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Insecticidal effect of lemongrass oil on behavioural responses and biochemical changes in cowpea weevil, *Callosobruchus maculatus* (Fabricius)

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Abstract

The cowpea weevil, *Callosobruchus maculatus* (Fabricius), is the most important postharvest insect pest of cowpeas throughout the tropics causing about 70% infestation within 6 months of storage. Control interventions have often been by way of synthetic chemical application but the negative impact of these chemicals on biotic and abiotic elements of the environment continues to motivate search for alternative control measures. The present study was conducted to evaluate insecticidal effect of essential oil (EO) from lemongrass, *Cymbopogon citratus* (DC.) Stapf, against the weevil. Air-dried lemongrass leaves were milled into powder, the EO was extracted by hydro-distillation using a Clevenger-type apparatus and the extract was dried over anhydrous sodium sulphate. Acute toxicity was determined by inhalation over a period of 24 h using five serial concentrations (0.62, 1.28, 2.50, 5.00 and 10.00 mg/ml) of the EO on filter papers. The median lethal concentration (LC_{50}) was determined from mortality data using probit analysis and three sublethal concentrations (1.41, 2.83 and 5.66 mg/ml) were used in subsequent antioviposition test and biochemical assays. Repellency against the weevils was tested using 2 ml 10 mg/ml EO concentration applied evenly on ½ disc of filter paper. Weevil homogenates were assayed for acetylcholinesterase (AChE), glutathione-S-transferase (GST), reduced glutathione (GSH), superoxide dismutase (SOD), lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Changes in the concentrations of total protein (TPR) and glycogen (GLY) as influenced by dosage and exposure time to the EO were also determined. The results showed that the EO had effective insecticidal properties against the pest. Weevil mortality was concentration- and time-dependent and 80% death rate was attained within 24 h of inhaling 10 mg/ml EO with a LC_{50} value of 7.07 mg/ml. The EO repelled a significantly higher number of weevils (66%) and fewer eggs were laid on cowpea seeds treated with the EO. The values of AChE ($P \leq 0.01$), GLY ($P \leq 0.001$), SOD ($P \leq 0.001$) and TPR ($P \leq 0.001$) reduced significantly with increasing EO concentrations giving an evidence of induced physiological stress in exposed weevils. Kinetics of the assayed enzymes and physiological implications for the storage pest were highlighted. In addition, potential of lemongrass EO as an alternative to synthetic chemicals was discussed.

Keywords: bioassay, *Cymbopogon citratus*, *Callosobruchcus maculatus*, enzymes, essential oil, insecticides.

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1. Introduction

Plant-derived insecticides are a group of naturally occurring protective agents that are usually safer to humans and with minimal residual effects in the environment compared to synthetic pesticides. Biopesticides contain alkaloids, rotenoids and pyrethrins and other groups of natural chemicals that could act as insecticides, deterrents or repellents (Adeyemi, 2010). These secondary metabolites protect plants against herbivores by acting as toxicants and interfering with reproductive system and other physiological processes in insect pests (Rattan, 2010). Essential oils (EOs) are natural volatile and strongly odorous compounds produced as secondary metabolites by aromatic plants. They were formed by complex volatile mixtures of chemical compounds, with predominance of terpene associated with aldehyde, alcohols and ketone deposited in various plant structures (Linares et al., 2005). Naturally, EOs protect plants by fighting against bacteria, viruses and fungi. They also act as insecticides by functioning as feeding deterrents, attractants or repellents (Bakkali et al., 2008). Some EOs have been reported to possess ovicidal and larvicidal properties against various insect pests (Cetin et al., 2004; Isman, 2000). Studies by Ayvaz et al. (2010) showed that EOs from oregano and savory were highly effective against *Plodia interpunctella* (Hübner) and *Ephestia kuehniella* Zeller, with 100% mortality obtained after 24 h at 9 and 25 µl/l. Kabera et al. (2011) also reported 100% mortality in maize weevil, *Sitophilus zeamais* (Motschulsky), treated with EOs of *Pelargonium graveolens* L'Hér. and *Cymbopogon citratus* (DC.) Stapf. *Cymbopogon citratus* commonly known as citronella grass or lemongrass, is a bunch of perennial grass growing to a height of 1 m with numerous stiff leafy stems arising from short rhizomatous roots with an economic lifespan of about 5 years (Carianne, 2005). Many ethnobotanical and medicinal uses of the plant have been documented in literature (Tripathi et al., 2009). The EO in lemongrass is biosynthesized in the rapidly growing leaves and stored in specific oil cells in the parenchymal tissues (Santoro et al., 2007; Luthra et al., 1999). The major constituent of lemongrass oil is citral (a mixture of geranial and neral) and other unusual active components include limonene, citronellal, ß-myrcene and geraniol (Schaneberg & Khan, 2002). Studies have shown that citral reduces attractivity of sex pheromone in the codling moth, *Cydia pomonella* (L.), thus acting as a mating disruptor (Hapke et al., 2001). Stored product insects constitute pest problems all over the world causing significant economic losses. The cowpea weevil, *Callosobruchus maculatus* (Fabricius), is a cosmopolitan field-to-store insect pest ranked as the principal post-harvest pest of cowpea in the tropics (Caswel, 1981). It causes substantial quantitative and qualitative losses manifested by seed perforation and reductions in weight, market value and germination ability of seeds (Oluwafemi, 2012). The use of synthetic insecticides to control pest infestation has impacted drastically on biotic and abiotic elements of the environment through excessive or indiscriminate use (Al-Zaidi et al., 2011). Some of the negative impacts are contamination of food and water sources, toxicity against beneficial insects and pesticide resistance in target insects (Kumar et al., 2008). A large number of synthetic pesticides exhibit potential for biomagnification, thereby, affecting food chains adversely (Gavrilescu, 2005; Linde, 1994). Detoxification is the metabolic process by which toxins or contaminated resources are changed into

less toxic or more readily excretable substances. Several defensive mechanisms and biochemical reactions are involved in detoxification processes against chemical intruders. These mechanisms predominantly involve either metabolic detoxification of the insecticide before it reaches its target site or the sensitivity of the target site changes so that it is no longer responsive to the active ingredient (Sun, 1992; Hama et al., 1987). A number of enzymes such as esterase, glutathione-S-transferase (GST), superoxide dismutase (SOD), reduced glutathione (GSH) and lactate dehydrogenase (LDH) participate in various defensive mechanisms (Nedal & Hassan, 2009). The present study investigated repellency and antioviposition activity of lemongrass oil against the cowpea weevil. It also assessed the effects on oxidative stress enzymes (SOD and GSH), xenobiotic detoxifying enzyme (GST), neurochemical enzyme (acetylcholinesterase: AChE) and energy metabolism biomolecules (glycogen and protein) in the insect pest with a view to establishing the insecticidal efficacy of the EO.

2. Materials and methods

2.1 Insect rearing

Callosobruchus maculatus was reared on cowpea seeds inside the Insect Physiology Laboratory, Faculty of Agriculture, Obafemi Awolowo University, Ile-Ife at 28 ± 2°C and 75 ± 5% RH. Cowpea seeds were bought from the local market and cleaned by handpicking. The pack of seeds was then kept in a deep freezer (sub-zero) for at least a week to eliminate infesting insects, mites or disease-causing microorganisms. At the end of the cold treatment, cowpea seeds were poured into 2 L glass jars, cowpea weevils were introduced and the containers were covered with muslin cloth for ventilation. The insects were reared for about three generations before newly-emerged adult weevils were selected for different experiments.

2.2 Extraction of essential oil from lemongrass

Fresh leaves of *C. citratus* were collected from Mokuro Road, Ile-Ife and authenticated at Ife Herbarium, Department of Botany, Obafemi Awolowo University. The materials were air-dried until crispy and milled to powder using a mill with rotatory knives for proper extraction. The essential oil was extracted from milled leaves following the procedure of hydro-distillation described by Papachristos and Stamopoulos (2004). Powdered *C. citratus* (500 g) was mixed in distilled water (3000 ml) and hydro-distilled for 6 h in a Clevenger apparatus. The oil was dried over anhydrous sodium sulphate (Na_2SO_4) to remove water molecules and refrigerated at 4°C for subsequent experiments.

2.3 Toxicity of lemongrass oil against adult *Callosobruchus maculatus*

The acute toxicity of *C. citratus* oil against *C. maculatus* was investigated by inhalation according to the method described by Ogunsina et al. (2011). Separate Whatman No. 1 filter papers were treated with different concentrations (0.62, 1.28, 2.50, 5.00 and 10.00 mg/ml) of *C. citratus* oil while

other filter papers were treated separately with ethanol and distilled water for comparison of results. The filter papers were air-dried for ethanol to evaporate after which each of them was placed inside 4.5 cm diameter Petri dishes. Ten newly emerged *C. maculatus* were transferred into each Petri dish which was then covered and its side sealed using a paper tape to prevent escape of vapour. Each treatment was replicated three times and insects were monitored for signs of toxicity and mortality at 1, 6, 12, 18 and 24 h after exposure. The number of insects that were dead at each observation hour was recorded per treatment.

2.4 Repellency test

A slight modification of the still-air olfactometer described by Weeks et al. (2011) was used to evaluate the action of *C. Citratus* EO against *C. maculatus.* A Whatman's No. 1 filter paper was divided into two halves; 2 ml 10 mg/ml essential oil was applied evenly on one while 2 ml 95% ethanol was applied on the other half using a micropipette. The two halves were allowed to air-dry for 5-10 min before they were attached lengthwise and placed inside the Petri dish. Twenty newly emerged adult *C. maculatus* were released in the middle of the Petri dish and proportion (%) of weevils that settled on each half of the filter paper disc was determined after 1 h as

$$\frac{\text{Number of weevils in the } x \text{ half of filter paper disc}}{\text{Total number of weevils introduced into the olfactometer per trial}} \times 100\%$$

If in a trial 30% of introduced weevils were found in the EO portion of the disc, it means that percent repellency by the oil was 70%. A total of 20 trials was carried out and percent repellency due to the essential oil and ethanol was recorded accordingly.

2.5 Anti-oviposition activity of lemongrass oil

The method described by Parugrug and Roxas (2008) was followed with little modification. Cowpea grains (50 g) were treated with three sublethal concentrations (1.41, 2.83 and 5.66 mg/ml) of the EO in triplicated 1.5 L glass jars. Jars containing grains for positive and negative control experiments were treated with distilled water and 95% ethanol, respectively. The jars were left opened long enough for the solvent to evaporate after which an aspirator was used to introduce 30 newly emerged C. *maculatus* into each of them. The jars were then covered with net material and left for 7 days. At the expiration of this period of oviposition, the weevils were removed and each grain per glass jar was examined carefully under a dissecting microscope to count the number of eggs laid on the surface. Eggs (about 0.75 mm long) are glued on the surface of seeds and they appear as whitish specks.

2.6 Preparation of insect homogenate

The weevils were reared on cowpea grains that had been treated with the three sublethal concentrations listed in subsection 2.5 and insect homogenate was prepared using the method of Upadhyay (2011). The insects (200 mg) were homogenized in 1.5 ml freshly prepared phosphate buffer (100 mM, pH 6.8) and the homogenate was centrifuged at 12,000 rpm for 20 min using Ice-cold

Bench Centrifuge (Searchtech 90-2). The supernatant was carefully collected in a clean vial and stored in a freezer until it was needed for biochemical assays.

2.7 Determination of total protein concentration in insect homogenate

Protein estimation was carried out using the method of Lowry et al. (1951). Insect homogenate (0.2 ml) was added to 2.1 ml alkaline copper reagent which was freshly prepared by mixing 2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4.5H_2O$ and 1% Na-K tartrate.$4H_2O$ (98:1:1 v/v/v). The mixture was vortexed and allowed to stand for 10 min followed by the addition of 0.2 ml Folin-Ciocalteu colour reagent. The resulting reaction mixture was vortexed and allowed to stand at room temperature in the dark for an hour after which absorbance of the solution was read at 550 nm against a reagent blank. The blank was made of 0.2 ml distilled water and appropriate volume of the diluents and colour reagent. The protein concentration of the homogenate was estimated from a standard curve obtained using Bovine Serum Albumin (BSA).

2.8 Estimation of reduced glutathione

Reduced glutathione (GSH) was measured according to the method of Beutler et al. (1963). One millimetre (1 ml) of the supernatant was added to 0.5 ml 10 mM Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid]) and 2 ml phosphate buffer (0.2 M, pH 8.0) was added. The yellow colour developed was read at 412 nm with a blank containing 3.5 ml phosphate buffer. A series of standards were also treated in similar manner and the amount of GSH was expressed in mg/200 mg homogenate.

2.9 Glutathione-S-transferase activity

Activity of Glutathione-S-transferase (GST) was determined as described by Habig et al. (1974). Into a clean, dry test tube was pipetted 2.7 ml sodium phosphate buffer (0.1 M; pH 6.5), 0.1 ml 30 mM reduced glutathione and 0.1 ml 30 mM 1-chloro-2,4-dinitrobenzene (CDNB). To the resulting mixture, 0.2 ml of insect hemolysate was added. The absorbance of the reacting mixture was measured at 340 nm at 15 s intervals for 3 min. Distilled water (0.2 ml) was used *in lieu* of hemolysate for the blank. The GST activity was calculated as below:

$$\text{GST activity} = \frac{\Delta A/\text{min} \times \text{DF} \times \text{V}}{\text{v} \times \varepsilon}$$

Where ε = extinction coefficient of CDNB (9.6 $mM^{-1}cm^{-1}$). $\Delta A/min$ = change in absorbance per minute. V = reaction volume. DF = dilution factor. v = sample volume.

2.10 Estimation of Superoxide dismutase

Superoxide dismutase (SOD) was estimated as described by McCord and Fridovich (1969). To 50 µL lysate, 75 mM Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM pyrogallol were added and thoroughly mixed. An increase in absorbance was recorded at 420 nm for 3 min. One unit of enzyme activity represented 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm. The activity of SOD was measured in unit/mg protein as expressed below:

$$\text{Increase in absorbance per minute } (\Delta A/\text{min}) = \frac{A_3 - A_0}{2.5}$$

Where A_0 = absorbance after 30 s. A_3 = absorbance after 150 s.

$$\% \text{ inihibition } = \frac{(\Delta A/\text{min})^{\text{Blank}} - (\Delta A/\text{min})^{\text{Test}}}{(\Delta A/\text{min})^{\text{Blank}}} \times 100\%$$

$(\Delta A/\text{min})^{\text{Blank}}$ = Increase in absorbance per minute for blank. $(\Delta A/\text{min})^{\text{Test}}$ = Increase in absorbance per minute for test sample. 1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the oxidation of pyrogallol.

2.11 Lactate dehydrogenase activity

The activity of Lactate dehydrogenase (LDH) in insect homogenate was assayed using Randox Diagnostic Kit (Randox Laboratories Ltd, Antrim, UK) as described by Weisshaar et al. (1975). Insect homogenate (0.02 ml) was pipetted into 1 cm cuvette at room temperature (25°C) and the reaction was initiated by adding 1.0 ml substrate. The initial absorbance was taken after 12 s at 340 nm and repeated first, second and third minute consecutively so as to obtain change in absorbance/min. The LDH activity was calculated as expressed below:

LDH activity (U/L) = 4127 × ΔA 340 nm/min

Where ΔA = change in absorbance.

2.12 Alanine aminotransferase activity

Alanine aminotransferase activity (ALT) was estimated according to the method of Reitman and Frankel (1957) using Randox Diagnostic Kit. The homogenate (0.1 ml) was pipetted into clean test tubes in triplicates and 0.5 ml of the buffer was added and mixed gently. The reaction mixture was incubated at 37°C for 30 min in a water bath. The reaction mixture was cooled and 0.5 ml 2.0 mM 2, 4-dinitrophenlhydrazine was added. The mixture was mixed properly and allowed to stand at room temperature for additional 20 min after which 5 ml 0.4 M NaOH solution was added and mixed thoroughly. The absorbance of the reaction mixture was taken at 546 nm against a blank prepared with distilled water (0.1 ml) in place of the sample. The enzyme activity (expressed in IU/L protein) was extrapolated from a calibration curve obtained from an absorbance-enzyme activity table of values provided by the kit manufacturer.

2.13 Aspartate aminotransferase activity

The procedure was similar to that described for aminotransferase (AST) except that the buffered substrate consisted of 100 mM phosphate buffer (pH 7.4), 100 mM L-aspartate and 2 mM α-ketoglutarate.

2.14 Acetylcholinesterase activity

Acetylcholinesterase (AChE) activity was determined according to the method of Ellman et al. (1961). Into microplate well was added, in triplicate, 240 µl buffer (0.1 M phosphate buffer, pH 8.0) and 20 µl insect homogenate. The reaction mixture was incubated in a dry bath incubator for 30 min at 37°C after which 20 µl 25 mM acetylthiocholine iodide and 20 µl 10 mM 5,5'-dithio-bis-[2-nitrobenzoic acid] were added. Change in absorbance at 30 s intervals

was measured spectrophotometrically for 4 min at 412 nm. The enzyme activity was calculated as follows:

$$\text{Acetylcholinesterase activity } (\mu \text{ mole/min/ml}) = \frac{\Delta A/\text{min} \times V}{v \times \Sigma \times d}$$

Where ΔA/min = change in absorbance per minute. V = total volume of reaction mixture. v = volume of test sample in reaction mixture. Σ = extinction coefficient of DTNB ($1.36 \times 10^4 mM^{-1} cm^{-1}$). d = light path length (1cm).

2.15 Estimation of Glycogen concentration

The insect glycogen was isolated and estimated according to the method of Oyedapo and Araba (2001). Insects (200 mg) were homogenized and transferred into separate test tubes containing 10 ml 30% (w/v) KOH. The tubes were heated at 70ºC until the tissues were fully digested. Distilled water was added to the resulting burgundy red suspension to make the volume 20 ml followed by the addition of 18 ml 95% (v/v) cold ethanol. The mixture was mixed by inversion and chilled with ice. A white flocculent precipitate of impure glycogen was formed and this was collected by centrifugation at 3,000 rpm for 15 min. The precipitate was dissolved in 15 ml 5% (w/v) cold trichloroacetic acid (TCA) and stirred vigorously after which it was heated at 85ºC for 5 min. The suspension was centrifuged at 5,000 rpm for 10 min and 25 ml cold 95% (v/v) ethanol was added to the supernatant, mixed by inversion and allowed to precipitate the glycogen. The precipitate was collected by centrifugation at 3,000 rpm for 10 min. The precipitated glycogen was dissolved in 5 ml distilled water and shaken thoroughly until fully dissolved. To the solution was added 12 ml 95% (v/v) ethanol, while mixing with stirring rod and a white flocculent precipitate of pure glycogen was formed. The precipitate was centrifuged at 2,000 rpm for 10 min and the residue obtained was kept in a deep freezer for analysis. The standard glycogen calibration curve was prepared by pipetting 0, 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working solution (250 µg/ml), in triplicates, into clean dry test tubes and volumes were adjusted to 1.0 ml with distilled water. To each of the tubes was added 0.5 ml concentrated HCl, followed by 0.5 ml 88% (v/v) formic acid and 2.0 ml Anthrone reagent (0.02 g in 68% (v/v) sulphuric acid). The tubes were transferred into boiling water for 10 min after which they were cooled under running water. The absorbance was read at 630 nm against the blank and the insect glycogen concentration was extrapolated from the standard calibration curve.

2.16 Statistical analysis

Percent mortality data were subjected to square root transformation before analysis of variance (ANOVA) was carried out and mean values were separated using Tukey's HSD test. Percent repellency data were also transformed and analyzed in a similar manner but mean values were separated using Fisher's LSD at 0.05 level of probability. On the other hand, count data obtained from anti-oviposition experiment were subjected to natural log transformation before ANOVA and mean values were separated using Tukey's HSD test. The median lethal concentration (LC_{50}) was determined

from mortality data using probit analysis in Microsoft Excel. Bioassay data were subjected to ANOVA procedure and mean values were separated using Tukey's HSD test.

3. Results and Discussion

Generally, insect mortality was dose- and observation time-dependent (Table 1). Mortality was reported among insects exposed to 10 mg/ml EO within 1 h of application and it increased to an excellent level of 80% within 24 h. The maximum efficacies attained by other concentrations were 25% (2.5 mg/ml and 5.0 mg/ml), 10% (1.28 mg/ml) and 15% (0.62 mg/ml). Ketoh et al. (2000) in an earlier study recorded > 90% mortality in *C. maculatus* within 24 h of exposure to EOs from two *Cymbopogon* spp. This report and the results obtained in the current study indicated that members of the genus *Cymbopogon* may possess good insecticidal properties against the cowpea weevil. The 24 h LC_{50} obtained for the EO against cowpea weevil was 7.07 mg/ml. This is an indication that toxicity of the EO against *C. maculatus* is superior to those of *Cordia millenii* Baker (Manjack) (LC_{50} = 36.3 mg/ml), *Zingiber officinale* Roscoe (Ginger) (LC_{50} = 37.5 mg/ml), *Xylopia aethiopica* (Dunal) (Negro pepper) (LC_{50} = 43.8 mg/ml), *Monodora myristica* (Gaertn.) (Nutmeg) (LC_{50} = 47.5 mg/ml) and *Allium sativum* L. (Garlic) (LC_{50} = 55.0 mg/ml) even at 96 h post-treatment (Edwin & Jacob, 2017). The lemongrass EO exhibited significant repelling and oviposition deterrence abilities against the cowpea weevil (Table 2). Approximately ⅔ of tested weevils was repelled by the oil and this effectiveness corroborates an earlier report (Jayasingha et al., 1999) establishing repelling efficacy of the essential oil against *Dacus dorsalis* (Diptera: Tephritidae). The anti-oviposition effect was dose-dependent with an average of 16 eggs laid on cowpea grains treated with 1.44 mg/ml EO. This was not significantly different from the number of eggs laid on grains without plant extract while significantly fewer eggs were laid when higher concentrations of EO were applied. This report is in agreement with Ketoh et al. (2000) where EOs of *C. citratus* and *C. nardus* (L.) Rendle elicited significant dose-dependent oviposition deterrence in *C. maculatus*. Plant EOs have also been reported to have ovicidal effect on eggs of *C. chinensis* (Dwivedi & Kumari, 2000; Pathak et al., 1997).

Table 1: Percent mortality of cowpea weevils exposed to serial concentrations of lemongrass oil over a period of 24 hours.

Time (h)	Concentration of essential oil (mg/ml)					Ethanol	Blank
	10.00	5.00	2.50	1.28	0.62		
1	15cA	0bB	0cB	0aB	0bB	0aB	0aB
6	35bA	15aAB	5bcBC	0aC	0bC	0aC	0aC
12	45bA	15aAB	10abcAB	10aAB	5abAB	5aAB	0aB
18	75aA	20aB	20abB	10aBC	5abBC	5aBC	0aC
24	80aA	25aAB	25aAB	10aB	15aB	5aB	5aB

Values with similar capital letters in the same row and similar small letters in the same column are not significantly different at 0.05 level of probability.

Table 2: The repelling efficacy (A) and antioviposition effect (B) of lemongrass oil against the cowpea weevil.

A	Proportion of cowpea weevils repelled (%)				
	Lemongrass oil (10 mg/ml)		Ethanol (extraction solvent)		$LSD_{0.05}$
	65.83 ± 0.32		34.17 ± 0.19		15.45
B	Average number of eggs laid on cowpea seeds				
	Concentration of lemongrass oil (mg/ml)			Ethanol	Distilled water
	5.66	2.83	1.41		
	0.33c	4.00b	16.00a	17.33a	22.67a

Values with similar letters in the same row are not significantly different at 0.05 level of probability.

This has been attributed to ability of the oils to penetrate egg chorion, thereby, creating the deleterious hypercarbic condition (Don-Pedro, 1989). The mean square value and contribution of each source of variation to activity of each biochemical entity that was quantified in the present study is presented in Table (3). Time post-exposure and concentration of essential oil had significant effect ($P \leq 0.001$) on activity of superoxide dismutase and total protein. They also affected activity of acetylcholinesterase, glycogen and lactate dehydrogenase significantly. However, these two experimental sources of variation did not have any significant effect on activity of alanine aminotransferase. Time post-exposure accounted for most of the variation in the activity of acetylcholinesterase (35%), aspartate aminotransferase (20%), alanine aminotransferase (11%), reduced glutathione (35%), glutathione-S-transferase (21%) and superoxide dismutase (47%) while concentration of essential oil accounted for a larger percentage in the case of glycogen depletion (75%), superoxide dismutase (28%) and total protein (65%).

Table 3: Mean square values from analysis of variance and percent contribution of each source of variation to biochemical reactions in cowpea weevils exposed to lemongrass oil.

Source of variation	df	Mean square value								
		AChE	AST	ALT	GLY	GSH	GST	LDH	SOD	TPR
Time post-exposure	3	0.001064*** (35.36%)	0.006382* (20.11%)	0.000709 (10.91%)	76.683** (9.17%)	0.002849*** (34.79%)	4.75×10^{-7}* (21.18%)	6.068* (20.45%)	0.175*** (46.97%)	2.360*** (17.53%)
Concentration of essential oil	4	0.000497** (2.20%)	0.001368 (5.75%)	0.000280 (5.74%)	469.846*** (74.88%)	0.000605 (9.85%)	1.28×10^{-7} (7.62%)	5.426* (24.39%)	0.077*** (27.66%)	6.535*** (64.75%)
Replication	1	0.000004 (0.04%)	0.003054 (3.21%)	0.000281 (1.44%)	24.613 (0.98%)	0.001172 (4.77%)	2.60×10^{-14} (0.00%)	0.000 (0.00%)	0.008 (0.72%)	0.000 (0.00%)
Error	39	0.000124 (42.58%)	0.002179 (70.94%)	0.000515 (81.91%)	12.124 (14.97%)	0.000401 (50.59%)	1.54×10^{-7} (71.20%)	1.583 (55.16%)	0.009 (24.65%)	0.231 (17.72%)
R^2		0.574	0.291	0.181	0.850	0.494	0.288	0.448	0.753	0.823
CV		46.60	13.21	10.41	19.96	27.83	49.10	20.69	34.19	16.16

*,**,*** significance at 0.05, 0.01 and 0.001 levels of probability, respectively. Percent contribution of each source of variation to changes in biochemical activity within exposed insects is presented in parentheses. AChE: Acetylcholinesterase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GLY: Glycogen, GSH: Reduced glutathione, GST: Glutathione-S-transferase, LDH: Lactate dehydrogenase, SOD: Superoxide dismutase, TPR: Total protein

This is an indication that the last three biochemical parameters were more sensitive to the active fraction of the EO. The *in vitro* biochemical activity of tested weevils, in both dose- and time-dependent situations, is presented in Tables (4) and (5), respectively. Activity of alanine aminotransferase and reduced glutathione did not vary with concentration of EO whereas reduction in acetylcholinesterase, aspartate aminotransferase and glutathione-S-transferase activity, and depletion of glycogen and total protein

were more pronounced at highest EO concentration of 5.66 mg/ml. The highest lactate dehydrogenase activity was recorded when 5.66 mg/ml EO was applied and the oil, irrespective of its concentration, clearly reduced the activity of superoxide dismutase**.** Activity of alanine aminotransferase remained comparable between the 6th and 24th hour of exposure whereas the suppression of glutathione-S-transferase by the EO waned with time post-treatment. Acetylcholinesterase and aspartate aminotransferase activity increased significantly after 18 h of inhalation while a similar trend was observed in superoxide dismutase activity after 12 h. Glycogen depletion increased with time post-exposure to the EO and protein depletion was more evident at the 6th hour of observation. Acetylcholinesterase is a serine protease found at cholinergic synapses where it terminates synaptic transmission by hydrolysis of the neurotransmitter acetylcholine. Some insecticidal agents have been reported to inhibit acetylcholinesterase activity, thereby, producing a deleterious effect on the insect as it causes excessive accumulation of the neurotransmitter. This results in hyperactivity, paralysis and eventual death of insect (Fournier & Mutero, 1994). During normal function of AChE, a serine-histidine-glutamate triad, located in the active site of the enzyme, catalyzes the hydrolysis of acetylcholine in a step-wise manner releasing choline and acetic acid (Quinn, 1987). Usually, acetylcholinesterase inhibitors attack the serine hydroxyl group in the enzyme active site and form a covalent bond (Mercey et al., 2012). The AChE inhibition observed in this study could be due to the formation of a complex at the enzyme active site but the inhibition was not irreversible as AChE activity increased significantly after 18 h of exposure to the EO.

Table 4: Activity of biochemical parameters determined in cowpea weevils exposed to serial concentrations of lemongrass oil.

Concentration of EO (mg/ml)	Biochemical activity								
	AChE	AST	ALT	GLY	GSH	GST	LDH	SOD	TPR
0	0.035a	0.380a	0.215a	26.049a	0.076a	0.0010a	6.294ab	0.450a	3.884a
1.41	0.027ab	0.363b	0.224a	17.426b	0.074a	0.0009a	5.877bc	0.213b	2.802b
2.83	0.024abc	0.347c	0.219a	12.486c	0.064a	0.0008a	6.157abc	0.248b	2.572b
5.66	0.014c	0.338d	0.223a	7.576d	0.062a	0.0005b	7.183a	0.227b	1.757c
n-hexane	0.019bc	0.378a	0.210a	23.683a	0.083a	0.0010a	4.895c	0.242b	3.850a

Values with similar letters in the same column are not significantly different at 0.05 level of probability. AChE: Acetylcholinesterase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GLY: Glycogen, GSH: Reduced glutathione, GST: Glutathione-S-transferase, LDH: Lactate dehydrogenase, SOD: Superoxide dismutase, TPR: Total protein.

Table 5: Activity of biochemical parameters determined at 6-hour intervals in cowpea weevils exposed to serial concentrations of lemongrass oil.

Time post-exposure (h)	Biochemical activity								
	AChE	AST	ALT	GLY	GSH	GST	LDH	SOD	TPR
6	0.016b	0.344b	0.224a	20.433a	0.057c	0.0006c	6.531ab	0.180b	2.394c
12	0.018b	0.354ab	0.210a	19.072a	0.075b	0.0007bc	5.204c	0.150b	2.762b
18	0.022b	0.328b	0.227a	14.520b	0.094a	0.0010a	6.910a	0.425a	3.277a
24	0.039a	0.388a	0.211a	15.750b	0.062bc	0.0010a	5.680bc	0.348a	3.459a

Values with similar letters in the same column are not significantly different at 0.05 level of probability. AChE: Acetylcholinesterase, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, GLY: Glycogen, GSH: Reduced glutathione, GST: Glutathione-S-transferase, LDH: Lactate dehydrogenase, SOD: Superoxide dismutase, TPR: Total protein.

The reduced glutathione (GSH), a free radical scavenger, is known to be an important cellular protectant against reactive oxygen metabolites in many insect cells that are concerned with pesticide detoxification (Büyükgüzel, 2009). The enhanced GSH level observed in this study within 18 h of inhaling fumes from the EO could be an adaptive mechanism which allows the insects to scavenge the free radical damaging systems thereby maintaining the integrity of the cell membrane and other biomolecules like proteins, polysaccharides and DNA. Glutathione-S-transferase (GST) which catalyzes the conjugation of GSH with a variety of electrophilic metabolites participates in defense against oxidative stress. It does this by detoxifying endogenous harmful compounds like hydroxyl alkenal and base propenal or DNA hydroperoxides and electrophilic xenobiotic, and is known to provide protection against oxidative/nitrosative stress by GSH-mediated process of reactive products of lipid peroxidation (Kim et al., 2003). Similar to GSH, the GST activity was induced and this might indicate an adaptive response to the oxidative stress triggered by the applied EO. This probably suggests involvement and activation of GST-dependent xenobiotic metabolism (Kolawole et al., 2011). The induction of GST is considered beneficial to handle environmental stress (van der Oost et al., 2003) while overexpression of GST could be an important means of cell protection during physiological stress. The GST stimulation in treated insects might be due to citral that is in the EO. It is a constituent of *C. Citratus* oil-made of mixture of geranial and neral stereoisomers. Citral was reported to possess a significant ability to suppress oxidative stress probably through induction of the endogenous antioxidant glutathione system (Nakamura et al., 2003). Superoxide dismutases (SODs) are metalloproteins with sufficient activity for dismutation of superoxide anions that are produced as a result of oxidative stress (Beedham et al., 1995) thereby protecting cells against damage. The SOD reacts with superoxide radicals and converts them to H_2O_2, catalyzed by catalase or GSH peroxidase (Kakkar et al., 1984). It could be inferred that the time-dependent increase in SOD activity in treated bean weevils was a possible survival mechanism in order to reduce possible oxidative (or toxic) stress posed by the applied essential oil (Al-Omar et al., 2004). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are key enzymes that catalyze the synthesis of glutamic acid from aspartic acid and alanine, respectively. These important enzymatic processes usually occur in the final stage of development in insects (Gowda & Ramaiah, 1976) and changes in transaminase levels are consistent with the sum total of anabolism and catabolism of protein. The time-dependent increase in AST activity observed in this study was a form of physiological response aimed at initiating amino acid synthesis with a view to overcoming damage caused by the EO-induced stress (Gowda & Ramaiah, 1976). Lactate dehydrogenase (LDH) is an important glycolytic enzyme that is present in almost all

animal tissues (Kaplan & Pesce, 1996). The enzyme helps in energy generation especially when a considerable amount of additional energy is required immediately (Nathan et al., 2006). A marginal reduction in LDH activity was observed when lower concentrations (1.41 and 2.83 mg/ml) of the EO were applied. The decrease denotes reduction in energy metabolism of the insect probably because of the effect of toxic phytoconstituents on membrane permeability, especially of the gut epithelium (Nathan et al., 2006). Reduction in LDH activity could also be due to a physiological disturbance at the molecular level as a result of depression or mutation of genes responsible for biosynthesis of the polypeptide chains that build LDH (Hassanien et al., 1996). However, a stress-induced marginal increase in LDH activity resulted when a higher concentration (5.66 mg/ml) of the oil was applied, apparently as a means of compensating for depleted protein. The depletion of glycogen in stressed organisms is associated with an increase in the utilization of food reserve (Sancho et al., 1998). The consistent concentration-dependent reduction in glycogen level reported in the current study is an evidence that treated weevils were under stress and they needed additional energy to match the increase in demand as a result of the physiological stress induced by the EO. Inhalation of the EO caused significant protein depletion in bean weevils and this was more evident within the first 12 h of exposure. Protein depletion correlates with break-down of proteins into amino acids, and their entrance into the Krebs cycle as keto acid. Thus, protein depletion in tissues plays a role in compensatory mechanisms under insecticidal stress to provide intermediates in the Krebs cycle (Shekari et al., 2008; Zibaee et al., 2008). Protein depletion in a stressed organism could also be attributed to inability of protein-synthesizing mechanisms to function properly. The subsequent increase in total protein level beyond 12th hour of inhalation could be due to ability of the insects to counter damaging effects of the EO through specific physiological mechanisms. The lemongrass oil exhibited effective insecticidal action against the cowpea weevils and could, therefore, be considered as an alternative agent for protecting cowpea seeds in storage, especially, by rural farmers in tropical and subtropical regions. Insecticides are costly and not sustainable in the long run due to environmental contamination whereas EOs contain a range of selective bioactive chemicals with insignificant or no harmful effect on non-target organisms and the environment (Vinayaka et al., 2010). The use of plant oil would be cost effective and sustainable, especially considering that this plant is easy to grow. In addition, the grass is safe to users as evidenced by the fact that it is used as culinary spices and herbs.

Author Contributions

BA conceptualised this work as part of SE's postgraduate Degree program. The research was co-designed and executed by BA, SE and OJ; BA and SE carried

out the biochemical assays while OJ and SE investigated the behavioural responses. SE, BA and OJ analysed obtained data and prepared the manuscript for publication.

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