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The potency of nanoemulsion of Clove oil and their main component Eugenol on some biochemical and histological aspects on *Spodoptera littoralis* (Boisd) (Lepidoptera: Noctuidae)

Mona N. Wahba[1] , Enase Adel Abdelatef[1] and Trandil F. Wahba[2]

[1]Plant Protection Research Institute, Agriculture Research Center, Dokki. Giza, Egypt.
[2]Insecticide Bioassay Department, Central Agricultural Pesticides Lab. (CAPL), Agriculture Research Center (ARC), Alexandria, Egypt.

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ABSTRACT
Although synthetic insecticides are most effective for controlling insects, their extensive application has hazardous effects on the environment and human health. Recently, nanotechnology techniques are open up novel applications in the field of agrochemical formulations. Nanoemulsion (NEs) attracted great attention in delivering and enhancing essential oils many poorly soluble in water. In this study, we evaluated the larvicidal activity of the clove oil and its main component eugenol, and both NE formulations against 4th instar larvae of *S. littorals* under laboratory conditions. NEs of clove oil and eugenol were prepared by high-energy ultrasonication, 5% EO was the optimal concentration for preparations. The droplet sizes of clove oil and eugenol NEs were 207nm and 54.68 nm and the polydispersity index (PDI) were 0.33 and 0.26, respectively. Generally, eugenol was more effective than clove oil, where the LC_{50} gradually decreased from 4.64 to 1.65 and 11.97 to 2.18 % after 1and 5days of exposure. Clove NE exhibited more toxicity than clove oil emulsion,the LC_{50} values were 1.77 and 2.18% at the end of the experiment. Besides, the eugenol NE formula demonstrated significant toxicity compared to the eugenol emulsion. Results noticed that all tested materials decreased protein levels in larvae of *S. littorals* by 46.31% and 31.91% with clove oil and clove NE. increases in aspartate aminotransferase (AST) activity were recorded with clove oil and clove NE but decreased activity with eugenol and eugenol NE. As well, alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activity decreased with all treatments. Also, the histological disturbance was estimated in this study as ultrastructural alterations in the midgut of the 4th instar larvae. histological examination showed a high distribution of this larvae's midgut after treatments with LC_{50} values of all tested materials, especially with NE. Midgut epithelial cells showed more deformation and have many vacuoles, some columnar and goblet cells were destroyed, and the peritrophic membrane was destroyed in many parts.

Keywords: clove oil, eugenol, nanoemulsion, *Spodoptera littoralis,* insecticidal activity, biochemical and histological studies.

1. Introduction
To secure food security for that enormous population of around 10 billion by 2050 (UN. 2015), it is required to ensure that agricultural production keeps speed with the ensuing food requirements. The need for the prevention and reduce crop losses from attacks of insects, weeds, microbial diseases, and other pests, is a crucial issue facing crop protection (Ghorab and Khalil, 2016). On the other hand, climate change causes a disadvantageous effect on pest species. Some pests have already increased their host range or distributions or at least in part as a result of climatic changes. FAO reported that multiple insect attacks have caused up to 40% of crop yields worldwide to fall. Over $220 billion is lost to plant diseases and at least $70 billion is lost to insects annually in the global economy (IPPC Secretariat, 2021).

Corresponding Author: Trandil F. Wahba, Insecticide Bioassay Department, Central Agricultural Pesticides Lab. (CAPL), Agriculture Research Center (ARC), Alexandria, Egypt.
E-mail: trandilwahba@gmail.com

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The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd, 1883) (Noctuidae: Lepidoptera), is the main pest of cotton and corn worldwide as this pest has been announced from 112 plant species with economic damage (Gordon, 1961; Şimşek *et al*., 2022). This insect is on the A2 quarantine list of the EPPO (European and Mediterranean Plant Protection Organization) (OEPP/EPPO, 2022). For the time being, insecticides currently on the market were unable to successfully control *S. littoralis*. Regular application of traditional pesticides against *S. littoralis*, has created the ideal conditions for the development of its resistance (Abd El-Kareem *et al*., 2022). Also, the pesticides had caused various issues in natural settings, such as toxicity against beneficial predators or parasites, additional considerable environmental pollution, health risks, and the accumulation of pesticide residues in plants (Abd El-Naby *et al*., 2022).

In recent years, increased attention to essential oils (EOs) or their bioactive compounds ex., monoterpenes, sesquiterpenes, and their oxygenated derivatives, aliphatic aldehydes, alcohols, esters, and phenol ethers (Khodaei *et al*., 2021). The application of essential oils EOs and their bioactive compounds are applied rapidly as biopesticides which rise dramatically to decrease the use of hazardous synthetic pesticides (Fernandes *et al*.,2020). For instance, clove oil has been studied as a powerful insecticide that is effective against a broad range of domestic insect pests (Jan *et al*., 2022) Indeed, the clove oil's main is eugenol at various percentages according to producing regions, harvesting time, and the plant part which oil is extracted from (Ikawati *et al*., 2022; Awad *et al*., 2022).

Eugenol was discovered to be active against polyphagous insects (Vargas-Méndez *et al*., 2019). Last investigations proved that clove oil and eugenol have the highest antifeedant and repellent activity as well as their insecticidal effects (Elbrense *et al*., 2022; Ikawati *et al*., 2021).

The main disadvantage of using essential oils as biopesticides under field conditions is their chemical instability in environmental conditions like air, sunlight, humidity, wind, and high temperatures which lead to rapid evaporation, degradation, water insolubility, and oxidation (Tia *et al*., 2021). To overcomes these disadvantages, many nanotechnological have been proposed as alternatives, especially NE systems. The NEs formula protects essential oils from rapid degradation and enhances their residue half-life by decreasing evaporation and facilitating the application and handling (Mustafa and Hussein, 2020). Essential oils NEs stability depends on many strategies as a mixture of the ingredients, low viscosity, and the smallest droplet size (Siddiqui *et al*., 2015).

The underlying mechanism of action of these EOs, their major components, and their NEs remain poorly understood because of the paucity of studies examining some biochemical targets and the biochemical changes they mediate, such as the total protein content, the activity of AST, ALT, and ALP (Hashem *et al*., 2020; Bajda *et al*., 2014). *S. littoralis* was used in this instance as a model organism. As well, insect midgut plays an important role in insect life. The midgut of insects is responsible for the production of enzymes and absorption of digestive products (Mishra *et al*., 2015) essential oils can play an important role and mortality as a result of stopping insects from feeding (Roel *et al*., 2010). Clove oil and eugenol can cause damage to the histological structure of midgut larvae of *S. littoralis.*

The objective of this research aimed to evaluate the toxicity of clove oil, eugenol and both NE formulations against 4th instar larvae of the cotton leafworm *S. littoralis.* In addition, assessment the effect of tested materials on the activity of transaminase (AST, ALT) and ALP on *S. littoralis* and effect on midgut membrane through the histological studies.

2. Materials and Methods

2.1. Analysis of essential oils

Clove essential oil analysis was performed using a gas chromatography-mass spectrometry (GC-MS) system (Hewlett Packard 5989B). Samples were injected after being diluted in 1 ml of diethyl ether. The GC column's dimethyl polysiloxane capillary column was 30m (0.25 mm in diameter, 0.25 ml in film thickness) HP-5MS (5% diphenyl). The following were the GC conditions: 240°C at 5°C/min programmed temperature, held for 1 min at this temperature, 200°C ion source temperature, and 300°C detector temperature. 205°C injector temperature, 90°C column temperature, and isothermal, then held for 1 minute. The carrier gas used was helium, which was used at a rate of 1 ml/min. Direct injection of the GC column's effluent into the MS ion source. Additionally, spectra were obtained in the EI mode at an ionization energy of 70 eV. Also programmed for a 5-second scan from 40 to 400 amu was the mass analyzer sector**.**

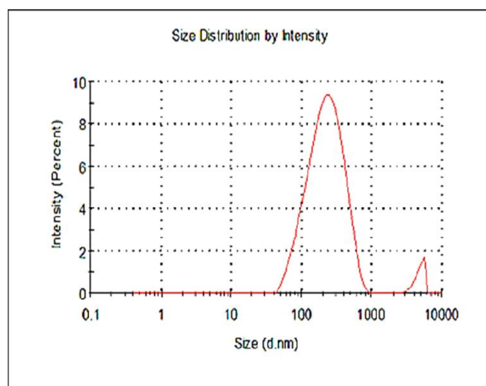

Fig. 1: Particle size distribution (nm) of Clove nanoemusion

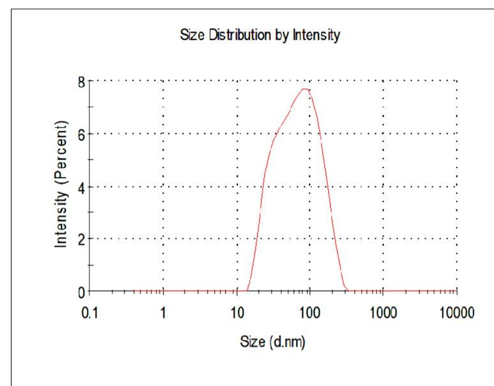

Fig. 2: Particle size distribution (nm) of Eugenol nanoemusion

2.2. NE preparation

Oil-in-water with the use of a non-ionic surfactant and deionized water, a NE containing clove essential oil and eugenol was created (tween 80) according to (Sugumar *et al*., 2013). Oil and surfactant were employed in different ratios of 1:1 and 1:1.5 (v/v). The first step is to create two coarse emulsions by adding the organic phase (oil 5%, v/v) gradually to the non-organic phase (5% tween 80 and 90%, v/v deionized water), and the second is to create a NE by adding the organic phase (essential oil 5%, to 5%, tween 80 and 90%, v/v deionized water) while magnetic stirring at about 800 rpm. The emulsions were then sonicated for 30 minutes at a 20Mz frequency. When dipped in coarse emulsions, the 13 mm-diameter sonicator probe had a diameter. The resulting NE was 10°C cooler than the first coarse emulsions. The ultrasonic high-energy emulsification technique produces heat. The emulsion sample beaker is kept in a rather large beaker that contains ice to help reduce this heat. They then gave the created NE a character.

2.3. NE Characterization

2.3.1. Droplet size and polydispersity index analysis

The particle size, viscosity, and polydispersity index (PDI) of a NE formulation were determined using a Zetasizer Nano ZS (Malvern Instruments, UK) at room temperature in the Central lab. Faculty of pharmacy Alex. University. To reduce the effects of multiple scattering, both NE specimens were diluted to 10% in deionized water before measurements. The diameter of the particle was measured as the average of three measurements in nm. clove NE was Particle size 207nm with PDI 0.33 (Figure1) and eugenol NE was Particle size 54.68nm with PDI 0.26 nm (Figure 2).

2.4. Insect rearing

A laboratory strain of cotton leafworm *S. littoralis* was obtained from the Central Laboratory of Pesticides at the Agricultural Research Center (ARC) in Cairo, Egypt, and was reared in the laboratory for several years without being exposed to insecticides. The population was kept at a temperature of 27±2 ºC and 65±5 RH. (El-Defrawi *et al*., 1964). Larvae were reared on castor oil leaves (*Ricinus communis* L.). The 4th instar larvae were selected for bioassays and biochemical assessments.

2.5. Bioassay studies

To assess the activity of different concentrations of clove, Eugenol, and two NEs. The larvae used in the experiments were newly molted 4th instar larvae by using the leaf dipping bioassay technique. Fresh castor bean leaves were immersed for 1 minute in each concentration. Then let it dry at a laboratory temperature. Each concentration was replicated three times and each replicate contained 10 larvae. Castor bean leaves treated with distilled water were fed to control larvae and then allowed to laboratory conditions (27 ±2 ºC and 65- 70% RH.). Mortality was recorded after 1 day up to 5 days of treatment. The adjusted mortality percentage of each compound was calculated statistically using

(Finney, 1971) from which the corresponding concentration probit lines (Ld-P lines) had 50% mortality, slope values tested.

2.6. Biochemical studies

2.6.1. Preparation of insects

Sample for biochemical analysis, *S. littoralis* 4th instar larvae after five days of all LC_{50} treatments, and control (Amin, 1998). The starved larvae were placed in clean glass jars. One gm of treated and untreated larvae was homogenized for 60 sec. in ice-cold 40 mM Tris-HCl (pH 7.4) and centrifuged at 4000 rpm for 15 minutes at 4 ºC. The resulting supernatants were filtered and stored at (-18 ºC) for subsequent use as an enzyme source.

2.6.2. Determination of enzyme activities

The activity of both enzyme AST and ALT was measured according to the method of (Henry *et al*., 1960), using an activity assay kit purchased from BIOLABO SAS, Les Hautes Rives 02160, Maizy, France.

AST activity was determined by transferring an amino group from L-aspartate to 2- α-ketoglutarate, which resulted in the formation of oxaloacetate and L-glutamate, and then reducing oxaloacetate to malate-by-malate dehydrogenase while simultaneously oxidising NADH to NAD+. ALT activity, the same as AST activity, was determined using a method based on the transfer of an amino group from L-alanine to 2- α- ketoglutarate with the formation of pyruvate, which was then reduced to L-lactate by LDH with simultaneous oxidation of NADH to NAD+. In both cases, the absorbance at 340 nm induced by NADH oxidation was proportional to the sample's ALT and AST activity. ALT and AST activity was determined as IU/mg protein and calculated as a percentage of the control.

The ALP activity is estimated according to (Klin, 1972) by using Biolabo Kits manufactured by Biolabo SAS, Maizy (France) company based on the principle DGKC German society of clinical chemistry (Tietz, 1999) and SCE Scandinavian society of clinical chemistry (Keiding *et al*.,1974). In this method, 10µl of the enzyme source was added to 1000 µl of 1 M diethanolamine buffer (pH =10) containing 0.5 mM magnesium ions (Mg chloride) and 10 mM p-nitrophenyl phosphate, mixed in the cuvette and incubated for 30 seconds. the activity was recorded after exactly 1, 2, and 3 minutes at a wavelength of 405nm. The alkaline phosphatase-specific activity was determined as IU /mg protein and calculated as a percentage of that of control activity.

2.6.3. Protein measurements

Protein was determined calorimetrically determined according to the Biuret method (Gornall *et al*., 1949), using an activity assay kit purchased from BIOLABO SAS, Les Hautes Rives 02160, Maizy, France. Aliquots (20 µl) of diluted protein solution were added to 1 ml of Biuret reagent for 1 h. The developed color was measured at 550 nm. A standard curve was produced using bovine serum albumin (BSA) as a reference protein.

2.7. Histological studies

The effect of all LC_{50}'s treatments, and control on the histological structure of the mid-gut of the 4th instar *S. littoralis* larvae was determined. After 5 days of treatment, 10 larvae of each treated and control group were dissected under a binocular microscope. The separated mid-gut was transferred into alcoholic Bouin's. As a fixative, the larvae were rinsed in a series of ethanol solutions to dehydrate and remove the yellow color of Bouin's solution. They were placed in 50% ethyl alcohol for 2 hours at 40 °C (two changes) before being left for 24 hours. The larvae were then subjected to a series of alcoholic treatments lasting 2 hours at room temperature, beginning with 80, then 90%, 96%, and finally 100%. Following dehydration, the larvae were placed in an amy1 acetate and colloidal solution to clear the tissues. Soft wax treatment was performed by placing them in vials containing equal parts fresh amy1 acetate solution and soft paraffin wax and leaving them at 50 °C for 24 hours. Microtome serial longitudinal sections at 6 microns were cut and mounted on clean slides with Mayer's albumin. Sections were mounted on glass slides, stained with haematoxylene, and counterstained with alcoholic eosin before being observed and photographed (Humason and Freeman, 1979).

2.8. Data analysis

Developmental research data have been collected and articulated by means (S.E.). The importance of mean variations between treatments and control was statistically measured using a variance analysis (ANOVA) at the probability level of 0.05 % with individual pairwise comparisons made using Tukey's HSD test using a Co-Stat program.

3. Result and Discussion

3.1. Chemical composition of clove oil

From the results of GC-MS analysis, the clove oil contained two main components caryophyllene (44.86%), and eugenol (20.97 %) (Table 1). Based on the provenly chromatographic data the major component of clove oils was eugenol and caryophyllene. (Ikawati *et al.*, 2022) found the largest component of clove was eugenol 89% and trans-caryophyllene 10%. Also, (Awad *et al*., 2022) mentioned that eugenol and caryophyllene represented (50.2%) and (19.3%) of clove oil content. Variability in clove oil main components may be due to a variety of factors such as geographical variants, environmental conditions, physiological varieties, genetic factors, and differences in EO extraction methods ((Zouirech *et al*., 2022; Fernandes *et al*., 2020; Ulanowska and Olas, 2021). The high level of eugenol, the one of major components of clove essential oil, increase its insecticidal activity. That was confirmed by our investigations which showed that eugenol displays a strong insecticidal effect.

3.2. Bioassay results

The larvicidal activity of clove oil and eugenol and its NE were investigated utilizing the feeding method. Accumulative mortalities for 4th instar larvae of *S. littorals* were calculated after 1,2,3,4 and 5 days post-treatment. The mortality rates of the treated.

Table 1: chemical component (%) of clove essential oils

Components	Area%	RT%
dimethylaminopyridine	20.8	19.81
Diethyl phthalate	0.63	20.14
Atrolactic acid	0.02	20.34
Eugenol	20.97	20.57
Caryophyllene	44.86	21.23
Tricyclo[7.1.0.0[1,3]]decane2carbaldehyde	0.05	21.42
Copaene	0.07	22.45
Aromadendrene	0.03	22.53
ent-α-selinene	0.05	22.83
alpha-Guajene	0.06	23.04
Farnesene	0.16	23.13
alpha. -Bulnesene	0.03	23.26
a-Cubebene	0.43	23.66
α-Calamenene	0.17	23.73
Peruviol	0.06	23.84
Isoeugenol	0.09	24.01
Alloaromadendrene oxide	0.32	25.19
Caryophyllene oxide	4. 65	25.31
5Pentadecen7yne,(Z)	0.05	25.65
à-acorenol	0.05	25.72
Bicyclo[8.2.0]dodecane, 11,11-dimethyl-	0.33	25.90
Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan9ol,4,4dimethyl	0.31	26.58
Squalene	0.08	29.06
nHexadecanoic acid	0.14	33.05
Linoleoyl chloride	0.94	33.69
Octadecanedioic acid	3.68	34.08
Linalyl isobutyrate	0.6	35.55
Oleic Acid	0.06	35.77

Larvae showed a positive correlation with tested materials concentrations. (Table 2). The calculated LC_{50} values showed that mortalities increased with increasing time of exposure. Generally, eugenol was more effective than clove oil, the LC_{50} gradually decreased from 4.64 to 1.65 and for 11.97 to 2.18 % for after 1and 5days of exposure. Clove NE exhibited more toxicity than clove oil emulsion. LC_{50} values were 5.16 and 3.04% after 2 days of exposure also LC_{50} values were 1.77 and 2.18% in respect at the end of the experiment. Besides, the eugenol NE formula demonstrated significant toxicity compared to the eugenol emulsion. The LC_{50} values were 3.52, 1.73%, and 1.65, 1.10% after 2 and 5 days, respectively. In the present work, the insecticidal effect of clove oil can be attributed to eugenol and caryophyllene as they are known for their insecticidal effects. In this regard, clove oil has a considerable effect on *S. littoralis* showing that mortality was recorded daily for 5 days post-treatment LC_{50} 2.48 ml/L (Yassin, 2013). When 4th instar larvae of *S. littoralis* larvae fed on castor leaves treated with clove oil 1% caused 92.67% mortality (Helaly *et al*., 2019).

Eugenol caused a significant reduction in *S. littoralis* larval growth after feeding in a treated diet with 500,1000 and 2000 mg/kg where growth inhibition was 86.2% after three days of treatment. the mortality of 2nd larval instar of *S. littoralis* was 60 % after feeding on a treated diet with 2000 mg/kg eugenol for 3 days (Abdelgaleil *et al*., 2020).

Table 2: Toxicity data for 4th instar larvae of *S. littorals* exposed to different concentrations of clove essential oil, Eugenol and their nanoemulsions via feeding bioassay method.

Materials	Exposure period (days)	LC_{50} (%)	Confidence limits		Slope± variance	ch^2	*P*-value
			lower	upper			
	1	11.97	5.45	175.134	1.06±0.30	0.10	1.00
	2	5.16	3.22	17.59	0.26±1.08	0.22	0.97
Clove oil	3	3.39	2.38	8.76	0.25±0.98	1.74	0.63
	4	3.26	2.67	6.65	1.40±0.27	1.88	0.63
	5	2.18	1.54	5.37	0.77±0.23	0.88	0.83
	1	8.56	4.34	79.46	0.98±0.27	1.00	0.80
	2	3.04	2.22	6.00	1.12±0.24	1.26	0.74
Nano-Clove oil	3	3.04	2.22	6.00	1.12±0.24	1.26	0.74
	4	2.97	2.08	7.34	0.93±0.24	3.00	0.38
	5	1.77	1.39	3.56	1.07±0.24	1.40	0.70
	1	4.64	3.20	10.33	1.40±0.28	1.96	0.58
	2	3.52	2.30	12.71	0.85±0.42	0.70	0.87
Eugenol	3	2.40	1.70	5.67	0.83±0.24	1.54	0.67
	4	2.13	1.60	3.91	0.92±0.24	2.11	0.55
	5	1.65	1.06	3.51	0.62±0.21	1.36	0.71
	1	4.50	2.78	46.10	0.72±0.24	1.22	0.75
	2	1.73	1.45	2.18	1.50±0.24	2.50	0.48
Nano-eugenol	3	1.55	1.34	1.85	1.76±0.25	5.16	0.16
	4	1.55	1.34	1.85	1.76±0.25	5.16	0.16
	5	1.10	0.81	1.37	1.13±0.23	6.26	0.10

3.3 biochemical studies

The 4th larvae instar of *S. littoralis* were treated with the LC_{50} concentration of tested materials for biochemical assays to evaluate the total protein the activities of AST, ALT and ALP. Latent biochemical effects are shown in Table (3). The protein levels were significantly decreased in all transactions when compared to the untreated control. Both clove oil and its NE decreased protein more than eugenol. Where they ranged from 56.55 mg/ml in the control to 30.36mg/ml for clove oil and 38.50 mg/ml for clove NE with Variation than control 46.31,31.91%, respectively. AST activities were greatly high in clove oil treatment followed by clove oil and clove NE 468.18 and 160.39 IU/L, respectively than in control 116.16 IU/L. On the other way, AST activities decreased significantly in eugenol, and eugenol NE treatment 42.00 and 52.00 IU/L. ALT activities were significantly decreased in all treatments which were 21. IU/L when under clove oil followed by 12.00, 12.40.and 11.00 IU/L in clove NE, eugenol, and eugenol NE treatments compared with in the control 53.93 IU/L with Variation than control was 60.76,77.74, 77.00 and 79.60%. There is no significant difference between ALP activity with clove oil and control but ALP activity decreased after treatments with other tested materials. Variation than control was 56.69, 57.98, and 60.12% with clove NE, eugenol, and eugenol NE, respectively. AST and ALT enzymes are indicated for the suitable proper functioning of the fat bodies in insects and which have similar to those in mammals' livers (Arrese and Soulages, 2010). Reductions in the activity of AST, ALT, and ALP, which are evidence of metabolic disorders. We suggest that EO and NE oil can inhibit the production of AST, ALT, and ALP in cells or prevent the enzymes from being released into the hemolymph (Bajda *et al*., 2014). Also, may decline ATP synthesis, -oxidation, Krebs cycle, oxidative phosphorylation, and other metabolic processes (Hashem *et al*.,2020). Which, our data in agreement with the LC_{50} of *Pimpinella anisum* NE EO significantly decreased AST more than *P. anisum* oil. That was in agreement with our finding that clove and eugenol NE decreases the activity of AST more that its oils. (El-Din *et al*., 2020) found 4th larval instar larvae of pink bollworms and spiny bollworms treated with LC_{50} of Jojoba oil, and flax oil significantly decreased total protein. (Huang *et al*., 2004) reported that the protein level was significantly influenced when 4th instar larvae of *S. litura* were fed with an artificial diet containing 1 ppm azadirachtin. Also, (El-Din *et al*., 2020) recorded that Jojoba oil and Flaxseed oil causes increases in AST and ALT activity in *Pectinophora gossypiella* and *Earias insulana* but reduction percentages were found in ALP. On the other hand, (Makarem *et al*.,2015) found Basil oil increased the activity of AST in contrast, anise and clove oils did not cause significant change. ALT activity increased in clove and basil oil.

Table 3: Enzyme activities of 4th instar larvae of *S. littorals* after feeding with clove essential oil, Eugenol and their nanoemulsions

Materials	**(TP) ±SE	* V	**AST ±SE	V	**ALT ±SE	V	**ALP ±SE	V
Control	56.5±0.2^{a}	-	116.1±6.8^{bc}	-	53.9±1.4^{a}	-	233.2±12.1^{a}	-
Clove oil	30.3±0.2^{e}	46.3	468.1±10.3^{a}	-301.4	21.1±1.1^{b}	60.7	217.2±3.9^{a}	6.8
Nano-Clove oil	38.5±.0.8^{d}	31.9	160.3±28.1^{b}	-37.2	12.0±1.1^{c}	77.7	101.0±6.8^{b}	56.6
Eugenol	52.0±1.1^{b}	8.0	42.0±1.1^{d}	63.9	12.4±0.1^{c}	77.0	98.0±1.7^{b}	57.9
Nano-eugenol	45.6±1.5^{c}	19.3	52.0±1.7^{cd}	55.4	11.0±0.2^{c}	79.6	93.0±1.7^{b}	60.1

*(VV) Variation (%) =[(control-treatment)/control] ×100
**(TP) Total protein (mg/ml), AST (IU/ mg protein) =Aspartate aminotransferase, ALT (IU/mg protein) =Alanine aminotransferase and ALP (UI/ mg protein) =alkaline phosphatases.
***The values (Mean ± SE) followed by different letters (superscript) with in a column indicate significant differences at Tukey's HSD test $p \leq 0.05$, Significant at 0.05% level.

Indeed, there were various effects of EOs and their nano on AST, ALT, and ALP activities that might be exerted on the synthesis or functional levels of these enzymes directly or indirectly by cells cytology.

3.4 Histological study

The midgut is the main origin of the insect digestive system where insects digested and absorbed ingested food. The control 4th larval instar of *S. littoralis* is illustrated in figure (3c). The Microscopic examination of the transverse section in untreated larvae midgut showed that. the midgut consisted of two layers longitudinal muscle and Circular muscle. Below that there was a single layer of simple epithelium composed of three main cell types, columnar epithelial cells, regenerative cells, and goblet cells. epithelium surrounded by a thin basement membrane which protects them from particles of food. epithelial cells were surrounded by gut lumen as well, these cells had a striated microvillus. LC_{50} treatment of clove oil emulsion caused some changes in cells of treated 4th instar larvae midgut figure (3A1), the epithelial cells separated from their basement membrane. Most columnar cells, goblet cells; microcells, and peritrophic membranes were ruined with some vacuolization of the cytoplasm. Some epithelial cells lost their nuclei and others appeared pyknotic. Larvae treated with clove NE damage was exceedingly pronounced in the midgut treated with nano clove figure (3A2). Midgut epithelial cells showed more deformation, some columnar cells were destroyed, epithelial cells have many empty vacuoles, and goblet cells were more destroyed, completely disappearing connective tissue. the peritrophic membrane was destroyed in many parts. LC_{50} treatment of eugenol emulsion caused changes in cells of treated 4th instar larvae midgut figure (3B1), epithelial cells were destroyed in many parts, some columnar cells disappeared, epithelial cells have many empty vacuoles, absent peritrophic membranes in many parts. Damage was exceedingly destruction pronounced in the midgut treated with nano eugenol figure (3B2), absent in the longitudinal muscle in many parts, several damages occurred in epithelial and columnar cells, appears several vacuoles, separation of the peritrophic membrane. The treatment of the 4th instar larvae of *S. littoralis* with LC_{50} of clove and eugenol caused some deformations in the midgut tissues of these larvae. but the treatment with both tested NEs produced high destruction of midgut tissue. The most digestion process happens in the midgut, so it is the most body part attacked with treated substances. The mid-gut tissues of the 4th instar larvae of *Pectinophora gossypiella* and *Earias insulana* exhibit vacuolization within the hypertrophied mucosal epithelial lining, blurred epithelial cells, and necrosis of nuclei after being treated with LC_{50} values of Jojoba oil and flaxseed oil (El-Din *et al*.,2020). Histological changes (vacuolated and necrotic epithelial cells) were observed in the midgut of *S. littoralis* larvae after treatment with *Azadirachtin indica* and *Citrullus* (Rawi *et al*., 2011). *Helicoverpa armigera* cotton bollworm larval midgut fed with a diet containing *Thevetia neriifolia* extract caused significant damage, shrinkage, distortion, and vacuolization of gut tissues and peritrophic membrane, disintegrating epithelial, goblet, and regenerative cells (Mishra *et al*., 2015). *Hyptis brevipes* extract caused detached on the peritrophic membrane midgut of *S. littoralis* larvae and the lumen was packed with pyknotic-nuclei-epithelial cells. wide destruction of the epithelium with cells lacking nuclei was also observed in the midgut of *S. littoralis* larvae (Sakr, 2014). Changes in the midgut of the 4th larval instar of *S. littoralis* treated with the LC_{30} of the essential oil of garlic *(Allium sativum*) and lemon (*Citrus limon*) were vacuolization of the epithelial layer, clumping of the nuclear chromatin material, and degeneration of the peritrophic membrane and microvilli. The loss of the cytoplasmic projections of the goblet cells, as well as vacuolization and degeneration of the columnar cells, clumping of the nuclear chromatin, breaking of microvilli, and nuclear chromatin (Ali *et al*., 2017).

5. Conclusions

Our study provided evaluates the larvicidal activity of the clove oil and its main component eugenol, and both NE formulations against 4th instar larvae of *S. littorals* under laboratory conditions. Generally, eugenol was more effective than clove oil. but NEs were more toxic than their oils. all tested materials decreased protein levels in *S. littorals* larvae. Clove oil and its NE increased AST transaminase activity but eugenol and its NE decreased activity. ALT transaminases and ALP activity decreased with all treatments. Histological examination showed a high distribution of this larvae's midgut after treatments with LC_{50} values of all tested materials, especially with NE. Our study suggested that, NEs can be taken into account as novel biopesticides. can be used as a sustainable and effective alternative in the control of *S. littoralis*.

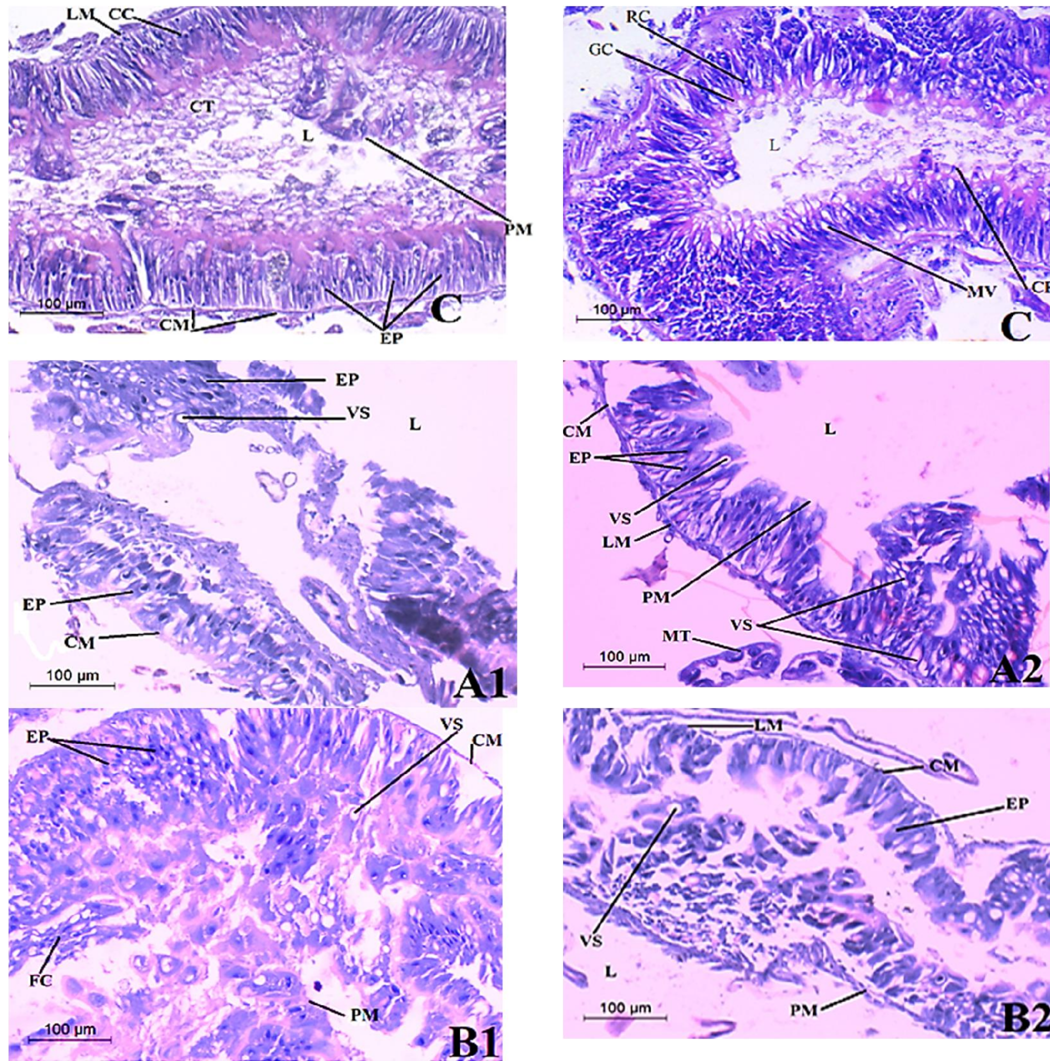

Fig. 3: Transverse section in the mid-gut of 4th larval instar of *Spodoptera littoralis,* **(c)** untreated larvae, **(A1)** larvae treated with LC_{50} of clove oil, **(A2)** larvae treated with LC_{50} of clove oil nano emulation, **(B1)** larvae treated with LC_{50} of eugenol, **(B2)** larvae treated with LC_{50} of eugenol nano emulation. Where, (CM) circular muscle; (LM) longitudinal muscle; (E) epithelial layer; (CC) Columnar cells; (CT) Connective tissue; (GC) Goblet cells; (RC) Regenerative cells; (L) lumen; (PM) peritrophic membrane; (VS) vesicles; (MV) microvilli; (CP) Cytoplasmic protrusions; (FC) free cells; (MT) Malpighian cell.

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