Antennal Responses, Repellency, and Toxicity of Essential Oil Formulations on the German Cockroach (Blattodea: Ectobiidae)

by

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Abstract

The toxicity, repellency, and the electroantennogram responses induced by six essential oil formulations and their components against the pyrethroid-susceptible (S) and resistant (D and E) strains of the German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae) were tested. This was achieved using the direct spray and continuous exposure assays, 2-choice olfactometer, flushing assays, and the electroantennogram technique, respectively. Essentria® All Purpose Insect Concentrate (Essentria®) generated the strongest antennal response (1-4 mV). Menthone generated the largest average antennal response (0.066 mV – 8.324 mV) of all the individual active components studied. Essentria® showed the highest average repellency (64.6%) across both assays. The cockroaches had the highest mortality after exposure to Excite R™ [median Lethal Time ($LT_{50)}$ = 3.772 hours], in the continuous exposure assay, and Essentria® (average LT_{50} = 1.343 minutes), in the direct spray assay. These results indicate that essential oil formulations and components and are toxic, repellent, and can be detected by the German cockroach.

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Introduction

Cockroaches and Humans

Cockroaches, especially the German cockroach, *Blattella germanica* (L.), are an example of how urbanization has resulted in increased human interaction with insects. Because of their capacity to adapt to many environmental situations, German cockroaches are ubiquitous (Schal et al. 1984). There are roughly 3500 different cockroach species, 30 of which are synanthropic, that is, live in close association with people (Atiokeng Tatang et al. 2017). During the winter, synanthropic cockroaches develop colonies in heated homes, however, they will reproduce outside in the summer (Roth and Willis 1957). Cockroaches are omnivorous by nature and can consume anything, which include human food, garbage, and sewage. As a result, they can pass infections on to us. Cockroaches carry approximately 100 different bacterium species (Schal and Hamilton 1990). *Blattella germanica*, for example, is a mechanical vector of *Escherichia coli*, which can cause infections in the stomach, urinary tract, and lungs in people (Schapheer et al. 2018).

Hypersensitivity to cockroaches occurs when cockroach allergens, present in exuviae, causes severe bronchial asthma (Schal and Hamilton, 1990). Sources of cockroach allergens include saliva, shredded skin, fecal contents, and other cockroach body parts. Cockroach allergens are thought to cause inflammation by activating epithelial cells in the lungs, which then create cytokines and chemokines that recruit inflammatory cells to heal the allergen-damaged

airway (Do et al., 2016). Epigenetics, via DNA methylation patterns, is a huge factor in the development of cockroach allergies. Some methylation patterns of DNA have been associated with characteristics of childhood asthma (Gomez 2019). In 1972, a link was discovered between cockroach extracts and asthmatic patients' respiratory problems. The prevalence of cockroach allergy in children and adults in the United States ranged from 17 to 41% in 2016 (Do et al., 2016). Cockroach allergens were found in 85% in the United States' inner cities, and 60% of children living in these cities were found to be allergic to cockroaches (Do et al., 2016). Similar studies were carried out in European and Asian urban cities. As of 2016, almost 25% of children in Poland were allergic to cockroaches, while 58% of asthmatic patients in Taiwan were allergic to cockroach allergen (Do et al., 2016).

Life Cycle of German Cockroaches

The German cockroaches develop by incomplete metamorphosis, as it lacks a pupal stage. Cockroaches mature from the egg stage through several nymphal stages before reaching adulthood. German cockroaches mate within the first 7-10 days after reaching adulthood. Males mate several times over their lives, whereas females generally only mate once. Within a few days of mating, an oothecae will form within the genital vestibulum of the female. The ootheca is a hard protective material that covers the eggs. It is left attached in the abdomen of the female cockroach for 20 – 30 days until the eggs are about to hatch (Cochran, 1999). Within 16 hours of formation, the ootheca is protruded fully and rotated 90º. The female provides resources for the eggs inside the oothecae, such as water and nutrition (Wang et al., 2021). An ootheca is 8 mm long, 3 mm tall, and 2 mm wide and typically contains 37-44 eggs. It has a keel running down its length that serves as an aperture for the eggs to hatch.

Nymphs go through between 5 and 7 instars. Females can go through one extra than males. This means that the male reaches sexual maturity quicker than the female. Environmental factors like exposure to CO_2, temperature, and diet can affect the number of instars the nymphs undergo (Wang et al. 2021). The rate of development of nymphs is faster when placed together (aggregated).

When German cockroaches reach adulthood, their color changes to light brown or tan and they have wings that cover their entire abdomen. At adulthood, the males have a slimmer abdomen compared to the female German cockroach. Olfaction is essential in the mating process of the German and many other cockroach species. It is needed for finding mate and copulating. To begin the mating behavior, the female displays a calling behavior. She releases volatile sex pheromones (Liang and Schal 1993a) to attract the male cockroach, after which they collide their antennae. Following that, the male detects a contact pheromone (a mixture of oxygenated methyl-branched hydrocarbons) on the female, prompting him to release his own pheromone, which initiates her to mount him and feed on his tergal gland. Copulation follows, which, if successful, can last up to 95 minutes.

Willis described the calling behavior in German cockroaches for the first time in 1970 (Willis 1970). Liang and Schal (1993b) discovered that under a photoperiod of 12L:12D, the number of unmated females who engaged in calling activity peaked just before the dark phase ended. The results of the study prompted them to propose that a very volatile sex chemical was responsible for attracting males during calling. The contact sex pheromone on the cuticle female German cockroach is made up of a mixture of oxygenated methyl-branched hydrocarbons, the most prevalent of which is 3,11-dimethylnonacosan-2-one (Eliyahu et al., 2004).

In the American cockroach, *Periplaneta americana* (L.), adult males have more antennal sensilla than adult females (Schaller 1978). However, antennal sexual dimorphism is absent in the nymphs. Schaller (1978) classified the American cockroach sensilla into wall-pore sensilla and no pore sensilla. The wall-pore sensilla was further grouped into single-walled (SW) and double-walled (DW) sensilla, which were classified into subtypes. There are three types of SW: SW-a, SW-b, and SW-c. SW-a and SW-c house two sensory neurons, in contrast to the four neurons SW-b contains, which makes up about 54% of the sensilla population. SW-a covers about 8% of the antenna sensillum population (Schaller, 1978). SW-b is divided into short and long types. The short type is majorly found in nymph and adult female antennae, while the long type is found majorly in the adult male antennae. The long type SW-b is suspected to be responsible for detecting the female sex pheromone (Schaller, 1978). This idea is supported by electrophysiological recordings where male larvae were shown to mildly detect female attractant, compared to adult males who responded strongly to the attractant (Schaller, 1978). There are few studies on the morphology of the German cockroach antennae.

Control of the German Cockroach

The control of German cockroaches relies very much on insecticides, even though they have developed resistance to virtually all of them. This is because insecticides are more effective and less costly compared to other methods of control. There are 18 classes of insecticides that have been used against the German cockroach (Wang et al. 2021). They include pyrethroids, organophosphates, neonicotinoids, oxadiazines, pyrroles, chlorinated hydrocarbons, phenylpyrazoles, amidinohydrazones, sulfonamides, macrocyclic lactones, insect growth regulators, spinosyns, isoxazolines, and ryanoids.

This review will focus more on pyrethroids because they are closely related to a natural insecticide, pyrethrins, which is a major component of one of the essential oil formulations used in this study. Pyrethroids act against the nervous system of insects. Pyrethroids bind to sodium voltage-gated channels, leaving them open and preventing them from transitioning from an activated state to an inactivated state (Field et al. 2017). This leads to prolonged and repetitive production of action potential (Narahashi 1971) causing hypersensitivity to stimuli (Vijverberg and vanden Bercken 1990). Pyrethroids also inactivate calcium voltage-gated channels. Deltamethrin causes inactivation of calcium channels in rats (Narahashi 1971).

Resistance occurs when a cockroach population develops metabolic, behavioral, or physiological mechanisms to resist or overcome insecticides, rendering it incapable of being controlled by a previously effective insecticide dosage (Cochran 1995). As a result of selection, the affected pest population has a survival advantage in the presence of insecticides. The first reported case of resistance development in *B. germanica* was in 1951, when a 2% solution of the cyclodiene insecticide, chlordane, a chlorinated hydrocarbon, no longer controlled the German cockroach (Heal et al. 1953). Reports of resistance later occurred in other field populations in various parts of the U.S. (Cochran 1995). Resistance to pyrethroids is wide-spread in German cockroach populations world-wide. The underlying mechanism behind this is due to increased levels of cytochrome P_{450}, esterase, glutathione S-transferase, and neuron insensitivity (knockdown resistance) (Hemingway et al. 1993). The Cytochrome P450 monooxygenase system detoxifies insecticides by oxidizing them, thereby converting to non-toxic forms (Scott 1999). Glutathione S-transferase (GST) detoxifies insecticides by catalyzing a nucleophilic attack by glutathione on the insecticide (Angelucci et al. 2005). This nucleophilic attack leads to the insecticide losing its functional group, thereby losing its toxicity in the process.

Carboxylesterase compounds are also used by the German cockroach to detoxify insecticides. This is achieved through hydrolyzing ester containing compounds like organophosphate and pyrethroid insecticides (Stankovic and Kostic 2017). Another way German cockroaches develop resistance to insecticides is by becoming insensitive to them at the neuronal level. This insensitivity arises because of a mutation in one of the amino acids that makes up the enzyme acetylcholinesterase (Siegfried and Scott 1992). Due to this mutation, acetylcholinesterase becomes unrecognizable to insecticides, like organophosphates, that inhibit its function of breaking down acetylcholine (Siegfried and Scott 1992).

Pyrethrin resistance emerged about the same time. German cockroaches were found to be resistant to 42 pesticide active components after 65 years (Zhu et al. 2016). Three different strains, insecticide-susceptible strain S, and two cross resistant strains D and E, of the German cockroach were used for this study based on their resistance profiles. Strain D and E were collected from manufactured homes and in daycare centers throughout Franklin County, North Carolina, USA, and are resistant to permethrin, chlorpyrifos, propoxur, and fipronil (Wu and Appel 2017).

The biological control of the German cockroach involves the use of bio-organisms like bacteria, fungi, viruses, nematodes, and parasitoids to control the German cockroach. This method of control is being explored due to the problems associated with the chemical method of control such as insecticide resistance and negative impacts on non-target organisms.

Essential Oils

Essential oils are a complex mixture of terpenes (hemiterpenes, monoterpenes, sesquiterpenes, and diterpenes) and terpenoids (aldehydes, ketones, alcohols, phenols, ethers, and

esters) that are biosynthesized and stored in the secretory granules in the internal and external parts of plants (El Asbahani et al. 2015, Sharma et al. 2021). Essential oils have insecticidal and fumigant effects against cockroaches (Ngoh et al. 1998, Hammond et al. 2000, Hubert et al. 2008, Dambolena et al. 2016).

Some essential oils have insecticidal, repellent, and fumigant properties. They function as insecticides by affecting the nervous systems of insects. Some of the insecticides that are the major constituents of the essential oil formulations used in this study function by inhibiting the activity of the enzyme acetylcholinesterase. Acetylcholinesterase catalyzes the break-down of acetylcholine, thereby preventing the continuous generation of action potentials in a neuron cell. Inhibiting this enzyme leads to the continuous firing of the neuron cell. The inhibition rates of alpha-pinene, p-cymene, limonene, 1,8-cineole, menthol, camphene, and β-Caryophyllene against acetylcholinesterase in the German cockroach have been investigated. Alpha-pinene had the greatest inhibition rate (85%), which was followed by p-cymene (17%), menthol (12%), 1,8-cineole (10%), and limonene (5%). The octopaminergic system is another target of essential oils in insects (Enan 1998). Octopamine serves as a neurotransmitter, neurohormone, and neuromodulator in insects (Evans 1981). The nervous system breaks down when the octopaminergic system is disrupted (Evans 1981). Eugenol, an essential oil compound, was revealed to have insecticidal effects on the American cockroach by binding to octopamine receptors in brain cells and causing calcium ions to be released (Enan 1998).

The repellency of some essential oil components has been investigated against the German cockroach. For example, limonene and 1-8, cineole had repellency, using the Ebeling choice box method, that ranged from 6% to 18%, and 1% to 22%, respectively, against adult male German cockroaches (Phillips 2009). However, using the harborage-choice method, the

repellency values of 1-8, Cineole ranged from 35% to 50%, while that of limonene ranged from 50% to 70%. Menthone also had a repellency that ranged from 5% to 38% in the same study using the Ebeling choice box. However, the repellency value of menthone ranged from 45% to 50% using the harborage-choice method (Phillips 2009). Alpha-pinene repellency values from the Ebeling choice method ranged from 55% - 62%. The repellency values of the essential oils differed depending on the experimental design. The values were higher in the harborage-choice method than in the Ebeling choice box method (Phillips 2009).

The exact mechanism of action of essential oil molecules as insect repellents is unknown. The use of essential oil components (EOCs), as an alternative to insecticides has gained relevance in recent years (Moretti et al. 2002).

Olfaction in the German cockroach

The antenna is the first site where odor detection takes place in insects. The German cockroach antenna is made up of a short scapus and a long flagellum, which is divided into about 150 annuli (Fuscà and Kloppenburg 2021). A higher level of development is coupled with an increase in the number of annuli (Ishii 1971). Each annulus has olfactory sensilla (OS) that house olfactory sensory neurons (OSNs), which detect odorants (Fusca and Kloppenburg 2021). The sensilla are divided into three groups based on the subtype and number of sensory neurons they contain. These groups are the trichoid sensilla, which responds to a wider range of chemicals, and two types of basiconic sensilla, grooved and perforated. The perforated basiconic sensilla contains two to four OSNs and primarily responds to alcohols, terpenes, aromatic compounds, and esters (detects specific odorants, just like the perforated basiconic sensilla).

Lockey and Willis (2015) compared the level of success of different antennae groups (left antenna, right antenna, and bilaterally symmetric antennae) of the American cockroach at finding an odor source (Lockey and Willis 2015). They found that the antenna's length, is critical for successfully finding a source. This was evidenced in experimental results showing that cockroaches with unilateral antennas performed similarly with their bilateral counterpart of equal antenna length, and cockroaches with longer antennas performed significantly better than those with shorter antennae.

The sensilla of cockroach antenna can respond differently to different compounds even of the same chemical class. For example, it was found that the responses of American cockroach antennal neurons that specifically detect aliphatic alcohols differ from one alcohol compound to another (Getz and Akers 1997).

The purpose of this thesis research was to examine the toxicity, repellency, and electroantennogram responses induced by six commercial essential oil formulations and their components against pyrethroid-susceptible and resistant strains of the German cockroach.

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Repellency and Antennal Responses Induced by Commercial Essential Oil Mixtures and Components in the German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae)

The German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae) is a major household and structural pest throughout the world (Wang et al. 2021). It can contaminate and consume human food during production, transportation, and storage, and can transmit diseases (Tang et al. 2019). German cockroach exuviae, feces, and bodies can cause allergic reactions in humans (Schal and Hamilton 1990). The prevalence of cockroach allergy in children and adults in the United States ranged from 17 to 41% in 2016 (Do et al. 2016). The German cockroach causes an annual economic loss due to structural damage and the cost of infestation management. As of 2022, it costs $650 per 3500 ft^2 of a house to control these cockroaches (Pomares 2022). Due to their economic importance and health effects, a variety of insecticides were developed to control German cockroaches. However, they have developed resistance to virtually all active ingredients in insecticides (Wang and Bennett 2006).

Continued application of insecticides like pyrethroids, carbamates, and organophosphates results in more insect resistance (Hemingway and Ranson 2000) and human poisoning (Carson 2002). Organophosphates are harmful to people (Altinok et al. 2006, Jaga and Dharmani 2003) and rainbow trout are harmed by methiocarbs. Non-target beneficial insects, including pollinators (honeybees) and biological control agents (parasitoids), are also affected (Palmquist et al. 2012). After bees were treated with 2.5 mg of deltamethrin per bee, the percentage of individuals who could return to their colony dropped from 90% to 9% (Palmquist et al. 2012).

Essential oils are plant-derived secondary metabolites that have insecticidal and repellent properties against cockroaches and other insects. Their insecticidal properties are well

understood, but the specific process by which they repel cockroaches is unknown. In general, repellents operate through the olfactory system (Boné et al. 2020), so it is possible that essential oils work through antennal perception to repel cockroaches. Previous research has revealed that some insects like mosquitoes can detect essential oils with their antenna (Zhu and Zeng 2006), however no research has been done to study if German cockroaches can do the same. Both mosquitoes and cockroaches are repelled by catnip essential oils, containing 4a-α,7-α,7a-β-nepetalactone (55%), 4a-α,7-β,7a-α-Nepetalactone (31.2%), and α-pinene (4.6%). This observation indicates that they may share a similar mode of perception and repellency (Schultz et al. 2006).

Insects use chemical cues to discover food, mates, and kin. These cues are transmitted to the brain via electrical signals, which are interpreted and converted into behavior responses. The electroantennogram (EAG) technique can be used to detect these signals (Olsson and Hansson 2013). For example, Nojima et al. (2005) used the EAG technique in conjunction with gas chromatography to identify blattellaquinone as the sex pheromone of the German cockroach. The EAG technique has also been used to monitor the responses of the American cockroach to their sex pheromones (periplanone-A and periplanone-B) (Nishino et al. 1983). In a study by Matos and Schal (2015), the EAG values of the male German cockroach in response to blattellaquinone ranged from 0.1 mV at 0.001 µg dosage to 0.5 mV at 100 µg dosage. The EAG responses of the American cockroach to several periplanone analogs ranged from 0.15 mV at 10^{-8} g to 2.3 mV at 10^{-4} g, according to Okada et al. (1990).

The purpose of this study was to determine if the German cockroach could respond to the vapor stimuli of five different commercial essential oil formulations via its antenna. The formulations were also tested for repellency against the German cockroach.

Materials and Methods

Insects

An insecticide-susceptible (S) and two insecticide-resistant German cockroach strains (D and E) were utilized. The susceptible strain has been kept in continuous culture at the urban entomology laboratory (Auburn University) for >37 years without insecticide exposure. The two resistant strains were collected from Franklin County, North Carolina, and are resistant to permethrins, chlorpyrifos, propoxur, and fipronil (Wu and Appel 2017). Each strain was maintained in separate 3.8-liter glass jars with cardboard harborage at a 27 ± 2°C, 40–70% RH, and a photoperiod of 12:12 (L: D) h. A 60 ml glass jar was provided with water and a cotton wick was affixed through the cap. Rat chow was fed to the colonies *ad libitum* (Purina 500l lab diet from Purina LabDiet, Inc. St. Louis, MO). Adult male German cockroaches were utilized in the study because they have a relatively consistent body mass and do not produce eggs and hence maintain steady hormone levels (Oladipupo et al. 2020).

Essential Oil Formulations

The commercial essential oil formulations, their manufacturers, and essential oil components used for this experiment are listed in Table 1. The formulations were all purchased from "Do My Own Pest Control" (Norcross, GA, USA). Acetone (Reagent ACS, Ward's Science, St Catherines, ON, Canada) was used to dissolve all the formulations. The five formulations, Essentria®, EcoVia™, Orange guard, ER-22™, and Garscentria are concentrated natural insecticides containing mixtures of essential oils (Table 1).

Essential Oil Components

Based on the essential oil formulations (Table 1) and their labelled constituents, eight pure essential oil components were selected to examine individually. These eight components were selected because they have been tested previously and were toxic and repellent against the German cockroach. These were purchased from Sigma-Aldrich (St. Louis, MO) and consisted of 5 cycloalkene compounds (caryophyllene, limonene, α-pinene, p-cymene, and camphene), 1 ketone compound (menthone), a cyclic alcohol compound (menthol), and an ether compound (cineole) (Fig. 1).

Electroantennography (EAG)

The electroantennography (EAG) technique (Fig. 1A) used was similar to that previously described by Chen and Fadamiro (2007). Adult male German cockroaches were collected from colony jars and lightly anesthetized with CO_2. Anesthetized adult males were placed in 60 mL plastic containers on ice to maintain their inactivity. For antennal dissection, a single male was removed from the container and placed on the stage of a microscope. The antenna of the cockroach was excised between the scape and pedicel using a microsurgical knife (Sharpoint™) purchased from Fine Science tools (North Vancouver, B.C, Canada); the distal most few flagellomeres were removed (Fig. 1B). The base (Fig. 1C) and tip of the antenna were inserted into respective glass electrodes filled with a 0.1 M KCL solution. Each glass electrode was connected to probes through chlorinated silver filaments to conduct current. Humidified air (≈65% RH), created by an air pump and forced to pass through a "Big Universal Trap" air purifier (Agilent, Santa Clara, CA, US), was directed over the antenna.

Electrical activity of the cockroach antenna was recorded with specialized EAG equipment and software. The electrodes were connected to a signal-conditioning amplifier and interface (INR-II, Syntech, Netherlands). The signal was processed by a data acquisition

controller (IDAC-4 Syntech, the Netherlands) and analyzed in EAG 2000 software. Depolarization was visualized with a computer monitor and the data recorded on a computer.

Each of the formulations and essential oil components was diluted in acetone to 1, 10, 100, and 1000 µg/µl and a 10-µl sample was applied to a piece of filter paper (2 cm × 1 cm, Whatman No. 1, Little Chalfont, Buckinghamshire, UK). The solvent was allowed to evaporate for 60 sec, after which the filter paper was inserted into a glass Pasteur pipette (15 cm long, VWR international, Radnor, PA, USA). The tip of the pipette was placed into a small hole (ca. 3 mm in diameter) in the wall of the glass tube directing humidified air towards the antenna. The stimulus was a 0.2 sec puff of air containing the vapor of a single essential oil formulation or component generated by an air stimulus controller. Air flowed constantly at 0.4 L/min via a glass tube positioned 2 cm away from the antenna and oriented towards the antenna's center. The inter-stimulus interval was approximately 2 min to allow the antenna to recover from the treatment (Chen and Fadamiro 2007b). Each concentration of a formulation and component was serially applied in a random order. Randomness was generated using an Excel spreadsheet (Microsoft, Redmond WA). A total of 108 (n = 18 per formulation) cockroach antennae were used to compare the EAG elicited by essential oil formulations and 144 antennae (n = 18 per component) were used for individual essential oil components.

The degree of antenna sensitivity of cockroaches depends on the circadian clock of the receptor neurons such that the neurons were most sensitive at the day (Saifullah and Page 2009). Because of this, the electroantennogram experiments were performed during the day.

Antennal length-response correlation:

The relationship between antennal length and magnitude (mV) of response was examined by correlating the electroantennogram responses of each strain at specific antennal length to a

single 0.2 sec puff of Essentria® at a concentration of 1000 µg/µl. The tip of the antenna was cut with a microsurgical knife at the desired lengths (0.3 cm, 0.6 cm, 0.9 cm, or 1.2 cm). The same EAG technique was followed.

Olfactometer assays.

A 2-choice olfactometer was used to assess the responses of the three German cockroach strains to the vapors of 6 commercial essential oil formulations. The testing apparatus was made up of 3 plastic Petri dishes (100 × 15 mm, each), which were serially connected by two 5 mL plastic syringes as seen in Fig. 2. The syringes, purchased from CVS Pharmacy, Inc. (Woonsocket, RI, US), had their plungers removed, leaving only the barrel. The barrels were cut on both ends, creating a tube. On the left and right sides of the middle dish, two holes (1.5 cm diameter) were drilled, and the syringe barrels inserted. The left and right dishes each had two holes (1.5 cm diameter), one that connects the barrel and one that connects the air inlet, drilled on their side that faced opposite the middle dish. The syringes fit into the drilled holes and linked the Petri dishes serially. The left and right Petri dishes were connected to independent air sources that generated constant flow of air at 0.4 L/min into the middle Petri dish where a single male cockroach was placed. Male adults were obtained as described above. The left and right Petri dishes were covered with aluminum foil to prevent the entry of light and provide potential harborage for the cockroach. The middle Petri dish was exposed to white fluorescent light (0.02 lux). A 40 mm diameter hole was cut in the center of the lid of the middle Petri dish to avoid any possible fumigation effects and to easily insert a cockroach (Phillips and Appel 2010). This design facilitated the movement of a cockroach away from the central Petri dish into either of the side Petri dishes. The treatment and control sides were alternated after every 20 trials. Each trial involved placing a male German cockroach into the middle Petri dish and recording the choice it

made. A choice was recorded when a cockroach entered either barrel toward one of the Petri dishes. No choice was recorded if a cockroach did not choose a side within 2 min. Cockroaches averaged ≈5 sec to make a choice.

The commercial essential oil products were diluted in acetone and tested at concentrations 1000 µg/µl, 1 µg/µl, and the high and low label rates. Essential oil or acetone control (10 µl) were pipetted onto a filter paper (0.5cm × 4.5 cm), allowed to evaporate for 60 sec, and placed on the left or right side of the middle Petri dish in the syringe barrel. Each test was replicated 10 times at each concentration for each strain (n = 720 per strain); a total of 2880 cockroaches were tested.

Data Analysis

Electroantennogram

For analysis, the absolute EAG value was obtained by Equation 1.

$$C = T - S \qquad (1)$$

Where C is the corrected EAG millivolt response value, T is the EAG value of the test chemical, and S is the EAG value of the dry solvent control for the same antenna. Analysis of variance (ANOVA) with a Tukey honestly significant difference (HSD) test was used to compare the corrected EAG response among German cockroach strains, formulations, and their components using JMP software (JMP, Cary, NC, USA). The base 10 logarithm of the concentration was taken to better visualize the graph. Significance was determined using $\alpha = 0.05$.

Olfactometer

Two-choice olfactometer data were analyzed using chi-square tests with $\alpha = 0.05$. The percentage repellency value was obtained by Equation 2.

$$\%\, R = (C \div T) \times 100 \qquad (2)$$

Where % R denotes the percentage of insects repelled by a test compound, C is the number of insects that chose the control, and T denotes the total number of insects used for each treatment. The difference in the number of insects who chose the control, and the treatment determines the significance of a repellency score. A large difference means the repellency is significant while a small difference means the repellency is non-significant.

RESULTS

Antennal length and EAG response

Antennal response (mV) was significantly positively correlated with antennal length ($r = 0.9$, $P = 0.083$) (Fig. 3).

Electroantennogram responses to essential oil formulations

Strains S, D, and E had average antennal responses of 0.629 ± 0.102 mV (mean ± standard error), 1.105 ± 0.389 mV, and 0.837 ± 0.219 mV, respectively, to the negative control air puff. The control response of each strain was significantly lower than the tested formulations.

For Essentria®, the greatest concentration, 1000 µg/µl, caused the strongest average antennal response (mean ± standard deviation: 4.041 ± 0.589 mV, range: 0.750 mV – 8.320 mV) compared to 10 µg/µl ($F_{3, 396} = 0.0011$, $P = 0.009$) and 1 µg/µl ($F_{3, 396} = 0.0011$, $P = 0.001$), which caused average responses of 2.509 ± 0.381 mV and 2.161 ± 0.343 mV, respectively. The concentration of the other formulations did not induce significantly different responses.

Strain E demonstrated significantly stronger EAG responses (3.587 ± 0.438 mV) to Essentria® than strain S (1.976 ± 0.206 mV) but were non-significant when compared to strain D (3.208 mV ± 0.444 mV) (Table 2). No other strains showed significant differences to Essentria®. Following exposure to EcoVia™, strain D had significantly greater EAG responses (0.661 ±

0.007 mV) than strain S (0.413 ± 0.051 mV, $F_{2, 71}$ = 8.0678, P = 0.0168) and E (0.319 ± 0.045 mV, ($F_{2, 71}$ = 8.0678, P = 0.0007) (Table 2). Strains S and E were not statistically different from each other (Table 2). Strain D had a significantly lower response (0.090 ± 0.026 mV) than strain S (0.114 ± 0.036 mV, $F_{2, 71}$ = 4.8967, P = 0.0371) and E (0.281 ± 0.067 mV, $F_{2, 71}$ = 4.8967, P = 0.0149) following exposure to Excite R™ In response to Garscentria, strains S (0.308 ± 0.083 mV), D (0.364 ± 0.059 mV), and E (0.450 ± 0.107 mV) were not significantly different (Table 2). In response to Orange Guard® (Orange Guard), strain D (0.213 ± 0.037 mV) had significantly greater responses than strain E (0.0977 ± 0.019 mV, $F_{2, 71}$ = 4.1123, P = 0.0283). However, strain D did not differ from strain S (0.199 ± 0.033 mV) (Table 2).

In strain S, Essentria® generated significantly stronger responses (1.976 ± 0.206 mV) than Excite R™ (0.114 ± 0.03 mV, $F_{5, 143}$ = 27.1781; P < 0.01), Garscentria (0.308 ± 0.083 mV, $F_{5, 143}$ = 27.1781; P < 0.01), Orange Guard (0.199 ± 0.033 mV, $F_{5, 143}$ = 27.1781; P < 0.01), and ER-22™ (0.128 ± 0.051 mV, $F_{5, 143}$ = 27.1781; P < 0.01) (Table 3). Essentria® elicited a significantly stronger (3.209 ± 0.444 mV) response in strain D than EcoVia™ (0.661 ± 0.007 mV, $F_{5, 143}$ = 30.1303; P < 0.01), Excite R™ (0.090 ± 0.026 mV, $F_{5, 143}$ = 30.1303; P < 0.01), Garscentria (0.364 ± 0.095 mV, $F_{5, 143}$ = 30.1303; P < 0.01), Orange Guard (0.213 ± 0.037 mV, $F_{5, 143}$ = 30.1303; P < 0.01), and ER-22™ (0.338 ± 0.067 mV, $F_{5, 143}$ = 30.1303; P < 0.01) (Table 3). Essentria® formulation generated a considerably higher response (3.587 ± 0.434 mV) in strain E than EcoVia™ (0.319 ± 0.045 mV, $F_{5, 143}$ = 38.0158; P < 0.01), Excite R™ (0.281 ± 0.067 mV, $F_{5, 143}$ = 38.0158; P < 0.01), ER-22™ (0.169 ± 0.034 mV, $F_{5, 143}$ = 38.0158; P < 0.01), Garscentria (0.450 ± 0.107 mV, $F_{5, 143}$ = 38.0158; P < 0.01), and Orange Guard (0.098 ± 0.019 mV, $F_{5, 143}$ = 38.0158; P < 0.01) (Table 3).

Electroantennogram responses to essential oil components

Each essential oil component induced antennal responses (Fig. 3). For all the strains, camphene at 1000 µg/µl elicited a significantly higher response (0.367 ± 0.091 mV) than 100 µg/µl (0.069 ± 0.025 mV, $F_{3, 71} = 7.411$, $P = 0.001$), 10 µg/µl (0.050 ± 0.013 mV, $P = 0.001$), and 1 µg/µl (0.094 ± 0.032 mV, $P = 0.003$) of the same chemical. Caryophyllene at 1000 µg/µl elicited a significantly higher response (0.237 ± 0.051 mV) than 100 µg/µl (0.056 ± 0.025 mV, $F = 7.385$, $P = 0.0027$), 10 µg/µl (0.061 ± 0.020 mV, $P = 0.0042$), and 1 µg/µl (0.038 ± 0.012 mV, $P = 0.0004$). Menthone at 1000 µg/µl elicited a significantly higher response (0.813 ± 0.106 mV) than 10 µg/µl (0.404 ± 0.061 mV, $F = 9.4807$, $P = 0.004$), and 1 µg/µl (0.215 ± 0.052 mV, $P < 0.0001$). The responses elicited by 1000 µg/µl of 1,8-cineole was greater than the responses (0.387 ± 0.091 mV) induced by 10 µg/µl (0.091 ± 0.018 mV, $F = 6.0474$, $P = 0.005$), and 1 µg/µl (0.084 ± 0.021 mV, $P = 0.003$). Menthol at 1000 µg/µl elicited a significantly higher response (0.436 ± 0.096 mV) than 10 µg/µl (0.118 ± 0.042 mV, $F = 5.722$, $P = 0.014$), and 1 µg/µl (0.051 ± 0.025 mV, $P = 0.01$) of the same compound. P-cymene at 1000 µg/µl elicited a significantly higher response (0.371 ± 0.099 mV) than 10 µg/µl (0.131 ± 0.044 mV, $F = 3.221$, $P = 0.041$), and 1 µg/µl (0.131 ± 0.044 mV, $P = 0.05$) of the same compound.

Strain S exhibited significantly higher reactions (0.291 ± 0.062 mV) than strain E (0.075 ± 0.025 mV, $F_{2, 71} = 6.3999$, $P = 0.0019$) to limonene (Table 4). Strain S also induced significantly higher responses (0.389 ± 0.077 mV) than D (0.115 ± 0.039 mV, $F_{2, 71} = 6.4287$, $P = 0.0069$) and E (0.121 ± 0.065 mV, $F_{2, 71} = 6.4287$, $P = 0.0085$) to menthol (Table 4). Strain D had significantly higher responses (0.245 ± 0.065 mV) than strain S (0.067 ± 0.012 mV, $F_{2, 71} = 5.4334$, $P = 0.0062$) to p-cymene. Strain D had significantly higher responses (0.313 ± 0.077 mV) to A-pinene than S (0.113 ± 0.021 mV) (Table 4). Strain D had significantly higher

responses (0.169 ± 0.048 mV) to caryophyllene than S (0.049 ± 0.020 mV, $F_{2, 71} = 5.4334$, $P = 0.0319$) (Table 4).

In strain S, menthone elicited significantly larger responses (0.504 ± 0.075 mV) than caryophyllene (0.049 ± 0.020 mV, $F = 12.442$; $P < 0.0001$), limonene (0.291 ± 0.062 mV, $P < 0.0001$), p-cymene (0.067 ± 0.012 mV, $P < 0.0001$), 1,8-cineole (0.171 ± 0.032 mV, $P < 0.0001$), camphene (0.076 ± 0.025 mV, $P < 0.0001$), and α-pinene (0.103 ± 0.022 mV, $P < 0.0001$) (Table 5). In the same strain, menthol elicited significantly higher reactions (0.389 ± 0.077 mV) than caryophyllene (0.049 ± 0.020 mV, $P = 0.0001$), p-cymene (0.067 ± 0.012 mV, $P = 0.0001$), camphene (0.076 ± 0.025 mV, $P = 0.0002$), α-pinene (0.103 ± 0.022 mV, $P = 0.0011$), limonene (0.291 ± 0.062 mV, $P = 0.0015$), and 1,8-cineol (0.171 ± 0.032 mV, $P = 0.034$) (Table 5). Similarly, limonene elicited significantly higher reactions (0.291 ± 0.062 mV) than caryophyllene (0.049 ± 0.020 mV, $P = 0.00115$), p-cymene (0.067 ± 0.012 mV, $P = 0.0263$), and camphene (0.076 ± 0.025 mV, $P = 0.0396$) in strain S. Menthone elicited significantly more responses (0.437 ± 0.101 mV) in strain D than menthol (0.115 ± 0.039 mV, $P = 0.0176$) (Table 5). The remaining active compounds were not significantly different from one another in all the strains. In strain E, there were no significant differences in antennal responses among the active components (Table 5).

Repellency of commercial essential oil formulations against* B. germanica*:

The repellency of six commercial essential oil products against *B. germanica* was evaluated using a 2-choice olfactometer. The repellency of Essentria®, at 1000 µg/µl, against strain S ($\chi^2_{1, 40} = 32$, $P < 0.0001$), D ($\chi^2_{1, 40} = 40$, $P < 0.0001$), and E ($\chi^2_{1, 40} = 40$, $P < 0.0001$) was 80%, 100%, and 100%, respectively, which were significant (Table 2). Repellency was 35%, 60%, and 50 % for strains S, D, and E, respectively, at the high label rate (44 .77 µg/µl) of

Essentria® (Table 6). Only strain D ($\chi^2_{1,40} = 4.82$, $P = 0.0280$) was significantly repelled by Essentria® at the high label rate (44.77 µg/µl) (Table 6). The percent repellency was 43% for strain S, 50% for strain D, and 45% for strain E at the Essentria® low label rate (3.917 µg/µl) which was non-significant. The repellency of strains S ($\chi^2_{1,40} = 6.8182$, $P = 0.0090$), D ($\chi^2_{1,40} = 7.5294$, $P = 0.0061$), and E ($\chi^2_{1,40} = 9.7568$, $P = 0.0018$) were significant at 1 µg/µl, with 60 %, 63 %, and 70 % of insects repelled respectively (Table 6).

At 1000 µg/µl, EcoVia™ repelled 37%, 68%, and 70% of strains S, D, and E, respectively; however, only strains D ($\chi^2_{1,40} = 24.14$, $P < 0.0001$) and E ($\chi^2_{1,40} = 28$, $P < 0.0001$) were significant (Table 6). The percent repellency against strain D was 13%, 20% against strain E, and 30% against strain S at the high label rate (15.38 µg/µl). At the low label rate (7.751 µg/µl), 23% of strains S and E were repelled significantly. Only 13 % of strain D were repelled, which was not significant. At 1 µg/µl, there was non-significant repellency in 33% of insects from strains S and D and 20% strain E (Table 6).

At 1000 µg/µl, Excite R™ repelled strains D, S, and E at 18%, 40%, and 50%, respectively. However, only strain S ($\chi^2_{1,40} = 5.7619$, $P = 0.0164$) and E ($\chi^2_{1,40} = 20$, $P < 0.0001$) were significantly repelled by Excite R™ (Table 6).

At 1000 µg/µl, ER-22™ repelled 43% of strain S, 18% of strain D, and 13% of strain E cockroaches; only S was determined to be significant ($\chi^2_{1,40} = 6.5455$, $P = 0.0105$) (Table 6). For strains S, D, and E, the percent repellency was 50%, 28%, and 13%, respectively, at the label rate (157.897 µg/µl); however, only strain S was significant (Table 2). At 1 µg/µl, strain D ($\chi^2_{1,40} = 9$, $P = 0.0027$) was significantly repelled at 48%, whereas strains S and E were not significantly repelled at 23% and 28 %, respectively (Table 6).

Orange guard repelled 20% of strain E, 38% of strain S, and 48% of strain D at 1000 µg/µl; however only strains S ($\chi^2_{1,40} = 5$, $P = 0.0253$) and D ($\chi^2_{1,40} = 5.7619$, $P = 0.0164$) were significant (Table 2). At 1 µg/µl, Orange guard was significantly repellent to only strain S ($\chi^2_{1,40} = 5$, $P = 0.0253$) (Table 2). Orange Guard does not have a high or low label rate (Table 6).

Discussion

There has been a recent surge in interest in the study and assessment of essential oil formulations for pest control due to problems resulting from the use of conventional insecticides (Moretti et al. 2002, Koul et al. 2008, Mossa 2016, Said-Al Ahl et al. 2017). In this study, we assessed the repellency of six essential oil formulations. We also examined the magnitude of EAG responses they induce in the antennae of three strains of the German cockroach to understand if there is a relationship between olfaction, insecticide resistance, and repellency. The electroantennogram responses of the cockroaches to different concentrations of the essential oil constituents were also tested.

The relationship between antennal length and antennal response was examined. According to our findings (Fig. 3), this link is positively correlated. After the antenna length was quadrupled, the German cockroach's response was 26 times its initial value (2.489 mV compared with 0.095 mV). This is likely due to longer antennae having more chemoreceptors than shorter ones in insects (Spaethe et al. 2007). American cockroaches with longer antenna have been shown to detect odor plumes more accurately than those with shorter antenna (Lockey and Willis 2015).

The antennal responses of the cockroach to essential oils were studied using the electroantennogram technique. EAG reactions of German cockroaches to synthetic insecticides

have also been examined in other investigations. The results of the EAG study show that Essentria® induced 13 and 18-fold greater EAG responses than Excite R™ and ER-22™, respectively. Essentria® also induced 5 to 6-fold greater response than EcoVia™ , Orange Guard, and Garscentria. The great difference in responses observed between Essentria®, Excite R™ and ER-22™ may have been due to their different major components. Essentria® has more major components that elicited high antennal response (menthone and menthol), compared to Excite R™ and ER-22. Excite R™ contains pyrethrins, while ER-22 contains geraniol and cedar oil. Although the antennal response of the German cockroach to pyrethrins has not been tested, previous studies have shown that *Aedes albopictus* had EAG responses that ranged from about 0.025 mV to 0.07 mV when exposed to pyrethrin (Yan et al. 2021). Future study should examine the antennal response of German cockroaches to pyrethrins. The major components of the formulations were screened, tested, and compared to one another. Menthone, which is found in Essentria® and Garscentria, elicited a 5-fold greater average response than caryophyllene, limonene, and camphene and a 2.5-fold greater response than cineole, menthol, A-pinene, and p-cymene. This observation may have been because menthone is a ketone compound. Ketone compounds have been shown to induce EAG responses in the American cockroach with values ranging from 0.1 mV to 0.6 mV (Nishino and washino 1976). More ketone compounds still need to be tested to ascertain whether they induce large antennal responses in the German cockroach.

Essentria® induced 5-fold greater responses than EcoVia™. This could be because, in comparison to Essentria®, its active components are primarily hydrocarbons (i.e., limonene, A-pinene, P-cymene, and camphene). Hydrocarbon compounds induced antennal responses on the American cockroach that ranged from 0.6 mV to 1.2 mV. These values are low in comparison to the antennal responses (0.5 mV to 2.5 mV) induced by mono-ketones in the American cockroach

(Nishino and Washio 1976, Saïd et al. 2005). Ketones are more effective than hydrocarbons in repelling mosquitoes, (Paluch et al. 2010). Future studies should investigate the responses induced by a series of ketone compounds on cockroaches. In our study on the antennal reactions of the German cockroach to the formulation ingredients, the ketone compound (menthone) caused stronger responses in all strains than the hydrocarbon compounds.

Even though they both contain menthone in the same concentration, Garscentria did not perform as well in the EAG trials as Essentria®, which could be due to the presence of geraniol in Garscentria. Some monoterpene alcohols, such as geraniol, inhibit olfactory neurons in the sensilla of the silk moth, *Bombyx mori* (Pophof 1997), thereby, reducing the level of electrical activity. The effect of geraniol on the antennal response of the German cockroach should be studied further.

The essential oils elicited a diversity of reactions to the essential oil formulations in the cockroach strains. Excite R™ induced significantly different responses in the pyrethrin-resistant strains D and E, with E eliciting responses that were noticeably stronger than D. This result is unexpected because both strains are resistant to the same chemical class (pyrethrins). A potential reason for this is that Excite R™ contains other components, that induce different antennal responses in strain D and E. Strain S had greater antennal response to EcoVia™ and ER-22™ than strain D. However, strain S had lesser response to Essentria® and Excite R™ than strain E. The other components of the oil formulations could have produced the observed variances.

We investigated the active components in the formulations. Strains D and E had similar responses to all the essential oil components, although they were both different from strain S, with S having 3-fold stronger responses to menthol and 4-fold greater responses to limonene than strain D. It could have been that strain D has fewer receptors to menthol and limonene than strain

S. Resistant strains of the German cockroach express fewer receptors to the insecticide they are resistant to compared to their susceptible counterparts. For example, German cockroaches resistant to cyclodiene insecticides had fewer receptors to this insecticide (Kadous et al. 1983). Strain D had 4-fold greater responses than S to P-cymene, and 3-fold greater responses to α-pinene, and caryophyllene than S. This could also have been a result of the difference in receptors to these components the strains have. It is likely that the lack of significant variations between strains D and E is related to their resistance to the same chemical class (Oladipupo et al. 2020). There were no significant differences in the strains' reactions to P-cymene, cineole, or camphene.

Results from the repellency assay show that all essential oil formulations examined, except for Garscentria, significantly repelled the three strains at one or more concentrations. Among the products studied, Essentria® had the highest percentage of repellency. It repelled 100% of strains D and E at 1000 µg/µl, and 80% repellency in strain S at 1000 µg/µl. All of these were significant. The repellency of the formulations at 1000 µg/µl were 3-fold greater than the label rates, which means that the label rate will not be enough to effectively repel cockroaches in real-world situations. Essentria®'s high repellency against the strains could be attributed to its menthone component. No essential oil formulation repelled the cockroaches beyond 70% at any concentration, except from Essentria®. Essentria® significantly repelled German cockroaches at the lowest concentration (1 µg/µl), but not at the high (44.77 µg/µl) or low (3.917 µg/µl) label rates. This pattern was also seen in other formulations tested, with lower rates repelling the cockroach more than greater ones in several cases. EcoVia™, for example, repelled strain S at 15.38 µg/µl and 1 µg/µl but not at 1000 µg/µl and 7.751 µg/µl, strain D at 1 µg/µl but not at 15.38 µg/µl and 7.751 µg/µl, and strain E at 7.751 µg/µl not at 15.38 µg/µl. At 1

µg/µl, Excite R™ strongly repelled strains D and E, but not at 32.11 µg/µl or 1.94 µg/µl. This could have been because the antenna of the German cockroach becomes acclimated to the high concentrations of vapor stimuli. Due to continual stimulation, sensory acclimation (adaptation) happens when the strength and frequency with which a neuron generates action potentials decreases with time, resulting in reduced behavioral response (Kaissling 1986). For example, the fifth instar of *Rhodnius prolixus* showed decreased repellent behavior to DEET after continuous exposure for five minutes (Sfara et al. 2011). The cockroach strains differed in repellency by essential oil formulations. Only Essentria®, EcoVia™, and Excite R™ significantly repelled strain E at 1000 µg/µl, 7.751 µg/µl, and 1 µg/µl, whereas strains S and D were repelled by all formulations, at 1000 µg/µl, 44.77 µg/µl, and 7.75 µg/µl, except Garscentria.

The German cockroach detects and responds to essential oil formulations and components, according to our findings. Our experiments also reveal that a positive correlation exists between antennal response and antennal length. Essential oil mixtures, on the other hand, may not be effective at repelling cockroaches. It is therefore necessary to examine their toxicity to insect pests.

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Table 1. Essential oil and their components. The components tested in the EAG experiment are bolded.

Essential Oil	Components	References
Rosemary	**P-cymene-0.2-44.02%** Linalool-0.25-20.5% Gamma-terpinene-1.02-16.62%, Thymol-1.81%, B-pinene-3.61-7.5%, **A-pinene-2.83-21.6%,** Eucalyptol-2.64%	Ozcan and Chalchat 2008 Salido et al. 2003 Jiang et al. 2011
Peppermint oil	**1,8-Cineole-3.75-13.5%, Limonene-3.29-6.8%, L-menthone-1.9-28.8%,** Menthofuran-1.90-8.9%, Neomenthol-2.83-3.8%, **Menthole-36.9-60.6%,** Carveone-3.82%, Methyl acetate-4.54-8.64% B-cubebe-1.305%	Clark and Menary 1981 Mahboubi and Kazempour 2014 Moghaddam et al. 2013
Thyme Oil	**P-cymene-7.76-43.75%,** Y-terpinene-4.20-27.62%, Thymol-21.38-60.15%, Carvacrol-1.15-3.04%, **B-Caryophyllene-1.30-5.28 %.**	Hudaib et al.2002 Imelouane et al. 2009
Cedar oil	A-cedrene-30.7% M-cymene-1.25% A-pinene-3.08-6.53% Sabinene-3.30% 3-carene-18.62% **P-cymene-1.27-3.68%** Limonene-2.69-9.74% A-terpineol-2.27%	Cheng et al. 2005
Cinnamon oil	Eugenol-74.9%, **B-Caryophyllene-4.1**%, Benzyl benzoate-3%, Linalool-2.5%, Eugenyl acetate-2.1%, Cinnamyl acetate-1.8%.	Schmidt et al. 2006

Table 2. Differences in the EAG responses to the essential oil formulations among strain S, D, and E. Blank cells are when one strain was insignificantly different from the other in response to the formulations.

Essential Oils	**Strains**			
Essentria®		S	D	E
	S	N.S.	N.S.	A
	D	N.S.	N.S.	N.S.
	E	a	N.S.	N.S.
EcoVia™	S	N.S.	a	N.S.
	D	a	N.S.	b
	E	N.S.	b	N.S.
Excite R™	S	N.S.	N.S.	a
	D	N.S.	N.S.	b
	E	a	b	N.S.
ER-22	S	N.S.	a	N.S.
	D	a	N.S.	N.S.
	E	N.S.	N.S.	N.S.
Garcentria	S	N.S.	N.S.	N.S.

[1] No comparison was significant	D	N.S.	N.S.	N.S.
	E	N.S.	N.S.	N.S.
Orange Guard	S	N.S.	N.S.	N.S.
	D	N.S.	N.S.	a
	E	N.S.	a	N.S.

Table 3. Differences in the EAG responses induced by the essential formulations in strain S, D, and E. Blank cells are when one the response induced by an essential oil on a strain was insignificantly different from the other essential oil.

Strain	Essential Oils						
		Essentria®	**EcoVia™**	**Excite R™**	**ER-22**	**Garcentria**	**Orange Guard**
S	**Essentria®**	N.S.	a	b	c	d	e
	EcoVia™	a	N.S.	N.S.	N.S.	N.S.	N.S.
	Excite R™	b	N.S.	N.S.	N.S.	N.S.	N.S.
	ER-22	c	N.S.	N.S.	N.S.	N.S.	N.S.
	Garcentria	d	N.S.	N.S.	N.S.	N.S.	N.S.
	Orange Guard	e	N.S.	N.S.	N.S.	N.S.	N.S.
D	**Essentria®**	N.S.	a	b	c	d	e
	EcoVia™	a	N.S.	f	N.S.	N.S.	N.S.
	Excite R™	b	f	N.S.	N.S.	N.S.	N.S.
	ER-22	c	N.S.	N.S.	N.S.	N.S.	N.S.
	Garcentria	d	N.S.	N.S.	N.S.	N.S.	N.S.
	Orange Guard	e	N.S.	N.S.	N.S.	N.S.	N.S.
E	**Essentria®**	N.S.	a	b	c	d	e
	EcoVia™	a	N.S.	N.S.	N.S.	N.S.	N.S.
	Excite R™	b	N.S.	N.S.	N.S.	N.S.	N.S.
	ER-22	c	N.S.	N.S.	N.S.	N.S.	N.S.
	Garcentria	d	N.S.	N.S.	N.S.	N.S.	N.S.
	Orange Guard	e	N.S.	N.S.	N.S.	N.S.	N.S.

Table 4. Differences in the antennal responses, between strain S, D, and E, to the components of the essential oil formulations. The blank cells are when one strain was insignificantly different from the other in response to an essential oil component.

Essential oil components	Strain	S	D	E
Menthone	**S**	N.S.	N.S.	N.S.
	D	N.S.	N.S.	N.S.
	E	N.S.	N.S.	N.S.
Menthol	**S**	N.S.	a	b
	D	a	N.S.	N.S.
	E	b	N.S.	N.S.
Camphene	**S**	N.S.	N.S.	N.S.
	D	N.S.	N.S.	N.S.
	E	N.S.	N.S.	N.S.
Caryophyllene	**S**	N.S.	a	N.S.
	D	a	N.S.	N.S.
	E	N.S.	N.S.	N.S.
Limonene	**S**	N.S.	N.S.	a
	D	N.S.	N.S.	N.S.
	E	a	N.S.	N.S.
A-pinene	**S**	N.S.	a	N.S.
	D	a	N.S.	N.S.
	E	N.S.	N.S.	N.S.
P-Cymene	**S**	N.S.	a	N.S.
	D	a	N.S.	N.S.
	E	N.S.	N.S.	N.S.
Cineole	**S**	N.S.	N.S.	N.S.
	D	N.S.	N.S.	N.S.
	E	N.S.	N.S.	N.S.

Table 5. Differences in the induced antennal responses by the essential oil components in strain S, D, and E. Blank cells indicate no significant difference in response induced by an essential oil component on a strain.

Strain	Essential Oil components								
		Menthone	**Menthol**	**Camphene**	**Caryophyllene**	**Limonene**	**A-pinene**	**P-cymene**	**Cineole**
S	**Menthone**	N.S.	N.S.	a	b	c	d	e	f
	Menthol	N.S.	N.S.	g	h	i	j	k	l
	Camphene	a	g	N.S.	N.S.	m	N.S.	N.S.	N.S.
	Caryophyllene	b	h	N.S.	N.S.	n	N.S.	N.S.	N.S.
	Limonene	c	i	m	n	N.S.	N.S.	o	N.S.
	A-pinene	d	j	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	P-cymene	e	k	N.S.	N.S.	o	N.S.	N.S.	N.S.
	Cineole	f	l	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
D	**Menthone**	N.S.	a	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Menthol	a	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Camphene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Caryophyllene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Limonene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	A-pinene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	P-cymene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Cineole	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
E	**Menthone**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Menthol	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Camphene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Caryophyllene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Limonene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	A-pinene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	P-cymene	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Cineole	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

Table 6: Pearson Chi square, percentage repellency, and *p*-values of oil formulations against strain S, D, and E. P.R means percentage repellency.

Essential Oil	Strain	1000 µg/µl	High label rate	Low label rate	1 µg/µl
Essentria®	***S***	a	N.S.	N.S.	b
	D	c	d		e
High label rate =44.77 µg/µl	***E***	f	N.S.	N.S.	g
Low label rate =3.917 µg/µl					
EcoVia™	***S***	N.S.	N.S.	N.S.	N.S.
	D	a			
High label rate =15.38 µg/µl	***E***	b	N.S.	c	N.S.
Low label rate =7.751 µg/µl					
Excite R™	***S***	a	N.S.	N.S.	N.S.
	D		N.S.	N.S.	N.S.
High label rate =32.11 µg/µl	***E***	b	N.S.	N.S.	N.S.
Low label rate =1.94 µg/µl					
ER-22	***S***	a	b	N.S.	N.S.
	D	N.S.	N.S.	N.S.	c
High label rate =44.77 µg/µl	***E***	N.S.	N.S.	N.S.	N.S.
Garcentria	***S***	N.S.	N.S.	N.S.	N.S.

High label rate =72.46 µg/µl	***D***	N.S.	N.S.	N.S.	N.S.
	E	N.S.	N.S.	N.S.	N.S.
Orange Guard	***S***	a	N.S.	N.S.	b
	D	c	N.S.	N.S.	N.S.
	E	N.S.	N.S.	N.S.	N.S.

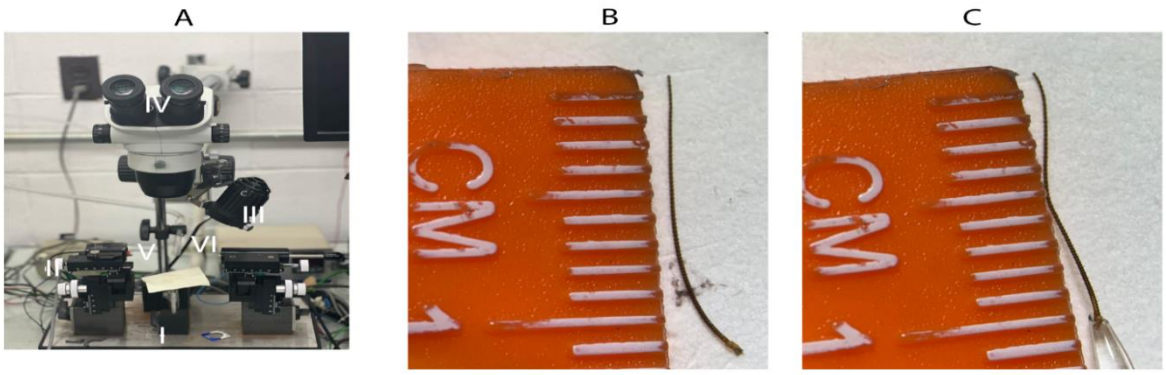

Figure 1 (A) Electroantennogram equipment **(B)** Antenna tip **(C)** Antenna base inserted into electrode

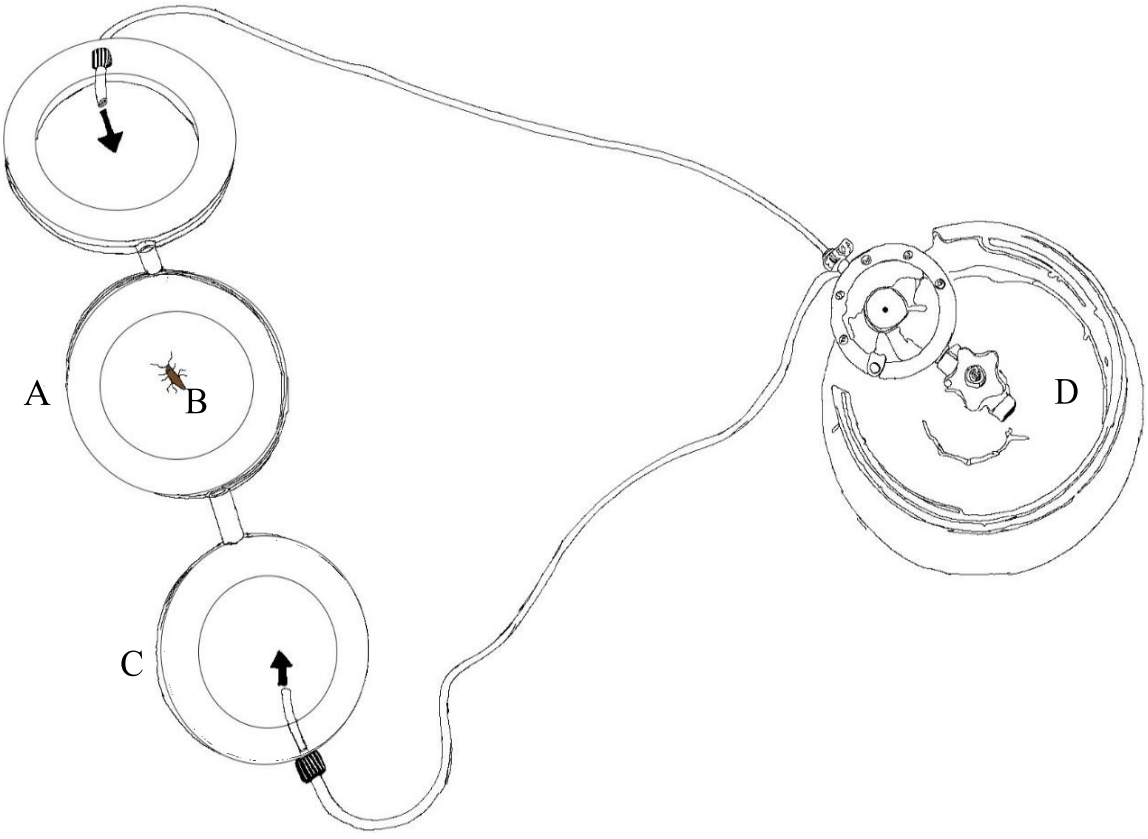

Figure 2: A 2-choice olfactometer set-up.

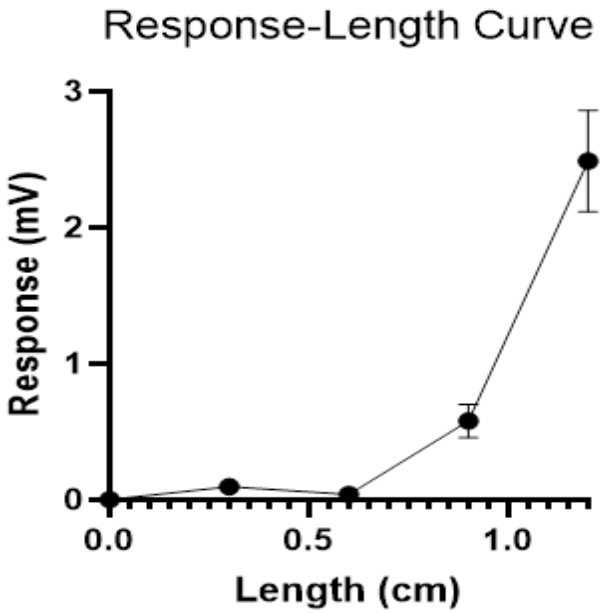

Figure 3: Correlation between antennal length and antennal response to Essentria®.

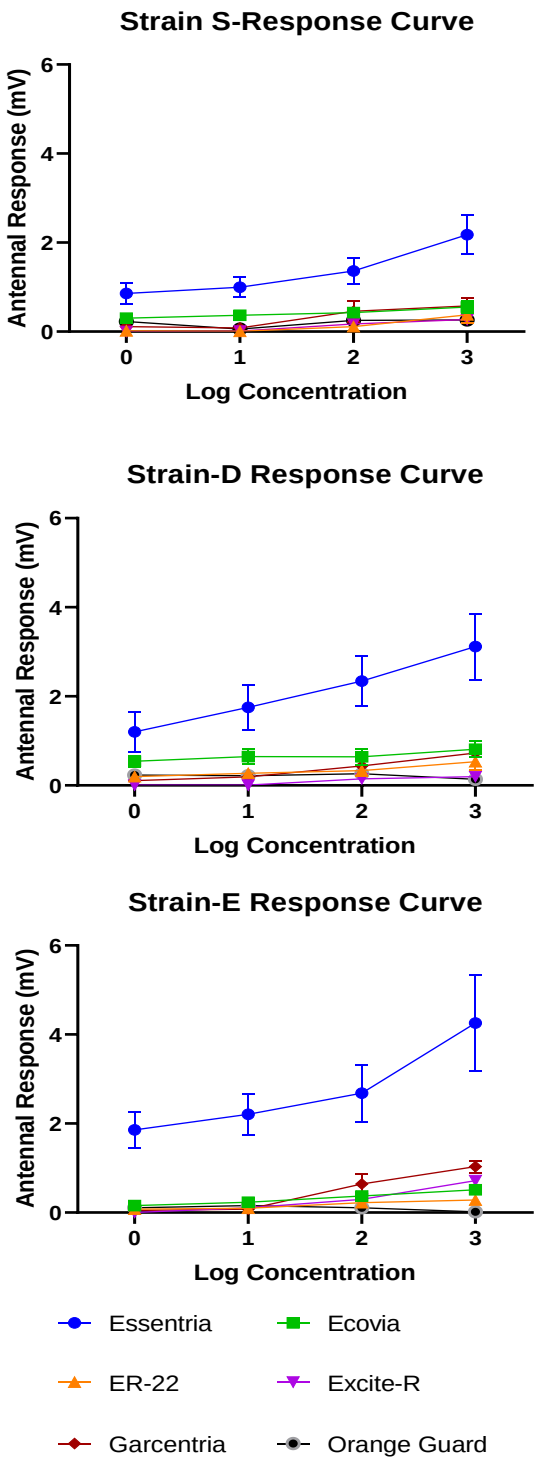

Figure 4: Electroantennogram responses (mean ± SE) of *B. germanica* to the 6 commercial essential oil formulations.

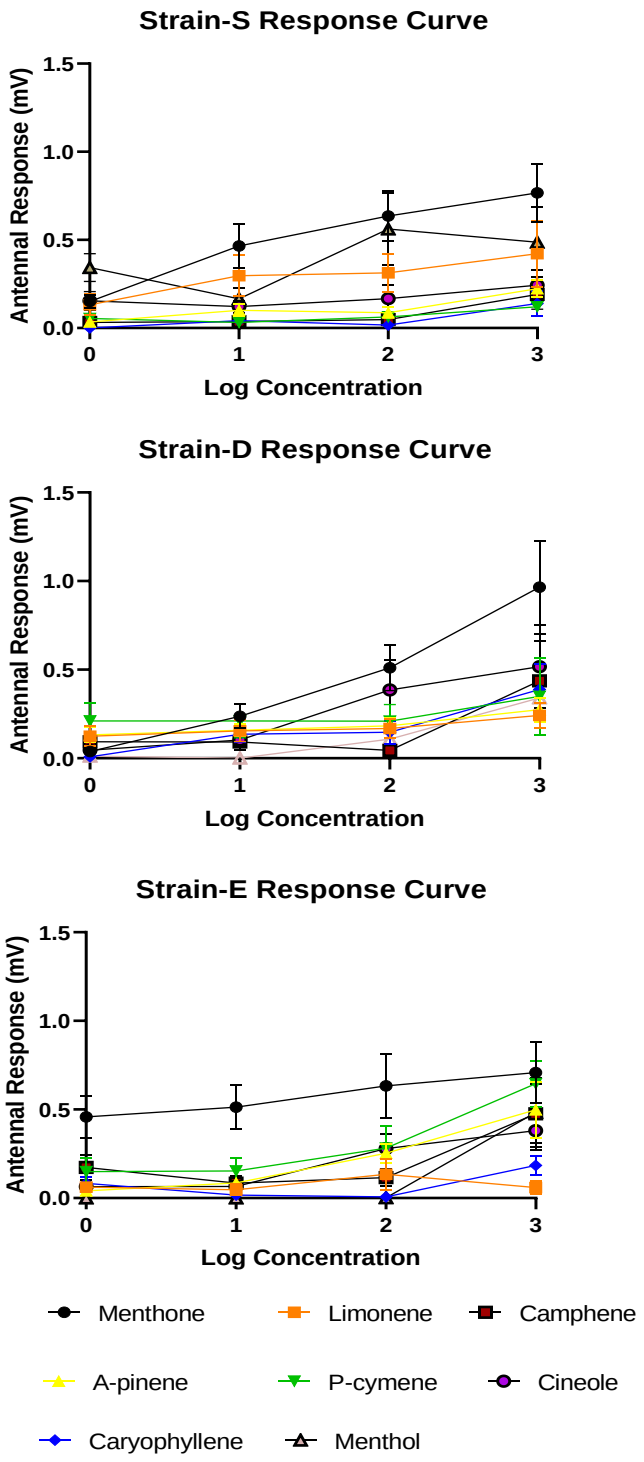

Figure 5: Electroantennogram responses (mean ± SE) of *B. germanica* to the 8 commercial essential oil components.

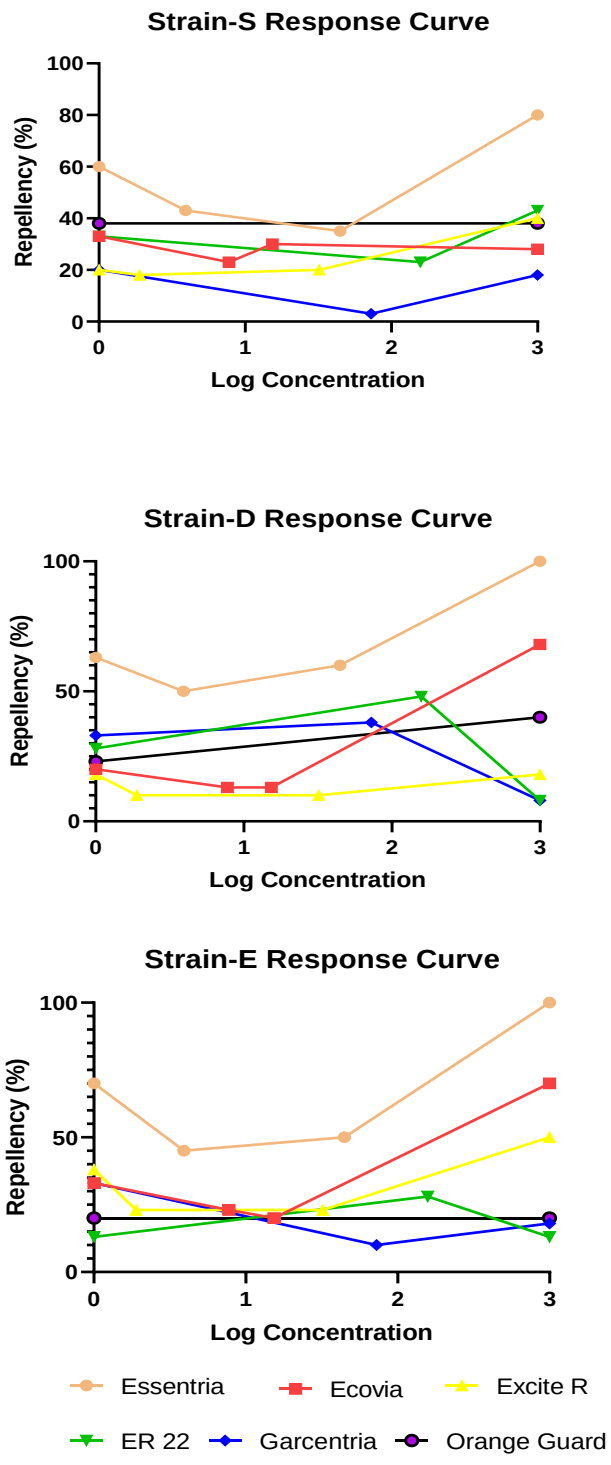

Figure 6: Repellency curve of *B. germanica* to the 6 commercial oil formulations.

Toxicity and Repellency of Five Essential Oil Formulations against the German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae)

The German cockroach, *Blattella germanica* (L.) (Blattodea: Ectobiidae), has thrived in urban settings because they have access to a highly fragmented, but resource rich habitat that has produced variation in various cockroach populations (Tang et al. 2019). Urban settings provide them with food, water, harborage, and warmth. German cockroaches are ubiquitous because of their capacity to adapt to many environmental situations (Schal et al. 1984). German cockroach infestations are more common in places that have poor sanitary conditions, which are widespread in low-income communities. They affect human health by spreading infections, interacting with our food, and developing asthma in children due to their body excretions (Schal and Hamilton 1990).

The use of insecticides to control German cockroaches is effective and less costly compared to other methods of control such as biological control (Wang et al. 2021b). However, insecticides pose a threat to non-target organisms, the environment, and to humans.

Essential oils are volatile compounds found in aromatic plants. Many essential oils are toxic to insect pests (Evans 1981). These essential oils target the insect nervous system and disrupt the octopaminergic system (Evans 1981). Essential oils also have repellent activities, however, the molecular process behind this is still unclear.

Essential oil components like p-cymene, limonene, α-pinene, 1,8-cineole, and menthone are toxic to the German cockroach (Jang et al. 2005, Phillips and Appel 2010, Yeom et al. 2013). For example, 1,8-cineole was tested to be toxic (LC_{50} = 6.8 mg/liter) against adult male German cockroach (Phillips and Appel 2010). This study assessed the toxicity of five essential oil

formulations against an insecticide-susceptible (S) and two insecticide-resistant German cockroach strains (D and E). Their repellency was also evaluated in this study using a different assay than the previous study.

MATERIAL AND METHODS

Insects

The susceptible strain has been kept in continuous culture at the urban entomology laboratory (Auburn University) for >37 years without insecticide exposure. The two resistant strains were collected from Franklin County, North Carolina, and are resistant to permethrins, chlorpyrifos, propoxur, and fipronil (Wu and Appel 2017). Each strain was maintained in separate 3.8-liter glass jars with cardboard harborage at a 27 ± 2°C, 40–70% RH, and a photoperiod of 12:12 (L: D) h. A 60 ml glass jar was provided with water and a cotton wick was affixed through the cap. Rat chow was fed to the colonies *ad libitum* (Purina 500l lab diet from Purina LabDiet, Inc. St. Louis, MO). Adult male German cockroaches were utilized in the study because they have a relatively consistent body mass and do not produce eggs and hence maintain steady hormone levels (Oladipupo et al. 2020).

Essential Oil Formulations

Water was used to dilute the formulations. Table 1 lists the formulation names, essential oil constituents, and manufacturers used in this trial. The formulations were all purchased from "Do My Own Pest Control" (Norcross, GA, USA).

Continuous exposure assay

The toxicity of the essential oil formulations against the focal strains of German cockroach was determined using a continuous exposure assay (Fig. 7). The design includes a flat

wooden board (6 × 12 inches) wrapped in Reynolds® Wrap aluminum foil (Lake Forest, IL, USA), with 2 ml of the high label rate of an essential oil formulation pipetted and spread uniformly on the wrapped board's surface. The surface of the board was allowed to dry, after which plastic cups (0.5 L) were placed on its surface. The tops of the cups were cut open to avoid possible fumigation effects. Each of the boards could hold three cups (Fig. 7). Each cup contained 6 cockroaches. To keep the cockroaches from escaping, Vaseline petroleum jelly (Trumbull, CT, USA) was used to coat the top 2 cm of the cups and the contact between the cup and the board. The number of insects that died was recorded every 15 minutes for the first hour, then every hour for the next 24 hours. If an insect did not respond after being nudged with forceps it was recorded as dead.

The experiment was carried out for the high label rates of each formulation, with each experimental set containing six boards (each with three cups) for each strain that served as replicates (n = 36 per strain). A negative control was a wrapped board treated with 2 ml water for each strain.

Direct spray assay

A spray assay was used to assess the direct toxicity of the high label rates of the essential oil formulations against the focal strains of the German cockroach. Six cockroaches were placed inside a 0.5 L glass mason jar, and immediately sprayed with 0.3 ml of either the high or low label rate of each formulation (Fig. 8). The number of dead cockroaches was recorded every minute for 30 minutes. The opening of each jar was coated with petroleum jelly, as specified in the continuous exposure assay section, to prevent the cockroaches from escaping.

The experiment was carried out for each formulation at each concentration (n = 6 jars per strain per concentration). Each strain had six jars as a negative control (water). The high and low

rates of each formulation were mixed in water as detailed in the ***Continuous exposure assay*** section.

Flushing assay

The repellency of essential oil formulations against the focus strains of the German cockroach was evaluated using a flushing assay. Adult male German cockroaches were collected from colony jars, briefly anesthetized with carbon dioxide, and placed in a cardboard box (12.5 cm × 8 cm × 1.5 cm). Anesthetization was done to prevent the cockroaches from escaping after placing them into the boxes. Once the cockroaches were placed in each box, the box was sealed with masking tape and the cockroaches were allowed to acclimate for 30 minutes. The essential oil formulations were sprayed into each box at 1000 µg/µl and the high and low label rates. After the formulation was sprayed into each box, a 2 cm length of tape was removed to provide the cockroaches an area to escape. Each box was placed in a plastic shoe box so the flushed cockroaches could be retained and counted. Over the course of 30 minutes, the number of cockroaches that left the boxes at was recorded every 1-2 minutes. Each box contained six cockroaches of one of the three strains. This was repeated six times (n = 36 cockroaches per strain)

Data analyses

Continuous exposure and direct spray assays:

For each strain, survivorship curves were generated for each concentration of every formulation. Survivorship for the residues or to direct spray were compared using the log rank (Mantel-Cox) test in GraphPad prism software (GraphPad software Inc, San Diego, CA, US).

Flushing assay:

The percentage repellency value was obtained by Equation 1.

$$\% R = (C \div T) \times 100 \qquad (1)$$

The LT_{50} values from the flushing assay were obtained using probit analysis from Polo plus software (LeOra Software LLC, Parma, Italy). The software is specifically designed to calculate the LT_{50} values after data that includes the number of insects used for the experiment, the number of insects dead at a particular time have been input. Significance was determined using $\alpha = 0.05$.

RESULTS

Toxicity in the continuous exposure assay

The cockroaches had the highest mortality after being exposed to Excite R™. They had the least mortality after being exposed to EcoVia™ (Fig. 9). The LT_{50} of Excite R™ was 3.83, 3.626, and 3.861 hours against strain S, D, and E, respectively (Table 7). This is in stark contrast to EcoVia™, where after 24 hours of exposure to the formulation, <10% of strain S, D, and E, died (Table 7). The LT_{50} of Essentria® was 16.426, 28.854, and 100.294 hours against strains S, D, and E, respectively (Fig. 9A). The LT_{50} value of ER-22 was 4.334, and 4.169 hours for strain D and E, respectively. Garscentria had no LT_{50} value (Table 7).

Strain S had significantly greater mortality ($\chi 2$ (1) = 9.926, P = 0.0016) to Essentria® than strain E (Fig. 9A). Strain S did not differ significantly from D (Fig. 9A). Strain D and E did not significantly differ from each other in mortality following exposure to Essentria®. Strain E was significantly more susceptible ($\chi 2$ = 9.574, P = 0.0020) to Garscentria than S, no other comparisons were significant (Fig. 9D). Strain E was more susceptible ($\chi 2$= 11.58, P = 0.0007) to ER 22 than strain S. Strain E did not differ significantly from D. Strain D was more susceptible ($\chi 2$= 4.238, P = 0.0395) than S to ER 22 (Fig. 9E).

EcoVia™ was the only formulation in which the strains did not differ significantly in terms of mortality (Fig. 9A–E).

Toxicity in the direct exposure assay

High label rate

The formulation's high label rates had an effect on the strains' mortality following direct spray. The LT_{50} of Excite R™ was 15.314, 5.433, and 0.837 minutes for strains S, D, and E, respectively (Table 8). The LT_{50} for ER 22 was 4.334 and 4.169 minutes for strains D and E, respectively. The LT_{50} of Essentria® against strain D was 1.343 minutes (Table 8). The LT_{50} value for EcoVia™ against strain E was 115.708 minutes. There were no LT_{50} values from exposure to Gascentria because of very low mortality (Fig. 9E).

For Garscentria and Essentria®, the strains' survivorship curves were not significantly different from each other (Fig. 10B and E). Strain S had greater mortality ($\chi 2$ (1) = 6.918, P = 0.0085*)* to EcoVia™ than strain E but not D. Strain D was more sensitive ($\chi 2$ = 14.64, P = 0.0001*)* to EcoVia™ than strain E (Fig. 10B). Strain E was more susceptible ($\chi 2$ = 18.42, $P <$ 0.0001*)* to Excite R™ than strain S (Fig. 10A). It was also more susceptible ($\chi 2$ = 8.286, P = 0.0040*)* than strain D (Fig. 10A). However, there were no significant differences between strains S and D to Excite R™. Strain S was more sensitive (χ^2 = 6.716, P = 0.0096) to ER-22 than strain D to ER-22 (Fig. 10D). It was likewise more vulnerable (χ^2 = 4.903, P = 0.0268) than E (Fig. 10D). However, strains D and E had no significant difference after exposure to ER-22.

Continuous exposure and direct spray comparison

When the mortality of the strains caused by continuous exposure is compared to the mortality caused by directly spraying the formulations, the direct spray test had a lower range of

LT_{50} values (0.83 minutes-115 minutes) than the continuous exposure test (3.83-100 hours) (Tables 7 and 8; Fig. 11).

Repellency using the flushing assay

The repellency against the German cockroach varied for each formulation at the different concentrations (Fig. 12). Overall, 1000 µg/µl concentration produced the highest average (12.9%) repellency compared to the average value of the all the label rates summed together (7.5%). Among the formulations tested, EcoVia™ produced the highest average repellency (15.5%), which was followed by Essentria® (13.8%), ER-22 (7%), Excite-R (5.6%), and Garscentria (3.5%). The strains differed in repellency, with strain S having the highest average repellency (11.6%), followed by strain E (9%), while strain D had the least average repellency (8.4%).

Discussion

The two assays used for measuring the toxicity of the essential oil formulations differed in their results, with the direct spray assay resulting in 2.5-fold lower average LT_{50} value (12.52 hours) than the continuous exposure assay (31.94 hours). This observation is due to the differences in the nature of the two assays. In the direct spray assay, each cockroach was exposed to a higher amount of formulation compared to the continuous exposure assay. This is because in the latter the formulations were spread evenly over a flat wood of surface area of 72 square inches, which reduced the amount of formulation present per unit area of the flat wood, while in the former assay, the cockroaches were exposed to most of the amount of formulation contained in a single spray.

In the continuous exposure assay, Excite R™ produced the fastest average LT_{50} value (3.772 hours), which was followed by ER-22 (4.251 hours), Essentria® (48.524 hours), and EcoVia™ (115.708 hours). Garscentria did not have any LT_{50} value because its average slope was close to zero (0.294). The strains differed in the LT_{50} values they had. Strain S had the least average LT_{50} value (10.132 hours), which was followed by strain D (12.271 hours), and strain E (56.008 hours). This is due to the fact that strain S was lab reared and was never exposed to any insecticide, which makes it have less resistant to various compounds compared to strain D and E that were previously exposed to different insecticides before being lab reared (Wu and Appel 2017).

In the direct spray assay, using the high label rates of the formulations, Essentria® gave the lowest LT_{50} value (1.34 minutes), which was followed by ER-22 (2.47 minutes), Excite R™ (7.194 minutes), and EcoVia™ (1237.37 minutes). Garscentria did not give any LT_{50} value because its average slope was close to zero (0.123). The strains differed in the LT_{50} values following direct spray. Strain E had the lowest average LT_{50} value (0.837 minutes), which was followed by strain D (3.401 minutes), and strain S (418.049 minutes). Using the low label rates of the formulations in the same assay, the formulations gave similar mortality results. This shows that the high concentration of the formulations is more toxic than the low concentration.

The repellency of the formulations was tested using the flushing assay. The repellency values were not as high as the two-choice olfactometer assay tested in the previous experiment that tested the repellency of the same set of formulations. This may have been due to differences in the way the assay worked. In the two-choice olfactometer repellency assay, the cockroaches encountered a constant stream of the vapor of the formulations compared to the flushing assay. The repellency values for this assay ranged from as low as 1% to as high as 44%. This was not

the case in the two-choice olfactometer assay, where the repellency ranged from 3% to 100%. In the flushing assay, EcoVia™ (15.5%) and Essentria® (13.5%) caused 2 fold-greater average repellency against the German cockroach than Excite R™ (7%), ER-22 (5%), and Garscentria (3.5%). Essentria® also had the highest average repellency compared to the rest of the formulations in the olfactometer assay. It also shows that regardless of the method of measuring repellency, Essentria® and EcoVia™ are more repellent to German cockroaches compared to Excite R™ER-22, and Garscentria.

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Table 7. LT_{50} values of each essential oil formulation at their high label rates against the focal strains in the continuous exposure assay. The empty cells do not have LT_{50} values (n = 36).

Insecticide	Cockroach Strain	LT_{50} (95% Cl)	Slope	SE	χ^2 (df)
Essentria®	**S**	16.426 (13.00 – 21.559)	1.432	0.206	5.695 (7)
	D	28.854 (23.564 – 42.608)	2.066	0.4	10.717 (8)
	E	100.294 (53.784 – 400.201)	1.069	0.261	2.326 (7)
EcoVia™	**S**		1.050	0.745	0.490 (3)
	D		0.178	0.554	0.348 (1)
	E	115.708 (53.225 – 1402.264)	1.053	0.346	0.988 (6)
Excite R™	S	3.838 (3.65 – 4.018)	11.686	1.597	0.637 (2)
	D	3.626 (3.480 – 3.771)	17.449	2.665	0.397 (1)
	E	3.861 (3.698 – 4.016)	15.950	2.337	0.815 (2)
Garscentria	S		0	0.338	0 (1)

	D		0.291	0.182	0.726 (2)
	E		0.593	0.232	1.097 (3)
ER-22	S		4.018	0.670	14.423 (3)
	D	4.334 (2.890 – 5.938)	1.877	0.244	5.9676 (4)
	E	4.169 (2.944 – 5.485)	2.221	0.250	4.9064 (4)

Table 8. LT_{50} values of each essential oil formulation at their high and low label rates against the focal strains in the direct spray assay (n = 36).

Insecticide	Cockroach Strain	LT_{50} (95% Cl)	Slope	SE	χ^2 (df)
Essentria®	**S**		1.768	0.238	30.327 (3)
High	**D**	1.343	1.121	0.181	1.956 (3)
= 44.7 µg/µl		(0.619 – 2.281			
	E		2.480	0.389	18.089 (3)
EcoVia™	**S**	1237. 375	0.679	0.118	4.551 (5)
High		(687.553 –			
= 15.38		2971.152)			
	D		0.377	0.096	7.2666 (4)
	E		0.686	0.359	0.737 (3)
Excite R™	**S**	15.314	1.376	0.172	4.125 (5)
High		(11.91 –			
= 32.11		20.291)			
µg/µl	**D**	5.433	0.967	0.146	6.957 (7)
		(3.883 – 7.285)			

	E	0.837 (0.157 – 1.660)	0.902	0.213	6.0964 (6)
ER-22 High = 44.77 µg/µl	**S**	1.458 (0.355 – 2.557)	1.564	0.250	12.886 (5)
	D	3.482 (1.543 – 5.307)	2.164	0.285	12.517 (4)
	E		2.595	0.307	24.368 (4)
Garscentria High = 72.46 µg/µl	**S**		0.263	0.267	0.878 (2)
	D		0.106	0.282	0.003 (1)
	E		0	0.308	0 (1)
Essentria® Low = 3.917 µg/µl	**S**		0.354	0.289	1.457 (2)
	D		0.604	0.287	4.4080 (2)
	E		0.446	0.261	1.744 (3)
EcoVia™ Low = 7.751 µg/µl	**S**	3851.9 (1310.8 – 46303)	0.801	0.203	3.839 (6)
	D		0.742	0.294	2.543 (6)

	E		0.645	0.506	2.517 (4)
Excite R™	**S**	1649.1	1.913	0.645	3.277 (6)
Low	**D**		1.517	1.028	0.876 (6)
= 1.94 µg/µl	**E**				

Table 9. Essential oil formulations and their extracts.

Essential oil Formulation	Manufacturer	Active Ingredient
Essentria® All Purpose Insect Concentrate (Essentria®)	Zoecon, schaumberg, IL, USA	• Rosemary oil-10% • peppermint oil-2%
EcoVia™ EC emulsifiable concentrate (EcoVia™)	Rockwell labs, Kansas City, MO, USA	• Thyme oil-20% • Rosemary oil-8% • 2-phenethylpropoinate-14%
Garscentria Insect and Pest Control	Bare Ground Solutions, Framingham, USA	• Garlic liquid-45% • Rosemary oil-10% • Peppermint oil-2% • Geraniol-5% • Wintergreen oil-5% • Vanillin-3% • Glycerin-5% • Water-25%
Excite R™	Zoecon, schaumberg, IL, USA	• Piperonyl butoxide-60% • Pyrethrins-6%
ER-22™	Renotech, North Bergen, NJ, USA	• Geraniol-2% • Cedar oil-2% • Sodium Lauryl sulfate-4%
Orange Guard	Orange Guard inc. Marina, CA, USA	D-Limonene- 5.8%

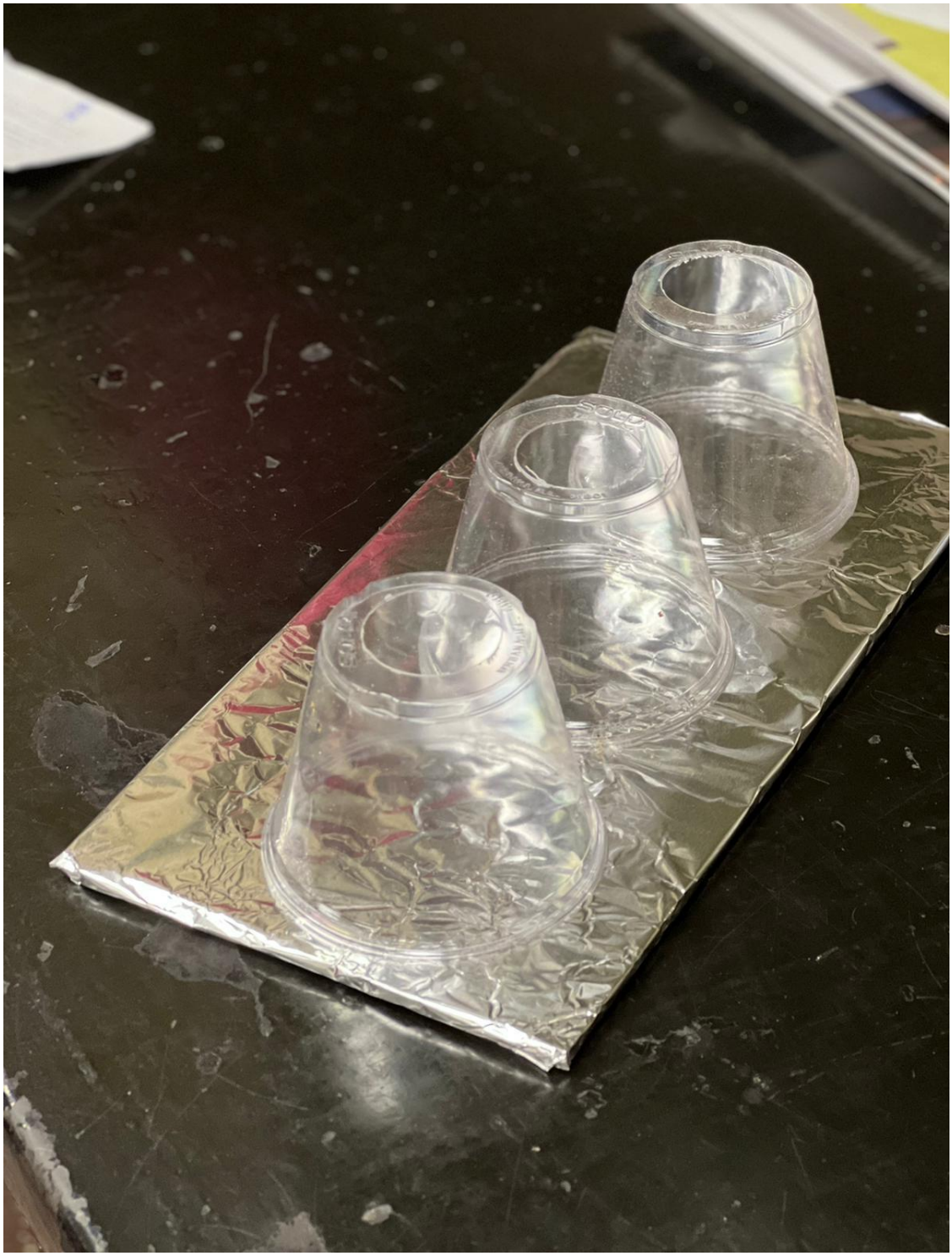

Figure 7. Toxicity experiment set up.

Figure 8. Mason Jars (0.5 L) each containing 6 cockroaches in the direct spray experiment.

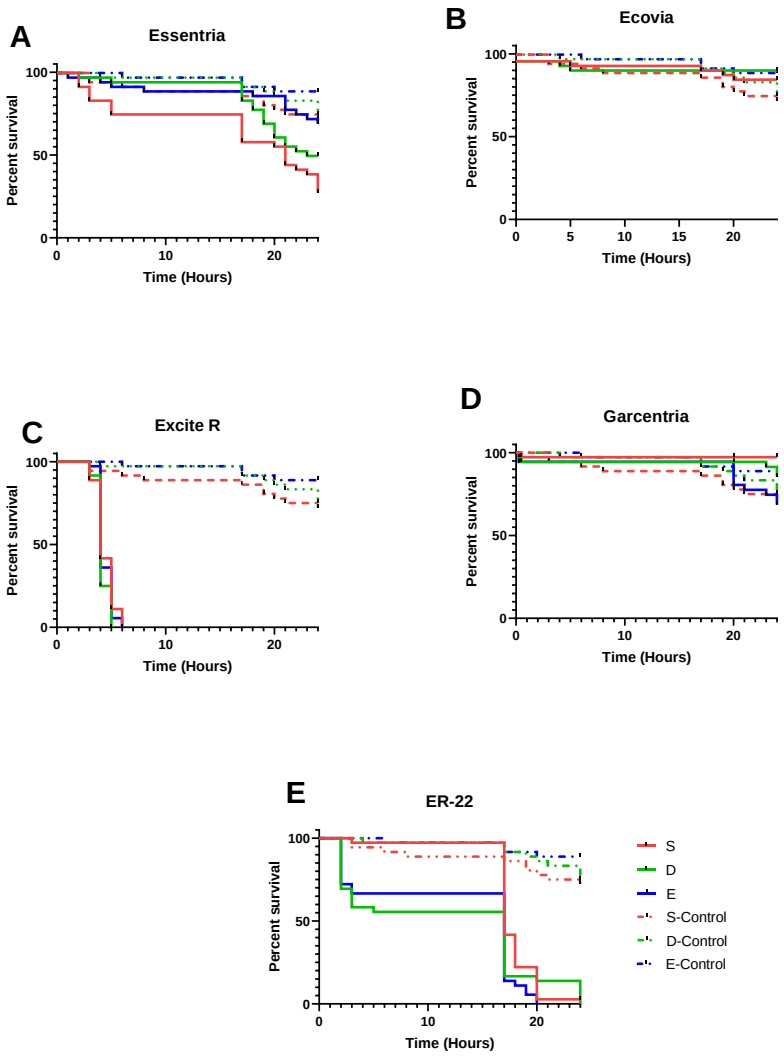

Figure 9. Survivorship of cockroach strains in the continuous exposure assay tested with the high label rates of (A) Essentria® (B) EcoVia™ (C) Excite R™ (D) Garscentria (E) ER-22. Log rank (Mantel-Cox) tests were performed to determine the differences among strains.

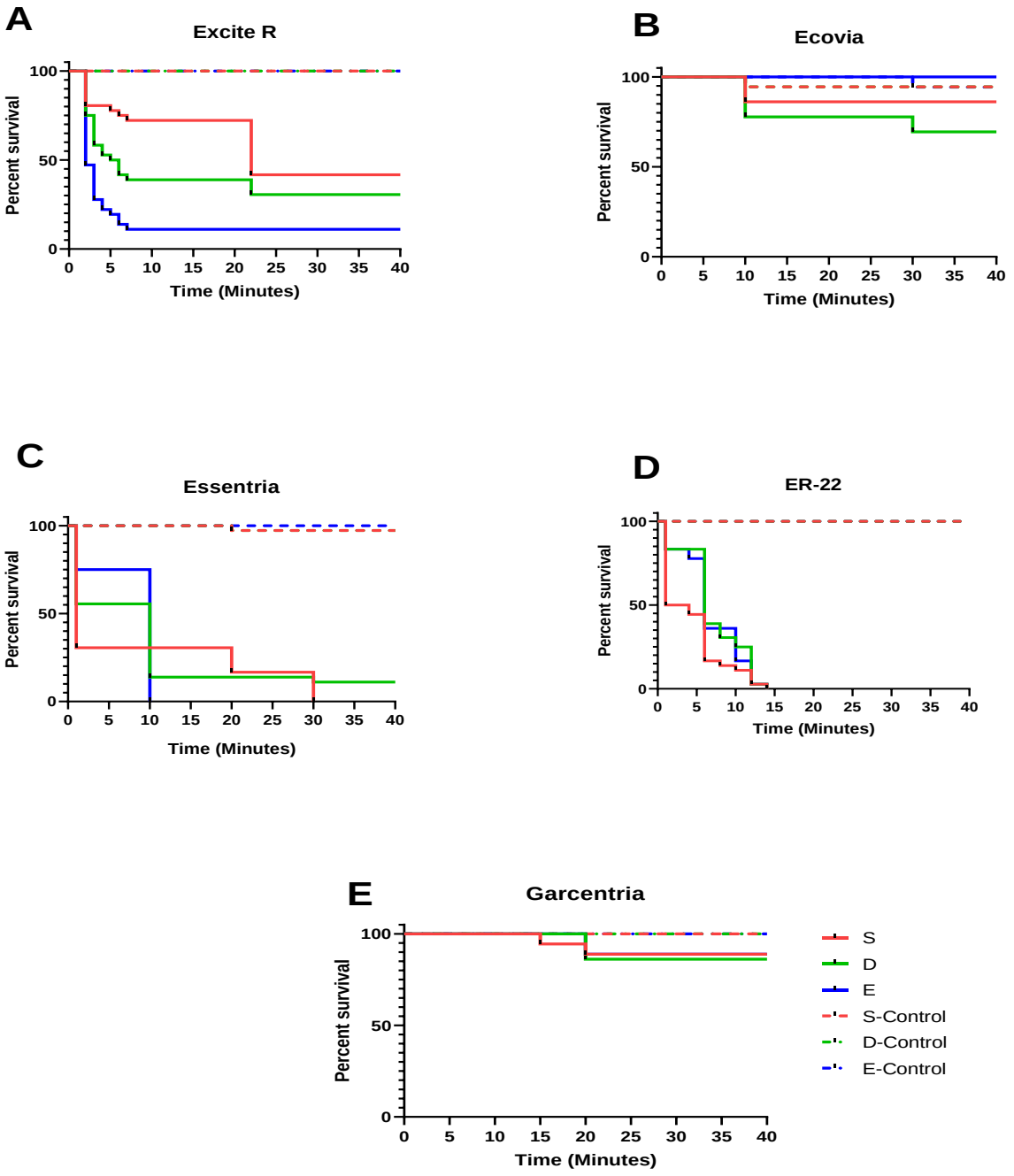

Figure 10. Survivorship of cockroach strains in the direct spray assay tested with the high label rates of (A) Excite R™ (B) EcoVia™ (C) Essentria® (D) ER-22 (E) Garcentria.

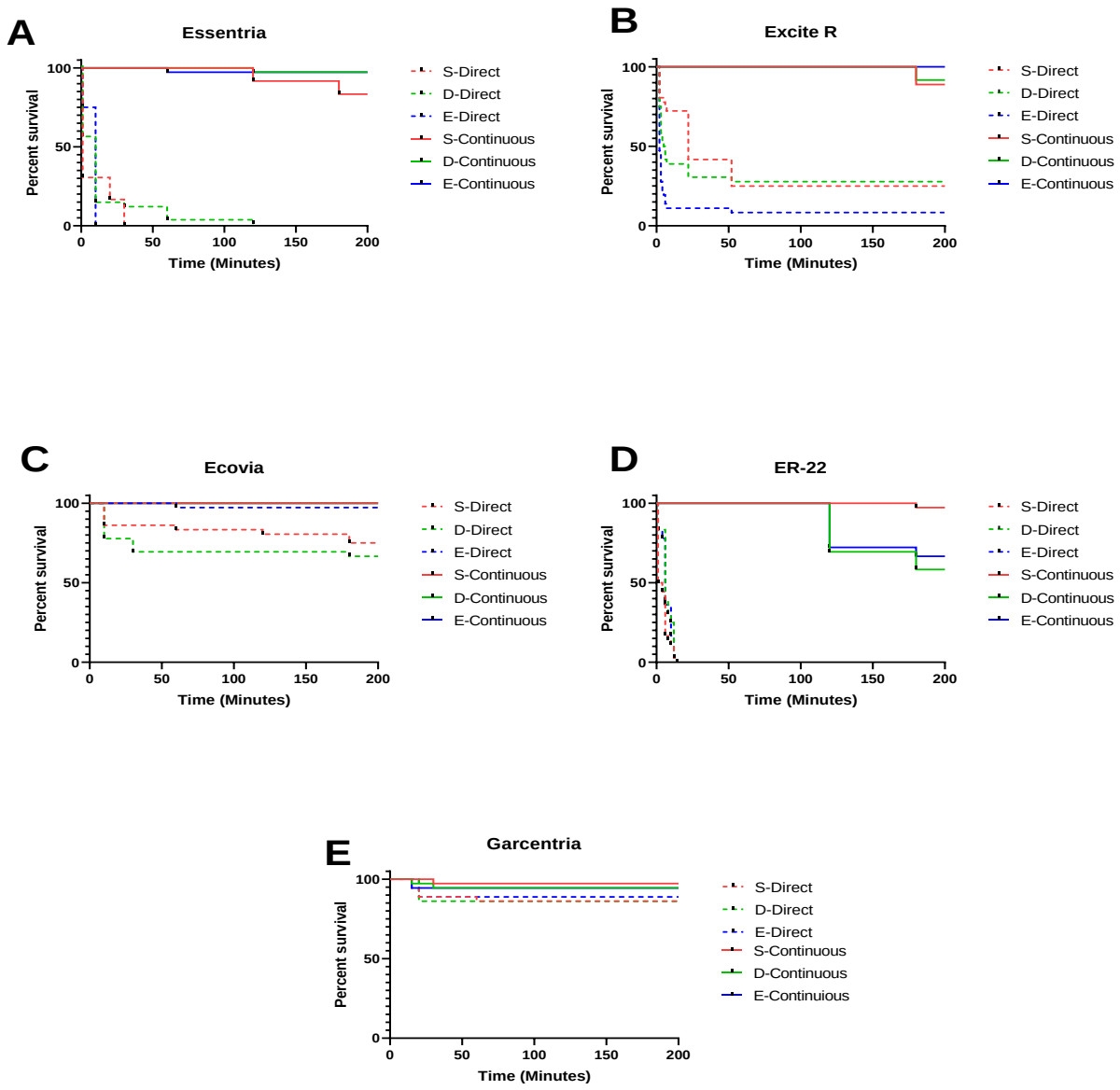

Figure 11. Survivorship of cockroach strains in the direct spray assay versus the continuous exposure assay tested with the high label rates of (A) Essentria® (B) Excite R™ (C) EcoVia™ (D) ER-22.

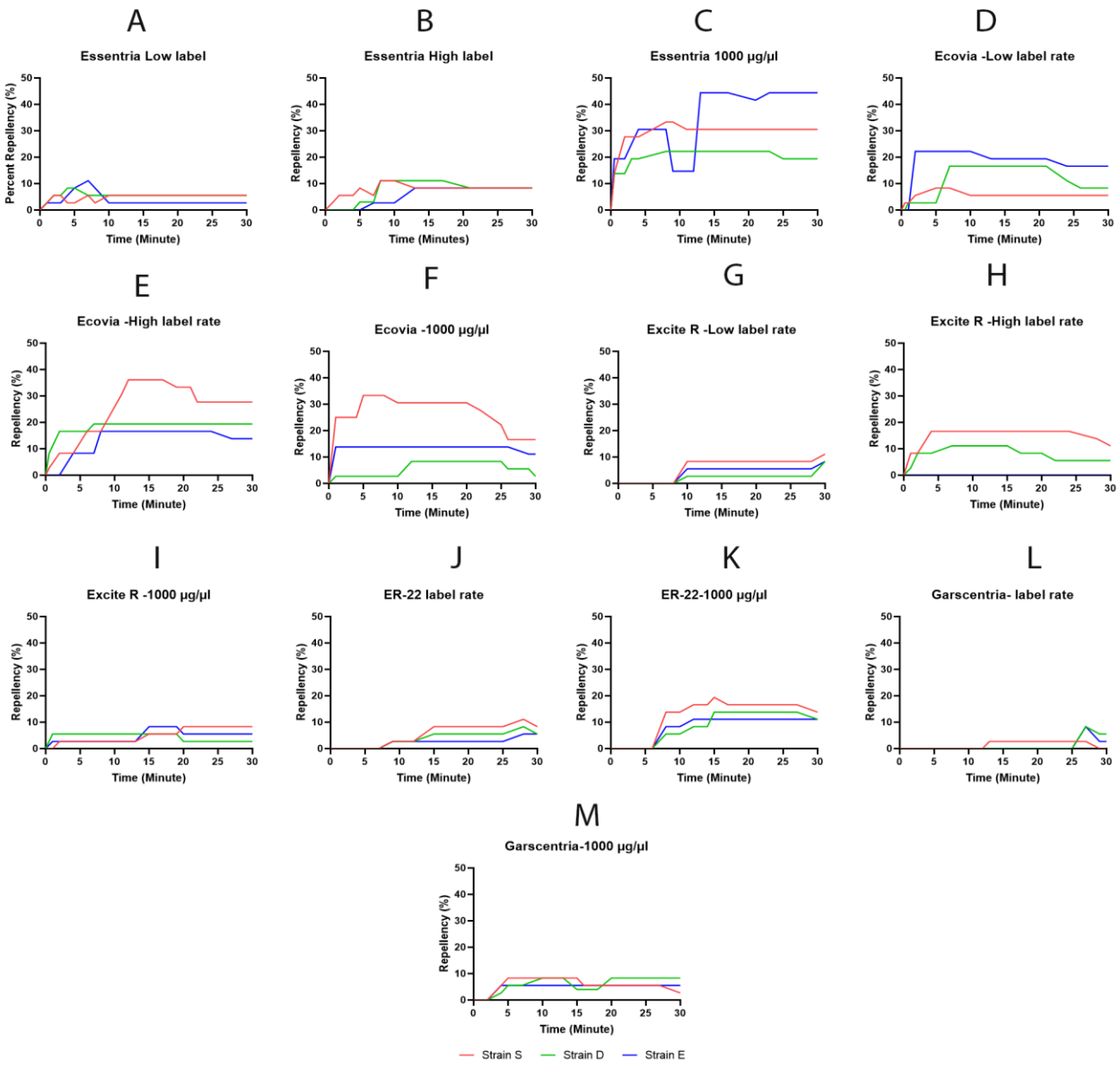

Figure 12. Repellency of the essential oil formulations against the German cockroach strains in the flushing assay tested with (A) low label rate of Essentria® (B) high label rate of Essentria® (C) 1000 µg/µl of Essentria® (D) low label rate of EcoVia™ (E) high label rate of EcoVia™ (F) 1000 µg/µl of EcoVia™ (G) low label rate of Excite R™ (H) high label rate of Excite R™ (I) 1000 µg/µl of Excite R™ (J) High label rate of ER-22 (K) 1000 µg/µl of ER-22 (L) High label rate of Garscentria (M) 1000 µg/µl of Garscentria.