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Insecticidal Activity and Biochemical Study of the Clove Oil (*Syzygium aromaticum)* Nano- Formulation on *Culex pipiens* L. (Diptera: Culicidae)

El Gohary E. El Gohary[1], Shaimaa M. Farag[1], Ahmed A. El-Sayed[2], Reham R. Khattab[2] and Dalia M. Mahmoud[1*]

1. Department of Entomology, Faculty of Science, Ain Shams University, Cairo, Egypt.
2. Photochemistry Department, Industrial Chemical Division, National Research Centre, Egypt.

[*]**Corresponding Author:Dalia.mahmoud@sci.asu.edu.eg**

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ABSTRACT

In the present study *Syzygium aromaticum* essential oil (EO) was extracted using water as a solvent and encapsulated within chitosan nanoparticles, which are characterized by Ultraviolet-Visible (UV-VIS) spectra, Infra-Red (IR), Transmission Electron Microscopy (TEM), and X-ray diffraction (XRD). The insecticidal activity for both *Syzygium aromaticum* bulk EO and encapsulated EO within chitosan nanoparticles was determined. Both of the two oil forms were assessed as a larvicide against third instar larvae of *Culex pipiens* by measuring LC values. Encapsulated nano-formulation EO showed higher toxicity (LC_{50} =20 ppm) than the bulk EO (LC_{50} =39 ppm). The biochemical changes were measured after treatment with both forms of EO. There is an increase in the activity of three enzymes (acid phosphatase, alkaline phosphatase, and glutathione-S-transferase)**,** while the activity of acetylcholinesterase was decreased. The obtained results revealed that water extract of *Syzygium aromaticum* EO and nano-encapsulated EO may be promising alternative larvicide for controlling *Culex pipiens* larvae in integrated pest management.

INTRODUCTION

Mosquitoes can transmit diseases to about 700 million people all over the world (**Taubes, 1997**). *Culex* species can transmit a nematode worm (*Wuchereria bancrofti*), which is responsible for filarial disease **(Holder, 1999).** About 3492 species of mosquito are recorded, 100 species of them are vectors and can transmit many diseases to humans and mammals **(Ghosh *et al.* 2012).** Mosquito control remains difficult since there is no drug or vaccine. *Culex* species control is an important step to prevent disease outbreaks. The control of the *Culex* species at the larval stage has been more convenient since the insect is more sensitive. Moreover, the insecticide application is in a defined area, which decreases environmental contamination. Synthetic insecticides were used for many decades to control mosquito larvae, which led to the development of insect resistance.

Recently there is great attention to botanical pesticides. Essential oils possess larvicidal, ovicidal, and repellent against various insect species **(Isman 2000; Cetin *et al.*, 2004).** Botanical pesticides constitute 1% of the world's insecticide **(Rozman *et al.*, 2007).** According to researches, there are more than 1500 plants that have an insecticidal effect (**Regnault *et al.*, 2012; Suresh *et al.*, 2018).** The main components of EOs are hydrocarbon terpenes, as well as the oxygenated terpenes **(Kayode and Afolayan, 2015).** EOs are a mixture of volatile and semi-volatile, odorous, secondary metabolites, soluble in organic solvents are extracted from different parts of plants **(Basak and Guha, 2017)**. Clove *Syzygium aromaticum* (L.) stands out among the plant species producing essential oils with insecticide potential for insect control **(Han *et al.*, 2006; Correa, 2011; Afonso *et al.*, 2012).** The composition of clove EO varies according to the plant part used for extraction from leaves, peduncle, and dried flower buds (**Oliveira *et al.*, 2009).** *Syzygium aromaticum* EO characterized by gas chromatography indicated eugenol as the major component (**Elzayat *et al*., 2018**). Essential oils pesticides can be used to reduce doses of pesticides used, decrease environmental contamination, and decrease risk to the consumers **(Anderson *et al.*, 2019).** Nanoparticles (NPs) can be classified based on the type of material to the semiconductor, metallic, and polymeric nanoparticles. Furthermore, this kind of formulation is expected to be more effective than bulk substances **(Rajendran and Sriranjini, 2008)** where showed better efficacy as a mosquito larvicide control and considered one of the well-documented techniques.

Recently, there has been great attention to nanotechnology to improve the effectiveness of botanical pesticides, **(Pant *et al.*, 2014; Khoshraftar *et al.*, 2019).**

The present study aimed to develop and characterize biodegradable nano encapsulated containing EO of *S. aromaticum* and assess the toxicity effect towards *Cx. pipiens* third instar larvae.

MATERIALS AND METHODS

Insect maintenance The colony of *Culex pipiens* was maintained in the insectary in Research training Center (RTC), Faculty of Science, Ain Shams University at 27 ± 2 °C, and 75% ± 5% relative humidity, and a 14L:10D hr of light-dark photoperiod. Adult insects were reared in standard wooden cages (75 cm × 60 cm × 60 cm). Mosquitoes were provided 10% sucrose for nourishment and take a blood meal from a pigeon according to (**Gerberg 1970; Kasap and Demirhan 1992**). The hatched larvae were fed on (Tetramin) daily.

***Syzygium aromaticum* essential oil extraction**

Syzygium aromaticum buds were collected from the local market (Cairo, Egypt) and identified by the Botany Department, Faculty of Science, Ain Shams University. The buds were washed and dried for 2 days and ground to a fine powder. 30 gm. from the powder was macerated for extractions in dark bottles with 200 ml water for three days with shaking then filtered, and lyophilized to produce 4.2 gm.

Encapsulation of *Syzygium aromaticum* using chitosan nanoparticles

Chitosan low molecular weight (L.M.W)(Merck), (1gm) was dissolved in 100 ml 2% aqueous acetic acid glacial under stirring sonication. One ml of *Syzygium aromaticum* essential oil was added to the chitosan solution under stirring. The mixture was sonicated till the solution become clear. Sodium Tripolyphosphate (TPP) (0.5 gm.) was dissolved in 50 ml distilled water and added drop by drop to the chitosan oil mixture with continuous stirring at room temperature to form chitosan nanoparticles according to **(Othman *et al.* 2018)**. The obtained turbid solution pale yellow color indicates the formation of chitosan nanoparticles with its encapsulated oil. The capsulated *S. aromaticum* chitosan- nano-particles (NPs) were centrifuge and freeze-dry to have capsulated *S. aromaticum* chitosan-NPs powder. Capsulated chitosan-NPs was confirmed and portrayed by UV-Spectrophotometer, Infra-Red (IR), Transmission Electron Microscopy (TEM), and X-ray diffraction (XRD).

Characterization of *Syzygium aromaticum* encapsulated with chitosan nanoparticles

Ultraviolet-Visible (UV-VIS) spectra

UV-VIS (Shimadzu spectrophotometer) has been used to follow the formation of *S. aromaticum* chitosan-NPs capsulated aqueous solution. The UV-Vis spectra were recorded between 100-800 nm.

Transmission Electron Microscopy (TEM)

The shape and size of chitosan-NPs were practically obtained using High-Resolution Transmission Electron Microscopy (HRTEM) JEOL (JEM-2100 TEM). Specimens for TEM measurements were prepared by placing a drop of colloidal solution on a 400 mesh Carbon coated copper grid and evaporating the solvent in air at room temperature.

Infra-Red (IR) spectroscopy

Attenuated total reflection (ATR-FT-IR) measurements were investigated using (8300 FT-IR Shimadzu Spectrophotometer) in the range from 4000 cm^{-1} to 400 cm^{-1}.

X-ray diffraction (XRD)

Crystallinity of samples was evaluated by wide-angle X-ray diffraction (WAXD) analysis using an XRD 7000 Shimadzu (Shimadzu, Kyoto, Japan) diffractometer operated with Cu Kα radiation (λ = 0.15418 nm). Diffraction patterns were recorded over a 2θ range of 5°–40° in continuous mode. The step size was 0.02°.

Bioassay test

The larvicidal activity of *S. aromaticum* chitosan-NPs was assayed against the 3rd larval instar of *Cx. pipiens* according to **(WHO, 2005),** by using five concentrations (150, 100, 75, 50, and 25 ppm), three replicates for each concentration. Twenty-five larvae for each replicate. Mortality was calculated by using Abbott's formula (**Abbott, 1925**). Lethal concentrations LC_{25} and LC_{50} were detected by (**Finney, 1971**).

Biochemical studies

The activity of four enzymes (acid phosphatase, alkaline phosphatase, acetylcholinesterase, and glutathione S-transferase) was measured in the untreated and

treated 3rd instar larvae of *Cx pipiens* with the *S. aromaticum* EO and encapsulated *S. aromaticum*.

Glutathione S-transferase was detected as described by the method of **Habig *et al.* (I974).** Acetylcholinesterase (AchE) activity was measured according to **Simpson *et al.* (1964)**, acetylcholine bromide (AchBr) was used as a substrate. The reaction mixture (200 µl enzyme, 0.5 ml AchBr (3 mM) and 0.5 ml 0.067 M phosphate buffer (pH7). The decrease in AchBr was read at 515 nm.

Acidphosphatase and alkalinephosphatase were determined according to **Powell and Smith (1954)**. The reaction mixture consisted of 1 ml carbonate buffer (pH10.4) for alkaline phosphatase or 1 ml citric buffer (pH 4.9) for acid phosphatase, 1 ml of 0.01 M disodium phenylphosphate (substrate), and 0.1 ml sample.

RESULTS

1. Characterization of *Syzygium aromaticum* encapsulated with chitosan nanoparticles

1.1. Ultraviolet-Visible (UV-VIS) spectra

The formation of chitosan-NPs has been monitored by UV-VIS spectroscopy. UV-VIS absorption spectrum of Chitosan-NPs is shown in Fig. (1). Chitosan-NPs colloidal solution showed absorption spectra around wavelength 330 nm.

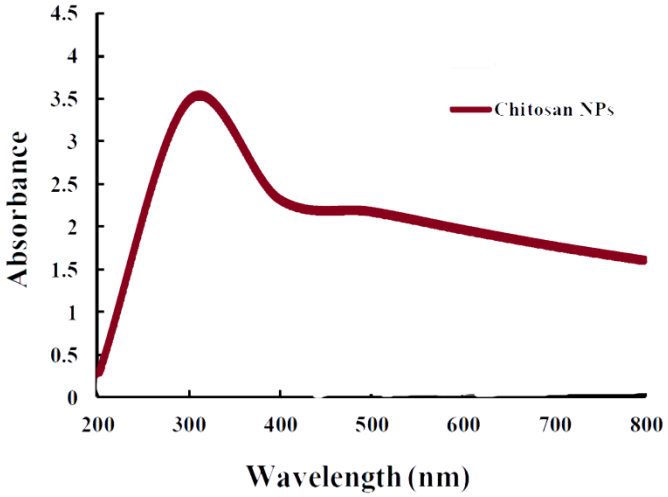


Fig. 1. UV-Vis spectra of chitosan nanoparticles

1.2. Transmission Electron Microscopy (TEM)

The Chitosan colloidal nanoparticles characterization has been confirmed by TEM as shown in Fig. (2).The synthesized Chitosan-NPs obtained have a relatively spherical shape with an average size of about 34-75 nm. The encapsulated *S. aromaticum* essential oil can be confirmed as dark parts within the more lighten chitosan nanoparticles. The average particle size was measured using the Image J program and it showed that the majority of the particle size around 34 and 75 nm.

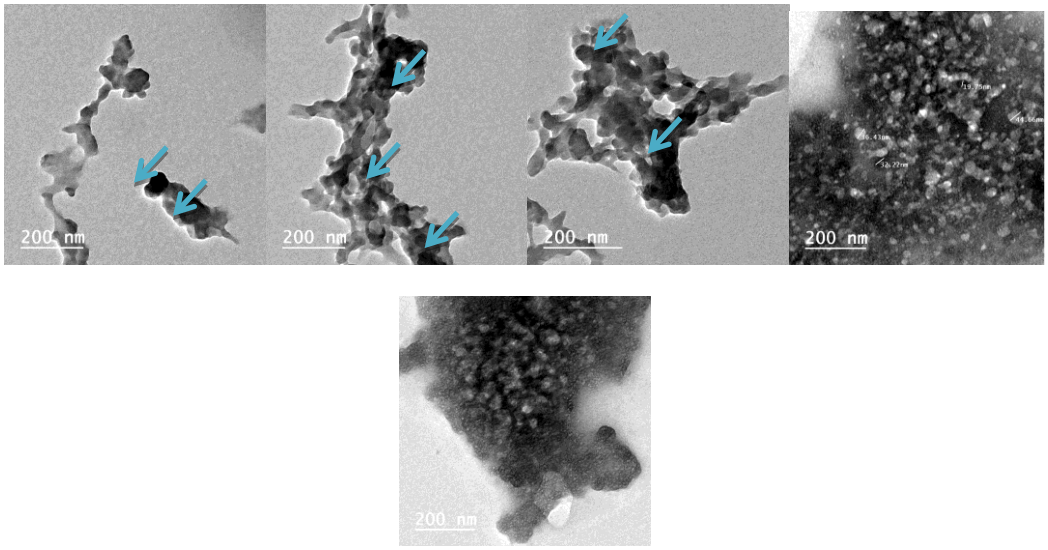


Fig. 2. TEM photomicrograph of *Syzygium aromaticum* chitosan nanoparticles encapsulated. The arrows refer to *S.aromaticum* essential oil

1.3. Infra-Red (IR) spectroscopy

Figure (3) shows Fourier Transforms Infrared spectra (FTIR) of chitosan nanoparticles and *S. aromaticum* chitosan-NPs. For chitosan nanoparticles in Fig. 3 (a), the peak of amide I (-NH_2 bending) shifted from 1647 to 1685 cm^{-1}, and new peaks appeared at1338(C- O -C stretch) and 1560 cm^{-1} (amide II),

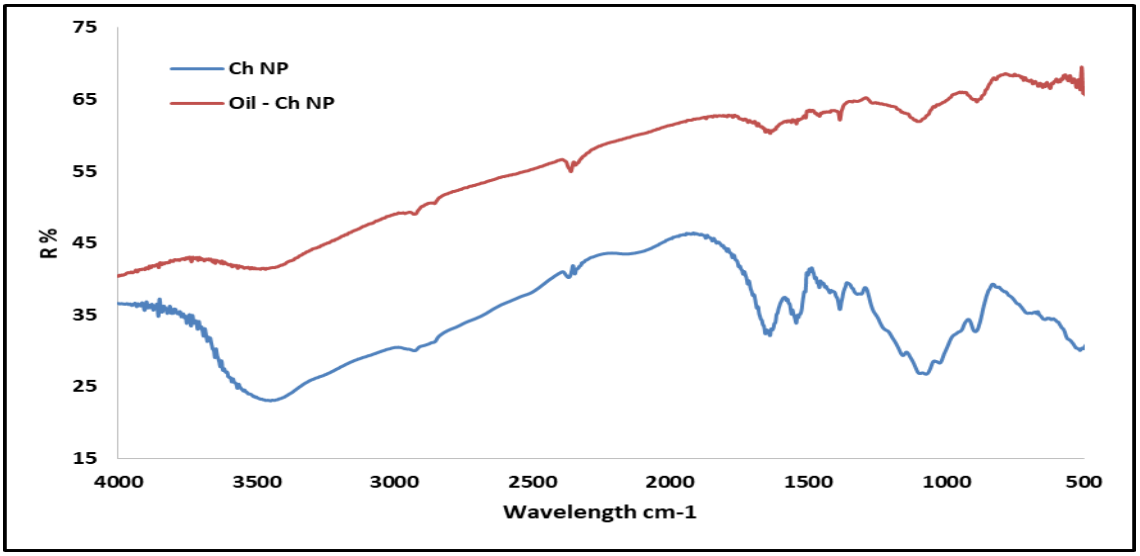
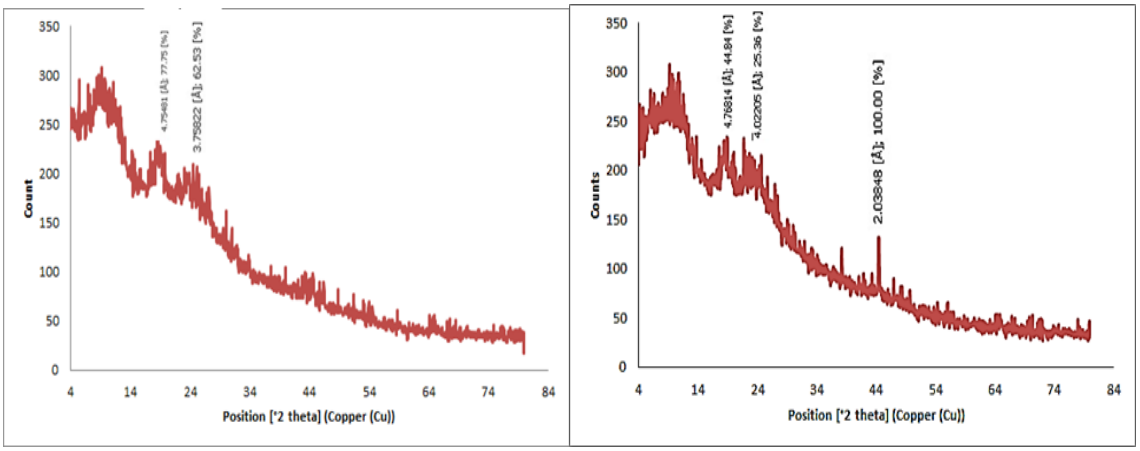


Fig. 3. Fourier Transforms Infrared spectroscopy (FTIR) spectra of (a) chitosan- nano-particles, (b) *Syzygium aromaticum* chitosan- nanoparticles

1.4. X-Ray Diffraction

The X-ray diffraction **(**XRD) study of chitosan nanoparticles with and without *S. aromaticum* extract was shown in (Fig. 4a). The diffraction pinnacle of unadulterated

chitosan which was generally seen at 20.20° has somewhat moved to a lower esteem (18.59°) in the present examination.



a-Chitosan- nanoparticles **b- *S. aromaticum*- chitosan-nanoparticles**

Fig. 4. X-ray diffraction **(**XRD) pattern of chitosan nanoparticles (a) and chitosan nanoparticles with *Syzygium aromaticum* (b) recorded in the 2h range of 20 θ –80 θ.

On the other hand, XRD studied showed the presence of characteristic diffraction peaks of *S. aromaticum* chitosan-NPs (Fig. 4b). It showed a main broad diffraction peak at 2θ estimations of 18.59° for the chitosan-NPs and other new peaks at 2θ = 20.00°, 24.50° & 44.40° referred to the effect of the capsulated.

2. Insecticidal activity

Table (1) represents the LC values of *S. aromaticum* EO and *S. aromaticum* chitosan-NPs on third instar larvae of *Cx. pipiens*. Data revealed that treated larvae were more susceptible to *S. aromaticum* chitosan-NPs followed by *S. aromaticum* EO. Based on LC_{50} *S. aromaticum* chitosan-NPs was more active (LC_{50} =20 ppm) and *S. aromaticum* EO was less active (LC_{50} =39 ppm). LC_{25} and LC_{95} also indicated that *S. aromaticum* chitosan-NPs was more effective (9 ppm and 146 ppm, respectively) than *S. aromaticum* EO (17 ppm and 304 ppm, respectively).

Table 1. Susceptibility of 3rd larval instar of *Culex pipiens* to *Syzygium aromaticum* EO and *S. aromaticum* chitosan-NPs 24 hr post-treatment.

Compounds	**LC values in ppm (95% C.I.)**		
	LC $_{25}$	**LC $_{50}$**	**LC $_{95}$**
***S. aromaticum chitosan*-NPs**	9 (7- 11)	20 (17- 23)	146 (102- 250)
***S. aromaticum* EO**	17 (13-21)	39 (33.5-45.9)	304 (222.7-465.8)

LC values = lethal concentrations values.
95% C.I.= Ninety-five percent confidence limit.

3. Biochemical studies

The activity of three enzymes acid phosphatase, alkaline phosphatase, and glutathione-S-transferase was significantly increased due to treatment with the *S. aromaticum* EO and *S. aromaticum* chitosan-NPs. The activity of acetylcholinesterase was decreased after treatment with both comparing to control as shown in Table (2).

Table 2. Effect of LC_{50} of the *Syzygium aromaticum* EO and *S. aromaticum* chitosan-NPs on acid phosphatase, alkaline phosphatase, acetylcholinesterase, and glutathione-S-transferase activity in the third instar larvae of *Culex pipiens*.

Treatment	**Acid phosphatase**	**Alkaline phosphatase**	**Acetylcholinesterase**	**Glutathione-S-transferase**
Untreated	2.26±0.6a	11.33±1.1a	38.63±1.04a	2.86±0.11a
LC_{50} of *S. aromaticum* EO	4.2±0.7b	15.3±1.2b	27.6±1.3b	3.17±0.12b
LC_{50} of *S. aromaticum* chitosan-NPs	8.3±1.0c	21.1±0.9c	24.83±1.4c	4.15±0.13ac

Data in Table (2) revealed that the activity of three enzymes (acid phosphatase, alkaline phosphatase, and glutathione-S-transferase) was significantly increased due to treatment with the *S. aromaticum* EO and *S. aromaticum chitosan*-NPs. The activity of acetylcholinesterase was decreased after treatment with *S. aromaticum* EO and *S. aromaticum chitosan*-NPs (27.6±1.3b and 24.83±1.4c, respectively) comparing to control (38.63±1.04a).

DISCUSSION

The absorption spectra of the colloidal solution of chitosan-NPs have wavelength 330 nm (**Ghadi *et al.*, 2014**), our study verified this result by characterization EO of

clove encapsulated with chitosan nanoparticles by Ultraviolet-Visible (UV-VIS) spectra. The nanoparticles formation, and its stability in the solution for about one month reflects that it was dispersed in the aqueous solution with no aggregation **(Divya and Jisha, 2018)**. They suggested that the nanoparticles formation and its stability in solution for about one month reflect that it was dispersed in the aqueous solution with no aggregation.

Zvezdova (2010) and Zidan *et al.* (2020) found that chitosan powder shows characteristic peaks at 3433 (-OH and -NH_2 stretching), 2920 (-CH stretching), 1647 (amide I), 1088 (C-O-C stretching), and 591 cm^{-1} (pyranoside ring stretching vibration), which was in consistence with the present obtained results. **Yoksan *et al.* (2010**) stated that implying the complex formation via electrostatic interaction between NH^{3+} groups of chitosan and phosphoric groups of TPP within the nanoparticles. Moreover, in comparison with the FTIR spectrum of chitosan nanoparticles, the addition of *S. aromaticum* EO resulted in a markedly decrease in intensity of the (-NH_2) and (-CH) stretching peaks at 3474 and 2930 cm^{-1} indicating an increase in the hydrogen bonds may be formed between the amino group of the nano chitosan and the hydroxylic groups come from the plant extracts. This result indicated that *S. aromaticum* extract is encapsulated into the chitosan nanoparticles.

According to **Zhao *et al.* (2011) and Jonassen *et al.* (2012)** , the pure chitosan has a high degree of crystallinity with well-characterized peaks at (2θ) of 20 and 10 degree associated with crystallographic planes (110) and (020), respectively related to non-deacetylated part of chitosan (chitin) The lower force displayed by the diffraction peaks of chitosan-NPs uncovered that they are indistinct. The tenancies of some other diffraction peaks corresponding to impurities were found in the XRD examples of chitosan-NPs showing their immaculateness. The ionic cooperation among TPP and –NH^{3+} of chitosan particles has brought about the development of chitosan-NPs **(Yoksan *et al.* 2010**) in the event of chitosan-NPs, the force of tops was expanded as an outcome of changing indistinct chitosan into solidified structure after response with TPP **(Jonassen *et al.*, 2012; Anand *et al.*, 2015)**.

The results obtained by using the X-ray diffraction to study of chitosan nanoparticles can be credited to the response of chitosan-NPs with TPP and the solidified structure chitosan-NPs, which was in great concurrence with the past reports **(Yoksan *et al.*, 2010; Ghadi *et al.*, 2014; Anand *et al.*, 2018**).

Insecticidal activity of *Syzygium aromaticum* EO and *S. aromaticum* chitosan-NPs on the third larval instar of *Cx pipiens* showed an increase in the mortality of insects by increasing the concentration levels of both oils. Although both oils have an insecticidal effect as a larvicide based on LC values, the results showed that *S. aromaticum* chitosan-NPs was more effective than S. *aromaticum* EO. This result was inconsistent with **Zohreh *et al*. (2020),** they indicated that nano-encapsulated formulation of *Plantago* extracts was more effective for controlling *Tribolium castaneum.* **Vahid *et al.* (2020)** also

found that nano-formulated *Lippia citriodora* was more toxic than *L. citriodora* EO to *Phthorimaea operculella.*

Smriti *et al.* (2020), tested pectin‑cedarwood essential oil nanocapsules on *Anopheles culicifacies* and they recorded its larvicidal activity as 98% mortality.

Detoxification enzymes in insects play an important role in the defense mechanism against foreign compounds **(Li and Liu, 2007).**

Biochemical studies of the present work revealed that there are increases in the activity of acid phosphatase, alkaline phosphatase, and glutathione-S-transferase after treatment with S. *aromaticum* EO and *S. aromaticum* chitosan-NPs. According to **Ranson *et al.* (1997)**, GST enzymes are a major family of enzymes that are associated with insecticide resistance. **Li *et al.* (2017)** recorded an increase in GST activity after treatment of destructor mites with *S. aromaticum*.

The increased alkaline phosphatase activity was similar to increased alkaline phosphatase activity which was recorded by **Wu, (1990)** after he treated the larvae of *Cx pipiens* with IGR diflubenzuron, he attributed that increase in activity to developmental disturbance. **Shekari *et al.* (2008).** Also attributed that increase to the involvement of this enzyme in the detoxification process.

Lopez and Pascual, (2010) stated that acetylcholinesterase can stop nerve communication at the neuromuscular junction in the nervous system. The LC_{50} of *S. aromaticum* EO and *S. aromaticum* chitosan-NPs were inhibited the activity of AChE in the present work which agreed with the results obtained by **Askar *et al.* (2016)** by recording inhibition in AChE in *Sitophylus oryzae* due to treatment with *S. aromaticum*. They attributed that inhibition to that *S. aromaticum* EO may interfere with the passage of pulses in the insect nervous system.

Previous works indicated that monoterpenoids in *S. aromaticum* cause insect mortality by inhibiting the acetylcholinesterase enzyme (**Lopez and Pascual, 2010). Mosleh *et al.* (2011)** found that organophosphorus insecticides showed a higher inhibiting effect of AChE than plant essential oils because organophosphorus is a specific inhibitor of cholinesterase.

CONCLUSION

The present study revealed that *S. aromaticum* EO *and S. aromaticum* chitosan-NPs have a larvicidal effect on the 3rd larval instar of *Cx pipiens*. Although *S. aromaticum* chitosan-NPs has more toxicity than *S. aromaticum* EO based on LC values but both of them have a toxic effect on *Cx pipiens* larvae. They also cause biochemical alterations in the tested insects. Both of *S. aromaticum* EO and *S. aromaticum* chitosan-NPs may be used as a larvicide for controlling *Cx pipiens* larvae in integrated pest management.

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الملخص العربى

دراسة السمية والتغيرات البيوكيميائية لتركيبة النانو من زيت القرنفل *Syzygium aromaticum* على بعوض *Culex pipiens* L (رتبة: ثنائية الاجنحة. Culicidae)

الجوهرى أ. الجوهرى[1]، شيماء م. فرج [1]، احمد ع. السيد[2]، ريهام ر. خطاب[2] و داليا م. محمود[1]

[1] قسم علم الحشرات- كلية العلوم- جامعة عين شمس- القاهرة

[2] قسم الكيمياء الضوئية- شعبة بحوث الصناعات الكيماوية-المركز القومي للبحوث- الدقي- الجيزة

في هذه الدراسة ،تم استخلاص الزيوت الاساسية بواسطة الماء ثم تغليف زيت القرنفل *Syzygium aromaticum* الأساسي بجسيمات نانوية من الكيتوزان. وقد تم توصيف الجسيمات النانوية من الكيتوزان باستخدام الأشعة فوق البنفسجية المرئية و المجهر الالكترونى و مطيافيه الاشعة تحت الحمراء وحيود الاشعة السينية. وقد تم تقييم كل من الزيت الاساسى للقرنفل *Syzygium aromaticum* وزيت القرنفل المغلف بالنانو كيتوزان كمبيدان لليرقات ضد الطور الثالث من بعوضة *Culex pipiens* عن طريق قياس القيم المميتة لليرقات LC، وقياس التغيرات الكيميائية الحيوية في اليرقات المعاملة. وقد أظهرت النتائج أن كلاً من زيت القرنفل الاساسى والمغلف لهما تأثير سام على يرقات البعوض و ان الزيت المغلف قد اظهر سمية أعلى من الزيت الاساسى. كما اظهرت نتائج الدراسة ان المعاملة بالزيتين يحدث تغيير في نشاط أربعة من الإنزيمات باجسام اليرقات المعاملة (acid phosphatase, alkaline phosphatase, acetylcholinesterase, and glutathione S-transferase)، مما يؤكد على التاثير السمى لهذين المبيدين. ومما سبق يتضح انه يمكن استخدام الماء كمستخلص للزيت الاساسى للقرنفل بجانب كلا من الزيت الاساسى للقرنفل *Syzygium aromaticum* وزيت القرنفل المغلف بالنانو كيتوزان لهما تأثير كمبيدات ليرقات الطور الثالث من بعوضة *Culex pipiens* ولهذا يمكن استعمالهما في نظام المكافحة المتكاملة للآفات.