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Inhibitory effects of oil extract of green Acalypha (*Acalypha wilkesiana*) on antioxidant and neurotransmitter enzymes in *Callosobruchus maculatus*

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Abstract

Background: *Callosobruchus maculatus* is an important insect pest of cowpea. The inhibitory effects of oil extract of *Acalypha wilkesiana* (Muell Arg.) leaves on antioxidant, neurotransmitter, and detoxifying enzyme in adult *Callosobruchus maculatus* (Fabricius) were evaluated under laboratory condition. The leaves of the plant were collected fresh and air-dried before the oil was extracted from them through cold extraction method while ethanol was used as solvent. Adult *C. maculatus* were exposed to 0.2, 0.4, 0.6, 0.8, and 1.0 ml of 0.5% concentration of the oil extract and were homogenized separately. The supernatants gotten from them were used as enzyme sources. The activities of SOD, CAT, GPx, AChE, CarEST and GST were observed with photospectrometer. GC-MS analysis was done to evaluate the active compounds present in the oil extract.

Results: The activities of the enzymes increased at lower dosages of the oil extract (0.2 and 0.4 ml) and reduced drastically at higher dosages of the oil (0.6, 0.8, and 1.0 ml). However, AChE activity was more affected by the oil extract as it was almost inhibited by the oil extract. The GC-MS showed that 114 compounds were present in the oil extract of the plant while acetaldehyde had the highest percentage (46.07%) of the total compounds.

Conclusion: Based on these findings, the oil extract of *A. wilkesiana* could be a good biopesticide for the control of adult *C. maculatus* instead of synthetic chemical insecticides.

Keywords: Plant extract, *Acalypha wilkesiana*, *Callosobruchus maculatus*, Antioxidant, Detoxification, Enzymes

Background

Callosobruchus maculatus is an important insect pest of cowpea, *Vigna unguiculata* (Walp). The insect is a cosmopolitan insect with ability to infest both on the field and in storage where its destructive activities multiply. Researches have shown that the insect is capable of causing more than 65% destruction of cowpea grains in storage and if left on unchecked it may cause total wastage of cowpea within 6 months of storage (Abd-Elhady, 2012; Ashamo, Odeyemi, & Ogungbite, 2013; Kedia, Prakash, Mishra, Singh, & Dubey, 2013; Oni, 2014; Shimomura et al., 2010). The ability of the insect to have many generations within a year makes its infestation on cowpea to be more pronounced (Kedia et al., 2013; Ogungbite, 2015; Oni, Ogungbite, & Ofuya, 2018).

Considering the importance of cowpea as main source of protein in the diet of families that cannot afford animal proteins, researchers have been combating the insect and other stored product insect pests with the use of synthetic chemical insecticides which were discovered in the late 1930s (Begum, Shaarma, & Pandey, 2013; Forim, Da-silva, & Fernandes, 2012; Isman, 2000, 2006). In recent decades, the adverse effects of many of these synthetic chemical insecticides both on human and environmental health called for their outright replacement with insecticides that have no or little adverse effects on humans and their habitat (Jaya, Prakash, & Dubey, 2012;

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Scott et al., 2003; Sivakumar, Chandrasekaran, Vijayaraghavan, & Selvaraj, 2010; Tripathi & Dubey, 2004).

Plant extracts and powders have for ages been the major weapons used by farmers against insect pests even before the discovery of synthetic chemical insecticides because they are believed to contain myriads of phytochemicals that are insecticidal in nature (Goławska & Łukasik, 2012; Goławska, Łukasik, Goławski, Kapusta, & Janda, 2010; Zibaee, 2011). Thousands of researches have been done on botanical base insecticides, thus many plant species have proven insecticidal and there are many publications on them. In spite of these many publications on botanical insecticides, Isman and Grieneisen (2014) opined that only few information are derivable from them because many of them failed to address the mode of actions of the plant base insecticides. Martins, Freire, Parra, and Macedo (2012), Rattan and Sharma (2011), and Zibaee and Bandani (2010) suggested that once the insecticidal potential of a botanical has been discovered, its effects on antioxidant, digestive, and detoxifying enzymes in insects should be evaluated and active compounds present in it should be determined as this will ensure the protection of non-target organisms.

Acalypha wilkesiana (Muell Arg.) green acalypha is a medicinal plant whose insecticidal potential of its extract had been proven (Oguntuase, 2018) but much work have not been done as regard its mode of action and active compounds present in it. This present work evaluated the inhibitory effects of *A. wilkesiana* oil extract on the antioxidant, detoxifying, and neurotransmitter enzymes in *Callosobruchus maculatus* and as well determined the active compounds present in it.

Materials and methods

Insect culture

The initial culture of *C. maculatus* used was collected from already infested cowpea grains in the Entomology Laboratory of the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure. The insects were sub-cultured on clean uninfested Ife-Brown cowpea variety inside a plastic container at laboratory temperature of 28 ± 2 °C and relative humidity of 75 ± 5%. The cover of the container was perforated and covered with muslin cloth to allow aeration and prevent the escape of the insect and entry of other insects that may be a parasitoid to the beetle (Ashamo et al., 2013).

Collection of plant material and extraction

The leaves of *A. wilkesiana* used were obtained from an open field inside the campus of Federal University of Technology Akure and were air-dried under shade before being pulverized into fine powder with Electric blender. The powder of the leaves was kept inside an air-tight plastic container and was kept till further use. The oil from the powder was extracted using cold extraction method while ethanol was used as solvent. The plant powder (500 g) was soaked in 1 l of ethanol for 4 days and the mixture was stirred vigorously to ensure even mixture. The oil extract was separated from the chaff of the powder with muslin cloth while the oil extract was separated from the solvent using rotary evaporator. The oil extract was kept inside air-tight container and placed inside refrigerator for subsequent use. From the main stock of the oil extract, 0.5% concentration of the oil extract was made and 0.2, 0.4, 0.6, 0.8, and 1 ml dosages were separately made from it.

Bioassay

Filter papers placed inside Petri-dishes were treated with 0.2, 0.4, 0.6, 0.8, and 1 ml dosages of 0.5% concentration of *A. wilkesiana* oil extract in five replicates. Two hundred fifty unsexed 0–24 old *C. maculatus* were placed in each of the Petri-dish and were left for 24 h. After 24 h, the live and dead *C. maculatus* were separated. The live insects were quickly demobilized by freezing and the wings were removed while the remaining body parts were homogenized in ice-cold buffer (25 mM potassium phosphate buffer, pH 7.2 EDTA, and 1 mM 2-mercaptoethanol). The homogenates were centrifuged at 10,000 g for 10 min at 4 °C (Bamidele, Ajele, Kolawole, & Akinkuolere, 2013). The floating lipids were carefully filtrated from the supernatants through funnel plugged with glass wool and the supernatants were kept in aliquots at temperature below – 4 °C for subsequent use.

Enzyme assays

Determination of catalase activity

Catalase, EC. 1.11.1.6 activity was assayed according to the method of Aebi (1984) by mixing 2.4 ml phosphate buffer (50 mM, pH 7.0), 10 μl of 19 mM H_2O_2 and 50 μl enzyme source (supernatant). The decrease in absorbance was measured at 240 nm over a 3-min period at 25 °C against the blank on a spectrophotometer. Two readings were taking at 0 and 3 min. Catalase (CAT) activity was calculated with the equation below:

$$\mathrm{CAT} = \frac{R_1 - R_2}{T}$$

Where R_1 is the initial reading at 0 min, R_2 is the final reading after 3 min, and the T is the time intervals.

Determination of superoxide dismutase activity

Superoxide dismutase (SOD) was assayed according to Beauchamp and Fridovich (1971) as described by Bamidele et al. (2013). The reaction mixture contained 1.17 μM riboflavin, 0.1 M methione, 0.2 μM potassium

cyanide (KCN), and 0.56 μM nitro blue tetrazolium salt (NBT) dissolved in 3 ml of 50 mM sodium phosphate buffer (pH 7.8). Three milliliter of the reaction medium was mixed with 1 ml of enzyme (supernatant) and the mixtures were illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes in a single row. Reaction was initiated at 30 °C for 1 h by the illumination. Identical solutions that were kept under dark served as blanks while absorbance was read at 560 nm in the spectrophotometer against the blank. SOD activity was expressed in units (U mg^{-1} protein). One unit is defined as the amount of change in the absorbance by 0.1 h^{-1} mg^{-1} protein. SOD activity was calculated with the equation below:

$$\begin{aligned} \text{SOD} &= R_4 \Big/ \text{A} \\ \text{A} &= R_1\left(50 \Big/ 100\right) \\ R_4 &= R_3\text{-}R_2 \\ R_3 &= \text{OD of sample} \\ R_2 &= \text{OD of blank} \\ R_1 &= \text{OD of reference} \end{aligned}$$

Where R_1 is the absorbance of the reference solution, R_2 is the absorbance of the blank, and R_3 is the absorbance of sample when enzyme has been added at a particular level.

Determination of glutathione peroxidase activity

The GPX, EC 1.11.1.9 activity was assayed with H_2O_2 as substrate according to the method of Paglia and Valentine (1967) as described by Bamidele et al. (2013). The reaction was monitored indirectly as the oxidation rate of NADPH at 340 nm for 3 min. Enzyme activity was expressed as micromoles of NADPH consumed per minute per milligram of protein, using an extinction coefficient of 6.220 M^{-1} cm^{-1}. A blank without enzyme was used as a control for the non-enzymatic oxidation of NADPH upon addition of H_2O_2 in 0.1 M Tris buffer, pH 8.0. Glutathione peroxidase (GPx) activity was calculated with the equation below:

$$\text{GPX} = \frac{2(\text{mRate}_s - \text{mRate}_b) \text{ x } V_{\text{Rxm}}}{6.22 \text{ x } V_s} \text{ x } \frac{\text{df}}{1}$$

Where mRate_s = – 1000 x ΔA_{340}/min of sample

mRate_b = – 1000 x ΔA_{340}/min of blank

6.22 = NADPH 340 nm millimolar absorption coefficient at 1 cm path length

V_{Rxm} = Volume of reaction mixture

V_s = Volume of sample

2 = correction for 2 mol GSH oxidized to 1 mol GSSG per mole NADPH

df = Sample dilution factor

Determination of AChE activity

The acetylcholine esterase (AChE) activity was determined by the method described by Ellman, Courtney, Andres, and Featherstone (1961) as described by Bocquene and Galgani (1998). Supernatants were used as enzymes while using acetylthiocholine iodide (ATCL) at 0.25 nM as substrate. Aliquots of enzyme (100 μl) and DTNB (100 μl of 0.01 M) were added to 0.1 M phosphate buffer (pH 7.4; 600 μl) then 100 μl phenylpropene test solutions prepared in absolute ethanol were added to the mixture. Control experiment was prepared by addition of 100 μl absolute ethanol. The mixtures were incubated at 35 °C for 15 min and 100 μl ACTL was added to start the reaction. Absorbance was measured at 412 nm using photo-spectrometer. The percentage AChE inhibition was calculated with the equation below

$$\text{AChE activity} = \frac{\Delta A_{412} \text{ x } Vol_T \text{x } 1000}{1.36 \text{ x } 10^4 \text{ x ligthpath x } Vol_s \text{ x [protein]}}$$

Where ΔA_{412} = change in absorbance (OD) per min, corrected for spontaneous hydrolysis

Vol_T = total assay volume (0.380 ml)

1.36×10^4 = extinction coefficient of TNB (M^{-1} cm^{-1})

Lightpath = microplate well depth (1 cm)

Vol_S = sample volume (in ml)

[protein] = concentration of protein in the enzymatic extract (mg ml^{-1})

Determination of carboxylesterase activity

The activity of carboxylesterase was determined according to Van Asperen (1962). Undiluted 100 μl of the homogenates and diluted 100 μl of the homogenates were briefly incubated with 1 ml of sodium phosphate buffer (20 mM, pH 7.0) containing 250 μM of naphthyl acetate for 30 min at 28 °C. Then, 400 μl of freshly prepared 0.3% fast blue B in 3.3% SDS were added to stop the enzymatic reaction and the color was allowed to develop for 15 min at 28 °C. The optical density of the sample was read at 430 nm against the reagent blank in Shimadzu UV-160A spectrophotometer.

Determination of glutathione transferase activity

Glutathione transferase (GST) catalytic activity was determined spectrophotometrically using Biochrome-4060 model spectrophotometer with the aromatic substrate, 1-chloro-2,4 dinitobenzene (CDNB) by monitoring the change in absorbance, due to thioether formation at 340 nm and 25 °C as described by Habig, Pabst, and Jokoby (1974). The assay mixture contained in a total volume of 1 ml which comprised 0.1 M potassium phosphate buffer at pH 7.4, 1 mM CDNB in ethanol, 1 mM GSH, and the enzyme solution. The absorbance at 340 nm of the complete assay mixture was monitored

against a control containing buffer instead of the enzyme and treated similarly. The product extinction coefficient was taken to be 9.6 mM^{-1} and defined as the amount of enzyme which catalyzed the formation of 1 μmol/min/mg protein. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. GST activity was calculated with the equation below:

$$\text{GST activity} = \frac{\Delta OD_{340nm}/\ \text{minx}\ 100\ \mu l}{2.99\ mM^{-1}\ \text{x}\ 10\mu l}\ \text{x}\ \frac{\text{Sample dilution}}{1}$$

Evaluation of the active compounds present in the leaves oil extract of *A. wilkesiana*

The aliquot used for the GC-MS qualitative characterization analysis was prepared by dissolving the oil extract of *A. wilkesiana* in 5 ml ethanol. The mixture was then separated for clear solvent layer by centrifuging at 4000 rpm. A qualitative characterization analysis of possible compounds present in the oil extract was carried out using GC-MS using scan mode. This analysis was performed using 7820A gas chromatograph coupled to 5975C inert mass spectrometer (with triple axis detector) with electron-impact source (Agilent Technologies). The stationary phase of separation of the compounds was HP-5 capillary column coated with 5% phenyl methyl siloxane (30 m length × 0.32 mm diameter × 0.25 μm film thickness) (Agilent Technologies). The carrier gas was Helium used at constant flow of 1.6 ml/min at an initial nominal pressure of 2.84 psi and average velocity of 46 cm/s. One microliter of the samples was injected in splitless mode at an injection temperature of 260 °C. Purge flow was 21.5 ml/min at 0.50 min with a total flow of 25.8 ml/min; gas saver mode was switched on. Oven was initially programmed at 60 °C (1 min) then ramped at 4 °C/min to 110 °C (3 min) then 8 °C/min to 260 °C (5 min) and 10 °C/min to 300 °C (12 min). Run time was 56.25 min with a 3 min solvent delay. The mass spectrometer was operated in electron-impact ionization mode at 70 eV with ion source temperature of 230 °C, quadrupole temperature of 150 °C, and transfer line temperature of 280 °C. Scanning of possible alkaloid compounds was from m/z 30 to 550 amu at 2.62 s/scan rate and was identified by comparing measured mass spectral data with those in NIST 14 Mass Spectral Library and literature.

Bioassay and statistical analysis

All the data obtained in this work were subjected to one-way analysis of variance and means were separated with Tukey honestly significant test. SPSS Version 21 was used for the analysis.

Results

Effects of oil extract of *A. wilkesiana* on the activities of antioxidant enzymes in *C. maculatus*

The effects of *A. wilkesiana* oil extract on the activities of SOD, CAT, and GPx were presented in Fig. 1. The effects of the oil extract were directly proportional to the dosage of the oil extract. Statistically significant differences existed between the treatments at $F_{5,\ 24} = 207.233$, $p < 0.0001$ (SOD), $F_{5,\ 24} = 106.932$, $p < 0.0001$ (CAT), and $F_{5,\ 24} = 1492.329$, $p < 0.0001$ (GPx). Tukey multiple comparison showed that for the SOD, significant difference did not existed between 0.2 ml of the oil extract and 0.8 ml and between 0.2 ml of the extract and control at $p = 0.239$ and 0.529 respectively. Also, there were no significant differences that existed between 0.4 ml and 0.8 ml of the extract at $p = 0.165$. For CAT, no significant difference existed between 0.2 ml and 0.6 ml of the oil extract as well as between 0.2 ml and the control at $p = 0.07$ and $p = 0.612$. Tukey multiple comparison showed that for the GPx, significant differences existed between all the treatments. The highest SOD activity was recorded at 0.3 ml (14.64 μmol/min/ml) of the extract while the lowest activity of 3.71 μmol/min/ml was recorded at 1.0 ml dosage of the oil extract and they were significantly ($p < 0.05$) different from all other treatments. The lowest CAT activity (2.74 μmol/min/ml) was recorded at 1.0 ml of the oil extract while the highest activity (5.79 μmol/min/ml) was recorded at 0.4 ml of the extract and was significantly different from others. At 0.4 ml, the highest activity of GPx (52.58 μmol/min/ml) was recorded while the lowest activity (20.06 μmol/min/ml) of the enzyme was recorded at 1.0 ml of the oil extract. Generally, the activities of the three enzymes increased above that of the control treatment with increase in dosage of the oil extract but drastically reduced at higher dosages (0.8 and 1.0 ml).

Effects of *A. wilkesiana* oil extract on activities of AChE and CarEST

Figure 2 showed the effects of *A. wilkesiana* oil extract at different dosages on activities of AChE and carboxylesterase (CarEST). The activities of the enzymes varied with the dosages of the oil extract. Statistically significant variations existed between the treatments at $F_{5,\ 24} = 2800.047$, $p < 0.0001$ (AChE) and $F_{5,\ 24} = 932.222$, $p < 0.0001$ (CarEST). Tukey multiple comparison showed that for AChE, significant differences existed between all the treatments while for CarEST significant difference did not existed between 0.2 ml of the oil extract and the control at $p = 0.223$. The activities of AChE decreased with increase in the dosage of the oil extract. The highest AChE activity was recorded in the control (0.41 μmol/min/ml) while the activity of the enzyme is almost inhibited as only 0.0012 μmol/min/ml activity of the enzyme was recorded

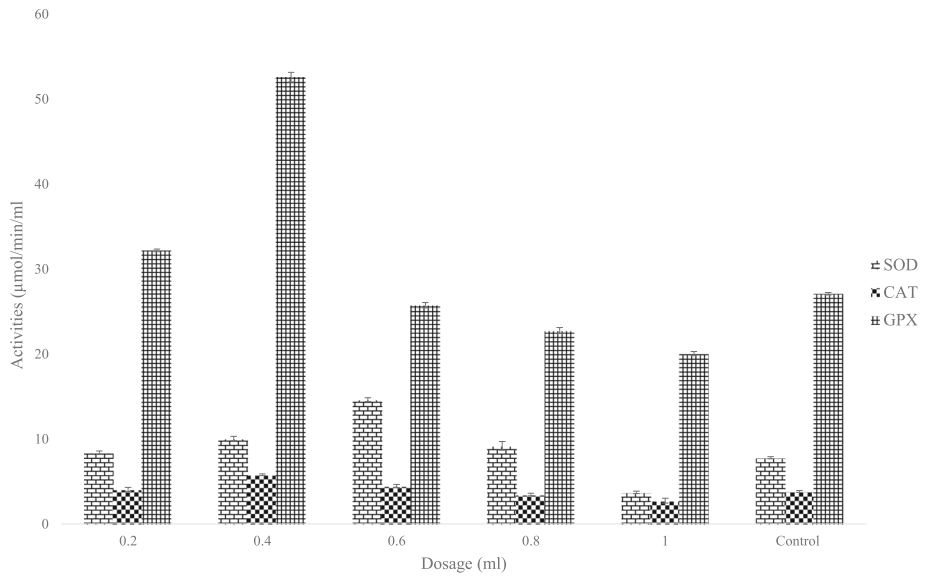

Fig. 1 Effect of oil extract of *A. wilkesiana* on the activities of antioxidant enzymes

and was significantly ($p < 0.05$) different. The activities of the CarEST increased with increase in the dosage of the oil extract but drastically reduced at higher dosages (0.8 and 1.0 ml). The highest activity (1.62 µmol/min/ml) of the enzyme was recorded at 0.4 ml of the oil extract while the lowest activity (0.62 µmol/min/ml) was recorded at 1.0 ml dosage and was significantly ($p < 0.05$) different from other dosages.

Effects of *A. wilkesiana* oil extract on the activities of GST

The effects of *A. wilkesiana* oil extract at different dosages on the activities of GST are presented in Fig. 3. The activity of the enzyme is dependent on the dosages of the oil extract. Significant statistical difference existed between the treatments at $F_{5,\ 24} = 2800.047$, $p < 0.0001$. Tukey multiple comparison showed that significant differences existed between the treatments except between 0.8 and 1.0 ml of the oil extract except at $p = 0.088$. The highest GST activity of 83.55 µmol/min/ml was recorded at 0.4 ml of *A. wilkesiana* oil extract while the lowest activity of the enzyme (38.15 µmol/min/ml) was recorded at 1.0 ml of the oil extract.

Active compound present in the leaves oil extract of *A. wilkesiana*

The active compounds present in the leaves oil extract of *A. wilkesiana* is presented in Table 1. One hundred fourteen active compounds were present in the oil

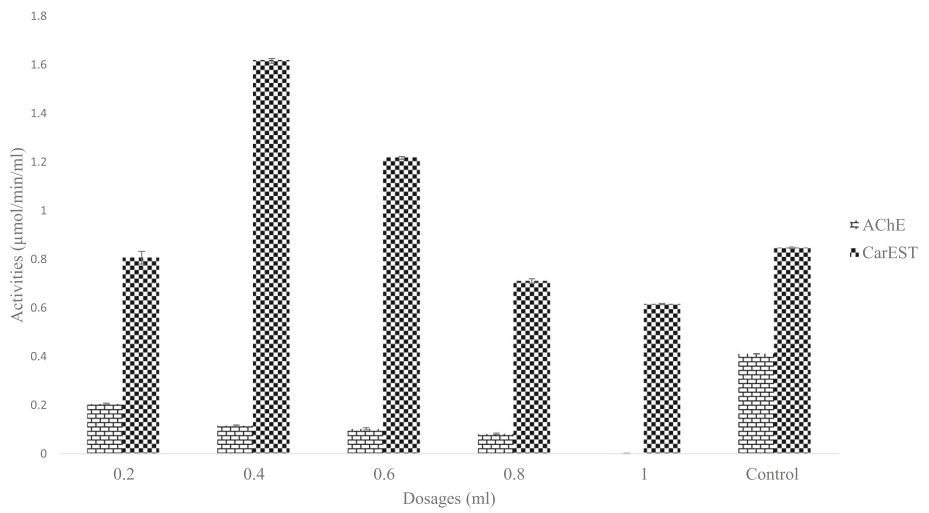

Fig. 2 Effects of oil extract of *A. wilkesiana* on activity of AChE and CarEST in adult *C. maculatus*

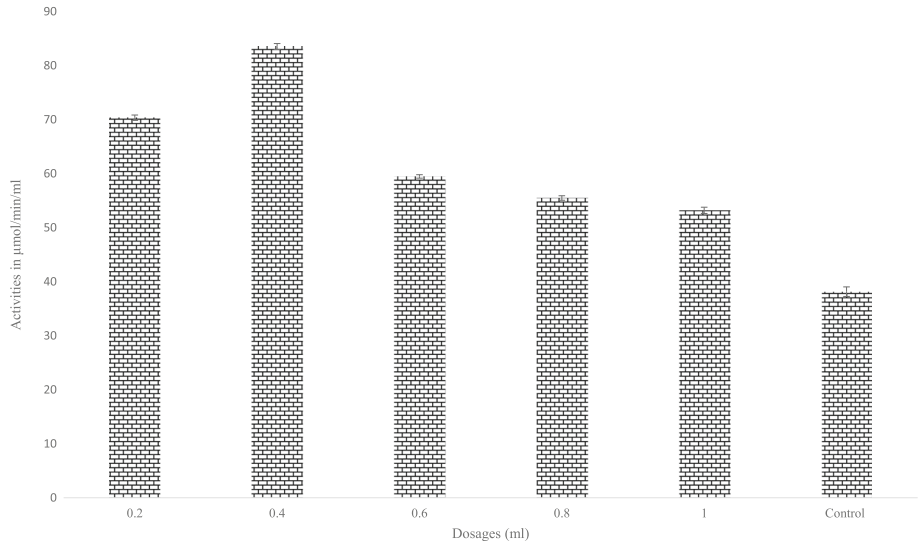

Fig. 3 Effect of oil extract of *A. wilkesiana* on GST in adult *C. maculatus*

extract of the plant. Acetaldehyde, Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-[1R-(1.alpha.,2.beta.,5.alpha.)]-, 3-Buten-2-one, 4-(6,6-dimethyl-1-cyclohexen-1-yl)-, and cyclopropyl carbinol are the five major compounds with 46.07, 3.23, 2.93, 2.85, and 2.72% respectively. The molecular structure and mass of the first five compounds with higher percentage in the oil extract were presented in Fig. 4a–e respectively.

Discussion

Inhibition of enzymes activities is a well-known instrument to block numbers of important biochemical and physiological processes leading to discovery of new metabolic pathways and in-depth knowledge of many kinetic mechanisms of enzyme-controlled reactions (Guerrero & Rosell, 2005). Thus, the development of potent enzyme inhibitors is now an important field of research in biopesticides and pharmaceutical researchers. In spite of many publications on botanical oil extracts and powders by stored product entomologists and other related scientists, only few numbers of the publications provided adequate information that could help in the formulation, commercialization, and acceptability of botanical base insecticides (Isman & Grieneisen, 2014). *A. wilkesiana* leaves oil extract being proven insecticidal by Oguntuase (2018) has not been evaluated for its inhibitory effects on different enzymes in adult *C. maculatus.* Thus, this work evaluated the inhibitory effect of oil extract of the plant on different enzymes in *C. maculatus* and as well evaluated the active compounds present in the oil extract.

The result of this work showed that the activity of the antioxidant, neurotransmitter, and detoxifying enzymes in adult *C. maculatus* varied with the dosages of the oil extract of *A. wilkesiana*. SOD is the first line of defense against toxic substance in insects, thus it prevents accumulation of oxygen free radicals (Kolawole, Olajuyigbe, Ajele, & Adedire, 2014). The dismutation ability of the enzyme enables it to remove superoxide radicals (O_2^-) to oxygen and hydrogen peroxide (H_2O_2) (Henry, Chao-Lin, Wen-Hui, Chia-Hsien, & Hong-Zin, 2006; Olawale, Ikechukwu, Grace, & Chidiebere, 2008). The variations in the activity of SOD at different dosages of *A. wilkesiana* revealed that the oil extract had induced some level of toxicity to the enzyme. Reactive oxygen species (ROS) are the contributors of oxidative stress that cause different diseases and disorders in insects (Buyukguzel, 2006). John, Kale, Rathore, and Bhatnagar (2001) reported that increase in SOD activity is an indicator for increase in ROS accumulation in insects. The overproduction of ROS leads to inability of the cell endogenous systems to neutralize them causing damage to proteins, lipids, mitochondria, and DNA of insects (Ali, 2012; Kolawole et al., 2014; Sankar, Telang, & Manimaran, 2012). Therefore, the decrease in the activity of SOD at higher dosage of oil extract of *A. wilkesiana* leaves implied that more ROS had accumulated in the cell of the insect due to inability of the enzyme to scavenge them. The decrease in the activity of SOD at higher dosages of the oil extract showed that 0_2^- and H_2O_2 had accumulated in the cell of the insect causing some levels of oxidative damages to the oil extract-stressed *C. maculatus* (Abdul Jaleel, Lakshmanan, Gomathinayagam, & Panneerselvam, 2008) . Wang, Wen, Chang, and Duan (2002) opined that scavenging ability of SOD is temporary and limited. Thus, this supported the reason why the activity of the enzyme increased with increase in the dosage of the oil extract and drastically decreased with further increase in dosage

Table 1 Active compounds present in the leaves oil extract of *A. wilkesiana* using GC-MS analysis

SN	Retention time (min)	Compound name/hit name	Quality ion (relative intensity, %)	Percentage total of all compound (% total)
1	7.135	Acetaldehyde	83	46.0737
2	25.766	Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]-	50	3.2297
3	24.318	3-Buten-2-one, 4-(6,6-dimethyl-1-cyclohexen-1-yl)-	27	2.9320
4	16.168	Cyclopropyl carbinol	53	2.8494
5	23.944	1,2-Hydrazinedicarboxaldehyde	47	2.7183
6	24.217	1-Undecanol	22	2.6824
7	24.686	4-Hydroxy-4-(4,6-dimethylcyclohex-3-enyl)butan-2-one	16	1.6914
8	5.580	Erythritol	80	1.6601
9	44.331	Testosterone cypionate	55	1.6126
10	21.041	p-Dioxane, methylene-	27	1.2305
11	23.024	4-Camphenylbutan-2-one	25	1.2291
12	25.131	Bicyclo[3.3.0]octan-2-one, 7-ethylidene-	50	1.2094
13	27.796	Hexadecanoic acid, ethyl ester	98	1.1615
14	18.970	3,4-Furandiol, tetrahydro-, trans-	59	1.1195
15	26.104	1-Methyl-4-[nitromethyl]-4-piperidinol	38	1.0566
16	17.973	Methanimidamide, *N*′-(2-cyanophenyl)-*N*,*N*-dimethyl-	53	0.8264
17	25.665	Propylamine, 3-(furan-2-yl)-1-methyl-	47	0.7989
18	23.350	Phenylephrine	47	0.7775
19	22.941	1,6-Octadiene, 3-ethoxy-3,7-dimethyl-	35	0.7611
20	24.478	Cyclohexanol, 2-methyl-3-(1-methylethenyl)-, acetate, (1.alpha.,2.alpha.,3.alpha.)-	52	0.7111
21	42.978	Acetamide, 2-(adamantan-1-yl)-*N*-(1-adamantan-1-ylethyl)-	30	0.7016
22	22.822	Benzyl alcohol, p-hydroxy-.alpha.-[(methylamino)methyl]-	50	0.6889
23	29.808	(E)-9-Octadecenoic acid ethyl ester	91	0.6631
24	27.487	n-Hexadecanoic acid	97	0.6578
25	35.025	3-Methyl-3,5-(cyanoethyl)tetrahydro-4-thiopyranone	40	0.6284
26	43.453	3,6-Bis-dimethylaminomethyl-2,7-dihydroxy-fluoren-9-one	1	0.6237
27	20.282	1,2-Ethanediol, 1-(2-phenyl-1,3,2-dioxaborolan-4-yl)-, [S-(R*,R*)]-	72	0.6152
28	13.990	Glutaraldehyde	45	0.6055
29	25.997	Spiro[4.5]decan-6-one	76	0.5935
30	41.886	MDMA methylene homolog	43	0.5678
31	25.368	3-Buten-2-ol, 2-methyl-4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]hept-2-yl)-	43	0.5674
32	9.242	Cycloserine	39	0.5460
33	24.555	4-Piperidinamine, *N*,1-dimethyl-	41	0.5387
34	24.893	Silane, dimethyldi-2-propenyl-	43	0.5337
35	51.816	2-Ethylacridine	38	0.5263
36	18.133	3-Bromo-1,2-propanediol	43	0.5166
37	24.959	Guanidineacetic acid	32	0.5133
38	23.659	Adenosine, 4′-de(hydroxymethyl)-4′-[*N*-ethylaminoformyl]-	43	0.4704
39	23.279	Bicyclo[2.2.1]heptan-2-one, 4,7,7-trimethyl-, semicarbazone	89	0.4526
40	21.682	2-Cyclopenten-1-one, 4-hydroxy-3-methyl-2-(2-propenyl)-	40	0.4506
41	28.983	Methanesulfonamide, *N*,*N*-dimethyl-	43	0.4307

Table 1 Active compounds present in the leaves oil extract of *A. wilkesiana* using GC-MS analysis *(Continued)*

SN	Retention time (min)	Compound name/hit name	Quality ion (relative intensity, %)	Percentage total of all compound (% total)
42	21.451	Acetonitrile, 2-(2H-tetrazol-2-yl)-	55	0.4223
43	22.395	Folic Acid	46	0.3989
44	22.282	Glucopyranuronamide, 1-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1, 4-dideoxy-4-(D-2-(2-(methylamino)acetamido)hydracrylamido)-, .beta.-D-	47	0.3885
45	25.861	trans-5-Methyl-2-isopropyl-2-hexen-1-al	35	0.3883
46	22.543	Diglycolamine	47	0.3645
47	14.121	2-Heptanamine, 5-methyl-	42	0.3536
48	31.855	9-Octadecenamide, (Z)-	98	0.3378
49	23.516	Adrenalone	50	0.3373
50	12.043	1-Pentanol, 4-amino-	47	0.3343
51	5.479	2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/–.)-	22	0.3273
52	30.110	Octadecanoic acid, 17-methyl-, methyl ester	98	0.3222
53	28.259	1H-Pyrazole, 4,5-dihydro-3-phenyl-	64	0.3211
54	26.810	Guanidine, N,N,N′,N′-tetramethyl-	35	0.3158
55	33.695	1-[.alpha.-(1-Adamantyl)benzylidene]thiosemicarbazide	43	0.3039
56	35.939	1-Methylene-2b-hydroxymethyl-3,3-dimethyl-4b-(3-methylbut-2-enyl)-cyclohexane	47	0.2937
57	12.667	2-Formylhistamine	39	0.2854
58	27.707	Arginine	38	0.2422
59	17.712	.+/–.-Tetrahydro-3-furanmethanol	28	0.2412
60	43.631	Ethyl 2-((diethoxyphosphoryl)oxy)-3,3,3-trifluoropropanoate	9	0.2323
61	9.426	Pent-3-enylamine	12	0.2318
62	26.520	3,3′-Bis(1,2,4-oxadiazolyl)-5,5′-diamine	50	0.2266
63	27.161	Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	38	0.2126
64	29.737	Linoleic acid ethyl ester	99	0.2114
65	11.569	Methylpent-4-enylamine	37	0.2079
66	43.744	1H-Indole-2-carboxylic acid, 6-(4-ethoxyphenyl)-3-methyl-4-oxo-4, 5,6,7-tetrahydro-, isopropyl ester	27	0.2068
67	17.824	*N*-Methylallylamine	47	0.2064
68	15.723	Tetrahydro-4H-pyran-4-ol	39	0.1836
69	5.960	1,2-Propanediol, 3-chloro-	47	0.1744
70	13.563	Ethyl oxamate	33	0.1702
71	34.146	4-Fluorohistamine	43	0.1625
72	13.177	*N*-Methyl-*N*-[2-cyanoethyl]-2-mercapto propylamine	47	0.1552
73	20.383	1,2,3,4-Butanetetrol, [S-(R*,R*)]-	5	0.1533
74	22.608	Carvone oxide, cis-	27	0.1501
75	42.551	Cefaclor	4	0.1489
76	18.311	*N*-Methoxy-1-ribofuranosyl-4-imidazolecarboxylic amide	38	0.1469
77	6.043	Ethyl hydrogen oxalate	38	0.1467
78	5.888	Xylitol	42	0.1399
79	3.437	3-Butyn-1-ol	9	0.1364
80	8.114	Bicyclo[3.1.0]hexan-3-one	47	0.1361
81	5.010	Topotecan	33	0.1228
82	10.934	1-Dodecanamine	28	0.1198

Table 1 Active compounds present in the leaves oil extract of *A. wilkesiana* using GC-MS analysis *(Continued)*

SN	Retention time (min)	Compound name/hit name	Quality ion (relative intensity, %)	Percentage total of all compound (% total)
83	4.470	Acetamide, 2-amino-*N*-(1-methylethyl)-	9	0.1101
84	42.112	*N*-(3-Methylbutyl)acetamide	9	0.1087
85	41.132	Cyclobutanol	9	0.1030
86	29.950	Butanamide	53	0.1015
87	10.530	Ethylene oxide	5	0.0856
88	4.880	Silane, methyl-	25	0.0818
89	4.595	2-Butynone, 1-acetyl-4-[1-piperidyl]-	35	0.0796
90	29.517	dl-Phenylephrine	50	0.0731
91	7.420	Glycyl-dl-alanine	9	0.0719
92	8.589	1-Propanol, 2-amino-, (.+/−.)-	28	0.0718
93	4.725	Dimethyl-(6-methyl-2-thioxo-[1,3,2]oxathiaphosphinan-2-yl)-amine	42	0.0704
94	33.310	azetidine, 1-methyl-3,3-dipentyl-	33	0.0670
95	5.758	L-Arabinitol	59	0.0663
96	31.612	2,4-Dimethylamphetamine	59	0.0631
97	27.321	2-Amino-1-(o-methoxyphenyl)propane	37	0.0606
98	31.001	2-Oxo-3-methyl-cis-perhydro-1,3-benzoxazine	37	0.0562
99	32.342	5-Aminoisoxazole	9	0.0550
100	5.164	3-Ethoxy-1,2-propanediol	40	0.0525
101	29.446	Propanamide, 3-(3,4-dimethylphenylsulfonyl)-	32	0.0515
102	41.435	1-Octadecanamine, *N*-methyl-	9	0.0482
103	37.868	1-(5-Bicyclo[2.2.1]heptyl)ethylamine	17	0.0374
104	9.729	dl-Alanine	9	0.0334
105	3.229	2-(Oxan-3-yl)ethanamine	38	0.0329
106	41.031	4,6-dimethyl-2-(propan-2-yl)-1,3,5-dithiazinane	5	0.0329
107	35.387	Benzaldehyde, 2-nitro-, diaminomethylidenhydrazone	25	0.0319
108	4.126	2-Hexynoic acid	12	0.0309
109	30.787	Phenethylamine, p,.alpha.-dimethyl-	32	0.0297
110	4.173	Sulfurous acid, diethyl ester	9	0.0293
111	39.073	4,6-dimethyl-2-propyl-1,3,5-dithiazinane	9	0.0290
112	41.530	Propan-1-one, 2-amino-1-piperidin-1-yl-	9	0.0251
113	38.639	S-[Tri-t-butoxysilyl]-2-mercaptoethylamine	9	0.0250

of the oil extract. Similar result was obtained by Kolawole et al. (2014) research in which SOD in *C. maculatus* exposed to different dosages of some botanical insecticides was first increased and decreased with further increase in concentration of the biopesticides. Aslanturk, Kalender, Uzunhisarcikli, and Kalender (2011), Gupta et al. (2010), and Wu et al. (2011) also reported decrease in SOD activity in mid-gut tissues of *Lymantria dispar* exposed to methidathion, an organophosphate insecticide.

The generation of SOD activity leads to conversion of superoxide radicals to less H_2O_2 which in turn induces CAT activity that perfectly reduces accumulation of H_2O_2 to water (Aslanturk et al., 2011; Kaur, Sohal, Arora, Kaur, & Kaur, 2014; Łukasik, Goławska, & Wójcicka, 2009). Aslanturk et al. (2011) opined that increase in activity of SOD would lead to increased H_2O_2 thus increase the activity of CAT. The increase in CAT activity in *C. maculatus* stressed with 0.2 and 0.4 ml of *A. wilkesiana* oil extract could be due to increased SOD activities at these dosages. Thus, this must have caused conversion of hydrogen peroxide to water and prevention of oxidative damage. Hence, it lowers the risk of hydroxyl radicals' formation through Fenton reaction (Fridovich, 1999; Kolawole et al., 2014). This finding supported the reports of Orr and Sohal (1994) that

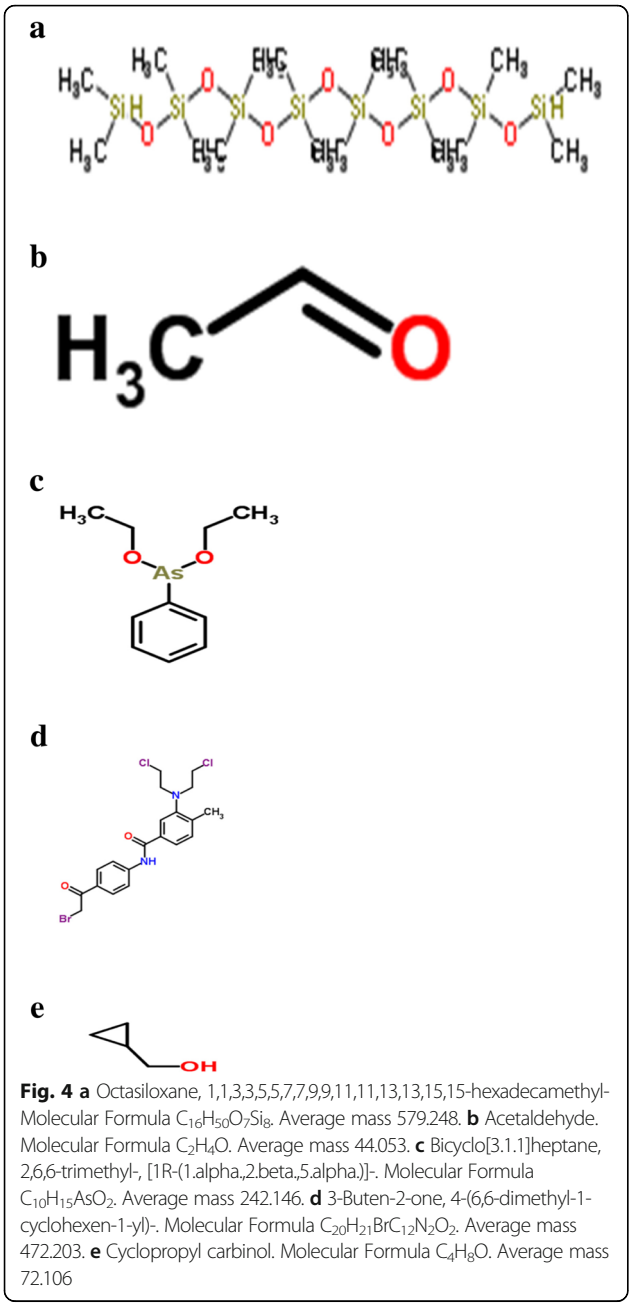

Fig. 4 **a** Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- Molecular Formula $C_{16}H_{50}O_7Si_8$. Average mass 579.248. **b** Acetaldehyde. Molecular Formula C_2H_4O. Average mass 44.053. **c** Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-, [1R-(1.alpha.,2.beta.,5.alpha.)]-. Molecular Formula $C_{10}H_{15}AsO_2$. Average mass 242.146. **d** 3-Buten-2-one, 4-(6,6-dimethyl-1-cyclohexen-1-yl)-. Molecular Formula $C_{20}H_{21}BrC_{12}N_2O_2$. Average mass 472.203. **e** Cyclopropyl carbinol. Molecular Formula C_4H_8O. Average mass 72.106

suggested that CAT protects cells against oxidative stress and extends lifespan of insects. However, it was noted in this work that further increase in the dosage of *A. wilkesiana* leaves oil extract significantly reduced the activity of CAT. This could be due to inability of the enzyme to catalyze the accumulation of H_2O_2 that resulted from SOD activity. This result acquiesced with the findings of Kaur et al. (2014) and Łukasik (2007). The result of this work revealed that activity of GPx increased at lower dosages of the oil extract and significantly reduced at higher dosages. The increase in the GPx activities could be associated to the increased CAT activity because it is when CAT is saturated that GPx, the second line of defense regulated by Selenium availability, is activated (Ali, 2012; Duntas, 2012). GPx catalyzes the glutathione-dependent reduction of lipid peroxides and hydrogen peroxide for detoxification at the membrane level into less reactive species by using GSH as substrate (Ali, 2012; Sankar et al., 2012). Thus, it prevents the progressive formation of free radicals and protects the cell against oxidative stress and lipid peroxidation (Sankar et al., 2012). Hence, the decrease in the activity of GPx indicates that there could be accumulation of lipid peroxides and hydrogen peroxides. This result agreed with the findings of Aslanturk et al. (2011) in which methidathion caused an increase in oxidative stress of *L. dispar* larvae.

Acetylcholine esterase (AChE) is an important neurotransmitter enzyme that degrades the molecule acetylcholine (ACh) to produce choline and acetate group. It is mainly found in the neuromuscular junctions and cholinergic synapses of the central nervous system where it terminates synaptic transmission (Kim et al., 2010; Purves et al., 2008). Thus, the choline produced by activity of AChE is recycled by being transported back to the nerve terminals for the synthesis of new Ach (Herholz, Weisenbach, Hilker, & Heiss, 2006; Kim et al., 2010). In this work, the activity of AChE significantly reduced when compared to the control and almost got inhibited by 1.0 ml of *A. wilkesiana* oil extract. Thus, this implies that the activity of the enzyme had been significantly inhibited by the oil extract and may have caused increase in the concentration of ACh that could lead to buildup of the neurotransmitter at nerve synapse and neuromuscular junctions (Fukuto, 1990; Rajashekar, Raghavendra, & Bakthavatsalam, 2014). The inhibitory effect of the oil extract on the activity of this enzyme can also be attributed to the active compounds it contained because active compounds of botanicals have been reported to have broad impact across the nervous system which is attenuated by modified acetyl choline and acetate function as suggested by Rajashekar et al. (2014). The result obtained from this study was in accordance with the results of Begum, Sharma, and Pandey (2010), Breuer, Hoste, De Loof, and Naqvi (2003), Ghoneim, Hamadah, and El-Hela (2012), Khan et al. (2003), Kim et al. (2008), Olmedo et al. (2015), and Prakash (2015) in which botanical-based insecticides were found to cause inhibition of AChE activities in insects.

In insects, CarEST is involved in many important physiological processes and plays an imperative role in the detoxification of insecticides to less toxic metabolites (Jones & Brancoft, 1986; Lassiter, Apperson, & Roe, 1995; Mukanganyama, Figueroa, Hasler, & Niemeyer, 2003; Shanmugavelu, Baytan, Chesnut, & Bonning, 2000; Tarigan, Dadang, & Harahap, 2016; Wheelock, Shan, & Ottea, 2005). Thus, it is used as

one of the most reliable biomarkers to assess the impact of insecticides on range of insects (Fourcy, Jumel, Heydorff, & Lagadic, 2002; Koodalingam, Mullainadhan, & Arumugam, 2011; Smirle, Zurowski, Lowery, & Foottit, 2010; Wheelock et al., 2005). In this research, the activity of this enzyme was found to increase at lower dosages (0.2 and 0.4 ml of 2% concentration of the oil extract) while it drastically reduced with higher dosages of the oil extract, reflecting that the enzyme activity was dose dependent. The dose-dependent activity of CarEST in the larvae of *Choristoneura rosaceana* exposed to neem oil (Smirle, Lowery, & Zurowski, 1996) while Ortego, López-Olguín, Ruiz, and Castañera (1999) reported the reduction in CarEST activity in the larvae of *Leptinotarsa decemlineata* exposed to limnoid. This result also agreed with the findings of Caballero, Lopez-Olguin, Ruiz, Ortego, and Castanera (2008), Koodalingam et al. (2011), Malahat, Jalal, and Alireza (2015), and Nathan et al. (2008). In contrast, Koodalingam et al. (2011) reported that the activity of CarEST in pupa of *Aedes aegypti* was not affected by the extract of *Sapindus emarginatus*. This variation could be due to the stages of insect used as reported by Koodalingam et al. (2011).

GST is a multifunctional enzyme responsible for the detoxification of many toxic substances in insects. It also catalyzes the conjugation of reduced glutathione and plays a vital role in detoxification of insecticides, thus rendering the less toxic (Rufingier, Pasteur, Lagnel, Martin, & Navajas, 1999; Tarigan et al., 2016). In this study, GST activity in adult *C. maculatus* was greatly reduced at higher dosages of the oil extract, thus the activity of the enzyme was dosage dependent. This result was supported by report of Van, Haubruge, Lognay, and Francis (2001) that stated that botanical extracts induce GST activity. War, Paulraj, Ignacimuthu, and Sharma (2013) reported that botanical extract have the ability to reduce total protein in insects, thus toxicity of plant extract is characterized by its ability to reduce the amount of total protein insects (Nath, Kalaivan, & Chung, 1997; Tarigan et al., 2016). Terrie (1984) reported that GST is a type of enzyme made of 85% protein and plays a critical role in the detoxification of toxic substances that enter and leave the body of insects. The decrease in the activity of GST at higher dosages of the plant oil extract could be that the protein content of the insect had been reduced. Similar result was reported by Ebadollahi, Roya, Jalal, Parisa, and Rahim (2013) in which botanical extract was found to cause low protein content in the body of *Tribolium castaneum* and in turn cause inhibition of GST. Furthermore, it was noted that most of the compounds found in the oil extract of *A. wilkesiana* oil extract were alkaloids. Thus, the effect of the oil extract of the plant could be due to these active compounds according to Yang, Zhao, Zhu, Fang, and Xia (2006) reports.

Conclusion

In conclusion, the oil extract of *A. wilkesiana* leaves appeared potent against the activities of all the antioxidant enzymes (SOD, CAT, and GPx), the esterases (AChE and CarEST), and the GST tested in this work. Thus, it means that oil extract of the plant has a multiple mode of action and it has affected all the enzymes evaluated. However, its inhibitory effects were more pronounced on the AChE activity as it almost inhibits the activity of enzyme. Based on these findings, oil extract of *A. wilkesiana* could be suggested as potential biopesticide for the control of *C. maculatus* infestation. More so, many of the compounds found in it are insecticidal in nature.

Abbreviations

AChE: Acetylcholine esterase; CarEST: Carboxylesterase; CAT: Catalase; GPx: Glutathione peroxidase; GST: Glutathione transferase; SOD: Superoxide dismutase

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Authors' contributions

The first and second authors designed the research while third and fourth authors joined them in carrying out the bench work. Second author carried out statistical analysis of data obtained and wrote the manuscript while the fifth author proof read the manuscript and make necessary adjustments. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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