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Characterisation and microbial activity of neem oil nano-emulsions formulated by phase inversion temperature method

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This study has been carried out to prepare neem oil-in-water nano-emulsions stabilized by Brij 30 surfactant using the phase inversion temperature (PIT) method at three different temperatures, i.e., 60, 75 and 80°C. Compositions of homogenous phase have been identified in the pseudo-ternary phase diagram. Among the total seventeen formulations, three formulations (NB1, NB2 and NB3) have been short-listed and characterized for emulsion size and viscosity. The selected formulations have shown emulsion size of 348-981 nm in diameter. The volume percentage ratio of Brij 30 to neem oil have shown significant effect on the droplet size of nano-emulsions. Formulations having lower concentration of Brij 30 have displayed a smaller emulsion droplet size (348 nm). The NB3 formulation (4% neem oil, 11% Brij 30 and 85% deionized water) has exhibited the highest stability after 60 days of storage. Antimicrobial study has shown that in contrast to raw neem and Ampicillin (synthetic drug), NB1 exhibited best result in terms of minimum inhibition concentration (MIC) reduction by 100% against *E-coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenus*.

Keywords: Antibacterial, Droplet size, Neem oil, Non-ionic surfactant, Phase inversion temperature, Ternary phase diagram

High energy or low energy dissipation methods are widely known for the formulation of nano-emulsions, differing by the amount of energy utilised. Mechanical devices generate large disruptive forces in high energy method, whereas low energy method modifies the physiochemical characteristics of the system to generate nano-sized particles[1]. Earlier, the high energy methods have the only choice for the researchers to develop nano-emulsions formulation due to ease of production[2]. Nevertheless, high energy methods included various mechanical devices such as high speed/high-pressure homogenisers, ultra-sonicators, and high shear stirrers. Thus, formulation using high energy methods has considered as an energy intensive process. Also, it has shown a poor utilization rate of energy in the formulation of nano-emulsion. Various literature shown that only about 0.1% of the total energy supplied gets utilized in producing nano-emulsions[3–5].

Presently, the low energy methods are widely considered as an eco-friendly technique which utilizes the internal chemical energy of the system itself. Moreover, it has become an appealing approach because of the requirement of components that are temperature sensitive such as pharmaceutical ingredients[6]. The low energy methods mainly include, phase inversion temperature (PIT), phase inversion composition (PIC), microemulsion dilution and surfactant phase emulsification[7].

Among the most preferred methods, the PIT method has demonstrated high emulsification efficiency and low polydispersity index (PDI) compared to PIC method[8]. PIT method comprises of stirring and gradual heating of surfactant, oil and water till phase inversion temperature (the temperature at which significant change in conductivity observed) followed by rapid cooling in an ice bath leading to formation of oil-in-water (o/w) nano-emulsion[9]. The temperature is the key parameter reported in the formation of o/w nano-emulsion using PIT method[10]. It has been reviewed that the PIT method provides temperature-driven phase inversion of emulsions. i.e., water-in-oil (w/o) to oil-in-water (o/w), stabilized using non-ionic surfactants (ethoxylated groups[11,12]. During heating, the dehydrated ethoxylated surfactants become hydrophobic and favour w/o emulsion system at elevated temperature[13–17]. Then w/o emulsion system has heated to temperature of PIT and upon rapid cooling, forms o/w emulsion system with a uniform small droplet size[18,19]. To get a deeper insight about the isotropic regions (single phases) formed in PIT

method, researchers have prepared pseudoternary phase diagrams (PTD) based upon their aqueous titration experiments. PTD also helps in evaluating a thermodynamically stable system and gives an idea about scale-up of proportions [20]. For a fixed volume percentage of oil, the formulation of single-phase boundary obtained at varying surfactant, co-solvent, and water compositions using low-energy method, has already been reported[21].

Neem oil is a deep yellow extract obtained from the seeds of neem tree (*Azadirachta indica*). These seeds are popularly used in India and in other South-East countries as versatile medicinal product due to its antibacterial, antifungal, antimalarial and insect growth inhibition properties, and in cosmetics due to its low toxicity *in vivo* studies[22, 23]. Nevertheless, neem oil in its raw form has exhibited poor water solubility, oxidation tendency and toxicity in high intake levels. In order to improve the effective utilization of neem oil, the application in the form of nano-emulsion has gained attention by various researchers[24]. Formulation of nano-emulsion prepared using neem oil or Karanj oil with Tween 80/Polyethylene glycol 400 as a surfactant and co-surfactant, respectively using emulsification method has produced smallest droplet size of 481 nm. A researcher has reported about the stability of nanoemulsion formulations of neem oil-water-nonionic surfactant (Lutensol® TO 6), prepared using high-speed homogenization[25]. Moreover, many authors have demonstrated the formulation of neem-based nano-emulsion with Tween 20 using ultrasonication[26,27]. PIT method to prepare nano-emulsion of natural oils such as soyabean oil, clove oil, nutmeg oil, cinnamon oil, retinyl palmitate oil, and sunflower seed oil have been researched widely[28-32]. However, to the best of our knowledge a study about preparing formulation of neem oil-based nano-emulsion using Brij 30 as a surfactant employing a low energy PIT method has not yet been reported. Brij 30 surfactant is a non-ionic surfactant, known for its low toxicity, biodegradability, and non-hazardous impacts on humans and the environment[33]. Moreover, Brij 30 is popularly known for its applications as an emulsifying agent, a penetration enhancer, a solubilizing agent, a wetting agent, and in the formulation of some drugs[34]. In addition to this, Jennifer *et al*. has reported use of Brij 30 based nano-emulsion as a carrier vehicle for hydrophilic drugs[35]. Apart from this, it was investigated that nano-emulsion favours pharmaceutical application due to significant reduction in consumption of active ingredients. Typical nature of the nano-emulsion, i.e., high-water solubility and capacity to solubilize hydrophilic and lipophilic compounds has credited to such a change in consumption of active ingredients[36]. Thus, a promising delivery system of nano-emulsion has led to its utilization in antimicrobial application. Various authors have demonstrated that nano-emulsion to be efficient for oral delivery of lipophilic drugs such as antibiotics, hormones, steroids, cytotoxics, diuretics and antifungals[37-39].

The objectives of the current study are summarized as follows: (i) preparation of neem oil nano-emulsion using Brij 30 by a low-energy PIT method and preparing pseudoternary phase diagram (ii) Studying their stability and screening the most stable nano-formulations (iii) Characterizing the screened nano-emulsions for emulsion size and viscosity. (iv) Evaluating the efficiency of the screened nano-formulation for their antimicrobial activity and comparison with raw neem oil and Ampicillin antibiotic drug.

Experimental Section

Materials

Neem oil (ρ = 0.949 g/mL; azadirachtin 250 ppm; μ = 4 cP) was purchased from Kama Ayurveda, Arya Vaidya Pharmacy, Coimbatore, India. De-ionized water was used for all experiments. Polyoxyethylene (4) lauryl ether (Brij 30) was purchased from LobaChemie, Mumbai, India. All chemicals were of analytical grade.

Construction of pseudoternary phase diagrams (PTD)

In order to construct PTD, neem oil and surfactant Brij 30 were mixed in different ratios (v/v%), i.e., 10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 4:6; 3:7; 2:8; 1:9 and 0:10. The prepared mixtures were stirred and heated to 50°C to attain equilibrium, and gradually cooled to room temperature. Next, water (5 v/v%) was added by titrating the mixtures of neem oil and surfactant until a 95% water content was obtained in the emulsion system. The components were stirred at 600 rpm, 27°C for 30 min using magnetic stirrer. Based on visual observation, the nano-emulsion systems were classified to be isotropic (transparent and single phase) or anisotropic (cloudy or two phase). The phase diagrams were constructed using ProSimTernaire software (France), version 1, phase

Table 1 — Composition (volume%) of neem oil, Brij 30 and deionized water.

Formulation	Neem oil	Brij 30	Deionized water	Phase inversion temperature°C
NB1	10	20	70	80
NB2	10	30	60	75
NB3	4	11	85	60

diagram plotter. Out of more than 200 formulations, based on the transparency and homogeneity of the resulting mixtures, three formulations were selected and denoted as NB1, NB2 and NB3.

Preparation of Nano-emulsions

Table 1 shows composition of three different formulation NB1, NB2 and NB3 and Fig. 1 shows their physical appearance. Preliminary experiments were carried out to determine the phase inversion temperature of these three formulations using electrical conductivity. Their electrical conductivity was measured using conductivity meter (Model: NU-317; Navyug, India). In order to determine electrical conductivity, each formulation containing neem oil, Brij 30 and water (0.01 N NaCl solution) was continuously stirred with the use of mechanical stirrer (Remi make magnetic stirrer, Model MS500; India) and heated at a rate of 1.5 °C per min. It was observed that conductivity of all three formulations, dropped rapidly with increasing temperature. The sharp change in conductivity considered as a PIT for individual formulation. The formulations were prepared by mixing neem oil, Brij 30, and deionized water (volume %) using magnetic stirrer at 600 rpm in a glass beaker and then heated using a water bath. Once the system reached its PIT, the mixture was rapidly cooled to 20-27 °C by keeping it in an ice bath. The emulsion was continuously stirred using a magnetic stirrer during the heating and cooling stages. The preparation of formulation was derived based on the method described by Rao and McClements[40] with correction in PIT, i.e., at PIT instead of above PIT. The emulsions prepared using PIT methods were further characterized for its emulsion size and viscosity.

Characterization of nano-emulsions

Emulsion size

Emulsion size of the selected formulations was determined using a dynamic light scattering (DLS) instrument (Malvern Mastersizer 2000, UK). The samples were diluted 40 times with deionized water to nullify multiple scattering effects. The growing rate of particle size of sample stored at 27°C was measured after 60 days. The experimental runs were taken thrice for each formulation and showed error ±5%.

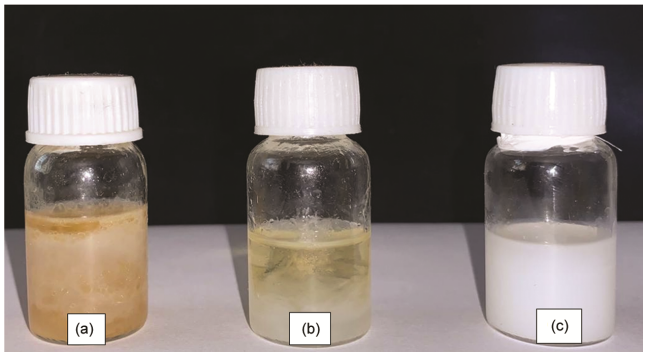

Fig. 1 — Appearances of nano-emulsion formulation steps (a) Neem oil and Brij 30 (b) Dispersion on water addition and (c) Nano-emulsion by PIT.

Viscosity

The viscosity of the nano-emulsion formulations (non-diluted) were determined using a digital rotational viscometer (Model: LMDV200; Labman, India) with L0 spindle at 25 °C at100 rpm. Sample reading was noted at zero shear once it reached equilibrium (after 2 min). The viscosity values were taken thrice for each formulation and showed error ±5%.

Antibacterial activity

The broth dilution method was used to evaluate the antibacterial activity of the nano-emulsions formulation. This method determined minimum inhibition concentration (MIC). MIC gives an account of the lowest concentration of antimicrobial agent and synthetic drugs that prevents visible growth of microorganism under specific conditions as observed by the unaided eye. In the present study, raw neem oil, synthetic drug(ampicillin) and three sets of neem oil nano-emulsions, namely NB1, NB2 and NB3 were tested for their antibacterial activity against bacterial strains, i.e., *E. coli*, *P. aeruginosa*, *S. aureus*, and *S. Pyogenus*. The bacterial strains were procured from the Institute of Microbial Technology, Chandigarh. One set of broth solution free of antibacterial agent was used as a standard medium and the second set was prepared by taking identical volumes of broth in antibacterial solution (raw neem oil, ampicillin and nano-emulsions). Both sets of test tubes were inoculated with a defined quantity of bacterial strains i.e., 108 organisms/ml. After incubation, presence of

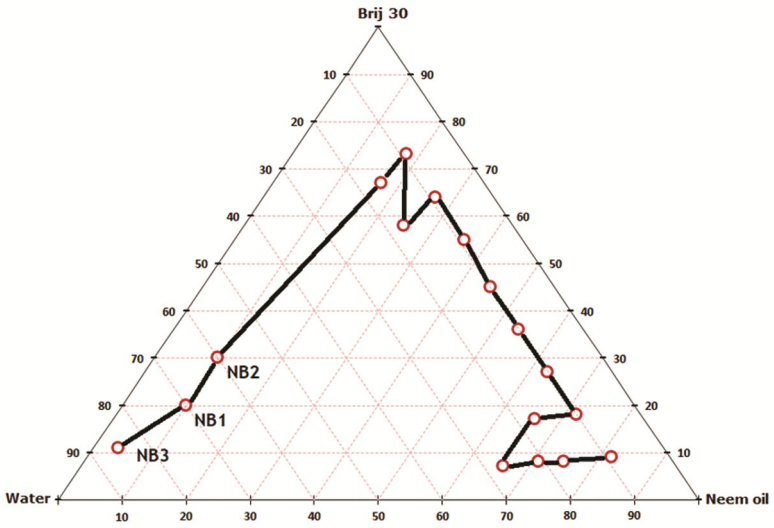

Fig. 2 — Ternary phase diagram of neem oil, Brij 30 and deionized water.

turbidity or sediment was used to analyzed the growth of bacteria.

Results and Discussion

Construction of Ternary Phase Diagram

Figure 2 shows the phase behaviour of three components in a ternary phase diagram. The phase diagram constructed by titration method using gradual addition of deionized water in a well-defined proportion to the neem oil and Brij 30 mixture. The points shown in the ternary phase diagram indicate single phase, slightly transparent and stable behaviour of the mixture on visual observation, whereas rest of the area refers to creaming and gel-like nature. Many researchers have used a similar titration method to determine precise nano-emulsion region[21,30,41]. Among the total seventeen points, three different compositions (NB1, NB2 and NB3) of neem oil, Brij 30 and deionised water have been selected based on minimum requirement of Brij 30 and neem oil. Then after, these shortlisted samples have been further utilized for the formulation of neem oil nano-emulsions using low energy method.

Effect of Surfactant on droplet size of nano-emulsion

Table 2 shows the effect of volume ratio of surfactant to oil on the size of emulsions in the selected neem oil nano-emulsion formulation. It can be seen that as the ratio of Brij 30 to neem oil in the formulation increases from 2 to 3, there is a gradual increase in the size of emulsion formed thereby. Liu *et al*. studied the size of emulsion formed in paraffin oil, water and chremophor (surfactant) system and observed a similar trend of increasing emulsion size upon increasing the surfactant to oil ratio[43]. Table 2 shows the effect of concentration of surfactant on size of emulsion formed. As the concentration of Brij 30 present in the nano-formulation increased from 11 vol% to 20 vol%, the droplet size of emulsion decreased from 555 nm to 348 nm. Then after, upon further increasing the surfactant concentration to 30% the droplet size of emulsion increased to 981 nm. Concentration of surfactant beyond critical micellar concentration might be attributed to such increment[42].

Table 2 — Effect of volume ratio of Brij30 to neem oil on size of the emulsion formed

Formulation	Brij30/Neem oil	Brij 30 (Vol.%)	Emulsion Size (nm)
NB1	2.00	20	348
NB2	3.00	30	981
NB3	2.75	11	555

Figure 3 shows the stability of three formulations examined after 60 days. All the formulations were observed to be visually stable at 27°C with no phase separation, creaming and sedimentation. Nevertheless, the DLS study showed increment in droplet size for NB1, NB2 and NB3 by 116, 15 and 12, respectively after 60 days. Ostwald ripening/ coalescence phenomenon might be attributed for the visible growth rate of droplet size over the period of storage time[43].

Viscosity

In order to characterize fluidity of neem oil nano-emulsion formulation, viscosity was reported.

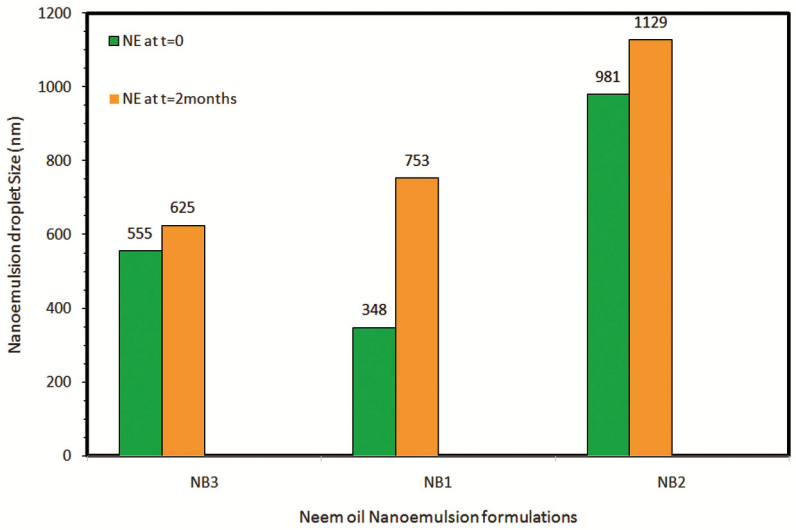

Fig.3 — Stability of droplet size over the period of 60 days.

Table 3 —Minimum inhibition concentration of raw neem oil, NB3, NB2, NB1 and synthetic drug for various bacterial strains.

	Minimum Inhibition Concentration (µg/ml)			
Sample	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. pyogenus*
Raw neem Oil	100	125	125	250
NB1 (348nm)	50	62.5	62.5	125
NB2 (981 nm)	62.5	125	125	200
NB3 (555nm)	62.5	100	100	150
Ampicillin (synthetic drug)	100	--	250	100

In this study, viscosity of three formulations, NB1, NB2 and NB3 were found to be 6.83 cP, 6.89 cP and 6.75 cP, respectively. The observed viscosity of nano-emulsion was about 72% higher than that of viscosity of raw neem oil (4 cP). Such an increment in viscosity was attributed to typical characteristics of Brij 30 surfactant (highly viscous) in the composition of nano-emulsion formulation. The non-ionic surfactant attracts water molecules into its cross-linked chain, leading to such an increment in viscosity of nano-emulsion formulations[9,44].

Antibacterial activity

Minimum inhibition concentration (MIC) represents the lowest concentration of a drug that prevents visible growth of bacteria. In the present study, the MIC of the screened nano-emulsion formulation (NB1, NB2 and NB3) were measured and then compared with raw neem oil and Ampicillin that are popularly used as antimicrobial agents. The results are shown in Table 3. It was observed that MIC of nano-emulsion formulation NB1, NB2 and NB3 were lesser than that of raw neem oil and synthetic drug for all strains. It was also observed that droplet size of nano-emulsion formulations significantly affects the MIC for all strains studied here (*E.coli*, *P. aeruginosa* and *S. aureus* and *S. pyogenus*). It was observed that, greater the emulsion droplet size of the prepared emulsion a larger amount of MIC had been registered for that particular formulation. The formulation, NB1 (having lowest emulsion droplet size) observed to be best suited as an antimicrobial agent as it had the lowest MIC. In regards to MIC, NB1 showed reduction in MIC by 100% against *E.coli*, *P. aeruginosa* and *S. aureus* and *S. pyogenus* when compared with that of raw neem. Similar findings were reported by Choupanian *et al*. They found that efficacy of azadirachtin, a main ingredient in the neem oil extract *(Azadirachta indica)* demonstrated a significant improvement in mortality rate of two serious pest species *Sitophilus oryzae (L.)* and *Tribolium castaneum (Herbst)* as compared to raw neem oil[45].

Conclusion

The ternary phase diagram has been classified in two regions. A single phase has been characterized by slightly transparent and stable, whereas rest of the area refers to creaming and gel-like nature. Considering the minimum requirement of Brij 30 and

neem oil, three different compositions (NB1, NB2 and NB3) of neem oil, Brij 30 and deionised water have been shortlisted. Upon decreasing the volume ratio of Brij30/neem oil (i.e., 3 to 2), droplet size of neem nano-emulsion has been reduced (981 nm to 348 nm). Increment in concentration of Brij 30 (11 vol% to 20 vol%), decreases the droplet size of nano-emulsion (555 nm to 348 nm). Then after, droplet size has been increased to 981 nm at higher concentration (30 vol % Brij 30). Furthermore, DLS study has shown increment in droplet size for NB1, NB2 and NB3 by 116%, 15% and 12%, respectively after 60 days. The viscosity of nano-emulsion is about 72% higher than that of viscosity of raw neem oil (4 cP). The MIC of nano-emulsion formulation NB1, NB2 and NB3 have been found lesser than that of raw neem oil and synthetic drug for all strains. In contrast to MIC of raw neem and Ampicillin (synthetic drug), NB1 has shown reduction in MIC by 100% against *E.coli*, *P. aeruginosa*, *S. aureus* and *S. pyogenus*.

Conflicts of interest

There is no conflict of interests among the authors

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