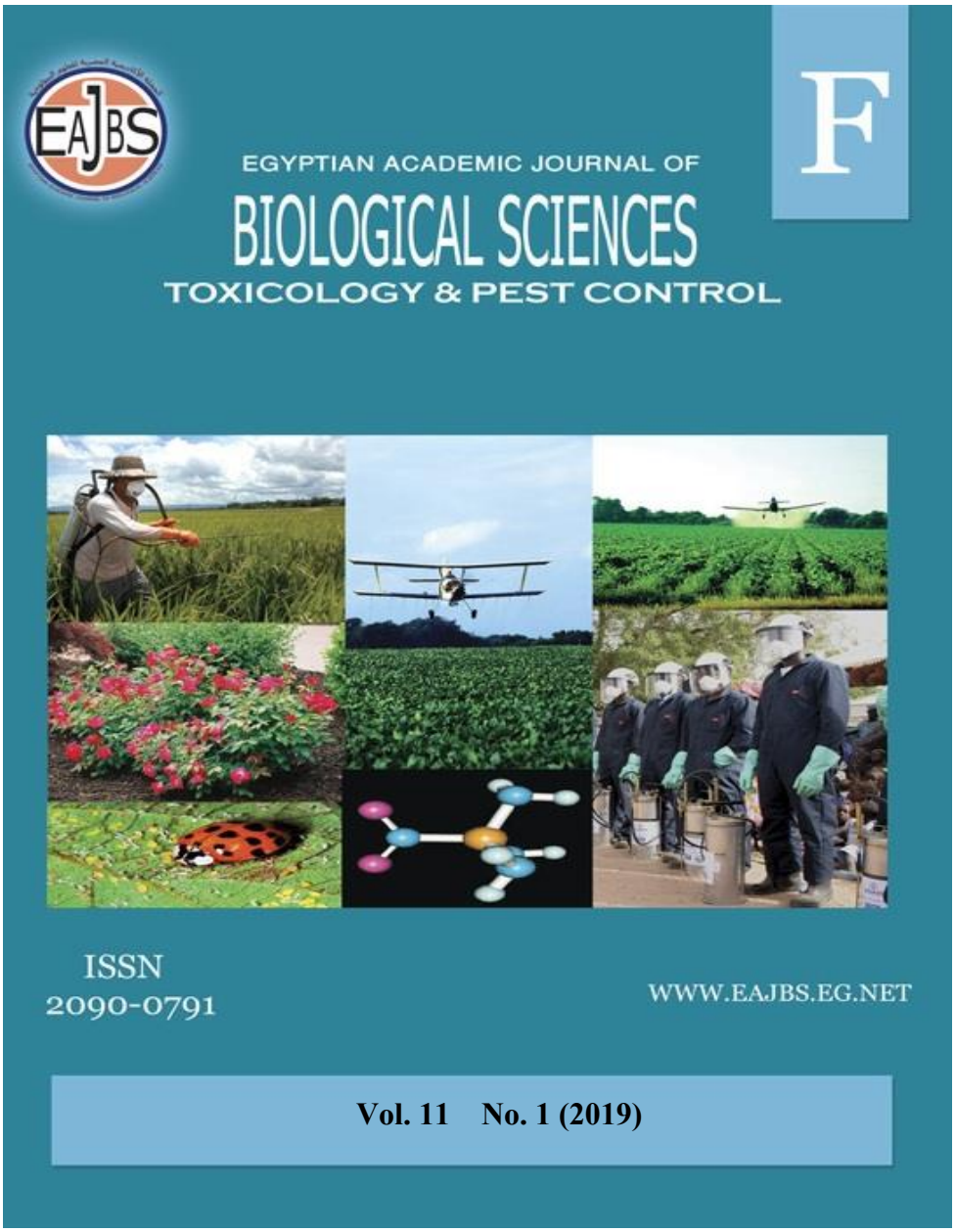

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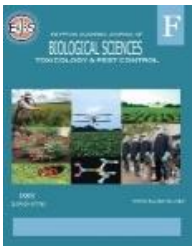

Genome-wide DNA Mutability and Biochemical Effects of Novel Insecticides in the Control of Date Palm Fruit Pest *Ephestia cautella* (Walker)

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ABSTRACT

Different pests attack date palm fruits during all the stages of maturity. Almond moth (*Ephestia cautella*) is a destructive insect of stored products, such as dates, wheat flour, and nuts. In the present study, the usage of novel groups of insecticides (Spinosyns, Diamide, Pyridalyl, and Azadirachtin) that are safe for humans and exert a relatively lower effect on the environment was assessed. In particular, the toxicological and biochemical impacts of five insecticides (Tracer, Radiant, Coragen, Pleo, and Achook) on the control of *E. Cautella* were evaluated, and the genome-wide DNA mutability caused by these insecticides was screened. A bioassay was performed in order to determine the LC_{50}value for each insecticide, the results of which revealed that Coragen with an LC_{50} value of 0.49 ppm was the most potent insecticide, followed by Radiant and Achook (with LC_{50} values of 1.51 and 1.73 ppm, respectively). In addition, the effect of these insecticides on vital enzymes was investigated. The data from this investigation revealed that the treatment with Radiant demonstrated stimulation in AST, ACP, ALP, and GST activity, and inhibition in AChE, ALT, and protease activity. The insects treated with Coragen exhibited an elevation in the activities of AChE, GST, ALP, ACP, ALT, and protease. Finally, DNA-level mutability caused by the insecticides was assessed by using RAPD-PCR analysis, and the results indicated Radiant as a genotoxic insecticide that caused large changes at the genomic-DNA level. On the other hand, Coragen exhibited the lowest mutability effect on insect DNA.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.) is an economically important crop with nourishing fruits; it is grown in Egypt and several other countries throughout the world. Egypt is the largest producer of dates (fruit) in the world (FAO 2016). Nevertheless, the amount of date fruit exported is <3% only, which is mainly due to an infestation of the fruit with certain pests (World Atlas 2018). Among these pests, an almond moth [*Ephestia cautella* (Walker) (Lepidoptera: Pyralidae)] is the major pest of the date fruits, which causes economic losses and critical damages to the fruit quality in the field as well as during the storage of the fruit. Larvae cause considerable damage to the fruit by feeding and/or contaminating the stored food with dead bodies and their own products, i.e. excreta, webbing, silk, and feces. This insect also infests flour, rice, maize, cereal grains, dry fruits such as fig, almond, raisins, pears, groundnuts, and walnuts, and confectionery items such as biscuits and chocolates (Girish 2017).

Conventional insecticides such as Malathion and Chlorpyrifos-methyl and fumigants such as methyl bromide (MB) and phosphine that were being used in the control efforts earlier poseda great danger to human health as well as to the environment. Therefore, the use of MB has been discontinued worldwide since the year 2015, under the terms of Montréal Protocol (UNEP 1998). Recently, several researchers have become devoted to seek alternatives to MB for being used against the insect pests in warehouses (Fields and White 2002; Robin 2013). These alternatives include novel groups of insecticides with novel modes of action that are safe for humans and exert a lower negative impact on the environment. Among the proposed modern insecticides, the insecticide groups/families such as Spinosyns, Diamide, Pyridalyl, and Azadirachtin have been investigated previously (Gavkare *et al*. 2013).

Spinosyns are a large family comprising unprecedented compounds produced during fermentation by two species of *Saccharopolyspora*. Several reports have confirmed their insecticidal potency, in addition to their broad-spectrum activities against all the stages of several lepidopterous pests (Rodriguez *et al*. 2016). At present, Spinosad Tracer® is the only insecticide registered for use against *E. Cautella* on date fruit in Egypt. Another member of the Spinosyn family, viz., Spinetoram Radiant®, has exhibited greater potency for a longer duration in comparison to Spinosad, as a control measure against certain insects (Dripps *et al*. 2008).

Among the Diamide insecticides, Chlorantraniliprole Coragen® is the insecticide with high controlling power, covering almost all the economically important Lepidoptera and other insects. It enters chewing pests through ingestion or contact, exhibiting substantial larvicidal activity (Su *et al*. 2017).

Pyridalyl Pleo® is a novel insecticidal class of compounds consisting of a phenoxy-pyridaloxy derivative structure that is not related to any other existing insecticide. This novel structure results in this insecticide exhibiting extraordinary activities against the larvae of various lepidopterans.

A natural pesticide, Azadirachtin Achook®, is derived from the Neem tree. It is a known repellent, and a potent anti-feedant, which disrupts the growth and development of insect larvae and sterilizes the adult insects (Chaudhary *et al*. 2017).

Generally, these different groups of insecticides cause defects in the vital and enzymatic systems of the insect physiology, through the interaction of the xenobiotic with the enzymes or transport proteins, thereby inhibiting the normal functions (McKinlay *et al*. 2012). In order to evaluate the effectiveness of the novel insecticides, several biochemical markers in the insects may be employed, such as the nerve conduction

enzyme acetyl cholinesterase (AChE), the detoxifying enzymes (Glutathione S-transferase, GST), and the hydrolytic enzymes (Alkaline Phosphatase, ALP; and Acid Phosphatase, ACP). ALP and ACP have been demonstrated to be correlated to the gradual growth and development of the larval tissues (Bream 2003). Transaminases (enzymes), such as glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT), are the strategic link between carbohydrate and protein metabolism (Etebari *et al.* 2005). Moreover, protease enzymes play a crucial role in the digestion of food in the insects, through the hydrolysis of peptide bonds in proteins, converting the latter into their respective amino acids (Terra and Ferriera2005). Therefore, the elucidation of insecticidal mechanisms by determining the activities of the nerve conduction enzymes, detoxifying enzymes, transaminases, and proteases is considered the main aspect of studying the toxicological effects of insecticides (Mohamed *et al*. 2016). Additionally, proteins serve as key organic constituents, play a vital role in the compensatory mechanisms in the insects during stress, and several activities of the insects are dependent on their protein metabolism (Khosravi and Sendi 2013).

Nevertheless, a great concern remains regarding the effects of insecticides on the genetic makeup of natural populations. The genetic effects include alterations in both the structure and the function of DNA, such as DNA breakage and DNA adducts, as well as several point mutations and large rearrangements (genotoxic effects) produced as a result of chemical exposure (Langie *et al.* 2015; Mokhtar and Atia, 2018). Randomly Amplified Polymorphic DNA (RAPD) serves as one of the most reliable DNA markers for detecting damages at the DNA level, as in the RAPD assay, the amplification stops at the site of mutation/damage (Lalrotluanga *et al.* 2011). The first study utilizing the RAPD assay for measuring genotoxic effects was performed by Savva *et al*. (1994). Additionally, the RAPD assay has been successfully used to detect the 'DNA effects' induced by radionuclides (Theodorakis and Shugart 1997), benzo (a) pyrene (Castano and Becerril, 2004), 4-*n*-nonylphenol and 17-β-estradiol (Atienzar *et al*. 2002), and metals such as lead, manganese, cadmium, and copper (Liu *et al*. 2005).

Therefore, the present study aimed to analyse five insecticides (Tracer, Radiant, Coragen, Pleo, and Achook) as alternatives to the conventional insecticides for controlling one of the most dangerous date fruit pest *E. cautella*.The present study investigated the impacts of these five insecticides on the toxicological and biochemical aspects of the insect, and their capability to cause genome-wide DNA alterations in the insect.

MATERIALS AND METHODS

Rearing of *E. cautella:*

The insects to be used in the experiments were collected from the infested date fruits obtained from traditional stores and date palm plantations in the Siwa oasis, Egypt. The fruits containing the insects were transferred to the laboratory. The larvae were collected from the fruits and maintained in glass jars by providing them with an artificial diet comprising crushed wheat, glycerin, sugar, and yeast (Lima *et al*. 2001) as a source of food until the emergence of adults. The emerged adults were collected each day by using a glass tube and were placed in glass cages with screen bottom to obtain the eggs. The eggs that fell through this screen bottom were collected each day in an open Petri dish and were transferred to plastic tubes to obtain the newly hatched larvae. The culture was maintained at 26 ±2°C, 65% ±5% R.H., and a photoperiod of 15:9 h Light:Dark cycles, until the emergence of larvae. This process was repeated for several generations of the insect.

Bioassay and Toxicity:

Different concentrations of each insecticide were prepared by diluting the emulsifiable concentrate formulation with water (1mL for each concentration), which were then mixed properly with 10g of artificial media in small Petri dishes (5cm). The treated artificial media were left to dry in air. Ten larvae among all those in the 3rd instar stage were placed on the Petri dish. Each concentration was replicated three times. The control was treated only with water and no insecticide. The larvae were left undisturbed to feed on the treated artificial media for 48 h, following which mortality counts were recorded. The LC_{50} values and the confidence limit were calculated using probit regression analysis in LDP-line software according to the method described by Finney (1971). This program is used to illustrate the relationship between the stimulus and the response in toxicological studies.

Biochemical Analysis:

The levels of enzyme activity and total protein were determined post 48 h of feeding of the insects (3rd instar-stage larvae) on the artificial media treated with different LC_{50} concentrations. The living larvae were collected and subjected to biochemical assays following starvation for about 4 h.

The control and treated samples were homogenized in 0.1 M (1/5 w/v) phosphate buffer (pH 7) and centrifuged at 10,000 rpm for 15min at 4°C. The supernatants obtained were subjected to assays for determining the activities of certain vital enzymes and the total soluble protein content using spectrophotometric techniques. The data were recorded and analyzed statistically using Student's *t*-test and Sigma Plot.

Total Protein:

The total protein content was determined by using the method of Bradford (1976) that involved the use of Coomassie Brilliant Blue.

Protease Activity:

Proteolytic enzyme activity was determined spectrophotometrically at 280 nm, as described by Ishaaya *et al*. (1971).

Acetyl cholinesterase (AChE) Activity:

The kinetic colorimetric determination of the acetyl cholinesterase activity was conducted using the method described by Ellman *et al*. (1961), with a few modifications as described by Bisso *et al.* (1991).

Glutathione S-transferase (GST) Activity:

The enzyme activity of Glutathione S-transferase was measured spectrophotometrically at 340 nm, according to the method described by Habig *et al*. (1974).

Alkaline phosphatase (ALP) Activity:

The concentration of ALP was determined according to the method described by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), using assay kits (Biosystems; kit no.11592).

Acid Phosphatase (ACP) Activity:

The colorimetric determination of the ACP activity was conducted according to the method described by Kind and King (1954), using assay kits (Biodiagnostic; kit no. AC1010).

Transaminase Activity:

The catalytic concentrations of the enzymes glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were measured according to the respective IFCC methods, using assay kits (Biosystems; kit no.11531 and 11533, respectively).

Molecular Analysis

DNA Extraction:

Genomic DNA was extracted from the control larvae as well as from the larvae belonging to each treatment at the LC_{50} stage, using the G-spin™ total DNA extraction mini kit in accordance with the manufacturer's instructions (iNtRON Biotechnology Inc.). The DNA concentrations were determined through spectrophotometric analysis.

RAPD Analysis:

RAPD-PCR was performed as described by Atia *et al*. (2017). A set of 15 random 10-mer primers was applied to the control as well as treatment samples, in order to assess the level of DNA damage in the unexposed *E. cautella* larvae as well as the larvae that were exposed to the insecticides used in the experiments. The amplification reaction was performed in a total volume of 25 µL which contained 1 X PCR buffer, 1.5 mM $MgCl_2$, 2mM dNTPs, 1 U Taq DNA polymerase, 10mM primer, and 25 ng of the template DNA.A master mix was prepared initially for all the samples to ensure accuracy and homogeneity of the reactions.

The PCR amplification was performed in a Perkin Elmer Gene Amp 9700 PCR system (PE Applied Biosystems). The amplification program was as follows: an initial denaturation cycle at 94 °C for 5 min, followed by 40 cycles at 94°C for 1min, 36°C for 1min, and 72°C for 1min 30 s, and a final extension at 72°C for 7 min. The PCR products were mixed with 5 µL of gel-loading dye (6 × dye: 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water), and spun briefly in a microcentrifuge prior to loading onto the gel.

The amplification products were resolved through electrophoresis using 1.5% agarose gel containing ethidium bromide (0.5µg/mL) in 1 × TBE buffer. A 100-bp DNA ladder and a 1-kb DNA ladder were used as standards for determining the molecular size. The PCR products in the gel were visualized under UV light and photographed using a gel documentation system (BioRad).

Data Analysis:

In order to conduct the biochemical data analysis, Principal Component Analysis (PCA)– a method used widely for reducing the dimensionality of high-dimensional data was implemented, followed by visualizing the results into two components as a scatterplot. ClustVis web tool was utilized for the visualization and clustering of the biochemical multivariate data (Tauno and Jaak 2015).

In order to conduct molecular data analysis, the generated/amplified bands were scored visually. The bands were scored based on their presence (1) or absence (0) in order to generate a binary data set. The percentage of polymorphism was calculated by dividing the number of amplified polymorphic bands by the total number of bands amplified by the same primer or primer combination. Jaccard's coefficient (Jaccard 1908) was utilized to estimate genetic similarity. A dendrogram was generated through the cluster analysis using the unweighted pair group method of the arithmetic averages (UPGMA) for each marker system.

RESULTS

Insecticidal Toxicity:

The toxicity responses of the insecticides included in the present study (Tracer, Pleo, Radiant, Achook, and Coragen) against the third instar-stage larvae of *Ephestia cautella* post 48 h of feeding on the artificial diet treated with investigated compounds are presented in Table 1.

The LC_{50} values obtained revealed Coragen as the most potent insecticide exhibiting an LC_{50} value of 0.49 ppm, followed by Radiant and Achook (LC_{50} values of 1.51 and 1.73 ppm, respectively). On the other hand, Tracer and Pleo exhibited weak toxicity, resulting in LC_{50} values of 13.8 and 17.88 ppm, respectively.

Table 1: The LC_{50} of tested insecticides on 3rd instar larvae of *E. cautella* after 48 hrs (on ppm basis).

Insecticides	LC_{50} ppm (95%CL)	Slop ±SE
Tracer	**13.80** (11.09 - 17.41)	**1.22** ± 0.196
Pleo	**17.88** (15.74 - 19.90)	**2.23** ± 0.293
Radiant	**1.51** (1.15 - 1.99)	**0.83** ± 0.085
Achook	**1.73** (1.43 - 2.031)	**1.62** ± 0.224
Coragen	**0.49** (0.402 - 0.59)	**1.79** ± 0.211

CL. confidence limit SE. standard error

Effects on Biomarkers:

The data concerning the effects of LC_{50} concentrations of the five insecticides investigated, on the activities of certain vital enzymes as well as on the total protein content in the homogenates of the third instar-stage larvae of *E. cautella*are presented in Table 2.

Table 2: Enzymes activity and total protein in the 3rd instars larvae homogenates of *E. cautella* after treatment with LC_{50} of tested Insecticides.

Insecticides	AChE (nmol/min/mg protein)		GST (µmol/min/mg protein)		GOT U/L		GPT U/L		ALP U/L		ACP U/L		Protease (O.D. units/min/mg protein)		Total protein (mg/ml homogenate)	
	Means ±S.E.	% Change	Means ±S.E.	% Change	Means ±S.E.	% Change	Means ±S.E.	% Change	Means ±S.E.	% Change	Means ±S.E.	% Change	Means ±S.E.	% Change	Means ±S.E.	% Change
Tracer	193.3^{ns} ±5.16	-0.77	0.053** ±0.001	+16.5	83.99*** ±6.26	-85.7	70.66*** ±5.06	-91.5	334.5*** ± 9.86	+69.1	60.8*** ± 0.66	+36.9	119.0** ±2.17	-12.2	4.62** ±0.09	-9.2
Pleo	175.5* ±9.49	-9.9	0.066*** ±0.001	+44.3	848.3*** ±16.2	+44.5	214.6*** ±1.98	-74.3	192.4^{ns} ± 6.52	-2.7	40.1*** ± 0.21	-9.8	166.2*** ±1.92	+22.6	4.11*** ±0.14	-19.2
Radiant	150.5*** ±6.91	-22.8	0.061*** ±0.002	+33.0	751.6** ±34.2	+28.0	770.7** ±7.51	-7.5	584.9*** ± 28.1	+195.8	66.8*** ± 0.45	+50.3	113.9** ±4.92	-15.9	4.49** ±0.13	-11.8
Achook	243.6*** ±11.7	+25.0	0.096*** ±0.005	+108.8	456.9** ±23.0	-22.2	807.6^{ns} ±28.2	-3.1	585.5*** ± 4.78	+196.1	57.5*** ± 0.34	+29.5	274.6*** ±14.4	+102.7	2.65*** ±0.14	-47.9
Coragen	527.5*** ±23.4	+170.8	0.089*** ±0.004	+94.4	544.8^{ns} ±43.4	-7.2	938.1** ±27.7	12.5	141.1*** ± 10.4	-28.7	58.1*** ± 1.19	+30.7	261.9*** ±14.3	+93.3	3.00*** ±0.13	-40.9
Control	194.8 ±3.69	----	0.046 ±0.001	----	587.0 ±30.8	----	833.6 ±15.5	----	197.7 ± 4.29	----	44.4 ± 0.51	----	135.5 ±6.16	----	5.09 ±0.10	----

Values: *<0.05; **<0.01; ***<0.001 ns: not significant.

% Change= [(sample value-control value)/control value]*100

Total Protein content and Protease activity:

The data from the assay for the determination of total protein content in the third instar-stage larvae of *E. cautella* revealed a significant decrease in the total protein content in the exposed larvae for all the investigated insecticides (−47.9%, −40.9%, −19.2%, −11.8%, and −9.2% for Achook, Coragen, Pleo, Radiant, and Tracer, respectively), relative to the total protein concentration in the control larvae (5.09 mg/mL).

An increase in the protease activity was observed under exposures to Achook, Coragen, and Pleo, with the activity values reaching 274.6 (102.7%), 261.9 (93.3%), and 166.2 (22.6%) units/min/mg of protein, respectively. Meanwhile, treatment with Radiant and Tracer significantly decreased the protease activity by 15.9% and 12.2%, respectively, in comparison to that in the control (135.5 units/min/mg of protein).

Acetylcholinesterase (AChE) Activity:

As for the AChE activity, the results indicated that the Tracer, Pleo, and Radiant treatments caused a decrease in the activity compared to that in the control. Significant reduction in the enzyme activity was observed for Radiant and Pleo (150.5 and 175.5 nmol/min/mg of protein, respectively), while Tracer exerted only a negligible effect on the Ache activity (−0.77%). On the other hand, significant stimulation in the activity of AChE was observed with Coragen and Achook (527.5 and 243.6 nmol/min/mg of protein, respectively). The percentage of change relative to the control sample reached +170% in case of Coragen exposure.

Glutathione S-transferase (GST) Activity:

In the case of GST activity, the data indicated that exposures to all the investigated insecticides caused significant stimulations in the enzyme activity. The enzyme activities observed under treatments with Achook, Coragen, Pleo, Radiant, and Tracer were 0.096 (+108.8%), 0.089 (+94.4%), 0.066 (+44.3%), 0.061 (+33.0%), and 0.053 (+16.5%) μmol/min/mg of protein, respectively [the values of percentage increase were obtained in comparison to the enzyme activity observed for the control (0.046 μmol/min/mg of protein)].

Alkaline phosphatase (ALP) Activity:

A highly significant increase in the ALP activity was observed to be caused by Achook, Radiant, and Tracer, with activity values of 585.5 U/L (196.1%), 584.9 U/L (195.8%), and 334.5 U/L (69.1%), respectively. On the other hand, Coragen caused an inhibition in the ALP activity, with the activity value reaching 141.1 (−28.7%) in comparison to that in the control (197.7 U/L). No significant change relative to control was recorded for Pleo [192.4 U/L (−2.7%)].

Acid phosphatase (ACP) Activity:

Data revealed that exposure to all the investigated insecticides resulted in the stimulation of the ACP activity, with the only exception of Pleo. The ACP activities observed were 66.8 (50.3%), 60.8 (36.9%), 58.1 (30.7%), and 57.5 U/L (29.5%) for the treatments with Radiant, Tracer, Coragen, and Achook, respectively. Pleo treatment inhibited the enzyme activity (40.1 U/L) by a small percentage (9.8%) compared to that observed in the control (44.4 U/L).

Glutamic Oxaloacetic Transaminase (GOT) Activity:

The treatment with Pleo and Radiant caused a significant stimulation in the GOT activity, from 587.0 U/L in the control to 848.3 (44.5%) and 751.6 U/L (28.0%), respectively. On the contrary, GOT activity was observed to be significantly lowered with Tracer and Achook exposures [83.99U/L (−85.7%) and 456.9 U/L (−22.2%), respectively] in comparison to the control value. No significant reduction in the enzyme activity was observed with Coragen treatment (544.8) compared to that in the control

(587.0 U/L).

Glutamic Pyruvic Transaminase (GPT) Activity:

The GPT activity observed in the control insects was 833.6 U/L. The enzyme activity was stimulated to 938.1 U/L (+12.5%) with Coragen exposure, while the enzyme activity was significantly decreased upon treatments with Tracer, Pleo, and Radiant [70.66 (−91.5%), 214.6 (−74.3%), and 770.7 U/L (−7.5%), respectively], compared to that in the control. It should be noted that the activity of GPT observed in the instars treated with Achook was 807.6 U/L (−3.1%), exhibiting no significant changes in comparison to control.

PCA Analysis:

The results of the principal component analysis (PCA), which was performed with eight biomarkers, revealed that the first (PC1) and the second (PC2) principal components explained 66.7% and 19.7% of the total variation in the biomarkers data, respectively (Fig.1).

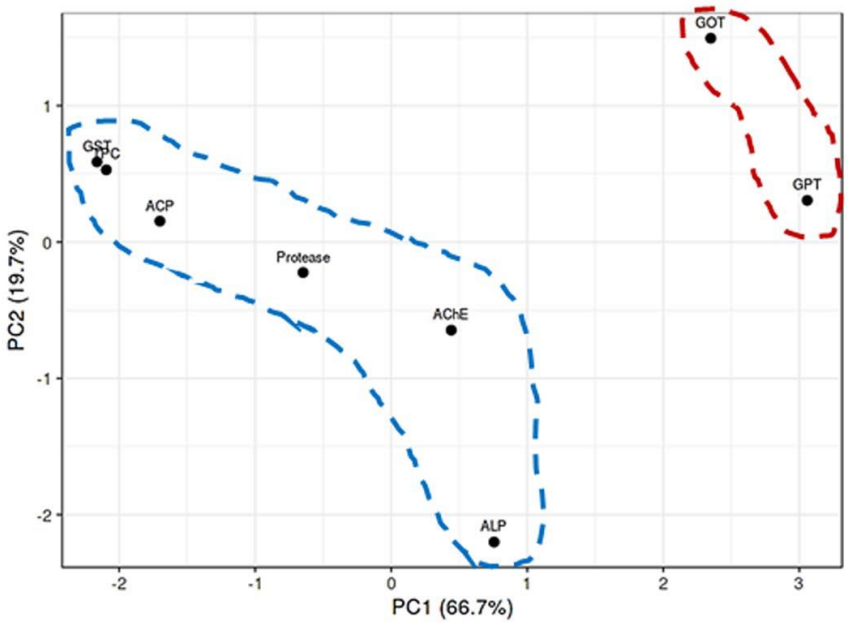

Fig. 1. Principal Component Analysis (PCA) of the 8 biomarkers in the treated *E. cautella* showing the two-dimensional (PC1 and PC2) plot.

RAPD Analysis and Phylogeny:

The DNA samples extracted from the third instar-stage larvae of *E. cautella* that were exposed to the five insecticides were further evaluated, using the RAPD-PCR analysis, for changes in their DNA in comparison to those in the untreated control larvae. The expected changes in the RAPD profile between the control and the insecticide treatments included variations such as the gain or loss of specific bands as well as the changes in the bands intensity.

Out of the fifteen RAPD primers that were used to investigate the impact of these five insecticides in terms of causing genome-wide DNA alterations, only ten primers exhibited polymorphic patterns, as certain bands, which were present in the control and other treatments, were absent in the Radiant-treated sample (Fig. 2).

In total, 118 fragments were amplified, with an average of 7.9 bands per primer. Out of the 118 amplicons, the 59 polymorphic amplicons with a percentage of polymorphism equal to 50% were scored. The number of bands per primer ranged from 4 to 12 bands across the 15 RAPD primers. The primers OP-B03 and OP-B04 generated the

highest number (12) of RAPD amplicons, while the primers OP-B05 and OP-A12 generated the least number (4) of RAPD amplicons (Table 3). The size of the amplified fragments ranged from ~140bp to ~1500bp.

A phylogenetic tree based on the scoring of the present or absent bands in the RAPD profile was constructed. The results demonstrated that Coragen and Achook were similar in their mutability effects, and Coragen exhibited the lowest mutability effect on the genome of *E. cautella*; the results were compared to the control sample (Table 4; and Fig. 3).

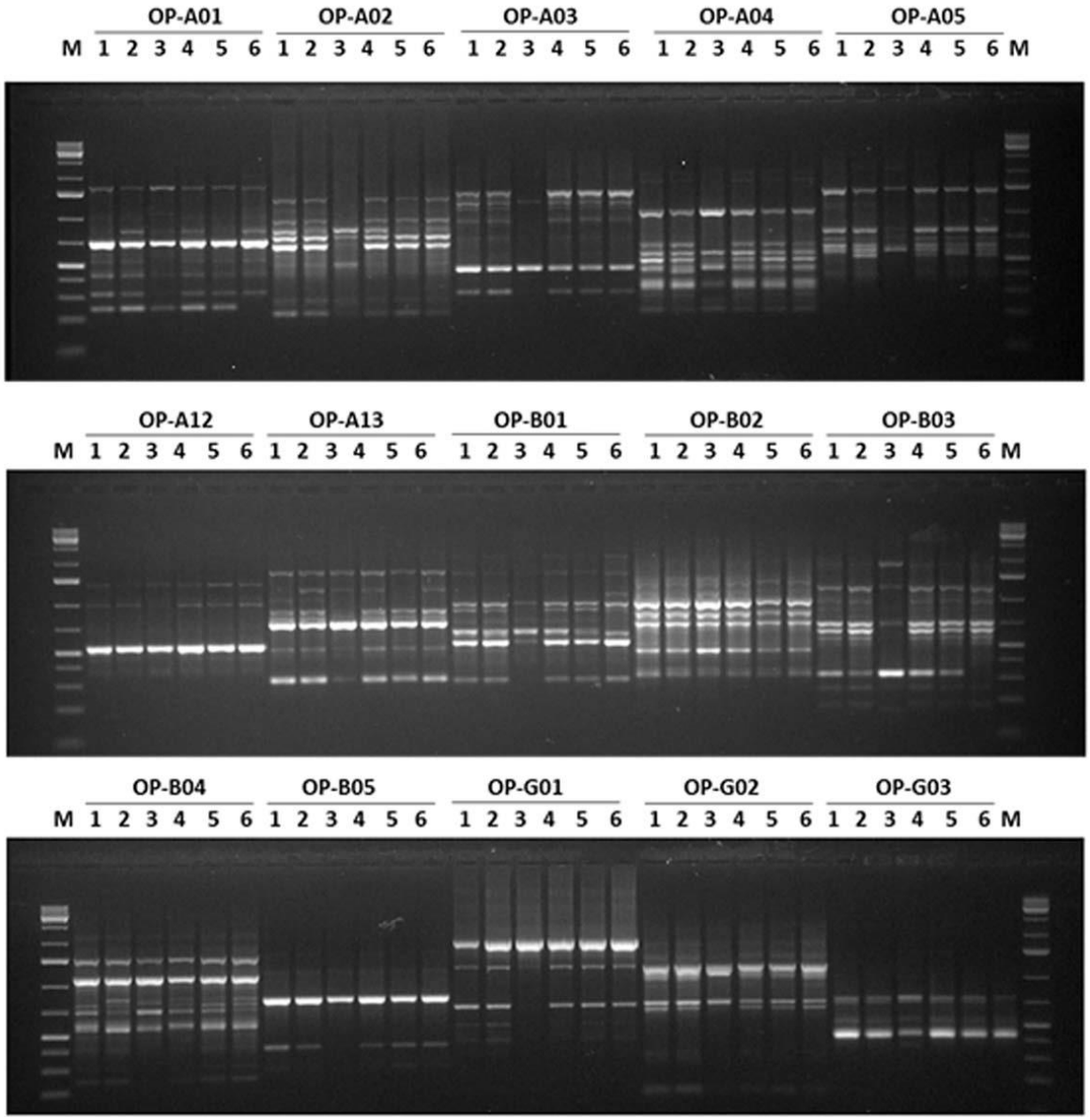

Fig. 2. Comparison of 15 RAPD-PCR profiles of 3rdinstar larvae of *E. cautella* control and treatments with the five insecticides. Lane on both the right and left side is the DNA size marker (100 bp plus). Samples order: 1: Tracer, 2: Pleo, 3: Radiant, 4: Achook, 5: Coragen and 6: Control.

Table 3: Primer Code, primer sequences, number of total bands, polymorphic bands, and percentage of polymorphism of RAPD primers.

Code	Primer Sequence	Number of bands		% of polymorphism
		Total	Polymorphic	
OP-A01	5'-CAGGCCCTTC-3'	8	4	50
OP-A02	5'-TGCCGAGCTG-3'	9	6	67
OP-A03	5'-AGTCAGCCAC-3'	7	4	57
OP-A04	5'-AATCGGGCTG-3'	11	6	55
OP-A05	5'-AGGGGTCTTG-3'	7	4	57
OP-A12	5'-TCGGCGATAG-3'	4	0	0
OP-A13	5'-CAGCACCCAC-3'	8	3	38
OP-B01	5'-GTTTCGCTCC-3'	9	4	44
OP-B02	5'-TGATCCCTGG-3'	9	3	33
OP-B03	5'-CATCCCCCTG-3'	12	6	50
OP-B04	5'-GGACTGGAGT-3'	12	7	58
OP-B05	5'-TGCGCCCTTC-3'	4	2	50
OP-G01	5'-CTACGGAGGA-3'	5	3	60
OP-G02	5'-GGCACTGAGG-3'	8	5	63
OP-G03	5'-GAGCCCTCCA-3'	5	2	40
Total		**118**	**59**	**50**
Average		**7.9**	**3.9**	

Table 4: Jaccard's similarity matrix based on RAPD analysis of the 3rd instars larvae of *E. cautella* treated with five Insecticides (Tracer, Radiant, Coragen, Pleo and Achook) as well as Control.

	Tracer	Pleo	Radiant	Achook	Coragen	Control
Tracer	1					
Pleo	0.977	1				
radiant	0.698	0.709	1			
Achook	0.943	0.958	0.747	1		
Coragen	0.967	0.953	0.683	0.946	1	
Control	0.928	0.934	0.667	0.926	0.951	1

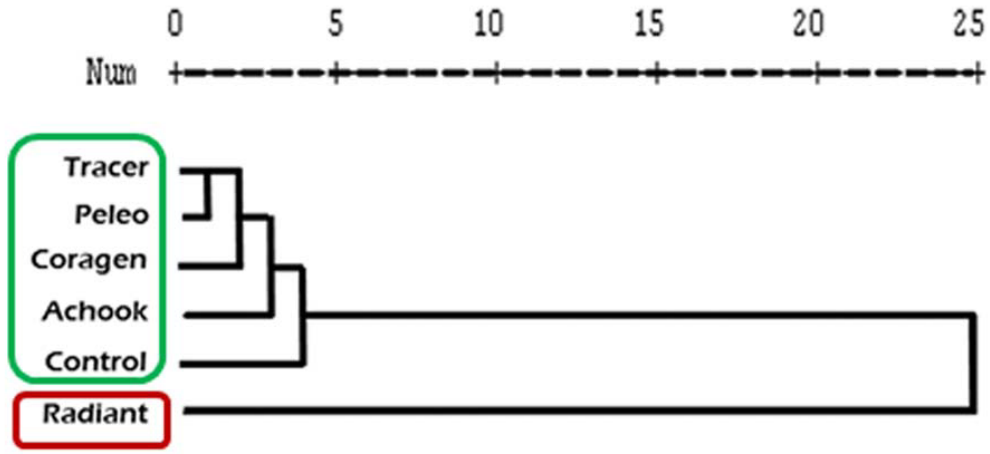

Fig. 3. UPGMA cluster analysis based on Jaccard's similarity coefficient of RAPD analysis of the 3rd instars larvae of *E. cautella* treated with five Insecticides (Tracer, Radiant, Coragen, Pleo and Achook) as well as Control.

DISCUSSION

The efficacy of insecticides is dependent on their modes of action, the insect species, developmental stage, the methods of treatment, and the number of days post-treatment (Rodriguez-Saona *et al.* 2016). In the present study, the results obtained from the investigation of the effects of five novel insecticides on the third instar-stage larvae of *E. cautella* under laboratory conditions indicated major advantages of these novel compounds in reducing the risks posed by conventional insecticides that exert higher toxicity on both mammals and environment.

Insecticidal Toxicity:

Two insecticides–Tracer (Spinosad) and Radiant (Spinetoram), belonging to the same class (Spinosyn) of compounds, which is exhibited a wide range of toxicity. This difference in the toxic effects of the same insecticide might be because of the systemic nature of such insecticides (Maienfisch *et al.* 2001), which might be influenced by the levels of their penetration and persistence in the insect (Nauen *et al.* 2003).

In case of Coragen (chlorantraniliprole) insecticide, an insecticide that has been observed to be safer for humans as well as for the environment (Han *et al.* 2012), the results obtained in the present study revealed that this insecticide possessed strong toxicity for the *E. cautella* larvae. This implied that the insecticide could be used for the control of *E. cautella* infestation in the stores as well as on the trees prior to harvesting the fruit, contributing to the development of integrated pest management (IPM).

The toxicity of Achook (azadirachtin) for the larvae of *E. cautella* was moderate in comparison to the other insecticides. However, it was obvious from the data that Achook exerted strong inhibitory effects on the growth of larvae, as confirmed by the stopped growth in its presence. This may be attributed to the altered feeding behaviour caused by the alteration in the neural control centres upon treatment with azadirachtin (Paranagama and *et al.* 2001). This may also have occurred because of the action of azadirachtin on the endocrine and neuroendocrine systems that regulate the developmental processes in insects (Mordue and Blackwell. 1993).

Although Pleo insecticide (pyridalyl) exhibited high insecticidal activity against various lepidopteran larvae, and was able to control a wide range of lepidopteran pests (Isayama *et al*. 2005), it appeared to exert the lowest toxic effects on the third instar-stage larvae of *E. cautella*, perhaps due to its highly selective toxicity, as it is well known that pyridalyl disrupts the essential functions that are specific to certain lepidopteran insects (Moriya *et al*. 2008).

Biochemical Analysis:

The effects of the insecticides on the natural levels of proteins and enzyme activities in the insects was evaluated in the present study, as both increasing or decreasing of these levels may affect the vital roles of these enzymes/proteins which may lead to disturbances in the physiological system of the insects.

Total Proteins:

The body of an insect contains thousands of different types of proteins, each with its own specific purpose. Nucleoproteins are essential for cell division, enzymes, and hormones. Moreover, proteins are important for energy production and for the control of several chemical reactions in the metabolism of the cells (Assar *et al*. 2012). Salgado (1997) indicated that most of the insecticides that are in use currently act on the target proteins involved in nervous system signalling, cellular respiration, or the growth and development process. Previously, Senthil-Nathan *et al*. (2008) observed that insecticides caused a reduction in the production of enzymes and other protein-based compounds. Therefore, lower levels of proteins would exert a negative effect on the insect pest.

Protease:

Proteases are the enzymes that hydrolyze the peptide bonds present in proteins. An increase in the protease activity was observed upon treatment with Pleo, Achook, and Coragen. This may represent a mechanism of adaptive physiological response through the reutilization of proteolytic products following the insecticide exposure (Ahmad *et al*.1998). A relation was observed between this increment in the protease activity and the high inhibition that occurred in the protein content in comparison to that observed with the other insecticides. In other words, the increment in the protease activity caused protein decomposition. Subsequently, the protein levels decreased upon treatment with both the insecticides. Proteases often function in cascade pathways, and this type of protease cascade occurs in the insect embryonic development and insect immune responses (Kanost and Clem 2012).Achook has also been reported to affect the larvae through starvation; therefore, the excess protease and GST might have been secreted or synthesized by the insects for protecting themselves from this damage (War *et al*. 2014).

Inhibition of the protease enzyme upon exposure to Spinosyn insecticides (Tracer and Radiant) could be attributed to the higher potency and efficacy of Spinosyn compared to the other insecticides to control the insect (Saleem *et al*. 2009). It is also possible that both of these insecticides caused alterations in the general processes of protein synthesis and degradation (Oppert *et al*.1993). Furthermore, any alteration in the protease activity would exert intensive effects on the insect growth and development (War *et al*. 2014).

Acetylcholinesterase (AChE):

Nerve synapses of insects contain a chemical mediator known as acetylcholine (ACh), through which the nerve impulses are transmitted from one nerve axon to another. Acetylcholinesterase is an enzyme that hydrolyzes acetylcholine to prevent the accumulation of the latter at nerve synapses as its accumulation results in disruption of nerve transmission ultimately leading to death (Das 2013).

The data obtained in the present study demonstrated that the effects of the treatment with the studied insecticides on the activity of acetylcholinesterase varied with the type of toxicant being analyzed. Only a slight decrease in the AChE activity was observed upon treatment with Tracer and Pleo, while the decrease in the activity was obvious in the case of Radiant. This inhibition might have been a result of the ability of Spinosyns to inhibit AChE in various parts of the nervous system, thereby disrupting the neurotransmission at that location (O'Brien *et al*. 1974). Krist (2010) stated that Spinosyn A affected the insect nervous system and neuronal activity through the excitation of motor neurons, causing muscle contractions, ultimately leading to paralysis and death.

On the contrary, an increase in the AChE activity was observed upon treatment with Achook, and a substantial increase was observed in the case of Coragen. This increase could be a result of the effect of Coragen on muscle contraction, or it could be a response mechanism for exposure to pesticides and might have been caused by the increased cholinesterase biosynthesis (Di-Marzio and Tortorelli 1994). Most of the pesticides are neurologically active compounds that exert a broad range of impact across the nervous system, and this impact is attenuated by modifications in the acetyl choline and acetate functions (Rajashekar *et al* .2014).

Glutathione S-transferase (GST):

Glutathione S-transferase (GST) represents a group of multifunctional proteins that play several roles in the detoxification process (Grant and Matsumura 1989). Insects are able to metabolize and degrade toxic substances in order to survive in a chemically unsuitable environment. However, their capacity of detoxification is dependent on variation among the species, on the developmental stage, as well as on the nature of insect's recent environment (Sívori *et al.*1997). On the basis of these facts, it is inferred

that the increase in the GST activity in the third instar-stage larvae of *E. cautella* which was observed in all the treatments might have been the result of the stress induced by the toxicity of the insecticides which led to an increase in the GST enzyme to resist the oxidative damage. These results were in agreement with the findings reported by War *et al*. (2014). Additionally, in the present study, a variation could be observed in the activity of GST for all the investigated insecticides. For example, there was a remarkable increment in the GST activity in case of Achook and Coragen compared to the other insecticides. This confirmed the selective toxicity of insecticides (Sívori *et al.* 1997).

Phosphatases:

Acid (ACP) and alkaline (ALP) phosphatases are hydrolytic enzymes that hydrolyze the phosphate monoesters under acidic or alkaline conditions, respectively (JandaandBenesova1991). It was obvious from the data obtained in the present study that the activity of ALP increased when Tracer, Radiant, and Achook were used. This could be explained by the role of this enzyme against these insecticides during the detoxification process (Zibaee *et al*. 2011). However, the ALP activity was decreased in the *E. cautella* larvae when treated with Coragen. This might have been due to the effect of this insecticide on the processes of evolution in the insect, as reported by Wu (1990). Moreover, the insecticide probably caused disruption in certain physiological processes in the midget, just as it was reported in a study by Klowden (2007) where it was stated that these enzymes are involved in the digestion and nutrient conduction in the midget of the insects. On the contrary, upon Pleo treatment, the activity was not affected, as the ALP enzymes might not detoxify this insecticide.

The increase in the ACP enzyme activity observed in the present study could be attributed to the increased number of lysosomes (Csikó sand Sass 1997) in response to the studied insecticides (Tracer, Radiant, Achook, and Coragen). Moreover, this observation might have been a result of interference of ACP with food digestion and absorption (Senthil-Nathan *et al*. 2004). In contrast, a non-significant decrease was observed in the ACP activity upon exposure to Pleo. This decline in the ACP activity could be a result of the effect of insecticides on phosphorous liberation for energy metabolism or on the rate of transport of the metabolites (Senthil-Nathan *et al.* 2005).

The changes in the activity of phosphatases could be altered according to the metabolism in the insect midgut, causing reduction or elevation in the extrication of the phosphate groups for energy production (Ramzi *et al.* 2014).

Transaminases:

The cuticle of insects consists of proteins and chitin. The building blocks for protein synthesis are obtained from the amino acid pool maintained mainly by the transformation between glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). These enzymes are essential for the formation of non-essential amino acids (Mordue and Goldsworthy 1973).

In the present study, GOT enzyme activity was observed to have increased upon treatments with Radiant and Pleo. The usage of these two insecticides might have exerted negative effects on the secretion of neurosecretory hormones (Abulyazid *et al* .2005).

On the contrary, Tracer, Achook, and Coragen caused a reduction in the GOT activity, which could be attributed to the imbalance in the levels of amino acids that fit the protein synthesis process.

On the other hand, GPT enzyme activity decreased for all the studied insecticides, with the only exception of Coragen which exhibited a slight increase in the activity level. The decline in the GPT activity could be due to a disturbance in the link between carbohydrate and protein metabolism (Tanani *et al*. 2016). It could also be because of low energy production. GOT and GPT transaminases play important roles in Kreb's cycle,

which is a high energy-producing cycle (Azmi *et al*. 1998). Therefore, the changes that occur in the normal levels of transaminase enzymes (GOT and GPT), either an increase or decrease, might be a result of the disturbance in the physiological system of the insect.

RAPD Analysis:

In the present study, Random Amplified Polymorphic DNA (RAPD) analysis was used as an effective approach to detect the genotoxicity of the studied insecticides against the third instar-stage larvae. One of the advantages of RAPD is that it allows a qualitative assessment of the DNA effects, and the nature of the variations in the profiles may only be speculated when the amplicons are analyzed using sequencing or any other suitable method. The exposure to compounds such as insecticides may cause damages to the stability of the genomic DNA template, such as mutations and rearrangements in the DNA structure, thereby causing changes in the RAPD profile, expressed in terms of presence or absence of certain bands or through variations in the band intensities **(**Yildirim and Agar 2016;Lalrotluanga 2011). Jones and Kortenkamp (2000) demonstrated that the changes that occurred in the RAPD profile due to genotoxic compounds related directly to the genome template stability and certain other parameters such as soluble protein levels, as these points of mutations promoted modifications in the structure of the cell molecules. In the present study, 15 RAPD primers were used against the third instar-stage larvae in order to detect the points of mutation. Ten primers exhibited polymorphic bands in the samples treated with Radiant, while the results for the other samples were similar to that of the control. Therefore, it was concluded that Radiant isa genotoxic insecticide that causes several changes in the genomic DNA. Furthermore, the results of the RAPD analysis were used for the construction of a dendrogram to determine the relationship between the five insecticides, and it was observed that Coragen and Achook were similar in their mutability effects and that Coragen exerted the lowest mutability effect on the insect DNA.

In summary, it may be stated that the present study, to the best of our knowledge, is the pioneer case-study that investigated and analyzed the genome-wide DNA mutability, biochemical effects, and the toxicity levels of five novel insecticides (Tracer, Radiant, Coragen, Pleo, and Achook) in relation to the control of the date palm fruit pest *Ephestia cautella*. It is proposed that the identified variations in the RAPD patterns may be due to the occurrence of structural changes or DNA damage or due to changes in the primer binding sites. The RAPD analyses have demonstrated their ability and effectiveness in detecting DNA damage/changes caused by the studied insecticides. The results of the genome-wide mutability, biochemical analysis, and insecticide toxicity corroborated each other. The slow development and disturbance in the activity of various enzymes observed in the present study could be explained by the reduction in the protein content that occurred for all the treatments with insecticides. This implied that protein biosynthesis might be inhibited or decreased under the toxic effect of the studied insecticides. Based on the results of the present study, it was confirmed that the mutagenic effects paralleled with protein content and insecticide toxicity.

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