5. SUMMARY

This study was carried out to estimate the toxic, biochemical and genotoxicity effects of three oils (citronella, mustard and sage) and their nanoemulsions on the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) and the greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae).

Transmission Electron Microscopy (TEM; Jeol, JEM-2100) and (FTIR) devices were used to determine the features of prepared oils nanoemulsions. To evaluate the efficiency of bulk oils and their nanoemulsions on *Spodoptera littoralis* and *Galleria mellonella*, bioassay tests were performed against second and fourth instar larvae and the LC_{25}, LC_{50}, and LC_{90} were calculated. Biochemical effects of bulk and nanoemulsions of tested oils on 4^{th} instar larvae whichb treated with LC_{50} concentrations were estimated the activities of Acetylcholine esterase (AChE), Glutathione S Transferase (GST) and Alpha esterases enzymes. To determine the mutagenic levels of the mustard, citronella and sage oils, and their nanoemulsions on the DNA of the fourth instar larvae of *S. littoralis* and *G. mellonella*, the ISSR marker system was used.

5.1 Preparation and characterization of oils nanoemulsions:

By using an ultrasonic cleaner set with the model WUC-DO3H 290W and a high energy ultrasonication probe (VCX750, 750W, 20 kHz) the nanoemulsions of citronella, mustard and sage oils were prepared. The characterization of oils nanoemulsions were estimated by Transmission Electron Microscopy (TEM; Jeol, JEM-2100) and (FTIR) devices.

5.1.1 **Electron microscopy examination of the studied nanoemulsions:**

The average sizes of oils nanoemulsions were 40, 50, and 100 nm for mustard, citronella and sage nanoemulsion, respectively.

5.1.2 Fourier Transforms Infrared (FTIR) measurements:

The chemical interactions between the ingredients, specifically the PEG alginate solution and the oils nanoemulsions, were detected.

Data proved that the mustard bulk oil contained 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 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).

In the case of citronella oil, data showed that 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, that the 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.

FTIR spectrum of Sage essential oil, 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 that the peaks cleared that 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.

5.2 The Efficiency of three tested oils bulk and their nanoemulsions against the two investigated insects:

5.2.1 Against 2nd and 4th instar larvae of *Galleria mellonella:*

5.2.1.1 Against 2nd instar larvae of *Galleria mellonella:*

Data 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%). While in the case of oils nanoemulsions when compared to bulk oils, the results showed that all of the tested nanoemulsions oils were extremely effective against wax moth larvae. The accumulative mortality was 100% for citronella nanoemulsion, followed by sage nanoemulsion at 96.67% and mustard nanoemulsion at 83.33% after 6 days of exposure time, respectively.

The LC_{25}, LC_{50}, and LC_{90} values and the slope of the different tested oils were estimated. 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.

5.2.1.2 Against 4th instar larvae of *Galleria mellonella:*

The observed data showed that the 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. While 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.

The LC_{50}, and LC_{90} values as well as the slope of the various tested oils in bulk and nanoemulsions were evaluated and 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.

5.2.2 Against 2nd and 4th instar larvae of *Spodoptera littoralis:*

5.2.2.1 Against 2nd instar larvae of *Spodoptera littoralis*:

The comparison between Mustard, citronella, and sage oils against 2nd instar larvae of *S. littoralis* was intended to see that the mustard oil was proved to be more effective in controlling *S. littoralis*, the mortality rate of *S. littoralis* were 96.67, 86.67, 80, 70 and 63.33% at concentrations of 10%, 5%, 2.5%, 1.25%, and 0.625%, respectively after 6 days exposure time. While for citronella oil the mortality rate were 80, 73033, 60, 50, and 40% followed by sage oil which caused mortalities percentages of 66.67, 63.33, 56.67, 50 and 46.67% at concentrations of 10%, 5%, 2.5%, 1.25%, and 0.625%, respectively after 6 days exposure time.

The highest cumulative larval mortalities were observed with the maximum concentration of 5% which produced 100 % larval mortalities after 5 days post-exposure to mustard nanoemulsion, followed by sage nanoemulsion 100% and citronella nanoemulsion 90% after 6 days post-treatment.

Data represented the LC_{50}, and LC_{90} values for mustard, citronella, and sage oil bulk and nanoemulsions against the 2nd instar larvae of *S. littoalis*. Results showed that the LC_{5o} values were 0.231, 1.898, and 2.500% for mustard, citronella, and sage, respectively. While they were 0.253, 0.266, and 0.962% for mustard citronella and sage nanoemulsions, respectively.

5.2.2.2 Against 4th instar larvae of *S. littoralis*:

Data showed that the highest tested concentration of 10 % of Mustard oil caused the highest mortality rate 96.67%, while, in the case of citronella and sage oils caused a mortality percentage of 76.67% after 6 days of exposure time. The other concentrations 5. 2.5, 1.25, and 0.625 % recorded 76.67, 70, 60, and 53.33 %

for mustard oil, respectively. While recorded 60, 53.33, 46.67, and 36.67%, respectively for citronella oil and 70, 66.67, 56.67, and 53.33% for sage oil. On the other hand the mortality percentages of larvae treated with mustard nanoemulsion were 100, 86.67, 80, 70, and 63.33% at concentrations 5, 2.5, 1.25, 0.625, and 0.3%, respectively. While the sage nano emulsion caused larvae mortality recorded 93.33, 86.67, 83.33, 73.33 and 66.67%, followed by citronella nanoemulsion which recorded 83.33, 73.33, 70, 63.33, and 43.33% at concentrations 5, 2.5, 1.25, 0.625 and 0.3%, respectively.

The LC_{50} values were 0.889, 3.358, and 0.995% for bulk mustard, citronella, and sage, respectively. On the other hand, LC_{50} values for mustard, citronella, and sage nanoemulsions were 0.657, 0.572, and 0.488, respectively.

5.3 Effect of tested oil bulk and nanoemulsions on some biochemical aspects:

5.3.1 The effect on *Galleria mellonella*:

The results showed significant differences in the activities of AChE, GST, and Alpha esterase values between conventional oils and their nanoemulsions 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.

5.3.2 The effect on *Spodoptera littoralis*:

The results indicated that there was a highly significant increase in stimulation in the case of mustard and citronella bulk and nanoemulsions in AChE activity, while there were no significant changes in the case of sage oil bulk and nanoemulsion compared with the control. On the other hand, there was a

significant decrease in GST enzyme for mustard, citronella bulk oil, and mustard nanoemulsion; in contrast, the GST values were significant increase in the case of sage bulk oil mustard, citronella and sage nanoemulsions. For alpha esterase activities there was a significant decrease in all treatments except mustard nanoemulsion which caused a significant increase in the enzyme activities compared with the control.

5.4 The results of genotoxicity tests / molecular analysis:

5.4.1 ISSR analysis:

To determine the mutagenic levels of the mustard, citronella, sage oils, and their nanoemulsions on the DNA of the fourth instar larvae of *Spodoptera littoralis* and *Galleria mellonella*, the ISSR marker system was used. The number of amplified DNA fragments per primer ranged from 8 bands (primer ISSR-9) to 15 bands (primers ISSR-5). The number of polymorphic bands (without Unique) per primer ranged from 5 bands (primer ISSR-9, primer ISSR-7 and primer ISSR-9) to 11 bands (primer ISSR-5). While the number of polymorphic bands (with Unique) per primer ranged from 5 bands (primer ISSR-9) to 12 bands (primer ISSR-5).

The treatments of mustard oil and mustard nanoemulsion resulted in absence of many bands in lanes 3 and 4 reflecting probable deletion mutation. In the same regard, the new bands that appeared in treatments citronella oil, citronella nanoemulsion and sage nanoemulsion could be attributed to possible insertion mutations.

5.4.2 Analysis of molecular phylogeny:

The analysis of the ISSR data revealed highly similar results to the cluster analysis. The results indicated that the LC_{50} of citronella oil, citronella nanoemulsion and sage oil were more similar to the control than the LC_{50} of mustard oil, mustard and sage nanoemulsions for the *Spodoptera littoralis*. While in the case of *Galleria mellonella* the results revealed that the LC_{50} of mustard,

mustard nanoemulsion and sage oil were more similar to the control than the LC_{50} of citronella, citronella nano emulsion and sage oil.

The results detected the genetic mutagenicity levels and screen the degree of polymorphism between treated and untreated larvae. Furthermore, established the relationship between the oils and their nanoemulsions, the ISSR study results were used to create a dendrogram. The results showed that the lowest mutagenic insecticidal effects on the *Spodoptera littoralis* DNA was observed in the LC_{50} of citronella, sage oils and their nanoemulsions treatment compared to the control. In contrast, the highest mutagenic effect was observed in the LC_{50} mustard oil and its nanoemulsion. For *Galleria mellonella* the results indicated that the lowest mutagenic insecticidal effects on the DNA were showed in the LC_{50} of mustard, sage oils and their nanoemulsions. While the highest mutagenic effects was observed in the LC_{50} of citronella bulk and nanoemulsion

5.5 Semi field experiment of the toxic effect of the tested oils, and their nanoemulsion against the cotton leafworm *Spodoptera littoralis* and on the tomato plants:

5.5.1 The effect of tested oils and their nanoemulsions on *Spodoptera littoralis*:

The results showed that the mustard oil was more effective than mustard nanoemulsion the mortality percentages reached 100% after 48 hours post treatment for bulk mustard oil. While the sage and citronella nanoemulsions were more effective than their bulk forms and safer to the tomato plants, the mortality recorded 73.33 and 86.76% for concentrations LC_{50} and double rate of LC_{50} for citronella nanoemulsion, and recorded 63.33 and 96.76 for concentrations LC_{50} and double rate of LC_{50} for sage nanoemulsion after 72 hours.. While the sage and citronella nanoemulsions were more effective than their bulk forms and safer to the tomato plants

5.5.2 Phytotoxicity of tested oils and their nanoemulsions on the tomato plants:

4.5.2.1 The morphological changes of the tested plants:

From the noticed data of the morphological changes in treated plants were derived that there were no phytotoxic effects of mustard and nanoemulsions of citronella, sage, and mustard. While there were phytotoxic effect of citronella and sage oils on the plants after 24 hour post treatment that they caused complete wilting in the treated plants.

4.5.3. Anatomical alterations of the tomato leaf:

Results indicated that the foliar application with mustard nano emulsion followed by citronella oil and sage oil respectively, recorded the highest values as exceeded that of other foliar application treatments and control in terms of the most studied anatomical features.

Regarding to the upper cuticle layer thickness, could be noticed that increment in the cuticle thickness especially, in case of mustard nano emulsion, mustard oil and citronella nanoemulsion treatments (13.20µ) comparing with the control (5.50µ).

As for the lower cuticle layer thickness, it was increased to reach its maximum values (11.00µ) with mustard nano emulsion and mustard oil treatments followed by citronella nanoemulsion (9.90µ) respectively, compared with the other treatments and control (4.40µ).

For the epidermis tissues, thickness of both upper and lower epidermis tissues were highly affected with different applied treatments. Here, upper epidermis tissue thickness value was increased to reach (31.90µ) with mustard nano emulsion, (26.40µ) and (23.65µ) with citronella nanoemulsion and mustard oil treatments respectively, which were the more effective treatments in the same

order compared with the control (22.00µ). As for lower epidermis thickness was (27.50µ) with mustard nano emulsion and (23.10µ) with citronella nanoemulsion but decreased to reach (16.50µ) with untreated plant, respectively.

With regard to the leaf blade thickness it was increased to reach its maximum values (245.30 and 239.80µ) with mustard and citronella nano emulsions treatments respectively, compared with control (135.30µ). For mesophyll tissues, Here, spongy tissue thickness was recorded (121.00µ) with sage nano emulsion which was the most effective treatment while decreased to reach (59.40µ) with the control. Meanwhile, palisade tissue thickness reached its maximum values (68.20µ) with mustard nano emulsion followed by citronella oil treatment (66.00µ) but decreased to reach (27.50µ) with control.

Regarding, vascular tissues (i.e., xylem and outer as well as inner phloem tissues) thickness of both xylem and phloem tissues were reached their maximum values with sage oil and mustard nano emulsion treatments. Here, xylem tissue thickness was (165.00µ) and (132.00µ) respectively, which were the more effective treatments in the same order compared with control (121.00µ). Also, phloem tissue thickness (outer and inner phloem) nearly behaved as the same as the xylem tissue thickness.

With regard to midrib anatomical features, could be noticed that increment in the midrib thickness especially, in case of sage oil treatment (1391.50µ) and (1155.00µ) with citronella nanoemulsion compared with the control (1077.45µ).

Of interest to note that these positive responses of different anatomical aspects to treatments especially, in case of mustard, citronella oil and sage nanoemulsions treatments compared with other treatments and the control were completely reversed upon enhancing vegetative growth of treated plants.