

Development and evaluation of a cream containing solid lipid nanoparticles loaded with Vitamin E

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Abstract: Solid lipid nanoparticles (SLNs) have several potential applications in the topical drug delivery. The current project aimed to prepare and characterize SLNs loaded with vitamin E for topical administration and incorporating the prepared SLNs in a cream base. Further, the permeation of prepared SLNs was studied through a synthetic membrane and the release profiles were compared with vitamin E cream. The prepared SLNs were subjected to stability studies at two different temperatures. Hot homogenization followed by dilution technique was used for the preparation of SLNs. In this project, PDMS membrane was used to mimic the skin for permeation studies. From the results of this study, it can be concluded that prepared SLNs had enhanced the permeation of vitamin E as compared to vitamin E cream.

Keywords: Solid lipid nano particles, skin permeation, vitamin E, PDMS.

INTRODUCTION

Skin aging is a progressive process that is a combination of intrinsic and extrinsic aging. Several theories are related to the skin aging and the most widely accepted is the free radical theory (Harman, 1956). Free radicals are reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$). They are produced in the human body due to metabolic activities in the body. These radicals are accumulated in our system and due to their reactive sites, they attack the cell membrane, DNA and, proteins resulting in undesirable appearances of lines and wrinkles. With age our body loses the ability to repair its cells and cellular functions are slow down that adds to the aging process. It is believed that anti-oxidative agents can prevent the attack of ROS on the body cells either by scavenging the free radicals or by inhibiting the chain propagation reaction of lipids. Thus, many cosmetic products are being marketed containing anti-oxidative agent like vitamin C or E claiming anti-wrinkles affects. However, the question arises is whether the claimed outcome after actual consumer use of the suggested activity could be demonstrated or no. There are several reasons for it. One of the main reason is the absorption of such active compounds through the skin. The stratum corneum (SC) is a natural barrier of the human skin. It not only protects the human body from extraneous materials and microbes but also poses a difficulty for the absorption of active biological compounds topically. Cosmetic preparations based on new type of carriers systems have a better scope than regular cosmetics as they not only protect the sensitive active ingredient from degradation but also enhances the cutaneous absorption (Hallan *et al*, 2021). Nanoparticles based on lipid systems are the most common type of carrier systems studied for topical

application (Argimón *et al.*, 2016). SLNs are sub-micron colloidal particles ranging from 10 to 1000 nm. They are composed of physiological lipid, dispersed in water or in aqueous surfactant solution. The core of SLN consisted of a lipid compound which remains solid not only at room temperature but also at body temperature. SLNs have certain unique properties such as nano sized particles, large surface area, better drug loading and, the interaction of phases at the interface. These properties make them attractive for their potential to improve the performance of pharmaceuticals (Kaul *et al*, 2018). The current focus of the new carrier systems like solid lipid nano particles is toward dermal applications. SLNs are widely used for cosmetics, since they show many favorable features such as adhesiveness, skin hydration, smoothness, skin penetration enhancement, modified release and protection of actives against degradation (Geszeke-Moritz, 2016). The effects of lipid nanoparticles on skin barrier properties have been well established. It had been reported that SLN forms an invisible, occlusive film with affinity for the stratum corneum (SC). This effect ensures the drug release for a prolonged time. The family of vitamin E consisted of tocopherol and tocotrienols (α , β , γ and δ). Of all these forms α -tocopherol is the biologically active component known. Due to its anti-oxidative properties it is considered as an effective molecule to prevent skin aging and reducing the appearance of lines and wrinkles. However, the solubility of the molecule is a question due to its hydrophobic nature. The compound is sensitive to oxygen, light and heat. A variable bioavailability is also known for vitamin E. The chemical stability can be overcome by using the esterified form of vitamin, α -tocopherol acetate (Reboul, 2017). Additional challenges associated with vitamin E can be overcome by incorporating it into a delivery system. Solid lipid nano particles are a very good candidate to prepare a stable delivery system through the skin with enhanced

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permeation and better stability (Badawi *et al.*, 2020); hence, the project was conceived.

The main objective of the current project was to investigate the feasibility of preparing solid lipid nano particles loaded with vitamin E. Optimization of the formulations and evaluation of the prepared SLNs. Preparation of vitamin E cream. Incorporation of the prepared SLNs into a cream base. Furthermore, to perform permeation studies using PDMS (Poly di-methyl siloxanes) membrane for the SLNs and vitamin E cream. In addition, studying the stability of the prepared SLNs at two different temperatures.

MATERIALS AND METHODS

Vitamin E acetate was bought from Sigma Aldrich, USA. Tween 80 (Sigma Aldrich, USA), oleic acid (Avonchem Ltd., UK), glyceryl mono stearate and glyceryl mono stearate (Amoco silicone, Belgium) and stearyl alcohol (Scharlau Germany) were all available in the college laboratory. All the other solvents and chemicals used were of analytical grade.

Preparation of SLNs

Various lipids were used in the preparation of SLNs including glyceryl mono stearate (GMS), glyceryl mono oleate (GMO), stearyl alcohol (SA) and oleic acid (OA). Similarly, tween 80 was used as the surfactant for the preparation of SLNs. Solid lipid nano particles were prepared by the hot homogenization technique followed by dilution method (Gazi *et al.*, 2018). Composition of SLNs containing surfactant, lipids and concentrations of vitamin E is summarized in table 1. For the oil phase, a weighed quantity of the lipid was heated to 100°C above its melting point and to the melted lipid a weighed quantity of vitamin E was added (Tripodi *et al.*, 2019). The aqueous phase consisted of tween 80 and water. It was heated to the same temperature of the oil phase and the two phases were mixed at the same temperature, water phase was added into the oil phase. The hot emulsion was homogenized for 5 minutes at 14000 (Bhalekar *et al.*, 2017) rpm during this time the emulsion temperature was maintained. Then the same hot emulsion was sonicated (Ultrasonic Homogenizer, 300V/T) for 5 minutes at 80 powers (Fouad *et al.*, 2015). The hot micro emulsion so produced was dispersed in water at room temperature under stirring (100 rpms) for 30 minutes. The volume ratio of the hot micro emulsion to water was 1:10 (Rostami, 2014). The prepared SLPs were refrigerated for 24 hours and then subjected to characterization.

Characterization of SLNs

Particle size (PS) and zeta potential (ZP)

Particle size, polydispersity index and zeta potential of SLNs was determined using the Zeta sizer (Marvern instruments Ltd, UK). Each nano dispersion was diluted

(1-10 ml) with deionized water (El-Housiny *et al.*, 2018) prior to the measurement. The mean particle size and the PDI was determined at the angle of 90°. Zeta potential was determined using the same instrument by measuring the surface charge of the particles. All the values were determined thrice and the average value was noted.

Determination of entrapment efficiency (%EE) & drug loading (%DL)

Entrapment of vitamin E in SLNs was determined using dialysis bag technique by measuring the concentration of the free drug in the dispersion medium (Vinod *et al.*, 2019). A cellulose membrane having a weight cut-off of 3500 dalton was used for this purpose. The membrane was soaked in the dispersion medium (Phosphate buffer 7.4 & absolute ethanol, 50:50) overnight prior to the dialysis. 3 ml of the sample SLNs was placed inside the membrane and it was tightly closed at both ends. This membrane was then soaked in 50ml of dispersion medium in a beaker. The beaker was covered to prevent the evaporation of medium and kept on a mechanical shaker (Sci chem Tech, UK) for 24 hours. Sample was collected from the receptor compartment and it was analyzed. The following equation was used to determine the amount of free vitamin E that had crossed the membrane.

$$EE \% = (W_{\text{initial drug}} - W_{\text{free drug}}) / W_{\text{initial drug}} \times 100$$

Where “W initial drug” is the mass of the initial drug used for the assay and “W free drug” is the mass of the free drug.

Drug loading efficiency (%DL) of vitamin E SLNs dispersion was determined using the following equation
$$\% DL = \frac{\text{Total weight of drug added} - \text{Weight of free drug}}{\text{Weight of lipid}} \times 100$$

Morphology of SLNs

Scanning electron microscopy (SEM) was used to visualize the prepared SLNs. Formulations F4 & F6 were selected for SEM. SEM (S 3700 N Hitachi, Japan) was used to capture images with Back Scattered Electrons (BSE) module at an intensity of 2 to 10 kV using magnification from 100 to 300 X. A drop of the emulsion was deposited on the slide and it was air dried before analysis.

FTIR analysis

Pure vitamin E, lipids, physical mixtures of vitamin E and lipids and, the prepared SLNs dispersions (blank & loaded) were also analyzed by using the FTIR spectrophotometer (Agilent Technologies, Cary 630) to confirm the compatibility between the lipid, vitamin E and selected SLNs formulations.

Permeation studies of SLNs

Permeability of the prepared SLNs was studied using PDMS (Poly di-methyl siloxane) on the Franz diffusion

cell (V3A-02 Peme Gear, USA) for 24 hours. The receptor compartments had a capacity of 5 ml with a diffusion area of 0.64 cm². The recipient compartment contained a mixture of Phosphate buffer pH 7.4 and absolute ethanol in the ratio of 50: 50 which was stirred at 600 rpm and temperature was maintained at 37±0.5°C with a thermostatic water pump (Haake SC 100, Thermo Fisher Scientific USA). The membrane was soaked overnight prior to permeation study in the receptor medium. At predetermined time intervals, 1.5 ml of the medium was removed and replaced by an equal volume of fresh receptor medium. Each experiment was performed in triplicate.

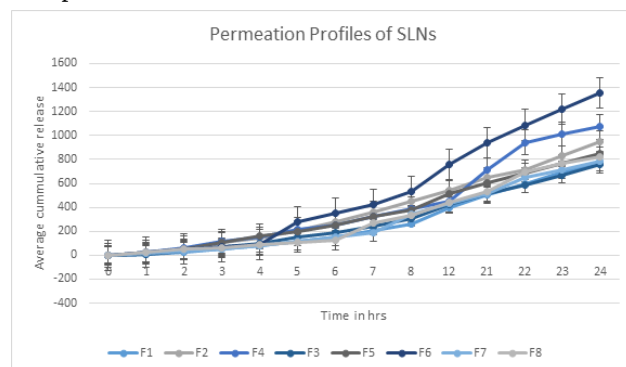


Fig. 1: Permeation profiles of SLNs formulations

Preparation of vitamin E cream

All the required ingredients were weighed accurately and the oil phase and water phase were heated separately to the same temperature before mixing. The generated cream was stirred until a smooth product was obtained at the room temperature.

Drug content estimation of vitamin E cream

The amount of vitamin E per gram was determined using the spectrophotometer (Shimadzu UV-1800, Japan) at 285 nm after suitable dilutions. The cream was sonicated with ethanol to extract vitamin E. The content of vitamin E was determined from the calibration curve.

Permeation studies of the vitamin E cream

For in vitro release study of vitamin E, cream an accurate amount of cream containing vitamin E equivalent to 2.6 mg was transferred on PDMS mounted on the Franz diffusion cell. The study was conducted for 24 hours. The rest of the conditions are the same as described above in the permeation of SLNs. The samples were analyzed using HPLC.

HPLC

In order to determine the amount of vitamin E released the collected samples of permeation studies were analyzed by HPLC (Shimadzu; Model DGU 20A3;SPD 20A;LC20 AD with dell-380 system).The following were the experimental conditions: column (C18 Bondclone-10 um-(LC Column 250 x 4.6 mm),mobile phase (methanol:

water, 8:2 v/v) (Pereira *et al.*,2104), flow rate 0.5 ml/min, and UV detection at 285 nm. The samples were filtered through 0.45 um (Millipore Filter, USA) and injected at the rate of 20uL.

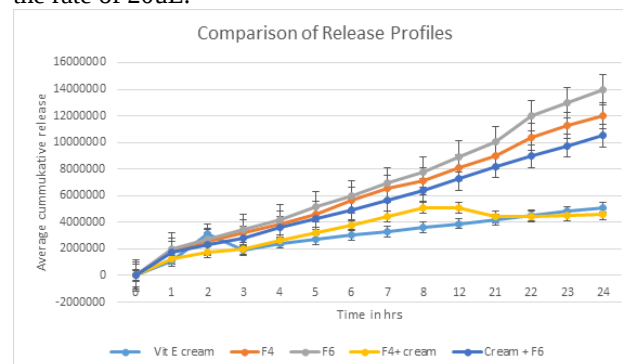


Fig. 2: Comparison of release profiles of vitamin E cream, F4, F6 & corresponding cream formulations.

Stability studies

The prepared SLNs dispersions were stored at room temperature (25°C) and refrigerated as well for stability studies. The particle size and zeta potential of SLNs were measured during this time interval and any other changes were noted.

RESULTS

Particle size (PS) and zeta potential (ZP) analysis

The prepared SLNs appeared from crystal clear to slightly hazy. The particle size of the blank formulations was found to be less than 100 nm. The particle size of the loaded SLNs was found between 40-700 nm. Results can be seen in table 2. It was observed that the loaded SLNs had a larger particle size than blank. This increase in particle size was an indicator of vitamin E incorporation. The results of SLNs analysis had shown a narrow size distribution with a low PDI (table 2). This low PDI indicated mono dispersity in SLNs dispersions. The nano dispersion were prepared with 5%, 7%, 9% and 11% tween 80. It was found that SLNs prepared with 5% tween were not stable even with low concentration of vitamin E. SLNs prepared with 7% and 9% tween had produced nano dispersion with almost similar particle size range so 7% tween 80 was selected due to economic and toxicity reasons. Zeta potential (ZP) for the optimized formulation of SLNs is shown in table 2. The ZP was relatively high and negative. As a general rule, the high ZP (>30 mV) value indicates a stable dispersion as aggregation is less likely to occur due to an electric repulsion. The negative charge is contributed by the lipid, as tween 80 is a nonionic surfactant and offers a steric repulsion.

Morphology of SLNs (SEM)

Surface electron microscopy was used to visualize the produced SLNs (fig.3). This visual examination indicated that the prepared particles were not entirely spherical but

slightly oblong. A slightly rough and cracked surface was observed for formulation F4 (fig. 4b) prepared using GMS as lipid matrix. For formulation F6, prepared with OA as lipid matrix (fig. 3a, b), the surface appeared to be smooth. For both type of lipids, no spikes were observed on the surface. No pits or depressions were observed which excluded the possibility of any leakage. In addition, these results also revealed that discrete particles were formed with a very slight aggregation. A high homogeneity can be inferred from SEM where individual particles almost look of the same size. SEM analysis confirmed the results of particle size analysis. In addition, formulation F4 & F6 had shown a low PDI indicating a homogeneous population of the particles.

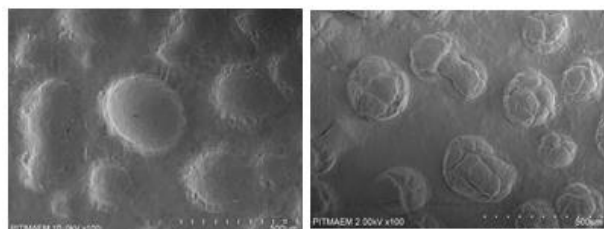


Fig. 3: SEM graphs of SLNs OA SLNs (a), GMS SLNs (b).

FTIR analysis

Infrared studies were carried out to confirm the compatibility between the lipid, vitamin E and selected SLNs formulations. The FTIR spectrum of vitamin E acetate (fig. 4) exhibited absorption bands at wavelengths 3746.551 cm^{-1} for -OH, 2923.320 and 2853.191 cm^{-1} for asymmetric and symmetric stretching vibrations of the CH_2 and CH_3 , for phenyl skeletal 14580 cm^{-1} , 1743.667 cm^{-1} for the ester group, 1206.392 cm^{-1} for carbon single bond (C-O) stretching and 1293.3 cm^{-1} for (C-O-C) methoxy group. For GMS (fig. 8) the peaks at 2913.338 cm^{-1} and 2847.855 cm^{-1} are attributed to C-H and CH_2 stretching groups present in the acyl chain of fatty acid. The absorption band at 3298.34 cm^{-1} is due to the O-H hydroxyl group present in the glycerol moiety, the peaks at 1316.89 cm^{-1} & 1389.565 cm^{-1} are due to C-C single bond, the peak at 1468.977 cm^{-1} indicates the C-H

stretch. The peak present at 1176.440 cm^{-1} indicates C-O bond. The characteristic peak at 1729.265 cm^{-1} is an indicative of C=O in the molecule. For oleic acid there is a band stretching between 3298.343 cm^{-1} to 2921.693 cm^{-1} , which is attributed to presence of $-\text{CH}_2$ group. The intense peak at 2921.693 cm^{-1} corresponds to the presence of symmetric and asymmetric $-\text{CH}_2$. The 2852.663 cm^{-1} peak is due to stacking of O-H group. The wide and intense band at 2473.142 cm^{-1} indicated the presence of -OH and the presence of the C=O is indicated by 1706.573 cm^{-1} peak, 1412.385 cm^{-1} absorption peak is due to angular deformation of the C-O-H, 1284 cm^{-1} indicated elongation of the C-O- and 966.173 cm^{-1} peak is due to angular deformation outside the O-H plane. The FTIR spectrum of lipids, vitamin E and their corresponding lipid mixture did not show any interaction as no characteristic peak disappearance or shifting was observed (fig. 4-a,b). For the blank formulations (GSM-b, OAB- b) a slight shifting of the C=O peak from 17000 cm^{-1} to 1650 cm^{-1} was observed. This might be due to the disruption of bonds due to heating and energy input during the preparation of SLNs. Similar, observations were made for the vitamin E loaded formulations (F4, F6). However, the $-\text{CH}_2$ peaks are diminished inside the formulations which may be attributed to intermolecular forces and re-arrangement of some molecules in SLNs. The characteristics peaks of vitamin E were diminished (fig. 4b) in the formulations as compared to the pure vitamin. Diminishing of vitamin E peaks in the formulations can be attributed to the embedding and molecular distribution of vitamin E into the lipid (Fouad EA *et al*, 2015). It can be concluded that the peak absence of vitamin E in SLNs confirmed the entrapment of the vitamin E inside the lipid (Ebrahimi HA *et al.*, 2015). To eliminate the doubt of vitamin E degradation due to heating during preparation pure vitamin E, OA and GMS were heated at 60 $^{\circ}\text{C}$ for 5 minutes and the FTIR was carried out. The FTIR spectrum of these heated mixtures (fig. 4b) showed characteristic peaks of lipids and vitamin E. This finding ruled out the doubt of drug degradation due to heating. HPLC results (fig. 5a) confirmed the FTIR findings. As characteristics peak of vitamin E showed

Table 1: Table1: Particle size, zeta potential, %EE & % DL of the optimized formulations

Formulations	Blank	Loaded					
	Mean diameter nm	Mean diameter nm			PDI	Zeta potential mv	Drug Loading % (DL)
	Freshly prepared	24 hrs	After 7 days	After 6 months	24hrs	24 hrs	
F1	19.76	439	404	588	0.39	-28.8	77
F2	99.5	404	588	615	0.367	-23.2	65
F3	178	218.5	108	263	0.22	-24.2	72
F4	34	104	104.8	105	0.24	-35.2	84
F5	38.08	41.2	41.4	41	0.22	-38.6	85
F6	40	69.08	69.6	69.3	0.25	-31.8	91
F7	78.82	213	255	879	0.20	-26.6	64
F8	61.47	746	766	831	0.311	-29.5	51

Table 2: Formulation of SLNs and Flux & Permeability coefficient for vitamin E through PDMS

Formulations	Lipid	% w/v	Concentration of vitamin E (%)	Surfactant	% w/v	Flux(J) ug/h/cm ²	Permeability coefficient
F1	GMO	1	0.5	Tween 80	11	50.4	0.504
F2	GMO		0.25		7	39.75	0.397
F3	GMS		0.5		7	50.70	0.507
F4	GMS		1		11	55.11	0.551
F5	OA		0.5		7	40.31	0.403
F6	OA		1		11	91.26	0.921
F7	SA		0.2		11	19.06	0.190
F8	SA		0.25		7	29.14	0.291

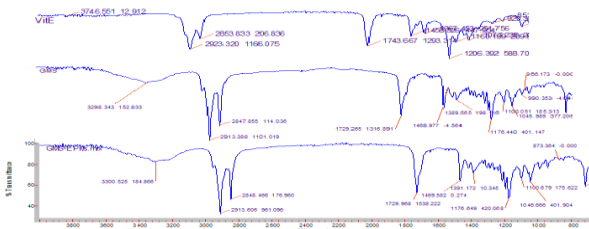
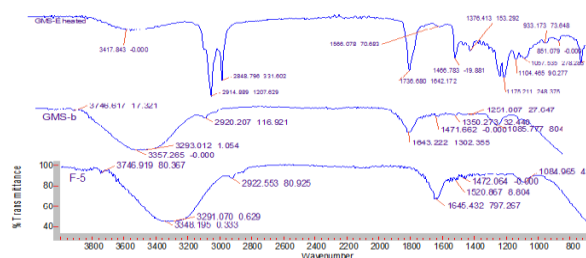
Table 3: R² Values of Zero order, Higuchi & Korsmeyer- Peppas model for vitamin E Formulations

Formulations	R ² Zero order	R ² Higuchi model	R ² Korsmeyer- Peppas model
F1	0.910	0.965	0.809
F2	0.97	0.974	0.844
F3	0.916	0.917	0.843
F4	0.976	0.957	0.800
F5	0.963	0.978	0.872
F6	0.977	0.962	0.871
F7	0.955	0.943	0.842
F8	0.967	0.978	0.856

absorbance at 285 nm (fig.5 b).

Permeation studies of the SLNs and vitamin E cream

The permeation study was performed using Franz diffusion cell and the cumulative percentage release is illustrated in fig. 1. It is evident from the release profile that prepared SLNs were capable of releasing vitamin E in a controlled manner over a period of 24 hours. For the convenience of application, the SLNs were incorporated into a stable cream base. The percentage release of vitamin E from the cream after 24 hours was 35%. The release pattern of vitamin E cream, SLNs+ cream base and SLNs alone is shown in fig. 2. Initially higher release of vitamin E was observed from the vitamin E cream but the release rate reduced with the passage of time. Analyzed samples of the cream showed a constant release rate after 12 hours. The amount of vitamin E released at the end of 24 hours from the prepared SLNs was much higher as compared to the cream (fig. 3). The percentage release of SLNs was found to be between 30-88 %. It was found that the SLNs loaded with vitamin E had enhanced the permeation which can be attributed to nano sized particles. The encapsulated vitamin E in SLNs had

**Fig 4(a):** FTIR graphs of vitamin-E, GMS, GMS-Vitamin E (physical mixture)- from top to bottom**Fig 4(a):** FTIR graphs of vitamin-E, GMS,GMS-Vitamin E (physical mixture)- from top to bottom

increased the contact of the vitamin E with stratum corneum producing improved permeability for the transdermal preparation (Wang *et al.*, 2019). Furthermore, presence of tween 80 enhanced the dissolution rate of vitamin Formulations prepared with oleic acid as lipid matrix (F5, F6) had shown the highest release (51% ,88 %). By comparing the release profiles between the SLNs and their corresponding cream formulations, the release of vitamin E at the end 24 hours was slower than that of nano dispersions indicating (fig. 2) that the incorporation of F4 and F6 into a cream base had further reduced the release. However, this release was significantly higher than the vitamin E cream. The highest release profile was observed from F6 (88%) followed by F4 (81%). The following was the release pattern
F6 > F4> F6+cream> F4+ cream> Vitamin E cream

The flux values for vitamin E were calculated and these indicated a high cumulative amount of vitamin E maintained permeation over a period of 24 hours (table 2). The prepared SLNs using tween 80 and GMO (F1, F2) through PDMS resulted in a flux of 50.454ug/cm²/h and

39.75 ug/cm²/h. Formulations F3 & F4 prepared with 1% GMS and 7% and 11% tween have 50.70 ug/cm²/h and 55.11 ug/cm²/h. Formulations F5 & F6 containing 1% OA and tween 80(7% and 11% tween) had showed a flux of 40.317 ug/cm²/h and 91.266 ug/cm²/h. Formulations F7 & F8 prepared with 1% SA and tween 80 (7% & 11%) resulted in a flux of 9.14 ug/cm²/h and 19.06 ug/cm²/h.

Stability studies

From the result of these studies, the following observations were made. The SLNs dispersion, which were stored at room temperature, were more likely to break or an increase in particle size was observed. Upon initial storing most of the SLNs, dispersions experienced an increase in droplet size with an exception to F6 and F4 that maintained the particle size up to 6 months. The instability was mostly observed in the systems with less concentration of the surfactant. In general, all the prepared SLNs with 11% tween 80 showed better stability. Formulations F7 & F8 had the least stability profile. At room temperature creaming and separation into distinct layer was observed with one week of storage. Creaming was observed for other formulations but they demonstrated better stability beyond six months.

DISCUSSION

The particle size is effected by various factors such as properties of lipid used, the concentration of surfactant, type of method used to prepare SLNs and processing conditions like speed of homogenization or time. The effect of these parameters is discussed below.

Effect of surfactant on droplet size/zeta potential (ZP)

Properties and concentration of the surfactant effects the PS and ZP. In all the prepared SLNs a significant decrease in droplet size was observed by increasing the concentration of tween 80. This decrease in particle size is due to the improved solubility of lipid and vitamin. Surfactants have the inherent property of reducing the interfacial tension between water and oil phase resulting in decreasing the amount of free energy required to deform and disrupt the droplets, hence reducing the particle size (Alexander *et al.*, 2020). Surfactants are known to form a protective layer around water and oil droplets in an emulsion system. This protective layer of surfactant prevents the coalescence because the presence of surfactant prevents aggregation and particle growth. In addition, to form the protective layer the surfactant must get adsorb in sufficient concentration around the droplet to prevent destabilization of an emulsion system. Therefore, it is very important to use the required amount of surfactant to maintain the particle size and to prevent the growth of the particles. It can be inferred from the results that 7-11 percent tween 80 was sufficient to produced nano sized particles with satisfactory stability.

Zeta potential is considered as an important surface characterization method to determine the stability. Tween 80 generated an electrostatic repulsion between the particles in the dispersion. An electrostatic repulsion between the particles with the same surface charge results in the prevention of aggregation and increases stability of the system. Increased stability of the system is attributed to the high negative value of zeta potential also, which electrostatically stabilizes SLNs dispersions. This steric layer shields the surface charge of SLNs and ensures their water dispersability. Moreover; with the high ZP values, the particles repel each other. This phenomenon in turn reduces their tendency to flocculate.

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Effect of vitamin E/lipid concentration on droplet size

The effect of the lipid and surfactant concentration on the particle size distribution of loaded SLNs prepared is summarized in table 1. The particle size ranged between 40 -700 nm. The particle size varies with the type of lipid used. Vitamin E loaded SLNs using OA and GMS had the

least particle size as compared to all other lipids (table 1). With the highest particle size observed in SLNs prepared with SA. This phenomenon could be attributed to the melting point of the lipid. It is well documented in the literature that the melting point of the lipid effects the average particle size i.e., increase in melting point increases the particle size particularly when high shear or hot homogenization technique is used to prepare SLNs (Souto *et al.*, 2019). Lipids with a high melting point increase the viscosity of the dispersed phase, this high viscosity of the dispersed phase is suggested as the reason for the large particle size (Tripodi *et al.*, 2019). This statement explains the least particle size observed in the case of oleic acid. Solid lipid had a higher melting point which results in slower lipid crystallization from the hot homogenized conditions resulting in an increased particle size (Ekambaram & Abdul 2011). In addition, lipid structure and crystallization rate which varies from one lipid to the other may effect the particle size. But this phenomenon was not observed in the case of GMS (M.P=50 °C & solid) and GMO (M.P=34°C & semi solid). It was observed that SLNs prepared with the GMS had shown better characteristics of physical appearance, particle size, zeta potential, PDI, %EE, loading and, stability than SLNs prepared with GMO. SLNs containing GMO (F1, F2) could not be prepared with a concentration of vitamin E more than 0.5 %. Decreasing the concentration of lipid or increasing the percentage of tween 80 had no significant effect on the stability as well. It was observed SLNs containing stearyl alcohol (F7, F8) could not be prepared with a high concentration of vitamin E. A concentration of vitamin E beyond 0.5 percent would result in complete breaking of the system even with 11 % tween 80. Reducing the amount of lipid had no significant effect on the stability of SLNs prepared with stearyl alcohol. This fact might be attributed to the physical properties of SA. Being solid makes it less soluble which had effected even distribution throughout the formulation. In addition, the loading capacity and entrapment offered by SA was least among all the SLNs. A similar behavior was observed in the release pattern of SA. This may be referred to the viscosity and the chain length of the lipid as well (El-Housiny *et al.*, 2018). Furthermore, this may be related to the melting point of the lipid which lead to crystallization from the hot homogenized circumstances causing growth in particle size (Azhar & Hamishehkar, 2016). The molecular weight, long chain and lipophilicity of vitamin E might be a contributing factor here. The SLNs prepared with oleic acid as oil phase showed a decrease of particle size diameter with the increase in tween 80 concentration. Similar, effect was observed for the entrapment efficiency and loading capacity. In fact, F5 & F6 showed the maximum entrapment efficiency and highest release profile. These two dispersions were most stable as well even with the highest amount of vitamin E (1%). This decrease in particle size due to increase concentration of

tween 80 might be the result of increased adsorption of surfactant at the interface and decreased interfacial tension. In addition, oleic acid being liquid was completely miscible with vitamin E, hence reducing the viscosity of this long chain molecule. Reduction in viscosity was reflected as reduced particle size and better entrapment. This also indicates that high shear forces due to homogenization were effective in disrupting the droplets leading to smaller particle size (Souto *et al.*, 2019).

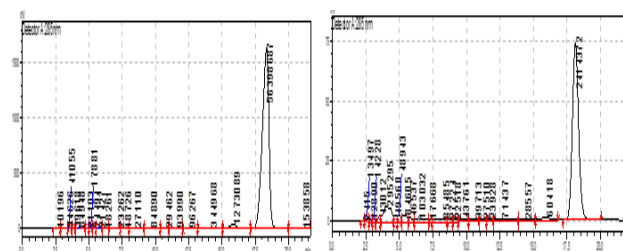


Fig. 5(a): HPLC peak of heated vitamin E & OA; **(b):** HPLC peak of F6

Entrapment efficiency (EE %) & drug loading (DL%)

The percentage entrapment efficiency of F5 and F6 was 85 & 91% respectively. This high % EE can be explained as follows: The enhanced incorporation of vitamin E in oleic acid can be due to the presence of tween 80. This high concentration of surfactant which may have caused the rapid quenching of the vitamin (Jafar *et al.*, 2015). Which might have resulted in the preparation of SLNs with a drug enriched shell-core model (Mishra *et al.*, 2018). In addition, oleic acid and vitamin E were completely miscible which in turn had a proportional effect on the entrapment. The high miscibility between oleic acid and vitamin E may have provided an additional space for the drug molecules to entrap. % EE of SLNs prepared with GMS was higher than in SLNs prepared with GMO. GMS is known for its emulsifying (HLB 3.8) and solubilizing properties. It is reported in the literature that the presence of a mixture of glycerides will act as a solubilizing agent for the lipophilic molecules (He *et al.*, 2019). If a mixture of mono-, di- and tri-glyceride is present more space is available for the incorporation of drug due to the defects of the crystal lattice (Ghan *et al.*, 2020). So that is why an enhanced entrapment was observed in the case of GMS as compared to GMO. Drug loading in SLNs is effected by lipid ratio, solubility, partition co-efficient and, structure of solid lipid matrix. In a lipid matrix drug is expected to be incorporated either in the core, shell or dispersed in the matrix uniformly. Crystallization of the lipid significantly effects the drug incorporation and the permeation rate. It is known that drug expulsion, thermodynamic instability and decrease drug loading rate is observed due to polymorphic modification of lipids (Kim *et al.*, 2017). A good solubility of drug in lipid ensures high loading. Solid lipids usually form a perfect lattice upon cooling. This effects the loading capacity as the solubility reduces

and drug get expels from the lattice (Emami *et al.*, 2018). This explains the good % EE but low drug loading in our case.

Permeation studies

SLNs for the topical drug delivery are known to enhance the permeability profile due to the small particle size. This enhanced effect is due to increase contact time with the membrane improving the delivery of the encapsulated system (Bayón-Cordero *et al.*, 2010). The change in the flux is the result of the interaction between vitamin E and tween 80 or the surfactant and the membrane (Patel KK *et al.*, 2019). Data obtained through permeation studies on the Franz diffusion cell is summarized in table 2. This data included permeation parameters like steady state flux (J), and permeability coefficient. A same molecule is expected to have same flux value across the membrane in the donor compartment regardless of the formulation. However; this is only true under ideal circumstances and if there is no interaction between the membrane and the formulation components. As given in table 2, flux values for vitamin E differ due to variability of surfactant concentration and type of lipid used. A significant variation is observed in flux by changing the concentration of tween 80. As this can be seen in formulation F1 & F2. F1 being prepared with 11% tween 80 had 6.7 % more value of flux. For formulation F3 and F4, F4 had enhanced the permeation by 4.4% than F3. F6 had enhanced the permeation by 6.9 % than F5. The least value of flux was obtained from F7 & F8. The highest release pattern was related to formulations prepared with oleic acid. (F5, F6). It is so proposed that the formulations of oleic acid had a high affinity for the membrane, which was demonstrated as higher partition and flux parameters. Moreover, oleic acid is liquid at room temperature, which may have led to better mobility at the temperature used for permeation studies. This also suggests a drug enriched core model of SLNs. A drug-enriched core is possible when a lipid fully solubilizes the drug. Glyceryl mono oleate (F1, F2) had shown a more controlled and slow release of vitamin E as compared to glyceryl mono stearate (F3, F4). This behavior can be explained as GMO produced less ordered crystals in the lipid matrix than GMS which lead to drug expulsion from the imperfect drug lattice which in turn had contributed to slow and controlled release. It is also reported that a slow release is expected from lipids with the low melting point (Nagraj *et al.*, 2020). Solid lipid particles are expected to form a lipid matrix with ordered crystals or a lattice structure contributing to prolonged release of lipophilic vitamin E. In our case a similar situation was observed between GMO and GMS. By referring to the similar argument the release profile of the other SLNs could be explained as well. The least permeation profile of stearyl alcohol can be explained as, it is possible that during the production of SLNs the solidification of lipid started before the solidification of drug-lipid melt around the lipid core

(Elgart *et al.*, 2012). Which lead to drug expulsion and a drug core with inadequate drug (Ezzati *et al.*, 2015). (Hackett *et al.*, 2013) had reported while studying the effect of lipids on the release profile of drugs that long chain fatty acids effects the release of lipophilic drugs and, stearyl alcohol is a long chain molecule. From the release profile, it can be concluded that formulations of SLNs had enhanced the permeation of vitamin E alone as compared to vitamin E cream. Vitamin E cream initially had a higher release but release had decreased over time. Cream + F6 had shown a higher release pattern than cream + F4. The release of vitamin E from cream + F6 is comparatively less than the F6 dispersion alone. This effect might be attributed to the excipients used to make the cream which might have had an inhibitory effect on the release of vitamin E. In addition, stearic acid, lanolin and mineral oil could have formed a solid matrix, which in turn had delayed or slow the release. The viscosity of the cream could be a contributing factor in slowing the release of vitamin. Drug partitioning in the outer phase reduces due to high viscosity which subsequently reduced the release of vitamin E in the outer medium. Moreover, the presence of mineral oil had caused occlusion to further retard the release of vitamin. Surfactants are known to enhance the penetration of biologically active compounds through the skin by interacting with the lipid matrix, which may result in alteration of the structure of stratum corneum (Kelidari *et al.*, 2015). Thus, the presence of tween 80 in SLNs had contributed to enhance the permeation of vitamin E. It is reported that spherical vehicles may penetrate the SC as intact particles (Aboud, *et al.*, 2016). As seen from SEM graphs particles were nearly spherical that might have synergized the permeation of vitamin E loaded particles. In brief, nano sized particles offer an enormous surface area that in turn had increased the percent cumulative amount of drug released. In order to predict and correlate the release of solutes from the SLNs in vitro studies, results must fit into a suitable mathematical model (Charles 2017). These mathematical models can also predict the release mechanism of the drug. The release kinetics of the SLNs was evaluated by fitting the data into various kinetic models like zero order, Higuchi and Peppas's. As presented in the table 3, it can be seen that the highest correlation and best fitted line was obtained for Higuchi model (R^2 nearly 1). Higuchi model of release kinetics had been reported in the literature for drug loaded systems like SLNs (Ligia *et al.*, 2017). From the R^2 value it could be inferred that the release of vitamin E was steady and continuous without any burst release. The linear fit indicated that the release was from a homogenous matrix and release was diffusion controlled (Lee and Yeo 2015). Researchers had used the n value for characterization of different release mechanism. A higher value of n between 0.5-1 or $n > 1$ indicates a non-Fickian model. Based on diffusion exponent from Korsmeyer-Peppas models, it can be concluded that release of vitamin E from SLNs

was non-Fickian and as per zero order kinetic, the release was independent of the concentration of vitamin E.

Stability studies

In determining the stability of a nano based dispersion droplet size plays a major role. As such, delivery systems can be separated, flocculated and, coalesced owing to the gravity. Nano dispersions prepared with the low concentration of tween 80 after storage showed a visible phase separation, which can be attributed to the aggregation of droplets and creaming. Dispersions prepared with large particle size are highly susceptible to creaming. The most common type of mechanism that effects an emulsion stability is Ostwald ripening and flocculation. SLNs in nanomeric range will have an enormous surface area, which in turn leads to thermodynamic instability and results in phase separation if surfactant is not present in sufficient concentration. The SLNs should be prepared with a narrow particle size range to avoid Ostwald ripening. The reason for phase separation in the prepared SLNs might be due to the high viscosity of vitamin E and the solid lipid as well. According to (Chan-Won Seo *et al.*, 2018) the high viscosity of the oil phase leads to larger droplets because the droplet disruption inside the homogenizer become inefficient. This phenomenon was confirmed in our case. Vitamin E being a viscous molecule demonstrated this behavior. In addition, the large amount of surfactant used must get adsorb at the interface because non adsorb surfactant is known to form micelles that decreases emulsion stability as it promotes droplet aggregation (Dannie *et al.*, 2020). Increased amount of lipid is associated with increase in particle size as well. Lipids in high concentration have a tendency to coalesce. According to Stoke's law, the difference in the densities of the internal and the external phase explains this behavior. It is reported that an increase in particle size of SLNs is due to the reduction in the diffusion of the solute molecules in the outer phase because of the increase viscosity in the lipid-solvent phase. Lower values of PDI indicates a narrow size distribution. SLNs initially prepared with the low PDI had shown a better stability. It was also observed with the storage over prolong period of time not only the particle size increased but PDI also increased indicating the formation of a heterogeneous system.

CONCLUSION

In the current study, solid lipid nano particles loaded with vitamin E were prepared using hot homogenization technique followed by dilution method. Vitamin E was successfully incorporated up to 1% concentration. The SLNs were characterized for particle size, PDI ZP, SEM and, FTIR. Entrapment efficiency and drug loading capacity was determined for SLNs. Permeation studies were performed using PDMS and Franz diffusion cell.

The prepared SLNs showed good stability at cold temperature. Formulations prepared with GMS and oleic acid maintained particle size for longer period of time. SEM micrographs revealed nearly spherical particles with a definite periphery. The change in the type of lipid seems to influence the particle size and permeation of vitamin E from SLNs. Furthermore, it can be presumed that nano sized particles had increased the permeability of vitamin E as compared to vitamin E cream. In addition, the release of the vitamin E was controlled or prolonged over 24 hours of time. The percent cumulative amount of vitamin E at the end of 24 hours was least from SLNs that had the maximum particle size. From the values of particle diameter, PDI and ZP it can be concluded that prepared SLNs had an acceptable physical and electrochemical stability and the particle size remain in the nanomeric range over a long period.

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REFERENCES

- Aboud HM, El komy MH, Ali AA, El Menshawe SF, and Elbary AA (2016). Development, optimization, and evaluation of carvedilol-loaded solid lipid nanoparticles for intranasal drug delivery. *AAPS Pharm. Sci. Tech.*, **17**(6): 1353–1365.
- Alexander A, Fedorets Dmitry V, Shcherbakov Leonid A, Dombrovsky Edward Bormashenko and Michael Nosonovsky (2020). Impact of surfactants on the formation and properties of droplet clusters. *J. American Chemical Society*, **36**(37): 11154-11160.
- Argimón Mauricio, Romero Mariano, Miranda Pablo, Mombrú Alvaro, Miraballes Iris, Zimet Patricia and Pardo Helena (2016). Development and characterization of vitamin A-loaded solid lipid nanoparticles for topical application. *J. Braz. Chem. Soc.* **28**(7): 1177-1184
- Azhar Shekoufeh Bahari L and Hamishehkar H (2016). The impact of variables on particle size of solid lipid nanoparticles and nanostructured lipid carriers; a comparative literature review. *Adv. Pharm. Bull.*, **6**(2): 143-151.
- Badawi N, El-Say K, Attia D, El-Nabarawi M, Elmazar M and Teaima M (2020). Development of pomegranate extract-loaded solid lipid nanoparticles: quality by design approach to screen the variables affecting the quality attributes and characterization. *ACS Omega.*, **5**(34): 21712–21721.

- Bayón-Cordero L, Alkorta I and Arana L (2019). Application of solid lipid nanoparticles to improve the efficiency of anticancer drugs. *Nanomaterials Basel*, **9**(3): 474.
- Bhalekar M, Upadhaya P and Madgulkar A (2017). Formulation and characterization of solid lipid nanoparticles for an anti-retroviral drug darunavir. *Appl. Nanosci.*, **7**(17): 47-57.
- Chan-Won Seo, Shin-Ho Kang, Yong-Kook Shin and Byoungseung Yoo (2018). Effect of homogenization pressure and supplementation with sucrose fatty acid ester on the physical properties of dairy cream-based emulsions. *Korean J. Food Sci. Anim. Resour.*, **38**(3): 476-486.
- Charles L Perrin (2017). Linear or nonlinear least-squares analysis of kinetic data. *Chem. Educ.* **94**(6): 69-672.
- Dannie JGP van Osch, Jaap van Spronsen, A Catarina C Esteves, Remco Tuinier abc and Mark Vis (2020). Oil-in-water emulsions based on hydrophobic eutectic systems. *J. Phys. Chem.*, **22**(4): 2181-2187.
- Ebrahimi HA, Javadzadeh Y, Hamidi M and Jalali MB (2015). Repaglinide-loaded solid lipid nanoparticles: Effect of using different surfactants/stabilizers on physicochemical properties of nanoparticles. *DARU J. Pharm. Sci.*, **23**(1): 46.
- Ekambaram P and Abdul HS (2011). Formulation and evaluation of solid lipid nanoparticles of ramipril. *JYP*, **3**(3): 216-220.
- Elgart A, Cherniakov I, Aldouby Y, Domb AJ, Hoffman A (2012). Lipospheres and pro-nano lipospheres for delivery of poorly water soluble compounds. *Chem Phys Lipids.*, **165**(4): 438-453.
- El-Housiny S, Shams Eldeen MA, El-Attar YA, Salem HA, Attia D, Bendas ER and El-Nabarawi MA (2018). Fluconazole-loaded solid lipid nanoparticles topical gel for treatment of pityriasis versicolor: Formulation and clinical study. *Drug Deliv*, **25**(1): 78-90.
- Emami J, Yousefian H and Sadeghi H (2018). Targeted nanostructured lipid carrier for brain delivery of artemisinin: Design, preparation, characterization, optimization and cell toxicity. *J. Pharm. Pharm. Sci.*, **21**(1s): 225s-241s.
- Ezzati Nazhad Dolatabadi J, Valizadeh H and Hamishehkar H (2015). Solid lipid nanoparticles as efficient drug and gene delivery systems: Recent breakthroughs. *Adv. Pharm. Bull.*, **5**(2): 151-159.
- Fouad EA, Yassin AE and Alajami HN (2015). Characterization of celecoxib-loaded solid lipid nanoparticles formulated with tristearin and softisan 100. *Trop. J. Pharm. Res.*, **14**(2): 205-210.
- Gazi AS and Krishnasailaja A (2018). Preparation & evaluation of paracetamol solid lipid nanoparticles by hot homogenization method. *J. Nanomed. Res.*, **7**(2): 152.
- Geszke-Moritz M and Moritz M (2016). Solid lipid nanoparticles as attractive drug vehicles: Composition, properties and therapeutic strategies. *Mater. Sci. Eng. C. Mater. Biol. Appl.*, **68**: 982-994.
- Ghan SY, Siow LF, Tan CP, Cheong WK and Thoo YY, (2020). Influence of soya lecithin, sorbitan and glyceryl monostearate on physicochemical properties of organogels. *Food Biophys.*, **15**: 386-395.
- Hackett MJ, Zaro JL, Shen WC, Guley PC and Cho MJ (2013). Fatty acids as therapeutic auxiliaries for oral and parenterals formulations. *Adv. Drug Deliv. Rev.*, **65**(10): 1331-1339.
- Hallan SS, Sguizzato M, Drechsler M, Mariani P, Montesi L, Cortesi R, Bjorklund S, Ruzgas T, Esposito E (2021). The potential of caffeic acid lipid nanoparticulate systems for skin application: in vitro assays to assess delivery and antioxidant effect. *Nanomaterials Basel.*, **11**(1): 171.
- Harman D Aging (1956). A theory based on free radical and radiation chemistry. *J Gerontol.*, **11**(3): 298-300.
- He J, Huang S, Sun X, Han L, Chang C, Zhang W and Zhong Q (2019). Carvacrol loaded solid lipid nanoparticles of propylene glycol monopalmitate and glyceryl monostearate: Preparation, characterization, and synergistic antimicrobial activity. *Nanomaterials Basel*, **9**(8): 1162.
- Jafar Ezzati, Nazhad Dolatabadi, Hadi Valizadeh and Hamed Hamishehkar (2015). Solid lipid nanoparticles as efficient drug and gene delivery systems: Recent breakthroughs. *Adv. Pharm. Bull.*, **5**(2): 151-159.
- Kaul S, Gulati N, Verma D, Mukherjee S and Nagaich U (2018). Role of nanotechnology in cosmeceuticals: A review of recent advances. *J. Pharm.*, 2018: 19.
- Kelidari HR, Saeedi M, Akbari J, Morteza-Semnani K, Gill P, Valizadeh H and Nokhodchi A (2015). Formulation optimization and *in vitro* skin penetration of spironolactone loaded solid lipid nanoparticles. *Colloids Surface B.*, (2015) **128**: 473-479.
- Khan S, Baboota S, Ali J, Khan S, Narang RS and Narang JK (2015). Nanostructured lipid carriers: An emerging platform for improving oral bioavailability of lipophilic drugs. *Int. J. Pharm. Investig*, **5**(4): 182-191.
- Kim CH, Lee SG and Kang MJ, Lee S & Choi YW (2017). Surface modification of lipid-based nanocarriers for cancer cell-specific drug targeting. *Int. J. Pharm. Investig*, **47**(3): 203-227.
- Lee JH and Yeo YL (2015). Controlled drug release from pharmaceutical nanocarriers. *Chem. Eng. Sci.*, **125**: 75-84.
- Lígia NM Ribeiro, Ana CS Alcântara, Gustavo H. Rodrigues da Silva, Michelle Franz-Montan, Silvia V. G. Nista, Simone R. Castro, Verônica M. Couto, Viviane A. Guilherme and Eneida de Paula1 (2017). Advances in hybrid polymer-based materials for sustained drug release. *Int. J. Polym. Sci.*, 2017: 16.
- Mishra V, Bansal KK, Verma A, Yadav N, Thakur S, Sudhakar K and Rosenholm JM (2018). Solid lipid nanoparticles: Emerging colloidal nano drug delivery systems. *Pharmaceutics*, **10**(4): 191.

- Nagraj BK and Aunshka DNPS (2020). Formulation and evaluation of microemulsion-based transdermal delivery of duloxetine hydrochloride. *IJPSN*, **13**(1): 4773-4782.
- Patel KK, Gade S and Anjum MM, Singh SK, Agarwal AK and Singh S (2019). Effect of penetration enhancers and amorphization on transdermal permeation flux of raloxifene-encapsulated solid lipid nanoparticles: An *ex vivo* study on human skin. *Appl. Nanosci.*, **9**(6): 1383-1394.
- Pereira, Gabriela, Guterres Sílvia, Balducci Anna, Colombo Paolo and Sonvico Fabio (2014). Polymeric films loaded with vitamin e and aloe vera for topical application in the treatment of burn wounds. *BioMed Res. Int.* **2014**(9). Article ID 641590.
- Reboul E (2017). Vitamin E Bioavailability: Mechanisms of intestinal absorption in the spotlight. *Antioxidants* (Basel). **6**(4): 95.
- Rostami E, Kashanian S and Azandaryani AH (2014). Preparation of solid lipid nanoparticles as drug carriers for levothyroxine sodium with in vitro drug delivery kinetic characterization. *Mol. Biol. Rep.*, **41**(5): 3521-3527.
- Souto EB, Doktorovova S, Zielinska A, Silva AM (2019). Key production parameters for the development of solid lipid nanoparticles by high shear homogenization. *Pharm. Dev. Technol.*, **24**(9): 1181-1185.
- Tao Xiaojun, Li Yu, Hu Qian, Zhu Li, Huang Zixuan, Yi Jiajin, Yang Xiaoping, Hu Jianzhuo and Feng Xing (2018). Preparation and drug release study of novel nanopharmaceuticals with polysorbate 80 surface adsorption. *J. Nanomater.*, pp.1-11.
- Tripodi E, Lazidis A, Norton IT and Spyropoulos F (2019). Production of oil-in-water emulsions with varying dispersed-phase content using confined impinging jet mixers. *Ind. Eng. Chem. Res.*, **58**(32): 14859-14872.
- Trivino A and Chauhan Harsh (2015). Drug-excipient compatibility for the formulation development of solid lipid nanoparticles. *Am. Pharm. Rev.*, **18**(2).
- Vinod L, Gaikwad Prafulla B, Choudhari Neela M and Bhatia Manish S Bhatia (2019). Characterization of pharmaceutical nanocarriers: *In vitro* and *in vivo* studies, in MG Alexandru (ed). *Nanomaterials for Drug Delivery and Therapy*, William Andrew Publishing, Boston, pp.33-58.
- Wang Y, Wang X, Wang X, Song Y, Wang X and Hao J (2019). Development of lidocaine microemulsions for transdermal delivery. *AAPS. Pharm. Sci. Tech.*, **20**(2): 63.