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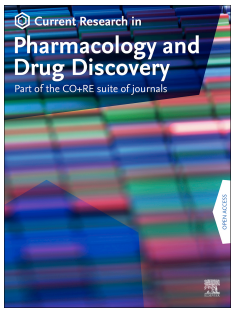

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Tocotrienols-Rich Naringenin Nanoemulgel for the Management of Diabetic wound: Fabrication, Characterization and Comparative *in vitro* Evaluations

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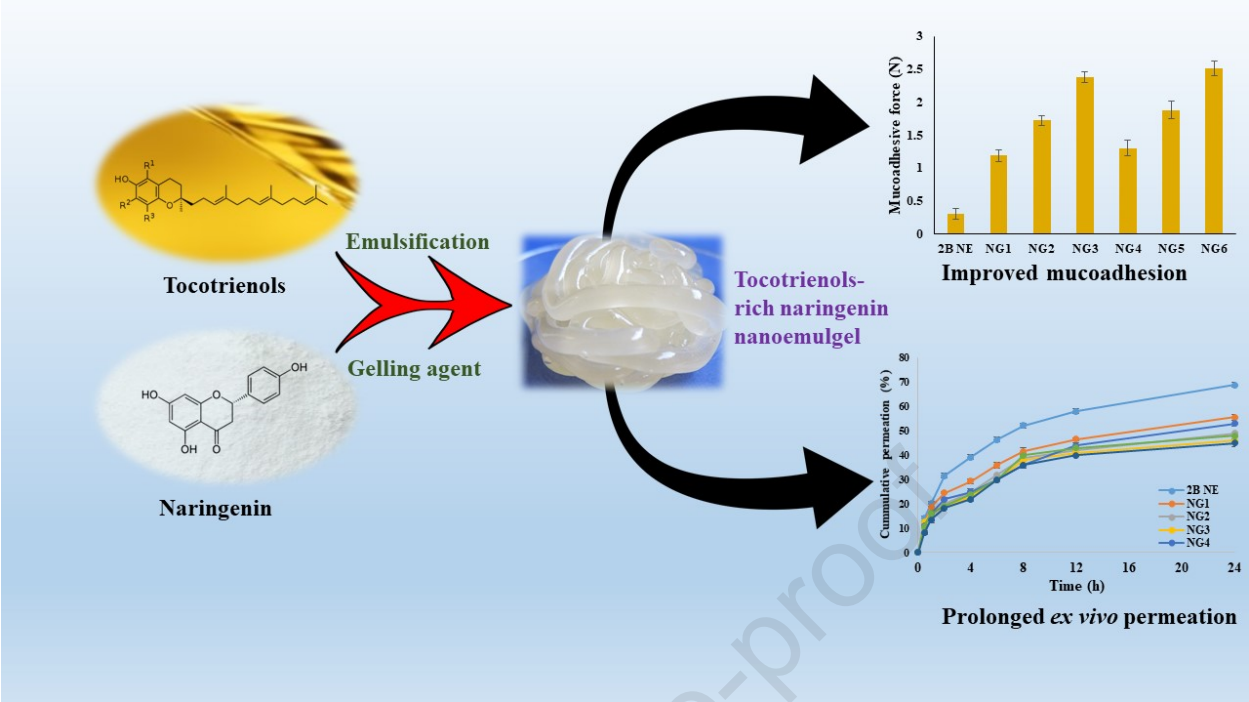

Tocotrienols-Rich Naringenin Nanoemulgel for the Management of Diabetic wound: Fabrication, Characterization and Comparative *in vitro* Evaluations

Abstract

The present research had been attempted to formulate and characterize tocotrienols-rich naringenin nanoemulgel for topical application in chronic wound conditions associated with diabetes. In due course, different phases of the nanoemulsion were chosen based on the solubility study, where combination of Capryol 90 and tocotrienols, Solutol HS15, and Transcutol P were selected as oil, surfactant, and cosurfactant, respectively. The nanoemulsions were formulated using the spontaneous emulsification method. Subsequently, Carbopols were incorporated to develop corresponding nanoemulgels of the optimized nanoemulsions. Thermodynamically stable optimized nanoemulgels were evaluated for their globule size, polydispersity index (PDI), surface charge, viscosity, mucoadhesive property, spreadability, *in vitro* release and release mechanism. Further, increasing polymer concentration in the nanoemulgels was reflected with the increased mucoadhesive property with corresponding decrease in the release rate of the drug. The optimized nanoemulgel (NG1) consisted of uniform dispersion (PDI, 0.452 ± 0.03) of the nanometric globules (145.58 ± 12.5) of the dispersed phase, and negative surface charge (–21.1 ± 3.32 mV) with viscosity 297,600 cP and good spreadability. *In vitro* release of naringenin in phosphate buffer saline revealed a sustained release profile upto a maximum of 74.62±4.54% from the formulated nanoemulgel (NG1) within the time-frame of 24h. Alternatively, the release from the nanoemulsion was much higher (89.17±2.87%), which might be due to lack of polymer coating on the dispersed oil droplets. Moreover, the *in vitro* release kinetics from the nanoemulgel followed the first-order release and Higuchi model with non-Fickian diffusion. Therefore, encouraging results in this research is evident in bringing a promising future in wound management, particularly associated with diabetes complications.

Keywords: naringenin; tocotrienols-rich fraction; nanoemulgel; diabetic wound healing; low-energy emulsification; Carbopol

1. Introduction

World Health Organization (WHO) defines diabetes as a chronic, metabolic disease characterized by elevated levels of blood glucose (or blood sugar), which leads to serious damage to the heart, blood vessels, eyes, kidneys, and nerves over time. As reported by WHO, the numbers of diabetic population has risen from 108 million adults in 1980 to 422 million in 2014 [1]. One of the major diabetes-associated complications is diabetic foot syndrome (DFS), as a consequence of wound healing issues in diabetes patients [2]. Chronic diabetic wounds and impairment in their healing are primary concerns for diabetic patients [3], where an increased level of glucose in the blood, decreased insulin level and concomitant ischemia of blood vessels in diabetic patients create obstacles towards the healing of the wounds in diabetic patients [4,5]. Consistent after-care and preventative measures are essential to improve prognosis and minimize the likelihood of amputation [2]. The main goal towards the treatment of diabetic wounds includes wound closure, where the treatment varies depending on the severity and vascularity of the wound [6]. Therefore, numerous efforts have been made to explore novel treatment strategies against the diabetic wound for a better tomorrow.

Tocotrienol, an element of vitamin E, is known to alleviate oxidative stress and inflammation in overcoming diabetes-associated complications [7]. It is said to possess four different isomers, α-, β-, γ-, δ, where they differ from one another at the chromanol ring in terms of position and methyl group present [8]. Tocotrienols are found naturally abundant in palm oil, annatto oil, rice bran oil as well as in wheat germ [8]. It possesses 40-60 times antioxidant potency than tocopherol whereby it was described to have superior anti-inflammatory, antiglycemic, anticholesterolemic, neuroprotective and cardioprotective activities [9]. On the other hand, naringenin- a plant flavonoid, mostly obtained from citrus fruits, such as grapefruit, orange, and lemons, is said to show potential role against several physiological complications combating metabolic syndromes including diabetes [10]. According to Kandhare *et al*., flavonoids are natural antioxidants that possess potential lipid peroxidation inhibitory properties [11]. Besides, flavonoids are known to exert eminent antioxidant, anti-inflammatory, antimicrobial and astringent properties, which are beneficial individually in different pathological conditions, whereas a combination of such actions helps in speedy healing of the wound. Consequently, antioxidants as well as radical trapping and scavenging properties of naringenin project towards an effective wound healing [11]. Role of naringenin and tocotrienol in would healing had been established in literature. Xu et al reported the angiogenic and cell migratory properties of tocotrienol in the healing of diabetic wound upon

local application when compared to the vehicle control animals [12]. Efficacy of naringenin on application over thermal-induced wound was found to be effective [13], whereas a recent article demonstrated the role of naringenin in the treatment of excision wound [14]. These evidences are describing the wound healing potential of these two natural components.

Further, there are various formulation approaches have been made for delivering therapeutics effectively in diabetic wounds through topical application. However, so far, the research outcomes are not satisfactory in terms of bringing a new formulation to control and treat diabetic wounds. There are tremendous scope to deliver therapeutic agents via novel tools in the drug delivery system, thus, the present study is attempted to deliver naringenin and tocotrienol using nanoemulsion platform, hypothesized to obtain a synergistic role of the two natural components in diabetic wound healing. Nanotechnology-based drug delivery using nanoemulsion platform has shown promising potential in the delivery of therapeutics through topical routes [10,15,16]. Homogenous dispersion of drug containing oil droplets in aqueous environment are known to provide aesthetic appeal by their appearance. These formulations are thermodynamically stable, whereas, droplets sizes of less than 200 nm potentiate permeation of the therapeutics easily from the rigid stratum corneum via transcellular and paracellular pathways [15,17]. To the best of our knowledge, there is no such attempt has been made to explore the combination of these two natural components in a single formulation targeting towards wound healing. Thus, an attempt has been made in this present study to develop and optimize a tocotrienols rich naringenin nanoemulgel, where the lipophilic naringenin was incorporated within the oil core of the nanoemulsion containing Capryol 90 and tocotrienols rich fraction (TRF) of palm oil. The optimized nanoemulsion was further converted to nanoemulgel using Carbopol, a biocompatible gelling agent, to achieve desired mucoadhesive property and retention of the drug to the target site for prolonged exposure and effective therapy. The developed formulations were characterized for size, zeta potential, PDI, thermodynamic stability, viscosity, pH and electrical conductivity. Finally, the mucoadhesion study, and *in vitro* release of the formulations in phosphate buffer were performed from the gel to establish considerable promise in improving healing of the diabetic wound in the future.

2. Materials and methods

2.1 Materials

Naringenin (purity ≥95%), Carbopol 934, Carbopol 940 and Solutol HS15 were procured from Sigma-Aldrich, USA. TRF was purchased from Sime Darby, Malaysia. Other chemicals, such as oleic acid, sunflower oil, olive oil, tween 20, tween 80, triethanolamine and sodium bicarbonate were obtained from Chemical Solutions Sdn. Bhd., Malaysia. Plurol oleique, labrafac, Transcutol P, Transcutol HP, Labrafil M2125 CS, Labrafil M1944 CS, Labrasol and Capryol 90 were gifted by Gattefosse, France for research, whereas Sefsol 218 was gifted from Nikko Chemicals Co. Ltd, Japan. Rest of the chemicals used in this research were of analytical grade.

2.2 Formulation of drug-loaded nanoemulsion

2.2.1 *Screening of components for tocotrienols rich naringenin nanoemulsion*

Appropriate selection of the excipients, the first step to formulate a nanoemulsion of the particular drug, was performed based on their solubility with various oils, surfactants, and co-surfactants. The drug should be solubilized in the oil phase to deliver the formulation in the desired form and simultaneously, any precipitation and instabilities can be avoided [18]. Firstly, excess of naringenin was measured and transferred into different glass vials containing 2mL of different oils (Labrafac, Sefsol 218, olive oil, sunflower oil, Capryol 90), surfactants (Labrafil M2125 CS, Labrafil M1944 CS, lauroglycol 90, tween 80, tween 20, labrasol, Solutol HS 15, oleic acid) and co-surfactants (ethanol, Carbitol, iso-propyl alcohol, Transcutol HP, Transcutol P). The respective vials were vortexed for a fixed period of time using vortex mixer (Harmony VTX-3000L), followed by incubation in shaking incubator at 37±2°C (Memmerth WNB 22) for 72 h to obtain equilibrium solubilization. After 72 h, the samples were centrifuged (Hettich Universal 320) at 15,000rpm for 30 minutes and the supernatant was strained. Any excess liquid component was blotted from the formed pellets, ensuring the presence of only precipitated solids. The precipitant was then diluted with methanol and quantified using UV spectrophotometer (Perkin Elmer Lambda XLS) at lambda max of naringenin, 289nm [19].

As TRF is a component of the formulation, solubility of naringenin was further evaluated in Capryol 90 (the oil that showed the highest solubility of naringenin) in the presence of TRF at different proportion to evaluate the influence of TRF in naringenin solubility in Capryol 90.

2.2.2 *Determination of the suitable composition of oil:surfactant:cosurfactant (oil:Smix)*

Based on the results of solubility studies, Capryol 90 was selected as the most suitable oil , whereas, surfactant and cosurfactant were Solutol HS15 and Transcutol P. The nanoemulsion formulations were then experimented with different compositions of excipients as described in Table 1.

Based on the ratios described in Table 1, the respective nanoemulsion was prepared by spontaneous emulsification method [20]. The respective formulations were then subjected to sonication using bath sonicator (Fisher Scientific FB15051) for 30 minutes at 37°C, followed by vortexing for 15 minutes until a homogenous mixture is formed.

2.2.3 *Preparation of drug-loaded nanoemulsion*

For the preparation of drug-loaded nanoemulsion, naringenin was first solubilized to the mixture of oils (Capryol 90 and TRF); thereafter, the surfactant and cosurfactant were added. Following mixing of the components, distilled water was added dropwise with continuous stirring to achieve 2mg of naringenin/mL nanoemulsion. The respective formulations were sonicated and vortexed. Drug-loaded formulations were further assayed for thermodynamic stability following the methods depicted below.

2.4 Characterization of drug-loaded nanoemulsion

2.4.1 *Thermodynamic stability studies*

Thermodynamic stability studies are essential to assess the physical stability of the nanoemulsion-based formulations, and it was performed at different stages of formulation development [21].

Centrifugation

The respective formulations were subjected to centrifugation at 5000rpm for 30 minutes. Signs of phase separation, cracking or creaming of the nanoemulsion was noted.

Heating-cooling cycle

The samples passed in the previous stage were stored successively at a temperature of 4°C and 40°C for 48 h at each temperature, consider as one cycle. Signs of phase separation, cracking or creaming of the nanoemulsion were recorded after each cycle until three cycles.

Freeze-thaw cycle

The respective nanoemulsions were stored for 48 h at each temperature, −20°C in the deep freezer and at room temperature (25±2°C), consider as one cycle. The study repeated for three cycles and signs of phase separation, cracking or creaming were recorded. Finally, after completion of the three stages of thermodynamic study, globule size, PDI and zeta potential were measured following the method described in section 2.4.2 and compared with the freshly prepared samples.

2.4.2 ***Globule size, PDI and zeta potential assessment of drug-loaded nanoemulsion***

The most popular technique for determination of globule size and size distribution involves a dynamic light scattering method, which could determine the size of the dispersed globules until 1 nm in diameter. Thus, Malvern ZetaSizer (NanoZS-90, Malvern Instrument, Worcestershire, UK) instrument was employed to determine the globule size, PDI and zeta potential of the developed formulations [18]. Before the respective measurements were made, all formulations were diluted with deionized distilled water for 50 times to reduce the multiple scattering effects. Light scattering was measured at a 90° angle at room temperature (25 ± 2°C). On the other hand, the same instrument was used to determine the surface charge of the globules at the same temperature.

2.4.3 ***Measurement of pH, refractive index and electrical conductivity of the developed formulations***

The topical formulation should be non-irritant, thus, the formulations should be of skin-pH friendly to avoid any allergic reactions or irritations [22]. A calibrated pH meter (Sartorius PB-10) was used to determine the pH of the nanoemulsion.

The refractive index (RI) value is also an important parameter to be determined as it reflects the isotropic nature of the formulation as well as explains the chemical interaction between the drug and the excipients [18]. Atago Rx-5000 refractometer was used to measure the refractive index by preparing a film of the respective nanoemulsion on the slide in a triplicate at 25°C [20].

To identify the *o/w* type of the respective nanoemulsions, an electrical current was passed through the respective formulations. Any deflection in the microprocessor-based conductivity

meter (Emtech Con 700) would confirm the presence of a continuous aqueous phase in the nanoemulsion [21].

2.5 Development of drug-loaded nanoemulgel

The stable optimized naringenin nanoemulsion was selected to formulate the respective nanoemulgel. The selected optimized naringenin nanoemulsion was dispersed into 1% *w/v*, 1.5% *w/v* and 2% *w/v* of Carbopol 934 gel bases. Similarly, gel formulations were developed using Carbopol 940. Naringenin nanoemulsion and gel base were mixed at 1:1 ratio with stirring for 10 minutes at 500 rpm using a magnetic stirrer [23]. Triethanolamine was used to adjust the pH of the formulation to 4.9-5.3 and the gel formulation was mixed thoroughly and uniformly to obtain the desired consistency of nanoemulgel containing 1mg of naringenin in 1mL nanoemulgel formulation. The gels were allowed to stand 24 h to eliminate any trapped air.

2.6 Characterization of drug-loaded nanoemulgel

The respective drug-loaded nanoemulgels were subjected to all the characterization tests as discussed earlier. Additionally, the following tests were performed on the formulated nanoemulgels.

2.6.1 *Spreadability test*

A topical preparation should possess good spreadability as it determines the therapeutic efficiency of the formulation. Spreadability can be defined as how readily a gel can spread over the site of application on skin and the affected area [22]. This experiment was performed following established method [24]. A weighed quantity of 350 mg of the respective gels was transferred onto a glass plate (10x5cm). Another glass plate of equivalent size was then dropped onto the gel from a distance of 5cm. After one minute, the diameter of the spreading area was measured and recorded.

2.6.2 *Viscosity measurement*

Brookfield Viscometer DV2T was utilized to measure the viscosity of the formulated nanoemulsions and nanoemulgels at physiological temperature (37°C). All measurements were done in triplicate with a fixed rotation speed using spindle 64 (LV-04).

2.6.3 *Ex vivo mucoadhesive study*

Ex vivo mucoadhesive study was performed to measure the force required to detach the formulation from goat-skin by using the Brookfield-LFRACT3 texture analyzer. The goat-skin was collected freshly from a local slaughterhouse. The skin was cleaned using cold tap water followed by removal of non-dermatome skin with the help of a scalpel. The skin was attached to the lower probe and approximately 250 µL of nanoemulsion/nanoemulgel was placed at the upper probe. The upper probe moved down with constant speed 0.1 mm/s and force of 0.5 N was applied during contact with skin. After 2 minutes, the probe moved upward and detachment force measured [25].

2.6.4 ***In vitro drug release study***

In vitro dissolution testing was done to evaluate the release of naringenin from the tocotrienols rich naringenin nanoemulsion and nanoemulgel formulations following established method [26]. A dialysis membrane (molecular weight cut off 12,000 Daltons) was used in this study in dissolution apparatus II USP (Electrolab Dissolution Apparatus EDT-08Lx). A 6mL of the respective drug-loaded nanoemulgels, drug-loaded optimized nanoemulsion and blank nanoemulgel were transferred into the activated dialysis membranes. The respective dialysis membrane was clipped from both ends so that the formulation could not come out. To establish sink conditions as well as to sustain permanent solubilization, the dialysis membrane was dipped in a 900 mL phosphate buffer solution at pH 7.4. The release study was carried out for 24 h at 37°C and 100 rpm. A 5 mL of aliquot was withdrawn at pre-determined time intervals of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24.0 h. At the same time, an equal amount of dissolution medium was replaced to maintain the sink condition. The respective aliquots or the diluted samples (wherever necessary) were filtered for UV spectrophotometer analysis at 298nm.

2.6.5 ***Determination of naringenin release kinetics***

A mathematical model was developed to determine the release kinetics as well as the mechanism of drug release of naringenin from the nanoemulgel formulations. As proposed by Dhawan and the team, three kinetic models could be used to study the release kinetics: zero-order, first-order and Higuchi's model [27]. Zero-order is defined as the cumulative percent of drug released versus time, first-order as log cumulative percentage of drug remaining versus time and Higuchi's model as the cumulative percent drug released versus square root of time. On the other hand, the Korsmeyer-Peppas model was used to determine the mechanism of drug release and was graphically represented as log cumulative percent drug release versus log time.

3. Results and discussion

3.1 Development of blank nanoemulsion

Formulating a stable nanoemulsion for hydrophobic agents, the drug is entrapped within the oil core of an *o/w* nanoemulsion, and thus, selection of suitable oil for solubilizing the lipophilic drug is an important step during formulation development [18]. To avoid any instabilities over time, typically drug precipitation, naringenin should be freely soluble in the oil. Firstly, the screening of the highest soluble oil for naringenin was tested and the obtained results were summarized in Figure 1. From the representation, it is evident that naringenin exhibited the highest solubility in Capryol 90 (59.991±3.49 mg/mL) among the different oils tested. It is suggested that the polarity of the lipophilic drug has an important role that favours its solubility in small or medium molar volume oils like monoglycerides or diglycerides or medium-chain triglycerides [21,28]. Hence, for the current experiment, Capryol 90 was chosen as the oil phase for the development of this nanoemulsion formulation. As we aimed to formulate tocotrienols rich nanoemulsion, thus TRF was also incorporated in the lipid phase. Therefore, to check the effect of TRF in the solubility of naringenin in Carpyol 90, we further investigated the solubility of naringenin in combinations of two lipid components. Thus, following the addition of tocotrienols to Capryol 90 in different ratios of 1:1, 2:1, 3:1 and 4:1 (Capryol 90:tocotrienol), moderation of naringenin solubility was found to be insignificant with the increased percentage of tocotrienols in the mixture.

Proper selection of surfactants is crucial as it guarantees a system that is not toxic when being administered topically or orally. For this research, non-ionic surfactants are prioritized as they are generally regarded as safe (GRAS), biocompatible and less distressed by pH or ionic strength modifications [29]. Based on the solubility studies, Solutol HS15 had been selected to be the choice of surfactant (Figure 1 (B)), while Transcutol P was selected as a cosurfactant (data not shown). Solutol HS15, chemically polyoxyethylene esters of 12-hydroxystearic acid, is a non-ionic amphiphilic surfactant that is comprised of fatty acids fused with end-capped methoxy polyethylene glycol and possesses low toxicity, outstanding biocompatibility as well as high performance [30]. Solutol HS15 has an HLB value of 15.2 and it is well known that the surfactants exhibiting HLB value between 8 to 17 could be able to produce a stable oil-in-water type of emulsion.

Co-surfactant is often used in combination with a surfactant to attain transient negative interfacial tension and fluid interfacial film. The use of co-surfactant is able to reduce the

interface's bending stress which brings about larger flexibility for the interfacial film to uptake different curvature needed to produce a stable nanoemulsion over a variety of compositions [17]. Transcutol, diethylene glycol monoethyl ether, is a common cosurfactant, widely explored in pharmaceutical products as well as in nutraceuticals, food and supplements. Transcutol has been proven to be an excellent skin permeation enhancer without significantly affecting the diffusion of permeants across the skin [31]. Contrarily, Panchagnula and Ritschel had shown that Transcutol can enhance skin retention of permeants whereas reduces it's systemic uptake [32]. This property of Transcutol is known to be an intracutaneous depot in which it increases the reservoir capacity of stratum corneum by causing intercellular lipids to swell. Incorporated therapeutics would then be able to hold up in these swollen lipids which creates an intracutaneous depot [32]. There are three grades of Transcutol; P, HP, and CG. In this research, only Transcutol P and HP are focused as they are both of pharmaceutical-grade whereby Transcutol CG is of cosmetic-grade. In the present experiment, we have selected Transcutol P for the development of nanoemulsion of naringenin as it shows the highest solubility of naringenin.

3.2 *Globule size, PDI and zeta potential assessment of blank nanoemulsion.*

To formulate and optimize a stable nanoemulsion, the globule size and distribution, as well as zeta potential values, are important characteristics, which may affect the *in vivo* stability of a nanoemulsion [19]. Nanosized globules are desirable as they assure faster and greater permeation of dug across absorption barriers due to their ability to provide a large surface area and high free energy [19]. Based on our results (Table 1), the mean globule size for the majority of blank nanoemulsions was found to be of less than 200 nm except for 3A and 4A. Formulations 1C, 2C, 3C, and 4C have similar ratios of cosurfactant and surfactant in their composition and only differ from one another in oil percentage. When comparing these four formulations, the globule size is increasing steadily with increasing oil concentration. This is in line with findings in the literature. where they found that upon increment in oil content in their emulsion composition, the globule sizes became larger following a reduction in the specific surface area [20,21,27]. In addition, increment in oil content may increase the rate of collision and coalescence frequency which in turn contributes to larger globule size [17]. PDI indicates the size distributions of globules in nanoemulsions in which smaller value reflects better size distribution and stability of the formulation [18]. All the blank nanoemulsions were found to have a PDI of less than 1.

Further, the determination of surface charge of any colloidal dispersion regulates the interaction between the dispersed particles. In general, the charge of the particle surface attracts the counter ions, and this layer of ions forms the Stern layer around the surface of the dispersed particles. Whenever there is movement of the particles in the solution, the double layer diffuses, where the electrical potential between the double layer boundary and dispersing medium is referred to as zeta potential [33]. This potential is often used to estimate globule's surface charge in the dispersion medium or as an indicator of globule stability. In this present experiment, the zeta potential of the nanoemulsion formulations were ranged from –4mV to –11mV. Theoretically, the nanoemulsions with low zeta potential value are unstable because of dispersion instability; however, different studies had shown stable nanoemulsion formulated with non-ionic surfactants with low surface charge value [21]. The same observation in our experiment reflected thermodynamically stable formulation, which had been discussed in the subsequent section.

3.3 *Preliminary studies to determine suitable compositions of oil:Smix*

Six nanoemulsions (1A, 1B, 1C, 2C, 3A, and 4A) among the twelve had shown instability during thermodynamic stability studies performed. Based on this preliminary study, six blank nanoemulsions had been deemed stable (2A, 2B, 3B, 3C, 4B and 4C) and stepped towards formulating naringenin-loaded nanoemulsions.

3.4 Development and characterization of naringenin loaded nanoemulsion

3.4.1 *Globule size, PDI and zeta potential assessment of drug-loaded nanoemulsions*

Based on the results shown in Table 2, it can be observed that the addition of naringenin resulted in an increase in globule size of all the nanoemulsions when compared with blank formulations, however, all the six drug-loaded nanoemulsions have a PDI less than 0.5, which indicates homogenous size distribution. Ideally, if the globule size is less than 200 nm with a PDI of <1, the formulation is said to be exemplary for topical delivery as rapid pore transport is supported through the providence of large surface area to transfer the entrapped drug through the skin barrier [23].

3.4.2 *pH, refractive index and electrical conductivity of drug-loaded nanoemulsions:*

All the drug-loaded nanoemulsions had achieved satisfactory pH, which is suitable for topical delivery. The relative index of the nanoemulsion was determined and compared to the refractive index of water (1.333). According to Gurpret and Singh, if a nanoemulsion has a refractive index similar to water, then the nanoemulsion it said to exhibit a transparent nature

[34]. Relating the statement to our results (Table 2), all six drug-loaded nanoemulsions can be considered to have transparency.

Conductivity measurements are based on the principle of water is a good conductor of electricity. This method is considered a qualitative method to identify the type of emulsion that a compound possesses. If the continuous phase of the emulsion is aqueous, there would be a deflection indicating that it is an *o/w* emulsion [23]. Based on the results in Table 2, it could be said that all the formulation are of *o/w* nanoemulsions.

3.4.3 *Thermodynamic stability studies*

Finally, six drug-loaded nanoemulsions were subjected to the same thermodynamic stability tests and there was no phase separation, creaming or cracking observed during centrifugation of the drug-loaded samples, which confirms the nanoemulsions' stabilities when subjected to mechanical force. Thereafter, all six drug-loaded nanoemulsions were run through heating and cooling cycles and freeze-thaw studies. In this stage, droplet size, PDI and surface charge are more consistent in 2B* and 3B* formulations. This indirectly confirms that 2B* and 3B* drug-loaded nanoemulsions are the most stable formulations among the six drug-loaded nanoemulsions tested.

3.5 Formulation of drug-loaded nanoemulgel

Based on the stability study, characterization parameters, lower droplet size, and lower size distribution, the 2B* formulation was chosen to convert into nanoemulgel with gelling agents, Carbopol 934 and Carbopol 940 and proceeded for evaluation of characterization parameters of 2B* drug-loaded nanoemulgel formulated using nanoemulsion and gel bases (using 1%, 1.5%, 2% *w/v* Carbopol 934 and 1%, 1.5%, 2% *w/v* Carbopol 940) at 1:1 ratio.

3.6 Characterization of drug-loaded nanoemulgel

3.6.1 *Globule size, PDI and zeta potential assessment of drug-loaded nanoemulgel*

One obvious change that could be observed in terms of the globule size was that the addition of the gelling agent could reduce the sizes of the dispersed globules tremendously. This finding is similar to that reported by Zheng et al., where they reported that the average globule size of the nanoemulgels to be significantly smaller than the nanoemulsions. The authors also reported that even with increasing gel base concentration, the globule size will still being maintained relatively small [35]. This observation supports our findings as shown in Table 3. Further, our results reflected the ability of the Carbopol gel matrix in enhancing nanoemulsions' thermodynamic stability by reducing the mesh size of the nanoemulgel

network. Concerning PDI, NG1-NG6 has shown relatively consistent value, which probably indicated that the addition of different percentage of Carbopol gels did not bring any drastic change in the size distribution of the globules as well. The zeta potential of the blank and drug-loaded nanoemulsions were found to be negative, which might be due to the presence of alcohol moiety in Transcutol [36]. However, this negative surface potential was further decreased when nanoemulgel was formulated. The zeta potential of the nanoemulgel was ranged from -17.2 ± 11.3 mV to -29.9 ± 4.43 mV, where concentration of Carbopol dependent decrease in surface charge is evident. This decreased zeta potential of the nanoemulgel might be attributed by the presence of carboxylate ions on the Carbopol molecules. The formation of the gel matrix by the polymer will further stabilize the dispersed oil droplets of the nanoemulsion as the polymeric barrier would prevent the dispersed droplets to come closer and indirectly help to stabilize the nanoemulgel [15].

3.6.2 *Spreadability test*

Spreadability is important for topical gels as it reflects how the gel readily spreads upon application onto the skin [37]. Based on the results in Table 3, it could be inferred that with the increase in gelling agent concentration in the formulation, the spreadability decreases. This finding is congruent with the reports by other researchers available in the literature [24,38]. According to Varma et al., a gel that possesses a spreadability of more than 2.4 cm can be classified as a fluid gel [24]. From our findings, it could be easily interpreted that the formulations are fluid gel in characteristics, which would be easily spread over the infected area.

3.6.3 *Measured pH of the developed nanoemulgels*

pH is an important characteristic when a pharmaceutical formulation is intended to apply on an open wound, as it affects the cellular processes in the wound in a direct and indirect manner [39]. The chronic wounds or wounds with impaired healing, such as diabetic foot ulcers are typically described to experience a shift in its pH, from acidic to neutral and alkaline [5,40]. According to Schneider *et al*., wounds tend to distort the skin's natural acidic environment and pH, which in turn expose the underlying tissue with neutral pH. Moreover, with the underlying tissue exposed, bacterial colonization may reside which may further shift to a higher pH. It is said that chronic leg ulcers are often populated with oral, resident and intestinal microorganisms [41]. One of the most common residing microorganisms in wounds is *Staphylococcus aureus,* which has been shown to form a chronic wounds. With that being said, our target on wound healing would be to reduce the pH of the wound. It had also been

reported that the application of acid onto the wounds greatly lowers the pH which then makes growth and proliferation of bacteria unfavourable [42]. With the incorporation of gelling agents, Carbopol 934 and Carbopol 940, nanoemulgels became slightly acidic in nature and further maintained the acidic pH of the desired range using triethanolamine. Therefore, it could be assumed that the formulation will be favourable to target wound healing. Furthermore, 3% citric acid had been studied to treat chronic wounds like diabetic foot ulcers and various types of nonhealing wounds [42], where this 3% citric acid solution was reported to have a pH range between 4.9 to 5.3. With this range as a reference, our formulated nanoemulgel were targeted and investigated to possess a pH of 4.9 to 5.3 (Table 3) for effective wound healing treatment.

3.6.4 ***Viscosity measurement***

The topical application of nanoemulsion to the skin is not preferable as a viscous system is required for longer retention at the site of application and therefore, the gelling agent was incorporated into the developed nanoemulsion to enhance the viscosity and suitability for topical delivery. The results displayed in Table 3 represents the expected outcome in regard to viscosity of the nanoemulgels to increase with increasing concentration of Carbopol. From the obtained results on the viscosity of the nanoemulgels, it could be observed that there is an increasing trend in viscosity of the formulations when the nanoemulsion is converted to gel. Thus, it could be assumed that the formulations will retain to the site of application for longer period of time to release the components to exert their physiological actions. The viscosity of the formulation could be correlated to the findings on spreadability, where it could be said that the increase in viscosity reduces the spreadability of the formulation.

3.6.5 ***Ex vivo mucoadhesive study***

Mucoadhesion is one of the most important parameters for topical delivery and promotes sustained release. *Ex vivo* mucoadhesion results revealed minimal adhesion of nanoemulsion compared to Carbopol nanoemulgel, however, nanoemulgel showed better mucoadhesive properties proportional to concentration and grade (Carbopol 934 to 940) as apparent from Figure 3. These results are coherent with the findings of Ashlani et al. where the authors reported that increase in the concentration of Carbopol in the gel led to the enhancement of mucoadhesion [43]. Additionally, Carbopol swells thousand times with higher mucoadhesion from its original volume when soaked in water and higher grade of Carbopol showed highest mucoadhesive property which may attribute to long polymeric chain and greater cohesive

force between goat-skin and formulated nanoemulgel [44]. Comparing the results of viscosity and mucoadhesive strength of the product, there is a liner relationship existing between the rheological factor and mucoadhesive strength. There is increase in mucoadhesive strength of the formulation with the increase in viscosity of the formulations within the limits of our experiments. Our results are in agreement to the existing literature [45]. Further, swelling property of Carbopol might help in absorbing the exudates from the wound environment.

3.6.6 *In vitro drug release study*

Dissolution studies were carried out using a dialysis membrane to compare the release of naringenin from the six different nanoemulgel formulations (NG1-NG6) against the drug-loaded nanoemulsion (2B*). Figure 4 represented the comparative release profile of naringenin from different nanoemulgels and the NE formulation.

Comparing the cumulative drug release outcome between the six nanoemulgels (NG1-NG6) it could be inferred that there is a declining trend of drug release with increasing concentration of gel bases. This is especially notable at the 24 h interval of drug release. For the nanoemulgels containing Carbopol 934; NG1, NG2, NG3, the cumulative drug release decreases from 74.62% (NG1) to 60.70% (NG3) with increasing Carbopol 934 concentration. Similar results were observed in the other three nanoemulgels containing Carbopol 940. At each concentration, the drug release was higher in Carbopol 934 containing nanoemugels as compared to nanoemulgels with Carbopol 940, which could be correlated to the viscosity of the nanoemulgels. It is suggested that Carbopol 934 gel bases exhibit a lower viscosity than Carbopol 940 and thus, is able to showcase a higher drug release [46]. The decrease in release rate in the nanoemulgels could be explained by the fact that the drug-loaded oil droplets are coated by the polymeric surface, and thus the drug had to travel a long and tortuous pathway to reach to the release media, thereby the release of the formulations was decreased in successive formulations.

3.6.7 *Mathematical modelling*

Mathematical modelling is an important aspect to analyze the release kinetic of a drug from formulation. The results obtained from the drug release study for all nanoemulgels as well as 2B* drug-loaded nanoemulsion was fitted to first-order release kinetics with R^2 value nearer to one (1). Further, first-order kinetics refers to that the release rate of drug from formulation largely depends on the drug concentration where the rate of drug release is proportional to the concentration of drug in the formulation [47].

It is said that there are a number of processes that regulates the release of drug from a controlled release dosage form. An initial "burst release" is often observed in polymer matrix-based formulation, where the drug release usually occur either through diffusion or erosion of the matrix [48]. The mentioned initial "burst release" is observed in Figure 4 in the first 2 h of drug release.

Higuchi model is one of the kinetic models that could be used to analyze or interpret a drug release mechanism in which diffusion dominates the drug release pattern [49]. Based on the release results, all formulations were showed to be best fitted with data in the Higuchi model with an R^2 value of >0.95. To further confirm the diffusion mechanism, all data were fitted into the Korsmeyer-Peppas model, which helps to determine the release pattern. This depends on the release exponent "*n*" which was estimated from the linear regression, whereby n=0.45 indicates Fickian diffusion, $0.45 < n < 0.89$ as non-Fickian transport, n=0.89 as case-II transport and n>0.89 indicates super case-II transport [50]. The present results showed that all formulations follow non-Fickian release mechanism except NG6, which represent that the release of naringenin from the NG1 to NG5 will be through diffusion and erosion, whereas release kinetics from NG6 will follow completely diffusion. Further, this can be correlated with the highest viscosity of NG6, which is in congruent to the literature where it had been mentioned high viscous formulation led to high diffusion rates compared to low viscous formulation [51].

Finally, the highest % naringenin release was observed with NG1 formulation when compared to other formulated nanoemulgels. Further, suitable droplet size, PDI, pH and additionaly lowest viscosity and highest spreadability values help us to consider NG1 as the optimized nanoemulgel formation for our future studies.

4. Conclusion

The novel tocotrienols rich naringenin nanoemulgel with suitable viscosity was successfully formulated and characterized for topical application. Here, in this present research two types of Carbomers, Carbopol 934 and Carbopol 940 were used to convert the nanoemulsion to nanoemulgel. From the obtained results, it can be concluded that the optimized nanoemulgel with 1% *w/v* Carbopol 934 (NG1) possessed nanodroplet size (145.58 ± 12.5 nm), desired viscosity (297,600 cP) and mucoadhesive property (1.19 N) with highest spreadability (4.2 cm x 4.4 cm) to apply on the skin surface, which will be suitable for the topical application on diabetes wound. *In vitro* drug release study had also confirmed the supremacy of the

addition of Carbopol gel to the nanoemulsion. The release pattern of the drug from the thermodynamically stable NG1 formulation followed first-order Higuchi model with >74% release of the drug (naringenin) occurred by diffusion and erosion method. The present study has affirmed that tocotrienols-rich naringenin nanoemulgel as a promising alternative for wound management particularly associated with diabetes complications via topical application. However, further studies are necessary to assess the efficacy and safety of the nanoemulgel using discrete study models.

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Disclosures

There is no conflict of interest and disclosures associated with the manuscript.

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Figure legends:

Figure 1. Solubility of naringenin in different (A) oils and (B) surfactants at 37±2°C. Data presented as mean ± SD

Figure 2. Representation of (A) particle size and PDI, and (B) zeta potential of 2B* drug-loaded nanoemulsion

Figure 3. Mucoadhesive strength of nanoemulsion (2B NE) and nanoemulgel (NG1, NG2, NG3, NG4, NG5, NG6). $^{\#}$represents significant difference from blank nanoemulsion ($p < 0.05$)

Figure 4. *In vitro* cumulative release of naringenin from nanoemulsion and six Carbopol nanoemulgels. Values are expressed as mean ± SD ($n = 3$)

Table 1: Compositions of different batches of nanoemulsion formulations with particle size, size distribution, and zeta potential of the blank nanoemulsions.

Formulation code	Oil (% *v/v*)		Surfactant (% v/*v*)	Cosurfactant (% *v/v*)	Water (% *v/v*)	Z-Average (d.nm)	PDI	Zeta Potential (mV)
	Capryol 90	TR F	Solutol HS 15	Transcutol P				
	Total oil: 5%							
1A	2.5	2.5	15	5	75	161.3 ± 0.8	0.198 ± 0.06	-9.18 ± 3.71
1B	2.5	2.5	20	5	70	156.4 ± 0.7	0.21 ± 0.05	-9.99 ± 4.51
1C	2.5	2.5	25	5	65	117.8 ± 0.9	0.511 ± 0.06	-11.4 ± 3.54
	Total oil: 7.5%							
2A	5	2.5	15	5	72.5	138.9 ± 0.6	0.235 ± 0.06	-6.81 ± 2.91
2B	5	2.5	20	5	67.5	150.4 ± 0.7	0.229 ± 0.01	-8.34 ± 5.49
2C	5	2.5	25	5	62.5	120.3 ± 0.5	0.531 ± 0.02	-9.25 ± 3.16
	Total oil: 10%							
3A	7.5	2.5	15	5	70	210.4 ± 0.5	0.402 ± 0.05	-5.36 ± 3.05
3B	7.5	2.5	20	5	65	180.6 ± 0.8	0.276 ± 0.03	-5.57 ± 3.98
3C	7.5	2.5	25	5	60	131.9 ± 0.6	0.193 ± 0.06	-7.65 ± 3.74
	Total oil: 12.5%							
4A	10	2.5	15	5	67.5	299.9 ± 1.1	0.316 ± 0.03	-5.79 ± 3.55
4B	10	2.5	20	5	62.5	176.8 ± 0.5	0.357 ± 0.04	-5.08 ± 3.30
4C	10	2.5	25	5	57.5	149.8 ± 0.9	0.315 ± 0.02	-7.36 ± 3.40

Notes: Data presented as mean ± SD.

Table 2: Characterization parameters of the drug-loaded nanoemulsions.

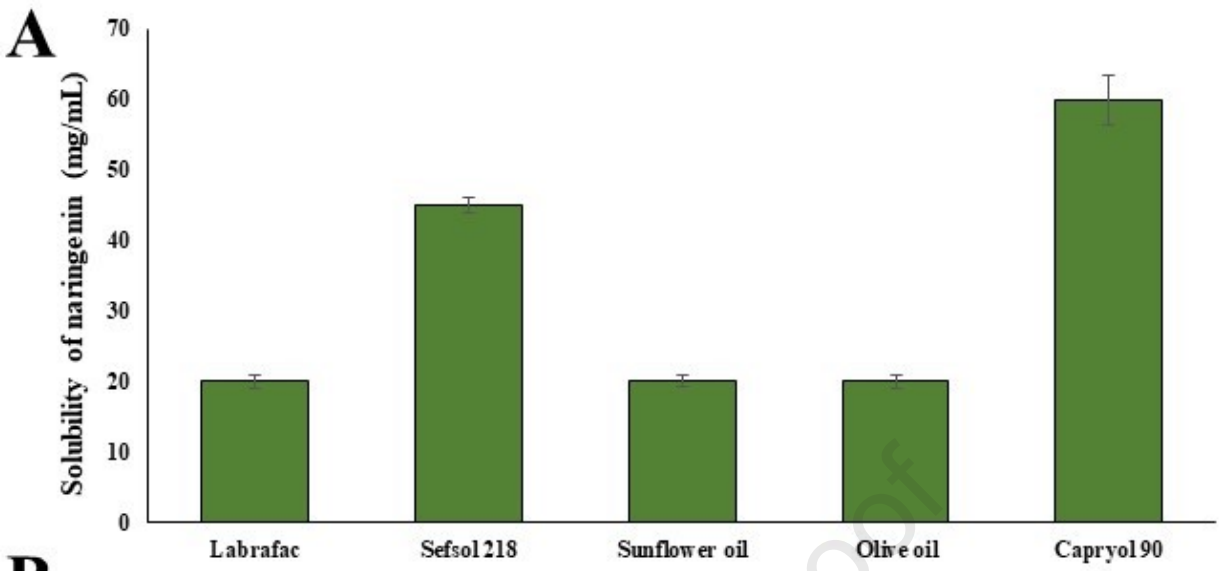
Formulation code	Z-Average (d.nm)	PDI	Zeta Potential (mV)	Refractive index	Conductivity (µS/cm)	pH
2A*	205.2 ± 1.3	0.449 ± 0.05	-7.80 ± 3.26	1.377 ± 0.5	8.239 ± 1.5	5.85 ± 0.4
2B*	162.7 ± 0.3	0.285 ± 0.02	-7.44 ± 3.25	1.381 ± 0.6	25.33 ± 0.4	6.12 ± 0.3
3B*	160.6 ± 0.5	0.216 ± 0.03	-5.57 ± 2.76	1.381 ± 0.4	22.543 ± 0.3	6.00 ± 0.6
3C*	188.3 ± 0.4	0.335 ± 0.03	-5.89 ± 2.93	1.386 ± 0.5	7.398 ± 0.3	5.97 ± 0.5
4B*	211.8 ± 1.5	0.292 ± 0.04	-5.44 ± 3.96	1.378 ± 0.5	44.14 ± 0.4	5.78 ± 0.4
4C*	183.04 ± 0.3	0.371 ± 0.03	-6.26 ± 3.25	1.382 ± 0.6	19.972 ± 0.4	5.73 ± 0.4

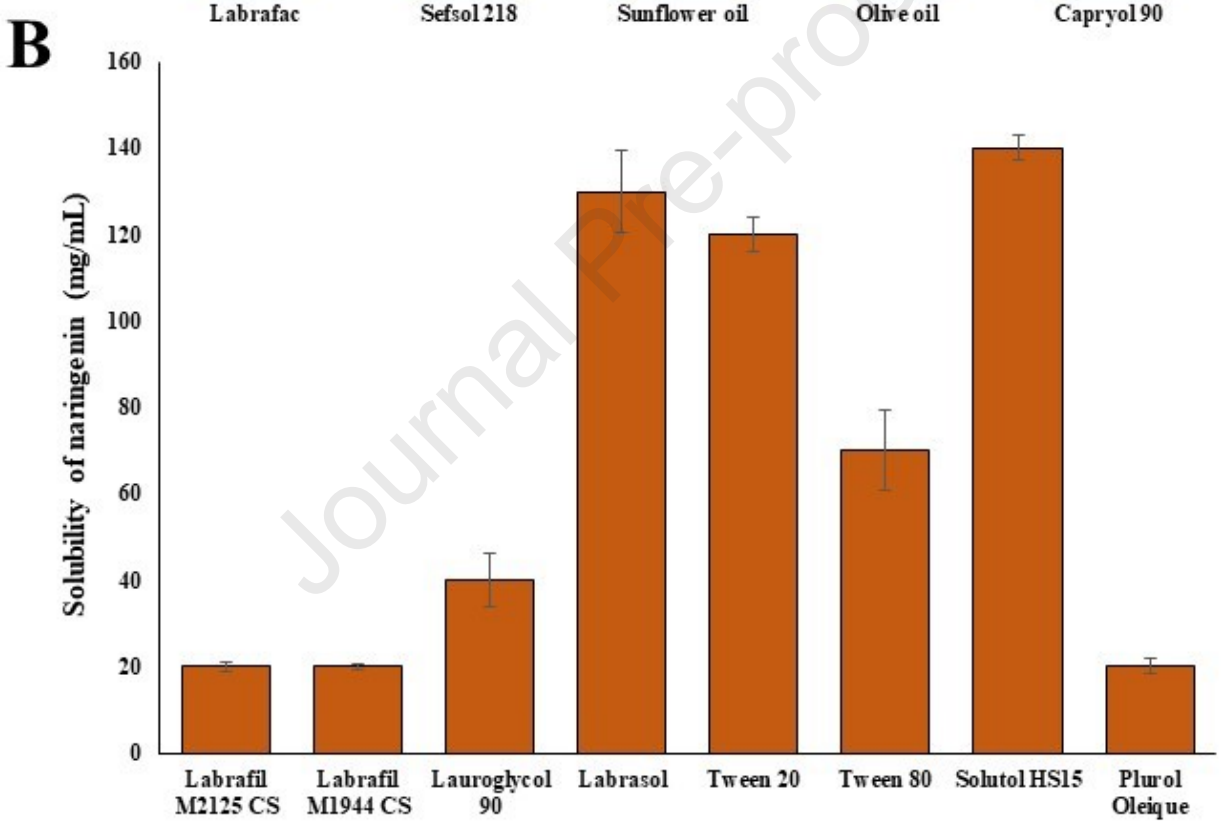
Data presented as mean ± SD.

Table 3: Characterization parameters of the respective naringenin nanoemulgels formulated with different gel bases at varying concentrations.

Formulation code	Gel base	(*w/v*)	Globule size (d.nm)	PDI	Zeta potential (mV)	Viscosity (cP)	pH	Diameter of spread (cm x cm)
NG1	Carbopol 934	1%	145.58 ± 12.5	0.452 ± 0.03	-21.1 ± 3.32	297,600	5.13 ± 0.6	4.2 x 4.4
NG2		1.5%	123.82 ± 9.6	0.432 ± 0.03	-26.9 ± 4.54	311,400	4.98 ± 0.5	3.7 x 3.5
NG3		2%	129.88 ± 11.4	0.449 ± 0.04	-29.9 ± 4.43	337,200	5.21 ± 0.7	3.6 x 3.6
NG4	Carbopol 940	1%	138.50 ± 13.7	0.384 ± 0.06	-17.2 ± 4.32	344,400	4.96 ± 0.6	3.5 x 3.6
NG5		1.5%	130.66 ± 7.5	0.433 ± 0.05	-21.2 ± 3.47	358,200	4.87 ± 0.4	3.3 x 3.4
NG6		2%	111.04 ± 8.6	0.439 ± 0.04	-22.5 ± 4.62	465,000	5.01 ± 0.4	3.1 x 3.2

Data presented as mean ± SD.

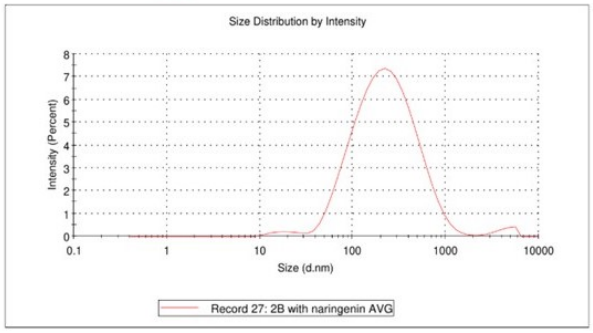




A

		Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 172.3	Peak 1:	280.3	96.6	219.9
PdI: 0.372	Peak 2:	4225	1.9	1049
Intercept: 0.903	Peak 3:	20.06	1.5	6.666

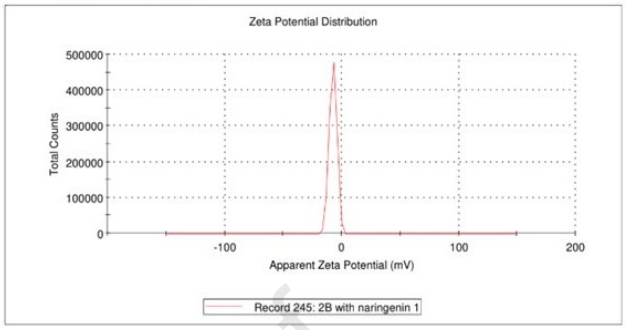
Result quality Good

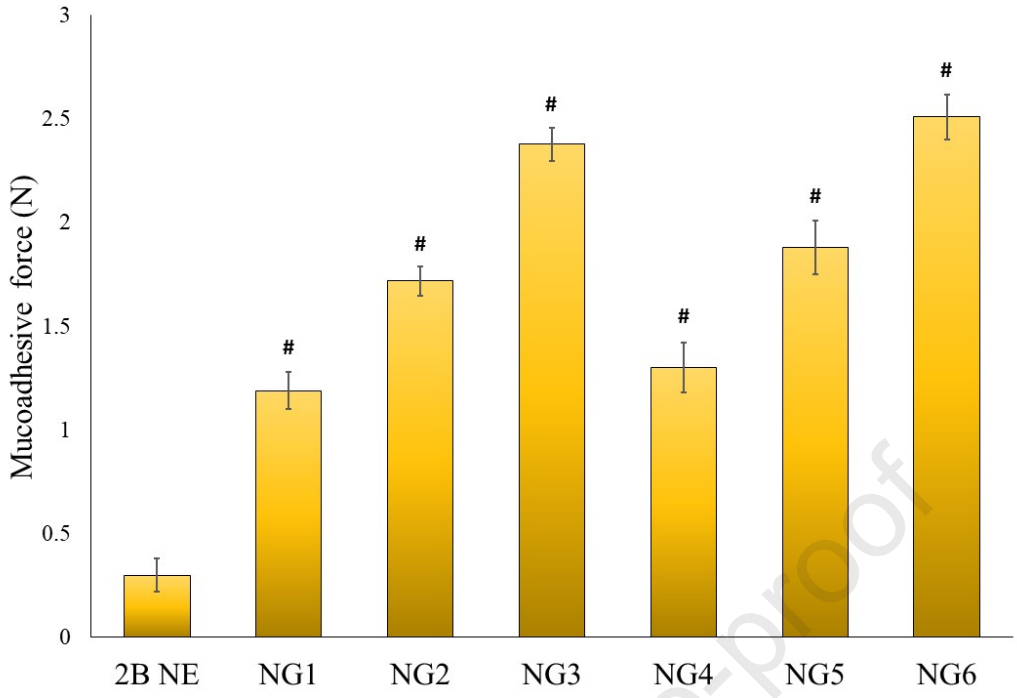


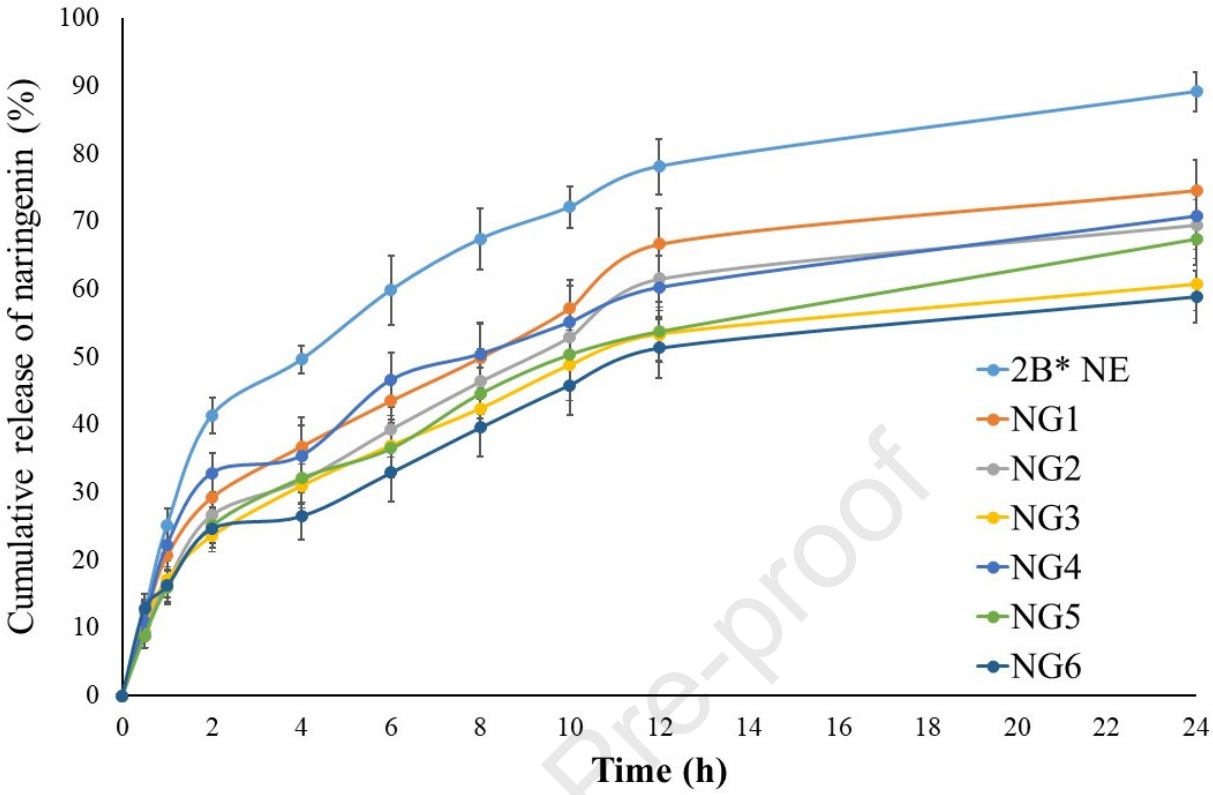
B

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -7.44	Peak 1:	-7.44	100.0	3.25
Zeta Deviation (mV): 3.25	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0966	Peak 3:	0.00	0.0	0.00

Result quality Good







Highlights:

- The thermodynamically stable optimized naringenin-loaded tocotrienol-rich nanoemulgels were fabricated using low-energy emulsification method.
- Developed nanoemulgel possesses nanometric globule size with good spreadability.
- Controlled *in vitro* release was obtained over a period of 24 h.
- First-order release and Higuchi model with non-Fickian diffusion was established in the *in vitro* release kinetic profile.
- This nanoemulgel could be a promising tool in the management of chronic wound condition.

AUTHOR DECLARATION TEMPLATE

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from bapi.gn@gmail.com.

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