Activity of the Antioxidant Defense System in a Typical Bioinsecticide- and Synthetic Insecticide-treated Cowpea Storage Beetle *Callosobrochus maculatus* F. (Coleoptera: Chrysomelidae)

Ayodele O. Kolawole[1], Folasade M. Olajuyigbe[1], Joshua O. Ajele[1] and Chris O. Adedire[2]

[1]Department of Biochemistry, The Federal University of Technology, Akure, Nigeria. [2]Department of Biology, The Federal University of Technology, Akure, Nigeria.

ABSTRACT: The non-enzymatic and enzymatic antioxidant defense systems play a major role in detoxification of pro-oxidant endobiotics and xenobiotics. The possible involvement of beetle non-enzymatic [α-tocopherol, glutathione (GSH), and ascorbic acid] and enzymatic [catalase (CAT), superoxide dismutase (SOD), peroxidase (POX), and polyphenol oxidase (PPO)] antioxidant defense system on the insecticidal activity of synthetic insecticides (cypermethrin, 2,2-dicholorovinyl dimethyl phosphate, and λ-cyhalothrin) and ethanolic plant extracts of *Tithonia diversifolia*, *Cyperus rotundus*, *Hyptis suaveolens* leaves, and *Jatropha Curcas* seeds was investigated. 2,2-Dicholorovinyl dimethyl phosphate (DDVP; 200 ppm, $LC_{50} = 13.24$ ppm) and *T. diversifolia* (20,000 ppm) resulted in 100% beetle mortality at 96-hour post-treatment. The post-treatments significantly increased the beetle α-tocopherol and GSH contents. Activities of CAT, SOD, POX, and PPO were modulated by the synthetic insecticides and bioinsecticides to diminish the adverse effect of the chemical stresses. Quantitative and qualitative allelochemical compositions of bioinsecticides and chemical structure of synthetic insecticides possibly account and for modulation of their respective enzyme activities. Altogether, oxidative stress was enormous enough to cause maladaptation in insects. This study established that oxidative imbalance created could be the molecular basis of the efficacy of both insecticides and bio-insecticides. Two, there was development of functional but inadequate antioxidant defense mechanism in the beetle.

KEYWORDS: *Callosobruchus maculatus* Cowpea storage beetles, oxidative stress, antioxidant enzymes, insecticides

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is a leguminous crop and a source of revenue that is popularly grown in various parts of tropical and subtropical regions of Africa. Seeds of the crop serve as a source of dietary protein to naturally and nutritionally complement starchy cereals and tuber crops where the per capital daily intake of animal proteins is low, thus alleviating protein malnutrition in humans.[1,2] The green parts of the plant can be used as vegetables or as fodder for cattle. Cowpea is susceptible to many insect pests both on the field and during storage.[3] The major constraint facing effective and efficient storage of the grain legume is the insect pest depredation. Cowpea storage beetle (CSB), *Callosobruchus maculatus* (Fabr.) (Coleoptera: Chrysomelidae), is a principal storage pest of cowpea and poses a great challenge to the process of assuring food security in developing tropical countries like Nigeria.[4,5] Infestation by this insect begins from the field when mature adults lay eggs on drying cowpea pods or seeds. The eggs hatch into larvae, which use their mouthparts to penetrate the pod wall or the seed testa. This feeding process ultimately

reduces the weight of the seeds and enhances mold growth, poor germination viability, and hampered seed marketability.[6] The post-harvest loss could be 100% within two to three months after harvest. Tropical temperatures and humidity are reported to be responsible for rapid increase in the number of insect population.[7] Unfortunately, Cowpea genome lacks insect resistant gene loci to be induced to produce insect-resistant cultivar.[8]

Over the years, diverse methods have been used to protect the stored cowpea against the pulse beetle (CSB). Hermetic storage, synthetic insecticides and/or plant materials application, gamma irradiation, and freezing and heating the beans are some of the documented control methods.[9,10] Substantial progress has been made on the effective and efficient control of this pulse beetle, which focused on the use of synthetic insecticides and plant products.[5,11] The use of insecticides remains the most effective means of controlling beetle in large-scale storages. Indiscriminate and misguided use and undesirable side effects have limited the usage of synthetic insecticides. Currently, plant materials as bioinsecticides have also proven their efficacy as insecticides.[12,13] The responses of the pulse beetle (CSB) to insecticides and bioinsecticides have been informative on ovicidal and oviposition effects, adult emergence, and avoidance and repellence to insecticides and the bioinsecticides. All these have not provided useful baseline information for strategizing effective control and resistance management. The main threat to the success of this control approach is the possible development of resistance to synthetic insecticides and bioinsecticides after prolong usage without prior and absolute understanding of the molecular basis of their respective efficacy. The current and reported understandings of the molecular basis of efficacy of the insecticidal plants and some selected insecticides on CSB have been inadvertently scanty. In CSB, study of acetylcholine esterase (AChE),[14] cytochrome P450,[8] and glutathione (GSH) transferase[15] is motivated by the perception that they have a role in insecticide or bioinsecticide toxicity.

The efficacy of the insecticidal plants and insecticides on the CSB could be based on several mechanisms to exert their toxic effect despite adaptive and compensatory responses that could be induced as a result of exposure. Some reports have posited that some pesticides and bergapten (an allelochemical) cause oxidative stress, characterized by exposure to in vivo excessive reactive oxygen species (ROS).[8,16,17] ROS is involved in the pathogenesis of some diseases. It is doubtful, however, in cowpea storage bruchid, if oxidative stress may be a consequence of detoxification enzymes' inhibitions, a separate effect, or a concurrent event. Inconsistent and preliminary results exist regarding this line of thought. Considerable work has been done on antioxidant enzyme systems and detoxification systems in insects. There have been no linkages of insects' antioxidant enzyme systems and detoxification enzymes.

Given the present suggestion that synthetic insecticides and plant-derived insecticides may cause oxidative stress, the present study was undertaken to characterize their role in oxidative stresses and cellular antioxidant defense mechanism in their possible toxicity or otherwise. Ethanolic extracts of *Tithonia diversifolia*, *Cyperus rotundus*, *Hyptis suaveolens* leaves, and *Jatropha Curcas* seeds as well as some selected synthetic insecticides on the bruchid non-enzymatic antioxidants contents and antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and polyphenol oxidase (PPO)] was studied.

Materials and Methods

Materials. Catechol, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), Pyrogallol, and *O*-phthalaldehyde were purchased from Sigma-Aldrich Chemie GmbH. Bradford reagent (#500-006) and BSA protein standard (#500-0206) were from Bio-Rad Laboratories. Cyperforce (Cypermethrin) was a product of Gharda Chemicals Ltd, and Master (λ-cyhalothrin) was purchased from Sinochem Ningbo Ltd. Sniper (2,2-dicholorovinyl dimethyl phosphate; DDVP) was manufactured by Hubei Sanonda Co. Ltd and supplied by Saro AgroSciences Limited. All other chemicals were of analytical grade. Absolute ethanol was used as a solvent in the preparation of the pesticides and biopesticide samples.

Preparation of plant-derived insecticides. Mature fresh leaves of *T. diversifolia*, *C. rotundus*, and *H. suaveolens*; and the seeds of *J. curcas* were collected from the Teaching and Research Farm of the Federal University of Technology, Akure, Nigeria in July 2012. The climatic condition of the location is tropical sub-Sahara. The plant materials were rinsed with autoclaved distilled water, air-dried to constant weight, and then ground into fine powder. Chemical extraction was carried out as described earlier.[18] Extracts were stored at −20°C prior to use. Here, extracted plant materials were referred to as bioinsecticides.

Insect culture. *C. maculatus* adults used for the establishment of the insect colony were obtained from a local market in Akure, Nigeria. The beetles were cultured on clean uninfested susceptible cowpea seeds in an ambient laboratory temperature of 25 ± 1°C, a relative humidity of 75 ± 5%, and a 12-hour photoperiod. New generation of CSB that was used for subsequent experiments was derived from the stock culture by infesting clean uninfested beans with 20 copulating pairs of teneral adult CSB.

Mortality response. In a typical mortality response study, disinfested cowpea seeds weighing 100 g put in sterile petri dishes (9 cm diameter) were serially treated with 2.0 mL of Cyperforce (cypermethrin), Master (λ-cyhalothrin), and Sniper (DDVP) at concentrations of 0, 50, 100, 150 and 200 ppm. In another set of dishes, the seeds were thoroughly mixed with an aliquot of 2 mL of 5,000, 10,000, 15,000, and 20,000 ppm ethanolic extracts of plant materials. The dishes were agitated manually for five minutes and then allowed to air-dry for four hours. Each experimental dish, prepared in triplicates, was infested with copulating adult beetle (20 males and 20 females). The mortality of the beetle because of the

insecticides and the bioinsecticides was monitored for four days. Here, the LC50 values were derived from the best-fit line obtained from regression equation (Y = % mortality; X = concentration) using Microsoft Excel (for Mac 2011).

Enzyme extraction and assays. Frozen 700 mg of whole insects were homogenized in ice cold buffer [50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA] and centrifuged at 10,000 g for 20 minutes at 4°C. Protein contents were quantified by Bradford's method, using bovine serum albumin as a standard.[19] Unless otherwise stated, Shimadzu UV 1800 Double Beam Spectrophotometer was used for all colorimetric assays, while Hitachi F-4500 Fluorescence Spectrophotometer was used for fluorimetric assays.

Determination ascorbic acid content. Ascorbic acid content was estimated by following exact procedure described by Omaye et al[20] where 1 g of insect was ground with 5 mL of 10% trichloroacetic acid (TCA) and the mixture was centrifuged at 5,000 g for 20 minutes at 4°C. An aliquot of 1.0 mL of 2,4-dinitrophenyl hydrazine-thio-urea-$CuSO_4$ (DTC) reagent was added to 0.5 mL of the supernatant, and the mixture was allowed to stand for three hours at 37°C. The reaction was terminated with 0.75 mL of ice-cold 65% H_2SO_4. After incubation at 30°C for 30 minutes, absorbance was read at 520 nm against blank. The result of the ascorbic acid content was expressed in milligrams per gram of fresh weight.

Determination reduced GSH content. The method of Ellman[21] was used for the assay. In brief, 200 mg of insects was homogenized with 2 mL of 5% (w/v) TCA in 1 mM EDTA and then centrifuged at 10,000 g for 20 minutes at 4°C. One milliliter of the reaction mixture, containing 150 µL extract, 800 µL of 0.1 M phosphate buffer (pH 8.0), and 50 µL of DTNB (0.01% in 0.1 M phosphate buffer, pH 8.0), was mixed thoroughly and then incubated at 25°C for 20 minutes. The absorbance of the reaction mixture was 412 nm. The GSH content was determined from a GSH standard curve, and the result was expressed in micrograms per gram of fresh weight.

Determination of α-tocopherol content. α-Tocopherol content was estimated as described elsewhere.[22] 2,2 Dipyridyl was used as chromophore, and absorbance was read at 520 nm. α-Tocopherol content was read from α-tocopherol standard curve and later expressed in micrograms per gram of fresh weight.

Determination of SOD activity. SOD activity was assayed according to the method of Fridovich,[23] which uses a tetrazolium salt to detect superoxide radicals generated by xanthine and xanthine oxidase system. The absorbance was monitored at 550 nm. SOD activity was expressed in units per milligram of protein. One unit is defined as the amount of change in the absorbance by 0.1 hours^{-1} mg^{-1} proteins.

Determination of CAT activity. CAT activity was determined as modified by Aebi.[24] The rate of H_2O_2 decomposition was spectrophotometrically monitored at 240 nm. Enzyme activities were calculated using 0.0394 mM^{-1} cm^{-1} as absorption coefficient at 240 nm. A unit of CAT activity is defined as the amount of enzyme required to decompose 1 mM of hydrogen peroxide in a minute. The CAT activity was expressed in units per milligram of protein.

Determination of POX activity. POX activity was assayed according to the protocol described by Kumar and Khan.[25] The reaction mixture comprised 2.0 mL of 0.1 M potassium phosphate buffer (pH 6.8), 1.0 mL of 0.01 M Pyrogallol, 1 mL of 0.005 M H_2O_2, and 0.5 mL of enzyme extract prepared from whole insect homogenate. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm. The POX activity was expressed in units per milligram of protein. One unit is defined as a change in the absorbance by 0.1 minute^{-1} mg^{-1} protein.

PPO activity. The method of Kumar and Khan[25] was used to estimate PPO activity in a reaction mixture containing 2 mL of 0.1 M potassium phosphate buffer (pH 6.0), 1 mL of 0.1 M catechol, and 0.5 mL of enzyme extract. The purpurogallin formed was read at 495 nm. PPO activity was expressed as units per milligram of protein (U = change in absorbance by 0.1 minute^{-1} mg^{-1} protein).

Statistical analysis. All data obtained were subjected to one-way analysis of variance (ANOVA) using the SPSS version 17.0 statistical software (SPSS). The means were separated using Duncan's New Multiple Range Test (DNMRT) at 5% probability level where significant differences existed between them.

Results

Beetle mortality. The result of percentage mortality of bioinsecticides and the synthetic insecticides used in this study on the adult *C. maculatus* after four days post-treatment is presented in Table 1. ANOVA revealed the significant difference between the mortality of CSB in the control and the treated samples. Among the synthetic insecticides, DDVP is the most potent with 100% mortality at 96 hours post-treatment. In all, 100% mortality of *C. maculatus* was observed in the group treated with *T. diversifolia* leaf extract at a concentration of 20,000 ppm, while *H. suaveolens* leaves have the least effect.

Ascorbic acid estimation. Information on the ascorbic acid content of the bruchid is presented in Table 2. Ascorbic acid content of all *C. maculatus*-treated groups decreased significantly ($P \leq 0.05$) compared to the control group. *C. rotundus*- and *H. suaveolens*-treated groups showed an increased ascorbic acid content after an initial decrease; however, the concentration of ascorbic acid decreased further in *C. maculatus* treated with *T. diversifolia* and *J. curcas* as the concentration of the extracts increased. The ascorbic acid content of the insecticide-treated groups decreased as the sample concentrations increased for DDVP and cypermethrin but increased for λ-cyhalothrin.

Reduced GSH content. The reduced GSH content of the cowpea seed beetle is shown in Table 3. There was a significant increase ($P \leq 0.05$) in the GSH content of *C. maculatus* group treated with *C. rotundus* and *H. suaveolens* compared to

Table 1. Percentage mortality of adult *Callosobrochus maculatus* in cowpea seeds treated with some bio-insecticides and insecticides.

(A) BIO-INSECTICIDES				
CONCENTRATION (ppm)	**MORTALITY OF *C. Maculatus* (%)**			
	C. Rotundus	***T. Diversifolia***	***H. Suavolens***	***J. Curcas***
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
5,000	75.00 ± 4.08[a]	90.00 ± 1.83[a]	16.25 ± 2.63[a]	86.25 ± 1.71[a]
10,000	85.00 ± 3.46[b]	93.75 ± 0.96[a]	17.50 ± 3.30[b]	87.50 ± 3.70[a]
15,000	88.75 ± 3.30[b]	96.25 ± 0.96[a]	21.25 ± 0.50[c]	91.25 ± 1.71[a]
20,000	91.25 ± 0.50[c]	100.00 ± 0.00[b]	22.50 ± 1.83[d]	95.00 ± 1.41[b]
(B) SYNTHETIC INSECTICIDES				
CONCENTRATION (ppm)	**MORTALITY OF *C. Maculatus* (%)**			
	DDVP	**CYPERMETHRIN**	**λ-CYHALOTHRIN**	
10	45.25 ± 8.55[a]	33.75 ± 8.55[a]	38.75 ± 4.80[a]	
50	61.75 ± 10.30[a]	46.25 ± 10.30[a]	51.25 ± 11.15[a,b]	
100	79.50 ± 2.90[b]	66.25 ± 6.30[b]	63.25 ± 8.55[b]	
150	92.00 ± 2.50[c]	83.75 ± 7.50[c]	81.25 ± 11.15[b,c]	
200	100.00 ± 0.00[d]	95.00 ± 0.00[d]	92.50 ± 7.50[c]	
LC$_{50}$ ppm	13.24	56.93	48.22	

Notes: The mean mortality values results were expressed as mean ± SEM (three replicates) determined by via one way ANOVA followed by Duncan's New Multiple Range Test (DNMRT). The mean values, in the same column with different superscripts are significantly different $P \leq 0.05$ while means with the same superscripts indicate non-significant changes.
Abbreviation: ppm, part per million.

the control. However, there was an initial decrease in the GSH content at 5,000 ppm in the group treated with *T. diversifolia* before the increase as the exposure concentration increases. The exogenous application of DDVP increased GSH content of the beetle.

α-Tocopherol estimation. The result shown in Table 4 represents the effect of varied concentrations of bioinsecticides

and synthetic insecticides on the α-tocopherol content of *C. maculatus*. *C. maculatus* group treated with 20,000 ppm *C. rotundus* had a 3% increase in α-tocopherol content compared with the control. This was after an initial decrease in the α-tocopherol content. *C. maculatus* group treated with *H. suaveolens* and *T. diversifolia* extracts had a significant increase ($P \leq 0.05$) in α-tocopherol content at 5,000,

Table 2. Effect of insecticides and bio-insecticides on Ascorbic acid content of adult *Callosobrochus maculatus*.

BIO-INSECTICIDES CONC. (ppm)	ASCORBIC ACID CONTENT (mg g^{-1} FRESH WEIGHT)			
	C. Rotundus	***T. Diversifolia***	***H. Suavolens***	***J. Curcas***
0	29.71 ± 0.10[a]	29.71 ± 0.10[a]	29.71 ± 0.10[a]	29.71 ± 0.10[a]
5,000	5.013 ± 0.10[e]	22.20 ± 0.60[b]	4.54 ± 0.10[e]	16.32 ± 0.50[b]
10,000	5.82 ± 0.50[d]	19.62 ± 0.60[c]	10.66 ± 0.40[d]	13.69 ± 0.50[c]
15,000	11.23 ± 0.10[c]	18.19 ± 0.90[d]	12.55 ± 0.50[c]	11.56 ± 0.40[d]
20,000	12.65 ± 0.60[b]	17.24 ± 0.10[d]	15.08 ± 0.10[b]	9.87 ± 0.20[e]
INSECTICIDES CONC. (ppm)	ASCORBIC ACID CONTENT (mg g^{-1} FRESH WEIGHT)			
	DDVP	**CYPERMETHRIN**	**λ-CYHALOTHRIN**	
0	29.71 ± 0.10[b]	29.71 ± 0.10[b]	29.71 ± 0.10[a]	
10	31.60 ± 0.40[a]	31.86 ± 0.50[a]	21.10 ± 1.00[c]	
50	23.14 ± 0.60[c]	22.27 ± 0.30[c]	21.37 ± 0.40[c]	
100	20.85 ± 0.40[d]	21.31 ± 0.20[d]	22.78 ± 0.20[b]	
150	18.17 ± 0.20[e]	19.24 ± 0.50[e]	22.54 ± 0.10[b]	
200	16.14 ± 0.50[f]	18.34 ± 0.20[f]	23.22 ± 0.60[b]	

Notes: The values were expressed as mean ± SEM (three replicates) determined by via one way ANOVA followed by Duncan's New Multiple Range Test (DNMRT). The mean values, in the same column, with different superscripts are significantly different $P \leq 0.05$ while means with the same superscripts indicate non-significant changes.
Abbreviation: ppm, part per million.

Table 3. Effect of insecticides and bio-insecticides on reduced Glutathione content of adult *Callosobrochus maculatus*.

BIO-INSECTICIDES CONC. (ppm)	GSH CONTENT (µg g⁻¹ FRESH WEIGHT)			
	C. Rotundus	*T. Diversifolia*	*H. Suavolens*	*J. Curcas*
0	47.53 ± 0.50^e	47.53 ± 0.50^d	47.53 ± 0.50^e	47.53 ± 0.50^d
5,000	83.11 ± 1.00^b	13.27 ± 0.80^e	58.90 ± 0.50^d	120.67 ± 0.90^a
10,000	120.90 ± 0.20^a	64.73 ± 0.60^b	61.44 ± 0.60^c	107.83 ± 1.70^b
15,000	71.70 ± 2.10^c	56.30 ± 0.70^c	76.91 ± 2.30^b	25.67 ± 1.40^e
20,000	65.47 ± 1.20^d	83.30 ± 0.90^a	80.95 ± 0.60^a	56.34 ± 0.70^c
INSECTICIDES CONC. (ppm)	GSH CONTENT (µg g⁻¹ FRESH WEIGHT)			
	DDVP	CYPERMETHRIN	λ-CYHALOTHRIN	
0	47.53 ± 0.5^d	47.53 ± 0.5^e	47.53 ± 0.5^f	
10	49.8 ± 0.6^c	58.8 ± 0.6^a	58.33 ± 0.5^a	
50	52.53 ± 0.4^b	58.40 ± 0.2^{a,b}	56.03 ± 0.8^b	
100	54.53 ± 0.7^a	57.70 ± 0.5^{b,c}	52.33 ± 0.4^c	
150	54.53 ± 0.7^a	57.23 ± 0.2^c	49.90 ± 0.2^d	
200	54.53 ± 0.5^a	56.33 ± 0.5^d	48.63 ± 0.5^e	

Notes: The values were expressed as mean ± SEM (three replicates) determined by via one way ANOVA followed by Duncan's Multiple Range Test (DNMRT). The mean values, in the same column, with different superscripts are significantly different $P \leq 0.05$ while means with the same superscripts indicate non-significant changes.
Abbreviation: ppm, part per million.

15,000, and 20,000 ppm when compared with the control value. α-Tocopherol values of the insects group treated with DDVP and cypermethrin decreased with the increase in insecticide concentrations compared with the values of the control group.

SOD activity. Effect of varied concentrations of synthetic insecticides and bioinsecticides on CSB SOD activity is presented in Figure 1. The result revealed a significant decrease ($P \leq 0.05$) in SOD activities of *C. maculatus* groups treated with the various bioinsecticides compared with the control. SOD activity of *C. maculatus* group treated with 20,000 ppm *H. suaveolens* (0.35 U mg⁻¹ protein) was 32 fold lower than the value of the control. The enzyme activity for all the groups treated with synthetic insecticides increased with increasing concentrations of all the treatments. For the synthetic insecticides, the SOD activity was enhanced at higher

Table 4. Effect of insecticides and bio-insecticides on α-Tocopherol content of adult *Callosobrochus maculatus*.

BIO-INSECTICIDE CONC. (ppm)	α-TOC CONTENT (mg g⁻¹ FRESH PROTEIN)			
	C. Rotundus	*T. Diversifolia*	*H. Suavolens*	*J. Curcas*
0	33.37 ± 0.40^b	33.37 ± 0.40^c	33.37 ± 0.40^c	33.37 ± 0.40^b
5,000	19.93 ± 0.40^c	33.87 ± 0.30^c	39.20 ± 0.80^b	35.67 ± 0.50^a
10,000	13.37 ± 0.30^d	12.47 ± 0.30^d	13.93 ± 0.60^d	33.90 ± 0.20^b
15,000	20.50 ± 0.30^c	37.07 ± 0.60^b	40.97 ± 0.30^a	24.23 ± 0.70^c
20,000	34.36 ± 0.30^a	41.13 ± 1.00^a	41.83 ± 0.30^a	33.27 ± 0.40^b
INSECTICIDE CONC. (ppm)	α-TOC CONTENT (mg g⁻¹ FRESH PROTEIN)			
	DDVP	CYPERMETHRIN	λ-CYHALOTHRIN	
0	33.37 ± 0.4^e	33.37 ± 0.4^d	33.37 ± 0.4^e	
10	62.23 ± 1.0^a	60.77 ± 0.7^a	125.60 ± 0.7^d	
50	57.50 ± 0.6^b	59.17 ± 0.3^b	127.87 ± 0.3^{c,d}	
100	56.17 ± 1.0^b	58.33 ± 0.2^b	128.90 ± 3.5^c	
150	44.03 ± 1.4^c	58.10 ± 0.9^{b,c}	132.43 ± 1.6^b	
200	37.40 ± 0.5^d	57.17 ± 1.0^c	138.00 ± 1.1^a	

Notes: The values were expressed as mean ± SEM (three replicates) determined by via one way ANOVA followed by Duncan's New Multiple Range Test (DNMRT). The mean values, in the same column, with different superscripts are significantly different $P \leq 0.05$ while means with the same superscripts indicate non-significant changes.
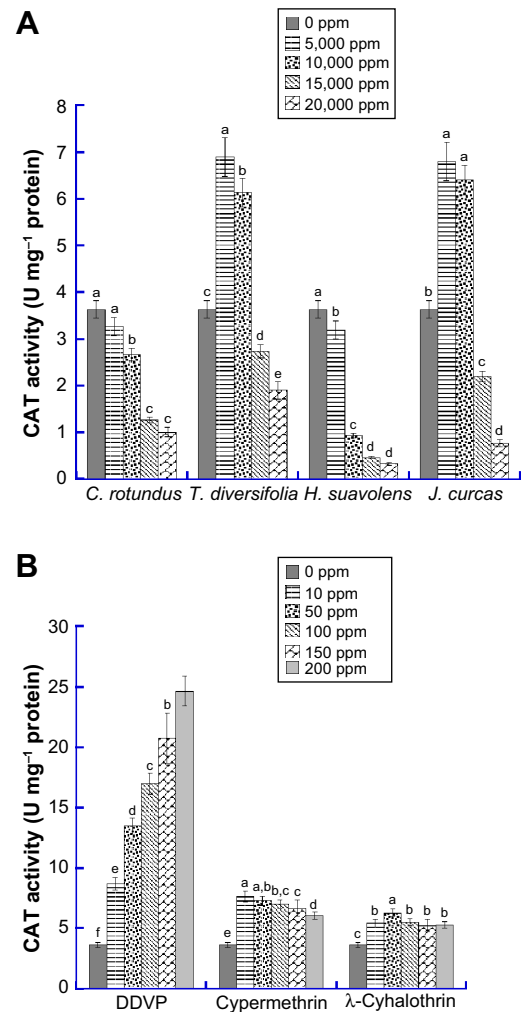**Abbreviation:** ppm, part per million.
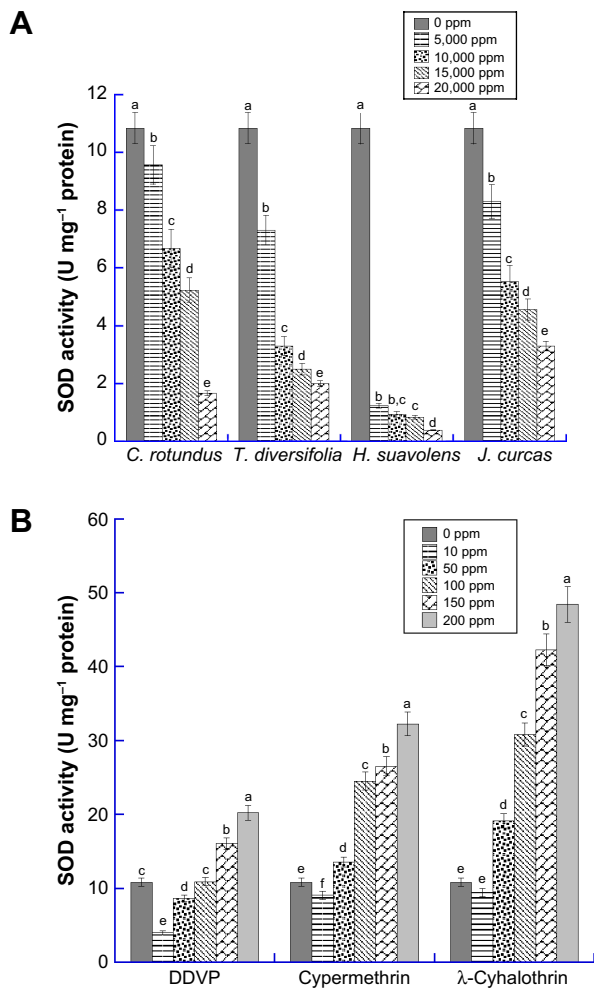
Figure 1. Level of Superoxide dis mutase (SOD) in the whole tissue homogenate of *C. maculatus* exposed to different concentrations of (**A**) bio-pesticides (*Tithonia diversifolia*, *Cyperus rotundus*, *Hyptis suavolens* leaves and *Jatropha curcas* seed) and (**B**) pesticides (DDVP, Cypermethrin and λ-cyhalothrin). The values are mean ± SD of triplicate determination of the results (U mg^{-1} proteins). Means within the same column followed by the same letter(s) are not statistically significant different using Duncan's Multiple Range Test (DMRT) at a 95% level of confidence ($P \leq 0.05$). Different letters indicate significant differences.

Figure 2. Level of Catalase (CAT) in the whole tissue homogenate of *C. maculatus* exposed to different concentrations of (**A**) bio-pesticides (*Tithonia diversifolia*, *Cyperus rotundus*, *Hyptis suavolens* leaves and *Jatropha curcas* seed) and (**B**) pesticides (DDVP, Cypermethrin and λ-cyhalothrin). The values are mean ± SD of triplicate determination of the results (U mg^{-1} proteins). Means within the same column followed by the same letter(s) are not statistically significant different using Duncan's Multiple Range Test (DMRT) at a 95% level of confidence ($P \leq 0.05$).

concentration (100–200 ppm) of the insecticides. However, at a concentration of 10 ppm, the activity value was higher in the control than SOD activity value of the insects treated with the synthetic insecticides.

CAT activity. The effect of varied concentrations of synthetic insecticides and bioinsecticides on CSB CAT activity is presented in Figure 2. A significant increase ($P \leq 0.05$) was observed for *C. maculatus* groups treated with *T. diversifolia* and *J. curcas* extracts at 5,000 and 10,000 ppm. At 20,000 ppm, CAT activities of *C. maculatus* groups treated with *C. rotundus* (0.85 U mg^{-1} protein), *T. diversifolia* (1.45 U mg^{-1} protein), *H. suaveolens* (0.14 U mg^{-1} protein), and *J. curcas* (0.75 U mg^{-1} protein) were 3.9, 2, 24, and 4.4 folds lower, respectively, compared with the values in the control group. There was increase in CAT activity in all the synthetic insecticide-treated groups

compared to the value of the control. The enzyme showed increased activity with increasing concentration of DDVP.

POX activity. Figure 3 shows the effect of varied concentrations of synthetic insecticides and bioinsecticides on CSB POX activity. *C. maculatus* group treated with 5,000 ppm *T. diversifolia* (3.65 U mg^{-1} protein) had a 28% increase in activity, whereas the group treated with 20,000 ppm *J. curcas* (2.99 U mg^{-1} protein) had 13% increase in POX activity compared with the control. The POX activity increased at higher concentrations of DDVP. Decrease in activity of the enzyme was obtained for cypermethrin-treated samples. The enzyme also exhibited increased activity in all concentrations of λ-cyhalothrin.

PPO activity. The result presented in Figure 4 shows the effect of varied concentrations of synthetic insecticides and
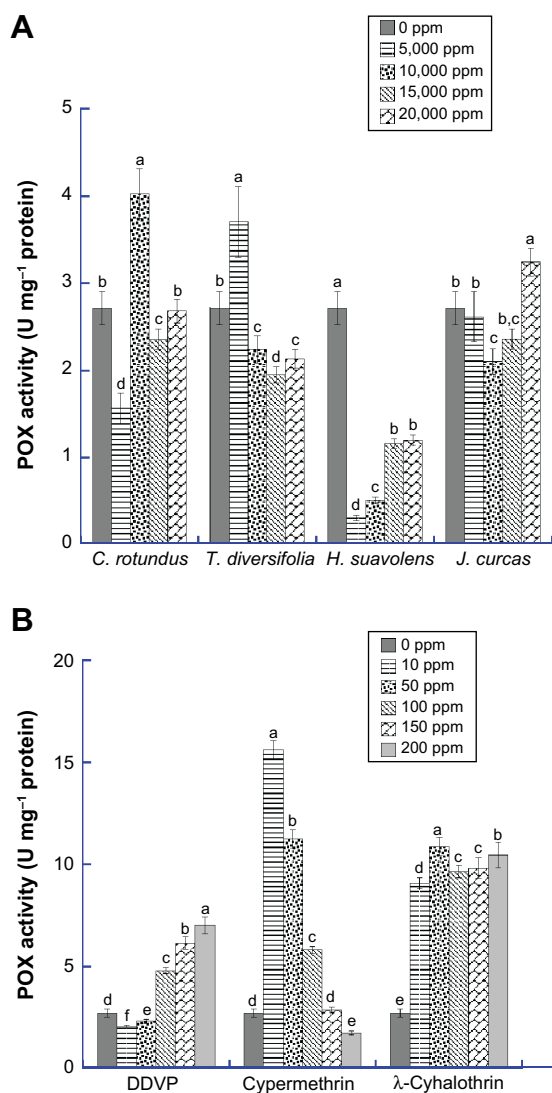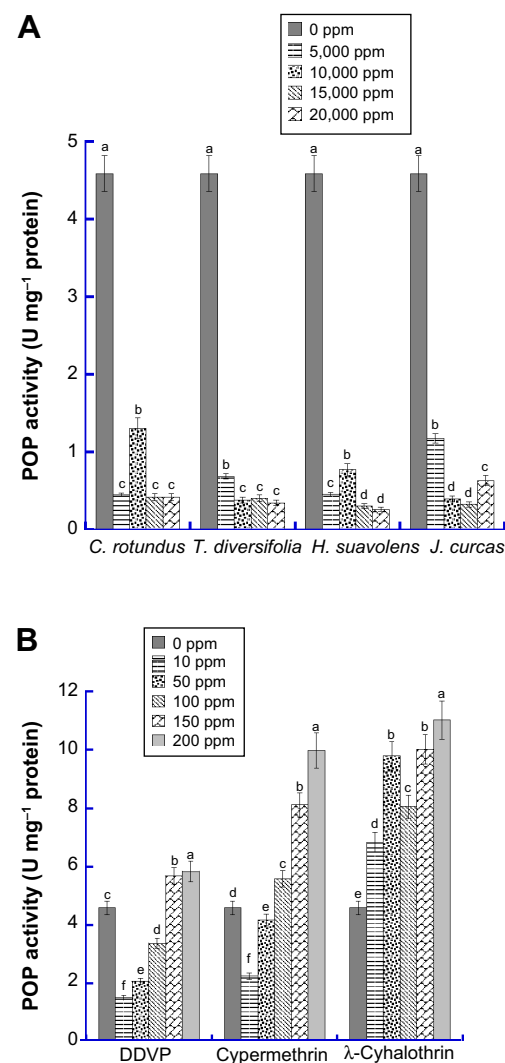
Figure 3. Level of Peroxidase (POX) in the whole tissue homogenate of *C. maculatus* exposed to different concentrations of (**A**) bio-pesticides (*Tithonia diversifolia*, *Cyperus rotundus*, *Hyptis suavolens* leaves and *Jatropha curcas* seed) and (**B**) pesticides (DDVP, Cypermethrin and λ-cyhalothrin). The values are mean ± SD of triplicate determination of the results (U mg⁻¹ proteins). Means within the same column followed by the same letter(s) are not statistically significant different using Duncan's Multiple Range Test (DMRT) at 95% level of confidence ($P \leq 0.05$).

Figure 4. Level of Polyphenol oxidase (PPO) in the whole tissue homogenate of *C. maculatus* exposed to different concentrations of (**A**) bio-pesticides (*Tithonia diversifolia*, *Cyperus rotundus*, *Hyptis suavolens* leaves and *Jatropha curcas* seed) and (**B**) pesticides (DDVP, Cypermethrin and λ-cyhalothrin). The values are mean ± SD of triplicate determination of the results (U mg⁻¹ proteins). Means within the same column followed by the same letter(s) are not statistically significant different using Duncan's Multiple Range Test (DMRT) at a 95% level of confidence ($P \leq 0.05$).

bioinsecticides on CSB PPO activity. It revealed a significant decrease ($P \leq 0.05$) in PPO activities of *C. maculatus* for all bioinsecticide-treated groups compared with the control. *C. maculatus* group treated with 20,000 ppm *C. rotundus* extract (0.30 U mg⁻¹ protein) had a 15.2 fold decreased PPO activity compared with the control. The enzyme activity in the insecticide-treated groups increased with increasing concentrations in all the samples.

Discussion
The results obtained in this study clearly demonstrate that bio-insecticides and synthetic insecticides have distinct effects on the survival/mortality of the beetles. While DDVP was the most potent among the synthetic insecticides with an LC_{50} of 13.43 ppm, the extract from *T. diversifolia* was the most potent among the bioinsecticides. Even though very high concentrations of bioinsecticides are required for them to be as effective as the synthetic insecticides in causing mortality, they are equally promising as the synthetic insecticides in insect control. The differences in the efficacy of the bioinsecticides might be a result of qualitative and quantitative composition of the secondary metabolites that characterized the ethanolic extract.[26–28] Ascorbic acid is a water-soluble antioxidant that is capable of scavenging activated oxygen species such as O_2^-, $OH^•$, and H_2O_2.[29] However, its efficiency in mopping up superoxide anions is an order of magnitude lower than that of SOD. The

decrease in ascorbic acid concentration may be ascribed to the increased usage of this non-enzymatic antioxidant to prevent the build-up of peroxides, which could have been generated in the insect in response to the bioinsecticides' and the synthetic insecticides' stresses. This result agrees with the work of Barbehenn et al[30] who reported a decline in lumenal ascorbate levels in the posterior midguts of *Malacosoma disstria* larvae when fed diets containing tannic acid. A reduction in the ascorbic acid concentration was also observed in all the tested tissues of *Aulocara ellioti* after it was fed wheat plant.[31] Ascorbic acid is also a substrate for redox enzyme ascorbate POX, a function that is particularly important in stress-resistant insects.[32]

GSH is a thiol group containing antioxidant that plays a very important role in the defense against ROS. GSH, together with ascorbate, participates in the protection against oxidative stress by its involvement in the ascorbate–GSH cycle, regulation of protein thiol-disulfide redox status, and reduction of H_2O_2 to water.[32] The exposure to the bioinsecticides might have brought about the induction of GSH synthesis. The inverse correlation between GSH and ascorbic acid observed in this study indicates that the increase in GSH content may be to compensate for the decline in the level of ascorbic acid. This inverse correlation also shows that intracellular GSH level appears to be a major determinant of the oxidative stress between the two indicators—GSH and ascorbic acid. All these suggest the possibility of cytotoxicity being mediated by oxidative stress. However, synthetic insecticides used do not appreciably alter the GSH content compared to bioinsecticides. The effect of bioinsecticides could be a result of the medley of allelochemicals that characterized the ethanolic extracts.[26] The response of the beetle GSH to the insecticides could demonstrate the insect sensitivity to xenobiotics and might be connected to enhance utilization of GSH as cosubstrate for GSH transferase or cofactor for GSH POX.[33]

α-Tocopherols are lipid soluble antioxidants that play an important role in defense against oxidative stress. Their function is to interact with the polyunsaturated acyl groups of lipids, stabilize membranes, and scavenge and quench various ROS and lipid soluble by-products of oxidative stress.[34] Various protective antioxidant roles of α-tocopherols have been documented in many species of organisms. In this study, the effect of *T. diversifolia* and *H. suaveolens* bioinsecticides on CSB α-tocopherols indicates a greater degree of protection that can allow the insect survive oxidative damage. However, the protection might not be adequate. The increase in α-tocopherols result agrees with the report of Barbehenn[35] who also observed an elevated α-tocopherols concentration in the midgut tissues of *Melanoplus sanguinipes* after being fed on diet containing 15% tannic acid. In some cases, the decrease in protection against oxidative stress was directly linked to lipid peroxidation and free radical formation.[36]

The change in (SOD activity at all concentrations of the bioinsecticides tested against CSB suggests that the bioinsecticides may possibly induce O_2^- in vivo. Synthetic

insecticides also enhanced SOD activity. The increase might not be unconnected to chemical structure of the synthetic insecticides. However, multivarious chemical components of bioinsecticides might have suppressed the SOD induction. SODs are the first line of defense against oxygen free radicals. This enzyme removes superoxide radicals (O_2^-) through the process of dismutation to oxygen and hydrogen peroxide (H_2O_2).[37,38] The ability of SOD to scavenge O_2^- is temporary and limited.[39] SOD is an antioxidant enzyme that can protect normal cells from ROS. The ability of this enzyme to overcome the toxic effects of ROS in insects has been documented.[40] It is also plausible to suggest that at higher concentration of the bioinsecticides, more O_2^- are generated, which may accumulate to an extent that may overwhelm the scavenging ability of the SOD enzyme, hence decreasing the activity at higher concentrations of the plant extract and synthetic chemicals. Decrease in activity of SOD could induce the accumulation of ROS, which in turn causes oxidative damage in the insecticide-stressed beetles. Decrease in SOD activity could also impair the O_2^- scavenging ability of the cells, thus favoring the accumulation of O_2^- and H_2O_2.[41] SOD activity for all the insecticide-treated groups increases with increased concentration. The increased insecticides concentration in all the treated samples stimulated the synthesis of SOD, resulting in higher dismutation of superoxide anion (O_2^-), thus preventing the production of hydroxyl radical (OH^-)—a highly reactive specie.

The possible reasons for the initial increase in the CAT activity of *C. maculatus* groups treated with *T. diversifolia* and *J. curcas* at the initial low concentration of 5,000 and 10,000 ppm compared with the control and later reduction in the activity at higher concentration are not clear. CAT has high turnover rate for hydrogen peroxide, and its activity is up-regulated by the H_2O_2 concentration;[42] it catalyzes the conversion of hydrogen peroxide to water and molecular oxygen. A higher CAT activity after exposure to bioinsecticides may signify an enhanced H_2O_2 removal and, hence, prevention of oxidative damage. This consequently lowers the risk of hydroxyl radicals formation via Fenton reaction.[43] CAT in the midgut of *Lymantria dispar* larvae fed on an unfavorable plant showed similar results.[44] Decreased activity of CAT was also detected because of high level of superoxide radical generation during oxidative stress in the acute stage of bacteriosis in *Galleria mellonella*.[45] CAT exhibited higher activity in all concentrations of DDVP, having activity as high as 91.7 U mg^{-1} protein at 200 ppm. This implied that beetles induce and utilize CAT as a major antioxidant enzyme against ROS generation. High CAT activity in DDVP-treated samples may explain the increase in SOD activity in DDVP. Reduction in CAT activity obtained for the pyrethroids (cypermethrin and λ-cyhalothrin) may be because of inhibition of the synthesis of CAT and impairment of its activity. It is fairly well documented that insects possess POX activities[46] in which POX breaks down H_2O_2 to oxygen and water.[47] The significant increase ($P \leq 0.05$) in the POX activity

obtained for *C. maculatus* groups treated with *T. diversifolia*, *J. curcas*, and *C. rotundus* extracts at various concentrations compared with the control (Fig. 3) might indicate increased chemical stress tolerance by the insects. Corona and Robinson[48] had earlier observed a significant increase in POX activity of honey bee *Apis mellifera* L., after exposure to thermal stress. The majority of the other treatment groups, however, showed a decrease in POX activity. This is similar to the report of Fu-Xian et al[49] after assessing the effect of thermal oxidation on lipid peroxidation and antioxidant enzyme activities of oriental fruit fly, *Bactrocera dorsalis*. Activity of POX decreased with increase in the concentration of cypermethrin. This may imply that cypermethrin exhibits inhibitory effects on the synthesis of POX. Unlike what was obtained for cypermethrin, POX has higher activity in samples treated with DDVP and λ-cyhalothrin, which may explain the higher activity values obtained for SOD in DDVP and λ-cyhalothrin, because POX catalyzes the decomposition of hydrogen peroxide.[50]

Measurable amount of PPO was found in stressed CSB in this study. PPO is an oxidoreductase that catalyzes the oxidation of a wide range of phenolic compounds using molecular oxygen.[51] PPO activity has been reported in insects, where they were found to be responsible for exoskeleton formation.[52] A decrease in PPO activities was observed for all the *C. maculatus* groups treated with all the bioinsecticides compared with the control. This suggests that the bioinsecticides inhibit PPO activity in *C. maculatus*. This is not unusual because all the allelochemicals that characterized both bioinsecticides and insecticides are made of phenolic rings. This result agrees with various reports of inhibition of PPO by substances from natural and non-natural sources. Isao et al[53] reported the inhibition of the tyrosinase enzyme of the aphid, *Melaphis chinensis* by extract from the leaves of *Rhus javanica* plant. Isao and Ikuyo[54] observed the inhibition of mushroom tyrosinase by anisaldehyde extracted from the seed of anise oil. Exoskeletons on the other hand serve to protect insects against desiccation and external injury. As this enzyme was significantly inhibited in this study, it may be postulated that body injury because of inhibition of exoskeleton production could be another possible explanation for the efficacy of these bioinsecticides against *C. maculatus*. Increase in the activity was also obtained for PPO in all the insecticide-treated samples. PPO functions in the oxidation of phenoxyl-end of pyrethroid insecticides.[55] This explains the higher activity values obtained for λ-cyhalothrin and cypermethrin (both pyrethroid), and it had been reported that oxidase provides resistance for pyrethroid.[56] PPO activity at 50 ppm λ-cyhalothrin was observed to be higher than activity at 100 ppm λ-cyhalothrin. Reason for this usual behavior is conjectural, but it can be assumed that the 50 ppm concentration favored the synthesis of the enzyme than situation obtained in the 100 ppm concentration.

Results of this study strongly support the hypothesis that antioxidant systems are modulated as a response to cytotoxicity mediated by oxidative stress from bioinsecticides and insecticides. There is a possibility of the bruchid developing specific adaptive response mechanism to combat the stresses. However, the present and respective efficacy is based on the chemical constituent, structure, and composition of either the bioinsecticides or the insecticides.

Author Contributions

Conceived and designed the experiments: AOK, JOA, COA. Analyzed the data: AOK, COA. Wrote the first draft of the manuscript: AOK, FMO, JOA, COA. Contributed to the writing of the manuscript: AOK, FMO, JOA, COA. Agree with manuscript results and conclusions: AOK, FMO, JOA, COA. Jointly developed the structure and arguments for the paper: AOK, FMO, JOA, COA. Made critical revisions and approved final version: AOK, FMO, JOA, COA. All authors reviewed and approved of the final manuscript.

REFERENCES

1. Oparaeke AM, Dike MC, Onu I. *Evaluation of Seed and Leaf Powders of Neem* (*Azadirachta indica* A. Juss) *and Pirimiphos-methyl for the Control of Callosobruchus maculatus* (F.) *in stored cowpea*. Vol 31. ESN Occasional Publication Maiduguri, Nigeria; 1998:237–242.
2. Steele WM, Allen DJ, Summerfield RJ. Cowpea (*Vigna unguiculata* (L.) Walp.), in Grain Legume Crops, ed. by Summerfield RJ, Roberts EH. Collins, London, UK. 1985:520–583.
3. Jackai LEN, Adalla CB. Pest management practices in cowpea: a review, in Advances in Cowpea Research, ed. by Singh BB, Mohan Raj DR, Dashiell KE, Jackai LEN. IITA/JIRCAS, Ibadan, Nigeria. 1997:240–258.
4. Zhu-Salzman K, Murdock LL. Cowpea: insects, ecology and control. An invited article for the Encyclopedia of Pest Management, published by Marcel Dekker; 2006:1–3.
5. Boeke SJ, Baumgart IR, Van loon JJA, Van huis A, Dicke M, Kossou DK. Toxicity and repellence of African plants traditionally used for the protection of stored cowpea against *Callosobruchus maculatus*. *J Stored Products Res*. 2004;40:423–438.
6. Appleby JH, Credland PF. Variation in responses to susceptible and resistant cowpea among West African populations of *Callosobruchus maculates* (Coleoptera: Bruchidae). *J Econ Entomol*. 2003;96:489–502.
7. Ohiagu CC. Storage of food grains in the savannah zones of Northern Nigeria. In: Menyonga JM, Bezunah T, Youdeowei A, eds. *Food Grain Production in Semi-arid Africa*. Ouagadougou: . Safgra; 1987:361–368.
8. Guo F, Lei J, Sun Y, et al. Antagonistic regulation, yet synergistic defense: effect of bergapten and protease inhibitor on the development of cowpea bruchid *Callosobrochus maculatus*. *PlosOne*. 2012;7(8):e41877.
9. Khalequzzaman M, Chowdhury FD. Evaluation of mixtures of plant oils as synergists for pirimiphos-methyl in mixed formulation against *Tribolium castaneum* (Herbst). *Online J Biol Sci*. 2003;3:347–359.
10. Govindan K, Jeyarajan NS, David PMM. Fly ash—excellent filler for black pepper, piper nigrum dust formulation against *Callosobruchus maculatus* (F). *J Biopesticides*. 2010;3(1):320–324.
11. Ileke KD, Oni MO. Toxicity of some plant powders to maize weevil, Sitophilus zeamais (Coleoptera: Curculionidae) on stored wheat grains. *African J Agric Res*. 2011;6:3043–3048.
12. War AR, Paulraj MG, Hussain B, Ahmad T, War MY, Ignacimuthu S. Efficacy of a combined treatment of neem oil formulation and endosulfan against *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). *Int J Insect Sci*. 2014;6:1–7.
13. Boeke SJ, Barnaud C, Van Loon JJA, Kossou DK, Van Huis A, Dicke M. Efficacy of plant extracts against the cowpea beetle, *Callosobruchus maculatus*. *Int J Pest Manage*. 2004;50:251–258.
14. Gbaye OA, Holloway GJ, Callaghan A. Variation in the sensitivity of *Callosobruchus* (Coleoptera: Bruchidae) acetylcholinesterase to the organophosphate insecticide malaoxon: effect of species, geographical strain and food type. *Pest Manage Sci*. 2012;68:1265–1271.
15. Kolawole AO, Ajele JO, Sirdeshmukh R. Studies on glutathione transferase of cowpea storage bruchid, *Callosobrochus maculatus* F. *Pesticide Biochem Physiol*. 2011;100:212–220.
16. Amin K, Hashem KS. Deltamethrin-induced oxidative stress and biochemical changes in tissues and blood of catfish (Clarias gariepinus): antioxidant defense and role of alpha-tocopherol. *BMC Veterinary Res*. 2012;2012(8):45.

17. Mostafalou S, Abdollahi M. Pesticides and human chronic diseases: Evidences, mechanisms, and perspectives. *Toxicol Appl Pharmacol*. 2013;268:157–177.

18. Adedire CO, Akinneye JO. Biological activity of tree marigold, *Tithonia diversifolia*, on cowpea seed, *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Ann Appl Biol*. 2003;144:185–189.

19. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem*. 1976;72:248–254.

20. Omaye ST, Turnbull JD, Sauberilich HE. Selected methods for the determination of ascorbic acid in animal cells tissues and fluids. *Methods Enzym*. 1979;62: 3–11.

21. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82:70–77.

22. Backer H, Frank O, De Angel B, Feingold S. Plasma tocopherol in man at various times after ingesting free or ocetylaned tocopherol. *Nutr Report Intern*. 1980; 21:531–536.

23. Fridovich I. Superoxide dismutase. *Adv Enzymol*. 1974;41:35–97.

24. Aebi H. Catalase in vitro. *Methods Enzymol*. 1984;105:121–126.

25. Kumar KB, Khan PA. Peroxidase and polyphenol oxidase in excised ragi (*Eleusine coracana* cv. PR 202) leaves during senescence. *Indian J Exp Botany*. 1982;20: 412–416.

26. Kolawole AO, Okonji RE, Ajele JO. *Tithonia diversifolia, Cyperus rotundus* and *Hyptis suaveolensis* ethanol extracts combinatorially and competitively inhibit affinity purified cowpea storage bruchid (*Callosobruchus maculatus*) glutathione S-transferase. *Arthropod–Plant Interactions*. 2011;5:175–184.

27. War AR, Paulraj MG, Hussain B, Buhroo AA, Ignacimuthu S, Sharma HC. Effect of plant secondary metabolites on *Helicoverpa armigera*. *J Pest Sci*. 2013;86: 399–408.

28. Isman MB, Miresmailli S, Machial CM. Commercial opportunities for pesticides based on plant essential oils in agriculture, industry and consumer products. *Phytochem Rev*. 2010;10:197–204.

29. Sies H. Role of reactive oxygen species in biological processes. *Klin Wochenschr*. 1991;69:965–968.

30. Barbehenn RV, Bumgarner SL, Roosen EF, Martin MM. Antioxidant defenses in caterpillars: role of the ascorbate-recycling system in the midgut lumen. *J Insect Physiol*. 2001;47:1095–1095.

31. Barbehenn R. Antioxidants in Grasshoppers: higher levels defend the midgut tissues of a polyphagous species than a graminivorous species. *J Chemi Ecol*. 2003;29: 683–702.

32. Shigeoka S, Ishikawa T, Tamoi M, et al. Regulation and function of ascorbate peroxidase isoenzymes. *J Exp Botany*. 2002;53:1305–1319.

33. Supratim R. Evaluation of protective role of ascorbic acid on flutamide-induced lipid peroxidation. *Int J PharmTech Res*. 2012;4:135–140.

34. Kamal-Eldin A, Appelqvist LA. The chemistry and antioxidant properties of tocopherols and tocotrienols. *Lipids*. 1996;31:671–701.

35. Barbehenn RV. Antioxidants in grasshoppers: higher levels defend the midgut tissues of a polyphagous species than a graminivorous species. *J Chem Ecol*. 2003;29:683–702.

36. Gopi R, Jalee CA, Lakshmanan GMA, Gomathinayagam M, Panneerselvam R. Differential effects of hexaconazole and paclobutrazol on biomass, electrolyte leakage, lipid peroxidation and antioxidant potential of *Daucus carota* L. *Colloids Surf. B: Biointerfaces*. 2007;60:180–186.

37. Henry WL, Chao-Lin K, Wen-Hui Y, Chia-Hsien L, Hong-Zin L. Antioxidant enzymes activity involvement in luteolin-induced human lung squamous carcinoma CH27 cell apoptosis. *Eur J Pharmacol*. 2006;534:2123–2130.

38. Olawale O, Ikechukwu NE, Grace TO, Chidiebere EU. Oxidative stress and superoxide dismutase activity in brain of rats fed with diet containing permethrin. *Biochemistry*. 2008;20:93–98.

39. Wang H, Wen C, Chang X, Duan C. Some detoxication mechanisms of different wheat varieties under cadmium treatment. *Acta Sci Circum*. 2002;22:523–528.

40. Suh HJ, Kim SR, Lee KS, Park S, Kang SC. Antioxidant activity of various solvent extracts from Allomyrina dichotoma (Arthropoda: Insecta) larvae. *J Photochem Photobiol B: Biology*. 2010;99:67–73.

41. Abdul Jaleel C, Lakshmanan GMA, Gomathinayagam M, Panneerselvam R. Triadimefon induced salt stress tolerance in *Withania somnifera* and its relationship to antioxidant defense system. *South Africa J Botany*. 2008;74:126–132.

42. Fornazier RF, Ferreira RR, Pereira GJG, et al. Cadmium stress in sugar cane callus cultures: effect on antioxidant enzymes. *Plant Cell Tissue Organ Cult*. 2002;71: 125–131.

43. Fridovich I. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen. *Annu N Y Acad Sci*. 1999;893:13–18.

44. Peric-Mataruga V, Blagojevic D, Spasic MB, Ivanovic J, Jankovic-Hladni M. Effect of the host plant on the antioxidative defence in the midgut of *Lymantria dispar* L. caterpillars of different population origins. *J Insect Physiol*. 1997;43: 101–106.

45. Dubovskiy IM, Martemyanow VV, Vorontsova YL, Rantala MJ, Gryzanova EV, Glupov VV. Effect of bacterial infection on antioxidant activity and lipid peroxidation in the midgut of *Galleria mellonella* L. larvae (Lepidoptera, Pyralidae). *Comp Biochem Physiol C*. 2008;148:1–5.

46. Mathews MC, Summers CB, Felton GW. Ascorbate peroxidase: a novel antioxidant enzyme in insects. *Arch Insect Biochem Physiol*. 1997;34:57–68.

47. Lee IM, Cook NR, Gaziano JM, et al. Vitamin E in the primary prevention of cardiovascular disease and cancer: the Women's Health Study: a randomized controlled trial. *JAMA*. 2005;294:56–65.

48. Corona M, Robinson GE. Genes of the antioxidant system of the honey bee: annotation and phylogeny. *Insect Mole Biol*. 2006;15:687–701.

49. Fu-Xian J, Wei D, Fei H, Jin-Jun W. Effects of thermal stress on lipid peroxidation and antioxidant enzyme activities of oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae). *Florida Entomol*. 2011;94:956–963.

50. Karthikeyan M. Induction of resistance in host against the infection of leaf blight pathogen (*Alternaria palandui*) in onion (Allium cepa var aggregatum). *Indian J Biochem Biophys*. 2005;42:371–377.

51. Mayer AM. Polyphenol oxidase in plants-recent progress. *Phytochemistry*. 1987;26: 11–20.

52. Halaouli S, Asther M, Sigoillot JC, Hamdi M, Lomascolo A. Fungal tyrosinases: new prospects in molecular characteristics, bioengineering and biotechnological applications. *J Appl Microb*. 2006;100:219–232.

53. Isao K, Kinst-Hor I, Nihei KI, et al. Tyrosinase inhibitors from Galls of Rhus javanica leaves and their effects on insects. *Zei fuer Natur C J. Biosci*. 2003;58: 719–725.

54. Isao K, Ikuyo K. Tyrosinase Inhibitors from Cumin. *J Agric Food Chem*. 1998; 46(12):5338–5341.

55. Friedman M. Chemistry, biochemistry, and dietary role of potato polyphenols. *J Agric Food Chem*. 1997;45:1523–1540.

56. Karunaratne SH. Insecticide cross-resistance in insects: review. *Ceylon J Sci*. 1998; 26:73–98.