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Formulation of anti-larval nanoemulsion: Impact of droplet size on larvicidal activity against malaria vectors in Chhattisgarh, India

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Mentha piperita (peppermint) essential oil nanoemulsion was prepared by low energy spontaneous emulsification method. GC-MS analysis revealed the composition of peppermint essential oil and menthol (45.2%) was the major bioactive compound along with menthone (15.39%), neomethol (8.1%), menthyl acetate (7.7%) and isomenthone (7.4%). Optimization of the nanoemulsion preparation process was done by Response Surface Methodology (RSM) with Central Composite Design (CCD). The droplet diameter and polydispersity index at optimized conditions (15% oil concentration, 25% surfactant concentration and Tween80 as surfactant) were 39.2 nm and 0.22 respectively. Optimized peppermint oil nanoemulsion (OPNE) was optically transparent, spherical in morphology and was stable for 6 months. OPNE formulation demonstrated dose, time and size-dependent larvicidal activity against malaria vectors with LC_{50} value of 48 ppm and 123 ppm against *Anopheles culicifacies* and *Anopheles stephensi* respectively. The LC_{50} values were 90 ppm and 163 ppm against *Anopheles culicifacies* and *Anopheles stephensi* correspondingly for the bigger droplet size formulation (PNE, droplet diameter: 129.6 nm) confirming the droplet size-dependent larvicidal activity of the nanoemulsion. The results of this study propose that peppermint oil-based nanoemulsion possibly be used as an eco-friendly larvicide for mosquito vector control strategies.

Keywords: Anopheles mosquitoes, Larvicidal activity, Nanoemulsion, Plant essential oil, Response Surface Methodology

Synthetic insecticides are the most important parts of the mosquito vector control program *i.e*., growth regulators for insects (*e.g.*, diflubenzuron, methoprene, *etc*.) and organophosphates (*e.g.*, temephos, fenthion, *etc*.). Repeated indiscriminate use of the synthetic pesticides has been reported to have a harmful effect on fish and other non-target organisms, and also they cause the increase in insecticide resistance of arthropods[1]. There are reports on malaria vector resistance to DDT (Organochlorine), Malathion (Organophosphate) and Deltamethrin (Pyrethroids) in Chhattisgarh state[2]. Synthetic insecticides also have caused environmental problems such as air, soil and groundwater pollution including toxicity to the aquatic ecosystem. Hence, scientists are looking for the development of more efficient and eco-friendly alternatives to conventional pesticides, which are safe to health of human and further to the environment and the non-target organisms.

Mosquitoes transmit diseases *i.e* Barmah Forest fever, chikungunya, dengue, dirofilariasis, Eastern equine encephalitis, filariasis, Japanese encephalitis, La Crosse encephalitis, malaria, Ross River fever, Saint Louis encephalitis, tularemia, Venezuelan equine encephalitis, West Nile virus, Western equine encephalitis, yellow fever and Zika fever[3,4]. They also cause a nuisance by biting which can lead to allergic reactions to humans. Hence, mosquito vector control is an essential requirement in control annoyance created by and the epidemic diseases spread by mosquitoes. Malaria is foremost public health concern of the Country and also globally. It is one of the life-threatening diseases and the causative organism is plasmodium parasites. Biting of the infected female anophelines transmits these parasites to human hosts. An estimated 219 million cases of malaria was spread over 90 various countries and resulted in death of 435,000 people in 2017. Although African countries share an unaccountably high score of the global burden of malaria, India shared 4% of the disease burden[5]. There are around one million malaria cases reported in India annually caused by *P. vivax* and *P. falciparum* with around 50% proportion of each[6]. Out of 58 anophelines found in India, Malaria is transmitted by 6 primary vectors and 3 secondary

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vectors. Of this *An. dirus* in the north-east jungles, *An. fluviatilis* is found in the foothills and plains, *An. minimus* in the foothill streams of the north-eastern states, *An. sundaicus* in the islands of Andaman and Nicobar, *An. culicifacies* in the rural areas and *An. stephensi* in the urban areas[7,8].

Plant essential oils constitute secondary metabolites extracted extensively from several plants. They contain bioactive compounds such as eugenol, linalool, menthol, cineol, estragole, caryophyllene, pinene, linalyl acetate, *etc*. which imparts antibacterial, antifungal, antioxidant, insecticidal and insect-repellent properties[9-15]. Due to hydrophobic nature of essential oils there is a need for formulation development before applying them for vector control program. Applications of plant essential oil nanoemulsions are novel and attractive strategies for controlling mosquito vector owing to their very low droplet size and large ratio of surface area to volume.

Nanoemulsions are the submicron size (droplet size ranges between 10 – 100 nm) dispersions of oil and water, which are immiscible liquids[16]. These sub-micron droplet size results in high physical stability, low turbidity and high ratio of surface area to volume as compared to conventional emulsions, hence making them an attractive system for vector control[17].

The objective of this study was to formulate *Mentha piperita* (peppermint) essential oil nanoemulsion with low droplet size and greater stability by optimizing variables using RSM, and to evaluate larvicidal activity against malaria vectors *An. stephensi* (urban vector) and *An. culicifacies* (rural vector) in Chhattisgarh, India.

Materials and Methods

Materials

Peppermint oil, Tween80 and Tween20 were obtained from HiMedia, India. Double distilled water (Millipore, USA) was used throughout the experiments.

Mosquito larvae

The larvae of malaria vectors *An. stephensi* and *An. culicifacies* were obtained from ICMR-National Institute of Malaria Research (NIMR) Field Unit, Raipur, Chhattisgarh. These larvae were maintained at 25-28°C, 90 ± 2% relative humidity with photoperiod of 12 h: 12 h of light and dark respectively.

Essential oil analysis by GC-MS

GC–MS (Perkin Elmer SQ8 C MS with Clarus 680 GC) was used to analyze constituents of peppermint essential oil. Elite Wax capillary column (30 M × 0.32 mM × 0.25 uM) was used with oven programming of 40 to 120 at the rate 3°C/min with 9 min hold and 120 to 140 at rate 2°C /min then 140 to 250 with of 5°C/min and final for hold 2 min. Helium was used as carrier gas with a flow rate of 1.5 mL/min and a sample injection split ratio of 1:60 and injector temperature of 240°C. Mass spectra were documented in the range of 40–450 amu with ionization energy of 70 eV, scan time of 0.8 sec and inter-scan delay of 0.01 sec. Ion source temperature 220°C and inlet line temp was 220°C.

Preparation of Nanoemulsion

Nanoemulsion was formulated using peppermint essential oil, Tween20 (HLB: 16.7) or Tween80 (HLB: 15) (non-ionic surfactants), and water by low-energy spontaneous emulsification method. First, by mixing the plant essential oil and surfactant, organic phase was prepared. Then, drop-wise addition of this organic phase to water was done, which was mounted onto magnetic stirrer with 500 rpm. All the formulated nanoemulsions were further characterized and stability was studied.

Experimental Design

RSM was used to explore the influence of oil concentration (X_1) and surfactant concentration (X_2) (numerical variables), and categorical factor such as surfactant type (X_3) on response variables such as nanoemulsion droplet diameter (Y_1) and the polydispersity index (PDI) (Y_2) of peppermint oil based nanoemulsions. This experiment was designed using CCD where each numeric factor (X_1 and X_2) was set to five different levels; 2 axial points ($-\alpha$ and $+\alpha$), 2 factorial points (+1 and -1) and the center point. For categoric factor X_3, the CCD was duplicated for each combination (two in this case, Tween20 and Tween80) of the categorical factor levels. Experiments with eighteen combinations were performed according to CCD (Table 1).

The predicted responses (nanoemulsion droplet diameter and polydispersity index) were indicated using a second order polynomial equation as function of oil concentration, surfactant concentration and surfactant type (independent variables) as follows (Eq. 1):

$$Z= \beta_0 + \beta_1 Y_1 + \beta_2 Y_2 + \beta_3 Y_3 + \beta_{11} Y_1^2 + \beta_{22} Y_2^2 + \beta_{33} Y_3^2 + \beta_{12} Y_1 Y_2 + \beta_{13} Y_1 Y_3 + \beta_{23} Y_2 Y_3 \quad \ldots (1)$$

where, Z represents response values, β_j, β_{jj} and β_{jk} denotes the linear, quadratic and interactive

Table 1 — Experimental design for peppermint oil nanoemulsion preparation.

Run No	Oil Conc. % (vol/vol)	Surfactant Conc. % (vol/vol)	Surfactant type	Droplet Diameter (nM)
1	15	25	Tween80	39.2
2	10	29.1421	Tween80	15.2
3	15	5	Tween20	800
4	2.92893	15	Tween20	12.5
5	5	25	Tween80	12.3
6	15	25	Tween20	52.2
7	17.0711	15	Tween80	432.4
8	5	25	Tween20	12.7
9	5	5	Tween20	499.8
10	15	5	Tween80	620
11	2.92893	15	Tween80	11.1
12	10	0.857864	Tween80	730
13	10	15	Tween80	289.3
14	10	0.857864	Tween20	760
15	10	15	Tween20	566.6
16	5	5	Tween80	68.7
17	17.0711	15	Tween20	368.2
18	10	29.1421	Tween20	19.8

coefficients values respectively with β_0 as a constant. Design expert (version 11) was used to calculate the coefficients values.

Characterization of nanoemulsion

Droplet diameter distribution

Measurements of nanoemulsion droplet diameter and the polydispersity index (PDI) were determined by using Beckman Coulter Delso Nano C instrument. Droplet diameter measurements were made by the DLS (Dynamic Light Scattering) technique. The submicron size range droplet in nanoemulsion undergoes Brownian motion, which results in variation of scattered light intensity. The DLS size analyzer records this fluctuation. Stokes-Einstein equation was used to calculate the hydrodynamic diameter (D_h) of the nanoemulsion formulation (Eq. 2)[18]:

$$D_h = \frac{k_B T}{3\pi\eta D_t} \quad \ldots (2)$$

where, T indicates absolute temperature, k_B indicates Boltzmann's constant, η indicates viscosity and D_t indicates translational diffusion coefficient. The formulated nanoemulsions were diluted a hundred times with double distilled water before droplet diameter analysis to invalidate the viscosity effect caused by the constituents of nanoemulsion and to diminish the effect of multiple scatterings.

Morphology by Transmission electron microscopy (TEM)

TEM was used to visualize the morphology of formulated nanoemulsion droplets. First, nanoemulsion formulations were suspended in water/ethanol and then solution was homogenized using ultrasonicator to prepare the dispersion. One drop of the dispersion was pipetted out and casted onto 200 meshes carbon-coated grids. Then, the carbon grid was air-dried and further fixed in a holder-containing specimen. Micrographs were taken by a TEM (Jeol/JEM 2100), which operated at 200 kV voltage.

Stability of nanoemulsion

Stability of the formulated nanoemulsions were assessed by incubating the nanoemulsion formulations at room temperature for prolonged storage and were observed for phase separation, flocculation or creaming (if any). The formulations were also studied for alteration in droplet size (if any) at regular interval of storage time.

Mosquito larvicidal activity

Dose-Response kinetics of killing

WHO protocol was used to assess the larvicidal activity of the optimized nanoemulsions[19]. Twenty larvae of either late third instar and early fourth instar *An. stephensi* and *An. culicifacies* were collected in bowls containing 200 mL of water and treated with a series of various concentrations of nanoemulsions in three replicates each. Control larvae without any nanoemulsion treatment were maintained in water. Test groups with only surfactant treatment were also kept to check the toxic effect of the surfactants on the larva if any. The beakers were held at room temperature (25-28°C), relative humidity of 75% with a photoperiod of 12 h: 12 h (light and dark, respectively). The exposure period for mosquito larvicidal activity was 24 h, hence the mosquito larvae were devoid of food supply during the study. Mortality of the larva was examined at regular interval of time exposed. If the larva failed to make movement after being probed with a needle in the cervical or siphon region after the exposure period, it was identified as dead larvae. During the exposure period, if the mosquito larvae pupated was found to be greater than 10%, then the test was discarded and repeated.

Statistical analysis

Data from all the three replicates of larvicide treatments were pooled for analysis. Abbott's formula was used to correct the mosquito larval mortality bioassay data (Eq. 3)[20]:

$$\text{Mortality (\%)} = \frac{X - Y}{X} \times 100 \quad \ldots (3)$$

where X and Y denotes the survival percentage in the control (untreated) and nanoemulsion treated sample respectively. Mean of all the replicates, standard deviation, standard error, and variance was calculated. The results of larvicidal activity were analyzed by ANOVA (One-Way) to establish the significant difference between the control and nanoemulsion treatments groups using SPSS (IBM SPSS Statistics V23.0) software. Results were contemplated to be statistically significant if the probability value was <0.05. LC_{50} value was calculated using Probit analysis from a regression line of log dosage–probit mortality at 95% confidence level[21].

Results and Discussion

GC-MS of peppermint essential oil

Bioactive component analysis using GC-MS showed that menthol was the major bioactive component of peppermint essential oil with the peak area of 45.2%. Other compounds found were menthone (15.39%), neomethol (8.1%), menthyl acetate (7.7%) and isomenthone (7.4%) (Fig. 1).

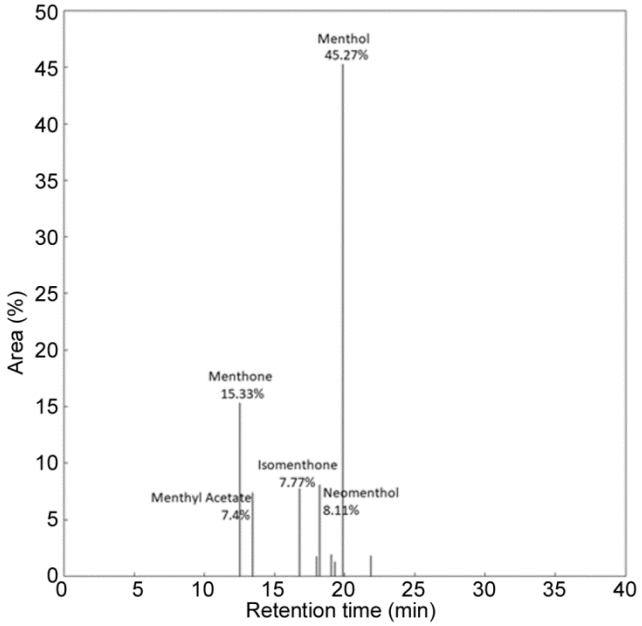

Fig. 1 — GC-MS spectra of the peppermint essential oil

Optimization of nanoemulsion preparation

Fitting the model

RSM is an important tool to optimize the effects of independent variables on the response values and to explore the relationship between them. Table 1 shows the effects of independent variables such as oil concentration, surfactant concentration and surfactant type on droplet diameter (Y_1) and the polydispersity index (Y_2) of peppermint oil nanoemulsions. Experimental data of RSM helped to compute the polynomial equation coefficients and to predict the values of response variables. RSM obtained regression equations for droplet diameter and the polydispersity index (response variables) are as follows: (Eqs. 4 & 5):

$$\text{Droplet Diameter} = +359.63 + 126.05Y_1 - 245.61Y_2 - 49.98Y_3 - 98.14Y_1Y_2 + 20.70Y_1Y_3 + 39.60Y_2Y_3 - 84.42\,Y_1^2 + 3.18Y_2^2 \quad \ldots (4)$$

$$\text{Polydispersity Index} = +0.3776 + 0.0691Y_1 - 0.0623Y_2 + 0.0105Y_3 - 0.0161Y_1Y_2 + 0.0083Y_1Y_3 - 0.0148\,Y_2Y_3 - 0.0932Y_1^2 - 0.0629Y_2^2 \quad \ldots (5)$$

Quadratic polynomial model for representation of experimental values for droplet diameter (Y_1) with 14.88 of model F-value and <0.0001 of p-value and in case of polydispersity index (PDI) (Y_2) with 6.86 of model F-value and 0.0004 of p-value was confirmed by ANOVA analysis. Lack of Fit p-value was 0.87 relative to the pure error test indicating that it is not significant and our model is valid statistically.

Effects of oil concentration, surfactant concentration and surfactant type on the droplet diameter and PDI of the nanoemulsion

Peppermint essential oil-in-water nanoemulsions were prepared using five levels of peppermint essential oil and non-ionic surfactant Tween20/ Tween80 (Table 1). To understand the influence of independent variables (*i.e.* peppermint oil concentration, Tween80/Tween20 concentration) on droplet diameter and PDI, 3D-response surface curves were plotted using the software Design Expert. By changing the values of oil concentration and surfactant concentration, while maintaining the surfactant type (*i.e.* either Tween20 or Tween80) constant, the curves were plotted. The impact of peppermint oil concentration and Tween80/Tween20 concentration on the droplet diameter of peppermint oil nanoemulsion formulations are illustrated in (Fig. 2A & B), which demonstrate the complex interaction between the independent variables. With

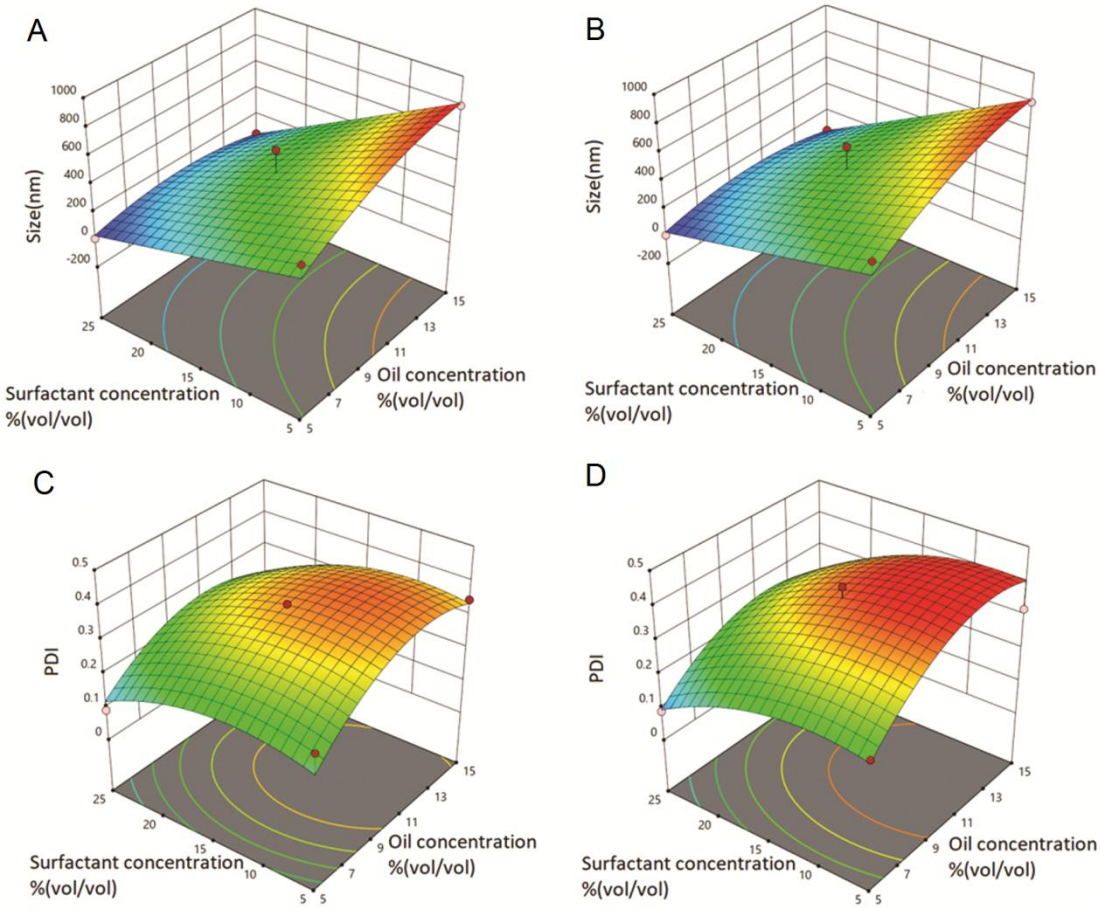

Fig. 2 — 3D response surface graph of (A) droplet diameter (nm) *vs* surfactant concentration (%) and oil concentration (%) for Tween20; (B) droplet diameter (nm) *vs* surfactant concentration (%) and oil concentration (%) for Tween80; (C) polydispersity index *vs* surfactant concentration (%) and oil concentration (%) for Tween20; and (D) polydispersity index *vs* surfactant concentration (%) and oil concentration (%) for Tween80

the increase in concentration of surfactant, a gradual reduction of droplet diameter was observed with the decrease in oil concentration.

The effects of surfactant concentration and oil concentration on the response value polydispersity index (PDI) of peppermint oil nanoemulsions is illustrated in (Fig. 2C & D). With an increase in concentration of Tween20/Tween80, polydispersity index decreased steadily with the decrease in concentration of peppermint oil. Higher concentration of oil resulted in higher values of PDI.

Using the desirability function of Design Expert Software, the numerical optimization was done further by selecting the goals to prepare nanoemulsion with low droplet size and PDI. The maximum desirability solution was chosen out of all the possible solutions. Optimized conditions for the preparation of peppermint oil nanoemulsion was 15% of peppermint oil, 25% of surfactant concentration and Tween80 as the surfactant. The values at these optimized conditions 39.2 nm and 0.22 of droplet diameter and PDI correspondingly corroborated well with the predicted response values.

Characterization of optimized nanoemulsion

Optimized peppermint oil nanoemulsion (OPNE) has a mono-modal and homogeneous droplet diameter distribution ranging from 10-200 nM with an average droplet diameter of 39.2 nM. The formulation was also optically transparent (Fig. 3).

OPNE droplets were observed to be of spherical in morphology (Fig. 4) as confirmed by Transmission Electron Microscopy (TEM). TEM micrographs also postulated additional information on droplet size *i.e.* the droplets were in the nano-metric size range between 10-100 nM.

Stability of nanoemulsion

OPNE was stable for 6 months (Fig. 5) when stored at room temperature. There was no separation of

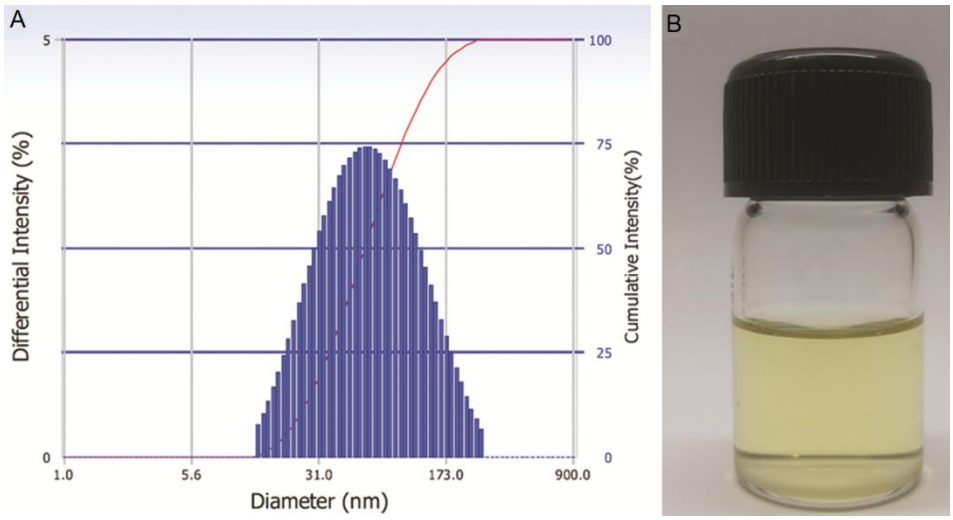

Fig. 3 — Droplet size distribution (A) and visual appearance; and (B) of optimized peppermint oil nanoemulsion (OPNE)

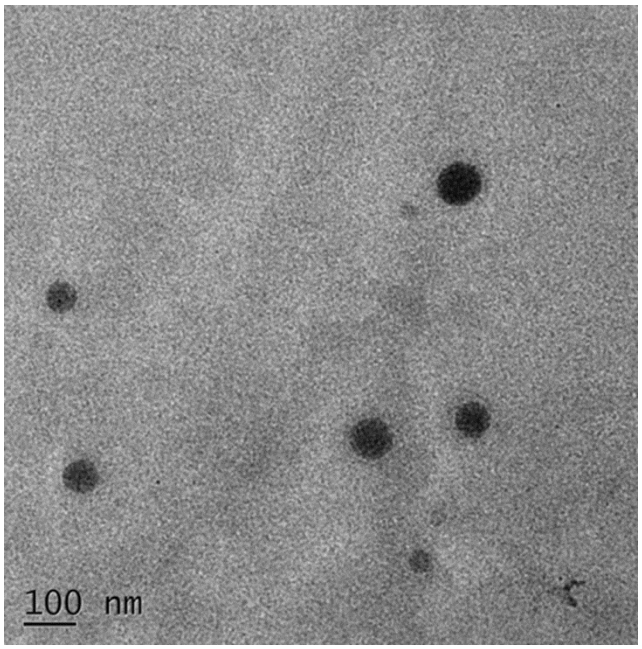

Fig. 4 — TEM image of optimized peppermint oil nanoemulsion (OPNE)

constituent phases, flocculation or creaming. Also, the droplet diameter of the formulation did not vary significantly with time.

Mosquito larvicidal activity

Dose-Response kinetics of killing

Due to the low droplet diameter and greater stability OPNE formulation was chosen for larvicidal activity against malaria vectors. OPNE demonstrated concentration-dependent death of Anopheles mosquito larva. The investigation was performed using various concentrations of the selected

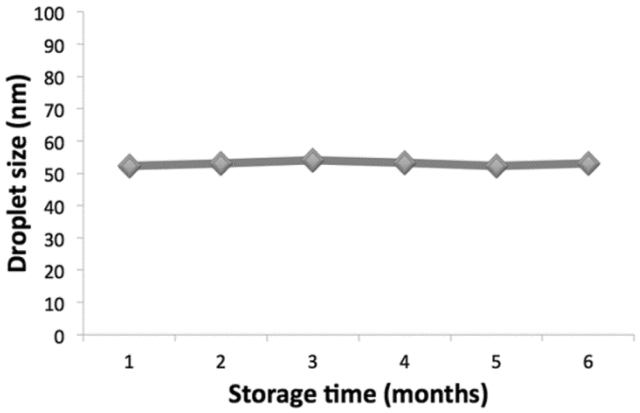

Fig. 5 — Stability of optimized peppermint oil nanoemulsion (OPNE)

nanoemulsion formulations corresponding to 25, 50, 100, 200 ppm, 250 and 500 ppm of essential oil composition to check the larvicidal effect after 24 h of incubation.

OPNE exhibited 35% mortality at 25 ppm against malaria rural vector *An. culicifacies*. Increase in concentration of nanoemulsion corresponding to 50, 100 and 200 ppm of oil resulted in 51.7%, 77.2% and 93.3% of mortality. Further increase in the peppermint oil concentration to 250 ppm caused 100% mortality of *An. culicifacies* larvae (Fig. 6). Results of the treatment groups incubated with surfactant only exhibited that all of the anopheles larvae were alive (result is not shown), which suggests that mortality in test groups was due to the essential oil. OPNE also showed concentration-dependent mortality of *An. stephensi* larva but the mortality rate was comparatively low (6.7%, 16.7%, 28.3%, 65.2% and 88.3% at 25, 50, 100, 200 and

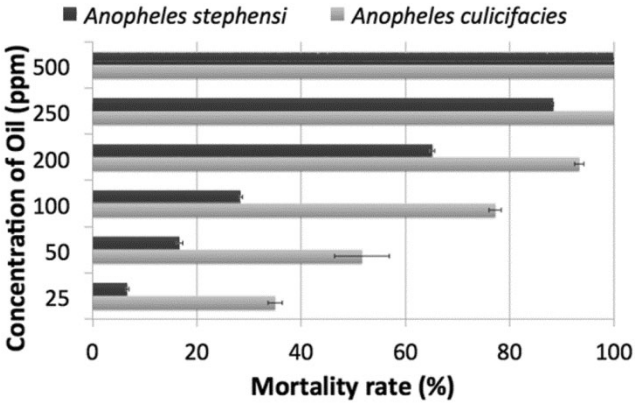

Fig. 6 — Dose dependent anopheles larvicidal activity of optimized peppermint oil nanoemulsion (OPNE)

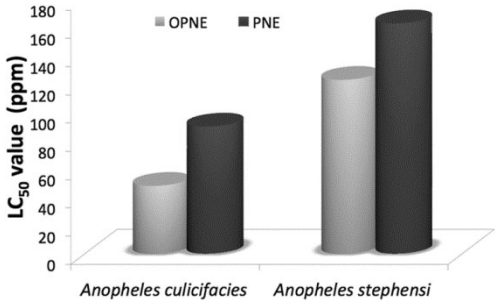

Fig. 7 — Droplet size dependent larvicidal activity of peppermint oil nanoemulsions OPNE (52.2 nm) and PNE (129.6 nm) against malaria vectors

250 ppm correspondingly) suggesting that OPNE formulation is a more efficient larvicidal agent against *An. culicifacies* than *An. stephensi.* One-Way ANOVA results on the mortality of malaria vector larva among the treated group with different concentration of nanoemulsion showed a significant difference on the mortality of mosquito larvae w.r.t the untreated control group. All the above cases exhibited less than 0.05 of P-value. So, it is established that plant essential oil based nanoemulsions are effective larvicidal agents.

Effect of nanoemulsion droplet size on larvicidal activity

LC_{50} value was determined using Probit analysis and it was calculated to be 48 ppm against *An. culicifacies* and 123 ppm against *An. stephensi* for OPNE respectively (Fig. 7). The figure also compares the LC_{50} value of optimized formulation OPNE with the corresponding peppermint oil nanoemulsion (PNE) with high droplet diameter (129.6 nM). The LC_{50} values were comparatively high *i.e.* 90 ppm and 163 ppm for *An. culicifacies* and *An. stephensi* correspondingly for PNE. These results demonstrate that both OPNE and PNE nanoemulsion formulations were efficient larvicidal agents against rural vector *An. culicifacies* than the urban vector *An. stephensi.* The mosquito larvicidal efficacy of the peppermint essential oil based nanoemulsions may be credited to the major bioactive compounds such as menthol and menthone in the peppermint essential oil (Fig. 1). The LC_{50} values were comparatively lower for OPNE formulation than PNE formulation indicating the higher larvicidal efficiency of the low droplet size formulation (OPNE) *i.e.* lower concentration of OPNE formulation is required to kill 50% of the malaria vector larva when compared to PNE formulation. So, it was confirmed that the nanoemulsions exhibit droplet size dependent larvicidal activity.

Discussion

Stable peppermint essential oil based nanoemulsion was formulated by low energy spontaneous emulsification method. RSM was a beneficial tool for optimizing oil concentration, surfactant concentration and surfactant type for preparation of nanoemulsion with lower droplet diameter and high stability. Quadratic polynomial model for representation of experimental values for droplet diameter and polydispersity index (PDI) had a greater model F-value and lesser P-value as confirmed by ANOVA analysis, suggesting the effects of all the independent variables to be greatly significant on the response variables[22]. Emulsifiers in the preparation of nanoemulsion, diminish the oil/water interfacial tension. Hence, escalation in concentration of surfactant results in a diminution of nanoemulsion droplet diameter[23,24]. Tween20 and Tween80 surfactants have lower molecular weight when compared to polymeric emulsifiers, which have high molecular weight. So, they are adsorbed to the droplets of oil in emulsion competently, minimizing the droplet diameter[25]. Also both Tween20 and Tween80 are high HLB (Hydrophile-lipophile balance) surfactants, hence, they were preferred in the preparation of the oil-in-water nanoemulsion. High HLB value of Tween20 and Tween80 leads to their diffusion from the organic phase (essential oil and Tween20/Tween80) to water and help in the formation of low droplet size emulsions. An additional advantage of both Tween20 and Tween80 is the non-ionic nature for which they stabilize the emulsion by stearic stabilization through deterrence of the bulky hydrophobic groups of the surfactants[26.]

Droplet size of nanoemulsion formulations was bigger when the oil concentration was more. This can be attributed to increase in viscosity. Also, higher oil concentration augments nanoemulsion droplet collision and aggregation which consequences in greater value of droplet size[27]. PDI is a dimensionless index of homogeneity of colloidal dispersions. Lower values of PDI indicate a homogenous and narrow size distribution, which can also contribute for a more representative distribution analysis[28].

The OPNE formulation demonstrated transparent optical property, which is due to the fact the nano-metric size droplet scatters light waves extremely weakly resulting in an optically transparent dispersion[29,30]. Very low droplet diameter enhances the nanoemulsion stability. The minimized droplet diameter resists the physical destabilization by coalescence, flocculation and gravitational separation. Brownian motion in low droplet size nanoemulsion is enough to avoid gravitational separation force[31].

Optimized nanoemulsion formulation OPNE demonstrated larvicidal activity against malaria vectors at a very low concentration, which was dependent on nanoemulsion droplet size and essential oil concentration. These larvicidal activity results corroborate with the study reported by Sugumar *et al*., (2014) *i.e*. eucalyptus oil nanoemulsion exhibited concentration dependent larvicidal activity of against filariasis vector *Culex quinquefasciatus*[32]. Nanoemulsion droplet diameter dependent mosquito larvicidal activity may be attributed to the higher ratio of surface area to volume in the nanometric range droplets in the nanoemulsions[16].

The findings of this study suggest that plant essential oil based nanoemulsion is cost effective and eco-friendly potent larvicide, which can possibly be used for mosquito vector control strategies. Formulated nanoemulsions exhibited better larvicidal property against *An. culicifacies* than *An. stephensi*. As about 75% of the total population of Chhattisgarh state of India lives in the villages, our findings may additionally provide eco-friendly strategies to control rural vector of malaria in Chhattisgarh, India in future.

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Conflict of interest

All authors declare no conflict of interest.

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