TOXICOLOGY OF PLANT ESSENTIAL OILS IN BED BUGS

by

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Dedicated to my family

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Bed bugs (*Cimex lectularius* L.) are globally important human ectoparasites. Their management necessitates the use of multiple control techniques. Plant-derived essential oils are extracts from aromatic plants that represent one of the alternative control measures for bed bug control, in addition to mechanical options and synthetic pesticides. However, there is limited information available on the efficacy and toxicology of plant essential oils against bed bugs. This project was designed with the aim to provide in-depth information on efficacy, toxicology and mode-of-action of essential oils and their insecticidal constituents in bed bugs. Initially, I evaluated topical and fumigant toxicity of fifteen essential oil components against adult male bed bugs of the Harlan strain (an insecticide susceptible strain). Neurological effects of the six most toxicologically active compounds were also determined. In both topical and fumigant bioassays, carvacrol and thymol were the most active compounds. Spontaneous electrical activity measurements of the bed bug nervous system demonstrated neuroinhibitory effects of carvacrol, thymol and eugenol, whereas linalool and bifenthrin (a pyrethroid class insecticide) produced excitatory effects. Further, I evaluated the efficacy and neurological impacts of a mixture of three neuroinhibitory compounds; carvacrol, eugenol and thymol in 1:1:1 ratio against adult male bed bugs of the Harlan strain. This mixture of monoterpenoids as well as the mixture of synthetic insecticides exhibited a synergistic affect in topical bioassays. In electrophysiology experiments, the monoterpenoid mixture led to higher neuroinhibitory effects, whereas a mixture of synthetic insecticides caused higher neuroexcitatory effects in comparison to single compounds.

In the next objective of my dissertation, I compared the efficacy of five plant essential oils (thyme, oregano, clove, geranium and coriander), their major components (thymol, carvacrol, eugenol, geraniol and linalool) and EcoRaider® (commercial product) between pyrethroid susceptible (Harlan) and field collected (Knoxville) bed bug populations. Initially, I found that the Knoxville strain was 72,893 and 291,626 fold resistant to topically applied deltamethrin (a pyrethroid class insecticide) compared to the susceptible Harlan strain at the LD_{25} and LD_{50} lethal dose levels, respectively. Synergist bioassays and detoxification enzyme assays showed that the Knoxville strain possesses significantly higher activity of cytochrome P450 and esterase enzymes. Further, Sanger sequencing revealed the presence of the L925I mutation in the voltage gated sodium channel gene. The Knoxville strain, however, did not show any resistance to plant essential

oils, their major components or EcoRaider® in topical bioassays (resistance ratios of ~ 1). In the final objective, I evaluated the efficacy of binary mixtures of above-mentioned essential oils or their major components or EcoRaider® with deltamethrin in susceptible and resistant bed bugs. In topical application bioassays, binary mixtures of essential oils or their major components or EcoRaider® and deltamethrin at the LD_{25} dose caused a synergistic increase in toxicity in resistant bed bugs. Further, I studied the inhibitory effects of major essential oil components on detoxification enzyme activities (cytochrome P450s, esterases and glutathione transferases). Detoxification enzyme assays conducted using protein extracts from bed bugs pre-treated with essential oil constituents showed that these compounds significantly inhibited cytochrome P450 activity in the resistant strain, but esterase and glutathione transferase activity were unaffected. No inhibition of detoxification enzyme activities was observed in the Harlan strain bed bugs pre-treated with essential oil constituents.

In conclusion, my dissertation research has created the foundation for utilization of natural products for bed bug management by (i) describing the efficacy of plant essential oils and their components against bed bugs, (ii) discovering synergistic interactions between essential oil components at the nervous system level, (iii) determining susceptibility of deltamethrin-resistant bed bugs to plant essential oils and their constituents and (iv) identifying synergistic effects of essential oils or their components on toxicity of pyrethroid insecticides and underlying mechanisms of this synergistic interaction.

CHAPTER 1. DISSERTATION OBJECTIVES AND GOALS

This dissertation is written in an article-based format and citations are listed according to the style used for each of the respective journals. There are four research chapters (2-5) consisting of published peer-reviewed articles (chapters 2 and 3) or soon to be published manuscripts (chapters 4 and 5). In addition to research chapters, Chapter 1 includes information on dissertation objectives and their major goals, and Chapter 6 includes description on the overall conclusions of my research and future directions. Following are the four research objectives of my dissertation:

Chapter 2: Toxicity and neurophysiological impacts of plant essential oil components on bed bugs (Cimicidae: Hemiptera)

This chapter was published in the journal Scientific Reports in March 2019. The main goals of this research were (i) determination of topical and fumigant toxicity of fifteen essential oil components against bed bugs and (ii) identification of neurological effects caused by the six most effective/ toxic constituents by performing electrophysiology experiments.

Citation:

Gaire, S., M. E. Scharf, and A. D. Gondhalekar. 2019. Toxicity and neurophysiological impacts of plant essential oil components on bed bugs (Cimicidae: Hemiptera). Scientific Reports 9 (1), 3961. https://doi.org/10.1038/s41598-019-40275-5 (News outlets on article: Purdue University, Morning Ag Clips, Phys.org & many others).

Chapter 3: Synergistic toxicity interactions between essential oil components against the common bed bug (*Cimex lectularius* L.)

This chapter was published in the journal Insects in February 2020. The main goals of this study were to determine the impacts of an equal ratio mixture of carvacrol, eugenol and thymol on their (i) efficacy against bed bugs and (ii) neuroinhibitory effects on the bed bug nervous system.

Citation:

Gaire, S., M. E. Scharf, and A. D. Gondhalekar. 2020. Synergistic toxicity interactions between plant essential oil components against the common bed bug (*Cimex lectularius* L.). Insects 11, 133. https://doi.org/10.3390/insects11020133

Chapter 4: Bed bugs, *Cimex lectularius* L. exhibiting metabolic and target site deltamethrin resistance are susceptible to plant essential oils

This chapter is formatted according to the style used for the journal Pesticide Biochemistry and Physiology. The main goals of this chapter were to; (i) determine deltamethrin resistance levels, and its mechanisms in a field strain of bed bugs which has an insecticide exposure history (ii) compare the effectiveness and quantify the resistance levels of five plant essential oils and their major constituents in the field-collected strain of bed bugs.

Chapter 4: Plant essential oils synergize deltamethrin toxicity in a resistant strain of the bed bug (*Cimex lectularius* L.) by inhibiting cytochrome P450 enzymes

This chapter is also formatted according to the style used for the journal Pesticide Biochemistry and Physiology. This study was designed with the aim of identifying potential synergistic interactions between binary mixtures of plant essential oils or their constituents and deltamethrin in insecticide susceptible and resistant bed bug populations. Additionally, the effects of essential oil components on detoxification enzymes activities of both bed bug populations were evaluated.

CHAPTER 2. TOXICITY AND NEUROPHYSIOLOGICAL IMPACTS OF PLANT ESSENTIAL OIL COMPONENTS ON BED BUGS (CIMICIDAE: HEMIPTERA)

This chapter was published in the journal Scientific Reports in March 2019. Citation:
Gaire, S., M. E. Scharf, and A. D. Gondhalekar. 2019. Toxicity and neurophysiological impacts of plant essential oil components on bed bugs (Cimicidae: Hemiptera). Scientific Reports 9 (1), 3961. https://doi.org/10.1038/s41598-019-40275-5 (News outlets on article: Purdue University, Morning Ag Clips, Phys.org & many others).

2.1 Introduction

Bed bugs (*Cimex lectularius* L.) are economically and medically important global human parasites. They feed on human blood and their bites can worsen psychological disorders, cause sleep deprivation and other health issues such as rashes, itching, allergies, and etc.[1]. The U.S. Center for Disease Control and Prevention (CDC) and the U.S. Environmental Protection Agency (EPA) consider bed bugs as a pest of significant public health importance[2]. A resurgence of bed bugs has occurred over the last 18 years and they continue to spread. One of the primary factors for their resurgence is due to the overuse of synthetic insecticides with similar modes of action, which has led to insecticide resistance development[3–6]. The application of synthetic insecticides within buildings or in indoor environments is also a public health concern due to the toxic effects that can result from prolonged exposure[7–9].

Integrated pest management (IPM) approaches have been proposed for the effective management of bed bugs. This strategy includes the use of multiple control tactics: resident education, bed bug monitoring using active and passive traps, non-chemical control (removal of infested furniture, heat treatments, use of mattress encasements etc.), along with the use synthetic and essential-oil based insecticides[10–12]. There is also an increased demand from the public for use of efficacious "green" products for urban pest management. Botanical insecticides, including essential oils are considered safe because of their low toxicity to humans and animals[13–14]. Plant-derived essential oils have emerged as a potential alternative option for the management of insect pests[15–16]. Because they pose a minimum risk, essential oil compounds are exempt from full EPA registration (Federal Insecticides, Fungicides, and Rodenticides Act-FIFRA, 40 CFR 152.25)[17]. Some of the drawbacks associated with the use of essential oils for pest control are: (i) short

residual life that necessitates frequent applications (ii) high volatility can lead to odor problems, which are sometimes unacceptable to residents, and (iii) field efficacy of these products is generally less documented for different insect pest species[15-16].

Essential oils are secondary metabolites derived from aromatic plants that are composed of complex mixtures of chemical constituents or components with different functional groups (e.g., phenols, aldehydes, acids, hydrocarbons, etc.)[18]. Recent studies have shown that plant-derived essential oils exhibit contact and fumigant toxicity against field populations of bed bugs[14,19,20]. However, these studies have not characterized the insecticidal activity of major constituents of essential oils against bed bugs. More than a dozen essential oil-based products are available commercially for indoor use, but only two products have been found effective for bed bug control[21]. Therefore, there is a need for conducting comparative baseline toxicity studies with bed bugs using major components or constituents of different plant essential oils (Table A.1) that have been shown to be efficacious against urban and agricultural insect pests[22–31].

There is also a significant knowledge gap regarding the effects of major or active components of essential oils on the insect nervous system[32–33]. The possible target sites for the essential oil components thymol, eugenol, and carvacrol are gamma-amino butyric acid (GABA), octopamine/tyramine and nicotinic acetylcholine (nACh) receptors, respectively[34–37]. Very few studies have documented electrophysiological responses induced by application of essential oil components to the nervous system of insects. Price and Berry[38] reported that the essential oil components eugenol, geraniol and citral are neurologically active against *Periplaneta americana* and *Blaberus discoidalis*. Similarly, Hertel et al.[39] found the plant essential oil components quassin and cinnamaldehyde to be neurologically active against *P. americana*. Recent *in silico* molecular docking studies with major chemical constituents of marigold essential oil (α-terpinolene, piperitone and piperitenone) suggested the neurotransmitter hydrolyzing enzyme acetylcholinesterase as the potential target site in bed bugs[14].

Given the knowledge gaps associated with the unavailability of comparative toxicity data for individual essential oil constituents against bed bugs, and their impacts on the nervous system, the objectives of this research were (i) to determine topical and fumigant toxicity of fifteen essential oil components against bed bugs and (ii) identify neurological effects caused by the six most effective constituents by performing electrophysiology experiments.

2.2 Materials and methods

2.2.1 Insects

The susceptible Harold Harlan strain of bed bug was used for all experiments. This strain was maintained at 25℃, 50±15% relative humidity, and a photoperiod of 12:12 (L: D) h. Bed bugs were fed weekly on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using the membrane feeding method[60]. Each week, 5th instar nymphs were separated from the main colony and reared in different jars. Newly emerged adult males were separated and used in all experiments. For toxicity evaluation, 8–10 d old adult males were used (average weight = ~2 mg per insect) that were fed 4–5 d before bioassays. However, for electrophysiology studies 10–15 d old adult males that were fed 7–8 d before evaluation were used. This starvation period allowed for clean dissections due to the absence of undigested blood in the foregut and midgut (Fig. 2.1b).

2.2.2 Chemicals

High purity essential oil components carvacrol, geraniol, eugenol, methyl eugenol, trans-cinnamaldehyde, citronellic acid, (±)-citronellal, α-pinene, linalool, R (+)-limonene, eucalyptol, (–)-terpinen-4-ol, and menthone were obtained from Sigma-Aldrich (St. Louis, MO), whereas thymol and (±)-camphor were obtained from Alfa Aesar (Hill, MA) (Table A.1). These active constituents are found in various aromatic plants (Table A.1). All fifteen essential oil components (Table A.1) were selected based upon the previous toxicity literature on different urban and agricultural pests[22–31]. The positive controls dichlorvos (≤ 100% purity) and bifenthrin (98% purity) were obtained from Sigma Aldrich and Chem Service Inc. (West Chester, PA), respectively. Analytical grade solvents such as acetone, ethanol and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Pittsburgh, PA). Buffer salts and other reagents used for preparation of HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered physiological saline were purchased from Sigma-Aldrich, Fisher Scientific and Avantor Performance Materials, LLC (Center Valley, PA).

2.2.3 Topical application

Initially, each essential oil component was diluted in acetone on a volume-to-volume basis to prepare stock solutions based on the density of each component (Table A.1). The only exceptions were thymol and (±)-camphor, which were prepared on a weight per volume basis due to their crystalline nature or form. The stock solutions were then serially diluted to prepare a range of dilutions (at least 5 for each component). Topical applications of different concentrations (volume range 0.5-1 µL) were made on the ventral metathorax using a 25 µL micro-syringe (Hamilton, Reno, NV) attached to a PB-600-1 repeating dispenser (Hamilton, Reno, NV). Insects were immobilized by attaching them dorsally to colored labelling tape (Fisher Scientific, Pittsburg, PA). Control groups were treated with acetone only. Technical grade bifenthrin dissolved in acetone (weight to volume basis) was used as a positive control. After treatment, insects (in groups of 10) were transferred into 35 x 10 mm Petri dishes with vents (Item number: 627161, Greiner Bio-One, Frickenhausen, Germany) lined with a single layer of Whatman # 1 filter paper (GE Healthcare UK Limited, Amersham Place, UK). Petri dishes were then placed in an environmental chamber with temperature, humidity and lighting conditions similar to those used for rearing. Initial bioassay experiments suggested that mortality caused by essential oil treatments did not significantly change between observation intervals of 24 and 48 h. Therefore, mortality scoring of all treatments was performed at 24 h post-treatment. Insects that were lying on their backs and/or were unable to move upon prodding were scored as dead. In total, three replicates were performed for each concentration (n = 30). The average weight of a single adult male bed bug used for bioassays was 2 mg. Hence, the topical lethal dose values are reported as µg/ mg body weight.

2.2.4 Fumigant exposure and quantification of evaporation for essential oil components

Filter papers (9 cm diameter, Whatman™ #1) (GE Healthcare UK Limited) were treated with essential oil component solution (volume range: 9.46-1892 µL) prepared in acetone as described under "Topical application" bioassays. Treated papers were placed in glass containers (473 mL Mason jars; Anchor Glass Container Corporation, Tampa, FL) after complete evaporation of acetone. Evaporation time varied from ~30 sec to 5 mins based upon insecticide volume that was applied to the filter paper. In case of dichlorvos, only 30-45 sec of evaporation time was required because the treatment volume of ~10–15 µL was much lower in comparison to that of

essential oil components. Ten adult bed bugs held in a mesh-covered glass scintillation vial (20 mL; W.W. Grainger, Inc., Lake Forest, IL) were then placed in mason jars along with treated filter papers. The mason jar was then sealed completely and transferred to an environmental chamber. Control insects were exposed to acetone treated filter papers. Acetone application volume for controls corresponded to the volume used for highest insecticide concentration or application volume of each tested compound. Three replicates (n = 30) were performed for each concentration. Mortality did not significantly change after the initial 24 h observation interval, as such all observations were recorded 24 h post exposure. Mortality was scored by following the same protocol described for topical bioassays. Fumigant lethal concentration values are expressed as amount of insecticide per liter air volume (mg/L).

To determine essential oil constituent or DDVP evaporation levels during the 24 h bioassay period, we first measured the weight (in grams) of untreated filter papers (W_0) on a Mettler AE 100 weighing scale (Mettler-Toledo, Inc., Columbus, OH). After that, acetone-diluted essential oil constituents or insecticides were applied to the filter paper and the weights of these treated filter papers were recorded after the acetone (solvent carrier) evaporation period (30 sec to 5 mins) described in the previous paragraph had elapsed (W_1). Control filter papers were treated with acetone only. Filter papers were then placed individually in sealed mason jars for 24 h. At 24 h, filter papers were weighed again (W_2). Three concentrations (low, medium and high) were used for determining evaporation percentage for each compound. They were representative of the entire range of concentrations tested in fumigant bioassays for each compound. Three independent replicates were performed for each concentration. The following formula was used for calculating percent evaporation:

$$\% \text{ Evaporation} = \frac{\text{Amount evaporated } (W_1\text{-}W_2)}{\text{Amount applied } (W_1\text{-}W_0)} \times 100$$

2.2.5 Electrophysiology equipment

The electrophysiology equipment used in this study was previously described by Gondhalekar and Scharf[61] and Feston[62]. The setup consists of three electrodes; recording, reference and ground (Fig. 2.1a). Recording and reference electrodes were mounted on suction electrode holders (Cat. No. 64-1035 Warner Instruments, Hamden, CT). Both electrodes were fabricated from ~ 4 cm lengths of 0.5 mm diameter gold wire (World Precision Instruments, Sarasota, FL)

and fitted within 1.0 mm borosilicate glass capillaries (Harvard Apparatus, Holliston, MA) that were pulled to a fine point with a Micropipette puller (Narishige Co., LTD, Tokyo, Japan). Capillaries were used only for single recordings. The ground electrode consisted of #2 steel pin (Catalog #1208B2 Bio Quip Products, Rancho Dominguez, CA) which was held by a Pin Vise (#162A The L.S. Starrett Company Athol, MA). All electrodes were connected to a model 4001 capacitance compensation head stage (Dagan Inc., Minneapolis, MN), which was connected to a Hum Bug 50/60 Hz Noise Eliminator (Quest Scientific Instruments Inc., North Vancouver, BC, Canada) and then a model EX-1 differential amplifier (Dagan Inc., Minneapolis, MN). The amplifier was interfaced with computerized digitizing hardware (PowerLab/ 4SP, ADInstruments, Milford, MA) and software that functioned as an eight-channel chart recorder (Chart version 3.5.7, ADInstruments, Milford, MA).

2.2.6 Dissections and neurophysiology recordings

Dissections were performed in 35 x 15 mm Petri dishes (Fisher Scientific, Hampton, NH) filled 2/3 of their volume with wax (Frey Scientific and CPO Science, Nashua, NH) (Fig. 2.1a) under a Leica S6D Greenough stereo microscope (Leica Microsystems Inc. Buffalo Grove, IL). Bed bugs were immobilized by four 0.15 mm stainless minutien pins (Carolina Biological Supply Company, Burlington, NC) during dissection (Fig. 2.1b). New Petri dishes and minutien pins were used for each recording. The general procedure described by Feston[62] was used for performing dissections. Each experimental bed bug was dissected via one longitudinal incision from the dorsal abdomen up to the thorax followed by two latitudinal incisions across the wing pads to expose the fused ganglion (Fig. 2.1b)[63]. One microliter of HEPES-buffered saline, pH 7.1 was pipetted into the insect hemocoel immediately after dissection. Fat bodies, gut and other thoracic and abdominal body tissues were removed for better visualization of the ganglion (Fig. 2.1b-c).

Baseline electrical or nerve activity recordings were performed in HEPES-buffered physiological saline (volume: 1.5-2µL; 185mM sodium chloride, 10 mM potassium chloride, 5 mM HEPES sodium salt, 5 mM calcium chloride, 5 mM magnesium chloride and 20 mM glucose; pH 7.1)[61,62,64]. The recording electrode, fitted with a pulled glass capillary and filled with HEPES-buffered saline, was placed in gentle contact with the fused ganglia (Fig. 2.1a-c) with the help of a micromanipulator (model MNJR, World Precision Instruments, Sarasota, FL). The reference electrode was prepared identically and placed in contact with the carcass (Fig. 2.1a). A ground

electrode was placed in the dissection dish outside the bed bug carcass, but in contact with physiological saline (Fig. 2.1a). The total electrical activity recording for each insect was done for 10 minutes (Fig. 2.2). For the first 5 mins, spontaneous pretreatment electrical activity (*i.e.*, baseline) was recorded by setting a threshold for the "counter" function on the Chart software (Fig. 2.2). The baseline electrical activity recording in physiological saline was briefly paused after the first 5 mins to enable application of 1 µL of essential oil component solution gently onto the ganglion. Multiple concentrations of essential oil constituents ranging from 0.5 to 5 mM were tested (approx. 0.5 to 5 mM or 3.75×10^{-12} to 4.25×10^{-10} µg of constituent per insect preparation). This solution was prepared by diluting essential oil components initially in DMSO (used for thymol, carvacrol, eugenol, citronellic acid and linalool dilution) or ethanol (used for (±)-camphor dilution) and then further dilutions were made in physiological saline containing 0.01% Tween 20. Recordings were resumed approximately 10–15 sec after the application of essential oil-containing solution. The waiting period of 10–15 sec was included to allow the ganglion to recover from the physical disturbance (if any) caused by application of 1 µL essential oil constituent solution. The threshold for the "counter" function remained constant for the 5 min pre-treatment and 5 min post-treatment nerve activity recordings (Fig. 2.2). For control recordings, a solution containing physiological saline + 0.1% DMSO or 0.1% ethanol + 0.01% Tween 20, but no essential oil component was used. To compare or see the effect of solvent controls on nervous activity, recordings were performed using physiological saline for the 5 min pre-treatment and 5 mins post-treatment recordings.

Departure ratios that represent deviation from the baseline electrical activity were calculated by dividing the total number of spike counts surpassing the threshold in post-treatment 5 min recordings (with essential oil constituents) with the total number of spike counts above threshold in 5 min of pre-treatment recordings (with physiological saline). Departure ratios that were significantly greater than "1.0" indicated neuroexcitatory action and ratios that were significantly less than "1.0" were indicative of neuroinhibition[61]. Similar procedures were followed to calculate departure ratios for solvent control preparations.

For the positive control treatment using bifenthrin (a pyrethroid insecticide), the same procedures were followed, however, the treatment volume was higher (2 µL). The use of a higher volume was necessary for bifenthrin based on preliminary experiments. In a preliminary study, 1 µL volume of 1.25–10 µM bifenthrin did not significantly excite the bed bug ventral nerve cord.

Each bed bug or dissection represented one replicate and ten replications were performed for each essential oil component or positive control (bifenthrin) concentration, solvent controls and physiological saline controls. The recordings in which bed bugs were dead during or after 10 minutes were discarded and a new recording was performed with a new insect preparation to account for the loss.

2.2.7 Topical bioassays to observe poisoning symptoms

To observe poisoning symptoms at the whole organism level caused by the six most toxic essential oil components, topical application bioassays were performed at the LD_{50} for each compound. Acetone-diluted compounds were applied to the metathoracic region using identical procedures outlined for "Topical application" bioassays. Control insects were treated with acetone. Poisoning symptoms exhibited by adult male bed bugs were observed at 2 and 4 h post treatment either directly in the bioassay Petri dish or under a microscope. Short videos (~30 secs) of bed bugs from various treatments were also recorded at the 2 and 4 h intervals and were used to confirm or cross-check the presence or absence poisoning symptoms. In total 30 insects were observed for each compound. Specifically, the presence or absence of three symptoms was observed: (1) hyperactivity (uncoordinated movement and wandering behavior), (2) paralysis (inability to walk or right themselves up on prodding) and (3) tremors (insects lying on their back and exhibiting involuntary leg spasms, twitching and quivering).

2.2.8 Statistical analysis

Probit analysis was performed on dose-mortality and concentration-mortality data of topical application and fumigant exposure bioassays to calculate LD_{50} and LC_{50} values, respectively and their 95% fiducial limit (FL)[65]. Relative median potency analysis was performed to statistically compare toxicity differences between the compounds[66,67]. The LD_{50} or LC_{50} values between different compounds are significantly different ($P < 0.05$) if confidence intervals (CIs) for toxicity ratios did not overlap with 1[66,67]. For the electrophysiology study, departure ratios calculated for essential oil components or bifenthrin were log transformed after adding the value one (1) to all departure ratios. The addition of "1" to all departure ratio values allowed us to obtain positive log transformed data, i.e., to prevent negative log transformed values. Log transformed

departure ratio data for different compounds were analyzed using linear regression to determine if they caused concentration-dependent increases or decreases in nervous system activity ($P<0.05$). Departure ratios determined for solvent controls (DMSO and ethanol) were statistically compared to the physiological saline treatment using two-sample t-tests with a Bonferroni adjusted significance level of $P<0.025$ (0.05 ÷ number of comparisons or tests)[68,69]. Bonferroni corrected two-sample t-tests were also used for conducting pairwise comparisons between log transformed departure ratio data for solvent controls and various concentrations tested for essential oil compounds or bifenthrin[68,69]. Relative median potency analysis of topical and fumigant toxicity was performed using SPSS Version 25. All other statistical analysis, including LD_{50} and LC_{50} estimation was done using Minitab Software Release 14.2 (Minitab Inc. State College, PA).

2.3 Results

2.3.1 Topical toxicity at 24 h

Acetone-diluted essential oil components were applied to the ventral metathorax of adult male bed bugs to determine their topical toxicity. Of the fifteen different components tested, carvacrol and thymol were relatively more toxic with LD_{50} values of 27.5 and 32.5 µg/mg body weight, respectively (Table 2.1). Both compounds showed similar levels of toxicity based on the relative median potency analysis (Table A.2). Similarly, carvacrol and thymol were significantly more toxic than citronellic acid, eugenol, geraniol, α-pinene, R (+)-limonene, linalool, eucalyptol, (–)-terpinen-4-ol, trans-cinnamaldehyde, menthone, (±)-citronellal, (±)-camphor and methyl eugenol (Table 2.1 and A.2). In the positive control treatment, the pyrethroid insecticide bifenthrin was ~72,000 times more potent than carvacrol with an LD_{50} of 0.000345 µg/mg body weight (Table 2.1 and A.2).

2.3.2 Fumigant toxicity at 24 h

Adult male bed bugs were exposed to vapors of essential oil components in sealed mason jars (volume of 473 ml) to determine their fumigant toxicity. Thymol was the most toxic compound with a LC_{50} value of 20.50 mg/L (Table 2.2). Carvacrol (LC_{50} = 46.3 mg/L) and linalool (LC_{50} = 51.2 mg/L) were less toxic than thymol followed by (±)-camphor, menthone, eucalyptol, (–)-terpinen-4-ol, trans-cinnamaldehyde, R (+)-limonene, α-pinene, (±)-citronellal, geraniol,

citronellic acid, eugenol and methyl eugenol based on the relative median potency analysis (Table A.3). Dichlorvos (DDVP), an organophosphate insecticide with fumigant properties was used as a positive control. DDVP was 445 times more potent (LC_{50} value of 0.0432 mg/L) than thymol (Table 2.2 and A.3).

Acetone (solvent carrier) applied to control filter papers evaporated completely (100%) during the 30 sec to 5 min drying time described in the methods section. Data on evaporation of different essential oil components for the 24 h bioassay duration are presented in Table 2.2. Percent evaporation was highest for eucalyptol (100%), whereas it varied from ~90% for thymol to < 1% for trans-cinnamaldehyde. When regression analysis was performed between compounds for which LC_{50} values were accurately determinable, i.e., the first 11 compounds shown in Table 2.2 and their percent evaporation values no significant correlation was observed ($P>0.05$; Fig. A.1). Similarly, regression analysis between the four most toxic fumigant compounds and evaporation percentage did not find any significant correlation ($P>0.05$; Fig. A.1).

2.3.3 Neurophysiology study

Spontaneous nerve activity recordings from the fused thoracic ganglion of adult male bed bugs demonstrated no neuroexcitatory or neuroinhibitory effects of solvent controls containing either 0.1% DMSO + 0.01% Tween 20 ($P = 0.790$) or 0.1% absolute ethanol + 0.01% Tween 20 ($P = 0.826$) in comparison to the HEPES-buffered physiological saline (PS) treatment (Fig. 2.3a). At the Bonferroni adjusted statistical significance level of $P<0.0125$ (i.e., 0.05 ÷ number of comparisons in two-sample t-tests) the concentration of 4 mM for both carvacrol ($P = 0.005$) and thymol ($P = 0.001$) caused significant neuroinhibition (Figs 2.3b and 2.3c). Eugenol exhibited significant neuroinhibitory effects at the 2 mM concentration ($P = 0.001$; Fig. 2.3d).

For linalool, the concentration of 4 mM ($P = 0.011$) produced significant neuroexcitatory effects ($P<0.0125$) (Fig. 2.3e). Citronellic acid (Fig. 2.3f) and (±)-Camphor (Fig. 2.3g) resulted in departure ratios that were >1 and were indicative of neuroexcitatory effects, however, none of the concentrations tested for these compounds caused a significant increase in nervous activity at the Bonferroni corrected significance levels of $P<0.01$ and $P<0.0125$, respectively. As expected, the positive control treatment with bifenthrin (a synthetic pyrethroid insecticide), caused significant neuroexcitation at the 10µM concentration ($P = 0.0001$; Fig. 2.3h).

Linear regression analysis showed that carvacrol and thymol caused a concentration-dependent decrease in spontaneous electrical activity of the nervous system ($P<0.05$, Fig. A.2). In contrast, citronellic acid, linalool and bifenthrin induced concentration-dependent increase in nervous activity ($P<0.05$, Fig. A.2). Eugenol and (±)-camphor did not show concentration-dependent changes in neurological activity ($P>0.05$, Fig. A.2), likely because their effects were bi-phasic (i.e., pronounced effects at intermediate concentrations in comparison to lower or higher concentrations; Figs. 2.3d and 2.3g).

2.3.4 Poisoning symptoms (non-quantitative) in bed bugs treated with plant essential oil components

Treatment of bed bugs with the solvent carrier (acetone) did not induce any poisoning symptoms such as hyperactivity, paralysis or leg tremors at 2 and 4 h after treatment (Table 2.3). However, hyperactivity, defined as uncoordinated movement and wandering behavior, was observed in bed bugs treated with five of the six most toxic essential oil components (carvacrol, thymol, eugenol, linalool and (±)-camphor) at the 2 h interval (Table 2.3). Citronellic acid treated insects did not show hyperactivity symptoms. Bed bugs treated with all six toxic plant essential oil components were paralyzed, i.e., they were unable to walk or right themselves upon prodding at the 4 h observation interval (Table 2.3). Paralysis was also observed in thymol and (±)-camphor treated insects 2 h after treatment. Leg tremors (involuntary leg spasms, twitching and quivering) were observed in knocked-down insects treated with thymol, linalool and (±)-camphor (Table 2.3). Death of treated insects was first observed ~ 6 hours after treatment with some of the compounds and hence observations on non-quantitative poisoning symptoms were not recorded after the 4 h observation interval.

2.4 Discussion

Initially we characterized the inherent toxicity of fifteen different plant essential oil components against bed bugs. Carvacrol and thymol were the most active compounds in topical application bioassays. Both compounds exhibited similar levels of contact toxicity and were 13–15 times more potent than the least toxic constituent, methyl eugenol in topical bioassays. Carvacrol and thymol were previously reported as being effective, with contact and fumigant toxicity against several insect pests including cockroaches, kissing bugs and house flies[22–26]. As

found in other insects, increased toxicity of carvacrol and thymol towards bed bugs might be due to two major properties: (i) they are saturated compounds (contain carbon-carbon single bonds outside the benzene ring) and (ii) the presence of functional hydroxyl groups on the benzene ring[22,25]. These structural properties may also have allowed thymol and carvacrol to penetrate rapidly through the cuticle, undergo slow detoxification and interact effectively with their target sites[22,25,40]. The lipophilicity of essential oil compounds is another important property that plays a role in penetration through the insect cuticle[22]. The LogP or octanol-water partition coefficients (higher values indicate greater lipophilicity)[25] for carvacrol were higher than thymol (Table A.1). Similarly, the LogP coefficient for the third most toxic compound in topical assays (citronellic acid) was higher than the LogP coefficient for eugenol (Table A.1). In previous studies, citronellic acid and eugenol have been shown to possess contact toxicity against *M. domestica* and *Tetranychus urticae*[22,28].

When considering the fumigant toxicity of essential oil constituents, thymol was most potent, followed by carvacrol, linalool, and (±)-camphor (Table 2.2). As stated in the previous paragraph, thymol and carvacrol have contact and fumigant toxicity against several insect species[22–26]. Fumigant effects of linalool have been demonstrated against *Thrips palmi*, *Plutella xylostella* and *B. germanica*[27,29,30]. Whereas, (±)-camphor was reported as having contact and fumigant action against the *P. xylostella*[30], but not against stored product pests[31].

Determination of 24 h evaporation levels for essential oil constituents revealed large variations among compounds. The amount of initially applied chemical that evaporated during the 24 h bioassay period ranged from <0.5% for trans-cinnamaldehyde to 100% for eucalyptol (Table 2.2). A series of regression analyses conducted between LC_{50} values and evaporation percentage showed no significant correlations ($P>0.05$; Fig. A.1). Interestingly, the constituents for which LC_{50} values were not determinable (geraniol, citronellic acid, eugenol and methyl eugenol) (Table 2.2) generally showed <5% evaporation during the 24 h bioassay period. However, for trans-cinnamaldehyde, which showed lowest evaporation percentage of 0.5%, a LC_{50} value was still determinable (Table 2.2). In the case of carvacrol, its evaporation estimate for the bioassay period was ~ 27%, but it was the second most toxic fumigant. In contrast, eucalyptol completely evaporated in 24 h, but was the sixth most toxic compound. Collectively, these results indicated that the fumigant toxicity of the tested essential oil components was not solely dependent on their volatility, but their inherent toxicity (i.e., unique target-receptor interactions) was likely a major

determining factor in their toxicity. Since fumigant toxicity is dependent on the exposure time, in the future it may be important to perform experiments to determine if compounds with low evaporation show increased toxicity against bed bugs in long duration bioassays (3–7 d) as shown by Feldlaufer and Ulrich[19] when using pure essential oils.

Several essential oil-based products have already been commercialized, especially for bed bug control. However, of the nine different natural compound products, only EcoRaider® (active ingredients: geraniol (1%), cedar extract (1%), and sodium lauryl sulfate (2%)) and Bed Bug Patrol® (active ingredients: clove oil (0.003%), peppermint oil (1%), and sodium lauryl sulfate (1.3%)) were reported to be effective in laboratory and field experiments conducted against bed bugs[11,21]. Carvacrol and thymol were the most active compounds in our assays but are not present in any of the essential oil-based products available for bed bug control. Therefore, based on the findings of this study there are opportunities to develop potentially efficacious essential oil-based formulations for use in bed bug IPM. Plant essential oils that contain high concentrations of effective compounds included in this study were found active against bed bugs and cockroaches[19–21,26]. Therefore, thyme (*Thymus vulgaris* L.) and oregano (*Origanum vulgare* L.) plant essential oils, which contain high amounts of thymol, and carvacrol, respectively (Table A.1) can be included in the formulation of natural product insecticides. The odor issue associated with the use of essential oils in indoor environments can be alleviated by formulating with inert carriers, surfactants, adjuvants and additives. Most prior work with commercial essential oil products has been performed by conducting direct spray and residual exposure bioassays, but no study has evaluated fumigant activity under field conditions. Thymol was more potent as a fumigant than any other essential oil constituent tested in this study. Therefore, thymol or thymol containing essential oils have the potential of being used as fumigants for bed bug control under field settings. For example, small bed bug infested items can be sealed in chambers or plastic bags with a paper or cloth impregnated with essential oils containing thymol[19].

Electrophysiology recordings were performed using the suction electrode technique to investigate the effects of essential oil components on the bed bug nervous system. Four of the six most active components identified collectively from topical and fumigant bioassays impacted baseline electrical activity of the bed bug nervous system. The neurophysiology data for carvacrol, thymol, eugenol and linalool provides a basis for understanding their toxicity against bed bugs. Bifenthrin (a positive control insecticide used in this study) and other synthetic pyrethroids modify

the gating characteristics of voltage-sensitive sodium channels that lead to a delay in their closure, and thereby cause a neuroexcitatory effect on the insect nervous system[41]. In this study, bifenthrin caused significant neuroexcitation of baseline nervous system activity. Effects of bifenthrin at the 10 µM concentration on the bed bug nervous system were similar with a study that employed the suction electrode electrophysiology technique against the mole crickets[42]. Both neuroexcitatory (linalool) and neuroinhibitory (carvacrol, thymol and eugenol) essential oil constituents were neurologically active at millimolar (mM) concentrations. The structural and chemical property differences between essential oil components and bifenthrin may have led to significant differences in toxicity at the nervous system level[40]. In this regard, higher lipophilicity of bifenthrin (LogP value of 6, Table A.1) in comparison to that of essential oil constituents may allow bifenthrin to effectively penetrate and interact with the membrane bound target site(s) within the nervous system at micromolar concentrations. Overall, low potency of neurological effects caused by essential oil compounds is consistent with their relatively lower topical and fumigant toxicity to different insect pest species and bed bugs. The effective concentration range or quantity of essential oil components (2 to 4 mM or 1.5×10^{-11} to 3.4×10^{-10} µg/insect or nerve preparation) necessary to produce statistically significant neurological effects was at least 1 billion times lower in comparison the topical LD_{50} estimates that ranged from 54–1120 µg/insect or 27–560 µg/mg body weight (Table 2.1). Large differences in effective quantities or doses of essential oil components between neurophysiology and whole organism bioassays were expected. This is because toxicants that are directly applied to nerves do not have to penetrate the cuticle, and thereby have less likelihood of being degraded or sequestered by detoxification enzymes before reaching their target site[40]. In bed bugs, detoxification enzymes expressed in the cuticle have been associated with rapid degradation of insecticides[4]. Therefore, different insecticides, including essential oil components are effective at lower concentrations when directly applied to the ventral nerve cord.

Neurological impacts of essential oil components against bed bugs were concentration-dependent for most test compounds ($P<0.05$; Fig. A.2). Similarly, Price and Berry[38] found concentration-dependent neurological effects of essential oil components on the ventral nerve cord of *P. americana* and *B. discoidalis*. The effective concentration ranges for essential oil constituents tested in this study were similar to those of Price and Berry for citral, eugenol and geraniol[38]. The neurological impacts of eugenol and (±)-camphor were not concentration-dependent and showed

a biphasic effect in our study (Figs. 2.3 and A.2). A previous study also revealed biphasic effects of geraniol on cockroach nervous system activity[38].

The three compounds that produced neuroinhibition were carvacrol, thymol and eugenol. Based on *in vitro* studies, carvacrol is known to inhibit *M. domestica* nAChRs[37] and its inhibitory activity was similar to dinotefuran (a neonicotinoid insecticide)[43]. In vertebrates, carvacrol can reversibly block the excitability of the rat sciatic nerve in a dose-dependent pattern[44]. However, in previous studies with insects, tyramine receptor[36], transient receptor potential-like (TRPL) channels[45] and GABA[46] were also proposed as potential target sites for carvacrol. Thymol has been shown to bind *D. melanogaster*, mouse and human GABA receptors[35,47,48]. It was also reported as a weak inhibitor of the acetylcholinesterase enzyme[49,50]. Eugenol, which is a phenolic compound, was previously reported to have neuroinhibitory effects on *P. americana* and *B. discoidalis*[38] and it was proposed to bind or interact with octopamine receptors in the insect nervous system[34,51].

Linalool produced neuroexcitatory effects on the bed bug nervous system (Fig. 2.3e). Linalool was initially reported to act as a reversible competitive inhibitor of the acetylcholinesterase enzyme[52]. However, in subsequent studies, it was concluded that linalool does not bind to neurotransmitter enzymes[53,54]. It also did not produce any effect on house fly [^{3}H]-TBOB ([3H]-t-butylbicycloorthobenzoate) binding and *P. americana* $^{36}Cl^{-}$ uptake studies[46]. Although, citronellic acid caused a concentration-dependent increase in nervous activity ($P<0.05$; Fig. A.2) and resulted in a 6–19% increase in activity of the nervous system, two-sample t-tests with Bonferroni adjustment revealed that none of the tested concentrations caused a statistically significant increase in nervous activity ($P>0.01$). Thus far, no target site or neurological impact data are available for citronellic acid. Lastly, camphor has been shown to inhibit catecholamine secretion by blocking nACHRs in bovine adrenal chromaffin cells[55]. In another study with stored product pests and *B. germanica*, camphor was a weak acetylcholinesterase inhibitor[54,56]. However, in this study the 6–15% increase in nervous activity induced by (±)-camphor at various concentrations was not statistically significant (Fig. 2.3g). Given these findings for citronellic acid and (±)-camphor, more sensitive electrophysiology techniques such as patch or two-electrode voltage clamping may be required to determine the actual neurological impacts of these constituents. Target site binding studies may also help in determining the neurotoxic nature of these compounds.

Bed bugs treated with the six most toxic plant essential oil components showed a range of poisoning symptoms such as hyperactivity, paralysis and leg tremors. Previously, Coats et al.[57] reported hyperactivity and leg tremors as common poisoning symptoms associated with essential oil constituents. In the Madagascar cockroach (*Gromphadorhina portentosa*), pulegone-1,2-epoxide (an essential oil component) caused hyperactivity and muscular spasms before eventual paralysis and death[58]. In general, neuroinhibitory insecticides (e.g., oxadiazines and avermectins) are known to cause flaccid paralysis, wherein the muscles become limp and are unable to contract due to reduction or loss of nerve activity[40,59]. In contrast, rigid paralysis is caused by neuroexcitatory insecticides (e.g. organophosphates, pyrethroids, neonicotinoids)[40,59]. Rigid paralysis occurs because of the overstimulation of nervous system activity that causes muscles to stay in a contracted state. However, such symptoms were not visually distinguishable in bed bugs treated with neuroinhibitory (carvacrol, thymol and eugenol) or neuroexcitatory (linalool) essential oil components.

In summary, baseline toxicity of essential oil components against bed bugs as reported here provides information for development of natural product insecticides that can be used in bed bug IPM. Electrophysiology data for the most active compounds from bioassays further verifies that certain essential oil constituents affect the normal functioning of the bed bug nervous system. Collectively, these results provide insights required for identifying the target or binding sites and mode-of-action of specific essential oil constituents.

Table 2.1 Mortality response of adult male bed bugs to topical application of essential oil components and bifenthrin.

Essential oil components	**N**	**LD_{50}[I], µg/ mg body weight (95% FL[II])**	**Slope ± SE**	**χ^2**	**d.f.**	***P* value**
Carvacrol	240	27.5 (25 – 30.5)a	2.67 ± 0.30	4.82	5	0.507
Thymol	240	32.5 (29.5 – 35)a	3.32 ± 0.47	2.33	5	0.801
Citronellic acid	270	49 (42 – 57)b	1.29 ± 0.15	4.05	6	0.669
Eugenol	270	52 (47 – 57.5)bc	2.20 ± 0.23	6.06	6	0.416
Geraniol	270	64 (55.5 – 73)bc	1.77 ± 0.19	10.27	6	0.113
α-Pinene	270	70.5 (62 – 79.5)cd	1.85 ± 0.20	3.89	6	0.690
R (+)-Limonene	240	91.5 (79.5 – 104)de	1.67 ± 0.20	6.28	5	0.280
Linalool	210	112 (94.5 – 130.5)e	1.59 ± 0.20	17.31	4	0.002
Eucalyptol	240	132 (118.5 – 146.5)ef	2.10 ± 0.25	7.67	5	0.175
(–)-Terpinen-4-ol	210	138.5 (125.5 – 153)efg	2.96 ± 0.44	3.62	4	0.459
trans-Cinnamaldehyde	330	138.5 (116.5 – 159.5)fg	1.15 ± 0.14	13.73	8	0.192
Menthone	240	165 (136.5 – 198)gh	1.10 ± 0.14	8.96	5	0.110
(±)-Citronellal	210	240 (211.5 – 273.5)h	1.81 ± 0.24	10.15	4	0.038
(±)-Camphor	210	515 (454 – 1121)i	3.27 ± 1.26	0.29	4	0.990
Methyl eugenol	180	560 (350 – 2655)j	0.76 ± 0.22	4.37	3	0.223
Positive control						
Bifenthrin	180	0.000345 (0.0003 – 0.000405)k	1.73 ± 0.25	0.68	3	0.877

[I] LD_{50} = median lethal dose necessary to kill 50% of individuals. [II] 95% FL = 95% fiducial limits. LD_{50} values with the same letter are not significantly different based on the relative median potency analysis (refer to Table S2 for details). Mortality in control groups was 0%, except in linalool (3.33 % mortality). Body weight of a single adult male bed bug used for bioassays was approximately 2 mg.

Table 2.2 The mortality response of adult male bed bugs exposed to vapors of essential oil components and dichlorvos and their corresponding percent evaporation values for the 24 h bioassay period.

Essential oil components	**N**	**LC_{50}[I], mg/L (95% FL[II])**	**Slope ± SE**	**χ^2**	**Df**	***P*-value**	**% Evaporation[III]**
Thymol	180	20.50 (17.70 – 23.18)a	2.19 ± 0.29	0.60	3	0.89	90 ± 4.86
Carvacrol	180	46.3 (37.8 – 54.9)b	1.37 ± 0.15	8.65	5	0.124	26.89 ± 4.23
Linalool	240	51.2 (41.3 – 70.0)b	1.09 ± 0.19	6.33	3	0.097	86 ± 6.77
(±)-Camphor	210	133.3 (106.9 – 157)c	1.93 ± 0.28	5.86	4	0.209	52.99 ± 23.95
Menthone	270	150.7 (132.3 – 169.3)cd	2.03 ± 0.23	4.70	6	0.58	60.22 ± 20.33
Eucalyptol	180	191.1 (168.3 – 213.8)d	2.77 ± 0.37	11.62	3	0.009	100
(–)-Terpinen-4-ol	210	388.3 (301.7 – 482.9)e	0.96 ± 0.13	4.04	4	0.40	24.43 ± 16.55
trans-Cinnamaldehdye	240	389.0 (304.5 – 482.9)e	0.90 ± 0.11	8.82	5	0.116	0.50 ± 0.50
R (+)-Limonene	270	454.0 (436.5 – 476.5)e	6.69 ± 1.09	14.63	6	0.023	73.11 ± 9.25
α-Pinene	300	488.8 (470.8 – 503.6)e	8.45 ± 1.10	23.71	7	0.001	87.36 ± 7.27
(±)-Citronellal	180	1474.6 (1047.7 – 2528.1)f	0.63 ± 0.11	5.00	4	0.286	21.57 ± 12.03
Geraniol[†]	180	ND					1.29 ± 0.53
Citronellic acid[†]	180	ND					4.48 ± 1.22
Eugenol[†]	180	ND					5.16 ± 2.73
Methyl eugenol[†]	180	ND					0.65 ± 0.17
Positive control							
DDVP	270	0.0432 (0.0397 – 0.0468)g	2.76 ± 0.32	4.08	6	0.665	95.95 ± 4.04

[I] LC_{50} = median lethal concentration (expressed as amount of essential oil constituents or insecticides per liter air i.e. mg/L) necessary to kill 50% of individuals. [II] 95% FL = 95% fiducial limits. Daggers ([†]) show essential oil components for which accurate LC_{50} values were not determinable (ND) because less than 30% mortality was observed at the highest concentration (2000 mg/L) that was testable. LC_{50} values with the same letters are not significantly different based on the relative median potency analysis (refer Table S3 for details). Mortality in control groups was 0%. [III]Percent evaporation values for the 24 h bioassay period. Acetone applied to control filter papers evaporated completely (100%) during the 30 sec to 5 min drying period described in the methods section.

Table 2.3 Poisoning symptoms observed in bed bugs topically treated with median lethal dose (LD_{50}) of six most toxic plant essential oil components.

Essential oil components	**Hours after treatment**	**Poisoning symptoms (0 = absent, + = present)**		
		Hyperactivity	**Paralysis**	**Leg tremor**
Control (acetone)	2	0	0	0
	4	0	0	0
Carvacrol	2	+	0	0
	4	+	+	0
Thymol	2	+	+	+
	4	0	+	+
Eugenol	2	+	0	0
	4	0	+	0
Citronellic acid	2	0	0	0
	4	0	+	0
Linalool	2	+	0	0
	4	0	+	+
(±)-Camphor	2	+	+	+
	4	0	+	+

Acetone treated insects did not display poisoning symptoms. However, poisoning symptoms such as “hyperactivity” (insects displaying uncoordinated movement and wandering behavior), “paralysis” (insects that were either unable to walk or knockdown insects that were unable to right themselves upon prodding) and “leg tremors” (insects lying on their back and exhibiting involuntary leg spasms, twitching and quivering), were observed in bed bugs treated with essential oil components.

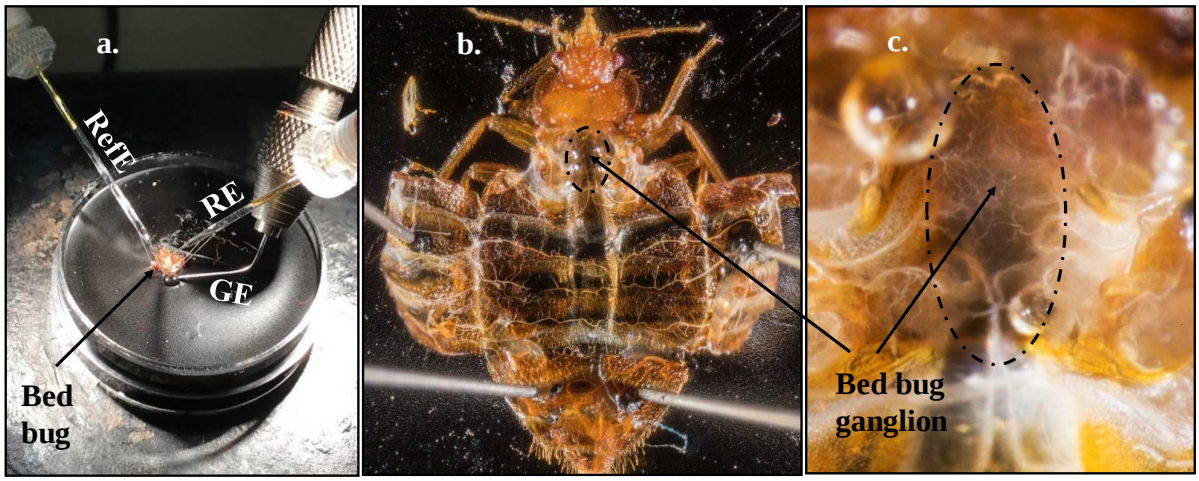


Figure 2.1 Electrophysiology recording set-up (suction electrode technique), dissected bed bug and its ganglion. (a) Recording electrode (RE) was placed in gentle contact with the fused thoracic ganglion, whereas the reference electrode (RefE) was placed in contact with the carcass. The ground electrode (GE) was placed in the Petri dish, but in contact with the external cuticle of the bed bug body in the presence of saline. (b and c) Fused thoracic and abdominal ganglion of the bed bug can be seen in the metathoracic region. Segmental nerves extend from the fused ganglion (see reference number 61 for a description of the bed bug ventral nerve cord).

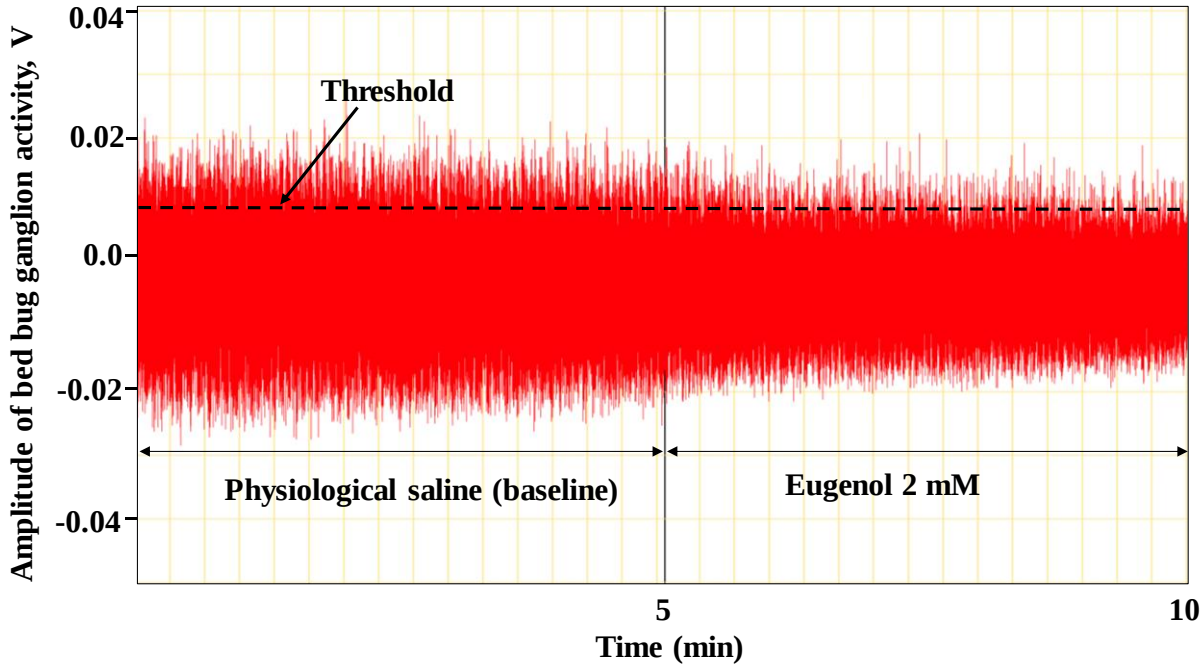
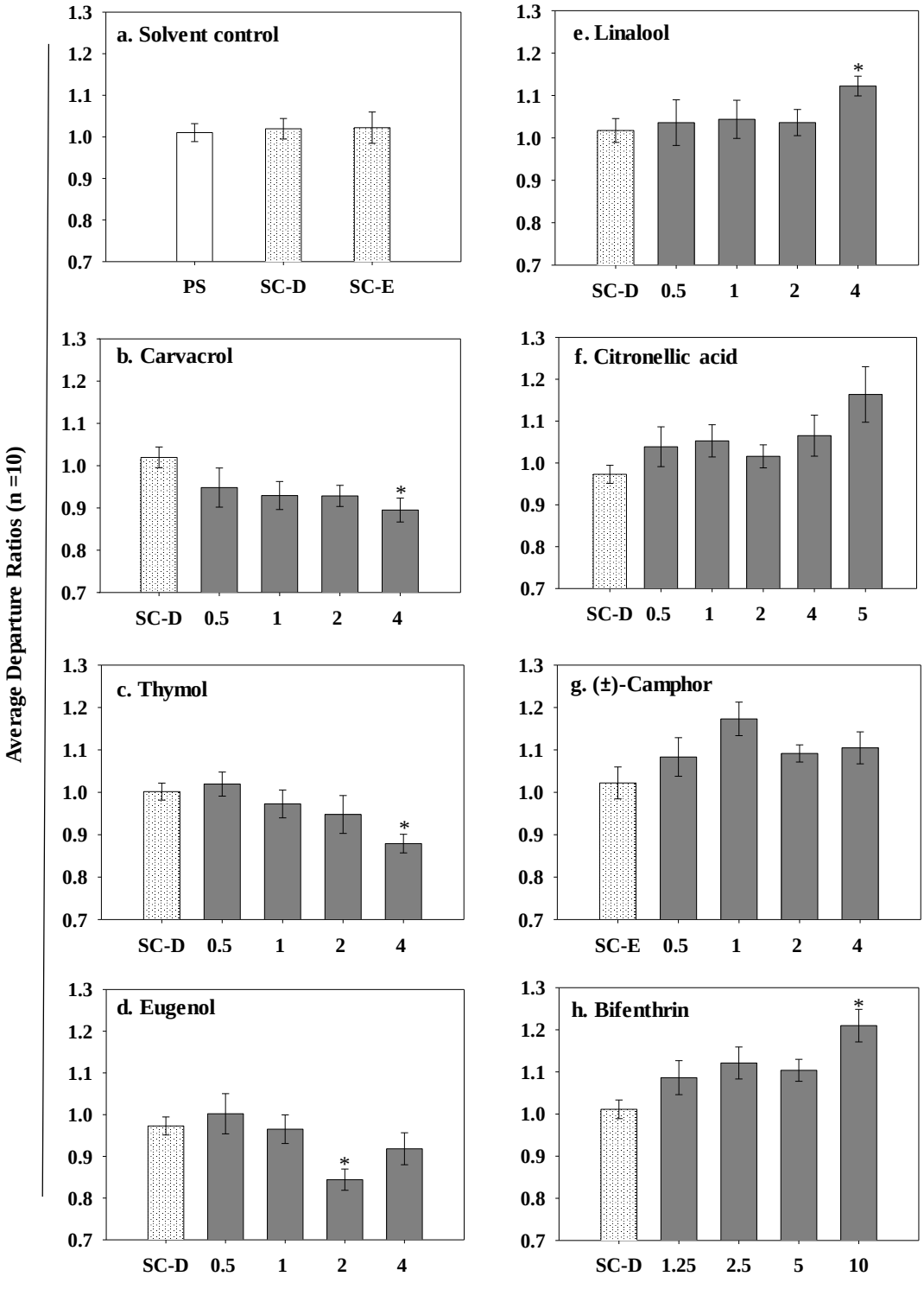


Figure 2.2 An example of 10-minute electrophysiological nerve activity trace from the Chart Software for 2 mM eugenol. Determination of spontaneous electrical activity bursts or spikes in pre-treatment or baseline recordings in physiological saline (for 5 mins) and post-treatment recordings in 2 mM Eugenol (5 mins) were enabled by setting the threshold using the "counter" function in the Chart software. The threshold was maintained constant between the pre-and post-treatment recordings. Data for the total number of spikes surpassing the threshold before and after treatment were used to calculate ratios representing a departure from baseline activity.

Figure 2.3 Neurophysiological effects of essential oil components, bifenthrin and solvent controls on the bed bug nervous system. Bars represent average departure ratios calculated by dividing the nervous activity spikes surpassing the threshold in post-treatment recordings (either with essential oil constituents or bifenthrin or solvent controls) with spike counts from physiological saline (PS) pre-treatment. Asterisks (*) in different graphs indicate significant differences from solvent control recordings (two-sample t-tests with Bonferroni corrected P-value i.e. 0.05 ÷ number of comparisons for each compound). (a) Solvent control treatments, PS + 0.1% dimethyl sulfoxide (DMSO) + 0.01% Tween-20 (SC-D) or PS + 0.1% absolute ethanol + 0.01% Tween 20 (SC-E) had no effect on nervous system activity ($P>0.025$). (b) Carvacrol (4 mM), (c) thymol (4 mM), and (d) eugenol (2 mM) exhibited a neuroinhibitory effect as indicated by departure ratios significantly below 1 ($P<0.0125$). (e) With departure ratios above 1, linalool (4 mM) led to significant neuroexcitation ($P<0.0125$), but (f) citronellic acid ($P>0.01$) and (g) (±)-camphor ($P>0.0125$) did not cause significant neurological impacts (h) The positive control treatment with bifenthrin (10 µM) caused significant neuroexcitation ($P<0.0125$).

Figure 2.3 continued



2.5 References

1. Eddy, C. & Jones, S. C. Bed bugs, public health, and social justice: part 1, a call to action. *J. Environ. Health* **73**, 8–14 (2011).
2. U.S. Centers for Disease Control and Prevention (CDC) and U.S. Environment Protection Agency (EPA). *Joint Statement on Bed Bug Control in the United States from the U.S. Centers for Disease Control and Prevention and the U.S. Environmental Protection Agency* (2010). https://stacks.cdc.gov/view/cdc/21750
3. Romero, A., Potter, M. F., Potter, D. A. & Haynes, K. F. Insecticide resistance in the bed bug: A factor in the pest's sudden resurgence? *J. Med. Entomol.* **44**, 175–178 (2007).
4. Zhu, F. *et al.* Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. *Sci. Rep.* **3**, 1456, doi; 10.1038/srep01456 (2013).
5. Romero, A. & Anderson, T. D. High levels of resistance in the common bed bug. *Cimex lectularius* (Hemiptera: Cimicidae), to neonicotinoid insecticides. *J. Med. Entomol.* **53**, 727–731 (2016).
6. Ashbrook, A. R., Scharf, M. E., Bennett, G. W. & Gondhalekar, A. D. Detection of reduced susceptibility to chlorfenapyr- and bifenthrin-containing products in field populations of the bed bug (Hemiptera: Cimicidae). *J. Econ. Entomol.* **110**, 1195–1202 (2017).
7. Diel, F. *et al.* Effects of permethrin on human basophils and lymphocytes in vitro. *Inflamm. Res.* **47**, 11–12 (1998).
8. Lei, W. *et al.* Assessment of the inhibitory effects of pyrethroids against human carboxylesterases. *Toxicol. Appl. Pharm.* **321**, 48–56 (2017).
9. Yang, Y. *et al.* Natural pyrethrins induces apoptosis in human hepatocyte cells via Bax- and Bcl-2-mediated mitochondrial pathway. *Chem-Biol. Interact.* **262**, 38–45 (2017).
10. Potter, M. F. The history of bed bug management-with lessons from the past. *Am. Entomol.* **57**, 14–25 (2011).
11. Wang., C., Singh, N. & Cooper, R. Efficacy of an essential oil-based pesticide for controlling bed bug (*Cimex lectularius*) infestations in apartment buildings. *Insects* **5**, 849–859 (2014).

12. Bennett, G. W., Gondhalekar, A. D., Wang, C., Buczkowski, G. & Gibb, T. J. Using research and education to implement practical bed bug control programs in multifamily housing. *Pest Manag. Sci.* **72**, 8–14 (2016).
13. Viciolle, E., Castilho, P. & Rosado., C. In vitro and in vivo assessment of the effect of *Laurus novocanariensis* oil and essential oil in human skin. *Int. J. Cosmetic. Sci.* **34**, 546–550 (2012).
14. Politi, F. A. S. *et al*. Insecticidal activity of an essential oil of *Tagetes patula* L. (Asteraceae) on common bed bug *Cimex lectularius* L. and molecular docking of major compounds at the catalytic site of ClAChE. *Parasitol. Res.* **116**, 415–424 (2017).
15. Isman, M. B. Botanicals insecticide, deterrents and repellents in modern agriculture and increasing regulated world. *Annu. Rev. Entomol.* **51**, 45–66 (2006).
16. Regnault-Roger, C., Vincent, C. & Arnason, J. T. Essential oils in insect control: low-risk products in a high-stakes world. *Annu. Rev. Entomol*. **57**, 405–424 (2012).
17. U.S. Environment Protection Agency (EPA). *Code of Federal Regulations. 40 CFR 152.25* (2013). https://www.epa.gov/minimum-risk-pesticides
18. Guenther, E. *The essential oils. Vol. II. The constituents of essential oils.* (D. Van Nostrand Company, Inc. New York. NY, 1949).
19. Feldlaufer, M. F. & Ulrich, K. R. Essential oils as fumigants for bed bugs (Hemiptera: Cimicidae). *J. Entomol. Sci.* **50**, 129–137 (2015).
20. Zha, C., Wang, C. and Li, A. Toxicities of selected essential oils, silicone oils, and paraffin oil against the common bed bug (Hemiptera: Cimicidae). *J. Econ. Entomol.* **111**, 170–177 (2018).
21. Singh, N., Wang, C. & Cooper, R. Potential of essential oil-based pesticides and detergents for bed bug control. *J. Econ. Entomol.* **107**, 2163–2170 (2014).
22. Rice, P. J. & Coats, J. R. Insecticidal properties of several monoterpenoids to the house fly (Diptera: Muscidae), red flour beetle (Coleoptera: Tenebrionidae), and southern corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* **87**, 1172–1179 (1994).
23. Jang, Y., Yang, Y., Choi, D., & Ahn, Y. Vapor phase toxicity of marjoram oil compounds and their related monoterpenoids to *Blattella germanica* (Orthoptera: Blattellidae). *J. Agr. Food Chem.* **53**, 7892–7898 (2005).

24. Moretti, A. N., Zerba, E. N. & Alzogaray, R. A. Behavioral and toxicological responses of *Rhodnius prolixus* and *Triatoma infestans* (Hemiptera: Reduviidae) to 10 Monoterpene alcohols. *J. Med. Entomol.* **50**, 1046–1054 (2013).
25. Phillips, A. K., Appel, A. G. and Sims, S. R. Topical toxicity of essential oils to the German cockroach (Dictyoptera: Blattellidae). *J. Econ. Entomol.* **103**, 448–459 (2010).
26. Gaire, S. *et al*. Insecticidal properties of essential oils and some of their constituents on the Turkestan cockroach (Blattodea: Blattidae). *J. Econ. Entomol.* **110**, 584–592 (2017).
27. Phillips, A. K. & Appel, A. G. Fumigant toxicity of essential oils to the German cockroach (Dictyoptera: Blattellidae). *J. Econ. Entomol.* **103**, 781–790 (2010).
28. Lee, S., Tsao, R., Peterson, C. & Coats, J. R. Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), two spotted spider mite (Acari: Tetranychidae), and house fly (Diptera: Muscidae). *J. Econ. Entomol.* **90**, 883–892 (1997).
29. Kim, K. H. *et al*. Fumigant toxicity of basil oil compounds and related compounds to *Thrips palmi* and *Orius strigicollis*. *Pest Manag. Sci.* **71**, 1292–1296 (2015).
30. Yi, C. G. *et al*. Toxicity of *Lavandula angustifolia* oil constituents and spray formulations to insecticide-susceptible and pyrethroid-resistant *Plutella xylostella* and its endoparasitoid *Cotesia glomerata*. *Pest Manag. Sci.* **72**, 1202–1210 (2016).
31. Abdelgaleil, S. A. M., Mohamed, M. I. E., Badawy, M. E. I. & El-arami, S. A. A. Fumigant and contact toxicities of monoterpenes to *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) and their inhibitory effects on acetylcholinesterase activity. *J. Chem. Ecol.* **35**, 518–525, (2009).
32. Tripathi, A. K., Upadhyay, S., Bhuiyan, M. & Bhattacharya, P. R. 2009. A review on prospects of essential oils as biopesticide in insect-pest management. *J. Pharmacognosy Phytother.* **1**, 52–63.
33. Jankowska, M., Rogalska, J., Wyszkowska, J. & Stankiewicz, M. Molecular targets for components of essential oils in the insect nervous system—a review. *Molecules* **23**, doi:10.3390/molecules23010034 (2018).
34. Enan, E. Insecticidal activity of essential oils: octopaminergic sites of action. *Comp. Biochem. Phys. C* **130**, 325–337 (2001).

35. Priestley, C. M., Williamson, E. M., Wafford K. A, & Sattelle D. B. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human $GABA_A$ receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. *Brit. J. Pharmacol.* **140**, 1363–1372 (2003).
36. Enan, E. E. Molecular response of *Drosophila melanogaster* tyramine receptor cascade to plant essential oils. *Insect Biochem. Molec.* **35**, 309−321 (2005).
37. Tong, F., Gross, A. D., Dolan, M. C. & Coats, J. R. The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor. *Pest Manag. Sci.* **69**, 775–780 (2013).
38. Price, D. N., & Berry M. S., Comparison of effects of octopamine and insecticidal essential oils on activity in the nerve cord, foregut, and dorsal unpaired median neurons of cockroaches. *J. Insect Physiol.* **52**, 309–319 (2006).
39. Hertel, W., Hertel, W. & Muller, P. Physiological effects of the natural products quassin, cinnamaldehyde and azadirachtin on *Periplaneta americana* (L.). *J. Appl. Entomol.* **130**, 323–328 (2006).
40. Yu, S. J. *The toxicology and biochemistry of insecticides*. (CRS Press, Taylor & Francis Group, Bacon Raton, FL, 2015).
41. Bradberry, S. M., Cage, S. A., Proudfoot, A. T. & Vale, J. A. Poisoning due to pyrethroids. *Toxicol. Rev.* **24**, 93–106 (2005).
42. Kostromytska, O. S., Buss, E. A. & Scharf, M. E. Toxicity and neurophysiological effects of selected insecticides on the mole cricket, *Scapteriscus vicinus* (Orthoptera: Gryllotalpidae). *Pestic. Biochem. Phys.* **100**, 27–34 (2011).
43. Kiriyama, K. & Nishimura, K. Structural effects of dinotefuran and analogues in insecticidal and neural activities. *Pest. Manag. Sci.* **58**, 669−676 (2002).
44. Joca, H. C. *et al*. Carvacrol decreases neuronal excitability by inhibition of voltage-gated sodium channels. *J. Nat. Prod.* **75**, 1511−1517 (2012).
45. Parnas, M. *et al*. Carvacrol is a novel inhibitor of *Drosophila* TRPL and mammalian TRPM7 channels. *Cell Calcium* **45**, 300−309 (2009).
46. Tong, F. & Coats, J. R. Effects of monoterpenoid insecticides on [^{3}H]-TBOB binding in house fly GABA receptor and $^{36}Cl^-$ uptake in American cockroach ventral nerve cord. *Pestic. Biochem. Phys.* **98**, 317−324 (2010).

47. Garcia, D. A *et al*. GABA released from cultured cortical neurons influences the modulation of t-[^{35}S] butylbicyclophosphorothionate binding at the $GABA_A$ receptor effects of thymol. *Eur. J. Pharmacol.* **600**, 26–31 (2008).
48. Waliwitiya, R., Belton, P., Nicholson, R. A. & Lowenberger, C. A. Effects of the essential oil constituent thymol and other neuroactive chemicals on flight motor activity and wing beat frequency in the blowfly *Phaenicia sericata*. *Pest Manag. Sci.* **66**, 277–289 (2010).
49. Jukic, M., Politeo, O., Maksimovic, M., Milos, M. & Milos, M. *In vitro* acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. *Phytother. Res.* **21**, 259–261 (2007).
50. Park, C. G *et al*. Insecticidal and acetylcholinesterase inhibitory activities of Lamiaceae plant essential oils and their major components against *Drosophila suzukii* (Diptera: Drosophilidae). *Ind. Crop. Prod.* **89**, 507–513 (2016).
51. Kostyukovsky, M., Rafaeli, A., Gileadi, C., Demchenko, N. & Shaaya, E. Activation of octopaminergic receptors by essential oil constituents isolated from aromatic plants: possible mode of action against insect pests. *Pest Manag. Sci.* **58**,1101–1106 (2002).
52. Ryan, M. F. & Byrne, O. Plant-insect coevolution and inhibition of acetylcholinesterase. *J. Chem. Ecol.* **14**, 1965–1975 (1988).
53. Lee, S. E. *et al*. Fumigant toxicity of volatile natural products from Korean spices and medicinal plants towards the rice weevil, *Sitophilus oryzae* (L). *Pest Manag. Sci.* **57**, 548–553 (2001).
54. Lopez, M. D. & Pascual-Villalobos, M. J. Mode of inhibition of acetylcholinesterase by monoterpenoids and implications for pest control. *Ind. Crop. Prod.* **31**, 284–288, (2010).
55. Park, T., Seo, H., Kang, B. & Kim, K. Noncompetitive inhibition by camphor of nicotinic acetylcholine receptors. *Biochem. Pharmacol.* **61**, 787–793 (2001).
56. Yeom, H. *et al*. Insecticidal and acetylcholine esterase inhibition activity of Asteraceae plant essential oils and their constituents against adults of the German cockroach (*Blattella germanica*). *J. Agr. Food. Chem.* **63**, 2241–2248 (2015).
57. Coats, J. R., Karr, L. L. & Drewes, C. D. Toxicity and neurotoxic effects of monoterpenoids: in insects and earthworms, in *Naturally Occurring Pest Bioregulators* (ed. Hedin, P. A.) 305-316 (ACS Symposium series, 1991).

58. Grundy, D. L. & Still, C. C. Inhibition of acetylcholinesterases by pulegone-1,2-epoxide. *Pestic. Biochem. Phys.* 23, 383-388 (1985).
59. Scharf, M. E., Neurological effects of insecticides and the insect nervous system, in *Encyclopedia of entomology* (ed. Capinera, J. L.) 2596-2607 (Springer, New York, NY, 2008).
60. Chin-Heady, E. *et al.* A quantitative analysis of a modified feeding method for rearing *Cimex lectularius* (Hemiptera: Cimicidae) in the laboratory. *Pest Manag. Sci.* **69**, 1115–1120 (2013).
61. Gondhalekar, A. D. & Scharf, M. E. Mechanisms underlying fipronil resistance in a multi resistant field strain of the German cockroach (Blattodea: Blattellidae). *J. Med. Entomol.* **49**, 122–131 (2012).
62. Feston, J. C. *The effects of ozone gas on the common bed bug (Cimex lectularius L.)*. (MS Thesis. Purdue University, West Lafayette, IN, 2015).
63. Usinger, R. L. Monograph of Cimicidae (Hemiptera - Heteroptera). (Thomas Say Foundation, College Park, MD, 1966).
64. Bloomquist, J. R. Excitation of central neurons by dieldrin and picrotoxinin in susceptible and resistant *Drosophila melanogaster* (Meigen). *Pestic. Sci.* **32**, 463-469 (1991).
65. Finney, D. J. *Probit analysis*. (Cambridge University Press, Cambridge, United Kingdom, 1971).
66. Robertson, J. L., Russell, R. M. Preisler, H. K. & Savin, N. E. Bioassays with arthropods, 2nd Ed. (CRC Press, Boca Raton, FL, 2007).
67. Benelli, G. *et al.* Mediterranean essential oils as effective weapons against the West Nile vector *Culex pipiens* and the *Echinostoma* intermediate host *Physella acuta*: what happens around? An acute toxicity survey on non-target mayflies. *Parasitol. Res.* **114**, 1011–1021 (2015).
68. Hsu, J. C. *Multiple Comparisons: Theory and methods*. (Chapman and Hall/CRC, New York, 1996).
69. Wilcox, R. R. New designs in analysis of variance. *Annu. Rev. Psychol.* **38**, 29–60 (1987).

CHAPTER 3. SYNERGISTIC TOXICITY INTERACTIONS BETWEEN ESSENTIAL OIL COMPONENTS AGAINST THE COMMON BED BUG (*CIMEX LECTULARIUS* L.)

This chapter was published in the journal Insects in February 2020. The main goals of this study were to determine the impacts of an equal ratio mixture of carvacrol, eugenol and thymol on their (i) efficacy against bed bugs and (ii) neuroinhibitory effects on the bed bug nervous system.

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3.1 Introduction

The common bed bug (*Cimex lectularius* L.) is one of two *Cimex* spp. that has resurged globally in the last two decades as a pest of public health and economic importance [1]. Several hypotheses have been proposed to explain the resurgence of common bed bugs (hereafter referred to as bed bugs), including increased travel by the public and the evolution of pyrethroid insecticide resistance in field populations [2–6]. More recently, some bed bug populations were shown to be highly resistant to various neonicotinoids [7] and possess reduced susceptibility to pyrrole compounds (i.e., chlorfenapyr) [5]. Difficulty in eliminating resistant bed bug populations demands a multi-faceted pest management approach that utilizes both chemical and non-chemical or alternative treatment options [8–10]. Plant essential oils, which are secondary metabolites derived from internal and external glandular cells on the leaves and stems of aromatic plants [11], are one of the alternative treatment options used for the control of bed bugs, cockroaches and many other urban and agricultural pests [12–19]. More than 20 plant essential oils and their components are listed as minimum risk pesticides by the Environmental Protection Agency and are exempt from registration requirements (https://www.epa.gov/minimum-risk-pesticides; located in 40 CFR 152.25 (f)). Due to this exemption, many essential oil-based products are readily available in the market for the control of bed bugs and other urban pests. However, out of the nine essential oil products tested by Singh et al. [15] against bed bugs, only two were efficacious. This finding by Singh et al. [15] suggested that more in-depth research on the toxicology of essential oils is

required to assist in the development of effective natural product formulations for bed bug and structural pest control in general.

Plant essential oils are composed of complex mixtures of monoterpenoids (generally referred to as essential oil components, compounds or constituents) with various functional groups, such as phenols, ketones, hydrocarbons, acids, etc. [11]. Of the various monoterpenoids tested in a recent study, the phenolic compounds carvacrol, eugenol and thymol were found to possess potent insecticidal activity against insecticide susceptible bed bugs when applied topically and/or as fumigants [20]. Furthermore, electrophysiology studies showed that these compounds also cause neuroinhibitory effects, i.e., suppression of the nervous system or nerve firing activity [20]. Additionally, target site studies conducted with carvacrol, eugenol and thymol suggested that they act on nicotinic acetylcholine (nACh), octapamine and gamma amino butyric acid (GABA) receptors, respectively [21–23].

Mixtures of two or more essential oil components exhibit synergistic, additive and/or antagonist toxicity effects in different insects, such as spider mites, cabbage loopers, house flies and nematodes [24–27]. Enhanced cuticular penetration caused by changes in pharmacokinetic properties (e.g., solubility and surface tension) of essential oil mixtures leads to synergistic action against the cabbage lopper [28,29]. While it is important to know all types of interactions between the monoterpenoids, synergistic toxicity interactions are more relevant from the perspective of pest management and the development of effective natural product formulations. Using synergistically interacting monoterpenoids in mixture products would allow us to achieve higher mortality by using similar quantities of active ingredients [26,28]. In bed bugs, the synergistic, additive or antagonistic effects of essential oil component mixtures on the insect nervous system and at the bioassay level have not been determined, thereby representing a knowledge gap.

Given the neuroinhibitory effect of carvacrol, eugenol and thymol on bed bug ventral nerve cord activity (i.e., a ganglionic mass or fused thoracic and abdominal ganglia, as termed by Usinger [30]) [20] and their ability to act on different neuronal target sites [21–23], we hypothesized that an equal ratio mixture of these three compounds would cause additive or synergistic toxicity effects and lead to a greater neurophysiological impact against bed bugs. To test these hypotheses, the objectives of our study were to determine the impacts of an equal ratio mixture of carvacrol, eugenol and thymol on their (i) efficacy against bed bugs and (ii) neuroinhibitory effects on the

bed bug nervous system. An equal ratio mixture of synthetic insecticides, bifenthrin (pyrethroid insecticide) and imidacloprid (neonicotinoid insecticide) was used as a positive control.

3.2 Materials and Methods

3.2.1 Insects

An insecticide-susceptible bed bug strain (Harlan) was used in this study. This strain was originally collected from the field in 1973 and has been maintained in the laboratory without insecticide selection pressure for more than 40 years. Insects were maintained in reach-in environmental chambers (Percival Scientific, Perry, IA, USA) at 25 °C temperature, 50% ± 15% relative humidity and a photoperiod of 12:12 (L: D) h. Insects were fed defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) using the membrane feeding method described by Chin–Heady et al. [31]. Topical application bioassays were performed with 8–10 days old adult males (average weight = ~2 mg per insect) that were fed 4–5 days before initiating the bioassays. For neurophysiology experiments, 10–15 days old adult males were used. They were starved for 7–8 days before dissection. Starvation for longer durations decreased the amount of undigested blood in their gut and resulted in cleaner dissections [20].

3.2.2 Topical Application Bioassays

The topical median lethal dose (LD_{50}) values of the individual compounds carvacrol, thymol, eugenol and bifenthrin for the same bed bug strain (Harlan) were previously determined by Gaire et al. [20]. The LD_{50} estimates of imidacloprid, the tertiary mixture (1:1:1 ratio) of carvacrol, eugenol and thymol and the binary mixture (1:1 ratio) of bifenthrin and imidacloprid were determined in this study. Before preparing a tertiary mixture, carvacrol and eugenol were individually diluted in acetone on a volume-to-volume basis to prepare stock solutions based on the density of each component (carvacrol = 0.976 g/mL, eugenol = 1.067 g/mL). However, a stock solution of thymol was prepared on a weight-per-volume basis, since it was in crystal form. Stock solutions of imidacloprid and bifenthrin (positive control) were also prepared in acetone (weight to volume basis) and then mixed in a 1:1 ratio. The range of test concentrations (at least 5 concentrations) of single and mixed components or insecticides were determined through preliminary screening (concentration range, carvacrol + thymol + eugenol: 4.1–41.66;

imidacloprid: 0.00025–003125; bifenthrin + imidacloprid: 0.0000775–0.000625 µg/mg body weight). For topical applications, insects were dorsally attached to the adhesive side of a colored label tape (Fisher Scientific) for immobilization. Insecticidal solutions (volume 0.5 µL) were applied topically on the ventral metathorax using a 25 µL syringe (Hamilton, Reno, NV, USA) attached to a PB-600-1 repeating dispenser (Hamilton). Control insect groups were treated with 0.5 µL of acetone. Treated and control insects were transferred into 35 × 10 mm Petri dishes (Greiner Bio-One, Frickenhausen, Germany) and placed in an environmental chamber. Mortality scoring of all treated insects was done at 24 h post-treatment. Insects that were lying on their backs and/or unable to move upon prodding were scored as dead. Mortality was also assessed 48 h post-treatment to ensure that insect recovery from intoxication symptoms did not occur. In total, three replicates were performed for each concentration in dose-response bioassays (n = 30; 10 adult males per replicate). Overall, 210–240 bed bugs were used for the determination of LD for each single compound or mixtures of the compounds.

3.2.3 Neurophysiology Equipment and Recording

Procedures followed for neurophysiology equipment setup, bed bug dissections and nervous system electrical activity recordings were adopted from an earlier study [20]. In brief, the neurophysiology equipment setup consisted of three electrodes, recording, reference and ground, which were connected to the model 4001 capacitance compensation head stage (Dagan Inc., Minneapolis, MN, USA). The head stage was further serially connected to noise eliminator, amplifier and digitizing computer software (i.e., Chart version 3.5.7, ADInstruments, Milford, MA, USA). The pulled glass capillary for the recording electrode was filled with HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid)-buffered physiological saline (pH 7.1) [20,32,33] and was placed in gentle contact with the fused ganglionic mass with the help of a micromanipulator (model MNJR, World Precision Instruments, Sarasota, FL, USA). The reference electrode was identical, but placed in contact with the carcass. A ground electrode was placed in the dissection dish outside the bed bug carcass.

Electrical activity recording with each insect was performed for 10 min (Figure 3.1). For the first 5 min, spontaneous pretreatment electrical activity (i.e., baseline) was recorded in physiological saline after setting a threshold level for the “counter” function on the Chart software (Figure 3.1). After 5 min, the recording was briefly paused to apply 1 µL of individual essential

oil components (carvacrol, eugenol or thymol), diluted to 0.5 mM in physiological saline containing 0.1% DMSO and 0.01% Tween 20 or their tertiary mixture (1:1:1), gently onto the ganglion. For the mixture, each individual essential oil component solution (carvacrol, eugenol and thymol) was prepared at 3-fold higher concentration (1.5 mM); then, equal volumes of each component were mixed to obtain the final 0.5 mM mixture solution. The threshold for the "counter" function on the Chart software was maintained at a constant level for the 5 min pre- and 5 min post-treatment nerve activity recordings (Figure 3.1). For solvent control recordings, a solution containing physiological saline + 0.1% DMSO + 0.01% Tween 20 was used. The effect of solvent controls on nerve activity was compared to recordings that were conducted only in physiological saline during the 5-min pre- and post-treatment intervals. To determine the effect of individual compounds or their mixture on nerve activity, "departure ratios" were calculated by dividing the total number of spike counts surpassing the threshold in post-treatment recordings with the total number of spike counts above the threshold in pre-treatment or baseline recordings [20].

For the positive control treatments, bifenthrin, imidacloprid and their equal ratio mixture were tested at a concentration of 5 µM. However, the treatment volume was higher (2 µL) because the 1 µL volume was not effective [20]. Nine to ten replications or nerve preparations were performed for physiological saline, solvent control, each essential oil compound and their mixtures and all positive control treatments with synthetic insecticides. Each bed bug represented one replicate. If the bed bug died during the ten-minute recording period, that replicate was discarded and a new recording was performed with a new insect.

3.2.4 Statistical Analysis

The dose-mortality data for the essential oil constituent mixture, the positive control mixture, and imidacloprid were analyzed by probit analysis to calculate the LD_{50} values and their 95% fiducial limits (FL) [34]. Probit analysis was done using Minitab Software Release 14.2 (Minitab Inc., State College, PA, USA, released 2005). To determine the expected LD_{50} and interaction between essential oil compounds or synthetic insecticides in a mixture, we used Hewlett and Plackett's model as per Tak et al. [26] and Tak and Isman [29].

$$E = (a \times LD_{50}(a)) + (b \times LD_{50}(b)) + (c \times LD_{50}(c)) + \ldots\ldots\ldots + (n \times LD_{50}(n))$$

where E is Hewlett and Plackett's expected LD_{50}, a is the proportion of compound A in the mixture and LD_{50}(a) is the LD_{50} of compound A, and so on. The interaction ratio was calculated by dividing the expected or theoretical LD_{50} value by the observed LD_{50}. An interaction ratio greater than 1.5 indicates a synergistic interaction, a ratio of 1.5 or less and greater than 0.5 indicates an additive interaction and ratios of 0.5 or less indicate antagonism.

$$\text{Interaction ratio (R)} = \frac{\text{Hewlett and Plackett's expected LD50 of mixture}}{\text{Observed LD50 of mixture}}$$

For the neurophysiology data, departure ratios calculated for all mixtures or individual compounds were log-transformed after adding a value of one (1). The addition of the value "1" was done to obtain positive log-transformed values [20]. First, log-transformed departure ratios determined for the solvent controls were statistically compared to the physiological saline treatment using a two-sample t-test with Bonferroni's adjusted significance level (0.05 divided by the number of comparisons or tests) [20,35,36]. Next, log-transformed departure ratio data for different mixtures and individual essential oil components or synthetic insecticides were compared to solvent controls using two-sample t-tests with Bonferroni's adjusted significance level. Lastly, the same test was used to compare departure ratio data for single essential oil components or insecticides with their respective tertiary or binary mixtures. Two-sample t-tests were performed using SPSS Version 25 (IBM Corp., Armonk, NY, USA, released 2017).

3.3 Results

3.3.1 Topical Toxicity

In all bioassays, <5% mortality was observed in acetone-treated bed bugs. The LD_{50} values of the individual compounds carvacrol, thymol, eugenol and bifenthrin, as determined by Gaire et al. [20], were 27.5, 32.5, 52 and 0.000345 µg/mg body weight, respectively. The LD_{50} value for imidacloprid was 0.0006 µg/mg body weight (Table 3.1). The tertiary mixture of carvacrol, thymol and eugenol caused a synergistic increase in bed bug mortality (interaction ratio of 1.96; Table 3.1). The mixture of bifenthrin and imidacloprid also showed synergism against bed bugs with an interaction ratio of 1.88 (Table 3.1).

3.3.2 Neurophysiological Effects of Mixtures

The solvent control treatment had no effect on bed bug nervous system activity in comparison to physiological saline ($p = 0.682$) (Figure 3.2a). When tested individually, the three essential oil components carvacrol, eugenol and thymol did not produce statistically significant inhibitory effects (i.e., no suppression of nerve firing activity) at the 0.5 mM concentration in comparison to the solvent control (carvacrol p-value = 0.435, thymol p-value = 0.468 and eugenol p-value = 0.918; Figure 3.2b). However, the mixture of the three essential oil components at the same 0.5 mM concentration inhibited spontaneous nerve firing by 12.44% when compared the solvent control ($p = 0.003$, two-sample t-test at Bonferroni's corrected significance level of $p < 0.0125$) (Figure 3.2b). When neuroinhibitory effects of the tertiary mixture were compared to impacts caused by individual compounds, statistically significant differences were observed for all compounds (two-sample t-test at Bonferroni's significance level of $p < 0.016$) (Figure 3.2b). More specifically, the neuroinhibitory potential of the tertiary mixture were 12%, 15% and 11% higher in comparison to the effects of the individual carvacrol, eugenol and thymol compounds, respectively (Figure 3.2b).

In the positive control treatment, the mixture of bifenthrin and imidacloprid at 5 µM produced significant neuroexcitation, i.e., a 25.94% increase in nerve firing activity compared to the solvent control treatment ($p = 0.001$, two-sample t-test at Bonferroni's significance level of $p < 0.016$) (Figure 3.2c). However, when these insecticides were tested individually at 5 µM, they did not cause statistically significant overstimulation or neuroexcitation in comparison to the solvent control treatment (bifenthrin p-value = 0.669 and imidacloprid p-value = 0.967; Figure 3.2c). In contrast, the neuroexcitatory effect of the bifenthrin and imidacloprid mixture was significantly higher than the impacts of the individual insecticides (two-sample t-test at Bonferroni's significance level of $p < 0.025$) (Figure 3.2c).

3.4 Discussion

Toxicity interactions between various compounds in insecticide mixtures are determined by a series of complex actions and counteractions between toxins and insect tissues [28]. Toxicity of insecticidal compounds or their mixtures is generally dependent upon cuticular penetration, activation of target sites and detoxification [28,37]. In this study, we observed that a tertiary

mixture of carvacrol, eugenol and thymol led to a synergistic increase in their topical toxicity levels against bed bugs. These bioassay findings correlated with electrophysiology results, wherein the same tertiary mixture caused a significant decrease in nerve firing activity of fused thoracic and abdominal ganglia in comparison to the effects caused by the individual compounds (carvacrol, eugenol and thymol) at 0.5 mM concentration. In the following subsections, factors responsible for synergism between essential oil components at the sub-organismal (nervous system) and organismal (topical bioassays) levels are discussed, along with the implications of these findings for natural product development and bed bug management.

3.4.1 Mechanisms of Synergism between Monoterpenoids

Previous studies showed that a binary mixture of camphor and 1,8-cineole exhibited enhanced cuticular penetration, leading to a synergistic increase in toxicity against cabbage looper larvae [28]. These changes in the cuticular penetration ability of camphor and 1,8-cineole mixture are caused by pharmacokinetic factors that reduce surface tension and increase their solubility [28]. In addition to cuticular penetration-related mechanisms of synergism, the synergistic interaction that we observed between the tertiary mixture of carvacrol, eugenol and thymol was likely caused by target site-associated factors, such as the ability of the monoterpenoids to act on different target sites within the insect nervous system. As shown in Table 3.2, carvacrol, eugenol and thymol bind to nACh, octopamine and GABA receptors, respectively [21–23,38–40]. Carvacrol, eugenol and thymol also have similar effects on suppressing nerve firing activity of the bed bug nervous system at specific concentrations (Table 3.2) [20]. In general, neurologically active insecticides kill insects by inhibiting or overstimulating the normal firing activity of the nervous system [41-45]. Therefore, the simultaneous action of the tertiary mixture constituents at different binding sites is at least partially responsible for the suppression of nerve firing activity and the increased mortality observed in topical bioassays. Furthermore, changes in solubility, decreased surface tension and altered lipophilicity of essential oil constituent mixtures may allow them to penetrate the nervous system membrane more effectively, thus leading to greater neurophysiological effects.

Since essential oil components are volatile and exhibit vapor toxicity against various urban and agricultural insect pests [12,20,46], the effects of monoterpenoid mixtures on their vapor toxicity levels need to be determined in the future. Lastly, an increasing body of literature suggests that plant essential oils containing monoterpenoids inhibit cytochrome P450s in different mosquito

species [47–49]. Thus, increased inhibition of detoxification enzymes by monoterpenoid mixtures could be yet another mechanism of synergism.

Topical bioassays and electrophysiology experiments that we conducted with *C. lectularius* using an equal ratio mixture of bifenthrin and imidacloprid (i.e., the positive control treatment), revealed a significant synergistic interaction between these two insecticides in whole organism bioassays and sub-organismal nerve activity recordings. Due to the differences in binding sites for pyrethroids (voltage-gated sodium channels) [44] and neonicotinoids (post-synaptic nAChRs) [42] and their neuroexcitatory actions (Table 3.2), it is expected that mixing insecticides from these two classes would cause a synergistic increase in activity toward target insect pests in comparison to either of the individual chemicals. Our findings regarding the synergism between pyrethroids and neonicotinoids were in agreement with the synergistic effects of a bifenthrin and imidacloprid mixture reported against mole crickets [43], wherein electrophysiology experiments showed that the mixture of bifenthrin and imidacloprid at a 10 µM concentration potentiated/synergized nerve firing activity of mole crickets and resulted in faster mortality in bioassays [43]. Neurological synergism between pyrethroids and other neuroexcitatory insecticides was also demonstrated in American cockroaches by Corbel et al. [50], who reported that a mixture of pyrethroid (permethrin) and carbamate (propoxur) insecticides drastically increased acetylcholine concentrations within the synaptic cleft.

3.4.2 Implications for Natural Product Development and Bed Bug Management

The use of synthetic organic insecticide mixtures is one of the strategies recommended for combating pesticide resistance in insect pests, including bed bugs [8,51]. Many laboratory and field-based studies with bed bugs showed that pyrethroid and neonicotinoid combination products exhibited higher efficacy against pyrethroid-resistant bed bugs and their eggs [52–54]. Several natural product insecticides containing a mixture of different essential oils (e.g., clove, cinnamon, cedar, peppermint, rosemary etc.) or their major constituents (e.g., eugenol and geraniol) are available in the market for bed bug control [15]. However, most of the available essential oil products are not effective against bed bugs [15], likely because they were formulated without considering synergistic, additive or antagonistic interactions that may occur either between different essential oils or their insecticidal components. The identification of the monoterpenoids carvacrol, eugenol and thymol, which interact synergistically and lead to increased toxicity against

insecticide-susceptible bed bugs, is thus an important finding for informing the development of efficacious plant essential oil-based products for urban pest control. In the future, similar studies could be conducted with insecticide-resistant bed bug strains to determine the feasibility of using mixtures of different monoterpenoids for their control. Although there are limitations associated with the use of essential oils for urban pest control, such as odor and short residual activity, nanoformulated essential oils have less odor, are less volatile and show prolonged residual activity [55,56]. Additionally, in pesticide-susceptible and resistant American cockroaches and mosquitoes, essential oils were shown to potentially synergize the toxicity of pyrethroid and carbamate insecticides. either by inhibiting P450 enzymes or by activating neurological target sites [48,49,57]. Therefore, future research should also explore the possibility of using monoterpenoids or essential oils as synergists for overcoming resistance to pyrethroids and other insecticides in bed bugs.

3.5 Conclusions

The present study and previous research [20] collectively provide new insights into essential oil constituents that can be formulated together in botanical insecticide products. Furthermore, the identification of increased neuroinhibitory effects of a tertiary mixture of carvacrol, eugenol and thymol on the bed bug nervous system further advances our understanding of the mechanisms of synergistic interactions between monoterpenoids. Increased cuticular penetration [28], as well as a greater alteration of nerve firing activity than just additive, appear to be the major mechanisms responsible for synergism between monoterpenoids and essential oil components.

Table 3.1 Topical toxicity of monoterpenoids, synthetic insecticides and their respective binary and tertiary mixtures against adult male bed bugs of the Harlan susceptible strain.

Treatments	N	Observed LD_{50} [I], µg/mg body weight (Fiducial limits)	Expected LD_{50} [II], µg/mg body weight	Ratio (Interaction)
Essential oil components				
Carvacrol [III]	–	27.5	–	–
Thymol [III]	–	32.5	–	–
Eugenol [III]	–	52	–	–
Carvacrol + thymol + eugenol	240	19 (17–21.5)	37.25	1.96 (Synergistic)
Synthetic insecticides				
Bifenthrin [III]	–	0.000345	–	–
Imidacloprid	210	0.0006 (0.0005–0.00075)	–	–
Bifenthrin + imidacloprid	210	0.00025 (0.00025–0.0003)	0.00047	1.88 (Synergistic)

[I] Observed median lethal dose (LD_{50}) refers to the lethal dose required to kill 50% of the population, as calculated from the probit analysis. [II] Expected LD_{50} refers to the estimated LD_{50} values from Hewlett and Plackett's model, as per Tak et al. [26] and Tak and Isman [29]. [III] LD_{50} values for carvacrol, eugenol, thymol and bifenthrin were adapted from Gaire et al. [20].

Table 3.2 Information regarding the target sites and neurological effects caused by plant essential oil components and synthetic insecticides used in the current study.

Essential Oil Components	**Target Site**	**Neurological Effect**	**Effective Concentrations and Insect Species**
Carvacrol	Nicotinic acetylcholine receptor (nAChR) [22]	Neuroinhibition [20]	4 mM in *Cimex lectularius* L. [20]
Thymol	Gamma-amino butyric acid receptor (GABA) [23]	Neuroinhibition [20]	4 mM in *C. lectularius* [20]
Eugenol	Octopamine receptor [21]	Neuroinhibition [20,41]	2 mM in *C. lectularius* [20]; 1 and 2 mM in *Periplaneta americana* L., *Blaberus discoidalis* Serville [41]
Synthetic insecticides			
Bifenthrin	Voltage-gated sodium channel [42]	Neuroexcitation [20,43]	10 µM in *C. lectularius* and *Scapteriscus vicinus* Scudder [20,43]
Imidacloprid	Nicotinic acetylcholine receptor [44]	Neuroexcitation [43]	10 µM in *S. vicinus* [43]

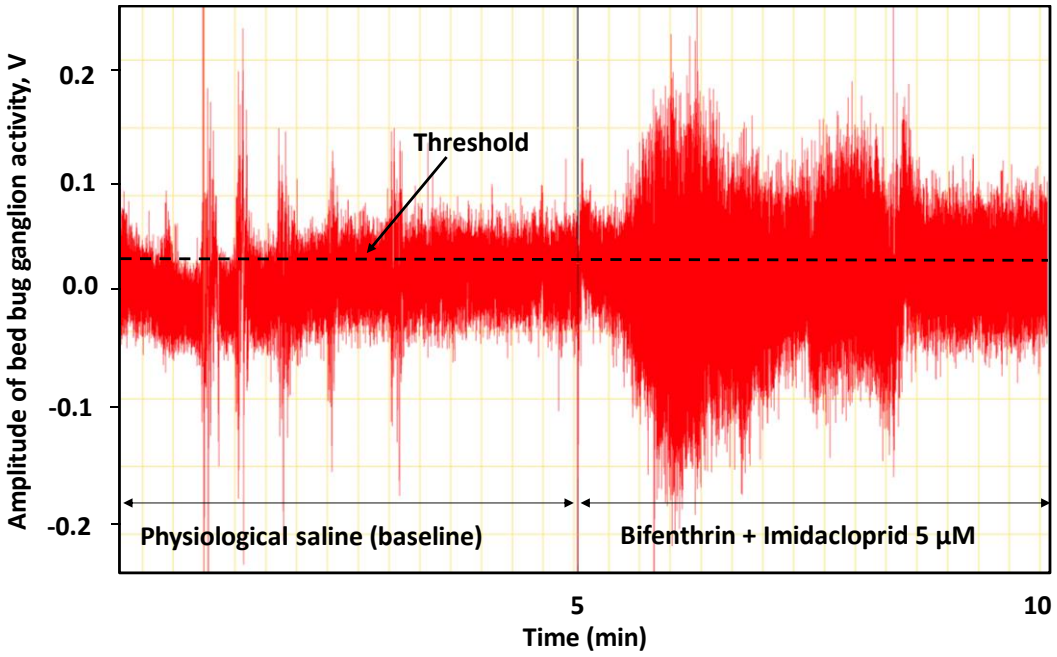
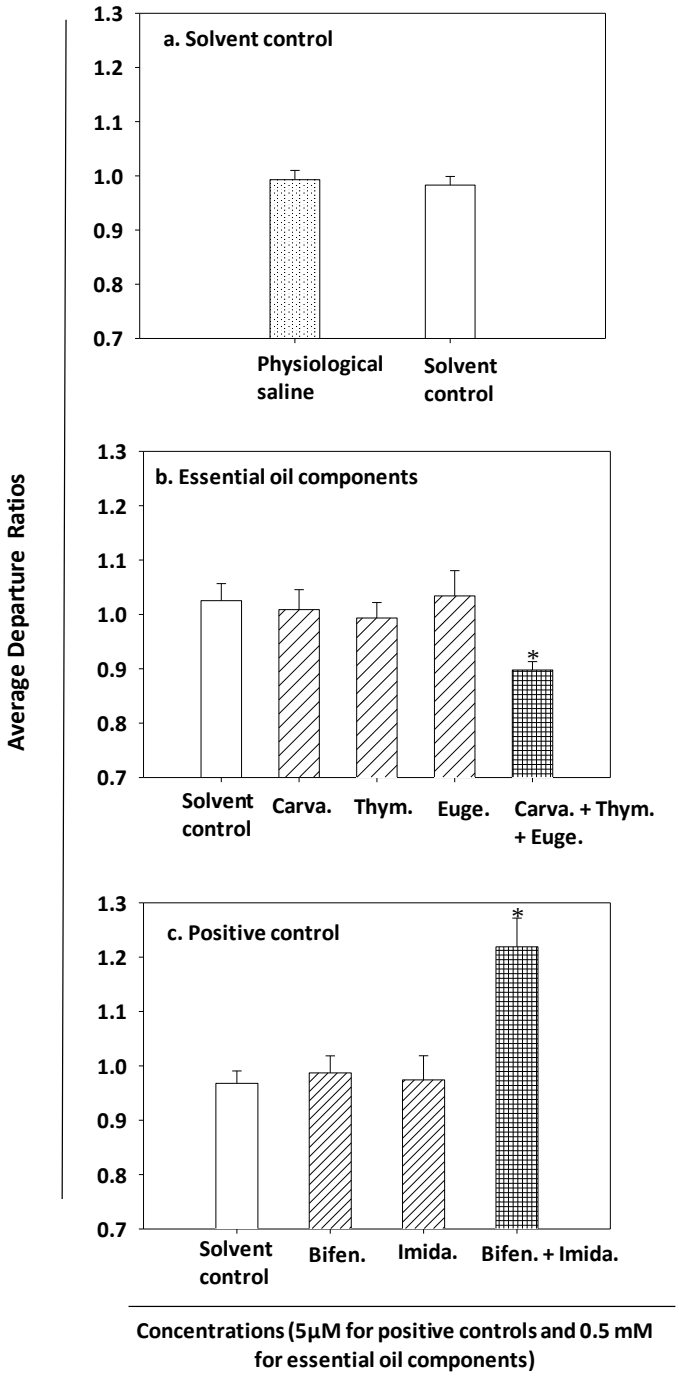


Figure 3.1 An example of a 10 min electrophysiological nerve activity recording for a 5 µM bifenthrin + imidacloprid mixture. Baseline spontaneous electrical activity recordings (pre-treatment) were performed in physiological saline for 5 min. After application of insecticide or mixture solutions to the nerve preparations, post-treatment recordings were performed for additional 5 min. The threshold was maintained at a constant level between the pre- and post-treatment recordings using the "counter" function in the Chart software.

Figure 3.2 Neurophysiological effects of solvent control, essential oil constituents and positive control treatments on bed bug nervous system activity. Asterisks (*) in different graphs indicate significant differences compared to the solvent control recordings (two-sample t-tests with Bonferroni's corrected p-value, i.e., 0.05 ÷ number of comparisons for each compound or mixture). (a) Solvent controls containing 0.1% dimethyl sulfoxide (DMSO) and 0.01% Tween 20 had no effect on nervous system activity ($p > 0.05$). (b) The essential oil component mixture of carvacrol (carva.), thymol (thym.) and eugenol (euge.) at 0.5 mM induced higher neuroinhibitory impacts than any of the individual compounds compared to the solvent control ($p < 0.0125$). (c) The positive control treatment mixture of bifenthrin (bifen.) and imidacloprid (imida.) at 5 µM induced significantly greater neuroexcitatory effects than either of the individual compounds in comparison to the solvent control ($p < 0.016$).

Figure 3.2 continued



3.6 References

1. Doggett, S.L.; Miller, D.M.; Lee, C.Y. *Advances in the Biology and Management of Modern Bed Bugs*; Wiley Blackwell: West Sussex, UK, 2018.
2. Romero, A.; Potter, M.F.; Potter, D.A.; Haynes, K.Y. Insecticide resistance in the bed bug: A factor in the pest's sudden resurgence? *J. Med. Entomol.* **2007**, *44*, 175–178.
3. Adelman, Z.N.; Kilcullen, K.A.; Koganemaru, R.; Anderson, M.A.E.; Anderson, T.D.; Miller, D.M. Deep sequencing of pyrethroid-resistant bed bugs reveals multiple mechanisms of resistance within a single population. *PLoS ONE* **2011**, *6*, e26228, doi:10.1371/journal.pone.0026228.
4. Zhu, F.; Gujar, H.; Gordon, J.R.; Haynes, K.F.; Potter, M.F.; Palli, S.R. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. *Sci. Rep.* **2013**, *3*, 1456, doi:10.1038/srep01456.
5. Ashbrook, A.R.; Scharf, M.E.; Bennett, G.W.; Gondhalekar, A.D. Detection of reduced susceptibility to chlorfenapyr-and bifenthrin-containing products in field populations of the bed bug (Hemiptera: Cimicidae). *J. Econ. Entomol.* **2017**, *110*, 1195–1202.
6. Hentley, W.M.; Webster, B.; Evinson, S.E.F.; Siva-Jothy, M.T. Bed bug aggregation on dirty laundry: A mechanism for passive dispersal. *Sci. Rep.* **2017**, *7*, 11668, doi:10.1038/s41598-017-11850-5.
7. Romero, A.; Anderson, T.D. High levels of resistance in the common bed bug. *Cimex lectularius* (Hemiptera: Cimicidae), to neonicotinoid insecticides. *J. Med. Entomol.* **2016**, *53*, 727–731.
8. Bennett, G.W.; Gondhalekar, A.D.; Wang, C.; Buczkowski, G.; Gibb, T.J. Using research and education to implement practical bed bug control programs in multifamily housing. *Pest Manag. Sci.* **2016**, *72*, 8–14.
9. Romero, A.; Sutherland, A.M.; Gouge, D.H.; Spafford, H.; Nair, S.; Lewis, V.; Choe, D.H.; Li, S.; Young, D. Pest management strategies for bed bugs (Hemiptera: Cimicidae) in multiunit housing: A literature review on field studies. *J. Integr. Pest Manag.* **2017**, *8*, 1–10.
10. Wang, C.; Eiden, A.; Singh, N.; Zha, C.; Wang, D.; Cooper, R. Dynamics of bed bug infestations in three low-income housing communities with various bed bug management programs. *Pest Manag. Sci.* **2018**, *74*, 1302–1310.

11. Guenther, E. *The Essential Oils. Vol. II. The Constituents of Essential Oils*; D. Van Nostrand Company, Inc.: New York, NY, USA, 1949.
12. Rice, P.J.; Coats, J.R. Insecticidal properties of several monoterpenoids to the house fly (Diptera: Muscidae), red flour beetle (Coleoptera: Tenebrionidae), and southern corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* **1994**, *87*, 1172–1179.
13. Isman, M.B. Botanicals insecticide, deterrents and repellents in modern agriculture and increasing regulated world. *Annu. Rev. Entomol.* **2006**, *51*, 45–66.
14. Phillips, A.K.; Appel, A.G.; Sims, S.R. Topical toxicity of essential oils to the German cockroach (Dictyoptera: Blattellidae). *J. Econ. Entomol.* **2010**, *103*, 448–459.
15. Singh, N.; Wang, C.; Cooper, R. Potential of essential oil-based pesticides and detergents for bed bug control. *J. Econ. Entomol.* **2014**, *107*, 2163–2170.
16. Feldlaufer, M.F.; Ulrich, K.R. Essential oils as fumigants for bed bugs (Hemiptera: Cimicidae). *J. Entomol. Sci.* **2015**, *50*, 129–137.
17. Gaire, S.; O'Connell, M.; Holguin, F.O.; Amatya, A.; Bundy, S.; Romero, A. Insecticidal properties of essential oils and some of their constituents on the Turkestan cockroach (Blattodea: Blattidae). *J. Econ. Entomol.* **2017**, *110*, 584–592.
18. Politi, F.A.S.; Nascimento, J.D.; da Silva, A.A.; Moro, I.J.; Garcia, M.L.; Guido, R.V.C.; Pietro, R.C.L.R.; Godinho, A.F.; Furlan, M. Insecticidal activity of an essential oil of *Tagetes patula* L. (Asteraceae) on common bed bug *Cimex lectularius* L. and molecular docking of major compounds at the catalytic site of ClAChE. *Parasitol. Res.* **2017**, *116*, 415–424.
19. Zha, C.; Wang, C.; Li, A. Toxicities of selected essential oils, silicone oils, and paraffin oil against the common bed bug (Hemiptera: Cimicidae). *J. Econ. Entomol.* **2018**, *111*, 170–177.
20. Gaire, S.; Scharf, M.E.; Gondhalekar, A. Toxicity and neurophysiological impacts of essential oil components on bed bug (*Cimex lectularius* L.). *Sci. Rep.* **2019**, *9*, 3961, doi:10.1038/s41598-019-40275-5.
21. Enan, E. Insecticidal activity of essential oils: Octopaminergic sites of action. *Comp. Biochem. Phys. C* **2001**, *130*, 325–337.
22. Tong, F.; Gross, A.D.; Dolan, M.C.; Coats, J.R. The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor. *Pest Manag. Sci.* **2013**, *69*, 775–780.

23. Priestley, C.M.; Williamson, E.M.; Wafford, K.A.; Sattelle, D.B. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human $GABA_A$ receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. *Br. J. Pharmacol.* **2003**, *140*, 1363–1372.
24. Ntalli, N.G.; Ferrari, F.; Giannakou, I.; Menkissoglu-Spiroudi, U. Synergistic and antagonistic interactions of terpenes against *Meloidogyne incognita* and the nematicidal activity of essential oils from seven plants indigenous to Greece. *Pest Manag. Sci.* **2011**, *67*, 341–351.
25. Gallardo, A.; Picollo, M.I.; Mougabure-Cueto, G. Lethal activity of individual and mixed monoterpenoids of geranium essential oil on *Musca domestica*. *Parasitol. Res.* **2015**, *114*, 1229–1232.
26. Tak, J.H.; Jovel, E.; Isman, M.B. Comparative and synergistic activity of *Rosmarinus officinalis* L. essential oil constituents against the larvae and an ovarian cell line of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). *Pest Manag. Sci.* **2016**, *72*, 474–480.
27. Wu, L.; Huo, X.; Zhou, X.; Zhao, D.; He, W.; Liu, S.; Liu, H.; Feng, T.; Wang, C. Acaricidal activity and synergistic effect of thyme oil constituents against carmine spider mite (*Tetranychus Cinnabarinus* (Boisduval)). *Molecules* **2017**, *22*, 1873.
28. Tak, J.H.; Isman, M.B. Enhanced cuticular penetration as the mechanism for synergy of insecticidal constituents of rosemary essential oil in *Trichoplusia ni*. *Sci. Rep.* **2015**, *5*, 12690, doi:10.1038/srep12690.
29. Tak, J.H.; Isman, M.B. Penetration-enhancement underlies synergy of plant essential oil terpenoids as insecticides in the cabbage looper, *Trichoplusia ni*. *Sci. Rep.* **2017**, *7*, 42432, doi:10.1038/srep42432.
30. Usinger, R.L. *Monograph of Cimicidae (Hemiptera-Heteroptera)*; Thomas Say Foundation: College Park, MD, USA, 1966.
31. Chin-Heady, E.; DeMark, J.J.; Nolting, S.; Bennett, G.; Saltzmann, K.; Hamm, R.L. A quantitative analysis of a modified feeding method for rearing *Cimex lectularius* (Hemiptera: Cimicidae) in the laboratory. *Pest Manag. Sci.* **2013**, *69*, 1115–1120.
32. Bloomquist, J.R. Excitation of central neurons by dieldrin and picrotoxinin in susceptible and resistant *Drosophila melanogaster* (Meigen). *Pestic. Sci.* **1991**, *32*, 463–469.

33. Gondhalekar, A.D.; Scharf, M.E. Mechanisms underlying fipronil resistance in a multi resistant field strain of the German cockroach (Blattodea: Blattellidae). *J. Med. Entomol.* **2012**, *49*, 122–131.
34. Finney, D.J. *Probit Analysis*; Cambridge University Press: Cambridge, UK, 1971.
35. Wilcox, R.R. New designs in analysis of variance. *Annu. Rev. Psychol.* **1987**, *38*, 29–60.
36. Hsu, J.C. *Multiple Comparisons: Theory and Methods*; Chapman and Hall/CRC: New York, NY, USA, 1996.
37. Sun, Y.P. Dynamics of insect toxicology-a mathematical and graphical evaluation of the relationship between insect toxicity and rates of penetration and detoxication of insecticides. *J. Econ. Entomol.* **1968**, *61*, 949–955.
38. Kostyukovsky, M.; Rafaeli, A.; Gileadi, C.; Demchenko, N.; Shaaya, E. Activation of octopaminergic receptors by essential oil constituents isolated from aromatic plants: Possible mode of action against insect pests. *Pest Manag. Sci.* **2002**, *58*, 1101–1106.
39. Garcia, D.A.; Vendrell, I.; Galofre, M.; Sunol, C. GABA released from cultured cortical neurons influences the modulation of t-[^{35}S] butylbicyclophosphorothionate binding at the $GABA_A$ receptor effects of thymol. *Eur. J. Pharmacol.* **2008**, *600*, 26–31.
40. Waliwitiya, R.; Belton, P.; Nicholson, R.A.; Lowenberger, C.A. Effects of the essential oil constituent thymol and other neuroactive chemicals on flight motor activity and wing beat frequency in the blowfly *Phaenicia sericata*. *Pest Manag. Sci.* **2010**, *66*, 277–289.
41. Price, D.N.; Berry, M.S. Comparison of effects of octopamine and insecticidal essential oils on activity in the nerve cord, foregut, and dorsal unpaired median neurons of cockroaches. *J. Insect Physiol.* **2006**, *52*, 309–319.
42. Soderlund, D.M.; Bloomquist, J.R. Neurotoxic actions of pyrethroid insecticides. *Annu. Rev. Entomol.* **1989**, *34*, 77–96.
43. Kostromytska, O.S.; Buss, E.A.; Scharf, M.E. Toxicity and neurophysiological effects of selected insecticides on the mole cricket, *Scapteriscus vicinus* (Orthoptera: Gryllotalpidae). *Pestic. Biochem. Phys.* **2011**, *100*, 27–34.
44. Tan, J.; Salgado, V.L.; Hollingworth, R.M. Neural actions of imidacloprid and their involvement in resistance in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Pest Manag. Sci.* **2008**, *64*, 37–47.

45. Scharf, M.E. Neurological effects of insecticides and the insect nervous system. In *Encyclopedia of Entomology*; Capinera, J.L., Ed.; Springer: New York, NY, USA, 2008; pp. 2596–2607.
46. Phillips, A.K.; Appel, A.G. Fumigant toxicity of essential oils to the German cockroach (Dictyoptera: Blattellidae). *J. Econ. Entomol.* **2010**, *103*, 781–790.
47. Waliwitiya, R.; Nicholson, R.A.; Kennedy, C.J.; Lowenberger, C.A. The synergistic effects of insecticidal essential oils and piperonyl butoxide on biotransformational enzyme activities in *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **2012**, *49*, 614–623.
48. Norris, E.J.; Johnson, J.B.; Gross, A.D.; Bartholomay, L.C.; Coats, J.R. Plant essential oils enhance diverse pyrethroids against multiple strains of mosquitoes and inhibit detoxification enzyme processes. *Insects* **2018**, *9*, 132, doi:10.3390/insects9040132.
49. O'Neal, S.; Johnson, E.J.; Rault, L.C.; Anderson, T.D. Vapor delivery of plant essential oils alters pyrethroid efficacy and detoxification enzyme activity in mosquitoes. *Pestic. Biochem. Phys* **2019**, *157*, 88–98.
50. Corbel, V.; Stankiewicz, M.; Bonnet, J.; Grolleau, F.; Hougard, J.M.; Lapied, B. Synergism between insecticides permethrin and propoxur occurs through activation of presynaptic muscarinic negative feedback of acetylcholine release in the insect central nervous system. *Neurotoxicology* **2006**, *27*, 508–519.
51. Tabashnik, B.E. Managing resistance with multiple pesticide tactics: Theory, evidence, and recommendations. *J. Econ. Entomol.* **1989**, *82*, 1263–1269.
52. Gordon, J.R.; Goodman, M.H.; Potter, M.F.; Haynes, K.F. Population variation in and selection for resistance to pyrethroid-neonicotinoid insecticides in the bed bug. *Sci. Rep.* **2014**, *4*, 3836.
53. Wang, C.; Singh, N.; Zha, C.; Cooper, R. Efficacy of selected insecticide sprays and aerosols against the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae). *Insects* **2016**, *7*, 5, doi:10.3390/insects7010005.
54. Hinson, K.R.; Benson, E.P.; Zungoli, P.A.; Bridges, W.C.; Ellis, B.R. Egg hatch rate and nymphal survival of the bed bug (Hemiptera: Cimicidae) after exposure to insecticide sprays. *J. Econ. Entomol.* **2016**, *109*, 2495–2499.

55. Oliveira, A.P.; Santana, A.S.; Santana, E.D.R.; Lima, A.P.S.; Faro, R.R.N.; Nunes, R.S.; Lima, A.D.; Blank, A.F.; Araujo, A.P.A.; Cristaldo, P.F.; et al. Nanoformulations prototype of the essential oil of *Lippia sidoides* and thymol to population management of *Sitophilus zeamais* (Coleoptera: Curculionidae). *Ind. Crops Prod.* **2017**, *107*, 198–205.
56. Gonzalez, J.O.W.; Gutierrez, M.M.; Ferrero, A.A.; Band, B.F. Essential oils nanoformulations for store-product pest control—Characterization and biological properties. *Chemosphere* **2014**, *100*, 130–138.
57. Jankowska, M.; Lapied, B.; Jankowski, W.; Stankiewicz, M. The unusual action of essential oil component, menthol, in potentiating the effect of the carbamate insecticide, bendiocarb. *Pestic. Biochem. Physiol.* **2019**, *158*, 101–111.

CHAPTER 4. BED BUGS, *CIMEX LECTULARIUS* L., EXHIBITING METABOLIC AND TARGET SITE DELTAMETHRIN RESISTANCE ARE SUSCEPTIBLE TO PLANT ESSENTIAL OILS

4.1 Introduction

Resistance to pyrethroids (e.g., deltamethrin, beta-cyfluthrin and d-allethrin) has been documented in two species of bed bugs, *Cimex lectularius* L. (bed bugs or common bed bugs) and *C. hemipterus* F. (tropical bed bugs) (Karunaratne et al., 2007; Romero et al., 2007; Adelman et al., 2011; Zhu et al., 2013; Gonzalez-Morales and Romero, 2018). The highest deltamethrin (type II pyrethroid) resistance ratio that was reported in *C. lectularius* was 20,000-fold (Gonzalez--Morales and Romero, 2018) whereas in the *C. hemipterus* it was 370,000-fold (Lilly et al. 2015). Bed bug populations of both species may possess multiple mechanisms that confer resistance to deltamethrin exposure, including the elevation of detoxification enzyme activity (cytochrome P450s, esterases and glutathione transferases), knockdown resistance (*kdr*) associated point mutations in the voltage-gated sodium channel gene, and reduced cuticular penetration (Yoon et al., 2008; Zhu et al., 2010a; Adelman et al., 2011; Zhu et al., 2013; Dang et al., 2015; Lilly et al., 2016a).

Given the array of mechanisms by which pyrethroid insecticides may fail to control *C. lectularius* or bed bug infestations, alternative management strategies are required which include treatments involving heat (Kells and Goblirsch, 2011), cold (Olson et al., 2013), steam (Puckett et al., 2013; Wang et al., 2018), desiccant dusts (Romero et al., 2009), carbon dioxide or dry ice (Singh et al., 2013), insecticide-treated mattress encasements (Jones et al., 2015), fumigants (Lehnert et al., 2011; Phillips et al., 2014), and plant essential oils (Singh et al., 2014; Zha et al., 2018; Gaire et al. 2019,2020). The public demand for plant essential oil-based or natural products is currently on the rise for the control of several insect pests, including bed bugs (Isman, 2006; Regnault-Roger et al., 2012; Singh et al., 2014).

Plant essential oils are extracts from aromatic plants that contain several insecticidal constituents (also referred as essential oil components or monoterpenoids), with various functional groups such as phenol, ketone, aldehyde, and alcohol (Guenther, 1949). More than 20 plant essential oils and their constituents are considered low risk insecticides and are exempt from EPA (Environmental Protection Agency) registration (https://www.epa.gov/minimum-risk-pesticides).

Because of this low risk status, many products are introduced to the market without rigorous toxicology and empirical efficacy studies. This may be the primary reason why only two of the nine essential oil-based products tested by Singh et al. (2014) were effective against field-collected bed bugs; however, where effective, these performed similarly to synthetic insecticides (Wang et al., 2014). Zha et al. (2018) later found that pure essential oils were effective against field-collected bed bugs that were moderately resistant to pyrethroid insecticides. Furthermore, plant essential oils and their constituents have been shown to possess contact and fumigant activity against the insecticide susceptible Harlan strain of bed bugs (Feldlaufer and Ulrich, 2015; Gaire et al., 2019,2020). However, to date, no toxicity bioassays have compared the efficacy of plant essential oils or their constituents against insecticide susceptible and field collected bed bug or *C. lectularius* strains. Generating data to bridge this gap in our knowledge is important for determining the feasibility of plant essential oil-based products to control pyrethroid resistant bed bugs, which are globally widespread (Zhu et al. 2010; Booth et al, 2015, 2018; Balvin and Booth, 2018; Holleman et al., 2019).

The goals of this study, which was conducted with bed bugs or *C. lectularius* were: (i) determining deltamethrin resistance levels and mechanisms in a field-collected strain (Knoxville), and (ii) assessing resistance levels to plant essential oils, their major constituents and a commercial essential oil product in the Knoxville strain.

4.2 Materials and methods

4.2.1 Bed bug strains

Experiments were performed on two strains of bed bugs, the Harlan lab strain, and the field-collected Knoxville strain. The Harlan strain was collected from the field in 1973 and is susceptible to all insecticides including pyrethroids (Doggett et al., 2018). As such, the Harlan strain was used as a baseline susceptible strain for all bioassays and enzyme activity determination experiments. Additionally, it has been previously shown to lack the *kdr*-associated target site mutations (Yoon et al., 2008). The Knoxville strain was collected from apartments in Knoxville, TN in 2013 and has a history of exposure to pyrethroid, chlorfenapyr and imidacloprid/beta-cyfluthrin treatments prior to its collection (Ashbrook et al., 2017). Furthermore, the Knoxville strain showed reduced susceptibility to bifenthrin (type I pyrethroid) containing products (Ashbrook et al., 2017). The

Knoxville strain was chosen for this study because it exhibited the highest level of deltamethrin-resistance among six field strains in preliminary topical application bioassays (Figure B.1). Strains were maintained at 25℃, 50±15% relative humidity, and a photoperiod of 12:12 h (L: D) in environmental chambers (Percival Scientific, Perry, IA). Bed bugs were fed weekly on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using the membrane feeding method (Chin-Heady et al., 2013).

4.2.2 Chemicals

Deltamethrin, a type II pyrethroid (purity 99.3%) was obtained from Chem Service Inc. (West Chester, PA). The insecticide synergists piperonyl butoxide (PBO), *S*,*S*,*S*-tributyl phosphorotrithioate (DEF) and diethyl maleate (DEM) were purchased from Sigma-Aldrich (St. Louis, MO). The solvent carrier acetone (analytical grade) and Triton X 100 were purchased from Fisher Scientific (Hampton, NH). Substrates and reagents used in enzyme assays were procured either from Sigma-Aldrich or Fisher Scientific.

Five pure plant essential oils; thyme oil (*Thymus vulgaris*), oregano oil (*Origanum vulgare*), clove oil (*Eugenia caryophyllata*), coriander oil (*Coriandrum sativum*) and geranium oil (*Pelargonium graveolens*) were purchased from Frontier Natural Products (Urbana, IA). The essential oil constituents with insecticidal activity; carvacrol, thymol, eugenol, geraniol and linalool were purchased either from Sigma-Aldrich or from Alfa Aesar (Hill, MA). The essential oil-containing commercial product EcoRaider® (active ingredients: geraniol (1%), cedar extract (1%), and sodium lauryl sulfate (2%)) was purchased from Reneotech, Inc. (Bergen, NJ).

4.2.3 Deltamethrin topical application bioassays

For toxicity evaluation, 7–10 days old adult males were used that were fed 3 days before performing bioassays. Topical application bioassays followed methods outlined in Gaire et al. (2019,2020). In brief, deltamethrin was serially diluted in acetone to prepare a range of more than five dilutions (Harlan: 0.001 – 0.1 mg/mL; Knoxville: 1 – 300 mg/mL). Topical applications of different concentrations (volume range 0.5 – 1 µL) were made on the ventral metathorax using a 25 µL micro-syringe attached to a PB-600-1 repeating dispenser (Hamilton, Reno, NV). Control groups were treated with acetone only. After treatment, insects (in groups of 10) were held in 35

x 10 mm Petri dishes (Greiner Bio-One, Frickenhausen, Germany) and placed in an environmental chamber under conditions identical to those used for rearing. Mortality scoring for all treatments was performed 24 h post-treatment. Insects that were lying on their backs and/or were unable to move upon prodding were scored as dead. In total, three replicates were performed for each concentration (n = 30). The deltamethrin dose-mortality data generated for Harlan and Knoxville adult males was subjected to probit analysis in SAS version 9.4 (SAS Institute, Cary, NC) to determine the lethal dose (LD) values (Finey, 1971). The topical lethal dose (LD) values are reported as µg/mg body weight based on the average mass of each strain (Harlan average weight = 5.35 mg per insect; Knoxville average weight = 3.81 mg per insect). Resistance ratios were calculated by dividing the deltamethrin LD_{25} or LD_{50} values for the Knoxville strain by LD_{25} or LD_{50} value for the Harlan strain.

4.2.4 Synergists bioassays

PBO, DEF and DEM are known synergists that inhibit detoxifying enzymes; specifically, cytochrome P450s, esterases, and glutathione transferases, respectively (Bernard and Philogène, 1993). All three synergists were diluted in acetone to prepare a 100 mg/mL concentration (Gonzalez-Morales and Romero 2019). They were topically applied to bed bugs (0.5 µL or 50 µg per insect) following the method described in section 4.2.3. Two hours after synergist or acetone application bed bugs of each strain (Harlan and Knoxville) were topically treated with their respective LD_{25} dose of deltamethrin (0.5 µL volume) (Table 4.1). Control bed bugs that were pre-treated with 0.5 µL of acetone or diluted synergist solution received a second topical treatment (0.5 µL) of acetone to ensure that the application of either two acetone treatments or synergist followed by acetone did not cause mortality. Mortality was scored after 24 h as previously described. Six replicates were performed for each treatment (10 insects per rep, n = 60). Two sample t-tests were performed to compare the effects of synergists on deltamethrin toxicity in both Harlan and Knoxville strains using SPSS Version 25 (Armonk, NY).

4.2.5 Detoxification enzymes assays

4.2.5.1 Protein preparations

Ten adult male bed bugs (10-15 days old) that were starved (i.e., not fed) post-eclosion to adulthood were homogenized in 1 mL ice cold 0.1 M sodium phosphate buffer (pH = 7.0). The homogenization buffer used for measuring cytochrome P450 activity also contained 0.3% Triton (vol./vol. basis) (Adelman et al., 2011; Romero and Anderson, 2016). Next, the insect homogenate was centrifuged at 10,000 rpm for 20 min at 4°C in a 5424 R centrifuge (Eppendorf North America, Hauppauge, NY). Resulting supernatants were used as the enzyme source for measuring detoxification enzyme activity. Bradford assays were performed to measure the protein concentration of each sample using bovine serum albumin (BSA) as a standard (Bradford, 1976). Protein concentration was measured in a PowerWave 340 spectrophotometer (BioTek Instruments Inc., Winooski, VT) at 595 nm.

4.2.5.2 Cytochrome P450 activity

Cytochrome P450-dependent *O*-deethylation activity was determined according to the method described by Anderson and Zhu (2004), Adelman et al. (2011) and Romero and Anderson (2016) with slight modification of emission and excitation wavelengths as per Valles et al. (1994). Assays were conducted in black walled 96-well micro plates (Corning Inc., Corning, NY) to prevent cross talk between wells. 7-ethoxycoumarin (7-EC) was used as the substrate for this assay along with a reaction mixture that included protein obtained from the Harlan or Knoxville strains (40 µL), 7-EC (50 mM), and the co-factor reduced NADPH (β-nicotinamide dinucleotide phosphate; 62.5 mM). For control reactions, protein was replaced by an equal volume of sodium phosphate buffer (Stumpf and Nauen, 2001). Relative fluorescence units were measured using a Spectramax m2e instrument (Molecular Devices, LLC, San Jose, CA) at an emission wavelength of 460 nm and excitation wavelength of 380 nm (Valles et al., 1994). The extinction coefficient for the end product, 7-hydroxycoumarin (4.44 M^{-1} cm^{-1}), was used for calculating specific activity, which was expressed as nmol/min/mg protein (Fang et al., 1997). Four replicates were performed for each strains. A two-sample t-test was used to compare the cytochrome P450 activity between strains.

4.2.5.3 Esterase activity

Esterase activity was measured using *p*-nitrophenyl acetate (*p*NPA) as a substrate following Wu et al. (1998). Initially, the reaction mixture was prepared by adding 50 µL of *p*NPA (0.2 M in acetonitrile) in 10 mL sodium phosphate buffer (pH = 7.0). Assays were conducted in clear 96-well microplates (Corning Inc., Corning, NY). Each treatment well received 10 µL of protein from either Harlan or Knoxville bed bugs and the same volume of sodium phosphate buffer was used in control or blank wells. Reactions were initiated by adding 225 µL of sodium phosphate buffer containing 1 mM *p*NPA to all wells. Immediately thereafter reactions were monitored at a wavelength of 405 nm every 20 sec for 5 minutes in a PowerWave 340 spectrophotometer. The extinction coefficient for the end product *p*-nitrophenol (6.53 mM^{-1} cm^{-1}) was used for calculating specific activity, which was expressed as nmol/min/mg protein (Wu et al., 1998). Six replicates were performed for each strain and, a two sample t-test was used to determine differences in activity between the two strains.

4.2.5.4 Glutathione transferase activity

Glutathione transferase activity was measured using chloronitrobenzene (CDNB) as a substrate following Wu et al. (1998). Assays were conducted in clear 96-well microplates. First, bed bug protein samples (10 µL) were added to both treatment and control wells. Next, two reaction mixtures were freshly prepared in 10 mL sodium phosphate buffer (pH 7.0). The first mixture (reaction mix 1) contained 5 mM reduced glutathione and 1 mM CDNB, whereas the second mix (reaction mix 2) contained everything except the co-factor reduced glutathione. Reaction mix 1 (225 µL) was then added to all treatment wells and reaction mix 2 (225 µL) was added to control wells. Reactions were monitored every 20 secs for 5 minutes at 344 nm wavelength in a PowerWave 340 spectrophotometer. The extinction coefficient of 9.5 mM^{-1} cm^{1} for the end product, *S*-(2,4-dintrophenyl) glutathione, was used for calculating specific activity (nmol/min/mg protein) (Wu et al., 1998). Six replicates were performed for each strain. Statistical differences in enzyme activity between strains were determined by performing a two-sample t-test.

4.2.6 DNA extraction and voltage-gated sodium channel mutation detection

Genomic DNA was extracted from 10 specimens per bed bug strain using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Germantown, MD). DNA was stored at -20°C until use. PCR

amplification of two genomic fragments, previously shown to possess voltage-gated sodium channel (VGSC) associated mutations (Yoon et al., 2008; Dang et al., 2015) was performed using primer combinations BBParaF1/ BBParaR1 (V419L) and BBParaF3/BBParaR3 (L925I, I936F) (Zhu et al., 2010). PCR products were purified using Exo-SAP-IT (Affymetrix Inc., Santa Clara, CA) and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Each fragment was unidirectionally sequenced, as the mutation sites were positioned such that base calling was unambiguous. Primers BBparaF1 sequenced the V419L region, and BBparaR3 sequenced the L925I and I936F regions. Sequencing was performed on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA) and the resulting chromatograms visualized using CLC Genomic Workbench (https://www.qiagenbioinformatics.com). The presence or absence of mutations was scored visually.

Individuals were identified as susceptible or resistant following Yoon et al. (2008) and Dang et al. (2015). Specifically, V419L – GTC = valine, CTC = leucine; L925I – CTT = leucine, ATT = isoleucine; I936F – ATT = isoleucine, TTT = phenylalanine. For each, the former amino acid represents the wild type (susceptible) state and the latter the mutant (resistant) state. Heterozygotes were identified by overlapping peaks at the respective position. Haplotype designations followed the methods of Zhu et al. (2010) and Balvin and Booth (2018).

4.2.7 Assessment of resistance to essential oils, their insecticidal constituents and EcoRaider in the Knoxville strain

Thymol, carvacrol, eugenol, geraniol and linalool were the most toxic essential oil components against the insecticide susceptible Harlan strain in a previous study (Gaire et al., 2019). The bioactivity of these five compounds were tested against the Knoxville strain by conducting topical dose-response bioassays and compared with previous data for the Harlan strain (Gaire et al., 2019). Topical toxicity of five plant essential oils (thyme, oregano, clove, geranium and coriander) was also determined against both Harlan and Knoxville strains by conducting dose-response bioassays. These five oils were selected because they are known to contain high abundance of abovementioned essential oil constituents (https://phytochem.nal.usda.gov/phytochem/search) that were most toxic to the Harlan strain (Gaire et al., 2019). The chemical composition of these five oils were analyzed using gas chromatography–mass spectrometry (GC-MS) according to

Gaire et al. (2017) with slight modification to re-verify that the respective compounds were present in high abundance in oils selected for this study (refer to Table B.1 footnotes for GC-MS methodology details). With GC-MS analysis it was confirmed that thyme, oregano, clove, coriander and geranium contained 45.34% thymol, 56.38% carvacrol, 89.87% eugenol, 66.26% linalool and 15.01% geraniol, respectively (Table B.1). An essential oil product (EcoRaider®) was also evaluated by performing topical dose-response bioassays against both strains. Topical application of essential oils, their major constituents and EcoRaider, and all data analysis methods were conducted as described for deltamethrin topical bioassays in section 4.2.3.

4.3 Results

4.3.1 Deltamethrin resistance levels in the Knoxville strain

The field-collected Knoxville strain was 72,893, and 291,626-fold resistant to topically applied deltamethrin in comparison to the susceptible Harlan strain, at the LD_{25} and LD_{50} levels, respectively (Table 4.1). Due to high level of deltamethrin resistance in the Knoxville strain, mortality achieved with the highest possible dose of deltamethrin was only 30%. Therefore, the probit estimated LD_{25} value and corresponding resistance ratios are relatively more accurate than the LD_{50} value and resistance ratios at 50% mortality level. Bed bug mortality in the control treatments was less than 6% in both strains.

4.3.2 Effects of synergists on deltamethrin toxicity

In both strains, less than 5% mortality was observed following treatment with either acetone or a synergist alone (PBO, DEF and DEM). However, pretreatment with the synergists PBO and DEF significantly increased deltamethrin toxicity (>90% mortality at the LD_{25} dose) in the resistant Knoxville strain in comparison to the deltamethrin-only treatment ($P < 0.01$, two sample t-test) (Fig. 4.1). Pretreatment with DEM did not cause a synergistic increase in toxicity of deltamethrin in the Knoxville strain ($P > 0.05$, two sample t-test) (Fig. 4.1). In the Harlan strain, pretreatment with PBO significantly decreased deltamethrin toxicity ($P < 0.05$, two sample t-test, Fig. 4.1), which represented an antagonistic effect. Neither DEF nor DEM caused a statistically significant change in deltamethrin toxicity in the Harlan strain ($P > 0.05$, two sample t-test) (Fig. 4.1).

4.3.3 Detoxification enzyme activity

Cytochrome P450 activity in the deltamethrin resistant Knoxville strain was significantly higher (~3.9-fold) compared to the susceptible Harlan strain ($P < 0.01$, two sample t-test) (Fig. 4.2). Esterase activity was ~1.5-fold significantly higher in the Knoxville strain than in Harlan ($P < 0.01$, two sample t-test) (Fig. 4.2). Lastly, glutathione transferase activity was also significantly higher (~1.25-fold) in the Knoxville strain relative to the Harlan strain ($P < 0.05$, two sample t-test) (Fig. 4.2).

4.3.4 Pyrethroid resistance-associated mutations in the voltage-gated sodium channel

Unambiguous gene sequences were produced for both amplified fragments of the sodium channel through Sanger sequencing. As expected, analysis of these gene fragments revealed only haplotype A (susceptible at both V419L and L925I *kdr*-mutation sites) in the deltamethrin susceptible Harlan Strain (Table 4.2). In contrast, the deltamethrin resistant Knoxville strain exhibited haplotypes A, B (susceptible at V419L, resistant at L925I), and individuals heterozygous at the L925I mutation site (Table 4.2). In both populations, all samples were susceptible for the I936F mutation (Table 4.2).

4.3.5 Resistance to essential oils, EcoRaider, and major constituents of essential oils in the deltamethrin-resistant Knoxville strain

Treatment of the deltamethrin resistant Knoxville strain with five different plant essential oils and EcoRaider revealed no evidence of resistance in comparison to the susceptible Harlan strain at the LD_{25} and LD_{50} levels (resistance ratio between 0.34 to 1.37) (Table 4.3). In the Knoxville strain, thyme, oregano and clove oils were most toxic, followed by coriander, geranium and EcoRaider (Table B.2). In the Harlan strain, thyme and oregano oils were equally active followed by coriander, clove, EcoRaider and geranium (Table B.2). With respect to essential oil constituents, carvacrol, eugenol, and thymol were equally active against the Knoxville strain, followed by geraniol and linalool (Table B.3). Further comparison of the essential oil constituent LD_{25} and LD_{50} estimate data for the Knoxville strain with previously determined LD_{25} and LD_{50} values for the Harlan strain (Gaire et al., 2019) revealed resistance ratios close to 1, except for linalool

(resistance ratios of 3.1 and 2.5 at the LD_{25} and LD_{50} levels, respectively) (Table 4.4). The mortality in the control group (acetone-treated) was less than 6%.

4.4 Discussion

This study revealed the presence of high-level deltamethrin resistance, influenced by both metabolic and target-site mechanisms, in the field-collected Knoxville strain. Further, this strain exhibited susceptibility to various plant essential oils, their major insecticidal components, and the essential oil-based product EcoRaider. In the following subsections, our findings on the magnitude of deltamethrin resistance and its mechanisms in the Knoxville strain are discussed. Additionally, we discuss potential factors that may have led to essential oil susceptibility in deltamethrin resistant bed bugs.

4.4.1 Deltamethrin resistance in the Knoxville strain

Pyrethroid resistance is reported as a primary reason for the resurgence of bed bugs in the early 2000's (Myamba et al., 2002; Boase et al, 2006; Moore and Miller, 2006; Romero et al., 2007). Since then, several studies have reported field-collected strains of bed bugs exhibiting pyrethroid resistance (Romero, 2018). In the present study, the field-collected Knoxville strain was 72,000 and 290,000 fold resistant to topically-applied deltamethrin at LD_{25} and LD_{50} levels, respectively, which is extremely high when compared to previous studies with bed bugs or *C. lectularius*. Previous studies have reported a 5,000–20,000 fold deltamethrin resistance ratios at the LD_{50} or LC_{50} level, in various field populations of the bed bugs collected from the United States (Romero et al., 2007; Adelman et al., 2011; Gonzalez-Morales and Romero, 2018). The Knoxville strain used in our study also has cross-resistance to another pyrethroid class insecticide, bifenthrin, and the pyrrole class insecticide chlorfenapyr (Ashbrook et al., 2017). Studies have shown that bed bugs possess multiple mechanisms that allow them to resist the insecticidal affects of deltamethrin and other pyrethroid class insecticides. Because the Knoxville strain was highly resistant to deltamethrin, we further determined metabolic and target site-based resistance mechanisms in this strain.

4.4.2 Mechanisms of deltamethrin resistance

The use of synergists such as PBO, DEF and DEM allows initial identification of the possible role of detoxification enzymes in bed bug insecticide resistance (Romero et al., 2009; Lilly et al., 2016a; Gonzalez-Morales and Romero, 2018). These synergists (PBO, DEF and DEM) are known to inhibit detoxifying enzymes that respectively include cytochrome P450s, esterases and glutathione transferases (Bernard and Philogène, 1993). We found that pre-application of PBO and DEF significantly increased deltamethrin toxicity in the Knoxville strain at the LD_{25} level, indicating the involvement of cytochrome P450s and esterases in resistance. To further confirm the role of detoxification enzymes in deltamethrin resistance we measured the activity of cytochrome P450s, esterases, and glutathione transferases in both bed bug strains. The Knoxville strain showed significantly higher activity of all three enzymes when compared to the Harlan strain. Cytochrome P450 and esterase enzyme activities corroborated with the synergist bioassay data. However, the results for glutathione transferase were contrasting, wherein synergist bioassays with DEM did not indicate the role of glutathione transferase in resistance, but enzyme assays showed that CDNB-conjugation glutathione transferase activity was higher in the field-collected Knoxville strain. Since glutathione transferase enzymes are phase II enzymes that act on xenobiotics that are modified by Phase I enzymes (Yu, 2015), their contribution to the overall resistant phenotype could be minor and hence we did not see significant synergism or toxicity effects in synergist bioassays with DEM.

The role of three detoxification enzymes has been demonstrated in insecticide resistant strains of *C. lectularius* and *C. hemipterus*, in addition to several other insect pest species (Romero et al., 2009; Lilly et al., 2016a; Karunaratne et al., 2007; Adelman et al., 2011; Romero and Anderson, 2016). However, in comparison to previous studies, the 7-EC deethylation cytochrome P450 activity was very high (390% or 3.9-fold higher than the susceptible Harlan strain) in the Knoxville strain. Two previous studies with *C. lectularius* reported 20–40% increases in 7-EC deethylation cytochrome P450 activity in resistant strains (Adelman et al., 2011; Romero and Anderson, 2016). In a strain of resistant red flour beetles, a brain-specific cytochrome P450 (*CYP6BQ9*), which showed 200-fold higher gene expression in microarray experiments, was responsible for metabolizing deltamethrin into 4-hydroxy deltamethrin (Zhu et al., 2010b). The Knoxville bed bug strain may possess a similar cytochrome P450-based resistance mechanism that allows it to detoxify deltamethrin to a more polar and relatively less-toxic metabolite. Gene knockdown (RNA

interference) experiments conducted with bed bugs or *C. lectularius* showed that four cytochrome P450's (CYP397A1, CYP398A1, CYP6DN1 and CYP4CM1), three cuticular proteins (c2, c10 and c13), and two ABC transporters (ABC8 and ABC9) were responsible for pyrethroid (beta-cyfluthrin) resistance (Zhu et al., 2013). Additionally, cuticular thickening has been linked to pyrethroid resistance in the *C. hemipterus* (Lilly et al., 2016b).

Although metabolic enzymes and reduced penetration mechanisms may play important roles in deltamethrin resistance, mutations in the voltage-gated sodium channel are also known to impart resistance to pyrethroid insecticides, including deltamethrin (Yoon et al., 2008; Dang et al., 2015). These *kdr*-associated mutations reduce or eliminate the ability of pyrethroid insecticides to disrupt sodium channel function (Davis et al., 2007; Dong et al., 2014). In our study, we found that the Knoxville strain population is composed of mixed haplotype A and B bed bugs, where A refers to wild type homozygotes, and hence susceptible to pyrethroids, and B refers to bed bugs possessing the L925I mutation either in the homozygous or heterozygous state (Zhu et al., 2010). No evidence for the presence of two other mutations (V419L and I936F) was found in the Knoxville strain. Haplotype A individuals comprised 10% of the screened Knoxville strain samples, 30% were heterozygous at L925I, and 60% were homozygous haplotype B. These results implicate the potential role of *kdr* mutations in deltamethrin resistance observed in the Knoxville strain. Previous studies have found the L925I mutation to be present in between 78%–100% of infestations that were screened (Dang et al., 2015; Palenchar et al., 2015; Durand et al., 2012; Booth et al., 2015; Balvin and Booth, 2018). The presence of haplotype A and B bed bugs within the Knoxville population likely resulted from the absence of insecticide exposure in the lab colony for the past 6–7 years (>30 generations). If *kdr*-associated mutations impart a fitness cost, removal of this selective pressure may facilitate the reappearance of the susceptible genotypes within the population.

4.4.3 Susceptibility of the Knoxville strain to plant essential oils and their active constituents

Plant essential oils contain complex mixtures of constituents and their composition may differ based on various factors, including the phenological state of the plant during sampling, plant part used for extraction, harvesting time, climatic and soil conditions, water level, and the presence of distinct chemotypic races of populations (Regnault-Roger et al., 2012). Therefore, we performed

GC–MS analysis of oils used in this study and confirmed the presence of major insecticidal constituents e.g., thymol, carvacrol, eugenol etc., in higher relative abundance (Table B.1). Bioassay findings revealed that the Knoxville strain, which possesses both metabolic and target site resistance mechanisms, was susceptible to all five essential oils (thyme, oregano, clove, coriander, geranium), and a commercially available natural product formulation (EcoRaider). Similarly, the Knoxville strain was also susceptible to individual compounds *viz.,* carvacrol, eugenol, thymol and geraniol, with slight tolerance to Linalool. However, it should be noted that the Knoxville strain was not resistant (resistance ratio = 0.98) to coriander oil that contains 66% linalool. Despite some exceptions, our results indicate that plant essential oils and their constituents are effective against bed bugs that possess metabolic and target site-based deltamethrin resistance.

We did not investigate the mechanisms responsible for essential oil susceptibility in the deltamethrin-resistant Knoxville strain. However, we predict that differences in target sites of deltamethrin and major plant essential oil constituents (e.g., carvacrol, eugenol and thymol) are partially responsible for susceptibility of the Knoxville strain, as observed in this study. In this regard, carvacrol, eugenol and thymol are known to act on the nicotinic acetylcholine, octopamine and gamma amino butyric acid receptors, respectively (Enan, 2001; Tong et al., 2013; Priestley et al. 2013). In recent years, plant essential oils have been shown to synergize toxicity of pyrethroid insecticides in resistant mosquitoes and tobacco cutworm by inhibiting detoxification enzymes (Norris et al., 2018; O'Neal et al. 2019; Ruttanaphan et al., 2019). Thus, future studies should consider the ability of plant essential oils to synergize deltamethrin toxicity in the resistant Knoxville bed bugs.

4.5 Summary and Conclusions:

This study revealed a high level of deltamethrin resistance in the Knoxville strain of the common bed bug. Synergist bioassays and enzyme assays support the involvement of detoxification enzymes in deltamethrin resistance. Additionally, DNA sequencing revealed the L925I *kdr*-associated mutation in the voltage-gated sodium channel gene as another deltamethrin resistance mechanism in the Knoxville strain. However, the Knoxville strain was completely susceptible to plant essential oils, their constituents, and an essential oil-based product. Overall, this study confirms that essential oils can be effective alternatives for the control of deltamethrin and/or pyrethroid resistant bed bugs. Nonetheless, other issues such as odor associated with the

use of essential oils and their short residual efficacy need to be addressed while formulating natural product insecticides for bed bug management.

Table 4.1 Resistance to deltamethrin in the field-collected Knoxville strain.

Strains	**N**	**LD_{25}[I], µg/mg body weight (FL95%)[II]**	**LD_{50}[I], µg/mg body weight (FL95%)[II]**	**Slope ± SE**	**χ^2**	**Df**	***P* value**	**LD_{25} resistance ratio[III]**	**LD_{50} resistance ratio[III]**
Harlan	270	5.36×10^{-5} ($2.24 \times 10^{-5} – 8.41 \times 10^{-5}$)	9.90×10^{-5} ($5.79 \times 10^{-5} – 1.72 \times 10^{-4}$)	2.51 ± 0.51	20.73	6	0.002	-	-
Knoxville	180	3.91 (ND)[IV]	288.71 (ND)[IV]	0.36 ± 0.20	2.383	3	0.496	72,893	291,626

[I]LD_{25} and LD_{50} = lethal dose necessary to kill 25% and 50% of individuals, respectively. [II]FL = Fiducial limits.
[III]Resistance ratio was calculated by dividing the LD_{25} or LD_{50} value of deltamethrin for Knoxville by LD_{25} or LD_{50} value of deltamethrin for Harlan. LD_{25} resistance ratio is relatively more accurate than the LD_{50} resistance ratio in this case because >30% mortality was never observed in the Knoxville strain even when using the highest possible dose of deltamethrin.
[IV]The acronym ND in parenthesis next to the deltamethrin LD_{25} and LD_{50} explains that 95% FLs were “not determinable”.

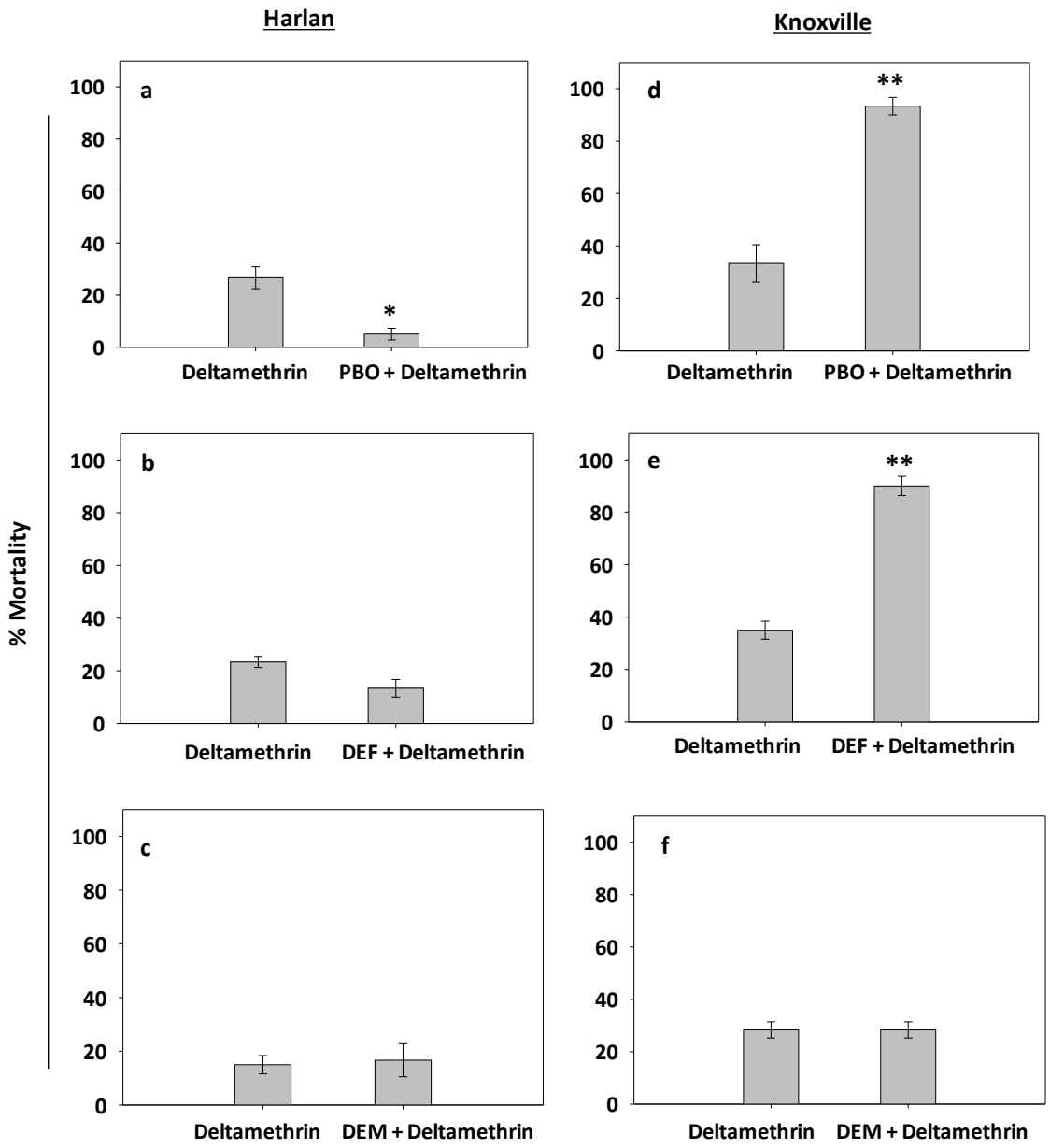


Figure 4.1 Effects of synergist on deltamethrin toxicity at respective LD25 doses for the Harlan and Knoxville strains. (a-c) There was an antagonistic effect of PBO pretreatment on deltamethrin toxicity in the Harlan strain. However, DEF and DEM pre-application did not cause significant change in deltamethrin toxicity. (d-f) PBO and DEF significantly increased toxicity of deltamethrin in the resistant Knoxville strain, however, the effect of DEM was non-significant. Double asterisk (**) indicates statistical significance at $P < 0.01$ and single asterisk (*) indicates significance at $P < 0.05$ (two sample t-test).

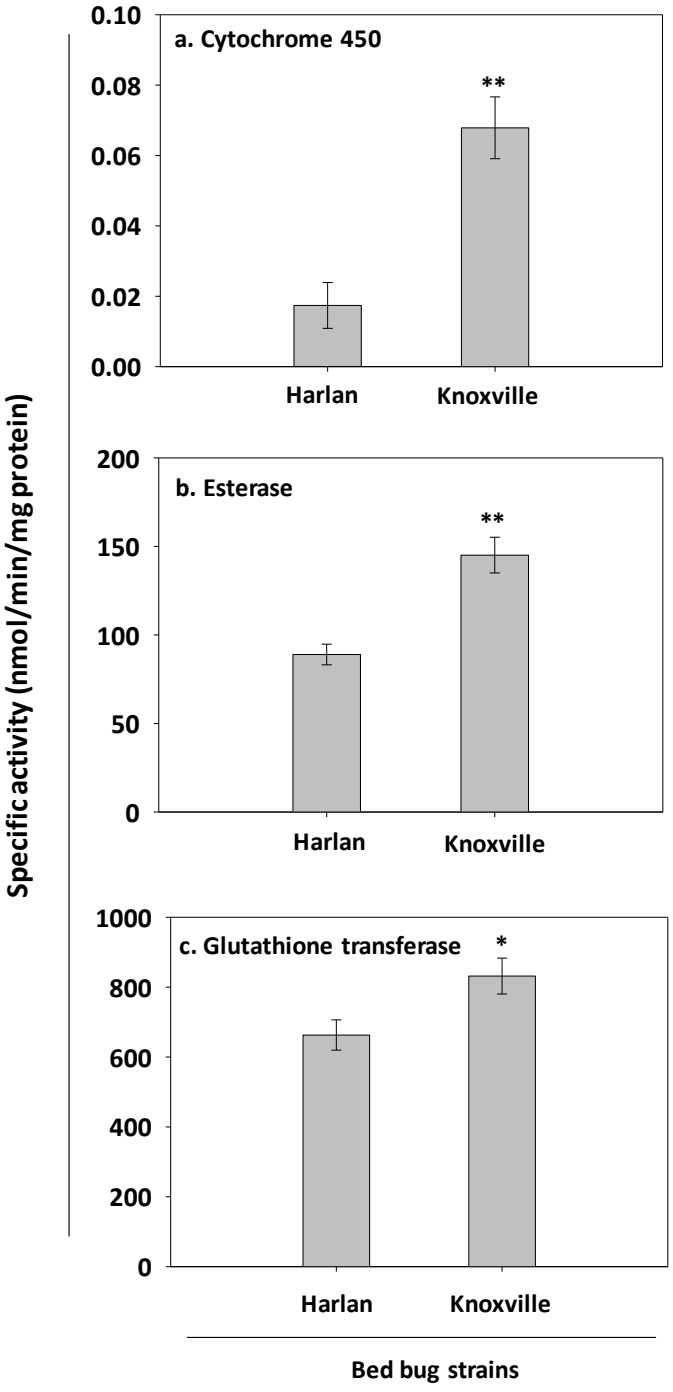


Figure 4.2 Detoxifying enzyme activities of the susceptible (Harlan) and resistant (Knoxville) strains. Significantly higher activities of all three detoxifying enzymes (cytochrome P450 (a), esterase (b) and glutathione transferase (c)) were observed in the deltamethrin resistant Knoxville strain in comparison to the susceptible Harlan strain. Double asterisk (**) indicates significance at $P < 0.01$ and single asterisk (*) indicates significance at $P < 0.05$ (two sample t-test).

Table 4.2 Frequency of kdr mutations across three previously identified mutation sites for the Harlan susceptible and Knoxville resistant strains.

Mutation sites	**Genotype[I]**	**Harlan**	**Knoxville**
V419L (Valine to Leucine at amino acid position 419)	SS (V419/V419)	100%	100%
	RS (L419/V419)	-	-
	RR (L419/L419)	-	-
L925I (Leucine to Isoleucine at amino acid position 925)	SS (L925/L925)	100%	10%
	RS (I925/L925)	-	30%
	RR (I925/I925)	-	60%
I936F (Isoleucine to Phenylalanine at position 936)	SS (I936/I936)	100%	100%
	RS (F936/I936)	-	-
	RR (F936/F936)	-	-

[I]SS indicates susceptible homozygotes, RS refers to resistant heterozygotes and RR refers to resistant homozygotes.

Table 4.3 Status of resistance to plant essential oils and EcoRaider in the deltamethrin resistant Knoxville strain.

Essential oils	**Strains**	**N**	**LD_{25}[I], µg/mg body weight (FL95%)[II]**	**LD_{50}[I], µg/mg body weight (FL95%)[II]**	**Slope ± SE**	**χ^2**	**Df**	***P* value**	**R. Ratio $_{25}$ [III]**	**R. Ratio $_{50}$[III]**
Thyme oil	Harlan	270	22.80 (20 – 25.42)	30.84 (27.85 – 34.20)	2.26 ± 0.25	5.56	6	0.474	-	-
	Knoxville	270	21.78 (14.43 – 27.55)	33.07 (25.98 – 42.25)	3.70 ± 0.62	12.34	6	0.054	0.955	1.07
Oregano oil	Harlan	240	19.25 (6.72 – 27.47)	30.65 (19.06 – 45.79)	3.33 ± 0.81	18.01	5	0.002	-	-
	Knoxville	210	26.50 (22.30 – 29.92)	35.17 (31.49 – 39.10)	5.52 ± 0.74	2.05	4	0.725	1.37	1.14
Clove bud oil	Harlan	270	37.94 (26.54 – 48.59)	98.31 (74.95 – 155.32)	1.63 ± 0.30	7.97	6	0.239	-	
	Knoxville	210	23.62 (18.63 – 27.82)	34.12 (29.39 – 38.84)	4.23 ± 0.55	3.36	4	0.498	0.622	0.34
Coriander oil	Harlan	240	55.70 (27.85 – 77)	86.91 (60.74 – 137.38)	3.51 ± 0.80	16.88	5	0.004	-	
	Knoxville	240	64.56 (29.65 – 84.25)	85.82 (56.95 – 113.64)	5.49 ± 1.35	20.80	5	0.001	1.15	0.98
Geranium oil	Harlan	270	79.25 (65.04 – 95.51)	150.65 (121.3 – 213)	2.42 ± 0.39	1.71	6	0.944	-	
	Knoxville	270	52.23 (42.25 – 60.89)	87.92 (76.37 – 102.88)	2.99 ± 0.39	5.28	6	0.507	0.659	0.58
EcoRaider	Harlan	270	63.36 (27.10 – 93.0)	104.11 (69.9 – 216.8)	3.14 ± 0.82	18.91	5	0.002	-	
	Knoxville	270	69.55 (47.50 – 88.97)	121.25 (94.7 – 166.6)	2.79 ± 0.46	13.11	7	0.069	1.09	1.16

[I]LD_{25} and LD_{25}= lethal dose necessary to kill 25% and 50% of individuals respectively.
[II]FL = Fiducial limits.
[III]Resistance ratio (R. ratio) was calculated by dividing the LD_{25} or LD_{50} value of essential oils or EcoRaider for Knoxville by LD_{25} or LD_{50} value of essential oils or EcoRaider for Harlan.

Table 4.4 Status of resistance to essential oil components in the Knoxville strain.

Essential oil components	Strain	N	LD_{25}[I], µg/ mg body weight (FL 95%) [II]	LD_{25}[I], µg/ mg body weight (FL 95%) [II]	Slope ± SE	χ^2	Df	P value	R. ratio$_{25}$ [III]	R. ratio$_{50}$[III]
Carvacrol	Harlan[IV]		8.06	13.75					-	-
	Knoxville	240	8.31 (6.48 – 9.50)	11.62 (10.28 – 12.90)	4.63 ± 0.57	8.85	5	0.115	1.03	0.84
Thymol	Harlan[IV]		9.93	16.25					-	-
	Knoxville	240	15.21 (13.71 – 16.46)	18.85 (17.49 – 20.36)	7.21 ± 0.89	4.31	5	0.504	1.53	1.16
Eugenol	Harlan[IV]		14.41	26					-	-
	Knoxville	270	11.69 (9.86 – 13.29)	16.67 (14.82 – 18.57)	4.37 ± 0.46	9.78	6	0.134	0.81	0.64
Geraniol	Harlan[IV]		16.45	32					-	-
	Knoxville	210	27.84 (23.88 – 31.08)	35.33 (31.71 – 39.13)	6.52 ± 0.90	3.26	3	0.353	1.69	1.10
Linalool	Harlan[IV]		28.91	56					-	-
	Knoxville	300	92.32 (79.30 – 111.90)	141.92 (115.58 – 271.34)	3.61 ± 1.04	1.91	4	0.751	3.19	2.51

[I]LD_{25} and LD_{50} = lethal dose necessary to kill 25% and 50% of individuals within a population.
[II]FL = Fiducial limits.
[III]Resistance ratio (R. ratio) was calculated by dividing the LD_{25} or LD_{50} value of essential oil constituents in Knoxville by LD_{25} or LD_{50} value of essential oil constituents for Harlan.
[IV]Harlan strain LD_{25} and LD_{50} values for essential oil components reported in this table are adopted from a previous study (Gaire et al., 2019) and converted as per the weight of bed bug mentioned in section 4.2.3.

4.6 References

Adelman, Z.N., Kilcullen, K.A., Koganemaru, R., Anderson, M.A.E., Anderson, T.D., Miller, D.M., 2011. Deep sequencing of pyrethroid-resistant bed bugs reveals multiple mechanisms of resistance within a single population. PLoS ONE 6, 10, e26228. https://doi.org/10.1371/journal.pone.0026228

Anderson, T.D., Zhu, K.Y., 2004. Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera: Chironomidae). Pestic. Biochem. Phys. 80, 54–64.

Ashbrook, A.R., Scharf, M.E., Bennett, G.W., Gondhalekar, A.D., 2017. Detection of reduced susceptibility to chlorfenapyr- and bifenthrin-containing products in field populations of the bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 110, 1195–1202.

Balvín, O., Booth, W., 2018. Distribution and frequency of pyrethroid resistance-associated mutations in host lineages of the bed bug (Hemiptera: Cimicidae) across Europe. J. Med. Entomol. 55, 923–928.

Bernard, B.C., Philogene, B.J.R., 1993. Insecticide synergists: role, importance, and perspectives. J. Toxicol. Env. Health 38, 199–233.

Boase, C.J., Small, G. and Naylor, R. (2006) Interim report on insecticide susceptibility status of UK bedbugs. Pro. Pest Cntrl. Summer, 6 − 7.

Booth, W., Balvín, O., Vargo, E.L., Vilímová, J., Schal, C., 2015. Host association drives genetic divergence in the bed bug, *Cimex lectularius*. Mol. Ecol. 24, 980–992.

Booth, W., Schal, C., Vargo, E. L., 2018. Population Genetics, in: Doggett, S.L., Miller, D.M., Lee, C.Y. (Eds.), Advances in the Biology and Management of Modern Bed Bugs ed. by Wiley Blackwell, West Sussex, UK, pp. 173–182.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chin-Heady, E., Demark, J.J., Nolting, S., Bennett, G., Saltzmann, K., Hamm, R.L., 2013. A quantitative analysis of a modified feeding method for rearing *Cimex lectularius* (Hemiptera: Cimicidae) in the laboratory. Pest Manag. Sci. 69, 1115–1120.

Dang, K., Toi, C.S., Lilly, D.G., Lee, C.Y., Naylor, R., Tawatsin, A., Thavara, U., Bu, W., Doggett, S.L., 2015. Identification of putative kdr mutations in the tropical bed bug, *Cimex hemipterus* (Hemiptera: Cimicidae). Pest Manag. Sci. 71, 1015–1020.

Davies, T.G.E., Field, L.M., Usherwood, P.N.R., Williamson, M.S., 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. IUBMB Life 59, 151–162.

Doggett, S.L., Miller, D.M., Lee, C.Y., 2018. Advances in the Biology and Management of Modern Bed Bugs. Wiley Blackwell, West Sussex, UK.

Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K., Zhorov, B.S., 2014. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem. Molec. 50, 1–17.

Durand R, Cannet A, Berdjane Z, Bruel C, Haouchine D, Delaunay P, Izri A, 2012. Infestation by pyrethroids resistant bed bugs in the suburb of Paris, France. Parasite 19, 381–387.

Enan, E., 2001. Insecticidal activity of essential oils: octopaminergic sites of action, Comp. Biochem. Phys. C 130, 325–337.

Fang, X., Kobayashi, Y., Halpert, J.R., 1997. Stoichiometry of 7-ethoxycoumarin metabolism by cytochrome P450 2B1 wild-type and five active-site mutants. FEBS Letters 416, 77–80.

Feldlaufer, M.F., Ulrich, K.R., 2015. Essential oils as fumigants for bed bugs (Hemiptera: Cimicidae). J. Entomol. Sci. 50, 129–137.

Finney, D.J., 1971. Probit Analysis. Cambridge University Press, Cambridge, United Kingdom.

Gaire, S., O'Connell, M., Holguin, F.O., Amatya, A., Bundy, S., Romero, A., 2017. Insecticidal properties of essential oils and some of their constituents on the Turkestan cockroach (Blattodea: Blattidae). J. Econ. Entomol. 110, 584–592.

Gaire, S., Scharf, M.E., Gondhalekar, A.D., 2019. Toxicity and neurophysiological impacts of plant essential oil components on bed bugs (Cimicidae: Hemiptera). Scientific Reports 9, 3961. https://doi.org/10.1038/s41598-019-40275-5

Gaire, S., Scharf, M.E., Gondhalekar, A.D., 2020. Synergistic toxicity interactions between plant essential oil components against the common bed bug (*Cimex lectularius* L.). Insects 11, 133. https://doi.org/10.3390/insects11020133

Gonzalez-Morales, M.A., Romero, A., 2019. Effect of synergists on deltamethrin resistance in the common bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 112, 786–791.

Guenther, E., 1949. The essential oils. Vol. II. The constituents of essential oils. D. Van Nostrand Company, Inc. New York. NY.

Holleman, J.G., Robison, G.A., Bellovich, I.J., Booth, W., 2019. Knockdown resistance-associated mutations dominate populations of the common bed bug (Hemiptera: Cimicidae) across the south central United States. J. Med. Entomol. 56, 1678–1683.

Isman, M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 51, 45–66.

Jones, S.C., Bryant, J.L., Sivakoff, F.S., 2015. Sublethal effects of ActiveGuard exposure on feeding behavior and fecundity of the bed bug (Hemiptera: Cimicidae). J. Med. Entomol. 52, 413–418.

Karunaratne, S.H.P.P., Damayanthi, B.T., Fareena, M.H.J., Imbuldeniya, V., Hemingway, J., 2007. Insecticide resistance in the tropical bedbug *Cimex hemipterus*. Pestic. Biochem. Phys. 88, 102–107.

Kells, S.A., Goblirsch, M.J., 2011. Temperature and time requirements for controlling bed bugs (*Cimex lectularius*) under commercial heat treatment conditions. Insects 2, 412–422. https://doi.org/10.3390/insects2030412

Lehnert, M.P., Pereira, R.M., Koehler, P.G., Walker, W., Lehnert, M.S., 2011. Control of *Cimex lectularius* using heat combined with dichlorvos resin strips. Med. Vet. Entomol. 25, 460–464.

Lilly, D.G., Dang, K., Webb, C.E., Doggett, S.L., 2016a. Evidence for metabolic pyrethroid resistance in the common bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 109, 1364–1368.

Lilly, D.G., Latham, S.L., Webb, C.E., Doggett, S.L., 2016b. Cuticle thickening in a pyrethroid-resistant strain of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae). PLoS ONE 11, e0153302. https://doi.org/10.1371/journal.pone.0153302

Lilly, D.G., Zalucki, M.P., Orton, C.J., Russell, R.C., Webb, C.E., Doggett, S.L., 2015. Confirmation of insecticide resistance in *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae) in Australia. Austral. Entomol. 54, 96–99.

Moore, D.J., Miller, D.M., 2006. Laboratory evaluations of insecticide product efficacy for control of *Cimex lectularius*. J. Econ. Entomol. 99, 2080–2086.

Myamba, J., Maxwell, C.A., Asidi, A., Curtis, C.F., 2002. Pyrethroid resistance in tropical bedbugs, *Cimex hemipterus*, associated with use of treated bednets. Med. Vet. Entomol. 16, 448–451.

Norris, E.J., Johnson, J.B., Gross, A.D., Bartholomay, L.C., Coats, J.R., 2018. Plant essential oils enhance diverse pyrethroids against multiple strains of mosquitoes and inhibit detoxification enzyme processes. Insects 9, 132. https://doi.org/10.3390/insects9040132

O'Neal, S.T., Johnson, E.J., Rault, L.C., Anderson, T.D., 2019. Vapor delivery of plant essential oils alters pyrethroid efficacy and detoxification enzyme activity in mosquitoes. Pestic. Biochem. Phys. 157, 88–98.

Olson, J.F., Eaton, M., Kells, S.A., Morin, V., Wang, C., 2014. Cold tolerance of bed bugs and practical recommendations for control. J. Econ. Entomol. 106, 2433–2441.

Palenchar, D.J., Gellatly, K.J., Yoon, K.S., Mumcuoglu, K.Y., Shalom, U., Clark, J.M., 2015. Quantitative sequencing for the determination of *kdr*-type resistance allele (V419L, L925I, I936F) frequencies in common bed bug (Hemiptera: Cimicidae) populations collected from Israel. J. Med. Entomol. 52, 1018–1027.

Phillips, T.W., Aikins, M.J., Thoms, E., Demark, J., Wang, C., 2014. Fumigation of bed bugs (Hemiptera: Cimicidae): effective application rates for sulfuryl fluoride. J. Econ. Entomol. 107, 1582–1589.

Priestley, C.M., Williamson, E.M., Wafford, K.A., Sattelle, D.B., 2003. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human $GABA_A$ receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. Brit. J. Pharmacol. 140, 1363–1372.

Puckett, R.T., Mcdonald, D.L., Gold, R.E., 2013. Comparison of multiple steam treatment durations for control of bed bugs (*Cimex lectularius* L.). Pest Manag. Sci. 69, 1061–1065.

Regnault-Roger, C., Vincent, C., Arnason, J.T., 2012. Essential oils in insect control: low-risk products in a high-stakes world. Annu. Rev. Entomol. 57, 405–424.

Robertson, J.L., Russell, R.M. Preisler, H.K., Savin, N.E.,2007. Bioassays with arthropods, 2nd Ed., CRC Press, Boca Raton, FL.

Romero, A., 2018. Insecticide resistance, in: Doggett, S.L., Miller, D.M., Lee, C.Y. (Eds.), Advances in the Biology and Management of Modern Bed Bugs ed. by Wiley Blackwell, West Sussex, UK, pp. 273–284.

Romero, A., Anderson, T.D., 2016. High levels of resistance in the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), to neonicotinoid insecticides. J. Medical Entomology 53, 727–731.

Romero, A., Potter, M.F., Haynes, K.F., 2009. Are dusts the magic bullet? Pest Manag. Prof. 77, 22–30.

Romero, A., Potter, M.F., Haynes, K.F., 2009. Evaluation of piperonyl butoxide as a deltamethrin synergist for pyrethroid-resistant bed bugs. J. Econ. Entomol. 102, 2310–2315.

Romero, A., Potter, M.F., Potter, D.A., Haynes, K.F., 2007. Insecticide Resistance in the bed bug: a factor in the pest's sudden resurgence? J. Med. Entomol. 44, 175–178.

Ruttanaphan, T., Pluempanupat, W., Aungsirisawat, C., Boonyarit, P., Goff, G. le, Bullangpoti, V., Isman, M., 2019. Effect of plant essential oils and their major constituents on cypermethrin tolerance associated detoxification enzyme activities in *Spodoptera litura* (Lepidoptera: Noctuidae). J. Econ. Entomol. 112, 2167–2176.

Singh, N., Wang, C., Cooper, R., 2013. Effect of trap design, chemical lure, carbon dioxide release rate, and source of carbon dioxide on efficacy of bed bug monitors. J. Econ. Entomol. 106, 1802–1811.

Singh, N., Wang, C., Cooper, R., 2014. Potential of essential oil-based pesticides and detergents for bed bug control. J. Econ. Entomol. 107, 2163–2170.

Stumpf, N., Nauen, R., 2001. Cross-resistance, inheritance, and biochemistry of mitochondrial electron transport inhibitor-acaricide resistance in *Tetranychus urticae* (Acari: Tetranychidae). J. Econ. Entomol. 94, 1577–1583.

Tong, F., Gross, A.D., Dolan, M.C., Coats, J.R., 2013. The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor. Pest Manag. Sci. 69, 775–780.

Valles, S.M., Yu, S.J., 1996. Insecticide resistance and resistance management detection and biochemical characterization of insecticide resistance in the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. 89, 21–26.

Wang, C., Singh, N., Cooper, R., 2014. Efficacy of an essential oil-based pesticide for controlling bed bug (*Cimex lectularius*) infestations in apartment buildings. Insects 5, 849–859. https://doi.org/10.3390/insects5040849

Wang, D., Wang, C., Wang, G., Zha, C., Eiden, A.L., Cooper, R., 2018. Efficacy of three different steamers for control of bed bugs (*Cimex lectularius* L.). Pest Manag. Sci. 74, 2030–2037.

Wu, D., Scharf, M.E., Neal, J.J., Suiter, D.R., Bennett, G.W., 1998. Mechanisms of fenvalerate resistance in the German cockroach, *Blattella germanica* (L.), Pestic. Biochem. Phys. 61, 53–62.

Yoon, K.S, Kwon, D.H., Strycharz, J.P., Hollingsworth, C.S., Lee, S.H, Clark, J.M., 2008. Biochemical and molecular analysis of deltamethrin resistance in the common bed bug (Hemiptera: Cimicidae). J. Med. Entomol. 45, 1092–1101.

Yu, S. J., 2015. The Toxicology and Biochemistry of Insecticides. CRS Press, Taylor & Francis Group, Bacon Raton, FL.

Zha, C., Wang, C., Li, A., 2016. Toxicities of selected essential oils, silicone oils, and paraffin oil against the common bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 111, 170–177.

Zhu, F., Gujar, H., Gordon, J.R., Haynes, K.F., Potter, M.F., Palli, S.R., 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Sci. Rep. 3, 1456. https://doi.org/10.1038/srep01456

Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M., Nauen, R., Harrison, D.A., Palli, S.R., 2010b. A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. P. Natl. Acad. Sci. USA 107, 8557–8562.

Zhu, F., Wigginton, J., Romero, A., Moore, A., Ferguson, K., Palli, R., Potter, M.F., Haynes, K.F., Palli, S.R., 2010a. Widespread distribution of knockdown resistance mutations in the bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), populations in the United States. Arch. Insect Biochem. 73, 245–257.

CHAPTER 5. PLANT ESSENTIAL OILS SYNERGIZE DELTAMETHRIN TOXICITY IN A RESISTANT STRAIN OF THE BED BUG (*CIMEX LECTULARIUS* L.) BY INHIBITING CYTOCHROME P450 ENZYMES

5.1 Introduction

Bed bugs (*Cimex lectularius* L.) are economically and medically detrimental urban pests (Doggett et al., 2018). Insecticides are one of the primary tools for the management of this pest (Lee et al., 2018). However, prolonged use of pesticides can cause bed bugs to develop resistance to multiple classes of insecticides including pyrethroids, neonicotinoids, and pyrroles (Romero et al., 2007; Adelman et al., 2011, Zhu et al., 2013; Romero and Anderson, 2016; Ashbrook et al., 2017; Caceres et al., 2019). Bed bug resistance to the type II pyrethroid insecticide, deltamethrin, has been extensively investigated (Romero, 2018). Similar to other pyrethroids, deltamethrin modifies the gating characteristics of voltage-sensitive sodium channels and leads to a delay in their closure (Dong et al., 2014). Target site mutations (i.e. knockdown resistance or *kdr*), elevation in the activity of detoxification enzymes such as cytochrome P450s, esterases and glutathione transferases, and reduced cuticular penetration have been reported as major mechanisms underlying the resistance of deltamethrin and pyrethroid in bed bugs (Adelman et al., 2011; Zhu et al., 2013; Koganemaru et al., 2013; Lilly et al., 2016a; Gonzalez-Morales and Romero, 2019).

Synergists are compounds that inhibit detoxification enzymes (e.g., cytochrome P450s, esterases and glutathione transferases) and thereby in some cases increase the toxicity of insecticides in several insect pest species (Bernard and Philogene, 1993). Synergists are commonly used to determine the involvement of detoxification enzymes in resistance (Bernard and Philogene, 1993). Laboratory studies have shown that synthetic synergists such as piperonyl butoxide (PBO), diethyl maleate (DEM), *S*,*S*,*S*-tributyl phosphorotrithioate (DEF), and triphenyl phosphate (TPP) can synergize deltamethrin toxicity in resistant bed bug populations (Romero et al., 2009; Lilly et al., 2016a; Gonzalez-Morales and Romero, 2019). However, only PBO and MGK-264 (N-(2-Ethylhexyl)-5-norbornene-2,3-dicarboximide) are commercially used as synergists for enhancing efficacy of pyrethroid insecticides against resistant bed bugs and other insect pests (Lee et al., 2018). These synergists (PBO and MGK-264) increase insecticide toxicity by inhibiting cytochrome P450 enzymes associated with metabolic resistance. In addition to these traditionally

known synergists, recent research has shown that various plant essential oils and their constituents are capable of synergizing pyrethroid (deltamethrin or permethrin or cypermethrin) toxicity against insecticide resistant and susceptible mosquitoes, tobacco cutworm, house flies, blow flies and fall armyworm (Gross et al., 2017; Silva et al., 2017; Changsang et al., 2018; Norris et al., 2018; O'Neal et al., 2019; Ruttanaphan et al., 2019; Suwannayod et al., 2019). Additionally, it has been shown that essential oils and their constituents inhibit different detoxifying enzymes in mosquitoes and tobacco cutworm (Norris et al., 2018; O'Neal et al., 2019; Ruttanaphan et al., 2019). However, the question as to whether plant essential oils and their constituents synergize pyrethroid (i.e., deltamethrin) toxicity in resistant bed bugs by inhibiting detoxification enzymes has remained unanswered, which is a major knowledge gap in effective control of resistant bed bug populations.

Plant essential oils are secondary plant metabolites from aromatic plants (Isman, 2006). More than 20 essential oils and their constituents are considered low risk pesticides by the Environmental Protection Agency (EPA) (https://www.epa.gov/minimum-risk-pesticides) and are exempt from registration. Essential oils are composed of complex mixtures of essential oil components or constituents (also referred as monoterpenoids). Pure essential oils and their constituents exhibit contact and fumigant toxicity against field-collected and lab populations of bed bugs (Feldlaufer and Ulrich, 2015; Singh et al., 2014; Politi et al., 2017; Zha et al., 2018; Gaire et al., 2019,2020). In a previous study, we found that bed bugs exhibiting metabolic and target site deltamethrin resistance (72,000-fold at the 25% mortality level) are completely susceptible to plant essential oils (Chapter 4). However, the various additive, synergistic or antagonistic toxicity interactions that could potentially occur between mixtures of deltamethrin and plant essential oils or their constituents are not known. Therefore, the objectives of this study were (i) to determine toxicity interactions exhibited by binary mixtures of deltamethrin with various plant essential oils or their constituents or EcoRaider® (a commercial essential oil-based product) in susceptible and resistant bed bug populations and (ii) to evaluate the ability of major essential oil constituents to inhibit detoxification enzymes in deltamethrin susceptible and resistant bed bug populations.

5.2 Materials and Methods

5.2.1 Bed bugs

Two strains of *C. lectularius*, Harlan and Knoxville were used. The insecticide susceptible Harlan strain has been reared in the laboratory without insecticide selection pressure for > 40 years (Doggett et al., 2018). The Knoxville strain was collected from apartments in Knoxville, TN in 2013 and has a history of exposure to neonicotinoid and pyrethroid class insecticides in the field (Ashbrook et al., 2017). In our previous research, the Knoxville strain showed 72,000-fold resistance (at the 25% mortality level) to deltamethrin, that was associated with higher activity of detoxification enzymes and the L925I or *kdr* mutation in the voltage-gated sodium channel (Chapter 4). Both strains were maintained at standard environmental conditions (temperature: 25℃, relative humidity: 50±15%, photoperiod: 12:12 (L: D) h) in reach-in growth chambers (Percival, Perry, IA). Bed bugs were fed weekly on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using the membrane feeding method (Chin-Heady et al., 2013). For topical application bioassays, 7–10 d old adult males were used that were fed 3 d before bioassays. For detoxification enzyme inhibition assays, 10–15 d old starved adult male bed bugs were used.

5.2.2 Chemicals

Thyme oil (*Thymus vulgaris*), oregano oil (*Origanum vulgare*), clove oil (*Eugenia caryophyllata*), coriander oil (*Coriandrum sativum*) and geranium oil (*Pelargonium graveolens*) were bought from Frontier Natural Products (Urbana, IA). The major essential oil constituents present in above oils; carvacrol, thymol, eugenol, geraniol and linalool (Chapter 4) were procured either from Sigma-Aldrich (St. Louis, MO) or from Alfa Aesar (Hill, MA). The EcoRaider® product (active ingredients: geraniol (1%), cedar extract (1%), and sodium lauryl sulfate (2%)) was purchased from Reneotech, Inc. (Bergen, NJ). Deltamethrin (purity 99.3%) was obtained from Chem Service Inc. (West Chester, PA). The detoxification enzyme inhibitors piperonyl butoxide (PBO), *S,S,S*-tributyl phosphorotrithioate (DEF) and diethyl maleate (DEM) were purchased from Sigma-Aldrich. The solvents acetone (analytical grade), and acetonitrile (analytical grade) and the surfactant Triton X 100 were bought Fisher Scientific (Hampton, NH). The enzymes substrates 7-ethoxycounmarin (7-EC), p-nitrophenol acetate (pNPA) and chlorodintrobenzene (CDNB) were obtained from Sigma-Aldrich. The NADPH (nicotinamide dinucleotide phosphate) regenerating

system Kit (solution A (10X) containing 26 mM NADP+, 66 mM glucose-6-phosphate and 66 mM magnesium chloride and solution B (100X) containing 40U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate, pH 5.5) was purchased from Promega (Madison, WI).

5.2.3 Topical application bioassay

The LD_{25} (lethal doses that kill 25% of test population) of deltamethrin, essential oils, essential oil components and EcoRaider® were identified for the Harlan and Knoxville strains in our previous study (Table 5.1; Gaire et al., 2019). These doses were prepared at 2x concentrations in acetone. Binary (1:1 ratio) mixtures of either deltamethrin + individual essential oils, deltamethrin + individual essential oil components, or deltamethrin + EcoRaider® were prepared by mixing equal volumes of respective stock solutions (2x of LD_{25} dose) for Harlan and Knoxville strains. For single compound treatments (deltamethrin, essential oils, essential oil constituents and EcoRaider®), insecticide solutions were prepared at respective LD_{25} concentrations for the Harlan and Knoxville strains (Table 5.1; Gaire et al., 2019).

Topical application bioassays with binary mixtures or single compounds at the LD_{25} dose were performed as per Gaire et al. (2019, 2020). In brief, insecticide solutions (0.5 µL/ insect) were applied topically on the ventral metathorax of bed bugs using a 25 µL micro-syringe attached to a PB-600-1 repeating dispenser (Hamilton, Reno, NV). Control bed bugs were treated with 0.5 µL of acetone. In total six replications (10 bed bugs per replicate) were performed for each mixture and individual compound treatment. After treatment, bed bugs (in groups of 10) were transferred to 35 x 10 mm petri dishes (Greiner Bio-One, Frickenhausen, Germany) and placed in an environmental chamber with temperature, humidity and lighting conditions identical to those used for rearing. Mortality scoring for all treatments was performed at 24 h post-treatment. Insects that were lying on their backs and/or were unable to move upon prodding were scored as dead.

5.2.4 Detoxification enzyme inhibition assays

5.2.4.1 Protein preparation

Two hours prior to homogenization, starved adult male bed bugs (10–15 days old) were topically treated with 0.5 µL of the respective LD_{25} dose of individual essential oil components (carvacrol, eugenol, geraniol, linalool and thymol in acetone) (Table 5.1), insecticide synergist as

positive controls (PBO, DEF, DEM @ 50 µg/insect in acetone), or solvent negative controls (acetone alone). After two hours, treated bed bugs from different groups were separately homogenized in 1 mL ice cold 0.1 M sodium phosphate buffer (pH = 7.0) containing 0.3% triton X 100 for cytochrome 450 activity measurement (Adelman et al., 2011; Romero and Anderson, 2016). Triton was not used for protein preparations that were intended to be used for conducting esterase and glutathione transferase assays. Homogenates were then centrifuged at 10,000 Xg for 20 min at 4°C in a 5424 R centrifuge (Eppendorf North America, Hauppauge, NY). The resulting supernatant from each treatment group was used as the enzyme source for measurement of detoxification enzyme activities. Protein concentration was determined using a Bradford assay with bovine serum albumin (BSA) as a standard (Bradford, 1976). Protein concentrations were measured in PowerWave 340 spectrophotometer (BioTek Instruments Inc., Winooski, VT) at 595 nm wavelength.

5.2.4.2 Cytochrome P450 assay

Cytochrome P450-dependent *O*-deethylation activity was determined by using 7-ethoxycoumarin (7-EC) as a substrate (Anderson and Zhu, 2004; Adelman et al., 2011; Romero and Anderson, 2016). Enzyme assays were conducted in 96-well black walled micro plates (Corning Inc., Corning, NY) to ensure that no cross-talk occurred between the wells. Initially, 2 µL of 100 mM 7-EC (in acetonitrile) and 40 µL of protein sample were added to both treatment and control wells. This was followed by the addition of 65 µL of freshly prepared mixture of sodium phosphate buffer (61.65 µL) and NADPH regeneration system (2.75 µL solution A + 0.6 µL solution B) to all sample wells. Control wells received only 65 µL sodium phosphate buffer. Next, plates were incubated for 30 min at 37°C while shaking at 400 rpm in MaxQ 6000 shaker (Thermo Fisher Scientific, Waltham, MA). Reactions were stopped by adding 120 µL of 50% (V/V) acetonitrile in 50mM TRIZMA-base buffer (pH: 10) to each well. Relative fluorescence units was measured using a Spectramax m2e instrument (Molecular Devices, LLC, San Jose, CA) at emission and excitation wavelengths of 460 nm and 380 nm, respectively (Valles et al., 1994). The extinction coefficient for the end product 7-hydroxycoumarin (4.44 M^{-1} cm^{-1}) was used for calculating specific activity, which was expressed as nmol/min/mg protein (Fang et al., 1997). Five biological replications were performed for each treatment.

5.2.4.3 Esterase assay

Esterase activity was measured using p-nitrophenyl acetate (pNPA) as a substrate according to Wu et al. (1998). Initially, the reaction mixture was prepared by adding 50 µL of pNPA (0.2 M in acetonitrile) in 10 mL sodium phosphate buffer (pH = 7.0; final concentration of 1 mM). In treatment wells, 10 µL of protein was used, whereas in control wells same volume of sodium phosphate buffer minus protein was added. Reactions were started by adding 225 µL of reaction mix to all wells. Reactions were monitored every 20 sec for 5 minutes at 405 nm wavelength in a PowerWave 340 spectrophotometer. The extinction coefficient of 6.53 mM^{-1} cm^{-1} for the end product, p-nitrophenol was used for the calculation of specific activity (Wu et al., 1998). Specific activity results were expressed as nmol/min/mg protein. In total, four biological replications were performed for each treatment.

5.2.4.4 Glutathione transferase assay

Glutathione transferase activity was measured using chlorodintrobenzene (CDNB) as a substrate according to Wu et al. (1998). Two different reaction mixtures were prepared in 10 mL sodium phosphate buffer. 'Reaction mix 1' contained 5 mM reduced glutathione and 1 mM CDNB whereas 'reaction mix 2' included everything except the co-factor, reduced glutathione. In each treatment and control well, 10 µL protein sample was added. Next, in the treatment well, 225 µL of the 'reaction mix 1' was added, whereas control wells received the same volume of 'reaction mix 2'. Reactions were monitored at 344 nm wavelength every 20 secs for 5 minutes in a PowerWave 340 spectrophotometer. The extinction coefficient for the end product, *S*-(2,4-dintrophenyl) glutathione) (9.50 mM^{-1} cm^{-1}) was used for calculation of specific activity, which was expressed as nmol/min/mg protein (Wu et al., 1998). In total, four biological replications were performed for each treatment.

5.2.5 Statistical analysis

Interactions between different binary mixtures of deltamethrin with essential oils or essential oil components or EcoRaider® were evaluated using co-toxicity factor analysis (Mansour et al., 1966).

$$\text{Co-toxicity factor} = \frac{\text{Observed \% mortality - Expected \% mortality}}{\text{Expected \% mortality}} \times 100$$

Observed percent mortality in the equation above refers to mortality obtained when using LD_{25} mixtures of deltamethrin + essential oil or deltamethrin + essential oil components or deltamethrin + EcoRaider®. Expected percent mortality represents the sum of mortality caused by LD_{25} dose of individual or single compounds present in the respective binary mixture. A positive co-toxicity factor of ≥+20 indicates a synergistic effect, a negative factor of ≤−20 indicates an antagonistic effect, and values between −20 and +20 imply an additive effect (Mansour et al., 1966).

Percent mortality data from the mixture and single compound treatments were statistically compared using analysis of variance (ANOVA followed by a post-hoc Tukey's HSD test). One way ANOVA followed by Dunnett's test was performed to compare the detoxification enzyme activities between essential oil constituents, PBO and acetone (solvent control) treatments. SPSS Version 25 software (IBM Corp., Armonk, NY) was used to perform statistical analysis.

5.3 Results

5.3.1 Toxicity interactions

Co-toxicity factor analyses showed that all six binary mixtures of essential oils + deltamethrin and EcoRaider + deltamethrin, that were tested against the Knoxville strain caused synergism (co-toxicity factor > +20) (Table 5.2). In the susceptible Harlan strain, synergistic increases in toxicity were observed only for the coriander oil + deltamethrin and geranium oil + deltamethrin mixtures (Table 5.2). Further, ANOVA analysis confirmed that binary mixtures of individual essential oils or EcoRaider with deltamethrin at the LD_{25} dose caused a significant increase in toxicity against the Knoxville strain (Fig. 5.1; $P < 0.01$, Tukey HSD test). However, in the susceptible Harlan stain, only coriander oil + deltamethrin and geranium oil + deltamethrin caused a synergistic increase in toxicity (Fig. 5.1; $P < 0.05$, Tukey HSD test).

Binary mixtures of all individual essential oil constituents (carvacrol, eugenol, geraniol, linalool and thymol) with deltamethrin caused a synergistic increase in toxicity against the resistant Knoxville strain (Table 5.3; co-toxicity factor > +20 and Fig. 5.2; $P < 0.01$, Tukey HSD test).

However, in the susceptible Harlan strain, only the mixtures of thymol + deltamethrin or carvacrol + deltamethrin led to a synergistic increase in toxicity (Table 5.3; co-toxicity factor > +20 and Fig. 5.2; $P < 0.05$, Tukey HSD test).

5.3.2 Detoxification enzyme inhibition

The 7-EC *O*-deethylation assay showed that PBO and all five essential oil components (carvacrol, thymol, eugenol, geraniol and linalool) significantly inhibited P450 activity (27–50% inhibition) in the Knoxville strain as compared to the solvent control (acetone) (Fig. 5.3; $P < 0.01$, ANOVA Dunnett's test). Inhibition of P450 activity, however, was not observed in the susceptible Harlan strain (Fig. 5.3; $P > 0.05$, ANOVA Dunnett's test). PNPA hydrolysis activity was only inhibited by the synergist DEF (Fig. 5.4, $P < 0.01$, ANOVA Dunnett's test) in both strains. However, in both strains, pre-treatment of bed bugs with essential oil components did not affect their esterase activity (Fig. 5.4, $P < 0.01$, ANOVA Dunnett's test) in comparison to acetone controls. In both bed bug strains, pre-treatment with the glutathione transferase inhibitor, DEM or essential oil constituents did not lead to inhibition of CDNB conjugation activity in comparison to the acetone treated controls (Fig. 5.5, $P > 0.05$, ANOVA Dunnett's test).

5.4 Discussion

To determine the utility the plant essential oils for bed bug insecticide resistance management, we evaluated the toxicity interactions between binary mixtures of deltamethrin with various plant essential oils, their insecticidal components, or an essential oil-based product. The five plant essential oils tested (thyme, oregano, clove, geranium and coriander) and their major components (thymol, carvacrol, eugenol, geraniol and linalool), significantly increased or synergized deltamethrin toxicity in the resistant Knoxville strain. However, in the susceptible strain, interactions between essential oils or their components and deltamethrin were either antagonistic, additive or synergistic. The enhancement of pyrethroid (e.g., deltamethrin and permethrin) toxicity by essential oils was variable (either synergistic, additive or antagonistic) in susceptible and resistant strains of mosquitoes (Gross et al., 2017; Norris et al., 2018; Chansang et al., 2018; O'Neal et al., 2019). However, in the tobacco cutworm, toxicity of binary mixtures of galangal oil or basil oil or their constituents and cypermethrin always lead to synergistic toxicity

interactions in both susceptible and resistant strains (Ruttanaphan et al., 2019). Variation of results among different studies and insect species indicates that the ratios of insecticides in a mixture, test insect species, and the presence/ absence of specific resistance mechanisms could be important determinants of various toxicity interactions.

Recent literature on mosquitoes suggests that plant essential oils synergize pyrethroid (e.g., permethrin and deltamethrin) toxicity by inhibiting either cytochrome P450 or glutathione transferase enzymes (Norris et al., 2018; O'Neal et al., 2019). Essential oil components such as linalool and 1,8-cineol when mixed with either cypermethrin or synergists (PBO, DEM and TPP) also inhibit cytochrome P450s and esterases in the tobacco cutworm; however, glutathione transferase inhibition varied based on the type of mixture and insect population (Ruttanaphan et al., 2019). The resistant Knoxville showed a significantly higher activity of detoxification enzymes (cytochrome P450, esterase and glutathione transferase) in our previous work (Chapter 4). Therefore, we evaluated the effects of bed bug pre-treatment with major essential oil constituents (thymol, carvacrol, eugenol, geraniol and linalool) on detoxification enzyme activities in both susceptible and resistant populations. All five essential oil components inhibited cytochrome P450 activity in the deltamethrin resistant-Knoxville strain and their inhibitory effects were similar to those caused by PBO. Even though the Knoxville resistant strain exhibited significantly higher esterase and glutathione transferase enzyme activities in comparison to the Harlan susceptible strain, essential oil components did not inhibit these enzymes. These results suggest that the observed synergistic toxicity interactions between deltamethrin and essential oil mixtures were at least partially caused by cytochrome P450 inhibition. Zhu et al. (2010) showed that RNAi mediated knockdown of brain-specific P450 expression increased deltamethrin susceptibility in the red flour beetle strain. Keseru et al. (1999) showed that high activity of PBO compared to other methylendioxyphenyl inhibitors was determined by its decreased conformational mobility and the steric block created by its long side-chain on the substrate access channel of the P450 enzyme. It is likely that essential oil components can inhibit cytochrome P450 enzymes of the resistant Knoxville strain in comparison to other detoxification enzymes.

We also observed that thymol and carvacrol synergized deltamethrin toxicity in the susceptible Harlan strain, however, these compounds did not inhibit any of the detoxification enzymes. Thymol and carvacrol are neurologically active compounds that act on gamma-aminobutyric acid (GABA) and nicotinic acetylcholine (nACh) receptors, respectively (Tong et

al., 2013; Priestley et al., 2013). It is possible that these compounds synergized deltamethrin toxicity in the Harlan strain by binding to different neuronal target sites and thereby causing an increased alteration (neuroinhibition or neuroexcitation) of the nervous system activity. Jankowska et al. (2019a,b) showed that menthol (one of the essential oil components found in mint oil) potentiated or synergized the activity of bendiocarb (a carbamate insecticide) in the American cockroach by activating octopamine receptors and protein kinase A. Similarly, a tertiary mixture of three neuroinhibitory compounds; thymol, carvacrol and eugenol significantly reduced the normal nerve firing activity of the susceptible Harlan strain of bed bugs and caused higher mortality in comparison to single compound treatments (Gaire et al., 2019,2020).

Mixtures of certain essential oil components exhibit synergistic toxicity interactions against insect pests due to enhanced cuticular penetration resulting from changes in pharmacokinetic factors such as surface tension and solubility (Tak and Isman, 2015,2017). Previous studies have shown that pyrethroid resistant bed bugs overexpress cytochrome P450, ABC transporter and cuticular genes and possess a thicker cuticle (Zhu et al., 2013; Lilly et al, 2016b). It is possible that the Knoxville strain may also possess reduced cuticular penetration as an additional mechanism for deltamethrin resistance. In such scenario, essential oil components could have potentially facilitated faster penetration of deltamethrin into the bed bug cuticle and its subsequent movement to the target site. However, additional molecular and pharmacokinetic studies are necessary to confirm this hypothesis.

Currently, several natural product insecticides that contain a mixture of two or more essential oils are available for the control of various insect pests including bed bugs. However, only a few of these products are efficacious against bed bugs (Singh et al., 2014). This evidence suggests that some of these products are likely formulated without conducting rigorous empirical efficacy studies and consideration of various toxicity interactions that could occur between mixtures of two or more essential oils or their components. In the study conducted by Singh et al. (2014), EcoRaider®, was the most effective essential oil-based product against a field population of bed bugs. In our study, this product also synergized deltamethrin toxicity in the resistant Knoxville strain. This finding has important implications for bed bug control and development of combination or mixture products. First, pending future efficacy testing and regulatory approvals, tank mixtures of essential oil products such as EcoRaider® and deltamethrin formulations show potential for effective management of bed bugs in the field. Secondly, combination products that

contain mixtures of various essential oils and pyrethroid insecticides can be developed for effective management of resistant bed bugs and other insect pests (e.g., mosquitoes). However, there are inherent challenges with the use of essential oils or their active components for urban pest control that need to be addressed before formulating these products. Essential oils are mostly effective against bed bugs through direct contact and as fumigants, but have a short residual effect due to their high volatility (Singh et al., 2014; Gaire et al., 2019). In addition, the strong odor associated with essential oils is not desirable to some people. However, nano and/or microencapsulated formulations of essential oils have reduced volatility and thereby have lowered odor issues and prolonged residual activity (Gonzalez et al., 2014; Oliviera et al., 2017). Therefore, nano- or microencapsulated formulations can be tested as carriers for pyrethroid and essential oil combination products in the future. Since bed bugs also exhibit high levels of resistance to many insecticides from the neonicotinoid class (Romero and Anderson, 2016; Caceres et al., 2019), the ability of essential oils or their constituents to enhance toxicity of neonicotinoid insecticides in resistant bed bug populations should be investigated as well.

Table 5.1 Lethal dose (LD25) values of deltamethrin, different essential oils and their insecticidal constituents, and EcoRaider® (an essential oil-based product) for the Harlan susceptible and Knoxville resistant strain of bed bugs from previous studies.

Treatments	**LD_{25} value (µg/mg body weight)**	
	Harlan strain	**Knoxville strain**
Deltamethrin	5.36×10^{-5}[a]	3.91[a]
Thyme oil	22.80[a]	21.78[a]
Oregano oil	19.25[a]	26.50[a]
Clove oil	37.94[a]	23.62[a]
Geranium oil	79.25[a]	52.23[a]
Coriander oil	55.70[a]	64.56[a]
Thymol	9.93[b]	15.21[a]
Carvacrol	8.06[b]	8.31[a]
Eugenol	14.41[b]	11.69[a]
Geraniol	16.45[b]	27.84[a]
Linalool	28.91[b]	92.32[a]
EcoRaider®	63.36[a]	69.55[a]

[a]Adapted from Chapter 4
[b]Adapted from Gaire et al. (2019)

Table 5.2 Topical toxicity of binary mixtures of plant essential oils and deltamethrin to the Harlan susceptible and Knoxville resistant bed bug strains.

Mixtures	**Strains**	**Mortality % (LD_{25} essential oil)**	**Mortality % (LD_{25} deltamethrin)**	**Expected % mortality of mixture**	**Observed % mortality of mixture**	**Co-toxicity factor[I]**	**Toxicity interaction**
Thyme oil + Deltamethrin	Harlan	46.66	28.33	74.99	48.33	-35.55	Antagonism
	Knoxville	26.66	15	41.66	96.66	132.02	Synergism
Oregano oil + Deltamethrin	Harlan	13.33	25	38.33	45	17.41	Additive
	Knoxville	21.66	20	41.66	90	116.03	Synergism
Clove bud oil + Deltamethrin	Harlan	16.66	25	41.66	36.66	-12.00	Additive
	Knoxville	50	20	70	91.66	30.94	Synergism
Coriander oil + Deltamethrin	Harlan	28.33	16.66	44.99	56.66	25.93	Synergism
	Knoxville	15	28.33	43.33	98.33	126.93	Synergism
Geranium oil + Deltamethrin	Harlan	28.33	16.66	44.99	55	22.24	Synergism
	Knoxville	23.33	28.33	51.66	98.33	90.34	Synergism

[I]A positive co-toxicity factor of +20 or higher indicates a synergistic effect, a negative factor of −20 or lower refers to an antagonistic effect, and values between −20 and +20 imply an additive effect (Mansour et al., 1966). For each bed bug strain and binary mixture, six replicates with 10 adult males per replicate (n = 60) were performed.

Table 5.3 Topical toxicity of binary mixtures of essential oil components and deltamethrin to the Harlan susceptible and Knoxville resistant bed bug strains.

Mixtures	**Strains**	**Mortality % (LD_{25} essential oil component)**	**Mortality % (LD_{25} deltamethrin)**	**Expected % mortality of mixture**	**Observed % mortality of mixture**	**Co-toxicity factor[I]**	**Toxicity interaction**
Carvacrol + Deltamethrin	Harlan	16.66	5	21.66	51.66	138.50	Synergism
	Knoxville	41.66	35	76.66	100	30.44	Synergism
Thymol + Deltamethrin	Harlan	6.66	13.33	19.99	46.66	133.41	Synergism
	Knoxville	10	23.33	33.33	61.66	84.99	Synergism
Eugenol + Deltamethrin	Harlan	10	16.66	26.66	8.33	-68.90	Antagonism
	Knoxville	23.33	36.66	59.99	85	41.69	Synergism
Geraniol + Deltamethrin	Harlan	15	28.33	43.33	28.33	-34.61	Antagonism
	Knoxville	3.33	35	38.33	80	108.71	Synergism
Linalool + Deltamethrin	Harlan	6.66	6.66	13.26	21.66	63.34	Synergism
	Knoxville	30	36.66	66.66	91.66	37.50	Synergism

[I]A positive co-toxicity factor of +20 or higher indicates a synergistic effect, a negative factor of −20 or lower refers to an antagonistic effect, and values between −20 and +20 imply an additive effect (Mansour et al., 1966). For each bed bug strain and binary mixture, six replicates with 10 adult males per replicate (n = 60) were performed.

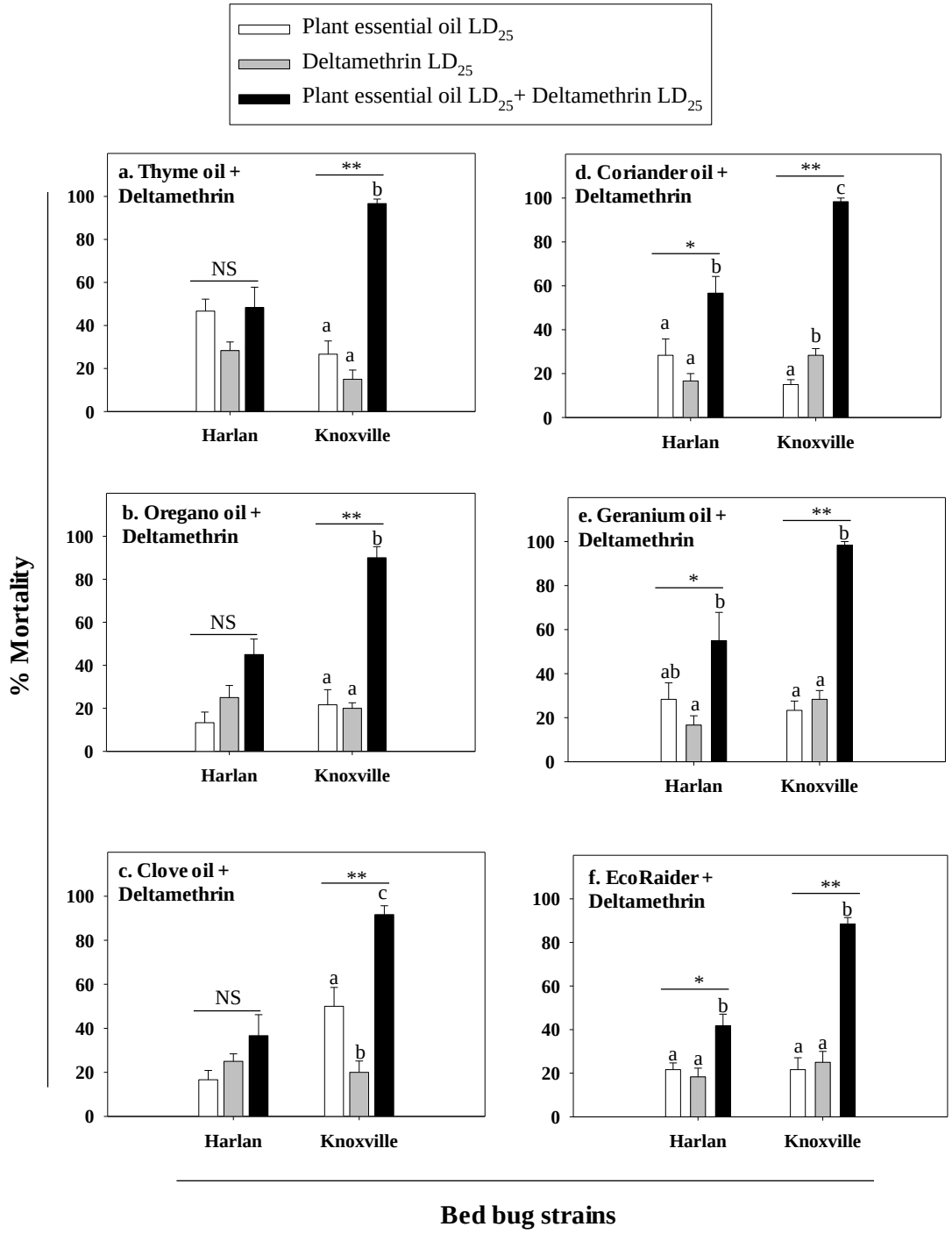


Figure 5.1 Percentage mortality observed in the Harlan susceptible and Knoxville resistant strain bed bugs treated with respective LD25 doses (see Table 1) of individual plant essential oils, EcoRaider, deltamethrin and their binary mixtures. NS refers to "statistically non-significant" differences in mortality among different treatments ($P > 0.05$). A single asterisk (*) indicates statistically significant difference between treatments at the $P < 0.05$ level and double asterisks (**) indicates statistical significance at the $P < 0.01$ level (ANOVA and Tukey's HSD test).

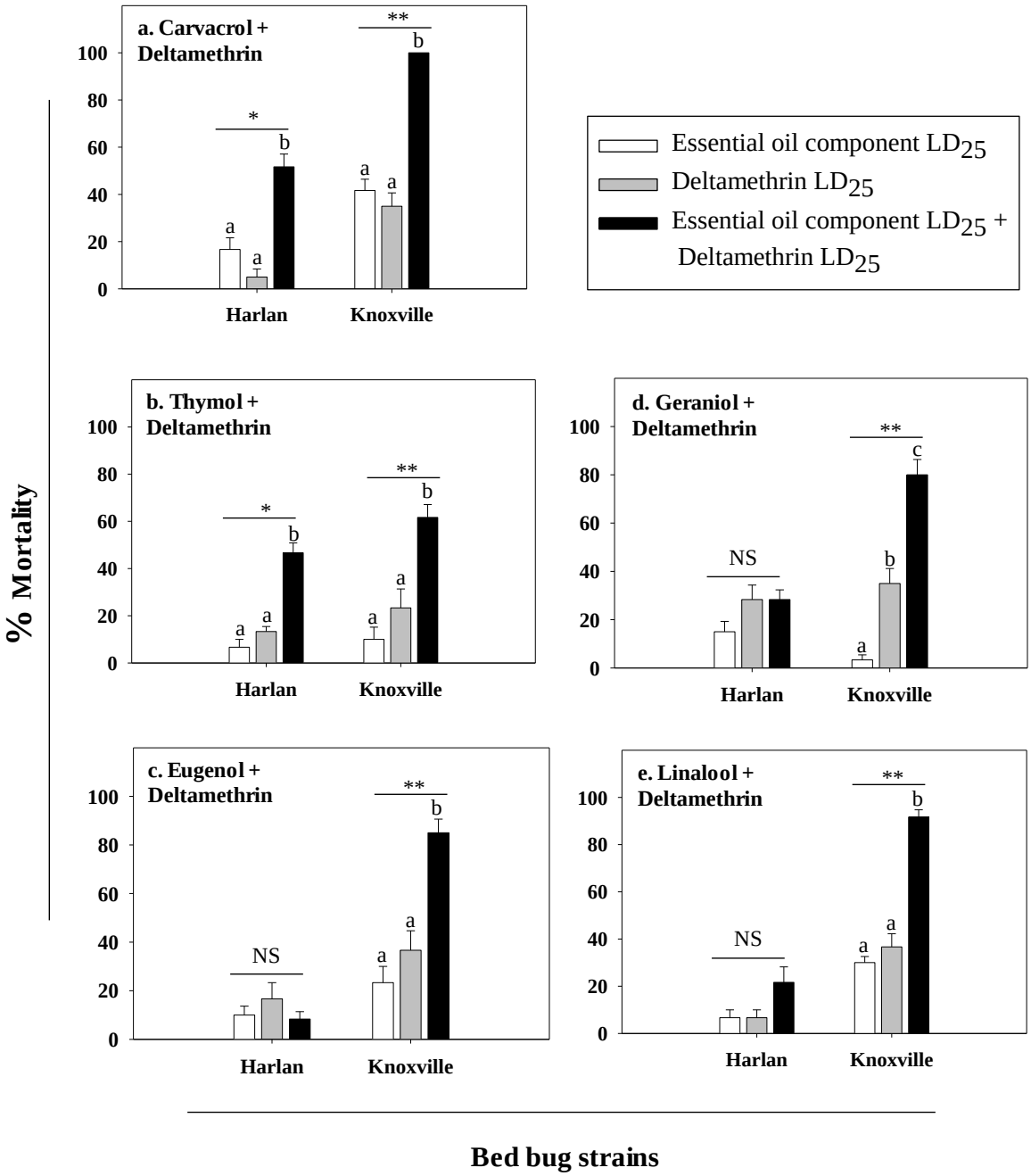


Figure 5.2 Percentage mortality observed in the Harlan susceptible and Knoxville resistant strain bed bugs treated with their respective LD25 doses (see Table 1) of individual plant essential oil constituents, deltamethrin and their binary mixtures. NS refers to "statistically non-significant" differences in mortality among different treatments ($P > 0.05$). A single asterisk (*) indicates statistically significant differences between treatments at the $P < 0.05$ level and double asterisks (**) represents statistical significance at the $P < 0.01$ level (ANOVA and Tukey's HSD test).

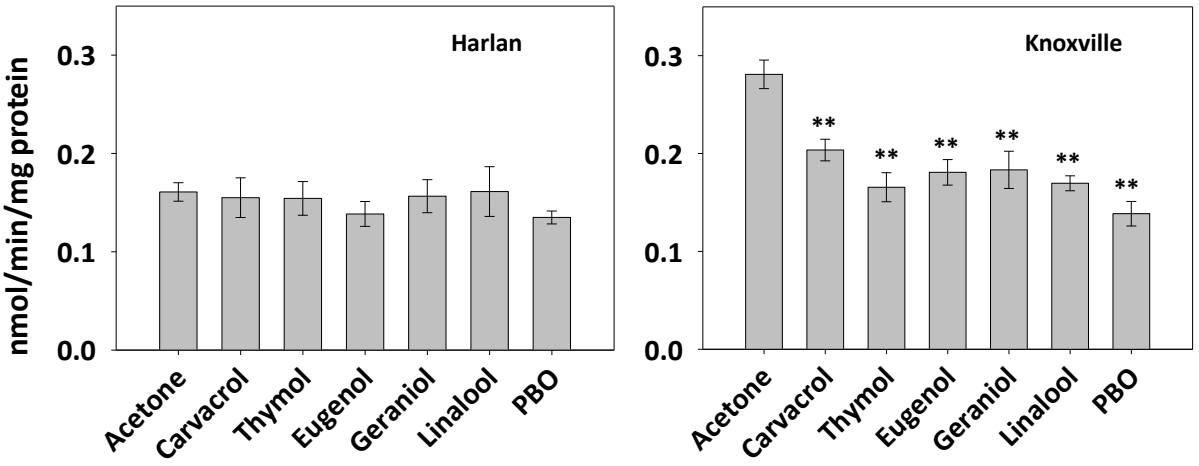


Figure 5.3 Effect of essential oil component and piperonyl butoxide (PBO) pre-treatment on cytochrome P450 enzyme activity (7-ethoxycoumarin hydroxylation) in the deltamethrin susceptible (Harlan) and resistant (Knoxville) bed bug strains. A double asterisk (**) represents statistical significance at the $P < 0.01$ level (ANOVA and Dunnett's test).

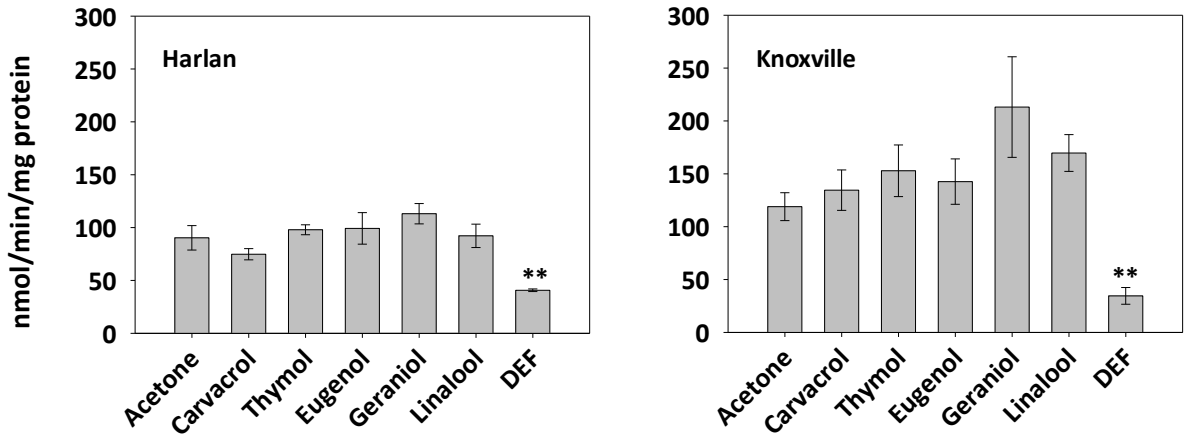


Figure 5.4 Effect of essential oil component and S,S,S-tributyl phosphorotrithioate (DEF) pre-treatment on esterase enzyme activity (p-nitrophenol acetate hydrolysis) in the deltamethrin susceptible (Harlan) and resistant (Knoxville) bed bug strains. A double asterisk (**) represents statistical significance at the $P < 0.01$ level (ANOVA and Dunnett's test).

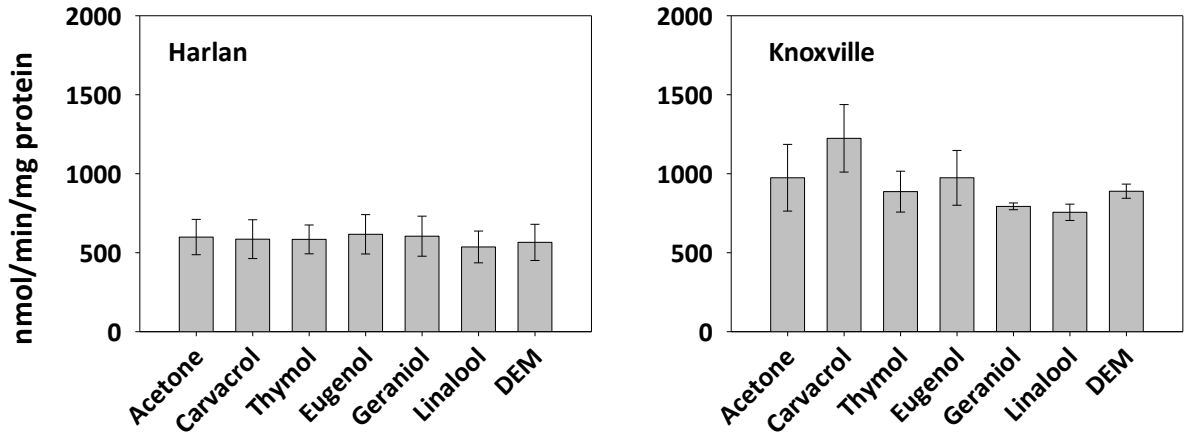


Figure 5.5 Effect of essential oil component and diethyl maleate (DEM) pre-treatment on glutathione transferase activity (chlorodintrobenzene conjugation) in the deltamethrin susceptible (Harlan) and resistant (Knoxville) bed bug strains. None of the essential oil component or the positive control (DEM) treatments caused significant inhibition of glutathione transferase activity in both strains (ANOVA and Dunnett's test; $P > 0.05$).

5.5 References

Adelman, Z.N., Kilcullen, K.A., Koganemaru, R., Anderson, M.A.E., Anderson, T.D., Miller, D.M., 2011. Deep sequencing of pyrethroid-resistant bed bugs reveals multiple mechanisms of resistance within a single population. PLoS ONE 6, 10, e26228. https://doi.org/10.1371/journal.pone.0026228

Anderson, T.D., Zhu, K.Y., 2004. Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera: Chironomidae). Pestic. Biochem. Phys. 80, 54–64.

Ashbrook, A.R., Scharf, M.E., Bennett, G.W., Gondhalekar, A.D., 2017. Detection of reduced susceptibility to chlorfenapyr- and bifenthrin-containing products in field populations of the bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 110, 1195–1202.

Bernard, B.C., Philogene, B.J.R., 1993. Insecticide synergists: role, importance, and perspectives. J. Toxicol. Env. Health 38, 199–233.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Cáceres, M., Santo-Orihuela, P.L., Vassena, C.V., 2019. Evaluation of resistance to different insecticides and metabolic detoxification mechanism by use of synergist in the common bed bug (Heteroptera: Cimicidae). J. Med. Entomol. 56, 1324–1330.

Chansang, A., Champakaew, D., Junkum, A., Jitpakdi, A., Amornlerdpison, D., Aldred, A.K., Riyong, D., Wannasan, A., Intirach, J., Muangmoon, R., Pitasawat, B., 2018. Synergy in the adulticidal efficacy of essential oils for the improvement of permethrin toxicity against *Aedes aegypti* L. (Diptera: Culicidae). Parasit. Vectors 11, 417. https://doi.org/10.1186/s13071-018-3001-7

Chin-Heady, E., Demark, J.J., Nolting, S., Bennett, G., Saltzmann, K., Hamm, R.L., 2013. A quantitative analysis of a modified feeding method for rearing *Cimex lectularius* (Hemiptera: Cimicidae) in the laboratory. Pest Manag. Sci. 69, 1115–1120.

Dang, K., Toi, C.S., Lilly, D.G., Lee, C.Y., Naylor, R., Tawatsin, A., Thavara, U., Bu, W., Doggett, S.L., 2015. Identification of putative kdr mutations in the tropical bed bug, *Cimex hemipterus* (Hemiptera: Cimicidae). Pest Manag. Sci. 71, 1015–1020.

Doggett, S.L., Miller, D.M., Lee, C.Y., 2018. Advances in the Biology and Management of Modern Bed Bugs. Wiley Blackwell, West Sussex, UK.

Dong, K., Du, Y., Rinkevich, F., Nomura, Y., Xu, P., Wang, L., Silver, K., Zhorov, B.S., 2014. Molecular biology of insect sodium channels and pyrethroid resistance. Insect Biochem. Molec. 50, 1–17.

Fang, X., Kobayashi, Y., Halpert, J.R., 1997. Stoichiometry of 7-ethoxycoumarin metabolism by cytochrome P450 2B1 wild-type and five active-site mutants. FEBS Letters 416, 77–80.

Feldlaufer, M.F., Ulrich, K.R., 2015. Essential oils as fumigants for bed bugs (Hemiptera: Cimicidae). J. Entomol. Sci. 50, 129–137.

Gaire, S., Scharf, M.E., Gondhalekar, A.D., 2019. Toxicity and neurophysiological impacts of plant essential oil components on bed bugs (Cimicidae: Hemiptera). Scientific Reports 9, 3961. https://doi.org/10.1038/s41598-019-40275-5

Gaire, S., Scharf, M.E., Gondhalekar, A.D., 2020. Synergistic toxicity interactions between plant essential oil components against the common bed bug (*Cimex lectularius* L.). Insects 11, 133. https://doi.org/10.3390/insects11020133

Gonzalez-Morales, M.A., Romero, A., 2019. Effect of synergists on deltamethrin resistance in the common bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 112, 786–791.

Gross, A.D., Norris, E.J., Kimber, M.J., Bartholomay, L.C., Coats, J.R., 2017. Essential oils enhance the toxicity of permethrin against *Aedes aegypti* and *Anopheles gambiae*. Med. Vet. Entomol. 31, 55–62.

Isman, M.B., 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. Annu. Rev. Entomol. 51, 45–66.

Jankowska, M., Lapied, B., Jankowski, W., Stankiewicz, M., 2019a. The unusual action of essential oil component, menthol, in potentiating the effect of the carbamate insecticide, bendiocarb. Pestic. Biochem. Phys. 158, 101–111.

Jankowska, M., Wiśniewska, J., Fałtynowicz, Ł., Lapied, B., Stankiewicz, M., 2019b. Menthol increases bendiocarb efficacy through activation of octopamine receptors and protein kinase A. Molecules 24, 3775. https://doi.org/10.3390/molecules24203775

Keseru, G.M., Kolossvary, I., Bertok, B., 1999. Piperonyl butoxide-mediated inhibition of cytochrome P450-catalysed insecticide metabolism: a rational approach. Pestic. Sci. 55, 1004–1006.

Koganemaru, R., Miller, D.M., Adelman, Z.N., 2013. Robust cuticular penetration resistance in the common bed bug (*Cimex lectularius* L.) correlates with increased steady-state transcript levels of CPR-type cuticle protein genes. Pestic. Biochem. Phys. 106, 190–197.

Lee, C.Y., Miller, D.M., Doggett, S.L., 2018. Chemical control, in: Doggett, S.L., Miller, D.M., Lee, C.Y. (Eds.), Advances in the Biology and Management of Modern Bed Bugs ed. by Wiley Blackwell, West Sussex, UK, pp. 285–310.

Lilly, D.G., Dang, K., Webb, C.E., Doggett, S.L., 2016a. Evidence for metabolic pyrethroid resistance in the common bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 109, 1364–1368.

Lilly, D.G., Latham, S.L., Webb, C.E., Doggett, S.L., 2016b. Cuticle thickening in a pyrethroid-resistant strain of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae). PLoS ONE 11, e0153302. https://doi.org/10.1371/journal.pone.0153302

Mansour, N.A., Elderfrawi, M.E., Toppozada, A., Zeid, M., 1966. Toxicological studies on the Egyptian cotton leafworm, *Prodenia litura*. VI. potentiation and antagonism of organophosphorus and carbamate insecticides. J. Econ. Entomol. 59, 307–311.

Norris, E.J., Johnson, J.B., Gross, A.D., Bartholomay, L.C., Coats, J.R., 2018. Plant essential oils enhance diverse pyrethroids against multiple strains of mosquitoes and inhibit detoxification enzyme processes. Insects 9, 132. https://doi.org/10.3390/insects9040132

O'Neal, S.T., Johnson, E.J., Rault, L.C., Anderson, T.D., 2019. Vapor delivery of plant essential oils alters pyrethroid efficacy and detoxification enzyme activity in mosquitoes. Pestic. Biochem. Phys. 157, 88–98.

Oliveira, A.P., Santana, A.S., Santana, E.D.R., Lima, A.P.S., Faro, R.R.N., Nunes, R.S., Lima, A.D., Blank, A.F., Araújo, A.P.A., Cristaldo, P.F., Bacci, L., 2017. Nanoformulation prototype of the essential oil of *Lippia sidoides* and thymol to population management of *Sitophilus zeamais* (Coleoptera: Curculionidae). Ind. Crop Prod. 107, 198–205.

Politi, F.A.S., Nascimento, J.D., da Silva, A.A., Moro, I.J., Garcia, M.L., Guido, R.V.C., Pietro, R.C.L.R., Godinho, A.F., Furlan, M., 2017. Insecticidal activity of an essential oil of *Tagetes patula* L. (Asteraceae) on common bed bug *Cimex lectularius* L. and molecular docking of major compounds at the catalytic site of ClAChE1. Parasitol. Res. 116, 415–424.

Priestley, C.M., Williamson, E.M., Wafford, K.A., Sattelle, D.B., 2003. Thymol, a constituent of thyme essential oil, is a positive allosteric modulator of human $GABA_A$ receptors and a homo-oligomeric GABA receptor from *Drosophila melanogaster*. Brit. J. Pharmacol. 140, 1363–1372.

Romero, A., 2018. Insecticide resistance, in: Doggett, S.L., Miller, D.M., Lee, C.Y. (Eds.), Advances in the Biology and Management of Modern Bed Bugs ed. by Wiley Blackwell, West Sussex, UK, pp. 273–284.

Romero, A., Anderson, T.D., 2016. High levels of resistance in the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), to neonicotinoid insecticides. J. Medical Entomology 53, 727–731.

Romero, A., Potter, M.F., Haynes, K.F., 2009. Evaluation of piperonyl butoxide as a deltamethrin synergist for pyrethroid-resistant bed bugs. J. Econ. Entomol. 102, 2310–2315.

Romero, A., Potter, M.F., Potter, D.A., Haynes, K.F., 2007. Insecticide Resistance in the bed bug: a factor in the pest's sudden resurgence? J. Med. Entomol. 44, 175–178.

Ruttanaphan, T., Pluempanupat, W., Aungsirisawat, C., Boonyarit, P., Goff, G. le, Bullangpoti, V., Isman, M., 2019. Effect of plant essential oils and their major constituents on cypermethrin tolerance associated detoxification enzyme activities in *Spodoptera litura* (Lepidoptera: Noctuidae). J. Econ. Entomol. 112, 2167–2176.

Silva, S.M., da Cunha, J.P.A.R., de Carvalho, S.M., Zandonadi, C.H.S., Martins, R.C., Chang, R., 2017. *Ocimum basilicum* essential oil combined with deltamethrin to improve the management of *Spodoptera frugiperda*. Cienc. Agrotec. 41, 665–675.

Singh, N., Wang, C., Cooper, R., 2014. Potential of essential oil-based pesticides and detergents for bed bug control. J. Econ. Entomol. 107, 2163–2170.

Suwannayod, S., Sukontason, K.L., Pitasawat, B., Junkum, A., Limsopatham, K., Jones, M.K., Somboon, P., Leksomboon, R., Chareonviriyaphap, T., Tawatsin, A., Thavara, U., Sukontason, K., 2019. Synergistic toxicity of plant essential oils combined with pyrethroid insecticides against blow flies and the house fly. Insects 10, 178. https://doi.org/10.3390/insects10060178

Tak, J.H., Isman, M.B., 2015. Enhanced cuticular penetration as the mechanism for synergy of insecticidal constituents of rosemary essential oil in *Trichoplusia ni*. Sci. Rep. 5, 12690. https://doi.org/10.1038/srep12690

Tak, J.H., Isman, M.B., 2017. Penetration-enhancement underlies synergy of plant essential oil terpenoids as insecticides in the cabbage looper, *Trichoplusia ni*. Sci. Rep. 7, 42432. https://doi.org/10.1038/srep42432

Tong, F., Gross, A.D., Dolan, M.C., Coats, J.R., 2013. The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor. Pest Manag. Sci. 69, 775–780.

Valles, S.M., Yu, S.J., 1996. Insecticide resistance and resistance management detection and biochemical characterization of insecticide resistance in the German cockroach (Dictyoptera: Blattellidae). J. Econ. Entomol. 89, 21–26.

Werdin González, J.O., Gutiérrez, M.M., Ferrero, A.A., Fernández Band, B., 2014. Essential oils nanoformulations for stored-product pest control - characterization and biological properties. Chemosphere 100, 130–138.

Wu, D., Scharf, M.E., Neal, J.J., Suiter, D.R., Bennett, G.W., 1998. Mechanisms of fenvalerate resistance in the German cockroach, *Blattella germanica* (L.), Pestic. Biochem. Phys. 61, 53–62.

Zha, C., Wang, C., Li, A., 2018. Toxicities of selected essential oils, silicone oils, and paraffin oil against the common bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 111, 170–177.

Zhu, F., Gujar, H., Gordon, J.R., Haynes, K.F., Potter, M.F., Palli, S.R., 2013. Bed bugs evolved unique adaptive strategy to resist pyrethroid insecticides. Sci. Rep. 3, 1456. https://doi.org/10.1038/srep01456

Zhu, F., Parthasarathy, R., Bai, H., Woithe, K., Kaussmann, M., Nauen, R., Harrison, D.A., Palli, S.R., 2010. A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. P. Natl. Acad. Sci. USA 107, 8557–8562.

CHAPTER 6. CONCLUSIONS

6.1 Conclusions, future directions and implication of results

I designed this PhD project with the aim to provide in-depth information on efficacy, toxicology and mode-of-action of essential oil compounds to the urban pest management industry and for utilizing natural products as a control tool for bed bug IPM. In my first dissertation objective (Chapter 2), I determined the efficacy of fifteen different individual essential oil constituents and evaluated the neurophysiological impacts of the six most toxic components in the insecticide susceptible Harlan strain. I found that thymol, carvacrol, citronellic acid, eugenol, linalool and (±)-camphor showed highest potency against bed bugs. In electrophysiology experiments, thymol, carvacrol and eugenol depressed bed bug nervous system activity, whereas linalool caused neuroexcitation. Further I would like to conduct more in-depth mode of action studies to find the actual target sites of these active components in bed bugs using voltage/patch clamping or other relevant techniques. In the second objective (Chapter 3), I found synergistic toxicity interactions between a tertiary (1:1:1 ratio) mixture of carvacrol, eugenol and thymol at the organismal (bioassay) and sub-organismal (nervous system) levels in the Harlan strain. Previous literature has shown that synergism between essential oil components occurred as a result of penetration enhancement due to change in pharmacokinetics factors such as solubility and surface tension (Tak and Isman, 2015). I would like to conduct similar study as per Tak and Isman (2015) to evaluate any penetration enhancement mechanism has played role in synergism between carvacrol, eugenol and thymol. I have identified the most toxic and synergistic essential oil components (e.g., carvacrol, eugenol and thymol) from chapter 2 and 3. Many of these toxic compounds are not included in currently available market products. I would like to lead an effort on the development of potential natural product insecticides by testing different formulations of these compounds in the field for bed bug control.

In the third objective (chapter 4), I compared the toxicity of five pure essential oils (thyme, oregano, clove, geranium and coriander), their major constituents (thymol, carvacrol, eugenol, geraniol and linalool), EcoRaider (market product), and deltamethrin in susceptible (Harlan) and field-collected (Knoxville) bed bug populations. The field-collected Knoxville bed bugs were >70,000-fold resistant to deltamethrin which provided me an opportunity to further characterize

enzymatic (high activity of detoxification enzymes) and target site (point mutations) based mechanisms of resistance in this strain. In contrast to deltamethrin, the field-collected Knoxville strain did not show resistance (resistance ratio ~ 1) to five essential oils and their major constituents, and EcoRaider, although it did take higher doses for the essential oil compounds to cause equivalent toxicity. Essential oils are highly volatile in nature and have short residual activity. Micro/Nano-formulations decrease the volatility of essential oils and thereby increase their residual activity (Gonzalez et al., 2014; Oliviera et al., 2017). My preliminary experiments in bed bugs with nano-formulated essential oils showed enhanced residual activity in comparison to non-formulated oils after six days.

In the last dissertation objective (chapter 5), I found that the abovementioned plant essential oils, their major components and EcoRaider synergized deltamethrin toxicity in the resistant Knoxville population. Using binary mixtures of plant essential oils and deltamethrin at the LD_{25} dose (a dose that kills 25% population) I was able to overcome resistance (mortality > 90%) in the Knoxville population. Further, I found that major components of plant essential oils inhibit cytochrome P450 enzyme activity in the Knoxville population. Since essential oil components inhibit cytochrome P450s in resistant bed bugs, I am very interested to further identify the effects of essential oil components on P450 gene expression. Additionally, P450 based antibodies coupled with confocal microscopy can be used to identify P450 isoforms that are differentially expressed due to pre-treatment with essential oil constituents (Balabanidou et al., 2016). There is evidence that insecticide resistance in insects can be mediated by microbes (Xia et al. 2018; Barnard et al. 2019). Plants essential oils possess antimicrobial properties (Akami et al., 2019). One important question to answer is whether these oils affect/eliminate those microbes and decrease insecticide resistance in bed bugs and other urban pest such as German cockroaches.

Bed bugs are resistant to many other pyrethroid insecticides as well as neonicotinoid class insecticides (Romero and Anderson, 2016; Caceres et al., 2019). I would like to determine the toxicity interactions of essential oils, their major constituents and EcoRaider with other pyrethroid and neonicotinoid insecticides. Determining the synergistically interacting mixtures is key for development of essential oils and synthetic insecticide mixture products. In the diagram below (Fig. 6.1), I have purposed the potential of developing new essential oil-based products based on the overall findings of my dissertation. These new products will be composed of most toxic essential oils/synergizing constituents (e.g., carvacrol, eugenol and thymol) and pyrethroids that

are formulated with nano-engineering techniques. The nano-formulation not only increased the residual toxicity, but it also potentially reduced the strong odors associated with essential oils.

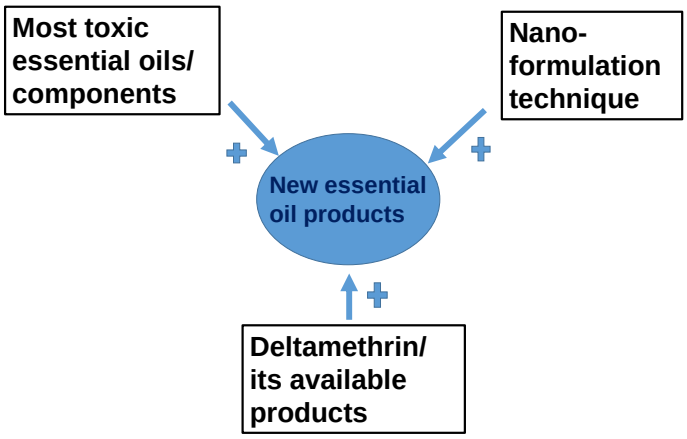


Figure 6.1 Potential for the development of new essential oil-based products based on findings presented in this dissertation. When the abovementioned hypothetical products are developed they would need further lab and field testing to determine their efficacy.

6.2 References

Akami, M., Njintang, N.Y., Gbaye, O.A., Andongma, A.A., Rashid, M.A., Niu, C.Y., Nukenine, E.N., 2019. Gut bacteria of the cowpea beetle mediate its resistance to dichlorvos and susceptibility to *Lippia adoensis* essential oil. Sci. Rep. 9, 6435. https://doi.org/10.1038/s41598-019-42843-1

Balabanidou, V., Kampouraki, A., Maclean, M., Blomquist, G.J., Tittiger, C., Juárez, M.P., Mijailovsky, S.J., Chalepakis, G., Anthousi, A., Lynd, A., Antoine, S., Hemingway, J., Ranson, H., Lycett, G.J., Vontas, J., 2016. Cytochrome P450 associated with insecticide resistance catalyzes cuticular hydrocarbon production in *Anopheles gambiae*. P. Natl. Acad. Sci. USA 113, 9268–9273.

Barnard, K., Jeanrenaud, A.C.S.N., Brooke, B.D., Oliver, S. v., 2019. The contribution of gut bacteria to insecticide resistance and the life histories of the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae). Sci. Rep. 9, 9117. https://doi.org/10.1038/s41598-019-45499-z

Cáceres, M., Santo-Orihuela, P.L., Vassena, C.V., 2019. Evaluation of resistance to different insecticides and metabolic detoxification mechanism by use of synergist in the common bed bug (Heteroptera: Cimicidae). J. Med. Entomol. 56, 1324–1330.

Oliveira, A.P., Santana, A.S., Santana, E.D.R., Lima, A.P.S., Faro, R.R.N., Nunes, R.S., Lima, A.D., Blank, A.F., Araújo, A.P.A., Cristaldo, P.F., Bacci, L., 2017. Nanoformulation prototype of the essential oil of *Lippia sidoides* and thymol to population management of *Sitophilus zeamais* (Coleoptera: Curculionidae). Ind. Crop Prod. 107, 198–205.

Romero, A., Anderson, T.D., 2016. High levels of resistance in the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae), to neonicotinoid insecticides. J. Medical Entomology 53, 727–731.

Tak, J.H., Isman, M.B., 2015. Enhanced cuticular penetration as the mechanism for synergy of insecticidal constituents of rosemary essential oil in *Trichoplusia ni.* Sci. Rep. 5, 12690. https://doi.org/10.1038/srep12690

Werdin González, J.O., Gutiérrez, M.M., Ferrero, A.A., Fernández Band, B., 2014. Essential oils nanoformulations for stored-product pest control - characterization and biological properties. Chemosphere 100, 130–138.

Xia, X., Sun, B., Gurr, G.M., Vasseur, L., Xue, M., You, M., 2018. Gut microbiota mediate insecticide resistance in the diamondback moth, *Plutella xylostella* (L.). Front. Microbiol. 9, 25. https://doi.org/10.3389/fmicb.2018.00025

APPENDIX A. SUPPLMENTARY INFORMATION FOR CHAPTER 2

Table A.1 Properties and plant sources of essential oil components used in the study

Functional group	**Essential oil components**	**Molecular formula[a]**	**Purity (%)**	**Molecular weight[a]**	**Density (g/ml) at 25°C[a]**	**Boiling point (°C)[a]**	**Vapor pressure at 25°C (mmHg)[a]**	**Partition Coefficient (LogP)**	**Plant sources with high proportion of respective compound[c]**
Phenol	Carvacrol	$C_{10}H_{14}O$	≥ 98	150.22	0.976	236	0.0296	3.49[b]	Red thyme oil (*Thymus vulgaris* L.)/ Oregano oil (*Origanum vulgare* L.)
	Thymol	$C_{10}H_{14}O$	≥ 99	150.22	0.965	232	0.016	3.3	Red thyme/ oregano oil
	Eugenol	$C_{10}H_{12}O_2$	99	164.20	1.067	254	0.0221	2.7	Clove bud oil (*Syzygium aromaticum* L.)
Acid	Citronellic acid	$C_{10}H_{18}O_2$	98	170.25	0.923	121-122	0.005	3[b]*	Citronella oil (*Cymbopogon winterianus* Jowitt)

Alcohol	Geraniol	$C_{10}H_{18}O$	98	154.25	0.879	229-230	0.03	2.5	Rose (*Rosa* × *damascena* Mill.) /citronella oil
	Linalool	$C_{10}H_{18}O$	97	154.25	0.87	194-197	0.17	2.84	Basil oil (*Ocimum basilicum* L.)
	(–)-Terpinen-4-ol	$C_{10}H_{18}O$	≥ 95	154.25	0.934	209[b]	0.04[b]	3.26	Tea tree oil (*Melaleuca alternifolia*)
Hydrocarbon	α-Pinene	$C_{10}H_{16}$	98	136.23	0.858	155-156	4.75	4.48	Pine tree oil (*Pinus cembra* L.)
	R (+)-Limonene	$C_{10}H_{16}$	97	136.23	0.844[b]	176-177	1.98[b]	4.2	Citrus oil (*Citrus nobilis*)
Ketone	Menthone	$C_{10}H_{18}O$	97	154.25	0.896	85-88	0.28[b]	3.05	Peppermint oil (*Mentha piperita* L.)
	(±)-Camphor	$C_{10}H_{16}O$	96	152.24	0.992	204	0.65	2.38[b]	Camphor oil (*Cinnamomum camphora* L.)
Ether	Eucalyptol	$C_{10}H_{18}O$	99	154.25	0.921	176-177	1.9	2.74[b]	Eucalyptus oil (*Alpinia kwangsiensis*)
Aldehyde	trans-Cinnamaldehyde	C_9H_8O	99	132.16	1.05	250-252	0.0289[b]	1.9	Cinnamon oil *(Cinnamomum zeylanicum* Blum)
	(±)-Citronellal	$C_{10}H_{18}O$	≥ 95	154.25	0.857	207	0.25	3.62	Citronella oil

Phenyl propanoid	Methyl eugenol	$C_{11}H_{14}O_2$	98	178.23	1.036	242-255	0.012	3.03	Basil oil
Chemical class	**Positive controls**								
Pyrethroids Type I	Bifenthrin	$C_{23}H_{22}ClF_3O_2$	98	422.87	1.212	453.2	< 1	6	
Organophosphate	Dichlorvos (DDVP)	$C_4H_7Cl_2O_4P$	≤100	220.98	1.41	234.1	0.0158	1.43	

[a]Properties presented in chemical "Safety Data Sheet" by respective company. [b]PubChem Open Chemistry database (https://pubchem.ncbi.nlm.nih.gov/). [c]Dr. Duke's Phytochemical and Ethnobotanical Databases (https://phytochem.nal.usda.gov). *refers computational prediction

Table A. 2 Relative median potency comparisons for topical LD50 estimates for essential oil components and bifenthrin.

Baseline essential oil components or insecticides	**Essential oil components or insecticides for comparison**	**Relative median potency ratios [(I)]**	**Confidence intervals (CIs)**	
			Lower limit	**Upper limit**
Carvacrol	Thymol	0.912	0.651	1.27
	Citronellic acid*	0.535	0.373	0.749
	Eugenol*	0.48	0.333	0.672
	Geraniol*	0.446	0.297	0.643
	α-Pinene*	0.351	0.229	0.512
	R (+)-Limonene*	0.284	0.18	0.422
	Linalool*	0.228	0.14	0.347
	Eucalyptol*	0.211	0.128	0.323
	(–)-Terpinen-4-ol*	0.203	0.118	0.323
	trans-Cinnamaldehyde*	0.173	0.103	0.27
	Menthone*	0.159	0.092	0.255
	(±)-Citronellal*	0.118	0.066	0.194
	(±)-Camphor*	0.044	0.02	0.083
	Methyl eugenol*	0.066	0.033	0.117
	Bifenthrin*	72076.002	8145.785	1058437.2
Thymol	Citronellic acid*	0.587	0.415	0.813
	Eugenol*	0.527	0.371	0.729
	Geraniol*	0.489	0.331	0.696
	α-Pinene*	0.385	0.257	0.553
	R (+)-Limonene*	0.311	0.202	0.456
	Linalool*	0.25	0.156	0.374
	Eucalyptol*	0.231	0.144	0.348
	(–)-Terpinen-4-ol*	0.222	0.133	0.348
	trans-Cinnamaldehyde*	0.19	0.116	0.291
	Menthone*	0.175	0.103	0.275
	(±)-Citronellal*	0.13	0.074	0.209
	(±)-Camphor*	0.048	0.023	0.089
	Methyl eugenol*	0.072	0.037	0.125
	Bifenthrin*	79060.069	8753.877	1191005.6
Citronellic acid	Eugenol	0.897	0.658	1.217
	Geraniol	0.832	0.594	1.149
	α-Pinene*	0.655	0.464	0.907

	R (+)-Limonene*	0.529	0.368	0.74
	Linalool*	0.425	0.289	0.601
	Eucalyptol*	0.393	0.265	0.56
	(–)-Terpinen-4-ol*	0.379	0.245	0.559
	trans-Cinnamaldehyde*	0.324	0.215	0.462
	Menthone*	0.298	0.191	0.439
	(±)-Citronellal*	0.221	0.138	0.331
	(±)-Camphor*	0.081	0.042	0.141
	Methyl eugenol*	0.123	0.07	0.198
	Bifenthrin*	134603.46	13625.857	2266382.6
Eugenol	Geraniol	0.928	0.669	1.275
	α-Pinene	0.73	0.523	1.004
	R (+)-Limonene*	0.59	0.415	0.818
	Linalool*	0.474	0.327	0.663
	Eucalyptol*	0.439	0.3	0.617
	(–)-Terpinen-4-ol*	0.422	0.277	0.616
	trans-Cinnamaldehyde*	0.361	0.244	0.508
	Menthone*	0.332	0.217	0.483
	(±)-Citronellal*	0.246	0.156	0.365
	(±)-Camphor*	0.091	0.048	0.156
	Methyl eugenol*	0.137	0.08	0.218
	Bifenthrin	150100.54	14971.335	2574398.3
Geraniol	α-Pinene	0.787	0.562	1.095
	R (+)-Limonene*	0.636	0.449	0.887
	Linalool*	0.511	0.356	0.714
	Eucalyptol*	0.473	0.327	0.664
	(–)-Terpinen-4-ol*	0.455	0.303	0.661
	trans-Cinnamaldehyde*	0.389	0.268	0.543
	Menthone*	0.358	0.238	0.517
	(±)-Citronellal*	0.265	0.172	0.388
	(±)-Camphor*	0.098	0.054	0.164
	Methyl eugenol*	0.148	0.088	0.23
	Bifenthrin*	161726.72	15431.96	2928808.8
α-Pinene	R (+)-Limonene	0.808	0.576	1.123
	Linalool*	0.649	0.459	0.899
	Eucalyptol*	0.601	0.422	0.836
	(–)-Terpinen-4-ol*	0.578	0.391	0.832
	trans-Cinnamaldehyde*	0.494	0.347	0.681

	Menthone*	0.454	0.308	0.649
	(±)-Citronellal*	0.337	0.224	0.485
	(±)-Camphor*	0.124	0.07	0.205
	Methyl eugenol*	0.188	0.115	0.288
	Bifenthrin*	205507.34	19101.794	3844703.4
R (+)-Limonene	Linalool	0.803	0.576	1.107
	Eucalyptol	0.743	0.53	1.029
	(–)-Terpinen-4-ol	0.715	0.491	1.022
	trans-Cinnamaldehyde*	0.612	0.439	0.832
	Menthone*	0.562	0.389	0.793
	(±)-Citronellal*	0.417	0.284	0.59
	(±)-Camphor*	0.154	0.089	0.248
	Methyl eugenol*	0.232	0.147	0.348
	Bifenthrin*	254236.23	22750.683	4985156.3
Linalool	Eucalyptol	0.925	0.674	1.269
	(–)-Terpinen-4-ol	0.89	0.627	1.257
	trans-Cinnamaldehyde	0.761	0.564	1.017
	Menthone*	0.7	0.498	0.971
	(±)-Citronellal*	0.519	0.367	0.717
	(±)-Camphor*	0.191	0.116	0.298
	Methyl eugenol*	0.289	0.191	0.42
	Bifenthrin*	316592.76	27008.329	6588016.8
Eucalyptol	(–)-Terpinen-4-ol	0.962	0.675	1.365
	trans-Cinnamaldehyde	0.823	0.607	1.104
	Menthone	0.757	0.537	1.053
	(±)-Citronellal*	0.561	0.396	0.778
	(±)-Camphor*	0.207	0.125	0.323
	Methyl eugenol*	0.312	0.206	0.455
	Bifenthrin*	342200.21	29059.56	7160424.7
(–)-Terpinen-4-ol	trans-Cinnamaldehyde	0.855	0.615	1.184
	Menthone	0.786	0.545	1.126
	(±)-Citronellal*	0.583	0.404	0.828
	(±)-Camphor*	0.215	0.13	0.339
	Methyl eugenol*	0.325	0.212	0.48
	Bifenthrin*	355549.9	29407.712	7679913.1
trans-Cinnamaldehyde	Menthone	0.919	0.676	1.248
	(±)-Citronellal*	0.682	0.503	0.913
	(±)-Camphor*	0.251	0.16	0.377

	Methyl eugenol*	0.38	0.263	0.531
	Bifenthrin*	415757.78	33831.377	9177737.7
Menthone	(±)-Citronellal	0.742	0.528	1.031
	(±)-Camphor*	0.273	0.17	0.42
	Methyl eugenol*	0.413	0.278	0.595
	Bifenthrin*	452261.46	36449.519	10093068
(±)-Citronellal	(±)-Camphor*	0.369	0.238	0.553
	Methyl eugenol*	0.557	0.387	0.786
	Bifenthrin*	609884.82	47124.797	14343916
(±)-Camphor	Methyl eugenol*	1.511	1.001	2.311
	Bifenthrin*	1654624.3	110713.93	46402083
Methyl eugenol	Bifenthrin*	1095413	78031.01	28471929

Asterisks (*) indicate components which show significantly higher or lower toxicity in comparison to the baseline essential oil components or insecticides. The LD_{50} estimates for components or insecticides are significantly different ($P < 0.05$) from the baseline compounds if confidence intervals for median potency ratios do not overlap with the value 1 (References 66 and 67). [(I)] Relative median potency ratios of < 1 indicate essential oil components or insecticides that are less toxic than the baseline compounds they are compared with, whereas ratios >1 indicate comparatively higher toxicity (References 66 and 67).

Table A. 3 Relative median potency comparisons for fumigant LC50 estimates for essential oil components and dichlorvos.

Baseline essential oil components or insecticides (I)	**Essential oil components or insecticides for comparison (I)**	**Relative median potency ratios (II)**	**Confidence intervals (CIs)**	
			Lower limit	**Upper limit**
Thymol	Linalool*	0.430	0.195	0.810
	Carvacrol*	0.397	0.165	0.786
	(±)-Camphor*	0.152	0.039	0.393
	Menthone*	0.133	0.035	0.332
	Eucalyptol*	0.123	0.031	0.310
	(–)-Terpinen-4-ol*	0.047	0.007	0.162
	trans-Cinnamaldehdye*	0.055	0.009	0.177
	R (+)-Limonene*	0.041	0.006	0.142
	α-Pinene*	0.050	0.008	0.168
	(±)-Citronellal*	0.018	0.000	0.128
	DDVP*	445.222	44.138	16552.345
Linalool	Carvacrol	0.924	0.487	1.683
	(±)-Camphor*	0.353	0.128	0.752
	Menthone*	0.309	0.118	0.621
	Eucalyptol*	0.286	0.107	0.573
	(–)-Terpinen-4-ol*	0.110	0.025	0.294
	trans-Cinnamaldehdye*	0.127	0.033	0.322
	R (+)-Limonene*	0.096	0.022	0.256
	α-Pinene*	0.116	0.027	0.301
	(±)-Citronellal*	0.042	0.002	0.214
	DDVP*	1034.877	79.023	58505.699
Carvacrol	(±)-Camphor*	0.382	0.147	0.803
	Menthone*	0.335	0.136	0.659
	Eucalyptol*	0.310	0.124	0.606
	(–)-Terpinen-4-ol*	0.119	0.030	0.305
	trans-Cinnamaldehdye*	0.138	0.038	0.335
	R (+)-Limonene*	0.104	0.026	0.265
	α-Pinene*	0.125	0.032	0.312
	(±)-Citronellal*	0.064	0.012	0.193
	DDVP*	1120.562	79.514	70970.060
(±)-Camphor	Menthone	0.877	0.442	1.718
	Eucalyptol	0.811	0.416	1.531

	(–)-Terpinen-4-ol*	0.311	0.118	0.658
	trans-Cinnamaldehdye*	0.361	0.147	0.741
	R (+)-Limonene*	0.272	0.104	0.560
	α-Pinene*	0.328	0.130	0.658
	(±)-Citronellal*	0.116	0.015	0.480
	DDVP*	2935.675	143.366	334383.574
Menthone	Eucalyptol	0.925	0.532	1.578
	(–)-Terpinen-4-ol*	0.355	0.148	0.689
	trans-Cinnamaldehdye*	0.412	0.186	0.773
	R (+)-Limonene*	0.310	0.132	0.583
	α-Pinene*	0.375	0.165	0.684
	(±)-Citronellal*	0.134	0.019	0.421
	DDVP*	3348.451	162.751	387920.248
Eucalyptol	(–)-Terpinen-4-ol*	0.383	0.171	0.711
	trans-Cinnamaldehdye*	0.445	0.214	0.800
	R (+)-Limonene*	0.336	0.153	0.600
	α-Pinene*	0.405	0.191	0.702
	(±)-Citronellal*	0.144	0.023	0.427
	DDVP*	3618.574	170.936	439980.384
(–)-Terpinen-4-ol	trans-Cinnamaldehdye	1.162	0.625	2.252
	R (+)-Limonene	0.875	0.487	1.547
	α-Pinene	1.056	0.605	1.828
	(±)-Citronellal*	0.378	0.105	0.944
	DDVP*	9438.613	319.082	1939854.938
trans-Cinnamaldehdye	R (+)-Limonene	0.753	0.406	1.317
	α-Pinene	0.909	0.507	1.551
	(±)-Citronellal*	0.327	0.08	0.84
	DDVP*	8123.118	294.933	1491225.139
R (+)-Limonene	α-Pinene	1.206	0.749	1.960
	(±)-Citronellal*	0.435	0.137	0.991
	DDVP*	10784.277	357.170	2301043.075
α-Pinene	(±)-Citronellal*	0.355	0.112	0.790
	DDVP*	8939.013	301.710	1854427.110
(±)-Citronellal	DDVP*	17610.837	503.306	4696063.162

Asterisks (*) indicate components which show significantly higher or lower toxicity in comparison to the baseline essential oil components or insecticides. The LC_{50} estimates for components or insecticides are significantly different ($P < 0.05$) from the baseline compounds if confidence intervals for median potency ratios do not overlap with the value 1 (References 66 and 67). [(I)] Relative median potency ratios of < 1 indicate essential oil components or insecticides that are less toxic than the baseline compounds they are compared with, whereas ratios >1 indicate comparatively higher toxicity (References 66 and 67).

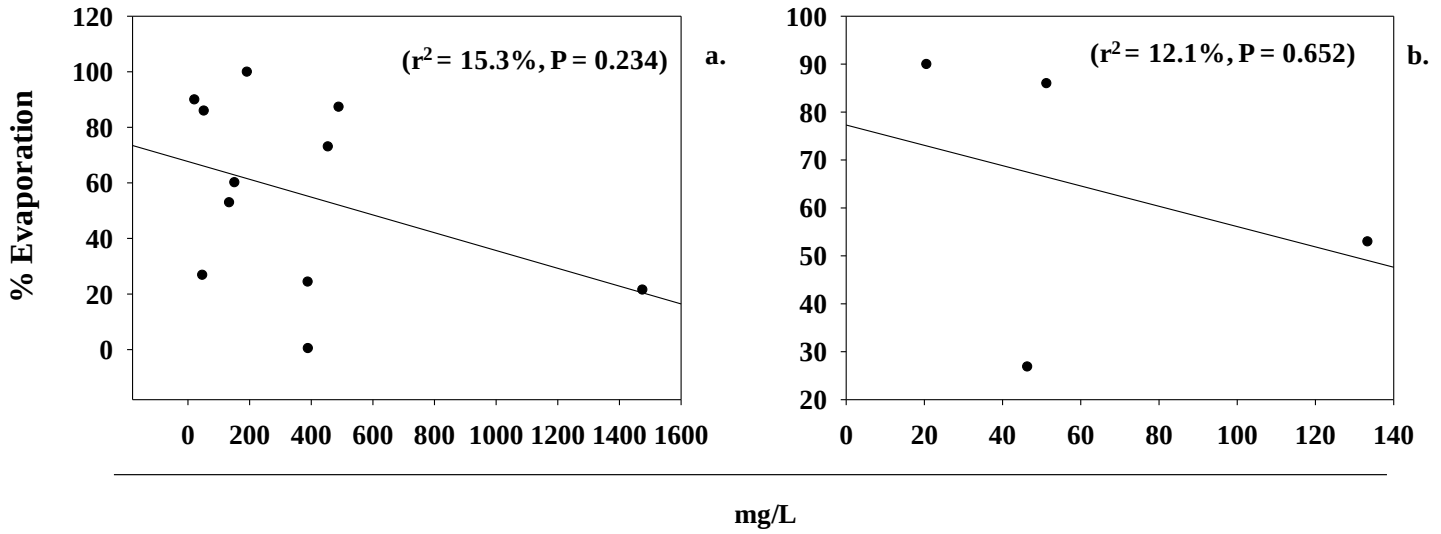


Figure A. 1 Regression analysis of fumigant LC50 values for essential oil components and their corresponding percent evaporation levels. Correlation coefficients (r2) and P-values were determined by regression analysis. (a) Regression analysis for 11 compounds for which we were able to determine LC50 values as shown in Table 2. There was no significant correlation between fumigant LC50 values and percent evaporation levels (P >0.05). The least toxic compounds (geraniol, citronellic acid, eugenol and methyl eugenol) were excluded from this analysis because their LC50 estimates were not determinable. (b) Regression analysis for the four most toxic fumigant compounds shown in Table 2 (thymol, carvacrol, linalool, and (±)-camphor. There was no significant correlation between fumigant LC50 values of these four most toxic compounds and their percent evaporation data (P >0.05).

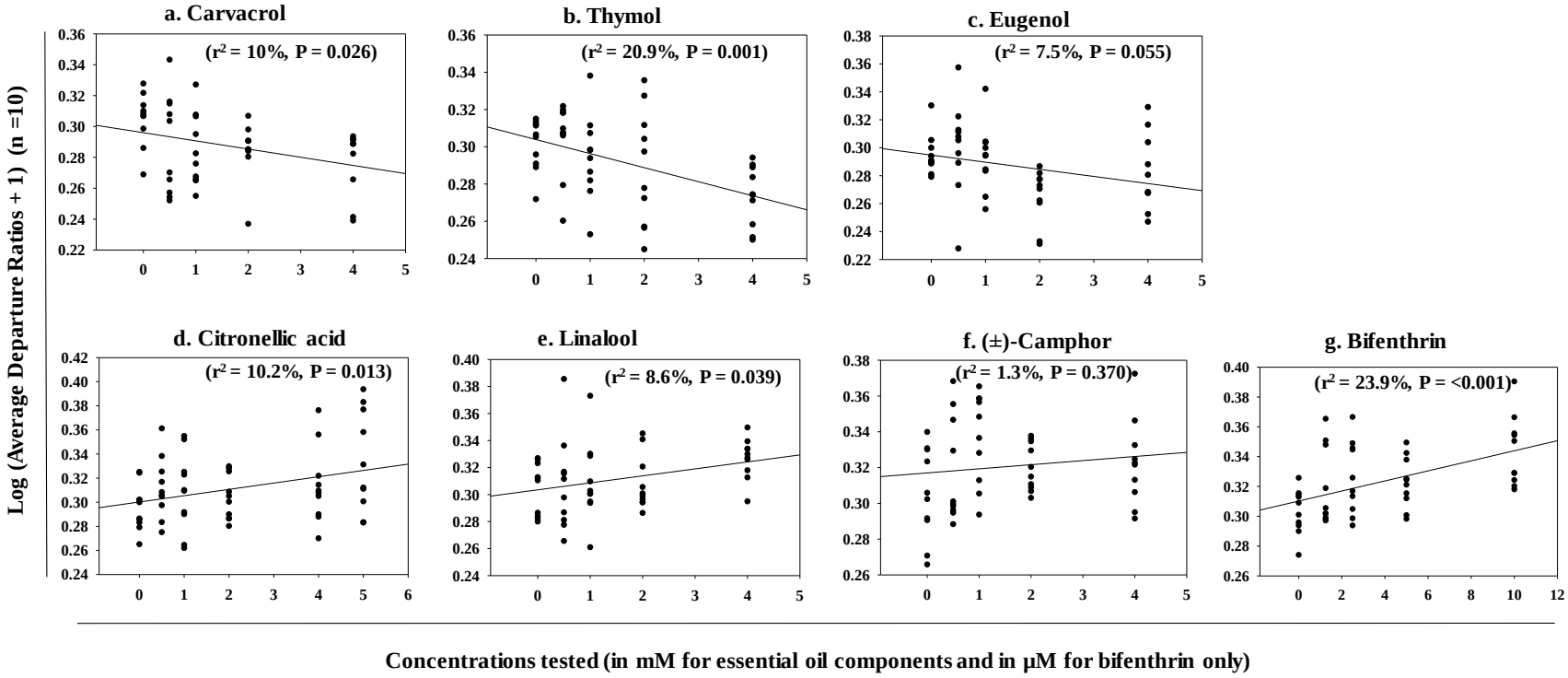


Figure A. 2 Regression analysis between concentrations of essential oil components or bifenthrin versus log transformed average departure ratio data from electrophysiology experiments. Correlation coefficients (r2) and P-values were determined by regression analysis. There was significant correlation between concentrations of carvacrol (a), thymol (b), citronellic acid (d), linalool (e) and bifenthrin (f) and their log transformed average departure ratio data ($P < 0.05$), thus indicating concentration-dependent effect of these compounds on the bed bug nervous system. However, eugenol (c) and (±)-camphor (f) concentrations were not correlated with log transformed average departure ratios ($P > 0.05$), likely because of their effects on the nervous system activity were biphasic i.e. pronounced effect at intermediate concentrations in comparison to lower or higher concentrations.

APPENDIX B. SUPPLMENTARY INFORMATION FOR CHAPTER 4

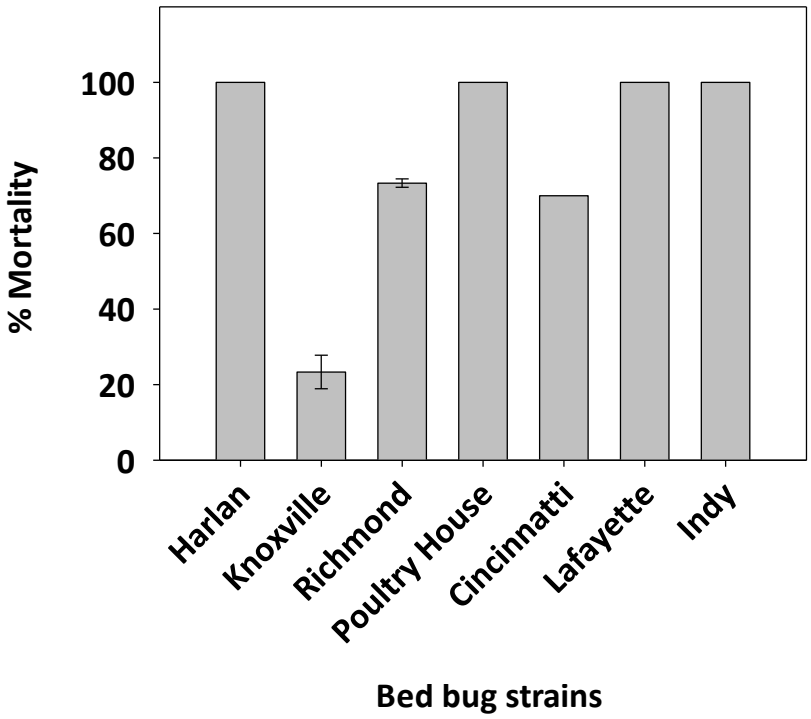


Figure B. 1 Initial screening of deltamethrin resistance status in different bed bug (Cimex lectularius L.) field strains. The Knoxville strain exhibited highest level of resistance (less than 25% mortality) in topical application bioassays at a dose of 10 mg/mL deltamethrin (volume 0.5 µL). Three replications (ten insects per rep, n = 30) were performed for each bed bug field strain and the susceptible Harlan strain.

Table B. 1 Chemical composition of different essential oils used in this study as determined by GC-MS analysis.

Essential oils	Chemical constituents	Retention time (min)	% of total*
Oregano oil	1 R-α-pinene	4.42	2.37
	(-)-β-pinene	4.98	1.18
	α-Terpinen	5.28	1.14
	O-cymene	5.36	22.73
	γ-Terpinen	5.72	7.97
	Linalyl aminobenzoate	6.11	2.24
	Thymol	7.98	3.41
	Carvacrol	8.10	56.38
Thyme oil	β -pinene	4.99	1.05
	α-Terpinen	5.29	1.31
	m-cymene	5.37	27.06
	γ-Terpinen	5.72	10.79
	Linalyl O-aminobenzoate	6.11	4.28
	(-)-Borneol	6.85	1.74
	1-Terpinen-4-ol	6.95	1.0
	Thymol	7.99	45.34
	Carvacrol	8.08	3.20
	Ledene oxide-(II)	17.90	1.13
	Eugenol acetate	18.46	1.14
Clove oil	Eugenol	8.64	89.87
	Caryophyllene	9.26	6.25
	Eugenol acetate	10.05	3.22
Coriander oil	1R-α-Pinene	4.43	8.81
	Camphene	4.59	2.43
	β-cymene	5.36	1.15
	β -terpinyl acetate	5.41	4.40
	γ-Terpinen	5.72	8.27
	Linalool	6.12	66.26
	(+)-Camphor	6.64	5.21
	β-myrcene	8.80	1.05
Geranium oil	Linalyl O-aminobenzoate	6.11	8.22
	Rose oxide	6.24	2.33
	Isomenthone-3-one	6.82	8.09
	(R)-(+)-β-citronellol	7.38	37.83
	Cis-Geraniol	7.63	15.01
	Citronyl formate	7.83	11.01
	Myrcene	8.088	3.28
	β-Bourbanene	8.94	1.69
	Caryophyllene	9.26	1.33
	(±)-Cadinene	10.91	1.60

*Relative abundance of essential oil constituents were identified using GC-MS analysis according to Gaire et al. (2017) with slight modification in dilution of essential oils. GC-MS analysis was carried out in Agilent 6890 N GC (Agilent Technologies, Santa Clara, CA) coupled with 5975B mass selective detector (Agilent Technologies). The column specifications and analysis procedures were same. Essential oils were diluted to 1:10,000 in carbon disulfide (Sigma-Aldrich, St. Louis, MO). Compounds were identified using NIST library (National Institute of Standard and Technology).

Table B. 2 Relative median potency comparisons of topical toxicity data of essential oils and EcoRaider on Knoxville and Harlan bed bug (C. lectularius) strains.

Strains	Baseline essential oils	Essential oils for comparison	Relative median potency ratios [I]	Confidence intervals (CIs)	
				Lower limit	Upper limit
Knoxville	Thyme oil	Oregano oil	0.974	0.593	1.565
		Clove oil	0.994	0.594	1.605
		Coriander oil*	0.404	0.174	0.714
		Geranium oil*	0.386	0.174	0.664
		EcoRaider*	0.287	0.111	0.535
	Oregano oil	Clove oil	1.021	0.604	1.702
		Coriander oil*	0.414	0.183	0.731
		Geranium oil*	0.396	0.183	0.681
		EcoRaider*	0.295	0.117	0.546
	Clove bud oil	Coriander oil*	0.406	0.183	0.712
		Geranium oil*	0.388	0.182	0.664
		EcoRaider*	0.289	0.118	0.530
	Coriander oil	Geranium oil	0.957	0.611	1.519
		EcoRaider	0.712	0.413	1.108
	Geranium oil	EcoRaider	0.744	0.454	1.134
Harlan	Thyme oil	Oregano oil	1.072	0.708	1.608
		Clove oil*	0.428	0.238	0.674
		Coriander oil*	0.370	0.186	0.616
		Geranium oil*	0.241	0.109	0.427
		EcoRaider*	0.310	0.149	0.533
	Oregano oil	Clove oil*	0.400	0.227	0.621
		Coriander oil*	0.345	0.178	0.567
		Geranium oil*	0.225	0.104	0.392
		EcoRaider*	0.290	0.142	0.490
	Clove bud oil	Coriander oil	0.863	0.561	1.277
		Geranium oil*	0.563	0.345	0.845
		EcoRaider	0.742	0.458	1.080
	Coriander oil	Geranium oil*	0.652	0.415	0.979
		EcoRaider	0.840	0.545	1.268
	Geranium oil	EcoRaider	0.840	0.545	1.268

Asterisks (*) indicate essential oils which show significantly higher or lower toxicity in comparison to the baseline essential oils or essential oil with highest level of toxicity (i.e., lowest LD_{50} value). The LD_{50} estimates for different oils are significantly different ($P < 0.05$) from the baseline oils if the confidence intervals for median potency ratios do not overlap with the value 1 (Robertson et al., 2007; Gaire et al., 2019).

[I] Relative median potency ratios of < 1 indicate essential oils that are less toxic than the baseline essential oils they are compared with, whereas ratios >1 indicate comparatively higher toxicity (Robertson et al., 2007; Gaire et al., 2019).

APPENDIX C. EFFICACY OF BINARY MIXTURE OF CARVACROL OR EUGENOL AND DELTAMETHRIN IN THE CINCINNATI STRAIN

Summary:

In my fifth chapter, I have found that plant essential oils and their major constituents increased deltamethrin toxicity in the highly resistant Knoxville strain of common bed bugs. To determine if this synergistic interaction would occur in other field strains of bed bugs that show resistance to deltamethrin, I conducted topical bioassays with binary mixtures of carvacrol + deltamethrin and eugenol + deltamethrin against the Cincinnati strain of common bed bugs. This strain was selected for conducting bioassays because it was the second most resistant strain in preliminary deltamethrin resistance screening bioassays (Fig. B.1). The mixture of carvacrol (at Knoxville LD_{25}) + deltamethrin (1 mg/mL) and eugenol (at Knoxville LD_{25}) + deltamethrin (1 mg/mL) killed 100% Cincinnati strain bed bugs (Fig. C.1) which was significantly greater than either of the individual compounds.

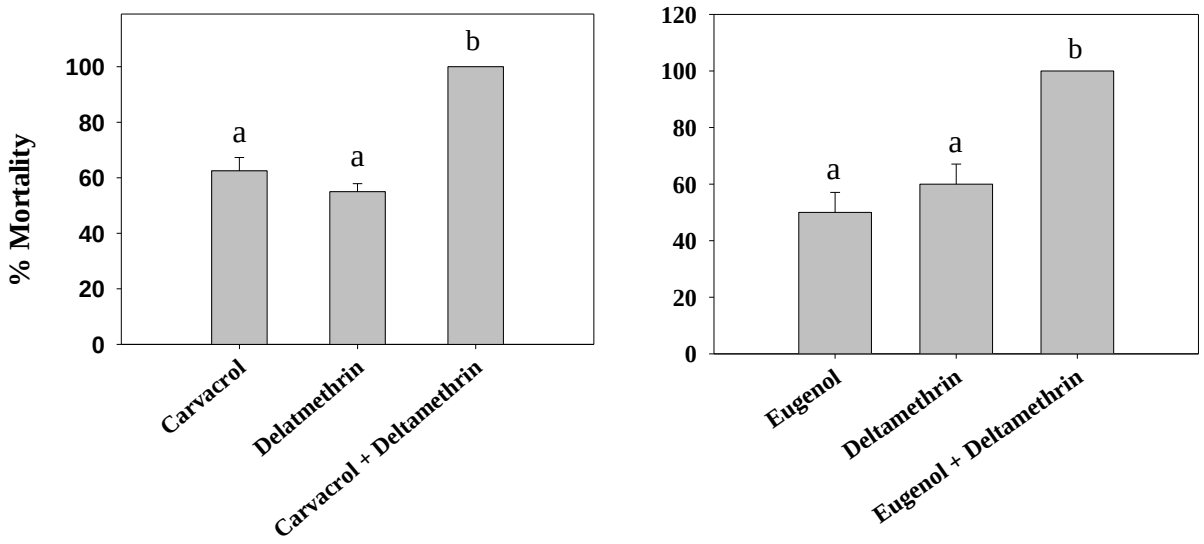


Figure C. 1 Percentage mortality observed in the Cincinnati strain treated with carvacrol, eugenol, deltamethrin and their binary mixtures. Within each graph bars connected with different letters indicate significantly different mortality response at the $P < 0.05$ statistical significance level (ANOVA, Tukey HSD test). Both carvacrol + deltamethrin and eugenol + deltamethrin mixtures caused significantly higher mortality ($P < 0.05$) in the deltamethrin-resistant Cincinnati strain.

APPENDIX D. INTERACTION OF MIXTURE OF ESSENTIAL OIL COMPONENTS IN BED BUGS

Summary:

The methodology for preparation of binary insecticide mixtures is described in chapter 3. The binary mixtures of neuroinhibitory components; carvacrol, thymol and eugenol resulted in synergistic interactions in the susceptible Harlan strain of the common bed bug. Citronellic acid and (±)-camphor led to somewhat concentration-dependent increase in nervous system activity of Harlan bed bugs, whereas linalool caused statistically significant neuroexcitation (Chapter 2). Therefore, I evaluated the efficacy of binary and tertiary mixtures of citronellic acid, (±)-camphor, and linalool against bed bugs. All mixtures except linalool + citronellic acid resulted synergistic interactions. Multiple mechanisms such as neurological potentiation and penetration enhancement might have led to synergistic toxicity interactions between essential oil compounds in the Harlan strain.

Table D.1 Toxicity interactions between binary and tertiary mixtures of essential oil components against the susceptible Harlan strain.

Essential oil component binary and tertiary mixtures	**n**	**LD_{50}[a], mg/insect (CI 95%)**	**LD_{50}[b], mg/insect**	**Ratio[c] (Interaction)**
Carvacrol + Thymol	240	0.035 (0.031 – 0.039)	0.06	1.71 (Synergistic)
Carvacrol + Eugenol	210	0.034 (0.031 - 0.039)	0.0795	2.33 (Synergistic)
Thymol + Eugenol	210	0.042 (0.039 – 0.047)	0.084	2.01 (Synergistic)
Linalool + Citronellic acid	300	0.158 (0.132 – 0.199)	0.161	1.01 (Additive)
Linalool + (±)-Camphor	210	0.200 (0.178 – 0.231)	0.627	3.135 (Synergistic)
Citronellic acid + (±)-Camphor	240	0.173 (0.146 – 0.213)	0.564	3.26 (Synergistic)
Citronellic acid + (±)-Camphor + Linalool	240	0.0753 (0.063 – 0.087)	0.3432	4.55 (Synergistic)

[a]Observed LD_{50}

[b]Theoretical LD_{50} (refer chapter 3 for details on data analysis)

[c]See chapter 3 for interaction ratio calculations

VITA

EDUCATION

2016-present **PhD Candidate, Entomology,** Purdue University, West Lafayette, IN
- Dissertation: Toxicology of plant essential oils on bed bugs (Hemiptera: Cimicidae) *(Advisor: Dr. Ameya Gondhalekar)*

2014-2016 **MS, Entomology,** New Mexico State University, Las Cruces, NM
- Thesis: Toxicity and repellency of essential oils on the Turkestan cockroach, (Blattodea: Blattidae) *(Advisor: Dr. Alvaro Romero)*

2009-2013BS, **Agriculture,** Tribhuvan University, Chitwan, Nepal

EXPERIENCE

2016-2020 **Graduate Research Assistant,** Department of Entomology, Purdue University, West Lafayette, IN

2016-2020 **Graduate Teaching Assistant,** Purdue Integrated Pest Management Correspondence Courses, Purdue University, West Lafayette, IN

2017-2020 **Graduate Teaching Assistant, Neurophysiology demonstration**
ENTM 6100: Toxicology of Insecticides, (2017, 19, 20), ENTM 35300: Insecticides and Environment (2019, 20)

2014-2016 **Graduate Research Assistant,** Department of Entomology, Plant Pathology and Weed Science (EPPWS), New Mexico State University (NMSU), NM

2016 **Graduate Teaching Assistant,** EPPWS, NMSU, Las Cruces, NM
EPWS 330: Economic Entomology and EPWS 420: Parasitology

2013-2014 **IPM Facilitator,** "3 Month Organic Offseason Vegetable Producer Training", Gothatar, Kathmandu and "IPM School, District IPM Farmers Association, Chitwan, Nepal"

AWARDS AND HONORS

2019 **Outstanding Doctoral Student Award,** Department of Entomology, Purdue University, West Lafayette, IN

2019 **Elanco Animal Health Graduate Endowment Travel Award,** Department of Entomology, Purdue University, West Lafayette, IN

2019 **Travel Award, Purdue Graduate Student Government,** Purdue University, West Lafayette, IN

2019 **Pest Management Foundation Scholarship,** National Pest Management Association

2019 **Bilsland Dissertation Fellowship,** The Graduate School, Purdue University, West Lafayette, IN

2019 **Norm Ehmann/Univar Scholarship,** Department of Entomology, Purdue University, West Lafayette, IN

2018 **Outstanding Service by a Student Award,** Department of Entomology, Purdue University, West Lafayette, IN

2018 **First Place, MUVE-Gradate level poster competition,** Entomological Society of America Annual Meeting, Vancouver, Canada

2018 **MUVE Shripat Kamble Urban Entomology Graduate Student Award for Innovative Research,** Entomological Society of America Annual Meeting, Vancouver, Canada

2018 **Megha Parajulee SONE Student Award for Academic Excellence,** Society of Overseas Nepalese Entomologists Annual Meeting, ESA, Vancouver, Canada

2018 **PhD Scholarship Award,** National Conference on Urban Entomology, Cary, NC

2018 **Travel Award,** The Graduate School, Purdue University, West Lafayette, IN

2018 **BASF Professional Pest Control Scholarship,** Department of Entomology, Purdue University, West Lafayette, IN

2017 **Second Place, Gradate level poster competition,** Entomological Society of America Annual Meeting, Denver, CO

2017 **MUVE Student Travel Award,** Entomological Society of America Annual Meeting, Denver, CO

2017 **Pi Chi Omega Founder's Scholarship,** Pi Chi Omega-The Professional Pest Management Fraternity

2017 **Oser Family Scholarship,** Department of Entomology, Purdue University, West Lafayette, IN

2016 **MS Scholarship Award,** National Conference on Urban Entomology, Albuquerque, NM

2016 **Travel Award for Prospective Ph.D. Student,** Department of Entomology, Purdue University, West Lafayette, IN

2016 **Dean's Awards of Excellence-Graduate**, College of Agriculture, Consumer and Environmental Sciences, NMSU, Las Cruces, NM

2015 **Salopek Foundation Endowed Scholarship,** EPPWS, NMSU, Las Cruces, NM

2012, 13 **University Merit Scholarship,** Tribhuvan University, Institute of Agriculture and Animal Sciences (IAAS), Rampur, Chitwan, Nepal

2011 **Third Place, District Level Inter Campus Elocution Competition,** Narayanghat Jaycees, Chitwan, Nepal

2010 **First Place, District Level Inter Campus Elocution Competition**, Birendra Multiple Campus, Chitwan, Nepal

2010 **First Place, Poem Competition,** Tribhuvan University, Institute of Agriculture and Animal Sciences (IAAS), Rampur, Chitwan, Nepal

RESEARCH

Grants:

1. Defining target sites for plant essential oil constituents used in agricultural, veterinary and structural insect pest management (PI: Ameya Gondhalekar, **CO-PI's: Sudip Gaire** and Wei Zheng) (Purdue University AgSEED Grant $50,000) (March 2019-Feb 2020) https://ag.purdue.edu/agseed/Pages/project.aspx?pid=96 ***(This proposal was also***

awarded by 2018 MUVE Shripat Kamble Urban Entomology Graduate Student Award for Innovative Research, ESA, Vancouver, Canada)

Disclosure (for Provisional patent):

1. **Gaire, S.** and A. D. Gondhalekar. Plant essential oils as synergists for the control for deltamethrin-resistant bed bugs **(Declined in Fall 2019)**

Peer-reviewed Publications:

1. Feston, J.*, **S. Gaire***, M. Fardisi, L. Mason and A. D. Gondhalekar. 2020. Determining baseline toxicity of ozone against an insecticide susceptible strain of the common bed bug, *Cimex lectularius* L. under laboratory conditions. Pest Management Science. (In Press)
2. Gaire, S., M. E. Scharf, and A. D. Gondhalekar. 2020. Synergistic toxicity interactions between plant essential oil components against the common bed bug (*Cimex lectularius* L.). Insects 11, 133. https://doi.org/10.3390/insects11020133
3. **Gaire, S.**, M. E. Scharf, and A. D. Gondhalekar. 2019. Toxicity and neurophysiological impacts of plant essential oil components on bed bugs (Cimicidae: Hemiptera). Scientific Reports 9 (1), 3961. https://doi.org/10.1038/s41598-019-40275-5 (*News outlets on article: Purdue University, Morning Ag Clips, Phys.org many others).*
4. **Gaire, S.,** M. O'Connell, F. O. Holguin, A. Amatya, S. Bundy and A. Romero. 2017. Insecticidal properties of essential oils and some of their constituents on the Turkestan cockroach (Blattodea: Blattidae). Journal of Economic Entomology 110 (2): 584-592.

Manuscript in preparation/ In Revision

1. **Gaire, S.** and A. Romero. **In Revision.** Comparative efficacy of residual insecticides against the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae) on different substrates. (**Insects**)
2. **Gaire, S.**, C. D. Lewis, W. Booth, M. E. Scharf, W. Zheng, M. D. Ginzel, and A. D. Gondhalekar. **To be submitted soon.** Bed bugs, *Cimex lectularius* L., exhibiting metabolic and target site deltamethrin resistance are susceptible to plant essential. (**Pesticide Biochemistry and Physiology**)
3. **Gaire, S.,** W. Zheng, M. E. Scharf and A. D. Gondhalekar. **To be submitted soon.** Plant essential oils synergized the deltamethrin on field collected bed bugs (*Cimex lectularius* L.) by inhibiting the cytochrome P450. **(Pesticide Biochemistry and Physiology)**

Side research projects:

1) Efficacy of nano-formulated essential oils against bed bugs (Team: **S. Gaire**, P. Vega, A. Gondhalekar, N. Mosier)
2) Population dynamics of bed bugs in caged free poultry house (Team: A. Gondhalekar, A. Ashbrook**, S. Gaire**).
3) Bed bugs effects on hen welfare and productivity in cage free housing systems (Team: M. Erasmus, Y. Dong, K. VanDeWater, A. Ashbrook, **S. Gaire** and A. Gondhalekar).

Industry insecticides/methods efficacy projects involved:

1) Efficacy of newly formulated baits on cockroaches in low income housing (Field: South Bend, Indianapolis) (more than 10 visits).
2) Identifying metabolism triggers in bed bugs with Phantom.
3) Identifying variation in feeding of bed bugs and its effect on its mortality.
4) Secondary mortality in bed bugs with chlorfenapyr.
5) Field evaluations of bed bugs traps.
6) More than seven laboratories based residual bioassays on cockroaches and bed bugs.

Books, proceedings and extension articles:

1. **Gaire, S. 2014.** Community Participation in Agriculture Development. Chitwan Post National Daily Newspaper, Nepal.
2. **Gaire, S. 2013.** Need of Agriculture and Forestry University(AFU). Chitwan Post National Daily Newspaper, Nepal.
3. **Gaire, S. et al. 2011.** A Reference Guide for Entrance of BScAg and BVSc & AH, Revised Edition. Chitwan, Nepal.

Special skills:

Computer: Microsoft Office (Word, PowerPoint, Excel), MINITAB, SAS, SPSS, SigmaPlot.
Instruments: Ethovision, GC-MS, Electrophysiology, Spectrophotometer, Spectrofluorometer, Confocal microscopy (currently working)
Public speaking: Elocution competition winner (in Nepal), moderator at conference/program
Language: Nepali, English, Hindi (moderate)

Scientific Presentations: In total 20 presentations

Invited presentation: 2 Award talks

1. **Gaire, S**., M. E. Scharf and A. D. Gondhalekar. 2018. Toxicity and neurophysiological impacts of essential oil components on bed bug (*Cimex lectularius* L.). National Conference on Urban Entomology. Cary, NC. (**Doctoral Award paper**)
2. **Gaire, S.,** M. O'Connell, F. Omar Holguin and A. Romero. 2016. Identification of botanically-derived repellents for Turkestan cockroaches using a video tracking system. National Conference on Urban Entomology, Albuquerque, NM. **(Master of Science Award paper)**

Submitted presentations (ESA and other scientific meetings): 11 talks and 7 posters

3. **Gaire, S.,** and A. D. Gondhalekar. 2019. Efficacy of plant essential oils, deltamethrin and their mixtures against field-collected bed bugs (Cimicidae: Hemiptera). Entomological Society of America Annual Meeting, St. Louis, MO. (Oral presentation)
4. **Gaire, S.,** and A. D. Gondhalekar. 2019. Susceptibility of deltamethrin-resistant bed bugs to various plant essential oils. Ohio Valley Entomological Association Annual Forum, Lexington, KY. (Oral presentation)
5. **Gaire, S.,** M. E. Scharf and A. D. Gondhalekar. 2018. Synergistic interactions between plant essential oil components and their impacts on bed bug (Cimicidae: Hemiptera)

nervous system. Purdue Pest Management Conference, West Lafayette, IN. (Poster presentation)

6. **Gaire, S.,** M. E. Scharf and A. D. Gondhalekar. 2018. Synergistic interactions between plant essential oil components and their impacts on bed bug (Cimicidae: Hemiptera) nervous system. Entomological Society of America Annual Meeting, Vancouver, BC, Canada. (Poster presentation)
7. **Gaire, S.,** M. E. Scharf and A. D. Gondhalekar. 2018. Synergistic interactions between plant essential oil components and their impacts on bed bug (Cimicidae: Hemiptera) nervous system. Ohio Valley Entomological Association Annual Forum, Indianapolis, IN. (Oral presentation)
8. **Gaire, S.,** M. E. Scharf and A. D. Gondhalekar. 2018. Toxicity and neurophysiological impacts of essential oil components on bed bug (*Cimex lectularius* L.). Health and Disease: Science, Technology, Culture and Policy Research Poster Session, Purdue University Chapter. (Poster presentation)
9. **Gaire, S.,** M. E. Scharf and A. D. Gondhalekar. 2018. Toxicity and neurophysiological impacts of essential oil components on bed bug (*Cimex lectularius* L.*)*. The Society of Sigma Xi, Purdue University Chapter. (Poster presentation)
10. **Gaire, S.,** M. E. Scharf and A. D. Gondhalekar. 2017. Toxicity and neurophysiological impacts of essential oil components on bed bug (*Cimex lectularius* L.*)*. Entomological Society of America Annual Meeting, Denver, CO. (Poster presentation)
11. **Gaire, S.,** M. E. Scharf and A. D. Gondhalekar. 2017. Toxicity and neurophysiological impacts of essential oil components on bed bug (*Cimex lectularius* L.*)*. Ohio Valley Entomological Association Annual Forum, Columbus, MO. (Oral presentation)
12. **Gaire, S.,** M. O'Connell, F. Omar Holguin and A. Romero. 2016. Toxicity and repellency of essential oils on the Turkestan cockroach (Blattodea: Blattidae). International Congress of Entomology, Orlando, FL. (Oral presentation)
13. **Gaire, S.,** M. O'Connell, F. Omar Holguin and A. Romero. 2016. Toxicity of essential oils on the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae). National Conference on Urban Entomology. Albuquerque, NM. (Oral presentation)
14. **Gaire, S.,** M. O'Connell, F. Omar Holguin and A. Romero. 2016. Botanically derived oils can kill the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae). Graduate Research and Arts Symposium. New Mexico State University, Las Cruces, NM. (Poster)
15. **Gaire, S.,** M. O'Connell, F. Omar Holguin and A. Romero. 2016. Toxicity and repellency of essential oils on the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae), Eighth International Conference, Nepalese Students Association, New Mexico State University, Las Cruces, NM. (Oral presentation)
16. **Gaire, S.,** M. O'Connell, F. Omar Holguin and A. Romero. 2016. Repellency of essential oils on the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae). Southwestern Entomological Society of America Annual Meeting, Tyler, TX. (Oral presentation)

17. **Gaire, S.,** and A. Romero. 2015. Toxicity and repellency of essential oil components on the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae). Entomological Society of America Annual Meeting, Minneapolis, MN. (Oral presentation)
18. **Gaire, S.** and A. Romero. 2015. Toxicity of essential oil components on the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae). Southwestern Entomological Society of America Annual Meeting, Tulsa, OK (Oral presentation)
19. **Gaire, S.** and A. Romero. 2015. Toxicity of essential oil components on the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae). Graduate Research and Arts Symposium, New Mexico State University, Las Cruces, NM. (Poster)
20. **Gaire, S.** and A. Romero. 2015. Quantifying the toxicity of essential oil components on the Turkestan cockroach, *Blatta lateralis* (Blattodea: Blattidae). Seventh International Conference, Nepalese Students Association, New Mexico State University, Las Cruces, NM. (Oral presentation)

SOCIAL SERVICE

University Academic Committee:

2019-2020 **Graduate Student Representative,** Curriculum and Student Relations Committee, College of Agriculture, Purdue University, West Lafayette, IN

Leadership in professional societies:

2019-2020 **President**, Society of Overseas Nepalese Entomologists
2019-2020 **Vice President,** Entomology Graduate Student Organization, Department of Entomology, Purdue University, West Lafayette, IN
2018-2019 **President-Elect**, Society of Overseas Nepalese Entomologists
2018-2019 **Treasurer,** Entomology Graduate Student Organization, Department of Entomology, Purdue University, West Lafayette, IN
2017-2019 **Executive Member, Fund Raising Subcommittee,** Ohio Valley Entomological Association
2017-2018 **Secretary**, Society of Overseas Nepalese Entomologists
2017-2018 **Treasurer,** Entomology Graduate Student Organization, Department of Entomology, Purdue University, West Lafayette, IN
2017-2018 **Executive member & Student representative,** Departmental IT Committee, Department of Entomology, Purdue University, West Lafayette, IN
2016-2018 **Member-At-Large**, Ohio Valley Entomological Association
2016-2017 **Treasurer**, Society of Overseas Nepalese Entomologists
2016 **Executive Member & NM state representative,** Students Affairs Committee and Insect Photo Salon Committee, SW Entomological Society of America
2015-2016 **Secretary,** Joined EPPWS and PES Department's Graduate Student Organization, NMSU, Las Cruces, NM

Leadership in community:

2017-2018 **President,** Nepali Society at Purdue, Purdue University, West Lafayette, IN
2016-2017 **Treasurer,** Nepali Society at Purdue, Purdue University, West Lafayette, IN
2014-2015 **Treasurer**, Nepalese Student's Association, NMSU, Las Cruces, NM

2014-2015 **Executive member,** Voice against Cancer, NMSU, Las Cruces, NM
2008-present **President**, Alumni Students Association, New Vision Academy, Chitwan, Nepal
2004-2005 **President**, Junior Red Cross Circle, New Vision Academy, Chitwan, Nepal

Professional society memberships:
2018-present Association of Nepalese Agricultural Professionals of Americas
2017-present Pi Chi Omega, The Professional Pest Management Fraternity
2016-present Ohio Valley Entomological Association
2015-present Entomological Society of America
2015-present Society of Overseas Nepalese Entomologists

Reviewer of the Journals:

- Journal of Economic Entomology (1)
- Pest Management Science (1)

Extension and outreach activities:

Extension activities:

1. Purdue Integrated Pest Management Correspondence Courses:
    - Involved in grading Pest Management Correspondence at a weekly basis for training Pest Management Professionals. **So far, I have graded ~8000 Urban IPM tests**.
    - Updated the course Food Pest Management with Dr. Gary Bennett in Spring 2019
2. Purdue Annual Pest Management Conference:
    - Planning committee executive member
    - Moderating the session
        - Student staff
        - Research presenters
3. Poultry house bed bugs management:
    - More than 20 visits to poultry houses in the Midwest.
    - Provides regularly scientific information on bed bugs management to poultry house managing group
    - Bed bug effects on hen welfare and productivity assessment
4. Low income multifamily housing:
    - More than 15 visits to low-income housing in Indiana and Illinois
    - Assess bed bugs and cockroach's population
    - Evaluate efficacy of newly formulated insecticides against cockroaches and bed bugs.
    - Provides regular cultural control information such as sanitation for housing authority and families
5. Science Communication graduate level course: Created the extension YouTube video on bed bug management using Purdue Video Express facility.
    YouTube Link: https://youtu.be/HGgc-50IWlo
6. Training of Training (TOT): Participated in 5 days training during undergraduate.

7. IPM Facilitator training: Participated in 16 weeks (once a week) training by District IPM Farmers Association, Chitwan Nepal.

Outreach for K-12 students and Bug bowl:

1. Observation zoo (bed bug life stages) and Petting zoo (caterpillars), Bug Bowl, Spring Fest, Purdue University (Aril 6-7, 2019)
2. Petting zoo. Bug Bowl, Spring Fest, Purdue University. (April 15, 2018)
3. Bed bug rearing and feeding activities, Experience Purdue Agriculture (September 11, 2017)
4. Cockroach Racing booth and Honey tasting booth, Bug Bowl, Spring Fest, Purdue University (April 8, 9, 2017)
5. Purdue Bug Barn 5th grade student visit (showed tobacco horn worm caterpillar/pupae, millipede and hissing cockroach) (March 1, 2017)
6. Brooks School Elementary Math and Science Night, Fishers, IN (showed tobacco horn worm caterpillar, pupae, adult and hissing cockroach adults) (February 15, 2017)
7. College of Agriculture Fish fry, Indiana State Fair ground, Indianapolis, IN, (showed caterpillar, bugs and petting zoo) (February 4, 2017)
8. Thomas Miller Elementary School, Purdue Bug Barn visit (showed hissing cockroach, and beetle) (Nov 17, 2016)

Volunteering:

Entomological Society of America (ESA) and other professional societies:

1. **2019 ESA Symposia Organizer:** Linking insect movement ecology with applied pest management. (Organizer: Jhalendra Rijal, Govinda Shrestha, **Sudip Gaire)**
2. **2019 ESA Organized Meeting Moderator and Organizer,** Society of Overseas Nepalese Entomologists Annual Meeting, St. Louis, MO, Nov 19th, 2019
3. **Judge,** MUVE Undergraduate Poster competition (MUVE 1), Entomological Society of America Annual Meeting, 2019
4. **Judge,** MUVE Undergraduate Poster competition, Entomological Society of America Annual Meeting, 2018
5. **2018 ESA Organized Meeting Moderator and Organizer,** Society of Overseas Nepalese Entomologists Annual Meeting, Vancouver, Canada, Nov 13th, 2018
6. **Judge,** Master Category, Ohio Valley Entomological Association Annual Forum, Oct 19th, 2018.
7. **Moderator:** Student paper competition session, National Conference on Urban Entomology, Cary, NC, 2018
8. **Presentation upload room**, Ohio Valley Entomological Association Annual Forum, Nov 4, 2016
9. **Photo Salon Judging**, South Western Branch ESA Meeting, Tyler, TX, Feb 22-25, 2016

Purdue University:

1. **Entomology Department seminar host:** Dr. Joel Coats (Iowa State University), Oct 24th, 2019.
2. **Judge:** Junior Life Sciences Award, Lafayette Regional Science and Engineering Fair- March 2, 2018
3. **Moderator**: Fumigation session, 2018 Purdue Pest Management Conference
4. **Student staff**: 2017, 2018, 2019 Purdue Pest Management Conference
5. **Judge:** Undergraduate Research and Poster Symposium, April 11, 2017, Purdue University, West Lafayette, IN (April 11, 2017)
6. **Judge:** Sigma Xi Senior Life Sciences (LS) Special Award, Lafayette Regional Science and Engineering Fair- March 3, 2017

New Mexico State University:

1. International Cultural Bazaar and Festivals 2014, 2015
2. Associated New Mexico State University Keep State Great Events 2014, 2015
3. Associated New Mexico State University Big Events 2015, 2016
4. Organic Pumpkin Planting and Selling 2014, 2015
5. Voice Against Cancer public booth 2014