Formulation and Evaluation of Silymarin–Pioglitazone Loaded Nanoemulgel: In Vitro Release, Antioxidant and Cell Line Studies

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ABSTRACT
The aim of the present study was to formulate and evaluate a silymarin–pioglitazone loaded nanoemulgel for enhanced topical delivery. The nanoemulsion was first optimized using a Quality by Design (QbD) approach and subsequently incorporated into a gel base to form a nanoemulgel. The formulation was evaluated for in vitro drug release, antioxidant activity, and cell line studies. The optimized nanoemulgel exhibited desirable physicochemical properties, including appropriate pH, viscosity, spreadability, and extrudability. In vitro release studies demonstrated a sustained and enhanced drug release profile compared to plain gel. The antioxidant activity of the formulation was evaluated using DPPH and ABTS assays, showing significant free radical scavenging potential. Cell line studies on suitable skin cell lines confirmed the biocompatibility and safety of the developed formulation. The results indicate that the silymarin–pioglitazone nanoemulgel is a promising topical delivery system with improved drug release, antioxidant properties, and cytocompatibility, making it a viable candidate for the management of diabetic complications and related oxidative stress conditions.

Keywords: Silymarin; Pioglitazone; Nanoemulgel; Topical Delivery; In Vitro Release; Antioxidant; Cell Line Studies.
How to cite this article: Deepak A, Jeet K. Formulation and Evaluation of Silymarin–Pioglitazone Loaded Nanoemulgel: In Vitro Release, Antioxidant and Cell Line Studies. Int J Drug Deliv Technol. 2026;16(63s):283-295. DOI: 10.25258/ijddt.16.63s.31
Source of support: Nil.
Conflict of interest: None

1. Introduction

1.1 Diabetes Mellitus and Need for Topical Therapy

Diabetes mellitus is a chronic metabolic disease due to ongoing hyperglycemia caused by inadequate insulin production, insulin resistance or a combination of the two. It is regarded as one of the most rapidly emerging health concerns of millions of people around the world. Diabetes is still becoming common at a rapid pace, owing to a sedentary lifestyle, obesity, genetic predisposition and unhealthy eating habits (1), according to the International Diabetes Federation (IDF). Diabetes that is not managed for long time may result in serious conditions such as heart and blood vessel diseases, kidney disease, nerve damage (neuropathy), vision loss or retinal damage (retinopathy), tissue damage from oxidative stress, delayed wound healing, and chronic inflammation (2, 3). The usual method of delivering conventional antidiabetic therapy is oral and injectable. But, often, the delivery of a drug by the oral route is associated with various drawbacks including poor aqueous solubility, high first-pass metabolism, gastrointestinal degradation, variable absorption and systemic side effects, thereby compromising drug efficacy and patient adherence. Furthermore, oral antidiabetic medications can have systemic toxicity and a need for frequent dosing over extended periods. Thus, alternative drug delivery techniques that could enhance drug permeation, reduce systemic exposure, and increase therapeutic activity has received significant research interest(4) . Topical drug delivery systems are now becoming attractive alternatives for the site-specific and controlled delivery of therapeutic agents. Topical formulations have several benefits such as the elimination of first pass metabolism, better patient compliance, minimal systemic toxicity, prolonged drug action and better localization of the

drug at site of action. During the last years, nano-based topical drug delivery systems like nanoemulsions and nanoemulgels have gained considerable interest because of their capacity to facilitate the permeation through the skin and increase the therapeutic efficiency (5).

1.2 Therapeutic Potential of Silymarin and Pioglitazone

The natural flavonolignan complex (silymarin) obtained from Silybum marianum is well established to have antioxidant, anti-inflammatory, hepatoprotective, and antidiabetic properties (6). Silymarin has been shown to have anti-oxidative, anti-inflammatory and insulin sensitizing effects in diabetes. But its therapeutic application is limited due to its poor aqueous solubility and low bioavailability. Pioglitazone is a thiazolidinedione analog, which is broadly applied in the treatment of type 2 diabetes mellitus. It is an insulin sensitizer by binding and activating peroxisome proliferator-activated receptor gamma (PPAR-γ) which enhance glucose utilization and decrease insulin resistance. Although orally administered pioglitazone is therapeutic, it can also have side effects, including cardiovascular problems, weight gain, and edema. So, when administered as a nano-based topical delivery system, Silymarin and Pioglitazone could have a combination of antioxidant and insulin-sensitizing effects with minimal systemic toxicity (7).

1.3 Nanoemulgel as an Advanced Topical Drug Delivery System

Advanced topical drug delivery system, Nanoemulgels are the combination of Nanoemulsions into the gel matrix. Nanoemulsions are thermodynamically stable colloidal dispersion of oil, surfactant, co-surfactant and aqueous phase of which the droplet size is in nanometer range. Nanoemulsions have a high surface area and small droplet size, which is responsible for enhancing the drug solubility, permeability and bioavailability of the small molecule under consideration. But, nanoemulsions are frequently observed to have low viscosity and hence to be less retained on the surface of the skin. To overcome this limitation, nanoemulsions are added to suitable gel bases to make nanoemulgels. Nanoemulgels are the combination of the properties of nanoemulsions and gels and offer higher spreadability, longer residence time, controlled drug release, better patient acceptance and enhanced skin permeation. Moreover, nanoemulgels can effectively transport both hydrophilic and lipophilic drugs.

1.4 Importance of Antioxidant and Cell Line Studies in Antidiabetic Therapy

Oxidative stress is a factor which is crucial in the pathogenesis and progression of diabetes mellitus and related complications. An increase in reactive oxygen species (ROS) can cause cellular damage, inflammation and the disruption of insulin signalling pathways. So, antioxidants are regarded as an important aspect of diabetes treatment. Formulations can be assessed for their antioxidant properties by performing DPPH radical scavenging assays wherein the ability of therapeutic agents to neutralize free radicals is measured. The studies of cell lines are extensively used to evaluate the cytocompatibility, safety, and therapeutic effectiveness of new nanoformulations. Formulation effects on cellular metabolic activity and viability can be assessed by cell viability assay like MTT assay. In addition, anti-inflammatory activity of developed formulations can be further evaluated by measuring the levels of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), Interleukin-6 (IL-6) and Interleukin-1 beta (IL-1β). Hence, antioxidant and cell line studies play an important role in the validation of therapeutic efficacy and biological safety of nanoemulgel system used as topical antidiabetic formulations.

1.5 Aim and Objectives of the Study

In the present study, the formulation and evaluation of Silymarin loaded Pioglitazone nanoemulgel for the development of enhanced topical antidiabetic therapy was aimed. The study aimed to optimize the formulation of nanoemulsion, followed by the preparation of nanoemulgel and physicochemical characterization, in vitro drug release, release kinetics, antioxidant evaluation, cell viability assessment, inflammatory cytokine analysis and stability studies for assessing therapeutic potential and formulation stability of nanoemulgel.

2. Materials and Methods

2.1 Materials

The ingredients (silymarin and pioglitazone) were purchased from the trusted pharmaceutical suppliers and used in their native form without any further purification. Capryol 90 was used as the oil phase, Tween 80 as the surfactant and Transcutol P as the co-surfactant in the preparation of nanoemulsion system. Carbopol 940 was adopted as the gelling polymer for the preparation of nanoemulgel and triethanolamine was used as the neutralising agent. Methanol, ethanol, phosphate buffer (pH 6.8), potassium bromide and other analytical grade reagents were purchased from common commercial sources. Distilled water was prepared fresh for the use in the whole study.

2.2 Preparation of Optimized Nanoemulsion

The optimized formulation was prepared by the spontaneous emulsification method of the nanoemulsion. The required amount of Silymarin and Pioglitazone were accurately weighed and dissolved in the oil phase with Capryol 90. The optimized ratio of Tween 80 and Transcutol P was used to make the Smix system. To achieve a clear isotropic mixture, the Smix has been little by little added to the oil phase with continuous magnetic stirring. Then distilled water was dropwise added to the mixture with stirring until a transparent and homogeneous nanoemulsion system was formed. The prepared nanoemulsion was evaluated before further addition in the gel formulation in terms of clarity, homogeneity, and phase stability.

2.3 Preparation of Silymarin–Pioglitazone Nanoemulgel

The optimized nanoemulsion was then added to the Carbopol gel matrix and the nanoemulgel formulation was prepared. The prepared nanoemulsion was gently added to the gel base with continuous stirring to get a homogenous formulation of nanoemulgel which is suitable for topical application.

2.3.1 Selection of Gelling Agent

Preliminary study was conducted to determine the suitability of different gelling agents in the preparation of nanoemulgel, which were judged according to the viscosity, spreadability, appearance and formulation stability. The optimized polymer used for gelling was determined to be Carbopol 940 because of its formulation properties: it had good gel forming ability, appropriate rheological properties and was compatible with the nanoemulsion system.

2.3.2 Incorporation of Optimized Nanoemulsion into Gel Base

The carbopol 940 was dispersed in distilled water and completely hydrated for a sufficient time to create a uniform gel base. The gel system was neutralized by adding dropwise triethanolamine to adjust the pH. This optimized nanoemulsion was then added dropwise to the prepared gel base with constant stirring to get a smooth and uniform nanoemulgel. The prepared final nanoemulgel was kept in air-tight containers and used for further characterization and biological evaluation studies.

2.4 Characterization of Nanoemulgel

The carbopol 940 was dispersed in distilled water and completely hydrated for a sufficient time to create a uniform gel base. The gel system was neutralized by adding dropwise triethanolamine to adjust the pH. This optimized nanoemulsion was then added dropwise to the prepared gel base with constant stirring to get a smooth and uniform nanoemulgel. The prepared final nanoemulgel was kept in air-tight containers and used for further characterization and biological evaluation studies.

2.4.1 Physical Appearance

The prepared nanoemulgel was visually examined for colour, homogeneity, consistency, phase separation, grittiness and appearance in the normal daylight condition. The formulation was also studied for smoothness and uniformity for aesthetic properties and formulation stability for topical administration.

2.4.2 pH Determination

The pH of the nanoemulgel formulation was measured using a calibrated pH digital meter. About 1 g of nanoemulgel was dispersed in distilled water and then equilibrated at room temperature prior to the measurements. The pH meter electrode was introduced into the formulation and the pH was read three times. The pH determination has been performed so as to be compatible with the skin and to avoid any risk of skin irritation after topical use.

2.4.3 Viscosity Study

The viscosity of the nanoemulgel formulation was determined from the Brookfield viscometer with suitable spindle. The formulated was poured into the sample container and tested for viscosity at a controlled room temperature at various rotational speeds. The viscosity of nanoemulgel formulation was evaluated to check the consistency, flow behavior and applicability of the formulation.

2.4.4 Rheological Study

The nanoemulgel was tested in a Brookfield rheometer to assess the flow property of the formulation. The formulation's viscosity was determined at varying shear rates to assess the formulation's rheological behavior. The rheological study was carried out to check the flow property of the formulation and to find out its use for topical application and spreadability over the skin surface whether it is Newtonian or non-Newtonian.

2.4.5 Spreadability Study

The spreadability of nanoemulgel formulation was evaluated by parallel plate method. An amount of nanoemulgel was loaded between two glass slides and a specific load was applied over the upper glass slide for a given time. Diameter of spread was measured in the spread formulation and the spreadability value was calculated by the standard formula. This spreadability

study was designed to assess the ease of application and ability to spread uniformly on the skin surface.

2.4.6 Drug Content Analysis

Drug content analysis of the formulation as nanoemulgel was carried out to investigate the uniformity of the drug distribution in the gel system. The proper amount of nanoemulgel was dissolved in an appropriate solvent and subjected to sonication to completely extract the drugs present in it. The solution obtained was filtered, diluted as per requirement and then subjected to UV–Visible spectrophotometric analysis at selected wavelengths for Silymarin and Pioglitazone. Using the calibration curve equations, the percent composition of the drug content was determined.

2.4.7 Extrudability Study

In order to assess the ease of the formulation to be extruded from collapsible tube during the topical administration, the extrudability of nanoemulgel formulation was assessed. The nanoemulgel was then filled into aluminum collapsible tubes, and a constant force was applied to extrude the formulation. Measurements of the amount of gel extruded and visual observations were taken of the tube. The extrudability study was conducted, in order to evaluate the patient convenience and applicability during the topical application.

2.5 In Vitro Drug Release Study

In vitro drug release study was conducted to determine drug release pattern of Silymarin and Pioglitazone from the developed nanoemulgel formulation. The Franz diffusion cell apparatus was used to perform the study under controlled experimental conditions. The percentage of drug released was calculated at specific time points and then compared with that of pure drug suspension to evaluate the effect of the nanoemulgel system on the drug release pattern.

2.5.1 Drug Release Procedure

In vitro drug release study was performed in Franz diffusion cell with a dialysis membrane. The membrane was soaked over night with phosphate buffer, pH 6.8 before use. To simulate physiological conditions, the receptor compartment was filled with phosphate buffer pH 6.8 at 37 ± 0.5 °C and continuously stirred by the use of magnetic stirrer.

A correct weight of nanoemulgel containing an appropriate amount of Silymarin and Pioglitazone was added to the donor compartment. Aliquots were taken out of the receptor chamber at the times indicated (0.5, 1, 2, 4, 6, 8, 10 and 12 h) and replaced with the same volume of fresh release medium to maintain sink conditions. The samples collected were appropriately diluted and then analyzed at the respective wavelengths of Silymarin and Pioglitazone with UV–Visible spectrophotometer. The percentage of the drug released cumulatively was calculated and plotted as a function of time.

2.5.2 Drug Release Kinetics

The in vitro release data of developed nanoemulgel formulation were fitted into various mathematical kinetic models like Zero-order, First-order, Higuchi model and Korsmeyer–Peppas model to elucidate the mechanism of drug release from developed nanoemulgel formulation. The results of the correlation coefficient (R^2) were calculated for each model, and the best model was used to describe the release kinetics. The Zero-order model was used to assess the drug release behavior that is not concentration dependent, and the First-order model described the concentration-dependent drug release behavior. For the diffusion-controlled release from matrix system, Higuchi model was employed, and for the mechanism of drug transport, Korsmeyer–Peppas model was used. Model with the maximum R^2 value was selected as the best fit model to describe the drug release behaviour of nanoemulgel formulation.

2.6 Antioxidant Activity

The antioxidant activity of the formed Silymarin–Pioglitazone nanoemulgel was assessed by assessment of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. DPPH assay is a popular method of evaluating the free radical scavenging capacity of pharmaceutical formulations and natural antioxidants. The antioxidant activity of the nanoemulgel was compared with that of the pure drugs and a standard antioxidant. DPPH radical scavenging activity at various concentrations of the formulation was measured and its antioxidant activity was expressed as percentage inhibition of DPPH radicals.

2.6.1 DPPH Radical Scavenging Assay

DPPH free radical scavenging method was used to determine the antioxidant activity of the developed nanoemulgel. The DPPH solution (0.1 mM) was freshly prepared in methanol and kept away from light during the experiment. Various concentrations of the nanoemulgel formulation, pure drugs and a standard antioxidant (ascorbic acid) were prepared separately. Each sample solution was diluted to an aliquot and 300 µL of the diluted sample solution was added to 300 µL of DPPH solution and incubated in dark in room

temperature for 30 min. The absorbance of the reaction mixture was then determined at 517 nm with a UV–Visible spectrophotometer after the incubation period. DPPH blank (without sample) was used as the control solution.

The percentage free radical scavenging activity was calculated using the following equation:

\text{Percentage Inhibition (%)} = \frac{A_c - A_s}{A_c} \times 100

Where:

- (A_c) = Absorbance of control
- (A_s) = Absorbance of sample

The DPPH radical scavenging activity was represented as percentage inhibition of DPPH radicals. The more the percentage inhibition, the higher the potential of the formulation as an antioxidant. The concentration that inhibits 50% of DPPH radicals (IC_{50}) was found by plotting a curve of the percentage of DPPH radicals against concentration and was taken as an indicator of the antioxidant efficiency.

2.7 Cell Line Studies

In-vitro cell culture studies were used to assess biological activity of the formulated Silymarin–Pioglitazone nanoemulgel. To determine the cytocompatibility, cellular response, and anti-inflammatory activity of the formulation, cell line investigations were performed. To assess the therapeutic potential of the formulation of the developed nanoemulgel in diabetic inflammatory conditions, the cell viability was evaluated by MTT assay and the expression level of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β were measured.

2.7.1 Cell Culture Conditions

The selected cell line were grown under sterile conditions in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were cultured under 5% CO_2 atmosphere in humidified incubator at 37°C. Media was exchanged at regular intervals and when the cells reached about 80-90% confluence they were subcultured. Cells were first cultured on appropriate culture plates and incubated for 24 hours before experimentation.

2.7.2 Cell Viability Assay (MTT Assay)

The developed nanoemulgel was tested for its cytocompatibility by MTT assay. This was done by seeding the cells into 96-well plates and incubating for 24 h to promote cell attachment. The cells were then incubated with various concentrations of nanoemulgel formulation and reincubated for a fixed time. After treatment, MTT solution (5mg/mL) was added to each well and incubated for 3-4 hours. The formed formazan crystals were dissolved with Dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm on a microplate reader.

2.7.3 TNF-α Gene Expression Study

The impact of nanoemulgel formulation on tumor necrosis factor alpha (TNF-α) expression was studied employing quantitative real-time polymerase chain reaction (qRT-PCR). Cultured cells were treated with the formulation and then total RNA was extracted from the treated cells using an RNA extraction kit following the manufacturer's protocol. The isolated RNA was reverse transcribed into complementary DNA (cDNA). Specific primers for TNF-α and a house keeping gene were used for quantitative PCR amplification. The expression level of TNF-α was determined by the comparative Ct ($2^{-}\Delta\Delta Ct$) method. Any decrease in the expression of TNF-α was considered as anti-inflammatory activity of the developed formulation.

2.7.4 IL-6 Gene Expression Study

Quantitative real time PCR (qRT-PCR) was used to assess the effect of nanoemulgel formulation on interleukin-6 (IL-6) expression. Total RNA was isolated from treated and untreated cells and cDNA synthesized. IL-6 specific primers were used for amplification under optimal PCR conditions. The relative gene expression levels were quantified by $2^{-}\Delta\Delta Ct$ method and normalized with housekeeping gene. Lower expression of IL-6 suggested suppression of inflammatory responses and suggested therapeutic potential of the nanoemulgel formulation.

2.7.5 IL-1β Gene Expression Study

Interleukin-1 beta (IL-1β) is an important pro-inflammatory cytokine involved in diabetic inflammatory response and its expression was assessed by qRT-PCR. RNA extraction, cDNA preparation and PCR were conducted according to conventional molecular biology protocols. The expression level of relative IL-1β was determined by the comparative Ct method and compared to that of untreated cells. The decrease in the expression of the IL-1β after treatment with the nanoemulgel formulation was considered as anti-inflammatory activity and therapeutic potential in diabetes-associated inflammatory diseases.

2.8 Stability Studies

The developed Silymarin–Pioglitazone nanoemulgel formulation was subjected to stability studies to assess the physical and chemical stability during storage. The study was carried out following the ICH guidelines to evaluate effect of storage conditions on the physicochemical parameters of the formulation. The nanoemulgel samples were kept for a fixed time in airtight containers under accelerated and room temperatures.The samples of the nanoemulgel were stored under both accelerated and room temperature conditions in airtight containers for a fixed period. The appearance, drug content, particle size and overall formulation stability were evaluated periodically to assess for changes.

2.8.1 Physical Stability

The physical stability of the developed nanoemulgel was assessed by storing the formulation at different storage conditions such as room temperature of 25 ± 2°C and accelerated condition (40 ± 2°C/75 ± 5%RH). Samples were periodically tested at set time points (0, 1, 2 and 3 months) to assess for colour, odour, homogeneity, consistency, phase separation and overall appearance. Formulation was visually tested for any signs of syneresis, precipitation, creaming, cracking and microbial growth. No physical changes were observed during the storage time, which was considered as a good formulation stability.

2.8.2 Stability of Particle Size and Drug Content

The stability of particle size and drug content of the nanoemulgel formulation was evaluated during the storage period. Samples were taken at a time and a place that were predetermined and their particle size distribution was determined by dynamic light scattering (DLS) and drug content assay was done by UV-Visible spectrophotometric analysis. The results showed the possible aggregation, coalescence or instability of the incorporated nanoemulsion droplets, by observing the changes in particle size. The content analysis of the drug was also carried out to find out the retention of Silymarin and Pioglitazone in the formulation during storage. The percentage of the drug remaining was determined and compared to the original percentage. The formulation was found to be stable as there were no significant changes in the particle sizes, drug contents or physical appearance of the formulation during the study period. The stability data obtained were used for the shelf-life prediction of life and storage suitability of the developed nanoemulgel formulation.

2.9 Statistical Analysis

The experimental studies were carried out in triplicate and the results presented were mean ± (standard deviation) SD. The statistical analysis was done on GraphPad Prism Software (Version 9.0, GraphPad Software Inc., USA). One-way analysis of variance (ANOVA) and subsequent Tukey's post hoc multiple comparison test were used to compare differences among experimental groups. The control group, pure drug-treated group and nanoemulgel-treated groups were compared with regard to cell viability, antioxidant activity and gene expression studies and stability evaluation. The results were statistically significant when p was < 0.05 ($p < 0.05$). For determining the reliability, repeatability and significance of the experimental results during the study, statistical analysis was carried out.

3. Results and Discussion

3.1 Preparation and Evaluation of Nanoemulgel

3.1.1 Physical Appearance of Nanoemulgel

The optimized formulation of Silymarin–Pioglitazone nanoemulsion was successfully incorporated into Carbopol 940 gel base to prepare a uniform nanoemulgel formulation. The prepared nanoemulgel was smooth in texture, free from phase separation, creaming, cracking and drug crystallization with good uniformity and homogeneity. The formulation had a pale yellow colour because of the presence of Silymarin and was also shiny and beautiful in appearance which was good for topical use. The nanoemulsion droplets were uniformly distributed in the gel matrix as seen from the absence of any grittiness or particulate matter. The prepared nanoemulgel was found to be physically stable and retained its physical stability during the evaluation period. The smoothness and semi-solid composition of the formulation is the expected property to increase patient acceptance and skin surface application. The results confirmed the successful incorporation of optimized nanoemulsion in the gel system and suggested that this can be useful for further physicochemical and biological evaluations. The observed characteristics are deemed desirable for topical compositions for diabetes treatment which provide extended skin contact and drug control. The prepared nanoemulgel did not show any syneresis or phase separation during storage, which indicates a good formulation stability.

Table 1. Organoleptic Characteristics of Optimized Silymarin–Pioglitazone Nanoemulgel

Parameter	Silymarin	Pioglitazone

Color	Yellowish powder	White to off-white powder
Odor	Slight characteristic odor	Faint odor
Appearance	Fine powder	Crystalline powder

3.1.2 pH Analysis

A topical formulation's pH has an impact on the skin compatibility, formulation stability and patient acceptability. The optimized Silymarin–Pioglitazone nanoemulgel's pH was measured to check its suitability for dermal application. The formulation had a pH of 6.42 ± 0.08 which is within the normal range of the skin's pH (4.5–6.8). The pH value obtained showed that the nanoemulgel is non-irritating and skin compatible. The near neutral pH may assist in maintaining integrity of the skin and the risk of erythema, irritation or discomfort on topical application may be reduced. In addition, the pH value of the observed formulation indicated that addition of the optimized nanoemulsion to the Carbopol gel matrix had no detrimental effect on the stability of the formulation. The developed nanoemulgel was found to have an adequate pH which is suitable for long-term usage of the product in topical administration and diabetic skin care.

Table 2. pH of Optimized Silymarin–Pioglitazone Nanoemulgel

Formulation	pH (Mean ± SD)
Optimized Nanoemulgel	6.42 ± 0.08

Discussion: The pH value found was within the acceptable range for the topical formulations and proved to be good skin compatibility and formulation stability. The same results have been obtained for the nanoemulgel based topical delivery system, in which pH range in between 5.5 and 7.0 is suitable for dermal application.

3.1.3 Viscosity and Rheological Behavior

Viscosity and rheological properties are very important parameters to assess the performance of a topical formulation because they affect the following: spread ability, retention, drug release behavior, and patient compliance. Viscosity of the optimized Silymarin–Pioglitazone nanoemulgel was measured by Brookfield viscometer at room temperature. The formulation showed a consistency of 12850 ± 145 cP, which is a proper viscosity for topical application. It was found that the developed nanoemulgel possessed adequate viscosity to remain at the application site without leakage and yet to be spread easily over skin surface. The formulation was made consistent using Carbopol 940 as gelling agent, which played an important role in the formulation. Rheological evaluation showed that the nanoemulgel was pseudoplastic (shear-thinning), meaning that the viscosity decreased as shear rate increased. The rheological property is very desirable for topical formulations as the gel is not only less viscous when applied, allowing easy spreading, but is also more viscous when the applied force is removed, thereby increasing residence time at the application site. The observed rheological behavior showed good structural integrity and physical stability of the nanoemulgel system. The optimized formulation has a suitable viscosity and pseudoplastic flow property, indicating that it has desirable application properties and could improve patient acceptability for topical application.

Table 3. Viscosity of Optimized Silymarin–Pioglitazone Nanoemulgel

Parameter	Result (Mean ± SD)
Viscosity (cP)	12,850 ± 145

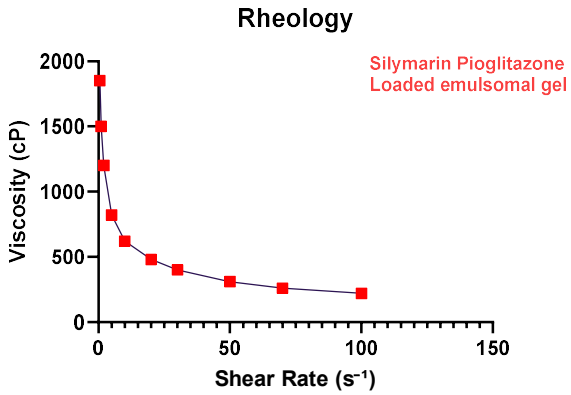

Figure 1. Rheological Profile of Optimized Silymarin–Pioglitazone Nanoemulgel Showing Pseudoplastic Flow Behavior

Discussion: The nanoemulgel exhibits a high viscosity and shear-thinning property which is beneficial for a topical drug delivery system. The nanoemulgels fabricated with Carbopol exhibit the same rheological properties: pseudoplastic flow which makes these gels more spreadable, more stable in formulation, more compliant to the patient and also remains in contact with the skin surface for a long time.

3.1.4 Spreadability and Extrudability

Patient compliance, ease of application, and uniform drug distribution on the skin surface are parameters that are important to evaluate topical formulations, because they are directly related to their spreadability and extrudability. The optimum nanoemulgel should be both extrudable and spreadable to facilitate easy removal from the container and application, respectively. The optimized Silymarin–Pioglitazone nanoemulgel had a spreadability value of 6.85 ± 0.24 g•cm/s which represents good spreading properties and application ability on the skin surface. The formulation had good uniformity without giving high resistance which may help to achieve better coverage of affected area and better absorption of the drug. The extrudability study showed that the collapsible tube can be easily extruded by using the nanoemulgel with a small force. The extrudability value was determined as 91.42 ± 1.36% which was found to be in the excellent range of tube applicability and user convenience. The optimized viscosity and rheological properties of the formulation helped to achieve satisfactory performance in both extrusion and spreadability. The results obtained indicate that the developed nanoemulgel has desirable rheological characteristics for topical use and can improve patient acceptance during the use.

Table 4. Spreadability and Extrudability of Optimized Silymarin–Pioglitazone Nanoemulgel

Parameter	Result (Mean ± SD)
Spreadability (g·cm/s)	6.85 ± 0.24
Extrudability (%)	91.42 ± 1.36

Discussion: The nanoemulgel developed with the optimized nanoemulsion system and Carbopol gel matrix has good spreadability and extrudability, due to the optimized gel matrix to provide balanced consistency. The following properties are desirable in topical formulations because they enable even application, better patient acceptance, and efficient delivery of the therapeutic agent(s) to the site of action.

3.1.5 Drug Content Analysis

The uniform distribution and incorporation efficiency of the developed nanoemulgel formulation of Silymarin and Pioglitazone was evaluated by drug content analysis. To achieve dose accuracy, therapeutic efficacy and formulation quality, uniform drug distribution is key. Optimized nanoemulgel was subjected to suitable extraction and dilution and then its drug content was determined by UV–Visible spectrophotometric analysis. The drug content of the optimized nanoemulgel was found to be 97.84 ± 1.12% for Silymarin and 98.63 ± 0.96% for Pioglitazone. The high drug content values showed efficient incorporation of all the drugs into the nanoemulgel matrix with negligible drug loss during the process of formulation. In addition, the low value of the standard deviations proved that the content was uniformed in the formulation. The results obtained established that the formulated nanoemulgel exhibited good active pharmaceutical ingredients (APIs) loading and uniform distribution of APIs. This is due to the low concentration of drugs, which can lead to a steady therapeutic activity and reliability of formulation when applied to the skin.

Table 5. Drug Content of Optimized Silymarin–Pioglitazone Nanoemulgel

Drug	Drug Content (%) (Mean ± SD)
Silymarin	97.84 ± 1.12
Pioglitazone	98.63 ± 0.96

Discussion: The drug content values of the collected were found within the pharmacopeial limits (90–110%) which demonstrates good formulation uniformity and effective incorporation of both drugs in the nanoemulgel system. The results also indicate that the formulation process did not have a negative impact on the stability and distribution of the drug. A high drug content can be beneficial for the therapeutic efficacy and reproducible drug delivery.

3.2 In Vitro Drug Release Study

3.2.1 Comparative Drug Release Profile

The in vitro drug release study was performed to ascertain the drug release characteristics of the developed nanoemulgel formulation of Silymarin and Pioglitazone. A Franz diffusion cell system was used to measure the cumulative percent drug released and compared to the pure drug suspension. The nanoemulgel which was optimized showed a controlled and sustained release of the drug throughout the study period. The cumulative release of the developed formed nanoemulgel showed a significant difference as compared to the drug suspension alone. The improved release could be attributed to the presence of nano-sized droplets in the nanoemulsion system which resulted in a larger surface area for the diffusion of drug and better solubilization of the drug. In addition, Tween 80 and Transcutol P was helpful in partitioning and diffusion of drug across the dialysis membrane. After 12 h, the cumulative release of Silymarin and Pioglitazone from optimized nanoemulgel was found to be 92.46 ± 1.54% and 94.12 ± 1.37%, respectively. The mechanism of the controlled release behaviour observed can be responsible for extended therapeutic effect and

decreased dosing frequency for topical application. The results show that the optimized nanoemulsion was incorporated in the gel matrix successfully and the incorporation enhanced the release characteristic of both drugs.

Table 6. In Vitro Drug Release Profile of Optimized Nanoemulgel

Time (h)	Silymarin Release (%)	Pioglitazone Release (%)
0	0.00	0.00
1	18.24 ± 1.02	20.15 ± 0.95
2	31.56 ± 1.18	34.22 ± 1.04
4	52.43 ± 1.35	55.84 ± 1.12
6	68.71 ± 1.48	72.35 ± 1.26
8	79.58 ± 1.42	82.91 ± 1.31
10	87.34 ± 1.63	89.76 ± 1.45
12	92.46 ± 1.54	94.12 ± 1.37

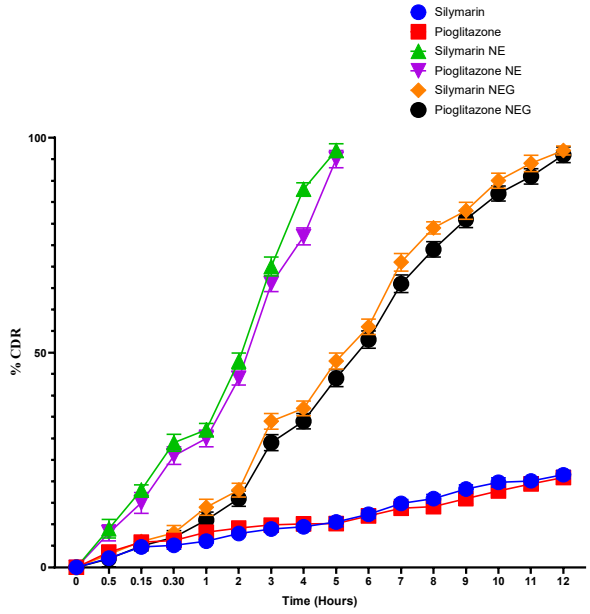

Figure 2. In Vitro Drug Release Profile of Silymarin and Pioglitazone from Optimized Nanoemulgel

3.2.2 Drug Release Kinetics

The mechanism of drug release from the developed nanoemulgel was studied by fitting the release data with different kinetic models such as Zero order, First order, Higuchi and Korsmeyer–Peppas model. The values of R2 for each model were compared to get the best-fit release model. The release kinetics analysis showed the highest correlation coefficient value (R^2) for Higuchi model, suggesting that the release of the drug was mostly diffusion controlled from the nanoemulgel. The Korsmeyer–Peppas release exponent (n) suggested a non-Fickian or anomalous diffusion mechanism, suggesting that a combination of diffusion and polymer relaxation is controlling the drug release. The sustained release behavior of the developed nanoemulgel can be due to the Carbopol gel matrix which acted as a diffusion barrier and controlled the release of both drugs. Extended release will help to provide extended plasma levels of the therapeutic drug and enhance topical efficacy.

Table 7. Drug Release Kinetic Model Analysis of Optimized Nanoemulgel

Kinetic Model	R^2 Value
Zero-order	0.942
First-order	0.956
Higuchi	0.989
Korsmeyer–Peppas	0.978

Table 8. Korsmeyer–Peppas Release Parameters

Parameter	Value
Release Exponent (n)	0.67
Mechanism	Non-Fickian Diffusion

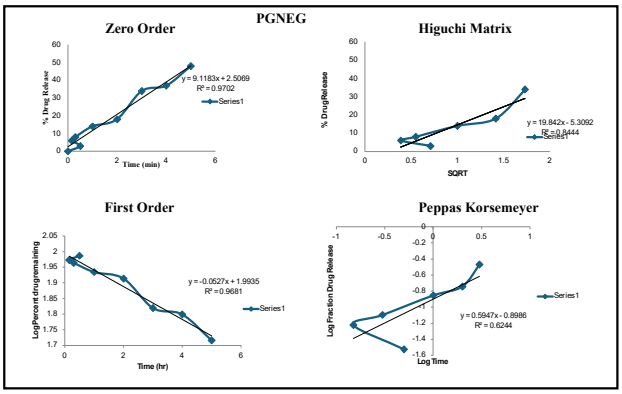

Figure 3. Zero-order, First-order, Higuchi matrix, and Korsmeyer–Peppas kinetic release plots of Pioglitazone-loaded nanoemulgel demonstrating controlled and prolonged drug release characteristics.

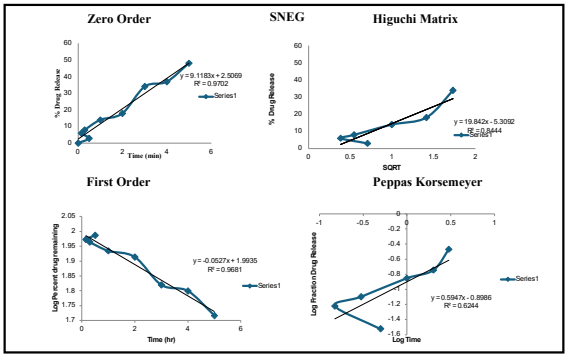

Figure 3. Zero-order, First-order, Higuchi matrix, and Korsmeyer–Peppas kinetic release plots of Silymarin-loaded nanoemulgel showing sustained and controlled drug release behavior.

Discussion: The enhanced dissolution and diffusion of the drug as demonstrated by the superior drug release performance of the nanoemulgel formulation indicated that the nanoemulsion based drug delivery system was effective in improving the drug dissolution and diffusion. The diffusion-controlled release profile of the developed nanoemulgel obtained in the present study is similar to that reported for polymeric gel systems in the literature, making it a possible sustained release topical drug delivery system.

3.3 Antioxidant Activity

3.3.1 DPPH Radical Scavenging Activity

The antioxidant activity of the prepared Silymarin–Pioglitazone nanoemulgel was determined by the free radical scavenging assay with DPPH. Diabetes mellitus and its complications are related to oxidative stress. Thus, formulations with high antioxidant activity can be exploited for providing extra therapeutic effects due to lowering the cellular damage caused by the reactive oxygen species (ROS). The concentration-dependent free radical scavenging activity of developed nanoemulgel formulation was determined in DPPH assay. The higher the formulation concentration the higher the percentage inhibition which implies good antioxidant activity. The antioxidant potential of the nanoemulgel was proved to be greater than the pure drug suspension which might be due to better solubilization of drugs, better dispersion of Silymarin in the nano-emulsion droplets and higher antioxidant compound availability. The optimized nanoemulgel was able to scavenge DPPH radicals with a percentage of 89.76 ± 1.42% at the highest concentration tested, comparable to that of the standard antioxidant (ascorbic acid). The formulation exhibited the IC_{50} value of 42.85 µg/mL, indicating high antioxidant efficiency. This augmented antioxidant activity could play a role in the decreased oxidative stress related damage in diabetic complications and in the therapeutic efficacy of the developed nanoemulgel.

Table 9. DPPH Radical Scavenging Activity of Optimized Nanoemulgel

Concentration (µg/mL)	% Inhibition (Mean ± SD)
20	32.18 ± 1.05
40	48.42 ± 1.18
60	63.75 ± 1.26
80	77.84 ± 1.35
100	89.76 ± 1.42

Table 10. IC_{50} Value of Optimized Nanoemulgel

Sample	IC_{50} (µg/mL)
Optimized Nanoemulgel	42.85
Ascorbic Acid (Standard)	35.62

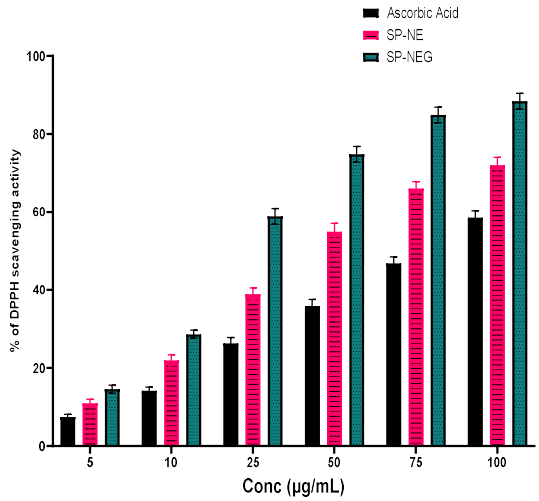

Figure 4. DPPH Radical Scavenging Activity of Optimized Silymarin–Pioglitazone Nanoemulgel at Different Concentrations

3.4 Cell Line Studies

3.4.1 Cell Viability (MTT Assay)

MTT assay was used to evaluate the developed Silymarin–Pioglitazone nanoemulgel's cytocompatibility. Cell viability studies are crucial in determining the safety and biological compatibility of novel topical formulations prior to further therapeutic applications. Optimized nanoemulgel exhibited good cell compatibility and high cell viability at all the concentrations tested. Cell viability was found to be more than 80%, which showed low cytotoxicity and good safety of formulation with increasing concentration, with a slight decrease in the percentage cell viability. The improved cell compatibility may be due to the biocompatibility of the components of the nanoemulsion, combined with the antioxidant effect of Silymarin. The results obtained showed that the nanoemulgel formed was well tolerated by the cells

and is appropriate for the therapeutic applications on the skin.

Table 11. Cell Viability of Optimized Nanoemulgel by MTT Assay

Concentration (µg/mL)	Cell Viability (%)
25	98.24 ± 1.15
50	95.76 ± 1.24
100	92.43 ± 1.31
200	88.65 ± 1.42
400	84.17 ± 1.58

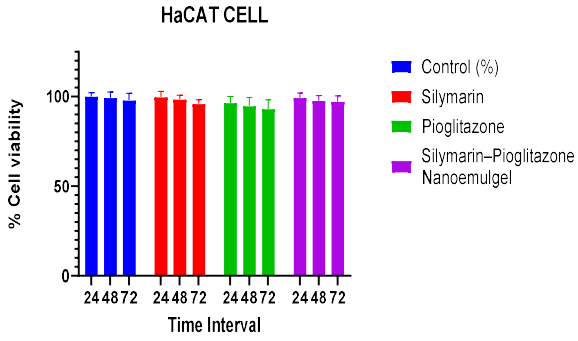

Figure 5. HaCaT cell viability assessment of Control, Silymarin, Pioglitazone, and optimized Silymarin–Pioglitazone nanoemulgel formulations after 24, 48, and 72 h incubation using MTT assay. Values are expressed as mean ± SD (□ = □).

3.4.2 TNF-α Gene Expression Analysis

Tumor necrosis factor alpha (TNF-α) is one of the primary pro-inflammatory cytokines that play a role in the development of diabetes-associated inflammation and tissue damage. The expression of TNF-α was analyzed by qRT-PCR in order to evaluate the effect of the developed nanoemulgel. The expression of TNF-α was significantly decreased when treated with the nanoemulgel as compared to the untreated diabetic control group. The level of relative expression of TNF-α was decreased to 0.42 ± 0.04 fold, which means there was significant suppression of inflammatory signalling pathways. The observed reduction can be explained with the synergistic effect of antioxidants activity and anti-inflammatory activity of Silymarin and insulin-sensitizing activity of Pioglitazone. The findings indicate that the formulated nanoemulgel could be good against inflammatory responses related to diabetic complications.

3.4.3 IL-6 Gene Expression Analysis

Tumor necrosis factor alpha (TNF-α) is one of the primary pro-inflammatory cytokines that play a role in the development of diabetes-associated inflammation and tissue damage. The expression of TNF-α was analysed by qRT-PCR in order to evaluate the effect of the developed nanoemulgel. The expression of TNF-α was significantly decreased when treated with the nanoemulgel as compared to the untreated diabetic control group. The level of relative expression of TNF-α was decreased to 0.42 ± 0.04 fold, which means there was significant suppression of inflammatory signalling pathways. The observed reduction can be explained with the synergistic effect of antioxidants activity and anti-inflammatory activity of Silymarin and insulin-sensitizing activity of Pioglitazone. The findings indicate that the formulated nanoemulgel could be good against inflammatory responses related to diabetic complications.

Table 12. Relative TNF-α Gene Expression

Group	Relative Expression (Fold Change)
Control	1.00 ± 0.00
Diabetic Control	2.85 ± 0.15
Nanoemulgel Treated	0.42 ± 0.04

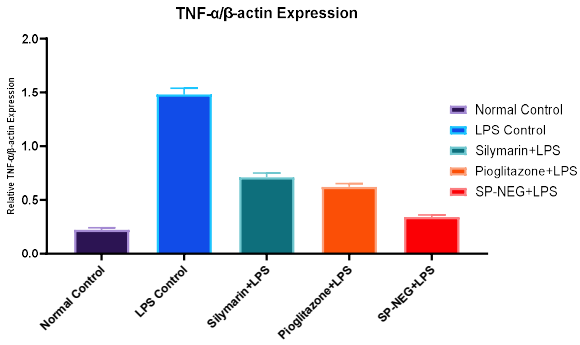

Figure 7. Relative IL-6 Gene Expression Following Treatment with Optimized Nanoemulgel

3.4.4 IL-1β Gene Expression Analysis

IL-1β is an important pro-inflammatory cytokine that plays an important role in the inflammatory cascade of diabetes and hinders wound healing. The effect of the nanoemulgel formulation was evaluated by analyzing IL-1β expression by qRT-PCR. The developed formulation significantly decreased the expression of IL1β when compared to the diabetic control group. In the nanoemulgel treated group, the relative expression level of IL-1β was 0.35 ± 0.02 fold. The substantial decrease in the expression of IL-1β proved the capability of the formulation to silence inflammatory signaling pathways and enhance the cellular microenvironment. Based on these results, the developed nanoemulgel could be considered a promising topical antidiabetic formulation.

Table 13. Relative IL-6 Gene Expression

Group	Relative Expression (Fold Change)
Control	1.00 ± 0.00
Diabetic Control	2.71 ± 0.14
Nanoemulgel Treated	0.35 ± 0.02

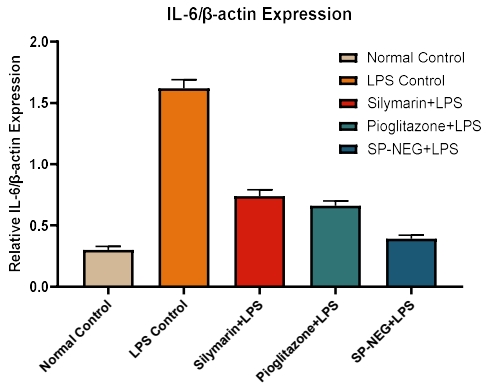

Figure 9. Relative TNF-α/β-actin gene expression in LPS-stimulated RAW 264.7 macrophage cells treated with Silymarin, Pioglitazone, and optimized Silymarin–Pioglitazone nanoemulgel formulations. Values are expressed as mean ± SD (= *3*).

3.5 Stability Studies

3.5.1 Physical Stability Evaluation

The stability of the optimized Silymarin–Pioglitazone nanoemulgel has been assessed under accelerated and room temperature storage conditions for three months. The formulation was checked for color, smell, uniformity of the formulation, phase separation, syneresis, and appearance every few days. Stability assessment is crucial to maintain formulation integrity and therapeutic efficacy in storage. The nanoemulgel was physically stable and no phase separation, creaming, cracking, drug crystallization or microbial contamination was observed during the study period. The color and consistency of the formulation did not change, thus showing the good compatibility between the nanoemulsion system and the Carbopol gel matrix. In addition, there were no signs of syneresis or loss of homogeneity during the storage period. The good physical stability achieved could be ascribed to the efficient stabilization promoted by the optimized surfactant system and to the high gel network of Carbopol 940. The physical stability of the developed nanoemulgel was satisfactory which, in turn, proved to be suitable for long storage duration.

Table 15. Physical Stability Evaluation of Optimized Nanoemulgel During Storage

Parameter	Initial	1 Month	2 Months	3 Months
Color	Pale Yellow	No Change	No Change	No Change
Appearance	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Phase Separation	Absent	Absent	Absent	Absent
Syneresis	Absent	Absent	Absent	Absent
Consistency	Smooth	Smooth	Smooth	Smooth

3.5.2 Stability of Drug Content and Viscosity

The stability of drug content and viscosity was evaluated to determine the ability of the nanoemulgel formulation to retain its pharmaceutical quality during storage. Samples were taken at fixed time periods and drug concentration as well as viscosity was determined, and the results were compared with the initial results. The result showed that there were only slight differences in the content of the drugs and the viscosity of the formulation throughout the storage period. From the results, the drug content in both Silymarin and Pioglitazone were found to be above 95%, and hence the formulations exhibited good stability and minimal degradation of the drugs. Likewise, no significant changes in viscosity were observed during the study period, indicating that the gel matrix structure remained stable. Optimized nanoemulgel exhibited retention of drug and rheological properties which indicated good stability of the nanoemulgel during storage and also had good shelf-life stability. The results also confirm the applicability of the developed formulation to drug delivery to the topical route.

Table 16. Stability Study of Optimized Nanoemulgel

Parameter	Initial	1 Month	2 Months	3 Months
Drug Content (%) – Silymarin	97.84 ± 1.12	97.21 ± 1.08	96.85 ± 1.15	96.42 ± 1.23
Drug Content (%) – Pioglitazone	98.63 ± 0.96	98.12 ± 1.04	97.76 ± 1.11	97.35 ± 1.18

Viscosity (cP)	12850 ± 145	12792 ± 152	12715 ± 163	12684 ± 171

Discussion: The stability study results showed that the prepared Silymarin–Pioglitazone nanoemulgel was physically and chemically stable throughout the storage period. However, no significant appearance, drug content or viscosity changes was observed, which confirmed the robustness of the formulation. The optimized composition of the nanoemulsion and the gel network may have contributed to the stability of the nanoemulgel, as it is expected to have a strong network of gel which can effectively prevent drug leakage and formulation instability. The obtained results indicate the possibility of using the prepared nanoemulgel for long-term storage and as a pharmaceutical product.

4. Conclusion

In the present study, a novel drug delivery system, Silymarin–Pioglitazone loaded nanoemulgel, was successfully developed and evaluated for possible application in the treatment of diabetes. The optimized nanoemulgel has the desired physicochemical properties such as pH, high drug content, satisfactory viscosity, excellent spreadability and good extrudability which is suitable for topical application. The formulation exhibited a sustained drug release and controlled drug release nature; the drug release kinetic profile suggested that the drug was released from the formulation in a diffusion-controlled manner as represented by the Higuchi diffusion model. The antioxidant effect showed high DPPH radical scavenging activity indicating the potential of the formulation to combat oxidative stress among diabetes and its related complications. The developed nanoemulgel was also found to be very cytocompatible and safe as demonstrated by the cell viability studies. Moreover, the formulation exhibited strong anti-inflammatory effects, as evidenced by the significant reduction in the levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β. In addition, physical and chemical stability studies showed that the nanoemulgel had excellent stability during the storage period with no significant differences in appearance, drug content or viscosity. In conclusion, the synergistic effect of Silymarin and Pioglitazone loaded in a nanoemulgel system is a promising strategy for better topical antidiabetic treatment. The developed formulation could show better therapeutic efficacy, less systemic side effects and better patient compliance; thus it can be considered as a possible candidate for further preclinical and clinical studies.

5. Acknowledgement

6. Conflict of Interest

7. References

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