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Chemical Composition of *Cymbopogon flexuosus* and *C. winterianus* Essential Oils and Their Insecticidal Potential Against the Coffee Berry Borer *Hypothenemus hampei*

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Abstract

Hypothenemus hampei is the most important insect pest of coffee in the world. *H. hampei* is controlled by using endosulfan and methyl bromide. However, using these two chemicals may pose pesticide resistance, bioaccumulation, and a threat to human health and the environment. This study evaluated the chemical compositions of essential oils from *Cymbopogon flexuosus* and *C. winterianus* and their insecticidal potential against *H. hampei* as an alternative to endosulfan and methyl bromide. The chemical profiles of the two essential oils showed that both constituted biochemically distinct monoterpenes and sesquiterpenes. The main compounds identified in *C. flexuosus* were citral, geraniol, and isoforms, representing 76.45% of the total composition, and other active ingredients were citronella, caryophyllene, citronellyl acetate, cadinene, linalool, and limonene. The composition of *C. winterianus* was citronella, nerol, and citronellol as principal components, corresponding to 66.47%, along with other active ingredients elemol, geranyl acetate, citronellyl acetate, germacrene, and limonene. Differential sensitivity of *H. hampei* adults was observed in the bioassay of two oils, where a dose-dependent increase in mortality was observed. Among two concentrations of both essential oils, 100% mortality was observed at 500 and 1000 ppm within 48 and 24 hours of treatment, respectively. The significant biological activity of both *Cymbopogon* species oils indicated a potential source for further development of botanical pesticides as an effective, useful alternative for currently used toxic insecticides. Additionally, essential oils of *C. flexuosus* and *C. winterianus* and their active ingredients are classified as non-residual insecticides and are eligible for a pesticide registration exemption.

Keywords: Botanicals, bioassay, *Cymbopogon spp.*, non-residual, toxic

Introduction

Coffee is a popular beverage consumed all around the Globe. Coffee cultivation generates income for many developing countries and grants them access to international markets (Ramalakshmi and Raghavan, 1999; Poltronieri and Rossi, 2016). Increasing global demand for coffee has remained promising, and rapid growth in the exporting countries shows strong potential for further expansion of niche markets in non-traditional consuming countries (ICO, 2015). The presence of invasive species in a trading commodity is often a risk that may cause non-native species incursion to new areas (Hallman, 2011). Unintentional introductions of alien species have been a major cause of significant damage to the economy of the country (Heather and Hallman, 2008).

Coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), is the most important insect pest of coffee, endemic to Central Africa and now has spread to almost every coffee-producing country in the world. Inadvertent transport of coffee with internal feeders like CBB from the place of processing and marketing facilitates the invasion of new areas (ICO, 2002). Hence, the goal of pest exclusion by phytosanitary quarantine measures becomes necessary to prevent the invasion of other areas. Chemical control of CBB can be achieved by using endosulfan and methyl bromide. However, the usage of these chemicals has been restricted/reduced due to the development of pesticide resistance, bioaccumulation, and threats to human health and the environment. Currently, methyl bromide is used as an insect fumigant for coffee shipments (Hollingsworth et al., 2013). However, methyl bromide is a harmful ozone-depleting substance (Aegerter and Folwell, 2001; UNEP, 2014).

Further, several concerning aspects of methyl bromide toxicity as a carcinogen, neoplastic or mutagenic and hence, toxicological studies have raised questions about its usage (Danse et al., 1984; Calvert et al., 1998; Budnik et al., 2012). The impending phase-out schedules were initiated according to the Montreal Protocol on 1 January 2005 for developed countries and 1 January 2015 for developing countries (UNEP 2014). Hence, irradiation of CBB in harvested coffee has been studied as an alternative to methyl bromide (Follett, 2018; Kiran et al., 2019; 2020.)

Previously, endosulfan, a broad-spectrum chlorinated cyclodiene insecticide, was used by coffee farmers for the control of CBB. However, this insecticide was banned globally due to human and environmental hazards and poses a severe threat to the lives of resident communities residing near coffee plantations (FAO, 2015; Jaramillo et al., 2006). The development of resistance to endosulfan has been another major issue regarding the chemical control of CBB (Brun et al., 1989).

Because of public concerns about the adverse effects of synthetic chemicals, there has been a growing interest in finding safer alternative protectants (Isman, 2006). Using natural plant protectants with pesticide activity is one alternative to synthetic insect-cides. Green pesticides have several advantages as they tend to have favorable ecotoxicological properties (Wink, 1993). Several studies have been conducted to use essential oils as botanical insecticides and to understand their mode of action for controlling various agricultural pests (Regnault-Roger, 1997; Isman, 2000).

Cymbopogon flexuosus (Nees ex Steud.) Wats. and *C. winterianus* Jowitt (Poales: Poaceae) are plants in the grass family that are well-documented for their insecticidal activity against various agricultural pests (Amer and Mehlhorn, 2006; Wiltz et al., 2007; Pinheiro et al., 2013; Gusmão et al., 2013; Pinto et al., 2015; Zhang et al., 2016; Tak et al., 2017).Both *C.flexuosus* and *C.winterianus* oils have been considered non-residual insecticides and are exempted under Section 25(b) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (Rust, 2010). The present study aimed to evaluate the chemical composition of essential oils of *C. flexuosus* and *C. winterianus* and determine the insecticidal activity of the essential oils on colonizing CBB adult females.

Methods

The aerial parts of *C. flexuosus* and *C. winterianus* were harvested from the garden maintained at the Department of Horticulture, University of Agricultural Sciences, Bengaluru, India. CBB-infested coffee fruits were collected from a plantation in the Chikkama-galuru region (13°26'41.4"N 075°48'29.5"E, 1067m elevation), Karnataka, India.

Extraction of Essential Oil

The harvested leaves were dried at room temperature, and essential oils were extracted by a hydro-distillation tech-nique using a modified Clevenger-type appa-ratus. The essential oils were stored in the laboratory in airtight containers covered with aluminum foil to protect the contents from light and refrigerated at 4°C until used for GC-MS MS analysis and bioassay studies.

Gas Chromatography-Mass Spectrometry

The chromatographic analysis of two essential oils, *C. flexuosus*, and *C. winterianus*, was done by GC-MS MS (Model GCMS TCE 8040, Shimadzu, Kyoto, Japan) equipped with DB 5 MS capillary column (30m × 0.25mm, 0.25µm film thickness). The Gas chromate-graphy analytical conditions were as follows: injector temperature at 250°C; initial oven temperature at 60°C for 2 minutes and ramped up to 250°C at a rate of 2°C held for 5 minutes; interface and ion source temperature at 250°C; carrier gas, Helium at gas flow at a rate of 1.00 mL/ minute; 1.0 µL sample injection with split ratio of 1:10. Ionization voltage of 70eV was used to operate electron impact ionization mass spectrophotometer. The sector mass analyzer was set to 45 to 500 m/z for measuring the total ion chromatogram. Compounds were identified by comparing retention time and mass spectra with the NIST library (Shimadzu, Kyoto, Japan).

Insect Rearing

The infested fruits were identified based on the presence of an entrance pinhole with pow-dery substance at the navel region of the fruit. After collection, the infested coffee fruits were maintained in a plastic tray (21 x 15 x 10 cm) covered with a nylon mesh in the laboratory. The tray was maintained in a growth chamber (GC-300TLH, Jeiotech, Korea) at 25±1ºC and 70% RH with 12L: 12D photoperiod.

CBB adult males and females differ in the ability of dispersal. The females with vestigial wings are the dispersal units in the species. In contrast, males lack vestigial wings and remain in the native berry. After attaining a dark cuticle, the colonizing adult females leave the native berry, searching for fresh berries for infestation (Vega et al., 2006, 2015a; Gómez et al., 2015). Due to these reasons, the present study is restricted to adult females as they are the only dispersal units in the species. The adult females who displayed active flight while emerging from infested coffee fruits were collected with the help of a camel hair brush for bioassay study.

Insecticidal Activity

The susceptibility of CBB colonizing females to essential oils of *C. flexuosus* and *C. winterianus* was tested using dose-response tests by direct contact application (Mendesil et al., 2012). Dilutions of essential oils were prepared in ethanol. The stock solution (10000 ppm) was prepared by mixing 0.10 mL essential oil with 9.90 mL ethanol, and concentrations for bioassay set at 100, 250, 500, and 1000 ppm (v/v) were designed by dilutions. Glass Petri dishes (9.5cm) lined with Whatman No. 1 filter paper were used as test chambers. 1 mL of solvent from each concentration was impregnated with

Whatman filter paper with the help of a micropipette. Ethanol alone was used as a control. The solvent was allowed to evaporate for 30 minutes at room temperature, and 1 ml of distilled water was added to each filter paper to facilitate the transfer of compounds to the insect body. Three replicates of 10 newly emerged CBB colonizing females were introduced separately in each treated container. The Petri dishes were closed with lids to prevent the escape of insects. The Petri dishes were kept in the growth chamber (GC-300TLH, Jeiotech, Korea) set at 25±1ºC and 70% RH with dark photoperiod. Colonizing females who were unable to make a coordinated movement, showing no leg or antennal movement when gently prodded, were scored as dead. Observations were made 24, 48, and 72 hours after treatment to determine end-point mortality under a stereo zoom microscope (Lawrence and Mayo, Trinocular Research Microscope).

Statistical Analysis

The data from the study were subjected to Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks followed by Dunn's multiple comparisons test because the data were not normally distributed. Logistic regression for LC50, LC90, and confidence interval calculations were made based on probit analysis by Finney (1971).

Results

Chemical Constituents of Essential Oils

The main compounds identified in *C. flexuosus* essential oil were monoterpenes, citral accounting for 39.27%, and geraniol and its isoforms of 37.19 % together, representing 76.45% oil composition. Other compounds include monoterpenes, i.e., DL-limonene, linalool, citronella, citronellyl acetate, and sesquiterpenes, i.e., n-decanal, *trans*-caryophyllene, α-humulene, germacrene, γ-cadinene, δ–cadinene, elemol, caryophyllene oxide, and 4 unknown compounds (Table 1).

A total of 18 compounds were identified from *C. winterianus* essential oil. Major compounds include monoterpenes, i.e., citronella (37.99%), nerol (17.32%), and citronellol (11.16%), accounting for a total of 66.47 % of the composition of the oil. Furthermore, monoterpenes, i.e., DL-limonene, linalool, citral, citronellyl acetate, geranyl acetate, and sesquiterpenes, i.e., germacrene-D, α-muurolene, β-elemene, δ–cadinene, α–cadinol, *tau*–cadinol, elemol and two unknown compounds were present. None of these constituents accounted for more than 5% of the oil (Table 1).

Table 1. Chemical composition of *Cymbopogon flexuosus* and *C. winterianus* essential oils

Cymbopogon flexuosus			***Cymbopogon winterianus***		
Compound	**RT**	**Area (%)**	**Compound**	**RT**	**Area (%)**
6-Methyl-5-hepten-2-one	8.122	0.50	DL-Limonene	10.053	2.53
DL-Limonene	9.999	0.91	Linalool L	14.038	0.60
4-Nonanone	12.232	0.21	Citronella	17.614	37.99
Linalool L	14.012	1.09	β-Citronellol	22.506	11.16
6-Octenal, 7-methyl-3-methylene	16.396	0.33	Nerol	24.405	17.32
Citronella	17.077	5.83	(E)-Citral	24.742	0.58
Isogeranial	17.566	3.29	Citronellyl acetate	29.888	4.92
n-Decanal	20.327	0.29	Phenol, 2-methoxy-3-(2-propenyl)-	30.304	1.46
(Z) –Citral	23.165	39.27	Geranyl acetate	31.853	6.01
trans-Geraniol	25.008	33.90	Germacrene-D	37.687	3.15
Citronellyl acetate	29.771	1.08	α-Muurolene	38.829	0.59
2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (E)	31.825	7.03	β-Elemene	39.226	0.71

Cymbopogon flexuosus			*Cymbopogon winterianus*		
Compound	**RT**	**Area (%)**	**Compound**	**RT**	**Area (%)**
trans-Caryophyllene	33.739	2.40	δ-Cadinene	40.094	2.31
α- Humulene	35.919	0.29	Elemol	42.385	5.44
Phenol, 2-methoxy-4-(1-propenyl)-, (Z)	36.278	0.65	(2E,4S,7E)-4-Isopropyl-1,7-dimethylcyclodeca-2,7-dienol	43.739	1.41
Germacrene-D	37.564	0.29	2-((2S,4aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydronaphthalen-2-yl)propan-2-ol	46.971	0.67
γ- Cadinene	39.652	1.09	Tau-Cadinol	47.59	0.61
δ- Cadinene	39.967	0.26	α- Cadinol	48.51	2.55
Elemol	42.131	0.62	**Total**		100
Caryophyllene oxide	43.759	0.66			
Total		100			

Insecticidal Activity of Essential Oils

The insecticidal efficacy of *C. flexuosus* and *C. winterianus* essential oils is presented in Figure 1.

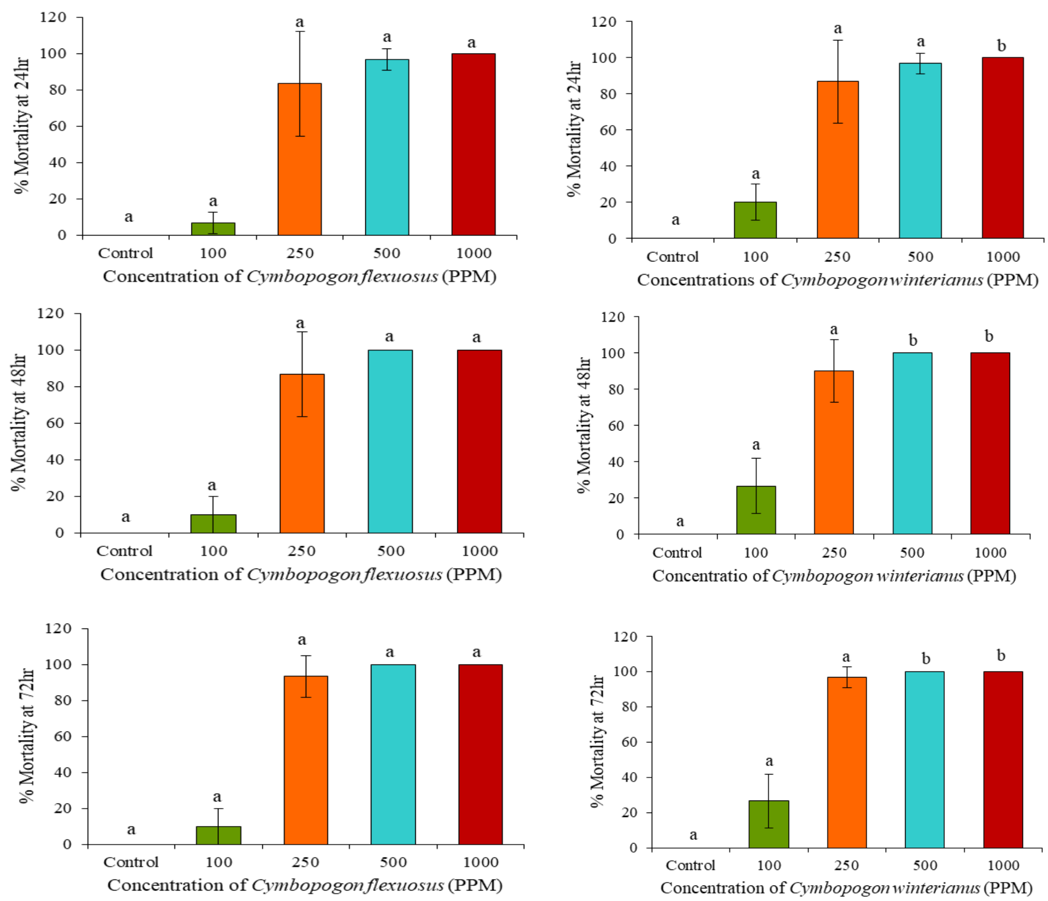

Figure 1. Insecticidal activity of *Cymbopogon flexuosus* and *C. winterianus* essential oils on colonizing *Hypothenemus hampei* adults. Data are mean ± SD three replicates. Bars with the same letter are not significantly different (p>0.05).

Overall percentages of adult mortality in both the essential oils were significantly different from that of control, where the increase in mortality was dose-dependent, i.e., mortality was increased with increased doses (*C. flexuosus*: 24 hours- df= 4; H= 12.11; P= 0.0015, 48 hours- df=4; H= 12.8; P= 0.0008, 72 hours- df= 4; H= 12.8; P= 0.0008; *C. winterianus*: 24 hours- df=4; H=12.36; P= 0.0008: 48 hours- df= 4; H= 13.08; P= 0.0001: 72 hours- df= 4; H= 13.08; P= 0.0002).

In both the essential oils, the lowest mortality was observed at 100 ppm, where mortality of CBB beetles did not exceed 50% even after 72 hours after treatment. The dose of 250 ppm resulted in mortality of beetles above 50% within 24 hours and reached above 90% in 72 hours after treatment. The 100% mortality of beetles was observed at 500 and 1000 ppm within 48 and 24 hours after treatment, respectively (Figure 1). LC50 and LC90 values from dose-response bioassay for *C. flexuosus* and *C. winterianus* at the time interval of 24, 48, and 72 hours of treatment are presented in Table 2.

Table 2**.** Lethal concentrations of *Cymbopogon flexuosus* and *C. winterianus* essential oils against colonizing *Hypothenemus hampei* adults.

Essential Oil	**Hr**	**Equation ± SE**	**Intercept ± SE**	**R^2**	**Predicted dose (ppm)**	
					LC_{50} (CI)	**LC_{90} (CI)**
Cymbopogon flexuosus	24	5.12 ± 0.49	-6.69 ± 1.24	0.98	180.03 (149.07- 217.43)	325.30 (264.50- 400.09)
	48	5.39 ± 1.15	-6.79 ±2.92	0.92	163.35 (139.10- 191.82)	268.70 (214.98- 335.84)
	72	5.30 ± 1.18	-6.46 ± 3.01	0.91	153.05 (131.34- 178.35)	237.69 (194.93- 289.81)
Cymbopogon winterianus	24	4.36 ± 0.46	-4.56 ± 1.17	0.98	152.50 (124.31- 187.09)	311.29 (242.68- 399.31)
	48	4.72 ± 1.01	-4.89± 2.57	0.92	134.67 (112.00- 161.92)	249.86 (191.22- 326.48)
	72	4.59 ± 1.03	-4.44 ± 2.63	0.91	126.36 (107.08- 149.11)	209.00 (163.83- 266.60)

LC_{50} and LC_{90}: lethal concentrations causing 50 and 90% mortality; CI, Confidence Interval

The pharmacological mechanism of actions of individual compounds involving the multiple systems reported in the insect system is presented in Table 3.

Table 3**.** Summary of a mode of actions of compounds of botanical origin involving multiple target sites reported in insect model systems

Active ingredients	**System**	**Mode of Action**	**Insect system**	**Reference**
Linalool, Limonene, Citral	Cholinergic system	Inhibition of AChE	*Acanthoscelides Obtectus, Sitophilus oryzae, Reticulitermes speratus, Gallaria mellonella*	Regnault-Roger et al. (1993), Lee et al. (2001), Seo et al. (2014), Keane and Ryan (1999)
Geranyl acetate, Citronellal,	Odorant Receptor System	Olfactory blocking through modulation of cation channel	*Anopheles gambiae, Drosophila*	Carey et al. (2010), Kwon et al. (2010)

Active ingredients	System	Mode of Action	Insect system	Reference
Linalool Geraniol, Geranyl acetate, Limonene		Olfaction blocking Similar to the action of DEET*	*Culex quinquefasciatus, Bombyx mori, Manduca sexta*	Syed and Leal (2008), Picimbon & Regnault-Roger (2008), Feng and Prestwich (1997)
Citral, Geraniol, Linalool	Neuronal activity (Post-hyperpolarization)	Reduction in the amplitude of action potential causing decrease in post-hyperpolarization	*Periplaneta americana, Blaberus discoidalis*	Price and Berry (2006) Huignard et al. (2008)
Citronellal	Octopaminergic system	Antagonistic activity on octopamine receptor	*Periplaneta Americana*	Enan (2001)

*DEET (N, Ndiethyl-3-methylbenzamide)

Discussion

The cumulative increase in resistance to one or more insecticides is a serious problem worldwide. Vega et al. (2015b) have identified a broad range of gene families involved in detoxifying insecticides in *H. hampei*. Genes identified in the draft genome study included 95 putative ATP-binding cassette transporter genes, 19 predicted glutathione S-transferase genes, and 54 full-length cytochrome P450s genes. Resistance to dieldrin gene (Rdl) and carboxylesterase genes involved in pyrethroid and organophosphate insecticide resistance. Vega et al. (2015b) identified that this molecular basis of insecticide resistance against several insecticides in CBB infers the search for alternative management methods.

The cryptic biology of CBB enables the pest to develop pesticide resistance readily. The obscure mating system involving a 10:1 female-to-male sex ratio within coffee fruits facilitates sibling mating. The resulting genetic inbreeding reduce the rapid spread of resistance allele in the female progeny. Populations of CBB resistant to endosulfan have already been recorded on the East Coast of New Caledonia, and the spread of resistant lines in unprocessed coffee berries might occur in other major coffee-producing regions based on the fast global dispersal of CBB (Brun et al., 1989; Brun et al., 1995). Thus, it is urgent to identify novel insecticides with multi-target effects essential to combat the increasing resistance rate in CBB. Therefore, several insecticides of plant origin have been tested and considered as an alternative strategy, as they are biodegradable, safe, and potentially suitable for use in the integrated management of CBB (Mawussi et al., 2009, 2012; Depieri et al., 2010; Mendesil et al., 2012; Santos et al., 2013; Celestino et al., 2016; Reyes et al., 2019).

Essential oils of aromatic plants are amongst the most promising insecticides that contain a complex group of secondary metabolites compared to synthetic pesticides and fumigants. Thus, resistance development is often delayed due to the varietal and complex mode of action by numerous bioactive compounds with multiple target sites. These aromatic compounds are complex mixtures in variable ratios constituted by a large number of monoterpene and sesquiterpene hydrocarbons, and their oxygenated derivatives are responsible for distinctive odor (Nerio et al., 2010; Gusmão et al., 2013).

Cymbopogon flexuosus and *C. winterianus* are two species of essential oil-bearing tropical grasses that are biochemically distinct with differences in active components. The toxic action of the compounds exerts a broad range of effects on insects, involving several mechanisms with specific target sites. Cholinergic, odorant receptors, octopaminergic system, and changes in neuronal activity are the major processes notably involved in the mechanism. Compounds such as linalool, limonene, citral, geranyl acetate, citronellal, and geraniol have been reported to possess multiple target sites affecting physiology in several insect species. The mode of action binds to odorant receptor proteins on specialized odorant receptor neurons in the antenna and maxillary palp (Ditzen et al., 2008).

Apart from inducing various toxicity levels, limonene, 3-octanone longifolene, and n-dodecane have been reported to attract a parasitoid of CBB, *Prorops nasuta* (Román-Ruíz, 2012). According to Burbano et al. (2012), lemonene and verbenone are known to repel Black twig borer, *Xylosandrus compactus* (Coleoptera: Curculionidae) in coffee. Essential oils of genus *Cymbopogon* have been shown to control several coleopteran pests in pre- and postharvest periods (Regnault-Roger et al., 1993; Ketoh et al., 2002; Scott et al., 2003; Nerio et al., 2009; Gusmão et al., 2013).

In our investigation, the effect of two essential oils was dose-dependent, where the percent mortality of CBB adults increased with increasing doses. Both the essential oils induced 100% mortality within 48 hours at higher doses. Safe treatment with quick action should be preferred to protect crop produce in the pre- and postharvest period. Our study has confirmed that both *C. flexuosus* and *C. winterianus* have effective biological activity against CBB and can be considered promising pesticides in the management of CBB.

Several studies on botanical insecticides and essential oils have shown to exert mortality in CBB. Toxicity of essential oils from *Ocimum canum* and *Aeollanthus pubescens* on CBB-induced LC50 at 320 and 220 ppm, respectively (Mawussi et al., 2009; 2012). Similar results have been reported by Santos et al. (2013), where essential oil from *Schinus terebinthifolius* with dilutions from 10-2 to 10-8 (v/v) resulted in 30-100% mortality. Celestino et al. (2016) reported that castor oil, *Ricinus communis,* induced LC50 at 3.49% (vv-1) against CBB.

In accordance with FIFRA, essential oils of *C. flexuosus* and *C. winterianus* and their active ingredients, i.e., geraniol and citronella, along with a few other compounds, are eligible for exemption from pesticide registration from the U.S. Environmental Protection Agency. These biopesticides are classified as non-residual insecticides under Minimum-Risk Pesticide Products and can be used in all raw agricultural food commodities (Rust, 2010; Baker et al., 2018). With the removal of hazardous insecticides such as methyl bromide and endosulfan from the market, the developing of safer and less expensive crop protectants from locally available plant materials is likely to rise in developing countries.

Conclusion

Results from the present study indicated a high mortality rate in short-term exposure of CBB adults to the essential oils of *C. flexuosus* and *C. winterianus*. The presence of various active ingredients in combine indicated their insecticidal potentiality against CBB in the laboratory. As an alternative to synthetic insecticides, using plant products can be more suitable as a sustainable control agent against CBB. Additionally, essential oils of *C. flexuosus* and *C. winterianus* and their active ingredients are classified as no residual insecticides and eligible for a pesticide registration exemption.

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Declaration

Author contribution

Venkatesha is the main contributor and corresponding author for this paper. Kiran is the co-author. Both authors read and approved the final paper.

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Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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