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Research Article
Nanoemulsion of Camphor (*Eucalyptus globulus*) Essential Oil, Formulation, Characterization and Insecticidal Activity against Wheat Weevil, *Sitophilus granarius*

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Abstract
Background and Objective: Insecticides are widely used to protect grains during stored against many insects, it caused serious adverse effects on grains, seeds, birds, humans and increase water, soil and environmental pollution. Therefore, nanotechnology including nanoformulations and green insecticides are interesting for control storage and planting grains insects. The current study was carried out for the first time to formulate nanoemulsion of camphor essential oil (EO) by ultrasonic and assess its insecticidal activity on wheat weevil, *Sitophilus granarius*. Acute and sub-chronic toxicity also were studied on male albino rats. **Methodology:** Camphor (*Eucalyptus globulus*) was obtained from leaves by hydrodistillation and analysis by GC/MS. Nanoemulsion of EO was formulated by ultrasonic and characterization. In addition, insecticidal activity and toxicological studies were investigated and statistical analysis by the log-probit software program Ldp Line® and SPSS version 18.0 for windows, respectively. **Results:** The EO to surfactant ratio was correlated to nanoemulsion droplet size and stability. Stable camphor EO formulation having a droplet of 99.0 nm was formulated after 40 min of sonication. Camphor nanoemulsion showed high insecticidal activity against wheat weevil, *Sitophilus granarius* with LC_{50} 181.49 $\mu g\ g^{-1}$ compared to 282.01 $\mu g\ g^{-1}$ of EO. The activity of EO could be due to the presence of 1,8-Cineole, β-cymene, D-limonene, α-pinene and α-terpineol which found in camphor EO by GC-MS analysis. Camphor nanoemulsion did not show any effect on germination or seedling growth. Acute and sub-chronic toxicity studies show no signs of toxicity and no biochemical alteration in liver biomarkers of male rats. **Conclusion:** These findings show that camphor nanoemulsion development as green and nano insecticide product. This study also concludes that the potential use of camphor EO nanoemulsion to protect grains against *Sitophilus granarius* and other insects.

Key words: Nanoemulsion, camphor, *Sitophilus granarius*, insecticidal activity, acute toxicity, biochemical, green pesticides

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Synthetic pesticides are widely used to control pests such as insect, fungi, acaroid, nematode, rodents, weeds and others. It is important in crop production to loss reduction in crops, food and for producing a sufficient and healthy food due to increasing human population[1]. Worldwide, about 2.5 million t of pesticides are used for crop protection annually. These pesticides caused damage reaches 100 billion dollars[2]. The excessive use of synthetic pesticides caused serious problems to human health, non-target organisms and ecosystem[3]. The most common problems in crop production are pesticide residues and pests resistance[4]. Insecticides are used to protect seeds against many insects during storage and planting. Although the beneficial role of these synthetic insecticides was reported, it can cause many adverse effects to seeds, birds and environment[5]. Consequently, it is important issue to research on alternative bio-pesticides from new, active and safe natural resources. Previous studies showed that essential oils (EOs) are among the most active bio-insecticide to control insect stored grains. It showed an abroad spectrum of activity against many insects with different sites and mechanism of toxic actions[3,6-8].

Currently, new trend is started for using the natural plant extracts as well as EOs as natural pesticides to control pests with nanoformulations[9,10] in green pest management[3]. These green and nanopesticides are safe, low to no mammalian toxicity and have many sites of toxic actions in pests, which lead to high selectivity and low resistance development[11,12].

Camphor, *Eucalyptus globulus* is one of the most active EOs with a wide range of biological activity against many pests. The oil from leaves contain some groups of plant secondary metabolites with high biological activity. For example, terpenoids and phenolic compounds had insecticidal, antibacterial, antifungal and antiviral activities with the different mechanism of action[13,14]. The main components in camphor leave EO from Algeria were oxygenated monoterpenes e.g., 1,8-Cineole, spathulenol and α-terpineol[15]. The major constituents of leaves EOs obtained from seven *Eucalyptus species* from Tunisia were identified by GC/MS. The major constituents were 1,8-Cineole (49.07-83.59%) and α-pinene (1.27-26.35%)[16]. The EO obtained from leaves of other plants of Genus *Eucalyptus* such as *Cinnamomum camphora* showed repellent and insecticidal activity against *Sitophilus oryzae* L. at concentrations from 250-1000 μg g^{-1} seeds. The EO at concentration 500 μg g^{-1} caused a significantly toxic effect to seed germination of wheat[17]. Camphor leaves EO also had strong fumigant (LC_{50} 2.5 mg L^{-1}) and contact toxicity (LD_{50} 21.25 μg/adult) against *Lasioderma serricorne*. The GC-MS analysis of this oil showed that D-camphor, linalool, cineole, and 3,7, 11-Trimethyl-3-hydroxy-6,10-dodecadien-1-yl acetate were the main components[18]. Camphor EO from the stem barks, leaves and fruits had highly fumigant and contact toxicity against *Tribolium castaneum* and *Lasioderma serricorne*[19]. Therefore, EOs are considered active, safe and alternative to synthetic insecticides to control storage insects without producing adverse effects on seed germination and ecosystem[3,12,20]. These oils have insecticidal activity against many insects due to the presence of many active components, which had different sites and mechanism of toxic action[3].

Nanotechnology including nanoformulations and green insecticides are interesting as new tools to control insects especially storage grain insects. It can use to avoid the problems resulting from synthetic insecticides, which did not contain toxic solvents and others synthetic chemical compounds. It had known low mammalian toxicity and more eco-friendly. Therefore, there is an urgent need for innovative strategies to developing new green insecticides based on EOs nanoformulations as an alternative to synthetic insecticides for control grains insects. In this nanotechnology, it is needed to develop nanoformulations with particles size ranged from 20-200 nm that improved insecticidal activity and emulsion stability[21].

This study was carried out for the first time to develop nanoemulsion of camphor essential oil by ultrasonic emulsification and evaluate its insecticidal activity against wheat weevil, *Sitophilus granarius*. Acute and sub-chronic toxicity of camphor essential oil nanoemulsion on male rats was also studied.

MATERIALS AND METHODS

All experiments were carried out of the year 2016 and 2017 in the laboratories of Environmental Toxicology Research Unit (ETRU), Department of Pesticide Chemistry, National Research Centre (NRC), 33 El Bohouth Street (Former El Tahrir St.), P.O. Box 12622, Dokki, Giza, Egypt and Department of Stored Products and Grains Pests, Plant Protection Research Institute, Agriculture Research Centre, Dokki, Giza, Egypt.

Chemicals: Chemicals and reagent used in this study were of reagent grades and obtained from the scientific distributors in Cairo, Egypt. Tween 20 (Polysorbate 20) was obtained from VWR International 201, Rue Carnot F-94126 Fontenay/Bois, France. All kits used for biochemical investigation including aspartate aminotransferases (AST; EC 2.6.1.1), alanine aminotransferases (ALT; EC 2.6.1.2), alkaline phosphatase (ALP; EC 3.1.3.1), lactate dehydrogenase (LDH; EC 1.1.1.27), protein and albumin were obtained from Biodiagnostic Co., 29 Tahrir Street, Dokki, Giza, Egypt.

Camphor essential oil: Essential oil of *Eucalyptus globulus* leaves was obtained by hydrodistillation by Clevenger apparatus (Garg Process Glass India Private Limited, Maharastra, India). The distillation continued until no more condensing oil could be considered. The essential oil was separated, dried over anhydrous sodium sulfate (Na_2SO_4), transfer to an amber glass flask and kept undercooling until used.

GC/MS analysis: Camphor (*Eucalyptus globulus*) EO was analyzed by a GC/MS, a TRACE GC Ultra Gas Chromatographs (THERMO Scientific Corp., USA), coupled with a Thermo mass spectrometer detector (ISQ Single Quadrupole Mass Spectrometer) at the Department of Medicinal and Aromatic Plants Research, National Research Centre. The GC-MS system was equipped with a TR-5MS column (30 m$\times$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^{-1} and a split ratio of 1:10 using the following temperature program: 60°C for 1 min, rising at 4.0°C min^{-1} to 240°C and held for 1 min. The injector and detector were held at 210°C. Diluted samples (1:10 hexane, v/v) of 1 μL of the mixtures were always injected. Mass spectra was obtained by electron ionization (EI) at 70 eV, using a spectral range of m/z 40-450. Identification of different constituents of camphor oil was determined by comparing the spectrum fragmentation pattern with those stored in Wiley, replib, nistdemo and Mainlib Mass Spectral Library data.

Nanoemulsion formation: Camphor nanoemulsion (Oil-in-water) (5%) was prepared using Polysorbate 20 as a non-ionic surfactant, camphor EO and deionized water. The organic phase was prepared by using different ratios of camphor EO and Polysorbate (1:1, 1:25, 1:1.5 w/w) while, deionized water was used as an aqueous phase. The organic phase was added to the aqueous phase and subjected to different sonication times (5, 10, 20 and 40 min) using Ultrasonic (Sonics and Materials, INC. 53 Church Hill RD. Newtown, CT USA). A probe diameter of 13 mm at a high frequency of 20 kHz and power output of 750 W were used. The energy was given through Sonicator probe and ice was used to reduce energy.

Characterization of nanoemulsion: Stability of camphor nanoemulsion and physico-chemical characterization were investigated. The stability to different stress including thermodynamics, centrifugation, heating, cooling and freeze cycles was evaluated as cited in the previous studies[22,23]. Different nanoemulsions were subjected to centrifugation at 10,000 rpm for 30 min at 25°C using Heraeus Labofuge 400R (Kendro Laboratory Products GmbH, Germany) and observed for phase separation. The stable formulations were subjected to the heating-cooling test, which has six cycles between refrigerator temperatures at 4°C for 48 h and 45°C for 48 h. then taken for the freeze-thaw stress test. In this test, nanoformulations were stored for two cycles; each cycle carried out by stored the nanoemulsion at -20°C for 48 h and at 25°C for 48 h. Finally, the stable formulation nanoemulsions were stored for 3 months at room temperature in closed tubes for other observation like phase separation or creaming. The observation was carried out every day in the first week, followed by every week upto 3 months. In this study, two nanoemulsions, sample A (EO: Tween 20 at ratio 1:1.25) and sample B (EO: Tween 20 at ratio1:1.5) with 40 min as sonication time were stable after the physico-chemical test, therefore, droplets size was done on these formulations (samples A and B).

Droplet size and transmission electron microscopy (TEM): Nanoemulsion droplet and distribution size were determined by dynamic light scattering (DLS) instrument (PSS, Santa Barbara, CA, USA) at 23°C, using the 632 nm line of a He-Ne laser as the incident light with angle 90°. Camphor nanoemulsions of sample A and B were found to be stable with the lowest droplet size diameter 171.7$\pm$0.478 and 99.0$\pm$0.605 nm, respectively. Therefore, the nanoemulsion with the lowest droplet (99.0$\pm$0.605 nm) was used for further TEM and insecticidal studies.

The morphology of camphor nanoemulsion was studied by transmission electron microscopy (model JEM-1230, Jeol, Tokyo, Japan). A drop of camphor nanoemulsion (sample B) was diluted with deionized water, transferred into a carbon-coated copper grid, then stained by phosphotungstic acid solution (2%, pH = 6.7) for 1 min. The replica was lifted to drying at room temperature (27°C) and then the image was visualized with TEM at 80 KV accelerating voltages.

Insecticidal activity

Insects: In the present study, adults of wheat weevil, *Sitophilus granarius* (Coleoptera: Curculionidae) was reared in the laboratory of Department of Stored Products and Grains Pests, Plant Protection Research Institute, Agriculture Research Centre, Dokki, Giza, Egypt. The insects reared for several generations on wheat grains as food. The wheat grains were sterilized at a temperature of 55°C for 6 h in order to eliminate any hidden infestation before using.

Bioassay test: Samples of wheat (10 g) were treated with different concentrations of camphor EO (250, 300, 350, 400 and 450 μg g^{-1}) and camphor nanoemulsion, sample B (150, 200, 250 and 300 μg g^{-1}). The wheat grains were put into 50 g glass jar then hand-shaken horizontally and vertically. Therefore, that every wheat grain might have a thin oil or nanoemulsion coating then 20 insect adults were introduced in each glass jar and 4 replicates were performed for each concentration. The jars were covered with the muslin cloth, secured with rubber bands and kept under conditions at 25°C and 65% RH. Results of mortality were recorded after 24, 48 and 72 h and during the experimental period (21 days). In this experiment, no mortality was recorded in control, therefore, we do not need any corrections of the percent of mortality. The toxic index of nanoemulsion or EO and reduction in F1-progeny were calculated by the following equation[24]:

$$\text{Toxicity index (TI)} = \frac{LC_{50} \text{ of nanoemulsion or EO}}{LC_{50} \text{ of EO}}$$

The compound has TI less than 1 (TI<1) have high toxicity:

$$\text{Reduction (\%) in F1-progeny} = \frac{\left(\begin{array}{c}\text{No. of adult emerged}\\ \text{in control}\end{array} - \begin{array}{c}\text{No. of adult emerged}\\ \text{in treatment}\end{array}\right)}{\text{No. adult emerged in control}} \times 100$$

Germination test: The germination test was carried out under laboratory conditions to evaluate the effect of camphor EO and nanoemulsion on germination of wheat grains at concentration equaled to LC_{50}, LC_{90} and LC_{99}. Three replicates of 20 seeds each were treated by camphor EO or nanoemulsion and placed separately on a surface of a layer of cotton wool in petri dish (6×1 cm) which wetted carefully with a tap water every day for 1 week. Germination of seeds was determined after 1 week of plantation by counting the viable seeds and the germination percentages were calculated for each treatment.

Toxicological studies

Animals: Male albino rats (*Rattus norvegicus*) weighing 100±5 g were obtained from the Animal Breeding House of the National Research Centre (NRC), Dokki, Giza, Egypt. Rats were kept in polypropylene cages, with free access to standard pellet diet, water *ad-libitum*, 12 h light/dark cycle, 22±2°C temperature and 48% humidity in the laboratory. The rats were acclimatized for 1 week before the start of the experiment. All the rats were kept according to the guidelines and welfare regarding animal protection approved by NRC Local Ethical Review Committee and were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals[25].

Acute oral toxicity: Rats were divided into three-groups, five rats of each. Group (I) was received distilled water (1 mL/rat) and served as a control. Group II received a single dose of the nanoemulsion of camphor oil (1 mL/rat) sample B by the oral route, this volume corresponding to 0.5 g camphor oil kg^{-1} body weight (average b.wt. 100 g/rat)[26]. This dose corresponding to 30 g of camphor oil in nanoemulsion/person if calculated based on average human weight (60 kg), which was equivalent to 600 mL of nanoemulsion/person. Group III received 1 mL/rat of EO (5% camphor oil) in corn oil. Signs of toxicity and mortality were recorded during the first 60 min and after 2, 3 and 4 h of oral treatment and daily for 14 days. In addition, food and water consumption were recorded daily during the experimental period.

Sub-chronic toxicity: Three groups of rats (n = 5) were used for sub-chronic studies. Groups of control (I), nanoemulsion (II) and normal oil (III) were received the same doses and treatments in acute oral toxicity study daily for 28 days. At the end of experimental period (28 days), rats fasted overnight, blood samples were collected and rats were sacrificed by cervical dislocation. Blood samples were left to clot in clean dry tubes and centrifuged at 3000 rpm (600xg) for 10 min at 4°C using Heraeus Labofuge 400R (Kendro Laboratory Products GmbH, Germany) to obtain the serum. The sera were kept in a deep freezer at -20°C until biochemical markers were analyzed within one week.

Liver dysfunction biomarkers were determined in serum according to the details given in the kit's instructions and performed by using a Shimadzu UV-VIS Recording 2401 PC (Japan).

Statistical analysis: Data were analyzed using SPSS version 18.0 for windows (SPSS Inc. 233 South Wacker Drive, 11th Floor Chicago, IL 60606-6412) and the statistical analysis was done by using one-way ANOVA followed by using *post hoc* multiple comparisons. The differences were statistically significant at $p<0.05$. All data were expressed as a mean±standard error (SE).

The results of bioassay were statistically analysis based on statistically analyzed by Finney[27] using the log-probit software program Ldp Line® model "Ehabsoft"[28].

RESULTS

Camphor (*Eucalyptus globulus*) EO was obtained by hydrodistillation and analysis by GC/MS. The MS spectra of EO constituents were identified based on data in GC/MS library (Table 1). The main compounds found in camphor EO were 1,8-Cineole (87.78%), β-cymene (7.77%), D-limonene (2.29%), α-pinene (1.04%) and α-terpineol (0.10%). These compounds represent 98.98% of EO components. The GC chromatogram, mass spectra and chemical structure of major chemical compounds in camphor EO were shown in Fig. 1 and 2. Other compounds also were found in camphor EO such as camphene, verbenol, terpinen-4-ol, 2α-pinene, limonene and other monoterpenoids.

Nanoemulsion of camphor EO was prepared in the current study by ultrasonic (Fig. 3). Twelve formulations have been prepared by using camphor EO (5%), tween 20 at different ratios (1:1, 1:25, 1:1.5 w/w) and deionized water with different sonication time (5, 10, 20 and 40 min). All emulsions were tested for stability and physicochemical e.g., centrifugation and thermodynamics. All formulations had unstable and showed different degrees of creaming except two samples, sample A (EO: Tween 20 at ratio 1:1.25) and sample B (EO: Tween 20 at ratio 1:1.5) at 40 min as sonication time (Fig. 3). Therefore, the distribution of droplets size was determined for these two emulsions. Results showed that the mean droplets size diameter was 171.7±0.478 nm and 90% of droplets size less than 282.4 nm of sample A, while sample B had 99.0±0.605 nm and 90% of droplets size less than 179.0 nm (Fig. 4).

Nanoemulsion of sample B was stable and unchanged during stored (3 months) with low droplet size diameter (99.0±0.605 nm). Therefore, further studies including characterization (TEM), insecticidal activity and toxicological studies were completed on sample B. The TEM image confirmed the results that show the spherical shape and a good dispersion of droplets nanoemulsion (Fig. 5).

Insecticidal activity of nanoemulsion and camphor EO were evaluated against wheat weevil, *Sitophilus granarius*. Results showed that nanoemulsion and camphor EO have

Table 1: Chemical compensation of camphor (*Eucalyptus globulus*) essential oil

No.	RT (min)	Area (%)	Compounds	Molecular formula	Molecular weight
1	4.71	1.04	α-Pinene	$C_{10}H_{16}$	136
2	7.39	7.77	β-Cymene	$C_{10}H_{14}$	134
3	7.48	2.29	D-Limonene	$C_{10}H_{16}$	136
4	7.65	87.78	1,8-Cineole	$C_{10}H_{18}O$	154
5	14.11	0.10	α-Terpineol	$C_{10}H_{18}O$	154
Total identification		98.98			

RT: Retention time

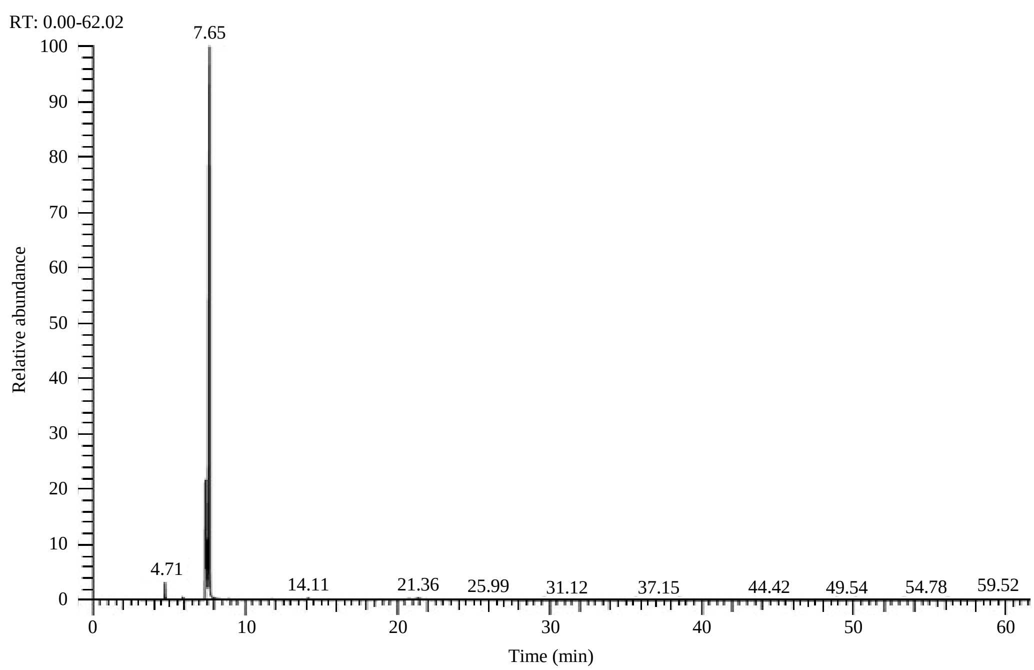

Fig. 1: GC chromatogram of the camphor (*Eucalyptus globulus*) essential oil

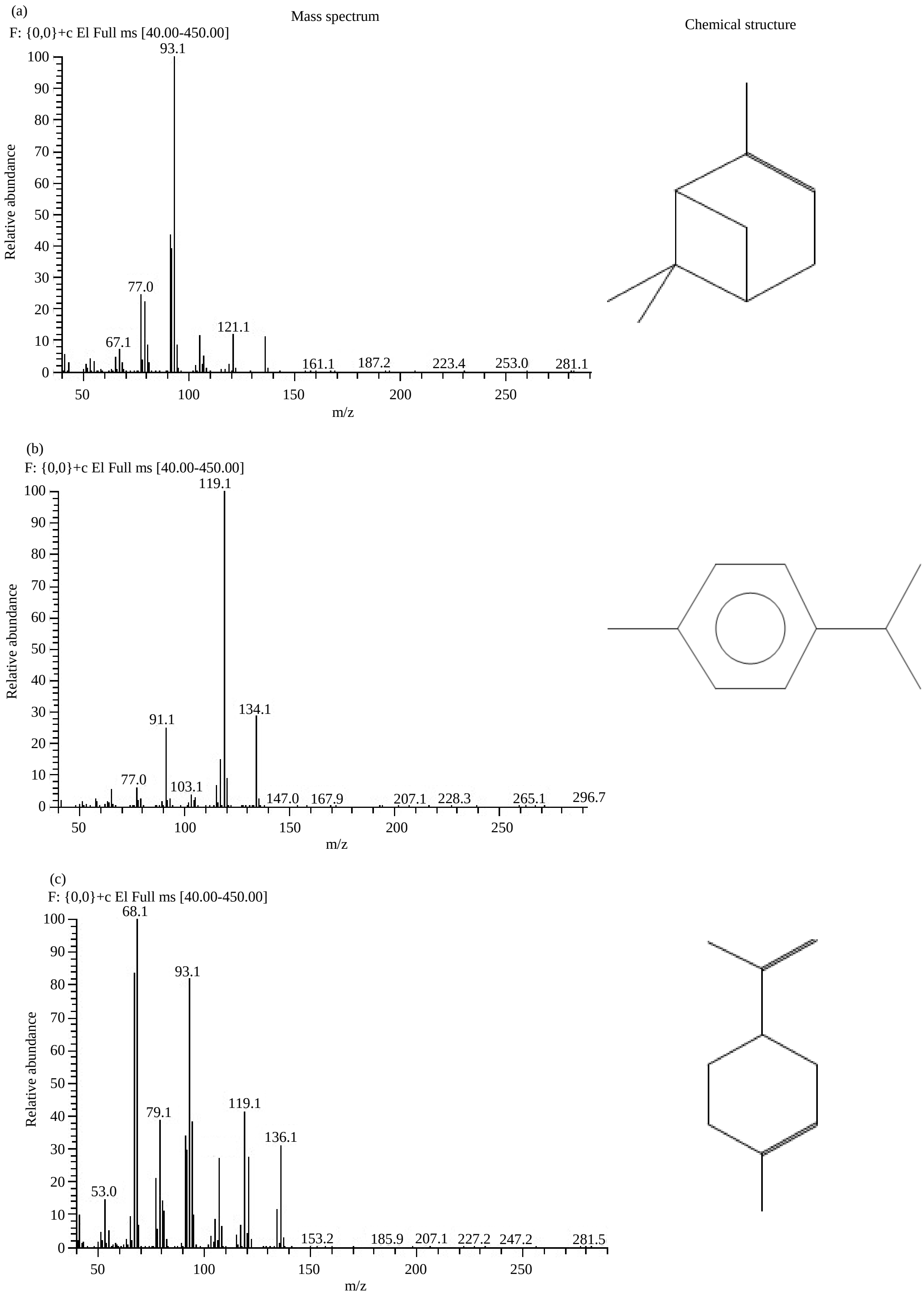

Fig. 2(a-e): Continue

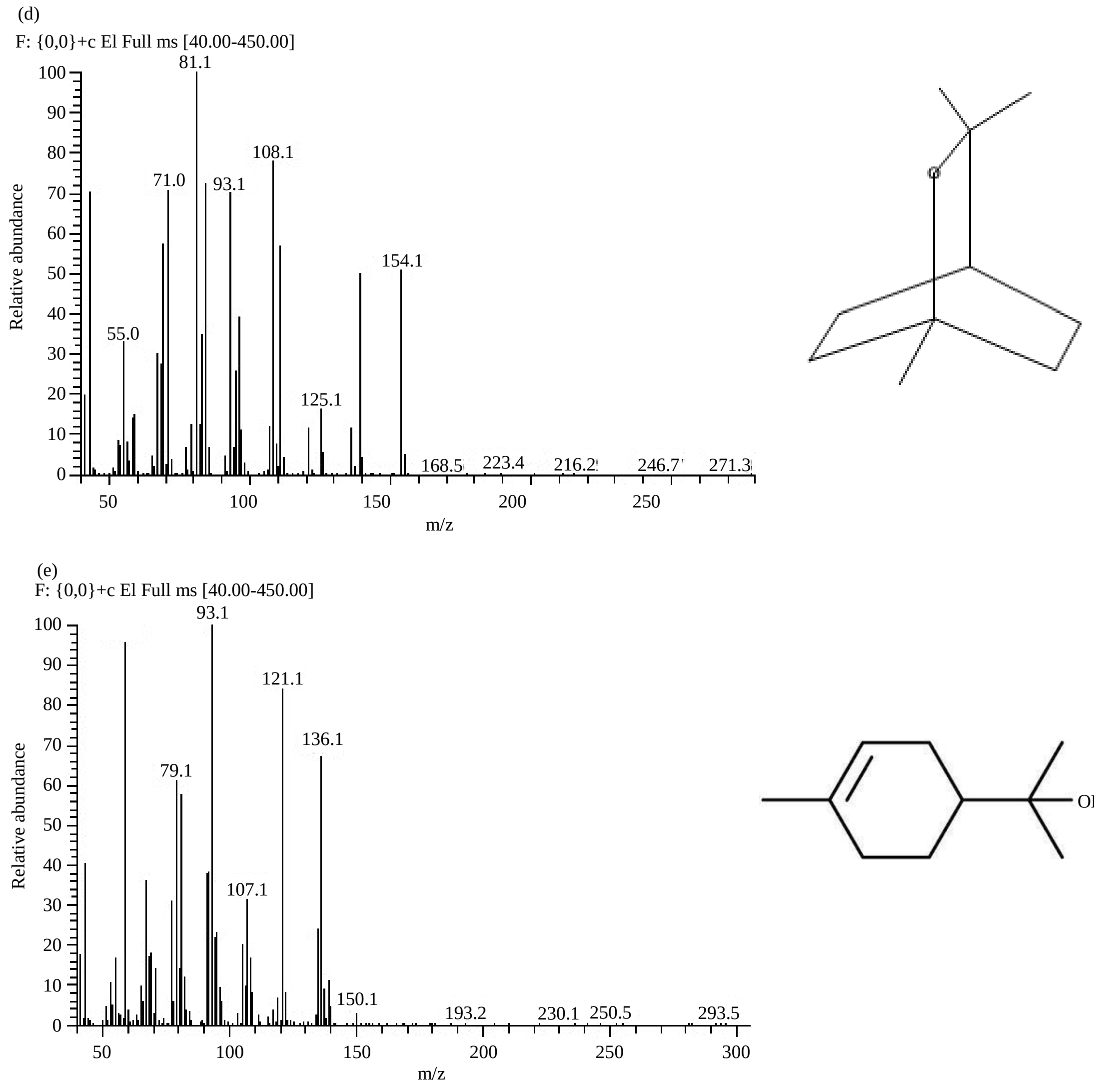

Fig. 2(a-e): Mass spectra and chemical structure of major chemicals compounds in camphor (*Eucalyptus globulus*) essential oil, (a) α-Pinene, (b) β-Cymene, (c) D-Limonene, (d) 1,8-Cineole and (e) α-Terpineol

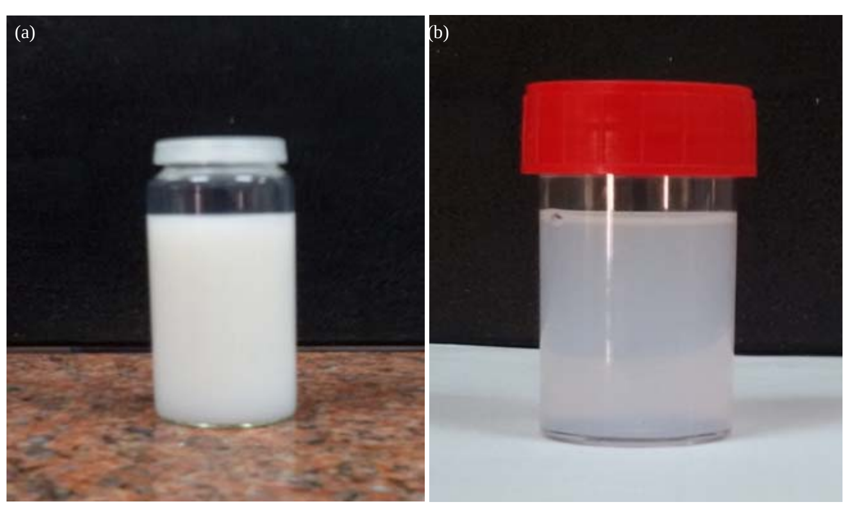

Fig. 3(a-b): Nanoemulsion (Oil-in-Water) of camphor essential oil (5%) obtained by (a) Ultrasonic and (b) After diluted with water

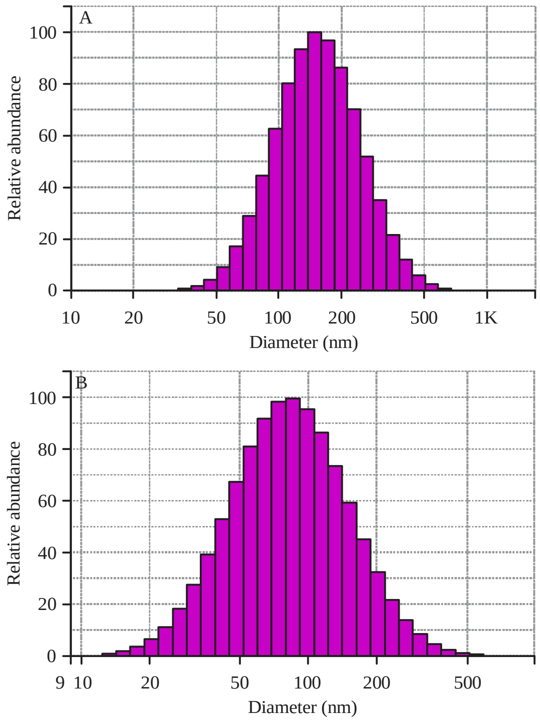

Fig. 4: Particle size distribution of camphor essential oil nanoemulsions, sample A Mean diameter = 171.7 nm and sample B Mean diameter = 99.0 nm

toxic effect against wheat weevil after, 24, 48 and 72 h of treatment (Fig. 6). The lethal concentration (LC_{50}) accounted 282.01 µg g^{-1} of EO and 181.49 µg g^{-1} of the nanoemulsion, respectively (Table 2). Nanoemulsion caused high mortality and toxicity was increased by 35.6% compared to the EO. The toxicity index (TI) accounted 0.644 based on LC_{50} after 72 h of exposure. However, the mortality in wheat weevil was increased with increasing exposure time and concentration of nanoemulsion or EO in a concentration-dependent manner. The mortality in wheat weevil account 85, 93.33, 100 and 100% after 21 days of exposure to nanoemulsion at concentration 150, 200, 250 and 300 µg g^{-1} and the reduction (%) in progeny (F1) account 51.5, 94, 98 and 100%, respectively (Fig. 7). In contrast, camphor EO showed the same effect with high concentrations ranged from 250-450 µg g^{-1}. The effect of nanoemulsion on germination of wheat grains was evaluated at different concentrations equaled to LC_{50}, LC_{90} and LC_{99} to study the adverse effect response. In the current study, both nanoemulsion and camphor EO did not show any effect on germination or seedling growth especially at the concentration of LC_{50} and LC_{90} (Fig. 8).

Nanoemulsion of camphor EO, showed high insecticidal activity against *Sitophilus granarius*. Therefore, this formulation was investigated for acute and sub-chronic

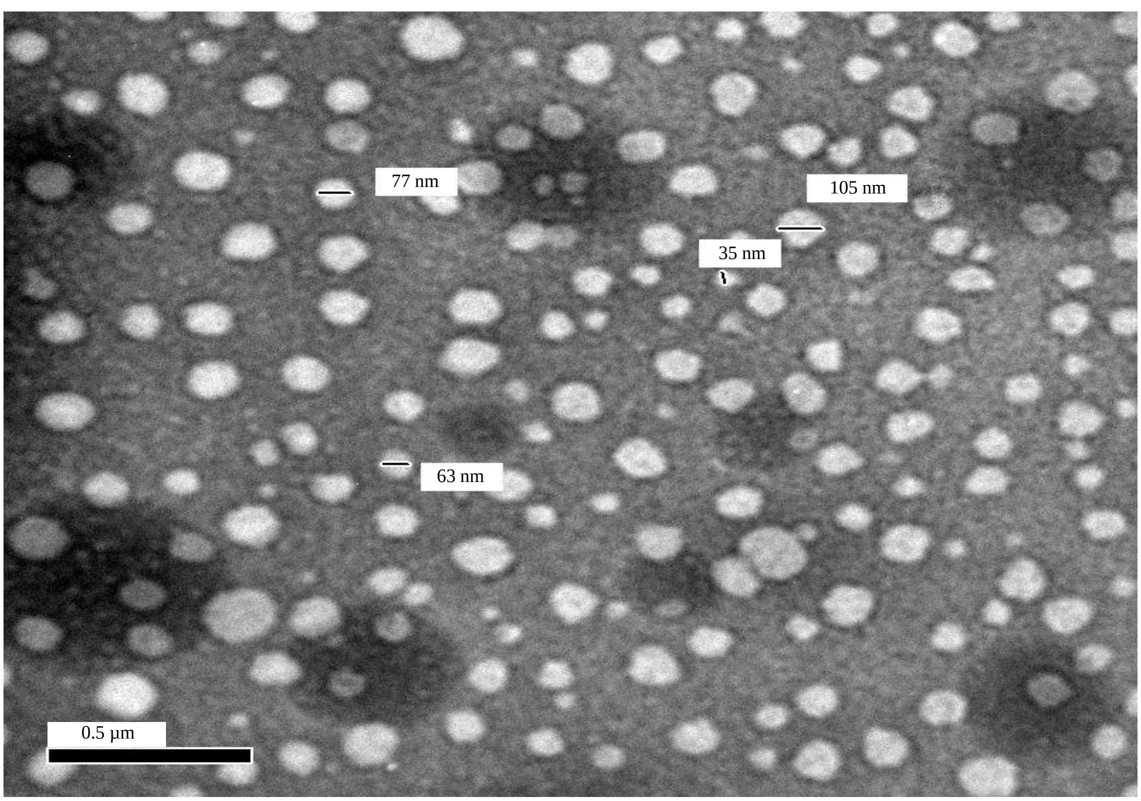

Fig. 5: Transmission electron microscopy (TEM) image of camphor oil nanoemulsion (sample B)

toxicity on male rats. In acute oral toxicity study, rats were received one single oral dose of nanoemulsion (5%) and camphor EO at the same dose. The selected dose is 0.5 g kg^{-1} b.wt., which corresponding to 30 g of camphor nanoemulsion/person/day and equivalent to 600 mL of nanoemulsion/person/day. There is no mortality or signs of toxicity in all treated rats during acute and sub chronic studies. Moreover, biochemical analysis of serum of male rats exposed to nanoemulsion showed insignificant changes in all liver dysfunction biomarkers such as AST, ALT, ALP, LDH, protein, albumin and globulin (Table 3, 4).

DISCUSSION

In the present study, GC/MS analysis of camphor (*Eucalyptus globulus*) EO showed 1,8-Cineole (87.78%), β-cymene (7.77%), D-limonene (2.29%), α-pinene (1.04%) and α-terpineol (0.10%) were the major chemical compounds. Many researcher analysis EO obtained from *Eucalyptus* genus by GC/M and identified the chemical compositions of EO. Maciel *et al.*[29] analysis the chemical composition of EOs obtained from three species plants of *Eucalyptus* genus in Brazil. They found that 1,8-Cineole (83.89%), (+) limonene (8.16%), o-cymene (2.93%) and α-pinene (4.15%) were the major constituents in *E. globulus* EO. The components of *E. globulus* leaf EO from Korea was analyzed by GC/MS. The major components in EO were1,8-Cineole (90.0%), (-)-α-pinene (2.2%), 1-α-terpineol (1.7%), 1-Isopropenyl-3-methylbenzene (1.5 %) , β-myrcene (0.6%), 2-β-pinene (0.6 %), (E)-pinocarveo (0.4%) and γ-terpinene (0.7%)[30]. Ghaffar *et al.*[31] also, evaluated chemical composition of seven *Eucalyptus* species from Pakistan. They found that 1,8-Cineole was the major constituent (56.5%) in *E. globulus* EO in addition to limonene (28.0%), α-pinene (4.2%), α-terpinol (4.0%) and globulol (2.4%). On the other hand, Iqbal *et al.*[32] reported that

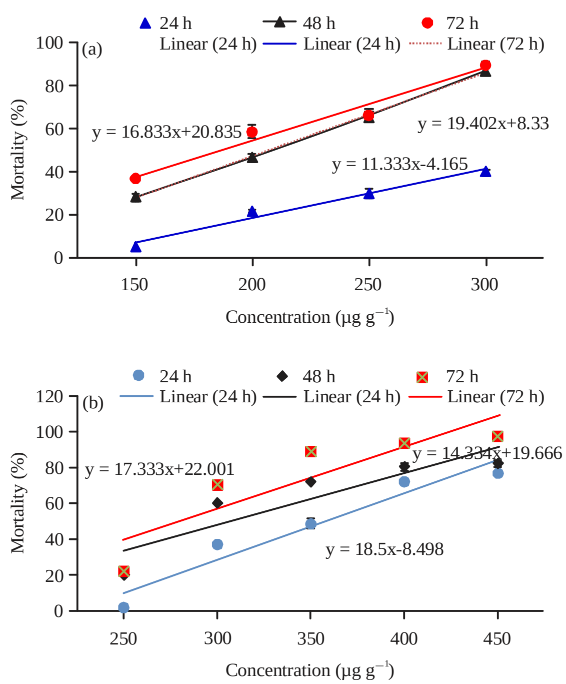

Fig. 6(a-b): Concentration-mortality response lines of (a) Nanoemulsion and (b) Camphor essential oil against wheat weevil (*Sitophilus granarius*) after 24, 48 and 72 h of exposure

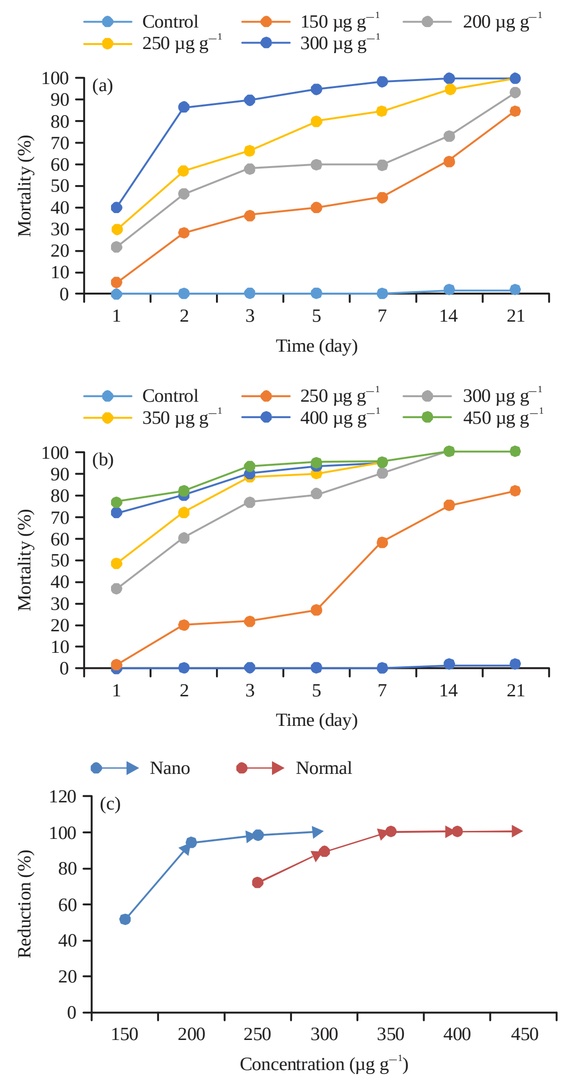

Fig. 7(a-c): Concentration-mortality response lines of (a) Nanoemulsion, (b) Camphor essential oil on wheat weevil (*Sitophilus granarius*) during 21 days of exposure and reduction (%) in (c) F1-progeny

Table 2: Toxic effect of nanoemulsion of camphor (*Eucalyptus globulus*) essential oil against wheat weevil (*Sitophilus granarius*) after 72 h of treatment

Treatments	LC_{50} (µg g^{-1})	LC_{90} (µg g^{-1})	Slope	LC_{50} (µg g^{-1}) Lower limit	LC_{50} (µg g^{-1}) Upper limit	LC_{90} (µg g^{-1}) Lower limit	LC_{90} (µg g^{-1}) Upper limit	Toxicity index (TI)	Toxicity increase (%)
EO	282.01	373.42	10.52	249.03	302.24	349.44	427.55	1	-
EO nanoemulsion	181.49	339.21	4.72	166.00	194.25	303.16	405.39	0.644	35.6

LC_{50}: Value represented the concentration of nanoemulsion or EO, which caused 50% mortality, TI: Toxicity index, TI = (LC_{50} of nanoemulsion or EO/LC_{50} of EO), high toxicity when TI less than 1, toxicity increase (%) = (TI of EO-TI of nanoemulsion)×100

Table 3: Effect of sub-chronic exposure to nanoemulsion of camphor (*Eucalyptus globulus*) essential oil on AST, ALT, ALP and LDH activities in sera of rats

Treatments	AST (U L^{-1})	ALT (U L^{-1})	ALP (U L^{-1})	LDH (U L^{-1})
Control	47.88 ± 2.67^a	34.33 ± 2.04^a	99.65 ± 4.08^a	198.28 ± 6.50^a
EO	48.78 ± 1.46^a	33.98 ± 1.32^a	102.77 ± 3.15^a	198.02 ± 7.63^a
EO nanoemulsion	49.89 ± 1.29^a	35.98 ± 2.45^a	104.44 ± 4.82^a	205.08 ± 8.45^a

Each value is a Mean of 5 rats±SE, values are shared the same superscripts letters not differ significantly at $p<0.05$

Table 4: Effect of sub-chronic exposure to nanoemulsion of camphor (*Eucalyptus globulus*) essential oil on concentrations of protein, albumin and globulin in sera of male rats

Treatments	Total protein (g dL^{-1})	Albumin (A) (g dL^{-1})	Globulin(G) (g dL^{-1})	A/G ratio
Control	6.54 ± 0.34^a	3.16 ± 0.09^a	3.38 ± 0.08^a	0.93
EO	6.65 ± 0.63^a	3.14 ± 0.06^a	3.51 ± 0.07^a	0.89
EO nanoemulsion	6.55 ± 0.75^a	3.25 ± 0.09^a	3.33 ± 0.06^a	0.98

Each value is a Mean of 5 rats±SE, values are shared the same superscripts letters not differ significantly at $p<0.05$

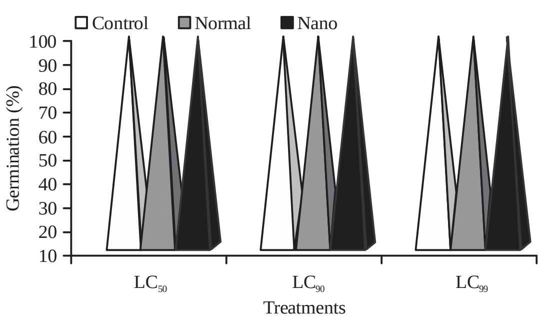

Fig. 8: Effect of nanoemulsion and camphor essential oil on germination (%) of wheat grains

1,8-Cineole (17.5%), α-pinene (1.7%) and α-phellandrene (1.1%) were the main components in *E. globulus* EO from Pakistan. The results in the current study are in almost agree with the results stated by earlier investigators. The differences in chemical components may be due to variations in environmental, climatic and geographical which effect on chemical composition of *E. globulus*.

Nanoemulsion of camphor EO was prepared in the current study by ultrasonic. Two nanoemulsions of camphor EO were stable, sample A (EO: Tween 20 at ratio 1:1.25) and sample B (EO: Tween 20 at ratio 1:1.5) at 40 min as sonication time. The mean droplets size diameter of these samples were 171.7 ± 0.478 nm of sample A and 99.0 ± 0.605 nm of sample B. This result showed that the ratio of EO to surfactant and time of sonication are an important factor in preparation nanoemulsion and stability. Nanoemulsion of sample B was stable and unchanged during stored (three months) with low droplet size diameter (99.0±0.605 nm). The TEM image confirmed the results that show the spherical shape and a good dispersion of droplets nanoemulsion of sample B. It has been reported that good nanoemulsion had droplets size between 20-200 nm[21]. The correlation between droplet diameters and stability also was reported by several studies[22-34]. Therefore, the stability of camphor EO nanoemulsion could be due to chemical and physical properties of nanodroplets[21]. Previous studies found that low droplets size of nanoemulsions obtained when hydrophile-lipophile balance (HLB) value of the surfactant couple coincides with required HLB value of the oil[35-37]. Moreover, the high correlation between droplets size, stability and attractive forces between droplets were reported[38].

The high insecticidal activity of nanoemulsion was recorded compared to camphor EO against wheat weevil, *Sitophilus granarius*. The LC_{50} accounted 282.01 µg g^{-1} of EO and 181.49 µg g^{-1} of the nanoemulsion against wheat weevil. This increase in insecticidal activity and toxicity of nanoemulsion could be due to low diameters of nanoemulsion droplets size (99.0 nm) which increase biological activity due to increased surface area of emulsion droplets. The insecticidal activity of camphor EO is due to the presence of 1,8-cineole, β-cymene, d-limonene, α-pinene and α-terpineol that found as major components by GC/MS analysis. Moreover, the minor compounds can play an important role in EO toxicity. Previous studies showed that insecticidal and biological activity of camphor EO could be due to the present of major constituents such as 1,8-Cineole, o-cymene, α-pinene, α-terpineol and (+) limonene[29,39]. It has been reported that *Eucalyptus globulus* and *Eucalyptus* spp.

have insecticidal activity against many insects such as *Sitophilus granarius* L.[40,41], *Sitophilus oryzae*[42], *Rhyzopertha dominica*, *Tribolium castaneum*[43,44], *Sitophilus zeamais*[45] and *Sitophilus zeamais*[46].

In the current study, both nanoemulsion and camphor EO did not show any effect on germination or seedling growth especially at the concentration of LC_{50} and LC_{90}.

The adverse effects of synthetic insecticides on human and their ecosystem are among the important problem worldwide. Therefore, several researchers are working to discover new compounds, especially from natural resources e.g., plant extracts and essential oils as green insecticides. Moreover, nanotechnology is playing an important role in preparing nanoformulations without using toxic solvents.

Results showed that no signs of toxicity or mortality in male rats exposed to nanoemulsion of camphor or the EO. Biochemical parameters also show insignificant changes in all liver biomarkers in serum of male rats. The liver is the mean organ in the body, play an important role in xenobiotic detoxification. It is the first target to toxic xenobiotic and their metabolites. Therefore, changes in liver function biomarkers are commonly used as biomarkers for liver toxicity and damage[47,48]. It has been reported that the increase in the activity of liver enzymes and change in concentrations of protein, albumin and globulin can be due to cell injury[49], hepatotoxicity and change in proteins biosynthesis. The results of the present study indicated that camphor nanoemulsion could consider non-toxic to mammals and can be used for control grains insects as green and nanoinsecticide. In future, further studies need to prepare other nanoformulations and evaluate at a long time.

CONCLUSION

Nanoemulsion of camphor EO with droplet diameter 99.0 nm was formulated by ultrasonic emulsification for 40 min and characterization. Small droplet size and stability of nanoemulsion was dependent on oil to surfactant ratio and sonication time. Nanoemulsion showed high insecticidal activity against wheat weevil, *Sitophilus granaries* with LC_{50} 181.49 $\mu g\ g^{-1}$. The activity of EO could be due to the presence of 1,8-Cineole, β-cymene, D-limonene, α-pinene and α-terpineol which found in camphor EO by GC-MS analysis. Camphor nanoemulsion did not show any effect on germination or seedling growth. Acute and sub-chronic toxicity studies showed no signs of toxicity or biochemical alterations in liver biomarkers of male rats. These findings showed that camphor nanoemulsion could be developed as a green and nanoinsecticide product.

SIGNIFICANCE STATEMENT

The current study was carried out to prepare the new nanoformulation of camphor essential oil by ultrasonic as stable, active and green insecticide to control wheat weevil, *Sitophilus granarius*. Camphor EO nanoemulsion shows high insecticidal activity with no toxic effects on germination or seedling growth of wheat grains. Nanoemulsion of camphor EO also, show no toxic or adverse effects on experimental animals. This study will help the researcher to prepare new formulations of natural product as green pesticides to control insect pests in green pest management.

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