Polymer-based encapsulation of peppermint oil (*Mentha piperita*) nanoemulsion and its effects on life and some physiological activities of honeybees *Apis mellifera* (Hymenoptera: Apidae)

Dalia Abd Allah Youssef[a], Sawsan M. Abdelmegeed[b]

[a]Department of Pests and Plant Protection, National Research Centre, Giza, [b]Department of Plant Protection, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

Correspondence to Dalia Abd Allah Youssef, PhD, Department of Pests and Plant Protection, National Research Centre, El-Behowth Street, PO Box 12311, Dokki, Cairo, 12622, Egypt
Tel: +20 3393 5044/+20 100 856 4887;
e-mail: daliayoussef88@yahoo.com

Received: 17 July 2021
Revised: 24 August 2021
Accepted: 30 August 2021
Published: 24 December 2021

Egyptian Pharmaceutical Journal 2021, 20:313–322

Background
Nanotechnology is thought to be a promising way to create more reliable and effective products in many fields, that is, biological pest-control methods. Biopesticides based on plant extracts, for example, essential oils, are often a complementary or alternative treatment option. The honeybee *Apis mellifera* is expected to be exposed for many applied pesticides. In this work, preparation and characterization of peppermint oil nanoformulation was carried out. The effects of prepared formulation were studied on adult workers of honeybee by oral and contact methods, in addition to evaluating some physiological parameters.

Objective
Honeybees are beneficial and economically important insects and have a major role in crop production because they are considered insect pollinators, representing 80% of insect pollinators, in addition to economic bee products such as honey, pollen, royal jelly, wax, propolis, and venom. Therefore, it is sensitive and is greatly affected by environmental changes, especially the pesticides used on those crops. Recently, nanoproducts appeared in fertilizers and pesticides and used them without knowing their effect on other living organisms in the surrounding environment, the most important of which are beneficial insects such as worker honeybees. The aim of the research was whether nanoparticles had a harmful effect on bees in terms of death rate and enzymatic activity.

Materials and methods
Four different concentrations of menthol extract and nanomenthol extract (1250, 2500, 3750, and 5000 ppm) were studied on the life of adult workers of honeybee by oral and contact methods (LC_{50} and LC_{90}). In addition, the effect of these treatments on physiological effects through chemical analysis was done to determine amylase enzyme, protein, and lipid contents.

Results and conclusion
Encapsulation of peppermint oil nanoemulsion using sodium alginate by cross-linking with calcium chloride was done successfully. Transmission electron microscopy images showed that nanocapsules exhibited a nearly spherical shape. Gas chromatography/mass spectrometry indicated no change in oil constituents after the loading process. Encapsulation efficiency and loaded-capacity percentages were evaluated. Mortality percentages for workers treated with peppermint nanoemulsion and crude emulsion were calculated and compared with control. The highest mortality occurred in nanoemulsion than crude oil, and increased at 5000 ppm for both treatments. LC_{50} value of nanoemulsion in oral treatment was 2629.85 ppm, but in contact treatment was 4246.84 ppm. While LC_{50} value of crude extract in oral treatment was 5471.13 ppm and in contact treatment was 11 895.65 ppm. Estimated amylase, total protein, and lipid contents in adult honeybees were significantly affected by different treatments. Nanopreparations are more toxic on honeybee workers than their crude materials. Both preparations (nano and bulk materials) have biochemical and physiological effects on honeybee works when exposed to them either by oral or contact treatments.

Keywords:
Apis mellifera, biological control, honeybees, LC_{50}, *Mentha piperita*, menthol extract, nanomaterials, peppermint

Egypt Pharmaceut J 20:313–322

1687-4315

Introduction

Mint plants are considered medicinal plants that are used in the production of some medicinal preparations to treat many diseases and have many benefits that have been known for a long time, while studies have shown

that it has a direct effect on the vasculature directly and blood pressure [1]. Recently, the world started using nanoparticles in most products such as medicine [2], agriculture, and crop protection [3], while it is not known what are the risks to humans, animals, and the environment in general [4]. Nanoparticles are used recently in many products such as pesticides to control many pests [5–7] Botanical insecticides provide a novel mode of action that can effectively control pests that have developed resistance to conventional pesticides. In order to reduce the impact of conventional synthetic pesticides, they have received increasing research attention. Biopesticides based on plant extracts, for example, essential oils, are often a complementary or alternative treatment option. They are used in crop production and integrated pest-management programs. If highly active compounds can be extracted from ready-existed plants, they can provide economic and effective pest control for small farmers in developing countries [8]. The development of green processes for nanoparticle synthesis is becoming a significant branch of nanotechnology. Because of coating process, incorporating essential oils into controlled-release nanoformulations prevents their rapid evaporation and degradation, improves stability, and allows using the lowest effective dose/application. In addition, this type of formulation is expected to be more efficient. Encapsulation of a natural liquid pesticide using sodium alginate (Na–Alg) as a control-releasing polymer after cross-linking with calcium chloride has been reported by several researchers. It was reported that alginate polysaccharides are characterized to be hemocompatible and do not participate in building up any organs of the human body. Encapsulating nanoparticle layers at the emulsion-droplet interface may be engineered to increase droplet stability and control the release kinetics [9]. Honeybee workers can be exposed for these pesticides when visiting the flowers, either through feeding or through contact [10–12]. Honeybee *Apis mellifera* is exposed for many environmental stresses in the world, such as pathogens, predators, parasites [13], high and low temperature, radiation [14], pesticides [15], and recently nanoparticles [16]. All these factors affected directly mortality, colony strength, broad, foraging, fecundity, fertility, and behaviors of bees in and out of their hive [15–17].

In this research, preparation and characterization of peppermint nanoformulation encapsulated in alginate-based nanoemulsion were carried out. The effect of these nanocapsules on the life of honeybee adult worker, *A. mellifera*, through feeding or through contact methods, was studied. LC_{50} and LC_{90} values were calculated. In addition, the effect of these treatments on some physiological parameters through chemical analysis was done to determine amylase-enzyme activity, protein, and lipid contents.

Materials and methods

Nanoemulsion preparation

Oil-in-water emulsification was the method used to prepare alginate nanocapsules followed by cross-linking using calcium chloride, using a modified method reported previously by Youssef [18]. Sodium alginate 3% (w/v) was prepared in distilled water at 50°C for 45 min. Peppermint oil was diluted by distilled water using Tween 80 as an emulsifier with mechanical stirring for 10 min. Briefly, sodium alginate oil-in-water nanocapsules were made by dropwise dispersion of diluted oil into alginate solution (1 : 2, v : v, respectively) under continuous mechanical stirring at room temperature. The emulsion thus formed was sonicated for 30 min using ultrasonic cleaner set, model WUC-DO3H 290 W and 60 Hz, and then sonicated for 2 min using a high-energy ultrasonic probe (model VCX 750, 750 W, 20 kHz). Calcium chloride (in the ratio of 2 : 10 $CaCl_2$ to alginate, respectively) was then added into the resulting emulsion and stirred for an additional 30 min, sonicated as mentioned previews, and remained overnight at room temperature for equilibration. The emulsion nanocapsules were obtained as dispersion in aqueous solution.

Transmission electron microscopy

The morphological shape of the prepared nanoformulation was carried out using transmission electron microscopy (TEM) (Jeol, JEM-2100, Musashino, Akishima, Japan). The nanocapsule suspension was diluted with distilled water and deposited onto a carbon-coated copper grid and stained with 1% phosphotungstic acid.

Gas chromatography/mass spectrometry

The gas chromatography/mass spectrometry (GC–MS) analysis of the crude and alginate nanocapsules were carried out using GC/MS instrument that stands at the Department of Medicinal and Aromatic Plants Research, National Research Center. Instrument: TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., Waltham, MA, USA), coupled with a thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer). The GC–MS system was equipped with a TG-5MS column (30 m×0.32-mm i.d., 0.25-

μm film thickness). Analyses were carried out using helium as carrier gas at a flow rate of 1.0 ml/min and a split ratio of 1 : 10 using the following temperature program: 60°C for 1 min, rising at 3.0°C/min to 240°C, and held for 1 min. The injector and detector were held at 240°C. Diluted samples (1 : 10 hexane, v/v) of 1 μl of the mixtures were always injected. Mass spectra were obtained by electron ionization (EI) at 70 eV, using a spectral range of *m/z* 40–450.

Efficiency of encapsulation

Loaded nanocapsules were evaluated for active ingredient contents (menthol in peppermint oil), this was done by refluxing of 0.75 g of loaded nanoemulsion with 10 ml of methanol at 65°C. Refluxing was carried out for 1 h to ensure complete extraction of encapsulated oil. The samples were then centrifuged at 10 000*g* for 10 min. The supernatant of methanol containing the extracted menthol was taken at a wavelength of 230 nm in a ultraviolet spectrophotometer, CHEM-7, using absolute methanol as a blank. Oil concentration was calculated by using of a calibration curve obtained from samples of menthol crystals. The encapsulation parameters were determined as follows:.

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

.

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

Honeybee *Apis mellifera* experiments

All experiments of honeybees were performed in apiary of Faculty of Agriculture, Ain Shams University. In total, 3000 adults of honeybee workers were divided into four groups. Each group was divided into four treatments using four concentrations in addition to the control (without treatment and fed only on sugar solution 50%). Each treatment (concentration and control) was divided into three replicates. Each replicate contained 50 adult workers.

The first two groups of workers were treated orally by feeding on sugar solution 50% with one of different concentrations of 1250, 2500, 3750, and 5000 ppm of one of both treatments (peppermint nanoemulsion or crude oil) and compared with control. The third and fourth groups were treated by spraying with the same concentrations of both treatments and compared with control insects that were sprayed by water [15].

The mortality of adult workers was calculated after 2 h, 1, 2, 4, 7, and 14 days by using the following equation:.

$$\%\,\text{Mortality} = \frac{\text{Number of dead workers in treatment} - \text{Number of dead workers in control}}{100 - \text{Number of dead workers in control}} \times 100$$

Toxicity of tested emulsions was estimated by LC_{50} and LC_{90} values, correlation, and regression values.

Effect of the tested nanoformulation- and bulk formulation of peppermint oil on certain biochemical activities of *Apis mellifera* larvae

The biochemical effects of peppermint oil either in bulk or nanoformulation were studied at the laboratory of Pests and Plant Protection Department, National Research Centre. The treatment procedure was carried out by feeding workers on sugar solution 50% mixed with the concentrations of 1250 and 5000 ppm of tested formulations. The workers were allowed to feed on treated solution for 4 days. On the fourth day, five adult workers of honeybee of each replicate in different treatments were dissected and the gut of the insects was isolated to estimate amylase-enzyme activity, total protein, and lipid contents.

Preparation of insect extracts

All samples of insects were homogenized using a volume of sodium phosphate buffer (0.2 M, pH 7.0) (0.01 g of sample/1 ml of buffer). The homogenates were centrifuged at 6000 rpm for 15 min/4°C and the supernatant was used as the source of desired evaluation. All samples were kept freezing for determination.

Total protein

Total protein determination was estimated by a colorimetric method using Protein-Biuret kit (Biogiagnostic Company, Giza, Egypt) by the method described by Mashal [19]. The principle of the method depends on the presence of alkaline cupric sulfate, the protein produces a violet color, the intensity of which is proportional to their concentration. The sample mixture contained 25 μl of sample and 1 ml of protein reagent (alkaline cupric sulfate). Standard mixture contained 25 μl of standard and 1 ml of

protein reagent. All samples were mixed well and incubated for 10 min at 37°C. The absorbance was read sample (A_{Sample}) and standard ($A_{Standard}$) against reagent blank at 550 nm. Color was stable for 1 h. Protein concentration was calculated according to the following equation:.

$$\text{Protein Concentration (g/dl)} = \frac{\text{Asample}}{\text{astandard}} \times 5$$

Total lipids

Total lipid determination was estimated using total lipids kit (Biogiagnostic Company) [19]. Lipids react with sulfuric, phosphoric acids, and vaniline to form a pink-colored complex. The sample mixture consists of 25 µl of sample and 1 ml of sulfuric acid concentrated, the standard sample consists of 25 µl of standard and 1 ml of sulfuric acid concentrated with mixing well. The tubes were covered with glass beads and let to stand in a boiling-water bath for 10 min, then cooled, and pipetted into dry test tubes. From the above solution, 50 µl of sample or standard was mixed with 50 µl of sulfuric acid concentrated and 1.5 ml of reagent 2, then mixed well, and let to stand at room temperature for 30 min in the dark. It was poured into dry cuvettes. The absorbance of sample (A_{Sample}) and standard ($A_{Standard}$) against reagent blank within 30 min at 545 nm. Total lipid concentration was calculated according to the following equation:.

$$\text{Total lipid concentration (mg/dl)} = \frac{\text{Asample}}{\text{Astandard}} \times 1000$$

α-Amylase

The test is based on the hydrolysis of starch by amylase and the blue–black complex that forms when iodine reacts with starch. The amount of starch, which remains at the end of the incubation period, is shown by the addition of an iodine solution, which produces a blue–black color. The amylase activity is measured by the difference in absorbance of the starch–iodine complex of the test against that of the reagent blank. The reaction mixture consists of 500 µl of buffered substrate (pH 7.0, 0.4 g/l) and then incubation at 37°C for 3 min. Adding 10 µl of sample, M=mix well and incubate at 37°C for exactly 7.5 min, then add 500 µl of working reagent, mix well, and then add 4 ml of distilled water. For blank tube, add all contents, except the sample. Read the absorbance of the sample (A_{sample}) and blank (A_{blank}) against distilled water at 660 nm. Amylase activity was calculated according to the following equation:.

$$\text{Amylase activity (U/L)} = \frac{\text{Ablank} - \text{Asample}}{\text{Ablank}} \times 1480$$

Statistical analysis

Data were subjected to statistical analysis by one-way analysis of variance using SPSS software (SPSS Inc. company, Illinois, Chicago, USA) (LSD test). A value of *P* value less than 0.05 was considered statistically significant.

Results

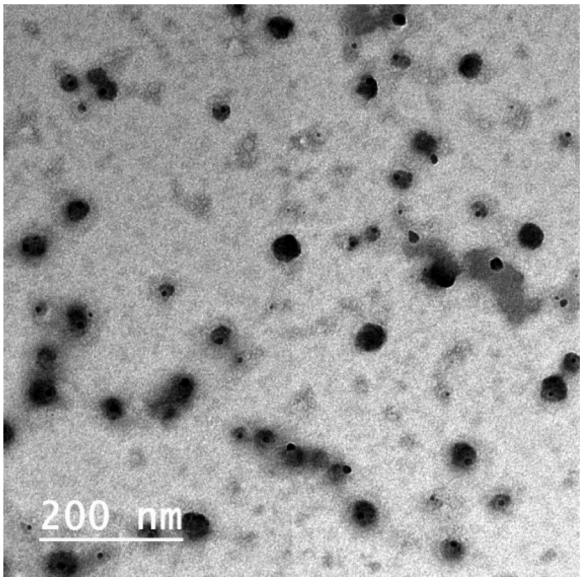
Electron microscopy examination

Oil-in-water emulsification was the method used to prepare peppermint nanoemulsion loaded in alginate polysaccharide and cross-linked with calcium chloride. TEM examination was carried out to determine the shape and size of the prepared nanocapsules. Desperation of oil emulsions containing Tween 80 in an aqueous alginate solution formed core and shell encapsulation. The inner core represents the oil and the shell represents the alginate that solidified by cross-linking of calcium chloride. Nanocapsule diameters were estimated at 40–100 nm (Fig. 1).

Gas chromatogram/mass spectrometry analysis of crude and alginate-loaded emulsion

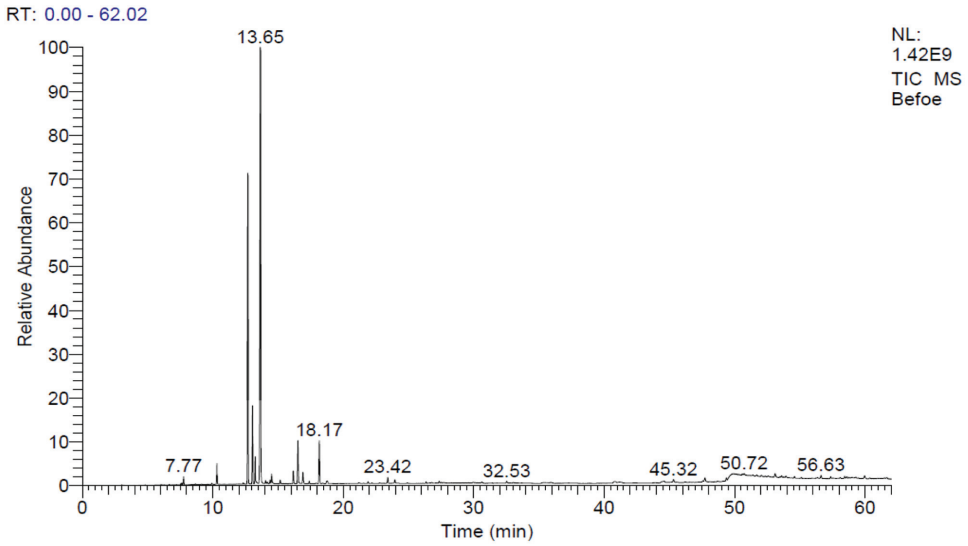
The chemical composition of peppermint oil (crude and alginate-loaded emulsion) was estimated and compared by GC/MS and illustrated in Figs 2 and 3

Figure 1



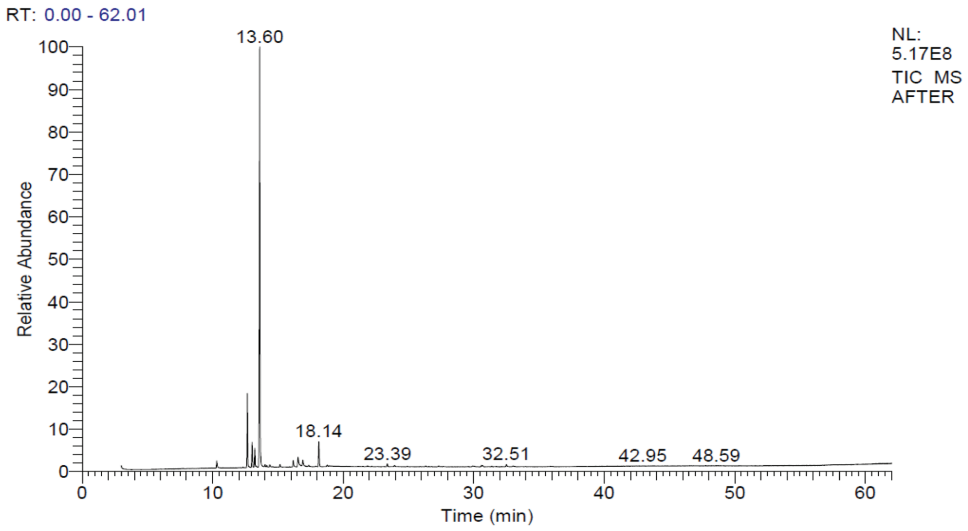
Transmission electron microscopy image of alginate-loaded peppermint nanoformulation.

Figure 2



Total ion chromatogram (TIC) of the major constituents of peppermint crude oil.

Figure 3



Total ion chromatogram (TIC) of the major constituents of alginate peppermint nanoemulsion.

and Table 1. The spectrum analysis determined the presence of five distinct peaks. Menthol, which is the abundance compound in the two extracts, represents 43.87 and 66.37% in crude and alginate-loaded emulsion, respectively, followed by p-menthone 28.99 and 12.26%, respectively. Isomenthone with 7.38 and 4.22%, respectively, followed by carvone 8.71 and 2.02%, for crude and alginate-loaded emulsion, respectively. Menthol acetate represents 4.41 and 4.84%, respectively. The last peak (0.36 and 0.42%) represented linalyl propionate. It was shown that eucalyptol was represented in crude oil (0.66%), but was absent in alginate-loaded emulsion.

Determination of nanoencapsulation efficiency (%EE) and loaded capacity (%LC)

The incorporation of peppermint oil into alginate nanocapsules has been studied in order to determine the encapsulation uniformity of prepared emulsion. The results of such procedure showed that the encapsulation efficiency (%EE) was 78.62±0.12%. Loading capacity (%LC) was 4.74±0.02%.

%Mortality of honeybee adult workers exposed to menthol and nanomenthol extract

The result in Tables 2 and 3 showed %mortality of honeybee adult workers exposed to different

concentrations of peppermint crude and nanoemulsion oil. The %mortality was affected by different treatments, concentrations, and exposed methods (oral or contact). The %mortality that occurred in nanoemulsion treatment was higher than crude emulsion, and increased at high concentration (5000 ppm) compared with other concentrations. As well as percentage mortality was not affected at the beginning of the experiment (after 1 h, 1, 2, 4, and 7 days), but lately high % mortality occurred 2 weeks posttreatment. In addition, the higher %mortality occurred in oral treatment than contact treatment.

Estimated % mortalities were 83.83, 77.15, 46.31, and 32.97% in treatments of nanoemulsion (oral), nanoemulsion (contact), crude emulsion (oral), and crude emulsion (oral) at 5000 ppm 14 days posttreatment, respectively.

Table 1 Gas chromatogram analysis of crude and alginate loaded emulsion

		Crude oil		Alginate loaded emulsion	
No.	Compounds	Rt	Area %	Rt	Area %
1	Eucalyptol=(1,8-Cineole)	7.77	0.66	–	–
2	L-LINALOOL	10.32	1.83	10.32	1.44
3	p-Menthone	12.69	28.99	12.66	12.62
4	Isomenthone	13.04	7.38	13.02	4.22
5	p-Menthan-1-ol	13.25	2.73	13.22	3.46
6	Menthol	13.65	43.87	13.60	66.37
7	Linalyl propionate	14.40	0.36	14.02	0.42
8	Estragole	14.52	0.94	14.39	0.49
9	Pulegone	16.18	1.34	16.18	1.23
10	Carvone	16.53	4.48	16.54	2.02
11	Piperitone	16.92	1.22	16.91	1.18
12	Menthol acetate	18.17	4.41	18.14	4.84
13	trans-Caryophyllene	23.42	0.69	23.38	0.60
14	α-Farnesene	23.96	0.38	23.94	0.26

LC_{50} and LC_{90} values of menthol and nanomenthol extract after treatment of honeybee adult workers

The data in Table 4 showed the LC_{50} and LC_{90} values, correlation, and regression at the end of the experiment after treatment with peppermint crude and nanoemulsions.

LC_{50} values were 2629.85, 4246.84, 5471.13, and 11 895.65 ppm of different treatments like peppermint nanoemulsion (oral), nanoemulsion (contact), crude oil (oral), and crude oil (contact), respectively. LC_{90} values were 7223.38, 5747.17, 16 676.32, and 90 913.18 ppm for the above-mentioned treatments, respectively.

Table 2 The percentage mortality honeybee workers after treatment of peppermint crude oil using oral and contact methods

		Mortality %					
Treatment	Conc. (ppm)	2 h	1 day	2 days	4 days	7 days	14 days
Oral	1250	0.00	0.00	0.00	0.00	0.00	0.00
	2500	0.04	1.55	1.06	1.68	1.05	18.66
	3750	3.50	4.86	3.61	2.41	3.05	32.46
	5000	6.25	6.88	5.71	6.05	3.98	46.31
Contact	1250	0.00	0.00	0.00	0.00	0.00	0.00
	2500	0.68	3.79	2.53	0.50	0.92	8.57
	3750	3.14	6.60	5.37	2.82	10.49	18.73
	5000	4.54	7.55	8.75	6.76	12.65	32.97

Mortality %=dead workers in treatment–dead workers in control/100–dead workers in control×100.

Table 3 The percentage mortality of honeybee workers after treatment of peppermint nanoemulsion using oral and contact methods

		Mortality %					
Treat	Conc. (ppm)	2 h	1 day	2 days	4 days	7 days	14 days
Oral	1250	0.00	0.00	0.00	0.00	0.00	21.37
	2500	0.22	0.84	0.25	1.07	2.05	38.41
	3750	1.70	1.07	2.88	3.26	4.56	66.80
	5000	10.81	18.09	32.49	32.89	35.81	83.83
Contact	1250	0.00	0.00	0.00	0.00	0.00	0.00
	2500	3.99	3.85	3.86	2.43	2.04	1.95
	3750	14.26	17.32	17.78	16.66	15.02	27.08
	5000	39.60	45.50	44.42	43.93	47.83	77.15

Mortality %=dead workers in treatment–dead workers in control/100–dead workers in control×100.

Table 4 LC_{50} and LC_{90} values of honeybees workers treated with peppermint crude and nanoemulsions 2 weeks posttreatment (oral and contact)

Treatment	LC_{50} (ppm/ml)	LC_{90} (ppm/ml)	Slope±SE	*R*
1	2629.85	7223.38	2.9206 ±0.318	0.9715
2	4246.84	5747.17	9.7543 ±1.072	0.9970
3	5471.13	16676.32	2.6478 ±0.639	0.9990
4	11895.65	90913.18	1.4510 ±0.371	0.9539

1. Nanomenthol extract (oral). 2. Nanomenthol extract (contact). 3. Menthol extract (oral). 4. Menthol extract (contact).

Physiological effects (biochemical analysis)

Data in Table 5 showed the effect of menthol nanoemulsion on amylase activity, total protein, and lipid contents in the digestive system of honeybee workers. The tested parameters were affected by different treatments and concentrations of peppermint nanoemulsion compared with control.

The amylase activity was significantly increased in different treatments (oral and contact) and different concentrations of treatments (5000 and 1250 ppm) than control (*F* value 93.75). On the other hand, the total protein and lipids were significantly lower in different treatments (oral and contact) and different concentrations (5000 and 1250 ppm) than control (*F* value 13.29 and 12.71), respectively.

Data in Table 6 showed the effect of crude peppermint oil on amylase activity, total protein, and lipid contents in the digestive system of honeybee workers. The amylase-enzyme activity, total protein, and lipid contents were affected by different treatments and concentrations of crude peppermint emulsion compared with control.

The amylase activity was significantly increased in oral treatment at concentrations of 5000 and 1250 ppm, as well as in contact treatment at concentration of 5000 ppm than both control and contact treatment at 1250 ppm (*F* value 997.95). The total protein was significantly increased in contact treatment at different concentrations of 5000 and 1250 ppm than oral treatment at tested concentrations (*F* value 18.18). The lipid contents were the lowest in oral treatment at 5000 ppm compared with other treatments.

Table 5 Effects of peppermint nanoemulsion on amylase activity, total protein and lipid contents in digestive system of honeybee workers

Treatment	Conc. (ppm)	Amylase (U/l)	Total protein (mg/dl)	Total lipids (mg/dl)
Oral	5000	660.13^{a} ±81.87	562^{c} ±111	22.03^{bc} ±2.65
	1250	164.84^{b} ±28.55	860^{b} ±53.5	24.62^{ab} ±1.92
Contact	5000	43.78^{bc} ±11.38	908^{ab} ±55.0	15.01^{c} ±1.44
	1250	61.18^{bc} ±6.13	989^{ab} ±60.0	21.61^{bc} ±2.19
Control		17.5^{c} ±5.64	1161.0^{a} ±119.6	32.66^{a} ±3.79
F value		93.75*	13.29**	12.71**
LSD		129.29	0.2795	8.3223

** Highly significant. Each value represent the means of three replicates (each composed of 10 mg/ml tissue homogenate)±SE. Values with different letters within the same row are significantly different ($P<0.05$) (analysis of variance) (LSD test).

Table 6 Effects of peppermint crude emulsion on amylase activity, total protein and lipid contents in digestive system of honeybee workers

Treatment	Concentration (ppm)	Amylase (U/l)	Total protein (mg/dl)	Total lipids (mg/dl)
Oral	5000	487.16^{a} ±12.03	819.0^{c} ±15.0	9.54^{d} ±1.89
	1250	62.22^{c} ±3.24	924.0^{bc} ±37.0	20.81^{c} ±2.78
Contact	5000	276.91^{b} ±16.57	1033.0^{b} ±37.0	41.11^{b} ±5.26
	1250	5.29^{d} ±2.16	1295.0^{a} ±110.0	53.69^{a} ±3.73
Control		13.24^{d} ±2.16	1021.0^{b} ±45.0	21.90^{c} ±1.15
F value		997.95**	18.18**	57.76**
LSD		30.846	0.1929	10.834

** Highly significant. Each value represent the means of three replicates (each composed of 10 mg /ml tissue homogenate)±SE. Values with different letters within the same row are significantly different ($P<0.05$) (analysis of variance) (LSD test).

Discussion

Recently, biopesticides were made from some plant oils [20]. They used leaves of *Menth avirids* (menthol) against *Phthorimaea operculella* [21]. They used neem, ginger, peppermint, garlic, and jojoba oils against larvae of *Spodoptera littoralis*. Lately Gokulakrishnan *et al.* [22], they used menthol against three species of Lepidoptera. The menthol oil was extracted from essential oil and proved to be effective for some insects [23]. They used synthetic menthol, thymol against *Tetranychus urticae*, and Abdelgaleil [24] found that menthol was effective and strong or toxic to adults of *Theba pisana.*

Recently, nanoparticles in pesticides were produced and used against insects [5–25]. Honeybee *A. mellifera* is a useful insect and is affected by

environmental changes, pesticides, and recently nanoparticle products that were used against many insects [10–16]. We studied the effect and toxicity of nanoparticles on honeybee *A. mellifera.*

TEM examination showed a successful encapsulation of peppermint oil using alginate polysaccharide and cross-linking with calcium chloride. The particle sizes were closed to those obtained by Lertsutthiwong [26], suggesting that using the high-energy ultrasonic probe is a major step to obtain small nanocapsules. Our suggestion agreed with those reported by the same authors. However, the authors reported that mechanical stirring only is insufficient to prepare nanocapsules in a uniform size, and smaller capsules can be obtained by increasing the time of sonication either before or after adding the oil to the sodium alginate. They added that sonication for 15 min after adding oil to sodium alginate resulted in nanocapsules (83.6 nm) that were about 50% smaller than those formed by sonication before adding oil to sodium alginate. It was clear that using of sonication after cross-linking by calcium chloride reduced the diameter of the nanocapsules.

GC/MS analysis indicated that menthol and menthone compounds were the abundance compounds in both extracts. Menthol is a cyclic monoterpene alcohol with the minty taste. Menthone is monoterpene with minty, structurally related to menthol; however, the alcohol group is oxidized to form ketones. It was clear that preparation of loaded nanoemulsion relatively did not affect on oil compounds, except the absence of Eucalyptol. The finding results are similar with those stated by Hawrył *et al.* [27]. They cleared that menthol is the most characteristic Mentha compound. Its highest concentration was in *M. piperita* var. Krasnodarskaja and other piperita varieties. The compound present in the largest amount overall was menthone, representing over 10% of the total. It was present at high concentrations in *M. piperita*, piperita var. Also, Yunilawati *et al.* [28] detected 17 compounds in peppermint oil. They added that menthol (39.79%) and menthone (35.69%) were the main components in peppermint oil.

Encapsulation uniformity indicated successful encapsulation sufficiently. The encapsulation efficiency (%EE) was 78.62±0.12%, and loading capacity (%LC) 4.74±0.02%. Lertsutthiwong *et al.* [26] prepared the same delivered system with turmeric oil. In such study, it was reported that loading capacity of 5.47 and 10.4% was obtained for processes with and without ethanol evaporation, respectively. They added that without ethanol evaporation, about 30% of the turmeric oil was lost during formation of the turmeric oil-loaded nanocapsules and solvent removal caused a total loss of about 42% of the turmeric oil, suggesting that evaporation process is responsible for loss of 14% of the total oil. Also, Jerobin *et al.* [29] stated that percentage-entrapment efficiency of neem oil loaded on alginate polymer varied considerably with the percentage loading. They added that entrapment efficiency decreased with an increase in neem-oil nanoemulsion loading. This might be attributed to the release of nanoemulsion to the external solvent (methanol).

The toxicity effect of both formulations (nano and crude oils) of peppermint oil was studied on honeybee worker *A. mellifera*. It was found that nanoemulsion had a higher effect than bulk oil. The obtained similar results were reported by Mohamed [30], who stated that nanoformulations of geranium and garlic oils had more effects on *S. littoralis* larvae than bulk ones. Also, Sharaby and El-Nojiban [31] arranged the toxicity values of certain oils in descending order based on LC_{50} values measured against *A*grotis *ipsilon as* garlic, mint, cumin, caraway, and parsley. Mint oil was the second most toxic essential oil, as seen. The LC_{50} value of peppermint oil as a contact poison on larvae was 0.032%, according to the researchers. The LC_{50} values as a stomach poison for larvae and pupal stages were approximately identical at 0.160 and 0.148%. This was in contrast with the results obtained in this investigation, as shown, the LC_{50} values of oral treatments were less than those of contact treatments.

In this investigation, a significant increase in amylase activity was recorded and these results were in agreement with those reported by Wang *et al.* [32]. They stated that the occurrence of *Bacillus* in bee guts, is directly related to the increase of amylase in the foregut of bees. Lee *et al.* [33] studied the relation between microbial community and food processing, and based on an analysis of metatranscriptomes, three major active bacterial classes were identified in the gut (gamma-Proteobacteria, Bacilli, and Actinobacteria), they thought that these bacteria were likely to be involved in the decomposition of complex macromolecules (e.g. polysaccharides). Wang *et al.* [32] added that *Bacillus* sp. commonly occur in the stomach of honeybees, *Bacilli* can produce enzymes, while *B*acillus *amyloliquefaciens* can produce amylase, lipase, and protease [34]. Consequently, we suggest that increasing of amylase levels after treatment can be explained by the increase of bee gut microbiota to compensate the lack of its production by the bee itself.

It was shown a significant reduction of total protein and total lipid after treatment with nanoemulsions, that was in parallel with the results obtained by Abdel-Hakim *et al*. [35]. They found that feeding the 4th larval instar of *A. ipsilon* on Neem Azal T/S reduced the larval total protein and lipid contents. Abd El-Aziz [36] reported that larvae of potato tuber moth *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) treated with eucalyptus, citronella, geranium, and marjoram essential oils increased total protein of the last larval instar. The increase in the total protein with oil treatments may be related to the increase of protein biosynthesis, and can be explained as a kind of detoxification mechanism. Shoukry *et al*. [37] stated that increasing or decreasing in the protein content accompanied with pesticide treatment related to the toxic and disturbance effect of the tested insecticides intrusive with the protein synthesis. The disturbance in protein by decreasing or increasing associated with inequity in the natural hormones of the treated insects. Protein production in an insect body is controlled by the endocrine glands and the hormonal system. Changes in protein level may be representing the balance between protein synthesis, transportation, storage, and deprivation of structural and nutrient functions throughout ontogeny in addition to the response to the exact physiological conditions.

There were variable effects of both nano and crude peppermint oils on honeybee workers when treated by oral or contact exposures, and that was clear on their effects on total lipid-content values. Significant reduction of total lipids resulted speedily by oral treatments. That was in parallel with the fondants of Amrutsagar and Joshi [38] who stated that insect repellent (allethrine) decreased lipid contents in cockroach insects (*Periplaneta americana*). Also, Lohar and Wright [39] observed a reduction in the amount of lipid in hemolymph, fat body, and oocyctes of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) females treated with malathion. They pointed out that reduction of lipid content may be attributed to the effect of insecticide on the adipokinetic hormone that controls lipid metabolism. It was found that many toxic agents cause disturbances of fats in different body organs of both invertebrate and vertebrate animals.

Conclusion

It can be concluded that peppermint oil can be encapsulated in sodium alginate polysaccharide and cross-linked with calcium chloride. As shown in TEM examination, nanocapsules were nearly spherical and less than 100 nm in size. GC/MS analysis represented no changes in oil compounds after loading on alginate polysaccharide. Content uniformity showed successful encapsulation by the testing-delivered system. It is clear that nanomaterials of peppermint oil have more toxic effect on honeybee worker (*A. mellifera*) than its crude form. As shown, the LC_{50} and LC_{90} values of nanoemulsion are less than those of bulk ones, even by oral or contact treatments. In addition, oral treatments were more toxic than contact ones. Also, both forms of emulsions have different effects on honeybee workers' biochemical aspects. Amylase activity was increased by both forms of emulations. Total protein contents significantly decreased by nanoemulsion treatment, especially by oral applications. However, bulk form affected total protein contents only by high-concentration oral treatment. Total lipid contents were affected by both treatments, but in different ways in consideration types of treatment and concentrations. Nanoemulsions decreased lipid contents, especially by contact treatments. In contrast, bulk emulsions significantly increased lipid contents by contact treatments.

Acknowledgements

The authors would like to thank the National Research Centre for the fund extended for this research. All honeybee experiments were carried out in apiary of Faculty of Agriculture, Ain Shams University. The authors are grateful to Prof. Dr Esam Mohamed Hob Allah (God mercy on him), Department of Environmental Microbiology, National Research Centre, for enabling his laboratory to do biochemical studies.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

References

1 Silva H. Current knowledge on the vascular effects of menthol. Front Physiol 2020; 11:298.

2 Mao1 B, Chen Z, Wang Y, Yan S. Silver nanoparticles have lethal and sublethal adverse effects on development and longevity by inducing ROS-mediated stress responses. Sci Rep 2018; 8:2445.

3 Gul1 HT, Saeed S, Khan FZA, Manzoor SA. Potential of nanotechnology in agriculture and crop protection. Appl Sci Busi Econ 2014; 1:23–28.

4 Tunçsoy BS. Toxicity of nanoparticles on insects. Adana Sci Tech Uni J Sci 2018; 1:49–61

5 Bhattacharyya A, Bhaumik A, Rani PU, Mandal S, Epidi TT. Nano-particles-A recent approach to insect pest control. Afr J Biotech 2010; 9:3489–3493.

6 Rouhani M, Samih MA, Kalantari S. Insecticide effect of silver and zinc nanoparticles against *Aphis neriiboyer* de fonscolombe (Hemiptera: Aphididae). Chilean J Agric Res 2012; 72:590–595.

7 Kitherian S. Nano and bio-nanoparticles for insect control. Res J Nanosci Nanotechnol 2017; 7:1–9.

8 Amoabeng BW, Gurr GM, Gitau CW, Nicol HI, Munyakazi L, Stevenson PC. Tri-trophic insecticidal effects of African plants against cabbage pests. PLoS ONE 2013; 8:1371–1382.

9 Dimetry NZ, Amin AH, Bayoumi AE, Abdel-Raheem MA, Youssef DA. Comparative toxicity between neem and pepper mint oils nano formulations against *Agrotis ipsilon* (Hufn.) larvae (Lepidoptera: Noctuidae). J Botanic Res 2019; 1:13–19.

10 Dağlioğlu1 Y, Kabakçi D, Akdeniz G. Toxicity of nano and non-nano boron particles on *Apis mellifera* (honey bee). Res J Chem Environ Sci 2015; 3:6–13.

11 Milivojević T, Glavan G, Božič J, Sepčić K, Mesarič T, Drobne D. Neurotoxic potential of ingested ZnO nanomaterials on bees. Chemosphere 2015; 120:547–554.

12 Özkan Y, Irende I, Akdeniz G, Kabakçi D, Sökmen M. Evaluation of the comparative acute toxic effects of TiO2, Ag-TiO2and ZnO-TiO2 composite nanoparticles on honey bee (*Apis mellifera*). J Int Environ Appl Sci 2015; 10:26.

13 Taha EH, Abdelmegeed SM. Effect of entomopathogenic nematodes, *Heterorhabditis bacteriophora* on *Galleria mellionella* in bee hive of *Apis mellifera*. J Biol Sci 2016; 16:197–201.

14 Sawires SG, Abdelmegeed SM. Morphological changes and colony activity in honeybee workers *Apis mellifera* produced from irradiated queens. Egypt Acad J Biol Sci (Entomology) 2016; 9:15–21.

15 Abdel razik MAA. Toxicity and side effects of some insecticides applied in cotton fields on *Apis mellifera*. Environ Sci Pollu Res 2019; 26:4987–4996.

16 Al Naggar Y. Effects of metal and metal oxide nanoparticles on honey bees (*Apis millefera* L.). J Aqua Pollu Toxic 2017; 1:1–3.

17 Abdelmegeed SM. Strong impact of five genetic and non-genetic factors exerting their effects on honey bee queens to increase bee honey production. Egypt Acad J Biol Sci (Entomology) 2015; 8:59–65.

18 Youssef DA. Improving properties of some botanical products using different nano-technological methods for controlling two lepidopterous pests [PhD Thesis]. Cairo, Egypt: Faculty of Agriculture, Ain Shams University; 2018. 222.

19 Mashal SAA. Effect of certain honey bee products as food subliments on the most important characteristics for mass rearing of three Trichogramma species (Hymenoptera: Trichogramatidea) [MSc thesis]. Cairo, Egypt: Faculty of Agriculture, Cairo University; 2020.

20 Sharaby A, Abdel-Rahman H, Moawad BS. Biological effects of some natural and chemical compounds on the potato tuber moth, Phthorimaea operculella Zell. (Lepidoptera: Gelechiidae). Saudi J Biol Sci 2009; 16:1–9.

21 Ismail SM, Shaker N. Efficacy of some essential oil against the immature stages of *Spodoptera littoralis*. Alex J Agric Res 2014; 59:97–103.

22 Gokulakrishnan J, Krishnappa K, Elumalai K. Effect of plant oil formulation against armyworm, Spodoptera litura (fab.), cotton bollworm, *Helicoverpa armigera* (hub.) and fruit borer, *Earias vitella* (fab.) (Lepidoptera: Noctuidae). Inter J Curr Life Sci 2012; 2:1–4.

23 Mohammadi M, Moayeri HRS, Kavousi A, Bolandnazar AR. Ovicidal and adulticidal effects of synthetic menthol, thymol and their mixtures against *Tetranychus urticae* (Acari: Tetranychidae). Persian J Acarol 2015; 4:125–136.

24 Abdelgaleil SAM. Molluscicidal and insecticidal potential of monoterpenes on the white garden snail, Theba pisana (Muller) and the cotton leaf worm, *Spodoptera littoralis* (Boisduval). Appl Entomol Zool 2010; 45:425–433.

25 Armstrong N, Ramamoorthy M, Lyon D, Jones K, Duttaroy A. Mechanism of silver nanoparticles action on insect pigmentation reveals intervention of copper homeostasis. PLoS ONE 2013; 8:1–9.

26 Lertsutthiwong P, Noomun K, Jongaroonngam-sang N, Rojsitthisak P, Nimmannit U. Preparation of alginate nanocapsules containing turmeric oil. Carbohyd Polym 2008; 74:209–214.

27 Hawrył MA, Krystyn SW, Ryszard ⊠, Małgorzata N, Klaudia S, Monika WH, *et al*. GC-MS fingerprints of mint essential oils. Open Chem 2015; 13:1326–1332.

28 Yunilawati R, Handayani W, Cahyaningtyas AA, Imawan C. Peppermint oil loaded on recycled paper as an antibacterial label for shrimp freshness. Earth Enviorn Sci 2021; 71:739–745.

29 Jerobin J, Sureshkumar RS, Anjali CH, Mukherjee A, Chandrasekaran N. Biodegradable polymer based encapsulation of neem oil nanoemulsion for controlled release of Aza-A. Carbohyd Polym 2012; 90:1750–1756.

30 Mohamed SSI. Applications of some bio-technological methods for controlling the most important pests of potatoes [PhD thesis]. Cairo, Egypt: Faculty of Science for Girls, Al-Azhar University; 2016. 306.

31 Sharaby A, El-Nojiban A. Evaluation of some plant essential oils against the black cutworm *Agrotis ipsilon*. Glob J Adva Res 2015; 2:701–711.

32 Wang M, Xu H, Yin G, Zhao W, He S. Diversity of bacteria in the honey stomach of Apis cerana and Apis mellifera during the rape blooming period. Chin J Appl Entomol 2014; 51:1567–1575.

33 Lee FJ, Rusch DB, Stewart FJ, Mattila HR, Newton IL. Saccharide breakdown and fermentation by the honey bee gut microbiome. Environ Microbiol 2014; 17:796–815.

34 Snyder A. *Bacillus amyloliquefaciens* strain. US patent application US 12/492, 816. Publication no. US20090324533. (2009).

35 Abdel-Hakim E, Salem NY, Sammour E, Ibrahim SS. Influence of three plant extracts on some biological and biochemical aspects of the black cutworm *Agrotis ipsilon* (Hufn.). Bull Natl Res Chem 2011; 36:223–237.

36 Abd El-Aziz MF. Bioactivities and biochemical effects of marjoram essential oil used against potato tuber moth *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae). Life Sci J 2011; 8:288–297.

37 Shoukry IF, Khalaf AA, Hussein KT, Khater KS. Toxicological evaluation of some botanical oils on biochemical aspects in the Indian meal moth *Plodia interpunctella* HB. (Lepidoptera: Pyralidae). Egypt J Biol 2003; 5:155–163.

38 Amrutsagar MV, Joshi SS. Insect repellent induced lipid diversions of *Periplaneta americana*. Int J Rec Sci Res 2017; 8:18370–18372.

39 Lohar MK, Wright DJ. Changes in the lipid content in haemolymph, fat body and oocytes of malathion treated Tenebrio molitor L. Adult females. Pak J Zool 1993; 25:57–60.