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Eucalyptus oil nano-emulsion encapsulated in chitosan beads as a new approach in control of *Culex pipiens* larvae

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Abstract
The current study designed to evaluate the effect of *Eucalyptus* oil on nanoscale capsulated in chitosan beads against the larvae of *Culex pipiens*. This cross-linking was described by using scanning electron microscopy, X-ray Diffraction, and Fourier Transform Infrared spectroscopy techniques. Mortality percentages of non-encapsulated *Eucalyptus* oil against tested larvae at different time intervals were 72.51, 83.91, 88.20, 97.36, and 98.00%, respectively with lethal concentration (LC_{50}) 52.390, 42.934, 33.545, 26.291, and 20.301µL.While the mortality percentages at a time-dependent study using the continuous release from encapsulated beads of nano-*Eucalyptus* oil were 80, 91.67, 98.3, 99.31, and 99.56%, respectively with lethal concentrations (LC_{50}) 0.734, 0.637., 0.5, 0.453. and 0.419 mg/l. We observed that the increased mortality was proportional to the concentration of the nano-emulsion in the beads. The controlled release of nano oil from chitosan beads making persistence to the active materials up to 5days of exposure to the encapsulated beads compared with the non-encapsulated oil. The results proved that the encapsulated beads possess interesting larvicidal activity that makes it suitable for application in integrated vector control strategies.

Keywords: *Eucalyptus* oil, nano-formulation, encapsulated chitosan beads, *Culex pipiens*, larvicidal activity

1. Introduction
Mosquitoes widely transmit pathogens that spread epidemic diseases among human beings and animals [12]. So it is the time for control by developing novel and developed mosquito control methods that are economically cheap and effective. The control methods should also be safe for non-target organisms and the environment.
Essential oils (EO) are aromatic volatile liquids extract from plants and have proved to be effective and eco-friendly for mosquito control, as these possess certain chemicals with unique larvicidal activity [24]. The *Eucalyptus* spp. leaves are rich in the compound cineole, which have good larvicidal and repellent property against mosquito vectors [10]. *Eucalyptus* oil was used as a natural insecticide; it is of great importance in the field of toxicological and environmental applications to overcome the problem of accumulative pest resistance [4]. But some of these oils generally show degradation after short periods due to some environmental factors such as light, accessibility to atmospheric oxygen and temperature [1].
Nano-formulation of the Essential Oil (EO) may resolve such problems by protecting it from degradation achieving a controlled release of EO and easy handling. So, the present study may be promising from the deduced data about essential oil nanoparticles chitosan encapsulated beads on the control of *Culex pipiens*. The application of nanoparticles is a developing area of nano- technology [9]. They often show noticeably different chemical, physical, and biological properties comparing with their macro scaled equivalents. Some advantages of nano-formulations are: improvement in the efficiency by reason of increasing the higher solubility in water, exposed surface area, stimulation of systemic activity owing to smaller particle size, higher mobility and lower toxicity due to the elimination of organic solvents compared to conventional insecticides, and their formulations [20]. Nanotechnology was utilized to develop new nano-pesticides employs nanoparticles (NP) which have one or more measurements in the order 10 – 1,000 nm [22].

Chitosan is mainly isolated from the shells of commercially harvested crustaceans because this is the most readily available material for large-scale manufacturing. Recent works obtaining chitosan from the insects. The Oriental hornet, *Vespa orientalis*, was the selected insect for chitin isolation, the procedure for extraction is close to that of crustacean sources, where the procedure includes subsequent steps [8, 27, 15, 17, 13].

This new source provides no limitation on availability to raw materials and on industrial production [2, 3]. Chitosan has a great possibility to be an alternative to synthetic stabilizers; it exhibits several remarkable biological activities such as biodegradability, biocompatibility, non-antigenicity, non-toxicity, and adsorption properties. Chitosan is often appeared to be GRAS (Generally Recognized as Safe) and bio absorbable [5].

Our research was designed to encapsulate *Eucalyptus* oil nano-emulsion into chitosan beads (NE-CH) for examining its larvicidal activity against *Culex pipiens* larvae and assess its persistence efficacy compared with non-encapsulated *Eucalyptus* oil. Characterization of the nano-emulsion encapsulated beads, its physicochemical properties, and ultra-structure properties were determined.

2. Materials and Methods

2.1. Insects

A) *Vespa orientalis* was gained from the Apiculture Research Department, Plant Protection Research Institute, Agricultural Research Center, and Dokki, Egypt.

B) The laboratory tested insect from a susceptible strain of *Culex pipiens* 3rd larval instar was brought as egg masses from the Research Institute of Medical Entomology, Giza, Egypt. Was reared in the laboratory for more than 10 generations (without chemical treatments)

2.2 Pure Chitosan preparation

Chitin was extracted from *Vespa orientalis* following the standard procedure mentioned in Majtán *et al.* [14] with certain modification in the deacetylation step mentioned by Nemtsev *et al.* [15]. Chitosan was solubilized in 1% acetic acid

2.3 Preparation of *Eucalyptus* oil nano-emulsion encapsulated in chitosan beads (NE-CH)

Eucalyptus Oil (*Eucalyptus globulus*, Cineole 60%) was purchased from the National Research Centre from the oils extract unit. Nano-emulsion formation oil in water nano-emulsion was prepared according to Sugumar *et al*., [23] with little modification. It was prepared in the ratio of 1:1 v/v using *Eucalyptus* oil (16%), Tween 80 (16%) and water (68%); Tween 80 is more effective in reducing the droplet radii as well as the stability than Tween 20. Initially, the emulsion was prepared by magnetic stirrer at 200 rpm then was subjected to ultrasonic emulsification using 20 kHz Sonicator (Sonics, USA) with a power output of 750 W for 10 min.

Wasp Chitosan solution (2% w/v) was solubilized in 1% acetic acid; the solution was filtered after complete mixing. Chitosan solution was mixed with different ratios of *Eucalyptus* oil nano-emulsion then was homogenized using the sonicator for 10 min. For beads formation this mixture was added into a solution of 5% sodium hydroxide using a gauge syringe (0.45×13 mm). The beads formed, were 1 mm in diameter and were kept in the drying solution for 30 min. Finally, the beads were washed with deionized water for three times.

2.4 Characterization of *Eucalyptus* oil nano-emulsion encapsulated chitosan beads (NE-CH) using

A. Scanning Electron Microscopy (SEM)

Chitosan beads with nano-emulsions were morphologically examined by SEM. Before analysing the sample it was sputtered with gold. The morphological structure of the coated sample was examined by the scanning electron microscope (FEI Quanta FEG 200).

B. X-ray Diffraction Studies

For characterization of the encapsulated beads we used a powdered X-ray diffractometer (Model: D8 Advance, BRUKER, Germany) by Ni filtered radiation. For scanning at the range of 10°–30° about 300 mg of the sample was deposited on the sample holder.

C. Fourier Transforms Infrared (FTIR)

To study the interactions between the chitosan and nano-emulsion beads the FTIR analysis was made to detect the functional groups present in the sample. Measurements concerning the dried beads were subjected to FTIR by potassium bromide method in a Nicolet 6700 FT-IR Spectrometer (Thermo Scientific Instruments Groups, Madison, Wisconsin).

2.5 Bioassay of Non-encapsulated *Eucalyptus* oil against *Culex pipiens* larvae

The efficiency of non-encapsulated *Eucalyptus* oil was estimated by testing its larvicidal activity against the 3rd instar larvae of *Culex pipiens* according to Kaura *et al.* [11]. In glass beakers, each containing 200 mL distilled water using the following concentrations (10, 20, 40, 80, and 100 µL). In the control group, larvae were introduced in the beaker containing only water (devoid of oil extract). The experiments were carried out in 3 replicates each contains 60 tested larvae i.e (n= 60) for each concentration of oil. The experimental conditions were optimized at 27°C with a 16:8-h light/dark cycle. The mortality percentage was recorded at 1, 3, 6, 12 and 24hr .The lethal concentrations that caused 50% mortality (LC_{50}) were calculated.

2.6 Bioassay of the pure wasp chitosan and *Eucalyptus* oil Nano-Emulsion Encapsulated beads (EN-CH) against *Culex pipiens* larvae

The larvicidal efficiency of (EN-CH) was evaluated against *Culex pipiens* larvae by using a standard protocol as stipulated by Zhang *et al.* [25]. Using encapsulated beads weighed in the ranges of (0.25- .0, 5- 0, 75 and 1 g) of beads. The mortality rate was calculated at the time interval 1, 3, 6, 12 and 24 hr. All experiments were carried out at the same optimized conditions mentioned before, and were replicated 3 times each has 60 tested larvae. The number of dead larvae was recorded and analysed to calculate the lethal concentration for each treatment.

2.7 Persistence effectiveness of *Eucalyptus* oil (non-encapsulated and encapsulated) formulations

The residual effect of *Eucalyptus* oil formulations (non-encapsulated and chitosan encapsulated beads) that recorded the highest mortality percentage was evaluated against 3rd

larval instar up to 5 days, according to Kaura *et al.* [11]. On each of the subsequent 5 days 60 freshly collected larvae were introduced continuously. The mortality was recorded every day. The experiments were replicated 3 times under the same environmental conditions of bioassay tests.

2.8. Statistical analysis
For bioassay test the LC_{50} values were estimated according to Finney [6] using; "LdPLine®"software, [http://embakr.tripod.com/ldpline/ldpline.htm].

3. Results
3.1 Characterization of *Eucalyptus* oil nano-emulsion Encapsulated in Chitosan beads (NE-CH).
The Scanning Electron Microscopy of (NE-CH) (Fig 1) appeared spherical with a slightly rough surface. The bead size was approximately 0.5 to 1 mm. The XRD patterns of (NE-CH) (Fig 2) indicated that the intensity of the crystalline peaks of chitosan at 10 º and 20.1º was found to be unchanged on loading with nano-emulsion.

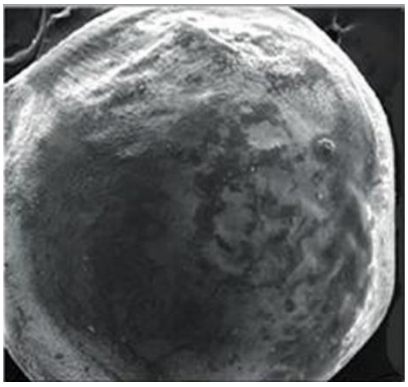

Fig 1: Scanning electron micrograph of *Eucalyptus* Nano-emulsion loaded beads (bar 200nm).

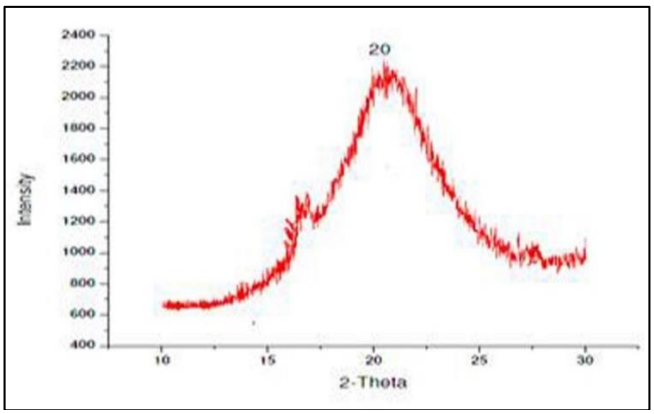

Fig 2: X-Ray Diffraction analysis of *Eucalyptus* Nano-emulsion loaded beads

The functional groups existing in both unloaded and loaded nano-emulsion were differentiated by the FTIR spectrum as observed in (Fig 3 and 4). For chitosan beads the major peaks were at around 3446.79 cm^{-1}, which referred to the stretching vibration of –OH groups. The peaks at 1629 cm^{-1} were attributed to the secondary amide C=O bond of the residual acetamido groups. There were minor changes in the peak intensities after encapsulation; the spectra of NE-CH revealed some a new peak at 2920 cm^{-1}, corresponds to the methylene group, and a peak at 2854 cm^{-1} due to a methyl group. Besides, the bending at 1452 cm^{-1} and the sharp peak at 1100 cm^{-1} is a result of C-H bending vibrations or –OH deformation of carboxyl groups.

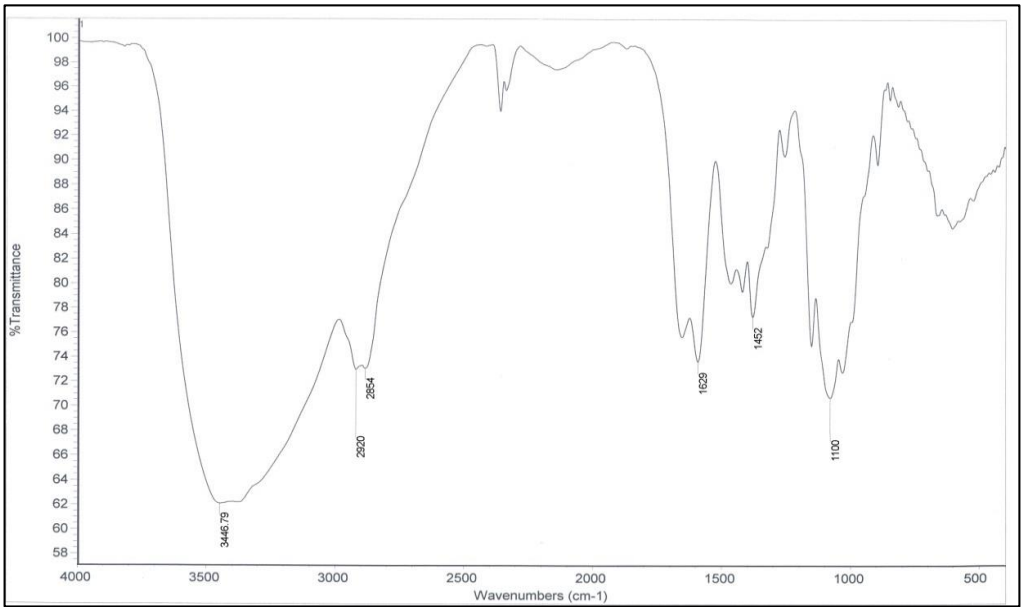

Fig 3: FTIR analysis of Nano- *Eucalyptus* loaded beads

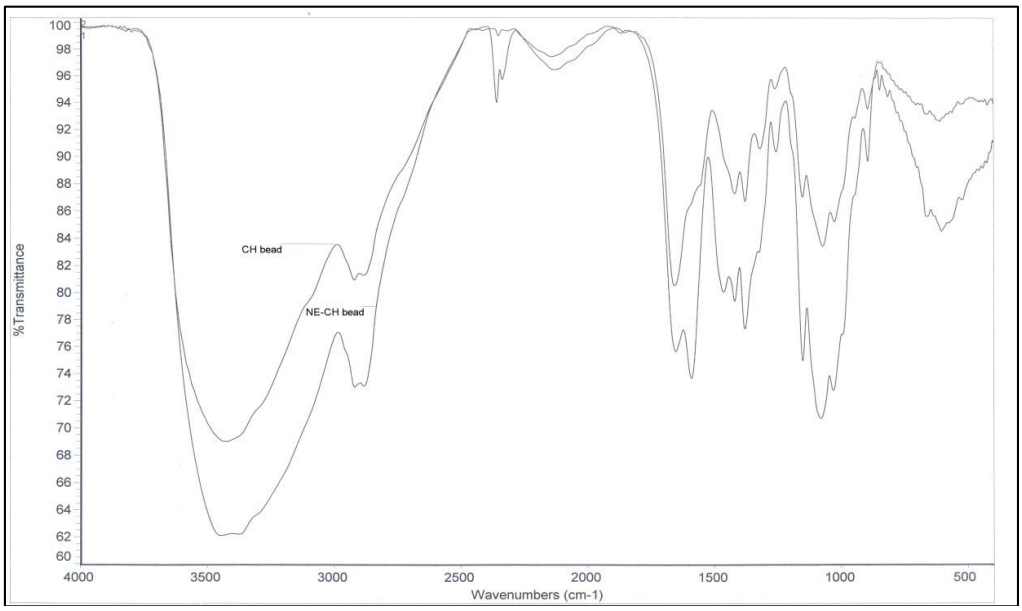

Fig 4: Overlay FTIR Spectra for CH bead and NE-CH bead.

3.2 Susceptibility of *Culex pipiens* to Non-encapsulated *Eucalyptus* Oil.

The mortality percentages of 3rd instar larvae of *Culex pipiens* were recorded by applying non-encapsulated *Eucalyptus* oil at different time intervals to be 72.51%, 83.91%, 88.20%, 97.36%, and 98.00%, respectively (Table 1). The calculated LC_{50} was 52.390, 42.934, 33.545, 26.291, and 20.301 µL, respectively (Table 2).

Table 1: Effect of different concentrations of non-encapsulated *Eucalyptus* oil against the 3rd larval instar of *Culex pipiens* treated at different time intervals.

Oil Concentrations (µL)	Observed mortality% (n=60)				
	1hr	3hr	6hr	12hr	24hr
Control	0	0	0	0	0
10	1.45	2.65	7.92	13.45	20.35
20	13.18	22.34	32.61	40.12	53.71
40	43.51	51.70	60.00	67.29	74.92
80	65.04	70.34	78.02	84.32	90.51
100	72.51	83.91	88.20	97.36	98.00
Slope	2.661+/- 0.2647	2.5693 +/- 0.2013	2.3979 +/-0.1889	2.5747 +/-0.1966	2.5111 +/-0.2027
χ 2	2.718	4.3232	2.4192	5.6207	4.2503
χ 2 tabulated	6	7.8	7.8	7.8	7.8
g	0.0378	0.0236	0.0238	0.0224	0.025

Control=distilled water.
n= 60 number of larvae tested.

Table 2: Lethal concentrations (LC_{50}) of non-encapsulated *Eucalyptus* oil against 3rd instar larvae of *Culex pipiens*.

Exposure time	LC_{50} Conc. µL	Fiducial limits	
		Lower limit (µL)	Upper limit (µL)
1hr	52.3907	25.1474	33.3526
3hr	42.934	34.237	48.2407
6hr	33.5451	29.5773	37.8698
12hr	26.2914	23.1634	29.5756
24hr	20.3013	17.6054	23.0401

3.3 Susceptibility of *Culex pipiens* larvae to Encapsulated *Eucalyptus* oil Nano-Emulsion

Treatment of the 3rd instar larvae of *Culex pipiens* with pure wasp chitosan as a control did not induce any larvicidal activity even after 48 h.

A time-dependent study for the larvicidal activity of encapsulated beads NE-CH against 3rd instar larvae of *Culex pipiens* using the continuous release from encapsulated beads of nano-*Eucalyptus* oil gave 80%, 91.67%, 98.3%, 99.31%, and 99.56% respectively (Table 3).The calculated LC_{50} was 0.734, 0.637, 0.5, 0.453 and 0.419 mg/l (Table 4). We observed that the mortality increased by increasing the concentration of *Eucalyptus* oil nano-emulsion in the beads.

Table 3: Effect of different concentrations of Encapsulated *Eucalyptus* oil Nano-Emulsion beads against the 3rd instar larvae of *Culex pipiens* at different time intervals.

Conc. gm	Observed mortality% (n=60)				
	1hr	3hr	6hr	12hr	24hr
Control	0	0	0	0	0
0.25	1.67	3.33	10.00	11.67	15.00
0.5	15.00	25.00	40.00	48.33	53.33
0.75	50.00	58.33	81.67	91.67	98.33
1	80.00	91.67	98.33	99.31	99.56
Slope	5.4769 +/- 0.7254	5.4134 +/- 0.6455	5.1266 +/- 0.5078	5.4167 +/- 0.5582	5.5138 +/- 0.5669
χ 2	2.3708	4.9658	5.1652	5.0599	9.1259
χ 2 tabulated	6	6	6	6	6
g	0.0674	0.0546	0.0378	0.0408	0.8931

Control= pure wasp chitosan dissolved in 0.5% acetic acid.
n= 60 number of tested larvae

Table 4: Lethal concentrations (LC_{50}) of Encapsulated *Eucalyptus* oil Nano-emulsion beads against 3rd instar larvae of *Culex pipiens*.

Exposure time	LC_{50} Conc.(gm)	Fiducial limits	
		Lower limit	Upper limit
1hr	0.734	0.675	0.802
3hr	0.637	0.584	0.693
6hr	0.500	0.454	0.545
12hr	0.453	0.410	0.496
24hr	0.419**	-	-

** Means g > 0.4, so lower and upper limits not be calculated.

3.4 Persistence effectiveness of *Eucalyptus* oil (non-encapsulated and encapsulated)

The persistence of activity of *Eucalyptus* oil formulation up to successive 5 days (24h-120h) was studied. The mortality percentage of non-encapsulated *Eucalyptus* oil remained approximately stable up to 2 days (48 hr) of exposure (98.39%, 94.0%). Followed by significant (P<0.05) sharp reduction 43.57%, 9.03%, 0.0%, respectively from 72 to 120 hr. On day 5(120 hr) there were no mortality had been recorded when larvae were introduced in the beaker. While, using *Eucalyptus* oil nano-emulsion encapsulated in chitosan beads (NE-CH) the residual efficiency of the encapsulated beads remained active till the day 5. Approximately the same mortality rate was recorded 99.56, 99.31, 99.23, 99, and 98.93%, respectively from 24 to 120hr (Fig 5).

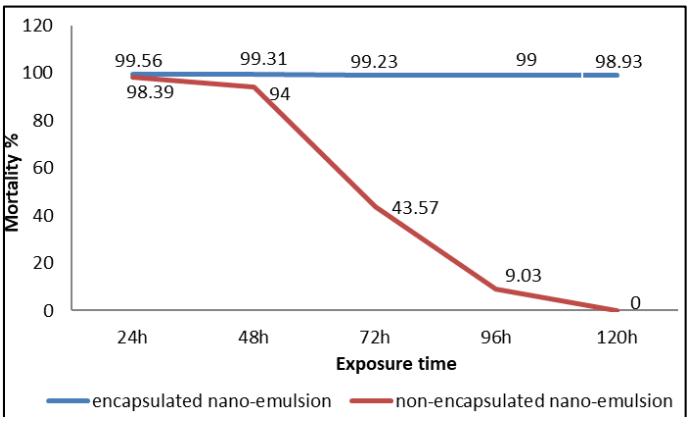

Fig 5: Persistence effectiveness of Eucalyptus oil (non-encapsulated and encapsulated) against Culex pipiens 3rd larval instar up to successive 5 days (24-120hr).

4. Discussion

Chitosan is widely interfering in many fields; biotechnology, water treatment, cosmetics, and food processing [18]. Many studies still work to enhance the extraction procedures, product characteristics and found new promising sources of chitosan [7, 15, 17 and 13]. In our research, we extracted chitosan from the oriental hornet *Vespa orientalis*, (Vespidae) which considers as an important pest in Egypt that attacking bee colonies to obtain honey and animal proteins [7].

The characterization of the chitosan polymer is very important according to the structure-properties relationship. the main problem facing many researchers is difficulties in solubilizing chitosan is as it hinders many applications including preparation and synthesis of chitosan nanoparticle compounds [13].

Data obtained from the characterization of (NE-CH) indicated that the scanning electron microscope analysis of nano-emulsion loaded Chitosan beads (NE-CH) appeared spherical with smooth surface, its size was approximately 0.5 to 1 mm that provides an indication for the stability and homogeneity of the droplet size in the emulsion, the same results were obtained by Sugumar *et al.* [23]. The XRD pattern of (NE-CH) unchanged on loading with nano-emulsion as it has the same peaks of chitosan at 20.1º due to its long-chain polysaccharide present in the biopolymer, the same observation reported by Pastor *et al.* and Wu *et al.* [16, 26]. The presence of the only peak at 20 º in both nano-emulsion chitosan beads and chitosan alone suggest that there was an interaction between these two components without any chemical modification in the crystalline structure. The FTIR spectrum that carried out in this study is very similar to that reported by Sirvaityte *et al.* [21] in which the chitosan source was from crustaceans indicating that the insect chitosan is very comparable to crustacean and commercial chitosan in the encapsulation of nano oils.

The larvicidal activity of non-encapsulated *Eucalyptus* oil at a time dependant study increased by increasing the oil concentration. The mortality percentage was represented in (Table 1 and 2) similar observation was recorded by Kaura *et al.* and Riat *et al.* [11, 19]. The persistence of its active material was concentration-dependent; higher mortality responses 98.39, 94.0% were recorded during the initial periods of treatment (as larvae were exposed to active components). The efficiency of its larvicidal activity was declined after 2 days of the exposure with mortality percentage reached to 0.0% our investigation is in accordance with Amaninder *et al.*, and Kaura *et al.* [1, 11].

Using the continuous release from encapsulated beads of nano-*Eucalyptus* oil (NE-CH), the mortality percentages were

mentioned in (Table 3 and 4) it was proportional to the concentration of nano-oil in the beads. Encapsulation process is made to ensure a sustained release of active compound into the larval media for a long time. The persistence efficiency and slow release of nano oil make the effectiveness of its active ingredients and larvicidal activity mainly stable with average mortality percentage 99% till 12hr. Even at a low concentration (0.25 g/l) gave 15% mortality after 24hr. This may be due to the disturbing effects inside the body of the larvae like a) fragmentation of the epithelium layer of the gut, b) gaps in microvilli, c) vanishing or deterioration of fat bodies. These structures are affected when they come in contact with *Eucalyptus* oil leading to their death [19].

5. Conclusion

The results obtained revealed that the encapsulation process is an appropriate method for entrapping essential oil. Such application will reduce the loss of the active ingredients that offer protection against environmental agents. It offers the possibility of a controlled release of essential oils. From the result of the persistence assessment of the encapsulated oil formulation, it is promising to incorporate in integrated pest management

6. Acknowledgements

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