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EVALUATING EFFECT OF PEPPER MINT OIL *(Mentha pipreta)* AND ITS NANO-FORMULATIONS ON SOME ENZYMATIC ACTIVITIES AND BIONOMICS OF COTTON LEAF WORM *Spodoptera littoralis* (BOISD.)

[146]

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ABSTRACT

Different formulations of pepper mint oil, *i.e*. bulk, nano-emulsion and nano-encapsulation were evaluated against 2nd and 4th instar larvae of the cotton leafworm *Spodoptera littoralis* (Boisd.). Their effects on enzymatic activities and bionomics were also investigated. Transmission Electron Microscopy (TEM) showed that the mean particle size of the prepared nano-emulsion ranged between 20-90nm. The encapsulation efficiency and loaded capacity percentages exhibited that distilled H_2O was more suitable than ethanol in preparation of nano-encapsulation particles. Bioassay treatments showed LC_{50}'s on 2nd instars larvae were 70.59, 12.23 and 21.72 ppm for pepper mint oil, pepper mint nano-emulsion and loaded nano-emulsion, respectively. While on 4th instars these values were 80.47, 26.14 and 67.69 ppm, respectively. LC_{90}'s on 2nd instars were 104.75, 33.97 and 30.14 for pepper mint oil, pepper mint nano-emulsion and loaded nano-emulsion, respectively. While on 4th instars these values were 177.66, 71.37 and 102.29 ppm. Toxicity index and relative potency proved that nano-emulsion was more effective than loaded nano-emulsion, while the bulk preparation was the least effective one. Results of enzymatic activities showed marked effects of the three formulations of mint oil, significant inhibitions were recorded for amylase, invertase, trehalase, protease and alkaline phosphatase, while significant increase in activities of cuticle phenoloxidase and chitinase. Effects of adding the three formulations to artificial diet of 2nd instar showed significant effects of the insect bionomics. Larvae durations, percentage mortalities, were increased as well as larval malformations. Also, pupal duration, percentage pupal mortality and pupal malformation were increased, while pupal weight was decreased. Adults longevity showed insignificant effects, while female fecundity and egg % fertility showed significant responce.

1- INTRODUCTION

Various natural products such as botanical pesticides showed promise alternative as to synthetic pesticides since it reduces negative impacts to human health and to environment. Natural products are compatible with the environmental components than synthetic pesticides. Botanical pesticides have been shown to posses feeding deterrence, repellency, toxicity and growth disruptive properties to numerous species and stages of insects of many orders. **(Dimetry et al 2013).**

Negative effects of conventional synthetic pesticides, biopesticides especially essential oils (EOs) were achieved when it used as complementary or alternative approach in integrated pest management programs **(Gonzlez et al 2013**). The pepper mint essential oil *Mentha pipreta* proved to be promising **(Ismail and Shaker, 2014)**. It con-

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tains active ingredient in form of essential oil (menthol and other components).The potential of menthol to control some arthropod pests have been reported **(Badawy et al 2010).** Despite the promising properties of essential oils, some problems related to EO, *i.e.* volatility, poor water solubility, and aptitude for oxidation should be cleared before use **(Moretti et al 2002)**. Potential applications of nano-materials in agriculture offer several advantages; *i.e.* delivery of nano-encapsulated pesticides for controlled release **(Ghormade et al 2011)**. In addition, it was found that nano-formulations affiliates to EOs as control release formulations. **(Martn et al 2010)**.

Preparing pesticides in nano-particles involve their encapsulated within polymers is very common **(Liu et al 2008; Perez-de-Luque and Rubiales, 2009)**. Natural polymers used were albumin (protein), gelatin (protein) **(Zwiorek et al 2004)**, alginate (saccharide), collagen (protein), chitosan (saccharide) and the milk protein a-lactalbumin **(Graveland-Bikker and De Kruif, 2006).**

Encapsulation of a natural liquid pesticide using sodium alginate (Na-Alg) as a controlled release polymer after cross linking with glutaraldehyde (GA) has been reported by several authors **(Kulkarni et al 2000)**. They reported that alginate polysaccharides are identified to be hemo-compatible and do not build up in any organs of the human body. Encapsulating nanoparticle layers at the emulsion droplet interface may be engineered to increase droplet stability and control of release kinetics.

The aim of this study is to evaluate the efficiency of the prepared nano-formulations of pepper mint oil encapsulated in alginate based nano-emulsion in order to investigate its insecticidal activity against larvae of the cotton leafworm *Spodoptera littoralis.* Also, the effect of the prepared nano-formulation on the insect in both bionomics were studied *i.e*. larval duration, weight, mortality, longevity, larval malformations and pupae. Also, fecundity and fertility were reported. In addition, the effect of the same nano- formulation on some enzymatic activities, *i.e*. Amylase, Trehalase, Invertase, Protease, Alkaline phosphatase, Phenoloxidase and Chitinase in both the 2nd and 4th instar larvae were evaluated.

2- MATERIAL AND METHODS

2-1- Chemicals used

Pepper mint oil was obtained from Oil Extraction Unit, (National Research Center)**.** Sodium alginate, calcium chloride and all chemicals used in enzymatic evaluation have been purchased from Sigma Chemical Co. (UK).

2-2- Test insect

The cotton leaf worm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae), was maintained for several generations in rearing units in the laboratories of the department of Pests and Plant Protection (National Research Center) under controlled conditions of 25±2°C and 65±5%RH.Larvae were fed on castor bean leaves, while moths were provided with 10% sucrose solutions. In case of treatment of larvae with tested formulations, the larvae were fed on an artificial diet according to the method described by **Shoery and Hale (1965)**.

2-3- Nano-emulsion preparation

Pepper mint oil was obtained from Oil Extraction Unit (National Research Center). The oil was extracted from the pepper mint*, Mentha pipreta* pepper mint oil, Tween 80 and distilled water were used in the preparation of emulsion by the modification of the method described by **Jerobin et al (2012**).

Nano-emulsion preparation were prepared by diluting the oil with distilled water in ratio 1:2 (oil : water, respectively), 2% Tween 80 was added as an emulsifier. The emulsion thus formed were sonicated 30 min using ultrasonic cleaner set, model WUC-DO3H 290W and 60 Hz, then sonicated for 1min using a high energy ultrasonication probe model VCX750, 750W, 20 kHz, and re-sonicated 30 min by the ultrasonic cleaner under cooling conditions.

2-4- Preparation of loaded Nano-emultions

Alginate nano-capsules were prepared using oil/water (o/w) emulsification, followed by crosslinking by calcium chloride and modified according to

the methods described by **Lertsutthiwong et al (2008)**. Distilled water was used instead of ethanol in this investigation. Also, high energy ultrasonication was used with stearing.

2-5- Characterization

To ensure that the prepared pepper mint oil formulations were became in the nano-sized particles, different methods of characterization were followed to measure the morphological shape, size, uniformity content and chemical interactions of the obtained nano-formulations and/or nanoparticles.

2-5-1- Transmission Electron Microscopy (TEM)

The morphological shapes of prepared nano-formulations were tested with Transmission Electron Microscopy (TEM) (Jeol, JEM-2100). The nano-capsule suspensions was diluted with distilled water and deposited onto a carbon-coated copper grid andstained with a1% phospho tungsten acid then examined by magnification (20000X) and photografed.

2-5-2- UV spectrophotometric examinations

Loaded nano-capsules were evaluated for the active ingredient contents (menthol in pepper mint oil) and this was done by refluxing of 0.75 grams the loaded nano-emulsions with 10 ml of methanol at 65°C. Refluxing was continued for 1 h, to ensure complete extraction of encapsulated oil. The samples were then centrifuged at 10,000xg for 10 min. The absorbance of methanol containing the extracted amount of menthol was taken at a wave length of 230 nm in a UV spectrophotometer, CHEM-7 using absolute methanol as a blank. Oil concentration was calculated with the use of a calibration curve obtained from samples of menthol crystals. The encapsulation parameters were determined as follows:

$$\% \text{ Encapsulation Efficiency (EE)} = \frac{\text{The amount of oil measured in the supernatant}}{\text{Total amount of oil}} \times 100$$

$$\% \text{ Loading Capacity (LC)} = \frac{\text{The amount of oil measured in the supernatant}}{\text{Total weight of nano- capsules}} \times 100$$

2-5-3- Fourier Transforms nfrared (FTIR) measurements

FTIR measurements were carried out to detect any chemical interactions between pepper mint oil nano-emulsion and Na-Alg or the cross linking agent (Ca Cl_2). This was carried out by taking following samples from pure bulk oil, oil nano-emulsion, loaded Nano-emulsion. The FTIR measurements were carried out using FTIR 6600, JASCO according to the method described by **Jerobin et al (2012)**.

2-6- Bioassay tests

Bioassays tests were carried out with 2nd and 4th instar larvae of *S. littoralis*. Samples of 100g semi-synthetic diet previously described by **Shorey and Hale (1965)** were treated. All prepared formulations were incorporated into the diet as aqueous dilutions at the desired concentrations during the preparation of the diet. Series of concentrations of each formulation were used to calculate the LC_{50} values. Such procedure was carried out just before gelling in order to avoid decomposition of the used materials. Media treated with distilled water and a drop of Tween 80 was used as control. All concentrations were prepared according to the active ingredient content in each formulation.

In case of 2nd instar larvae, 5gm of treated semi-synthetic diet for each concentration was added in plastic cups of 120ml in capacity. Serial concentrations of pepper mint oil containing 30, 60, 90 and 120 ppm menthol for bulk oil. For nano-emulsion and loaded nano-emultion formulations, serial concentrations of 5, 10, 20, 30, and 40 ppm were added to the targeted medium. Ten of 2nd instar larvae were then transferred to each cup with four replicates, of 10 larvae /replicate.

Efficiency of the prepared formulations was tested on 4th instars larvae individually. Glass tubes of 10 cm height were used. One piece weighted 1gm of treated diet was cut by the cork borer and was added in each glass tubes and each larva was transferred to each tube with four replicates, of 10 larvae /replicate. Pepper mint oil was prepared in the concentrations of 30, 60, 120 and 280 ppm menthol. Nano- emulsion and loaded nano-emultion were prepared in concentrations of 30, 60, 90 and 120 ppm menthol.

Those cups and glass tubes were incubated at 25 ± 2℃ and 65 -70% R.H. Larval mortality was recorded daily throughout 4 days after treatment and adjusted for control. Concentrations mortality regression lines were corrected and ploted in form of log/probit relation and the LC_{50} values were calculated using Ld-p line program according to **Finney, (1971)**.

2-7- Effect of pepper mint oil bulk and nano-formulations on certain enzymatic activity

The biological effects of prepared nano-compounds compared with their bulk form were studied on the 2nd insar larvae of *S. littoralis*. Emerged 2nd instars larvae were treated with concentrations of 10 ppm menthol for pepper mint formulations. Forty larvae were used for each concentration and transferred separately in glass tubes as mentioned in bioassay tests. The treated larvae were exposed to the treated diet until pupation. Untreated diet was used as a control by adding distilled water with a drop of Tween 80. Records were made daily of living and dead individuals. The duration of larval and pupal stages were recorded. Pupae of all test larvae were weighed on the first day after pupation. Fecundity was determined by rearing each couple of emerged moths and calculating the number of eggs deposited by each female and hatchability percentage of produced eggs were calculated.

2-7-1- Preparation of enzyme extracts

Both of 2nd and 4th instars larvae were homogenated in a volume of potassium phosphate buffer (0.2M, pH 7.0) equal to 4 times their weight. Individuals of 4th instars larvae were dissected and gut canals were removed and collected for determination of amylase, invertase, trehalase, protease and alkaline phosphatase enzyme activities. Cuticle samples were collected for the phenol oxidase and chitinase enzyme activities. The homogenates were centrifuged at 10,000xg for 15 min/ 4℃ and the supernatant was used as the enzyme source.

2-7-2- Determination of carbohydrate hydrolyzing enzymes activities

The activities of three carbohydrate hydrolyzing enzymes were determined, *i.e.* amylase (which hydrolyzing the starch), invertase (which hydrolyzing the sucrose) and trehalase (which hydrolyzing the trehalose). It was selected such three enzymes to reflex the effect of the studied bulk and nano-formulations on the physiological functions of the digestive system of the affected instar larvae.

The determination procedure of the three mentioned enzymes was carried out according to the method described by **Ishaaya and Swiriski (1976).** Such method was based on the digestion of starch, and sucrose by amylase, and invertase, respectively. The free aldhydic group of glucose formed after starch and/or sucrose digestion were determined using 3,5dinitrosalicylic acid reagent. The enzymatic activity was expressed as µg glucose released/ min/mp protein.

2-7-3- Determination of Protease activity

The proteolytic activity was determined by the casein digestion method described by **Ishaaya et al (1971)**. Enzymatic activity was expressed by measuring the resultant optical density at 280 nm and the O.D. multiplicated by 10^3.

2-7-4- Determination of alkaline phosphatase activity

Alkaline phosphatase activity was determined according to the method described by **(Powell and Smith (1954).** The produced colour was measured immediately by spectrophotometer at 510 nm. The enzymatic activity is expressed as µg phenol released/min/mg protein. The quantification of enzyme activity was carried out through standard calibration curve used phenol as standard.

2-7-5- Determination of Chitinase activity

Chitinase was assayed using 3,5-dinitrosalicylic acid reagent to determine the free aldehydic groups of hexoaminase liberated on chitin digestion according to the method described by **Ishaaya and Casida (1974).** The specific activity of chitinase is expressed as µg N-acetylglucosamine (NAGA) released /min/mg protein.

2-7-6- Determination of phenoloxidase activity

Phenoloxidase activity was determined according to a modification of **Ishaaya et al (1971)**, in a reaction mixture consisting of 0.5 ml sodium phosphate buffer (0.1 M, PH 7), 200 µl enzyme solution and 200 µl catechol solution 2%. Prior to the initiation of the reaction, the substrate and other ingredients of the reaction mixture were separately incubated at the optimum temperature of the reaction (25°C). Enzyme reaction was initiated by adding catechol solution. Then after exactly 1 min, the

optical density was determined. Zero adjustment was against sample blank. The phenol oxidase activity was determined as O.D. units × 10^3 at an absorbance of 405 nm using UV spectrophotometer, CHEM-7.

2-7-7- Determination of total protein

Total proteins were determined by the method of **Bradford (1976)**. The resultant color of protein – dye complex was read at 595 nm using UV spectrophotometer, CHEM-7.

2-7-8- Statistical analysis

Data were subjected to statistical analysis by one way analysis of variance (ANOVA) using SPSS software (Tukey test). A value of $p < 0.05$ was considered statistically significant. Percentage Values were transformed to Arcsin before statistical analyses.

2-8- Effect of pepper mint oil nano-formulations on bionomics of the second instar larval of *S. littoralis*

The biochemical effects of the prepared pepper mint oil bulk and nano-frmulations (nano-emulsion and loaded nano-emulsion) were studied throughout the determination of certain enzymatic activities, *i.e*. amylase, trehalase, invertase, protease, alkaline phosphatase, phenoloxidase and chitinase in both of 2^{nd} and 4^{th} treated instar larvae of *S. littoralis*. Both instar larvae were treated by their corresponding calculated LC_{50} values of each formulation.

The treatment procedure was carried out by mixing the concentration of each LC_{50} value of each formulation by the semisynthetic diet. After then, the 2^{nd} and/or 4^{th} instar larvae were allowed to feed the treated diet during 4 days. At the fourth day of treatment the live larvae of both tested in stars were selected to perform the enzyme extraction procedure.

3- RESULTS AND DISCUSSION

3-1- Characterization of the prepared nano-formulations

3-1-1- Electron microscopy examination

In order to study the morphological shapes and sizes of the prepared nano- formulations (nano-emultions and loaded nano- emultions) all nano - samples of pepper mint oils were examined by TEM. **Fig. (1).** Pepper mint oil nano-emulsions were almost spherical in shape with smooth surface as showen by the TEM images **Fig. (1a)**. The mean particle sizes were ranged between 20 – 90 nm. Preparation of alginate nano-capsules containing pepper mint oil were carried out using a multi-step process of o/w emulsification and gelification. Desperation of oil emulsions containing Tween 80 in an aqueous alginate solution caused a formation of core and shell encapsulation. The inner core represents the oil and the shell represents the alginate that solidified by cross linking by calcium chloride **Fig. (1b)**. Regarding to this results, **Lertsutthiwong et al (2008)** prepared alginate nano-capsules containing turmeric oil by diluting turmeric oil with ethanol or acetone in a concentration of 1% (w/v). They reported that drop wise addition of diluted turmeric oil in sodium alginate containing Tween 80 resulted in immediate turbidity with a forming of larger nano-capsules in diameter (373 nm) than those formed with ethanol as a solvent (263 nm). In our study it was followed the same method by dissolving pepper mint oil in ethanol. As shown in **Fig. (1c)** loaded nano- particles diameter ranged 70-100 nm and they were smaller than those gained with **Lertsutthiwong et al (2008)**. We referred that to the using of high energy ultrasonic probe and the sonication twice times before and after cross linking gelishing by calcium chloride.

In this study it was carried out a diluted emulsion using distilled water by adding the surfactant to form the oil emulsion before drop wising in the alginate solution that was the same method used by **Jerobin et al (2012)**. To gain the small size of capsules and a good emulsion in the absence of solvent it was used both cleaner sonicator and the high energy ultrasonic probe which didn't used by **Lertsutthiwong et al (2008)**. After loading on alginate, capsules diameters were bigger than those of emulsion particles and increased to 40-100 nm for loaded pepper mint nano-emulsion. The particle sizes were closed to those obtained by **Lertsutthiwong et al (2008)** suggesting that using the high energy ultrasonic probe is a major step to obtain the small nano-capsules. Our suggestion agreed with those reported by the same authors. However, the authors reported that mechanical stirring only is insufficient to prepare nano-capsules in a uniform size, and increasing time of sonication either before or after adding the oil to the sodium alginate resulted in smaller capsules.

For example, the sonication for 15 min after adding oil to sodium alginate resulted in nano-capsules (83.6) that were about 50% smaller than those formed by sonication before adding oil to sodium alginate.

Using of sonication after cross linking by calcium chloride participated in reducing the diameter of nano-capsules in the absence of ethanol. In addition, it was indicated that loaded nano-capsules prepared using ethanol showed bigger size than those prepared by water dispersion emulsion. Similar finding was reported by **Lertsutthiwong et al (2008)** who found that the size of the prepared nano-capsules affected by using of high energy sonication, sonication time (before and after calcium chloride cross linking) and the more dilution using distilled water the smaller nano-capsular size.

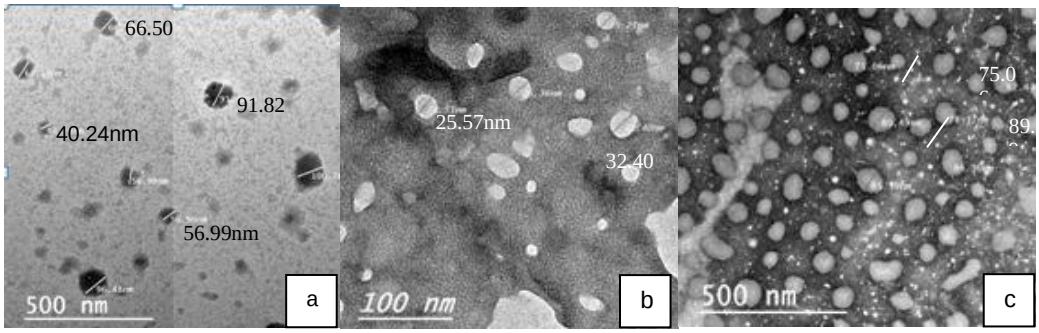

Fig. 1. Images of Transmission electron microscopy of the prepared pepper mint nano-Formulations, Pepper mint nano-emulsion (a);alginate loaded pepper mintnano-emulsion prepared using distilled water(b) and alginate loaded pepper mint nano-emulsion prepared using ethanol(c).

3-1-2- Determination of nano-encapsulation efficiency (EE) and loaded capacity (LC)

The incorporation of pepper mint oil into alginate nano-capsules has been studied. The nano-capsules were prepared using ultrasonic emulsification technique using alginate as the encapsulation material and pepper mint oil as the active ingredient. Such step was carried out to prevent the rapid evaporation, degradation and enhance their stability. The results of such procedure showed that the encapsulation efficiency (%EE) increased with loaded emulsion prepared with distilled water than those prepared with using ethanol; the EE were 75.53%and 21.49%, respectively **(Table 1)**.

On the other hand, loading capacity (%LC) showed the same significant differences between the two treatments, they were 1.99% and 1.40%, respectively. These results were similar to those obtained by **Lertsutthiworg et al (2008)** they prepared the same delivered system with turmeric oil. In such study it was reported that loading capacity of 5.47 and 10.4 % was obtained for processes with and without ethanol evaporation, respectively. They added that without ethanol evaporation, about 30% of the turmeric oil was lost during formation of the turmeric oil-loaded nano-capsules and addition of solvent removal to the process caused a total loss of about 42% of the turmeric oil, suggesting that the evaporation process is responsible for loss of 14% of the total oil.

Table 1. Values of encapsulation efficiency and loaded capacity of the prepared pepper mint oil nano-encapsulation using distilled water and ethanol

Parameter	Treatment	Mean±s.d.%	Min.	Max.	F value	Sig.
%EE	Distilled water	75.53±0.88	74.58	76.31	1236.00	0.001
	Ethanol	21.49±2.48	19.58	24.30		
%LC	Distilled water	1.99±0.15	1.87	2.15	26.87	0.007
	Ethanol	1.40±0.13	1.26	1.52		

3-1-3- Fourier Transforms Infrared (FTIR) measurements

The chemical interactions among the ingredients *i.e*. sodium alginate (Na-Alg) and pepper mint oil nano-emulsions were studied in order to understand the stability of nano-emulsion inside the matrix system.

FTIR spectrum as shown in **Fig. (2)** confirmed significant peaks in samples of pepper mint pure oil (curve a) at: 3424.94 cm^{-1}corresponding to hydroxyl group, 2870.52- 2924.00 cm^{-1}ascribed to methyl group, 1045.23- 1097.30 wave length cm^{-1} attributed to (C-O) bond and 1372.1 cm^{-1} corresponding to isopropyl group. These peaks were closed to those shown by **Al-Bayati, (2009)** who compared with them as he reported that the major groups of pepper mint oil from *Mentha longifolia* L. leaves were at: 3362 (O-H), 2855 – 2924 cm^{-1} (methyl group), 1025-1045 cm^{-1} (C-O bond) and 1368 (isopropyl group). Samples of pepper mint nano-emultion were not changed to much than the pure pepper mint samples and their peaks were represented at: 3412.42 (O-H), 2872.45 – 2924.52 cm^{-1} (methyl group), 1051.01 – 1106.94 cm^{-1} (C-O bond) and 1371.14 cm^{-1} (isopropyl group). Samples of Na-Alg loaded peppermint oil nano-emultion showed more shafting of its major peaks but with keeping not changed in the range represented by the pure oil samples indicating that they have been kept in the same property.

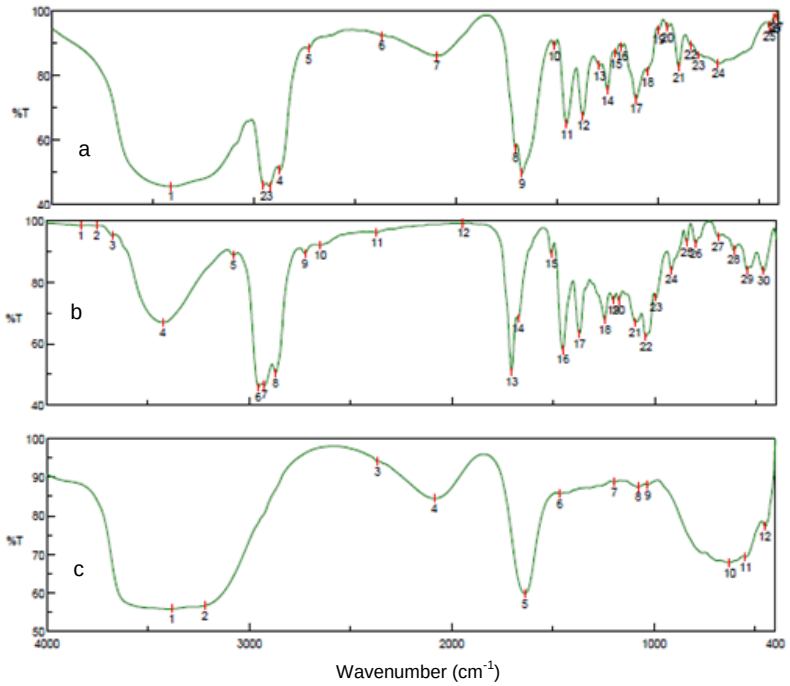

Fig. 2. FTIR spectra of pure pepper mint oil (a); Pepper mint oil nano-emulsion (b) and Peppe rmint-loaded alginate nano-capsules(c)

3-2- Efficiency of pepper mint oil bulk and its nano-formulations against 2nd and 4th instar larvae of *S. littoralis*

The results related to the effect of pepper mint oil in different formulation forms, *i.e*. bulk, nano-emulsions and loaded nano-emulsion against 2*nd* and 4*th* instar larvae *S. littoralis* are listed in **(Table, 2)**. Results in **Table (2)** show the values of LC_{50} and LC_{90}, slope and regression calculated from the plotted toxicity regression lines and both of toxicity indexes and relative potency of the different tested preparations of the pepper mint oil formulations. Based on the LC_{50} values of the tested formulations against the 2*nd* instar larvae of *S. littoralis*, it is clear that nano-preparations increased the toxic effect.

Table 2. LC_{50}, LC_{90} of the pepper mint oil nano-formulations against 2nd and 4th instar larvae of *S. littoralis*

Larval instar	Formulation	LC_{50} ppm (Fudicial limits)	LC_{90} ppm (Fudicial limits)	Slope	Regression	Toxicity index	Relative Potency
2 nd	Bulk	70.59 (63.35 –77.52)	104.75 (93.74 –124.08)	7.48 ± 1.11	0.98	17.33	1
	Nano emulsion	12.23 (8.60 – 15.08)	33.97 (26.09 -58.58)	2.89 ± 0.61	0.98	100	5.77
	Loaded nano emulsion	21.72 (19.98 –23.34)	30.14 (27.67 – 34.16)	9.00 ± 1.23	0.99	56.31	3.25
4th	Bulk	80.47 (68.29 –94.21)	177.66 (144.59 –241.40)	3.73 ± 0.48	0.99	32.48	1
	Nano emulsion	26.14 (21.56 –34.64)	71.37 (48.11 – 178.76)	2.94 ± 0.63	0.99	100	3.08
	Loaded nano emulsion	67.69 (60.47 –74.63)	102.29 (90.611 – 125.20)	7.15 ± 1.18	0.99	38.62	1.19

*ppm expressed as active ingredient (menthol)

In addition, it was found that nano-emulsion was the most effective formulation than both bulk and loaded nano-emulsion. The LC_{50} values for 2nd instar larvae were 70.59 ppm for bulk, 12.23 ppm for nano-emultion and 21.72 ppm for loaded nano-emulsion, LC_{90} values were 30.14, 33.97 and 104.75 ppm for loaded nano-emulsion, nano-emulsion and bulk oil, respectively. The relative potency was 3.25 folds with loaded nano-emulsion and reached 5.77 with nano-emulsion. In addition, it was shown that slope values were 7.48, 2.89 and 9.00 and toxicity index 17.33, 100 and 56.31 for bulk, nano-emulsion and loaded nano-emulsion, respectively. Regression values were 0.98 for both bulk and nano-emulsion and were 0.99 for loaded nano-emulsion**.** In this respect, many researchers reported the effectiveness of plant essential oils against insects. For example, it was found that some medicinal plants essential oils are larvicidal to the third instar larvae of *S. littoralis*. **Ismail and Shaker, (2014)** reported that the LC_{50} values of pepper mint oil on newly hatched larvae of *S. littoralis* were 1.04 ppm after 24h and 0.79 ppm after 48h. The insecticidal effect of some essential oils was estimated by **Isman, et al (2006).** They reported that oil of *Mentha arvensis* produced at least 50% mortality to 4th instar larvae of *Spodoptera litura.*

Pepper mint oil and its corresponding preparations were tested against the fourth instar larvae **(Table, 2)**. It was found that such instar was more tolerable to all of the tested preparations compared to 2nd instar larvae. LC_{50} values were 80.42, 26.14 and 67.69 ppm, while LC_{90} values were177.66, 71.37 and 102.29 ppm for bulk, nano-emulsion and loaded nano-emulsion respectively. The obtained similar results were reported by **Mohamed (2016)** who stated that the nano-formulations of geranium and garlic oils had more effects on *S. littoralis* larvae than bulk ones.

3-3- Effect of the pepper mint oil bulk and its nano-formulations on cartain enzymes activities

The biochemical effects of nano-formulations compared to the bulk oil were tested against 2nd and 4th instars larvae of *S. littoralis*. The tested larvae were treated by the calculated LC_{50} values of each formulation prepared from pepper mint oil. Enzymatic activities were recorded after 4 days post treatment. Results of biochemical effects of nano-formulations are listed in **(Table 3)**.

In 2nd instar larvae of *S. littoralis* bulk oil of pepper mint was the most effective treatment on the specific activity of α-amylase (1.59 µg glucose /min/mg protein) when compared to the control which was 4.99 µg glucose /min/mg protein with reduction of -68.14% with respect to the control. Reduction of the enzyme activity reached to 2.62 and 2.57µg glucose /min/mg protein for pepper mint nano-emulsion and loaded nano-emulsion, respectively, with reduction in the enzyme activity reached **-**47.49% and **-**48.50%, respectively compared to control value. Results of biochemical effects of nano-formulations on 4th instars larvae followed the same trend of the 2nd instar larvae. There was a significant reduction in the α- amylase

enzyme activity when compared with the control 14.16 µg glucose /min/mg protein. The most affected larvae were those treated with bulk pepper mint oil, followed by pepper mint nano-emulsion and then pepper mint loaded nano-emulsion. Their determined anzymatic activities were 3.87 (-72.67%), 5.93 (-58.12%) and 9.47 (-33.12%), respectively.

Reduction on the specific activity of invertase was also observed. The maximal suppression of gut enzyme activity was obtained by loaded formulation from pepper mint nano-emulsions that was reduced to 2.35 (-79.15%) µg glucose /min/ mg protein compared with the control value 11.27 µg glucose /min/ mg protein. There was significantly and mnjbulk pepper mint and pepper mint nano-emulion, respectively. Similar results were obtained by 4th instars larvae showed decreasing in the invertase activity, the recorded decreasing of activities were 31.28 (-59.85%), 38.23 (-50.93%), and 56.80 (-27.10%) for bulk pepper mint oil, pepper mint nano-emulsion and pepper mint loaded nano-emulsion, respectively, compared to the control value (77.91 µg glucose /min/ mg protein).

The activity of trehalase was also significantly reduced by 2nd instars larvae to reach 17.21 (-45.30%), 16.81 (-46.57%) and 15.86 (-49.59%) µg glucose /min/ mg protein for bulk pepper mint oil, loaded nano-emulsion and pepper mint nano-emulsion, respectively. These results were in agreement with those obtained by **Rao, et al (1999**). The same reduction was observed by 4th instars larvae and trehalase activities were arranged in descending order as follows: 93.38 (-50.81%), 90.82 (-52.15%) and 88.38 (-53.44%) for bulk pepper mint oil, pepper mint nano-emulsion and pepper mint loaded nano-emulsion, respectively compared to control value189.82 µg glucose /min/ mg protein. These reductions of digestive enzymes may be due to the plant secondary metabolites. It may cause cytotoxicity in epithelial cells **(Rharrabe et al 2009)**. **Hill and Orchard, (2005)** referred the reduction of enzymatic activities by the lack of food intake.

Results in the same table shows that protease activity in the 2nd instar larvae seemed to be affected to some extent by all tested compounds. The most affected larvae were those treated by pepper mint nano-emulsion (7.48U/min/mg protein) (-64.35%). The protease activity resulted from other treatments reached 12.29 (-41.42%) and 15.87 (-24.36%) U/min/mg protein for pepper mint loaded nano-emulsion and bulk pepper mint, respectively, compared to the control value (20.98 U/min/mg protein).Also, the 4th instars larvae recorded inhibition in protease activity by all tested formulations.

The determined specific activity was 24.75 (-72.95%), 25.02 (-72.66%) and 29.32 (-67.96%) for bulk pepper mint oil, pepper mint loaded nano-emulsion and pepper mint nano-emulsion, respectively compared to the control value (91.51 U/min/mg protein).

The activity of alkaline phosphatase was reduced in the 2nd instar larvae by treatments of pepper mint nano-emulsion and loaded pepper mint nano-emulsion, *i.e.* 18.81 (-31.17%) and 18.93 (-30.74%) µg phenol /min/mg protein, respectively. While, bulk pepper mint oil showed increasing in the enzyme activity that was 34.14 (+24.92%) µg phenol /min/mg protein, compared to the control value (27.33 µg phenol /min/mg protein). These results are parallel to those obtained by **Osman et al (2015)** they clearly indicated that the alkaline phosphatases activities were highly significantly reduced at all-time interval post–treatment of *S. littoralis* larvae treated with nano-zinc oxide. Significant decrease in alkaline phosphatase activity when 4th instars larvae treated with the same formulations. The control larvae recorded 128.38µg phenol /min/mg protein. Results of treatments could be arranged in a descending order according to the inhibition of the enzyme activity.

In case of 2nd instar phenoloxidase, all tested formulations exhibited a significant increase reached 37.42 (+36.72%), 48.05 (+75.56%) and 53.10 (+94.01%) U/min/mg protein for pepper mint nano-emulsion, pepper mint loaded nano-emulsion and bulk pepper mint, respectively, compared to control value (27.37 U/min/mg protein). Also, 4th instars larvae showed an increasing of phenol oxidase activity, it was found an increasing in the enzyme activity when compared to the control (38.87 U/min/mg protein). The specific activity could be arranged in an ascending order according to the increasing of the enzyme activity as follows: 68.12 (+75.25%), 68.94 (+77.36%) and 73.46 (+88.99%) for pepper mint loaded nano-emulsion, pepper mint nano-emulsion and bulk pepper mint oil, respectively. The same finding was reported on neem oil by **Farrag et al (2015)**. The insect increase immune response and attack these foreign particles. Also, **(Santoyo and Aguilar, 2012)** stated that in invertebrate immunology have documented a complex array of host defenses. These defenses include phagocytosis. **Gilmour (1961)** suggested the function of phenol peroxidase in hardening and darkening of the insect cuticle.

Chitinase showed a significant increase the 2nd instar larvae when compared with the control value (11.95 µg NAGA/min/mg protein). The activity could be arranged in a descending order as follows: 18.11(+51.55%), 12.49 (+4.52%) and 12.39 (+3.68%) for bulk pepper mint, pepper mint nano-emilsion and pepper mint loaded nano-emulsion, respectively. **(Retnakaran and Grant, (1985)** clarified that chitin senthyies polymeriz to form discrete chitin microfibrils then covalently bound to proteins. Structural integrity is essential for the polymerization of the oligosaccharides just before the second polymerization start. Inhibition to the enzyme that catalysing the polymerization of UDP-N-acetyl glucosamine to chitin causing inhibition to protease that activates the chitin synthase zymogen and inhibits ecdysone metabolizing enzymes. This stimulates chitinase production which results in molting disruption.

In contrary the 4th instars larvae showed decrease in the enzyme activity of chitinase. Both bulk and nano- emulsion treatments showed decreasing activity 20.02 (-20.33%) and 24.89 (-0.96%), respectively. Treatment with loaded nano-emulsion showed the highest percentage of increase 46.46 (+84.88%) when compared to the control 25.13 µg NAGA/min/ mg protein. Inhibition of chitinase activity can be attributed to the properties of the pepper mint oil as a growth regulator were originally inappropriately timed and poorly coordinated moulting processes, the resulting perturbation of moulting and metamorphosis leads to death, usually because the insects cannot escape from the exuvie **(Liburd et al 2000)**.

Table 3. Specific activity of some enzymes in the 2nd and 4thinstar larvae of *S. littoralis* treated with different prepared formulations of the pepper mint oil

Larval Instar	Formula	Enzymatic Specific activity and percentage of increase or decrease						
		Amylase: µg glucose /min/mg protein	**Invertase µg glucose /min/ mg protein**	**Trehalase µg glucose /min/ mg protein**	**Protease: U/min/mg protein**	**Alkaline phosphatas: µg phenol /min/mg protein**	**Phenol oxidase U/min/mg protein**	**Chitinase: µg NA-GA/min/ mg protein**
2nd	Bulk	1.59±0.08^{c} (-68.14)	6.13±0.26^{c} (-45.61)	17.21±0.40^{b} (-45.30)	15.87±0.46^{b} (-24.36)	34.14±0.74^{a} (+24.92)	53.10±1.57^{a} (+94.01)	18.11±0.75^{a} (+51.55)
	Nano-emulsion	2.62±0.04^{b} (-47.49)	9.48±0.28^{b} (-15.88)	15.86±0.48^{b} (-49.59)	7.48±0.11^{d} (-64.35)	18.93±0.61^{c} (-30.74)	37.42±0.36^{c} (+36.72)	12.49±0.26^{b} (+4.52)
	Loaded Nano-emulsion	2.57±0.07^{b} (-48.50)	2.35±0.06^{d} (-79.15)	16.81±0.23^{b} (-46.57)	12.29±0.34^{c} (-41.42)	18.81±0.25^{c} (-31.17)	48.05±0.62^{b} (+75.56)	12.39±0.63^{b} (+3.68))
	Cont.	4.99±0.23^{a}	11.27±0.19^{a}	31.46±1.36^{a}	20.98±0.42^{a}	27.33±0.64^{b}	27.37±0.41^{d}	11.95±0.25^{b}
	F	77.07**	250.64**	99.82**	66.20**	81.37**	107.52**	25.85**
4th	Bulk	3.87±0.11^{d} (-72.67)	31.28±0.68^{d} (-59.85)	93.38±0.77^{b} (-50.81)	24.75±0.30^{b} (-72.95)	36.79±0.44^{d} (-71.34)	73.46±1.09^{a} (+88.99)	20.02±0.28^{c} (-20.33)
	Nano loaded	5.93±.01^{c} (-58.12)	38.23±0.72^{c} (-50.93)	90.82±4.56^{b} (52.15)	29.32±1.43^{b} (-67.96)	48.85±0.47^{c} (-61.95)	68.94±0.72^{b} (+77.36)	24.89±0.55^{b} (-0.96)
		9.47±0.26^{b} (33.12)	56.80±0.74^{b} (-27.10)	88.38±3.56^{b} (-53.44)	25.02±1.02^{b} (-72.66)	83.42±3.65^{b} (-35.02)	68.12±0.96^{b} (+75.25)	46.46±0.47^{a} (+84.88)
	Cont.	14.16±0.49^{a}	77.91±1.90^{a}	189.82±4.28^{a}	91.51±2.94^{a}	128.38±0.4^{a}	38.87±0.57^{c}	25.13±0.41^{b}
	F	338.36**	564.07**	417.89**	427.67**	767.70**	168.56**	740.71**

*Each value of the specific activity represents the mean of 3 replicates ± s.d. Values between brackets represent the percentage of increase or decrease compared to the control larvae. Values with different letters within the same column are significantly different (p. <0.05).

4- Effects of pepper mint oil nano-formulations on bionomics of the second larval instar of *S. littoralis*.

Table (4) shows the effects of adding three formulations of mint oil (bulk, nano-emulsion and loaded nano-emulsion) at subleethal dose (10 ppm menthol) to artificial dite which fed to 2nd larval instar of *S. littoralis*. Their effects on bionomics have been evaluated. Statistical analyses showed significant difference. The larval duration was prolonged in the three treatments than in control (15.10 ± 0.14 days). Means of larval duration were 22.52 ± 0.61, 21.74 and 22.89 ± days for bulk, nano-emulsion and loaded nano-emulsion, respectively. Results indicated that mint oil may be inhibited digestive enzymes and caused retardation of larval duration. These phenomena are in harmony with our findings in enzymatic activities. These results showed that digestive enzymes were inhibited when larvae were treated with different the three formulations of mint oil.

The percentages of larval mortalities were increased in the three tested formulations in comparison with control (5.00%). The maximum mortality (50%) was occurred in larvae treated with loaded nano-emulsion, followed by nano-emulsion (32.50%), While, bulk oil produced (17.50%). These results indicated that mint oil in all formulations caused different larval mortalities. The larvae fed on different formulations produced malformed larvae (4.33 and 5.0%) for bulk oil and loaded nano-emulsion, respectively.

From the above mentioned results it is well recognized that retardation of growth and high percentage mortality, and malformation of larvae stage was due to adding mint oil. **Renolds et al (1985)** attributed the retardation in the larval growth to the fact that the insects in general spent a considerable amount of energy to detoxify the allele chemicals (secondary plant metabolites) present in food to overcome the effect of treatment. Also, similar results were stated by **Sourguir et al (2013)** who reported that monoterpenes, the major components of plant essential oils act as neurotoxicant and act on acetyl cholinesterase enzyme activity and blocking octopamine receptors in insects.

Pupal duration was also prolonged with regards to control treatment (10.77 ± 0.32 days) while mean duration of the three treatments were 12.90 ± 0.53, 12.25 ± 0.25 and 11.0 ± 0.44 day for bulk oil, nano-emulsion and loaded nano-emulsion, respectively. A significant decrease was occurred in pupal weight in the three formulations in comparison with control. While means of pupal weight were 316.25 ± 11.18, 304.0 ± 9.45 and 300.0 ± 11.55 mg for bulk oil, nano-emulsion and loaded nano-emulsion, respectively. Also, significant parentage mortality between resulted pupae was occurred. The highest mean percentage mortality was 52.63% for loaded nano-emulsion followed by nano-emulsion treatment 26.09% and 20% for bulk oil, while control treatment was 6.35%. The percentage of pupal malformation showed significant difference between means. The highest percentage was 20.0% produced from loaded nano-emulsion, followed by nano-emulsion treatment (18.18%) and bulk oil treatment (4.76%), while control treatment produced 2.50% malformed pupae.

It could be stated also that adding menthol to 2nd instar larvae with different formulations caused prolongation in pupal duration, decrease in pupal wight, increasing in percentage of pupal mortality as well as increasing in percentage of pupal malformation. The foregoing results were in agreement with **Mohamed, (2016)** on *S. littoralis*.

Adult longevity of the resulting adult was insignificantly affected by the three treatments. Means adult longevity were decreased than control (10.10 ± 0.87 days). While means in the three treatments were 9.67 ± 2.03, 8.75 ± 0.69 and 7.56±0.69days for bulk oil, nano-emulsion and loaded nano-emulsion, respectively. Fecundity of resulted adult females showed significant decreasing in mint oil formulations in comparison with. The maximum decrease was occurred in loaded nano-emulsion (633.30 eggs / female), followed by nano-emulsion (650 eggs/female). While bulk oil female fecundity was 1091 eggs/female. Regarding egg fertility which represented by percentage of egg hatchability, results showed significant difference between mean percentages of hatchability. In formulation of loaded nano-emulsion no eggs were hatched. While other two formulations percentages of hatchability were 50.42% and 67.85% for nano-emulsion and bulk mint oil, respectively.

From the aforementioned results it could be stated that 2nd instar larvae fed on loaded nano-emulsion resulted females produced the least mean numbers of eggs and non of them hatched Therefore, this formulation seemed to be the most preferable formation of mint oil. The same trend was obtained by **Klingauf et al (1982)** who stated that *Mentha piperita* oil decreased the fertility of

female moths of *S. littoralis*. **Ismail (2014)** was in agreement with the present findings. The symptoms of toxicity included stop of feeding, delay or prevention of pupation, blackening the body, failure of molting to the next larval instar; formation of larval-prepupal intermediates and malformed pupae.

Table 4. Effect of pepper mint oil nano-formulation on certain biological aspects of the 2nd instar larvae of *S. littoralis*

Formula / Parameter	Bulk oil	Nano-emulsion	Loaded nano-emulsion	Control	Mean/F
Larval duration (days)	22.52±0.61^{a}	21.74±0.24^{a}	22.89±0.50^{a}	15.10±0.14^{b}	123.77**
%Larval mort. (*)	17.50 (24.97±2.19^{c})	32.50 (35.07±1.76^{b})	50.00 (46.45±1.45^{a})	5.00 (13.1±1.85^{d})	49.17*
% Malformations in larvae (*)	4.35 (11.27±1.76^{a})	0.0 (0.00^{a})	5.00 (12.73±1.53^{a})	2.56 (7.63±3.91^{a})	3.83^{NS}
Pupal duration (Days)	12.90±0.53^{a}	12.25±0.25^{a,b}	11.00±0.44^{a,b}	10.77±0.32^{b}	5.11**
Pupal wt/mg	316.25±11.18^{b}	304.00±9.45^{b}	300.00±11.55^{b}	386.10±13.20^{a}	14.95 **
% Pupal mortality (*)	20.00 (26.33±0.63^{c})	26.09 (30.67±0.78^{b})	52.63 (46.70±0.35^{a})	6.35 (14.10±1.42^{d})	230.80**
% Malformations in pupa (*)	4.76 (12.87±0.78^{b})	18.18 (25.10±0.40^{a})	20.00 (26.57±0.43^{a})	2.50 (9.87±0.98^{b})	148.69**
Adult longevity (days)	9.67±2.03^{a}	8.75±0.69^{a}	7.56±0.69^{a}	10.00±0.87^{a}	1.57^{NS}
Fecundity	1091.00±149.00^{a,b}	650.00±73.60^{b}	633.30±16.67^{b}	1441.00±110.00^{a}	12.90**
% Hatchability (*)	67.85 (55.57±0.38^{b})	50.42 (45.00±0.35^{c})	0.00 (0.00^{d})	92.50 (74.70±0.64^{a})	604.600**

(*) Arcsin transformation of percentage. NS= Not significant. * Significant. **Highly significant.

Each value represent the means of four replicates (each composed of 10 larvae) ±s.e.. Values with different letters within the same row are significantly different (P.< 0.05) (ANOVA) (Tukey test)

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تقييم تأثير زيت النعناع و صوره النانوية على بعض الأنشطة الانزيمية والمظاهر البيولوجية لدودة ورق القطن

[146]

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الكلمات الدالة: زيت النعناع، التحضيرات الثانوية، الاختبارات الحيوية، إنزيمات دودة ورق القطن

الموجـــــز

تم دراسة تقييم كفاءة زيت النعناع وبعض صوره النانوية (الصورة الطبيعية– مستحلب النانو– المستحلب المحمل) على العمر الثاني والرابع ليرقات دودة ورق القطن. وكذلك تأثيرها على النشاط الانزيمي وبعض المظاهر الحياتية ليرقات العمر الثاني.

وقد أظهرت نتائج الفحص بالمجهر الألكتروني النفاذ أن أحجام الجزيئات النانوية تتراوح بين 20–90 نانوميتر. أظهرت نسبة كفاءة تكوين الكابسول والسعة التحميلية أن استخدام الماء كان الافضل عند تكوين كابسولات النانو. كما أظهرت نتائج التقييم الحيوي للمستحضرات الثلاثة أن التركيزات نصف المميتة (LC_{50}) للعمر الثاني لليرقة كانت 70,59–12,23– 21,72 جزءاً بالمليون للصورة الطبيعية، مستحلب النانو، والمستحلب المحمل علي الترتيب. بينما للعمر الرابع كانت تلك القيم 80,47– 26,14 67,69 جزءً بالمليون على الترتيب. كما كانت قيم LC_{90} للعمر الثاني 104,75– 33,97– 30,14 جزءاً بالمليون وللعمر الرابع 177,66– 71,37–102,29 جزءً بالمليون للمستحضرات السابق ذكرها على الترتيب. أظهرت نتائج دليل السمية والكفاءة النسبية أن مستحلب النانو كان الاكثر كفاءة يليه المستحلب المحمل وأقلهم كفاءة زيت المستحضر الطبيعي.

أظهر تأثير المعاملات الثلاثة على النشاط الانزيمي لليرقات المعاملة حدوث تثبيط لانزيمات الاميليز، الانفرتيز، التريهاليز، البروتييز، الفوسفاتيز القاعدى، بينما حدث زيادة معنوية في نشاط انزيمى الكيوتيكل (الفينول أوكسيديز– الكيتينيز). أظهرت نتائج تأثير معنويا لمستحضرات الثلاثة المضافة لغذاء يرقات العمر الثاني علي بعض المظاهر الحياتية لهذه اليرقات والحشرات الكاملة. حيث حدث زيادة في فترة الاعمار اليرقية وزيادة في نسب الموت ونسب التشوهات، أيضاً حدث طول فترة طور العذراء ونسب الموت ونسب التشوهات، بينما حدث انخفاض في أوزان العذارى. لم يكن هناك تأثيراً معنوياً على طول فترة الحشرات الكاملة، بينما كان التأثير معنوياً على كفاءة الاناث التناسلية ونسب خصوبة البيض.

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