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Limonene-based Self-nanoemulsifying System: Formulation, Physicochemical Characterization and Stability

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ABSTRACT

Objectives: The aim of current research was to formulate limonene self-nanoemulsified delivery system (SNEDS) by spontaneous emulsification method. **Methods:** The optimization was carried out through the construction of a pseudo-ternary phase diagram. Limonene-based self- nanoemulsifying system was optimized by evaluating its droplet characteristic; namely; size, polydispersity, zeta potential, in addition, its morphology was assessed through the use of transmission electron microscopy. Moreover, the formulation stability under different storage conditions for three months was examined. **Results:** The obtained results showed that the optimized limonene-based self-nanoemulsifying system was characterized by a small droplet size, low polydispersity index, high percentage transmittance and optimal zeta potential with uniform spherical droplets. The selected formulation with 50% w/w limonene, 40% w/w Tween 80 and 10% w/w propylene glycol had a droplet size of 113.3±1.18nm with bluish transparent appearance and a zeta potential value of -19.13±0.38 mV. The developed formula was stable against pH change. The stored limonene-based SNEDS showed acceptable stability at 4°C and 0°C compared to 25°C. **Conclusion:** The formulated self-nanoemulsifying system showed an improved aqueous dispersibility, patient acceptability and stability of limonene, representing a promising carrier for lipophilic drugs.

Key words: D-Limonene, Essential oil, Formulation, Nanoemulsion, Self-nanoemulsifying system.

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INTRODUCTION

Phytomedicine have witnessed a tremendous progress and the world now is moving toward the use of herbal medicine or phytomedicine to strengthen the physiological systems. Essential oils extracted from plants are mixtures of volatile compounds, containing hundreds of bioactive chemical constituents mainly mono- and sesquiterpenoids and phenylpropanoids. These oils exert antifungal, antibacterial, gastroprotective, anticancer and many other therapeutically effects.[1] Several studies have focused on the benefits of these phytochemicals and their effect on human health. For example, the minimum inhibitory concentration of oregano essential oil against multiple drug-resistant *Escherichia coli* was 0.5µl ml^{-1}. Thyme essential oil obtained from *Thymus vulgaris* L. strongly inhibited the growth of clinical strains of *Staphylococcus, Enterococcus, Escherichia* and *Pseudomonas genus.*[2]

D-limonene (1-methyl-4-isopropyl-cyclohexene) is a cyclic monoterpene of molecular formula $C_{10}H_{16}$. It is the major constituent of the essential oil extracted from the peels of citrus fruits (family Rutaceae). It has a pleasant citric fragrance and widely used for flavoring food and personal care items. It is an optically active compound that has two enantiomeric forms; R and S, where the R-(+) is the one known as D-limonene (Figure 1).[3] A study was conducted to evaluate the difference in the bioactivity of limonene enantiomers and the results showed that (+) limonene was active against 25 different Gram positive and Gram negative bacterial species and eight different fungal species more than the (-) limonene.[4] D-limonene is classified by the Federal regulations as safe (GRAS).[5]

The therapeutic effects of limonene have been studied extensively, including antioxidant, anti-inflammatory, antidiabetic, anticancer and gastroprotection. Espina *et al.* reported that limonene provided a bactericidal activity against *Escherichia coli.*[6] Limonene also exhibits antifungal activity, which was evaluated by Chee *et al.* where the minimum inhibitory concentration (MIC) of limonene against Trichophyton rubrum was 0.5% v/v with a fungicidal activity.[7] The anti-inflammatory effect of limonene was further explored in D'Alessio *et al.* study which demonstrated the ability of D-limonene to significantly reduce inflammation scores when compared to ibuprofen by decreasing tumour necrosis factor alpha (TNf-α) and interleukin 6 (IL-6) concentrations.[8] Plant extracts are frequently assessed for their chemotherapeutic and chemo preventive effects, especially limonene that has been studied for its chemotherapeutic effects against different types of cancer. Yu *et al.* showed that D-limonene inhibited the growth of the lung cancer cells and the transplanted tumor in mice, by inducing apoptosis and promoting autophagy.[9] The anticancer effect of D-limonene and berberine against human gastric carcinoma cell line MGC803 was approved by Zhang *et al.* where D-limonene alone inhibited the growth of MGC803 cells and its combination with berberine showed remarkable synergistic anticancer effect through reactive oxygen species production, cell-cycle arrest and apoptosis induction via mitochondria-mediated intrinsic pathway.[10] D-limonene is a volatile oil, unstable in the presence of high temperature, light, air or humidity and thus it requires special preparation and formulation. Ghasemi *et al.* used protective encapsulation method for this bioactive compound, where it was encapsulated into whey protein and pectin at different pH values to form stable nanocomplexes.[11] However, high energy methods apply disruptive forces and the level of energy required to produce nanoscaled droplet size is very high and thus high cost, also it has been claimed that nanoemulsion preparation using high energy method requires higher oil-to-surfactant ratio unlike the one prepared by low energy methods.[12]

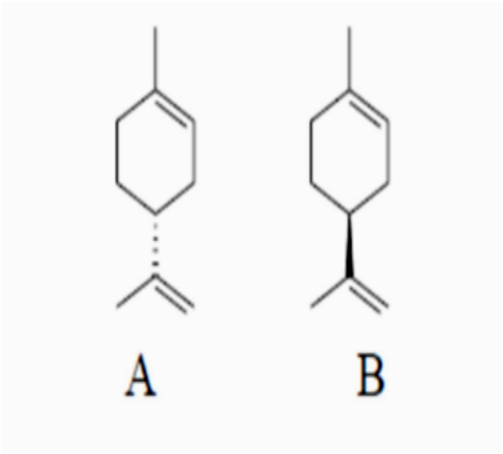

Figure 1: Structure of R-(+) limonene (A) and S-(–) limonene (B).

The aim of this study is to formulate limonene self-nanoemulsifying delivery system by spontaneous emulsification method. The formulation will be carried out through the construction of a pseudoternary phase diagram. Limonene-based self-nanoemulsifying system will be optimized by evaluating its droplet characteristics; namely; size, surface potential and polydispersity index. The surface morphology of the globules will be assessed through the use of transmittance electron microscopy. In addition, the formulation stability in different storage conditions for three months will be examined.

MATERIALS AND METHODS

Materials

R-(+)-Limonene, propylene glycol, polyethylene glycol 400 (PEG 400), PEG-40 hydrogenated castor oil (Cremophor®RH 40) and Tween®80 were purchased from Sigma Co.(Sigma-Aldrich, Steinheim, Switzerland). PEG-8 caprylic/capric glycerides (Labrasol®), oleoyl polyoxyl-6 glycerides (Labrafil®M 1944 CS), diethylene glycol monoethyl ether (Transcutol HP®) were kindly donated by Gattefosse Co. (Lyon, France). All other reagents and solvents used were of analytical grade.

Preliminary screening of surfactants and co-surfactants

The self-emulsification ability of various surfactants and the selection of the suitable surfactant were based on its emulsification power. Surfactants were screened according to method described by Date and Nagarsenker with some modifications.[13] Briefly, equal weights of each surfactant were added to limonene. The mixture was then heated at 45±2°C with controlled mixing (30 rpm) using magnetic stirrer till homogenization. Sample of the mixture was diluted with deionized water to 100ml in stoppered conical flask. The emulsification was judged according to the numbers of flask inversions required to produce a uniform emulsion. Emulsions were kept for 2h and then their transmittance was measured at 638.2nm (Jasco V-730 spectrophotometer) using deionized water as a blank. Then, a mixture of co-surfactant, the selected surfactant and limonene at a ratio of 1:2:3 respectively were mixed at 45±2°C using a magnetic stirrer (30 rpm) with controlled temperature for complete homogenization. Sample of the mixture was diluted with deionized water to 100 ml in stoppered conical flask. The self-emulsification was judged as previously described.

Construction of pseudoternary phase diagram

Constructing a pseudoternary phase diagram is a vital tool in the preparation of any lipid-based formulation that is able to self-emulsify in the body to form a thermodynamically stable nanocarrier. It will assess different phase behavior data of the formulation between different components. Limonene, the selected surfactant and co-surfactant were used to construct the ternary phase diagram using Chemix school software version 7.0 (Arne Standnes, Bergen, Norway). The concentrations of surfactant and oil varied between 30% and 70% w/w and that of co-surfactant ranged from 0% to 30 % w/w according to Date and Nagarsenker.[13] The mixtures were prepared by mixing surfactant/co-surfactant then the oil was added to the mixture and homogenized on a magnetic stirrer at 45±2°C for 15 min.[14] From each mixture, 50 mg were diluted to 50 ml with deionized water. The aqueous dispersions that are transparent to slightly bluish with a particle size of 200 nm or less were considered in the accepted region of the phase diagram.[14] The droplet size determination was carried out using Zeta sizer 2000 (Malvern Instruments, UK).

Preparation of limonene-based self-nanoemulsifying delivery system

The selected formula was prepared by mixing surfactant and co-surfactant and then adding the oil drop wise. Heating the mixture for 15 min on a magnetic stirrer at 30 rpm with controlled temperature at 45±2°C was carried out. The preparations were stored in sealed glass vials at room temperature for further investigations.

Study of SNEDS characteristics

Color, transparency, phase separation and homogeneity of the preparation at room temperature were observed for any changes that might occur. All tests were performed in set of three. Turbidity measurement was performed as the following; prepared formulation was diluted into 100ml with deionized water. The percentage transmission was measured spectrophotometrically at 638.2 nm. In addition, the self-emulsification time of the prepared SNEDS was determined by adding 1 ml of the preparation to 300ml deionized water at 37±0.5°C in a beaker stirred at 30 rpm. The time required for this concentrate to form a homogeneous clear bluish mixture is recorded as the emulsification time. The selected formulation was visually assessed and classified accordingly to the following criteria; rapidly forming emulsion having a clear or bluish appearance (grade A), rapidly forming, slightly less clear emulsion, having a bluish white appearance (grade B), fine milky emulsion formed within two minutes (grade C), dull, greyish white emulsion having slightly oily appearance that is slow to emulsify longer than two minutes (grade D), or poor minimal emulsification with large oil globules present on the surface (grade E).[15] In order to determine the formulation the cloud point, the prepared formulation was diluted with deionized water in ratio of 1:100 then placed in a water-bath (FALC, WB-MF24, Treviglio (BG) – Italy) with gradual increase in the temperature at a rate of 1°C/min. At the cloud point, the drop in sample percentage transmittance was measured spectrophotometrically at 638.2 nm.[14]

Moreover, the surface morphology and globule size of the optimized limonene-based SNEDS were observed by TEM (Jeol, JEM-100 CX electron microscope). The sample was diluted with water (1:1000) then a drop was placed on the copper grid. Excess sample was removed with filter paper. The grid was dried at room temperature and then observed using TEM. Zeta potential, droplet size and polydispersity index (PDI) were measured at 25±2°C and at 90° scattering angle by Zeta sizer 2000 (Malvern Instruments, UK) after the dilution of the formulation to 100 ml with deionized water.

Evaluation of the self-nanoemulsifying delivery system

The robustness of the preparation to dilution was performed through diluting the formulation 50, 100 and 1000 times with different media, namely; deionized water, 0.1N HCl (pH 1.2) and phosphate buffer

(pH 7.4). The diluted preparations were stored for 12h at 25±2°C and observed for any signs of phase separation or precipitation.[16] In addition the Thermodynamic stability studies were performed as following testing the formulation stability. The prepared formulation after dilution to 100ml with deionized water was exposed to centrifugation at 5000 rpm for 30 min to observe any changes in its homogeneity. In addition, stability was challenged through six freeze–thawing cycles between refrigerator temperature (4±0.5°C) and (25±0.5°C) with storage at each temperature for 48 hr.

Stability upon storage

The stability of the formulation was tested through its storage at room temperature at 25±2°C, at 4±2°C and at 0±2°C for three months and monitored for any signs of separation or precipitation. Limonene based SNEDS samples were assessed every four weeks and analyzed for clarity, particle size, PDI and zeta potential.

Statistical analysis

Results were expressed as mean±standard deviation (n=3) and compared for statistical significance using t-test or one-way-analysis of variance (ANOVA) test. p-value<0.05 was considered to be significant using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA).

RESULTS

Selection of surfactants and co-surfactants

The choice of surfactant and co-surfactants was based on the optical clarity of the nano-dispersion, reflected by high transmittance, where the intensity of the scattered light passing through dispersion increases with the presence of optical homogeneities in the medium. The percentage transmittance of the limonene preparations using different vehicles are represented in Figure 2, revealed that the preparation with Tween®80 as surfactant and propylene glycol as co-surfactant showed highest percentage transmittance compared to other vehicles, 76.7±0.135% and 50.57 ±0.16% respectively with p-value < 0.05 (Figure 2).

Pseudoternary phase diagram of limonene-based SNEDS

After selecting the optimum components to formulate limonene SNEDS, the pseudoternary phase diagram was constructed. Table 1 shows the different compositions of phase diagram mixtures meanwhile Figure 3 illustrates the pseudoternary phase diagram of limonene SNEDS. In the current investigation, although at a ratio of 1:1 (oil: surfactant) the nanoemulsifying ability was present, when the surfactant/co-surfactant mixture was at a ratio of 4:1, the nanoemulsion region was wider. Formulation A10 with 50% w/w limonene, 40% w/w Tween 80 and 10% w/w propylene glycol had the smallest droplet size (113.3±1.18nm) with bluish transparent appearance was chosen for the further studies.

Physiochemical characteristics and evaluation of limonene-based SNEDS

The physiochemical characteristics of the optimized limonene-based SNEDS are presented in Table 2. The percent transmittance after dilution with deionized water was 92.56% with a clear bluish to slightly white solution. With a simple agitation at 37±0.5°C, the self-emulsification time of limonene-based SNEDS was 37 sec as shown in Table 2. Visual observations showed that the formulation was found to be grade A. Formulation was diluted with 0.1N HCl, phosphate buffer pH 7.4 and deionized water to 50-, 100- and 1000 folds and showed no signs of precipitation, separation or cloudiness for 12h except for one with deionized water that

Table 1: Composition of SNEDS formulations constructing phase diagram.

Formula	Limonene (%w/w)	Tween®80 (%w/w)	Propylene glycol (%w/w)	Appearance	Size (nm)*
A1	30	70	0	Transparent with oil droplets	83.56±15.4
A2	30	60	10	Transparent with some oil droplets	87.45±6.2
A3	30	50	20	Turbid	377.3±48.5
A4	30	40	30	Turbid	242.1±12.5
A5	40	60	0	Bluish transparent	143.2±1.08
A6	40	50	10	Bluish transparent	119.2±0.801
A7	40	40	20	Less clear	167.8±22.94
A8	40	30	30	Less clear	176.9±0.83
A9	50	50	0	Slightly less clear	151.4±0.89
A10	50	40	10	Bluish transparent	113.3±1.18
A11	50	30	20	Less clear	179.1±1.55
A12	60	40	0	Less clear	154.4±2.27
A13	60	30	10	Turbid	166.8±2.14
A14	70	30	0	Turbid	178.8±4.35

* Results are expressed as mean±SD (n=3)

Table 2: Physiochemical characteristics of limonene-based SNEDS.*

Formula	Percentage Transmittance	Droplet size (nm)	PDI index	Zeta potential (mV)	Self-emulsification time (sec)	Cloud point (°C)
A10	92.57±0.98	113.30±1.18	0.211±0.007	-19.13±0.38	37±2.52Grade (A)	72±1.73

*Results are expressed as mean±SD (n=3)

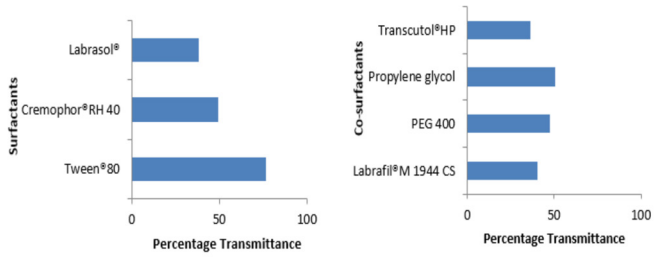

Figure 2: Preliminary screening of different surfactants and co-surfactants based on their percentage transmittance.

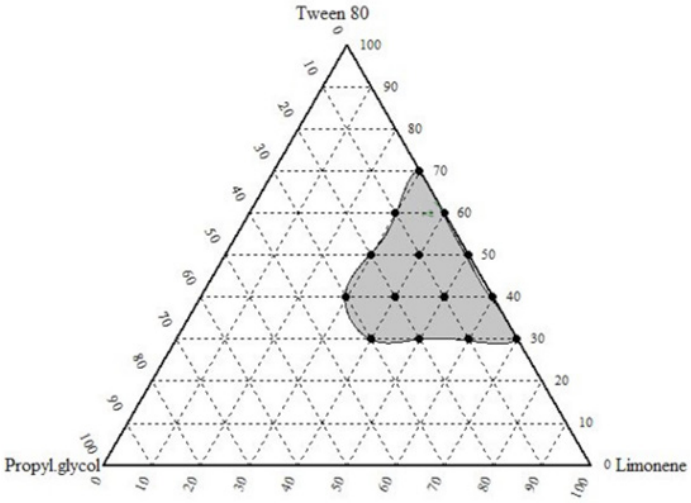

Figure 3: Pseudoternary phase diagram of limonene-based SNEDS. The shaded region indicates the nanoemulsion region.

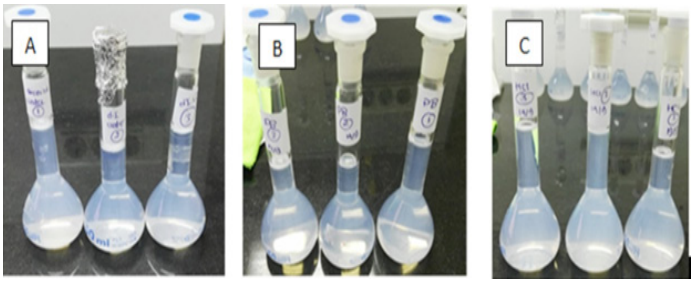

Figure 4: Limonene based SNEDS after 24hr dilution with (A) deionized water, (B) phosphate buffer, pH7.4 and (C) 0.1N HCl, pH 1.2.

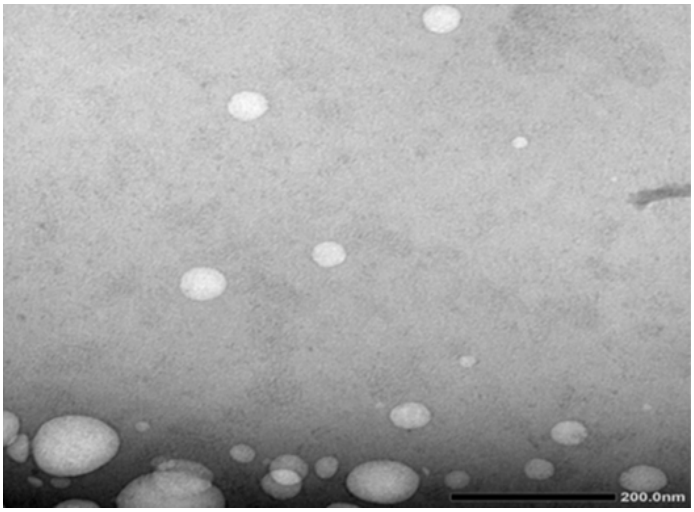

Figure 5: Photoelectromicroscopic image limonene-based SNEDS using transmission electron microscopy.

showed slight creaming which was reversible with simple flask inversion (Figure 4).

The formulation showed high stability and no signs of precipitation or phase separation under drastic stressful conditions, specifically, centrifugation at 5000 rpm for 30 min, six cycles of freeze-thawing between temperature 4°C and 25°C. The optimized limonene-based SNEDS was stable despite the increase in temperature up to 72°C and its percentage transmittance didn't change and remained above 90%. The morphology of the limonene-based SNEDS was observed using TEM. As the microphotograph represented in Figure 5, globules after dilution showed spherical shape with diameter less than 200 nm and appeared as bright particles with dark surrounding. In the current study, limonene-based SNEDS had small droplet size less than 200nm as shown in Table 2 and Figure 5. The polydispersity index for the formulation was 0.211 with a unimodal distribution and with a zeta potential value of - 19.13±0.38 mV Figure 6.

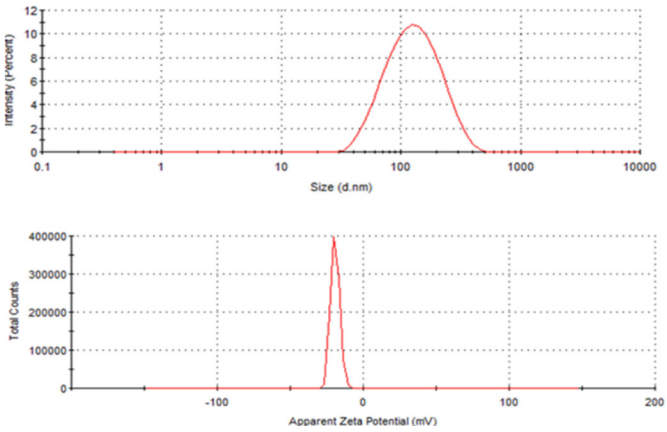

Figure 6: Droplet size distribution and zeta potential of optimized limonene-based SNEDS (formula A10).

Storage stability testing

Limonene-based SNEDS didn't show any change in its visual appearance upon storage, there was no precipitation or flocculation or phase separation. Globule size, PDI, zeta potential and percentage transmittance remained stable at both 4±2°C and 0±2°C within three months storage period; however storage at room temperature (25±2°C) didn't favor the stability of the formulated limonene-based SNEDS as size and PDI increased from 113.6 ±1.18 nm and 0.211±0.007 to 167.73± 29.44 nm and 0.88 ±0.01, respectively as illustrated in Figures 7 and 8.

DISCUSSION

Self-nanoemulsifying drug delivery system is widely used in the pharmaceutical field. This system safely and effectively increases drug bioavailability.[17] Natural components have received great interest due to their low toxicity, pharmacological activity and economical availability.[18]

In the current study, three non-ionic surfactants were of HLB in the range of 12-16 and such values resulted in the formation of a stable o/w nanoemulsion. Aqueous dispersions with high transmittance were

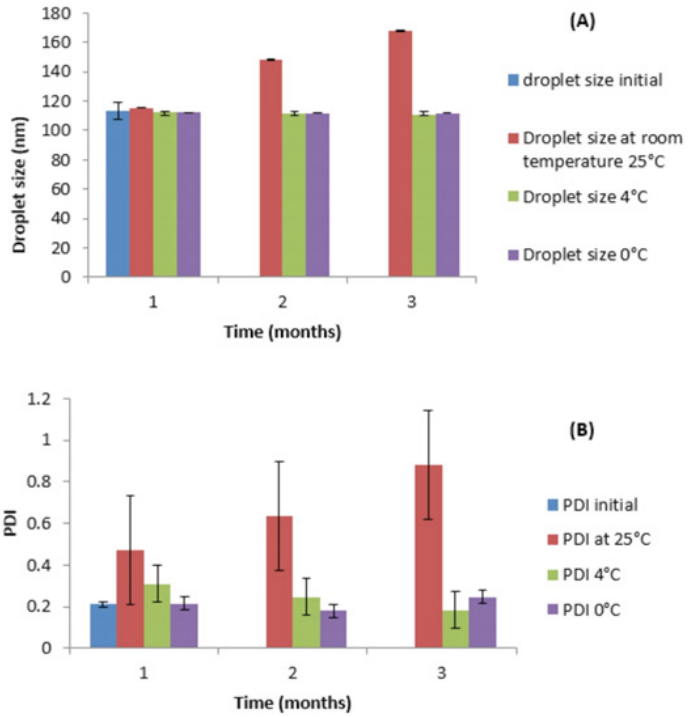

Figure 7: Droplet size (A) and PDI (B) of limonene-based SNEDS for three months storage period at different temperatures.

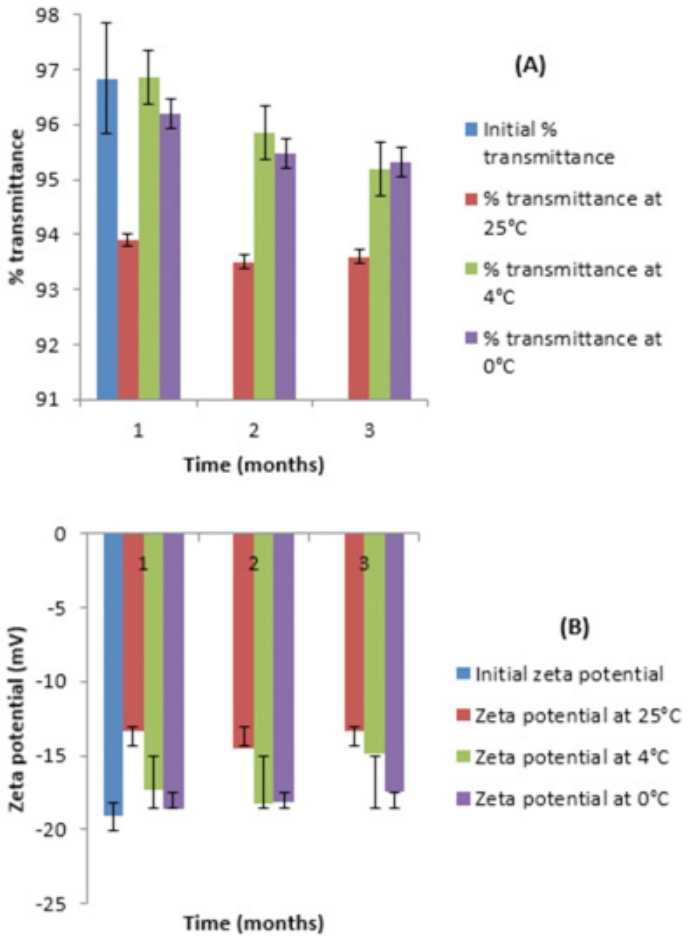

Figure 8: Percentage transmittance (A) and zeta potential (B) of limonene-based SNEDS for three months storage period at different temperatures.

classified as optically clear and oil droplets were assumed to be in a nanodispersion state.[19] Tween®80 exhibited the highest emulsification power compared (p-value <0.0001) thus it was selected for the formulation of the SNEDS.

Addition of co-surfactants is believed to enhance the emulsification ability of the surfactant, reduces its concentration, as well as providing more stability.[20] Besides, co-surfactant decreases the bending stress of the interface and enhances its flexibility and allows its occurrence at different curvatures.[21] The highest emulsification ability was reported for propylene glycol followed by PEG400. Although the addition of co-surfactant didn't increase the percent transmittance of the dispersions, yet the incorporation of short-to medium chain co-surfactants enhanced nanoemulsion region, stability and obtained nanoemulsion at low surfactant concentration.[22] On the pseudoternary diagram, the shaded region at ratio 4:1 was the nanoemulsion region indicating a good self-nanoemulsifying capability. This could be due to the incorporation of co-surfactant and its effect on the penetration of the oil phase into the hydrophobic region of the surfactant which decreased the interfacial tension and increased fluidity of the interface and the system entropy as previously reported by Akhtar *et al.*[23] Thus, formula A10 with 50% w/w limonene, 40% w/w Tween®80 and 10% w/w propylene glycol was selected.

After dilution, the formulation showed a clear bluish to transparent solution. This result was in accordance with that reported by Gaikwad.[24] The purpose of the determination of self-emulsification time is to study the efficiency of nanoemulsion formation.[16] With a simple agitation at 37±0.5°C, the self-emulsification time of limonene-based SNEDS was 37 sec and the nanoemulsion was grade (A). The SNEDS was subjected to several folds of dilutions with different media, aiming at mimicking the *in-vivo* conditions that the formulation might encounter.[16] The tested dilutions showed no signs of precipitation, separation or cloudiness for 24h except for one with deionized water that showed slight creaming which was reversible with simple flask inversion. This creaming doesn't strike a problem since nanoemulsions with their small droplet size are subjected to Brownian motion rather than gravitational forces. An analogous phenomenon was observed by Klang *et al.*[25] This stability against pH change is mainly attributed to the presence of non-ionic surfactants.[26] Cloud point temperature study should always be tested at temperature above 37°C corresponding to the body temperature. The turbidity can be explained by the dehydration of the polyethylene oxide group in a non-ionic surfactant causing phase separation and a drop in the percentage transmittance. Limonene-based SNEDS was stable up to 72°C.

Transmission electron microscopy illustrated the spherical shape of the nanodroplets with diameter less than 200 nm indicated well packed and stable interfacial film and thus lower surface tension and rapid absorption of drug. Similar results were reported by Kazi *et al.* where droplet size was found to be around 150 nm having spherical shape.[27]

Another crucial parameter is polydispersity index (PDI), which reflected positively on formulation physical stability. Our results were less than 0.5 which indicates uniformity of droplet size distribution of limonene within the formulation and affirms its homogeneity.[28] Zeta potential reflects the degree of repulsion between adjacent, similarly charged particles, high zeta potential assures colloidal stability. The formulation had zeta potential value of -19.13±0.38 mV. The obtained results are in agreement with Dou *et al.* who prepared stable brucine SNEDS formulation with a zeta potential value of -15.76±0.42 mV.[29]

The main advantage for using SNEDS is its stability, where the small size of the droplets act as Brownian particles and don't interact with each other, thus providing stability for months. Limonene-based SNEDS didn't change in visual appearance upon storage at different temperatures. However, it didn't favor the storage at room temperature due to the movement of the dispersed droplet through the continuous phase

increasing the opportunity for droplet collisions. This change in the physical characteristics is due to temperature. It also affects film compressibility by changing the solubility of the surfactants in the bulk phase. These results are in agreement with those of Eid *et al*. where the olive oil SNEDS stored at 4, 25 and 40°C for six months, showed good stability at 4°C compared to 25°C and 40°C.[30]

CONCLUSION

In the current investigation, limonene-based SNEDS was developed using low energy emulsification method. The optimized formulation was characterized by the smallest droplet size (113.3 nm), with a unimodel size distribution indicated by the lowest PDI (0.211), highest percentage transmittance (92.57%) and optimal zeta potential (-19.13 mV). The SNEDS preparation was robust to different media and volumes of dilutions. The preparation was stable during the three month of storage at temperature 0 and 4°C, without showing any signs of phase separation, precipitation or flocculation. The data accumulated from this chapter suggested that self-nanoemulsifying drug delivery systems are potential tool for liquid lipophilic components such as essential oils. A liquid formulation such as SNEDDS is probably more suitable to be used for essential oil to enhance its stability and patient acceptability.

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CONFLICT OF INTEREST

The author reports no conflict of interest.

ABBREVIATIONS

GRAS: Generally recognized as safe; **HCl:** Hydrochloric acid; **HLB:** Hydrophilic lipophilic balance; **IL-6:** Interleukin 6; **MIC:** Minimum inhibitory concentration; **O/W:** Oil in water; **%w/w:** Percentage weight per weight; **PEG:** Polyethylene glycol; **PDI:** Polydispersity index; ***p*-value:** Probability value; **rpm:** Round per minute; **SNEDS:** Self-nanoemulsifying delivery system; **SNEDDS:** Self-nanoemulsifying drug delivery system; **TEM:** Transmission electron microscopy; **TNF-α:** Tumor necrosis factor alpha.

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