

RESEARCH ARTICLE

Lemon Essential Oil Nanoemulsion containing Ascorbyl Tetraisopalmitate: Investigating the Relationship Between Surfactant HLB and Size

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ABSTRACT

Vitamin C (Vit-C) and lemon essential oil have several notably benefits for human skin physiology such as stimulating the biosynthesis of collagen, reduce acne and dark spot. But, they poorly bypass the epidermis stratum corneum barrier. Also, lemon essential oil has high vapor pressure. To address these challenges, our aim was preparing and size optimizing a lipophilic version of Vit-C (Ascorbyl tetraisopalmitate) and lemon essential oil by encapsulation of them inside of nanoemulsion droplets (used as oil phase of nanoemulsion), for improving skin diffusion and delivery across the stratum corneum cells. Lemon essential oil was characterized with gas chromatography-mass spectroscopy. Multiple regression analysis was used for obtaining of the HLB required of mix Lemon essential oil and Vit-C. The results revealed that the concentration ratio of Tween 80 to Tween 85 is important for achieving nano-range particle size with good stability. Particle size was determined by Dynamic light scattering method. FT-IR showed encapsulation of Vit-C and lemon essential oil inside of nanoemulsion droplets. The pH of final formulation (F1) was 4.8. Current study indicated that the stable lemon essential oil nanoemulsion contain Vit-C is a good candidate for increasing dispersity and protection of lemon essential oil and Vit-C in water.

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INTRODUCTION

Vitamin C (Vit-C) is an essential micronutrient for humans [1] that has several notably benefits for human skin physiology such as stimulating the biosynthesis of collagen, reduce acne and dark spot [2, 3]. Vit-C is one of the main promoters of collagen formation. The main cutaneous collagens are types I and III. These types of collagens are less synthesized with aging. Vit-C is a potent antioxidant and a cofactor for a family of biosynthetic and gene regulatory enzymes that induce collagen synthesis (e.g., lysyl and prolyl hydroxylase) [1, 3-5]. In addition, Vit-C can block melanin production and

improved hyperpigmentation [6].

Due to instability of ascorbic acid and first pass effect, oral supplementation of Vit-C is not suitable delivery dosage form to increase concentration in target tissue (e.g., skin tissue) [7]. Also, as a topical solution of Vit-C, passing through the epidermis stratum corneum barrier is poor [2]. Because the stratum corneum cells at skin tissue are closely arranged, it is difficult for the water-soluble type of Vit-C to skin penetrate. Delivery through the stratum corneum cells prefer hydrophobic substances [6]. Also, water soluble of Vit-C has poorly stable and easily oxidized to dehydroascorbic

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acid, and subsequently lose its activity [6]. Oil soluble of Vit-C is more stable than water soluble form. Encapsulation of the fat-soluble type of Vit-C inside of surfactant barrier in nanoemulsion can prevent oxidation by oxygen.

There are various commercial formulations on the market that used oil soluble of Vit-C [8]. Formulations containing esters ascorbyl tetraisopalmitate (ATIP) or tetrahexyldecyl ascorbate at concentrations of 2 to 3% produced commercially by several companies. ATIP is approved as a quasi-drug active in Japan at 3% concentration [9]. ATIP as a functional ingredient for skin brightening at 2% concentration is registered in Korea [9]. So, in this study, 2% concentration of ATIP was selected. ATIP as highly stable form of Vit-C has high efficiency for radiance (whitening), wrinkles (anti-oxidant) and softness (collagen level) of the skin [10].

Previous studies showed that lemon (*Citrus limon* L.) essential oil has antioxidant effect [11, 12], antifungal [13], antibacterial [14], and antiviral [15] activities. Our previous studied showed that ointment of lemon essential oil has very effective active ingredient for induce collagen synthesis [16]. Poor dispersity and highly vapor-pressure of lemon essential oil are two mainly drawback of it for application in formulation. By encapsulation it inside nanoemulsion particles, can be overcome on these challenges.

In the most topical delivery system of antioxidant whitening ingredients (e.g., Vit-C, Kojic acid and so on) through the skin barrier, the three factors of stratum corneum breakthrough, epidermal penetration and dermal deposition are essential to be effectively done to inhibition of melanin production [6]. One of the best carriers for transfer therapeutic agent across the stratum corneum cells is the nanoemulsion drug delivery systems.

Nanoemulsions (NEs) are two immiscible liquids (as liquid and oil phase) that utilized an appropriate surfactant(s) for stabilization of systems [17, 18]. Nanoemulsions with smaller size are transparent that are different from microemulsion or coarse emulsion with milky white color [19]. Their long-term physical stability is related to apply higher surfactant concentration than microemulsion and select a proper HLB (according to the HLB required of oil(s)), which impairs conventional destabilization phenomena like creaming, sedimentation and coalescence

[20]. Oil in water nanoemulsion (NE) capable to solubilize very hydrophobic agent (e.g., ATIP) [17, 21] that significantly increase the rate of drug dissolution and systemic bioavailability [22].

Oil phase of nanoemulsion, as carrier, is excellent candidate for hydrophobic drugs [21]. Oils or essential oils of herbal plants, as oil phase of nanoemulsion, can provide an additional property such as antioxidant effect, antibacterial activity or so on.

Use Vit-C and lemon essential oil as the oil phase of nanoemulsion have several benefits such as:

1- It protect of Vit-C (Ascorbyl tetraisopalmitate) and lemon essential oil from oxidation by encapsulation of them inside the surfactant barrier of nanoemulsion droplets.

2- Achieved very good dispersion of oily active ingredient such Ascorbyl tetraisopalmitate and lemon essential oil in water.

3- Lemon essential oil and Vit-C have many benefits, for examples skin anti-aging activity [23, 24], induce increasing collagen synthesis [3, 16] and reducing tissue bacterial infection [16, 25].

HLB of surfactant effect on nanoemulsion size and stability. The HLB required of oil needed for determination of proper surfactant. In some formulations, when two or more oils are mixed, the HLB required of final oil mixture determines according to oil mixing ratio. Also, HLB of more than one surfactant determine with a mixing ratio of them. For achieved desire HLB related to the HLB required of oil mixture, two surfactants with different HLB can be used. Select exact the HLB required is essential to achievement nanoemulsion with lower particle size and stable solution. In this study, we investigated and optimized surfactant ratio for adjusted the HLB of formulation to close to HLB required of Lemon essential oil nanoemulsion contained vitamin C (CLONE).

Our aim was the design and development of Lemon essential oil nanoemulsion for protecting Vit-C from harsh environmental condition along with increasing deeply delivery to epiderm and derm of skin tissue.

MATERIALS AND METHODS

Materials

Lemon essential oil was obtained from Barij Essence Pharmaceutical Co. (Kashan, Iran). Vitamin C (Ascorbyl Tetraisopalmitate) was purchased from Nikko Chemical Co., LTD (Tokyo,

Japan). Polysorbate 80 and Polysorbate 85 (Tween 80 and Tween 85) and other chemical materials were obtained from Merck Chemicals Co. (Darmstadt, Germany).

Characterization of lemon essential oil

Gas Chromatography

Gas Chromatography-Mass spectrometry (GC-MASS) instrument (GC 6890) was applied for determination of the most constituents of Lemon essential oil, according to the method described in our previous study [21]. A GC-MASS with a BPX5 column (30 m×250 µm inner diameter and film thickness of 250 nm) was applied for separation of Lemon essential oil compounds. A Mass Spectrometer (model MS 5973N) was coupled to gas chromatography device for detection of constituents. The initial oven temperature was set to 50°C for 5 min before injection, and increase to 240°C gradually at a rate of 3°C/min. Then, the temperature was increase immediately to 300°C at a rate of 15°C/min and maintained for 3 min at this temperature. Helium gas (99.999%) was applied as carrier gas with a flow of 0.5 mL/min. The split ratio was 1:35 and the injector temperature was set at 250°C. Fragment mass was recorded at 70 eV ionization energy at 40 to 500 m/z with a scan time of 1 s.

Refractive index

Refractive index was measured using an Abbetype refractometer (ATAGO, Berlin, Germany). Briefly, 3 milliliters of lemon essential oil were applied on stage of the refractometer and refractive index was determined at room temperature (25 °C).

Preparation of Lemon oil nanoemulsion contained Vitamin C

Lemon essential oil nanoemulsion contained Vitamin C (CLONE) was prepared by the low-energy method. For preparation of CLONE,

Lemon essential oil and Vit-C as the oil phase, Tween 80 and Tween 85 as surfactant mixture and distilled water as the aqueous phase were applied for preparation of formulations.

Briefly, 100 µl of Vitamin C (ATIP) and 100 µl of Lemon essential oil were mixed together by stirrer at 200 RPM at room temperature. Then, 550 to 1250 µl surfactants were added, and up to 100 % (v/v) by distilled water to final volume solution (5000 µl). Final solution, stirred at 800 RPM for 60 minutes.

HLB Optimization

A general regression analysis was applied for obtaining an equation for optimization of HLB by Minitab[®] 21.4.1 (64-bit) software, v. 8 (Mathworks Ltd., USA). In this study, 12 experiments (Table 2) with two independent factors (concentration of Tween 80 and Tween 85) were used (Table 1). To decrease the complexity of preparation condition, concentrations of Lemon essential oil and Vit-C along with stirrer time and speed were applied as fixed factors. Also, water has no effect on HLB, so it was not considered as an influencing parameter.

The following independent formulation parameters were taken into account (see Tables 1 and 2):

C1 (Independent Factor): Tween 85 (% v/v)

C2 (Independent Factor): Tween 80 (% v/v)

C3 (Dependent Factor): Particle size (nm)

Construction of Pseudo-ternary phase diagram

To determination of the region of nano- and microemulsion size of formulations, as visually, 12 samples were prepared. Unlike conventional pseudo-ternary phase diagram, Tween 80, Tween 85 as surfactants, and distilled water as aqueous phase were used as variable parameters due to indicated HLB impact. The pseudo-ternary phase diagram was obtained by Statistica[™] software, v. 10 (Humborg, Germany).

Table 1. Lemon essential oil constituents was listed.

No.	Retention Time [min]	Peak Area [%]	Components	KI Type	KI Adams	Type of Materials
1	11.38	3.38	α-Pinene	935	939	MH
2	13.46	2.83	Sabinene	976	975	MH
3	13.74	16.46	β-Pinene	982	979	MH
4	14.28	1.18	Myrcene	993	991	MH
5	16.30	1.80	o-Cymene	1032	1026	MH
6	16.57	58.30	Limonene	1037	1029	MH
7	17.99	10.95	γ-Terpinene	1065	1060	MH
8	27.15	1.10	Z-Citral	1251	1238	MO
9	28.58	1.61	Geranial	1281	1267	MO

Table 2. Coefficients

Term	Coef	SE Coef	T-Value	P-Value
Constant	-1900	877	-2.17	0.067
Tween 80	10.46	1.08	9.70	0.000
Tween 85	-3.79	4.50	-0.84	0.428
Tween 85*Tween 85	0.04607	0.00713	6.46	0.000
Tween 80*Tween 85	-0.03822	0.00478	-8.00	0.000

Stability test

The accelerate stability tests including, heating-cooling three cycles (12 h), and freeze-thaw three cycles (12 h), and also, centrifugation at 14000 RPM for 30 min was carried out.

F1 formulation was evaluated visually for turbidity, flocculation, phase separation and creaming. Also, F1 formulation store one month at incubator at 40°C.

Particle size

The dynamic light scattering (HORIBA, SZ-100, Japan) technique was applied for the determination of hydrodynamic diameter of formulations at room temperature. The average particle size (d50) was taken as particle size of samples. Also, Zeta Potential was determined with this device.

pH measurement

pH of F1 formulation was determined by a pH meter (ZAG CHEMIE Co., Tehran, Iran) at room temperature.

Fourier Transform Infrared Spectroscopy

Lemon essential oil, Vit-C, micelle (nanoemulsion without lemon essential oil and Vit-C) and CLONE were analysed via Fourier Transform-Infrared Spectroscopy (FT-IR) in the spectroscopic range of 400-4000 cm^{-1} (Thermo Nicolet Avatar 370, USA) with the potassium bromide (KBr) disk method.

Statistical analysis

Statistical analyses and general regression analysis were done by Minitab[®] 21.4.1 (64-bit) software, v. 8 (Mathworks Ltd., USA). Parameter effect of surfactant HLB on the size of formulations was analysis by Statistica[™], v 10 (Humborg, Germany).

RESULTS AND DISCUSSION

Refractive index Lemon Essential oil

Refractive index of Lemon essential oil was 1.4720 at 25°C by reflectometer.

Lemon essential oil characterization

Identified compounds in Lemon essential oil using GC-MASS analysis are listed in Table 1. The results from GC-Mass showed that Limonene (58.30%), β -Pinene (16.46%), γ -Terpinene (10.95%), α -Pinene (3.38%) and Sabinene (2.83%) were the five major ingredients.

Multiple Regression for HLB Optimization

The size of all formulations was listed in Table 3. Multiple regression mathematic method was applied for the HLB required optimization of CLONE formulation.

In this study, the X variables are the Tween 80 and Tween 85, and also, the response variable is the size. According to our aim, we have determined that we want optimization of HLB to obtained minimize the size and find a mathematical function to forecast size based on HLB by adjusting the concentrations of two independent variables (Tween 80 and Tween 85 concentrations).

Figure 1 shows that regression model is statistically significant ($p < 0.001$) and has an R-squared value of 96.13%. The comments section demonstrated that variables were included in the model. In this case, after optimization the model includes concentrations of Tween 80 and Tween 85, along with one polynomial term Tween 85² and one interaction term Tween 80*Tween 85.

The effects report in Figure 2 graphically shows all of interaction and main effects that are in the regression model. The lines are curved when there exists a polynomial term to fit a curve. In this model, the Tween 80*Tween 85 interaction is significant. This result demonstrate that the effect one variable has on size depends on the volume of the other variable. If Tween 85 is set at a low volume (135 ml), increasing Tween 80 increases the size. Also, when Tween 85 is at a high volume (500 ml), increasing Tween 80 volume reduces the size.

From tables coefficients (Table 2) and analysis of variance (Table 3), the terms Tween 80, Tween80² and Tween 80*Tween 85 have significant ($p < 0.05$). These results indicate that both predictors have a

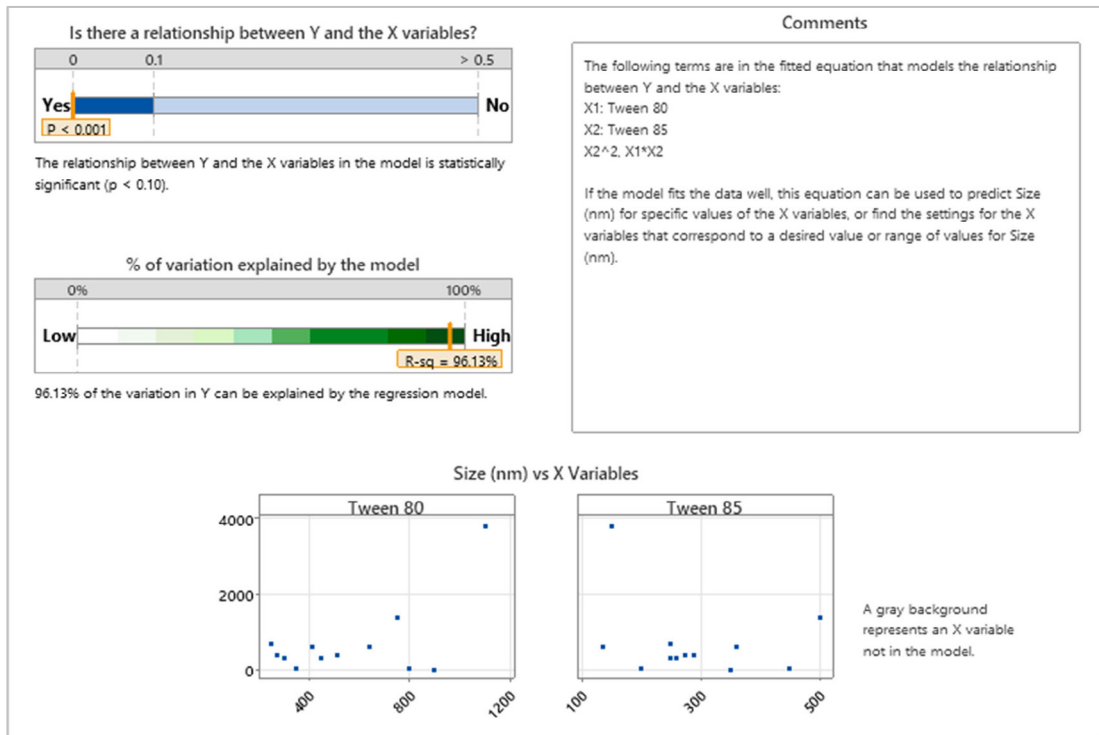


Fig. 1. Effect of Tween 80 and Tween 85 on the particle size.

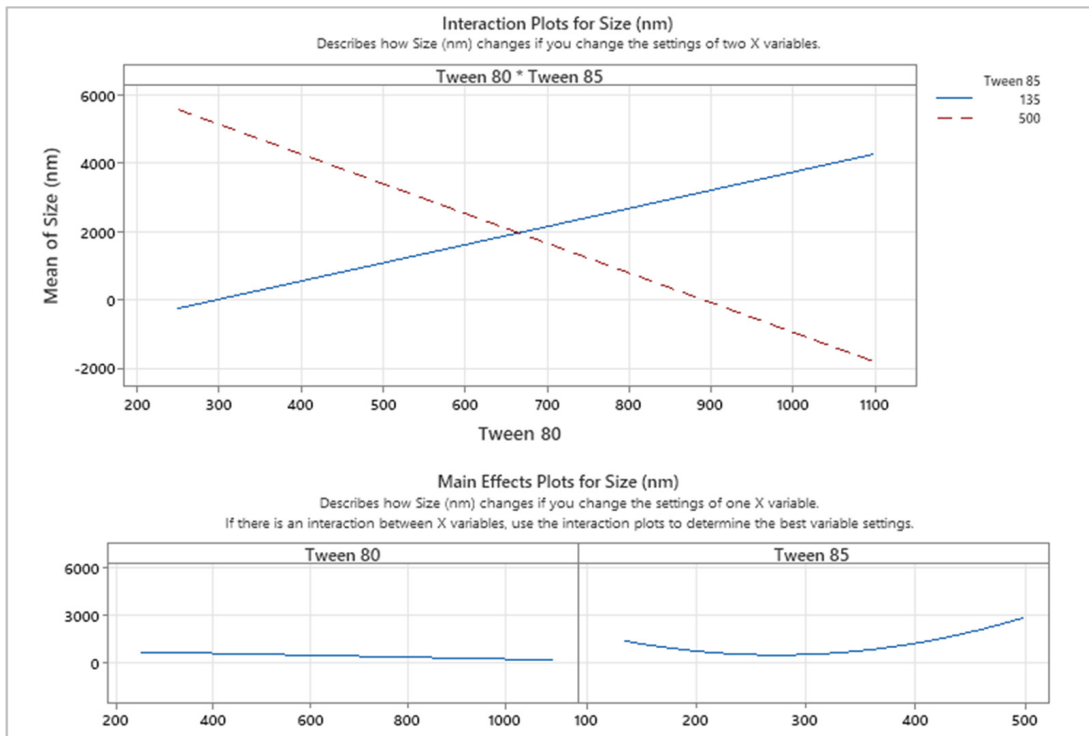


Fig. 2. Interaction plots (up) and main effects plots (bottom) for particle size was shown.

statistically significant effect on size. But, in case of Tween 85, p-value is greater than 0.05, which indicates there is not enough evidence to conclude that the term Tween 85 is related to the response. R^2 and R^2 (adj) values are 96.13% and 93.92% (Table 4), respectively, that indicate the model provides (regression equation) an adequate fit to the data (Table 5).

The Pareto chart (Figure 3) shows that the effects in terms Tween 80, Tween 80* Tween 85 and Tween 80² are statistically significant (p<0.05). Tween 80 has the largest effect due to it extends the farthest. The effect of Tween 85 is the smallest due to it extends the least.

Normal probability plot (Figure 4) shows the points generally follow a straight line that we can accept the assumption that the residuals are normally distributed.

The residuals versus fits (Figure 5) demonstrate the points fall randomly on both sides

of 0, with no recognizable patterns in the points and we can accept that residuals are randomly distributed and have constant variance. The residuals versus order plot illustrations no trends or patterns in time order. Thus, the residuals are independent from one another.

Regression Equation

$$\text{Particle Size (nm)} = -1900 + 10.46 \text{ Tween 80} - 3.79 \text{ Tween 85} + 0.04607 \text{ Tween 85} * \text{Tween 85} - 0.03822 \text{ Tween 80} * \text{Tween 85}$$

As Figure 6 shown, the best zone for achieving particle size at the nano-range is where volume of Tween 80 was twofold of Tween 85 (e.g., Tween 80: 400 ml compare to Tween 85: 200ml). So, estimated HLB for mix Lemon essential oil with Vit-C is around 13.5 to 14.

Pseudo-ternary phase diagram study

Ternary phase diagram (Figure 7) consists of

Table 3. Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression	4	11397144	2849286	43.47	0.000
Tween 80	1	6170169	6170169	94.13	0.000
Tween 85	1	46482	46482	0.71	0.428
Tween 85*Tween 85	1	2732599	2732599	41.69	0.000
Tween 80*Tween 85	1	4194506	4194506	63.99	0.000
Error	7	458828	65547		
Total	11	11855973			

Table 4. Model Summary.

S	R-sq	R-sq(adj)
256.021	96.13%	93.92%

Table 5. Fits and Diagnostics for All Observations.

Obs	Tween 80 (µl)	Tween 85 (µl)	Size (nm)	Fit	Resid	Std Resid
F1	350	200	18	168	-150	-0.67
F2	300	250	320	301	19	0.08
F3	415	135	626	625	1	0.00
F4	275	275	386	526	-140	-0.65
F5	250	250	698	256	442	1.95
F6	800	450	20	326	-306	-1.48
F7	640	360	627	590	37	0.16
F8	510	290	386	554	-168	-0.72
F9	450	260	320	461	-141	-0.59
F10	900	350	13	-214	227	1.73
F11	750	500	1390	1229	161	1.33
F12	1100	150	3780	3763	17	0.41

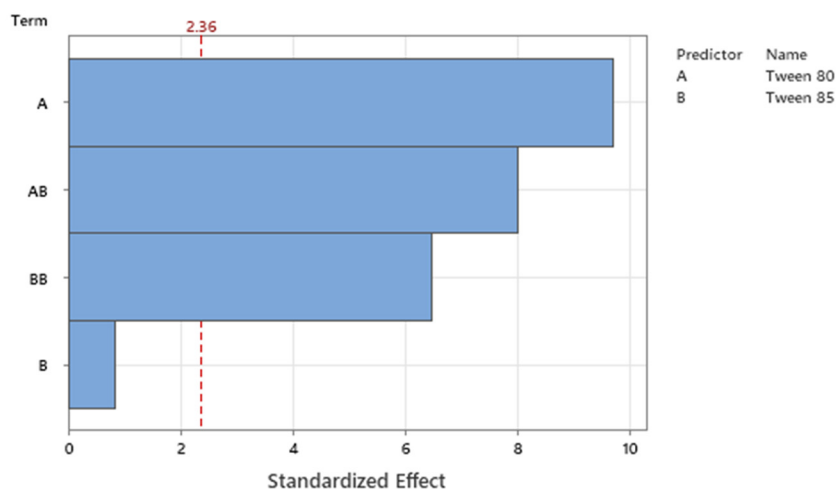


Fig. 3. Pareto chart of the standardized effects was shown. A: Tween 80, and B: Tween 85 concentrations (response is size, $\alpha = 0.05$).

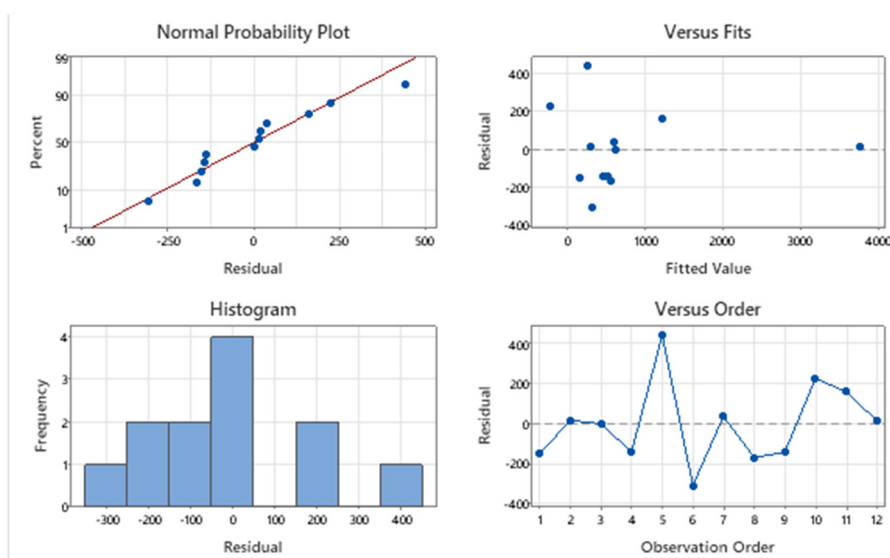


Fig. 4. Normal probability plots for particle size (nm) were shown.

Tween 80, Tween 85 and water with 100% of each component. The blue sphere is stable nano- and microemulsion (Figure 7). According to the ternary phase diagram, it was showed that an appropriate ratio of Tween 80 and Tween 85 concentrations is essential for the formation of stable emulsions due to improve the hydrophilicity of the oil in water phase, and also, reducing the particle size. This data approved by previous studies [26, 27].

Droplet size

Average particle size (d_{50}) of formulations was

determined by DLS (Table 1). Formulation F1, F6 and F10 demonstrated nano- range particle size. F1 was selected as optimized formulation for the next tests. Also, zeta potential of F1 was 0.1 mV that it is because of usage of nonionic surfactant at nanoemulsion formulation.

FT-IR Analysis

To identify the functional groups and present the drug in the formulation, the FT-IR method was used [28]. Figure 8 illustration the FT-IR spectra of Lemon essential oil, Vit-C, free micelle (without

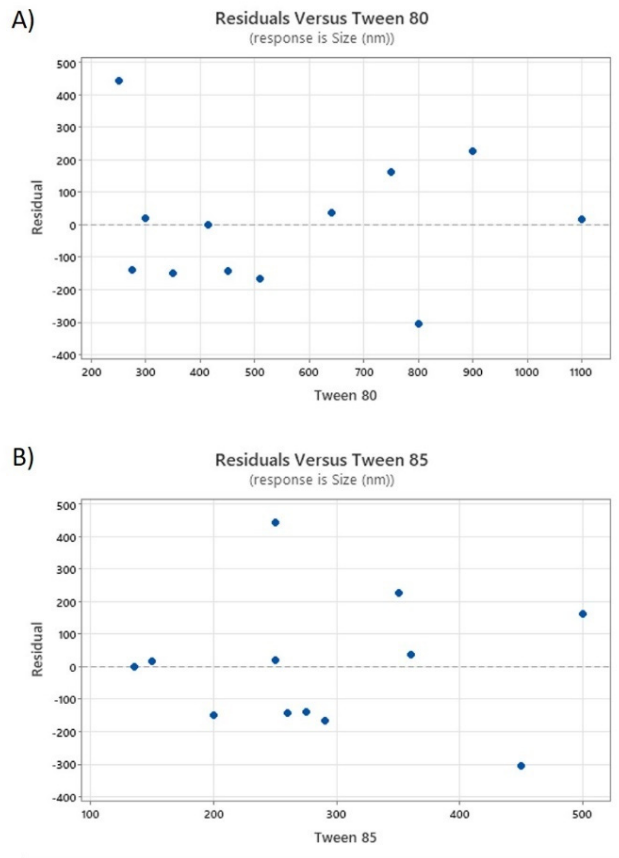


Fig. 5. Residual versus (A) Tween 80 and (B) Tween 85 response to particle size was shown.

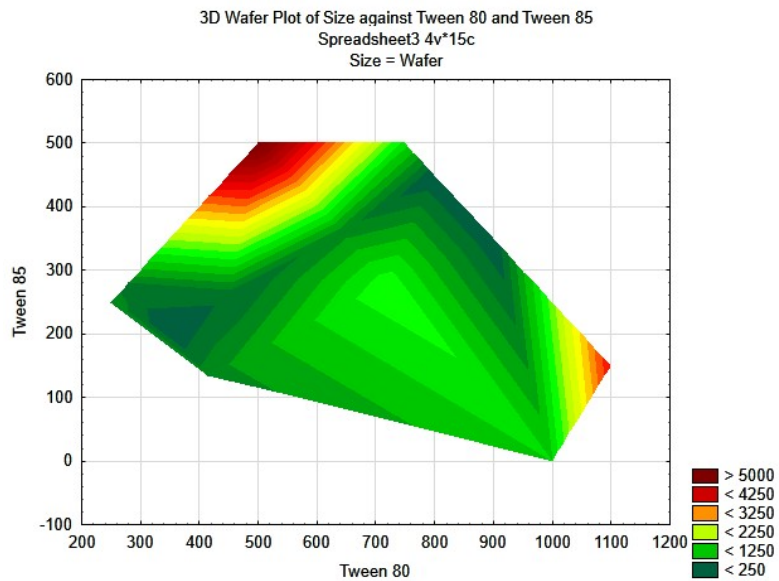


Fig. 6. Effect of different concentrations of Tween 80 and Tween 85 on particle size was demonstrated.

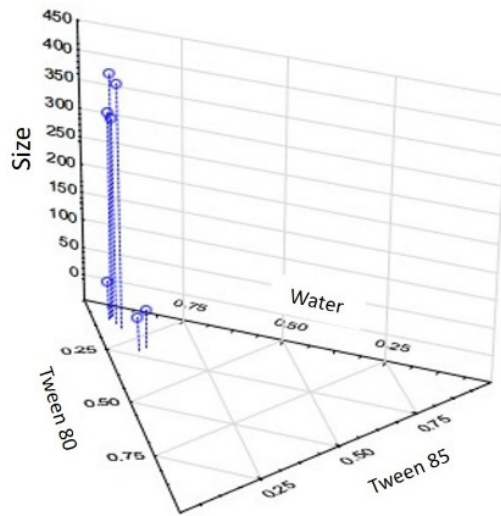


Fig. 7. Pseudo-ternary phase diagram depicting the oil in water nano- and microemulsion region. In this graph, particle size against Tween80, Tween 85 and water was demonstrated.

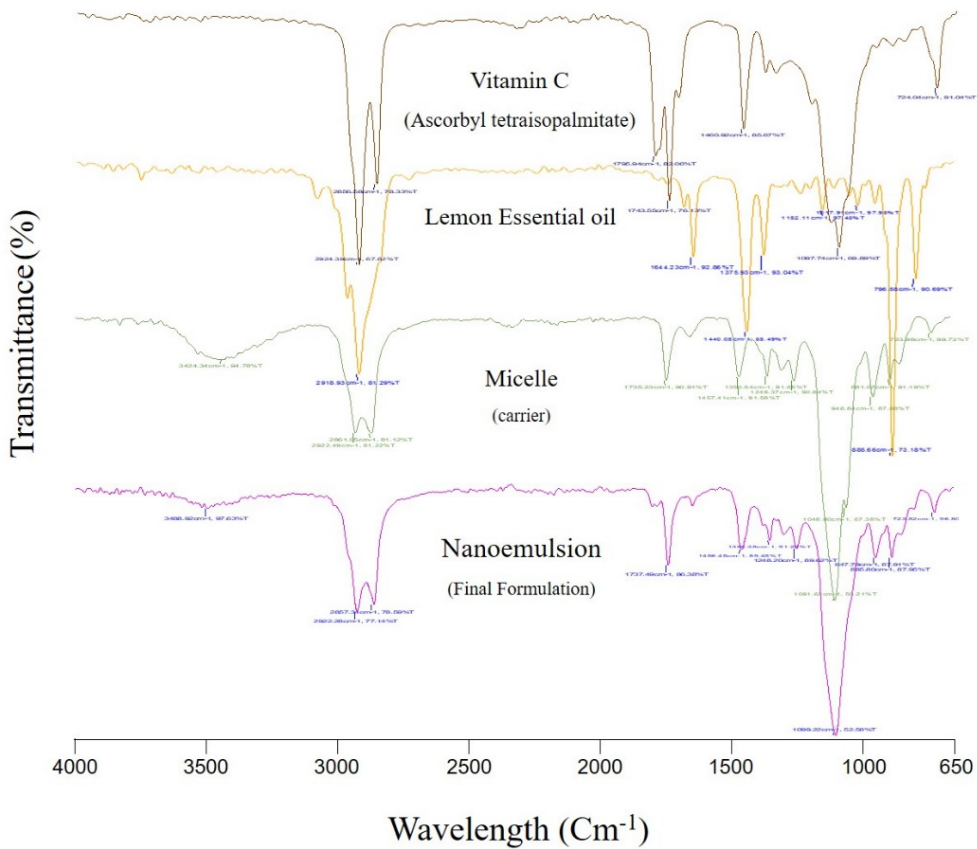


Fig. 8. FT-IR graph of vitamin C, lemon essential oil, Micelle (carrier without vitamin C and lemon essential oil) and Lemon essential oil nanoemulsion contained vitamin C (CLONE).

Lemon essential oil and Vit-C) and CLONE (Lemon essential oil contained Vit-C).

The FT-IR spectrum of Vit-C (ATIP) is demonstrated in Figure 4. The absorption bands in the region of 2924.399 cm^{-1} and 2856.58 cm^{-1} is related to stretching of $-\text{CH}_2$ of Vit-C. The bands in the region of 1795.94 cm^{-1} and 1743.55 cm^{-1} are due to C=O stretching of ester and double bond C=O or C=C stretching mode in ascorbic acid, respectively. The characteristic band of 1460.92 cm^{-1} is due to CH_3 . The absorptions in the regions 1377.67 , 1337.52 , 1201.63 , 1128.92 , and 1097.74 cm^{-1} are due to C-O-C bond in ascorbyl tetraisopalmitate. Also, esterified palmitic acid showed absorption in the region 724.04 cm^{-1} [29].

Lemon essential oil is a mixture system with overlapping absorptions of functional groups [30]. So, the peaks of pure Lemon essential oil at 796.58 (limonene and α -pinene), 885.66 (β -pinene), and 990.63 (γ -terpinene) cm^{-1} were characterized by previous study [31]. Thus, the stretching vibrations of terpenoid components of pure Lemon essential oil were shown at the bands around 1375.93 and 1440.05 cm^{-1} are related to C-H functional group [32]. The peaks of 1017.91 and 1152.11 cm^{-1} is related to C-O stretching vibration of tertiary alcohols [33]. In addition, band peaks of 885.66 cm^{-1} and 796.58 cm^{-1} are corresponded to C=H bending vibration of Alkene) [34, 35].

Moreover, at micelle (final formulation without Lemon essential oil and Vit-C), a broad absorption peaks in the range of $3100\text{--}3700\text{ cm}^{-1}$ are corresponded to O-H of Tween 80 and Tween 85 [36]. Also, strong bands were observed at 2861.05 cm^{-1} and 2922.49 cm^{-1} are attributed to the aliphatic $-\text{CH}_2$ asymmetric and symmetric of the hydrocarbon chain positions in Tween 80 and Tween 85 at formulation [37]. The absorption band at 1735.23 cm^{-1} is ascribed to C=O stretching vibrations, respectively [36]. The band peaks at 1457.41 cm^{-1} and 1646 cm^{-1} are attributed to the framework vibration of O-heterocyclic ring at both Tween 80 Tween 85 [38]. The band at $1,353\text{ cm}^{-1}$ is corresponded to C-H bending in $-\text{CH}_3$ and $-\text{CH}_2$. The band at 1091.62 cm^{-1} is related to the C-O asymmetric stretching vibration in the ester bond (O-C=O). The bands at 881.07 cm^{-1} and 946.84 cm^{-1} are attributed to symmetrical extension vibration of C-O-C in $(\text{CH}_2\text{CH}_2\text{O})_n$ and that at 723.98 cm^{-1} is due to plane vibration of $(-\text{CH}_2-)_n$ ($n>4$) [38].

The CLONE spectrum was very similar to

micelle, due to high concentrations of surfactant mixture (Tween 80 and Tween 85) to compare with low concentrations of Lemon essential oil and Vit-C. However, CLONE demonstrated Lemon essential oil and Vit-C peaks. Increase intensity of peak of CLONE at 2922 cm^{-1} at compare with micelle peak at this region is related to synergic effect of absorbance of Lemon essential oil and Vit-C peaks. The shoulder peak in the region of 1798.00 cm^{-1} of CLONE is related to Vit-C. The adsorption peak below 800 cm^{-1} was weak, similar to the Vit-C spectrum. More, the peaks with weaker intensity than Vit-C between 1099 cm^{-1} and 1644 cm^{-1} were detected [39]. Changes of the adsorption peaks were revealed by comparing the spectra of Lemon essential oil, Vit-C and CLONE that may be related to the simple interaction of functional groups in the CLONE formulation [39].

Stability studies

The accelerate stability tests including, heating-cooling cycles, freeze-thaw cycles and centrifugation tests demonstrated that F1 had good physical stabilities without any visually turbidity, flocculation, phase separation and creaming. Also, F1 formulation showed one-month stability at 40°C .

DISCUSSION

The topical drug delivery systems at compare to orally drug delivery, have revealed great preclinical results for skin problems [40]. It is expected that topical to compare oral drug delivery has lower adverse side effect [41]. Also, it needed to the lower required drug administered dose when it applies topically at compared with orally. So, topically administered drug led to reduce side effect of drug and eliminating first-pass effect.

In this study, a novel topical formulation of Vit-C and Lemon essential oil at nanoemulsion system was developed. The previous studies revealed the improved efficacy of nanoemulsion in topical delivery systems, compared with the bulk form [42]. A possible reason for increasing efficacy in topical delivery based on nanoemulsion is increased skin permeability [42], because of the presence of surfactants and the high surface area of the oil droplets [43]. In this study, formulations were prepared by low-energy method and their size was determined by DLS method.

HLB of surfactants is important for preparation of stable nanoemulsion with nano-range size.

In oil in water (o/w) nanoemulsion systems, the HLB required is higher than 10. For achieved to this value, two surfactants with various HLB were applied usually. HLB of Span 80, Span 20, Tween 85, Tween 80 and Tween 20 is 4.3, 8.6, 11, 15 and 16.7, respectively. The combination of Span and Tween give the milky and cloudy appearance of nanoemulsion. According to previous our work, Tween 80 and Tween 85 were selected for obtaining visually clear nanoemulsion [44].

The results from multiple regression analysis (size versus volume (ml) of Tween 80 and Tween 85 showed that Tween 80 has more effect on size due to the HLB effect (Figures 5). Also, the amount of water does not effect on the size of particles. The o/w nanoemulsion or w/o nanoemulsion types are depended on the continues phase (the base fluid) [21]. At nanoemulsion system, continues phase must be larger than dispersed phase. Here at the o/w nanoemulsion system, water as continues phase, can be not affected on the HLB required of mixed Lemon essential oil and Vit-C.

Droplet size of three formulations (F1, F6 and F10) was in nano-rang size and F1 (as selected formulation) was stable (at least for 30 days) in a dark place. The results showed that the particle size of CLONE decreased with the increase concentration ratio of Tween 80 to Tween 85 (HLB adjustment). So, final HLB is important for size and stability. In this study, the HLB required of formulations was related with the HLB required of lemon essential oil and Vit-C. The calculated HLB of F1 formulation (with nano size and good stability) is 13.54. In previous our study, garlic oil nanoemulsion was prepared by low energy method [18]. For preparation garlic oil nanoemulsion, 20% (v/v) and 5% (v) of Tween 85 and Tween 80 were used, respectively. The HLB required of garlic oil in this formulation was 11.8 [18]. In our previous study, nanoemulsion of *Cinnamomum zeylanicum* essential oil was developed with Tween 80 (HLB required was 15) [45].

In other work, we prepared nanoemulsions of *Mentha piperita* essential oil, *Zataria multiflora* essential oil and nanoemulsion from mixing of them [46]. Results showed that the HLB required of achieving nanometer particles was 16.7.

The physiological pH of the stratum corneum of skin tissue is 4.1 to 5.8 [47]. pH of F1 formulation is 4.8 that is near to pH of skin tissue. Formulations with a pH around 5 are beneficial for the skin due to:

- a) Maintain or even fortify the skin barrier (e.g., stratum corneum),
- b) Support the natural skin flora
- c) Helps to reduce the applicable concentration of preservatives and stabilizes at many cosmetic active ingredients.

So, pH of these formulations is good for topical application.

Tweens are non-ionic surfactants that are widely apply as excipients in oral, topical and injectable formulations [48]. Utilization of polysorbate surfactant (Tween) in pharmaceutical products results in favorable encapsulation efficiency [49]. In addition, the LD₅₀ (abbreviation for “lethal dose, 50%”) of Tweens at drug delivery system as orally is 25 g/kg that showed they are safe and well tolerable [50]. Also, at nanoemulsion formulation system, it provides lower particle size.

The accelerated stability tests, including, heating-cooling and freeze-thaw cycles along with centrifugation showed good physical stability of F1 formulation without phase separation, flocculation, creaming, and turbidity. Also, the long-time stability tests demonstrated that F1 formulation was stable for one month at 25°C in a dark place. More, F1 formulation was stable for one month in an incubator at 40°C. Our previous study is consistent with the results of the present study [17, 18].

CONCLUSION

In nanoemulsion drug delivery systems, the HLB required is important for achieving nano-range particle size with good stability. In the present study, nano-range formulations include F1, F6 and F10 were prepared by low energy method. The results showed that the HLB required of Lemon oil nanoemulsion and Vit-C mixture (determined by the multiple regression method) is 13.54. Among prepared formulations, F1 (as called NOVO EMULEC) was selected for future *in vitro* and *in vivo* studies.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

1. Carr, A.C. and S. Maggini, Vitamin C and Immune Function. *Nutrients*, 2017. 9(11): p. 1211. <https://doi.org/10.3390/nu9111211>
2. Gref, R., et al., Vitamin C-squalene bioconjugate promotes epidermal thickening and collagen production in human

- skin. *Scientific Reports*, 2020. 10(1): p. 16883. <https://doi.org/10.1038/s41598-020-72704-1>
3. Boo, Y.C., Ascorbic acid (vitamin C) as a cosmeceutical to increase dermal collagen for skin antiaging purposes: emerging combination therapies. *Antioxidants*, 2022. 11(9): p. 1663. <https://doi.org/10.3390/antiox11091663>
 4. Lis, D.M. and K. Baar, Effects of different vitamin C-enriched collagen derivatives on collagen synthesis. *International journal of sport nutrition and exercise metabolism*, 2019. 29(5): p. 526-531. <https://doi.org/10.1123/ijsnem.2018-0385>
 5. Boyera, N., I. Galey, and B.A. Bernard, Effect of vitamin C and its derivatives on collagen synthesis and cross-linking by normal human fibroblasts. *International Journal of Cosmetic Science*, 1998. 20(3): p. 151-158. <https://doi.org/10.1046/j.1467-2494.1998.171747.x>
 6. Lv, X., Z. Wu, and X. Qi, High skin permeation, deposition and whitening activity achieved by xanthan gum string vitamin c flexible liposomes for external application. *International Journal of Pharmaceutics*, 2022. 628: p. 122290. <https://doi.org/10.1016/j.ijpharm.2022.122290>
 7. Rozman, B. and M. Gasperlin, Stability of vitamins C and E in topical microemulsions for combined antioxidant therapy. *Drug delivery*, 2007. 14(4): p. 235-245. <https://doi.org/10.1080/10717540601067786>
 8. diligent, s. vitamin c serum in oil. 2023 [cited 2023 August 4]; Available from: <https://skindiligent.com/products/vitamin-c-serum-in-oil>
 9. Bastianini, M., M. Sisani, and A. Petracci, Ascorbyl Tetraispalmitate Inclusion into γ -Cyclodextrin and Mesoporous SBA-15: Preparation, Characterization and In Vitro Release Study. *Cosmetics*, 2017. 4(3): p. 21. <https://doi.org/10.3390/cosmetics4030021>
 10. Jeon, J.-S., et al., Simultaneous Detection of Glabridin, (-)- α -Bisabolol, and Ascorbyl Tetraispalmitate in Whitening Cosmetic Creams Using HPLC-PAD. *Chromatographia*, 2016. 79(13): p. 851-860. <https://doi.org/10.1007/s10337-016-3104-2>
 11. Lin, L.-Y., et al., Lime (*Citrus aurantifolia* (Christm.) Swingle) Essential Oils: Volatile Compounds, Antioxidant Capacity, and Hypolipidemic Effect. *Foods*, 2019. 8(9): p. 398. <https://doi.org/10.3390/foods8090398>
 12. Misharina, T.A. and A.L. Samusenko, Antioxidant properties of essential oils from lemon, grapefruit, coriander, clove, and their mixtures. *Applied Biochemistry and Microbiology*, 2008. 44(4): p. 438-442. <https://doi.org/10.1134/S0003683808040182>
 13. Białoń, M., et al., The Influence of Chemical Composition of Commercial Lemon Essential Oils on the Growth of *Candida* Strains. *Mycopathologia*, 2014. 177(1): p. 29-39. <https://doi.org/10.1007/s11046-013-9723-3>
 14. Yazgan, H., Y. Ozogul, and E. Kuley, Antimicrobial influence of nanoemulsified lemon essential oil and pure lemon essential oil on food-borne pathogens and fish spoilage bacteria. *International journal of food microbiology*, 2019. 306: p. 108266. <https://doi.org/10.1016/j.ijfoodmicro.2019.108266>
 15. Arena, M.E., M.R. Alberto, and E. Cartagena, Potential use of Citrus essential oils against acute respiratory syndrome caused by coronavirus. *Journal of Essential Oil Research*, 2021. 33(4): p. 330-341. <https://doi.org/10.1080/10412905.2021.1912839>
 16. Valizadeh, A., et al., Preparation and Comparison of Effects of Different Herbal Oil Ointments as Wound-Healing Agents. *Cells Tissues Organs*, 2019. 207(3-4): p. 177-186. <https://doi.org/10.1159/000503624>
 17. Valizadeh, A., et al., Levofloxacin nanoemulsion gel has a powerful healing effect on infected wound in streptozotocin-induced diabetic rats. *Drug Delivery and Translational Research*, 2021. 11(1): p. 292-304. <https://doi.org/10.1007/s13346-020-00794-5>
 18. Keshvadi, M., et al., Comparative study of antibacterial inhibitory effect of silver nanoparticles and garlic oil nanoemulsion with their combination. *Biointerface Res. Appl. Chem*, 2019. 9(6): p. 4560-4566. <https://doi.org/10.33263/BRIAC96.560566>
 19. Valizadeh, A., et al., Increased antibacterial activity of Cinnamon Oil Microemulsion in Comparison with Cinnamon Oil Bulk and Nanoemulsion. *Nanomedicine Research Journal*, 2018. 3(1): p. 37-43.
 20. Yousefpoor, Y., et al., Current challenges ahead in preparation, characterization, and pharmaceutical applications of nanoemulsions. *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology*, 2023. 15(6): p. e1920. <https://doi.org/10.1002/wnan.1920>
 21. Valizadeh, A., et al., Design and development of novel formulation of Aloe Vera nanoemulsion gel contained erythromycin for topical antibacterial therapy: In vitro and in vivo assessment. *Journal of Drug Delivery Science and Technology*, 2022. 74: p. 103519. <https://doi.org/10.1016/j.jddst.2022.103519>
 22. Yu, H. and Q. Huang, Improving the Oral Bioavailability of Curcumin Using Novel Organogel-Based Nanoemulsions. *Journal of Agricultural and Food Chemistry*, 2012. 60(21): p. 5373-5379. <https://doi.org/10.1021/jf300609p>
 23. Bertuzzi, G., et al., Antioxidative action of Citrus limonum essential oil on skin. *Eur. J. Med. Plants*, 2013. 3: p. 1-9. <https://doi.org/10.9734/EJMP/2013/1987>
 24. Mumtaz, S., et al., Aging and its treatment with vitamin C: a comprehensive mechanistic review. *Molecular Biology Reports*, 2021. 48(12): p. 8141-8153. <https://doi.org/10.1007/s11033-021-06781-4>
 25. Mumtaz, S., et al., Evaluation of antibacterial activity of vitamin C against human bacterial pathogens. *Brazilian Journal of Biology*, 2021. 83: p. e247165. <https://doi.org/10.1590/1519-6984.247165>
 26. Bazylińska, U., J. Kulbacka, and K.A. Wilk, Dicapalic ionic surfactants in fabrication of biocompatible nanoemulsions: Factors influencing droplet size and stability. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 2014. 460: p. 312-320. <https://doi.org/10.1016/j.colsurfa.2013.12.042>
 27. Ke, Z., X. Hou, and X.-B. Jia Design and optimization of self-nanoemulsifying drug delivery systems for improved bioavailability of cyclovirobuxine D. *Drug design, development and therapy*, 2016. 10, 2049-2060 DOI: <https://doi.org/10.2147/dddt.s106356>
 28. Gurpreet, K. and S. Singh, Review of nanoemulsion formulation and characterization techniques. *Indian Journal of Pharmaceutical Sciences*, 2018. 80(5): p. 781-789. <https://doi.org/10.4172/pharmaceutical-sciences.1000422>
 29. Yadav, M.G., et al., Production of 6-O-l-Ascorbyl Palmitate by Immobilized *Candida antarctica* Lipase B. *Applied Biochemistry and Biotechnology*, 2018. 184(4): p. 1168-1186. <https://doi.org/10.1007/s12010-017-2610-5>
 30. Ali, H., K.D. Safa, and S. Rezazadeh, Nanoemulsion of *Silybum marianum* Seeds Extract: Evaluation of

- Stability, Antioxidant and Antibacterial Activity. *Colloid Journal*, 2023. 85(2): p. 287-297. <https://doi.org/10.1134/S1061933X22600178>
31. Nunes, J.C., et al., Effect of green tea extract on gelatin-based films incorporated with lemon essential oil. *Journal of Food Science and Technology*, 2021. 58(1): p. 1-8. <https://doi.org/10.1007/s13197-020-04469-4>
 32. Hasani, S., S.M. Ojagh, and M. Ghorbani, Nanoencapsulation of lemon essential oil in Chitosan-Hicap system. Part 1: Study on its physical and structural characteristics. *Int J Biol Macromol*, 2018. 115(115): p. 143-151. <https://doi.org/10.1016/j.ijbiomac.2018.04.038>
 33. Permanasari, A.A., et al., The Potential of Waste Cooking Oil B20 Biodiesel Fuel with Lemon Essential Oil Bioadditive: Physicochemical Properties, Molecular Bonding, and Fuel Consumption. 2021, 2021. 16(3): p. 10. <https://doi.org/10.9767/bcrec.16.3.10493.555-564>
 34. Berechet, M.D., et al., Composition of the essential oil of Rosa damascena Mill. cultivated in Romania. *Rev. Chim*, 2015. 66(12): p. 1986-1991.
 35. Benoudjit, F., L. Maameri, and K. Ouared, Evaluation of the quality and composition of lemon (Citrus limon) peel essential oil from an Algerian fruit juice industry. *Algerian Journal of Environmental Science and Technology*, 2020. 6(4): p. 1575-1581.
 36. Bide, Y., M.A. Fashapoyeh, and S. Shokrollahzadeh, Structural investigation and application of Tween 80-choline chloride self-assemblies as osmotic agent for water desalination. *Scientific Reports*, 2021. 11(1): p. 17068. <https://doi.org/10.1038/s41598-021-96199-6>
 37. Batool, R., S. Ayub, and I. Akbar, Isolation of biosurfactant producing bacteria from petroleum contaminated sites and their characterization. *Soil & Environment*, 2017. 36(1): p. 35-44. <https://doi.org/10.25252/SE/17/20992>
 38. Li, X.-H., et al., Inhibition by tween-85 of the corrosion of cold rolled steel in 1.0 M hydrochloric acid solution. *Journal of Applied Electrochemistry*, 2009. 39(7): p. 1125-1135. <https://doi.org/10.1007/s10800-008-9770-5>
 39. Deng, M., et al., Tea saponins as natural emulsifiers and cryoprotectants to prepare silymarin nanoemulsion. *LWT*, 2022. 156(156): p. 113042. <https://doi.org/10.1016/j.lwt.2021.113042>
 40. Raina, N., et al., New Insights in Topical Drug Delivery for Skin Disorders: From a Nanotechnological Perspective. *ACS omega*, 2023. 8(22): p. 19145-19167. <https://doi.org/10.1021/acsomega.2c08016>
 41. Idrees, N., et al., Hemodialysis for treatment of levofloxacin-induced neurotoxicity. *Hemodialysis International*, 2019. 23(2): p. E40-E45. <https://doi.org/10.1111/hdi.12687>
 42. Ghiasi, Z., et al., Enhancing analgesic and anti-inflammatory effects of capsaicin when loaded into olive oil nanoemulsion: An in vivo study. *International Journal of Pharmaceutics*, 2019. 559: p. 341-347. <https://doi.org/10.1016/j.ijpharm.2019.01.043>
 43. Dragicevic, N. and H.I. Maibach, Percutaneous penetration enhancers physical methods in penetration enhancement. 1 ed. 2017: Springer. <https://doi.org/10.1007/978-3-662-53273-7>
 44. Raffaelli, V., et al., Levofloxacin-induced visual hallucinations: A case report and review of the literature. *J Health Soc Sci*, 2018. 3(1): p. 85-89.
 45. Ghanbariasad, A., et al., Nanoformulating Cinnamomum zeylanicum essential oil with an extreme effect on Leishmania tropica and Leishmania major. *Journal of Drug Delivery Science and Technology*, 2021. 63: p. 102436. <https://doi.org/10.1016/j.jddst.2021.102436>
 46. Osanloo, M., et al., Antibacterial potential of essential oils of Zataria multiflora and Mentha piperita, micro- and nano-formulated forms. *Iran J Microbiol*, 2020. 12(1): p. 43-51. <https://doi.org/10.18502/ijm.v12i1.2517>
 47. Proksch, E., pH in nature, humans and skin. *The Journal of Dermatology*, 2018. 45(9): p. 1044-1052. <https://doi.org/10.1111/1346-8138.14489>
 48. Garidel, P., C. Hoffmann, and A. Blume, A thermodynamic analysis of the binding interaction between polysorbate 20 and 80 with human serum albumins and immunoglobulins: A contribution to understand colloidal protein stabilisation. *Biophysical Chemistry*, 2009. 143(1): p. 70-78. <https://doi.org/10.1016/j.bpc.2009.04.004>
 49. Song, J., et al., Formulation and Evaluation of Celastrol-Loaded Liposomes. *Molecules*, 2011. 16(9): p. 7880-7892. <https://doi.org/10.3390/molecules16097880>
 50. Additives, E.P.o.F. and N.S.a.t. Food, Scientific Opinion on the re-evaluation of polyoxyethylene sorbitan monolaurate (E 432), polyoxyethylene sorbitan monooleate (E 433), polyoxyethylene sorbitan monopalmitate (E 434), polyoxyethylene sorbitan monostearate (E 435) and polyoxyethylene sorbitan tristearate (E 436) as food additives. *EFSA Journal*, 2015. 13(7): p. 4152. <https://doi.org/10.2903/j.efsa.2015.4152>