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Larvicidal Activity of Certain Natural Essential Oils and Their Nanoemulsions against *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Abstract

To compare the insecticidal activity of natural essential oils of citronella, mustard, sage, and their nanoemulsions against the second and fourth instar larvae of greater wax moth, *Galleria mellonella*, the tested natural oils were converted into nano emulsions that were formulated and bioassay evaluated. The results indicated that LC_{50} values were 0.266, 0.553, and 0.791% for mustard, citronella, and sage oils, respectively. While in case of mustard, citronella, and sage nanoemulsions LC_{50} values were 0.226, 0.501, and 0.238%, respectively when tested on the 2nd instar larvae. While on the 4th instar larvae the LC_{50} values were 5.944, 1.454, and 2.609% for mustard, citronella, and sage oils, respectively. On the other hand, LC_{50} values were 0.663, 0.504, and 0.700% for mustard, citronella, and sage nanoemulsions, respectively. These results proved that essential oils nanoemulsions were more effective than conventional oils. Biochemical changes induced in 4th instar larvae at the treatment with LC_{50} of tested essential oils and their nanoemulsions were also studied. According to the findings, all examined essential oils elevated the activities of acetylcholinesterase (AChE) and decreased the activity of glutathione S-transferase (GST) in all treatments except citronella nanoemulsion where it caused significant increase. In addition, there were significant decrease in the activity of alpha esterases enzymes for all treatments except sage nanoemulsion which increase the enzymes activity of the tested larvae compared with the control.

Keywords: Citronella, mustard, sage oils, mortality, biochemical studies.

Introduction

Bees are an essential component of human food security because honey bees (*Apis mellifera* L.) are among of the most significant managed pollinators of many economically important plants worldwide **(Topitzhofer *et al.*, 2019),** in addition to their useful products like pollen, royal jelly, bee wax, and honey. Honey bee hives are also infested with pest insects such as the greater and lesser wax moths which they are among the most destructive enemies of honey bee hives. Also, they attack and destroy beeswax combs, especially those that are in storage, causing significant losses to hives all over the world **(Crane, 1990; NBN Atlas, 2017)**. Moths lay their eggs in the hive, develop into larvae, eat in their way through the honeycombs, and trap emerging bees in addition to wreaking havoc. Due to this, honeycombs are destroyed, and causes the weak colonies to degrade **(Kwadha *et al.*, 2017).**

Several chemicals and non-chemicals have been used to control the wax moth on stored beeswax combs. Use of chemical and fumigant insecticides such as sulphur dioxide, acetic acid, formic acid, para dichloro benzene (PDCB), methyl bromide or phosphine are harmful to bee populations **(Whitcomb, 1967; Calderone, 2000)**. Certain chemicals were used to control wax moth, like PDCB, contaminate honey bee products, such as honey and wax **(Wallner, 1991)**.

The use of several botanical products as effective alternatives to synthetic and chemical pesticides has drawn much attention in recent years. These plant products are reported to be more effective, less expensive, biodegradable and safe for mankind and environment, than their synthetic counterparts, which are environmentally persistent and toxic to nontarget organisms including humans eliciting many unidentified diseases after bioaccumulation. Therefore, alternatives to conventional pesticides are required to be developed from the active ingredients of plant origin **(Sorour, 2021)**. Despite the essential oils' promising properties, some issues with them need to be resolved before use, including volatility, poor water solubility, and a propensity for oxidation **(Moretti *et al.*, 2002)**. The delivery of nano-encapsulated pesticides for controlled release is just one of the benefits of using

nanomaterials in agriculture **(Ghormade *et al.*, 2011)**. In addition, it was found that nano-formulations affiliates to EOs as control release formulations **(Martin *et al.*, 2010)**.

In this study, the toxicity of some essentials oils extracted from three local plants citronella oil (*Cymbopogon sp*), mustard oil (*Brassica Nigra*), and sage oil (*Salvia officinalis*) in their conventional forms compared with their nanoemulsions, were evaluated, on both the 2^{nd} and 4^{th} instar larvae of *Galleria mellonella* L. under laboratory conditions. In addition, the effect of the same bulk and nano-formulations on some enzymatic activities, i.e. acetylcholinesterase, glutathione S-transferase and nonspecific esterases in 4^{th} instar larvae were estimated.

Materials and Methods

2.1. Essential oils used:

Citronella oil (*Cymbopogon sp*), mustard oil (*Brassica Nigra*), and sage oil (*Salvia officinalis*) were purchased from the Oil Extraction Unit at the National Research Center in Egypt.

2.2. Chemicals for enzymes determination:

- Bovine albumin standard was obtained from Stan bio Laboratory (Texas, USA).
- Commasie Brilliant Blue G-250 was obtained From Sigma (Sigma Chemical Co.) .
- P-nitro anisole was purchased from Ubichem Ltd. in Hampshire and has a purity of 97%.
- Nicotinamide adenine dinucleotide phosphate (reduced form, NADPH) was from BDH chemicals Ltd. (Poole, England).
- The remaining chemicals were of high quality and were obtained from reputable local companies.

2.3. Nanoemulsion preparation:

The oil was diluted with distilled water in a ratio of 1:2, and 2% Tween 80 was added as an emulsifier to create the nanoemulsion by the modification of the method described by **Jerobin *et al.*, (2012)**. The formed emulsion was sonicated for 30 minutes at 60 Hz using an ultrasonic cleaner set with the model WUC-DO3H 290W. Afterwards, it was sonicated for 1 minute with a high energy ultrasonication probe (VCX750, 750W, 20 kHz), then resonicated for 30 min by the ultrasonic cleaner under cooling conditions **(Yousef *et al.*, 2018)**.

2.4. Preparation of loaded nanoemulsion:

Alginate nanocapsules were made by using oil in water (o/w) emulsification, proceeded by cross-linking using Polyethylene glycol (PEG) **(Lertsutthiwong *et al.*, 2008)**. PEG alginate was dissolved in distilled water at 50°C for 45 minutes to produce a PEG alginate solution (10%, w/v). Tween 80 was used as an emulsifier to dilute the tested oils with distilled water while mechanical stirring continued for 10 min. In an essence, the PEG alginate O/W emulsion was made by drop wise dispersion of diluted oil into appropriate volume of alginate solution 1: 1 oil to alginate under continuous mechanical stirring at room temperature. The emulsion formed was sonicated for 30 min using ultrasonic cleaner set, model WUC-DO3H 290 W and 60 Hz and then sonicated for 4min using a high energy ultra-sonication probe model VCX 750, 750 W, 20 kHz) **(Yousef *et al.*, 2018).**

2.5. Characterization:

A variety of characterization techniques were used to determine the morphological shape, size, consistency content, and chemical interactions of the obtained nanoformulations and/or nanoparticles in order to confirm that the prepared tested oils were transferred into nanosized particles.

2.5.1. Transmission Electron Microscopy (TEM):

By using Transmission Electron Microscopy (TEM; Jeol, JEM-2100), the morphological shapes of the prepared nanoformulations were examined. The nano capsule suspensions was diluted with distilled water and deposited onto a carbon coated copper grid and stained with a 1% phospho tungsten acid, then results examined by magnification (20000X) and photographed.

2.5.2. Fourier Transforms Infrared (FTIR) measurements:

To identify any chemical interactions between the cross-linking agent (PEG) and oils nanoemulsions, FTIR measurements were performed. This was carried out by taking following samples from pure bulk oils, oils nano emulsions. FTIR 6600, JAS-CO was used to perform the FTIR measurements in accordance with the methodology explained by **Jerobin *et al.*, (2012)**.

2.6. Test insect:

In laboratory conditions (25 ± 5 °C, 65 ± 5% RH), larvae of the greater wax moth, G. mellonella, were reared on an artificial diet. The artificial diet contents were; wheat flour 250g, wheat bran 1000g, milk powder 100g, yeast powder 50g, honey 175 ml, black honey 400 ml, and glycerol 175 ml **(Metwally *et al.*, 2012)**. In a plastic tube, the proper amount of the aforementioned dry ingredients (wheat flour, wheat bran, milk powder, and yeast powder) was weighed and thoroughly mixed. Appropriate quantity of honey, black honey and glycerol (liquid ingredients) were measured and mixed thoroughly in a plastic beaker. The dry ingredients were mixed with liquid ingredients for uniform distribution of liquid ingredients over dry ingredients. The prepared artificial diet was used for the rearing of larvae of *G. mellonella*. For egg hatching, The egg mass transferred in a plastic jar (1 liter capacity) and an artificial diet. Initially, larvae were reared in a plastic jar (1 liter capacity) containing an artificial diet for the first week of larval development because they take up little space. Afyer that, larvae were transferred to a round plastic container (2-liter capacity) with artificial diet. On metal shelf, the containers were arranged. During this period, fresh diet was provided for the healthy growth of larva. For healthy, late instar larvae were transferred into a

separate plastic container (2-liter capacity,) with an artificial diet for pupation. Larvae were left to pupate in the artificial diet without being disturbed. Afterwards, the adults were transferred into a different plastic container with a 2-liter capacity provided with the accordion papers for egg laying. The egg masses adhered to were cut and used for additional research.

2.7. Bioassay tests:

Different concentrations of each oil solution were prepared10, 5, 2.5, 1.25, and 0.625% (v/v) for conventional oils and 5, 2.5, 1.25, 0.625, and 0.3% (v/v) for oils nanoemulsions oils. Thirty grams of artificial diet were used in each concentration and divided into three equal replicates in 500 ml glass jars. From each concentration, one ml was taken and added to 10 g of artificial diet. After water drying, the treated artificial diet was infested by ten of second and fourth instar larvae of *Galleria mellonella*. Three replicates of untreated larvae as a control. Mortality rates were noted at days 1, 2, 3, 4, 5, and 6, and corrected based on **the Abbott formula (1925).** Probit analysis was performed for calculating LC_{25}, LC_{50}, and LC_{90} after 6 days post-treatment according to **Finney (1971)**.

2.8. Determination of the effect of tested oils bulk and nanoemulsions on certain enzymatic activity:

The 4th instar larvae of *G. mellonella* were used to examine the biochemical activites of prepared nano compounds compared to their bulk forms. The fourth instar larvae were treated with LC_{50} values of tested oils. According to the results of the bioassay tests, forty larvae were used for each concentration and transferred separately into glass jars. 24 hours period of treatment exposure was done to the treated larvae. Untreated diet was served as a control by adding distilled water according to the method of **Sorour (2021)**.

2.8.1. Apparatus:

The fourth instar larvae of *G. mellonella* were homogenized for biochemical analysis in a chilled glass Teflon tissue homogenizer (ST – 2 Mechanic-Preczyina, Poland). After homogenation, supernatants were kept in a deep freezer at -20ºC till use for biochemical assays. Double beam ultraviolet / visible spectrophotometer (spectronic 1201, Milton Roy Co., USA) was used to measure absorbance of colored substances or metabolic compounds.

2.8.2. Preparation of insects for analysis:

The insects were prepared as described by **Amin (1998)**. In distilled water (50 mg/ml), they were homogenized. A chilled centrifuge was used to centrifuge homogenates at 8000 rpm for 15 min at 2 °C. The deposits were thrown away, and the supernatants, which is referred as enzyme extract, can be stored at least one week without appreciable loss of activity when stored at less than 0 °C.

2.8.3. Determination of acetylcholinesterase activity:

A substrate called acetylcholine bromide (AChBr) was used to measure the acetylcholinesterase activity in accordance with the procedure described by **Simpson *et al.*, (1964**). The reaction mixture included 200 µl of the enzyme solution, 0.5 ml of 0.067 M phosphate buffer (pH 7), and 0.5 ml of AChBr (3 mM). It took exactly 30 minutes for the test tubes to be incubated at 37 °C. The test tubes received 1 ml of alkaline hydroxylamine, which was made up of an equal volume of 2 M hydroxylamine chloride and 3.5 M NaOH. The next step involved adding 0.5 ml of HCl (1 part concentration of HCl and 2 parts of ΔH_2O). Just after a vigorous shake, the mixture is given two minutes to stand. Then, 0.5 ml of the ferric chloride solution (0.9 M $FeCl_3$ in 0.1 M HCl) was added and thoroughly mixed. At 515 nm, AChE's hydrolysis caused a decrease in AChBr, which was measured.

2.8.4. Determination of glutathione S-transferase (GST):

Glutathione S-transferase (GST) catalyzes the conjugation of reduced glutathione (GSH) with 1-chloro 2,4-dinitrobenzene (CDNB) via the -SH group of glutathione. The conjugate , S-(2,4-dinitro-phenyl)-L-glutathione could be detected as described by the method of **Habig *et al.*, (I974)**.The reaction mixture consisted of 1 ml of the potassium salt of phosphate buffer (pH6.5),100µl of GSH and 200µl of larval homogenate. The reaction started by the addition of 25µl of the substrate CDNB solution.The concentration of both GSH and CDNB was adjusted to be 5mM and 1mM, respectively. Enzyme and reagents were incubated at 30˚C for 5 min. The increment in absorbance at 340 nm was recorded against blank containing everything without the enzyme to determine the nanomole substrate conjugated/min/larva using a molar extinction coefficient of 9.6/mM/Cm.

2.8.5. Determination of nonspecific esterases:

According to **Van Asperen (I962)**, alpha esterases (α -esterases) and beta esterases (β -esterases) were calculated using α-naphthyl acetate or β-naphthyl acetate as substrates, respectively. The reaction mixture consisted of 5ml substrate solution ($3x10^{-4}$M α-or β-naphthylacetate, 1% acetone and 0.1M phosphate buffer, pH7) and 20µl of larval homogenate made up the reaction mixture. The mixture was incubated for exactly 15 min at 27˚C, then 1 ml of diazoblue color reagent (prepared by mixing 2 parts of 1% diazoblue B and 5 parts of 5% sodium lauryl sulphate) was added. The developed color was read at 600 or 555 nm for α- and β-naphthol produced from hydrolysis of the substrate, respectively. α-and β-naphthol standard curves were prepared by dissolving 20 mg α- or β-naphthol in 100 ml phosphate buffer, pH7 (stock solution), standard curves for α- or β-naphthol were created. Ten milliliters of stock solution were diluted up to 100 ml by the buffer. Aliquots of 0.1, 0.2, 0.4, 0.8 and 1.6 ml of diluted solution (equal to 2, 4,8,16 and 32 µg naphthol) were pippeted into test tubes and completed to 5 ml by phosphate buffer. One milliliter

of diazoblue reagent was added and the developed color was measured as mentioned before.

2.9. Statistical analysis:

SAS (Statistic Analysis Software) **(SAS 1999)** was used to statistically analyze. All data were presented as means and standard errors. By using a one-way analysis of variance (Danken) ($P \leq 0.05$), the statistical significance of differences between the various study groups was assessed. Duncan's multiple range tests was used to distinguish between means (to find differences between means of treatments at significant levels of ($P \leq 0.05$). According to **Finney (1971)**, LC_{25}, LC_{50}, and LC_{90} were calculated using probit analysis.

Results and discussion:

3.1. Characterization of the prepared nanoemulsions:

3.1.1. Electron microscopy examination:

Using nano-samples of the tested oils, TEM analysis was performed to determine the morphological sizes and shapes of the prepared nanoformulations (loaded nanoemulsions) **(Sanghi and Verma 2009)**. **Fig. (1)**.

The mean particle sizes ranged between 30 and 60 nm, with an average size of 40 nm for mustard nanoemulsion **(Fig.1 (A)**). While for citronella nanoemulsion the particle sizes were ranged between 50 and 100 nm with an average size of 50 nm **(Fig.1 (B))** and the mean particle sizes were ranged between 100 and 200 nm, with an average size of 100 nm for sage nanoemulsion **(Fig.1 (C))**.

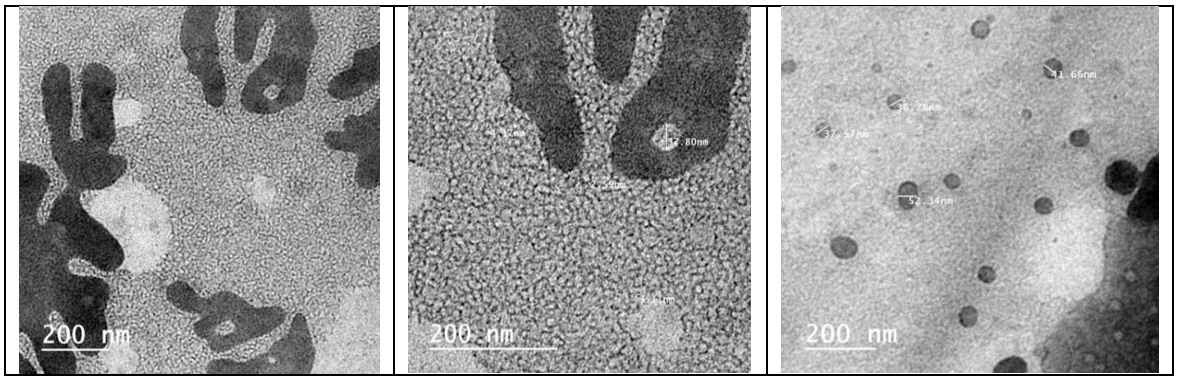


Fig.1(A) TEM images of the prepared mustard nanoemulsion.

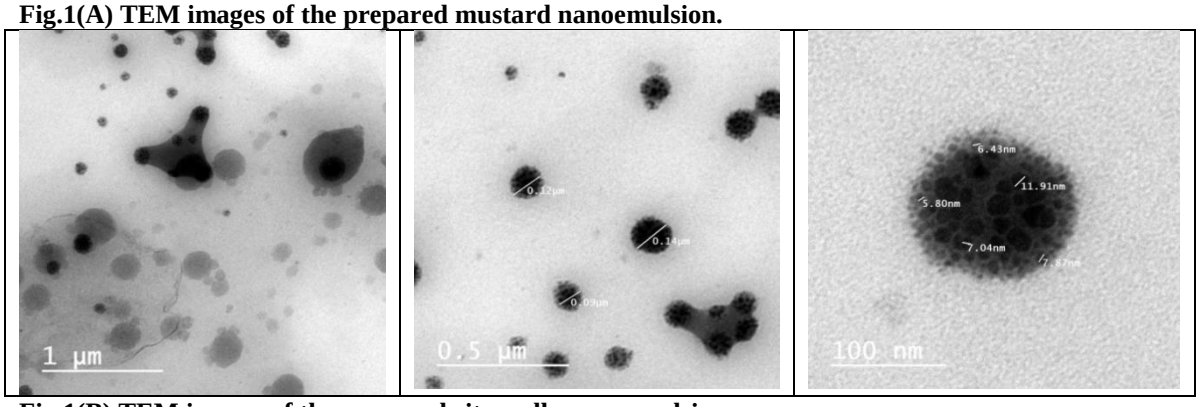


Fig.1(B) TEM images of the prepared citronella nanoemulsion.

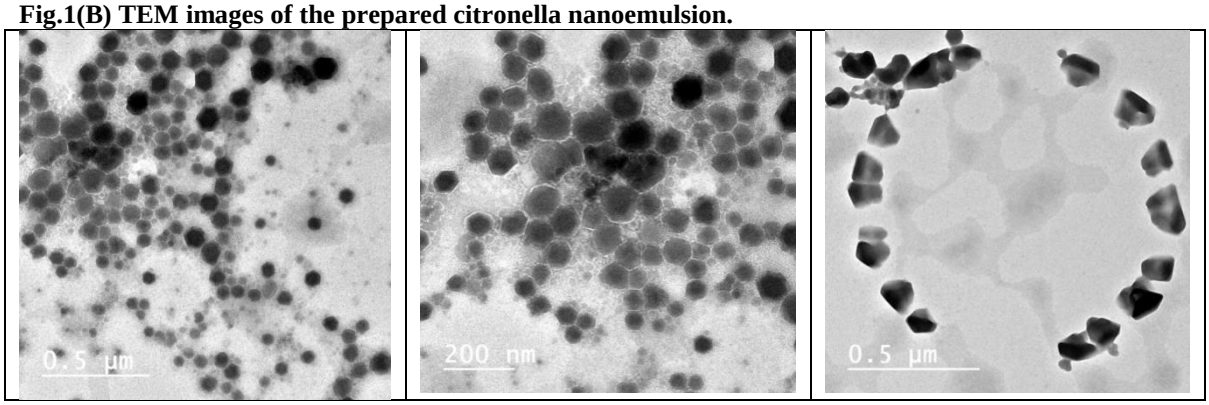


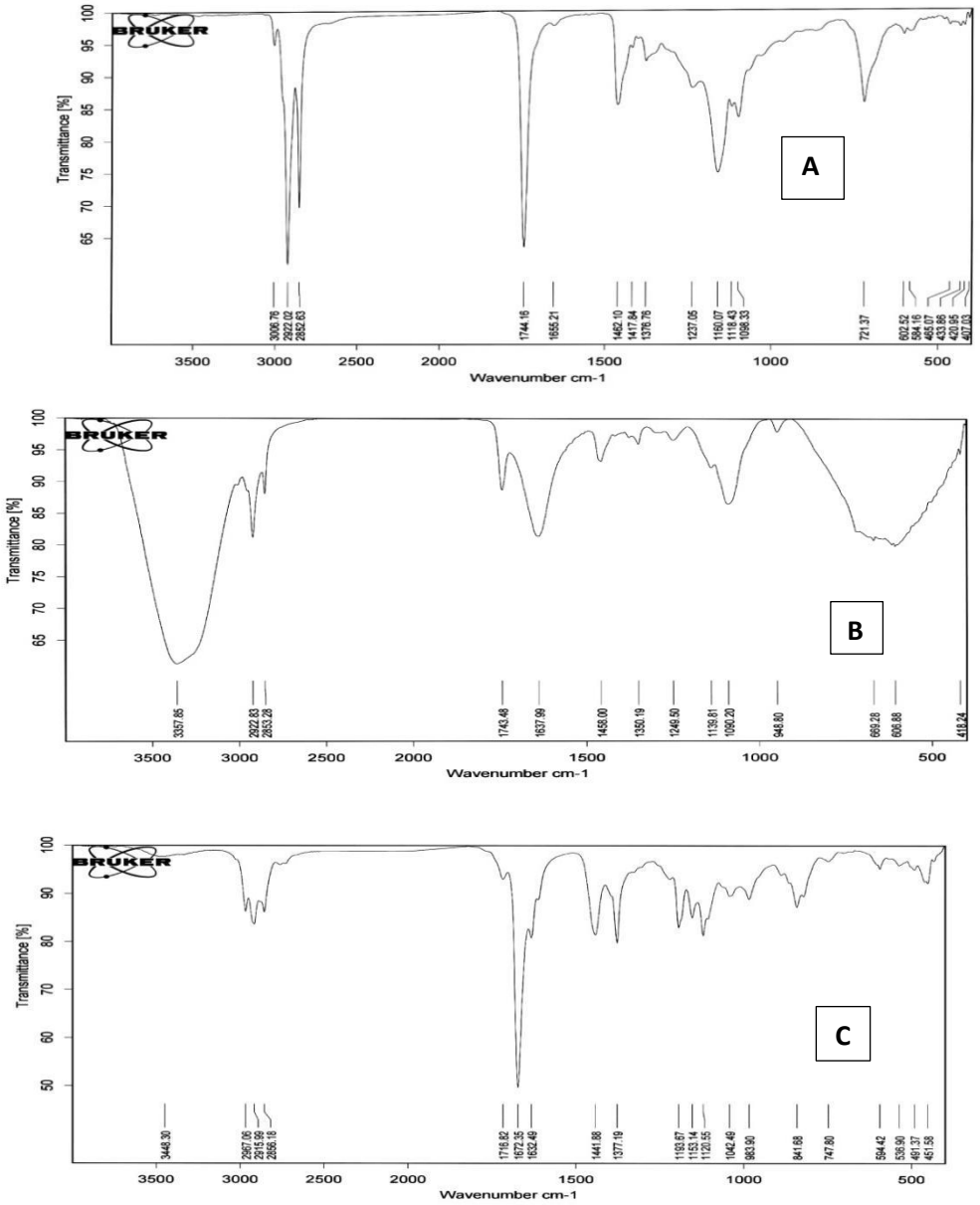
Fig.1 (C) TEM images of the prepared sage nanoemulsion.

3.1.2. Fourier Transforms Infrared (FTIR) measurements:

To comprehend the stability of the nanoemulsion inside the matrix system, the chemical

interactions between the ingredients, specifically the PEG alginate solution and the oils nanoemulsions, were investigated **(Fig. 2)**.

FTIR spectrum as shown in **Fig.2 (A)** was proven significant peaks in samples of mustard bulk oil at: 3006.76 cm^{-1} as CAH stretching vibration of the cis-double bond, 2852.63 - 2922.02 cm^{-1} corresponding to (C – H) bond, 1744.16 cm^{-1}ascribed to aldehyde (CHO), and 1655.21 cm^{-1} corresponding to aromatics group. While in the case of mustard nanoemulsion that showed in **Fig.2 (B)** determined significant peaks at: 3357.85 cm^{-1} corresponding to the (N – H) bond, 2853.28 – 2922.3 cm^{-1} ascribed to the (C - H) bond and 1743.48 cm^{-1} corresponding to aldehyde (CHO) These peaks are similar to those reported by **Zahir *et al.*, (2017)** who noted that at room temperature, the band between 2854.7 and 2925.8 cm^{-1} exhibits the asymmetric and symmetric (C - H) stretching vibrations ofthe aliphatic CH_2, the band between 3006.0 and 3007.0 cm^{-1} exhibits the (C - H) stretching vibration of the cis-double bond, and 3473 cm^{-1} is attributed to the (O - H) stretching vibration of hydroperoxide.



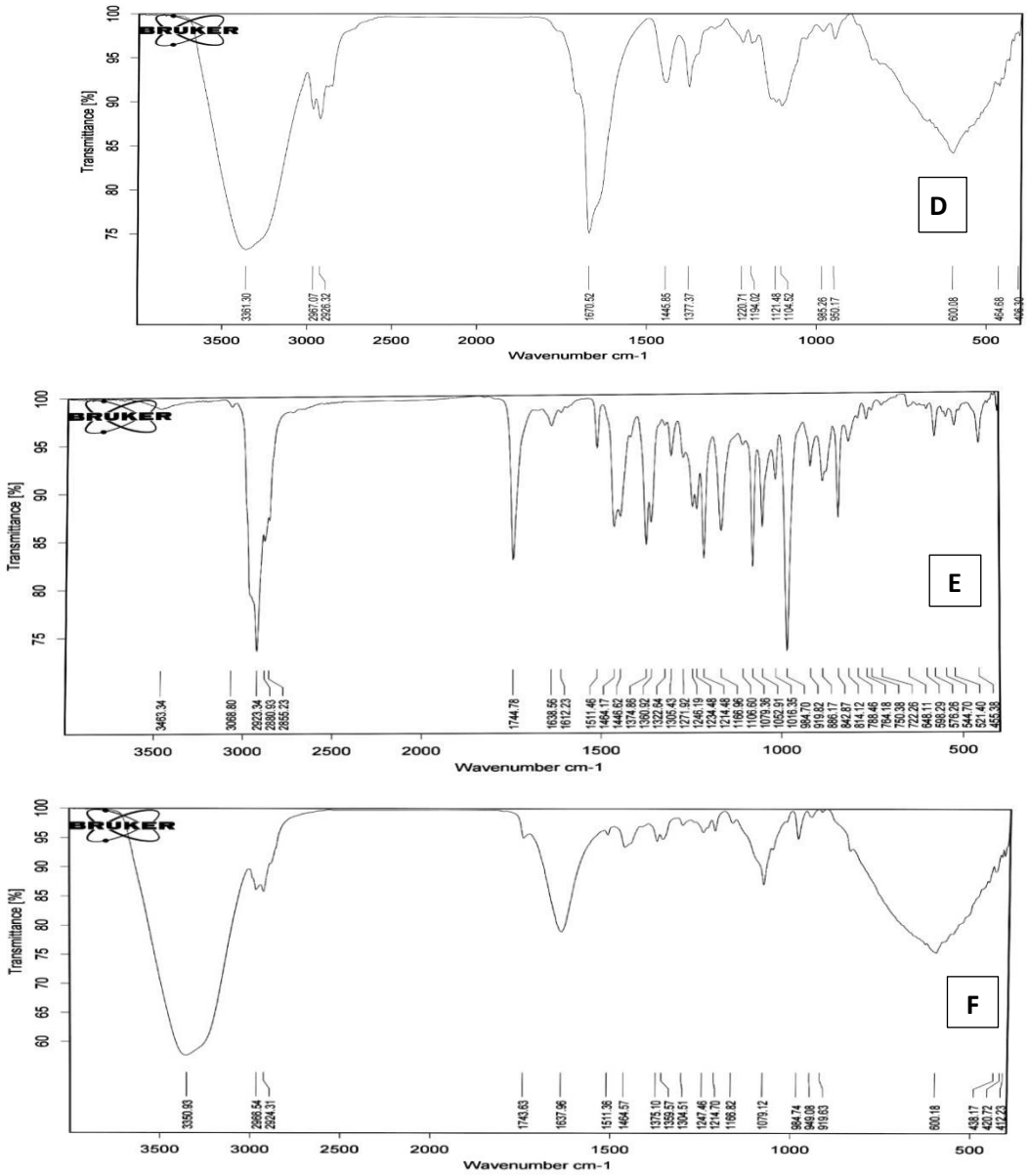


Fig.2 (A): Bulk mustard oil. (B): Mustard oil nanoemulsion. (C) Bulk citronella. (D) Citronella oil nanoemulsion. (E): Bulk sage. (F): Sage nanoemulsion.

In order to understand the stability of nanoemulsions, the chemical interactions between the ingredients, namely PEG and tested oils, were examined. Significant peaks in samples of the FTIR spectrum, as depicted in **Fig. 2 (C)**, confirm significant peaks in citronella oil, which were at 3448 cm^{-1} (O-H) bond, 2856 – 2967 cm^{-1} (alkane group) (C – H) bond, 1716 cm^{-1} acid (R COOH) and 1672 cm^{-1} (Aromatic group). Samples of citronella nanoemulsion were not changed too more than the pure Citronella, as shown in **Fig.2 (D)** samples and their peaks were represented at 3361 cm^{-1} (O-H) bond, 2926 – 2967 cm^{-1} (alkane group) (C – H) bond and 1670 cm^{-1} Alkene group (C= C) bond. This outcome is very consistent with earlier research by **Rihayat *et al.*, (2020)**, who reported that the total attenuated reflectance IR spectrum concludes the percentage of transmittance corresponding to the wave number. According to the hydroxyl polymer group (OH), there is a strong broad peak in the range 3600-3200 cm^{-1}, specifically at 3365.78 cm^{-1}. Other strong, branching peaks in 2935–2915 cm^{-1} range are appropriate for stretching methyl C and methylene, where the majority of aliphatic alkyl groups are seen. The medium peak at 2719.63 cm^{-1} confirms that compounds with terminal C aldehyde carbonyls have been stretched. The peaks in the 1,750–1705 cm^{-1} Aldo, keto, ester, and/or acid (CO) stretch are distinct and sharp. Unsaturated olefinic was a result of the strong and relatively narrow absorption peak at 1668.43 cm^{-1}. Group (C - C) Methylene C H (1485-1445 cm^{-1}), symmetrical bend CH methyl (1380-1371 cm^{-1}), and stretch of aryl-O H (1270-1230 cm^{-1}) all showed sharp and strong peaks. Additional functional groups with moderate peaks include the C O bends (1140-1050 cm^{-1}), simple OH stretches (1200-1000

cm^{-1}), and trans-unsaturated CH=CH (910-860 cm^{-1}). At 825.53 cm^{-1}, a moderate peak that represents the strain or tri-substitution of alkene (C H) is found. Aromatics and vinyl C H groups are both associated with low vibrations in the 750–660 cm^{-1} range.

FTIR spectrum of Sage essential oil, shown in **Fig.2 (E)** presented peaks at 3463 cm^{-1}, which could be assigned to the (O–H) alcohol bond, 3068 cm^{-1} (C=C-H) alkene bond, from 2880 to 2923 cm^{-1} that could be (C-H) alkane bond and 1744 cm^{-1} that could refer to an ester (RCOOR). While in the case of sage nanoemulsion samples, there were no differences much from the pure sage as shown in **Fig.2 (F)** which presented peaks at 3350 cm^{-1} which could be (N-H) amines bond, from 2924 to 2966 cm^{-1} that referred to (C-H) alkane bond and 1743 cm^{-1} that could be ester (RCOOR) and (C=O) stretching vibration in carbonyl group. These findings concur with those of **Oliveira *et al.*, (2016)**, who claimed that the analysis of the sage sample revealed bands that might be related to lipids: at 3304 cm^{-1}, also possibly related to OH groups vibrations at 2980 cm^{-1} and 2904 cm^{-1}, also possibly related to ethanol, but this last band may also be related to asymmetric stretching CH_2 vibration of lipids. There was no information on the band at 2118 cm^{-1}. The band at 1644 cm^{-1} might be connected to protein stretching C=O vibration, carboxylic acid, or amide. There was no identification of the bands at 1454 cm^{-1} and 1418 cm^{-1}. The bending symmetric CH_3 (CO) vibration of 1,8-cineole may be the cause of the band at 1385 cm^{-1}. There were two bands that were likely caused by the bending of the CH_2 vibration at 1328 cm^{-1} and 1276 cm^{-1}, respectively. Possible causes of the bands at 1086, 1044, and 877 cm^{-1} include carbohydrates. The first two of these bands might be connected to polysaccharides. The 1,8-cineole's (C-O-C) symmetric stretching could also be the cause of the band at 1086 cm^{-1}, the OH groups' vibration could also be the cause of the band at 1044 cm^{-1}, and the stretching vibration of the 1,8-cineole's (CH_2) CH_2 or ethanol could also be the cause of the band at 877 cm^{1}.

3.2. Efficiency of three tested oils bulk and their nanoemulsions against 2nd and 4th instar larvae of *Galleria mellonella:*

Data in **Table (1)** cleared that citronella essential oil recorded the highest larval mortality (80%), followed by sage essential oil (76.67%), and finally, a mustard essential oil which recorded (73.33%) larval mortality compared to control with no mortality (0.0%). These findings corroborated those of **Mohamed *et al.*, (2014)** whoevaluated the larvicidal activity of essential oils derived from two essential plants Marjoram, *Origanum majorana* and Lemon Grass, *Cymbopogon proximus* against early fourth instar larvae of the greater wax moth, *Galleria mellonella* L. the results indicated that the percentage of *G. mellonella* larval mortality increased with exposure duration and concentration. On the other hand, toxicity is influenced by exposure duration, essential oil type, and concentration. According to the findings, the essential oil of *Origanum majorana* caused 4% mortality in the insect population after 24 hours at the lowest concentration (0.625%), compared to 100% mortality at the highest concentration (5%) after 96 hours. The *Cymbopogon proximus* essential oil caused 4% mortality in the insect population after 24 hours at the lowest concentration (0.625%), compared to 90% mortality at the highest concentration (5%) after 96 hours.

Table 1. Efficiency of tested bulk oils against 2nd instar larvae of *Galleria mellonella:*

Bulk oils	Conc.% (v/v)	Accumulative Mortality % after Indicated Days					
		1	2	3	4	5	6
Mustard	10	40	50	53.33	63.33	66.67	73.33
	5	30	40	43.33	56.67	63.33	70
	2.5	23.33	36.67	40	53.33	60	63.33
	1.25	20	30	36.67	50	56.67	60
	0. 625	16.67	23.33	30	46.67	53.33	56.67
Citronella	10	23.33	40	50	63.33	70	80
	5	16.67	30	43.33	60	63.33	73.33
	2.5	13.33	26.67	40	50	56.67	66.67
	1.25	13.33	23.33	33.33	46.67	50	56.67
	0. 625	10	20	30	40	46.67	53.33
Sage	10	30	43.33	50	56.67	70	76.67
	5	26.67	30	43.33	53.33	63.33	66.67
	2.5	23.33	26.67	40	46.67	53.33	60
	1.25	20	23.33	30	40	43.33	53.33
	0. 625	10	23.33	30	33.33	43.33	50

Table 2. Efficiency of nanoemulsions of tested oils against 2nd instar larvae of *Galleria mellonella:*

Oil's nanoemulsions	Conc.% (v/v)	Accumulative Mortality % after Indicated Days					
		1	2	3	4	5	6
Mustard	5	60	63.33	66.67	73.33	76.67	83.33
	2.5	40	53.33	60	63.33	66.67	73.33
	1.25	33.33	46.67	53.33	60	63.33	66.67
	0. 625	30	43.33	50	53.33	56.67	60
	0. 3	26.67	36.67	43.33	50	53.33	56.67
Citronella	5	63.33	80	86.67	93.33	96.67	100
	2.5	33.33	43.33	46.67	53.33	63.33	80
	1.25	30	40	43.33	50	60	73.33
	0. 625	26.67	33.33	40	50	53.33	66.67
	0. 3	20	30	36.67	46.67	50	63.33
Sage	5	43.33	56.67	70	86.67	93.33	96.67
	2.5	43.33	53.33	60	66.67	70	83.33
	1.25	36.67	46.67	53.33	60	66.67	76.67
	0. 625	26.67	43.33	53.33	56.67	63.33	73.33
	0. 3	23.33	40	50	56.67	60	66.67

Data in **Table (2)** showed the accumulative mortality of the essential oils nanoemulsions tested on 2nd instar larvae of wax moth. These findings showed that, when compared to bulk oils, all of the tested nanoemulsions oils were extremely effective against wax moth larvae. The accumulative mortality was 100% for citronella, followed by sage at 96.67% and mustard at 83.33% after 6 days of exposure time, respectively. Among the essential oils, citronella oil accomplished well against wax moth larvae with a 100 % mortality, which is consistent with earlier research by **Ramesh *et al.*, (2022)**, who estimated the efficacy of some plant products (citronella, neem and garlic oil) against third instar larvae of *Galleria mellonella* L., the results proved that there are significant differences in *G. mellonella* larvae mortality and it peaked at day 14 after treatment, ranging from 53.33 to 76.67%; citronella oil at 3% resulted in the highest mortality (42.67%), while garlic at 4% extract had the lowest mortality (28.00%). Next in terms of mean mortality was 3% neem oil (38.67%).

According to **Table (3)**, the observed larval mortality showed high toxicity against *G. mellonella* 4th instar larvae as it increased gradually with exposure time and concentration. However, citronella essential oils had a significant toxic effect on the tested larvae, and the mortality percentages reached 70%. Sage essential oil came in the second with a mortality percentage of 56.67%, and mustard essential oil came in the third with a mortality percentage of 53.33% after 6 days, according to the results. These outcomes resembled those of **Elbarky *et al.*, (2015)** who demonstrated the impact of four distinct concentrations of the tested oils' peppermint, *Mentha piperita* L., geranium, *Pelargonium graveolens* L., and basil, *Ocimum basilicum* L., on the fourth larval instar of *G. mellonella* at various time intervals. The results demonstrated that all concentrations tested had a toxic effect on the treated larvae compared to the control.

Table 3. Efficiency of tested bulk oils against 4th instar larvae of *Galleria mellonella:*

Bulk oil	Conc. (v/v)	Accumulative Mortality % after Indicated Days					
		1	2	3	4	5	6
Mustard	10	16.67	26.67	33.33	43.33	46.67	53.33
	5	16.67	20	26.67	36.67	43.33	50
	2.5	10	20	23.33	33.33	36.67	43.33
	1.25	6.67	13.33	20	26.67	33.33	36.67
	0. 625	3.333	10	16.67	20	30	33.33
Citronella	10	23.33	33.33	46.67	60	70	70
	5	16.67	33.33	40	43.33	46.67	56.67
	2.5	13.33	23.33	30	36.67	40	53.33
	1.25	10	16.67	23.33	30	36.67	50
	0. 625	6.67	13.33	20	26.67	30	43.33
Sage	10	10	16.67	23.33	33.33	46.67	56.67
	5	6.667	13.33	16.67	26.67	36.67	50
	2.5	3.333	6.67	10	16.67	26.67	46. 67
	1.25	0	3.333	6.667	10	23.33	43.33
	0. 625	0	0	3.33	6.67	20	33.33

Table 4. Efficiency of nanoemulsions of tested oils against 4th instar larvae of *Galleria mellonella:*

Oil's nanoemulsions	Conc. (v/v)	Accumulative Mortality % after Indicated Days					
		1	2	3	4	5	6
Mustard	0.5	23.33	30	36.67	46.67	53.33	66.67
	0.25	16.67	23.33	30	43.33	50	63.33
	0.125	13.33	20	26.67	40	46.67	53.33
	0.06	10	16.67	23.33	33.33	43.33	50
	0.03	6.67	10	20	23.33	36.67	43.33
Citronella	5	36.67	40	63.33	80	83.33	90
	2.5	30	36.67	46.67	50	56.67	66.67
	1.25	20	26.67	33.33	40	46.67	60
	0. 625	16.67	20	26.67	33.33	40	53.33
	0. 3	13.33	16.67	23.33	30	36.67	46.67
Sage	5	13.33	20	26.67	43.33	56.67	76.67
	2.5	10	16.67	20	40	43.33	63.33
	1.25	6.67	13.33	16.67	30	40	53.33
	0. 625	3.333	10	13.33	26.67	33.33	46.67
	0. 3	0	6.67	10	23.33	26.67	43.33

The percentage of larval mortality increased vertically with concentration and horizontally with exposure time. The highest percentage of total larval mortality (100%) was obtained with a concentration of 2.5% of *Ocimum basilicum*, while the lowest percentage (18%) was recorded at a concentration of 0.625 of the same oil. Data in **Table (4)** illustrated that *Galleria mellonella* mortality was induced by mustard, citronella, and sage nanoemulsions at all concentrations.

However, the citronella nanoemulsion caused mortality of 90% at a concentration of 5%, whereas the sage and mustard nanoemulsions caused mortality of 76.67% and 66.67%, respectively. These results support those of **Said *et al.*, (2019)**, who assessed the toxicity of five natural essential oils at four concentrations (5-10-15-20%) each: Camphor (*Eucalyptus globules*: Myrtaceae) and lavender (*Lavandula angustifolia*: Labiatae) - Clove (*Syzygium aromaticum*: Myrtaceae) - Mint (*Mentha spp*. Labiatae) - rosemary (*Rosmarinus officinalis*: Labiatae) against the third instar of *G. mellonella*. The results showed that the tested essential oils were highly toxic to the tested larvae, but lavender and rosemary essential oils were particularly toxic to the tested larvae; after 48 hours, the mortality rates for lavender essential oil and eucalyptus essential oil were 90%, and 100%, respectively.

Table 5. Lethal concentrations of tested oils bulk and their nanoemulsions against 2nd instar larvae of *Galleria mellonella*:

Oils		Day*	Lethal concentrations % (v/v) and their95% confidence limits			Slope ± SE
			LC_{25}	LC_{50}	LC_{90}	
Bulk	Mustard	6	0.005 (0.001 - 0.042)	0.266 (0.032 - 2.180)	491.369 (59.967 - 4026.281)	0.392 ± 0.466
	Citronella	6	0.052 (0.014 - 0.185)	0.553 (0.154 – 1.987)	50.062 (13.927 – 179.955)	0.655 ± 0.283
	Sage	6	0.059 (0.015 – 0.232)	0.791 (0.200 – 3.122)	110.288 (27.947 – 435.227)	0.599 ± 0.304
Nanoemulsions	Mustard	6	0.017 (0.004 – 0.068)	0.226 (0.057 – 0.890)	29.575 (7.506 – 116.524)	0.612 ± 0.304
	Citronella	5	0.126 (0.064 – 0.250)	0.501 (0.253 - 0.994)	6.925 (3.495 – 15.487)	1.283 ± 0.151
	Sage	5	0.035 (0.013 - 0.096)	0.238 (0.088 - 0.646)	8.924 (3.292 – 24.191)	0.876 ± 0.221

***: days after treatmen.**

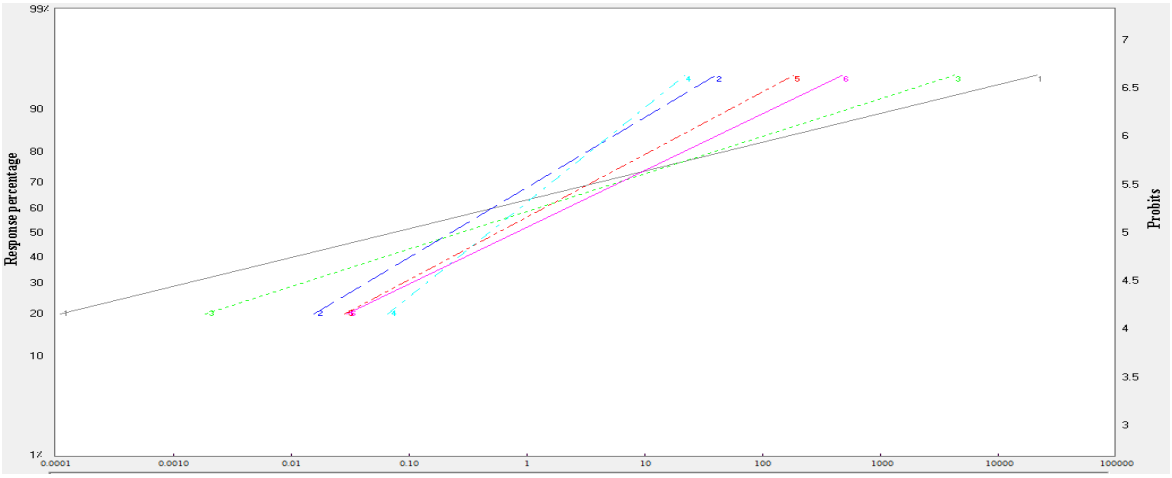


Fig. (3): LDP line of oils and their nanoemulsions against 2nd instar larvae of *G. mellonella*.

The results related to the effect of mustard, citronella, and sage oils in different formulation forms, bulk, and nano-emulsions against 2nd instar larvae *G. mellonella* are listed in **Table (5)**. These results showed LC_{25}, LC_{50}, and LC_{90} values and the slope of the different tested oils. It is evident from the LC_{50} values of the formulations tested against *G. mellonella* larvae in the 2nd instar that nano preparations increased the toxic effect. In addition, it was found that nanoemulsions were more effective formulations than bulk. The LC_{50} values for 2nd instar larvae were 0.266, 0.553, and 0.791% for mustard, citronella, and sage oils, respectively. While LC_{50} values were 0.226, 0.501, and 0.238% for mustard, citronella, and sage nanoemulsions, respectively. **Mohamed *et al.*, (2014)** assessed the larvicidal activity of essential oils obtained from marjoram, *Origanum majorana*, and lemon grass, *Cymbopogon proximus*, on early 4th instar larvae of the greater wax moth. Following a comparison of the estimated LC_{10}, LC_{50}, and LC_{90} values, it was discovered that *Origanum majorana* essential oils are more toxic to *G. mellonella* than *Cymbopogon proximus* essential oils.

Table (6) show the LC_{25}, LC_{50}, and LC_{90} values as well as the slope of the various tested oils in bulk and nanoemulsions. The findings showed that against *G. mellonella* larvae of the 4th instar, oils nanoemulsions were more effective than conventional oils. The values of LC_{50} were 5.944, 1.454, and 2.609% for mustard, citronella, and sage oils, respectively. But LC_{50} values were 0.663, 0.504, and 0.700% for mustard, citronella, and sage nanoemulsions, respectively. **Said *et al.*, (2019)** evaluated the effect of four concentrations (5-10-15, and 20%) of five essential oils: lavender–camphor–mint– clove - rosemary on the 3rd instar larvae of the greater wax worm. The results showed that the lavender and rosemary essential oils had LC_{50} values of 7.11 and 6.45%, respectively, while eucalyptus, clove, and mint were less toxic, with LC_{50} values of 9.45, 11.45, and 13.61%, respectively.

Table 6. Lethal concentrations of tested oils bulk and their nanoemulsions against 4th instar larvae of *Galleria mellonella* after 6 days of treatment:

Oils		Lethal concentrations % (v/v) and their95% confidence limits			Slope ± SE
		LC_{25}	LC_{50}	LC_{90}	
Bulk	Mustard	0.196 (0.033 - 1.159)	5.944 (1.003 - 35.235)	3906.255 (659.006 - 23154.318)	0.455 ± 0.394
	Citronella	0.071 (0.015 - 0.340)	1.454 (0.303 - 6.981)	453.208 (94.406 - 2175.687)	0.516±0.348
	Sage	0.328 (0.112 - 0.965)	2.609 (0.888 - 7.671)	134.093 (45.614 - 394.196)	0.756±0.239
Nanoemulsions	Mustard	0.031 (0.006 - 0.152)	0.663 (0.133 - 3.294)	229.399 (46.153 - 1140.201)	0.505±0.355
	Citronella	0.102 (0.044 - 0.237)	0.504 (0.217 - 1.170)	10.443 (4.499 - 24.239)	1.008±0.187
	Sage	0.081 (0.026 - 0.252)	0.700 (0.226 - 2.163)	41.650 (13.478 - 128.705)	0.726±0.250

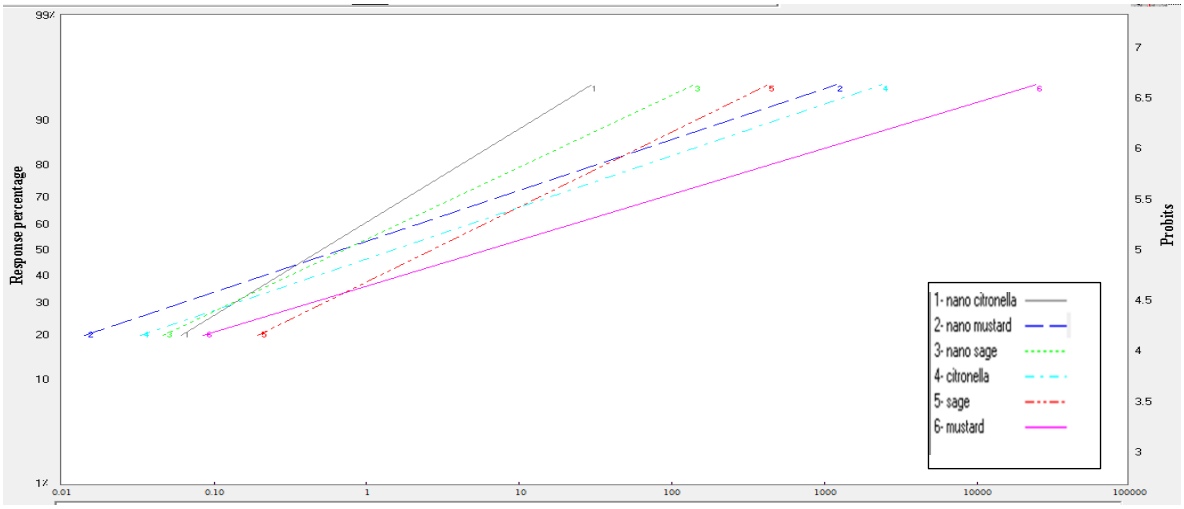


Fig. (4): LDP line of oils and their nanoemulsions against 4th instar larvae of *G. mellonella*.

3.3. Effect of tested oil bulk and nanoemulsions on biochemical aspects of *Galleria mellonella*:

Table 7. Effect of tested plant essential oils on the certain enzymatic activity of *Galleria mellonella*:

Tested oils		Enzymatic activity (Mean ± SD)		
		AchE (ug AchBr/min/g.b.wt)	GST (mmol sub. Conj./min/g.b.wt)	Alpha esterases (ug α-naphthol/min/g.b.wt)
Bulk	Mustard	213.33^{a} ± 15.95	28.33^{e} ± 6.03	781.33^{e} ±43.50
	Citronella	102.33^{d} ± 7.51	47.0^{e} ± 4.36	986.33^{c} ±12.06
	Sage	123.33^{c} ± 7.64	134.67^{d} ± 5.03	894.67^{cd} ±39.15
Nanoemulsions	Mustard	160.67^{b} ± 11.02	244.00^{c} ± 18.73	820.0^{de} ±40.0
	Citronella	82.67^{e} ± 6.81	431.33^{a} ±19.01	978.33^{c} ±36.86
	Sage	111.33^{cd} ±10.02	342.33^{b} ±31.56	2407.67^{a} ± 55.54
Control		71.33^{e} ± 9.07	367.67^{b} ± 29.26	2270.67^{b} ±113.14

Values with different letters are significantly different at P< 0,05 (Duncan test).

Results in **Table (7)** showed significant differences in the activities of AChE, GST, and Alpha esterase values between conventional oils and their nanoemulsions control compared with control. The data confirmed that all tested essential oils increased the AChE activity and decreased GST in all treatments except citronella nanoemulsion which caused a significant increase, and there was a significant decrease in Alpha esterases enzymes for all treatments except sage nanoemulsion which increased the enzymes of *G. mellonella* larvae compared to the control. The averages activity of AChE were 71.33, 213.33, 102.33, and 123.33 (ug AChBr/min/g.b.wt) for control, mustard, citronella, and sage, respectively, while they were 160.67, 82.67, 111.33 and 71.33 (ug AChBr/min/g.b.wt) for mustard, citronella, sage nanoemulsions, and control, respectively. The average means of GST were 28.33, 47.0, 134.67, and 367.67 (mmol sub. Conj./min/g.b.wt) for the three oils and control, respectively. While the outcomes of average means of GST were 244.0, 431.33, 342.33, and 367.67 (mmol sub. Conj./min/g.b.wt) for oils nanoemulsions and control. Regarding alpha esterases, results in **Table (7)** indicate the averages which were 781.33, 986.33, 894.67, and 2270.67 (ug α-naphthol/min/g.b.wt) for bulk oils and control, respectively. But in the case of oils, nanoemulsions results were 820.0, 678.33, 2407.67, and 2270.67 **(**ug α-naphthol/min/g.b.wt) for mustard, citronella, sage nanoemulsions, and control, respectively. These findings coincide with that reported by **Said *et al.*, (2019)** who assessed the biochemical parameters (total protein, alkaline phosphatase, and acetylcholinesterase) in the third instar larvae fed on treated artificial diets containing LC_{50} of lavender, camphor, mint, clove, and rosemary oils for 48 hours. The analysis of the data revealed that treated and control larvae had significantly different levels of total protein, alkaline phosphatase, and AChE. The findings demonstrate that, when compared to the control, all of the tested essential oils increased the total protein content and decreased alkaline phosphatase in the tested larvae.

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نشاط بعض الزيوت الطبيعية ومستحلباتها النانوية كمبيدات لليرقات ضد يرقات دودة الشمع الكبيرة *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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تمت مقارنة نشاط الزيوت الطبيعية مثل السترونيلا والخردل والمريمية ومستحلباتها النانوية كمبيدات حشرية ضد يرقات العمر الثاني والرابع لدودة الشمع الكبيرة *Galleria mellonella* ، و ذلك بتحويل الزيوت الطبيعية المختبرة إلى مستحلبات نانوية تمت صياغتها وتقييمها لإجراء إختبارات التقييم الحيوى. أشارت النتائج إلى أن قيم التركيز المميت ل 50% من التعداد (LC_{50}) كانت 0.266 و 0.553 و 0.791٪ لزيوت الخردل والسترونيلا والمريمية على التوالي. بينما قيم LC_{50} في حالة المستحلبات النانوية لزيوت الخردل, السترونيلا والمريمية كانت 0.226 و 0.501 و 0.238٪ على التوالي عند معاملة يرقات العمر الثاني. بينما كانت قيم التركيز المميت النصفي ليرقات العمر الرابع هى 5.944 و 1.454 و 2.609٪ لزيوت الخردل والسترونيلا والمريمية على التوالي. بينما كانت قيم LC_{50} 0.663 و 0.504 و 0.700٪ للمستحلبات النانوية لزيوت الخردل والسترونيلا والميرمية على التوالي. أثبتت هذه النتائج أن مستحلبات النانو للزيوت الأساسية كانت أكثر فعالية من الزيوت فى الصورة التقليدية. كما تمت دراسة التغيرات البيوكيميائية التى تحدث في يرقات العمر الرابع المعاملة بقيم LC_{50} للزيوت الأساسية المختبرة ومستحلباتها النانوية. أظهرت النتائج أن جميع الزيوت الأساسية المختبرة تسببت فى زيادة معنوية فى محتوى إنزيم الأستيل كولين إسترايز (AChE), ونقص معنوى فى محتوى إنزيم الجلوتاثيون S− ترانسفيراز (GST) في جميع المعاملات باستثناء مستحلب النانو لزيت السترونيلا الذي أدى إلى زيادة معنوية فى مستوى الإنزيم ، كما كان هناك انخفاض معنوي في مستوى إنزيمات ألفا إستيرايز فى جميع المعاملات باستثناء مستحلب النانو لزيت المريمية الذي أدى إلى زيادة مستوى إنزيم الالفا إستيريز فى اليرقات المختبرة مقارنة بالكنترول.