Plant essential oils synergize various pyrethroid insecticides and antagonize malathion in *Aedes aegypti*

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Abstract. Pyrethroid resistance is a significant threat to agricultural, urban and public health pest control activities. Because economic incentives for the production of novel active ingredients for the control of public health pests are lacking, this field is particularly affected by the potential failure of pyrethroid-based insecticides brought about by increasing pyrethroid resistance. As a result, innovative approaches are desperately needed to overcome insecticide resistance, particularly in mosquitoes that transmit deadly and debilitating pathogens. Numerous studies have demonstrated the potential of plant essential oils to enhance the efficacy of pyrethroids. The toxicity of pyrethroids combined with plant oils is significantly greater than the baseline toxicity of either oils or pyrethroids applied alone, which suggests there are synergistic interactions between components of these mixtures. The present study examined the potential of eight plant essential oils applied in one of two concentrations (1% and 5%) to enhance the toxicity of various pyrethroids (permethrin, natural pyrethrins, deltamethrin and β-cyfluthrin). The various plant essential oils enhanced the pyrethroids to differing degrees. The levels of enhancement provided by combinations of plant essential oils and pyrethroids in comparison with pyrethroids alone were calculated and synergistic outcomes characterized. Numerous plant essential oils significantly synergized a variety of pyrethroids; type I pyrethroids were synergized to a greater degree than type II pyrethroids. Eight plant essential oils significantly enhanced 24-h mortality rates provided by permethrin and six plant essential oils enhanced 24-h mortality rates obtained with natural pyrethrins. By contrast, only three plant essential plants significantly enhanced the toxicity of deltamethrin and β-cyfluthrin. Of the plant essential oils that enhanced the toxicity of these pyrethroids, some produced varying levels of synergism and antagonism. Geranium, patchouli and Texas cedarwood oils produced the highest levels of synergism, displaying co-toxicity factors of > 100 in some combinations. To assess the levels of enhancement and synergism of other classes of insecticide, malathion was also applied in combination with the plant oils. Significant antagonism was provided by a majority of the plant essential oils applied in combination with this insecticide, which suggests that plant essential oils may act to inhibit the oxidative activation processes within exposed adult mosquitoes.

Key words. pyrethroids, plant essential oils, enhance, synergize, malathion.
**Introduction**

Synergists are important components of some insecticide formulations and prevent the detoxification of active ingredients of insecticides (Metcalf, 1967; Yu, 2015). These compounds function by inhibiting the enzymatic pathways that broadly metabolize and aid in the excretion of particular toxicants encountered by insects within their environments (Metcalf, 1967). When used properly, synergists increase the overall toxicity of insecticidal active ingredients by increasing the bioavailability of the active compounds to interact with the target in the insect. Moreover, synergists can be utilized to reclaim the efficacy of certain insecticidal chemicals to which some insect populations have become resistant (Darriet & Chandre, 2011; Somwang et al., 2011; Gao et al., 2012). If insecticide resistance to select modes of action is mediated by upregulated detoxification mechanisms, synergists may inhibit these processes. In this scenario, synergists may facilitate similar insecticidal activity in both insecticide-resistant and insecticide-susceptible strains of mosquito.

Plant essential oils and their constituents represent promising additives to insecticides. Numerous studies have demonstrated that plant terpenoids act at a variety of molecular targets (Miyazawa & Kameoka, 1997; Mills et al., 2004; Lopez & Pascual-Villalobos, 2010; Tong & Coats, 2010; Anderson & Coats, 2012; Gross et al., 2017b). Many of these targets are distinct from mammalian targets and therefore these constituents should be safer for humans and non-target animals (Isman, 2000). Furthermore, because of their novel mechanisms of action, there is little chance of the development of cross-resistance with compounds currently available on the market. Moreover, multiple studies have demonstrated the ability of plant essential oils to enhance the efficacy of different insecticidal chemistries (Joffe et al., 2012; Gross et al. 2017a). Although this mechanism has not been fully characterized, the present authors hypothesize that this enhancement is caused, in part, by the inhibition of the degradation of these insecticides by detoxification enzymes. As the development of new insecticidal active ingredients is costly and prohibitive (Sparks & Lorsbach, 2017), plant essential oils may represent a relatively cost-effective means of increasing the efficacy of various insecticidal formulations. Moreover, many of these chemistries are recognized as possessing low levels of toxicity to mammals and other non-target organisms, and are not likely to be persistent in the environment (Isman, 2000).

Because of the relatively small size of the mosquito control industry and the importance of mosquito control in countries with scant economic resources, there is little economic incentive to create new insecticidal technologies (Hemingway et al., 2006; Knapp et al., 2015). Instead, mosquito control research and development rely, in large, on the repurposing of agricultural pest control chemistries (Mnzava et al., 2015). Of the wide variety of agrochemicals available in agricultural pest control, only select chemistries are ideal for mosquito control. Currently, only four classes of insecticide are available for the control of mosquitoes. The persistence of synthetic insecticides in the environment and the toxicity of these chemistries to mammals and non-target organisms are important characteristics that limit the viability of mosquito control insecticides. Novel chemistries for the control of public health vector insect species must also result in rapid knock-down in the target species, particularly in the context of active pathogen transmission, and must rapidly intervene and kill adult mosquitoes (Nauen, 2007). Moreover, the relatively few available and approved mosquito control chemistries are beginning to fail in the field as wild mosquito populations continue to become resistant to these classes of insecticide.

Gross et al. (2017a) demonstrated that many essential oils enhance permethrin efficacy to a higher degree than piperonyl butoxide (PBO) in two species of mosquito. Moreover, according to the findings of previous work (Norris et al., 2015), the baseline toxicity levels of these oils are not sufficient to completely account for the enhanced efficacy of mixtures of plant essential oils and synthetic insecticides against adult female mosquitoes. The present study explored the potential of plant essential oils to enhance the insecticidal effects of diverse synthetic pyrethroids and natural pyrethrins. Plant essential oils were applied in combination with a pro-insecticide, malathion, which requires oxidative activation to exert a toxic effect (Wickham, 1998), to test whether plant essential oils inhibit the oxidative activation of this insecticide.

**Materials and methods**

**Insect rearing**

Adult female *Aedes aegypti* (Liverpool strain) (Diptera: Culicidae) were reared according to standard laboratory procedures maintained by the Medical Entomology Laboratory at Iowa State University. In short, mosquito colonies were maintained at a constant temperature of 30°C and relative humidity (RH) of 80±10% and provided with 10% sucrose water pads *ad libitum*. Colony cages were provided with weekly bloodmeals of defibrinated sheep blood (Hemostat Laboratories, Inc., Dixon, CA, U.S.A.) to promote the development of eggs. Eggs were collected on paper towels once per week and deposited into aluminium pans for hatching. Larvae were fed TetraMin® fish flakes (Tetra, Inc., Blacksburg, VA, U.S.A.) every other day and larval water was replaced twice per week. Pupae were collected and subsequently separated based on size. Female pupae were collected and kept in 12-oz (355-mL) deli cartons, at a density of approximately 50 females per carton. Unmated adult female mosquitoes were used at 2–5 days post-eclosion in all studies.

**Topical application of insecticide and insecticide–plant essential oil mixtures**

The technical grade synthetic insecticides and natural pyrethrins used for this study were obtained from a variety of sources. Permethrin Z:E 40:60 and natural pyrethrins (20% pure in ethanol) were obtained from EcoSMART Technologies, Inc. (Roswell, GA, U.S.A.). Deltamethrin, β-cyfluthrin and malathion were obtained from Sigma Aldrich Corp. (St Louis, MO, U.S.A.). To obtain dose–response data for each insecticide, varying concentrations of synthetic insecticides were applied to individual adult female mosquitoes in a volume
of 0.2 μL per mosquito. Plant essential oils were obtained from Berje, Inc. (Carteret, NJ, U.S.A.). All insecticidal active ingredients and mixtures were applied in acetone throughout this study. Treated mosquitoes were kept in an environmental chamber at a constant temperature of 28 °C and RH 80 ± 10%. Concentrations that caused between 5% and 95% mortality at 24 h after the initial exposure were chosen. Gas chromatography–mass spectrometry data for the oils included in this study are provided in Table S1.

After LD25 (lethal dose required to produce 25% mortality) values had been calculated for each of the synthetic insecticides, mixtures of a discrete dose of synthetic insecticide, which corresponded to the LD25, and either 1% or 5% plant essential oil by weight were made. This facilitated the screening of plant essential oil mixtures at both low and high doses. Because enhancement by plant essential oils may be mediated by either synergistic processes (e.g., enhancement of cuticular penetration, inhibition of monooxygenase detoxification of synthetic insecticides, etc.) or additive toxicity, both low and high plant essential oil dosage mixtures were screened to assess the effects of plant terpenoids applied in combination with synthetic and natural insecticides.

### Data analysis

**LD20 and LD25 value characterization.** The lethal dosages required to produce 25% mortality (LD25) and 50% mortality (LD50) at 24 h were calculated in SAS Version 9.4 (SAS Institute, Inc., Cary, NC, U.S.A.) using a PROC PROBIT model with OPTC correction. At least five concentrations of each insecticide producing mortality rates of 5–95% at 24 h were chosen. A minimum of three biological replicates were used for theoretical dose–response calculation to ensure accurate characterizations of synthetic insecticide toxicity. A minimum of at least 750 Ae. aegypti mosquitoes were used for each insecticide to guarantee the reproducibility of these studies. When χ²-values were high (P < 0.05), a heterogeneity co-factor was applied to the data to provide for a better fit of the data (PROBIT, SAS Version 9.4).

**Oil-alone, insecticide-alone and mixture toxicity studies**

For each combination of plant essential oil and insecticide, a minimum of three replicates were performed with Ae. aegypti using two concentrations of oil (1% and 5% w/v) paired with the LD25 of each insecticide. To assess synergism by plant essential oils in each mixture, mosquitoes were also exposed to 0.2 μL plant essential oils alone at both the 1% and 5% w/v concentrations. For this study, patchouli, Origanum (i.e. Oregano), cedarwood [Texas type (CWT)], clove leaf, clove bud, cedarwood [Moroccan type (CWM)], basil and cinnamon bark oils were used. For each plant essential oil and insecticide mixture at both the 1% and 5% plant essential oil concentrations, three replicates of, respectively, a 1% oil alone, 5% oil alone and an LD25 of each insecticide application were also performed. This strategy was intended to account for the percentage effects of each of these components (i.e. LD25 insecticide alone, 1% oil alone, 5% oil alone, 1% oil + LD25 insecticide, 5% oil + LD25 insecticide) across biological replicates. This form of analysis takes into account any minor changes in effect among different biological cohorts and prevents cohort bias in the analysis. To assess statistical differences among the 24-h mortality rates provided by 1% and 5% plant essential oil + LD25 insecticide compared with the LD25 insecticide applied alone, a Student’s t-test was performed (α = 0.05) to assess changes in toxicity that occurred with the addition of the plant essential oils compared with the insecticide alone. Piperonyl butoxide was used throughout this study as a commercially used pyrethroid synergist.

#### Percentage enhancement

Toxicity was calculated as a percentage of the average mortality of Ae. aegypti exposed to each plant essential oil and synthetic insecticide or natural pyrethrin mixture. Percentage enhancement was calculated using a method similar to that used by Gross et al. (2017a) relying on the following equation for each replicate:

\[
\text{Percentage Enhancement} = \left( \frac{\text{toxicity of mixture}}{\text{toxicity of synthetic insecticide or natural pyrethrins}} \right) \times 100
\]

A Student’s t-test (α = 0.05) was performed to evaluate whether the percentage enhancement caused by each plant essential oil was statistically significant compared with PBO. All statistical analyses were performed using SAS Version 9.4.

#### Synergism calculations

The synergy of various components with the plant essential oil and insecticidal mixtures was characterized using a previously established method (Mansour et al., 1966). In short, the co-toxicity factor was calculated using the equation:

\[
\text{Co} = \frac{\text{observed % mortality} - \text{expected % mortality}}{\text{expected % mortality}} \times 100
\]

with expected mortality calculated as the sum of the percentage mortalities achieved by, respectively, the pyrethroid alone and the plant essential oil. The co-toxicity factor can be used to assess whether an antagonistic, additive or synergistic relationship exists among components within a mixture compared with the individual components. Values lower than −20 suggest an antagonistic relationship, values between −20 and 20 suggest an additive character, and values greater than 20 suggest a synergistic character. Values of less than −15 but greater than −20 or greater than 15 but less than 20 were considered to indicate trends towards antagonistic and synergistic characters, respectively.

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Results

The LD$_{25}$ and LD$_{50}$ values for *Ae. aegypti* exposed to each insecticide were calculated [Table 1; permethrin data published previously in Norris et al. (2015)]. The most toxic insecticide for *Ae. aegypti* in this study was β-cyfluthrin, with an estimated LD$_{50}$ value of 0.03 µg/g mosquito. This was followed by permethrin, deltamethrin, malathion and natural pyrethrins, with LD$_{50}$ values of 0.42 µg/g, 0.58 µg/g, 1.42 µg/g and 6.21 µg/g mosquito, respectively. The LD$_{25}$ values for β-cyfluthrin, deltamethrin, permethrin, malathion and natural pyrethrins were 0.004 µg/g, 0.01 µg/g, 0.19 µg/g, 1.02 µg/g and 2.87 µg/g mosquito, respectively. A 207-fold range in LD$_{50}$ values and a 718-fold range in LD$_{25}$ values from the least toxic to the most toxic insecticide screened were observed.

### Table 1. Dose–response data for *Aedes aegypti* topically exposed to a panel of insecticides.

| Insecticide         | n  | LD$_{25}$ (µg/g mosq.) | LD$_{50}$ (µg/g mosq.) | Slope (SE) | 95% CI (of LD$_{50}$) | χ$^2$-value (d.f.) |
|---------------------|----|------------------------|------------------------|-----------|------------------------|-------------------|
| Permethrin          | 1300 | 0.19                    | 0.42                   | 1.93 (0.3) | 0.3–0.5                 | 129.6 (43)        |
| Natural pyrethrins  | 850  | 2.87                    | 6.21                   | 2.015 (0.3) | 4.5–8.6                 | 98.5 (27)         |
| β-cyfluthrin        | 1575 | 0.004                   | 0.03                   | 0.84 (0.15) | 0.02–0.06               | 199.6 (51)        |
| Deltamethrin        | 1525 | 0.01                    | 0.58                   | 0.39 (0.08) | 0.2–5.2                 | 120.0 (50)        |
| Malathion           | 900  | 1.02                    | 1.42                   | 4.73 (0.73) | 1.3–1.6                 | 92.3 (28)         |

CI, confidence interval; SE, standard error.

### Combinations with permethrin

Mortality rates in *Ae. aegypti* induced by exposure to mixtures of plant essential oils and permethrin are shown in Fig. 1. The 24-h percentage mortality caused by the LD$_{25}$ of permethrin alone ranged from 12.8 ± 3.0% to 48.6 ± 5.0% in trials associated with each plant essential oil. The plant essential oil most toxic to *Ae. aegypti* in this experiment was basil, which provided mortality rates of 60.0 ± 8.0% and 92.0 ± 4.0% in the 1% and 5% oil-alone challenges. The oil least toxic to *Ae. aegypti* was CWT, which provided mortality rates of 1.6 ± 1.0% and 3.2 ± 1.0% at 24 h in the oil-alone challenges. Among the LD$_{25}$ permethrin +1% plant essential oil exposures, a significant increase in mortality compared with that of the LD$_{25}$ of permethrin applied alone was observed for both geranium (P = 0.03) and basil (P = 0.0022) oils. In the LD$_{25}$ permethrin +5% plant essential oil exposures, multiple oils produced significant increases in *Ae. aegypti* mortality compared with the LD$_{25}$ permethrin alone exposure. Patchouli (P = 0.042), Origanum (P = 0.015), clove leaf (P = 0.0279), CWT (P = 0.0047), geranium (P = 0.003), cinnamon bark (P = 0.0002), basil (P = 0.0019) and CWM (P = 0.0145) oils all produced mortality in *Ae. aegypti* that was higher than that expected by the LD$_{25}$ of permethrin alone. Piperonyl butoxide did not significantly increase the mortality of permethrin at either the 1% or 5% levels applied in combination with permethrin. Numerous plant essential oils and PBO also caused significant synergism when applied to *Ae. aegypti* in combination with permethrin (Table 2). Piperonyl butoxide, patchouli, clove bud, geranium and cinnamon bark oils caused significant synergism when applied to *Ae. aegypti* in combination with permethrin at the 1% concentration. Three plant essential oils caused significant synergism when applied in combination with permethrin at the 5% level: CWT, cinnamon bark and CWM oils all caused significant synergism of this pyrethroid. By contrast, some oils caused significant antagonism. Although it was synergistic when applied in combination with permethrin at the 1% level, PBO was antagonistic at the 5% level. *Origanum*, clove leaf and CWT oils were all antagonistic when applied at the 1% level, and clove leaf and basil oils were antagonistic at the 5% level in combination with permethrin.

### Combinations with natural pyrethrins

A different trend was observed in the effects on *Ae. aegypti* of plant essential oil combinations with natural pyrethrins (Fig. 2). Again, levels of plant essential oil toxicity to *Ae. aegypti* observed in the natural pyrethrins trial were similar to those in the previous experiments with permethrin, with CWT being the least toxic and basil the most toxic plant essential oil when applied alone at the 1% and 5% concentrations. Moreover, the LD$_{25}$ of natural pyrethrins produced mortality in *Ae. aegypti* with low variability ranging from 8.0 ± 4.0% to 24.7 ± 2.0% in all plant essential oil trials. Although numerical increases in 24-h mortality were observed for many oils applied in combination with the LD$_{25}$ of natural pyrethrins, only patchouli oil (P = 0.0288) produced a significant increase in mortality in *Ae. aegypti* at the 1% level when applied in tandem with natural pyrethrins. Piperonyl butoxide was not capable of enhancing the mortality caused by natural pyrethrins at this level. Multiple plant essential oils (applied at 5%) significantly increased 24-h mortality in *Ae. aegypti* over the LD$_{25}$ of natural pyrethrins alone. The essential oils were those of patchouli (P = 0.0255), *Origanum* (P = 0.0032), CWT (P = 0.0074), geranium (P = 0.0147) and cinnamon bark (P = 0.042), which caused mortality rates of, respectively, 98.0 ± 1.0%, 53.0 ± 6.0%, 30.0 ± 4.0%, 41.3 ± 4.0% and 48.0 ± 13.5% at 24 h. Although combinations of other oils with natural pyrethrins induced numerically higher mortality in *Ae. aegypti* than some of these oils, the differences were not statistically significant. Significant synergism was also noted for plant essential oils applied to *Ae. aegypti* in combination with natural pyrethrins (Table 3). Piperonyl butoxide, patchouli oil and clove leaf oil all produced significant synergism when applied at the 1% level in combination with natural pyrethrins. Patchouli, CWT, geranium and cinnamon bark oils all produced significant synergism when applied at the 5% level in combination with natural pyrethrins. Again, numerous oils produced
**Fig. 1.** Percentage mortality in adult female *Aedes aegypti* at 24h after application of each synergist or plant essential oil in combination with permethrin. *, statistically significant difference in percentage mortality caused by the 5% synergist/plant essential oil + LD25 permethrin combination compared with the LD25 permethrin-alone application. †, statistically significant difference in percentage mortality caused by the 1% synergist/plant essential oil + LD25 permethrin combination compared with the LD25 permethrin-alone application. PBO, piperonyl butoxide; CWT, Texas cedarwood; CWM, Moroccan cedarwood.

**Table 2.** Synergism and antagonism of an LD25 of permethrin by piperonyl butoxide (PBO) and select plant essential oils applied to *Aedes aegypti* adult females. Values lower than −20 suggest an antagonistic relationship, values between −20 and 20 suggest an additive character, and values greater than 20 suggest a synergistic character.

| Synergist/plant oil  | Oil/synergist 1% | Oil/synergist 5% |
|----------------------|-----------------|-----------------|
|                      | Mortality, %    | Mortality, %    |
| Synergist/oil alone  | Expected  | Observed  | Co-toxicity factor | Expected  | Observed  | Co-toxicity factor |
|----------------------|---------------|-------------|-------------------|---------------|-------------|-------------------|
| PBO                  | 21.1          | 2.0         | 23.1              | 36.0          | 55.8        |                    |
| Patchouli            | 26.7          | 6.7         | 33.4              | 42.7          | 27.8        |                    |
| Origanum             | 28.8          | 22.0        | 50.8              | 28.8          | 28.8        | −43.3             |
| Clove bud            | 24.0          | 4.0         | 28.0              | 36.0          | 24.0        | 35.0              |
| Clove leaf           | 48.6          | 2.0         | 50.6              | 38.9          | 48.6        | 50.0              |
| Texas cedarwood      | 32.0          | 1.6         | 33.6              | 4.0           | 32.0        | −88.1             |
| Geranium             | 12.8          | 0.0         | 12.8              | 29.6          | 12.8        | 17.3              |
| Cinnamon bark        | 18.4          | 4.0         | 22.4              | 27.2          | 18.4        | 36.0              |
| Basil                | 33.3          | 60.0        | 93.3              | 80.0          | 33.3        | 92.0              |
| Moroccan cedarwood   | 34.0          | 2.0         | 36.0              | 43.0          | 34.0        | 50.0              |

- Light grey shading refers to synergistic co-toxicity factors.
- Dark grey shading refers to antagonistic co-toxicity factors.
- No shading corresponds to additive co-toxicity factors.

Significant levels of antagonism when applied at either of these two concentrations in combination with natural pyrethrins.

**Combinations with deltamethrin**

When applied in combination with the LD25 of deltamethrin, select plant essential oils produced higher 24-h mortality in *Ae. aegypti* than the LD25 of deltamethrin alone (Fig. 3). *Aedes aegypti* mortality after exposure to the LD25 of deltamethrin ranged from 22.0 ± 6.0% to 35.0 ± 2.7% across all plant essential oil challenges. No plant essential oil caused a significant increase in mortality in *Ae. aegypti* at 1% in combination with an LD25 of deltamethrin. Three plant essential oils [patchouli (*P* = 0.0342), geranium (*P* = 0.011) and cinnamon bark (*P* = 0.027)] at 5% and in combination with an LD25 of deltamethrin caused significant increases in mortality in *Ae. aegypti* compared with deltamethrin applied alone. Mortality rates of 65.3 ± 9.3%, 46.4 ± 6.5% and 43.0 ± 3.7% were observed in *Ae. aegypti* exposed to mixtures of the LD25 of deltamethrin with patchouli, geranium and cinnamon bark oils, respectively. Although some plant essential oils caused significant synergism of type II pyrethroids, significantly less
synergism was noted for these pyrethroids compared with type I pyrethroids (Tables 3 and 4). In summary, only two oils caused significant synergism of deltamethrin. Piperonyl butoxide, clove leaf oil and CWM oil all caused significant synergism of deltamethrin and only when applied at the 1% concentration.

Combinations with β-cyfluthrin

The increases in mortality caused by plant essential oils applied in combination with β-cyfluthrin were considerable for select oils (Fig. 4). Two plant essential oils caused significant increases in Ae. aegypti mortality when applied in combination with the LD$_{25}$ of β-cyfluthrin at the 1% level. Both patchouli ($P = 0.0138$) and Origanum ($P = 0.0165$) oils caused significant enhancement compared with the LD$_{25}$ of β-cyfluthrin applied alone. Patchouli ($P = 0.00155$), Origanum ($P = 0.0001$), CWT ($P = 0.012$) and cinnamon bark ($P = 0.0134$) oils caused significant increases in mortality in Ae. aegypti compared with the LD$_{25}$ of β-cyfluthrin alone when applied in combination with the LD$_{25}$ of β-cyfluthrin at the 5% level. Mortality rates of $66.0 \pm 6.0\%$, $56.0 \pm 3.0\%$, $51.2 \pm 7.0\%$ and $53.3 \pm 6.5\%$ were observed in Ae. aegypti at 24 h after application of combinations of 5% patchouli, Origanum, CWT and cinnamon bark oils.
Synergism of pyrethroids by plant oils

Fig. 3. Percentage mortality in adult female *Aedes aegypti* at 24h after application of each synergist or plant essential oil in combination with deltamethrin. *, statistically significant difference in percentage mortality caused by the 5% synergist/plant essential oil + LD25 deltamethrin combination compared with the LD25 deltamethrin-alone application. PBO, piperonyl butoxide; CWT, Texas cedarwood; CWM, Moroccan cedarwood.

Table 4. Synergism and antagonism of an LD25 of deltamethrin by piperonyl butoxide (PBO) and select plant essential oils at various concentrations applied to *Aedes aegypti*. Values lower than −20 suggest an antagonistic relationship, values between −20 and 20 suggest an additive character, and values greater than 20 suggest a synergistic character.

| Synergist/plant oil          | Oil/synergist 1% |                     | Oil/synergist 5% |                     |
|------------------------------|------------------|---------------------|------------------|---------------------|
|                              | Mortality, %     | Co-toxicity factor  | Mortality, %     | Co-toxicity factor  |
|                              | Synergist/       |                     | Synergist/       |                     |
|                              | 1%               | Expected            | 5%               | Expected            |
|                              | oil alone        | Observed            | oil alone        | Observed            |
| PBO                          | 26.0             | 3.0                 | 29.0             | 42.5                |
| Patchouli                    | 30.7             | 6.7                 | 37.4             | 36.0                |
| Origanum                     | 31.0             | 0.0                 | 31.0             | 31.0                |
| Clove bud                    | 29.1             | 1.7                 | 30.8             | 33.1                |
| Clove leaf                   | 22.0             | 1.2                 | 23.2             | 30.0                |
| Texas cedarwood              | 26.9             | 4.6                 | 31.5             | 30.9                |
| Geranium                     | 28.0             | 2.0                 | 30.0             | 33.6                |
| Cinnamon bark                | 27.0             | 3.2                 | 30.2             | 26.0                |
| Basil                        | 35.0             | 50.2                | 85.2             | 44.0                |
| Moroccan cedarwood           | 29.6             | 0.8                 | 30.4             | 36.8                |

| Co-toxicity factor | PBO    | 46.6 | 26.0  | 22.2  | 48.2  | 43.0  | −10.8 |
|--------------------|--------|------|-------|-------|-------|-------|-------|
|                    | Patchouli | 7.5  | 29.1  | 30.3  | 59.4  | 39.4  | −9.9  |
|                    | Origanum | 0.0   | 31.0  | 38.0  | 69.0  | 52.0  | −24.6 |
|                    | Clove bud | 29.3  | 22.0  | 40.0  | 62.0  | 45.0  | −27.4 |
|                    | Clove leaf | 7.5  | 29.1  | 30.3  | 59.4  | 39.4  | −33.7 |
|                    | Texas cedarwood | −1.9 | 26.9  | 4.5   | 31.4  | 29.7  | −5.4  |
|                    | Geranium | 12.0  | 28.0  | 19.2  | 47.2  | 46.4  | −1.7  |
|                    | Cinnamon bark | −13.9 | 27.0  | 32.2  | 59.2  | 43.0  | −27.4 |
|                    | Basil   | −48.4 | 35.0  | 60.3  | 95.3  | 43.0  | −54.9 |
|                    | Moroccan cedarwood | 21.1 | 29.6  | 14.4  | 44.0  | 29.6  | −32.7 |

Light grey shading refers to synergistic co-toxicity factors.
Dark grey shading refers to antagonistic co-toxicity factors.
No shading corresponds to additive co-toxicity factors.

respectively, and the LD25 of β-cyfluthrin. Only the patchouli and *Origanum* oils were found to cause significant synergism of β-cyfluthrin. Significant antagonism was noted for combinations of plant essential oils applied at the 5% level in combination with type II pyrethroids. Six oils were antagonistic in combinations with deltamethrin and seven oils were antagonistic in combination with β-cyfluthrin at this level.

**Combinations with malathion**

The application of malathion in combination with varying concentrations of plant essential oils did not cause increases in *Ae. aegypti* mortality as significant as those achieved with the various combinations of plant essential oils and pyrethroids (Fig. 5). No oils caused increased mortality when applied at 1% in combination with the LD25 of malathion in comparison with malathion alone. However, PBO caused reduced mortality when applied at 1% in combination with malathion alone (*P* = 0.008). Only one oil, patchouli (*P* = 0.0004), caused a statistically significant increase in mortality when applied at 5% in combination with the LD25 of malathion, producing 70.0 ± 5.0% mortality at 24 h after application. Again, plant essential oil toxicity levels were similar to those observed with these oils in the previous pyrethroid combination studies (Figs 1–4). Malathion was significantly antagonized by almost all plant essential oils, with all but...
Fig. 4. Percentage mortality in adult female Aedes aegypti at 24h after application of each synergist or plant essential oil in combination with β-cyfluthrin. *, statistically significant difference in percentage mortality caused by the 5% synergist/plant essential oil + LD25 β-cyfluthrin combination compared with the LD25 β-cyfluthrin-alone application. PBO, piperonyl butoxide; CWT, Texas cedarwood; CWM, Moroccan cedarwood.

one oil antagonizing malathion when applied to Ae. aegypti in combination with insecticide at both the 1% and 5% levels (Table 5). This antagonism was also caused by PBO, with co-toxicity factors of −83.3 and −69.4 when applied at the 1% and 5% levels, respectively. In general, the levels of antagonism of plant essential oils were lower than that caused by PBO for both application concentrations. The one exception to this was geranium oil at the 5% level, which produced a co-toxicity factor of −75.0 compared with the −69.4 caused by PBO. For the oils that did not produce antagonism, a purely additive co-toxicity factor was calculated for CWM and clove leaf oils applied in combination with malathion at the 1% and 5% concentrations, respectively.

Percentage enhancement

Another metric, percentage enhancement, was used to evaluate the degree to which plant essential oils increased the toxicity of pyrethroids or malathion when applied to Ae. aegypti in combination at 1% (Fig. 6) or 5% (Fig. 7). At the 1% level, PBO positively enhanced the effects of all the pyrethroids tested in Ae. aegypti by 93.5 ± 40.0%, 178.6 ± 66.0%, 70.2 ± 40.0% and 16.7 ± 19.0% when applied in combination with permethrin, natural pyrethrins, deltamethrin and β-cyfluthrin, respectively. Piperonyl butoxide caused a negative enhancement when applied at 1% in combination with malathion (−86.0 ± 6.0%). The majority of the oils screened performed similarly to PBO when applied at the 1% concentration with the respective insecticide. The exceptions to this were patchouli (P = 0.001) and CWM (P = 0.002) oils, which caused statistically higher percentage enhancements of malathion than PBO. A number of oils produced statistically lower levels of enhancement than PBO when applied in specific combinations with various insecticides. These included Origanum (P = 0.0009), clove leaf (P = 0.042), CWM (P = 0.0308) oils when applied in combination with permethrin; all oils except patchouli (P < 0.05) oil in combination with natural pyrethrins; Origanum (P = 0.0461) and cinnamon bark (P = 0.0249) oils in combination with deltamethrin, and patchouli (P = 0.0173), Origanum (P = 0.0049) and clove bud (P = 0.0252) oils in combination with β-cyfluthrin. At the 5% level, many plant essential oils significantly outperformed PBO. For example, every plant essential oil outperformed PBO in combination with at least one insecticide, with the exception of clove leaf oil. Some oils, such as patchouli oil, outperformed PBO in combination with three of the four pyrethroids screened. Summaries of the P-values for oil–insecticide combinations that produced statistically higher enhancement values compared with PBO and the corresponding insecticide are provided in Appendix S1.

Discussion

The LD50 and LD25 values of various insecticides applied topically to adult female Ae. aegypti were successfully determined in this study in order to later evaluate the synergistic potentials of plant essential oils compared with that of PBO. The LD50 values obtained in this study for each insecticide were similar to those reported in the literature (Agramonte et al. 2017; Norris et al. 2015). In general, no insecticide LD50 differed from values reported in the literature by greater than one order of magnitude. More than 700 mosquitoes were used to calculate a robust LD25 value for each insecticide to be utilized in subsequent experiments. Table 1 highlights the dose–response statistics for each insecticide utilized in this study. Of the insecticides screened initially, β-cyfluthrin was the most toxic insecticide applied to mosquitoes. This was followed by deltamethrin, permethrin, malathion and natural pyrethrins. Type II pyrethroids were significantly more toxic than type I pyrethroids in terms
Synergism of pyrethroids by plant oils

Fig. 5. Percentage mortality in adult female Aedes aegypti at 24 h after application of each synergist or plant essential oil in combination with malathion. *, statistically significant difference in percentage mortality caused by the 5% synergist/plant essential oil + LD\textsubscript{25} malathion combination compared with the LD\textsubscript{25} malathion-alone application. †, statistically significant difference in percentage mortality caused by the 1% synergist/plant essential oil + LD\textsubscript{25} malathion combination compared with the LD\textsubscript{25} malathion-alone application. PBO, piperonyl butoxide; CWT, Texas cedarwood; CWM, Moroccan cedarwood.

Table 5. Synergism and antagonism of an LD\textsubscript{25} of \(\beta\)-cyfluthrin by piperonyl butoxide (PBO) and select plant essential oils at various concentrations applied to Aedes aegypti. Values lower than −20 suggest an antagonistic relationship, values between −20 and 20 suggest an additive character, and values greater than 20 suggest a synergistic character.

| Synergist/Plant Oil | \(\beta\)-cyfluthrin | Synergist/oil alone | Expected | Observed | Co-toxicity factor | \(\beta\)-cyfluthrin | Synergist/oil alone | Expected | Observed | Co-toxicity factor |
|---------------------|----------------------|--------------------|----------|----------|--------------------|----------------------|--------------------|----------|----------|--------------------|
| PBO                 | 22.9                 | 4.0                | 26.9     | 26.3     | −2.2              | 22.9                 | 26.4                | 49.3     | 30.9     | −37.3              |
| Patchouli           | 28.0                 | 4.0                | 32.0     | 44.0     | 37.5              | 28.0                 | 50.4                | 78.4     | 66.0     | −15.8              |
| Origanum            | 21.3                 | 2.0                | 23.3     | 36.7     | 57.5              | 21.3                 | 42.0                | 63.3     | 56.0     | −11.5              |
| Clove bud           | 24.7                 | 1.7                | 26.4     | 19.3     | −26.9             | 24.7                 | 40.0                | 64.7     | 21.3     | −67.1              |
| Clove leaf          | 28.0                 | 2.0                | 30.0     | 33.2     | 10.7              | 28.0                 | 44.0                | 72.0     | 40.6     | −43.6              |
| Texas cedarwood     | 27.2                 | 3.0                | 30.2     | 32.8     | 8.6               | 27.2                 | 4.0                 | 31.2     | 51.2     | −54.1              |
| Geranium            | 29.0                 | 3.5                | 32.5     | 33.0     | 1.5               | 29.0                 | 24.0                | 53.0     | 37.0     | −30.2              |
| Cinnamon bark       | 24.0                 | 2.7                | 26.7     | 22.7     | −15.0             | 24.0                 | 24.0                | 48.0     | 53.3     | 11.0               |
| Basil               | 24.0                 | 40.0               | 64.0     | 30.0     | −53.1             | 24.0                 | 46.0                | 70.0     | 37.0     | −47.1              |
| Moroccan cedarwood  | 23.2                 | 2.6                | 25.8     | 24.8     | −3.9              | 23.2                 | 16.0                | 39.2     | 32.8     | −16.3              |

Light grey shading refers to synergistic co-toxicity factors.
Dark grey shading refers to antagonistic co-toxicity factors.
No shading corresponds to additive co-toxicity factors.

of LD\textsubscript{25} and LD\textsubscript{50} values. For each insecticide screened, the PROBIT model sufficiently described the dose–response data for each insecticide to be used in the subsequent synergism explorations. Heterogeneity co-factors were applied to the models as \(\chi^2\)–values were high, but this is most likely to be attributable to the large number used for each insecticide.

A panel of pyrethroids in combination with either PBO, a synergist that is currently used to enhance the toxicity of various insecticidal ingredients for the control of mosquitoes, or plant essential oils was screened. In the present study, PBO did not cause significant increases in type I pyrethroid mortality in Aedes aegypti at the 1% rate and produced increases in mortality exerted by natural pyrethrins only at the 5% application concentration. This may be attributable to the application protocol utilized in this study or to the concentration at which PBO was applied. Numerous studies have shown that the efficacy of PBO as a synergist is dependent on the concentration at which it is applied (Hewlett, 1969; Guglielmone et al., 1999; Dehkordi et al., 2017; Nikpour et al., 2017). This is further evidenced by the inability of PBO to increase the toxicity of both type II pyrethroids screened in this study (deltamethrin and \(\beta\)-cyfluthrin). It is possible that the application concentrations in this study were too low for PBO to be effective as a
synergist for type I and II pyrethroids. By contrast, numerous plant essential oils were capable of increasing the efficacy of both type I and II pyrethroids at these application concentrations. A majority of plant oils produced significant increases in the mortality rates achieved by both permethrin and natural pyrethrins, and many oils produced a higher percentage mortality than PBO. Plant essential oils were less likely to increase the toxicity of type II pyrethroids than that of type I pyrethroids. Among the oils that most significantly increased the toxicity of type II pyrethroids, patchouli oil was the only one to increase the toxicity of both deltamethrin and β-cyfluthrin. This may reflect its intrinsic toxicity as it was noted to be one of the most toxic plant essential oils screened in a previous study (Norris et al., 2015). Other oils that caused significant increases in type II pyrethroid toxicity were Origanum, cinnamon bark, CWT and geranium oils. Of these oils, cinnamon bark and Origanum oils were also noted to be among the most toxic plant essential oils screened previously against *Ae. aegypti* adults (Norris et al., 2015). Texan cedarwood and geranium oils may contribute to the toxicity of deltamethrin and β-cyfluthrin, respectively, by synergistic interactions as they were not among the most toxic oils (Norris et al., 2015).

However, PBO applied in combination with an LD$_{25}$ of malathion at a concentration of 1% caused a significant decrease in 24-h mortality compared with the LD$_{25}$ of malathion applied alone. A lower percentage mortality of malathion in combination with PBO may be produced by the inhibition of oxygenases, which are required to activate this pro-insecticide into malaoxon (Hoffman et al., 1954; Rai & Roan, 1959; Forsyth & Chambers, 1989; Dos Santos et al., 2010). A majority of oils either decreased the efficacy of malathion or produced no statistically significant increase in the 24-h mortality produced by malathion. This may reflect the inhibition of detoxification enzymes by plant essential oils, which limits the toxicity of malathion in a similar manner to the effect observed with combinations of this insecticide and PBO. At the 5% level, only patchouli oil caused a statistically significant increase in mortality in *Ae. aegypti* when applied in combination with an LD$_{25}$ of malathion. This increase in toxicity may have been caused by the intrinsic toxicity of patchouli oil as was observed when this oil was combined with pyrethroids.

Enhancement levels (as percentages) caused by PBO or plant essential oils were also characterized for each of the insecticides. Establishing the percentage enhancement is another method of quantifying the increased efficacy of insecticides applied in combination with plant essential oils, and accounts for both additive and synergistic interactions between agents within a mixture (Gross et al. 2017a). Using this metric, it is apparent that plant essential oils significantly enhance the effects of a wide variety of insecticides to a higher degree than PBO in many cases. In general, percentage enhancement was more pronounced for plant essential oils applied at the 5% level in combination with various pyrethroids. This may be attributable to their intrinsic toxicity at the 5% application concentration. In addition, enhancement occurred more frequently for type I pyrethroids applied in combination with plant essential oils compared with type II pyrethroids. From an adult mosquito control perspective, this metric may be the most informative.
Fig. 7. Percentage enhancement of mortality ± standard error of the mean (SEM) in adult female *Aedes aegypti* at 24 h post-application of a mixture of 5% synergist or plant essential oil compared with the LD_{50} of insecticide (synthetic insecticide or natural pyrethrins) alone. Each bar represents the average percentage enhancement for 5% plant essential oil in combination with the LD_{50} of each insecticide listed above. The average percentage enhancement for each mixture was compared with the percentage enhancement caused by 5% piperonyl butoxide (PBO) + each insecticide via a one-way ANOVA with a Student–Newman–Kuel test (α = 0.05). *, statistically significant percentage enhancement (increased or decreased enhancement). CWT, Texas cedarwood; CWM, Moroccan cedarwood.

As increased toxicity may be the most practical and desirable endpoint.

Finally, to explore whether plant essential oils synergize these insecticides, the co-toxicity factor was used as a means of assessing the synergistic potentials of these various oils when applied in mixtures with various insecticides. Tables 2–6 highlight the various co-toxicity factors of each insecticide screened with plant essential oils and insecticides or plant essential oils applied alone at the LD_{50} level or the 1% or 5% levels, respectively. As discussed in previous work, co-toxicity factors greater than 20 indicate a synergistic interaction between two toxicants present in an insecticidal mixture, values between 20 and −20 indicate a purely additive interaction, and values below −20 suggest an antagonistic interaction (Mansour *et al.*, 1966). From these data, it is evident that many plant essential oils synergized diverse insecticides for *Ae. aegypti*. Piperonyl butoxide synergized the toxicity of both permethrin and natural pyrethrins at the 1% but not the 5% concentration. At the 5% concentration, PBO acted more antagonistically with co-toxicity factors of −24.46 and −14.1 for permethrin and natural pyrethrins, respectively. Interestingly, PBO did not synergize type II pyrethroids in this study (deltamethrin and β-cyfluthrin) at either the 1% or 5% application level when used in combination with these insecticides in *Ae. aegypti*. In general, plant essential oils were also less likely to synergize the type II pyrethroids used in this study; however, a number of oils caused synergism for select type II pyrethroids. This highlights a potential advantage of utilizing plant essential oils rather than PBO in future insecticidal formulations. As resistance to deltamethrin and other type II pyrethroids builds, plant essential oils may represent promising avenues for the development of new insecticides. Esterase- and oxygenase-mediated clearance of pyrethroids is less pronounced for type II pyrethroids in general (Craig *et al.*, 1957). The lesser potential of plant essential oils to synergize type II pyrethroids may reflect the lower levels of enzymatic clearance or detoxification of these insecticides. This difference in type I vs. type II pyrethroid metabolism is further evidenced by the differential synergism caused by PBO applied in combination with either a type I or type II pyrethroid. In previous work, PBO synergized natural pyrethrins (a type I pyrethroid) almost 300-fold, whereas it enhanced deltamethrin (a type II pyrethroid) only 10-fold (Soderlund & Casida, 1977; Casida *et al.*, 1983). In the present study, lower levels of type II pyrethroid synergism caused by PBO and plant oils were evident. Piperonyl butoxide and a majority of plant essential oils caused significant antagonism when applied in combination with malathion at both the 1% and 5% concentrations. The antagonism of this insecticide by plant essential oils in a similar manner to PBO further indicates that plant essential oils may act to inhibit oxidative processes important for the activation of this pro-insecticide.

Multiple oils enhanced the toxicity of diverse pyrethroids in this study. Of these oils, patchouli was the most successful as it synergized permethrin, natural pyrethrins and β-cyfluthrin at the 1% concentration. Numerous other plant essential oils also successfully synergized various pyrethroids at the 5% concentration. Of the oils, CWT and geranium oils were the most successful synergists against multiple types of pyrethroid. When applied at the 5% concentration in combination with various...
pyrethroids, CWT oil synergized three of the four pyrethroids screened; only deltamethrin was not synergized by this plant essential oil. Moreover, this oil consistently showed the highest co-toxicity factor of all the plant essential oils screened. Finally, all plant essential oils screened caused significant antagonism of malathion when applied in combination with the LD_{25} of malathion. This was observed when plant essential oils were combined with malathion at both the 1% and 5% concentrations. This result may indicate that particular terpenoids in CWT and geranium oils act to interfere with detoxification processes, leading to higher bioavailability of the topically applied pyrethroid. This in turn could lead to the higher percentage mortality and account for the synergism seen in this study.

Multiple other studies have suggested that plant essential oils enhance the toxicity of various permethrin and natural pyrethrins (Joffe et al., 2012; Gross et al., 2017b). The mechanism of this enhancement has not been fully explored, but it has been suggested that various components within plant essential oils prevent the detoxification of pyrethroids and other insecticides. Previous studies demonstrated that select plant essential oil terpenoids and plant extracts significantly inhibited detoxification enzymes (Waliwitiya et al., 2012; Carreño Otero et al., 2018) and hence it is possible that the terpenoids in the plant essential oils screened in this study work in a similar manner. The present study further confirms this finding with whole organism toxicological endpoints, such as 24-h mortality and co-toxicity factors. The observation of the antagonism of malathion further suggests that plant essential oils act to inhibit detoxification processes, specifically monooxygenases, in exposed *Ae. aegypti*. As malathion requires activation by monooxygenases, the observed antagonism of malathion strongly supports the premise that these activation processes are inhibited or down-regulated. Although promising synergistic plant oils were identified, no plant essential oil caused significant antagonism of all pyrethroids and antagonism of malathion. Instead, the ability of

Table 6. Antagonism of an LD_{25} of malathion by piperonyl butoxide (PBO) and select plant essential oils at various concentrations applied to *Aedes aegypti*. Values lower than −20 suggest an antagonistic relationship, values between −20 and 20 suggest an additive character, and values greater than 20 suggest a synergistic character.

| Synergist/plant oil | Oil/synergist 1% |   |   |   | Oil/synergist 5% |   |   |   |
|---------------------|-----------------|---|---|---|-----------------|---|---|---|
|                     | Mortality, %    |   |   |   | Mortality, %    |   |   |   |
| Synergist/expected  | Co-toxicity     |   |   |   | factor          |   |   |   |
| factor              | Malathion       |   |   |   | Malathion       |   |   |   |
|                     | oil alone       |   |   |   | oil alone       |   |   |   |
|                     | Expected        |   |   |   | Expected        |   |   |   |
|                     | Observed        |   |   |   | Observed        |   |   |   |
| PBO                 | 30.0            | 6.0 | 36.0 | 6.0 | −83.3          | 30.0 | 32.0 | 62.0 | 19.0 | −69.4 |
| Patchouli           | 31.0            | 5.3 | 36.3 | 27.0 | −25.6          | 31.0 | 72.0 | 103.0 | 70.0 | −32.0 |
| Origanum            | 28.0            | 7.4 | 35.4 | 20.0 | −43.5          | 28.0 | 40.0 | 68.0 | 56.0 | −17.6 |
| Clove bud           | 31.0            | 3.2 | 34.2 | 20.0 | −41.5          | 31.0 | 50.0 | 81.0 | 34.0 | −58.0 |
| Clove leaf          | 24.0            | 4.6 | 28.6 | 10.0 | −65.0          | 24.0 | 40.0 | 64.0 | 58.0 | −9.4  |
| Texas cedarwood     | 31.0            | 4.0 | 35.0 | 16.0 | −54.3          | 31.0 | 4.0 | 35.0 | 26.0 | −25.7 |
| Geranium            | 30.0            | 10.0 | 40.0 | 20.0 | −50.0          | 30.0 | 10.0 | 40.0 | 10.0 | −75.0 |
| Cinnamon bark       | 29.3            | 4.4 | 33.7 | 18.7 | −44.5          | 29.3 | 28.0 | 57.3 | 45.3 | −20.9 |
| Basil               | 32.0            | 38.0 | 70.0 | 36.0 | −8.6           | 32.0 | 50.0 | 82.0 | 38.0 | −53.7 |
| Moroccan cedarwood  | 29.0            | 8.4 | 37.4 | 36.0 | −3.7           | 29.0 | 16.0 | 45.0 | 25.0 | −44.4 |

- Light grey shading refers to synergistic co-toxicity factors.
- Dark grey shading refers to antagonistic co-toxicity factors.
- No shading corresponds to additive co-toxicity factors.
of novel, safe synergists as an alternative means of increasing insecticide efficacy may be both viable and responsible.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. $P$-values for comparisons of enhancement percentages (oils with piperonyl butoxide).

Table S1. Gas chromatography–mass spectrometry data for the oils included in this study.

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