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RESEARCH ARTICLE

Linezolid-Loaded Nanoparticle for Topical Administration of Diabetic Foot Treatment: Formulation, *In Vitro* and *Ex Vivo* Characterization

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Linezolid-Loaded Nanoparticle for Topical Administration of Diabetic Foot Treatment: Formulation, In Vitro and Ex Vivo Characterization

SUMMARY

The aim of this research is to formulate a topical gel containing nanoparticles for diabetic foot ulcers (DFU). In this respect, the nanoparticle formulations were prepared using the spontaneous emulsification technique. Linezolid (LZD)-loaded nanoparticle formulation exhibited low average particle size (PS) of 195.27±5.42 nm, low polydispersity index (PI) of 0.214±0.019, high zeta potential (ZP) of 20.57±0.35 mV and high drug entrapment efficiency (EE) of 99.746±0.021%. To enhance topical residence time, the LZD-loaded nanoparticles were dispersed in a gel formulation using Methocel TM K4M (HPMC) and Carbopol® 974 P NF. The formulated gels demonstrated favorable characteristics, including an appropriate pH value, suitable mechanical performance, and desirable viscosity and spreadability for topical application. All formulations displayed pseudoplastic flow and typical gel-type mechanical spectra at the specified frequency value. Moreover, the developed formulation achieved sustained drug release as intended for these systems. During ex vivo drug diffusion studies, 0.007±0.004% of LZD was found in receptor phase, indicating a local effect. The optimum formulation was stable for six months. The initial findings suggest that the formulated topical gel containing LZD-loaded nanoparticles holds promise as an effective drug delivery system for DFU management. However, further comprehensive investigations are required to substantiate this hypothesis.

Key Words: *Diabetic foot ulcer, linezolid, nanoparticle, gel*

Diyabetik Ayak Tedavisinin Topikal Uygulaması için Linezolid Yüklü Nanopartikül: Formülasyonu, In Vitro ve Ex Vivo Karakterizasyonu

ÖZ

Bu çalışmanın amacı, diyabetik ayak ülseri (DFU) tedavisi için nanopartikül içeren bir topikal jel formülasyonu geliştirmektir. Bu bağlamda, nanopartikül formülasyonları, spontan emülsifikasyon tekniği kullanılarak hazırlandı. Linezolid (LZD) yüklü nanopartikül formülasyonu, düşük partikül boyutu (PS) 195.27±5.42 nm, düşük polidispersite indeksi (PI) 0.214±0.019, yüksek zeta potansiyel (ZP) 20.57±0.35 mV ve yüksek enkapsülasyon etkinliği (EE) %99.746$_{\pm 0.021}$ gösterdi. Topikal kalma süresini artırmak için, LZD yüklü nanopartiküller, MethocelTM K4M (HPMC) ve Carbopol® 974 P NF kullanılarak bir jel formülasyonunda disperse edildi. Formüle edilen jeller, uygun pH değeri, uygun mekanik özellik ve topikal uygulama için istenen viskozite ve yayılabilirlik gibi olumlu özellikler sergiledi. Tüm formülasyonlar psödoplastik akış sergiledi ve belirtilen frekans değerinde tipik jel tipi mekanik spektrumlar sergiledi. Ayrıca geliştirilen formülasyon, bu sistemler için amaçlandığı gibi sürekli ilaç salımı sağladı. Ex vivo ilaç difüzyon çalışmaları sonucunda LZD'nin %0.007$_{\pm 0.004}$'ünün reseptör fazında bulunması lokal etkinin bir göstergesidir. Optimum formülasyon 6 ay boyunca stabil bulundu. İlk bulgular, LZD yüklü nanopartikülleri içeren formülasyonun, DFU tedavisi için umut vadeden etkili bir dozaj şekli olduğunu göstermektedir. Ancak, bu hipotezi desteklemek için daha kapsamlı araştırmalar gerekmektedir.

Anahtar Kelimeler: *Diyabetik ayak ülseri, linezolid, nanopartikül, jel.*

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INTRODUCTION

Diabetic foot ulcer (DFU) is a severe health problem with the increasing prevalence of diabetes. DFU is a multifaceted disease from various risk factors such as peripheral neuropathy, peripheral arterial disease, foot deformities, minor trauma, and compromised infection resistance (Rayate et al., 2023; Loera-Valencia et al., 2022). Topical administration of antimicrobial agents becomes crucial due to the heightened susceptibility to infections associated with this disease (Ezhilarasu et al., 2020).

Linezolid (LZD) is an antibiotic used to treat various bacterial infections. It belongs to the oxazolidinone class of antibiotics and inhibits protein synthesis in bacteria (Traunmüller et al., 2010). The molecular formula of LZD is $C_{16}H_{20}FN_3O_4$, and its molecular-weight, melting point, and LogP values are 337.351 g/mol, 181.5–182.5 C, and 0.9, respectively. LZD is sparingly soluble in water, its solubility at 25°C is approximately 3 mg/mL. It is more soluble in acidic solutions. LZD is a weak base, and its pKa values are reported to be approximately 1.8 (Fernandes et al., 2020). It is used in the treatment of gram-positive bacteria and mycobacteria. It is effective against gram-positive aerobic bacteria, gram-negative anaerobic bacteria and gram-positive anaerobes (Chen et al., 2020; Basetti et al., 2014). Due to the reported side effects of systemic LZD (such as nausea, vomiting, headache, dizziness, allergic reactions, diarrhea and abdominal discomfort), there is a need to develop a topical formulation that does not carry the previously mentioned risks in clinical practice.

The topical route of drug administration offers benefits such as reduced side effects, improved patient compliance and avoidance of the first-pass effect. Despite the limitations of poor skin permeability for some drugs, using nanoparticular drug delivery systems and other innovative formulation approaches can help overcome these barriers and improve the effectiveness of topical drug delivery (Wagas et al., 2022). Drug research aims to find new and effective ways of delivering medications. This situation includes prolonging their effectiveness, targeting specific areas, controlling release, preserving fragile drugs until absorption, and enhancing overall treatment efficiency (Wagas et al., 2022; İlhan et al., 2022).

Nanoparticles (NPs) offer several advantages in various applications, including DFU healing. One of the critical advantages of NPs is their versatility and ability to be used in different ways. They can be designed to have controlled sizes and unique physiochemical properties, making them suitable for particular purposes (Ezhilarasu et al., 2020; Jifar et al., 2021). A significant advantage of NPs in DFU healing is their larger surface area to volume ratio. This property allows for better cell adhesion, which is crucial for tissue regeneration. NPs can penetrate the skin more effectively, reaching deeper layers and ensuring better drug absorption. Furthermore, encapsulated drugs within NPs can be released in a sustained and controlled manner. This sustained release allows for a continuous and prolonged therapeutic effect, promoting the healing process over an extended period. The delivery rate of the encapsulated drugs can also be adjusted by altering the distribution of NPs, providing flexibility and customization in treatment. In summary, using NPs in wound healing treatments offers advantages such as targeted drug delivery, enhanced cell adhesion, sustained release of encapsulated drugs, and the ability to modify the delivery rate. These advantages make NPs an attractive approach to improve DFU healing outcomes (Ezhilarasu et al., 2020; Rajendran et al., 2018). Eudragit® (EUD) was chosen to prepare NPs. It is a non-biodegradable, cationic copolymer derived from acrylic and methacrylic acids or their esters. EUD RS contains a low percentage of quaternary ammonium groups, typically ranging from 4.5% to 6.8%. This polymer is insoluble in water under physiological pH conditions but exhibits swelling properties, making it suitable for dispersing active ingredients. EUD polymers have garnered

interest for their excellent stability, consistent release rates of active substances, and favorable mucosal tolerability, making them attractive candidates for various research applications (Rençber et al., 2016; Yenilmez, 2017).

Nevertheless, frequent administration is required for topical NP formulations due to their brief residence time on the skin. Semi-solid systems can be employed to extend the residence time of NPs. Topical gels provide numerous benefits, including easy administration to the skin, reduced frequency of application, enhanced patient compliance, and improved comfort compared to conventional dosage forms (Rençber & Karavana, 2020). Natural, synthetic or semi-synthetic polymers are used for gels, especially for controlled release. Hydroxypropyl methylcellulose (HPMC) is a nonionic, water-soluble synthetic polymer obtained from cellulose that is extensively utilized to formulate semi-solid preparations. Recognised for its lack of toxicity, non-irritating nature, exceptional mucoadhesive properties and swelling capacity, HPMC serves as a versatile ingredient in various formulations (Ghosal et al., 2011; Pan et al., 2023). Carbopol® polymer or carbomer, is a synthetic, high molecular-weight polymer derived from acrylic acid. Cross-linked acrylic acid polymers that swell in water to form transparent or slightly opaque gels. Carbopol® is known for its ability to increasing the viscosity of aqueous solutions at low concentrations, offering excellent rheological control. Additionally, it is pH-sensitive and can form gels over a wide pH range, making them versatile in various formulations (Shin et al., 2000; Kim et al., 2003).

The present work aims to develop a topical gel formulation containing NPs that can effectively alleviate the symptoms of DFU. This approach is designed to minimize the risk of undesirable side effects associated with existing oral formulations available on the market. The developed formulation was characterized for particle size (PS), polydispersity index (PI), zeta potential (ZP), morphology, drug entrapment efficiency (EE), pH, mechanical properties, viscosity, spreadability, flow, *in vitro* drug release, *ex vivo* drug diffusion, and stability.

MATERIAL AND METHODS

LZD was donated by MS Pharma, Turkey. EUD RS 100 was donated by Karadeniz Chemical Company (Karadeniz, Turkey). Carbopol® 974 P NF was obtained from Noveon (Cleveland, OH). Methocel™ K4M was kindly gifted by Colorcon (Dartford, UK). All other chemicals used were of analytical grade.

Preparation of NPs

The NPs were prepared using the spontaneous emulsification technique. A solution containing 2.5% EUD RS 100 and 0.6% LZD was dissolved in 25 mL of ethanol. The alcoholic solution was slowly dripped into 50 mL of distilled water with continuous stirring. The resulting NPs were stirred for 48 hours at room temperature (Rençber et al., 2016).

Characterization of NPs

Determination of mean PS, PI, and ZP values for the formulations was carried out using the Dynamic Light Scattering method (Malvern NanoZS, Malvern Instruments, Malvern, UK) at ambient temperature.

The morphological analysis of both the blank and LZD-loaded NPs was carried out using a Scanning Electron Microscope (SEM) (Carl Zeiss 300VP). Before SEM analysis, the samples underwent a coating process with a thin layer of gold, facilitated by the QUORUM Q150 RES device.

The LZD content in the samples was measured spectrophotometrically (BMG Labtech- Clariostar) at 283 nm. The drug entrapment efficiency was determined through a validated spectrophotometric method involving ultracentrifugation of NP dispersion for 90 seconds at 15,000 rpm (Hettich Mikro 200R). The supernatant obtained after centrifugation was utilized for LZD analysis via spectrophotometry to determine the quantity of free drug. The quantity of encapsulated LZD was determined by subtracting the amount of free LZD from the total LZD content present in the dispersion (n=5, Equation 1) (Rencber & Tanrıverdi 2018).

$$\text{Encapsulation efficiency (EE) (\%)} \frac{\text{Total amount of LZD - The amount of free LZD}}{\text{Total amount of LZD}} \times 100 \quad \text{(Equation 1)}$$

Preparation of gel formulations

To prepare HPMC gels, varying proportions (2-3%) of Methocel™ were slowly added to distilled water with a magnetic stirrer. The gels were refrigerated until a transparent solution was achieved, typically within 24 hours.

To prepare Carbopol® gels, different ratios (1-2%) of Carbopol® 974 NF were kept in distilled water for 24 hours to ensure homogeneous swelling. After 24 hours, the pH was adjusted by adding triethanolamine, and transparent gels were obtained.

Gels prepared with different polymers in different ratios were evaluated for parameters such as pH, viscosity, mechanical properties, and rheological analysis. NPs with low viscosity were dispersed in gel formulations selected to give the appropriate viscosity for topical application.

The necessary amounts of Carbopol® 974 P NF, and Methocel™ were allowed to blend for 24 hours. Subsequently, the gels incorporating Carbopol® were neutralized with triethanolamine. The NP dispersion was introduced into the pre-prepared gels and stirred for 1 hour at ambient temperature. The concentration of LZD in the gels was set at 6 µg/mL as the conclusive level. Details regarding the composition of the gel formulations are outlined in Table 1.

Table 1. Composition of gel formulations

Formulation Code	Content of Formulation	Polymer Ratio (%)	LZD NP
F1	Carbopol® 974 P NF	1	-
F2	Carbopol® 974 P NF	1.5	-
F3	Carbopol® 974 P NF	2	-
F4	Methocel™ K4M	2	-
F5	Methocel™ K4M	2.5	-
F6	Methocel™ K4M	3	-
F1 + LZD NP	Carbopol® 974 P NF	1	+
F4 + LZD NP	Methocel™ K4M	2	+

Characterization of gel formulations

Macroscopic evaluation of gels: Macroscopic features of the gels, including color, uniformity, transition from clarity to opacity, and the identification of residues or phase separation, were evaluated through visual observation over a 48-hour duration.

Determination of pH: To evaluate the compatibility of the prepared gels with topical surfaces, pH measurements were carried out at room temperature using a pH meter (Hanna Edge, USA).

Mechanical properties of gels: The mechanical characteristics were evaluated, and assessed using a software-controlled penetrometer (TA-TX Plus, Stable Micro System, UK) operating in texture profile analysis (TPA) mode at temperatures of 25±0.5°C and 32±0.5°C. The formulations were transferred into a beaker, and the analytical probe (Perspex P/10) of the penetrometer was pressed into the formulations twice at a consistent speed to a certain depth. These compressions were performed at room temperature, with a 15-second interval between each compression. The mechanical parameters were derived from the force-time curves obtained during the analysis (n=6) (Rençber & Karavana 2020; Jones et al., 1997; Rencber et al., 2021).

Viscosity measurement of gels: The viscosity of the gel formulations was measured using a Brookfield viscometer (Brookfield Ametek DV2T Viscometer, Brookfield Engineering Laboratories, Inc., USA) at 25°C. The measurements were performed using a

rotational viscometer with a spindle 27. Viscosity parameters were measured at various rotations per minute (rpm) with a 1-minute equilibration time at each rpm. Samples were applied to the lower plate using a spatula to ensure proper shearing of the formulation (n=3).

Spreadability studies of gels: The spreadability of gel formulations was measured at 25°C and 32°C using a software-controlled penetrometer (TA-TX Plus, Stable Micro System, UK, Prob: TTC Spreadability Rig). The gel formulation was carefully added to a beaker, taking particular care to avoid the formation of bubbles. The force, quantified in Newtons, was monitored throughout the testing period, and spreadability was assessed by computing the area under the curve (AUC).

Flow behavior analysis of gels: The rheogram for the gels was generated using a Brookfield Viscometer (Brookfield Ametek DV2T Viscometer, Brookfield Engineering Laboratories, Inc., USA) at 25±1°C, employing spindle 27. Shear stress was measured by incrementally increasing the shear rate from 0.5 to 100 rpm.

In vitro drug release studies: The release of LZD from both NPs and gels containing NP was evaluated utilizing a dialysis bag submerged in PBS at 32±0.5°C. The system was continuously stirred with a magnetic stirrer for 24 hours. The drug content in the receiving solution was quantified through a validated spectrophotometric method. Sink conditions were upheld in the receptor compartment throughout the *in vitro* release studies (n=5).

Release kinetics: Five kinetic models were chosen to describe the release profiles of the prepared systems. Thus, the kinetic models were investigated using a computer program for empirical analysis.

Ex vivo drug diffusion study: The permeation study of LZD-NP-loaded gels was conducted using Franz diffusion cells. Sheep ear skin, sourced from a local slaughterhouse, was carefully mounted onto the diffusion cells following established protocols (Huong et al., 2009; Bayldon et al., 2014; Reddy et al., 2019). The donor and receptor chambers were filled with 1 mL of the formulation and 20 mL of PBS, respectively. The cells were kept at a temperature of 32±0.5°C with continuous magnetic stirring. Samples were collected at designated time intervals over 24 hours and analyzed using a validated spectrophotometric method. Sink conditions were maintained in the receptor compartment throughout the *ex vivo* drug diffusion studies (Senyiğit et al., 2010; Padula et al., 2019).

Stability studies: For stability evaluations, the optimum gel formulations containing NP were stored at 4±1°C in a refrigerator and at 25±2°C with a relative humidity of 60% for a period of 6 months in a stability cabinet (Nuve ID 300, Ankara, Turkey). The gels were scrutinized for alterations in macroscopic appearance, pH, and mechanical properties (n=3).

Statistical data analysis: All experiments were performed at least three times and data are expressed as mean values ± standard deviation. Statistical data analysis was performed using the Student's t-test with a minimum significance level of $p<0.05$.

RESULTS AND DISCUSSION

Preparation of NPs

NPs were prepared through the spontaneous emulsification technique, as outlined by Rençber et al. (Rençber et al., 2016). An inherent advantage of this method lies in its ability to circumvent the use of harmful organic solvents. The resulting NP formulation exhibited a clear and consistent appearance.

Characterization of NPs

Determination of PS, PI and ZP

Particle size is a critical aspect of characterizing nanoparticle properties, given that particle size significantly influences various nanoparticle characteristics. The size of nanoparticles affects the penetration of drug molecules into the stratum corneum. It has been shown that the penetration and retention of nanoparticles into the skin depends on

the particle size. The optimal size range for topical application and improvement of topical delivery is around 100-700 nm (Xiang et al., 2023; Liu et al., 2018). PS is crucial for drug delivery and clearance. The PS of blank NP and LZD-loaded NP were found to be 184.93±4.87 nm and 195.27±5.42 nm, respectively (Table 2). The PS value slightly increased with the addition of LZD ($p < 0.05$). Similar results of 182.6±8.4 and 225.1±7.4 nm were obtained by Puglia et al. (Puglia et al., 2016).

The PI of blank NP and LZD-loaded NP were found 0.170±0.030 and 0.214±0.019, respectively (Table 2). The increase in PDI value of LZD-loaded NP was significant ($p < 0.05$) compared to blank NPs, but the PDI value was still around 0.3. PI of blank NP and LZD-loaded NP was low (PI<0.3), showing that this method of preparation resulted in appropriate size distribution, a narrow dispersity, high physical stability and uniform system (Table 2) (Aksu et al., 2014; Behbahani et al., 2017).

The stability of NPs is significantly indicated by the ZP, which is a crucial factor. The ZP of blank and LZD-loaded NP was found to be 26.40±1.04 and 20.57±0.35 mV, as represented in Table 2. ($p < 0.05$). The incorporation of LZD into nanoparticles appears to cause a significant decrease in zeta potential, which can be attributed to the preferential adsorption of counter ions or hydrogen ions on the nanoparticle shell with the addition of the active substance (Behbahani et al., 2017). The findings support a previous study by Salatin et al. (Salatin et al., 2017), who determined that NPs made with Eudragit have a positive charge surface.

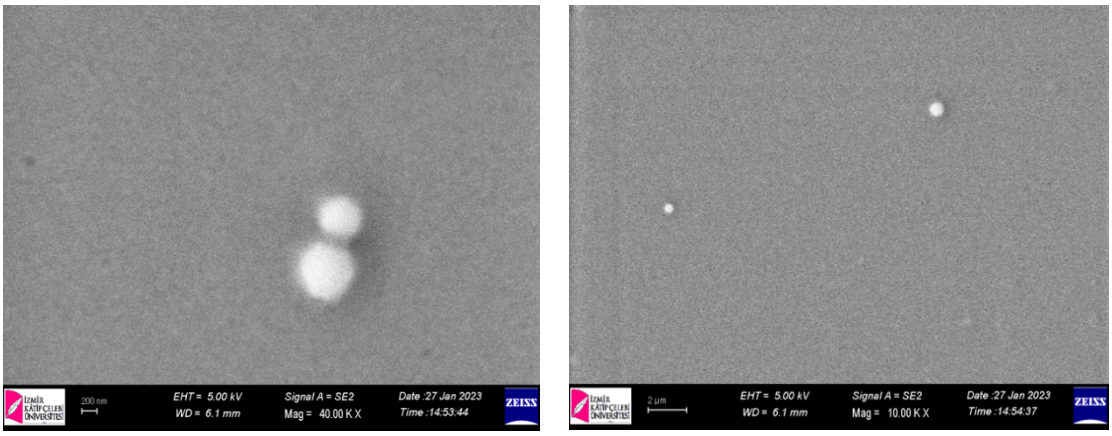
Table 2. PS, PI, and ZP of NP formulations

	PS (nm) ± SD	PI ± SD	ZP (mV) ± SD
Blank NP	184.93±4.87	0.170±0.030	26.40±1.04
LZD-loaded NP	195.27±5.42	0.214±0.019	20.57±0.35

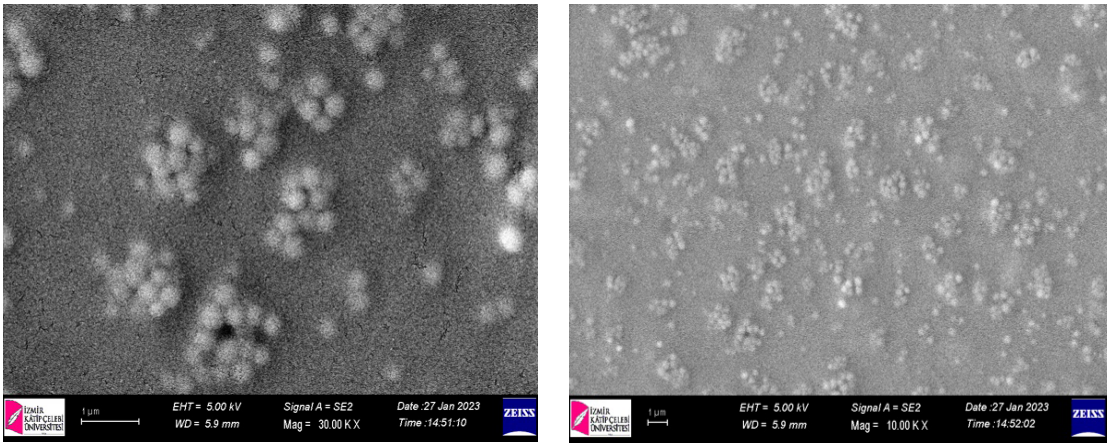
Morphology analysis

The SEM analysis of blank and LZD-loaded NP formulation showed spherical-shaped as shown in

Figure 1. The average particle size aligns with the results obtained using the Nano-ZS Zetasizer.



Blank NP formulation (at 40.00x and 10.00x magnifications)



LZD-loaded NP (at 30.00x and 10.00x magnifications)

Figure 1. SEM photographs of the NPs at 40.00x, 30.00x and 10.00x magnifications

Drug entrapment efficiency

The concentration of LZD in the aqueous phase was measured using a validated spectrophotometric method. The calibration curve for LZD exhibited linearity within the concentration range of 20-150 µg/mL, with a correlation coefficient (r^2) of 0.9999. The detection, and limit of quantification for LZD were determined to be 3.170, and 9.607 µg/mL, respectively.

As expected, the prepared LZD-loaded NPs exhibited a high drug EE of 99.746±0.021%. This elevated EE% is likely attributed to the high solubility of LZD in the selected solvent. The high EE% implies an outstanding level of protection against environmental stressors, including oxygen, moisture, pH fluctuations, and temperature variations (Bertrand et al., 2023). In the study of Enazy et al. (Enazy et al., 2023) in 2023, the EE of polymeric NP was 99.1%. The optimized NP dispersion was then introduced into the gel.

Preparation of gel formulations

In the present study, gels were successfully formulated using Methocel™ K4M (HPMC) and Carbopol® 974 P NF improve in terms of the patient compliance and comfort. Methocel™ is a biodegradable, polar, non-toxic, and non-irritant polymer with water solubility that undergoes swelling upon contact with a solution, leading to the formation of a gel mass (Yousaf et al., 2021; Rençber et al., 2019; Feroz & Dias 2021). It has received approval from the FDA for utilization in controlled-release formulations (Feroz & Dias 2021). Carbopol®, a poly(acrylic acid) (PAA) polymer, possesses good biocompatibility, biodegradability, and low toxicity. This polymer

is valuable as mucoadhesive agent, demonstrating controlled drug release profiles (Huei-Jen et al., 2006; Surassmo et al., 2015).

Characterization of gel formulations

Macroscopic appearance of gels

The gel formulations were observed to have a translucent appearance, providing a smooth feel upon application and exhibiting homogeneity.

Determination of pH

The pH of the formulation is significant for topical drug delivery systems. A small change in the pH of the topical formulation can cause skin irritation during application (Wagas et al., 2022; Rençber et al., 2019; Rençber & Tanrıverdi, 2018). The gel formulations exhibited pH values ranging from 6.15±0.107 to 7.23±0.092. The pH values of F1 + LZD NP and F4 + LZD NP were found to be 5.94±0.038 and 5.99±0.137, respectively (Table 3). The results suggest that the prepared gels are suitable, as their pH values are close to the normal pH of the skin.

Table 3. pH values of formulations

Formulation Code	pH ± SD
F1	6.15±0.107
F2	6.35±0.038
F3	6.28±0.065
F4	6.39±0.006
F5	6.44±0.072
F6	7.23±0.092
F1 + LZD NP	5.94±0.038
F4 + LZD NP	5.99±0.006

Mechanical properties of gel formulations

Mechanical properties (hardness, compressibility, adhesiveness, elasticity, and cohesiveness) were examined using software controlled penetrometer (Table 4).

The hardness is an essential parameter in TPA. This property refers to the force necessary to achieve a specific deformation in semi-solid systems. Hardness and compressibility characterize the stress and work needed to dispense a sample from the container and apply it to the desired site. For topical applications, low hardness and low compressibility for easy removal from the container are desirable. Furthermore, the literature demonstrates a correlation between viscosity and hardness (Jones et al., 1997; Toksoy et al., 2013; Rençber et al., 2019; Rençber et al., 2021). Gels prepared with Carbopol® and Methocel polymers were evaluated separately to decide the ideal polymer ratio. Both gel hardness and compressibility increased as a function of growing polymer concentrations. It was observed that the F1 formulation had the lowest hardness and lowest compressibility value among the gels prepared with Carbopol®. Among the gels prepared with Methocel, the F4 formulation had the lowest hardness and compressibility value (Table 4). Therefore, among the gel formulations, F1 and F4 formulations were selected as optimum formulation for both groups. After loading the nanoparticles into the gels, an increase in the hardness values of the gel formulations was observed. This situation can be explained by concentration-dependent effects on the formulations' viscosity. This is also supported by the literature (Karavana et al., 2012). F4 + LZD NP had lower hardness value, lower compressibility and the lower viscosity value than F1 + LZD NP (Table 4, Figure 2). Significant differences were observed in the values of hardness and compressibility ($p<0.05$).

Elasticity signifies the speed at which a deformed sample reverts to its original, undeformed state. Lower numerical values obtained through Texture Profile Analysis (TPA) indicate more excellent elasticity (Rençber et al., 2021). Among gel formulations containing NPs, F4 + LZD NP formulation has the most appropriate elasticity value. After loading the NPs into the F4 coded gel formulation, an decrease in the elasticity values of the gel formulation was observed. This situation is supported by the literature (Karavana et al., 2012).

Table 4. Mechanical properties of the gels

Code	Temperature	H (g) ± SD	C (g·sec) ± SD	A (g·sec) ± SD	E ± SD	Ch ± SD
F1	25°C	0.133±0.002	0.511±0.008	-0.380±0.026	1.026±0.046	1.045±0.020
	32°C	0.104±0.007	0.309±0.084	-0.256±0.072	0.854±0.038	1.060±0.047
F2	25°C	0.157±0.006	0.634±0.071	-0.478±0.063	1.055±0.015	1.052±0.022
	32°C	0.121±0.016	0.202**±0.**032	-0.190±0.088	1.009±0.083	1.053±0.066
F3	25°C	0.153±0.003	0.564±0.083	-0.398±0.077	1.006±0.018	1.089±0.042
	32°C	0.201±0.011	0.840±0.207	-0.665±0.200	1.014±0.029	1.047±0.047
F4	25°C	0.013±0.000	0.048±0.004	-0.042±0.003	0.877±0.008	1.127±0.012
	32°C	0.009±0.000	0.026±0.000	-0.022±0.000	0.891±0.000	1.238±0.000
F5	25°C	0.017±0.001	0.064±0.002	-0.068±0.001	0.909±0.007	1.149±0.009
	32°C	0.012±0.000	0.038±0.004	-0.047±0.011	0.870±0.021	1.195±0.014
F6	25°C	0.020±0.001	0.076±0.005	-0.087±0.008	0.939±0.018	1.119±0.014
	32°C	0.013±0.000	0.033±0.002	-0.053±0.007	0.913±0.016	1.187±0.004
F1 + LZD NP	25°C	0.181±0.004	0.503±0.008	-0.206±0.011	1.012±0.056	1.043±0.047
	32°C	0.144±0.003	0.216±0.005	-0.206±0.011	1.049±0.068	1.033±0.058
F4 + LZD NP	25°C	0.038±0.001	0.068±0.009	-0.163±0.001	0.973±0.013	1.063±0.001
	32°C	0.031±0.002	0.060±0.014	-0.136±0.013	0.990±0.049	1.140±0.006

*H: Hardness, C: Compressibility, A: Adhesiveness, E: Elasticity, Ch: Cohesiveness

Viscosity Measurement

The viscosity of the gel formulations was assessed using a Brookfield viscometer at a temperature of 25°C. The viscosity value increased significantly with increasing the hardness and compressibility of gels (Jones et al., 1997).

In gel formulations, Carbopol® and Methocel create a physically bonded network through the formation of junction sites, contributing to the mechanical strength of the gel. Incorporating of NPs in the F1 formulation resulted in a slight reduction in gel viscosity. This effect may be attributed to the low viscosity of the NPs and potential electrostatic interactions with the gel network (Saez et al., 2019). Contrastingly, the inclusion of NPs in the F4 formulation led to a slight increase in gel viscosity. This situation suggests no apparent interactions between the NPs and Carbopol® chains. The viscosity of F4+LZD NP increased slightly with the increase of particle size of NP. This situation is compatible with the literature (Liu et al., 2008). The viscosity values were ranked as F1>F1+LZD NP>F4+LZD NP>F4, respectively. These findings are consistent with TPA studies.

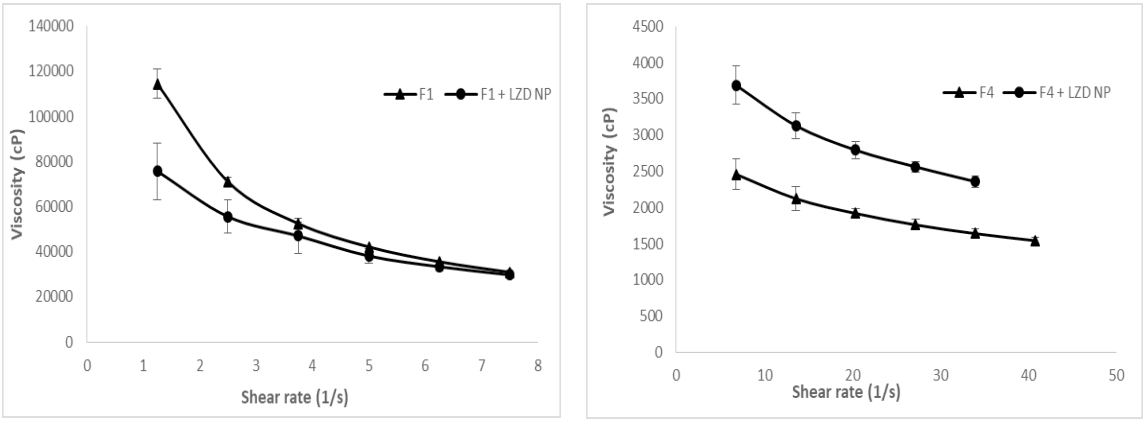


Figure 2. Viscosity values of the optimum formulations

Spreadability studies

Spreadability is a crucial property of gels as it reflects the behavior of the gel when applied to the skin. It is the term used to describe the ease with which the gel spreads over the skin surface (Nikumbh et al., 2015). Spreadability plays a crucial role in ensuring the stability and ease of application of topical gels (Hussain et al., 2016; Pathan et al., 2018). Moreover, spreadability has a direct impact on the therapeutic efficacy of the drug. It promotes the uniform application of the gel on the skin, contributing to increasing patient acceptance (Ergin et al., 2023; Lesieur et al., 1993). A gel's suitability for skin is determined by its effective spreadability, which is closely linked to its resilience against external forces. Endurance, measured by the force applied to surface deformation, is interconnected with spreadability, representing the overall structural response to external forces. Even if two products exhibit similar firmness, their skin application may differ. Hence, firmness and shear work are commonly assessed in tandem (Ergin et al., 2023).

The spreadability of the gel depends upon the polymer type, polymer rate, hardness, and viscosity of the gel. The spreadability of optimum gels selected from each group (Carbopol® and Methocel™ gels) is shown in Table 5. When the spreadability data are analysed, as the viscosity value increases in the formulations, the required work and firmness of the gels also increase. As this component increases, the spreadability of the prepared gel decreases. The spreadability of the prepared gels was significantly reduced with increasing the viscosity of formulations. The obtained result aligns with expectations and is consistent with findings reported in the literature ($p \geq 0.05$) (Ergin et al., 2023; Garg et al., 2022).

Table 5. Spreadability of gels

Formulation Code	Temperature	Work of Shear ± SD (g.sec)	Firmness ± SD (g)	Stickiness ± SD (g)
F1	25°C	421.398±18.118	491.771±30.280	-482.154±20.481
	32°C	451.562±13.284	528.920±13.779	-528.807±14.529
F4	25°C	39.480±3.582	61.785±6.006	-97.646±9.460
	32°C	48.311±1.014	79.946±1.705	-125.096±2.517
F1 + LZD NP	25°C	339.866±2.104	359.980±9.031	-314.640±0.437
	32°C	367.459±21.572	394.207±49.815	-334.006±37.946
F4 + LZD NP	25°C	65.556±1.918	111.845±3.141	-158.497±26.781
	32°C	98.202±9.279	201.515±34.822	-300.152±49.413

Flow properties

The flow property of a semi-solid formulation is crucial as it significantly influences the success of the formulation as a drug delivery system. The flow curves showed a decrease in the viscosity of all formulations with increased shear rate. All the prepared formulations were identified to exhibit a non-Newtonian pseudoplastic (shear-thinning) behavior, as evidenced by their observed flow curves (Figure 3). Our findings demonstrated sheer thinning, in line with the literatures (Rençber et al., 2019; Rençber & Karavana 2020; Mariane et al., 2023). Furthermore, as can be seen from the graphs, when the effect of LZD NP addition in gel formulations was examined, a decrease in viscosity values was observed in the F1 formulation and an increase in the F4 formulation. These findings are consistent with TPA studies.

The flow curves showed a decrease in the viscosity of all formulations with an increase in shear rate. This reflects the shear-thinning behavior of a pseudoplastic (non-Newtonian) system (Figure 2).

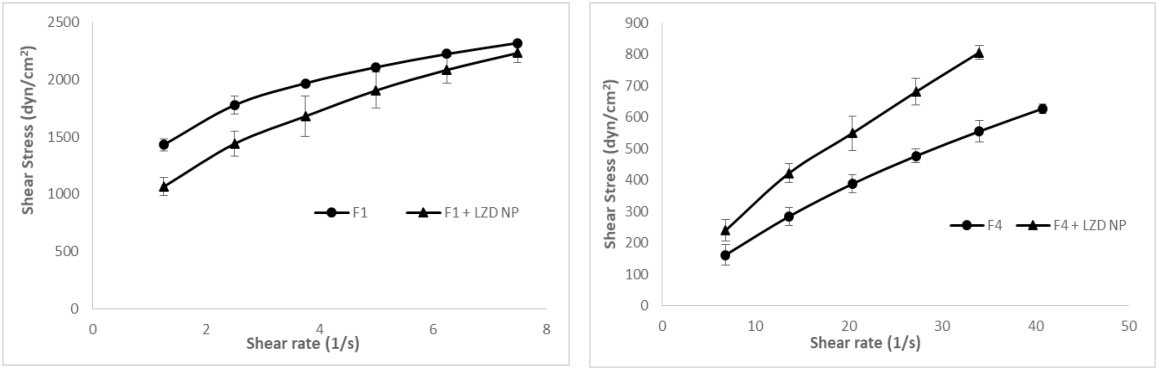


Figure 3. Flow curve of gel formulations

In vitro drug release studies

Figure 4 illustrates the *in vitro* release of LZD from NP and gel formulations containing NP over 24 hours, conducted using a dialysis bag. At the end of 8 hours, the LZD NP reached 84.86% release, the F1+LZD NP reached 100% release, and the F4+LZD NP reached 80% release. As a result, the release profile of gel formulations containing NP was found to be close NP dispersion. This is thought to be due to the rapid erosion of Carbopol® 974 P NF and HPMC K100M, which are polymers soluble in distilled water, in aqueous media, resulting in increased LZD release.

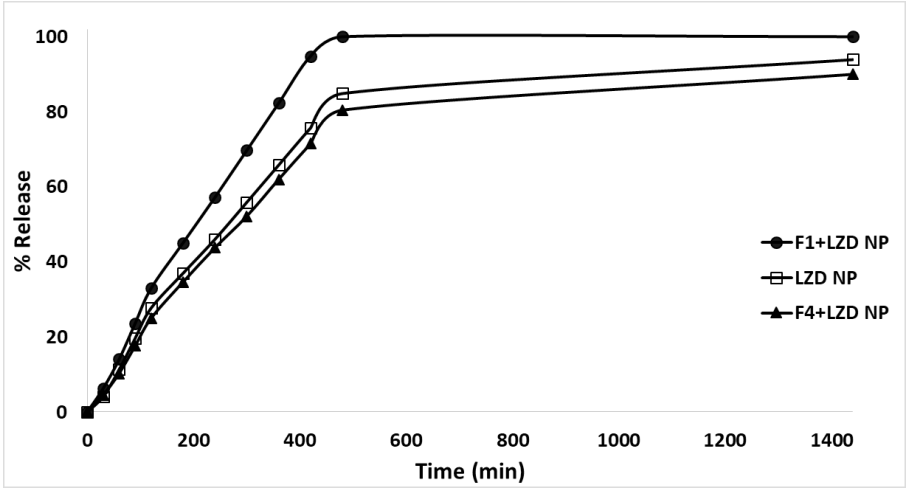


Figure 4. *In vitro* release profile of NPs

Release kinetics

Kinetic models are analyzed to determine their impact on the stability and release kinetics of drugs. Commonly used mathematical models are the zero-order kinetic model, first-order kinetic model, Higuchi model, Hixson-Crowell model and Korsmeyer-Peppas model. First order describes release from systems where the release rate is concentration dependent. Higuchi describes the release of drugs from the insoluble matrix as the square root of a time-dependent process based on Fickian diffusion. The Hixon–Crowell cube root law describes the release from systems where there is a change in the surface area and diameter of the particles or tablets. The zero-order describes the systems where the drug release rate is independent of its concentration.

As given in Table 6, according to the r^2 values obtained, LZD release kinetics from LZD NP, F1+LZD NP, F4+LZD NP formulations was determined as a zero-order kinetic model. Therefore, it can be said that the same amount of LZD is released from the formulation per unit time. The release is time-dependent and concentration-independent. This finding agrees with the study conducted by Dandagi et al. (Dandagi et al., 2020) in which zero-order release kinetics were obtained from LZD-loaded niosome gel. Although different release kinetics (Higuchi) have been reported for LZD in the literature, this result was obtained using chitosan NPs (Alkholief et al., 2023).

Table 6. The evaluation of the release kinetic of LZD from formulations

	Zero order			First order			Higuchi			Hixon-Crowell			Korsmeyer-Peppas		
Code	**r^2**	**n**	**M**	**r^2**	**n**	**m**	**r^2**	**n**	**m**	**r^2**	**N**	**M**	**r^2**	**N**	**m**
LZD NP	0.9935	2.2773	0.1767	0.7165	0.7255	0.0031	0.9483	14.561	4.1629	0.7722	1.5432	0.007	0.9799	0.7988	1.033
F1+LZD NP	0.9966	2.3352	0.2226	0.7049	0.8084	0.0031	0.9439	5.2245	18.619	0.7792	1.6663	0.0075	0.9924	0.6156	0.9959
F4+LZD NP	0.9955	1.8188	0.1675	0.7301	0.7114	0.003	0.9453	14.013	3.936	0.7786	1.5114	0.0069	0.9907	0.7676	1.0081

Ex vivo drug diffusion study

Based on *in vitro* characterization studies, the F4+LZD NP formulation exhibited suitable hardness, compressibility, elasticity, and viscosity. To determine whether LZD would accumulate or penetrate the skin, diffusion cells were used in *ex vivo* drug penetration and permeation studies on tissue. In these studies, 0.007±0.004% of F1+LZD NP was detected in the receptor compartment. The low drug permeability results obtained are consistent with the fact that gels are semi-solid formulations and drug release is based on swelling and drug diffusion. Furthermore, topical drug delivery systems such as the developed gel do not cause systemic effects, making it a safe method of application for children and pregnant women. Therefore, the optimized gel formulation was determined to have the potential to deliver LZD by topical application through the skin (Singh et al., 2016; Okur et al., 2018; Ay Şenyiğit et al., 2021).

Stability studies

Stability studies for the optimal gel formulation were conducted at temperatures of 4±1°C and 25±2°C for 3 months. No significant changes in the macroscopic appearance, pH, and mechanical properties of the gels were observed during this period ($p \geq 0.05$) (Table 7-Table 8).

Table 7. pH results of the stability study (n=3)

	Formulation Code	pH±SD	pH±SD	pH±SD	pH±SD
		Beginning	1. month	2. month	3. month
5±2°C	F4	6.39±0.006	6.37±0.006	6.37±0.006	6.37±0.006
	F4+LZD NP	5.99±0.006	5.98±0.010	5.97±0.006	5.98±0.01
25±2°C/ %60±5	F4	6.39±0.006	6.38±0.006	6.37±0.006	6.37±0.006
	F4+LZD NP	5.99±0.006	5.98±0.006	5.98±**0.006**	5.97±**0.01**
40±2°C/%75±5	F4	6.39±0.006	6.37±0.006	6.36±0.006	6.34±0.006
	F4+LZD NP	5.99±0.006	5.95±0.006	5.93±0.011	5.90±0.006

Table 8. Mechanical properties of gel formulations throughout stability

Code	Months	Stability Conditions	H (g) ± SD	C (g·sec) ± SD	A (g·sec) ± SD	E ± SD	Ch ± SD
F4	Beginning	-	0.013±0.000	0.048±0.004	-0.042±0.003	0.877±0.008	1.127±0.012
F4 + LZD NP		-	0.038±0.001	0.068±0.009	-0.163±0.001	0.973±0.013	1.063±0.001
F4	1. month	5±2°C	0.013±0.000	0.047±0.002	-0.040±0.004	0.865±0.001	1.127±0.030
		25±2°C/ %60±5	0.015±0.000	0.045±0.002	-0.039±0.003	0.874±0.009	1.176±0.023
		40±2°C/%75±5	0.015±0.000	0.046±0.002	-0.038±0.000	0.857±0.013	1.120±0.044
		5±2°C	0.039±0.000	0.062±0.002	-0.157±0.002	0.969±0.010	1.055±0.004
F4 + LZD NP		25±2°C/ %60±5	0.038±0.000	0.065±0.002	-0.158±0.003	0.975±0.002	1.054±0.021
		40±2°C/%75±5	0.039±0.001	0.059±0.001	-0.149±0.005	0.948±0.32	1.030±0.040
F4	2. month	5±2°C	0.014±0.000	0.042±0.005	-0.041±0.003	0.904±0.097	1.127±0.005
		25±2°C/ %60±5	0.013±0.000	0.046±0.009	-0.039±0.012	0.890±0.083	1.121±0.076
		40±2°C/%75±5	0.015±0.000	0.046±0.014	-0.034±0.015	0.900±0.020	1.117±0.105
F4 + LZD NP		5±2°C	0.038±0.000	0.059±0.007	-0.158±0.001	0.974±0.002	1.155±0.009
		25±2°C/ %60±5	0.039±0.000	0.062±0.002	-0.156±0.003	0.963±0.083	1.159±0.008
		40±2°C/%75±5	0.040±0.001	0.056±0.002	-0.149±0.003	0.962±0.025	1.144±0.013
F4	3. month	5±2°C	0.015±0.000	0.045±0.003	-0.042±0.002	0.902±0.081	1.116±0.068
		25±2°C/ %60±5	0.014±0.000	0.046±0.004	-0.040±0.004	0.917±0.111	1.129±0.024
		40±2°C/%75±5	0.015±00.00	0.045±0.014	-0.036±0.003	0.916±0.028	1.131±0.001
		5±2°C	0.039±0.001	0.064±0.011	-0.158±0.002	0.968±0.012	1.058±0.017
F4 + LZD NP		25±2°C/ %60±5	0.039±0.000	0.059±0.009	-0.157±0.000	0.948±0.003	1.042±0.003
		40±2°C/%75±5	0.040±0.001	0.039±0.006	-0.148±0.003	0.933±0.026	1.050±0.006

*H: Hardness, C: Compressibility, A: Adhesiveness, E: Elasticity, Ch: Cohesiveness

CONCLUSION

This study involved the preparation and evaluation of gel formulations containing NPs with LZD to treat DFU. The formulations were assessed for various properties, including PS, PI, ZP, morphology, drug entrapment efficiency, pH, mechanical properties, viscosity, spreadability, flow, *in vitro* drug release, *ex vivo* drug diffusion, and stability. The prepared NP showed a PS of 195.27±5.42 nm, PI of 0.214±0.019, a ZP of 20.57±0.35 mV and EE of 99.746±0.021%. The rheological analysis of the gels indicated a pseudoplastic flow and exhibited typical gel spectra. Furthermore, texture analysis demonstrated that the developed gel formulations had appropriate consistency. The optimized F4+ LZD NP showed sustained drug release over eight hour. In the *ex vivo* drug diffusion studies, it was determined that 0.007±0.004% of LZD permeated into the receptor phase, indicating a local effect.

The novelty and contribution of this research lie in the formulation of a topical gel incorporating nanoparticles for the treatment of DFU. The formulated gels demonstrated favorable characteristics, including an appropriate pH value, suitable mechanical

performance, and desirable viscosity and spreadability for topical application. The initial findings suggest that the formulated topical gel containing LZD-loaded NPs holds promise as an effective drug delivery system for DFU management. However, additional investigations, such as cytotoxicity assessments and *in vivo* animal studies, would be benefit for further evaluation.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Concept – S.R., H.H.A.; Design – S.R., H.H.A.; Supervision – S.R.; Resources – S.R., H.H.A.; Materials – S.R., H.H.A.; Data Collection and/or Processing – Y.P., H.H.A., S.R.; Analysis and/or Interpretation – Y.P., H.H.A., S.R.; Literature Search – Y.P., S.R., H.H.A.; Writing – S.R., H.H.A.; Critical Reviews – Y.P., H.H.A., S.R.

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