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Research Article

NANOFORMULATIONS OF KARANJ AND NEEM OIL: PHASE EQUILIBRIUM AND LARVICIDAL ACTIVITY

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ABSTRACT

The increasing risk of environmental pollution and health problems in the day-to-day life due to the usage of synthetic pesticides make the role of nanoemulsions very significant. The study is aimed at development of eco-friendly nanoemulsion of non-edible oil such as Karanj oil and Neem oil by using the Tween80 and PEG400 as surfactant and cosurfactant respectively. The phase behaviour of ternary mixtures and nanoformulations was studied by visual observation. The nanoemulsion was found to be stable for the period of one month at 30˚C . The nanoemulsions were characterized by various physicochemical properties such as viscosity, surface tension, pH, droplet size, polydispersity index etc. The Larvicidal activity was studied under 24h of exposure against *Aedes agypti* and *Culex quinquefasciatus* larvaes. Rheological study shows the pseudoplastic flow behaviour.

Keywords: Nanoemulsions, Karanj oil, Neem oil, Phase behaviour, Larvicidal activity.

1. INTRODUCTION

A pesticide is a substance or mixture of substances intended for preventing, destroying, repelling or lessening the damage caused by pest [1, 2] which includes insecticide, herbicides, bactericides, fungicides, insect repellents, animal repellents, nematocides [3, 4]. Many chemical pesticides are poisonous to human and animals, and cause considerable damage to ecosystem. Thus, many of the chemical pesticides are removed from the market due to high toxicity and hazardous nature. The application of this causes acute or chronic toxicity [5, 6], risk to the central nervous system [7-9], cancer, teratogenicity such as birth defects [10-12], tumor growth (Oncogenecity) [13-15], skin damage (allergic sensation) [16, 17], respiratory problems and liver damage. Moreover, the residues of pesticides are detected in the breast milk [18-20]. It emphasizes the importance of natural and biopesticides. They can be an excellent alternative to synthetic pesticides and serve as means to reduce negative impacts to human health and the environment.

Biopesticides are the naturally occurring substances which control pest by non-toxic mechanisms [21]. They are reported as the biodegradable, economically safer, eco-friendly, target specific and sustainable in integrated pest management (IPM) programs [22]. These pesticides include plant extracts, essential and vegetable oils, hormones, pheromones and toxins from the organic origin also include many aspects of pest control such as microbial [23], entomophagous nematodes, plant based and metabolites [24].

The aromatic characteristics of vegetable oils provide various functions for the plants including (i) attracting or repelling insects, (ii) protecting themselves from heat or cold; and (iii) utilizing chemical constituentsin the oil as defence materials. The Neem, Karanj and Castor are the non-edible vegetable oils which show the insecticidal and larvicidal activity. The various products of Neem such as Neem leaf extract, Neem seed kernel extract, Neem cake extract, Neem oil emulsion and Neem in combination with other plant extract or oils for control of verity of pests [25, 26]. Karanj seed oil has a light brown colour, moderate viscosity, and distinctive aroma with quick absorption on application. Karanj oil, organic leaf extract of Karanj tree, and methanolic and aqueous seed extract of Karanj seed oil have shown the potential to act as oviposition deterrents,antibacterial, antifungal, mosquito repellent and larvicidal against a wide range of insects [27, 28]. It is proved that the synergic effect of various oils gives good results than the individual [29, 30].

Nanoformulations have the ability of improving biological activity of lipophilic compounds by increasing the surface area per unit of mass [25, 31]. Additionally to high kinetic stability, low viscosity and optical transparency make them very attractive systems for many industrial applications [32, 33]. The nanoformulations of single and mixture of edible oils with artificial surfactants as well as natural surfactants are effective for pesticidal activity. Extraction of oil from fruits and seeds of Neem (*Azadirachtaindica*), Castor (*Ricinus cammunis*) and Karanj (*Pongamia glabra*) trees are explored for their larvicidal activity against vector of filaria, *Cx. quinquefasciatus, Aedes agypti* mosquitos [34, 35]. As a continuation of our earlier research work, we have decided to formulate non-edible oils using combination of surfactants.

The objective of present study is to prepare nano-emulsions of Karanj oil and Neem oil to investigate their larvicidal activity. The screening of surfactant and cosurfactant was done by determining particle size and transmittance. From pseudoternary phase diagram the amount of surfactant and cosurfactant was optimized. This optimized concentration of surfactants and oil form nanoformulation. The emulsion stability studies were carried out at 30°C±0.5°C for one month. The prepared nanoemulsion was characterized for droplet size, polydispersity index, morphology (TEM) and physicochemical properties such as viscosity, Surface tension and pH. The stable emulsion was tested for their rheological and larvicidal properties.

2. MATERIAL AND METHODS

2.1. Material

Karanj oil and Neem oil were procured from local Ayurvedic pharmacy. Tween20, Tween60, Tween 80, Span20, Span80, PEG400 surfactants were obtained from Oxford Lab Fine chem LLP, Palghar. Methanol was procured from SD Fine Chemicals, Mumbai. Double distilled water was used for experimental work.

2.2. Methods

2.2.1. Screening of Surfactants

Different types of hydrophilic surfactants were screened for nanoemulsion formulations, which included Tween 20, Tween60, and Tween80. The oil percentage was maintained constant and the amount of surfactant was varied to get the stable formulations. The stability of this emulsion was observed on the basis of droplet size and percentage transmission at 560nm, using UV-visible spectrophotometer (Shimadzu 2450) against distilled water as a blank. The surfactant which formed a stable emulsion with highest percent transmittance and exhibited colloidal stability over 24h was selected for further study [36].

2.2.2. Screening of Cosurfactant

Cosurfactants were screened for their emulsification capacity by mixing them individually at different ratios with the selected oil phase and surfactant. Briefly, the amount of oil phase and surfactant (Tween80) was kept constant with 1:1 w/w ratio. Different cosurfactants used here include Span20, Span80 and PEG400. The relative percent of cosurfactant was varied to get a stable emulsion. The formulation with minimum particle size and maximum colloidal stability was chosen for further study.

2.2.3. Construction of pseudoternary phase diagram

Microemulsion technique was adopted for the formulation of Karanj and Neem oil formulations. The initial step while preparation of any microemulsion is the development of the pseudoternary phase diagram. The emulsification capacity of screened surfactant and cosurfactant was the basis for construction of pseudo-ternary phase diagram. Components were chosen for phase diagram are given in table 1.

Table 1: Components of phase diagram

Component I	Component II	Component III
Oil phase Karanj oil and Neem oil	S_{mix}phase Tween80 and PEG400	Aqueous phase Distilled water

Initially, Surfactant was blended with cosurfactant in the weight ratios of 2:1, 1:1 and 1:2. The aqueous titration method was used for the construction of the pseudoternary phase diagrams, which involves stepwise addition of water to each weight ratio of oil and surfactants, and then vortexing the components on the vortex mixer at 30°C . The nanoemulsion phase was identified as the region in the phase diagram where clear, easily flowable, and transparent formulations were obtained based on the visual observation. Ten different combinations of different weight ratios of oil and S_{mix}, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1 were taken. The apex of triangular graph indicates 100% concentration of the eachcomponent. The pseudo-three-component phase diagram represented the aqueous phase, the other represented the oil phase, and the third represented a mixture of surfactant and cosurfactant at a fixed weight ratio (S_{mix}). The graphs were plotted as a triangular graph to obtain a ternary or a pseudoternary diagram using Chemix School 3.51 software, Arne standnes USA.

2.2.4. Preparation of nanoemulsion

After identification of maximum microemulsion region from the phase diagram study, the blend of surfactant and cosurfactant (S_{mix}) with the desired ratio was prepared. The nanoemulsion was prepared under external agitation with the help of magnetic stirrer. The optimised S_{mix} from the phase diagram was taken with Karanj and Neem oil and then added to the aqueous phase with constant stirring. The resultant mixture was stirred for 2h at 1200rpm on magnetic stirrer and subsequently sonicated in ultrasonic bath (Ultrasonics, 30 kHz; MS Manufacturer, India) for 15 min at 30°C to obtain the nanoemulsion. The percentage composition is shown in the table 2.

Table 2: Percentage (w/w) compositions of Karanj-Neem oil formulation (KN)

Formulation	Oil(w/w%) (Karanj+Neem)	Surfactant(w/w%) (Tween80:PEG400)	Distilled water (w/w%)	Stability
KN formulation	20%	40%	40%	100% Stable

KN formulation: Karanj-Neem oil emulsion

2.2.5. Physicochemical Properties

2.2.5.1. Viscosity

The viscosity of the nanoemulsion was determined by using a 'Dial reading Brookfield viscometer' (Model: RVT#60768, AMETEK India) at room temperature. The sample was equilibrated for 2 minutes and then reading was taken.

2.2.5.2. pH

The pH of the formulation was found out by a pH meter (Model-Meter EQ-610) at 30°C. The device was calibrated at pH 4.0. The reading was repeated three times for each formulation.

2.2.5.3. Surface tension

The surface tension of nanoformulation was measured by Kruss K6 tensiometer (Kruss, UK) equipped with platinum plate using the du Nouy ring method. The plate was cleaned by heating it to red-orange colour with a gas burner prior to using; Deionized water was used for calibration, with the surface tension 72-73mNm^{-1}. The reproducibility between the measurements was ± 0.2mNm^{-1}. The readings were taken three times for each formulation.

2.2.6. Nanoemulsion particle size measurement

The mean droplet size distribution of Karanj-Neem oil nanoemulsion was determined by dynamic light scattering using (DLS) method using Nano book Plus PALS (Brookhaven Instrument, NY, USA). Measurements were made in triplicate at 30°C with fixed angle at 90° of 10% diluted formulations.

2.2.7. Transmission Electron Microscopy

The samples of nanoformulation were characterized by transmission electron microscopy (TEM). The formulation was diluted in 1:10 ratio with distilled water and was mixed with a drop of 2% w/v uranyl acetate solution. A drop of this was placed onto carbon-coated grid and was dried to form a thin film. The extra liquid was drained off using filter paper. The grid was air dried off using a transmission microscope with accelerating voltage of 120 kV. The micrographs of the sample were captured at different magnification.

2.2.8. Stability studies

The formulation were prepared and were stored at 30°C for one month and was evaluated for appearance, droplet size and polydispersity index.

2.2.9. Rheological Characterization

Rheological properties of emulsions were measured using a dynamic shear rheometer (Physica MCR 301, Anton Paar, Graz, Austria) operated by "Rheocompass™ software" equipped with EC200. About 1 mL of oil or emulsion was placed on a 50 mm diameter parallel plate geometry measurement cell. Steady-state flow measure-ments were performed at 30°C in the range from 0.1S^{-1} to 100S^{-1} (shear rate). The rheological parameters (shear stress, shear rate, and apparent viscosity) were recorded.

2.2.10. Larvicidal Activity

The most efficient and simpler way to control the mosquito population is larviciding. In the present paper the larvicidal activity of stable formulations were tested using twenty larvae of each mosquito species at 25±3°C with a photoperiod of 12 h light and12 h dark. Initially series of the trial experiments (non-replicated) were conducted with various formulations to optimize the dose with a geometric factor of 2.0. Test formulations were composed of Karanj oil emulsion (K), Neem oil emulsion (N), Karanj-Neem oil formulation (KN) where concentration varies from 0.32 to 3.20 % (v/v). Each measurement is performed in three replications. The number of dead larvaes was counted after 24 h of exposure.

3. RESULTS AND DISCUSSION

3.1. Screening criteria for Surfactants

The proper selection of surfactant becomes a crucial factor in nanoformulation of pesticides. The non-ionic surfactants are relatively less toxic than their counter-parts and typically have low CMCs. The emulsification capacity of surfactant was evaluated on the basis of percent transmittance of formed emulsion and its colloidal stability on storage. The surfactant which shows maximum transmittance and stability was selected. Maximum transmittance is due to optical clarity of the emulsion, as a scattering of incident light is more in opalescent dispersion compared to the transparent dispersions. The intensity of light passing through such dispersion is attributed to the scattering of light, which occurs due tothe absence of optical homogeneities in the medium. Hence, % transmittance is indirectly related to the relative droplet size of the emulsion and thus could be used to predict size in qualitative terms. Based on this underlying principle, aqueous dispersions with high transmittance (lower absorbance) were considered optically clear, and oil droplets were thought to be in a state of nano-dispersion [37]. The surfactant Tween80 showed maximum percent transmittance and better stability on storage with the oil phase (depicted in table 3).

3.2. Screening of cosurfactant

Use of single surfactant hardly achieves the negative interfacial tension between the two immiscible phases. Hence, there is a need to add a cosurfactant as it reduces the bending stress between the interface and provides sufficient flexibility to the interfacial film [38]. From the observation Cosurfactant PEG400 which showed maximum percent transmittance (table 4) and stability is used for further study.

Table 3: Surfactant selection based on its emulsification capacity

Oil Phase (20%)	Surfactant (40%)	% Transmittance	Stability
Karanj oil + Neem oil	Tween 20	56.5±1.2	Unstable
Karanj oil + Neem oil	Tween 60	75.9±0.6	Stable
Karanj oil + Neem oil	Tween 80	95.6±0.33	Stable

**%Water = 40%; % Transmittance expressed as mean ± SD, n=3.*

Table 4: Cosurfactant selection based on its emulsification capacity

Sr. No.	Oil phase+ Surfactant (50%)	Cosurfactant (10%)	% Transmittance	Stability
1	Karanj oil + Neem oil + Tween 80	Span 20	55.5±0.33	Unstable
2	Karanj oil + Neem oil + Tween 80	Span 80	65.5 ±0.56	Unstable
3	Karanj oil + Neem oil + Tween 80	PEG400	94.5 ±0.33	Stable

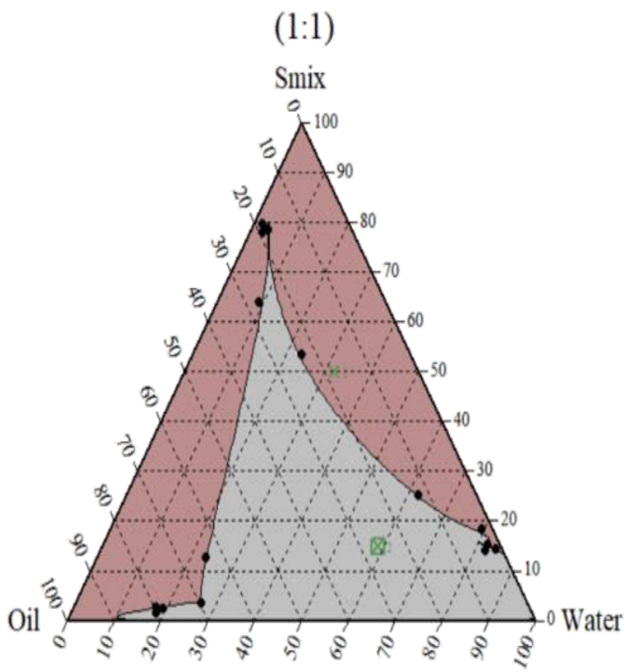
**%Water = 40% ;% Transmittance expressed as mean ± SD, n=3.*

3.3. Construction of pseudoternary phase diagram:

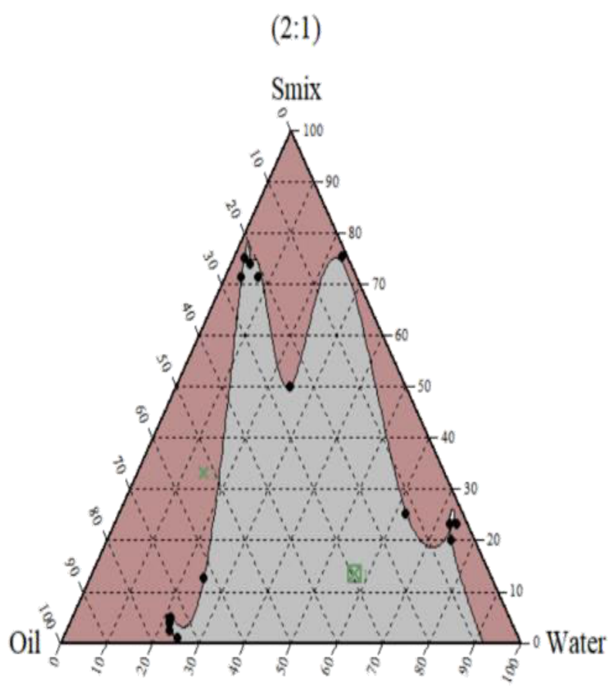
The existence of nanoemulsion formation zone can be illustrated with the help of the pseudoternary phase diagram. The order of mixing of various components is not expected to influence the formation of nano-emulsion if the system is indeed thermodynamically stable (path-independent). Phase diagrams were constructed using Karanj-Neem as the oil phase and Tween80 and PEG400 as the surfactant and cosurfactant, respectively.

When cosurfactant was added to surfactant in equal amounts, a higher nanoemulsion region was observed. It is attributed to the reduction in the interfacial tension and increased fluidity of the interface at S_{mix} 1:1 (Fig.1a). The maximum concentration of oil that could be solubilized, was only 31% (w/w) at 60% (w/w) of S_{mix}. On further increasing the surfactant concentration i.e., at the ratio of S_{mix}2:1 (Fig.1b), the nanoemulsion region increased in size as compared to the region in S_{mix} 1:1. In case of S_{mix} 1:2, area covered for nanoemulsion was less which may be due to the less amount of surfactant.

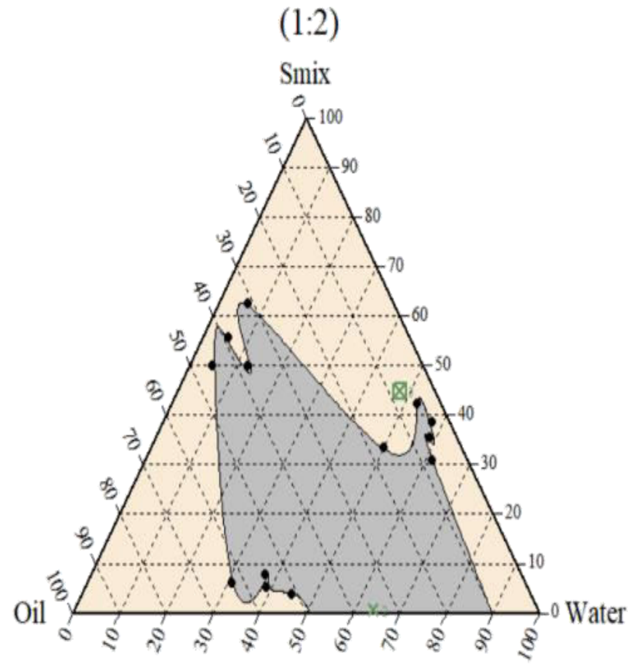
The surfactant and cosurfactant mass ratio had been found to play the key role in influencing the phase properties, *i.e.,* size and position of the nanoemulsion region. S_{mix} 2:1 showed the maximum area as compared to the other ratios [39, 40]. This effect was assigned to the differences in the packing of surfactant and cosurfactant at the o/w interface. From the particle size data it is also showed that in the 2:1 S_{mix} ratio getting stable formulations [41]. 1:2:1 ratio of oil: surfactant: cosurfactant suggested that the oil constitutes the inner phase of the nanoemulsion droplets, which is consistent with a direct o/w type formulations [42].



(a)



(b)



(c)

Fig. 1: Pseudoternary phase diagrams indicating o/w nanoemulsion region of Karanj-Neem (oil), water, Tween 80 (surfactant), and PEG 400 (cosurfactant) at different S_{mix} ratios indicated in a. (S_{mix} 1:1) b. (S_{mix} 2:1), c. (S_{mix} 1:2)

3.4. Physicochemical Properties

3.4.1. Viscosity

The viscosity of formulation is about 27mPa.s. which is efficient for spreading and spraying on crops due to low viscosity.

3.4.2. pH

The pH of the formulation was found to be 6.65 that is in neutral range because non-ionic nature of Tween80.

3.4.3. Surface tension

The surface tension of nanoformulation was found significantly lower than that of the water, (72mNm^{-1} at 30°C). The formulation of Karanj and Neem has 32.45 mNm^{-1}surface tension.

3.5. Nanoemulsion particle size measurement:

The small size and low polydispersity of nanoemulsions are the outstanding advantages as compared to conventional emulsions therefore the researchers focused on the optimization of the various functionalities of nanoemulsions [43, 44]. Another aim in nanoemulsion optimization is to improve overall stability by achieving the minimum droplet size and/or polydispersity [45]. Fig.2a and Fig.2b shows the evolution of the variations in mean droplet size and polydispersity under different storage periods. After prepartion of nanoemulsion, the mean droplet size of nanoemulsion was 481.2nm; moreover mean droplet size did not dramatically change after 7, 14, 21, 28 and 35 days of storage at room temperature. Low value of polydispersity index indicated the uniformity in formulation.

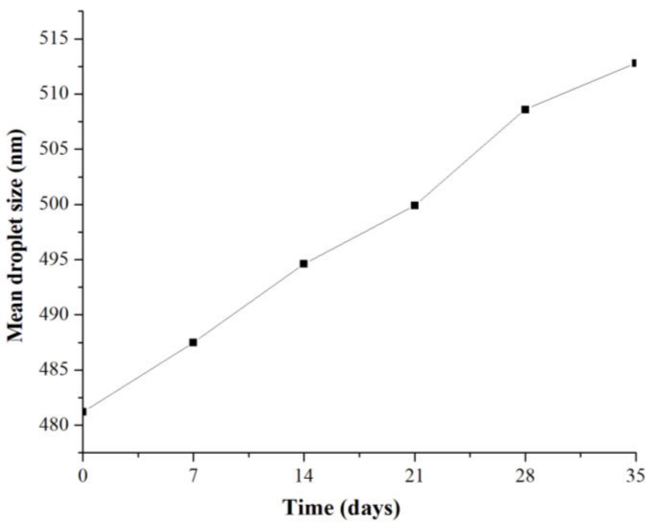


Fig. 2a: Variations in mean droplet size for pesticide nanoemulsion under different storage periods

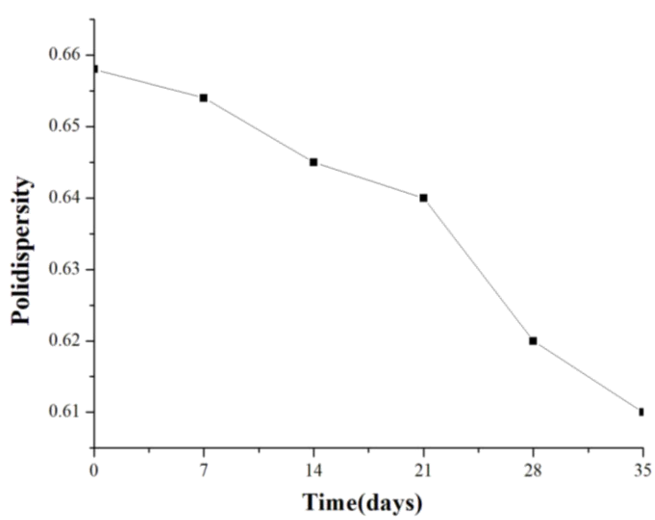


Fig. 2b: Variations in mean polydispersity for pesticide nanoemulsion under different storage periods

3.6. Transmission Electron Microscopy

Nanoformulation was evaluated morphologically based on TEM images as shown in Fig.3. The TEM images showed that the nanoemulsion droplets were spherical in shape and represent a typical appearance of oil-in-water nanoemulsion. The resultant compared with that of globule size analysis showed nanometric range, varying from 50-100nm.

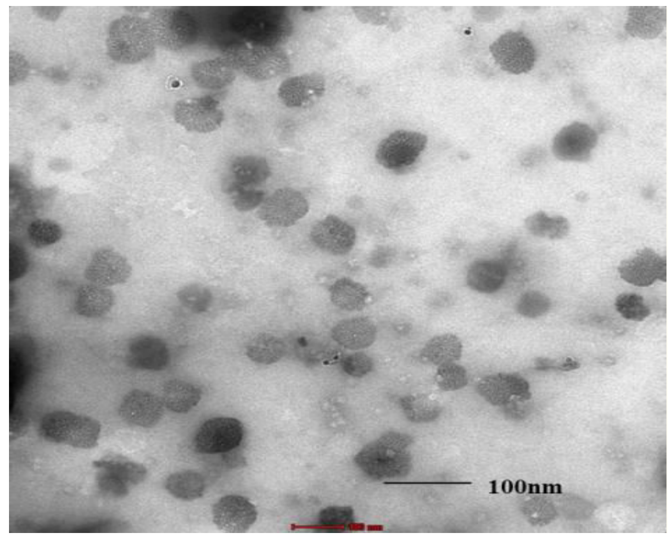


Fig. 3: TEM images of KN formulation

3.7. Stability Study

No phase separation and apparently change in clarity were observed on the nanoformulation. The physical stability of the optimized nanoformulation was investigated after 35 days of storage at room temperature with changes in mean droplet size and polydispersity index. The results after 35 days stability studies showed negligible alteration in droplet size and polydispersity index as compared to initial measurements (Table 5).

Table 5: Stability Data of KN Formulation at Initial and 35-Days time point at room temperature

Parameters	Initial	35days
Mean droplet size(nm)	481.2±6.4	512.8±4.6
Polydispersity index	0.658±1.22	0.61±2.06

3.8. Rheological Characterization

The rheological properties of the Karanj-Neem formulation was evaluated to understand its viscoelastic properties [46]. The rheogram shows concave curve (fig.4) indicating that as the shear rate increases, viscosity decreases. This proves that the nanoemulsion shows non-Newtonian flow behaviour [47, 48]. The reason of high viscosity might be attributed to the entangled polymeric chain at low shear rate. This typical property of shear thinning systems favors the applicability of nanoemulsions on crops.

3.9. Larvicidal activity

The results of the larvicidal activity of nanoemulsion are depicted in Table 6. Third-instar larvae of *Aedes agypti* and *Culex quinquefasciatus* were treated with different concentrations of Karanj and Neem formulation. The mortality rate of *Aedes* and *culex* increases with an increase in the concentration of emulsions. Table 7 depicts the larvicidal data of Karanj oil emulsion (K) and Neem oil emulsion (N) with same amount of surfactants and oil. This single oil emulsions compared with combined effect of Karanj-Neem oil emulsion. The formulation of KN with a maximum 3.20% (v/v) showed 100% mortality after 24h of exposure. From the study, it was observed that the combination of Karanj and Neem oil emulsions were found to be more effective than their individual treatment against all the mosquito larvae tests.

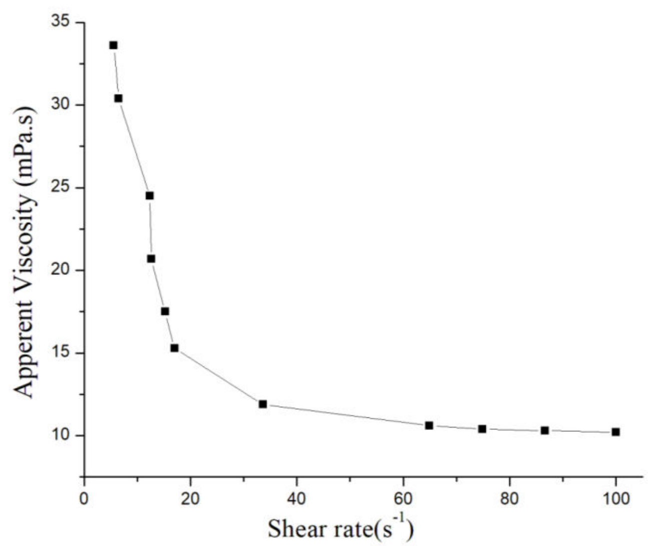


Fig. 4: Viscosity measurement of formulated emulsion

Table 6: Larval Mortality Data of combination of KN nanoemulsion against different Mosquito species

Emulsion Concentrations (v/v%)	KN Formulation Corrected Larval mortality at 24h (%) *	
	Aedes Agypti	*Culex quinquefasciatus*
0.32	22(0.88)	2(0.33)
0.64	37(0.33)	8(0.33)
0.96	43(0.33)	10(0.57)
1.28	47(0.33)	17(0.33)
1.60	55(0.57)	28(0.66)
1.92	67(0.33)	37(0.33)
2.24	73(0.33)	50(0.57)
2.56	83(0.33)	62(0.33)
2.88	98(0.57)	78(0.66)
3.20	100	100
Untreated control	0.00	0.00

**Mean of three replications; Values in parentheses are standard errors. KN Formulation: Karanj-neem oil emulsion*

Table 7: Larval Mortality Data of K and N nanoemulsions against different Mosquito species

Emulsion Concentrations (v/v%)	K Formulation Corrected Larval mortality at 24h (%) *		N Formulation Corrected Larval mortality at 24h (%) *	
	Aedes Agypti	*Culex quinquefasciatus*	*Aedes Agypti*	*Culex quinquefasciatus*
0.32	10(0)	2(0.33)	3(0.33)	0(0)
0.64	20(0.57)	2(0.33)	7(0.57)	2(0.33)
0.96	25(0.57)	5(0)	12(1.20)	8(0.33)
1.28	28(0.66)	7(0.33)	18(0.33)	12(0.57)
1.60	37(0.33)	12(0.33)	23(0.57)	15(0)
1.92	37(0.33)	13(0.57)	28(0.33)	18(0.57)
2.24	40(0.57)	15(0.57)	35(0.33)	20(0.57)
2.56	47(0.33)	27(0.33)	33(0.33)	25(0.33)

2.88	47(0.33)	32(0.33)	48(0.57)	33(0.57)
3.20	57(0.66)	37(0.57)	50(0.33)	37(1.20)
Untreated control	0.00	0.00	0.00	0.00

**Mean of three replications; Values in parentheses are standard errors. K Formulation: Karanj oil emulsion ; N Formulation: Neem oil emulsion*

4. CONCLUSION

The nanoemulsion containing combination of Karanj and Neem oil, Tween80: PEG400 and distilled water was successfully obtained by the emulsification method. A nanoemulsion with smallest droplet size of 481.4nm and 0.658 polydispersity index was found to be more effective in controlling mosquito larvae. Rheological study reveals the pseudoplastic flow behaviour that is shear thinning nature of formulation. Karanj oiland Neem oil nanoformulation can be good, economical, biodegradable and eco-friendly alternative to other pesticides for the control of vector-borne diseases.

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Conflict of interest

None declared

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