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TOXICITY OF CLOVE OIL AS INFLUENCED BY THE SIZE OF GERMAN COCKROACH *BLATTELLA GERMANICA* L.

Douglas J H Shyu[1,3], Yu-Ming Yen[2,3] and Lekhnath Kafle[2,*]

[1]Department of Biological Science and Technology; [2]Department of Tropical Agriculture and International Cooperation, National Pingtung University of Science and Technology, 1, Shuefu Rd, Neipu, Pingtung 912301, Taiwan
([3]Authors with equal contributions)
*Email: kafleln@gmail.com (corresponding author): ORCID ID 0000-0002-5817-9483

ABSTRACT

German cockroaches *Blattella germanica* L are one of the most common urban pests. Varieties of methods are being used to control this, including spraying, baiting and repellents. Many of these synthetic toxicants cause public health concerns and environmental hazards. There is a need to search for ecofriendly alternatives and clove oil is one such option. There is a need to understand the toxicity of clove oil as influenced by the size of cockroaches, and hence this study was conducted. Cockroaches respond differently to topically applied chemicals on their thorax, wings, and abdomen. Clove oil (16 µl) took significantly longer time to kill smaller-sized cockroaches (004 g). With an increasing topical application of clove oil, the LT_{50} values decreased. Moreover, cockroaches showed special behaviour such as shrinking abdomen, exhausted gut, or feces, decreased wing thickness, and wing shaking. Thus, this study reveals that clove oil toxicity depends on the body size, and altered the structure of the tissues in cockroaches.

Key words: *Blattella germanica*, urban pest, clove oil, topical application, toxicity, LT_{50}, dose mortality response, special behaviour, wing thickness, thorax, abdomen, relationship

The German cockroach *Blattella germanica* (L.) (Dictyoptera: Blattellidae), is an important pest of industry and urban areas. Its faeces and exuviae trigger an allergic reaction in asthmatic individuals (Schal and Hamilton, 1990; Phillips and Appel, 2010). Moreover, it acts as a vector for many bacteria, viruses, and protozoa that may cause disease in either humans or wildlife. It has an incredible multiplication ability and a short lifecycle, which increases the possibility of developing insecticide resistance (Phillips and Appel, 2010). For the last few decades, many pesticides have been recommended for controlling *B. germanica* in Taiwan (Linet al., 2008; Huet al., 2020). The toxicity of these to humans or animals is as serious concern due to their side effects when applying in food storage, to surrounding buildings, and within human habitats. As a result, the development of botanical insecticides, which are highly volatile and usually less toxic to humans or the environment, has become an alternative (Yeomet al., 2018). In previous studies, Philips and Appel[1] indicated that the 1, 8-cineole (-)-menthone, (+)-*a*-pinene, and (-)-*B*-pinene had fumigation effects. The trans-cinnamaldehyde, thymol, carvacrol and eugenol had topical toxicity to *B. germanica.* Eugenol in clove oil had great efficacy against cockroaches through contact toxicity (Enan, 2001). Neupaneet al. (2020) found that the contact toxicity of clove oil could provide a 95% mortality when applied @ 4.0 ml/ cm^2; furthermore, it also repelled 80% cokroaches at 2.0 ml/ cm^2. Clove (*Syzygium aromaticum*), mainly produced in tropical areas contains an abundance of oxygenated monoterpenes, for instance, eugenol, eugenyl acetate, β-cariofilen, and α-humulen (Chaiebet al., 2007; Yeomet al., 2018). Eugenol is a short hydrocarbon chain that belongs to the methoxyphenol $C_{10}H_{12}O_2$; 2- methoxy-4-(2-propenyl) phenol, this substance comprises 80- 95% of the clove oil. The eugenyl is a pale, clear, yellow compound that can dissolve in some organic solvents such as acetone, and it is slightly soluble in water. It has been proven through previous studies to have insecticidal capabilities (Pramod et al., 2010; Kafle and Shih, 2013). Eugenyl acetate was observed to have higher antioxidant activity after esterification, when compared to crude essential oil (Vanin et al., 2014). This study determines the effects of two doses of clove oil as influenced by the body size of *B. germanica* under laboratory conditions.

MATERIALS AND METHODS

The cockroaches were reared at the Integrated Pest Management (IPM) Laboratory, National Pingtung University of Science and Technology, Taiwan following the earlier methods (Neupaneet al., 2020). The cockroaches were maintained in polymer containers (588x 418x 330 mm) containing wooden harborages as their shelters. Water was provided in a 50 ml vial with absorption cotton, and chicken-flavored dog food pellets (Uni-president Enterprises Crop., Taiwan) were provided as feed. The rearing environment was 25± 5°C, 60± 10% RH, with a photoperiod of 12:12 (L: D) hr (Neupane et al., 2020). To ensure the test cockroaches were healthy and normal, 200 adult cockroaches were isolated from the rearing container and placed in another rearing container for two days without feed or water. After two days, any dead or abnormal cockroaches were removed, and feed and water were provided for the next two days. From those isolated cockroaches, only males of small (0.04± 0.005 g) or larger (0.05± 0.005 g) body weight were used for further testing.

Clove oil (99%) used in this study was purchased from a local company (Aladdin Ltd., Kaohsiung, Taiwan). It was applied topically on the thorax, wings, and abdomen. The treatment duration, behaviour, and effects on wing tissues were monitored. The cockroach samples were kept in a freezer for 2 min, to make them unconscious. The test cockroaches were weighed individually on a microbalance (Balance Classic Plus PB403-S/ FACT; 625 East Bunker Ct Vernon Hills, IL 60061 United States; Mettler Toledo). The 8 µl or 16 µl of clove oil was directly pipetted (4642090, Finnpipette™inn, Thermo Fisher Scientific Inc.) onto the wing, abdomen, and thorax. All the pipetting was conducted under room temperature and humidity. The time from the topical application of the clove oil to the death of the cockroaches were recorded individually. To confirm the mortality of the tested cockroaches, their antenna and stylus were brushed for two sec, using a fine brush, three times. If they did not show any movements or responses, then those were counted towards the mortality record. Each experiment was replicated thrice. The cockroach's wings treated with clove oil were collected after 24 hr and they were processed using surgical scissors into an appropriate size (0.1x 3 cm). The wings were examined through 20x laser electron microscopy (Keyence, VL-X1100, NPUST) and the measurements for length and thickness were analyzed using the software (VK-X1000 Series, Keyence corporation). The mortality period and the thickness measurement data were analyzed by ANOVA, with SPSS version 22.0 software (SPSS Institute, 2013) and all data presented as mean± SE compared using Tukey's HSD test (SPSS Institute, 2013). LT_{50} was processed by StatPlus (2020).

RESULTS AND DISCUSSION

The time was recorded after application on the thorax, abdomen and fore wing to the cockroach's death. If no cockroaches died during the test period of 40 min, those were counted as surviving. No deaths were reported in the control. As regards thorax- mortality was observed for the treated period of the smaller size (0.04 mg), treated by topically applying three different amounts of clove oil (0, 8 and 16 µl) to the thorax. The time taken to kill was significantly different across the treatments. Those treated with 16 µl clove oil died in a significantly shorter time than those treated with 8 µl. The control (without any clove oil application) reported no deaths ($F=1178.614$, $DF= 2$, $p< 0.001$); larger sized cockroaches (0.05 g) treated with 16 µl, reported a similar trend ($F=509.543$, $DF= 2$, $p<0.001$). When data for the treatment period of either topically applied 8 or 16 µl on the smaller and larger cockroaches were compared, the time of treatment taken to kill either small or large ones was not significantly different for both doses (8 µl: $F=2.325$, $DF= 28$, $p= 0.139$; 16 µl: $F=3.669$, $DF= 28$, $p=0.0066$) (Table 1). The 8 µl dose revealed the least LT_{50} at 14.97± 0.22 and 16.23± 0.32 min in smaller and larger cockroaches, respectively. When the amount was

Table 1. Effects of topical application of clove oil to the thorax, abdomen, and wings on the mortality of *B. germanica*

Cockroach body wt. (g)	Clove oil applied (µl) Mean (±) SE*		
	0 µl (Control)	8 µl	16 µl
Thorax			
0.04 g	40± 0 aA	15.36± 2.02 bA	11.72± 2.22 cA
0.05 g	40± 0 aA	16.88± 3.29 bA	10.68± 3.19 cA
Abdomen			
0.04 g	40± 0 aA	21.4± 3.74 bA	5.77± 3.11 cA
0.05 g	40± 0 aA	23.99± 2.63 bA	4.97± 1.66 cB
Wings			
0.04 g	40± 0 aA	15.36± 2.02 bA	11.72± 2.22 cA
0.05 g	40± 0 aA	16.88± 3.29 bA	10.68± 3.19 cA

increased to 16 µl, the LT_{50} decreased 1.2 and 1.77 folds, and the LT_{50} were 11.22± 0.24 and 9.79± 0.35, for the smaller and larger cockroaches, respectively (Table 2).

As regards abdomen, mortality was observed with smaller cockroaches (0.04 mg), treated by topically applying three dosages (0, 8 and 16 µl) and the time taken was significantly different; those treated with 16 µl died in a significantly shorter time, while the control reported no death (F=681.883, DF= 2, p> 0.001); larger size cockroaches (0.05 mg) treated with 16 µl clove oil reported a similar trend (F=681.883, DF= 2, p> 0.001). When the effect of treatment period with 16 µl dose with smaller and larger males were compared, the time it took to kill was not significantly different (F=7.098, DF= 26, p= 0.013); 8 µl dose with larger cockroaches took a shorter time to die, and when comparing the smaller and larger ones there was no significant difference (F=1.964, DF= 28, p=0.172). The 8 µl dose revealed the least LT_{50} value at 20.45± 0.39 and 23.63± 0.30 min, in smaller and larger cockroaches, respectively. With increase to 16 µl the LT_{50} value decreased five and six folds for smaller and larger cockroaches, respectively; the values became 4.53± 0.33 and 4.46± 0.16, respectively (Table 2).

As regards wing, time taken to kill was significantly different across treatments; 16 µl dose led to death in significantly shorter time while the control group reported no death (F=296.576, DF= 2, p> 0.001); with the larger sized cockroaches a similar trend was observed (F=235.426, DF= 2, p> 0.001). With 16 µl dose, the time to kill small or large cockroaches was significantly different (F=5.314, DF= 28, p= 0.029); and 8 µl dose with larger cockroaches took a shorter time to die, but when comparing the smaller and larger ones, there was no significant difference (F=5.473, DF= 28, p=0.27) (Table 2). The dosage of 8 µl revealed the least LT_{50} at 22.34± 34 and 17.47± 0.52 min in smaller and bigger cockroaches, respectively. When the dose increased to 16 µl the LT_{50} value decreased 2.2 and 1.2 folds for smaller and larger ones, respectively, and these were 10.76± 0.50 and 14.00± 0.29, respectively (Table 2). With the doses (0, 8 and 16 µl) were topically applied to the fore wings, the thickness of wings significantly decreased, however, there was no significant difference between 8 and 16 µl (F=64.503, DF= 2, p> 0.001) (Table 3).

Table 2. LT_{50} values of clove oil at 40 min after treatment, when applied topically to the thorax, on the mortality of *B. germanica*

Treated parts	LT_{50} (min) (Mean± SE)*	
	Small (0.04 g)	Big (0.05 g)
8 µl		
Thorax	14.97± 0.22	16.23± 0.32
Abdomen	20.45± 0.39	23.63± 0.30
Wing	22.34± 34	17.47± 0.52
16 µl		
Thorax	11.22± 0.24	9.79± 0.35
Abdomen	4.53± 0.33	4.46± 0.16
Wing	10.76± 0.50	14.00± 0.29

*LT_{50} (min) is the length of time required to kill 50% (StatPlus, 2020)

Upon topical application, the cockroaches displayed various behavioural responses depending on the location of the application. With 16 and 8 µl applied on the wings, cockroaches began shaking their wings, whilst scratching the stylus or cercus. Colour alternation of the wings with decreased thickness, as well as a burning reaction were observed (Fig. 1); yet, these showed the ability to walk immediately after application. Thorax applications had similar behavioural responses to that of the wing applications; however, with thorax applications, frequent cleaning of the antennae and the inability to walk was observed especially when treated with 16 µl. When treatments were applied to the abdomen, abdomen shrinkage and the expelling of the faeces or digestive organs was observed. In all applications i.e., wings, thorax, and abdomen, at both doses, leg extension was observed as an immediate response.

Clove oil has been tested as a toxicant against many agricultural or urban pests (Ho et al., 1994; Perrucci et al., 1995; El Hag et al., 1999; Trongtokit et al., 2005; Kim et al., 2021). The present study found that clove oil is toxic to *B. germanica*, even at relatively low dosages. Neupane et al. (2020) indicated that clove oil is composed of eugenol acetate and eugenol. There was a hypothesis posed in earlier studies, that these bioactive mixtures were absorbed by the cockroach's cuticle and released into the nervous system. These compounds may be up taken into the tracheal system (Appel et al., 2004; Cheng et al., 2008; Kafle and Shih, 2013). During this study, it was observed that some cockroaches showed difficulty with breathing, shaking the stylus, shrinkage

Table 3. The effect of applying to the wings on the alteration of wing thickness of *B. germanica*

Clove oil applied (µl)	Wing thickness (Mean± SE)*
0 *u*l (Control)	8.79± 0.24a
8 *u*l	6.74± 0.06b
16 *u*l	5.99±0.22b

*Means within the same column followed by same letter are not significantly different (p> 0.05) (Tukey's HSD test; SPSS, 2013)

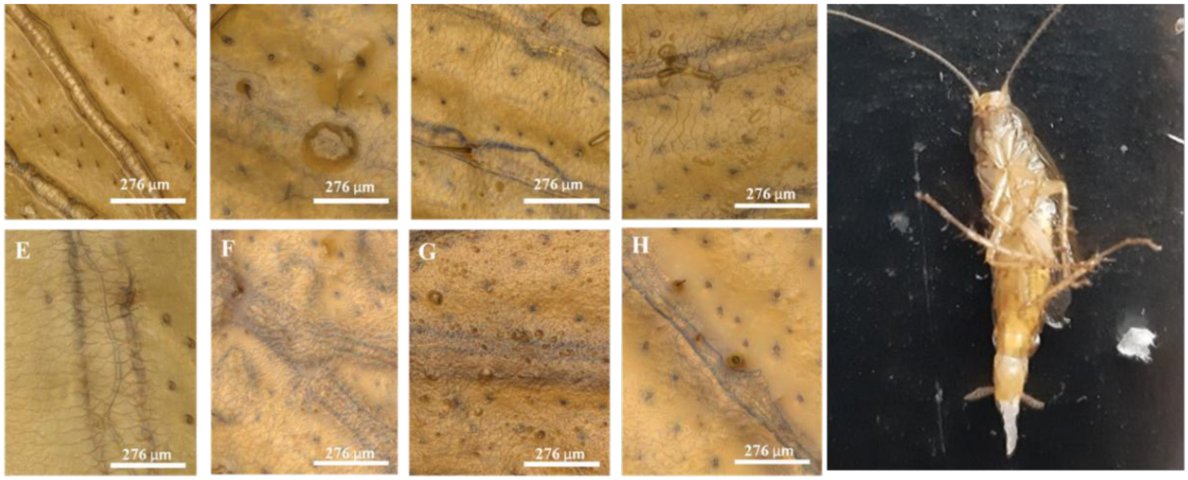

Fig. 1. Symptoms after treatments. (A). Control group, (B). Colour alternation after treating with 8 µl, (C) and (D). Burning reaction after treating with 8 µl, (E). Control group, (F). Colour alternation after treating with 16µl, (G) and (H). Burning reaction after treating with 16 µl, (I). Behaviour after treatment with abdomen

of the abdomen, and contraction of anal areas; even showing the expelling of their digestive organs, when clove oil was applied to the abdomen. The compounds from clove oil could kill or repel *B. germanica* (Neupane et al., 2020). During this study, some behaviour such as waving of the wings, and paralysis were observed; subsequently, the cockroaches twitched and died. Enan (2001) indicated that the octopaminergic system in insects was affected by the eugenol, and this can be one of the tactics for the control of carpenter ants- *Camponotus pennsylvanicus* (DeGeer). Furthermore, clove oil changed the heart rate and the levels of cAMP in the nervous system of cockroaches (Kafle and Shih, 2013; Neupane et al., 2020). To explain this phenomenon, Isman (2006) highlighted octopamine as one of the neurotoxic compounds in insects, which is associated with the action of eugenol.

Lange and Orchard (1986) investigated the mechanism of the effect of neurons in the modulation of contractions of locust's visceral muscles via cAMP. The increasing octopaminergic DUMOV (dorsal unpaired median neurons projecting to the oviducts) caused the raising of cAMP in the oviducts of *Locusta migratoria*. Furthermore, octopamine also resulted in the raising of cyclic AMP within this system with the side-effect of enhancement of the phosphodiesterase inhibitor, IBMX, indicating the presence of an octopamine-sensitive adenylate cyclase. Therefore, the gradual increase of octopamine indirectly evoked contractions, and the inhibition of myogenic contractions. One possible explaination for the observed twitching and abdominal shrinkage in cockroaches could be attributed to this factor, but this still did not explain why they expelled their digestive organs. Cockroaches absorbed gas into their respiratory system and the toxic compound was released into the nervous system (Cheng et al., 2008; Phillips and Appel, 2010; Kafle and Shih, 2013). (Andersen, 2004) reported that the chlorinated tyrosine content in the wings was lower compared to both the thorax and abdomen. Thus, results indicated that the topical application of clove oil to *B. germanica* has altered the thickness of wings and stimulated abnormal behaviour patterns. It might help us to understand how insects might react to the application of essential oils to different body parts. Furthermore, an amount of the clove oil could be precisely applied in later experiments.

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AUTHOR CONTRIBUTION STATEMENT

In this study, Lekhnath Kafle and Douglas J. H. Shyu conceptualized and designed the study, Yu-Ming Yen conducted the study, analyzed the data and prepared a draft of the manuscript, while Lekhnath Kafle and Douglas J. H. Shyu provided resources for the study and revised the manuscript.

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CONFLICT OF INTEREST

No conflict of interest.

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