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Evaluation of Acaricidal Activity of *Cinnamomum camphora* (F. Lauraceae) Essential Oil Nanoemulsion Against Cattle Tick *Rhipicephalus microplus*

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Abstract The present study aims at preparing nanoemulsions of plant essential oil *Cinnamomum camphora* as acaricidal agent against cattle tick *Rhipicephalus microplus* larvae. In the initial step, the phytochemical screening of oil was done through gas chromatography and mass spectrometry depicting major compounds as α-pinene (53.99%) and camphor (20.89%) in *C. camphora* oil. Different ratios of *Cinnamomum camphora* oil and surfactant Tween 80 (oil:surfactant-1:0.25, 1:0.5, 1:1, 1:1.5, 1:2 and 1:2.5) were used for preparing stable nanoemulsion through ultrasonication approach. Thermodynamic stability analysis exhibited that CA3 ratio (1:1) to be the most stable which was further characterized using Dynamic light scattering study through Nano zeta sizer indicating droplet size of 101.60 nm, polydispersity index as 0.50 and zeta potential value measured as -8.59 mV. Spectral analysis through Fourier Transform Infrared spectrometry for functional group interaction and Transmission electron microscopy for morphology of nanoemulsion droplets was also done for CA3 nanoemulsion. Acaricidal activity of stable nanoemulsion was evaluated against *R. microplus* larvae using larval packet assay and repellency rod test with concentration ranging from 500 ppm to 20000 ppm and 500 ppm to 25000 ppm respectively. Highest concentration (20000 ppm) showed 77% mean mortality with LC_{50} (3364.46 ppm) after 24 hour. CA3 showed promising repellent activity that ranged from 27% to 87% at various concentrations at different time intervals. These findings proved that *Cinnamomum camphor* essential oil nanoemulsion could be developed and used against *R. microplus* larvae as a safer, greener and nanoacaricide product.

Keywords C*innamomum camphora* Essential Oil, Nanoemulsion, Ultrasonication, Larvae, *Rhipicephalus microplus*

1. Introduction

Cattle ticks, or *Rhipicephalus microplus*, are major concerns leading to clinical condition known as Bovine Parasitic Sadness by hematophagism in addition to causing weight loss, anaemia, reduction in milk and meat production, and inoculating hosts with toxins and protozoa like *Babesia bovis*, *B. bigemina*, and rickettsia *Anaplasma marginale* through saliva, [1,2]. Additionally, the expansion of livestock in a region is hampered by cattle ticks because maintaining the health of cattle herds increases the expense of acaricides, labour, equipment, and facilities [3].

Tick control methods primarily include the use of chemical acaricides, which is the most common approach for tick management. Chemical acaricides have

detrimental consequences, including the selection of resistant tick populations and adverse effects on animals, living beings, and the environment [4]. Because of the numerous major drawbacks of chemical acaricides, embracing alternative solutions could very well help to mitigate them.

Extensive research has been conducted on plant-based natural products with acaricidal activities against many species of ticks [5,6] including acaricide resistant species. As natural products contain a variety of active molecules with different modes of action, they can delay the emergence of resistance [7]. Recently, several promising efforts have been made to use plant essential oils as herbal treatments, and green-fabricated nanoemulsions have been developed using a nanotechnology approach. They offer numerous therapeutic advantages in a variety of areas, such as routes of administration, site specificity, and increased therapeutic effect, making them appealing to researchers. Essential oils are complex mixtures of volatile phytoconstituents rich in terpenes, and oxygenated compounds obtained through hydro-distillation of aerial or subaerial plant parts such as flowers, seeds, leaves, roots. They have been extensively screened and established for diverse bioactivities viz. insecticidal, anticancer, antioxidant, antimicrobial etc. [8]. Besides, they have wide applications in clinical, medical, pharma industry, perfumery, food preservation and flavor addition [9].

Cinnamomum camphora, a member of the Lauraceae family, is a representative of East Asian tropical and subtropical evergreen broad leafed vegetation with exceptional economic, ornamental, and ecological value. *C. camphora* essential oil has anti-oxidant, anti-inflammatory, anti-microbial, and insecticidal attributes [10]. The main objective of this study was to formulate *C. camphora* essential oil based nanoemulsion by high energy approach (ultrasonication). Stability and characterization studies of the formulated CA3 nanoemulsion were also done. Further, the developed CA3 nanoemulsion was evaluated for its acaricidal and repellent activity against *Rhipicephalus microplus* larvae.

2. Materials and Methods

C. camphora (white) essential oil extracted from bark wood, provided by Allin exports, India. Tween 80 was purchased from Sigma Aldrich, India. All experiments were done in deionised and Milli-Q (Millipore Corporation) water.

2.1. GC-MS Analysis

GC-MS was used to examine the constituents of *C. camphora* essential oil (Shimazdu GCMS-QP2010 Plus). Helium gas was used as the carrier gas, with a flow rate of 1 ml/min. A split ratio of 1:100 was used to inject the samples. The injector temperature was 260ºC, while the detector temperature was 275ºC. Mass spectra were collected over a 40-650 amu range with ionisation energy of 70 eV and an ion source temperature of 220ºC. The oil compounds were detected by matching the obtained mass spectra data with the NIST MS search version on the Wiley library.

2.2. Preparation of *C. camphora* Nanoemulsion

C. camphora essential oil, non-ionic surfactant Tween 80 (HLB-15), and water were used to develop the nanoemulsion. All emulsion preparations used the same concentration of *C. camphora* essential oil (10%). The coarse emulsion was made by combining oil and surfactant in the aforementioned v/v ratios: 1:0.25 (CA1), 1:0.5(CA2), 1:1(CA3), 1:1.5(CA4), 1:2(CA5), and 1:2.5 (CA6), followed by addition of water on magnetic stirrer. After that, the coarse emulsion was ultrasonically emulsified with a 20 kHz Sonicator (Sonics & Materials, Inc., USA) with a maximum power output of 750 W. Sonicator probe was dipped symmetrically into coarse emulsion and sonication was performed at 10-3 pulse emulsification time. The formulated nanoemulsion was then characterized, and the emulsion's stability was investigated. The pH of nanoemulsions was also measured. The entire characterization procedure was carried out at room temperature.

2.3. Dynamic Light Scattering Study

The Nano Zetasizer (Malvern) was used to determine the droplet size, polydispersity index (PDI), and zeta potential of a nanoemulsion. The size of droplets was determined using the dynamic light scattering technique. The intensity of scattered light varies as the nanosized droplets undergo Brownian motion in the emulsion formulation. The dynamic light scattering technique measures the intensity fluctuation in scattered light. Prior to the experiment, all formulations were diluted with milli-Q (Millipore corporation) double distilled water to eliminate the effect of viscosity caused by the ingredients and to reduce the multiple scattering effects.

2.4. Transmission Electron Microscopy

Transmission electron microscopy was used to examine the morphology and structure of the CA3 nanoemulsion. To conduct TEM experiments, a drop of nanoemulsion (negatively stained with 1% phosphotungstic acid) was placed on a copper grid and allowed to dry. A transmission electron microscope was used to capture TEM micrographs (Tecnai G2-20 FEI Netherland).

2.5. FTIR Analysis

FT-IR spectroscopic analysis was performed. For

FT-IR analysis, the Perkin Elmer spectrum1 FT-IR instrument from the United States was used. It makes use of scanning, which was done in the 4000-500 cm^{-1} range.

2.6. Acaricidal Activity

Female engorged *R. microplus* ticks were collected from naturally infested cattle on unorganized local farms in the Agra region. To avoid any negative interference, these animals did not obtain any acaricidal treatment in the 50 days that followed the start of the study. The engorged female ticks were placed in plastic bottles and immediately transported to the laboratory, where the larval bioassays were conducted with minor modifications to the Larval Packet assay (LPT).The FAO-recommended LPT protocol was used to evaluate the acaricidal activity of CA3 nanoemulsion against *R. microplus* larvae. In this study, tick larvae aged 7-14 days were used. Hatching vials with the highest larval eclosion rate (90-100 percent) was preferred and placed in the center of a porcelain tray, which was then filled with water and soap, preventing their escape. With the help of paintbrush, 100 larvae were gently relocated to clean Whatman No.1 filter paper packets impregnated with test CA3 nanoemulsion (500 ppm to 20000 ppm) and control.

Percentage mean mortality of larvae were calculated as % Mean mortality= dead larvae/total larvae*100

Repellency activity was performed through simulated glass rod assay [11] with slight modification in which a 12" glass rod was fixed in vertical position in a stand. Filter paper was wrapped over the top half of the rod which was half impregnated with the 100µl of the test concentration and half was not (non- impregnated). Glass rod with impregnated and non-impregnated papers was air dried for 30s before conducting the repellency bioassay. After impregnation, approx. 30 tick larvae were placed on the glass rod and observations were made after 5 min, 1 h and after 3 h. Larvae were counted after each interval of time. Tests were performed at room temperature. Repellency was calculated against larvae. Nanoemulsion was tested at the concentration range of 500 ppm to 25000 ppm along with control.

2.7. Statistical Analysis

Lethal concentrations (LC) to kill 50% of larvae and their respective 95% confidence intervals (CI) were calculated by Probit analysis with the help of software. The analysis was done in triplicates.

3. Results and Discussion

Cinnamomum camphora (white) essential oil was analyzed *via* gas chromatography- mass spectrometry. Eighteen monoterpene compounds (Figure 1 and Table 1) were identified in 100% relative area. The major compound found in our study was α-pinene (53.99%), followed by camphor (20.89%), Isobornyl acetate (15.76%) and 1,8 cineole (6.17%). Study of Wang et al. [12] reported the presence of camphor (14.29% and 17.58%) in methanol and chloroform extract of *C. camphora* xylem which was lower than the percentage of *C. camphora* essential oil. The chromatogram obtained by the various researchers exhibited several similar monoterpenes such as ß-pinene, camphene, α-phellandren and terpinene, however, major constituents differed which was noted as D-camphor by several authors [13,14] whereas, few reports also mentioned 1,8-cineole or linalool or eugenol as the main compounds [15,16]. Wanyang et al. [17] investigated these differences in detail.

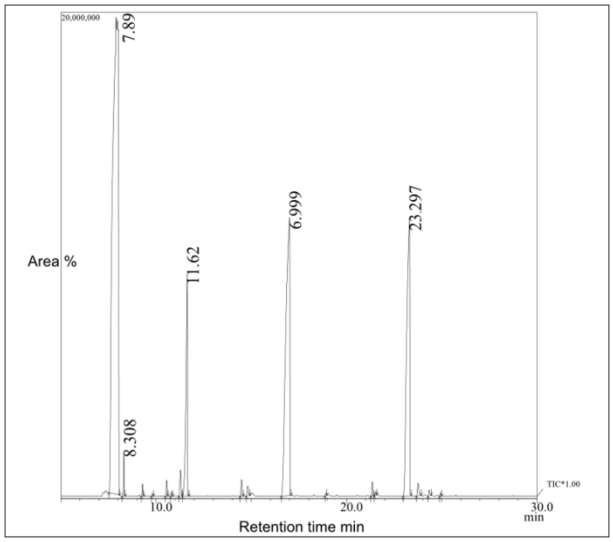

Figure 1. GC-MS Chromatogram of *Cinnamomum camphora* essential oil

Table 1. Phytocompounds in *Cinnamomum camphora* essential oil

Retention time (min)	Relative area (%)	Phytocompounds
7.89	53.99	α-pinene
11.62	6.17	1,8 cineole
16.99	20.89	Camphor
23.29	15.76	Isobornyl acetate

Out of six oil: surfactant ratios, the CA3 nanoemulsion, (ratio 1:1) performed best in terms of stability as observed till 90 days at 4ºC in thermodynamic stability analysis, whereas other ratio showed creaming and phase separation. Table 2 displays the droplet size, polydispersity, zeta potential and pH of the nanoemulsions after 10-3 minutes of ultrasonic emulsification. The droplet diameter (101.60 nm) was observed in a CA3 nanoemulsion with a 1:1 (v/v) ratio of oil (10% of total emulsion volume), whereas PDI was found to be 0.50 with surfactant (10% of total emulsion volume). Tween 80 was used as a surfactant because of its

high hydrophilic-lipophilic balance (HLB-15) and adaptability for oil-in-water emulsions. Tween 80, as a small molecule surfactant, is also far more effective than polymers in lowering droplet diameter due to its rapid adsorption onto the droplet surface [18]. There was a decrease in the mean droplet diameter of the nanoemulsion with increasing surfactant concentration. Droplet diameter and PDI 101.60 nm and 0.50 were measured in nanoemulsions with oil and surfactant ratios of 1:1 (v/v) CA3 nanoemulsion. These findings support previous findings that minimum droplet size could be obtained at lower oil surfactant ratios [19]. Surfactants lowers the free energy needed for preparing nanoemulsions at the oil/water interface [20]. The oil concentration in the formulation was 10% of total emulsion volume and water served as the continuous phase. There was a negligible decline in droplet size as surfactant concentration was increased further. As a result, the formulation was optimized at a 1:1 oil-surfactant ratio. Additional characterization and application studies were conducted using a 1:1 CA3 nanoemulsion. The droplet size distribution, polydispersity and zeta potential of the CA3 1:1 (v/v) ratio formulation are shown in Figure 2. Zeta value of the 1:1 nanoemulsion formulation was found to be -8.59 mV at an emulsion native pH of 4.5. Non-ionic surfactant was used to stabilize droplets in the CA3 nanoemulsion. As a result, the final value of droplet charge is extremely small. However, Ghosh et al. [21] found mean droplet size 254 nm size, 0.254 PDI and pH value 4.46 for (CF1) 1:1 ratio of nanoemulsion of (*C. zeylanicum* oil: Tween 80) after ultrasonic emulsification for 30 min. These results were contradictory to our results which may be due to the reasons like method used for nanoemulsion preparation, plant species and sonication time as well.

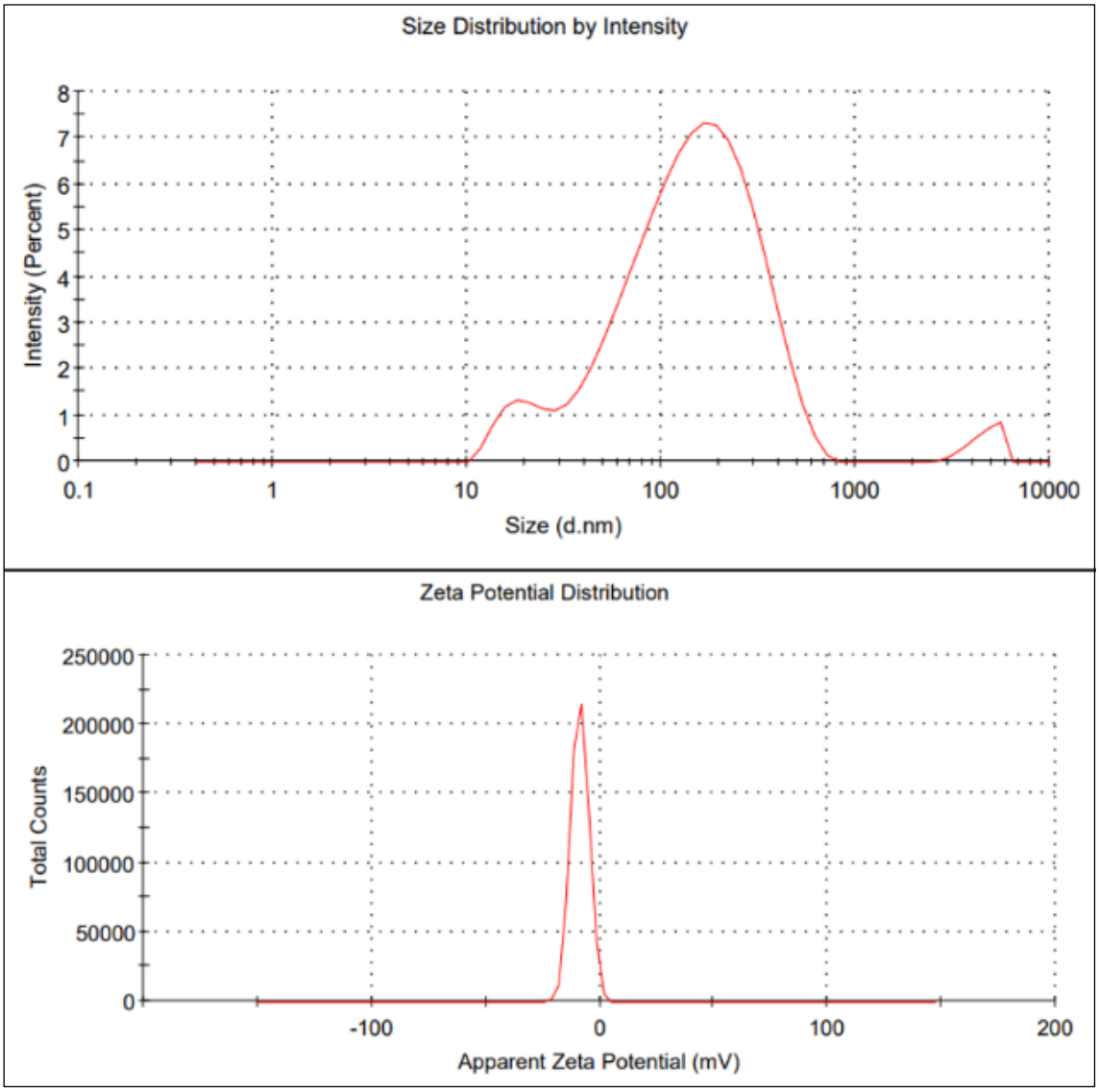

Figure 2. DLS graph showing droplet size distribution, polydispersity index and zeta potential of *Cinnamomum camphora* essential oil nanoemulsion (CA3, Ratio 1:1)

Table 2. Droplet size distribution, polydispersity index, zeta potential and pH of *Cinnamomum camphora* essential oil nanoemulsion (Ratio CA3 1:1)

Nanoemulsion code	Droplet size	PDI	Zeta potential	pH
CA3 nanoemulsion	101.60 nm	0.50	-8.59 mV	4.5

Transmission electron microscopy was used to examine the morphology of a nanoemulsion (TEM). Figure 3 depicts a TEM micrograph of CA3 nanoemulsion (1:1 ratio) where droplets were homogeneously dispersed with spherical in shape and between 20- 50 nanometric range in size. The droplet size data provided through TEM analysis correlates with the droplet size range attained by Nanozetasizer. This study finds support from the previously documented reports where Oil -in-water nanoemulsion droplets exhibited spherical morphology [21-23].

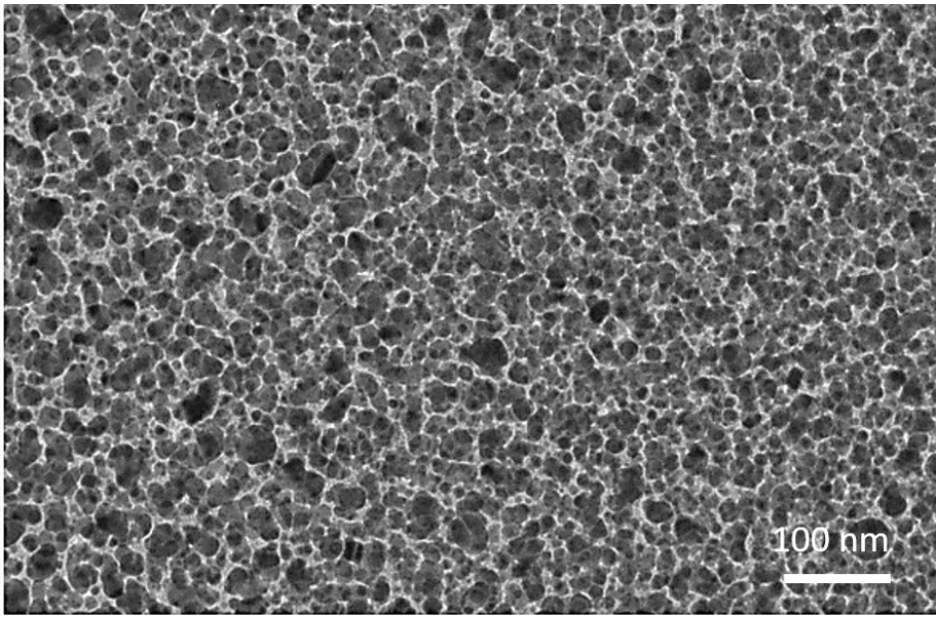

Figure 3. TEM micrograph of *Cinnamomum camphora* essential oil nanoemulsion (CA3, Ratio 1:1)

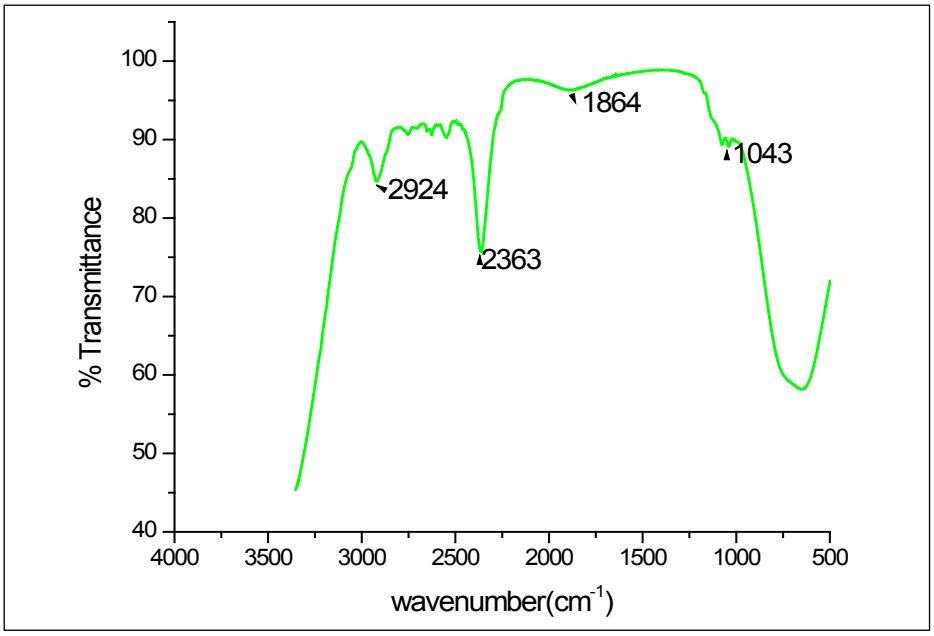

Figure 4. FTIR of *Cinnamomum camphora* essential oil nanoemulsion (CA3, Ratio 1:1)

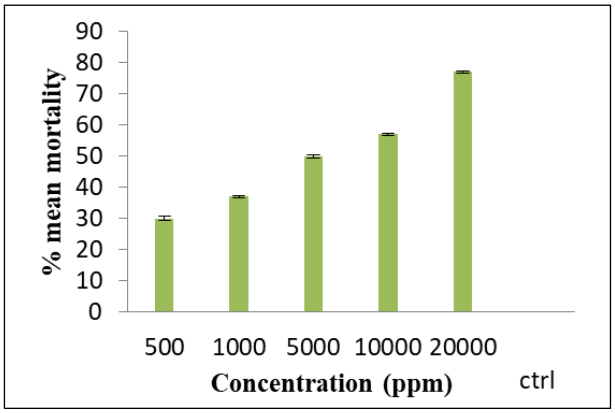

Figure 5. Percent mean mortality of *R. microplus* larvae exposure to *Cinnamomum camphora* essential oil nanoemulsion (CA3) at various concentrations ($p>0.05$)

In FTIR analysis of CA3 nanoemulsion, there was no distinguishable peak in the spectrum. Despite the fact that a smooth and widened peak at 2363 cm-1 was observed, indicating the hydrophilic interaction (Figure 4). Other peaks were found at 2924 cm-1 (alkane C-H stretch), 2363 cm-1 (strong carbon dioxide O=C=O stretching), 1864 cm-1 (aromatic C-H bending) and 1043 cm-1 (ester C-O stretch). FTIR study further validates the outcome of the GC-MS analysis. In contrast to our study, thymol nanoemulsion also showed similar kind of FTIR graph with shifts in wavelength [24]. This difference in peak spectra could be due to the difference in oil and the percentage of phytocomponents present in the oil used in the studies.

Acaricidal activity of CA3 nanoemulsion was tested against *R. microplus* larvae. Results depicted that this nanoemulsion has acaricidal potential against 7- 14 day old larvae after 24 hour treatment in larval packet assay. CA3 nanoemulsion caused 77% mortality at the highest concentration shown in Figure 5. Larval mortality was observed to be concentration dependent manner. All the concentrations were simultaneously prepared for acaricidal assay and were stable. Nanoemulsion stability was increased by energy used during the ultrasonication process. Sonication time and surfactant concentration largely played an important role in establishing the stability of nanoemulsions. Higher amount of surfactant had a direct relation to emulsion's stability [21].

Table 3 showed LC_{50} value of CA3 nanoemulsion was calculated to be 3364.46 ppm. Present results are in substantial agreement with the findings of Monteiro et al. (2017) [25]. Further, present study is comparable to a previously published report by Diaz et al. (2019) [26] where *Cinnamomum zeylanicum* oil nanoemulsion in mixture (mixture of essential oils from allspice, cinnamon, and cumin) showed highly toxic (100%) effect against 10-day old *R. microplus* larvae. High toxicity may be due to the age of larvae and the technique employed during nanoemulsion preparation in the study. This could be because of the additive effect of three different oils [27]. Difference in the *Cinnamomum* species could be the reason having different major compounds found in phytochemical analysis. This contradiction in study may be caused due to the presence of major compounds and their interaction among the essential oil of *Cinnamomum camphora.*

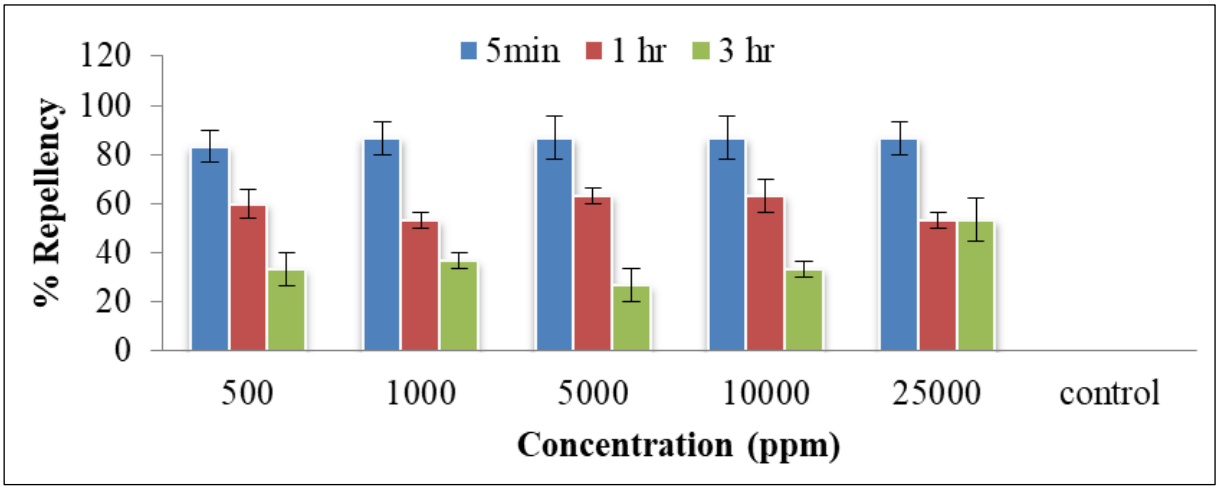

Figure 6. % Repellency of CA3 nanoemulsion at different concentration at different interval of time (5 min, 1 hr and 3 hr)

Table 3. Acaricidal activity of *Cinnamomum camphora* essential oil nanoemulsion (CA3) against *R. microplus* larvae

Regression equation	LC_{50} (LFL-UFL) (ppm)	R^2	X^2
Y=0.69x+2.56	3364.46 (1027.836-11013.078)	0.915	0.879

Repellent study was done on freshly cultured larvae hatched in the laboratory under simulated conditions. Percentage repellency was tested at the concentration ranging from 500 ppm to 25000 ppm respectively as shown in Figure 6. At the lowest concentration, the repellency percentage was higher at the first time interval (just after 5 min placing the larvae) as the exposure time increased from 5 min to 1 h the repellency percentage recorded as to be 60%. After 3h, repellency was decreased to less than 33%. At the highest concentration (25000 ppm), the repellency percentage was 86% at 5 min. and there was similar reduction observed after 1 h and 3hr in the percentage repellency was 53%. Therefore, as the concentration increases, repellency % also increases.

Whereas, inverse relation was observed with the exposure time from 5 min to 3 h wherein, repellency percentage decreased gradually till 5000 ppm however, with further increase in concentrations range to 25000 ppm, retention time was increased leading to better protection. Extensive literature is available on repellent activity against agricultural pests, mosquitoes, mites and lice. However, only trace studies are conducted on *R. microplus* using essential oil nanoemulsion employing high energy approach.

4. Conclusions

In the present study, green approach was employed to develop herbal nanoemulsion with promising acaricidal potential. CA3 (oil:surfactactant -1:1 ratio) nanoemulsion exhibited good mortality against *R. microplus* larvae with LC_{50} value 3364.46 ppm. The bioefficacy of CA3 nanoemulsion could be due to the presence of phytopotentials that found in *Cinnamomum camphora* essential oil in GC-MS analysis. Acaricidal activity of the CA3 nanoemulsion was exhibited due to the presence of major compound; α-pinene and camphor of the C. *camphora* (bark) essential oil by GC-MS analysis. These findings proved that *C. camphora* essential oil nanoemulsion could be used against *R. microplus* larvae as a safer, greener and nanoacaricide product.

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