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Bioinsecticides Induce Change in Biochemical and Immunological Parameters of *Spodoptera Littoralis Larvae*

Badriah M. K. Asiri

Department of Biology, Faculty of Science for Girls, King Abdulaziz University, Jeddah, Saudi Arabia

Email address:
gibriel2003@gmail.com

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Abstract: The cotton leaf worm, *Spodoptera littoralis*, is a major pest in Egypt causes severe quantitative and qualitative losses of cotton and other economic crops. This study examines the effect of *Caltropis proceraa* and *Atriplex halimus extracts* on the 4nd instar larvae. This study was carried out to identify the effect plant extracts on biochemical parameters and differential and total haemocyte counts of *S. littoralis* after treated with LC_{25} of *Caltropis proceraa* and *Atriplex halimus extracts* for 48 hours (treated with each extract in separate). Marked biochemical changes, however, being recognized in pest as marked decrease in total lipids, total protein and glucose contents. The activities of both aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) are also being highly affected. Four types of haemocytes marked by; prohaemocytes (PRs), plasmatocytes (PLs), granulocytes (GRs) and oenocytoids (OEs). The percentage of PRs decreased in insects fed on leaves treated with *Caltropis proceraa* and *Atriplex halimus* plant. The percentage of PLs increased, while the percentage of GRs decreased in all tested insects treated. The percentages of oenocytoids (OEs) increased in insect fed treated compared with control. Results indicated that total Haemocyte Counts (THCs) of insects fed on leaves treated significantly decreased in all insects treated.

Keywords: *Spodoptera Littoralis* Larvae, *Caltropis Proceraa*, *Atriplex Halimus*, Biochemical, Immunological Parameters

1. Introduction

In Egypt many crops and various vegetables attacked by numerous insect pests. Among these, the lepidopterous insects in general and the cotton leafworm *Spodoptera littoralis* (Boisd) in particular are the most damaging. It is extremely polyphagous and always inflicts excessive damage when it occurs in masses during certain years, commonly referred to as cotton worm monsoons [1]. The intensive use of chemical control measures to manage this pest in particular on cotton has resulted in the development of resistance to almost all classes of insecticides used [2]. The cotton leaf worm, *S. littoralis* (Boised) is swarming polyphagous, foliage feeding insect that is distributed throughout the world. This insect is one of the major cotton pests which causes considerable damage to many of cotton crops [3-5]. Many different countries search for less dangerous pesticides by using the naturally occurring herbs that can be applied effectively in habitats [6-9]. Many studies have reported that plants are one of the richest sources which can be used for pest control [10.11]. In Egypt, attempts have been made to monitor insecticidal activity of different plant extracts against many insects [9.12].

The aim of the present investigation is to determine insecticidal effects of two different organic solvents of *Caltropis proceraa* and *Atriplex halimus* against the 4th larval instar of *S. littoralis*. The present study was also extended to evaluate the most potent promising plant extracts post formulation on biochemical and Immunological parameters of the 4th larval instar of *S. littoralis*

2. Material and Methods

2.1. Insect Maintenance

A colony of the cotton leaf worm *Spodoptera littoralis* (Family: Lepidoptera) was maintained in the laboratory for many generations at 25±2°C according to Ghoneim [13]. It was obtained from Central Laboratory of insecticide, Agriculture Research Center Dokki Giza. Experiments were

carried out on the 4th instar larvae.

2.2. Botanical Plants

Caltropis procera (Asclepiadaceae) plant was collected from the fields of Giza governorate, Egypt, but *Atriplex halimus* (Chenopediaceae) was collected from Borg El-Arab on the Mediterranean coast. These plants were kindly identified by the Botany Department, Faculty of Science, Cairo University. The whole overground parts of these plants were left to dry in the air and then in an oven at 50°C.

2.3. Plant Extract Preparation

The whole overground parts of these plants were left to dry in the air and then in an oven at 50°C and powdered by a mixer. Each plant powder was then extracted with methanol (70%) by soaking at room temperature (25±3°C). The solvents were distilled off under vacuum and the crude extract residues were assayed as aqueous solutions.

The extracts of *C. procera* and *A. halimus* were carried out by Freedman et al. [14] method with some modification. The crude extract was transferred quantitively to a clean and weighted flask and kept in the refrigerator until used for toxicological investigation. Consider the crude extract as 100% a known weight of the crude was added to a similar volume of the solvent (acetone) to obtain the stock solution. A stock solution of each extract was made prior to use. The different concentrations of each plant extract were tested on the 4th instar larvae of *S. littoralis*. The Leaf dipping technique was used. The same sizes of fresh castor bean leaves for *S. littoralis* were dipped in each tested concentration of plant extracts and in the control for 20 seconds and left to dry. The dried leaves were put singly in plastic cups. Tens insects were transferred to each cup and allowed to feed on the treated leaves for one day. Three replicates for each concentration were done. After 24h surviving pests were transferred to clean cups and supplied daily with untreated leaves. A group of insects was left unexposed under the same laboratory conditions as a control. Mortality was recorded according to Abbout [15]. Also, development duration of instars was observed and calculate according to Dempster [16]. Malformation of different stages of pests was observed and the percentage of deformation was calculated. Mortality values after exposure for 24h were analyses by probit analysis (LDP line) to obtain LC_{50} LC_{90} and slopes for each extract according to the method adopted by Finney [17]. The most effective plant extracts were selected for further experiments.

2.4. Biochemical Studies and Sampling

After feeding on treated the leaves with sublethal concentration (LC_{25}) of the two extracts for 48 hours, alive insects were collected and allowed to feed on leaves after 1, 2 and 10 days in the case of *S. littoralis*. The number of pests (larvae) was taken and subjected directly for biochemical assays.

The collected pests after each time inter interval. The homogenates were centrifuged at 3500 r p m. For 10 minutes and the supernatants were filtered through glass wool to remove fatty materials and kept in deep freezer at-20°C till the use for determination of glucose, total protein and lipid concentration and aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) activities. aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) activities were determined according to the method of Reitman and Frankel [18]. Total protein was determined according to the method of Weichselbaum [19]. Glucose concentration was determined according to the method of Trinder [20]. and Total lipids were determined according to the method of Zollner and Kirsch [21]. Three samples used in each experiment

2.5. Immunological Studies

After feeding on treated the leaves with sublethal concentration (LC_{10}) of the extracts for 48 hours, alive insects were collected and allowed to feed on normal leaves after 48 hours for *S. littoralis larvae*. A specific number of pests were taken and subjected directly for Immunological studies.

2.5.1. Differential Haemocyte Counts (DHCs)

After 48 hrs of feeding on treatedleaves, the haemolymph samples were withdrawn from the coxal corium according to Hoffmann [22]. Haemocytes (blood films) were stained by Giemsa stain and examined by light microscopy. The haemocytes are classified according to the classification scheme.

Various haemocytes were differentially counted by examining approximately 100 cells per slide. 10 slides prepared from 10 locusts / count. The percentages of haemocyte types were calculated by the formula:

$$= \frac{Number\ of\ each\ haemocyte\ type \times 100\%}{Total\ number\ of\ haemocytes\ examined}$$

2.5.2. Total Haemocyte Counts (THCs)

The oozed haemolymph was collected directly into Thoma- white blood cell diluting pipette to the mark 0.5. Diluting solution (NaCl- 4.65 g, KCl- 0.15 g, $CaCl_2$- 0.11 g, crystal violet- 0.05 g and acetic acid- 1.25 ml/liter distilled water) was taken up to the mark 11 on the pipette (dilution is 20). The mixture was dispensed to both chambers of the counting slide (the chamber depth is 1.0 mm) and the total number of cells were counted according to the formula of Jones [23]:

$$\frac{Haemocytes\ in\ 1\ mm\ squares\ X\ dilution\ X\ depth\ of\ chamber}{Number\ of\ 1mm\ squares\ counted}$$

2.6. Statisitical Analyses

Date obtained were analyzed by student (t) test according to the equation of Dixon and Massay [24]. Significant difference were established at $P<0.05$ and $P<0.01$ levels.

3. Results

3.1. Effect Plant Extracts on the 4th Larval Instar of the Cotton Leaf Worm Spodoptera Littoralis 24 Days After Treatment

From the data recorded in Table (1), it was observed that, the two plant extracts had an effect on the cotton leaf worm, *S. littoralis*. Generally, the percentages of larvae mortality were higher by using *Atriplex halimus* as extract than *Caltropis proceraa*. The LC_{50} values were 10.4 and 8.8 ppm for *Atriplex halimus* and *Caltropis proceraa* extracts, respectively. Also, The LC_{90} values were 13.2 and 16.2 ppm for *Atriplex halimus* and *Caltropis proceraa* extracts, respectively.

Table 1. *Toxicological evaluation of plant extract against Spodoptera littoralis larvae After 24 days.*

	Toxicity of match Ppm			Slop Function
	LC_{25}	LC_{50}	LC_{90}	
Caltropis procera	7.6	10.4	16.2	2.8
Atriplex halimus	4.8	8.8	13.2	2.2

3.2. Biochemical Effects

3.2.1. Effect of the Tested Extracts on ASAT Activity

The activity of aspartate aminotransferase in the whole body homogenate of the 4th instar larvae of *S. littoralis* was determined after treatment with LC_{25} extracts of *Caltropis procera and Atriplex halimus*. The present results in Table (2). The data recorded revealed, increase in the level of the enzyme activity throughout all the tested time intervals after exposure to LC_{25} of the two tested extracts. As compared to the control level the percentage of changes at the 1st, 5th and 10th days were 5.45%, 11.8% and 17.9%, respectively after exposure to *Atriplex halimus* extract and 10.72%, -21.1% and 32.17% respectively post exposure to *Atriplex halimus*.

Table 2. *Mean activity of ASAT in Spodoptera littoralis larvae treated with the extract of Caltropis procera and Atriplex halimus.*

Plant extracts	Dose mg/ml	Exposure period (day)					
	LC25	1 Mg/ml	Change %	5 Mg/ml	Change %	10 Mg/ml	Change %
	Control	22 ±1.3		18±0.64		16.2±0.41	
Caltropis procera	7.6 ppm	23.2 ± 0.62	5.45	20.13±0.7*	11.8	19.1±0.8*	17.9
Atriplex halimus	4.8 ppm	24.36±1.1*	10.72	21.8±0.8**	-21.1	21.5±0.6**	32.17

*Significant P<0.05 ** highly significant P<0.01, Values are presented as Mean± SD.

Table 3. *Mean activity of ALAT in Spodoptera littoralis larvae treated with the extract of Caltropis procera and Atriplex halimus.*

Formulated extract of	Dose mg/ml	Exposure period (day)					
	LC25	1 Mg/ml	Change %	5 Mg/ml	Change %	10 Mg/ml	Change %
	Control	16.6±0.5		14.4±0.5		12.11±0.19	
Caltropis procera	7.6	15.12±0.12	-8.9	13.2±0.15*	-8.3	12.5±0.25	3..22
Atriplex halimus	4.8	14.2±0.11**	-14.46	12.12±0.2**	-15.8	13.1±0.16	8.18

Values are presented as Mean± SD. *Significant P<0.05,** highly significant P<0.01

3.2.2. Effect of the Tested Formulated Extracts on ALAT Activity

The data recorded in table (4) show, the effect of LC_{25} of both plants induced a marked decrease in ALAT activity on the 1st and 5th days. This was followed by an increase in the 10th days for the two tested formulated extracts. The percentage of change at 1, 5 and 10 days were 14.46%, 15.8% and 8.18%, respectively in case of *Caltropis procera* extracts and as compared with the control.

Table 4. *Effect Caltropis procera and Atriplex halimus extract on the total protein concentration of Spodoptera littoralis larvae.*

Plant extract	Dose mg/ml	Exposure period (day)					
	LC25	1 Mg/ml	Change %	5 Mg/ml	Change %	10 Mg/ml	Change %
	Control	43.15±0.76		46.6±0. 44		43.23±0.4	
Caltropis procera	7.6	39.6±0.4**	-8.22	39.8±0.66**	-14.72	36.14±0.115**	-16.4
Atriplex halimus	4.8	38.67±0.8**	-10.38	38.7±0.326**	-16. 95	35.13±0.32**	-18.73

Values are presented as Mean± SD *Significant P<0.05, ** highly significant P<0.01

3.2.3. Effect of the Tested Extracts of Total Protein

The data in table (4) show, the effect of LC_{25} extracts of *Caltropis procera* and *Atriplex halimus* on the concentration of total protein of the 4th instar larvae of *S. littoralis*. From the data recorded, it was shown that, there was a highly significant decrease in the level of total protein of treated larvae post exposure to LC_{25} of *Caltropis procera* and *Atriplex halimus* extract extracts throughout all the tested time intervals. The percentage of changes reached its maximal after 10 days -16.4% and -18.73%) for both extracts *altropis procera* and *Atriplex halimus* extract, respectively.

3.2.4. Effect of the Tested Extracts on Glucose Content

Data presented in table (5) shows the effect of LC_{25} extracts of *Caltropis procera* and *Atriplex halimus* on glucose concentration of 4th instar larvae of *S. littoralis*.

The data recorded shows a significant decrease of glucose content throughout all the tested periods. The recorded values significantly decreased by-14. 65%- 16.17% and -26.24% post the 1st, 5th and 10th days of *altropis procera* exposure. On the other hand, the post exposure to the dose level LC_{25} of *Caltropis procera* extract showed a more highly significant decrease in *S. littoralis* larvae glucose level on the 1st and 10th days, the recorded values being decreased by -17.16% and -30.11%, respectively as compared to the control level.

Table 5. *Effect Caltropis procera and Atriplex halimus extract on glucose concentration of Spodoptera littoralis larvae.*

Formulated extract of	Dose mg/ml	Exposure period (day)					
	LC_{25}	1 Mg/ml	Change %	5 Mg/ml	Change %	10 Mg/ml	Change %
	Control	68.2±0.43		53.8±0.98		62.10±0.68	
Caltropis procera	7.6	58.21±0.42**	-14. 65	45.1±1.02**	-16.17	45.8±0.73**	-26.24
Atriplex halimus	4.8	56.5±0.62**	-17.16	42.1±0.73**	-21.82	43.4±0.68**	-30.11

Values are presented as Mean± SD.. *Significant P<0.05, ** highly significant P<0.01

Table 6. *Effect Caltropis procera and Atriplex halimus extract on total lipid concentration of Spodoptera littoralis larvae.*

Formulated extract of	Dose mg/ml	Exposure period (day)					
	LC25	1 Mg. ml	Change %	5 Mg/ml	Change %	10 Mg/	Change %
	Control	9.61±0.43		8.82±0.32		7.52±0.0713	
Caltropis procera	7.6	8.1±0.42**	-15.62	6.8±0.21**	-22.9	5.54±0.14**	-26.33
Atriplex halimus	4.8	7.21±0.4**	-24.97	5.8±0.42**	-34.42	4.62±0.44**	-38.56

Values are presented as Mean± SD., *Significant P<0.05, ** highly significant P<0.01

3.2.5. Effect of the Tested Extracts on Total Lipid

Table (7) shows the effect of LC_{25} of *Caltropis procera* and *Atriplex halimus* extracts on the concentration of total lipid of 4 thinstar larvae of *S. littoralis*. The data recorded revealed, highly significant decrease in the level of total lipid post treatment with LC_{25} of *Caltropis procera* and *Atriplex halimus* throughout all the tested time intervals. The maximal percentage of reduction was reached after 10 days (--38.56% and -38.56%) for both *Caltropis procera* and *Atriplex halimus* extracts, respectively.

Table 7. *Differential haemocyte counts (DHCs) of S Spodoptera littoralis larvae fed on leave at 48 hr post-injection.*

Experimental plants	Haemocyte type %●			
	Prohaemocytes	Plasmatocytes	Granulocytes	Oenocytoids
Control	13.6	51.2	32	6
Caltropis procera	2.5	62.3	20.4	7.20
Atriplex halimus	0.0	65.2	17.4	8.30

3.3. Immunological Studies

3.3.1. Differential Haemocyte Counts (DHCs)

The present results in table 7 showed that the percentages of different haemocyte types of adults (2-4 days) fed on leaves treated with plant extracts. The percentage of Prohaemocytes (PRs) decreased in insects fed on leaves treated with *Caltropis procera* (2.5%) compared to the control (13.6%). PRs disappeared In insects fed on leaves with *Atriplex halimus*.

The results also recorded that the highest number of these Plasmatocytes (PLs) was in insects fed on *Atriplex halimus*. (65.2%) than control insects (51.2). Granulocytes (GRs) percent decreased when insects fed on *Caltropis procera* and *Atriplex halimus*. (20.4&17.4 respectively) compared with control insects (32). The percentages of Oenocytoids (OEs) increased in insect fed on on *Caltropis procera* and *Atriplex halimus*. (7.20± 8.3 respectively) compared with control (6).

3.3.2. Total Haemocyte Counts (THC)

The present results in Figure (1) showed the effects of LC_{25} of *plant extracts* on *S. littoralis* adults. The THC values showed a significant decrease ($P < 0.05$) with all adults fed on leaves treated with *Caltropis procera* and *Atriplex halimus* (7885±31.2, 6779±19.4 cells/mm^3, respectively) compared with controls (8665±34.5 cells/mm).

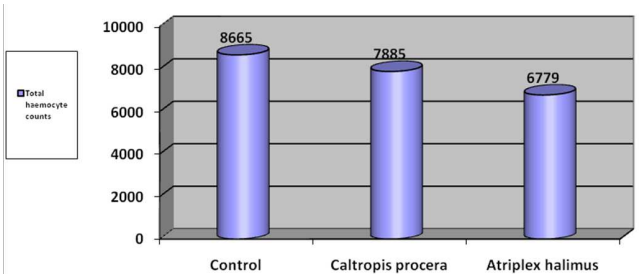

Figure 1. *Effects of LC_{25} of extract of Caltropis procera and Atriplex halimus on total haemocyte counts of Spodoptera littoralis.*

4. Discussion

The present results showed that The LC_{50} values were 10.4 and 8.8 ppm for *Atriplex halimus* and *Caltropis proceraa*

extracts, respectively. Also, The LC_{90} values were 13.2 and 16.2 ppm for *Atriplex halimus* and *Caltropis proceraa* extracts, respectively. The maximum effect was given with *Atriplex halimus* followed by *Caltropis proceraa*. This is in accordance with the findings of other investigators using different plant insecticides, including *A. indica* [5, 25]. The high insecticidal potency of *Atriplex halimus* than the extract of *Caltropis proceraa* can be attributed to several factors, including plant specific differences of the extracted active ingredients, types of the extracted products, differences in their mode of action, Method of penetration and the behavioral characteristics of the studied pests [26]. The activity is due to the presence of saponin components [7] triterpenoid components [26]. Alkaloids components [27] Tannins compounds [28] although the effect seems to be very specific dependent.

The present data obtained from the biochemical effect of the tested extracts of *Atriplex halimus and Caltropis proceraa* at a sublethal dose also confirms different degrees of action on total protein content, total lipid content, glucose content and transaminases activity of *S. littoralis* 4th larval instar.

In the present study, the activity of ALAT of both insects decreased and the activity of ASAT increased throughout all the tested periods. Elevation of ASAT after exposure to different toxic agents in invertebrate and vertebrate animals has been investigated by Bakry et al. [29]. In the present study, the greater and continuous release of ASAT might be due to the necessity of enhanced domination of aspartic acid in the process of gluconeogensis especially under conditions of impaired carbohydrate metabolism and/or a potential induced damage to parenchymal cells as reported by Rawi et al., [7]. On the other hand, the higher decrease in ALAT activity compared to that of ASAT suggests that with the use of formulating extract of both plants, the reaction involving oxaloacetate seems to gain more importance than other pyruvate [7].

Data obtained showed that the tested dose level of *Atriplex halimus and Caltropis proceraa* the recorded values in pest showed marked decreases of the total protein content. Extensive work has been carried out in order to determine how various toxic agents affect protein synthesis. A diminution in the rate of ATP synthesis and inhibition of RNA synthesis is also the main causes of decreased total protein content [30]. Also, Ahmed *et al*. [31, 32] and Rawi et al. [6] reported that protein leakage during intoxication may arise from reduced body weight. Conversion of protein into amino acids, degradation of protein to release energy or to the direct effect of the tested extracts on the amino acid transport of the cell.

The behavioral pattern changes of glucose level post treatment showed significant decreases in the past. These findings coincide with those of Abdo El-Ela et al. [33]. Chitra and Reddy [34] showed reduction in carbohydrate content of different instar larvae when treated with *Ammi majus, Apium graveolens, Melia azedarach* and *Vince rosea* extracts. Also, as reported by another investigator [35]. Amylase is the most sensitive enzyme to the action of several molluscicddes. Otherwise the inhibition of the enzyme activity will in turn reduce glucose level in both pests through decreasing the hydrolytic rate of glycogen.

Data obtained in the present work disclosed a significant reduction in the lipid contents of the pest throughout all the tested periods. These findings are in agreement with those obtained by Mostafa [36] that *Melia azedarach* and *Vince rosea* have significant inhibition in the lipid content in the case of the 3rd nymphal instar of *S. gregaria* and 2nd larval instar of fruit fly *C. capitata.* Anitha et. al. [37] indicated that histopathological changes are one of the most definitive indicators of fat changes.

Circulating haemocytes have important positions in the immune system, metabolism, detoxification, and play a decisive role in the defense of xenobiotics or microbial infection [38]. This work proceeded to the impact of *Caltropis proceraa* and *Atriplex halimus* extracts on differential and total haemocyte count of adult locust Four types of haemocytes were found in locust fed on each experimental plant (PRs, PLs, GRs and OEs).

The present results investigated that normal locusts fed on leaves treated with *Atriplex halimus* extracts (high protein diet) had the highest percentage of PLs (65.2%) and the highest percentage of GRs (17.4%) was found in normal locusts fed on leaves treated with *Atriplex halimus* sorghum (high carbohydrate diet). Szymaś and Jędruszuk, [39] stated that GRs cells may act as storage cells in insect. Further, Following *Spodoptera littoralis* fed on leaves with plant extracts, the PLs increased and the GRs decreased at post-injection in all treatments than control. The increase in PLs was attributed to the release of sessile haemocytes after infection, and this can be attributed the decline in GRs for their involvement in phagocytosis and the formation of nodules. This concept is supported by the observations of Anandakumar and Michael,[40] on silk worm larvae infected with *Bti.* Hillyer, et al. [41] Shed light on GRs which play an important role to phagocytose bacteria and showed that a reduced GRs population in insects may affect their capacity to clear high infection levels. In addition, insects fed with protein might invest more resources in certain types of haemocytes (e.g. Grs or PLs) at the expense of others types (Alaux et al.,[42]). Also, Hillyer, et al. [41] suggested that the decrease of PRs after injection may be explained with the transformation of PRs (stem cells) into PLs and GRs which are needed in phagocytosis and nodule formation. From our observation to haemocyte pathological conditions we suggested that toxins secreted by injected bacteria cause lysis of haemocytes or induce programmed cell death.

The low values presented by OEs in *Spodoptera littoralis* fed on leaves treated with plant extracts in this study was confirmed by Silva, et al.[43] who reported the absence of quantitative alterations of OE in response to pathogens in the haemocoel of *Anastrepha oblique* larvae. It is known that the OE cells are involved in the production of prophenoloxidase, an enzyme that actively participates in the mechanisms of defense in insects [44].

It is expected to be accompanied with a higher protein and higher resistance to disease phenotypic higher concentrations. Food natural protein is an important factor in the function of the cellular system of the insect haemolymph whereas lack of protein in the diet causes significant changes in the function and structure of the cellular phone system from haemolymph [39]. Overall, The total number of haemocytes (THC) closely and positively associated with the rate of phagocytosis and the formation of nodules. The total number of haemocytes in haemolymph more likely to reflecting the immune system's ability for dealing with pathogens or chemical molecules [29]. The present results indicated that THCs decreased significantly after treated with plant extracts than controls. Similar observations were also reported by Anandakumar and Michael, [40] Who observed that about 15.3% of the total haemocytes was decreased after a bacterial infection in the silkworm larvae, compared with the normal worms. They pointed out that the decline in the total THCs worm infected with bacteria due to the depletion of prohaemocytes, which represents more than the total census lowered comparison to normal. Banville, et al. [45] mentioned that the immune response requires a high degree of resources to maintain the optimum level of infection control.

5. Conclusion

It was concluded that the extract of *Caltropis proceraa* and *Atriplex halimus* against *S. littoralis* larvae and Marked biochemical changes however, being recognized in pest as marked decrease in total lipids, total protein and glucose contents. The activities of both ALAT and ASAT are also being highly affected. Finally, we can conclude that, *S. littoralis* adults feed on leaves treated with *Caltropis proceraa* and *Atriplex halimus* had considerable variation in the differential and total haemocyt counts. In conclusion, the present data showed that the emulsifiable concentrate of *Caltropis proceraa* and *Atriplex halimus* has high toxicity on *S. littoralis*

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