy showed that it was significantly greater in pyrethroid-tolerant mosquitoes than their susceptible counterparts (Wood *et al.*, 2010).

The peritrophic membrane of the insect digestive system is a complex tissue composed of proteins, glycosaminoglycans, and chitin that serves as an integral part of the controlled enzymatic degradation and absorption of food. It also provides an exclusionary barrier to bacteria, viruses, and damaging mechanical elements (Lehane, 1997; Tellam *et al.*, 1999,

Hegedus *et al.*, 2009; Kuraishi *et al.*, 2011). In this way, the peritrophic membrane minimizes the impact of negative biotic and abiotic factors in the insect diet.

#### *Target Site Insensitivity*

Target site insensitivity was defined as the failure of a toxicant to bind to the target due to alteration in the structure or accessibility to that target site (Brooks, 1976). Target site insensitivity may also be conferred by a reduction in the total number of receptors for a particular chemical. One well-known target site resistance mechanism in the house fly is knockdown resistance (*kdr*), which confers resistance to DDT and pyrethroids (Harrison, 1951; Farnham, 1973, 1977). Jiang *et al.* (2011) conducted a survey of resistance in Colorado potato beetle *Leptinotarsa decemlineata* from four areas that had developed resistance to five conventional insecticides, and discovered that target site insensitivity mutation in the AChE and LdVssc1 genes conferred resistance to carbamates and pyrethroids, respectively. These results are consistent with other studies which revealed that CPB resistance to carbamates is associated with the gene mutation of amino acid in the AChE, and CPB resistance to pyrethroids is caused by a single amino acid substitution L1014F in the voltage-sensitive sodium channel (Ben'kovskaya *et al.*, 2008; Zichová *et al.*, 2010; Lee *et al.*, 1999; Clark, *et al.*, 2001). Further direct evidence for the occurrence of target-site resistance in the brown plant hopper, *Nilaparvata lugens* to a neonicotinoid insecticide was provided by Liu *et al.* (2005) who discovered a strong correlation between the frequency of a single point mutation in two subunits of AChE and the level of resistance to imidacloprid.

#### **Enhanced Detoxification**

The insect detoxification activity of xenobiotics including insecticides mainly involves three metabolic enzyme families: cytochrome P450s, glutathione S-transferases (GSTs) and esterases.

P450s are phase 1 detoxification enzymes which participate in the metabolism of xenobiotic compounds such as pesticides to more water soluble derivatives. GSTs, phase 2 enzymes of insecticide metabolism, mainly conjugate the metabolites formed in phase 1, making them even more water soluble and aiding in their excretion from the organism. The esterase family of enzymes can hydrolyse ester bonds which are present in a wide range of insecticides and make them non- or much less toxic. These modes of action will be discussed in detail in Chapter Three.

### 1.3 Synergists including the botanical lead molecule dillapiol

To overcome insecticide resistance, various insecticide resistance management strategies, including employing synergists or pesticide mixtures, have been adopted (Gunning *et al.*, 1998). Synergists are also of interest as sparing agents for costly new insecticides and for rapidly metabolized botanical insecticides. In addition, there is a crossover into pharmaceutical therapies. For example, synergists may be of use for sparing and reducing the rate of drug resistance development.

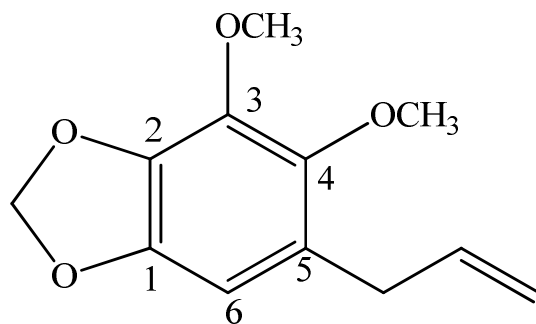
Synergists have been used commercially for about 50 years and have contributed significantly to improve the efficacy of insecticides by broadening their bioactivity spectrum, countering resistance development, increasing effective commercial lives, and mitigating the residual effects of persistent and highly toxic products by reducing the application dose.

The most frequently used synergist is piperonyl butoxide (PBO) (Fig. 1-2), which was initially synthesized in the 1940s by Herman Wachs (Jones, 1999). Although PBO was originally described as ‘a new safe insecticide’ (Wachs *et al.*, 1950), its major application has been as a synergist for pyrethrins. Today, it is used in combination with many pesticides on the market as a powerful synergist for controlling resistant pest populations. Zimmer *et al.* (2011) investigated three common synergists towards lambda-cyhalothrin resistance in 27 pollen beetle populations

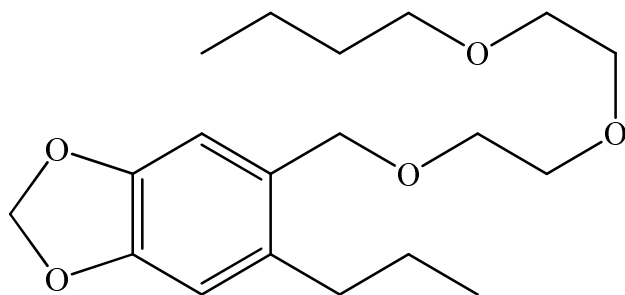
and found that PBO reduced the pyrethroid-resistance dramatically in resistant strains, but showed no synergistic effect in the highly susceptible strain.

It is well established that PBO, containing a methylenedioxyphenyl and a polyalkoxy side chain as synergophoric groups, is an effective synergist due to its ability to inhibit the monooxygenase activity of cytochrome P450s, the detoxifying enzymes in target organisms (Casida, 1970; Jao *et al.*, 1974). In recent years, PBO has been flagged for toxicological concerns and although it is still registered, it may be removed entirely in the future. Due to the suspected sub-acute and chronic toxicity of PBO (Sarles *et al.*, 1949; Bond *et al.*, 1973; Fujitani *et al.*, 1992, 1993; Tanaka *et al.*, 2009; Kawai *et al.*, 2009), there has been mounting commercial interest in alternative synergists. One of these alternatives is the plant based insecticide synergist -dillapiol (5-allyl 6,7-dimethoxy-1,3-benzodioxole) and its derivatives.

The phenylpropanoid dillapiol, which was first found in the Indian dill, *Anethum sowa*, is a natural synergist which is nearly as effective as PBO as a pyrethrin synergist. Over the years, dill seed oil has been produced using various conventional techniques. It can be obtained by steam or hydro distillation of the aerial parts and it also can be obtained in relatively pure form by using supercritical carbon dioxide process technology or by fractional high vacuum distillation (Maia *et al.*, 1998; Nautiyal *et al.*, 2011).



Chemical Formula: C<sub>12</sub>H<sub>14</sub>O<sub>4</sub>  
Molecular Weight: 222.24



Chemical Formula: C<sub>19</sub>H<sub>30</sub>O<sub>5</sub>  
Molecular Weight: 338.44

Fig. 1-2 Molecular structure of dillapiol (Top) and PBO (Bottom)

### *Botanical Sources of Dillapiol*

Dillapiol is a potent naturally occurring synergist which has been found in a variety of tropical plant species from 6 different families (Table1-1). Two species of Lauraceae, *Ocotea cymbarum* and *Aniba*, were determined to contain dillapiol (Mahran *et al.*, 1992; Silvia *et al.*, 1982). A well-known species of the Apiaceae family is the Indian dill plant, *Anethum sowa*, which was the first Indian species in which dillapiol was found with a concentration in the essential oil up to 35% (Tomar *et al.*, 1979; Nautiyal *et al.*, 2011). More than eight other species, belonging to eight genera, were found to contain dillapiol in the essential oil that ranged from 0.2% to 65%. (Fatope *et al.*, 2006; Tsyganov *et al.*, 2007; Singh *et al.*, 2010; Marongiu *et al.*, 2007; Pateira *et al.*, 1999; Velasco-Negueruela *et al.*, 2003; Jabrane *et al.*, 2009, 2010; Jassbi *et al.*, 2005; Gross *et al.*, 2009).

In the Piperaceae, the genera *Piper* and *Peperomia* produce dillapiol. The genus *Piper* is an important source of secondary metabolites with insecticidal activity. Dillapiol has been detected in about ten species including *P. permucronatum*, *P. hostmanianum*, *P. guineense*, and *P. aduncum* etc. collected from Brazil, Malaysia, Cuba, Costa Rica and other areas in the world. Only one species of *Peperomia*, *P. pellucida*, produces dillapiol identified in the oil (Silva *et al.*, 1999).

*Protium* is the principal genus in the Burseraceae family. The essential oils obtained by steam distillation (leaves and resin) from *Protium* species were analyzed to show that the resin oil of *P. heptaphyllum* March consisted mainly of monoterpenes and phenylpropanoids, including dillapiol (Siani *et al.*, 1999).

Zouari *et al.* (2011) studied *Malva aegyptiaca* (Malvales: Malvaceae) and the results indicated that dillapiol was the major compound (55.15%) in the essential oils. Greger *et al.* (1981) and Soerensen *et al.* (1969) reported that dillapiol was found in several species of the *Asteraceae* including *Artemisia* spp. and *Erigeron* spp.

Table 1-1 Summary of species in which dillapiol was detected.

Family	Genus	Species	Collection area (organs)	Dillapiol Content	Reference	
Piperaceae	<i>Piper</i>	<i>P. permucronatum</i>	Brazil (leaves)	54.7%	Morais <i>et al.</i> , 2007	
		<i>P. hostmanianum</i>		7.66%		
		<i>P. guineense</i>	S. Tome e PrôÂncipe (leaves)	44.8%	Martin <i>et al.</i> , 1998	
		<i>P. aduncum</i>	Malaysia (leaves)	64.5%	Jantan <i>et al.</i> , 1994	
			Costa Rica (leaves and branches)	61.8%	Ciccio <i>et al.</i> , 1997	
			Cuba (leave)	82.2%	Pino <i>et al.</i> , 2004	
			North Brazil (leavea and branches)	30-90%	Almeida <i>et al.</i> , 2009	
			Fiji (leaves and branches)	58%	Maia <i>et al.</i> , 1998	
		<i>P. banksii</i>	Queensland (leaves)	Detected	Loder <i>et al.</i> , 1972	
		<i>p. hispidun</i>	Jamaica (fruits)	Detected	Burke <i>et al.</i> , 1986	
		<i>P. marginatum</i>	Jamaica (fruits)	Detected		
		<i>P. novae-hollandiae</i>	Jamaica (fruits)	Detected		
		<i>P. umbellatum</i>	Jamaica (leaves)	Isolated		Bernhard <i>et al.</i> , 1978
		<i>Peperomia</i>	<i>P. pellucida</i>	Brazil (leave and branches)	39.7%	Silva <i>et al.</i> , 1999
		Lauraceae	<i>Ocotea</i>	<i>O. cymbarum</i>	Amazonas (leaves)	Isolated
<i>Aniba</i>	<i>A. ferra</i>		Amazonas (wood)	Isolated	Dias <i>et al.</i> , 1982	
<i>Anethum</i>	<i>A. sowa</i>		India (seeds)	34.05%	Nautiyal <i>et al.</i> , 2011	
Apiaceae	<i>Carum</i>	<i>C. bulbocastanum</i>	India (fruits)	44.6%	Singh <i>et al.</i> , 2010	
	<i>Crithmum</i>	<i>C. maritimum</i>	Portugal (leaves)	0.2%	Marongiu <i>et al.</i> , 2007	
			Sardinia Island (leaves)	64.2%		
	<i>Ruthiopsis</i>	<i>R. herbanica</i>	Spain (leaves and branches)	21.3%	Velasco - Negueruela <i>et al.</i> , 2003	
	<i>Ridolfia</i>	<i>R. segetum</i>	Tunisia (roots)	47.4%	Jabrane <i>et al.</i> , 2010	
	<i>Bunium</i>	<i>B. caroides</i>	Iran (leaves and branches)	10.2%	Jassbi <i>et al.</i> , 2005	
	<i>Daucus</i>	<i>D. carota</i>	Tunisia (roots)	46.6%	Jabrane <i>et al.</i> , 2009	
	<i>Foeniculum</i>	<i>F. vulgare</i>	Israel (leaves and branches)	65%	Gross <i>et al.</i> , 2009	
Burseraceae	<i>Protium</i>	<i>P. heptaphyllum</i>	Amazonas (resins)	16%	Siani <i>et al.</i> , 1999	
Malvaceae	<i>Malva</i>	<i>M. aegyptiaca</i>	Tunisia(leaves and branchse)	55.15%	Zouari <i>et al.</i> , 2011	
Asteraceae	<i>Artemisia</i>	<i>A. absinthium</i>	Vienna (seeds)	Detected	Greger <i>et al.</i> , 1981	
	<i>Erigeron</i>		Tasmania (not available)	Detected	Soerensen <i>et al.</i> , 1969	

## *Bioactivity of Dillapiol*

### a) Pesticidal Activity (Table 1-2)

Bernard *et al.* (1989) investigated the *in vivo* activity of dillapiol on the gut microsomal polysubstrate monooxygenases (PSMOs) in the European corn borer (ECB), *Ostrinia nubilalis* and found that dillapiol was one of the most potent inhibitors of epoxidase activity, showing 50%-75% inhibition. As a single additive in meridic diet (100 µg/g), dillapiol can also cause acute toxicity in ECB larvae and pupae, leading to a cumulative mortality of 92- 95% and inhibiting their growth (Bernard *et al.*, 1990). Insecticidal and growth-reducing properties of dillapiol against mosquito larvae were investigated and the result showed that dillapiol caused 92% mortality of mosquito larvae at 0.1 ppm (Bernard *et al.*, 1995).

The 96% larvicide mortality of the mosquitoes *Anopheles marajoara* and *Aedes aegypti* (malaria and dengue vectors, resp.) was observed when treated with essential oil of *P. aduncum* (Almeida *et al.*, 2009). The LC<sub>50</sub> for both mosquito larvae species was 52.7 and 42.9 ppm for 24 and 48 h. The insecticidal activity of dillapiol against adult mosquitoes resulted in 100% insect mortality at the concentration of 100 ppm for 30 min with aerosol application. The essential oil from *P. aduncum* also produced 100% mortality in yellow mealworm *Tenebrio molitor* larvae (Fazolin *et al.*, 2007) and in adult cowpea weevils *Callosobruchus maculatus* (Pereira, 2006).

Fungicidal activity of dillapiol against *Clinipellis pernicioso* (witches' broom) was detected by Almeida *et al.* (2009) who showed that the basidiospores were completely inhibited at concentrations above 0.6 ppm. The action of dillapiol resulted in the emergence of plasmolized basidiospores.

The antifungal activity of dill oil in which dillapiol was a major component inhibited the growth of *Candida albicans* and some molds that cause crop or food damage but demonstrated

weak contact toxicity to aphids (Majekodunmi *et al.*, 2006). An evaluation of the anti-acarid effect of essential oil (95% dillapiol, hydro-distilled from *P. aduncum*) determined that 100% mortality of *Rhipicephalus (Boophilus) microplus* larvae was caused by 0.1 mg/ml (Silva *et al.*, 2009).

b) Synergistic Activity (Table 1-3)

The methylenedioxyphenyl (MDP) group is the functionality responsible, in part, for the ability of dillapiol to act as a potent synergist. In this regard, dillapiol has been shown to function by interacting with the cytochrome P450 thus inhibiting the activity of many polysubstrate monooxygenases (PSMOs) responsible for metabolism of toxins in insects (Bernard *et al.*, 1989; Belzile *et al.*, 2000). Dillapiol can synergize not only natural insecticides, for instance, pyrethrum (Mukerjee *et al.*, 1979), azadirachtin (Bertrand, 1992), and tenulin (Bernard *et al.*, 1990) but also synthetic insecticides, including synthetic pyrethroids, several carbamates and organochlorines (Handa *et al.*, 1974; Mukerjee *et al.*, 1979; Parmar *et al.*, 1983).

Bernard *et al.* (1990) reported that mixtures of dillapiol with either phenylheptatriyne (PHT), tenulin or both were significantly less toxic to ECB than dillapiol alone but the insecticidal activity of these allelochemicals was greatly enhanced by it. The reduction of growth of ECB larvae reared on diets containing dillapiol ranged from 58% to 75% of controls. The study of the synergistic activity of dillapiol and its derivatives combined with the phototoxin  $\alpha$ -terthienyl found that the synergistic activities varied between 0.9-2.4 against mosquito larvae *Aedes atropalpus* in a 1:5 insecticide/synergist ratio (Belzile *et al.* 2000).

Table 1-2: The pesticidal activity of dillapiol and its derivatives

	Species	Concentration	Duration	Activity	Reference
	<i>Aedes atropalpus</i> .	0.1 ppm	24 h	92% mortality	Bernard <i>et al.</i> , 1996
	<i>Ostrinia nubilalis</i>	100 mg g <sup>-1</sup>	35 d	92-95% mortality	Bernard <i>et al.</i> , 1990
Insect	<i>Aedes aegypti</i>	10-500 mg mL <sup>-1</sup>	24 h	LC <sub>50</sub> : 365 mg mL <sup>-1</sup>	Morais <i>et al.</i> , 2007
	<i>Anopheles marajoara</i>	100 ppm	48 h (larvae)	100% mortality	Almeida <i>et al.</i> , 2009
	<i>Aedes aegypti</i>		30 min (adult)	100% mortality	
Fungus	<i>Clinipellis perniciosa</i>	0.6-1 ppm	24 h	100% basidiospores inhibition	
Mite	<i>Rhipicephalus microplus</i>	0.1 mg mL <sup>-1</sup>	24 h	100% mortality	Silva <i>et al.</i> , 2009

Table 1-3 The synergistic activity of dillapiol and its derivatives

Name of Insect	Insecticide	Insecticide Concentration	Synergist Ratio	Duration	Factor of Synergism	Reference
<i>Aedes atropalpus</i>	alpha-terthienyl	LC <sub>50</sub> : 0.006 ppm	5:1	4 h: light 20 h: dark	6	Belzile <i>et al.</i> , 2000
	piperine	LC <sub>50</sub> : 8.6 ppm		24 h	2.6	
	asimicin	LC <sub>50</sub> : 0.0012 ppm		24 h	2.7	
	rotenone	LC <sub>50</sub> : 0.016 ppm		24 h	3.7	
<i>Ostrinia nubilalis</i>	tenulin	1000 µg g <sup>-1</sup>	10:1	35 d	2.1	Bernard <i>et al.</i> , 1990
	PHT*	100 µg g <sup>-1</sup>	1:1	35 d	1.25	
	<i>Cedrela odorata</i>	0.03%	0.6:1-1:1	11 d	significant	Scott <i>et al.</i> , 2004
<i>Drosophila melanogaster</i>	pyrethrum	0.01- 0.16 mg mL <sup>-1</sup>	1:10-11.6:1	24 h	11.6	Jensen <i>et al.</i> , 2006
<i>Tribolium castaneum</i>	pyrethrum	N/A	1:5	N/A	2.3-4	Mukerjee <i>et al.</i> , 1982
	carbaryl	N/A	1:5	N/A	4.6	Tomar <i>et al.</i> , 1979
<i>Choristoneura rosaceana</i>	tansy oil	0.1%-10%	10:1	24 h	1.6	Larocque <i>et al.</i> , 1999
<i>Helicoverpa armigera</i>	deltamethrin	LD <sub>50</sub> :1.08 µg larvae <sup>-1</sup>	1:5	72 h	18.6	Ashraf <i>et al.</i> , 2010

\* phenylheptatriyne

A synergistic phagostimulant effect of dillapiol toward an insect species was reported by Larocque *et al.* (1999), who showed that the presence of dillapiol with tansy essential oil can increase the feeding activity of the oblique-banded leafroller (OBLR), *Choristoneura rosaceana* (Harris) (Lepidoptera: Tortricidae) larvae compared to the control. If confirmed, dillapiol could be mixed with natural insecticides such as *Bacillus thuringiensis* Berl. to increase ingestion.

c) Therapeutic Activity (Table 1-4)

Good anti-inflammatory activity (17%) of dillapiol against rat paw edema was shown to be comparable with indomethacin (38%) (Parise-Filho *et al.*, 2011). Dillapiol is also promising for the use in combination with many anticancer drugs, because it markedly decreases the cell resistance against antimetabolic drugs (Konyushkin *et al.*, 2010). According to the study by Tsygenov *et al.* (2007), isoapiol and isodillapiol exhibit antiproliferative activity, i.e., arrest the cell division of sea urchin embryo at concentrations of 5-20  $\mu\text{mol L}^{-1}$ . The oil of *Crithmum maritimum* L, which contains dillapiol (64.2%), produced the most active antifungal activity against dermatophyte strains with the minimal inhibitory concentration values ranging from 0.08-0.32  $\mu\text{L mL}^{-1}$  (Marongiu *et al.*, 2007).

The antibacterial property of dillapiol were tested with root oil from *Ridolfia segetum* which was dominated by dillapiol (47.4%) and revealed that *R. segetum* root oil exerted a significant antibacterial activity (Jabrane *et al.*, 2009).

Table 1-4 The therapeutic activity of dillapiol

Bioactivity	Target	Result	Duration	Reference
Anti-inflammatory	Mouse edema	20-35% reduction at 50 mg kg <sup>-1</sup> (body weight)	120 min	Roberto <i>et al.</i> , 2011
Antibacterial	Common human pathogenic bacteria	MIC: 1.25-5 mg mL <sup>-1</sup>	24 h	Jabrane <i>et al.</i> , 2009
Antimicrobial	Additional 14 species <sup>a</sup>	MIC: 1.25-5 mg mL <sup>-1</sup>	24 h	Jabrane <i>et al.</i> , 2010
Antifungal	<i>Candida albicans</i>	MIC: 32 µg mL <sup>-1</sup>	24 h	Majekodunmi <i>et al.</i> , 2006
Antimitotic activity	Sea urchin embryos	EC <sub>50</sub> : 1 µmole.L <sup>-1</sup>	—	Konyushkin <i>et al.</i> , 2010
Antiproliferative activity	Sea urchin embryos	5-20 µmole.L <sup>-1</sup>	—	Tsyganov <i>et al.</i> , 2007

<sup>a</sup> include *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *S. aureus*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Streptococcus pneumoniae*, *Salmonella typhimurium* *Shigella* spp., *E. coli* and *E. faecalis*, and environmental strains *P. aeruginosa* and *S. aureus*.

## 1.4 Pesticide synergists as a tool to control pesticide resistance

Synergists have been used commercially for more than 60 years in agriculture and have contributed significantly to improve the efficiency of insecticides by broadening their bioactivity spectrum, countering resistance development, increasing effective commercial lives, and mitigating the residual effects of persistent and highly toxic products by reducing the application dose. The development of those compounds grew out of a need in the late 1930s and early 1940s for extending the usefulness of the naturally derived insecticide pyrethrum, which was considered a strategic insecticide against mosquitoes and other disease-carrying insects (Wachs, 1947). Chemicals that were developed had little intrinsic pesticidal activity of their own; however, they did increase the effectiveness of a given dose of pyrethrins and were thus called synergists, such as sesamin, sesamol, safrole and so on. With the development of medicinal chemistry, various kinds of synergist have been found or synthesized.

Generally, about 4 different kinds of compounds can act as insecticidal synergists including methylenedioxyphenyl (MDP) compounds, such as sesamin, dillapiol, and PBO (Fig. 1-3); N-alkyl compounds, such as SKF525A and MGK264 (Fig. 1-4 A); propargyl, for example, RO5-8019 and verbutin (Fig. 1-4 B), and other compounds, like avidin and N-ethylmaleimide. The major kind is the MDP compounds including many naturally occurring lignans, such as safrole, sesamin and dillapiol, as well as semi-synthetic ones, for example, PBO, which is the most commonly used synergist in today's markets.

a) Naturally occurring compounds

b) Semi-synthetic compounds

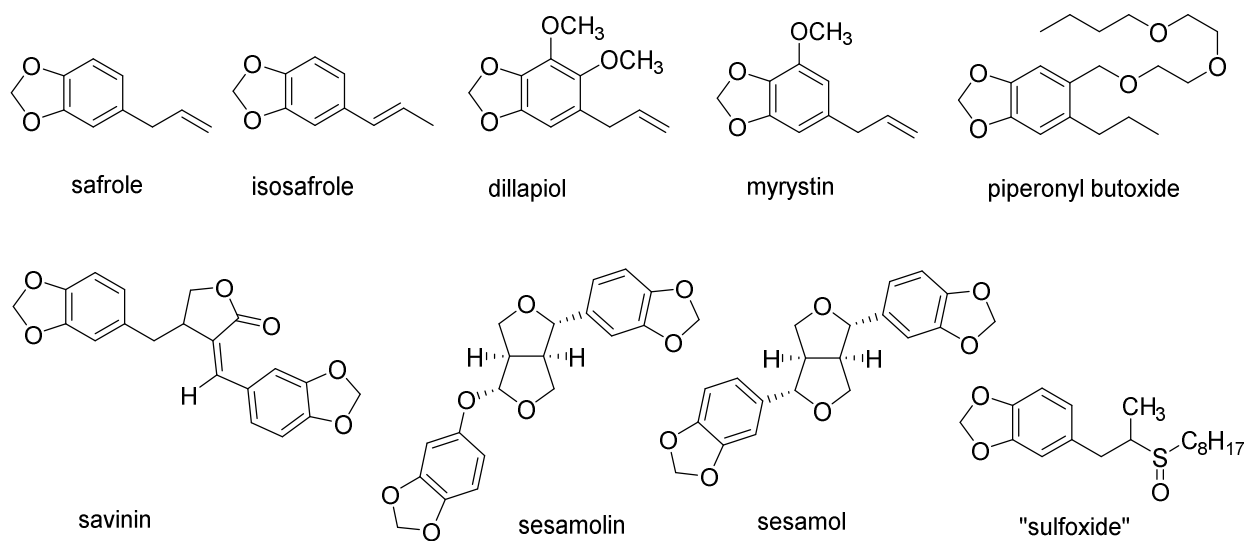
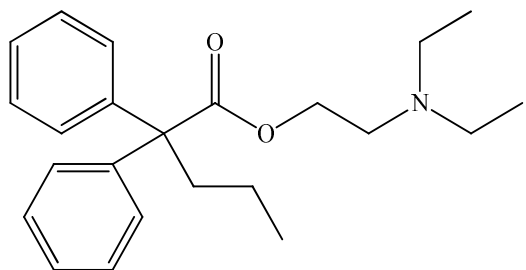
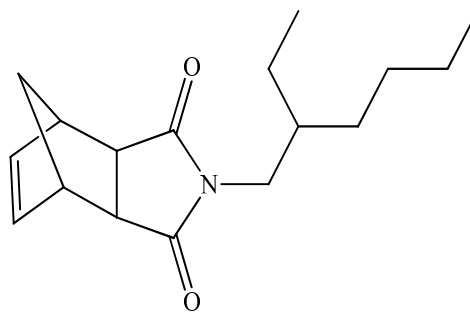


Fig. 1-3 Examples of natural (a) and semi-synthetic (b) MDP compounds as synergists

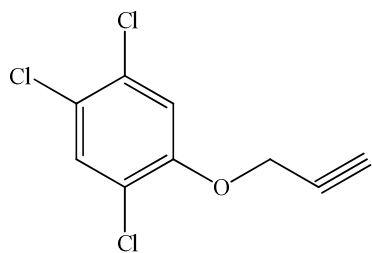


SKF 525A

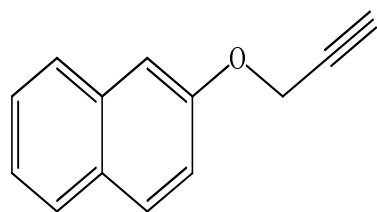


MGK264

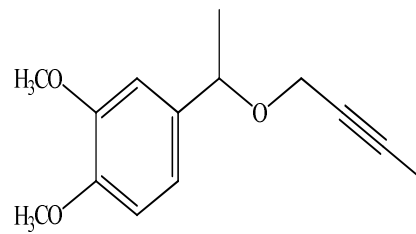
A



RO 5-8019



Naphthyl propynyl ether



Verbutin

B

Fig. 1-4 N-alkyl Ccompounds (A) and Propargyl ethers (B) as insecticide synergist.

The main function of a pesticide synergist is to enhance the efficiency of pesticide. In 1942, the structure and synergistic effects of many compounds were tested by Haller *et al.* (1942). The results indicated that several compounds were synergistic and the authors noted the similarity of chemical structure in the presence of MDP groups. Unfortunately, these products had low solubility in petroleum solvents and were not exploited commercially.

To develop commercial synergists, various compounds with the MDP group in their structure were tested and confirmed for their synergistic insecticidal activity (Casida *et al.*, 1970; Mukerjee *et al.*, 1979; Bernard *et al.*, 1989; Almeida *et al.*, 2009; Khot *et al.*, 2008), and the most successfully developed one is piperonyl butoxide (PBO). Today, it is used in many pesticides on the market as a powerful synergist for control of resistant pest populations. Appearing in over 1500 U.S. Environmental Protection Agency (U.S. EPA)-registered products, PBO is one of the most commonly registered pesticides in terms of the number of formulas in which it is present (Krieger, 2010).

#### *Mode of action of insecticide synergists*

Many xenobiotics, including insecticides, are detoxified from one or more of the three major groups of enzymes: microsomal oxidases, esterases, and glutathione-S-transferases (GST). The synergistic activity of different synergists was related to the direct inhibition effect of these synergists on the metabolic enzymes in the insects.

#### *Effect on Poly-substrate Monooxygenases*

The P450 monooxygenases are a large and functionally diverse family of enzymes that carry out the initial oxidation of a wide variety of lipophilic compounds. A major function of cytochrome P450 in insects is that they are key mediators of the hydroxylation and epoxidations

required for the efficient destruction and elimination of xenobiotics, including insecticides, before they are absorbed in the gut tissue. Two different mechanisms involved in the biochemical reaction of P450s could result in insecticide resistance development:

1. Over expression of P450 genes: Several research studies revealed that the over expression of multiple P450 genes in insecticide resistant strains is a common phenomenon, and the interaction of multiple insecticide resistance P450 genes may be responsible for the development of resistance (Liu *et al.* 2015). For example, CYP6D1 is responsible for monooxygenase-mediated resistance to pyrethroid insecticides in the housefly, and CYP6D1 is ubiquitously expressed in adults with 10-fold higher levels found in the resistant strain compared to susceptible strains (Liu *et al.*, 1998).
2. Gene induction: Gene induction refers to the induced expression of some genes by exogenous and endogenous compounds. The induction of P450s and their increased activity showed the possibility that induction is involved in the adaptation of insects to the the detoxification of insecticides and the development of insecticide resistance (Terriere, 1984; Poupardin *et al.*, 2008; Scharf *et al.*, 2001; Pridgeon *et al.*, 2002).

The effective cytochrome P450 inhibitor is regarded as the major approach to confront insecticide resistance in insect pests. Perry *et al.* (1970) and Matthews *et al.* (1970) first demonstrated that the moiety of methylenedioxyphenyl (MDP) group interacts with the P450 monooxygenase oxidase system in a manner not explained by a typical enzyme-substrate relationship using house flies. MDP compounds referred to a group of chemicals such as PBO, dillapiol or other MDP moiety related secondary compounds. These studies demonstrated an apparent reduction in microsomal P450 levels prepared from insects treated *in vivo* with PBO.

Many study results revealed that MDPs can interact with cytochrome P450 to develop either competitive or non-competitive inhibition and inhibit the activity of the poly-substrate monooxygenases (Dahl *et al.*, 1985; Delaforge *et al.*, 1985; Franklin, 1976; Hodgson *et al.*, 1973; Murray *et al.*, 1989), thereby reducing the oxidative breakdown of other pesticides such as pyrethrum and the synthetic pyrethroids (Casida, 1970). For example, MDP compounds such as PBO have been used extensively as synergists with the pyrethroid and carbamate pesticides, groups of pesticides whose detoxifying metabolism in insects is P450-mediated (Haley, 1978). The P450-MDP metabolite complex formed during reaction is extremely stable, since even in sub-cellular microsomal preparations the enzyme remains inhibited, and the effects of *in vivo* interaction can be demonstrated *in vitro* (Raffa *et al.*, 1985) (Fig. 1-5).

It has also been known that some MDP compounds can act as both inhibitors and inducers of P450 activity in mammals as well as in insects (Hodgson *et al.*, 1974; Adams *et al.*, 1995; Marcus *et al.*, 1986; Kinsler *et al.*, 1990). The biphasic effect of MDP compounds in insects was tested by Marcus *et al.* (1986) who reported the increase of CYP450 levels in preparations of midgut microsomes of sixth-instar southern armyworm larvae (*Spodoptera eridania*) which were exposed to each of five MDP compounds. Isosafrole and the 4-bromo-S-methoxy derivative are particularly effective inducers, which cause the largest increases in CYP-450 (1.8-fold).

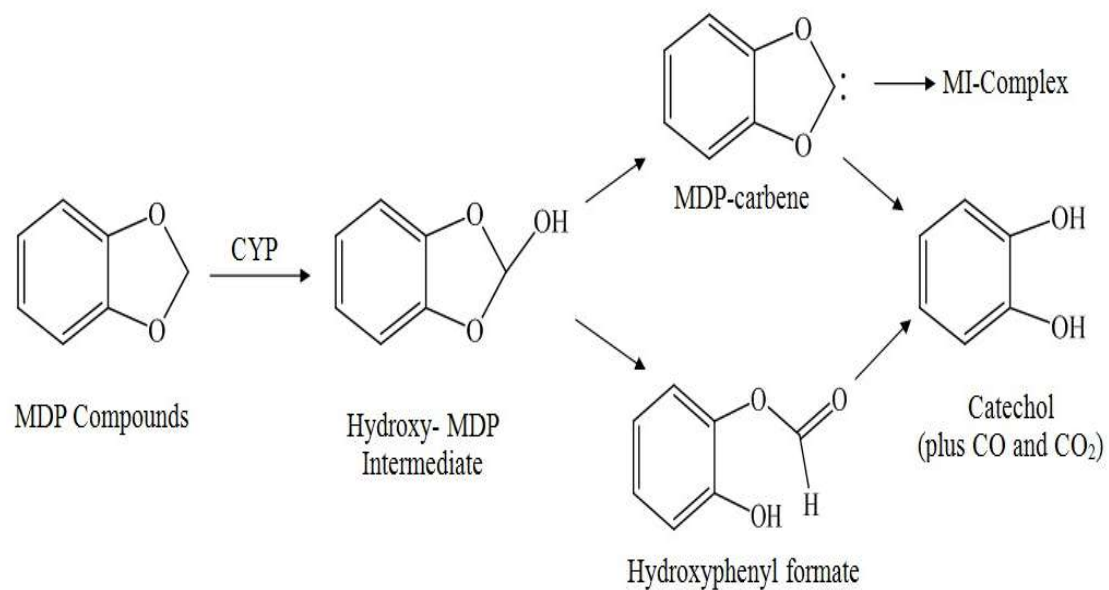


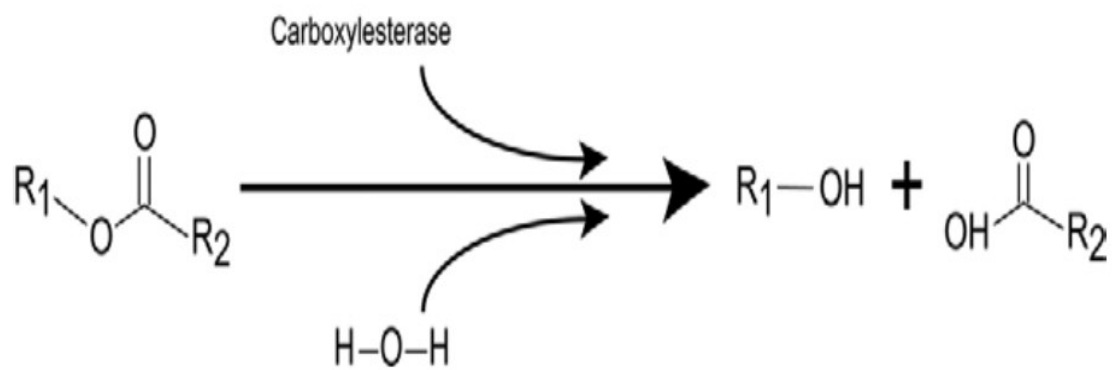
Fig. 1-5 Oxidation of MDP compounds at the methylenedioxy carbon to form the hydroxy-MDP intermediate that can generate the MDP-carbene resulting in the formation of the inhibitory MI-complex. Alternately, hydroxy-MDP may be hydrolysed to the hydroxyphenyl formate derivative that decomposes to the catechol, releasing in the process carbon monoxide and carbon dioxide. (Adopted from Ioannides, 2008)

### *Effect on Esterases*

The esterases are a very large class of enzymes which include three groups: arylersterases (E.C.3.1.1.2.), cholinesterases (E.C.3.1.1.8.), and carboxylesterases (E.C. 3.1.1.1.). Carboxylesterases, most esterases belonging to (Punta *et al.* 2012), are one of the most common proteins found in nature (Ollis *et al.* 1992, Hotelier *et al.* 2004) and are capable of hydrolysing a wide range of substrates (Scheme 1-1).

Esterases involved in insecticide metabolism include carboxyl esterases, phosphotriester hydrolases, carboxylamidases and epoxide hydrolases. These enzyme systems can confer significant levels of resistance to many diverse groups of insecticide, including organophosphates (OPs), carbamates and pyrethroids.

The role of esterase in pyrethroid resistance was studied in the final larval instar of different strains of the cotton bollworm by El-Latif *et al.* (2010). Native polyacrylamide gel electrophoresis (PAGE) displayed important differences in the midgut esterase isozyme pattern between the susceptible and the pyrethroid-resistant strains. Out of the 10 esterase isozymes observed, the susceptible strain lacked three bands, E2, E6 and E10 that were found in the resistant strains. An *in vivo* inhibition study was conducted by topical application of PBO and followed by biochemical analysis. The native PAGE revealed the E6 band which is present in both resistant strains. It disappeared at about 4 h post treatment but was present again at about 10h post treatment which indicated that PBO-esterase binding is a rather slow and non permanent process. This native PAGE also revealed that the *in vivo* esterase inhibition caused by PBO was due to the binding of the synergist with the E6 isozyme which was not present in the susceptible strain.



Scheme 1-1 Basic carboxylesterase hydrolysis reaction. (Adopted from Montella *et al.* 2012)

The same result was obtained from a study conducted by Khot *et al.* (2008). In the peach-potato aphid, enzyme purification studies demonstrated that a single esterase isozyme (E4) was highly over expressed in organophosphates (OP)-resistant strains. E4 isozyme was purified from the resistant strains and AChE was used as the reference. In the "esterase interference" assay, E4 and E4 plus PBO are incubated with a serial dilution of azamethiphos. AChE was then added to aliquots and following a short incubation, the AChE activity was determined. The results demonstrated that there was a reduction in the ability of E4 to protect the AChE from azamethiphos when the E4 had been pre-incubated with PBO. This indicated that PBO blocked the isozyme E4.

#### *Effect on Glutathione S-transferase*

Glutathione S-transferases (GSTs) are one of the major families of detoxification enzymes. In insects, there are two types of GSTs which are classified according to their location within the cell: microsomal and cytosolic. The microsomal GSTs are trimeric, membrane-bound proteins, which have not been implicated in the metabolism of insecticides. The majority GSTs of insects are soluble dimeric proteins found in the cytosol. Insect cytosolic GSTs have been assigned to at least seven classes: Delta, Epsilon, Mega, Sigma, Theta, Zeta (Chelvanayagam *et al.*, 2001; Ranson *et al.*, 2001) and unclassified (Ding *et al.*, 2003). The members of the Delta and Epsilon classes are insect-specific and believed to be the most important in detoxification and insecticide resistance (Hemingway and Ranson, 2000).

Elevated GST activity has been associated with resistance to all the major classes of insecticides (Prapanthadara *et al.*, 1993, Huang *et al.*, 1998; Vontas *et al.*, 2001). In many cases, the individual GST enzymes involved in resistance have been identified and GSTs have been

implicated by association only, i.e. an increase in GST activity, detected using model substrates, in insecticide-resistant strains of insect vs. their susceptible counterparts.

Wang *et al* (2014) detected significant elevated GST activity in an insecticide-resistant strain of Colorado potato beetle, and identified two compounds which effectively inhibited GST activity. One is a flavonoid, taxifolin, extracted from *Picea mariana* Mill. (Pinales: Pinaceae) and *Picea banksiana* Lambs. cones, and *Larix laricina* Du Roi (Pinales: Pinaceae) bark; another compound was a lignan, (+)-lariciresinol 9-p-coumarate, obtained from *P.mariana* cones, and *L. laricina* and *Abies balsamea* L. (Pinales: Pinaceae) bark. By contrast, the investigation of the effect of synergists in another insect GST has shown different results (Willoughby *et al.*, 2007). The *Drosophila melanogaster* genome with PBO treatment was studied and a subset of GST genes was identified. After 4 h of exposure to PBO, five out of 37 GST genes were induced. The increased production of GST enzymes by PBO exposure has the potential to increase insecticide tolerance if these enzymes are capable of insecticide metabolism.

## 1.5 Rationale and objective

In previous work from our lab, dillapiol was analyzed for its *in vitro* inhibition of human cytochrome P450 3A4 (CYP3A4) via a fluorometric microtitre plate assay. This is a relatively high throughput assay facilitated by a commercially available cloned enzyme. The results indicated that dillapiol was a potent inhibitor at 23.3 times higher the concentration of the positive control CYP3A4 inhibitor ketoconazole (Budzinski *et al.*, 2000). Recently our collaborating medicinal chemistry research group (Durst lab) has successfully synthesized a set of fifty dillapiol analogs which were made available for the present study. A first step in this thesis was to evaluate the CYP3A4 inhibition of the analogs in this very reproducible and well controlled

assay. Based on the *in vitro* results, my next step was to develop Structure Activity Relationships (SAR) to predict features of the most active inhibitors. Since dillapiol was also reported to be as effective as PBO as a pyrethrin synergist, the dillapiol-analogs were evaluated to determine if the analogs can successfully cause synergism *in vivo* with insects.

Therefore, the overall objective of this thesis was to find novel, highly active synergists based on dillapiol analogs for insect pest control and possibly selected biopharmaceutical applications, and to develop an understanding of their mode of action. The influence of selected derivatives of dillapiol on two insect pests, ECB, and CPB, was studied. The specific objectives for the study were:

Objective 1. The inhibition of human CYP 3A4 in a rapid screening assay was used for high throughput assessment of P450 inhibition of available dillapiol analogs in order to identify potential candidate synergists based on their inhibition of monooxygenase activity; Based on CYP3A4 assay results, Gaussian modeling techniques were used to develop a new quantitative structure-activity relationship model (QSAR).

Objective 2. The topical and ingestion assays were conducted to evaluate the synergistic activity of selected dillapiol analogs combined with pyrethrum with the oligophagous Colorado potato beetle (CPB). Greenhouse or field applications to potato plants were undertaken in order to assess the activity of the original essential oil as an organic synergist for pyrethrum. Since resistant Colorado potato beetle more rapidly metabolizes insecticides compared to the susceptible strain, it was hypothesized that dillapiol analogs would show greater synergistic activity with the resistant strain than with susceptible ones.

Objective 3. ECB larvae were employed as a lepidopteran insect model to evaluate 1) the synergistic activity of six selected dillapiol analogs with pyrethrum in comparison to the synergists PBO, and dillapiol; 2) the intrinsic toxicity of the synergists PBO, dillapiol and the most active ester and ether analogs with an ECB growth inhibition bioassay.

Objective 4. The mode of action of selected dillapiol analogs on the detoxification enzyme system of oligophagous and polyphagous insects was undertaken to determine underlying mechanisms of synergism in insects.

Objective 5. A pilot study was conducted to determine the dysregulation of insect metabolism of the insecticide alone and in combination with dillapiol analogs in resistant and susceptible Colorado potato beetle, and European corn borer by ultra high performance chromatography quadrupole time-of-flight mass spectrometry (UPLC- QTOF/MS).

## **CHAPTER 2 INHIBITION OF CYTOCHROME 3A4 BY DILLAPIOL, ITS ANALOGS AND THEIR QSAR RELATIONSHIP**

### Preface

The major goal of the present chapter was to screen active CYP3A4 inhibitors of semi-synthesized dillapiol analogs, and construct a 3D-QSAR model with available IC<sub>50</sub> data in order to predict molecular structures with high cytochrome P450 inhibition activity. The work involved equal contributions by three authors. PhD student Ana Francis Carballo from Professor Durst's medicinal chemistry lab synthesised all tested compounds; I conducted all CYP3A4 assays with analogs, verified the data and obtained accurate IC<sub>50</sub> data which was employed to construct a 3D-QSAR model by Nathalie Pineda and Professor Cesar Compadre at the University of Arkansas. While this chapter does not include entomological studies, the interdisciplinary work achieved here were essential to the future entomological research in chapters 3 to 6.

### 2.1 Introduction

Cytochrome P450 3A4 (CYP3A4), the major form of P450 expressed in adult human liver (Shimada *et al.*, 1994), comprises about 30% of the total hepatic P450 content, and is involved in the metabolism of about 50% of commonly prescribed drugs (Guengerich, 1999). Compounds that inhibit CYP3A4 could significantly increase plasma levels of administered drugs and improve clinical efficacy of therapeutics. Studies have shown that furanocoumarin derivatives identified from grapefruit (*Citrus paradisi* Macfad) juice strongly inhibit the catalytic activity of CYP3A4, and the two prominent furanocoumarins in the juice, bergamottin and 6',7'-

dihydroxybergamottin have been demonstrated as important mechanism-based inhibitors of CYP3A4 in the grapefruit juice (Kakar *et al.*, 2004; Paine *et al.*, 2004) .

Methylenedioxyphenyl compounds (MDPs) are an important group of phytochemicals found in many plants and oils, including sesame seeds, pepper, parsley etc. Naturally occurring MDPs can act as inhibitors of CYP enzymes. Extracts of goldenseal, *Hydrastis canadensis* L. (Ranunculales: Ranunculaceae), contain the MDP-based alkaloids hydrastine which can effectively inhibit human CYP3A4 (Chatterjee and Franklin, 2003). Two MDP-carrier lignans, (-)-clusin and (-)-dihydroclusin, isolated from the medicinal plant *Piper cubeba* L.f. (Piperales: Piperaceae), were determined as potent and selective mechanism-based inhibitors of CYP3A4 (Usia *et al.*, 2005).

As a naturally occurring MDP compound found in many plant families, dillapiol is a demonstrated CYP3A4 inhibitor by our research group (Budzinski *et al.*, 2000). When dillapiol analogs were combined with the botanical larvicide, alpha terthienyl, differences based on the QSAR modeling of toxicity and molecular structure confirmed that modifications of dillapiol leads to improved synergistic activity (Belzile *et al.*, 2000). To gain further insight into dillapiol and its analogs as pharmaco-enhancing agents, a more comprehensive series of dillapiol analogs was prepared semi-synthetically by collaborators, Ana Francis Carballo Arce Ph. D. and Professor Tony Durst (Chemistry Department, University of Ottawa), exploring a range of semi-synthetic modifications of the parent molecule.

In this chapter, the objective was to determine the inhibition of CYP P450 3A4 by a set of 52 dillapiol analogs evaluated using a highly uniform and purified cloned commercial enzyme preparation (BD Gentest, ON, Canada). The median inhibitory concentrations (IC<sub>50</sub> values) obtained from this method were predicted to allow the discovery of highly significant QSAR models using

Gaussian techniques. These models presented here were developed in collaboration with Nathalie Pineda and Professor Cesar Compadre (College of Pharmacy, University of Arkansas), and provided insight into the potent inhibitory properties of the molecule.

To meet the submission requirement of the journal *Tetrahedron*, the compounds in the present chapter were named as number or number plus low-case letter. In order to be consistent in the following chapters, the original codes were displayed in the parentheses.

## 2.2 Materials and methods

### 2.2.1 Chemicals

Dillapiol used in the present study was obtained via steam distillation of the fruit of *Piper aduncum* L. (Piperales: Piperaceae) collected in the Sarapiquí region of Costa Rica. A typical steam distillation was used for the extraction of crude oil which was then purified by column chromatography, and the purity of dillapiol was determined by proton NMR. A typical distillation of 3 kg of fruit when steam distilled with 3 L of water yielded between 30 and 35 g (1 to 1.2%) of essential oil with more than 95% dillapiol purity. This material was considered sufficiently pure for transformation to the various intermediates and final products. Sessamol, safrol, and piperonal (Fig. 2-1) were purchased from Sigma- Aldrich (Oakville, ON, Canada) and used as starting materials for synthesis. Standard well known semi-synthetic chemical transformations were employed to produce the various analogs. The structures of the desired products were verified by their  $^1\text{H}$  and  $^{13}\text{C}$  NMR. A total of 52 analogs (Supplement I) were synthesized, and the compounds were dissolved into methanol to make a serial dilution of concentrations in order to determine the  $\text{IC}_{50}$  of each analog.

### 2.2.2 Cytochrome CYP3A4 assay

Enzyme inhibition assays were conducted with a cloned CYP3A4 isozyme (BD Gentest, ON, Canada). The method described by Budzinski *et al.* (2000) was adopted in the present study. Three different solutions were prepared: 1) a 100 mL of Solution A containing distilled water, 43 mL, NADPH ( $\beta$ -nicotinamide adenine dinucleotide phosphate) (20 mM), 6 mL, DBF (dibenzylfluorescein) (200  $\mu$ M), 1 mL and potassium phosphate buffer solution (0.5 M, pH 7.4), 50 mL 2) a 90 mL Solution B containing distilled water, 65 mL, CYP3A4, 1 mL, and a potassium phosphate buffer solution (0.5 M, pH 7.4), 24 mL; 3) a 90 mL Solution C was identical to Solution B except that the enzyme was heat denatured, and it acted as the reading background to adjust the active assay value.

Assays were performed in clear bottom, opaque-welled microtiter plates (Corning Costar, Corning, NY). Wells were designated as either "Control," "Blank," "Test," or "Test-Blank." Control wells consisted of Solution A, B and methanol; blank wells consisted of Solution A, C and methanol; test wells consisted of Solution A, B and dillapiol analogs at a particular concentration; and test-blank wells consisted of the Solution A, C and corresponding compounds. A volume of 100  $\mu$ L of solution A was added to all wells, and followed by 10  $\mu$ L of analogs solution or methanol only. A volume of 90  $\mu$ L of Solution B was added into half of the test wells, and the same volume of Solution C was added into the remaining half of the test wells. A Millipore Cytofluor 4000 Fluorescence Measurement System (Applied Biosystems, Foster City, CA) set to 485 nm excitation filter (20-nm bandwidth) was used to analyze each plate after 20 mins with endpoint reading.

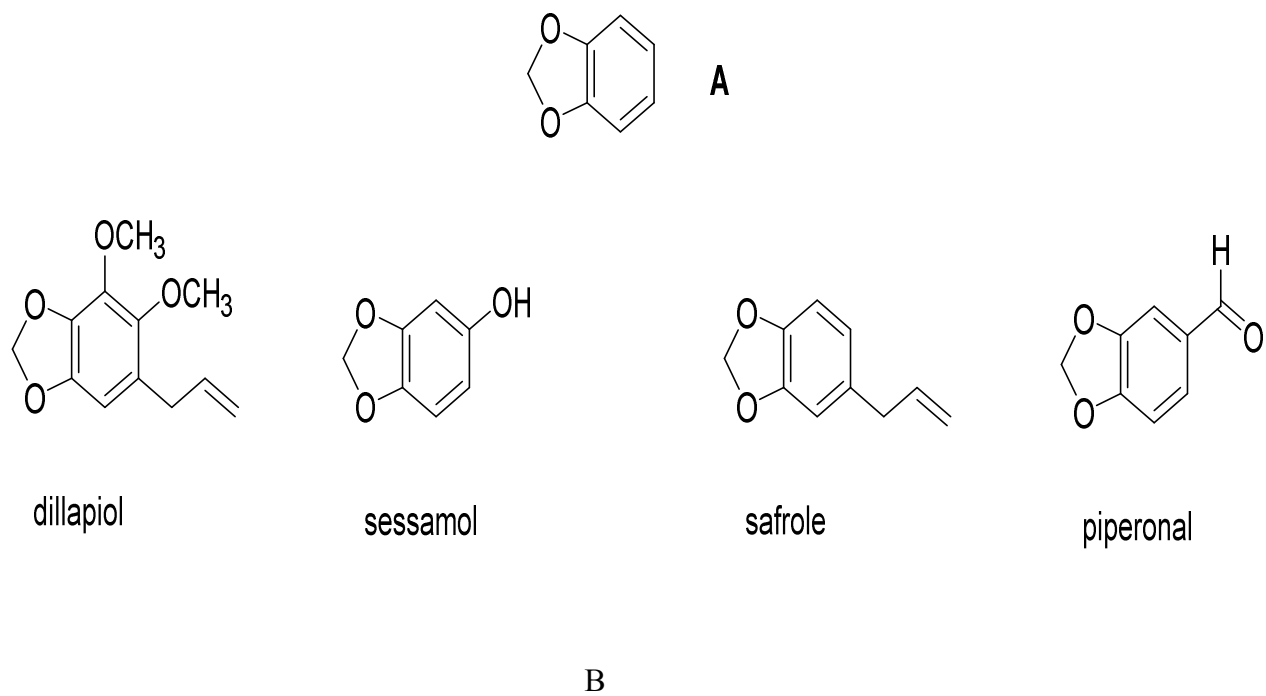


Fig. 2-1 The molecular structure of methylenedioxybenzene (MDP) moiety (A) and four compounds (B) used as the starting materials to synthesize the 52 analogs.

DBF, the substrate in the assay, contained a benzyle group on the fluorescein moiety which can be cleaved by CYPs and converted to fluorescein benzyl ester. The produced ester compound could be detected under 485 nm wavelength. When the CYP inhibitor is present, the production of fluorescein ester is blocked, therefore no fluorescence can be detected. Percent inhibition calculations were based on differences in fluorescence between the control/blank wells and test/test blank wells. All assays were performed under gold fluorescent lighting. Methanol only served as the control. Ketoconazole (Calbiochem, ON, Canada, Cat# 420600) at 10  $\mu\text{g/mL}$  was selected as the positive control.

In order to clarify the relationship between molecular structure and the  $\text{IC}_{50}$  values of CYP3A4 inhibition, analogs were selected and grouped by structural similarity based on dillapiol and sessamol as starting materials.

### 2.2.3 3D-QSAR modeling construction

#### *Dataset*

From the synthesized dillapiol and sessamol analogs a total of 47 molecules were considered to develop the Comparative Molecular Similarity Indices Analysis (CoMSIA) model. The training set included 35 molecules and the rest were considered as the testing set. For the modeling studies, the  $\text{IC}_{50}$ 's for the inhibition of cytochrome CYP450 3A4 were expressed as  $\log 1/\text{IC}_{50}$  ( $\text{pIC}_{50}$ ).

#### *Structures and molecular alignment*

Dillapiol and sessamol analogs were constructed by modifying the crystal structure of compound ACXOW obtained from the Cambridge Crystallographic Data Centre (Allen, 2002). The modifications were made using standard bonds and angles and minimized in two steps, first using the steepest descendent method and then the Powell method (Pick et al., 2008) as

implemented in the program SYBYLX2.1.1 (Certara, St. Louis, MO 63101, USA). In some cases systematic conformational analyses were performed to locate the lowest energy conformation of the molecule. Atomic charges were calculated using the semi-empirical quantum chemistry program MOPAC, also included in SYBYLX 2.1.1, using PM3 method. All the compounds were aligned using dillapiol as the reference molecule and the methylenedioxybenzene moiety (Fig 2-1 A) as the common fragment. The alignment of the training set was shown in Fig 2-2.

### *CoMSIA studies*

CoMSIA (Comparative Molecular Similarity Indices Analysis) is a form of 3D-QSAR that is based on the assumption that changes in the structure of similar molecules are related with its properties, and that these properties could be described by fields (Klebe *et al.*, 1994). CoMSIA was used to calculate the similarity index fields, the aligned molecules were placed in a 3D cubic lattice with a grid spacing of 2 Å. Steric, electrostatic and hydrophobic descriptors were generated using the  $sp^3$  atom with charge +1 and radius of 1 Å. Similarity indices ( $A_{F,K}$ ) for the molecule  $j$  and the atom  $i$  at grid point  $q$  were calculated according to:

$$A_{F,K}^q(j) = - \sum_{i=1}^n w_{probe,k} w_{i,k} e^{-\alpha r_{iq}^2}$$

Where  $W_{i,k}$  is the actual value of the physicochemical property  $k$  of atom  $i$ ;  $W_{probe,k}$  indicates the probe atom with radius 1 Å;  $\alpha$  is the attenuation factor, with a default value of 0.3;  $r_{iq}^2$  is the mutual distance between the probe atom at grid point  $q$  and atom  $i$  of the test molecule.

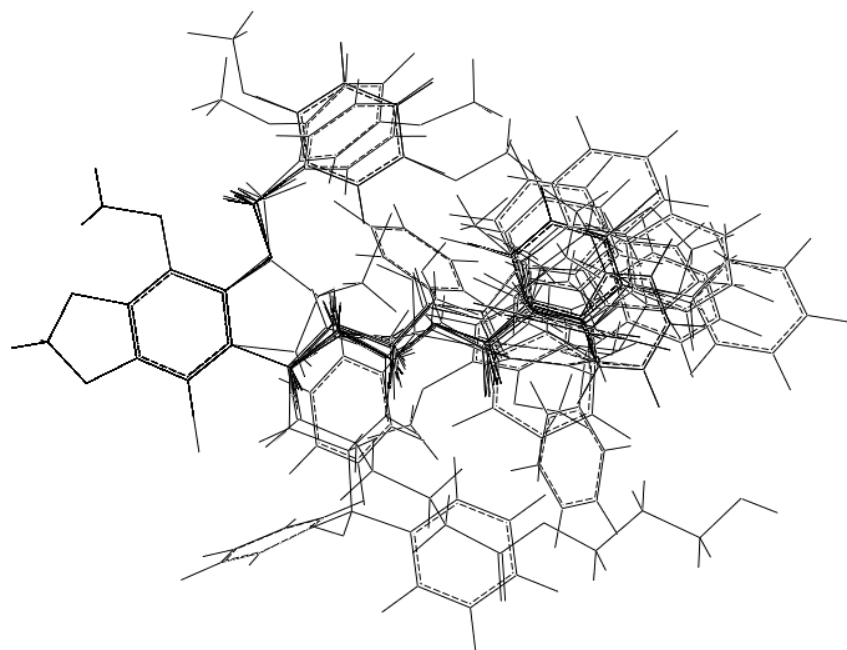


Fig. 2-2 Aligned training set of molecules used to construct the CoMSIA fields

## 2.2.4 Inhibition calculation and statistical analysis

Percent inhibition values were calculated based on differences in fluorescence between the control/control-blank wells and test/test-blank wells by the formula:

$$[1-(\text{Test } t=X-t=0)-(\text{Test blank } t=X-t=0) / (\text{Control } t=X-t=0)-(\text{Control blank } t=X-t=0)]*100$$

Where  $t=X$  and  $t=0$  are the reading values of the control/control-blank/test/test-blank wells at the end and at the beginning of running  $X$  min, respectively. The median inhibition percent ( $IC_{50}$ ) of each analog was determined with logarithmic curves plotted by different concentration and percent inhibition.

The dillapiol-relative inhibition activity was obtained by the formula:

$$IC_{50} \text{ of dillapiol} / IC_{50} \text{ of tested compound.}$$

Statistical analysis was undertaken using Graphpad Prism 5.01 software. The significant differences between dillapiol and individual compounds were determined using one-way analysis of variation (ANOVA) and Bonferroni post-hoc tests. Considering that the aim of the research was to find the analogs possessing higher CYP3A4 inhibition activity than dillapiol (positive control), the analogs which showed more than two times  $IC_{50}$  value higher than dillapiol were labelled as "/" (Table S-1). Statistical analysis of confidence intervals (C.I.) and range of  $IC_{50}$  values observed were carried out using GraphPad Prism 5 (GraphPad Prism<sup>®</sup>).

## 2.3 Results

### 2.3.1 Cytochrome CYP3A4 inhibition activity

A set of fifty-two analogs were prepared and successfully tested in the CYP3A4 assay. The full set of analog inhibition results are presented as inhibition activity relative to dillapiol activity = 1 (Fig. 2-3). The primary data set including mean IC<sub>50</sub> values, dillapiol-relative inhibition activity, and 95% confidence intervals (C.I.) are provided in supplementary data (Sup. II). Although the raw IC<sub>50</sub> values of 39 out of 52 analogs showed a lower trend (ie. higher inhibition) than that of dillapiol (9.2 μM), the statistical analysis revealed that only 17 analogs possessed significantly greater CYP3A4 inhibition activity than dillapiol (d. f. = 52, 106; F= 167.3; p<0.05). These analogs are highlighted with \* in the Fig. 2-3, 2-4, 2-5, Sup. II, Table 2-1 and 2-2. The two most effective CYP3A4 inhibitors were AF45 and AF53 with IC<sub>50</sub> values of 43- and 47-fold lower than that of dillapiol, respectively.

A group of seventeen dillapiol esters was prepared via Scheme 2-1. Hydroboration of dillapiol, **1** gave mainly the expected primary alcohol **2** (AF28) along with minor amounts of the secondary alcohol **3** (AF54). Both isomers were esterified with either different sized aliphatic or aromatic acids, and the compounds were characterized by both <sup>1</sup>H and <sup>13</sup>C NMR and high resolution mass spectrometry which assured their structure assignments. The IC<sub>50</sub> values and dillapiol-relative activity of 17 dillapiol esters were shown in Table 2-1 and Fig 2-4, respectively.

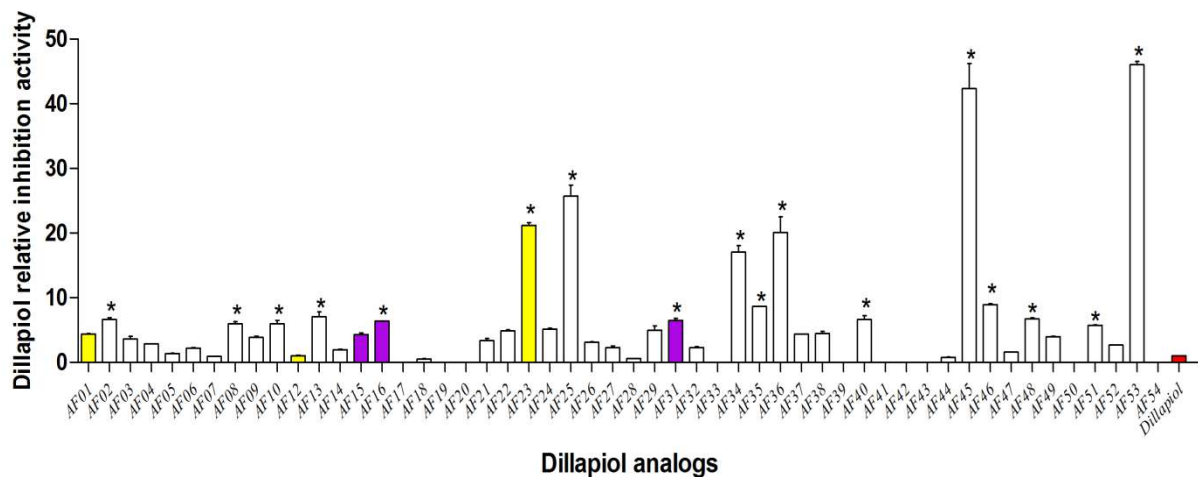
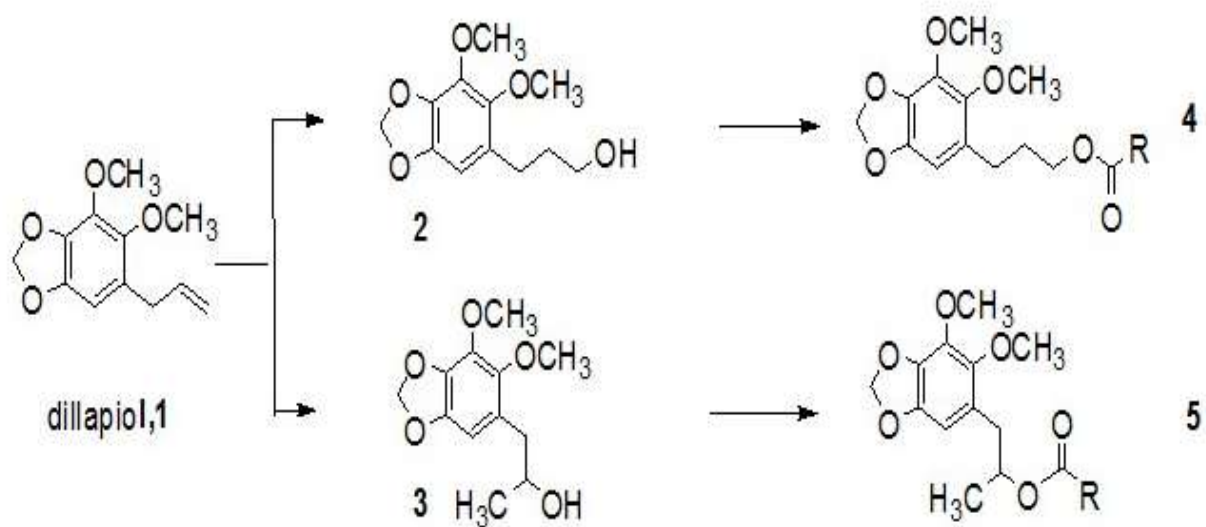


Fig. 2-3 CYP3A4 inhibition activity  $\pm$  Standard error (S.E.) of dillapiol analogs relative to dillapiol=1. The bars with asterisk indicate the significantly higher inhibition activity compared to dillapiol (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ). The yellow and purple bars represent either ester or ether analogs, respectively, selected for further insect biological testing. The parent compound, dillapiol was shown in red bar.



Scheme 2-1. Preparation of esters starting with dillapiol

Table 2-1 Inhibition of CYP3A4 by esters derived from dillapiol via alcohols **2** and **3**.

#	R	IC <sub>50</sub> (μM) (95% C.I.)	#	R	IC <sub>50</sub> (μM) (95% C. I.)
<b>1</b> (dillapiol)	-	9.2 (8.3-10.1)	<b>2</b> (AF28)	/	/
<b>3</b> (AF54)	/	/			
<b>4a</b> (AF12)	CH <sub>3</sub>	9.3 (6.6-11.9)	<b>4b*</b> (AF21)	C(CH <sub>3</sub> ) <sub>3</sub>	2.8 (1.7-3.9)
<b>4c*</b> (AF10)	C(CH <sub>3</sub> )=CHCH <sub>3</sub>	1.6 (1.0-2.1)	<b>4d</b> (AF04)	C <sub>5</sub> H <sub>11</sub>	3.3 (3.1-3.4)
<b>4e*</b> (AF22)	<i>c</i> C <sub>6</sub> H <sub>11</sub>	1.9 (1.6-2.2)	<b>4f</b> (AF06)	(CH <sub>2</sub> ) <sub>3</sub> Ph	4.1 (3.7-4.5)
<b>4g</b> (AF01)	Ph	2.1 (2.0-2.2)	<b>4h*</b> (AF13)	<i>o</i> -Cl-Ph	1.3 (0.71-2.0)
<b>4i*</b> (AF08)	<i>p</i> -Cl-Ph	1.6 (1.2-1.9)	<b>4j*</b> (AF02)	<i>o,p</i> -diCl-Ph	1.4 (1.22-1.6)
<b>4k</b> (AF03)	3,4-methylenedioxy- Ph	2.6 (1.3-4)	<b>4l</b> (AF09)	( <i>t</i> ) CH=CH-Ph	2.4 (2.0-2.8)
<b>4m*</b> (AF29)	CH <sub>2</sub> Ph	1.9 (0.46-3.4)	<b>4n*</b> (AF23)	CHPh <sub>2</sub>	0.43 (0.4-0.47)
<b>5a</b> (AF50)	CH <sub>3</sub>	/	<b>5g</b> (AF52)	Ph	3.5 (2.77-4.21)
<b>5n*</b> (AF53)	CHPh <sub>2</sub>	0.2 (0.15-0.24)			

\* indicates that the compound inhibited CYP3A4 to a higher degree than dillapiol; Ph=phenyl

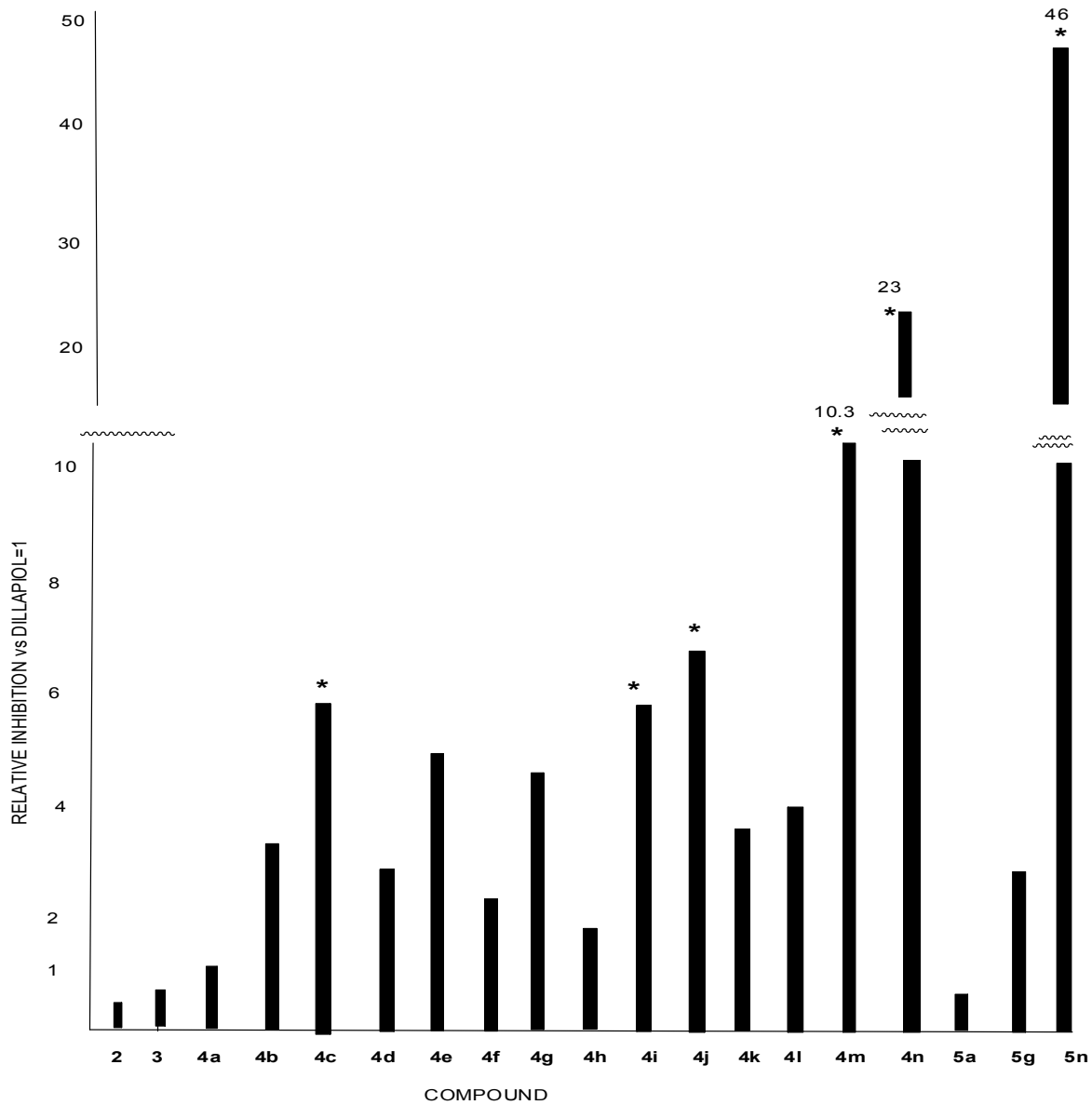
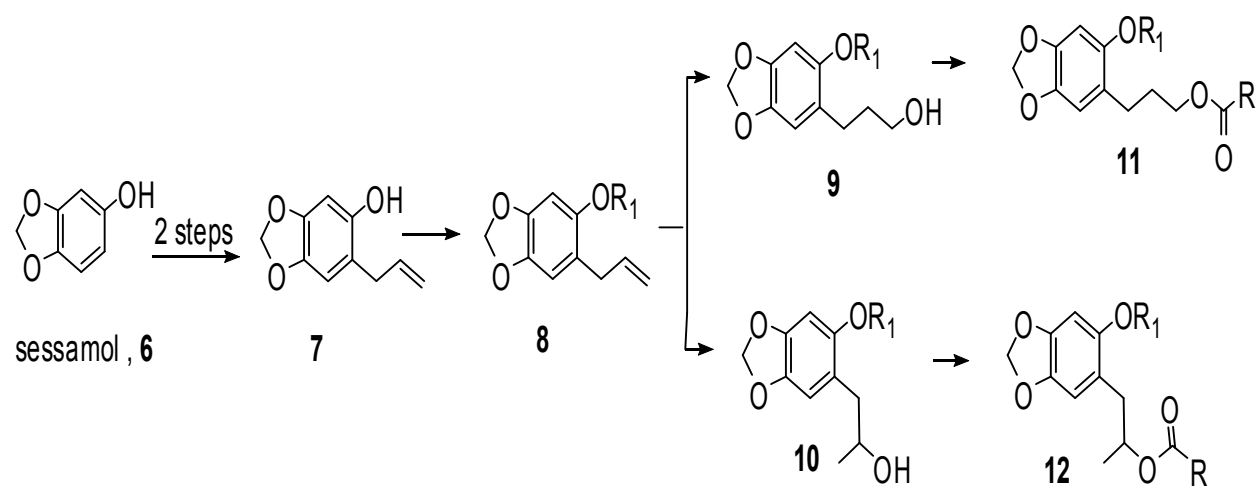


Fig. 2-4 Inhibition of CYP3A4 relative to dillapiol =1 by esters derived from dillapiol via alcohols 2 and 3.

Additional esters and several ethers were prepared starting with sessamol in order to investigate the importance of the 3- and 4- methoxy groups (Scheme 2-2). Sessamol, **6**, was converted into its ortho-allyl derivative by reaction with allyl bromide in the presence of potassium carbonate. The resultant allyl ether underwent a clean Claisen Rearrangement by heating to 190 °C in decalin to give the ortho-allylated phenol **7**. Subsequent O-alkylation, provided the ether **8** which upon hydroboration yielded the primary and secondary alcohols **9** and **10**, respectively. Acylation of these compounds produced the general formula **11** and **12**. The CYP3A4 inhibition data of nine esters belonging to the family **11** and **12** and four of the intermediate ethers **8** are shown in Table 2-2 and Fig.2-5.



Scheme 2-2 Preparation of sessamol analogs

Table 2-2 IC<sub>50</sub> and 95% C.I. of CYP3A4 inhibited by esters derived from sessamol.

#	R	IC <sub>50</sub> (μM)		#	R1	R2	IC <sub>50</sub> (μM)	
		(95% C.I.)					(95% C.I.)	
<b>1</b> dillapiol	-	9.2 (8.3-10.1)		<b>11a</b> (AF38)	CH <sub>3</sub>	Ph	2.1	(1.5-2.7)
<b>8a</b> (AF19)	H	/		<b>11b</b> (AF47)	CH <sub>2</sub> -Ph	Ph	5.8	(5.4-6.2)
<b>8b</b> (AF39)	CH <sub>3</sub>	/		<b>11c*</b> (AF25)	CH <sub>2</sub> -Ar	Ph	0.36	(0.26-0.46)
<b>8d</b> (AF16)	CH <sub>2</sub> -Ph	1.4 (1.4-1.5)		<b>11d</b> (AF14)	CH <sub>3</sub>	<sup>t</sup> CH=CH-Ph	17.2	(13.6-20.8)
<b>8e*</b> (AF15)	CH <sub>2</sub> -Ar	2.2 (1.6-2.7)		<b>11e</b> (AF18)	CH <sub>2</sub> -Ph	<sup>t</sup> CH=CH-Ph	4.7	(4.0-5.5)
				<b>11f*</b> (AF36)	CH <sub>3</sub>	CH-Ph <sub>2</sub>	0.47	(0.25-0.69)
				<b>11g*</b> (AF46)	CH <sub>2</sub> Ph	CH-Ph <sub>2</sub>	1.0	(0.97-1.08)
				<b>11h</b> (AF48)	CH <sub>2</sub> Ph	CH <sub>2</sub> -Ar <sup>&amp;</sup>	1.4	(1.2-1.5)
				<b>11i*</b> (AF45)	CH <sub>2</sub> Ar	CH-Ph <sub>2</sub>	0.22	(0.09-0.35)

Ar = 2, 5-dimethoxyphenyl; Ar<sup>&</sup> = 3, 4-methylenedioxy-phenyl; Ph = phenyl; <sup>t</sup> = trans

\* noted that the compound inhibited CYP3A4 to a lesser degree than dillapiol.

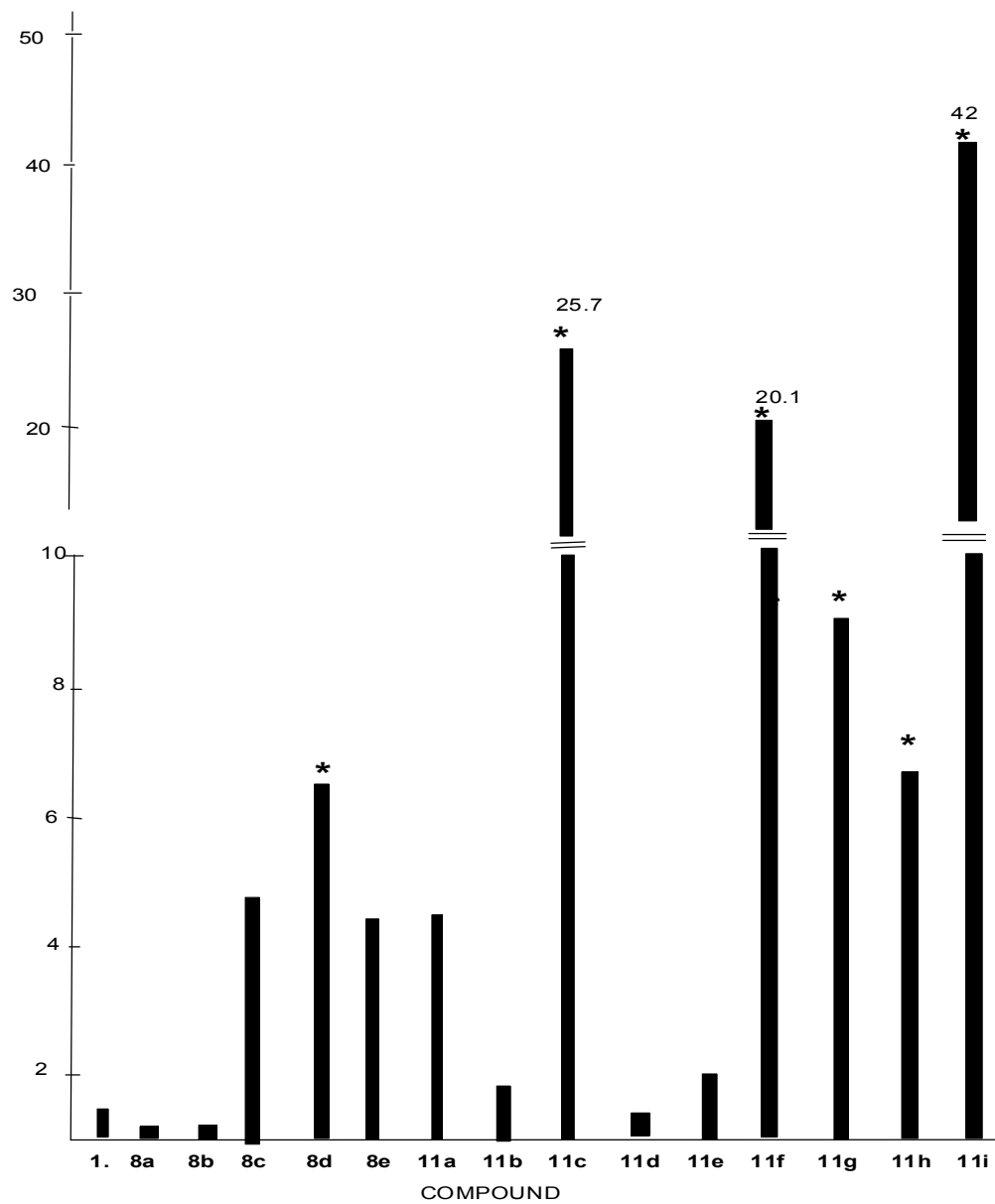


Fig. 2-5 Inhibition of CYP3A4 of compounds derived from sessamol  
relative to dillapiol =1.

### 2.3.2 QSAR model

The optimal CoMSIA model was obtained using steric, electrostatic and hydrophobic field descriptors. The steric field descriptor explains 17.4 % of the variance; the electrostatic contributes 38.3 %, while the hydrophobic descriptor accounts for 44.2 %. The observed and predicted activities used in the model, and the residual values for the training set were calculated, and values are shown in Table 2-3 and Fig. 2-6. In order to validate the 3D-QSAR model, a set of analog molecules were used. Inhibitory activities were predicted for the testing set shown in Table 2-4.

#### *CoMSIA contour maps*

The most active compound 5n (AF53) in the model ( $pIC_{50}=6.70$ ) was embedded in the CoMSIA contour displays. The contour maps for the hydrophobic fields, electrostatic field and steric field were shown in Fig. 2-7, 2-8 and 2-9, respectively.

Relative contributions	
CoMSIA electrostatic	0.383
CoMSIA steric	0.174
CoMSIA hydrophobic	0.442

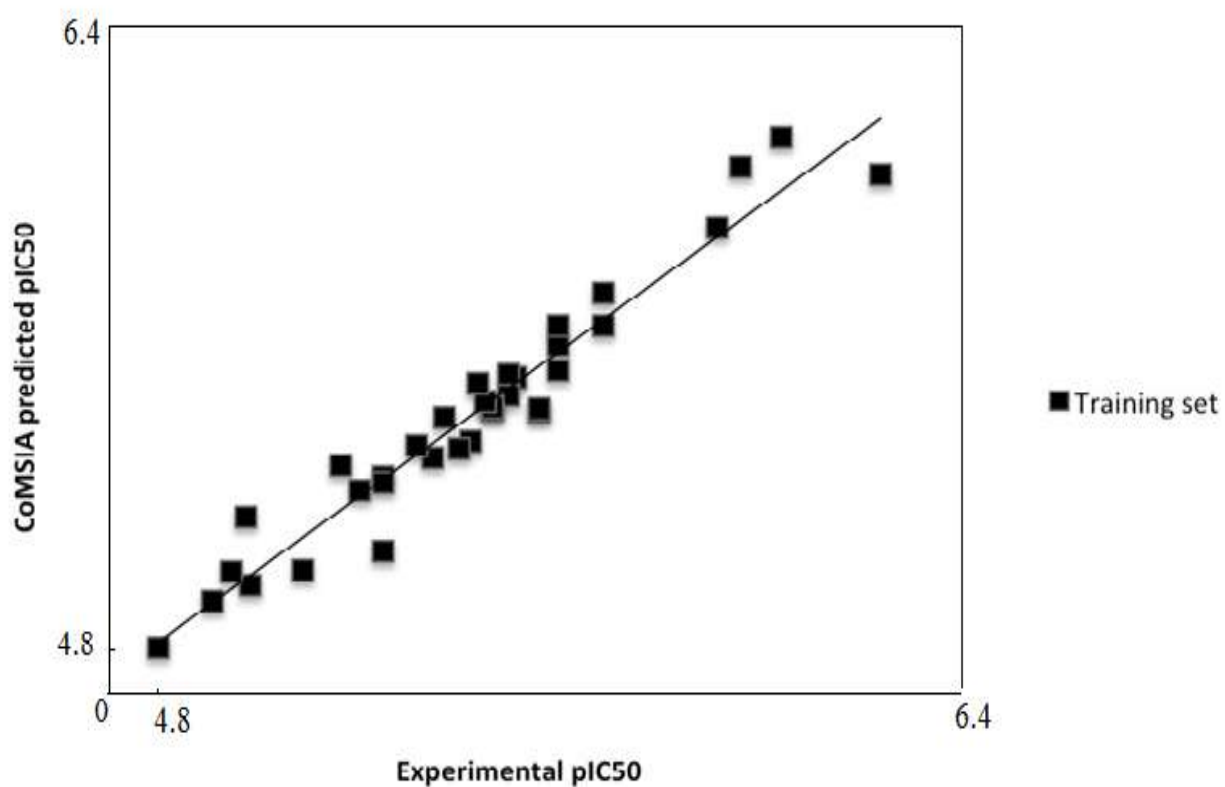


Fig 2-6. Plot of the experimental and predicted PIC<sub>50</sub> of 35 training analogs for CoMSIA analysis with relative contribution portion of three major parameters.

Table 2-3 The observed and predicated inhibitory concentration (pIC<sub>50</sub>) of 35 dillapiol analogs used as training analogs for developing 3D-QSAR model. CoMSIA residuals were included.

#	Code	Obs. Log (pIC <sub>50</sub> )	Pred. Log (pIC <sub>50</sub> )	CoMSIA Residuals	#	Code	Obs. Log (pIC <sub>50</sub> )	Pred. Log (pIC <sub>50</sub> )	CoMSIA Residuals
1	<b>5n</b> (AF53)	6.70	6.45	0.25	19	<b>AF 49</b>	5.64	5.73	-0.09
2	<b>11c</b> (AF25)	6.44	6.57	-0.13	20	<b>4l</b> (AF09)	5.62	5.54	0.09
3	<b>11f</b> (AF36)	6.33	6.47	-0.14	21	<b>4k</b> (AF03)	5.59	5.51	0.08
4	<b>AF34</b>	6.27	6.26	0.01	22	<b>4b</b> (AF21)	5.55	5.61	-0.06
5	<b>AF35</b>	5.97	5.93	0.04	23	<b>AF26</b>	5.52	5.48	0.04
6	<b>13</b> (AF35)	5.97	6.04	-0.07	24	<b>4d</b> (AF04)	5.48	5.52	-0.04
7	<b>8d</b> (AF16)	5.85	5.86	-0.01	25	<b>9b</b> (AF27)	5.39	5.16	0.23
8	<b>14</b> (AF40)	5.85	5.78	0.07	26	<b>AF32</b>	5.39	5.41	-0.02
9	<b>4j</b> (AF02)	5.85	5.93	-0.08	27	<b>4f</b> (AF06)	5.39	5.39	0.00

<b>10</b>	<b>4i</b> (AF08)	5.80	5.64	0.17	<b>28</b>	<b>11e</b> (AF18)	5.33	5.37	-0.04
<b>11</b>	<b>4c</b> (AF10)	5.80	5.65	0.15	<b>29</b>	<b>4h</b> (AF13)	5.28	5.45	-0.17
<b>12</b>	<b>15</b> (AF24)	5.74	5.75	-0.01	<b>30</b>	<b>16a</b> (AF05)	5.18	5.09	0.09
<b>13</b>	<b>5a</b> (AF50)	5.72	5.76	-0.04	<b>31</b>	<b>dillapiol</b>	5.04	5.04	0.00
<b>14</b>	<b>4e</b> (AF22)	5.72	5.69	0.03	<b>32</b>	<b>4a</b> (AF12)	5.03	5.28	-0.25
<b>15</b>	<b>AF37</b>	5.68	5.65	0.03	<b>33</b>	<b>16b</b> (AF07)	4.99	5.09	-0.10
<b>16</b>	<b>11a</b> (AF38)	5.68	5.64	0.04	<b>34</b>	<b>AF44</b>	4.94	4.99	-0.05
<b>17</b>	<b>4g</b> (AF01)	5.68	5.65	0.03	<b>35</b>	<b>2</b> (AF28)	4.80	4.83	-0.03
<b>18</b>	<b>8e</b> (AF15)	5.66	5.67	-0.01					

---

Table 2-4 Predicted and observed inhibitory concentration (pIC<sub>50</sub>) of the testing set of analogs in order to evaluate the accuracy of developed 3D-QSAR model.

	<b>Pred. Log (pIC<sub>50</sub>)</b>	<b>Obs. Log (pIC<sub>50</sub>)</b>	<b>CoMSIA Residuals</b>
4m (AF29)	5.54	5.72	-0.18
4h (AF13)	5.59	5.89	-0.3
4n (AF23)	6.08	6.37	-0.29
5g (AF52)	6.05	5.46	0.59
11b (AF47)	6.3	5.24	1.06
11d (AF14)	5.55	4.76	0.79
11g (AF46)	6.78	6.00	0.78
11h (AF48)	6.5	5.85	0.65
11i (AF45)	6.6	6.66	-0.06
15 (AF31)	5.44	6.15	-0.71
8b (AF39)	5.13	5.72	-0.59

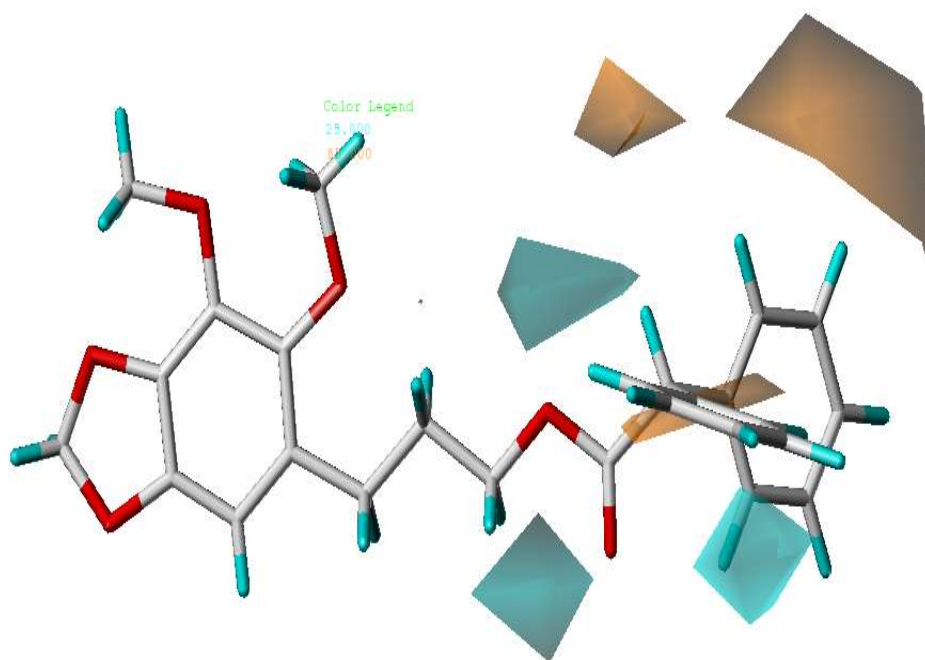


Fig 2-7. CoMSIA contour hydrophobic map of 5n (AF53). Cyan/orange colors indicate the regions where hydrophobic/hydrophilic groups would enhance the activity respectively. Orange color shows favored areas while cyan shows disfavored. The graphic is fixed to display 25% and 85% colors respectively.

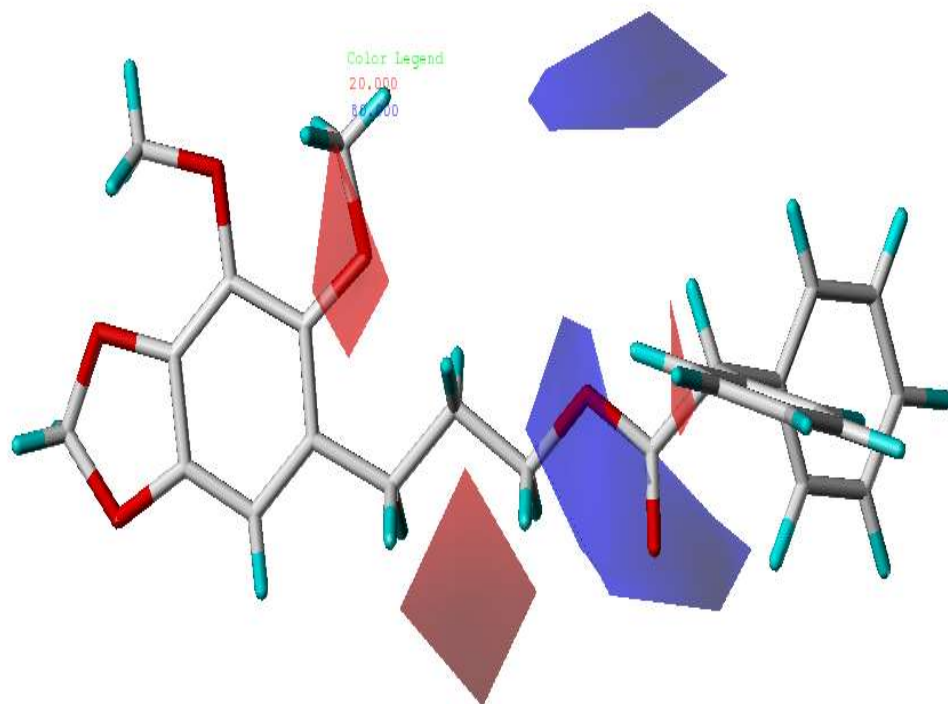


Fig 2-8. CoMSIA contour map for electrostatic fields of 5n (AF53). Blue and red depict favorable and disfavored sites for positively/negatively charged groups. This graphic used 20%/ 80% display colors.

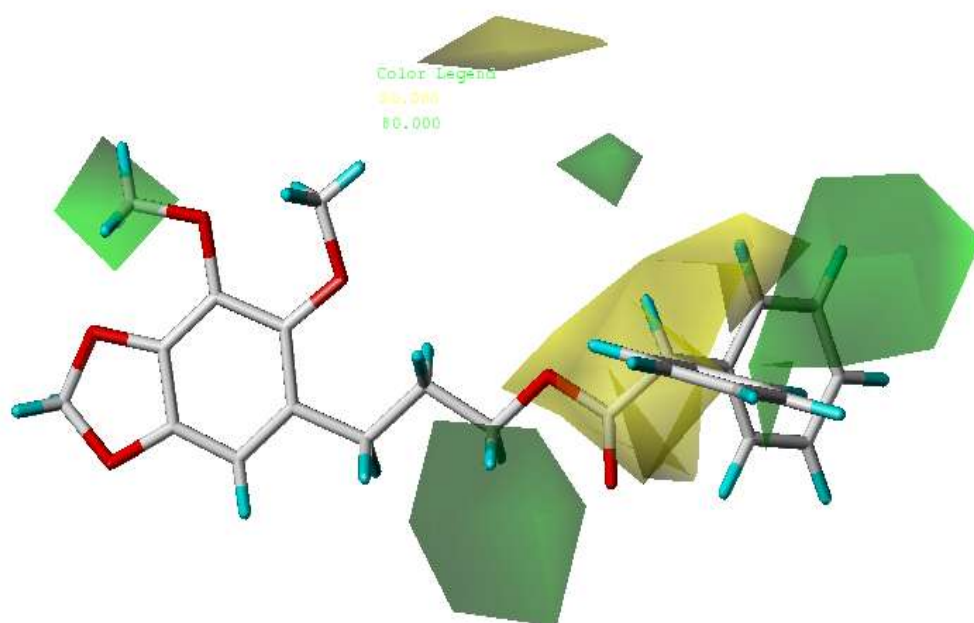
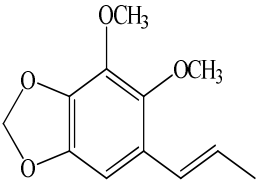
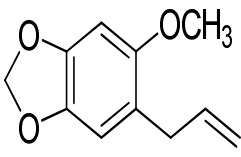
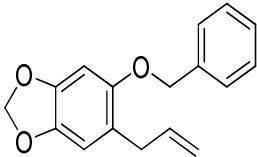
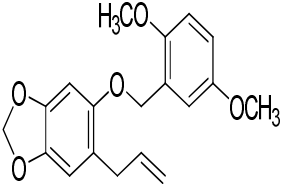
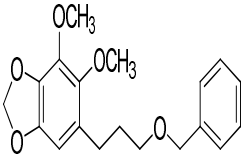
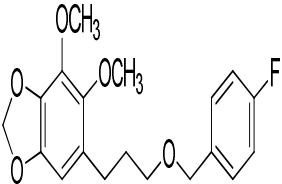


Fig 2-9. CoMSIA contour steric map of 5n (AF53). Sterically favored/disfavored areas display in green/yellow color respectively. The graphic is fixed in 80/ 50% display level.

Table 2-5. Inhibition of CYP3A4 by ethers derived from sessamol and dillapiol

Compound	Code	IC <sub>50</sub> (μM) (Relative to dillapiol=1)	Compound	Code	IC <sub>50</sub> (μM) (Relative to dillapiol=1)
	1 (dillapiol)	9.2 (1)		8b (AF39)	/
	8d (AF16)	1.4 (6.4)		8e (AF15)	2.2 (4.3)
	16a (AF05)	6.7 (1.4)		16b (AF07)	10.3 (0.9)

## 2.4 Discussion

Plant is a rich source of biologically active natural products and plant-derived compounds selected for use in medicine or agriculture, which are often structurally optimized to improve their activity. There is usually an advantage to modifying the lead molecule and designing analogs that will increase the activity and reduce negative impacts of the parent compound. The first step of the present study was to synthesize and screen dillapiol analogs by CYP 3A4 isozyme inhibition for comparison with dillapiol.

Within the tested analogs, both the alcohols **1** and **2** were less potent than dillapiol in inhibiting P450 3A4. The data in Table 2-1 and Fig. 2- 4 showed that for the esters derived from the primary alcohol **2**, an increase in size of the R group, particularly if this is near the ester function, results in increased P450 3A4 inhibition. For example, the acetate **4a** (AF12) (R=CH<sub>3</sub>) had essentially the same inhibitory effect as dillapiol. The change in R from CH<sub>3</sub> (**4a** AF12) to C<sub>5</sub>H<sub>11</sub> (**4d** AF04) and cyclohexyl (**4e** AF22) resulted in increased inhibition by factors of 2.8 and 4.8, respectively. The benzoyl ester (**4g** AF01) was 4.4 times more potent inhibitor than the methyl ester; the addition of chlorine substituents to the aromatic ring further significantly increased the potency of these compounds 6-fold (**4h** AF13, **4i** AF08, and **4j** AF02). The greatest change was observed when a hydrogen in the benzyl group (**4m** AF29) was replaced by a second aromatic ring to yield R = benzhydryl (**4n** AF23). This change resulted in increased inhibition by factors from 5 to more than 21. The benzhydryl ester, **5n** (AF53), of the secondary alcohol **5** was twice as active (47 times more potent than dillapiol).

Based on the available data, comparison of the same esters derived from dillapiol and sessamol suggests that the removal of the 3-methoxy group in dillapiol did not have a significant effect on the inhibition of the 3A4 enzyme. For example the benzoyl esters **4g** (AF01) and **11a**

(AF38), inhibited this enzyme to essentially the same extent; the same conclusion can be drawn when one compares data for the benzhydryl esters **4n** (AF23) and **11f** (AF36) each of these compounds were approximately 20 times as effective as dillapiol. The replacement of the 4-methoxy group in the sessamol derivatives by a benzyl group, (**11b** AF47 and **11g** AF46) lowers the inhibitory activity of these compounds by a factor of 2-3.

The change from ester to ether was only briefly investigated. Based on the limited data available, a comparison of the phenyl acetate **4m** (AF29) and the two benzyl ethers **17** (AF31) it appeared that the ethers are somewhat less potent than the corresponding esters. A series of ether, **8a-8e** derived from sessamol were also evaluated. These compounds where R is CH<sub>3</sub>, C<sub>6</sub>H<sub>13</sub>, CH<sub>2</sub>Ph and CH<sub>2</sub>-(2,5-dimethoxyphenyl) showed only modest potency in comparison to dillapiol with the methyl ether showing a 50% decrease, while the hexyl ether and the benzyl ether had a 6.4 and 4.8 fold increase relative to dillapiol. The methoxy derivatives **8a** (AF19), which lacks the second methoxy group present in dillapiol, was somewhat less potent than dillapiol. Replacement of the methoxy group by benzyl (**8d** AF16) or 2,5 dimethoxybenzyl (**8e**, AF15) gave compounds that were 4 to 7 times more potent in inhibiting CYP3A4 than dillapiol.

Qualitative analysis of the inhibition data for a variety of esters indicates that larger acyl group attached to the alcohols **4**, **5**, and **11** and **12** gave higher in vitro inhibition of CYP3A4. Additionally, it appears that the same esters derived from the secondary alcohols, for example **5n** (AF53) was approximately twice as potent as those obtained from the isomeric primary alcohols, **4n** (AF23). Removal of one of the two methoxy groups in dillapiol yielded compounds such as **8**, **11** and **12**. This did not result in a significant decrease of the potency of derivatives compared to the corresponding dillapiol analogs. In the case of the ethers **8c**, **-8e** the replacement of the remaining methoxy group by other, “bulky” substituents such as the hexyl or benzyl groups resulted in an

increase in the inhibitory property of these compounds relative to dillapiol. This suggests that the reaction of the allyl derivative **7** with “bulky” alkylating agents such as benzhydrol and conversion of such compounds to several of the esters of the type **11** and **12** should yield more potent inhibitors than those shown in Table 2-1. Interestingly, CoMSIA showed that the effect of the “bulky” groups in the inhibitory effects was a mix of hydrophobic (44.2 %) and steric (17.4%) effects.

The importance of the QSAR relationship based on the current group of compounds is that it points to new structures with predicted higher P450 3A4 inhibition. For example, the hydrophobic CoMSIA contour suggests that the activity would significantly increase if we made changes that improve the hydrophobicity over the orange color (Fig. 2-7). According to the electrostatic CoMSIA contour (Fig. 2-8), the blue color indicates regions where electron deficiency enhanced the biological activity, while the red color represented areas where the electron rich moieties were favorable for the activity.

It has been determined that CYP6 clade of enzyme, the major cytochrome P450 family of insects which conducts the detoxification in many insect pests, belong to the same clade as CYP3 family of humans and other mammals (Feyereisen, 2006). In the present study, many dillapiol analogs exhibited higher inhibition levels with the human CYP3A4 than the parent compound. It provided a number of molecules which show potential as insecticide synergists. The analogs to be tested were grouped as either ester compounds, AF01, AF12, and AF23 for the first generation synergists, or ether compounds, AF15, AF16, and AF31 for the second generation synergists. I expected that similar inhibition trends could be observed in the insect biological testing.

# **CHAPTER 3 DILLAPIOL AND ITS ANALOGS AS PYRETHRUM SYNERGISTS FOR CONTROL OF OLIGOPHAGOUS COLORADO POTATO BEETLE, LEPTINOTARSA DECEMLINEATA (SAY)**

## Preface

In this chapter, Chapter 4 and 5, analogs selected from chapter 2 were used to determine their potential in insect models. Specifically, the synergistic activity, intrinsic toxicity, and mode of action of selected analogs were determined with both oligophagous Colorado potato beetle and polyphagous European corn borer. All experiments were conducted by myself with the exception of the intrinsic toxicity bioassay (in Chapter 4) which was undertaken with Emerson Harkin, an undergraduate student of University of Ottawa.

## 3.1 Introduction

The widespread insect pest, the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a leaf beetle originally native to the southwestern United States and Mexico, and was first described by Thomas Say, who found the beetle on buffalo bur, *Solanum rostratum* Dunal, in Missouri and Arkansas in 1824. Today the beetle has migrated throughout much of North America, Europe, parts of Asia, and parts of Central America (Capinera, 2001). The severe damage potential of this insect pest combined with its rapid ability to develop resistance to any newly introduced insecticide has made it one of the most important pests of agricultural crops.

CPB is an oligophagous insect that feeds exclusively on the foliage of cultivated and wild plants in the Solanaceae (Hsiao, 1985). It is a major insect pest on potato, tomato (*Solanum lycopersicum* L.), eggplant (*S. melongena* L.), but rarely on pepper (*Capsicum annuum* L.) (Capinera, 2001). In some cases, CPB can adapt its host range by accepting locally abundant *Solanum* species (Mena-Covarrubias *et al.*, 1996; Horton *et al.*, 1998a, 1998b) which confer

increased survival probabilities to those beetle populations. As a gluttonous leaf feeder, the damage caused by CPB can greatly reduce yield and even kill plants. During the whole life cycle, both adults and larvae feed on the same plant foliage, but the fourth instar larvae are responsible for most (~77%) of total leaf consumption (Capinera, 2001). Potato crops could be completely defoliated if CPB larval densities are high enough (Hare, 1980).

CPB is notoriously difficult to control, and many control strategies available in integrated pest management have been employed. The major techniques attempted include: 1) cultural control, including common cultural practices such as crop rotation, manipulation of planting time and crop varieties; 2) physical control, such as digging plastic-lined trenches along a field border in order to intercept post-diapause CPB colonizing the crop in the spring; 3) biological control by using a number of biological organisms that feed on or infect CPB to reduce CPB populations; 4) plant resistance, for example, selection for toxic secondary metabolites, mainly glycoalkaloids, which are present in solanaceous plants including potato, and confer plant resistance to herbivorous insects (Tingey and Sinden, 1982; Sinden *et al.*, 1991; Pelletier *et al.*, 2013). Unfortunately, most of these methods have had limited success and potato and vegetable growers still rely heavily on the method of last resort in integrated pest management (IPM): chemical control. This has been used as the major approach to control CPB for more than 160 years, and has caused rapidly developed resistance of CPB to almost all insecticides used for its control.

The first case of insecticide resistance in CPB populations was noted with the organochlorine insecticide, DDT, in the 1950s (Quinton, 1955) and cross-resistance was observed soon afterwards with other chlorinated hydrocarbons (Hofmaster *et al.*, 1967). Over the past five decades CPB developed resistance to the organophosphates and carbamates (Forgash, 1985) as well as virtually all classes of insecticides used for its control (Kuhar, 2013). Research has demonstrated that

insecticide synergists, such as PBO and S, S, S-tributylphosphorotrithioate (DEF) can decrease CPB insecticide-resistance (Mohammadi Sharif et al., 2007).

In the previous chapter, the structure activity relationships for a large number of analogs of the natural compound dillapiol were developed using a cytochrome P450 assay, which is useful in predicting molecules that may be useful in reducing the rapid rate of insecticide metabolism in CPB. In this chapter, I systematically examined the possibility of restoration of susceptibility to insecticides in CPB using a selected number of these compounds as resistance modulators. Six analogs were carefully selected, which included three ester compounds with inhibitory activity greater than dillapiol (first generation analogs) and three ether compounds also with enhanced activity, but which were stable to hydrolysis by insect esterases (second generation analogs) (Fig. 3-1).

## 3.2 Material and methods

### 3.2.1 Source of chemicals applied

The insecticide applied in the present study is pyrethrum which has been used for centuries as a natural pesticide and it is one of the most commonly used non-synthetic insecticides in certified organic agriculture (Isman, 2006). The commercial pyrethrum was grown in Kenya and extract was obtained from Whitmire Micro-Gen (BASF, St. Louis, MO). The extract was purified by supercritical fluid extraction with CO<sub>2</sub> at Loyalist College (Belleville, ON, Canada) (Kramp, 2010).

As mentioned in Chapter 2, dillapiol was prepared by steam distillation from the fruit of *Piper aduncum*. The crude oil was purified using column chromatography. The purity was >95% as determined by NMR. Six analogs were carefully selected, which included three ester compounds (first generation analogs) and three ether compounds (second generation analogs) (Fig. 3-1).

For insect toxicity bioassays, all chemicals were dissolved in acetone and the different solutions were prepared by combining pyrethrum and dillapiol or the analogs at a 1:5 (w:w) ratio.

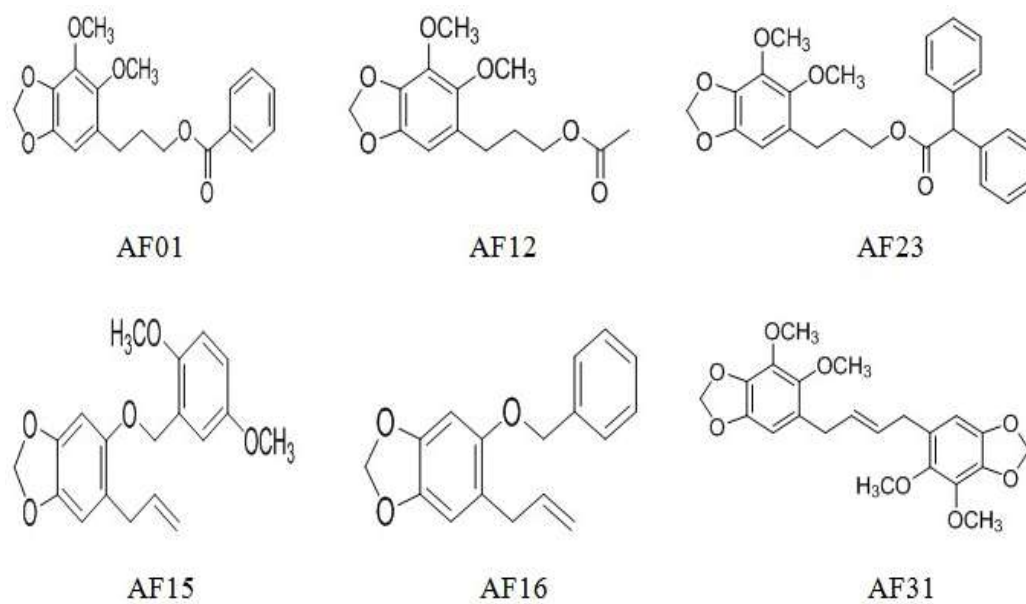


Fig. 3-1 Selected dillapiol analogs for testing insecticide synergistic activity. AF01 AF12 and AF23 are ester analogs; AF15, AF16, and AF31 are ether analogs.

### 3.2.2 Laboratory bioassay

#### *Insects:*

Laboratory bioassay experiments were carried out with two Colorado potato beetle (CPB) *Leptinotarsa decemlineata* Say strains: 1) Insecticide-resistant CPB (RS-CPB) were obtained from the Department of Entomology, Michigan State University, East Lansing, MI, USA, and maintained without exposure to insecticides for > 20 generations at the Southern Crop Protection and Food Research Centre (SCPFRC), Agriculture and Agri-Food Canada (AAFC), London, ON laboratory prior to use in the insect and biochemical assays. 2) The insecticide-susceptible CPB (SS-CPB) were reared for >150 generations without exposure to insecticides at AAFC London. Both CPB strains were reared on greenhouse grown potato *Solanum tuberosum* (var. Kennebec) foliage and maintained in an insectary at  $27 \pm 1^\circ\text{C}$ ,  $65\% \pm 5$  RH and a photoperiod of 16:8 L:D.

#### *Insect Bioassays*

Two insecticide exposure methods, topical and ingestion, were used to test the synergistic potential of dillapiol analogs against CPB with a pyrethrum discriminating concentration (DC) and a range of pyrethrum concentrations.

The topical application bioassay was conducted by cutting 4 cm diameter leaf discs from potato leaves collected from greenhouse-grown plants. Five second instar CPB larvae were transferred to each leaf disc and a 1  $\mu\text{L}$  dose of the test solutions was applied to the dorsal thoracic region of the larvae using a 5  $\mu\text{L}$  micro-applicator (Hamilton Robotics, Reno, NV). CPB larvae treated with acetone only served as controls. The treated as well as control larvae were held under the same condition in a holding room at  $27 \pm 1^\circ\text{C}$ ,  $65\% \pm 5$  RH and a photoperiod of 16:8 L: D. Mortality was recorded at 24 and 48 h by gently touching the larvae with a probe to assess response and mobility. Moribund larvae were scored as dead.

For the ingestion method, each leaf disc was dipped for 3 s into the same solutions prepared for the topical method and then held in the fumehood for 20 min to ensure adequate drying of the solutions before transferring the leaf disc to a Petri dish with a filter paper disc (Whatman #1) underneath. Leaf discs dipped into the acetone alone served as the control treatment. Five second instar CPB larvae were placed on each leaf disc and the petri dishes were put into the holding room. Mortality was assessed at 24h as described previously.

Bioassays were replicated three times for each test concentration. The highest concentration of each synergist applied in the combination was also tested singly to determine the inherent toxicity to CPB in both topical and ingestion treatments.

Discriminating concentration (DC) bioassays were used to compare the synergistic potential of dillapiol and its analogs with two diagnostic concentrations of pyrethrum. Pyrethrum concentrations of 10 and 5 ppm for SS-CPB second instar larvae, and 50 and 10 ppm for RS-CPB second instar larvae with the topical and ingestion treatments, respectively, were chosen, since each produced less than 100% mortality among all treatments. The two pyrethrum concentrations were tested alone as the unsynergized treatment.

The 50% lethal concentration ( $LC_{50}$ ) of pyrethrum alone or pyrethrum plus each of the different synergists was determined at 24 h and 48 h. A serial set of six concentrations of the different solutions were made by the half-half dilution method. The degree of synergism was estimated using a synergism ratio (SR), calculated by dividing the  $LC_{50}$  of pyrethrum alone by the  $LC_{50}$  of pyrethrum plus synergists at a 1:5 ratio.

At least three separate series of bioassays were run with the SS and RS strains at each of 4-5 concentrations for each pyrethrum/synergist combination giving a minimum of 30 larvae (3

bioassays x 2 replicates/bioassay x 5 larvae/replicate) per concentration tested. Pyrethrum concentrations were selected based on preliminary trials to provide a range of greater than 0 and less than 100% mortality.

Observed mortality was corrected for natural mortality using Abbott's correction (Abbott 1925). Results for a series were discarded or the series repeated if control mortality exceeded 10% for 2 day bioassays.

### 3.2.3 Greenhouse trial

A greenhouse trial was used to compare the synergistic and residual activity of PBO and dillapiol in combination with pyrethrum with a more realistic application method. The pyrethrum and pyrethrum-dillapiol or PBO combinations were formulated with tetrahydrofurfuryl alcohol (THFA; Penn Specialty Chemicals, Memphis, TN) and the emulsifier prepared from ethoxylated castor oil Alkamuls EL-719 (A-719; Rhodia, Cranbury, NJ). Formulations were prepared with pyrethrum alone at 6.4%, or pyrethrum at 0.64% in combination with PBO or dillapiol. The formulation blank for all experiments consisted of 10% (wt: wt) A-719 and 90% (wt: wt) THFA. The addition of pyrethrum or synergists in the formulation reduced the amount of THFA proportionally, but the A-719 amount remained constant. One hour before application, 5 mL of four different formulations were diluted in water 40X to obtain a concentration of pyrethrum at 10X or 100X the LC<sub>50</sub> concentration determined for the SS-CPB strain (16 ppm pyrethrum and 80 ppm dillapiol).

In the greenhouse at AAFC London, three pots of potato plants were placed in a triangle and 70 mL of each formulation was sprayed by hand using a misting sprayer. Leaves, one leaf per level, were sampled from top, middle and bottom of plants at 1, 6 and 24 h post treatment. Leaves were placed in the Petri dishes described in the previous laboratory bioassay section, and five 2<sup>nd</sup> instar

SS-CPB larvae were carefully transferred onto the top of the leaf. The Petri dishes were put into the holding room. Mortality was assessed at 24 h as described previously.

#### 3.2.4 Microplot field trial

A microplot trial was used to compare the synergistic and residual activity of PBO and dillapiol in combination with pyrethrum under more realistic field conditions. Microplots, 2.25 m x 0.9 m, 15 in total were planted with 2 rows x 5 potato (var. Kennebec) in early June 2012 at AAFC London. The formulations applied for the field trial were identical with those prepared for the greenhouse trial. In mid-July, the plants were sprayed with 560 mL of each solution for the entire plot (40 mL / plant). Ten leaflets per treatment, each from a different plant, were collected from the foliage at 1, 6, 12, and 24 h post-application. The leaflets were placed in a plastic Petri dish (9 cm) lined with damp filter paper, labeled with date and treatment, and transported to the lab in a cooler with freezer packs. Nine out of ten leaflets were separated randomly into three different replicates, each in one Petri dish. Five second instar larvae were placed on each leaflet replicate and the Petri dish were held in a growth room at 16:8 h photoperiod, 25°C daytime, 20°C night, and 50% RH. Mortality was checked at 1, 6, 12 and 24 h post treatment.

#### 3.2.5 Statistical Analysis

Probit analysis (SAS Institute, 2001) of the data generated was then completed to develop regression lines and determine the  $LC_{50}$  values and 95% confidence interval (C.I.) for both the SS- and RS-CPB for each pyrethrum/synergist combination.

The field and greenhouse trials were analyzed by two-way ANOVA and Bonferroni post hoc tests (GraphPad Prism 5). The log-transformed data and Arcsin-transformed data were subjected to the general linear model procedure to test the significant differences between individual fractions.

### 3.3 Results

#### 3.3.1 Laboratory Bioassay

##### *Synergistic effect of dillapiol analogs against SS-CPB*

The LC<sub>50</sub> values for pyrethrum with or without dillapiol determined with 2<sup>nd</sup> and 4<sup>th</sup> SS-CPB instar larvae (Table 3-1) were used to select a concentration of 5 ppm pyrethrum for the ingestion method and 10 ppm for the topical method with the 2<sup>nd</sup> instar SS-CPB larvae while 50 ppm pyrethrum was chosen for both topical and ingestion treatment against 4<sup>th</sup> instar larvae to combine at a synergist ratio of 1:5 with dillapiol and the 1st generation analogs (ester compounds) AF01, AF12, and AF23.

After 24 h, AF23 was the only compound to significantly synergize pyrethrum compared to pyrethrum alone by the topical method, but its inhibitory activity was not statistically higher than dillapiol-pyrethrum combination. Similarly, AF23 and dillapiol both significantly increased the CPB mortality compared to the unsynergized pyrethrum by the ingestion method (d.f. =4; 10; F=10.64; p<0.05) (Fig. 3-2). All analogs had greater synergistic activity at half the concentration applied with the ingestion method (5 ppm), compared to the topical method (10 ppm), with the exception of AF01 (Fig. 3-2).

Table 3-1 The LC<sub>50</sub> value for pyrethrum (Py) with or without dillapiol (95% C.I.) and synergism ratio (SR) with 2<sup>nd</sup> and 4<sup>th</sup> instars SS-CPB larvae (24h).

	Insecticide with or without synergists	Topical		Ingestion	
		LC <sub>50</sub> (95% CI) (ppm)	SR	LC <sub>50</sub> (95% CI) (ppm)	SR
2 <sup>nd</sup> instar larvae	Py	9.3 (8.2-10.7)	--	3.7 (2.1-6.4)	--
	Py+dillapiol	3.7 (2.3-6)	2.5	1.8 (1.1-2.8)	2.1
4 <sup>th</sup> instar larvae	Py	115 (89-199)	--	113 (87-139.3)	--
	Py+dillapiol	36.8 (18.5-78.3)	3.1	34.7 (25.4-56.9)	3.3

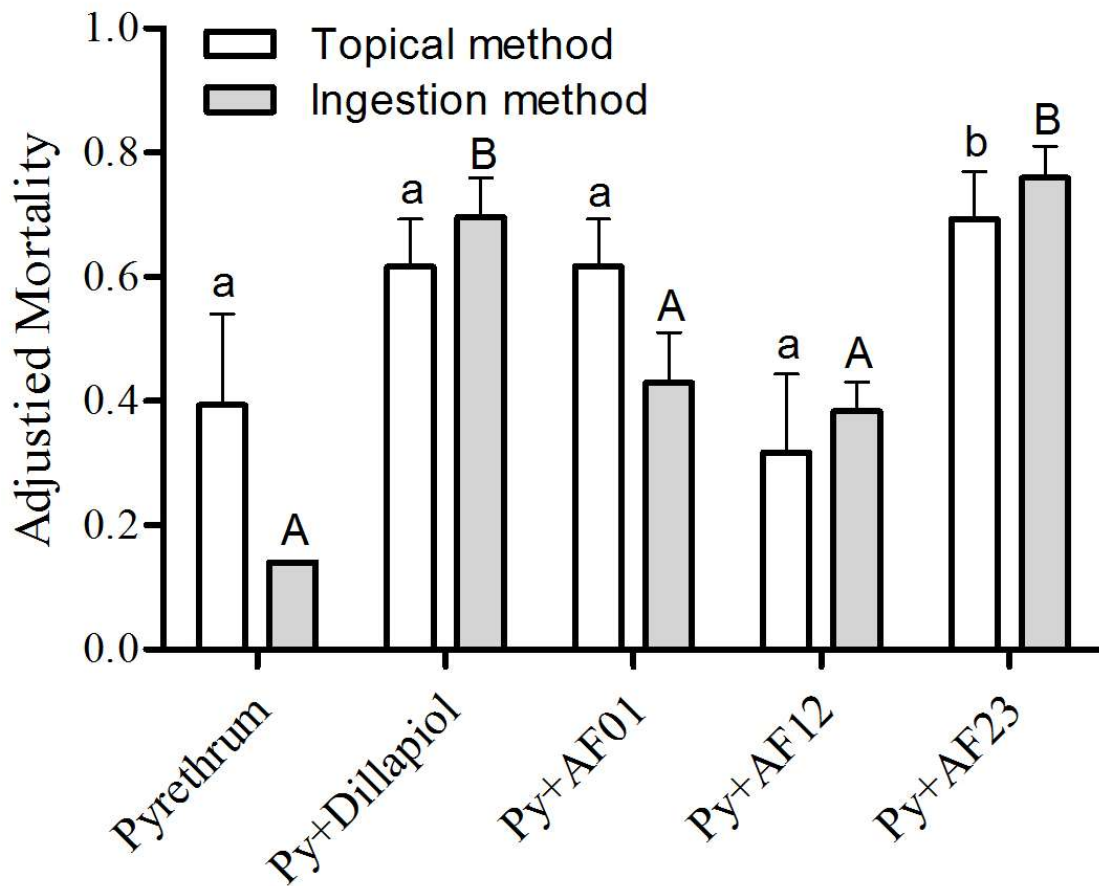


Fig. 3-2 Comparison of 24 h adjusted mortality ( $\pm$  S.E.) for pyrethrum with or without dillapiol or 3 ester analogs with 2<sup>nd</sup> instar SS-CPB larvae by topical (10 ppm) and ingestion (5 ppm) treatment method. Bars with different letters indicate a significantly higher mortality for the pyrethrum-synergist combination compared with pyrethrum alone (Low-case letters for topical treatment, while capital letters for ingestion treatment) (Two-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ )

With the topical treatment, dillapiol was the only compound to significantly synergize pyrethrum at both 24 and 48 h post treatment. AF23 produced significantly greater mortality than pyrethrum singly at 24 h, but not at 48 h, while the other two ester analogs, AF01 and AF12, had no synergistic activity or toxicity (d.f. = 4, 10; F=30.1; p<0.05) (Fig. 3-3). When 4<sup>th</sup> instar larvae were treated by the ingestion method, all tested compounds showed significantly higher mortality than pyrethrum alone both at 24 and 48 h post treatment, with the exception of AF12 at 24 h.

When 4<sup>th</sup> instar larvae were treated with same concentration of pyrethrum plus analogs using the two different application methods, all the synergistic activities by ingestion (Fig. 3-4) were greater than by topical method (Fig. 3-3), especially at 48 h post treatment. The results with the topical method of pyrethrum/analog combination produced lower mortality after 48h than that after 24 h, with the exception of AF01. This phenomenon indicated that some larvae can recover by metabolising toxic compounds over time, thus allowing recovery and survival, but the same results were not detected by ingestion (Fig. 3-4).

Among all tested ester analogs, AF23 was the only compound that produced comparable pyrethrum synergistic activity to dillapiol against both 2<sup>nd</sup> and 4<sup>th</sup> instar SS-CPB larvae. Considering the variety of esterase enzymes in insects which play a key role in detoxifying xenobiotics, especially ester compounds, the three ether compounds, AF15, AF16, and AF31, considered to be less susceptible to esterase, were selected to test the synergistic activity with pyrethrum using 2<sup>nd</sup> instar SS-CPB.

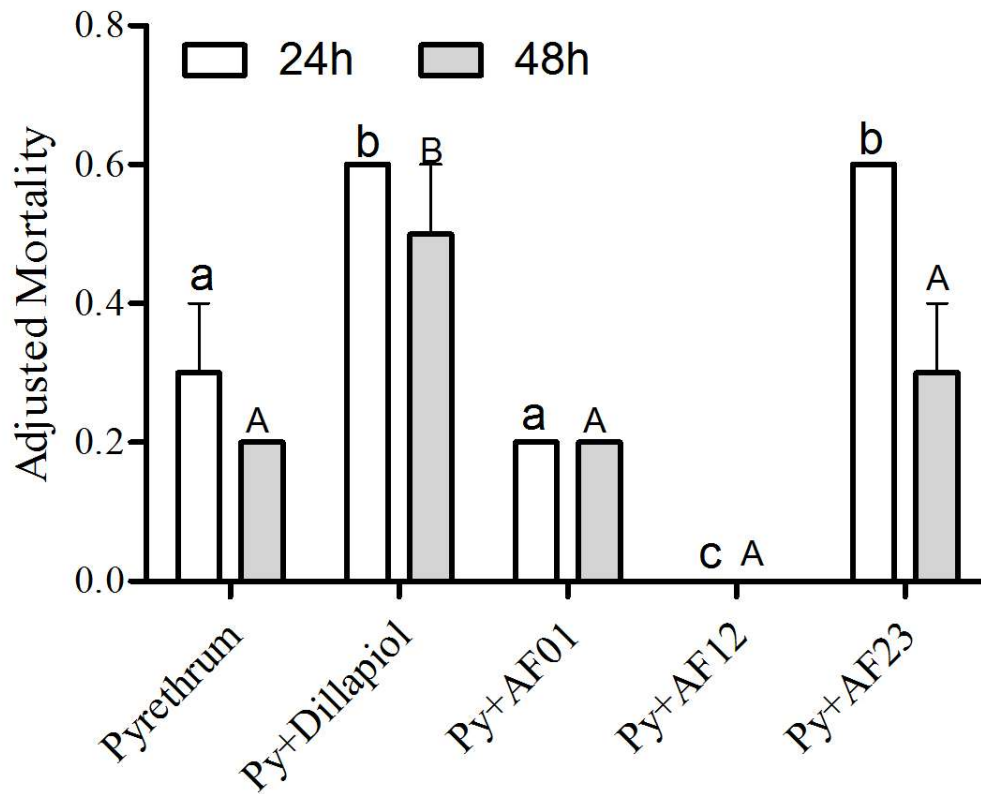


Fig 3-3 Comparison of adjusted mortality ( $\pm$  S.E.) for pyrethrum or pyrethrum (Py)/ dillapiol or 3 ester analogs combination treated with 4<sup>th</sup> instar SS-CPB larvae by topical treatment method (50 ppm) at 24 and 48 h. Bars with different letters indicate a significantly different mortality for the pyrethrum-synergist combination compared to pyrethrum alone at the same time point (lowercase letter for 24 h post-treatment, and capital letter for 48 h post-treatment) (Two-way ANOVA and Bonferroni post-hoc tests  $p < 0.05$ ).

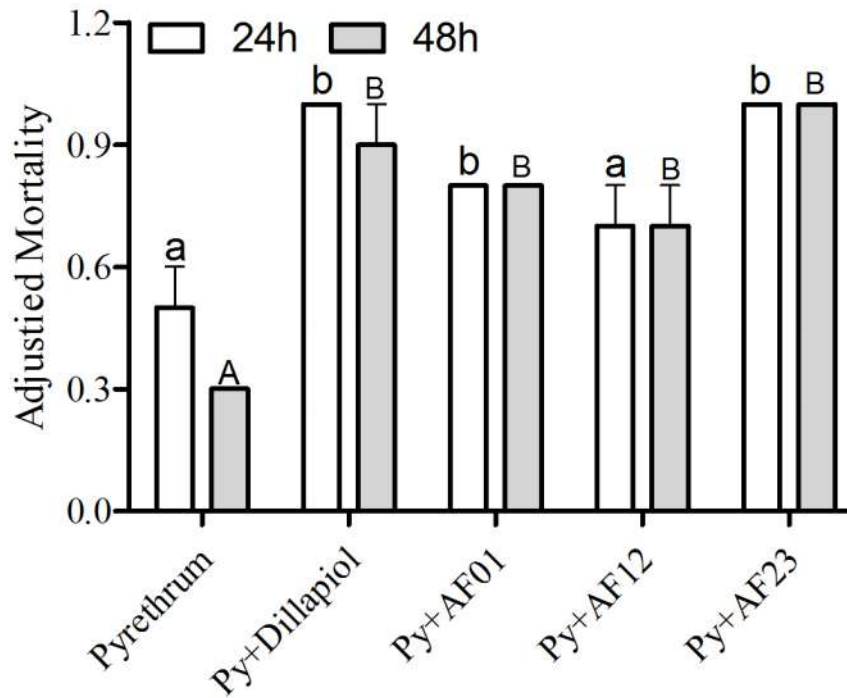


Fig 3-4 Comparison of adjusted mortality ( $\pm$  S.E.) for pyrethrum or pyrethrum (Py) / dillapiol or 3 ester analogs combination treated with 4<sup>th</sup> instar SS-CPB larvae by ingestion treatment method (50 ppm) at 24 and 48 h. Bars with different letters indicate a significantly different mortality for the pyrethrum-synergist combination compared to pyrethrum alone at the same time point (lowercase letter for 24 h post-treatment, and capital letter for 48 h post-treatment) (Two-way ANOVA and Bonferroni post-hoc tests  $p < 0.05$ ).

When the LC<sub>50</sub> values for pyrethrum plus the ether compounds were determined (Table 3-2), it was found that all three ether compounds produced weaker synergistic effects than dillapiol when the insects were treated by the topical method. In contrast, two tested ether compounds, AF15 and AF16, produced a significant synergistic effect with pyrethrum with a SR ratio of 3.9 and 4.6, respectively, while AF31 was determined to possess comparable synergistic activity to dillapiol with a SR of 2.3 by the ingestion method.

*Synergistic effect of dillapiol analogs against RS-CPB*

When 2<sup>nd</sup> instar RS-CPB larvae were tested with the selected analogs, the LC<sub>50</sub> of pyrethrum alone was determined to be 667 and 225 ppm when treated by the topical and ingestion methods, respectively (Table 3-3). Based on the LC<sub>50</sub> value for both methods, 50 and 30 ppm of pyrethrum were initially chosen to combine with the different analogs at a 1:5 ratio to test the relative synergistic activity of all compounds with topical and ingestion methods, respectively. However, the preliminary bioassay by the ingestion method produced 100% mortality of all larvae except those treated with AF 01 and AF12 at 24 h (Data not shown). Therefore, the subsequent pyrethrum concentration was decreased to 10 ppm for ingestion treatment.

Table 3-2 24 h LC<sub>50</sub> values (95% C.I.) for pyrethrum (Py) with or without ether analogs and corresponding synergism ratio (SR) determined with second instars SS-CPB larvae treated by topical and ingestion methods. The different letters behind 95% C.I. indicate significant difference of LC<sub>50</sub> value compared to pyrethrum alone due to the overlap of 95% C.I.

Insecticide with or without synergists	Topical		Ingestion	
	LC <sub>50</sub> (95% C.I.) (ppm)	SR	LC <sub>50</sub> (95% C.I.) (ppm)	SR
Pyrethrum	9.3 (8.2-10.7) a	/	3.7 (2.1-6.4) a	/
Py+dillapiol	3.7 (2.3-6) b	2.5	1.8 (1.1-2.8) a	2.1
Py+AF15	5.6 (3.8-9.9) a	1.7	0.96 (0.47-1.5) b	3.9
Py+AF16	5.5 (2.9-22) a	1.7	0.8 (0.3-1.4) b	4.6
Py+AF31	4.8 (3-10.4) a	1.9	1.6 (1.1-2.3) a	2.3

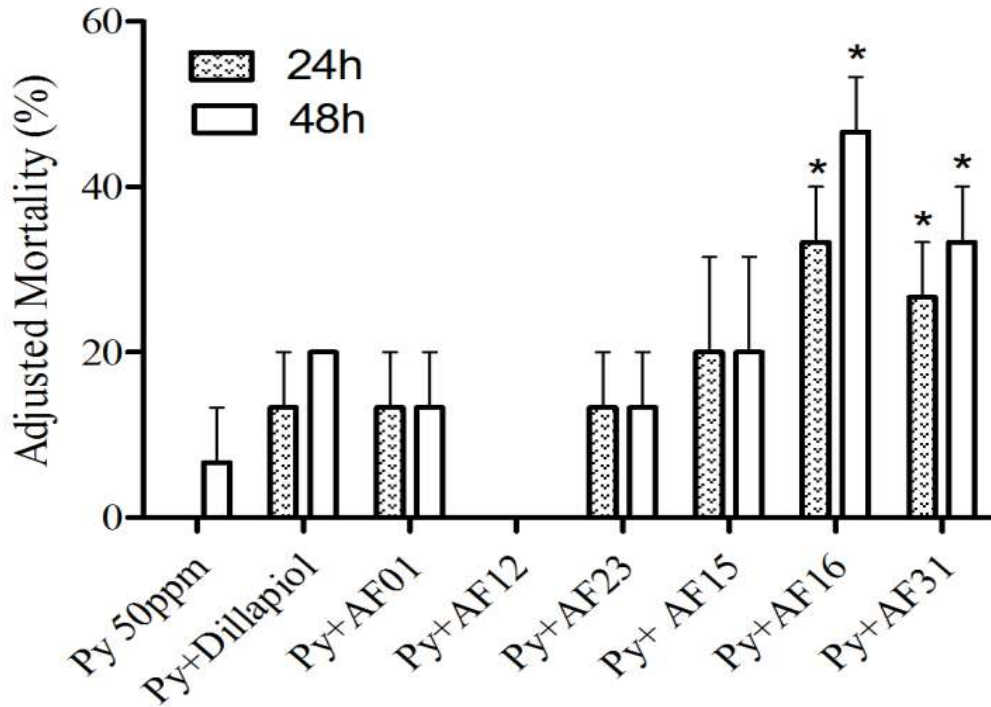


Fig. 3-5 Comparison of adjusted mortality ( $\pm$  S.E.) for pyrethrum or pyrethrum (Py)/ dillapiol or 6 analogs combination treated with 2<sup>nd</sup> instar RS-CPB larvae by topical treatment method (50 ppm) at 24 and 48 h. Bars with asteriks indicate a significantly different mortality for the pyrethrum-synergist combination compared to pyrethrum alone at the same time point (Two-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

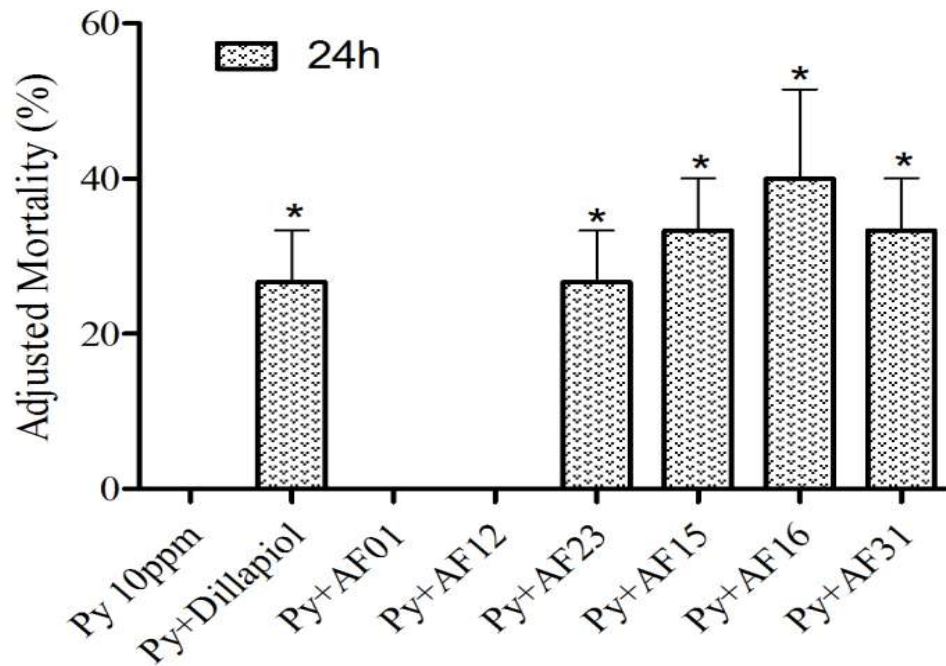


Fig. 3-6 Comparison of 24 h adjusted mortality ( $\pm$  S.E.) for pyrethrum (Py) with or without dillapiol or 6 analogs with 2<sup>nd</sup> instar RS-CPB larvae by ingestion treatment method (10 ppm). Bars with asterisks indicate significantly greater mortality of pyrethrum-synergist combinations compared to pyrethrum singly (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ )

The DC bioassays determined that the two ether dillapiol analogs, AF16 and AF31, produced significantly (d.f. =7, 16; F=4.98; p<0.05) greater mortality at 24 and 48 h than pyrethrum alone when applied topically to RS-CPB larvae (Fig. 3-5). All other dillapiol analogs increased mortality, but not significantly more than pyrethrum alone (p>0.05). When RS-CPB larvae were fed potato leaf discs pretreated with 10 ppm pyrethrum plus synergists, there was significantly (d.f. =7,16; F=7.51; p<0.05) higher mortality with dillapiol, the AF23 ester analog and all three ether analogs, AF15, AF16 and AF31, compared to pyrethrum alone (Fig. 3-6). Ingestion of the esters, AF01 and AF12, did not significantly increase mortality (p>0.05). These bioassays indicate that the three ether compounds possess greater potential as pyrethrum synergists than the ester compounds.

When dillapiol and the AF16 and AF23 analogs were combined with six pyrethrum concentrations at a 5:1 ratio, the resulting LC<sub>50</sub> values was lower, in all cases, than pyrethrum alone for both topical and ingestion treatments and the synergism ratio (SR) ranged from 4 to 27 (Table 3-3). All compounds showed a higher SR by ingestion rather than by topical exposure. The SR values of dillapiol, AF23 and AF16 tested in the ingestion assay were 15.8, 20 and 27, respectively. These compounds lowered the pyrethrum concentration required for RS-CPB control to a level effective against the SS-CPB (LC<sub>50</sub>= 3.7 ppm with 95% C.I. between 2.1-6.4 ppm) (Table 3-2). Dillapiol and the selected analogs therefore restored the pyrethrum susceptibility of the RS-CPB strain.

Table 3-3 24 h LC<sub>50</sub> values and 95% confidence intervals (C.I.) for pyrethrum (Py) alone or in combination with dillapiol or two analogs against 2<sup>nd</sup> RS-CPB instar larvae by topical or ingestion treatment. The different letters behind 95% C.I. denote the significant difference LC<sub>50</sub> values compared to other treatments due to the non-overlap of 95% C.I.

Insecticide with or without synergists	Topical Treatment		Ingestion Treatment	
	LC <sub>50</sub> (95% C.I.) (ppm)	SR	LC <sub>50</sub> (95% C.I.) (ppm)	SR
Py alone	635 (584.8- 691.8) a	N/A	225.5 (182.8-282.5) a	N/A
Py + Dillapiol	97.5 (76.5- 121.9) b	6.5	14.3 (3-21.8) b	15.8
Py + AF23	153.5 (132.7-185.8) c	4.1	11.3 (3-22.7) b	20
Py+ AF16	93.4 (81.6-107.4) b	6.8	8.3 (6.2-14.1) b	27

### 3.3.2 Greenhouse trial

The greenhouse trial results (Fig. 3-7) indicated that when pyrethrum at 160 ppm was combined with dillapiol or PBO (1: 5 ratio), the two synergized formulations showed similar activity to pyrethrum alone at a 10-fold higher concentration (1600 ppm) after 6 h (d.f.=6,12;  $F=2.34$ ;  $P=0.098$ ). All formulations retained high insecticide activity up to 6 h but the activity declined with longer exposure times. By 24 h, the residual activities of the two formulations with synergists was significantly less active than pyrethrum alone at 1600 ppm, and produced similar mortality (40 to 50%) with no statistical difference between PBO and dillapiol ( $p<0.05$ ) (Fig. 3-7).

### 3.3.3 Field trial

To test the bioactivity of dillapiol under more realistic field conditions, a microplot trial was performed and included PBO as the positive control. All three tested formulations demonstrated similar trends indicating that the residual bioactivity was very short. All tested formulations produced mortality from 30 to 40% with no significant difference among them after 6 h post-treatment, while the pyrethrum at 1600 ppm maintained activity up to 12 h which was significant greater than dillapiol but not PBO treatment. The bioactivity of synergized formulations was significantly reduced after 12 h compared to pyrethrum alone, but no statistical difference was observed between PBO and dillapiol formulations (d.f.= 2, 12;  $F= 3.4$ ;  $p= 0.1$ ) (Fig. 3-8). Since no difference was determined between the 1600 ppm pyrethrum and the pyrethrum + dillapiol combination (160 ppm pyrethrum + 800 ppm dillapiol) up to 6 h ( $p> 0.05$ ), the level of active ingredient could be reduced to 160 ppm pyrethrum by synergizing with dillapiol and moderate to complete control of insecticide-susceptible populations on the plants could be obtained.

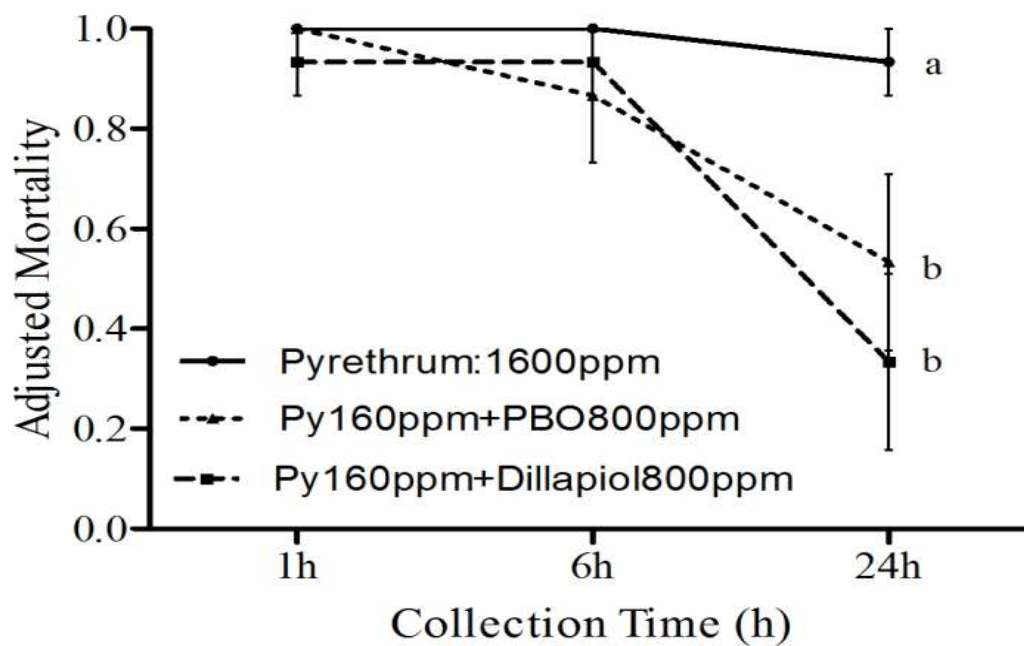


Fig. 3-7 Adjusted mortality  $\pm$  S.E. for SS-CPB 2<sup>nd</sup> instar larvae at 1, 6, and 24 h post application of pyrethrum or pyrethrum-synergists combinations on greenhouse potato plants. Two-way ANOVA and Bonferroni post-hoc tests were performed. Different letters indicate a significant difference from the 1,600 ppm pyrethrum treatment or other formulation ( $p < 0.05$ ).

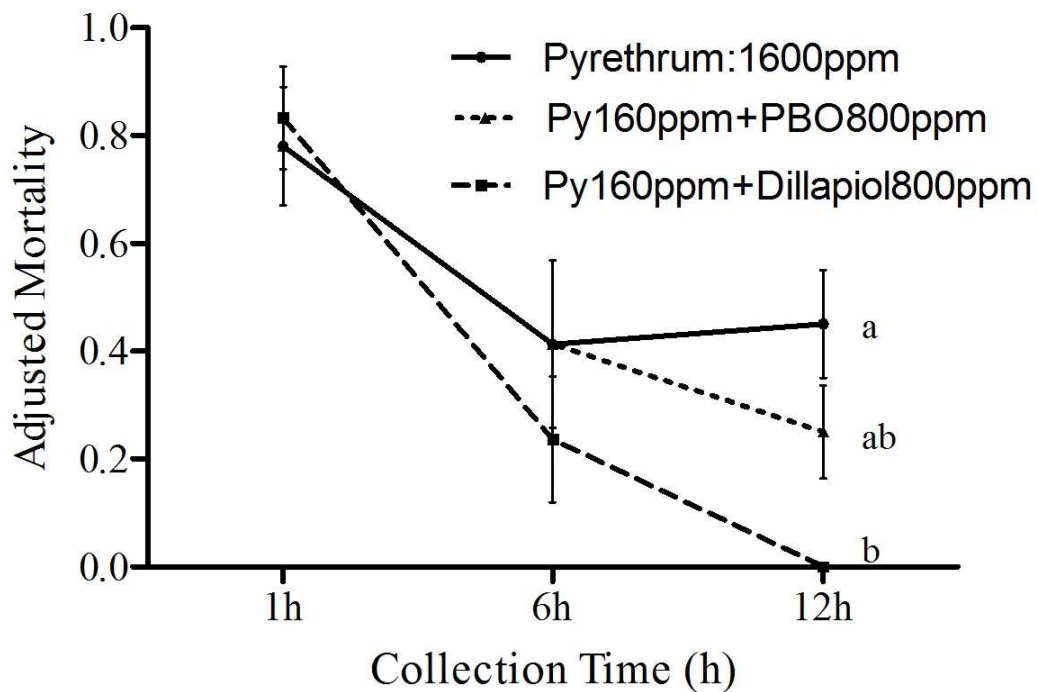


Fig. 3-8 Percent mortality  $\pm$  S.E. for SS-CPB 2<sup>nd</sup> instar larvae at 1, 6, and 12 h post application of pyrethrum or pyrethrum-synergists combinations on microplot potato plants. Two-way ANOVA and Bonferroni post-hoc tests were performed. Different letters indicate a significant difference from the 1,600 ppm pyrethrum treatment or other formulation ( $p < 0.05$ ).

### 3.4 Discussion

The dillapiol synergists were highly effective against the insecticide-resistant strain of CPB. Under laboratory conditions, dillapiol performed as a pyrethrum synergist that effectively reduced the amount of insecticide required up to 15.8-fold for insecticide-resistant Colorado potato beetle. The synergist effect with dillapiol was greater with the RS-CPB than with the susceptible strain as the synergism ratio was determined to be 6.5-fold versus 2.5-fold (topical treatment), and 15.8-fold versus 2.1-fold (ingestion treatment). The same trend was observed using CPB 2<sup>nd</sup> instar larvae of both strains (Table 3-2, 3-3). These results supported my hypothesis that the selected compounds would show greater synergistic activity with the resistant strain than with the susceptible one. Similarly, Zimmer *et al.* (2011a, b) found that PBO reduced the pyrethroid-resistance dramatically in lambda-cyhalothrin resistant strains of *Meligethes aeneus* (Coleoptera: Nitidulidae) pollen beetles, but showed no significant synergistic effect in the highly susceptible strain.

With respect to the ester analogs, only AF23 showed comparable activity to dillapiol in both mortality assays against 2<sup>nd</sup> and 4<sup>th</sup> SS-CPB or 2<sup>nd</sup> RS-CPB instar larvae (Fig.3-2, Fig. 3-4, Fig. 3-5, and Fig. 3-6). The relatively strong activity of AF23 compared to AF01 and AF12 is likely due to its decreased susceptibility to the esterase enzymes by increased steric hindrance at the carbonyl carbon. Since esters are susceptible to esterase-induced hydrolysis, the synergistic activity of comparable ether compounds successfully proved that altering this structure created a more active molecule. All three ether compounds produced commensurate or greater synergistic activity to dillapiol by ingestion treatment for both SS- and RS-CPB larvae (Table 3-2, Fig. 3-6), while two of these, AF16 and AF31, were more active than the lead molecule, dillapiol, in the topical mortality assay (Fig. 3-5). There is a greater synergism ratio of the ether AF16 (27)

compared to the ester AF23 (20) and the relative to dillapiol (15.8) (Table 3-3) which indicated that ester bonds in the molecule could be the factor that renders ester analogs a lower synergistic bioactivity in the present study. The ether bond in structural analogs deserves more attention when designing or selecting potential synergists in future research.

The differences in toxicity observed for the dillapiol analogs in topical and ingestion assays strongly supported my hypothesis that a greater effect of the synergist (combined with pesticide) would be detected with the ingestion method than that with the topical method (Fig. 3-5 and 3-6). The variations are probably due to differences in toxicokinetics. For example, the  $LC_{50}$ 's are lower for the same treatments applied by ingestion rather than topically for both SS-, and RS-CPB larvae (Table 3-2, 3-3). The robust insect cuticle is a greater barrier to penetration (Vincent and Wegst, 2004) than the gut. The one exception is dillapiol itself, which showed comparable or higher synergistic activity than other tested analogs with topical treatment against SS-CPB (Fig. 3-2, 3-3; Table 3-2), but not with the ingestion methods. This may be explained by noting that dillapiol is an essential oil compound extracted from fruits of *P. aduncum* with smaller molecular weight than the analogs. Essential oil compounds like dillapiol are known to easily pass through the insect cuticle and gain access to the functional site for toxicity (Regnault-Roger *et al.* 2012). Several other essential oil components, including eugenol, pulegone and citronellal can also readily penetrate the blowfly (*Chrysomya megacephala* Fabricius) cuticle and interfere with flight muscle central nervous function (Waliwitiya *et al.* 2012). A very recent and detailed study (Tak 2015) showed similar results observed by GC-MS analysis which detected the penetration-enhancing effects of 1, 8-cineole extracted from rosemary (*Rosmarinus officinalis* L.) when topically applied with camphor against third instar larvae of cabbage looper (*Trichoplusia ni* Hübner).

The potential of dillapiol as a pyrethrum synergist for organic agriculture was tested for the first time. Under both greenhouse and field conditions, the pyrethrum/dillapiol combination was as effective as pyrethrum/PBO formulation and a 10-fold higher pyrethrum concentration for up to 6 hours. The toxicity of combinations decreased rapidly during the treatment mainly due to the degradation of the pyrethrum under sunshine, but dillapiol achieved comparable bioactivity to PBO during all treated time points which indicated that dillapiol can act as the potential pyrethrum synergist to replace PBO for the insect pest control of the organic farms.

In conclusion, among six dillapiol analogs, three ester analogs, selected as the first generation synergists, displayed bioactivity lower than expected which led to the design of the second generation compounds, comprised of three ether analogs with molecular structure more resistant to esterase hydrolysis by insects. The toxicity bioassay results revealed that the second generation compounds have greater potential as effective synergists. Three ether analogs demonstrated stronger pyrethrum synergistic activity against both SS- and RS-CPB. The modification of the dillapiol structure improved the synergism ratio from 15.7 with dillapiol to 27 with AF16. With the high synergism ratio, dillapiol and tested analogs can successfully restore the susceptibility of RS-CPB to pyrethrum. Finally the natural compound dillapiol showed great potential as a pyrethrum synergist not only in the laboratory, but also demonstrated comparable synergistic activity to PBO under both greenhouse and field conditions, thus suggesting the feasibility as a novel PBO replacement for organic farming.

# **CHAPTER 4 DILLAPIOL AND ITS ANALOGS AS PYRETHRUM SYNERGISTS FOR CONTROL OF POLYPHAGOUS EUROPEAN CORN BORER, OSTRINIA NUBILALIS (HUBNER)**

## **4.1 Introduction**

European corn borer (ECB), *Ostrinia nubilalis*, Hübner (Lepidoptera: Pyralidae), is a highly polyphagous moth, which was accidentally imported into North America with broom corn, *Sorghum bicolor* L. (Poales: Poaceae), in 1909. Its host plant range includes over 223 reported species (Lewis, 1975), but in North America, the most economically important host is corn *Zea mays* L., and it has caused and continues to cause the highest level of concern to sweet corn growers since this insect species was introduced (Hudon *et al.*, 1989).

ECB is a tunneling insect which feeds initially on leaves after the larvae hatch and then bores into the stems of plants causing early breakage of tassels and stalks. Subsequent feeding on conducting tissues disrupts the nutrient and water flow of plants (Martin *et al.*, 2004) which leads to stalk lodging and impairs ear development leading to yield loss. In Canada, ECB is an established sweet corn pest which feeds in the developing corn ear (Hudon and McLeod, 1994). Its yearly widespread occurrence in Ontario and Quebec, the provinces that produce 85% of the sweet corn in Canada, creates high economic damage in this crop (Agriculture and Agri-Food Canada, 2012). ECB not only reduces the quality of ears for sale as fresh market products, but small larvae may remain in kernels of sweet corn resulting in the chances of increased contamination during processing. Sweet corn ear damage tolerance levels vary depending on the processor or fresh market outlet. Some processors can tolerate up to 20 percent ears if it is only

tip damage, but only up to 5% if it is side kernel damage before the quality of the final food products is affected.

To control this insect pest, several management practices were adopted by the sweet corn stakeholders in Ontario and Quebec (Agriculture and Agri-Food Canada, 2012) including: 1) planting resistant hybrids to avoid the damage of ECB, for example, the hybrids producing the defensive phytochemical DIMBOA (2,4-dihydroxy-7-methoxy-1, 4- benzoxazin-3-one) (Ostrander and Coors 1997) or having increased stalk strength (Martin *et al.* 2004); 2) physical practices to prevent ECB reproduction, including crop residue removal, infested material elimination, or *Bt*-corn cultivation; and 3) the use of insecticides such as, acetamiprid (neonicotinoid), carbaryl (carbamate), chlorantraniliprole (anthranilic diamide), and cypermethrin (pyrethroids) etc. (Pest management regulatory agency label database, 2014). Insecticides remain the major control strategy; however, the use of up to 10 spray applications per year leads to significant environmental contamination and risks of insecticide residues in food.

The use of synergists is one way to reduce the amount of insecticides used in sweet corn and their associated risks. In the present chapter, the objective was to test the synergistic activity of six selected dillapiol analogs with pyrethrum in comparison to the synergists PBO, and dillapiol with ECB larvae. In addition, the intrinsic toxicity of the synergists PBO, dillapiol and the most active ester and ether analogs was evaluated with ECB growth inhibition bioassay.

## 4.2 Material and methods

The insecticide and synergists employed in the present study were identical to the ones used in Chapter 3 with CPB larvae. Pyrethrum or each synergist was dissolved either in acetone (topical treatment) or ethanol (ingestion treatment and growth inhibition bioassay) to make a final concentration solution at a 1:5 (pyrethrum/ synergist) ratio.

#### 4.2.1 Insect source

Eggs of insecticide-susceptible ECB were provided by University of Guelph Ridgetown Campus, ON, Canada. The larvae were reared with meridic diet containing wheat germ (Guthrie *et al*, 1985) and maintained without exposure to insecticides for > 10 generations in a growth incubator at University of Ottawa, ON, Canada, at  $25\pm 1^\circ\text{C}$ ,  $60\% \pm 5$  RH and a photoperiod of 16:8 L: D, prior to use in the bioassay.

Two insecticide exposure methods, topical and ingestion, were used to test the synergistic potential of dillapiol analogs against ECB with a pyrethrum discriminating concentration (DC) and a range of pyrethrum concentrations. A growth inhibition bioassay was used to test the intrinsic toxicity of selected compounds with ECB larvae.

#### 4.2.2 Insect bioassay

The topical application bioassay was conducted by applying 5  $\mu\text{L}$  of each solution on the dorsal thoracic segments of second instar larvae (hatched 5-6 days) using a 5  $\mu\text{L}$  micro-applicator (Hamilton Robotics, Reno, NV). Larvae dosed with acetone only served as the control. Five larvae were used per replication. After treatment, larvae were held in the growth incubator with fresh diet at  $25\pm 1^\circ\text{C}$ ,  $60\pm 5\%$  RH and a photoperiod of 16:8 L: D. Mortality was assessed after 24 h by gently touching the larvae with a probe to assess response or mobility. Moribund larvae were scored as dead.

The oral ingestion treatments were prepared by adding the pyrethrum ethanol solution at different concentrations with or without synergist into the meridic diet mixture. Diet containing ethanol (0.5%) only served as the control treatment. Five 2<sup>nd</sup> instar larvae were transferred to individual cups (1 oz; Frontier Agricultural Sciences, DE, United States) containing two treated diet cubes and covered with translucent lids. All larvae were held in the growth incubator at the

conditions described above. Mortality was recorded at 24, 48 and 72 h by gently touching the larvae with a probe to assess response or mobility. Moribund larvae were scored as dead.

#### 4.2.3 Growth inhibition bioassay

The toxicity of the synergist was tested by measuring the impact of selected synergists on several ECB growth and development parameters including larval mortality and weight gain, days to pupation, mortality during pupation, sex ratio of ECB moths, and total mortality by the adult stage. Based on the preliminary bioassay results, AF23 and AF16 were selected for the ECB toxicity tests as they were the best ester and ether analogs, and compared their intrinsic toxicity with PBO and dillapiol. Synergist treated diet was prepared by adding the ethanol dissolved synergists into the fresh diet mixture to obtain a final synergist concentration of 100 ppm and ethanol concentration at 0.5% (wt/wt) in the diet. Newly hatched larvae were gently transferred to each growth cups (1 oz) containing one treated diet cube. Three replicate trials with ten larvae for each synergist were performed. The larval weight was recorded after 4, 10 and 12 days of feeding and the other growth and development parameters were recorded during the lifecycle.

#### 4.2.4 Statistic Analysis

Adjusted mortality was calculated by Abbott's correction (Abbott 1925). Statistical analysis was undertaken using GraphPad Prism 5.01 software, and the log-transformed and probit-transformed data were subjected to the general linear model procedure to determine the  $LC_{50}$  and 95% C.I. value of each solution and whether there was a significant difference among the  $LC_{50}$  values. A significant difference between individual solutions for DC and growth inhibition assay was tested using two-way ANOVA and Bonferroni post-hoc tests.

## 4.3 Results

### 4.3.1 Toxicity of selected pyrethrum synergists applied topically to ECB 2<sup>nd</sup> instar larvae

The LC<sub>50</sub> of pyrethrum alone was determined to be 195.4 ppm for a topical treatment of 5µL (Table 4-1). A discriminating concentration (DC) bioassay was conducted with the pyrethrum concentration set at 50 ppm and mixed with each of the synergists at a 1:5 ratio. After 24 h treatment, all tested solutions, except AF12 and AF31, produced significantly greater mortality than unsynergized pyrethrum, while AF12 was the only analog that did not display statistically higher activity at 48 h post treatment. It was also the only compound that possessed significantly lower synergistic activity than PBO, the positive control, at both 24 and 48 h post treatments (d. f. =18, 18; F=2.33; p<0.05) (Fig. 4-1).

The full range of concentrations was applied to determine the LC<sub>50</sub> of pyrethrum plus each synergist (Table 4-1) and results showed that the synergism ratio (SR) of the tested compounds ranged between 1.1 (AF12) to 2.6 (PBO). AF 01 was the sole analog with an LC<sub>50</sub> that was not different from that of pyrethrum alone at 24 h post treatment, and all synergists, except AF12 and AF15, possessed an LC<sub>50</sub> value similar to PBO as determined by overlap of the 95% C.I.s. Two ester analogs, AF01 and AF23 showed similar synergistic activity compared to the three ether compounds.

Table 4-1 The 24 h LC<sub>50</sub> (95% C.I.) and slope ( $\pm$ S.E.) for pyrethrum (Py) with or without synergists against 2<sup>nd</sup> instar ECB larvae. Synergism ratio (SR) was determined for the topical treatment relative to Py alone. Lower case letters for LC<sub>50</sub> values denote no significant difference (same letter) between the treatment and pyrethrum alone while upper case letters denote no significant difference (same letter) between Py+PBO and the treatment, determined by the overlap of 95% C.I.s.

		LC <sub>50</sub> (95%C.I.) (ppm)	Slope ( $\pm$ S.E.)	SR
Insecticide	Pyrethrum	195.4 (175.8-219.3) a	5.04 ( $\pm$ 0.22)	/
Positive control	Py+PBO	74.1 (44.8-97.5) bA	10.86 ( $\pm$ 1.65)	2.6
Mother compound	Py+Dillapiol	89.5 (68.7-114.8) bA	5.30 ( $\pm$ 0.42)	2.2
	Py+AF01	118.3 (80-163.3) bA	7.342 ( $\pm$ 0.70)	1.7
Ester Analogs	Py+AF12	173 (146.9-204.2) aB	14.38 ( $\pm$ 1.27)	1.1
	Py+AF23	82.2 (72.9-93.1) bA	6.30 ( $\pm$ 0.29)	2.4
	Py+AF15	121.9 (103.7-142.6) bB	2.97 ( $\pm$ 0.22)	1.6
Ether Analogs	Py+AF16	111.9 (94.2-133) bA	8.37 ( $\pm$ 0.71)	1.7
	Py+AF31	86.3(66.8-109.1) bA	5.98 ( $\pm$ 0.47)	2.3

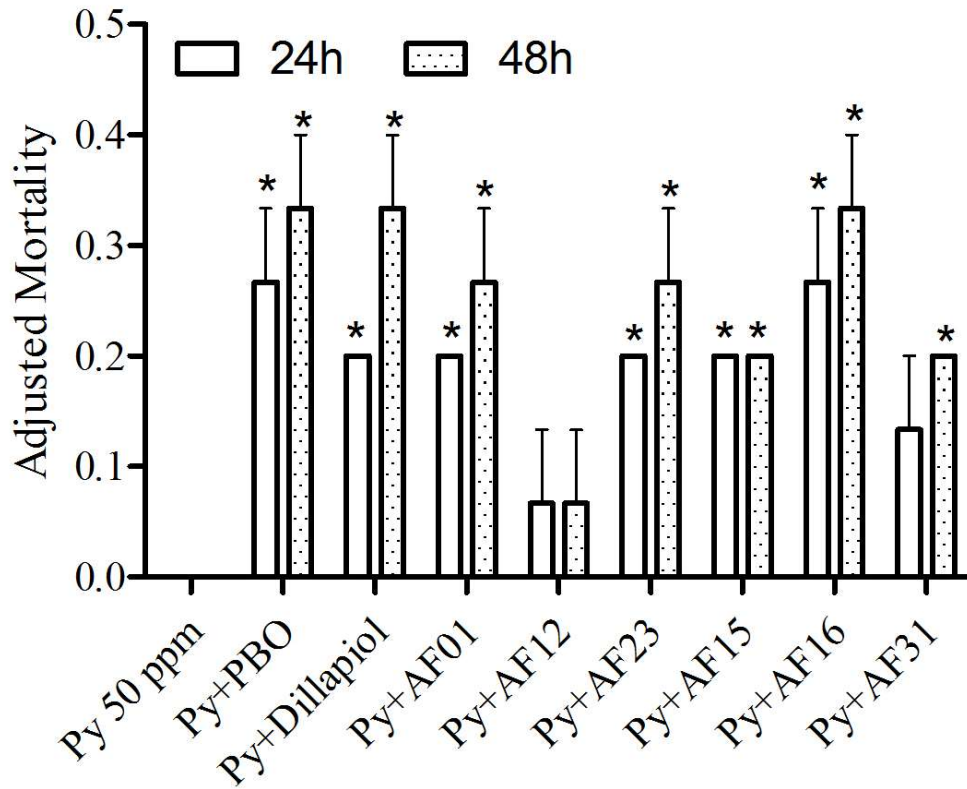


Fig. 4-1 24 and 48 h 2<sup>nd</sup> instar ECB larvae mortality  $\pm$  S.E. treated topically with eight synergists combined with pyrethrum (Py) (50 ppm) at a 5:1 ratio. Bars with an asterisk on the top indicate significantly greater mortality compared with pyrethrum (Two-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

#### 4.3.2 Toxicity of selected pyrethrum synergists ingested by ECB 2<sup>nd</sup> instar larvae

When the ECB larvae consumed pyrethrum treated diet, the pyrethrum alone LC<sub>50</sub> at 24, 48, and 72 h decreased from 884 ppm at 24h to 91.8 ppm at 72 h (Table 4-2), while the SR of the PBO combination increased from 0.9 at 24 h to 2.8 at 72 h. By contrast, the dillapiol combination had a higher SR of 3.8 at 24 h which declined to 1.8 at 72 h. The SRs determined with the analogs combinations were mixed, as the highest SR for AF16 was observed at 72 h, and for AF23 at 24 h post treatment (Table 4-2).

The DC bioassay was conducted to compare the synergism of all selected compounds combined with a final pyrethrum concentration at 100 ppm in the diet (Table 4-2). At 24 h, none of the tested analog combinations showed significantly higher activity compared with pyrethrum alone, while at 48 and 72 h, all analogs produced significantly higher mortality than unsynergized pyrethrum (d.f.= 14, 32; F=6.07; p<0.05). Dillapiol, AF23 and two ether compounds, AF15 and AF16, displayed similar synergistic activity to PBO, the positive control. However, two out of three ester compounds, AF01 and AF12 were determined to possess significantly weaker synergistic activity compared to all of other tested analogs ( d.f. = 7, 36; F= 28.6, p<0.001).

Table 4-2 The 24, 48, and 72 h LC<sub>50</sub> (95% C.I.) and slope ( $\pm$  S.E.) for pyrethrum (Py) with or without synergists against 2<sup>nd</sup> instar ECB larvae by ingestion method. Synergism ratio (SR) was determined relative to Py alone. Lower case letters for LC<sub>50</sub> values denote no significant difference (same letter) between the treatment and pyrethrum alone while a upper case letter denotes no significant difference (same letter) between Py+PBO and the treatment determined by the overlap of 95% C.I.s.

	24h			48h			72h		
	LC <sub>50</sub> (95%C.I.) (ppm)	Slope ( $\pm$ S.E.)	SR	LC <sub>50</sub> (95%C.I.) (ppm)	Slope ( $\pm$ S.E.)	SR	LC <sub>50</sub> (95%C.I.) (ppm)	Slope ( $\pm$ S.E.)	SR
Py	884 (688-1188.5) a	2.4 ( $\pm$ 0.14)	/	179 (143.9-221.8) a	2.0 ( $\pm$ 0.13)	/	91.8 (73-117) a	2.8 ( $\pm$ 0.21)	/
Py+ PBO	1004.7 (417.8-10715) aA	2.1 ( $\pm$ 0.34)	0.9	109.6 (77.3-150.3) aA	3.5 ( $\pm$ 0.35)	1.6	32.4 (13.5-54.2) bA	2.8 ( $\pm$ 0.29)	2.8
Py+ Dillapiol	236 (175.8-358.9) bB	3.2 ( $\pm$ 0.26)	3.7	71.2 (60.9-82) bA	4.01 ( $\pm$ 0.13)	2.5	51.5 (29.7-75.5) aA	4.5 ( $\pm$ 0.35)	1.8
Py +AF16	337 (249-503.5) bA	2.3 ( $\pm$ 0.18)	2.6	106 (86.9-128.8) bA	2.7 ( $\pm$ 0.17)	1.7	27.8 (15.9-38.6) bA	4.5 ( $\pm$ 0.35)	3.3
Py +AF23	266.1 (165.2-673) bA	3.0 ( $\pm$ 0.41)	3.3	96.8 (71.6-130.3) bA	4.1 ( $\pm$ 0.37)	1.8	42.3 (27.7-57.4) bA	3.7 ( $\pm$ 0.24)	2.2

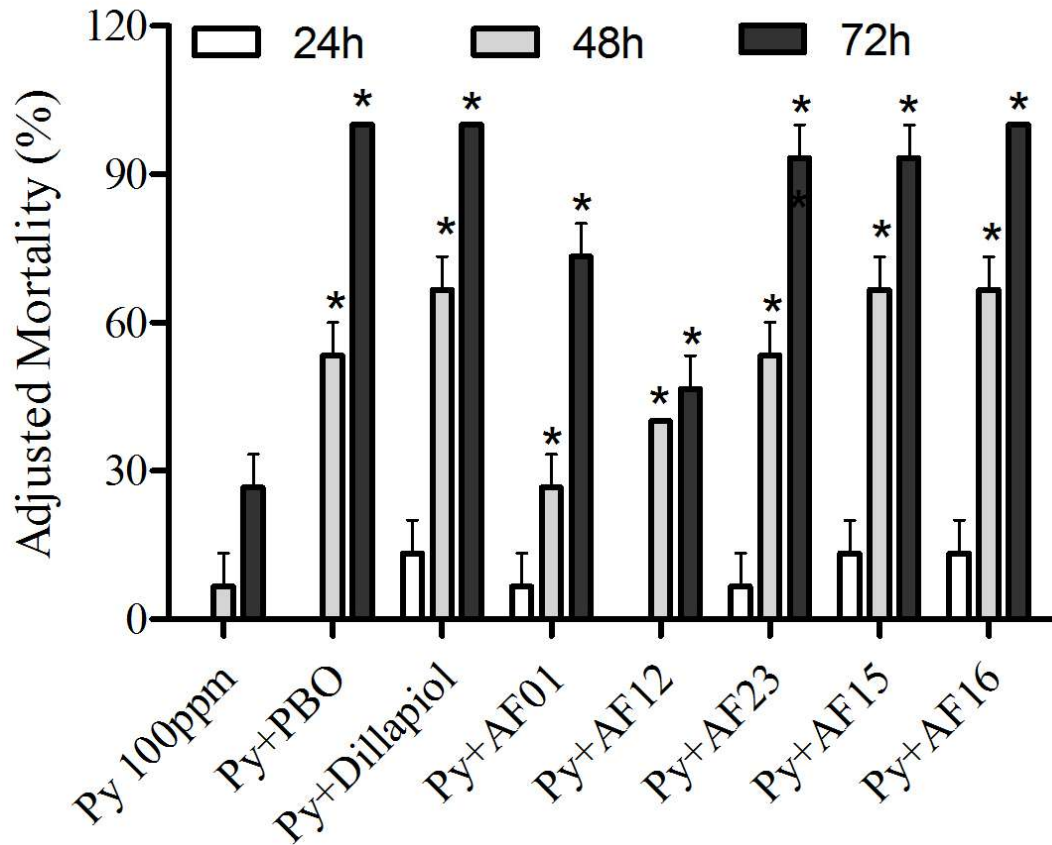


Fig. 4-2 24, 48 and 72 h 2<sup>nd</sup> instar ECB larvae mortality  $\pm$  S.E. orally treated with seven synergists combined with pyrethrum (Py) (100 ppm) at a 5:1 ratio. Bars with asterisks on the top indicate significantly greater mortality compared with pyrethrum (Two-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

### 4.3.3 Growth inhibition activity of selected compounds

The effect of the synergists ingested in the diet recorded several alterations in growth and development parameters in the ECB lifecycle.

Significant ECB larval weight differences between treated groups were observed after 4, and 10 days compared to control group, with the exception of PBO at 4 days (d.f.= 4, 566;  $F=12.34$ ;  $p<0.001$ ). However, after 12 days of feeding, the ECB larval weights in all treated group were not statistically different from the control group, with the exception of those fed AF23 (Fig. 4-3).

During the larval development stage, the weights of PBO fed larvae were not significantly different compared to those in the control diet group, except at day 10, while dillapiol and AF16 significantly influenced the larval growth after 4, and 10 days feeding. The ester analog AF23 significantly impacted the larvae weight as measured at each measurement date (d.f. = 4, 569;  $F=11.9$ ;  $p<0.0001$ ) (Fig. 4-3). In terms of the effects on larval growth, PBO and the ester analog AF23 influenced the weight gain for 10 days, while dillapiol and the ether analog AF16 negatively impacted the weight gain only during the first 4 days followed by a positive effect afterwards.

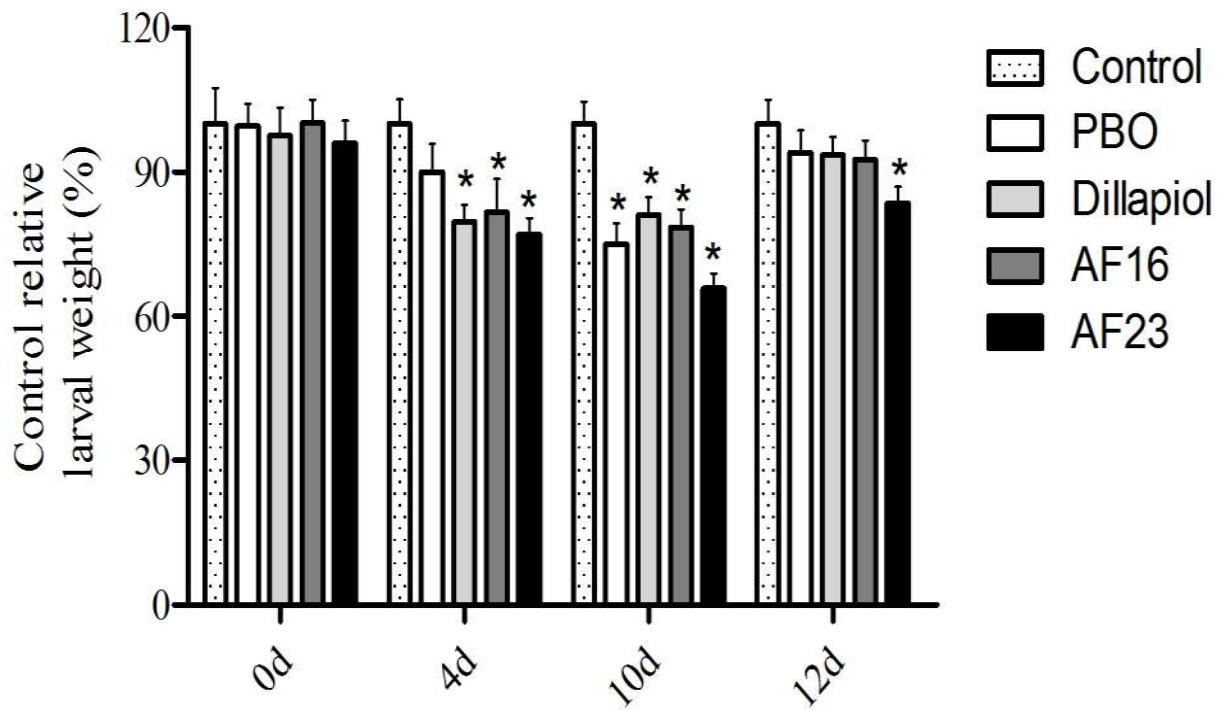


Fig. 4-3 The larval weight ( $\pm$  S.E.) of ECB relative to control group when fed diet containing ethanol (control) or synergists during larval development. Bars with an asterisk on the top indicate significantly lower larval weight compared with control group (Two-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

Mortality induced by the four synergists occurred during both the larval and pupal development stages. PBO and the ester analog AF23 produced significantly higher mortality during both the larval and pupal stages compared to the control, dillapiol and the ether analog AF16 (d.f. = 4, 20;  $F=20.97$ ;  $p<0.001$ ). The highest and lowest pupal mortality were observed in AF23 and AF16 groups at 29.7 % and 0%, respectively, compared to the control group at 8.3% (Fig. 4-4). The lifecycle mortality produced by dillapiol (3.3%) and AF23 (29.7%) was completely attributed to the mortality that occurred during the pupal stage. Dillapiol fed ECB had a lower mortality compared to all other treatments, followed by the control and AF16 treatments. ECB fed PBO (27.6%) and the ester analog AF23 (29.7%) had the highest mortality (Fig. 4-4).

The sex ratio for male: female of ECB adults was influenced by all synergists compared to the control group (Fig. 4-5). The greatest shift in the sex ratio was observed in PBO treatment where there was 0.41:1 male per female compared to the control group where male: female ratio was 1.86:1. In contrast, dillapiol produced a minor sex ratio difference of 1.36:1, while AF16 and AF23 treatments decreased the ratio to 0.77:1 and 0.73:1, respectively.

The days to pupation in the dillapiol and AF16 groups was not significantly different from control group, while PBO and AF23 significantly delayed the pupation (d.f. = 4,124;  $F=8.389$ ;  $p<0.05$ ) (Fig. 4-6 A). Similarly, the pupae weight of all larvae from synergist-treated groups was increased but not statistically greater than control (d.f. =4; 124;  $p>0.05$ ) (Fig. 4-6 B).

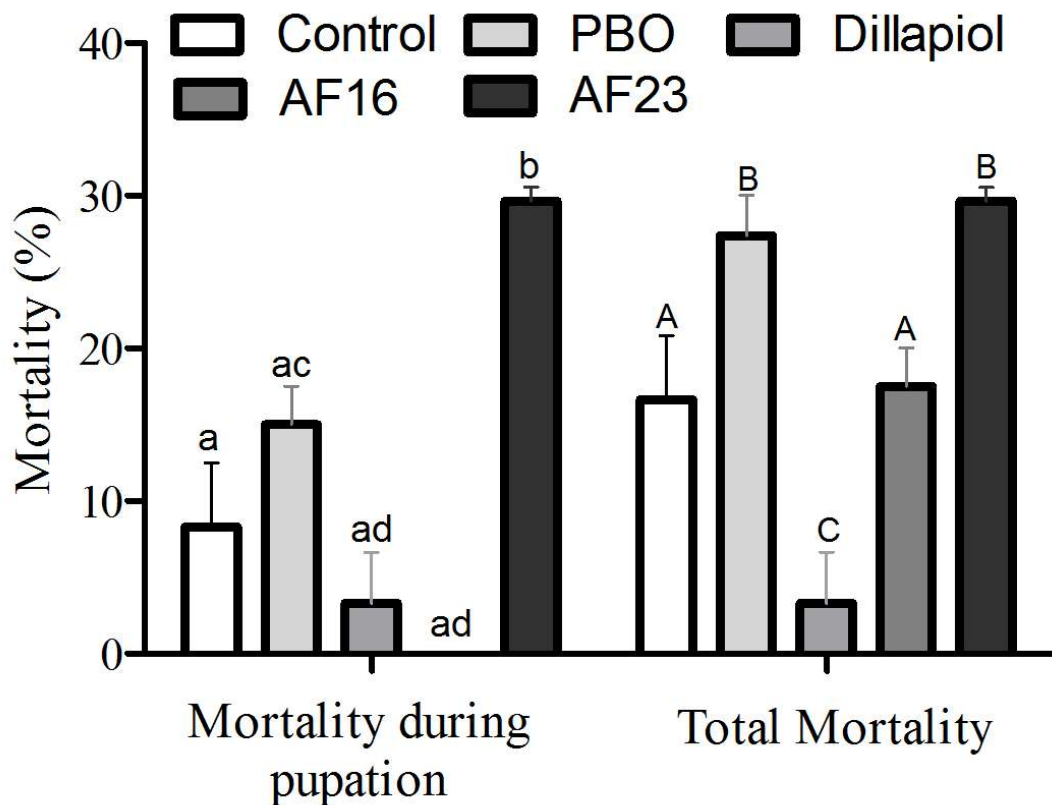


Fig. 4-4 Percentage mortality ( $\pm$ S.E.) during pupation and the total mortality of five larval groups fed diet containing ethanol (control) or synergists. Bars with different letters (lower case letters for mortality during pupation; upper case letters for total mortality) on the top indicated the significantly different mortality produced by the treatment compared with others (Two-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

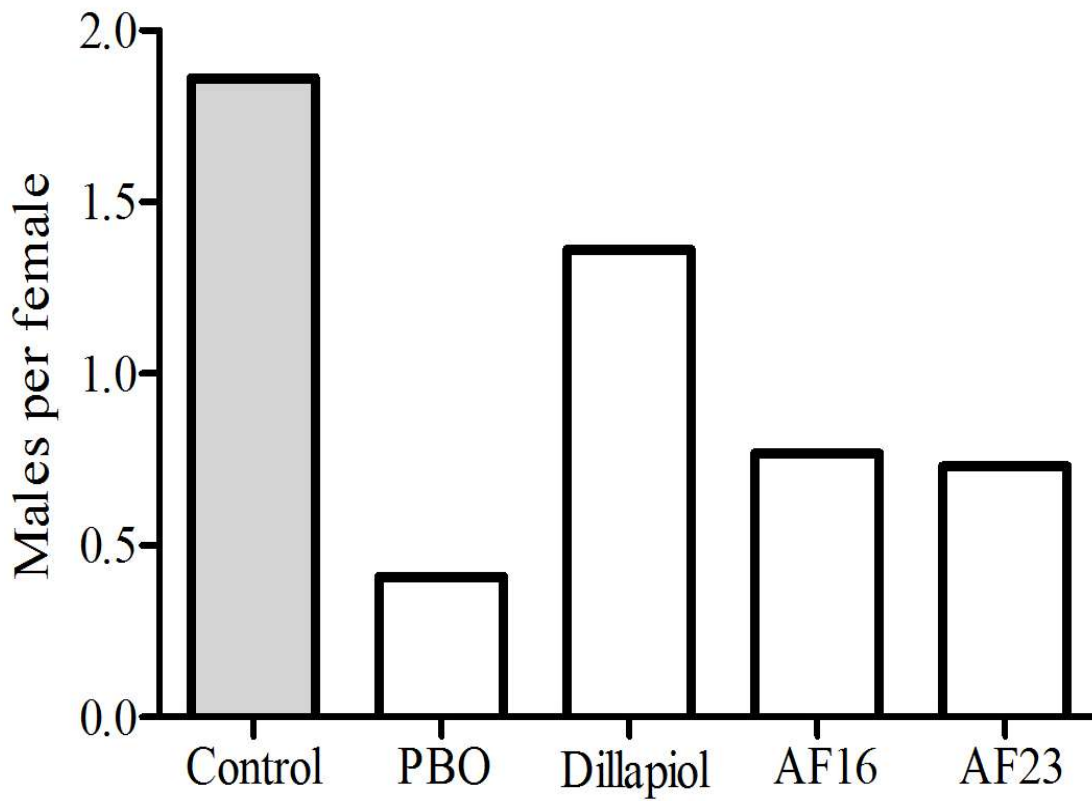


Fig. 4-5 Sex ratio (male: female) of emerged ECB adults after larvae were fed diet containing synergists or ethanol (control).

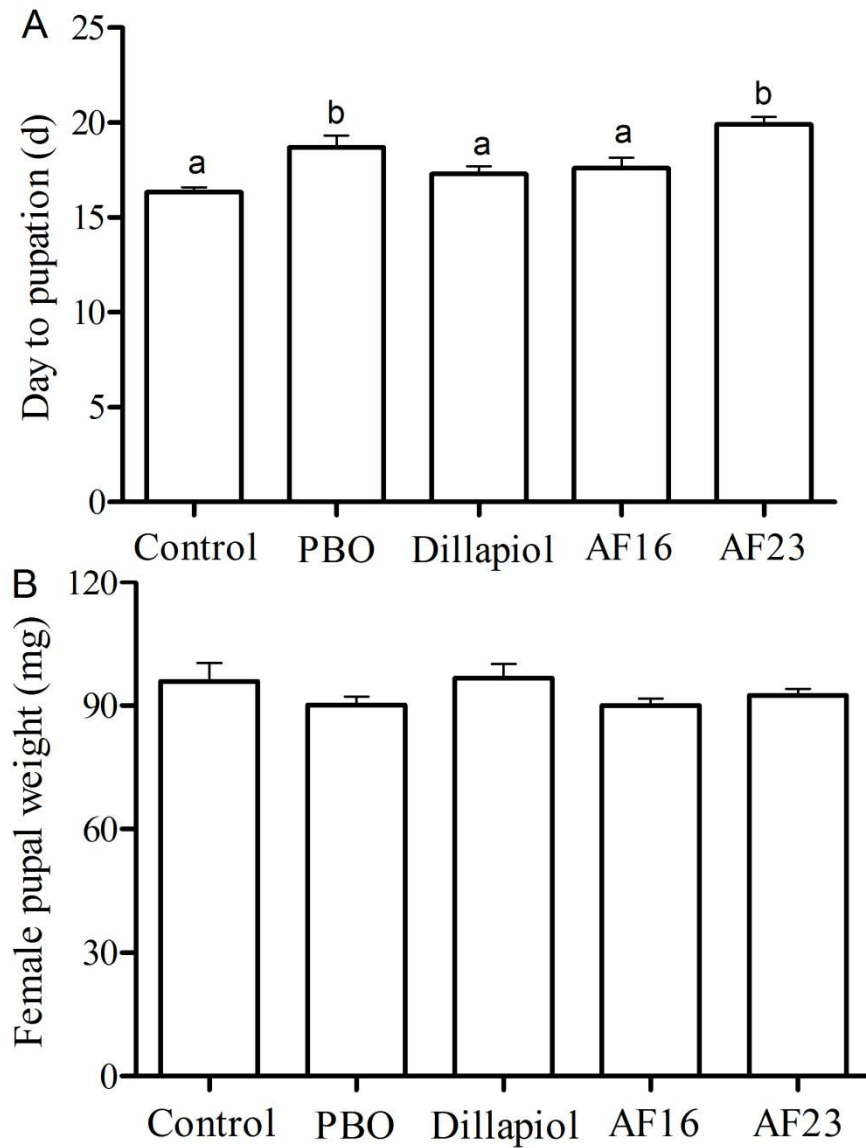


Fig. 4-6 Days to pupation ( $\pm$ S.E.) (A) and average female pupal weight ( $\pm$ S.E.) (B) for ECB fed diet containing ethanol (control) or synergists. Bars with different letters on top indicated a significant difference related to other treatments (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ). No significant difference was detected among treatments for pupal weights ( $p > 0.05$ ).

#### 4.4 Discussion

The synergistic activity of pyrethrum combined with PBO, dillapiol and three ester and three ether analogs of dillapiol using the polyphagous ECB as test organism was comparable to enzyme inhibition measured previously (Chapter 2). The ester AF12, which showed the lowest CYP3A4 inhibition activity with a dillapiol-relative inhibition ratio (DRIR) =1 among all selected analogs (Chapter 2), also displayed weaker synergistic activity than most of the other analogs in both topical and oral treatments with the ECB (Table 4-1; Fig. 4-2). By comparing the CYP3A4 DRIR (Refer to Supplement II) with the pyrethrum synergism ratio (SR) or  $LC_{50}$  value of pyrethrum combined with synergists, a similar trend can be seen where analogs with a higher DRIR have higher pyrethrum synergism ratios by topical treatment (Table 4-1).

When ECB larvae were fed pyrethrum at 100 ppm plus synergists in the diet, ether analogs showed toxicity comparable to or higher than, the three ester analogs, as well as PBO and dillapiol (Fig. 4-2). The  $LC_{50}$  of pyrethrum plus PBO, dillapiol, AF16, and AF23 determined by the ingestion method observed that PBO, the positive control synergist, exhibited the lowest and highest SR after 24 and 72 h feeding, respectively. This may be explained by the recognized antifeedant property of PBO, such that the lower SR determined at 24 h could be caused by the avoidance of the diet by ECB larvae. Starvation forced the larvae to eat the pyrethrum/PBO incorporated diet which gradually induced a higher SR ratio by 72 h. This result is not a surprise considering that PBO has been determined to be a strong antifeedant for corn rootworm (Mullin *et al.*, 1991), Colorado potato beetle and other beetle species (Nawrot and Harmatha, 1994; Mullin *et al.*, 1997). By contrast, the SR of dillapiol gradually decreased from 24 h to 72 h. This result could be caused by the volatile nature of dillapiol as an essential oil (Regnault-Roger *et al.*, 2012).

The toxicity of PBO, dillapiol, AF16 and AF23 as measured by growth and development parameters determined that PBO produced the greatest mortality in ECB (Fig.4-4), comparable to what has been demonstrated previously (Food and agriculture organization of the United Nations, 2012), and the ester analog AF23 produced comparable mortality to PBO during the larvae development. In contrast, dillapiol and the ether analog AF 16 had low or no detectable toxicity during the ECB larval stage. Both dillapiol and AF16 caused larval weight loss during the first four days but by 12 days, the weights were similar to the control group (Fig. 4-3). Neither of these two compounds significantly delayed the pupation compared to the control (Fig. 4-6 Top), and dillapiol had the lowest sex ratio shift followed by AF16 (Fig. 4-5). Unfavorable environments, for example weather and insecticide use, can cause ECB larval growth and development delays, and the sex ratio (m: f) is often decreased (Sadasivam and Thayumanavan, 2003). To confirm this, White *et al.* (2014) collected ECB moths from several locations in the Midwestern United States where transgenic *Bt* maize is widely used and found female-biased sex ratios consistent with the present study where PBO produced the highest mortality and greatest female-biased sex ratio (Fig. 4-5).

In conclusion, all selected analogs possess significant pyrethrum synergistic activity comparable to PBO, except AF01 applied as a topical treatment. Ether analogs showed comparable or stronger synergistic activity compared to ester analogs which was consistent with the toxicity bioassay results with CPB (Chapter 3). The larvae growth inhibition bioassay revealed that PBO had the strongest intrinsic toxicity with ECB larvae, followed by ester analog AF23. Dillapiol was the safest synergist among four tested compounds, while the ether analog AF16, the best pyrethrum synergist against both oligophagous CPB (Chapter 2) and polyphagous ECB, produced similar safety with dillapiol.

## **CHAPTER 5 MODE OF ACTION OF DILLAPIOL AND ITS ANALOGS AS PYRETHRUM SYNERGISTS**

### 5.1 Introduction

In insects, several major enzyme systems involved in xenobiotic metabolism, and considered in this report are the cytochrome P450s, esterases, and glutathione S-transferases.

Insect cytochrome P450s are of particular interest because of their critical biological function as the first phase in the detoxification of xenobiotics, such as insecticides and plant toxins. To successfully survive in a chemically unfavorable environment, insects display varying capacities to detoxify insecticides and xenobiotics they encountered. Under the high selection pressure from repeated insecticide exposure insects evolve resistance to insecticides. A number of studies have demonstrated that resistance is in part due to the enhanced capacity of metabolizing insecticide and this is directly linked to the increased expression and induction of P450 gene expression in insects (Terriere *et al.*, 1983, 1984; Zhu *et al.*, 2008a, 2008b, 2008c; Festucci-Buselli *et al.*, 2005; Liu *et al.*, 2011). In phase 1 metabolism, cytochrome P450s convert lipophilic toxins to more hydrophilic metabolites by oxidation, typically leading to addition of OH groups.

In insects, phase 2 metabolism is normally mediated by glutathione S transferase which conjugates the hydrophilic product of phase one metabolism with glutathione, creating a more water soluble excretable product. Insect glutathione S-transferases (GST) have been of interest for more than 50 years (Ketterman, 2011). To date, GSTs have been confirmed to be involved in development of resistance of several insecticide groups, including organophosphates and organochlorines by quantitative and/or qualitative changes of enzyme activity to increase

insecticide metabolism (Hemingway and Ranson, 2000; Li *et al.* 2007). GSTs are involved in the development of pyrethroid resistance by 1) conjugation of pyrethroids (Prapanthadara *et al.* 1998; Jirajaroenrat *et al.* 2001), and 2) their secondary enzyme activity, glutathione peroxidase activity, conferring protection against oxidative stress, which is the by-product of pyrethroid toxicity (Vontas *et al.* 2001, 2007).

Esterases deserve attention because they are often involved in resistance development to the leading chemicals that are extensively used in insect pest control programs (Li *et al.*, 2007; Nauen 2007). Young *et al.* (2006) presented evidence to show the over production of esterase isozymes which metabolize and sequester pyrethroid insecticides in resistant *Helicoverpa armigera* (Hübner) larvae. A similar study demonstrated the exceptionally high level of resistance to deltamethrin in field strain of *H. armigera* in different parts of India was partially conferred by the enhanced esterase activities (Kranthi *et al.*, 2001).

Although it is not the only mechanism of insecticide resistance, the metabolic resistance derived by the above major detoxification enzymes is a crucial problem in insect populations because it has been correlated with resistance to the major insecticide classes. Resistance by these mechanisms is also known to cause heritable cross-resistance among insecticide classes and other chemicals used for insect control (Carvalho *et al.*, 2010; Farnsworth *et al.*, 2010; Oakeshott *et al.*, 2005; Van-Leeuwen *et al.*, 2010). To combat metabolic resistance, chemical synergists can be successfully employed, such as PBO, diethyl maleate (DEM), and S,S,S-tributyl phosphorotrithioate (DEF) which are inhibitors of cytochrome P450 monooxygenases, GSTs, and hydrolases, respectively. The mode of action of the majority of synergists is to block the metabolic systems that would otherwise break down insecticide molecules.

In this chapter, the impacts of the selected dillapiol analog synergists studied in chapter 3 on three major detoxification enzymes of oligophagous Colorado potato beetle and polyphagous European corn borer were investigated. The inhibition activity of the tested compounds was compared with standard inhibitor of each enzyme used as a positive control.

## 5.2 Material and methods

### 5.2.1 Enzyme sources

To conduct the enzyme assay, fourth instar larvae of CPB or ECB were used as a source of enzymes for *in vitro* and *in vivo* biochemical assays to test the enzyme inhibitory activity of each compound. The *in vitro* assay measured enzyme inhibitory activity in a reaction system containing pooled CPB or ECB larval enzyme homogenate and the synergist, while the *in vivo* assay measured pooled enzyme activity of twenty CPB or ECB larvae that had been topically pre-treated with the synergist 24 h prior to enzyme homogenate preparation. The enzymes for the different treatments were normalized to the protein concentration as determined by BCA protein assay (Thermo Scientific, ON, Canada).

### 5.2.2 Cytochrome P450 assay

Preparation of monooxygenase enzyme was performed using the method of Liu and Scott (1998) with slight modification. Specifically, twenty CPB or ECB 4<sup>th</sup> instar larval midguts and fat bodies were dissected and the gut food bolus was carefully removed. The collected tissues were transferred to 1 mL of freshly prepared homogenization medium (HM). The HM was composed of 50 mL glycerol (40%), 1 mL ethylene diamine tetra acetic acid (EDTA) (100 mM), 1 mL dithiothreitol (DTT) (10 mM), 1 mL phenylmethyl sulfonylfluoride (PMSF) (100 mM), 1 mL phenylthiourea (PTU) (100 mM), and 71 mL 0.1M sodium phosphate (NaP) buffer with pH

7.5 for a total volume of 100 mL. The insect tissue and HM solution was homogenized at 1000×g for 1 min and centrifuged (Avanti J-26XP Beckman, Mississauga, ON, Canada) at 10,000×g for 20 min at 4°C. The supernatant was collected and centrifuged (Optima XE, Beckman, Mississauga, ON, Canada) at 100,000×g for 1 h at 4°C. The pellet was re-dissolved in a 2 mL re-suspension medium (RM). RM was prepared with 50 mL glycerol (40%), 1 mL EDTA (100mM), 1 mL DTT (10mM), 1 mL PMSF (100mM), and 47 mL NaP buffer (0.1M, pH 7.5) for a total volume of 100 mL. The microsome homogenate solution was stored at -80°C until use.

The microsomal P450 activity was determined using the 7-methoxyresorufin *O*-demethylation (MROD) assay, where methoxyresorufin is converted to resorufin by monooxygenases in the presence of NADPH and oxygen (Liu and Scott, 1998). The resorufin produced was measured fluorometrically in a 96 well microplate spectrofluorometer (Applied Biosystems, Foster City, CA) at 530/590 nm excitation/emission. Each microplate well contained 202 µL of reaction mixture which included 10 µL enzyme (1.5 mg/mL protein content), 10 µL synergist solution (5 µg/mL in methanol for CPB enzyme, and 1 mg/mL in methanol for ECB enzyme), 0.4 µL methoxyresorufin (1 mM), 2 µL NADPH (0.01 M) and 179.6 µL potassium phosphate buffer (0.1 M, pH 7.8). The percent inhibition by dillapiol or analog was determined relative to resorufin produced in the presence of the methanol control. PBO at the same concentration was used as the positive control.

### 5.2.3 Glutathione S-transferase (GST) assay

The enzyme preparation method was the same as that used for the monooxygenase enzyme homogenate preparation except that HM and RM were replaced by a sodium phosphate (Sigma-

Aldrich) buffer (pH 7.8, 0.1M sodium phosphate), and following the centrifugation at 100,000g for 1 h, the supernatant rather than pellet was collected and stored at -80°C as the enzyme stock.

The inhibitory activity of GST was determined by measuring the change in absorbance at 340 nm produced by the reaction of the glutathione thiol group of GSH (Sigma-Aldrich) with the 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich) substrate (Habig *et al.*, 1974) using a Cytofluor 4000 Fluorescence Measurement System 96 well microplate reader (Applied Biosystems, Foster City, CA). In each well, 128 µL of solution A (50 µL of 0.1M sodium phosphate buffer, pH 6.5, 70 µL of 1.5 mg/mL enzyme and 8 µL synergist dissolved at 1mg/mL in methanol) were added and incubated for 10 min at 30°C. The absorbance was measured at 340 nm for 7 min as soon as an additional 172 µL of solution B (163 µL of 0.1 M sodium phosphate buffer, 3 µL GSH and 6 µL CDNB) were added to each well. The IC<sub>50</sub> of diethyl maleate (DEM) (Sigma-Aldrich, St. Louis, MO), an inhibitor of GSTs (Wang *et al.*, 2014), was determined with the same volume in the reaction system and used as positive control. Boiled enzyme was used as the negative control.

#### 5.2.4 Esterase assay

Esterase activity was determined by measuring the change in absorbance when the substrate  $\alpha$ -naphthyl acetate is hydrolyzed to  $\alpha$ -naphthol that then couples with a diazonium to form a colored complex detected at 605 nm. The assay was performed using the same enzyme source and same microplate assay described previously in section 5.2.3. The substrate solution was prepared by mixing 780 µL 0.02 M sodium phosphate buffer (pH 7.0) and 10 µL  $\alpha$ -naphthyl acetate (25 mM). In each well, 2.5 µL of synergist (10 mg/mL in acetone), 50 µL enzyme and 197.5 µL substrate solution were added. The plate was shaken and incubated for 15 min at 30°C. The absorbance was read at 605 nm 15 min after 41 µL Diazo Blue B was added to each well.

S,S,S-tributyl phosphorotrithioate (DEF) (Sigma-Aldrich, St. Louis, MO), an inhibitor of esterase (Yang *et al.*, 2004) was used as positive control at 10 mg/mL with the same volume as tested synergist in reaction system. Boiled enzyme served as the negative control.

### 5.2.5 Statistical Analysis

All enzyme inhibition results were compared by one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests. The median inhibition concentration (IC<sub>50</sub>) value of each analog was calculated by plotting the percentage enzyme inhibition versus log concentration of the insecticide. Statistical analysis of confidence intervals (C.I.) and range of IC<sub>50</sub> values observed were carried out using GraphPad Prism 5.

## 5.3 Results

### 5.3.1 Cytochrome P450 inhibitory activity

#### *5.3.1.1 CPB cytochrome P450 inhibitory activity of selected synergist compounds*

Among all tested synergists, the ester compounds, AF01 and AF23, inhibited the *in vitro* RS-CPB monooxygenase activity as effectively as PBO at 5 µg/mL (d.f. = 8, 18; F=168.7; p<0.05), while dillapiol, AF12, AF15, AF16 and AF31 were less active (p>0.05) (Fig. 5-1). When RS-CPB were topically pretreated with PBO, dillapiol and AF16, the monooxygenase activity decreased significantly (d.f. = 4, 10; F=1052; p<0.05) (Fig. 5-2). The cytochrome P450 activity of RS-CPB larvae was more than four times greater than that of the SS-CPB but *in vivo* treatment with dillapiol and AF16 effectively inhibited this increased activity. PBO displayed weaker *in vivo* inhibition activity than both dillapiol and AF16 (Fig. 5-2).

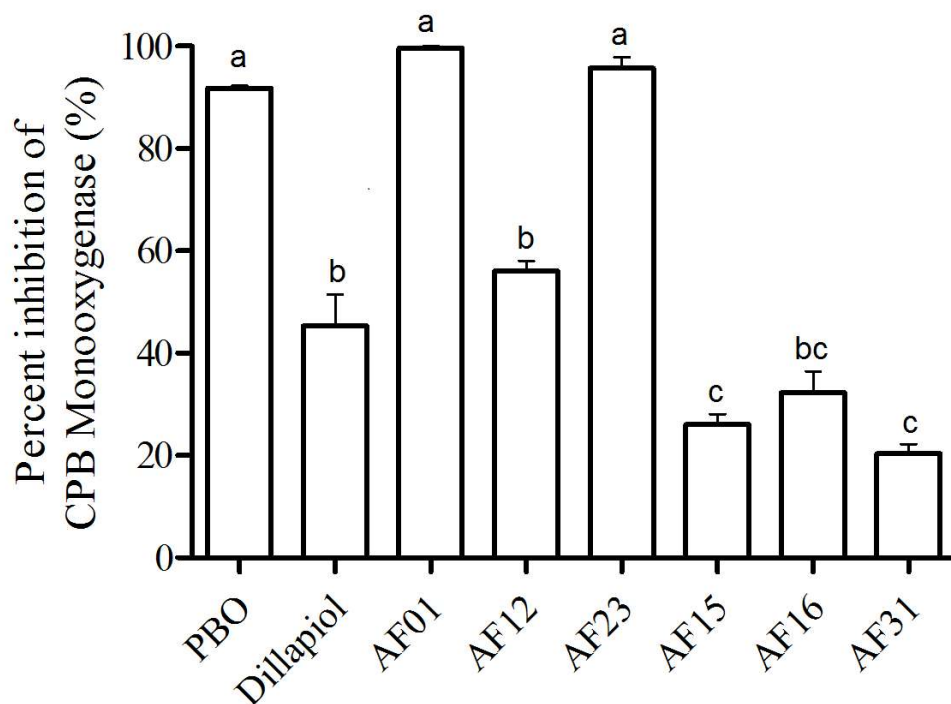


Fig. 5-1 Percent inhibition of *in vitro* MROD monooxygenase activity  $\pm$  S.E. for RS-CPB microsomes treated with eight synergists at 5  $\mu\text{g}/\text{mL}$  relative to the control (methanol). Different letters on the top of each bar denote that monooxygenase activity of synergists are significantly different from that of PBO or other synergists (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

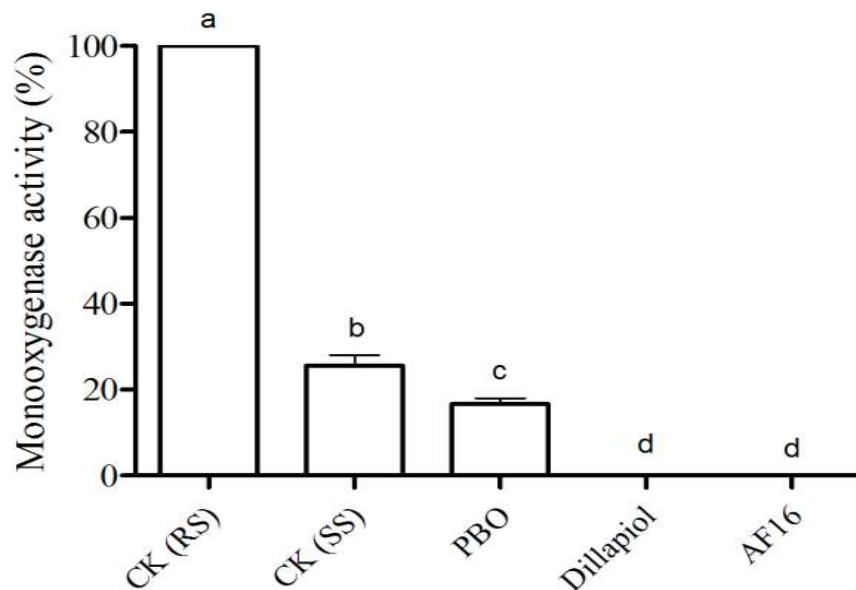


Fig. 5-2 Percent *in vivo* MROD monoxygenase activity  $\pm$  S.E. for RS- and SS-CPB pre-treated with acetone (CK) compared to RS-CPB treated with 3 synergists at 10 mg/mL. Different letters on the top of the bars denote that monoxygenase activity of synergists are significantly different from that of CK or other synergists (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

### 5.3.1.2 ECB cytochrome P450 inhibitory activity of selected synergist compounds

The effect of synergists on P450 monooxygenase activity was determined with MROD *in vivo* and *in vitro* assay (Fig.5-3, 5-4). Two ether compounds, AF15 and AF16, effectively inhibited P450 monooxygenase *in vitro* activity more than 80% at 1 mg/mL, and showed significantly greater inhibition activity than PBO (d.f.= 9, 20; F=214.9; p<0.001). Dillapiol and AF01 possessed comparable P450 *in vitro* inhibition activity to PBO, and no statistical difference was observed between PBO, dillapiol and AF01 (p>0.05) (Fig.5-3). AF16 completely inhibited P450 activity at 1 mg/mL. Subsequently the IC<sub>50</sub> of AF16 was determined to be 15 µM which is 11 times lower than that of PBO at 160 µM by the MROD *in vitro* assay, while AF 01, an ester analog, possessed similar IC<sub>50</sub> with PBO (Table 5-1).

The same trend was observed with *in vivo* testing in which the activity of cytochrome P450 from ECB pretreated with four tested compounds, PBO, dillapiol, AF16, and AF23, was significantly suppressed by up to 90% (d.f.= 4,10; F= 6742; p< 0.05) (Fig. 5-4). AF16 showed the strongest inhibition activity of all synergists tested. The P450 activity of dillapiol and AF23 treatment were statistically lower than that of the control, but neither one was as active as PBO.

Table 5-1 The IC<sub>50</sub> (95% C.I.) and slope (± S.E.) determined for four most active ECB P450 inhibitors in present study. Different letters after the 95% C.I. value denote the significant difference from each other due to the overlap of IC<sub>50</sub> values.

<b>Synergist</b>	<b>IC<sub>50</sub> (95% C.I.) μM</b>	<b>Slope (± S.E.)</b>
PBO	160 (130-220) a	42.52 (± 1.84)
Dillapol	380 (330- 470) b	71.54 (± 4.82)
AF16	15 (10- 20) c	57.22 (± 3.66)
AF01	140 (120-150) a	107.1 (± 3.11)

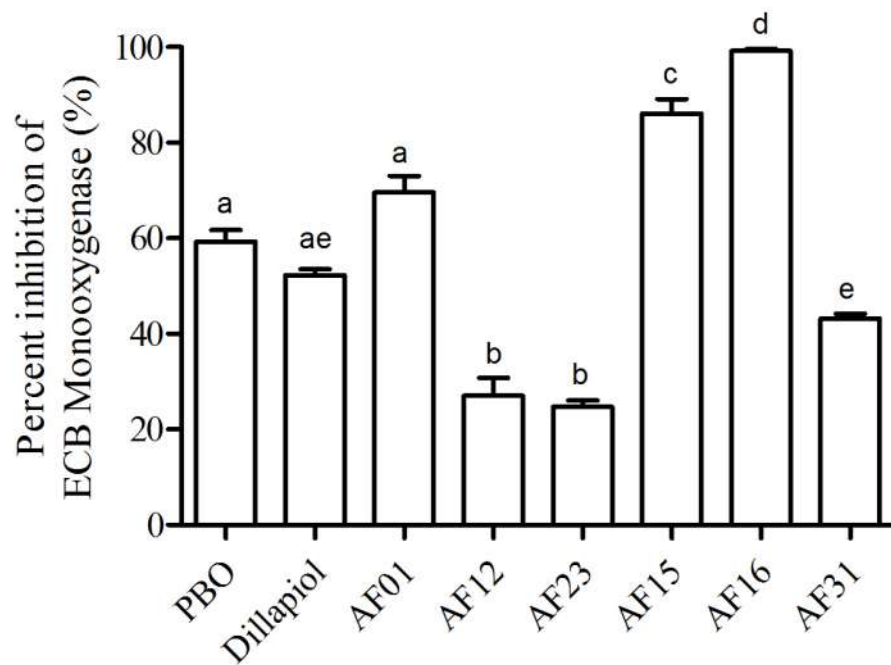


Fig. 5-3 Percent inhibition of *in vitro* MROD monooxygenase activity ( $\pm$  S.E.) for ECB microsomes treated with eight synergists at 1 mg/mL relative to ECB treated with methanol (control). Different letters on the top of the bars denote that monooxygenase activity of synergists are significantly different from that of other treatments (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

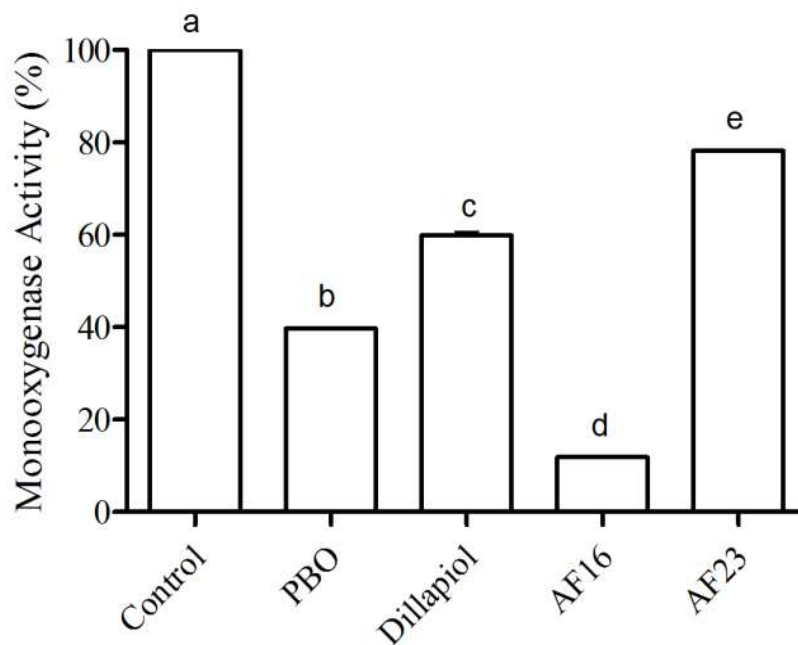


Fig. 5-4 Percent *in vivo* MROD monoxygenase activity ( $\pm$  S.E.) for ECB pre-treated with Control (acetone) or four synergists at 10 mg/mL. Different letters on the top of the bars denote that monoxygenase activity of synergists are significantly different from that of other treatments (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

## 5.3.2 Inhibition of GST activity

### *5.3.2.1 CPB GSTs inhibitory activity of selected synergist compounds*

All dillapiol analogs at 1 mg/mL significantly inhibited *in vitro* GST activity (d.f. = 8, 18;  $F=41.75$ ;  $p<0.05$ ) (Fig. 5-5). AF16, the most effective pyrethrum synergist (Chapter 3), displayed the highest GST inhibitory activity compared to all tested compounds. Ether compounds AF16 and AF31, possessed greater GST inhibitory activity than PBO ( $p<0.05$ ). The  $IC_{50}$  of AF16 was determined to be 0.23 mM, 180 times lower than the diethyl maleate (DEM)  $IC_{50}$  of 42 mM (Fig. 5-7).

RS-CPB GSTs activity was determined to be two fold greater than SS-CPB (Fig. 5-6). RS-CPB pre-treated with different analogs had significantly reduced *in vivo* GST activity (d.f. = 5, 12;  $F= 254.7$ ;  $p<0.05$ ) except for those pre-treated with PBO (Fig.5-6). The GSTs activity in dillapiol-treated RS-CPB insects was significantly lower than in untreated SS-CPB ( $p<0.05$ ), and no statistical difference of the GST activity was observed between SS-CPB and RS-CPB pretreated with AF23. AF16 showed moderate *in vivo* GST inhibition activity compare to PBO and other synergists.

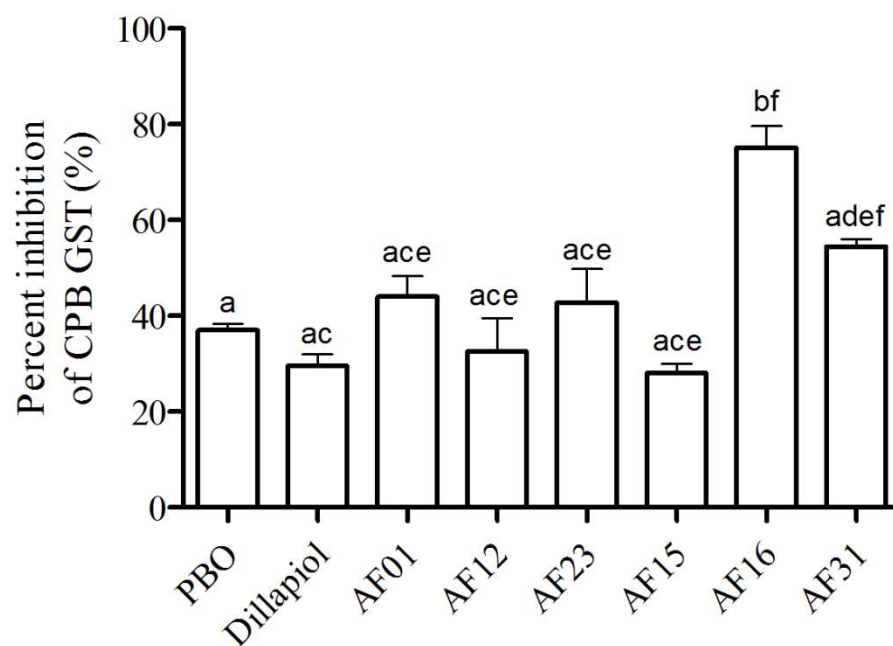


Fig. 5-5 Percent inhibition of *in vitro* GSTs activity  $\pm$  S.E. for RS-CPB enzyme homogenate treated with 8 synergists at 1 mg/mL relative to control (methanol treated). Different letters on the top of the bars denote that percent inhibition of GSTs activity is significantly different. (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

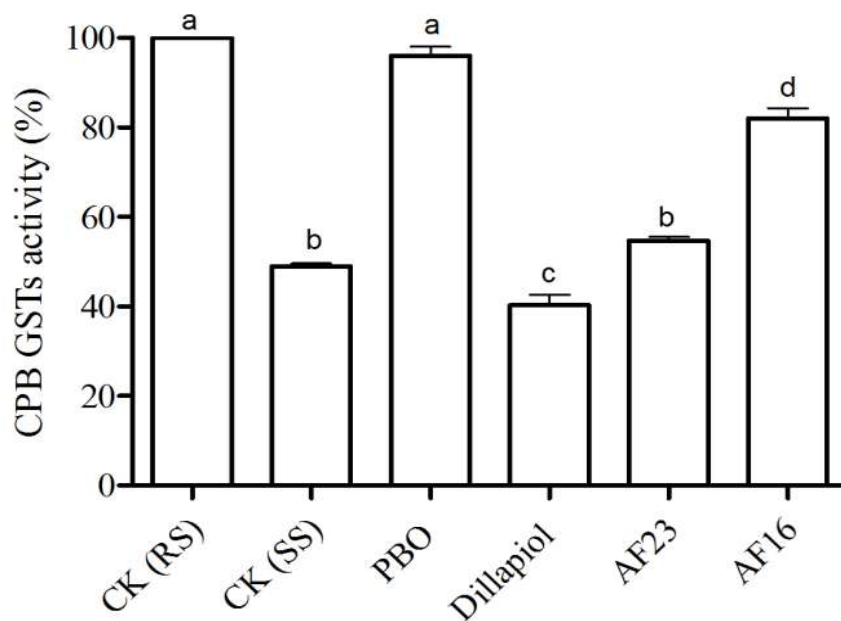


Fig. 5-6 Percent *in vivo* GSTs activity  $\pm$  S.E. for RS- and SS-CPB pre-treated with acetone (CK) or RS-CPB pre-treated with 4 synergists at 10 mg/mL. Different letters on the top of the bars denote that GSTs activity of CK or synergists are significantly different (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

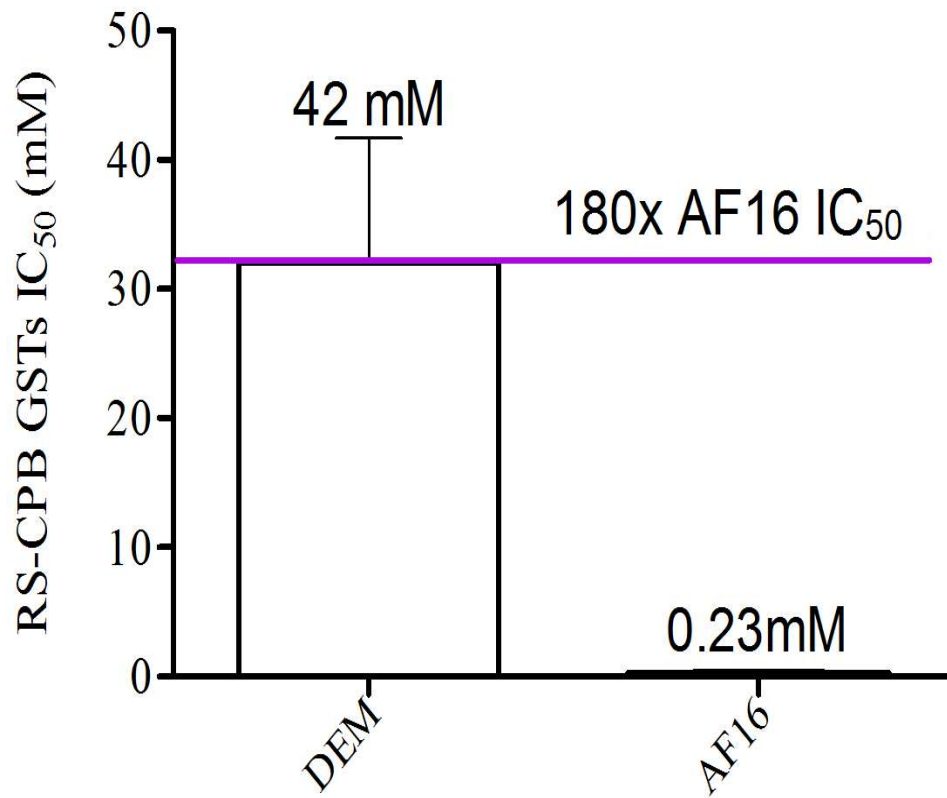


Fig. 5-7  $IC_{50} \pm$  S.E. of DEM and AF16 to inhibit *in vitro* GST of RS-CPB

### 5.3.2.2 ECB GSTs inhibitory activity of selected synergist compounds

GSTs inhibition activity of selected compounds was determined using CDNB as the substrate as previously described in 5.2.2.1. All selected compounds significantly inhibited the *in vitro* GSTs activity at 1 mg/mL compared to the control, except PBO, dillapiol and AF12 (d.f. = 7, 15; F=190.5; p<0.05) (Fig. 5-8). AF01, AF23 and AF15 showed moderate GST inhibition activity with 40 to 50 percent inhibition, and AF16 reduced *in vitro* GSTs activity more than 80% (Fig. 5-8). The IC<sub>50</sub> of AF16 was determined to be 0.04 (± 0.006) mM which is 575 times lower than DEM (IC<sub>50</sub> of 23 (± 4.4) mM) (Fig. 5-10). AF23 is the only compound that can significantly inhibit *in vivo* GSTs activity compared to control (d.f. =4, 10; F=17.17; p<0.05) (Fig. 5-9).

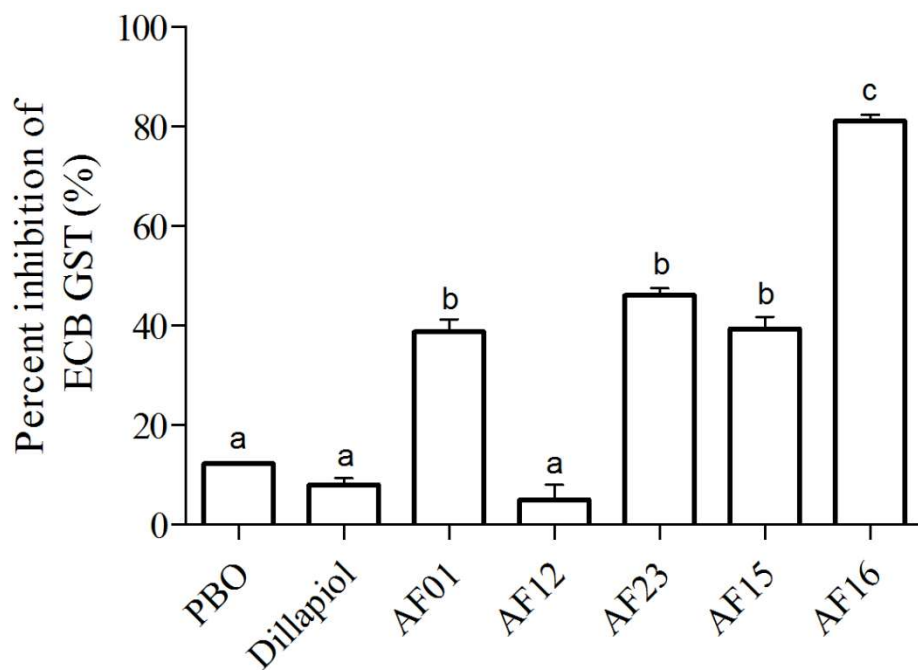


Fig. 5-8 Percent inhibition of *in vitro* GSTs activity  $\pm$  S.E. for ECB enzyme homogenate treated with 7 synergists at 1 mg/mL. Different letters on the top of the bars denote that GSTs activity of synergists are significantly different (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

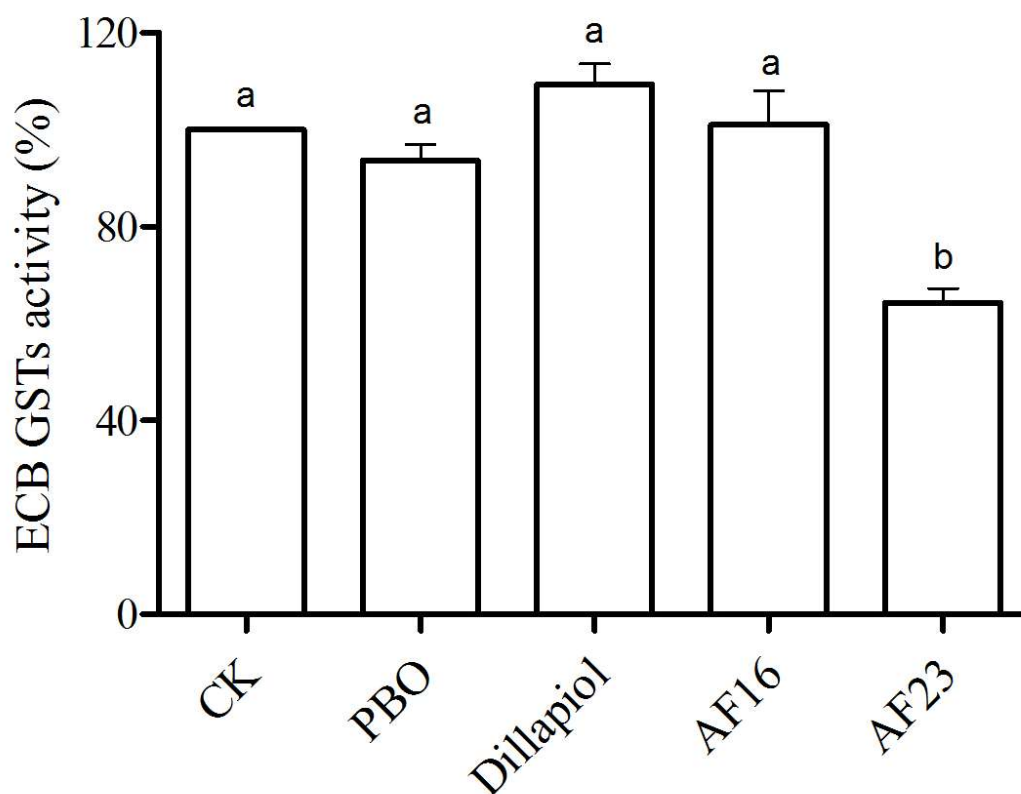


Fig. 5-9 Percent *in vivo* GSTs activity  $\pm$  S.E. for enzyme homogenate of ECB pre-treated with acetone (CK) and 4 synergists at 10 mg/mL. The bar with a letter on the top denotes that GSTs activity of the CK or synergist is significantly different (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

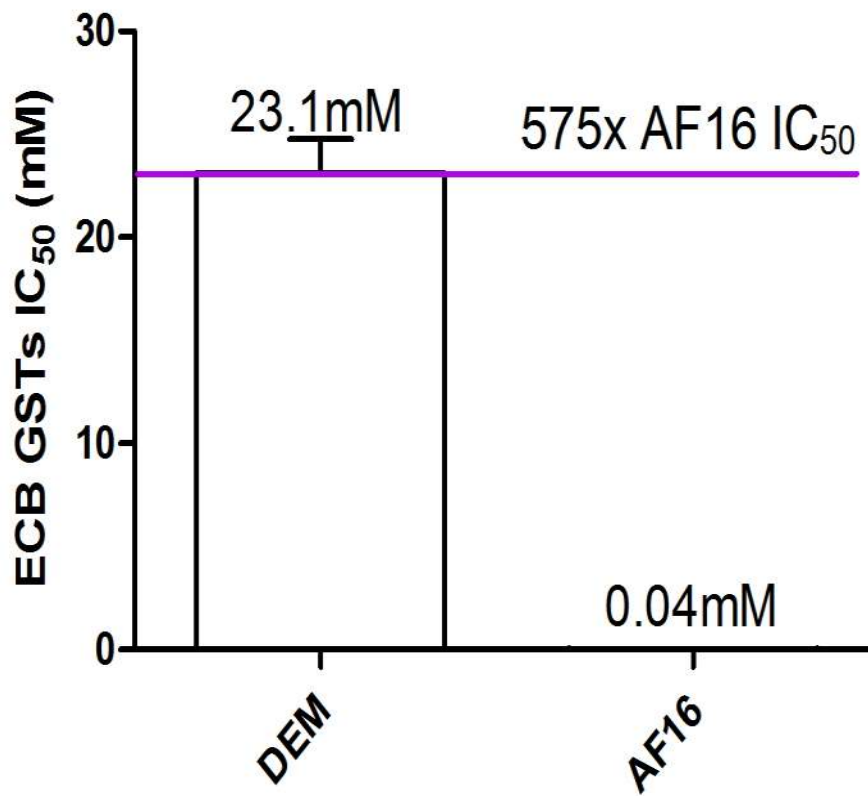


Fig. 5-10  $IC_{50} \pm$  S.E. of DEM and AF16 to inhibit *in vitro* GST of ECB

### 5.3.3 Inhibition of esterase activity

#### 5.3.3.1 CPB esterase inhibitory activity of selected synergist compounds

Most of the compounds had low activity as CPB esterase inhibitors. Of the tested compounds, the ester dillapiol analog AF12 was the only compound which displayed comparable *in vitro* esterase inhibition activity with DEF (d. f. = 8, 18; F=16.73; p< 0.05) (Fig. 5-11), while AF23 was the only ester compound that significantly lowered *in vivo* esterase activity in pretreated RS-CPB (d. f. = 5, 12; F= 10.84; p<0.05) (Fig. 5-12). No difference in esterase activity was observed between RS-CPB and SS-CPB (Fig. 5-12). Although there is no statistical difference in esterase inhibition activity between DEF and AF12 at 10 mg/mL, the IC<sub>50</sub> of DEF was determined to be 88.5 (± 19.5) µM, 5.1 times lower than that of AF12 with IC<sub>50</sub> of 454 (± 33) µM.

#### 5.3.3.2 ECB esterase inhibitory activity of selected synergist compounds

Eight synergists were tested for their ability to inhibit ECB esterases with *in vitro* and *in vivo* assay using a conventional esterase assay (Fig. 5-13 and Fig. 5-14). All synergists, except AF01 and AF15, possessed comparable inhibition activity with DEF while AF16 was the strongest inhibitor among all tested compounds at 10 mg/mL, except DEF (d.f. = 7, 16; F= 5.159; p<0.05) (Fig.5-13). However, the *in vitro* IC<sub>50</sub> of AF16 was determined to be 64 (±4.8) µM which is 36% lower than the DEF IC<sub>50</sub> of 99.7 (±29) µM. When insects were pretreated with different synergists, no significant *in vivo* esterase inhibition activity was detected after 24 h post treatment for all tested compounds (Fig 5-14).

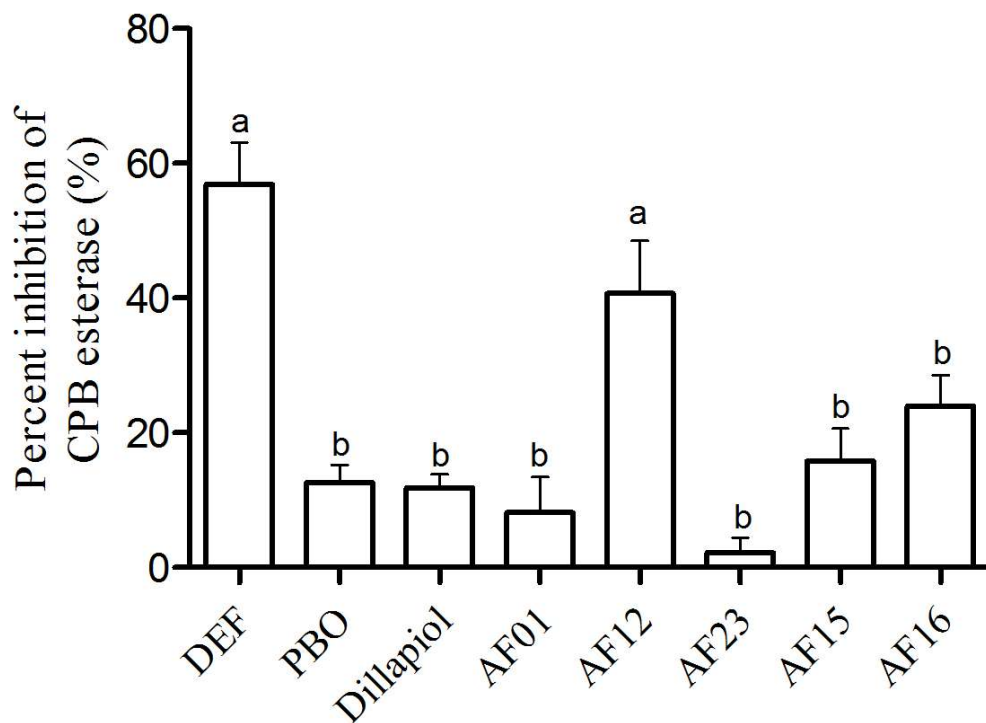


Fig. 5-11 Percent inhibition of *in vitro* esterase activity  $\pm$  S.E. for RS-CPB enzyme homogenate treated with 8 synergists at 10 mg/mL relative to acetone treated control. Different letters on the top of the bars denote that esterase activity of synergists are significantly different (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

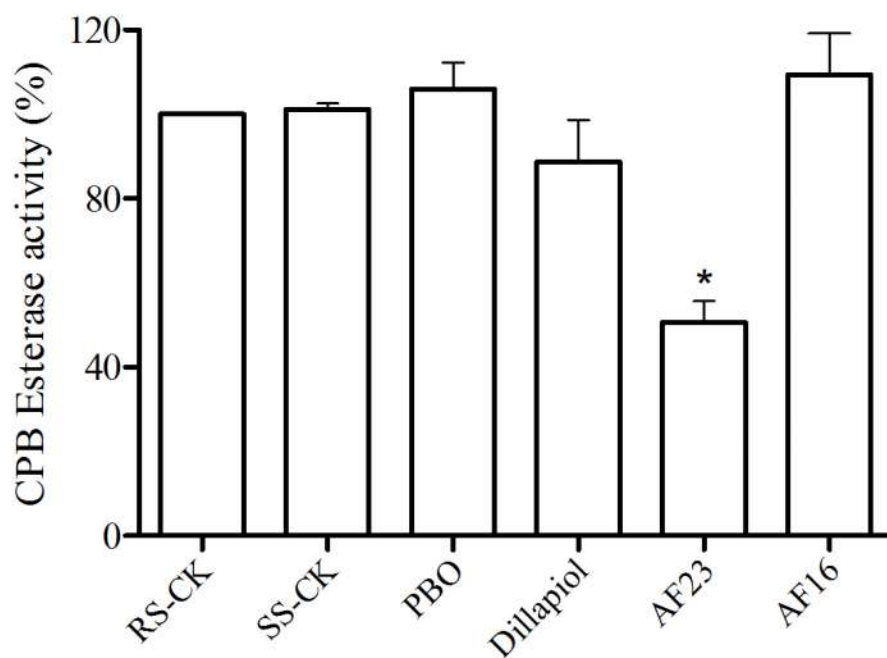


Fig. 5-12 Percent *in vivo* esterase activity  $\pm$  S.E. for RS- and SS-CPB pre-treated with acetone (CK) and RS-CPB pretreated with 4 synergists at 10 mg/mL. Bar with an asterisk indicates that the synergist has significantly lower esterase activity than CK or other synergists (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

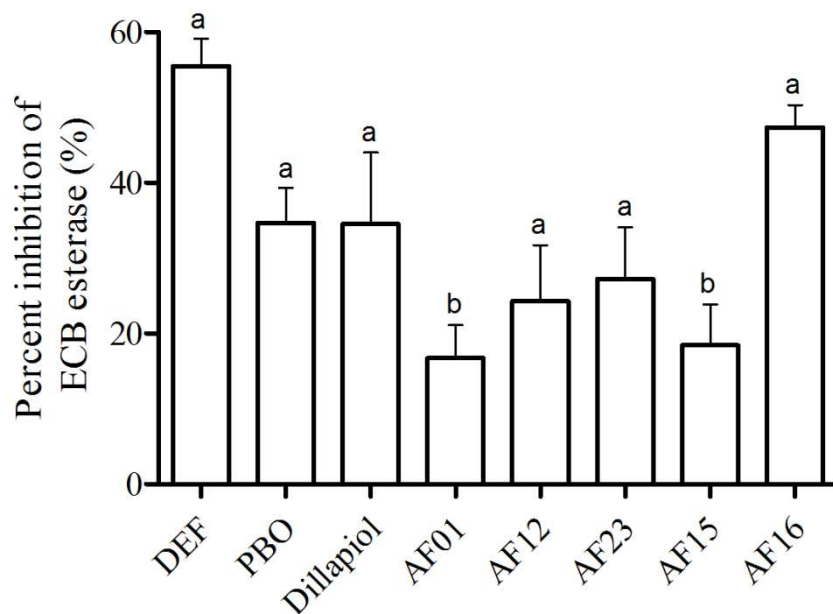


Fig. 5-13 Percent inhibition of *in vitro* esterase activity  $\pm$  S.E. for ECB enzyme homogenate treated with eight synergists at 10 mg/mL relative to acetone treated control. Different letters on the top of the bars denote that esterase activity of synergists are significantly different (One-way ANOVA and Bonferroni post-hoc tests,  $p < 0.05$ ).

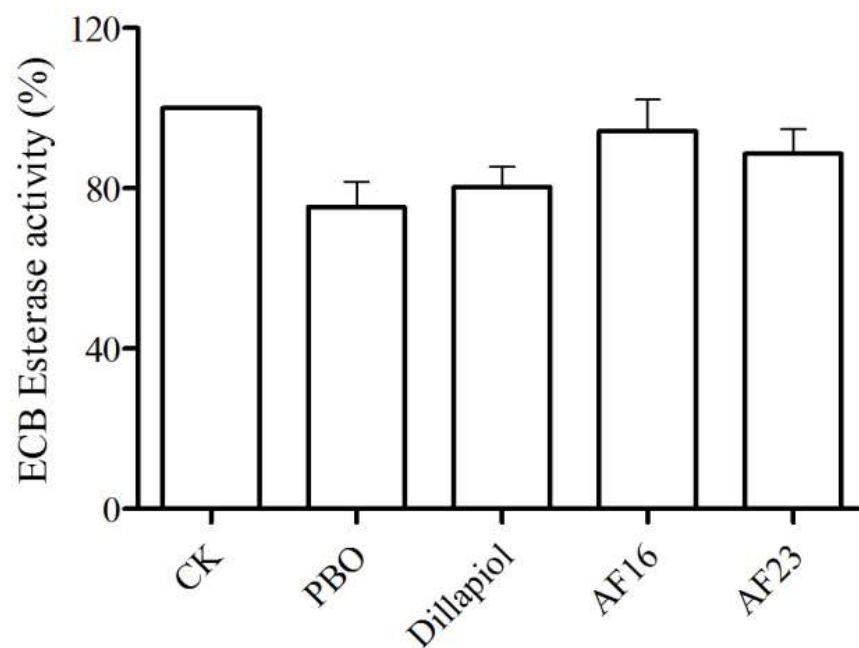


Fig. 5-14 Percent *in vivo* esterase activity  $\pm$  S.E. for enzyme homogenate of ECB pretreated with acetone (CK) or four synergists at 10 mg/mL. All treatments couldn't show any significant difference from each other (One-way ANOVA and Bonferroni post-hoc tests,  $p > 0.05$ ).

## 5.4 Discussion

MDP compounds have frequently been shown to be effective P450 inhibitors (Heckel, 2014). As expected, all the tested compounds at an exceptionally low concentration of 5  $\mu\text{g}/\text{mL}$  showed monooxygenase inhibition with methoxyresorufin as a substrate for RS-CPB and at 1  $\text{mg}/\text{mL}$  for ECB P450. The ester analogs, AF01 and AF23, possessed P450 inhibitory activity similar to PBO during *in vitro* testing (Fig. 5-1) but the ether compounds were less effective with CPB P450s. By contrast, two of the ether analogs, AF15 and AF16, were the strongest inhibitors of *in vitro* ECB P450s and AF16 possessed 11- fold greater inhibitory activity than PBO (Fig. 5-3).

Since the inhibitory activity of MDP compounds involves an initial metabolic activation by the P450 enzyme that leads to the formation of a carbene-iron complex (Ortiz de Montellano and Correia, 1995), it is possible that certain P450 enzymes have either a low affinity for the tested MDP compounds or a capacity to metabolize them, and thus will not be inhibited which could explain the reason that some tested MDP compounds are more effective than others for both the specialist CPB and the generalist ECB P450s.

The different P450 capacities of both insects could be due to the structural difference of enzymes between feeding strategies. The polyphagous insects, like ECB, possess wider broad substrate specificities of cytochrome P450 monooxygenases to cope with the wide diversity of xenobiotics it encounters among its numerous host plants. Li *et al.*, (2004) compared the P450 protein CYP6B1 from the specialist *P. polyxenes* and CYP6B8 from the generalist *H. zea* based on a molecular difference. They discovered that replacing three aromatic residues of the constrained CYP6B1 active site with small hydrophobic residues significantly expanded the volume of the predicted CYP6B8 catalytic site and conferred a capability to metabolize a more diverse range of xenobiotics to the generalist CYP6B8 protein than the specialist CYP6B1 protein. Since *P.*

*polyxenes* is well adapted to feed on xanthotoxin-containing plants, CYP6B1 cloned from the *P. polyxenes* midgut, appeared to be selective in the metabolism of linear furanocoumarins (Ma *et al.*, 1994), and are inducible by furanocoumarins (Harrison *et al.*, 2001), while in generalist insects, different kinds of P450 proteins are responsible for detoxifying different xenobiotics. The metabolic difference of CYP6B8 and CYP321A1 expressed in *H. zea* was compared and homology models developed for them indicated that CYP6B8 has a narrow active site cavity extending from the substrate access channel with a very narrow access to the ferryl oxygen atom. In contrast, CYP321A1 has a more spacious cavity allowing larger molecules to access the heme-bound oxygen which allowed CYP321A1 to metabolize larger and more rigid substrates (angelicin,  $\alpha$ -naphthoflavone etc.) at rates several-fold faster than CYP6B8, even though the latter is more effective at metabolizing the more flexible insecticide substrates (diazinon, and cypermethrin) (Rupasinghe *et al.*, 2007). The molecular mechanisms underlying detoxification enzyme in the orangeworm *Amyelois transitella* (Walker) is that CYP6AB11, which can metabolize PBO, contains a doughnut-like constriction over the heme that excludes aromatic rings on substrates and allows only their extended side chains to access the catalytic site (Niu *et al.*, 2011).

*In vivo* synergism is often used to suggest a P450-mediated detoxification. In the present study, *in vivo* inhibitory activity of MDP compounds insect P450 monooxygenases was successfully determined (Fig 5-2; 5-4) demonstrating that an ether analog of dillapiol, AF16 effectively suppressed the P450 activity in both RS-CPB and ECB. The effectiveness of MDP compounds as P450 inhibitors is metabolism-dependent, thus the *in vivo* activity of those compounds can be delayed or decreased.

It has been known for many years that GSTs have a broad binding specificity with possible functions in intracellular transport processes. Therefore, over expression of insect GSTs would

increase resistance to cytotoxicity. For example, Lipke and Kearns (1959) found that there was a glutathione (GSH)-dependent enzyme in both insecticide-susceptible and -resistant insects with similar specificity, but larger quantities in resistant insects. In the present study, GSTs activity of RS-CPB was elevated 100% compared to SS-CPB but the increased GST activity of RS-CPB was completely inhibited by dillapiol, a natural compound (Fig. 5-6). As several other studies have demonstrated, some natural plant chemicals including taxifolin, (+)-lariciresinol 9'-p-coumaratequercetin, ellagic acid, apigenin, and tannic acid, etc., act as potent insect GST inhibitors (Wang *et al.*, 2014; Ahmad and Pardini, 1990; Lee, 1991; Yu, 2002). This activity may due to an “arms race” counter adaptation of plants, designed to circumvent GST-mediated metabolism of insect herbivores which reduces toxicity of plant defense compounds (Li *et al.*, 2007). However, dillapiol did not have the same effect on susceptible ECB larvae *in vivo* (Fig. 5-8). On one hand, AF23, the best ester compound pyrethrum synergist, suppressed *in vitro* and *in vivo* GSTs activity in both the specialist CPB and the generalist ECB, while on the other hand, AF16, the best ether compound pyrethrum synergist, strongly inhibited *in vitro* GST activity in CPB and ECB, but not *in vivo*. DEM is two times more effective at suppressing *in vitro* GSTs of polyphagous ECB than with oligophagous RS-CPB, while AF16 is 5.7 times more active as *in vitro* GST inhibitor of ECB than CPB. In the present study, the best pyrethrum synergistic activity by analog AF16 against both oligophagous CPB and polyphagous ECB could be explained by its potent inhibitory activity of GST and/ or Cyp P450s.

In both oligophagous and polyphagous insects, all tested synergists did not significantly reduce the *in vitro* esterase inhibition activity compared with DEF, the standard esterase inhibitor. These findings are not surprising since the compounds were not designed as esterase inhibitors. Similarly Joffe *et al.* (2012) found that no esterase inhibition was identified with seven natural

compounds including dillapiol oil in a housefly esterase assay. For CPB, no elevated esterase activity was detected in the resistant strain (Fig. 5-12), therefore esterase enzyme might not play a key role in the resistance mechanism of the RS-CPB strain.

In summary, dillapiol, the natural MDP compound, can partly or completely inhibit P450 enzyme activity of both oligophagous and polyphagous insects, and CPB GST activity. All selected analogs can effectively inhibit *in vitro* and *in vivo* P450 monooxygenases activity for both oligophagous CPB and polyphagous ECB which is consistent with the CYP3A4 inhibitory activity. The best pyrethrum synergist, AF16, also displayed significantly greater P450 inhibition activity than PBO, i.e. AF16 is eleven times more effective than PBO to inhibit ECB *in vitro* P450 activity. The inhibition assay with GSTs produced the surprising result that AF16 exhibited a 180-fold (CPB) and 575-fold (ECB) lower  $IC_{50}$  than DEF, the standard inhibitor of GST. To our knowledge, this is the first report of MDP related compounds showing significant GST inhibition activity.

# **CHAPTER 6 DYSREGULATION OF INSECT METABOLISM STUDIED BY ULTRAPERFORMANCE LIQUID CHROMATOGRAPHY QUADRUPOLE TIME OF FLIGHT MASS SPECTROMETRY**

## **Preface**

The present chapter was a pilot study of the effect of synergists on insects using novel metabolomics methods. The study provided results on alteration of insect metabolom which were induced by pyrethrum singly or pyrethrum/ synergist combinations, as detected with UPLC-QTOF/MS. The main purpose of the present study was to verify the applicability of UPLC-QTOF/MS with insect synergists and to test for appropriate methods for insect metabolomic investigation. Although preliminary and requiring further replication, this chapter provides evidence of the applicability of metabolomics to the field.

### **6.1 Introduction**

Metabolomics, the most recent of the “omics” technologies, is the systematic study of the downstream small-molecule metabolites produced by specific cellular processes in the organism (Bennett, 2005). The metabolite concentrations are sensitively influenced by perturbations that affect metabolite biosynthesis, and react to changes of enzyme capacity in which they are substrates (Zamboni and Fischer, 2012). Therefore, the metabolic variation caused by stimuli such as diet, environment or chemical intervention can be monitored over time (Lindon *et al.*, 2007; Nicholson *et al.*, 1999).

The metabolomics approach was first adopted for entomologic research in 1990 to analyze the metabolic effects of parasitism on larvae of the tobacco hornworm, *Manduca sexta* Linnaeus

(Lepidoptera: Sphingidae) (Thompson *et al.*, 1990). To date, ento-metabolomics studies have increased steadily but the publication numbers are still fairly low compared to medical applications (< 50) (Snart *et al.*, 2015). Published studies have mainly focused on the several topics, including insect-bacterial symbiosis (Wang *et al.*, 2010), behavioral ecology (Lenz *et al.*, 2001), parasitism (Thompson *et al.*, 1990), temperature-dependent stresses (Michaud and Denlinger, 2007; Kostal *et al.*, 2011a, b), etc. Only a few studies have focused on pesticide exposure, for example, the impact of imidacloprid on the metabolic networks in honeybee larvae *Apis mellifera* (Hymenoptera: Apidae) (Derecka *et al.*, 2013). In these reported ento-metabolomics studies (Snart *et al.*, 2015), GC-MS and LC-MS were the major approaches used in 13 experimentations, while 1D or 2D NMR was used in eight research papers. One study (Rivera-Perez *et al.*, 2014) used HPLC-FD to test mevalonate and juvenile hormone pathways in the mosquito, *Aedes aegypti* Linnaeus in Hasselquis (Diptera: Culicidae), and in another study, Zhang *et al.* (2012) employed MALDI-TOF to assess diapause-induced metabolic alterations associated with photoperiod and energy storage in *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae).

Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS) is a newly developed technique that provides rapid and efficient access to detailed information of specific components within multi-component mixture. Taking advantage of high resolution and efficiency, UPLC-QTOF/MS has been increasingly adopted to analyze metabolites in many areas such as environmental, biomedical or food safety (Fraser *et al.*, 2013).

In the present study, a pilot insect metabolomics study using UPLC-QTOF/MS was conducted to determine metabolomic effects of selected synergists. Metabolomics was used to

assess altered phenotypes induced by selected synergist compounds in three insect tissues, hemolymph, fat body and gut, of oligophagous RS-, SS-CPB and polyphagous SS-ECB.

## 6.2 Methods and Material

### 6.2.1 Sample preparation

The insecticide, pyrethrum, the synergists, PBO and dillapiol, and a negative control (acetone), were selected as the chemical materials to test the metabolite alteration of insects topically treated with pyrethrum or pyrethrum/ synergist. All tested chemicals were dissolved in acetone to prepare a final concentration of 100 ppm pyrethrum for pyrethrum singly and pyrethrum/ synergist at a 1:5 ratio.

To ensure that the same amount of each compound was applied to all larvae, a topical treatment was employed. Five  $\mu\text{L}$  of each solution was gently spread on the dorsal thorax of each 4<sup>th</sup> instar CPB or ECB larvae which were fed regular meridic diet during the procedure. One hour post-treatment each insect was dissected to obtain the three different tissues. Hemolymph was collected by gently pulling up and cutting down with a scalpel one of the middle pair of thoracic legs near the thoracic cuticle. Approximately 10  $\mu\text{L}$  of hemolymph was collected from each insect with a disposable 10  $\mu\text{L}$  micropipette. After a pool of 100  $\mu\text{L}$  hemolymph from approximately 10 insects in each treatment was collected, 10  $\mu\text{L}$  mixed hemolymph was taken out and mixed with 50  $\mu\text{L}$  of either methanol (MeOH) or acetonitrile (ACN), or 99.9% MeOH: ACN (1:1) +0.1% formic acid (FA) for metabolite extraction. The samples were centrifuged at 10,000  $\times g$  for 20 min at 4°C. Supernatant was filtered by syringe PTFE 8 mm disc diameter, in preparation for analysis.

The same larvae were then used for preparing the gut and fat body metabolite extraction after hemolymph collection. The gut and fat body were dissected out, and carefully separated. A

set of five guts or fat bodies were immediately transferred to a glass vial containing 2 mL cold MeOH or ACN, or 99.9% MeOH: ACN (1:1) +0.1% FA. The tissue solution was homogenized for 1 min at 1000×g, followed by centrifuging at 10,000×g for 20 min at 4°C. Supernatant was filtered into a HPLC vial by syringe PTFE 8 mm disc diameter (Fig. 6-1).

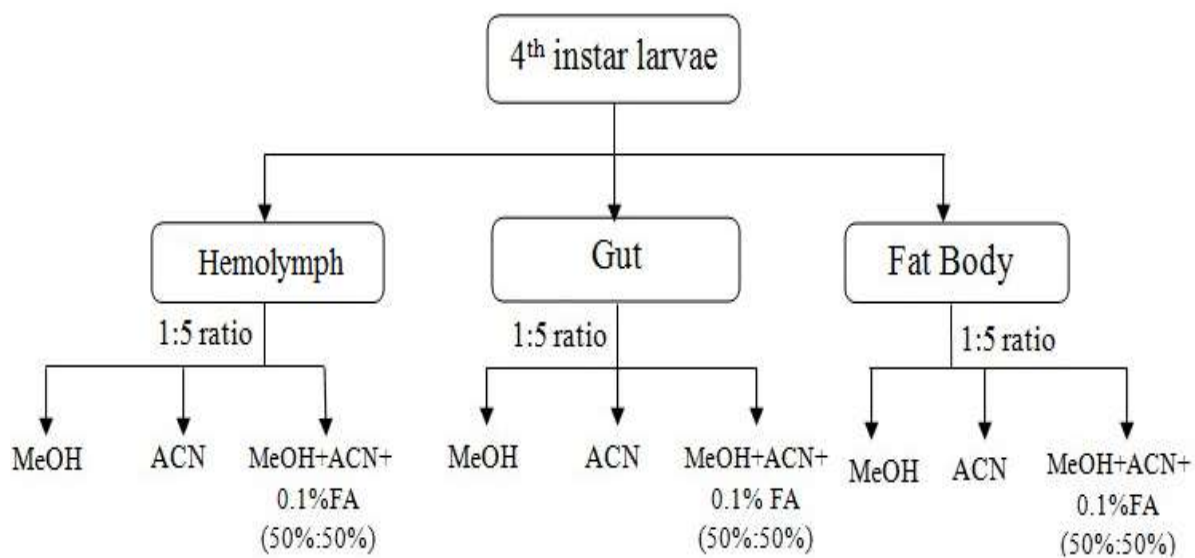


Fig. 6-1 The diagram of sample preparation for testing the metabolites in various insect tissues with different solvent systems prior to analyses by UPLC-QTOF/MS.

### 6.2.2 UPLC-QTOF/MS analysis

Ultra performance-LC analysis was performed on Waters Acquity UPLC (Department of Biology, University of Ottawa) consisting of a vacuum degasser, an autosampler, a binary pump, and a column oven. Separations were performed on an Acquity UPLC<sup>®</sup> BEH C18 column (2.1 mm × 50 mm, i.d. 1.7 μm, Waters Corp, Milford, USA). The analytical column was maintained at a temperature of 50 °C and the mobile phases were composed of A (MeOH, or ACN, or MeOH+ACN+0.1% FA) and B (water) each containing 0.1% formic acid. A solvent gradient system was as follows: 5-100% A from 0-5 min with a flow rate of 0.5 mL/min, with an injection volume of 2 μL. QTOF analysis was performed in both positive and negative ion modes (positive mode was used for analysis). QTOF data were collected in the MSe mode from m/z 100-1500. All the data were acquired using an independent reference lock mass via the LockSpray<sup>TM</sup> interface to ensure accuracy and reproducibility during the QTOF analysis.

### 6.2.3 Statistical Analysis

Following the UPLC-QTOF scan, the raw data were analyzed using MassLynx (V4.1 SCN918, Waters Corp). Metabolite signal intensities were detected within 0.25-5 min. the marker intensity threshold was set at 1000 counts within a mass window of 0.05 and noise elimination level of 8. The intensity of each signal normalized with respect to the total ion count to generate a data matrix. Marker Lynx software (MassLynx, V4.1, SCN918, Waters Corp) was used for multivariate analysis to investigate the differences in metabolite signal intensity between samples. Principal component analysis (PCA) provided a 2D score that plot on an x and y axis, the two principal components that encompass the most variance within the data set, providing a visual overview of all of the samples and their potential groupings.

### 6.3 Results

The preliminary results for metabolite variation within pyrethrum or pyrethrum/synergist treated ECB and CPB was evaluated by comparing and analyzing the metabolite signals produced from UPLC-QTOF/MS. Based on these results, methanol was chosen as the optimal solvent for the remainder of the research (Supplement III) due to the better peak symmetry compared to the other two solvent systems (MAF and ACN). Similarly, the insecticide concentration (100 ppm) applied to each insect was determined based on the successful detection of pyrethrins (Fig. 6-2, Sup. IV) and dillapiol at a threshold that did not cause mortality (Sup. V).

To discover the effect of dillapiol on the amount of active pyrethrum in insects, the profiles of six main components of pyrethrum were carefully searched in metabolomes of RS-CPB gut treated with pyrethrum or pyrethrum/dillapiol combination. Three major ones, cinerin I, II, and jasmolin I, were successfully detected in pyrethrum/dillapiol-treated sample, while none of the six components was discovered in pyrethrum singly treated samples (Fig. 6-2). This indicated that the combination of pyrethrum with dillapiol protected pyrethrum from being metabolized to some extent by the insects.

To determine if there were metabolite differences between RS-CPB induced by different treatments, the chromatographs of three hemolymph groups, including acetone control, pyrethrum, and pyrethrum+dillapiol, were compared (Fig. 6-3). Two peaks (observed at 1.63 and 1.73 min) circled with black rectangle, as well as several other metabolites indicated by small peaks in the control treatment, were observed to be absent in both the pyrethrum, and pyrethrum/dillapiol groups, while a new metabolite, likely a pyrethrum derivative was visible at 3.46 min in pyrethrum/dillapiol treatments which was not detected in the other two groups. The two peaks observed between 1.63-1.73 min in the control group have been identified as leaf derived glycoalkaloids. The absence of these plant compounds in larvae pre-treated with pyrethrum or

pyrethrum/dillapiol was explained by the observation that the feeding was greatly reduced when larvae were applied with insecticide or insecticide/ synergist combination solution.

When the hemolymph metabolomes of SS- and RS-CPB were compared, the untreated RS-CPB controls were clearly separate. The metabolomes of RS-CPB after treatment with pyrethrum or pyrethrum+dillapiol were visibly shifted towards SS-CPB with the same treatment and were displaced far away from the RS-CPB controls (Fig. 6-4). They were also clearly closer to the SS-CPB controls suggesting that the treatments had altered the metabolome to a state similar to the susceptible strain. With the small number of replicates, it was not clear if there was a difference between dillapiol or pyrethrum/ dillapiol.

The metabolomics results for different tissue samples extracted from either ECB or CPB with methanol showed visible differences (Fig. 6-5). The metabolomes of tissues from ECB and CPB were completely separated. The metabolome of hemolymph, gut and fat bodies extracted from RS-CPB showed hemolymph was distinct from other tissues but gut and fat body of RS-CPB could not be clearly discriminated. Taking into account the laborious and time consuming work to separate the gut and fat body tissues, it was determined that the sample preparation for the study in the future would use gut and fat body tissue mixtures only.

In terms of metabolite alterations in the ECB hemolymph treated with acetone or pyrethrum or pyrethrum/PBO, several peaks or metabolites were induced by pyrethrum treatment, but those peaks could not be detected in either the control and pyrethrum/PBO groups. This indicated that the metabolite shift caused by the uptake of the insecticide could be altered by co-applying a synergist (Sup. VI). Further molecular identification of those compounds absent in two treatments may reveal an even greater number of mechanisms responsible for the synergistic activity.

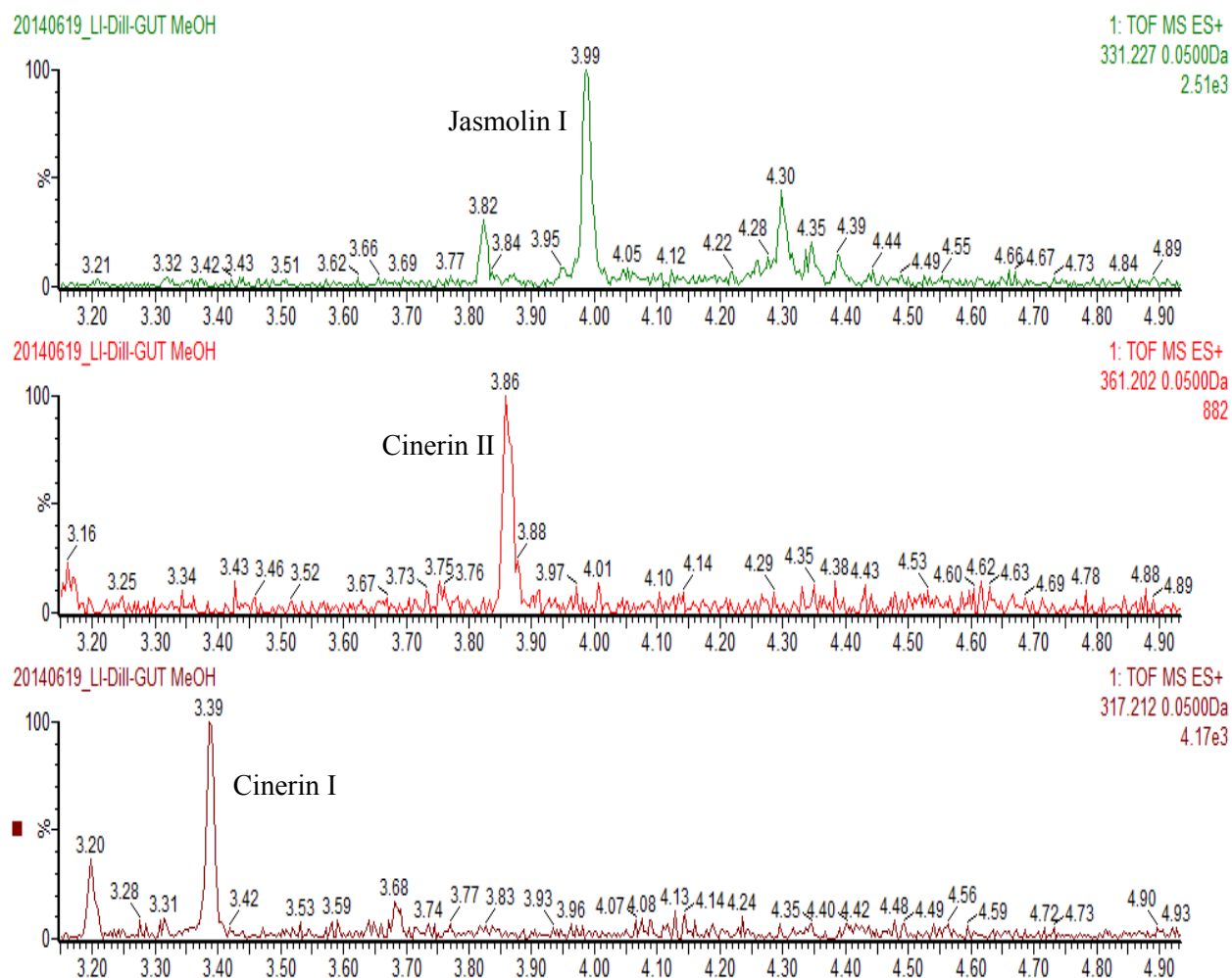


Fig.6-2 The UPLC-QTOF/MS chromatographs of several major pyrethrins detected in 4<sup>th</sup> instar RS-CPB gut 1 h after topical treatment with pyrethrum plus dillapiol.

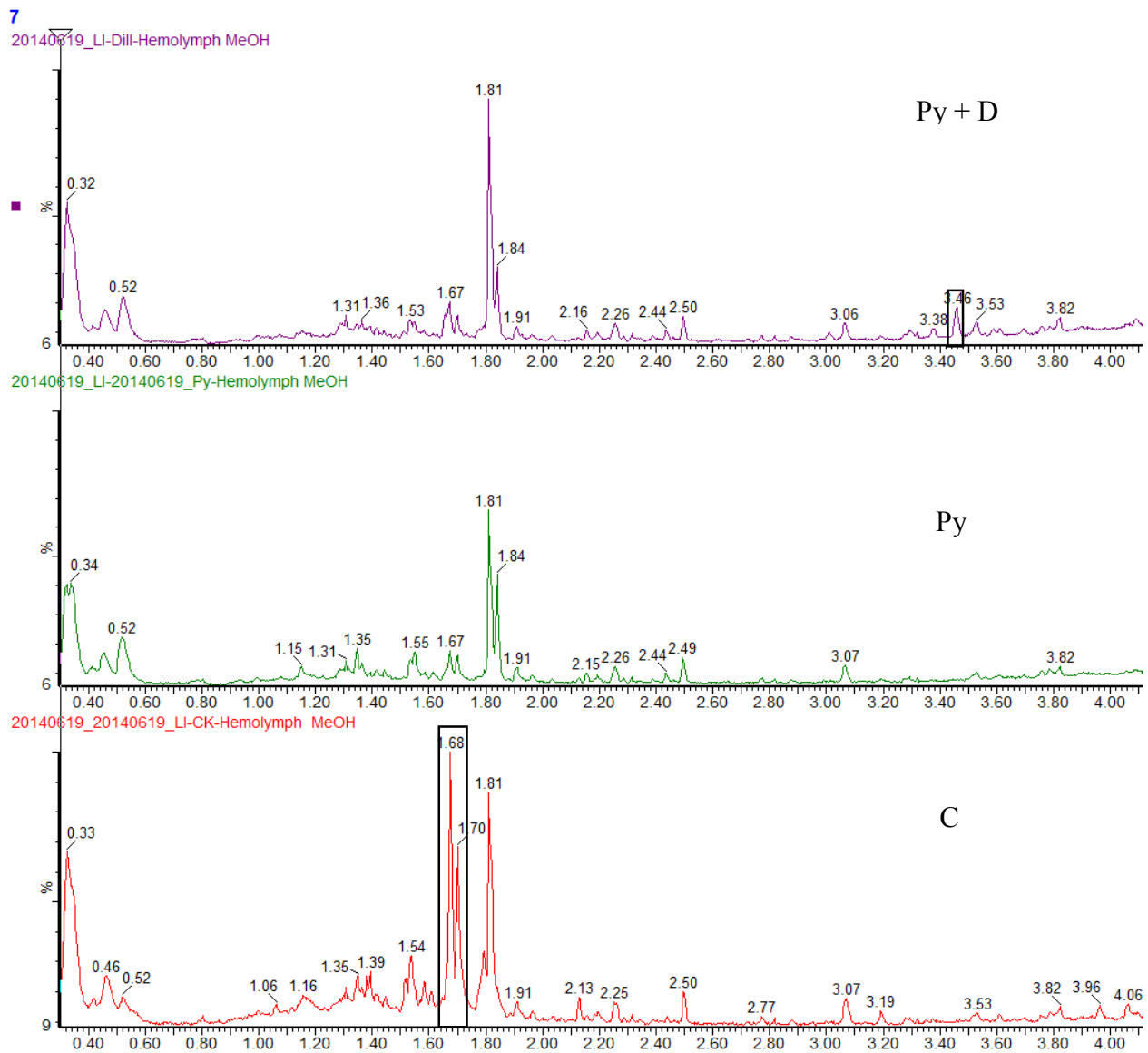


Fig. 6-3 The UPLC-QTOF/MS chromatographs with metabolite profiles of 4<sup>th</sup> instar RS-CPB hemolymph 1 h after topical treatment with acetone (C), pyrethrum (Py), or pyrethrum plus dillapiol (Py+D). The box in the control at Rt 1.64- 1.74 min shows peaks due to leaf derived glycoalkaloids.

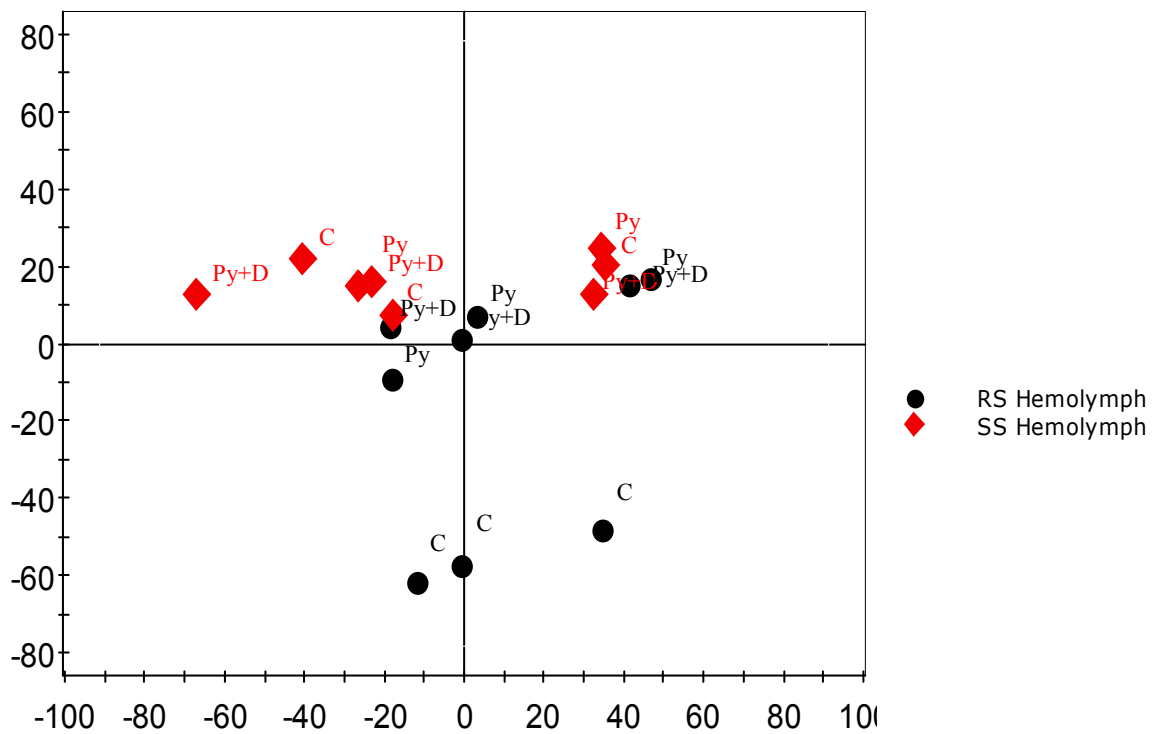


Fig. 6-4 Principal component analysis showing the metabolome of hemolymph in SS- (red) and RS-CPB (black) exposed to different treatments, control (C), pyrethrum (Py) or pyrethrum + dillapiol (Py+D). The first two principal components are shown.

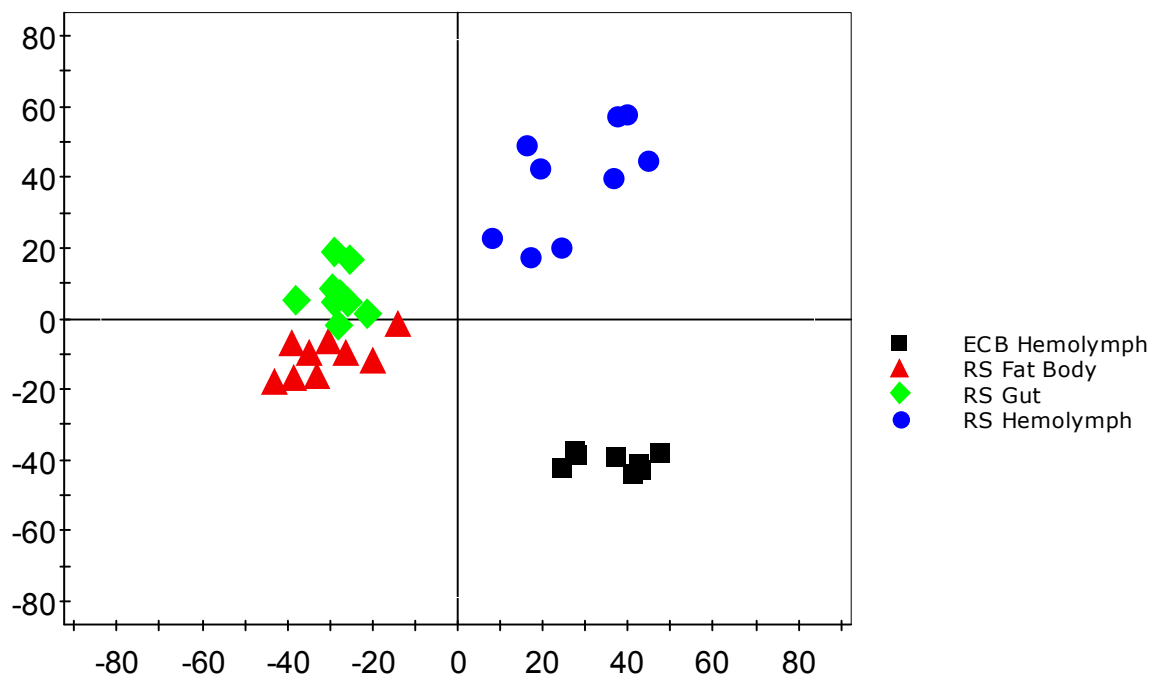


Fig. 6-5 A principal component analysis of the metabolome of combined treatments of insect tissues (ECB hemolymph; RS-CPB hemolymph, gut and fat body).

## 6.4 Discussion

The highly sensitive chromatographic resolution and the outstanding mass accuracy of the UPLC-QTOF/MS system can provide new insights into the metabolomic changes produced by treatment with target chemicals. The present study demonstrates that UPLC-QTOF/MS is a suitable technique to study the insect metabolomes of this thesis, since the metabolomes in different treatments were successfully separated and the chromatographic profiles clearly indicated the differences in specific metabolites between insect tissues induced after pre-treating with selected compounds.

Methanol was selected as the optimal solvent in which most of target chemicals were detected and successfully separated. The observation of the main components of pyrethrins (Sup. IV) and two other synergists in related tissues samples at 1 h post treatment made further research possible for dynamic function testing on target compounds based on different treating time.

The ability of untargeted global analyses coupled with high-resolution quantitative analyses by UPLC-QTOF/MS has great potential for future metabolomics investigations on this system and for different insect species.

## CHAPTER 7 GENERAL DISCUSSION

### *7.1 Cytochrome 3A4 inhibition activity of dillapiol and its analogs*

The present study determined that a set of new dillapiol analogs have great potential as CYP3A4 inhibitors. Among the 52 tested analogs, two compounds possessed significantly greater CYP3A4 inhibition activity than dillapiol itself (> 40 times). Although not tested side by side here, they had five times greater inhibitory activity than the reported value for paradisin C, a recognized CYP3A4 inhibitor isolated from grapefruit (Ohta *et al.*, 2002). By comparing the IC<sub>50</sub> values of the two dillapiol analogs tested here with several CYP3A4 inhibitor compounds reported by Stresser *et al.* (2000), dillapiol analogs had greater inhibitory activity than 15 out of 21 known CYP3A4 inhibitors, thus indicating their potential as phamarcoenhancers.

The qualitative analysis (QSAR) determined that larger acyl groups attached to the alcohol of the ester analogs conferred higher *in vitro* inhibitory activity than smaller groups, while the two methoxy groups on the dillapiol molecule did little to influence the CYP3A4 inhibitory activity of dillapiol analogs.

The QSAR model developed with the current group of compounds has been tested as a good predictive model and pointed to new structures with predicted higher P450 3A4 inhibitory activity for future research.

### *7.2 Pyrethrum synergistic activity and ability of selected dillapiol analogs to reverse resistance*

The potential of the dillapiol analogs as a pyrethrum synergist and for susceptibility restoration was successfully determined in the present study. To evaluate the pyrethrum synergistic activity and relative mechanism of analogs, the oligophagous CPB and the

polyphagous ECB were used as the insect models. Three ether compounds displayed synergistic activity greater than or comparable to ester analogs for both CPB and ECB. It is highly probable that the ester had reduced activity due to metabolism by insect esterases. Therefore, an important finding is that ether compounds are a better choice for producing active analogs.

A relatively stronger pyrethrum synergistic activity by dillapiol analogs was observed through ingestion compared to topical treatment for both the oligophagous and polyphagous insects. These results were in good agreement with the observations of fairly low synergism ratios (<2-fold) detected in the PBO or DEF/ insecticide combinations against a susceptible *Drosophila melanogaster* strain compared to a resistant strain (>12-folds) (Nguyen *et al.*, 2007). In the present study, a SR of 15.8-, and 27-fold for the natural compound dillapiol and its ether analog AF16, respectively, were determined with resistant CPB larvae, indicating that mixing pyrethrum with these synergists significantly improved its efficacy against the resistant CPB larvae on the same scale as the susceptible CPB. In other words, dillapiol and its analogs can successfully restore pyrethrum susceptibility of RS-CPB. The restoration to insecticide efficiency by MDP compounds was also observed in other insect pests, such as the pyrethroid-resistant strain *Helicoverpa armigera* (Hubner) (Young *et al.*, 2005), and the human ectoparasite mite, *Sarcoptes scabiei* (Pasay *et al.*, 2009).

In addition to the ability to decrease of the amount of insecticide usage required for controlling insect pests, synergists have also been reported to be able to delay insecticide control failure and prolong the useful life of those insecticides. This principle was first successfully demonstrated by Moorefield (1960), who found that carbamate resistance in house flies under carbaryl exposure was 194 times higher than those with carbaryl-PBO exposure after 20 generations. This was consistent with another study 20 years later that showed carbaryl-PBO

selected houseflies exhibited only 3-fold resistance over 20 generations as compared to the 25-fold increase with carbaryl alone (Wilkinson, 1983). In another similar study, PBO synergized deltamethrin was used in the selection of yellow fever mosquito *Aedes aegypti* (L.) larvae, and produced 89% resistance reversion after 24 generations (Kumar *et al.*, 2002). In the field, the increased number of susceptible individuals in the population can dilute the ratios of resistant individuals. Furthermore, the resistant individuals can have decreased properties of resistance when they adapt to an environment with no or low insecticides (Yang *et al.*, 2014). This could be achieved faster by employing the insecticide/ synergist combination in the field.

The determination of intrinsic toxicity of pyrethrum/synergist combinations allowed for a comparison of PBO, dillapiol and selected dillapiol analogs with ECB larvae. The results reinforced the feasibility of dillapiol and its analogs as substitutes for PBO, in part because 1) dillapiol has been regarded as safe by GRAS (Generally Recognized as Safe, 2013); 2) the dillapiol ether analog, AF16, was less toxic than PBO to ECB larvae growth and development (Chapter 4) and 3) the analogs showed strongest pyrethrum synergistic activity for both CPB (Chapter 3) and ECB (Chapter 4).

The variation in synergism of dillapiol analogs between the SS-CPB and RS-CPB is most likely conferred by the substantial difference in detoxification enzyme levels. In susceptible insects, the constitutive levels of the major metabolic enzyme systems are low when exposure to insecticides occurs, therefore the use of synergists may provide less insecticide activity enhancement (IRAC, 2011). However, in the resistant insects, synergists can significantly enhance insecticide efficacy due to the inhibition of the resistant insect's enhanced levels of metabolic enzymes.

The biochemical assays employed in this research with selected dillapiol analogs unexpectedly discovered that dillapiol MDP analog, AF16, inhibited CPB and ECB *in vitro* GST activity at 180 and 560 times higher than GST standard inhibitor DEM, respectively. A weak GST inhibitory activity was observed for PBO and dillapiol as confirmed by Joffe (2011) who concluded that PBO, dill oil, and saffron oil showed low or no GST inhibition activity.

As one of the major insect metabolic enzymes, GST was best known for the ability to catalyze the conjugation of the GSH with xenobiotic substrates for detoxification, but recently, the crucial role of insect GST in the DDT-resistance development was elicited in unprecedented detail using a vector mosquito *Anopheles funestus* insect model (French-Constant, 2014). The over expression of the *GSTe2* gene, responsible for encoding the GST enzyme, was sufficient to cause DDT resistance in tested mosquito strains. The following study (Riveron *et al.*, 2014) suggested that pre-selection with DDT can also confer subsequent *GSTe2*-mediated resistance in mosquito to the pyrethroid insecticides designed to replace the use of DDT itself.

To date, the cross-resistance between DDT and certain pyrethroids was regarded to be associated with knock-down resistance (*kdr*) alleles of the *para*-encoded sodium channel target (Brenques *et al.*, 2003; Ndiath *et al.*, 2012). The elucidation of the role of GST in insect resistance development is a vital addition to the better understanding of the underlying resistance mechanism. Therefore, there is potential for GST inhibitors, such as AF 16 in the present study, to be further used for both resistance mechanism research and commercial product development.

Dillapiol has previously been determined to be an effective pyrethrum synergist in laboratory bioassays (Tomar *et al.*, 1979; Liu *et al.*, 2014). In the present study, the insecticidal potency of pyrethrum/ dillapiol formulation was evaluated for the first time in both greenhouse

and field trials. The insecticide-synergist formulation produced comparable efficiency to pyrethrum/ PBO formulation for both greenhouse and field trials (Chapter 3) which indicates that dillapiol could be a suitable PBO replacement as pyrethrum synergist, especially for organic farming. Organic farming is based on a simple principle that strictly respects natural balances, and therefore synthetic chemicals are prohibited. As a natural insecticide with fairly low mammal toxicity, pyrethrum was one of the few products authorized for organic farming (Organic Material Review Institute, OMRI, 2015). In contrast, PBO, the semi-synthetic synergist, has been removed from the OMRI list as an acceptable product for organic farming. As a natural component coupled with the synergistic property that dramatically reduces the amount of insecticide usage, the application of dillapiol/pyrethrum formulation will be a benefit for both organic farmers and environmentally conscious consumers.

According to Statistics Canada, Canada's organic market was worth an estimated \$3.7 billion in 2012, and 58% of Canadians buy organic products every week (COTA, 2013). Although synthetic chemicals have been excluded from organic farming, organic farms cannot operate in isolation because certain common agricultural inputs, including pesticides previously used in the conventional operation can remain in the soil or water for decades, a continuing risk for the current organic products. According to a CBC News analysis of data supplied by Canadian Food Inspection Agency (CFIA, 2014), nearly half of the organic fresh fruits and vegetables tested across Canada in the past two years contained some pesticide residue.

To partially address the insecticide residue issue in the environment, synergists with low or no intrinsic toxicity should be employed. The MDP compound, AF 16, designed with a simple molecular structure without problematic halogenation is a compound with fairly low toxicity but high pyrethrum synergistic activity and offers a possible PBO alternate for conventional agriculture.

### 7.3 Future research

Based upon the biological and biochemical bioassay results, MDP analogs tend to possess pharmaceutical and insecticidal synergistic activity. The successful QSAR model construction provided hints for further research.

#### 7.3.1 Design and prepare new analogs

According to the results, the benzyl ether analog AF16 which contains an allyl moiety and an aryl ring B substituent at C3, displayed higher biological and biochemical activity. Therefore, a series of 20-30 dillapiol analogs should be designed by modifying the allyl moiety or aryl ring B of AF16 based on the QSAR model. The pharmaco-enhanced activity can then be evaluated with human cytochrome CYP3A4 assay. It is anticipated that the preparation of these analogs would allow determination of whether this approach is likely to lead to significantly more active compounds than the currently investigated ether compound, AF16.

The biological evaluation of new analogs would be conducted in a similar fashion to those tested in the present study with SS- and RS- CPB. The main goals include a) increasing efficacy of resistant Colorado potato beetle control; b) reduction of active ingredient insecticide use; c) providing a replacement for PBO.

#### 7.3.2 Metabolism research for new synthesized dillapiol analogs

In order to better evaluate safety and efficacy of new synthesized dillapiol analogs, the biochemical and physiological effects and mode of action of the potential synergists can be determined using new methods in metabolomics, in which the entire metabolome of the target insect can be assessed using the developed method in the pilot study with UPLC-QTOF/MS. This would provide direct information of the effects of synergistic treatments on physiology and

sites of action. Discriminant statistical analysis allows an unbiased discovery of key metabolites that are biomarkers of effectiveness.

## REFERENCES

- Abbott WS. 1925. A method for computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18: 265-167.
- Adams NH, Levi PE, and Hodgson E. 1995. Regulation of cyto- chrome P450 isozymes by methylenedioxyphenyl compounds – an updated review of the literature. *Rev. Biochem. Toxicol.* 11: 205-222.
- Ahmad S, and Pardini RS. 1990. Mechanisms for regulating oxygen toxicity in phytophagous phytophagous insects. *Free Radical Bio. Med.* 8: 401-413.
- Agriculture and Agri-food Canada. 2012. Crop profile for sweet corn in Canada. Ottawa, ON, Canada.
- Allen FH. 2002. The Cambridge Structural Database: a Quarter of a Million Crystal Structures and Rising. *Acta Crystallographica Section B* 58 (3 Part 1): 380-88. doi:10.1107/S0108768102003890.
- Almeida RRP, Souto RNP, Bastos CN, Da Silva MHL, and Maia JGS. 2009. Chemical variation in *Piper aduncum* and biological properties of its dillapiol-rich essential oil. *Chem. Biodivers.* 6:1427-1434
- Alyokhin AV, Baker M, Mota-Sanchez D, Dively G, and Grafius E. 2008. Colorado potato beetle resistance to insecticides. *Am. J. Potato Res.* 85: 395-413.
- Alzahrani AM. 2009. Insects Cytochrome P450 enzymes: evolution, functions and methods of analysis. *Glob. J. Mol. Sci.* 4:167-179.
- Andrei CC, Brax-Filho R, and Gottlieb OR. 1988. Allylphenols from *Ocotea cymbarum*. *Phytochem.* 27(12): 3992-3993.
- Arensburger P, Megy K, Waterhouse RM, Abrudan J, and Amedeo P. 2010. Sequencing of *Culex quinquefasciatus* establishes a platform for mosquito comparative genomics. *Science* 330: 86-88.
- Awolola TS, Brooke BD, Hunt RH, and Coetze M. 2002. Resistance of the malaria vector *Anopheles gambiae s.s.* to pyrethroid insecticides, in south-western Nigeria. *Ann. Trop. Med. Parasitol.* 96(8): 849-852
- Bass C, Field LM 2011. Gene amplification and insecticide resistance. *Pest Manag Sci* 67: 886-890.
- Bailey JC, Scott-Dupree CD, Tolman JH, Harris CR, and Harris BJ. 2005. Alternative agents for control of European corn borer and Corn flea beetle on sweet corn. *J. Veg. Sci.* 11(1):27-46

- Belzile AS, Majerus SL, Podeszinski C, Guillet G., Durst T, and Arnason JT. 2000. Dillapiol derivatives as synergists: Structure-activity relationship analysis. *Pestic. Biochem. Phys.* 66(1): 33-40
- Ben'kovskaya G, Udalov M, and Khusnutdinova E. 2008. The genetic base and phenotypic manifestations of Colorado potato beetle resistance to organophosphorus insecticides. *Russ. J. Genet.* 44: 553-558
- Bernard CB, Arnason JT, Philogene BJR, Lam J, and Waddell T. 1989. Effect of lignans and other secondary metabolites of the Asteraceae on the mono-oxygenase activity of the European corn borer. *Phytochem.* 28(5):1373-1377
- Bernard CB, Arnason JT, and Philogene BJR. 1990. *In vivo* effect of mixtures of allelochemicals on the life cycle of the European corn borer, *Ostrinia nubilalis*. *Entomol. exp. appl.* 57:17-22
- Bernard C.B., Krishnamurthy H.G., Chauret D, Arnason JT, and Philogene BJR. 1995. Insecticidal defenses of *Piperaceae* from the neotropica. *J. Chem. Ecol.* 21 (6):801-814
- Bernhard HO, and Thiele K. 1978. Isolierung von 1-Allyl-2, 3-dimethoxy-4, 5- methylendioxybenzol (= Dillapiol) aus *Heckeria umbellata* (L.) Kunth (Piperaceae) *Helvetica Chimica. Acta.* 61 (6): 2273-2274
- Bishop BA, and Grafius EJ. 1996. Insecticide resistance in the Colorado potato beetle. In P. Jolivet and T. H. Hsiao (eds.), *Chrysomelidae biology*. SBP Academic Publishing, Amsterdam.
- Bond H, Mauger K, and DeFeo JJ. 1973. The oral toxicity of pyrethrum, alone and combined with synergists. *Pyreth. Post* 12: 59
- Brattsten LB. 1979. Ecological significance of mixed-function oxidations. *Drug Metab. Rev.*, 10:35-58.
- Bregues C, Hawkes NJ, Chandre F, McCarroll L, Duchon S, Guillet P, Manguin S, Morgan JC, and Hemingway J. 2003. Pyrethroid and DDT cross-resistance in *Aedes aegypti* is correlated with novel mutations in the voltage-gated sodium channel gene. *Med. Vet. Entomol.* 17(1):87-94
- Brown TM, Bryson PK, and Payne GT. Synergism by propynyl aryl ethers in permethrin-resistant tobacco budworm larvae, *Pestic. Sci.* 46 (1996) 323-331.
- Brooks GT. 1976. Penetration and distribution of insecticides. In: *Insecticide biochemistry and physiology*. Wilkinson, C.F. (Ed). Plenum press, New York, Pp.3-58

- Budzinski JW, Foster BC, Vandenhoeck S, and Arnason JT. 2000. An *in vitro* evaluation of human cytochrome P450 3A4 inhibition by selected commercial herbal extracts and tinctures. *Phytomedicine* 7(4): 273-282.
- Burke B, and Nair M. 1986. Phenylpropene, benzoic acid and flavonoid derivatives from fruits of Jamaican *Piper* species. *Phytochem.* 25(6): 1427-1430
- Canada Organic Trade Association. 2013. Canada's organic market: National highlights, 2013. (Retrieved on May 8, 2015) [http://www.filierebio.qc.ca/Filierebio/Documents/Canada\\_s%20Organic%20Market\\_%202013.pdf](http://www.filierebio.qc.ca/Filierebio/Documents/Canada_s%20Organic%20Market_%202013.pdf)
- Capinera JL. 2001. Handbook of Vegetable Pests, Academic Press, New York
- Carvalho RA, Azeredo-Espin AM, and Torres TT. 2010. Deep sequencing of New World screw-worm transcripts to discover genes involved in insecticide resistance. *BMC Genomics* 11: 695.
- Casagrande RA. 1987. The Colorado potato beetle: 125 years of mismanagement. *Bull. Entomol. Soc. Am.* 33: 142-150.
- Casida JE. 1970. Mixed function oxidase involvement in the biochemistry of insecticide synergists. *J. Agric. Food Chem.* 18: 753-772
- CBC. 2011. Pesticides found in Canadian organic produce. (Retrieved on May 8, 2015) <http://www.cbc.ca/news/canada/manitoba/pesticides-found-in-canadian-organic-produce-1.985584>
- Chatterjee P, and Franklin MR. 2003. Human cytochrome p450 inhibition and metabolic-intermediate complex formation by goldenseal extract and its methylenedioxyphenyl components. *Drug Metab. Dispos.* 31 (11):1391-1397
- Chelvanayagam G, Parker MW, and Board PG. 2001. Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. *Chem Biol Interact* 133: 256-260.
- Ciccio JF. 2005. Essential Oil from the Leaves of *Piper augustum* from “Alberto M. Brenes” Biological Preserve, Costa Rica. *J. Essent. Oil Res.* 17(3): 251-253
- Clark JM, Lee SH, Kim HJ, Yoon KS, and Zhang AG. 2001. DNA-based genotyping techniques for the detection of point mutations associated with insecticide resistance in Colorado potato beetle *Leptinotarsa decemlineata*. *Pest Manag. Sci.* 57: 968-974.

- Dahl AR, and Bechtold WE. 1985. Deposition and clearance of a water-reactive vapor, methylphosphonic difluoride (difluoro), inhaled by rats. *Toxicol. Appl. Pharmacol.* 81: 58-66
- Da Silva MHL, Zoghbi MGB, Andrade EHA, Maia JG, Maia JGS. 1999. The essential oils of *Peperomia pellucida* Kunth and *P. circinnata* Link var. *circinnata*. *Flavour Fragr. J.* 14: 312-314
- Davidson G. 1957. Insecticide resistance in *Anopheles sudaicus*. *Nature* 180(4598):1333-1335.
- Daviss B. 2005. Growing pains for metabolomics. *The Scientist.* 19(8):25-28
- De Moraes SM, Facundo VA, Bertini LM, Cavalcanti ESB, Dos Anjos Jf, Ferreira SA, de Brito ES, and de Souza Neto MA. 2007. Chemical composition and larvicidal activity of essential oils from *Piper* species. *Biochem. Syst.Ecol.* 35: 670-675
- Derecka K, Blythe MJ, Malla S, Genereux DP, Guffanti A, Pavan P, Moles A, Snart C, Ryder T, Ortori CA, Barrett DA, Schuster E, and Stoger R. 2013. Transient exposure to low levels of insecticide affects metabolic networks of honeybee larvae. *PLoS ONE* 8(7): e68191. doi:10.1371/journal.pone.0068191
- Dias SMC, Fernandes JB, Maia JGS, Gottlieb OR, Gottlieb HE, Fernandes JB, Maia JGS, Gottlieb OR, and Gottlieb HE. 1982. Neolignans from an *Aniba* species. *Phytochem.* 21(7):1737-1740
- Ding Y, Ortelli F, Rossiter LC, Hemingway J, and Ranson H. 2003. The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genomics* 4(1): 35.
- Dix KJ. 2010. Distribution and pharmacokinetics. *Hayes' Handbook of pesticide Toxicology* 1: 271-284
- Djouaka RF, Bakare AA, Coulibaly ON, Akogbeto MC, Ranson H, Hemingway J, and Strode C. 2008. Expression of the cytochrome P450s, CYP6P3 and CYP6M2, are significantly elevated in multiple pyrethroid resistant populations of *Anopheles gambiae* s.s. from Southern Benin and Nigeria. *BMC Genomics* 9:538
- DuRant JA. 1990. Influence of temperature on spring emergence of European corn borer moths (Lepidoptera: Pyralidae). *J. Agric. Entomol.* 7(3): 259-264
- El-Latif AOA, and Subrahmanyam S. 2010. Pyrethroid synergists suppress esterase-mediated resistance in Indian strains of the cotton bollworm, *Helicoverpa armigera* (Hübner). *Pesticide Biochem. Physiol.* 97: 279-288

- Farnham AW. 1973. Genetics of resistance of pyrethroids-selected house flies, *Musca domestica* L. *Pestic. Sci.* 4:513-520
- Farnham AW. 1977. Genetics of resistance of houseflies (*Musca domestica* L.) to pyrethroids. 1. knock-down resistance. *Pestic. Sci.* 8:631-636
- Farnsworth CA, Teese MG, Yuan G, Li Y, Scott C, Zhang X, Wu Y, Russell RJ, Oakeshott JG 2010. Esterase-based metabolic resistance to insecticides in heliothine and sopodopteran pests. *J. Pest Sci.* 35: 275-289.
- Faruque MD. 2008. A comparison of univoltine and multivoltine European corn borer (*Ostrinia nubilis* Hübner): life history characters, *Bt* toxin susceptibility, parasitoid impact, and population pattern. Ph.D. thesis. The Pennsylvania state univerisity.
- Fatope MO, Marwah RG, Onifade AK, Ochei JE, and Al Mahroqi YKS. 2006. <sup>13</sup>C NMR analysis and antifungal and insecticidal activities of Oman dill herb oil. *Pharm. Bio.* 44(1):44-49
- Ferro DN. 1993. Potential for resistance to *Bacillus thuringiensis*: Colorado potato beetle (Coleoptera: Chrysomelidae) a model system. *Am. Entomol.* 39: 38-44.
- Festucci-Buselli RA, Carvalho-Dias AS, de Oliveira-Andrade M, Caixeta-Nunes C, Li HM, Stuart JJ. 2005. Expression of Cyp6g1 and Cyp12d1 in DDT resistant and susceptible strains of *Drosophila melanogaster*. *Insect Mol. Biol.* 14: 69-77.
- Feyereisen R. 2012. Insect CYP genes and P450 enzymes, in: L.I. Gilbert (Ed.), *Insect Molecular Biology and Biochemistry*, Elsevier Academic Press, Oxford, pp. 236-316.
- ffrench-Constant RH. 2014. Insecticide resistance comes of age. *Genome Biology.*15:106
- Field LM, Blackman RL, Tyler-Smith C, Devonshire AL. 1999. Relationship between amount of esterase and gene copy number in insecticide-resistant *Myzus persicae* (Sulzer). *Biochem J.* 339: 737-742.
- Food and agriculture organization of the United Nations. 2012. *FAO specifications and evaluation for agricultural pesticides: Piperonyl butoxide.*
- Forgash, A.G. 1985. Insecticide resistance in the Colorado potato beetle. In Ferro, D.N. and R.H. Voss (eds.) *Proceedings of the Symposium on the Colorado potato beetle, XVII International Congress of Entomology.*

- Franklin MR. 1976. Methylenedioxyphenyl insecticide synergists as potential human health hazards. *Environ. Health Perspectives* 14: 29-37
- Fraser K, Lane GA, Otter DE, Hemar Y, and Quek SY. 2013. Analysis of metabolic markers of tea origin by UHPLC and high resolution mass spectrometry. *Food Res. Int.* 53: 827–835
- Fujitani T, Ando H, and Fujitani K. 1992. Sub-acute toxicity of piperonyl butoxide in F344 rats. *Toxicology.* 72: 291-298
- Fujitani T, AdaYT, and Yoneyama M. 1993. Hepatotoxicity of piperonyl butoxide in male F344 rats. *Toxicology.* 84:171-183
- Georghiou GP, Ariaratnam V, Pasternak ME, and Lin CS. 1975. Organophosphorus multiresistance in *Culex pipiens quinquefasciatus* in California. *J. Econ. Entomol.* 68(4):461-467.
- Greger H. 1981. Sesamin-type lignans as chemical markers within *Artemisia*. *Biochem. System. Ecol.* 9 (2/3):165-169.
- Gross M, Lewinsohn E, Tadmor Y, Bar E, Dudai N, Gross M, Cohen Y, Friedman J, and Gross M. 2009. The inheritance of volatile phenylpropenes in bitter fennel (*Foeniculum vulgare* Mill. var. *vulgare*, Apiaceae) chemotypes and their distribution within the plant. *Biochem. Syst. Ecol.* 37(4): 308-316
- Guengerich FP. 1999. Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* 39: 1-17
- Gunning RV, Moores GD, and Devonshire AL. 1998. Inhibition of resistance-related esterases by piperonyl butoxide in *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Aphis gossypii* (Hemiptera: Aphididae), Chapter 13, pp. 215-237. In D. G. Jones (ed.), Piperonyl butoxide. Academic, London, United Kingdom
- Guthrie WD, Jarvis JL, and Reed GL. 1985. Development of a third backup system for European corn borer (Lepidoptera: Pyralidae) egg production. *J. Econ. Entomol.* 78:497-499
- Haley T. 1978. PBO, [ $\alpha$ -(2-butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene: a review of the literature. *Ecotox. Environ. Safety* 2:9-31
- Hare, JD. 1980. Impact of defoliation by the Colorado potato beetle on potato yields. *J. Econ. Entomol.* 73: 69-73.

- Harrison CM. 1951. Inheritance of resistance to DDT in the house fly, *Musca domestica* L. *Nature* 167: 855-856
- Harrison TL, Zangerl AR, Schuler MA, and Berenbaum MR. 2001. Developmental variation in cytochrome P450 expression in *Papilio polyxenes* in response to xanthotoxin, a hostplant allelochemical. *Arch. Insect Biochem. Physiol.* 48: 179-189.
- Head G, Hoy CW, and Hall FR. 1995. Direct and indirect selection on behavioral response to permethrin in larval diamondback moths (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 88: 461-469
- Heckel DG. 2014. Insect detoxification and sequestration strategies. *Annu. Plant Rev.* 47:77-114
- Hegedus T, Aleksandrov A, Mengos A, Cui L, Jensen TJ, and Riordan JR. 2009. Role of individual R domain phosphorylation sites in CFTR regulation by protein kinase A. *Biochim. Biophys. Acta.* 1788:1341-1349
- Hemingway J, Hawkes NJ, McCarroll L, and Ranson H. 2004. The molecular basis of insecticide resistance in mosquitoes. *Insect. Biochem. Mol. Biol.* 34: 653-665
- Hemingway J, and Ranson H. 2000. Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol.* 45: 371-391.
- Hodgson E, and Kulkarni AP. 1974. Interactions of pesticides with cytochrome P450. *ACS Symp. Ser.* 2: 14-38.
- Hofmaster RN, Waterfield RL, and Boyd JC. 1967. Insecticides applied to the soil for control of eight species of insects on Irish potatoes in Virginia. *J. Econ. Entomol.* 60 (5):1311-1318.
- Horton DR, and Capinera JL. 1988a. Effects of host availability on diapause and voltinism in a non-agricultural population of Colorado potato beetle (Coleoptera: Chrysomelidae). *J. Kans. Entomol. Soc.* 61: 62-67.
- Horton DR, Capinera JL, and Chapman PL. 1988b. Local differences in host use by two populations of the Colorado potato beetle. *Ecology* 69: 823-831.
- Hotelier T, Renault L, Cousin X, Negre V, Marchot P, and Chatonnet A. 2004. ESTHER, the database of the alpha/beta-hydrolase fold superfamily of proteins. *Nucleic Acids Res* 32: 145-147.

- Hsiao TH. 1985. Ecophysiological and genetic aspects of geographic variations of the Colorado potato beetle. In Ferro, D.N., Voss, R.H. (eds.) Proceedings of the Symposium on the Colorado potato beetle, XVII International Congress of Entomology.
- Huang HS, Hu NT, Yao YE, Wu CY, Chiang SW, and Sun CN. 1998. Molecular cloning and heterologous expression of a glutathione S- transferase involved in insecticide resistance from the diamondback moth, *Plutella xylostella*. *Insect Biochem. Mol. Biol.* 28: 651-658
- Hudon M, LeRoux EJ, and Harcourt DG. 1989. Seventy years of European corn borer (*Ostrinia nubilalis*) research in North America. *In* Russell GE. (ed.). Agricultural Zoology Reviews. Vol. 3. Intercept, Wimborne, Dorset, UK.
- HudonM, and McLeod DGR. 1994. European corn borer. Section 12.16, p. 173- 174. In: R.J. Howard, J.A. Garland and W.L. Seaman (Eds.). Diseases and pests of vegetable crops in Canada. Canadian Phytopath. Soc. Entomol. Soc. Canada. M.O.M. Printing Ltd., Ottawa, ON, Canada.
- Ioannides C. 2008. Cytochromes P450: Role in the metabolism and toxicity of drugs and other xenobiotics. Royal Society of Chemistry. Cambridge, UK.
- Isman M.B. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annu. Rev. Entomol.* 51:45-66
- Jabrane A, Jannet HB, Harzallah-Skhirib F, Mastouri M, Casanova J, and Mighri Z. 2009. Flower and Root Oils of the Tunisian *Daucus carota L. ssp. Maritimus* (Apiaceae): Integrated Analyses by GC, GC/MS, and <sup>13</sup>C-NMR Spectroscopy, and in vitro Antibacterial Activity. *Chem. & Biodivers.*6: 881-889.
- Jabrane A, Jannet HB, Mastourib M, Mighri Z, and Casanova J. 2010. Chemical composition and in vitro evaluation of antioxidant and antibacterial activities of the root oil of *Ridolfia segetum* (L.) Moris from Tunisia. *Nat. Prod. Res.* 24(6): 491-499
- Jallow MFA, and Hoy CW. 2005. Phenotypic variation in adult behavioral response and offspring fitness in *Plutella xylostella* (Lepidoptera: Plutellidae) in response to permethrin. *J. Econ. Entomol.* 98: 2195-2202.
- Jallow MFA, and Hoy CW. 2006. Quantitative genetics of adults behavioral response and larval physiological tolerance to permethrin in diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 99:1388-1395.

- Jallow MFA, and Hoy CW. 2007. Indirect selection for increased susceptibility to permethrin in the diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 100:526-533.
- Jao LT, and Casida JE. 1974. Insect pyrethroid-hydrolyzing esterases. *Pestic. Biochem. Physiol.* 4:465-472.
- Jantan IB, Ahmad AS, Ahmad NA, and Ali NA. 1994. A comparative study of the essential oils of five *Piper* species from peninsular Malaysia. *Flavour Frag. J.* 9:339-342
- Jassbi AR, Mehrdad M, Soleimani M, Mirzaeian M, and Sonboli A. 2005. Chemical composition of the essential oils of *Bunium elegans* and *Bunium caroides*. *Chem. Nat. Compd.* 41(4): 415-417.
- Jensen HR, Scott IM, Sims SR, Trudeau VL, and Arnason JT. 2006. The effect of a synergistic concentration of a *Piper nigrum* extract used in conjunction with pyrethrum upon gene expression in *Drosophila melanogaster*. *Insect Mol. Biol.* 15(3):329-339
- Jiang WH, Guo WC, Lu WP, Shi XQ, Xiong MH, Wang ZT, and Li GQ. 2011. Target site insensitivity mutations in the AChE and LdVssc1 confer resistance to pyrethroids and carbamates in *Leptinotarsa decemlineata* in northern Xinjiang Uygur autonomous region. *Pest Biochem. Physiol.* 100:74–81
- Jirajaroenrat K, Pongjaroenkit S, Krittanai C, Prapanthadara L, and Ketterman AJ. 2001. Heterologous expression and characterization of alternatively spliced glutathione S-transferases from a single *Anopheles* gene. *Insect Biochem. Molec. Biol.* 31:867-875.
- Joffe T, Gunning RV, Allen GR, Kristensen M, Alptekin S, Field LM, and Moores GD. 2012. Investigating the potential of selected natural compounds to increase the potency of pyrethrum against houseflies *Musca domestica* (Diptera: Muscidae). *Pest Manag. Sci.* 68: 178-184.
- Jones DJ. 1999. The insecticide synergist: Piperonyl butoxide. Academic Press. London, UK pp.3
- Jurenka RA, Subchev M, Abad JL, Choi MY, and Fabrias G. 2003. Sex pheromone biosynthetic pathway for disparlure in the gypsy moth, *Lymantria dispar*. *Proc. Natl. Acad. Sci. USA*, 100: 809-814.
- Kakar SM, Paine MF, Stewart PW, and Watkins PB. 2004. 6',7'-Dihydroxybergamottin contributes to the grapefruit juice effect. *Clin. Pharmacol. Ther.* 75: 569-579.
- Kapoor IPS, Singh B, Singh G, de Heluani CS, de Lampsasona MP, and Catalan CAN. 2010. Chemistry and antioxidant activity of essential oil and oleoresins of black caraway (*Carum bulbocastanum*) fruits: Part 69. *J. Sci. Food Agric.* 90: 385-390

- Kawai M, Saegusa Y, Jin M, Dewa Y, Nishimura J, Harada T, Shibutani M, and Mitsumori K. 2009. Mechanistic Study on Hepatocarcinogenesis of Piperonyl Butoxide in Mice. *Toxicol. Pathol.* 37: 761-769.
- Ketterman AJ, Saisawang C, and Wongsantichon J. 2011. Insect glutathione transferases. *Drug Metab. Rev.* 43(2): 253-265
- Khot AC, Bingham G, Field LM and Moores GD. 2008. A novel assay reveals the blockade of esterases by piperonyl butoxide. *Pest Manag. Sci.* 64: 1139-1142
- Klebe G, Abraham U, and Mietzner T. 1994. Molecular Similarity Indices in a Comparative Analysis (CoMSIA) of Drug Molecules to Correlate and Predict Their Biological Activity. *J. Med. Chem.* 37 (24): 4130-4146.
- Konyushkin LD, Godovikova TI, Vorontsova SK, Tsyganov DV, Karmanova IB, Raihstat MM, Firgang SI, Pokrovskii MA, Pokrovskii AG, Semenova MN, and Semenov VV. 2010. Polyalkoxybenzenes from plant raw materials 4\*. Parsley and dill seed extracts in the synthesis of polyalkoxy-3,5-diaryl-1,2,4-oxadiazoles with antiproliferative activity. *Russ. Chem. Bulletin, International Edition.* 59(12): 2268-2275
- Kostal V, Korbelova J, Rozsypal J, Zahradnickova H, and Cimlova J. 2011a. Long-term cold acclimation extends survival time at 0 degrees C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. *PLoS ONE* 6:e25025.
- Kostal V, Zahradnickova H, and Simek P. 2011b. Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proceedings of the National Academy of Sciences of the USA* 108: 13041– 13046.
- Kramp KL. 2010. Targeted extraction of select natural products using supercritical CO<sub>2</sub>. Ph.D thesis. University of Ottawa.
- Kranthi K, Jadhav D, Wanjari R, Kranthi S, and Russel D. 2001. Pyrethroid resistance and mechanisms of resistance in field strains of *Helicoverpa armigera* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 94: 253-263.
- Kuhar TP, Kamminga K, Philips C, Wallingford A, and Wimer A. 2013. Chemical control of potato pests. In P. Giordanengo, C. Vincent and A. Alyokhin (eds.), *Insect pests of potato: Global perspectives on biology and management*. Elsevier, Waltham, MA. pp. 375-397

- Kumar S, Thomas A, Sahgal A, Verma A, Samuel T, and Pillai MKK. 2002. Effect of the synergist, piperonyl butoxide, on the development of deltamethrin resistance in yellow fever mosquito, *Aedes aegypti* L. (Diptera: Culicidae). *Arch. Insect. Biochem. Physiol.* 50:1-8.
- Kuraishi N, Matsui M, Ota H, and Chen SL. 2011. Specific separation of *Polypedates braueri* (Vogt, 1911) from *P. megacephalus* (Hallowell, 1861) (Amphibia: Anura: Rhacophoridae). *Zootaxa* 2744: 53-61
- Lapied B, Penetier C, Apaire-Marchais V, Licznar P, and Corbel V. 2009. Innovative applications for insect viruses: towards insecticide sensitization. *Trends Biotechnol.* 27(4): 190-198
- Larocque N, Vincent C, and Belanger A. 1999. Effects of tansy essential oil from *Tanacetum vulgare* on biology of oblique-banded leafroller, *Choristoneura rosaceana*. *J. Chem. Ecol.* 25(6):1319-1330
- Lee D, Park Y, Brown TM, and Adams ME. 1999. Altered properties of neuronal sodium channels associated with genetic resistance to pyrethroids. *Mol. Pharmacol.* 55: 584-593
- Lee K. 1991. Glutathione S-transferase activities in phytophagous insects: induction and inhibition by plant phototoxins and phenols. *Insect Biochem.* 21:353-62
- Lee SH, Dunn JB, Marshall CJ, and Soderlund DM. 1999. Molecular analysis of *kdr*-like resistance in a permethrin-resistant strain of Colorado potato beetle. *Pestic. Biochem. Physiol.* 63: 63-75.
- Lee SH, Kang JS, Min JS, Yoon KS, and Strycharz JP. 2010. Decreased detoxification genes and genome size make the human body louse an efficient model to study xenobiotic metabolism. *Insect Mol Biol.* 19: 599-615.
- Lehane MJ. 1997. Peritrophic matrix structure and function. *Annu. Rev. Entomol.* 42: 525-550
- Lenz EM, Hagele BF, Wilson ID, and Simpson SJ. 2001. High resolution <sup>1</sup>H NMR spectroscopic studies of the composition of the haemolymph of crowd- and solitary-reared nymphs of the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* 32: 51-56.
- Lewis LC. 1975. Natural regulation of crop pests in their indigenous ecosystems and in Iowa agrosystems: bioregulation of economic insect pests. *Iowa State J. Res.* 49: 435-445
- Li CX, Dong YD, Song FL, Zhang XL, Gu WD, and Zhao TY. 2009. Copy amplification of *estalpha2/ estbeta2* and correlation between esterase gene copy number and resistance to insecticides in the field *Culex pipiens pallens* strains collected from Beijing, China. *J. Med. Entomol.* 46: 539-545.

- Li X, Baudry J, Berenbaum MR, and Schuler MA. 2004. Structural and functional divergence of insect CYP6B proteins: From specialist to generalist cytochrome P450. *Proc. Natl. Acad. Sci.* 101: 2939-2944.
- Li X, Schuler MA, and Berenbaum MR. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Ann. Rev. Entomol.* 52: 231-253.
- Lindon JC, Holmes E, and Nicholson JK. 2007. Metabonomics in pharmaceutical R&D. *FEBS J*, 274:1140-1151.
- Lipke H, and Kearns CW. 1959a. DDT dehydrochlorinase. I. Isolation, chemical properties, and spectrophotometric assay. *J. Biol Chem* 234: 2123-2128.
- Lipke H, and Kearns CW. 1959b. DDT dehydrochlorinase. II. Substrate and cofactor specificity. *J. Biol Chem* 234: 2129-2132.
- Liu N, Li M, Gong Y, Liu F, and Li T. 2015. Cytochrome P450s -Their expression, regulation, and role in insecticide resistance. *Pestic. Biochem. Physiol.* doi:10.1016/j.pestbp.2015.01.006
- Liu N, Li T, Reid WR, Yang T, and Zhang L. 2011. Multiple cytochrome P450 genes: their constitutive overexpression and permethrin induction in insecticide resistant mosquitoes, *Culex quinquefasciatus*. *PLoS ONE* 6:e23403.
- Liu N, and Scott JG. 1998. Increased transcription of CYP6D1 cause cytochrome P450 mediated insecticide resistance in house fly. *Insect Biochem. Mol. Biol.* 28(8): 531-535
- Liu SQ, Scott IM, Pelletier Y, Kramp K, Durst T, Sims SR, and Arnason JT. 2014. Dillapiol: a pyrethrum synergist for control of the Colorado potato beetle. *J. Econ. Entomol.* 107(2):797-805.
- Liu Z, Williamson M, Lansdell S, Denholm I, Han Z, Millar NS. 2005. A nicotinic acetylcholine receptor mutation conferring target-site resistance to imidacloprid in *Nilaparvata lugens* (Brown planthopper). *PNAS* 102 (24): 8420-8425.
- Loder JW, and Nearn RH. 1972. Constituents from *Piper banksii*. *Phytochem.* 11:2645-2646
- Ma R, Cohen MB, Berenbaum MR, and Schuler MA. 1994. Black swallowtail (*Papilio polyxenes*) alleles encode cytochrome P450s that selectively metabolize linear furanocoumarins. *Arch. Biochem. Biophys.* 310: 332-340.

- Maia JGS, Zoghbi MGB, Andrade EHA Santos AS, da Silva MH, Luz AIR, and Bastos CN. 1998. Constituents of the essential oil of *Piper aduncum* L. growing wild in the Amazon region. *Flav. Frag. J.* 13:269-272
- Marcus CB, Murry M, Wang C, and Wilkinson CF. 1986. Methylendioxyphenyl compounds as inducers of cytochrome P450 and monooxygenase activity in the Southern armyworm (*Spodoptera eridania*) and the rat. *Pestic. Biochem. Physiol.* 26:310-322
- Marongiu B, Piras A, Porcedda A, Tuveri S, Maxia E, Goncalves MJ, Cavaleiro C, and Salgueiro L. 2007. Isolation of *Crithmum maritimum* L. volatile oil by supercritical carbon dioxide extraction and biological assays. *Nat. Product Res.* 21(13):1145-1150
- Martin S, Darrah L, Hibbard B. 2004. Divergent selection for rind penetrometer resistance and its effects on European corn borer damage and stalk traits in corn. *Crop Sci.* 44:711-717
- Martins AP, Salgueiro L, Vila F, Cañigueral S, Adzet J, Tomi A, and Casanova T. 1998. Essential oils from four *Piper* species. *Phytochem.* 49: 2019-2023.
- Matthews HB, Skrinjaric-Spoljar M, and Casida JE. 1970. Insecticide synergist interactions with cytochrome P-450 in mouse liver microsomes. *Life Sci.* 9: 1039-1048.
- Melander AL. 1914. Can insects become resistant to sprays? *J. Econ. Entom.* 7: 167-173
- Mena-Covarrubias J, Drummond FA, and Haynes DL. 1996. Population dynamics of the Colorado potato beetle (Coleoptera: Chrysomelidae). *Environ. Entomol.* 25 (1): 68-77.
- Michaud MR, and Denlinger DL 2007. Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J. Comparative Physiol. B* 177: 753-763.
- Montella IR, Schama R, and Valle D. 2012. The classification of esterases: an important gene family involved in insecticide resistance- A Review. *Mem. Inst Oswaldo Cruz, Rio de Janeiro.* 107(4): 437-449
- Moorefield HH. 1960. Resistance of carbamate insecticides. *Misc. Publ. Entomol. Soc. Am.* 2:151
- Mukerjee SK, Saxena VS, and Tomar SS. 1979. New methylendioxyphenyl synergists for pyrethrins. *J. Agric. Food Chem.* 27:1209-1211

- Mullin CA, Alfatafta AA, Harman JL, Everett SL, and Serino AA. 1991. Feeding and toxic effects of floral sesquiterpene lactones, diterpenes and phenolics from sunflower (*Helianthus annuus* L.) on western corn rootworm. *J. Agric. Food Chem.*, 39(12):2293-2299
- Mullin CA, González-Coloma A, Gutiérrez C, Reina M, Eichenseer H, Hollister B, and Chyb S. 1997. Antifeedant effects of some novel terpenoids on Chrysomelidae beetles: comparisons with alkaloids on an alkaloid-adapted and nonadapted species. *J. Chem. Ecol.* 23 (7):1851-1866
- Murry M. 2012. Toxicological Actions of Plant-Derived and Anthropogenic Methylenedioxyphenyl-Substituted Chemicals in Mammals and Insects. *J. Toxic. Environ. Health.* Part B, 15(6): 365-395
- Nauen R. 2007. Insecticide resistance in disease vectors of public health importance. *Pest Manag. Sci.* 63: 628-633.
- Nautiyal OP, and Tiwari KK. 2011. Extraction of Dill Seed Oil (*Anethum sowa*) Using Supercritical Carbon Dioxide and Comparison with Hydrodistillation. *Ind. Eng. Chem. Res.* 50, 5723–5726
- Nawrot J. and Harmatha J. 1994. Natural products as antifeedants against stored product insects. *Postharvest News and information.* 5, 17N-21N.
- Ndiath MO, Sougoufara S, Gaye A, Mazonot C, Konate L, Faye O, Sokhna C, and Trape JF. 2012. Resistance to DDT and pyrethroids and increased *kdr* mutation frequency in *An. gambiae* after the implementation of Permethrin-treated nets in Senegal. *PLoS ONE* 7(4): 10.1371/annotation/855f784c-b8db-449c-9de8-83b6404a049d. doi: 10.1371/annotation/855f784c-b8db-449c-9de8-83b6404a049d
- Newcomb RD, Campbell PM, Ollis DL, Cheah E, Russell RJ, and Oakeshott JG. 1997. A single amino acid substitution converts a carboxylesterase to an organophosphorus hydrolase and confers insecticide resistance on a blowfly. *Proc. Natl. Acad. Sci.* 94: 7464-7468.
- Nicholson JK, Lindon JC, and Holmes E. 1999. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 29:1181-1189.
- Niu GD, Rupasinghe SG, Zangerl AR, Siegel JP, Schuler MA, and Berenbaum MR. 2011. A substrate-specific sytochrome P450 monooxygenase, CYP6AB11, from the polyphagous navel orangeworm (*Amyelois transitella*). *Insect Biochem. Mol. Biol.* 41:244-253

- Nguyen SN, Song C, and Scharf ME. 2007. Toxicity, synergism, and neurological effects of novel volatile insecticides in insecticide-susceptible and -resistant *Drosophila* strains. *J. Econ. Entomol.* 100 (2): 534-544.
- Oakeshott JG, Johnson RM, Berenbaum MR, Ranson H, Cristino AS, and Claudianos C. 2010. Metabolic enzymes associated with xenobiotic and chemosensory responses in *Nasonia vitripennis*. *Insect Mol. Biol.* 19 (Suppl. 1): 147-163
- Ohta T, Nagahashi M, Hosoi S, and Tsukamoto S. 2002. Dihydroxybergamottin caproate as a potent and stable CYP3A4 inhibitor. *Bioorg. Med. Chem.* 10: 969-973
- Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolov F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J, Sussman JL, Ver- schueren KHG, and Goldman A. 1992. The alpha/beta hydrolase fold. *Protein. Eng.* 5: 197-211.
- Onstad DW. 2014. Insect resistance management: biology, economics, and prediction. Elsevier Ltd. London NW 17BY, UK. pp: 22-25
- Organic Material Review Institute. 2015. OMRI Canada product list. Retrieved on May 7, 2015. [http://www.omri.org/sites/default/files/cor\\_pdf/OMRI-CanadaPL-March2015-p1.pdf](http://www.omri.org/sites/default/files/cor_pdf/OMRI-CanadaPL-March2015-p1.pdf)
- Ortiz de Montellano PR, and Correia MA. 1995. Inhibition of cytochrome P450 enzymes. In T. Omura, Y. Ishimura, and Y. Fujii-Kuriyama (Eds.), *Cytochrome P-450* (2nd ed.) New York, NY: Plenum Press. pp. 305-364
- Ostrander B, and Coors J. 1997. Relationship between plant composition and European corn borer resistance in three maize populations. *Crop Sci* 37: 1741-1745
- Paine MF, Criss AB, and Watkins PB. 2004. Two major grapefruit juice components differ in intestinal CYP3A4 inhibition kinetic and binding properties. *Drug Metab. Dispos.* 32: 1146-1153.
- Parise-Filho R, Pastrell M, Camerlingo CEP, Silva GJ, Agostinho LA, de Souza T, Magri FMM, Ribeiro RR, Brandt CA, and Polli MC. 2011. The anti-inflammatory activity of dillapiole and some semisynthetic analogues. *Pharm. Biol.* 49 (11): 1173-1179
- Pasay C, Arlian L, Morgan M, Gunning R, Rossiter L, Holt D, Walton S, Beckham S, and Mc Carthy J. 2009. The effect of insecticide synergists on the response of Scabies mites to pyrethroid acaricides. *PLoS Negl Trop Dis* 3(1): e354. doi:10.1371/journal.pntd.0000354

- Pateira L, Nogueira T, Antunes A, Venâncio F, Tavares R, Capelo J, and Pateira L. 1999. Two Chemotypes of *Crithmum maritimum* L. from Portugal. *Flavour Fragr.J.* 14:333-343
- Pelletier Y, Horgan FG, and Pompon J. 2013. Potato resistance against insect herbivores: Resources and opportunities. In P. Giordanengo, C. Vincent and A. Alyokhin (eds.), *Insect pests of potato: Global perspectives on biology and management*. Elsevier, Waltham, MA. pp. 439-462
- Perry AS, and Buckner AJ. 1970. Studies on microsomal cytochrome P-450 in resistant and susceptible house flies. *Life Sci.* 9: 335-50.
- Pest management regulatory agency label database. 2014 (Retrieved on May 18, 2015).  
<http://pr-rp.hc-sc.gc.ca/pi-ip/index-eng.php>
- Pick A, Muller H, Wiese M. 2008. Structure-activity relationships of new inhibitors of breast cancer resistance protein (ABCG2). *Bioorg. Med. Chem.* 16(17): 8224-8236.
- Pino JA, Marbot R, Bello A, and Urquiola A. 2004. Essential oils of *Piper peltatum* (L.) Miq. and *Piper aduncum* L. from Cuba. Instituto de Investigaciones para la Industria Alimenticia, Havana, Cuba. *J. Essent. Oil. Res.* 16, 124-126.
- Plapp FW Jr. 1984. The genetic basis of insecticide resistance in the house fly: Evidence that a single locus plays a major role in metabolic resistance to insecticides. *Pestic. Biochem. Physiol.* 22: 194-201
- Poupardin R, Reynaud S, Strode C, Ranson H, Vontas J, and David JP. 2008. Cross-induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito *Aedes aegypti*: impact on larval tolerance to chemical insecticides. *Insect Biochem. Mol. Biol.* 38: 540-551
- Prapanthadara L, Koottathep S, Promtet N, Hemingway J, and Ketterman AJ. 1996. Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (Species B). *Insect Biochem. Mol. Biol.* 26: 277-285.
- Pridgeon JW, Appel AG, Moar WJ, and Liu N. 2002. Variability of resistance mechanisms in pyrethroid resistant German cockroaches (Dictyoptera: Blattellidae), *Pestic. Biochem. Physiol.* 73: 149-156.
- Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Bournsnel C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer ELL, Eddy SR, Bateman A, and Finn RD. 2012. The Pfam protein families databases. *Nucleic. Acids Res.* 40: D290-D301.

- Putri SP, Nakayama Y, Matsuda F, Uchikata T, Kobayashi S, Matsubara A, and Fukusaki E. 2013. Current metabolomics: practical applications. *J. Biosci. Bioeng.* 115(6): 579-589
- Quinton RJ. 1955. DDT-resistant Colorado potato beetles? Proceedings of the North Central Entomological Society of America: 94-95.
- Raffa KF, Berryman AA, Simasko J, Teal W, and Wong BL. 1985. Effects of grand fir monoterpenes on the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae), and its symbiotic fungus. *Environ Entomol.* 14: 552-556.
- Ranson H, Rossiter L, Orтели F, Jensen B, Wang X, Roth CW, Collins FH, and Hemingway J. 2001. Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochem. J.* 359: 295-304.
- Raymond M, Chevillon C, Guillemaud T, Lenormand T, and Pasteur N. 1998. An overview of the evolution of overproduced esterases in the mosquito *Culex pipiens*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 353: 1707-1711.
- Regnault- Roger C, Vincent C, and Arnason JT. 2012. Essential oils in insect control: low-risk products in a high-stakes world. *Annu. Review of Entomol.* 57: 405-424
- Rivera-Perez C, Nouzova M, Lamboglia I, and Noriega FG. 2014. Metabolic analysis reveals changes in the mevalonate and juvenile hormone synthesis pathways linked to the mosquito reproductive physiology. *Insect Biochem. Mol. Biol.* 51: 1-9
- Riveron JM, Yunta C, Ibrahim SS, Djouaka R, Irving H, Menze BD, Ismail HM, Hemingway J, Ranson H, Albert A, and Wondji CS. 2014. A single mutation in the *GSTe2* gene allows tracking of metabolically based insecticide resistance in a major malaria vector. *Genome Biol.* 15:R27
- Roberts DR, Chareonviriyaphap T, Harlan HH, and Hshieh P. 1997. Methods for testing and analyzing excito-repellency responses of malaria vectors to insecticides. *J Am Mosq Control Assoc.* 13(1): 13-17.
- Rupasinghe SG, Wen Z, Chiu TL, and Schuler MA. 2007. *Helicoverpa zea* CYP6B8 and CYP321A1: Different molecular solutions to the problem of metabolizing plant toxins and insecticides. *Protein Eng. Des. Sel.* 20: 615-624.
- Sadasivan S, and Thayumanayan B. 2003. Molecular host plant resistance to pests. Marcel Dekker, Inc New York, USA pp.217

- Sarles MP, Dove WE, and Moore D.H. 1949. Acute toxicity and irritation tests on animals with the new insecticide, piperonyl butoxide. *Am. J. Trop. Med.* 29: 151-166
- Scharf ME, Parimi S, Meinke LJ, Chandler LD, and Siegfried BD. 2001. Expression and induction of three family 4 cytochrome P450 (CYP4) genes identified from insecticide-resistant and susceptible western corn rootworms, *Diabrotica virgifera*. *Insect Mol. Biol.* 10: 139-146.
- Schuler MA. 2011. P450s in plant-insect interactions. *Biochim. Biophys. Acta* 1814:36-45
- Scott IM, Jensen H, Nicol R, Lesage L, Bradbury R, Sanchez-Vindas P, Poveda L, Arnason JT, and Philogène BJR. 2004. Efficacy of *Piper* (Piperaceae) extracts for control of common home and garden insect pests. *Hortic. Entomol.* 97(4): 1390- 1403
- Scott IM, Leduc RI, Burt AJ, Marles RJ, Arnason JT, and Foster BC. 2006. The inhibition of human cytochrome P450 by ethanol extracts of North American botanicals. *Pharm. Biol.* 44(5): 315-327
- Shimada T, Yamazaki H, Mimura M, Inui Y, and Guengerich FP. 1994. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.* 270: 414-23
- Shimoyama Siani A, Ramos MFS, Menezes-de-Lima O, Ribeiro-Dos-Santos R, Fernandez-Ferreira E, Soares R, Rosas EC, Susunaga G, Guimaraes A, Zoghbi M, and Henriques M. 1999. Evaluation of anti-inflammatory-related activity of essential oils from the leaves and resin of species of *Protium*. *J. Ethnopharmacol.* 66: 57-69
- Silva WC, Souza MJR, de Souza MJR, de Souza HEM, Heinzen H, Cesio MV, Mato M, Albrecht F, de Azevedo JL, and de Barros NM. 2009. Toxicity of *Piper aduncum* L. (Piperales: Piperaceae) from the Amazon forest for the cattle tick *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae). *Vet. Parasitol.* 164: 267-274
- Sinden SL, Cantello WW, Sanford LL, and Deahl KL. 1991. Allelochemically mediated host resistance to the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). *Mem. Entomol. Soc. Can.* 157: 19-28.
- Snart CJP, Hardy ICW, and Barrett DA. 2015. Entometabolomics: applications of modern analytical techniques to insect studies. The Netherlands Entomological Society Entomologia Experimentalis et Applicata 155: 1-17

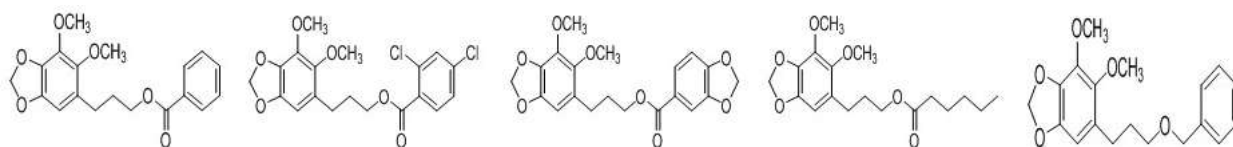
- Soerensen JS, and Soerensen NA. 1969. Studies related to naturally occurring acetylene compounds:XXXV, Investigation of *Erigeron* spp. from the Australian Mountains and Tasmania. *Aust. J. Chem.* 22:751-760
- Stresser DM, Blanchard AP, Turner SD, Erve JC, Dandeneau AA, Miller VP, and Crespi CL. 2000. Substrate-dependent modulation of CYP3A4 catalytic activity: analysis of 27 test compounds with four fluorometric substrates. *Drug Metabol. Dispos.* 28(12): 1140-1148.
- Tak JH. 2015. A study on insecticide synergy of plant essential oil constituents against *Trichoplusia ni*. Ph. D thesis. The University of British Columbia.
- Tanaka T, Takahashi O, Oishi S, and Ogata A. 2009. Effects of piperonyl butoxide on spontaneous behavior in F1-generation mice. *Toxicol. Ind. Health.* 25(7): 489-497
- Tellam RL, Wijffels G, and Willadsen P. 1999. Peritrophic matrix proteins. *Insect Biochem. Mol. Biol.* 29:87-101
- Terriere LC. 1983. Enzyme induction, gene amplification, and insect resistance to insecticides, in: G.P. Georgiou, T. Saito (Eds.), *Pest Resistance to Pesticides*, Plenum Press, New York, , pp. 265-297.
- Terriere LC. 1984. Induction of detoxification enzymes in insects, *Ann. Rev. Entomol.* 29: 71-88.
- Thompson SN, Lee RWKf, and Beckage NE 1990. Metabolism of parasitized *Manduca sexta* examined by nuclear-magnetic-resonance. *Arch. Int. Biochem. Physiol.* 13:127-143.
- Thompson SN 2001. Parasitism enhances the induction of glucogenesis by the insect, *Manduca sexta* L. *Int. J. Biochem. Cell B.* 33: 163-173.
- Tingey WM, and Sinden SL. 1982. Glandular pubescence, glycoalkaloid composition, and resistance to the green peach aphid, potato leafhopper, and potato fleabeetle in *Solanum berthaultii*. *Am. J. Potato Res.* 59: 95-106.
- Tomar SS, Maheshwari ML, and Mukerjee SK. 1979. Synthesis and synergistic activity of dillapiole based pyrethrum synergists. *J. Agric. Food Chem.* 27(3): 547-550
- Tsyganov DV, Yakubov AP, Konyushkin LD, Firgang SI, and Semenov VV. 2007. Polyalkoxybenenes from plant sources 2. \*Synthesis of isoxazoline analogs of combretastatin from natural allyl (methylenedioxy)methoxybenenes. *Russian Chemical Bulletin, International Edition.* 56(12): 2460-2465

- Usia T, Watabe T, Kadota S, and Tezuka Y. 2005. Metabolite-cytochrome P450 complex formation by methylenedioxyphenyl lignans of *Piper cubeba*: mechanism-based inhibition. *Life Sci.* 76(20): 2381-2391.
- Van- Leeuwen T, Vontas J, Tsagkarakou A, Dermauw W, and Tirry L 2010. Acaricide resistance mechanisms in the two-spotted spider mite *Tetranychus urticae* and other important Acari: a review. *Insect Biochem. Mol. Biol.* 40: 563-572.
- Velasco-Negueruela A, Pérez-Alonso MJ, Pérez de Paz PL, Palá-Paúl J, and Sanz J. 2003. Analysis by gas chromatography–mass spectrometry of the essential oils from the aerial parts of *Rutheopsis herbanica* (Bolle) Hans. & Kunk, gathered in Fuerteventura (Canary Islands). *J. Chrom. A.* 984: 159-162
- Vincent JFV, and Wegst UGK. 2004. Design and mechanical properties of insect cuticle. *Arthropod. Struct. Dev.* 33 (3): 187-199
- Vinson SB, and Law PK. 1971. Cuticular composition and DDT resistance in the tobacco budworm. *J. Econ. Entomol.* 64: 1387-1390
- Vontas JG, Small GJ, and Hemingway J. 2001. Glutathione S- transferases as antioxidant defence agents confer pyrethroid resistance in *Nilaparvata lugens*. *Biochem. J.* 357: 65-72.
- Vontas J, David JP, Nikou D, Hemingway J, Christophides GK, Louis C, and Ranson H. 2007. Transcriptional analysis of insecticide resistance in *Anopheles stephensi* using cross-species microarray hybridization. *Insect Mol. Biol.* 16: 315-324
- Wachs H, Jones H, and Bass L. 1950. New safe insecticides. *Advances in Chemistry Series* 1: 43-47.
- Waliwitiya R, Belton P, Nicholson RA, and Lowenberger CA. 2012. Plant terpenoids: acute toxicities and effects on flight motor activity and wing beat frequency in the blow fly *Phaenicia sericata*. *J. Econ. Entomol.* 105(1): 72-84
- Wang Y, Carolan JC, Hao F, Nicholson JK, Wilkinson TL, and Douglas AE. 2010. Integrated metabonomic-proteomic analysis of an insect-bacterial symbiotic system. *J. Proteome Res.* 9: 1257-1267.
- Wang ZL, Zhao Z, Abou-Zaid MM, Arnason JT, Liu R, Walshe-Roussel B, Wayne A, Liu SQ, Saleem A, Caceres LA, Wei Q, and Scott IM. 2014. Inhibition of insect Glutathione S-transferase (GST) by conifer extracts. *Arch. Insect Biochem. Physiol.* 87(4): 234-249

- Whalon ME, and Ferro DN. 1998. Bt-potato resistance management., In M. Mellon and J. Rissler (eds.), Now or never: Serious new plans to save a natural pest control. Cambridge: UCS. pp. 107-136.
- White JA, Burkness EC, and Hutchison WD. 2014. Biased sex ratios, mating frequency and *Nosema* prevalence in European corn borer, at low population densities. *J. Appl. Entomol.* 137(3): 195-201
- Wilkinson CF. 1983. The role of mixed function oxidases in insecticide resistance. In Georghiou GP, Saito T. (eds): Pest Resistance to Pesticides. New York: Plenum, pp 175-205.
- Willoughby L, Batterham P, and Daborn PJ. 2007. Piperonyl butoxide induces the expression of cytochrome P450 and glutathione S-transferase genes in *Drosophila melanogaster*. *Pest Manag. Sci.* 63: 803-808
- Wood OR, Hanrahan S, Coetzee M, Koekemoer LL, and Brooke BD. 2010. Cuticle thickening associated with pyrethroid resistance in the major malaria vector *Anopheles funestus*. *Parasit. Vectors* 3: 67
- Yang ML, Zhang JZ, Zhu KY, Xuan T, Liu XJ, Guo YP, and Ma EB. 2009. Mechanisms of organophosphate resistance in a field population of oriental migratory locust, *Locusta migratoria manilensis* (Meyen). *Arch. Insect Biochem. Physiol.* 71(1): 3-15.
- Yang Y, Wu Y, Chen S, Devine GJ, Denhoim I, Jewess P, and Moores GD. 2004. The involvement of microsomal oxidases in pyrethroid resistance in *Helicoverpa armigera* from Asia. *Insect Biochem. Mol. Biol.* 34: 763-773
- Yang Y, Dong B, Xu H, Zheng X, Tian J, Heong K, and Lu Z. 2014. Decrease of insecticide resistance over generations without exposure to insecticides in *Nilaparvata lugens* (Hemipteran: Delphacidae). *J. Econ. Entomol.* 107(4): 1618-1625
- Young S, Gunning RV, and Moores GD. 2005. The effect of PBO on pyrethroid resistance-associated-esterases in *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). *Pest Manag. Sci.* 61: 397-401
- Young SJ, Gunning RV, and Moores GD. 2006. Effect of pretreatment with piperonyl butoxide on pyrethroid efficacy against insecticide resistant *Helicoverpa armigera* (Lepidoptera: Noctuidae) and *Bemisia tabaci* (Sternorrhyncha: Aleyrodidae). *Pest Manag. Sci.* 62: 114-119
- Yu SJ. 2002. Substrate specificity of glutathione-S-transferases from the fall armyworm. *Pestic. Biochem. Physiol.* 74: 41-51

- Zamboni N, and Fischer SM. 2012. High-throughput, high-efficiency metabolome profiling using the Agilent 6550 iFunnel Q-TOF LC/MS system. Agilent Technologies Inc. USA
- Zhang L, Harada K, and Shono T. 1997. Genetic analysis of pyriproxifen resistance in the housefly, *Musca domestica* L. *Appl. Entomol. Zool. (Jpn.)* 32: 217-226.
- Zhang Q, Lu YX, and Xu XH. 2012. Integrated proteomic and metabolomic analysis of larval brain associated with diapause induction and preparation in the cotton bollworm, *Helicoverpa armigera*. *J. Proteome Res.* 11: 1042-1053.
- Zhu F, Feng JN, Zhang L, and Liu N. 2008a. Characterization of two novel cytochrome P450 genes in insecticide-resistant house-flies. *Insect Mol. Biol.* 17: 27-37.
- Zhu F, Li T, Zhang L, and Liu N. 2008b. Co-up-regulation of three P450 genes in response to permethrin exposure in permethrin resistant house flies, *Musca domestica*. *BMC Physiol.* 8:18
- Zhu F, and Liu N. 2008c. Differential expression of CYP6A5 and CYP6A5v2 in pyrethroid-resistant house flies, *Musca domestica*. *Arch. Insect Biochem. Physiol.* 34: 147-161.
- Zichová T, Kocourek F, Salava J, Nad'ová K, and Stará J. 2010. Detection of organophosphate and pyrethroid resistance alleles in Czech *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) populations by molecular methods. *Pest Manag. Sci.* 66: 853-860.
- Zimmer CT, and Nauen R. 2011a. Cytochrome P450 mediated pyrethroid resistance in European populations of *Meligethes aeneus* (Coleoptera: Nitidulidae). *Pestic. Biochem. Physiol.* 100: 264-272
- Zimmer CT, and Nauen R. 2011b. Pyrethroid resistance and thiacloprid baseline susceptibility of European populations of *Meligethes aeneus* (Coleoptera: Nitidulidae) collected in winter oilseed rape. *Pest. Manag. Sci.* 67(5): 599-608
- Zouari N, Fakhfakh N, Zouari S, Sellami M, Abid M, Ayadi MA, Zaidi S, and Neffati M. 2011. Volatile and lipid analyses by gas chromatography/mass spectrometry and nutraceutical potential of edible wild *Malva aegyptiaca* L. (Malvaceae). *Int. J. Food Sci. Nutr.* 62(6): 600- 608

## SUPPLEMENTARY DATA



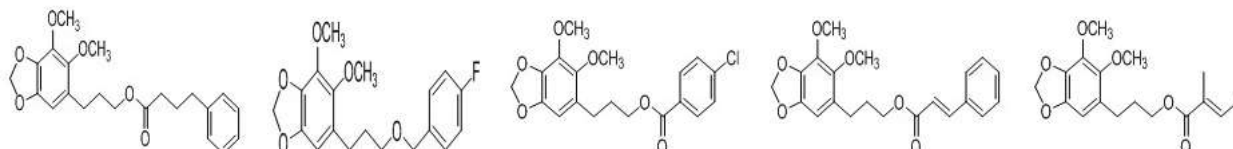
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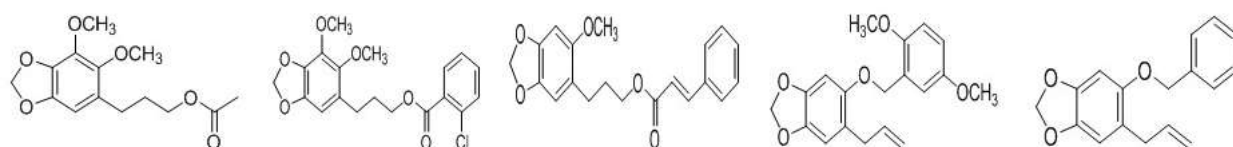
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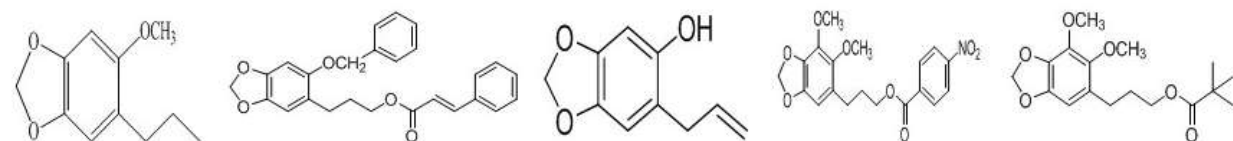
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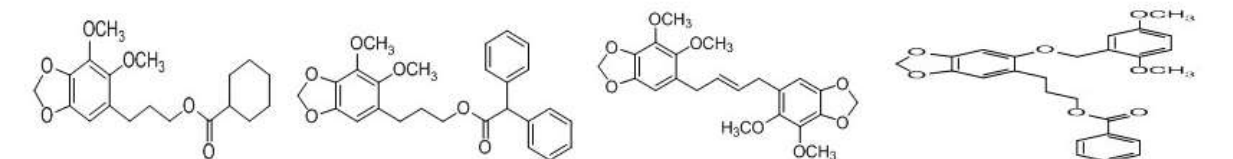
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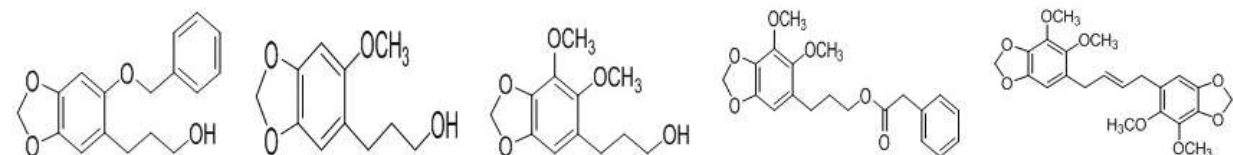


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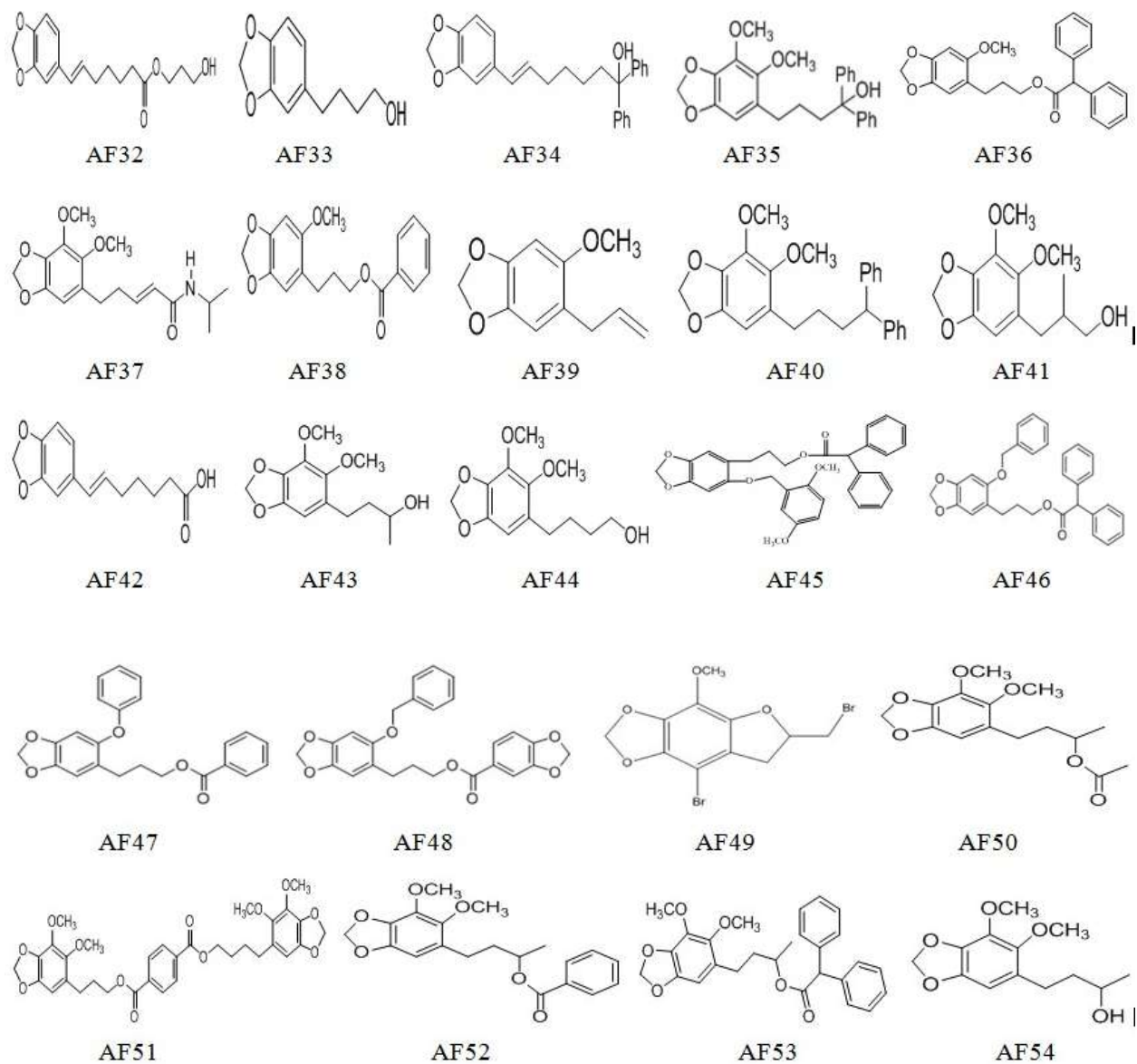
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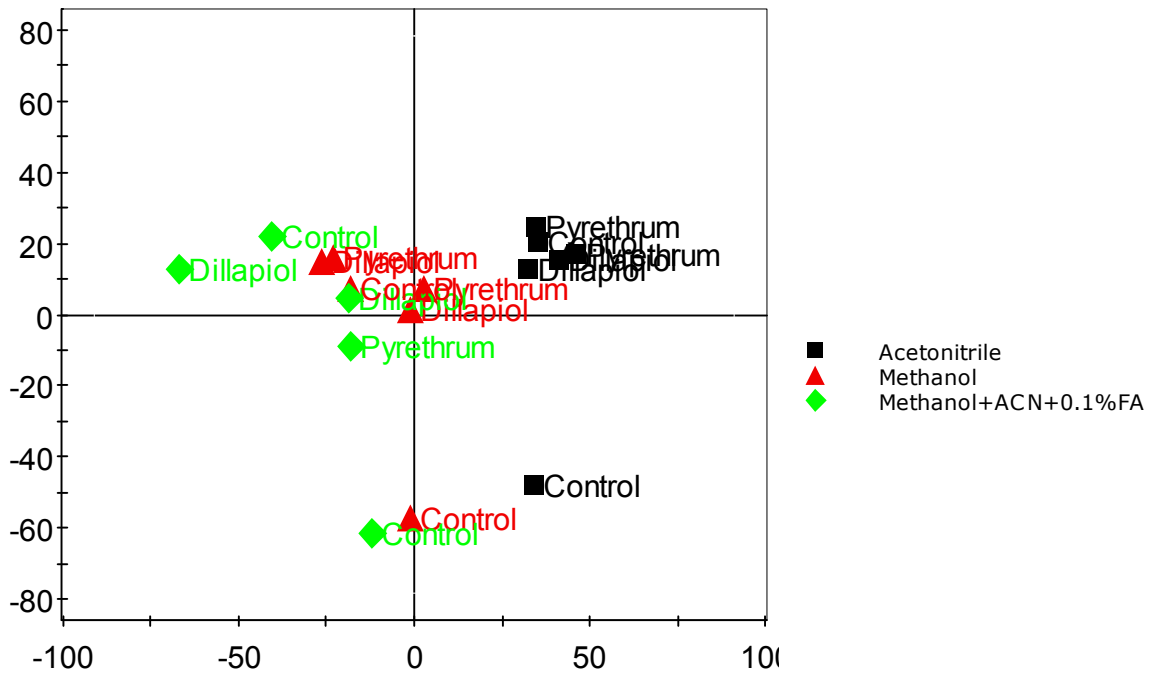


Sup. I Molecular structure of 52 dillapiol analogs.

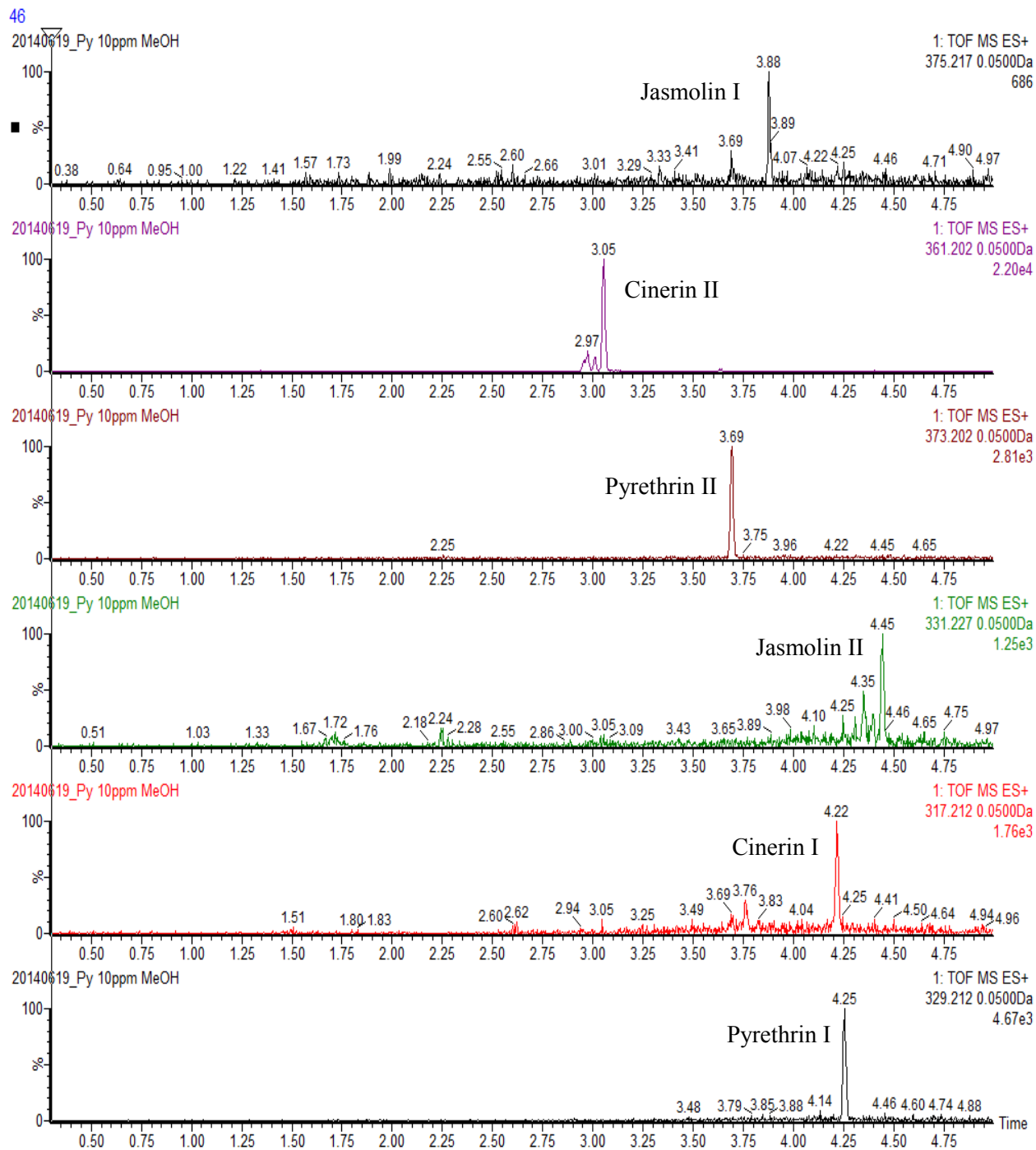
Sup. II IC<sub>50</sub> (95% C.I.) and dillapiol-relative inhibition activity (95% C.I.) of 52 dillapiol analogs.

Analog	IC <sub>50</sub> (μM) and 95% C.I.	Dillapiol-relative inhibition activity and 95% C.I.	Analog	IC <sub>50</sub> (μM) and 95% C.I.	Dillapiol-relative inhibition activity and 95% C.I.
<b>AF01</b>	2.1 (1.98-2.22)	4.4 (4.15-4.65)	<b>AF02*</b>	1.4 (1.15-1.65)	6.6 (5.36-7.84)
<b>AF03</b>	2.63 (1.27-4.00)	3.5 (1.73-5.48)	<b>AF04</b>	3.25 (3.13-3.37)	2.82 (2.69-2.98)
<b>AF05</b>	6.67 (6.04-7.29)	1.38 (1.22-1.51)	<b>AF06</b>	4.14 (3.73-4.54)	2.2 (1.95-2.45)
<b>AF07</b>	10.34 (9.51-11.16)	0.89 (0.82-0.96)	<b>AF08*</b>	1.56 (1.19-1.94)	5.9 (4.42-7.45)
<b>AF09</b>	2.38 (1.95-2.82)	3.877 (3.11-4.63)	<b>AF10*</b>	1.55 (1.03-2.07)	5.9 (3.69-8.21)
<b>AF12</b>	9.22 (6.55-11.88)	1.03 (0.75-1.32)	<b>AF13*</b>	1.33 (0.71-1.958)	7.03 (3.80-10.27)
<b>AF14</b>	4.73 (3.97-5.49)	1.94 (1.55-2.31)	<b>AF15</b>	2.15 (1.58-2.72)	4.3 (3.16-5.44)
<b>AF16*</b>	1.44 (1.43-1.46)	6.37 (6.22-6.51)	<b>AF17</b>	/	/
<b>AF18</b>	17.2 (13.59-20.81)	0.53 (0.39-0.68)	<b>AF19</b>	/	/
<b>AF20</b>	/	/	<b>AF21</b>	2.78 (1.70-3.86)	3.37 (1.95-4.78)
<b>AF22</b>	1.91 (1.59-2.23)	4.83 (4.07-5.59)	<b>AF23*</b>	0.43 (0.40-0.47)	21.2 (19.41-22.99)
<b>AF24</b>	1.8 (1.55-2.05)	5.13 (4.59-5.76)	<b>AF25*</b>	0.36 (0.26-0.46)	25.73 (18.64-32.83)

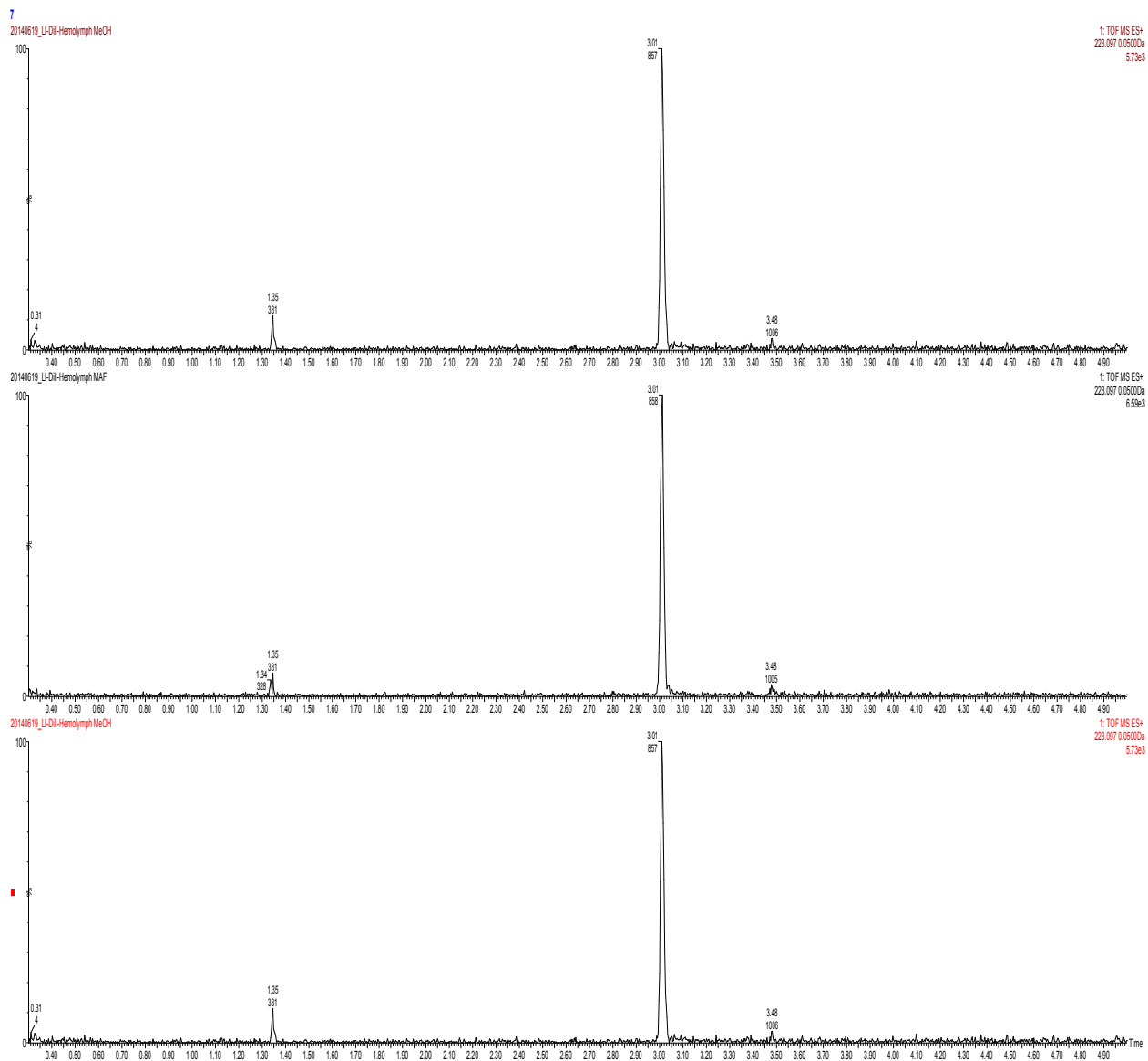
<b>AF26</b>	3 (2.75-3.25)	3.1 (2.85-3.35)	<b>AF27</b>	4.05 (2.28-5.83)	2.27 (1.26-3.27)
<b>AF28</b>	16 (13.88-18.12)	0.57 (0.42-0.71)	<b>AF29</b>	1.93 (0.46-3.39)	4.97 (2.0-7.93)
<b>AF31*</b>	1.44 (1.14-1.74)	6.47 (5.22-7.72)	<b>AF32</b>	4.07 (3.19-4.94)	2.27 (1.75-2.78)
<b>AF34*</b>	0.54 (0.40-0.69)	17 (12.63-21.37)	<b>AF35*</b>	1.06 (1.03-1.10)	8.63 (8.35-8.92)
<b>AF36*</b>	0.47 (0.25-0.70)	20.07 (9.74-30.4)	<b>AF37</b>	2.13 (1.99-2.28)	4.33 (4.05-4.62)
<b>AF38</b>	2.07 (1.45-2.70)	4.47 (3.1-5.84)	<b>AF39</b>	/	/
<b>AF40*</b>	1.4 (0.91-1.9)	6.62 (4.18-9.05)	<b>AF41</b>	/	/
<b>AF42</b>	/	/	<b>AF43</b>	/	/
<b>AF44</b>	11.6 (10.94-12.26)	0.79 (0.75-0.83)	<b>AF45*</b>	0.22 (0.09-0.35)	42 (25.55-59.11)
<b>AF46*</b>	1.03 (0.97-1.08)	8.93 (8.36-9.51)	<b>AF47</b>	5.82 (5.43-6.21)	1.57 (1.47-1.67)
<b>AF48*</b>	1.38 (1.25-1.51)	6.7 (6.04-7.36)	<b>AF49</b>	2.32 (2.13-2.51)	3.97 (3.64-4.29)
<b>AF50</b>	/	/	<b>AF51*</b>	1.6 (1.49-1.73)	5.8 (5.5-5.92)
<b>AF52</b>	3.49 (2.77-4.21)	2.7 (2.41-2.84)	<b>AF53*</b>	0.2 (0.15-0.24)	46.9 (43.8-48.2)
<b>AF54</b>	/	/	<b>Dillapiol</b>	9.18 (8.26-10.1)	1



Sup. III A 2D plot showing the clustering of metabolite profile from different insect tissues extracted with different solvents

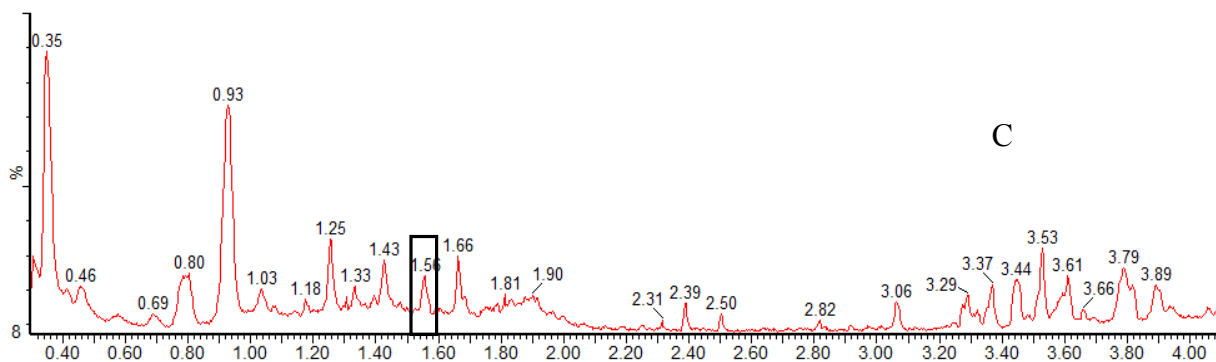
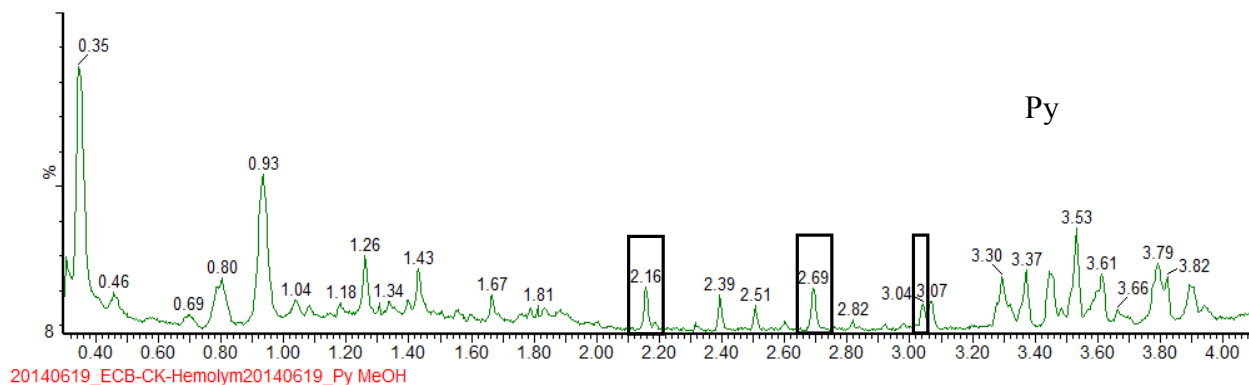
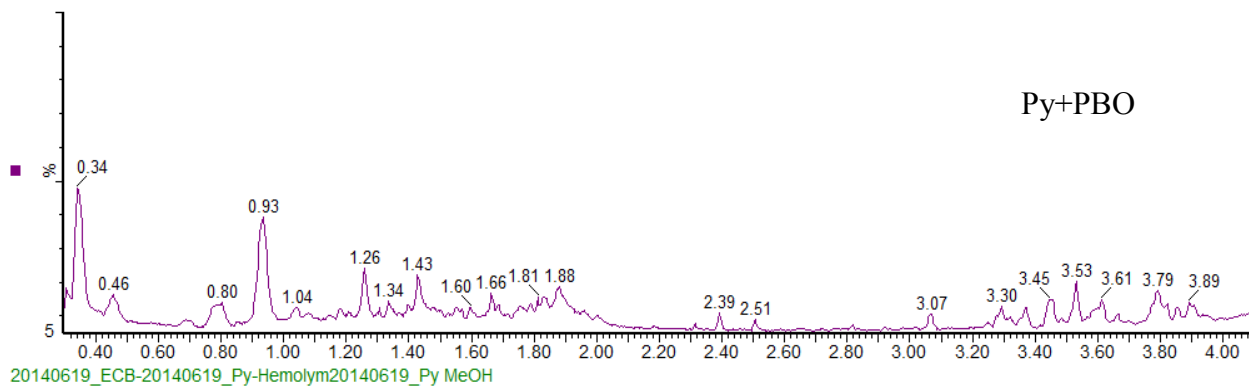


Sup. IV The UPLC-QTOF/MS chromatographs of six pyrethrins compounds detected in pyrethrum standard samples.



Sup. V The chromatographs of dillapiol detected in the hemolymph of 4<sup>th</sup> instar RS-CPB 1 h post topic treatment with pyrethrum/ dillapiol extracted with three different solvent systems.

20140619\_ECB-PBO-Hemolym20140619\_Py MeOH



Sup. VI The UPLC-QTOF/MS chromatographs with metabolite profiles of 4th instar SS-ECB hemolymph 1 h post topic treatment with acetone (C), pyrethrum (Py), or pyrethrum plus PBO (Py+PBO). The boxes showed the major different metabolite peaks in different samples.