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ORIGINAL RESEARCH

Alopecia Management Potential of Rosemary-Based Nanoemulgel Loaded with Metformin: Approach Combining Active Essential Oil and Repurposed Drug

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Introduction: Androgenetic alopecia (AGA) is a multifactorial and age-related dermatological disease that affects both males and females, usually at older ages. Traditional hair repair drugs exemplified by minoxidil have limitations such as skin irritation and hypertrichosis. Thus, attention has been shifted to the use of repurposing drugs. Metformin is an anti-diabetic drug, that can promote hair follicle regeneration via upregulation of the hair-inductive capability. Hence, the current study aims to fabricate a safe and effective nanoemulsion to improve metformin efficacy in targeting AGA.

Methods: Rosemary oil was selected as the oily phase due to its ability to increase blood flow and hair growth. Rosemary-based nanoemulsions were statistically optimized by Box-Behnken experimental design, loaded with metformin, and incorporated into a hydrogel to form a nanoemulgel. Metformin-loaded nanoemulsions were assessed for their diametric size, uniformity, zeta potential, and metformin characteristics within the formulated nanosystem. The nanoemulgel was then evaluated in terms of its pH, percentage drug content, and in-vitro release performance. In-*vivo* study assessed the nanoemulgel's ability to augment hair growth in rats.

Results: The experimental design displayed that using 50%w/w, 20%w/w, and 10%w/w of Cremophor®, Labrafil®, and deionized water, respectively, resulted in nanoemulsion formulation with the smallest globule size (125.01 ± 0.534 nm), unimodal size distribution (PDI=0.103), negative surface charge (−19.9 ± 2.01 mV) with a spherical morphological structure. Rosemary-based nanoemulgel displayed acceptable physicochemical characterizations namely; a neutral pH value of 6.7±0.15, high drug content (92.9± 2.3%), and controlled metformin in-vitro release. Besides, the formulated nanoemulgel significantly increased the number of hair follicles in the animal model compared with other controls and tested groups.

Conclusion: The designed nanoemulgel is a promising approach for treating androgenic alopecia.

Keywords: androgenic alopecia, emulgel, metformin, nanoemulsion, rosemary oil, quality by design

Introduction

Androgenetic alopecia (AGA) is among the most frequent types of progressive hair loss disorders in men and women. AGA affects up to 50% and 80% of white men by the age of 50 and 70, respectively.[1] AGA has a multifactorial and polygenetic etiology related to hormonal imbalance and insufficient blood flow to the scalp. The binding of androgens to their androgen receptors forms a hormone-receptor complex, which commences the signaling cascade of regulating hair growth causing hair follicle shrinkage and thinning.[2] Testosterone in the dermal papilla is rapidly converted to the more

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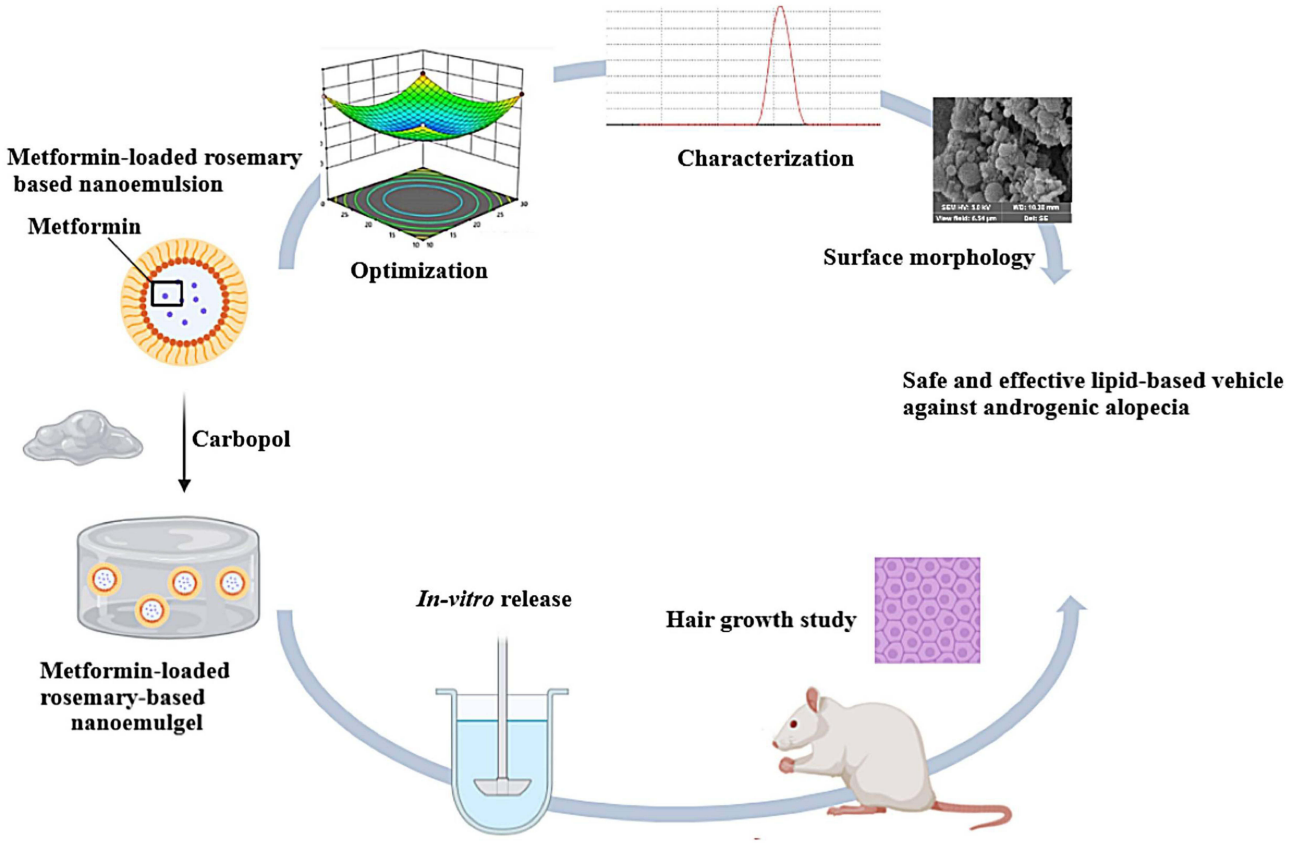
Graphical Abstract



potent dihydrotestosterone form which has a higher affinity to androgen receptors under the action of type II 5α-reductase. Inhibiting reductase enzyme can prevent testosterone conversion to dihydrotestosterone making it an effective treatment option for AGA.[3] Androgenetic alopecia reduces patients' quality of life and lowers their self-confidence, thus designing an efficient treatment is extremely desirable.

Even though androgenic alopecia is a common disorder, a limited number of drugs are approved for its management. Minoxidil has been utilized for decades to treat hair loss by affecting follicular cells, improving hair growth, and decreasing hair loss. Nevertheless, topical minoxidil solution has been known for its ability to cause irritant contact dermatitis.[4] Hypertrichosis is another limitation of using minoxidil. Herein, attention turned to other medications that may have safer profiles or what is known as repurposing drugs. Drug repurposing is the application of a drug for another indication different from the therapeutic one than originally approved. This strategy received growing interest among pharmaceutical companies and scientists as an alternative replacement for synthesizing new chemical moieties.[5] Metformin is a biguanide antihyperglycemic drug that is used for the treatment of type 2 diabetes mellitus. It has recently been reported that metformin can augment the cellular proliferation of human dermal papilla and outer root sheath cells. It can further increase Ki-67 expression in the ex-vivo hair follicle organ culture. It can significantly increase AMP-activated protein kinase (AMPK) phosphorylation, suppressing β-catenin degradation and improving its nuclear accumulation, resulting in hair regeneration induction.[6] Furthermore, Sun et al stated that metformin could stimulate alkaline phosphatase activity of cultured self-assembled three-dimensional aggregates of epidermal and dermal cells (DCs), upregulate both the protein and mRNA expression levels of molecular markers, and improve the survival rate of reconstituted hair follicles.[7] Therefore, metformin can be considered a promising candidate to treat alopecia.

Treating androgenic alopecia can be carried out topically or systemically in which the former is preferred to limit non-specific adverse effects. Nevertheless, the limited penetration of traditional topical formulations can minimize the therapeutic efficacy of the applied drug. Henceforth, incorporating drugs into a nanocarrier system can potentially improve drug percutaneous delivery owing to their advantages mainly the nano-diametric size that can penetrate through hair follicles and the interaction of the system with skin.[8] Vogt et al demonstrated that particles of 40 nm penetrated hair follicles and were absorbed by Langerhans cells in human skin, whereas particles of 750 and 1500 nm produced aggregates that were maintained on the follicle openings' surface layers. Thus, it is possible to create selective particle vectoring for hair follicles by controlling the particle size.[9] Therefore, a suitable nanocarrier system needs to be meticulously designated which should possess a high affinity to hair follicles to deliver more therapeutic cargos to the target cells or tissues attaining maximal therapeutic efficacy. Moreover, the nanocarrier should have a safe profile in order not to cause any undesirable effects at the application site.[10] Nanoemulsion (NE) is one of the most attractive nanocarrier systems owing to its nano-size range (<200 nm), safety profile, and ability to control drug release and its permeation through the skin. It was stated previously that ex-vivo permeation of terbinafine hydrochloride from nanoemulsion revealed almost total skin permeation of 97% in 6 hours. In contrast, commercial products reached only 57% permeation simultaneously, which was linked to the small droplet size that can penetrate the skin easily.[11]

In the current study, *Rosmarinus officinalis* (Rosemary) essential oil was used as the oily phase due to its ability to stimulate hair growth.[12] It consists of 2% volatile oil, rosmarinic acid, carnosol, and caffeic acid, which can boost microcapillary perfusion. In addition, its content of 2-methoxy carnosic acid suppresses type II 5α-reductase activity which is usually utilized to treat androgenic alopecia.[13] Further, rosemary oil is well known for its anti-bacterial and antioxidant effects which are required in scarring alopecia.[14] It was claimed previously that rosemary-based gel enhanced hair development in length, thickness, and bulb diameter by approximately 1.2 times.[15] Thus, our study aimed to load metformin into rosemary-based nanoemulsion gel to upgrade metformin anti-hair loss activity and lower its required dose against androgenic alopecia. Additionally, the study aimed to improve drug permeation through the skin with minimal adverse effects. Box-Behnken design was proposed to optimize the nanoemulsion formulation. The optimized formulation was characterized regarding its physicochemical characteristics, surface morphology, and metformin state within the formed nanoemulsion as well as system stability. Additionally, metformin-loaded rosemary-based nanoemulgel was fabricated using Carbopol® 934 and characterized regarding its physicochemical characteristics. Furthermore, the prepared metformin-loaded rosemary-based nanoemulgel was assessed clinically in the alopecia model in rats.

Materials and Methods

Materials

Carbopol 934 was purchased from Shanghai Xietai Chem Co. (Shanghai, China). Cremophore EL (Castor oil polyoxyethylene ether, Ethoxylated castor oil, PEG-35 castor oil, polyoxyl 35 castor oil, polyoxyl 35 castor oil) and propylene glycol (1,2-Propanediol, Propylene glycol), were purchased from Sigma Aldrich (Steinheim, Switzerland). Labrafil (Oleoyl polyoxyl-6 glycerides) and Labrasol® (PEG-8 Caprylocaproyl polyoxyl-8 glycerides NF) were kindly donated by Gattefosse Co. (Lyon, France). Metformin was a kind gift from Benta pharma industry, Lebanon. *Rosmarinus officinalis*, a Rosemary oil, of 100% purity, was purchased from a local pharmacy, Lebanon. All other chemicals and solvents used were of analytical grade.

Methods

Preliminary Screening of Surfactants and Co-Surfactants

The selection of the suitable surfactant and co-surfactant was based on the measurement of percentage transmittance according to the method described by Mehanna et al.[10] Briefly, 300 mg of each surfactant, namely, Cremophor, labrasol, and Tween 80 were added to an equal weight of Rosemary oil. The mixture was heated at 45±2°C and mixed on a magnetic stirrer at 800 rpm till complete homogenization. Certain weight of the isotropic mixture was then diluted with ultra-pure water. The emulsification was assessed according to percentage transmittance measured at 638.2 nm (Jasco V-730 spectrophotometer). The surfactant that formed a dispersion with the highest percent transmittance was selected.

The selected surfactant and the lipid phase were used to evaluate the effectiveness of the co-surfactants to improve the system's emulsification capability. The selected surfactant was mixed individually with each of the tested co-surfactants (Transcutol, propylene glycol, and Labrafil) and then added to the lipid phase. The mixture was then mixed at 45±2°C on a magnetic stirrer till complete homogenization. Then, 50 mg from each mixture was diluted with ultra-pure water and kept for 2 hours. The transmittance of each dispersion was measured as previously described.

Preparation and Optimization of Metformin-Loaded Rosemary-Based Nanoemulsion

Nanoemulsions were prepared utilizing the spontaneous emulsification method. Rosemary oil was first warmed to 45°C on a magnetic stirrer followed by the dropwise addition of the aqueous phase containing the selected surfactant and co-surfactant. The mixture was mixed at 600 rpm under the same temperature until a homogenous dispersion was formed. The amount of surfactant, co-surfactant, and deionized water that produced nanoemulsion with the smallest size, and polydispersity index (PDI) were selected for nanoemulsion formulation.

Metformin-loaded rosemary-based nanoemulsion was prepared by the same procedure where metformin was dissolved in the aqueous phase before adding the latter into the oily phase. In all the formulations the amount of metformin was fixed at 10 mg and the oily phase at 10%w/w.

Metformin-loaded rosemary-based nanoemulsions were optimized using Box-Behnken response surface methodology experimental design (Design-Expert® Software Version 11). This design is crucial while developing new formulations since it defines and determines the relationship between independent variables and formulation responses, moreover, it determines the levels of those formulation variables that can generate the best response. The design was based on 3 factors each at 2 levels. The independent variables studied were weight ratios (%w/w) of surfactant (X1), co-surfactant (X2), and deionized water (X3) with their low, medium, and high levels for preparing 17 formulations as detailed in Table 1. The studied responses were particle size (Y1) and polydispersity index (Y2). Moreover, 3D response surface graphs were plotted to depict the effects of the predetermined factors on the measured responses.

The prepared nanoemulsions were filled into vials and the lyophilization technique was as follows: freezing at −77 °C for 24 h in a freezer; primary drying at −38 °C for 8 h; then, the shelf temperature was raised to −22 °C for 15 h; secondary drying at 22 °C for 10 h in a lyophilizer (Freeze-dryer, Labconco, China). The chamber pressure was maintained at 13.3 Pa and the temperature of the cold trap was −53.3 °C during the whole lyophilization procedure.

Physicochemical Characterization of Metformin-Loaded Rosemary-Based Nanoemulsion

Droplet Size, Polydispersity Index (PDI), and Zeta Potential

The droplet size, polydispersity index (PDI), and zeta potential of the metformin-loaded rosemary-based nanoemulsion system were measured using the Zetasizer 2000 device (Malvern Instruments, Malvern, UK). The system was diluted 100 times with ultra-pure water before being measured at ambient temperature and a scattering angle of 90 degrees.

Table 1 Independent Variables and Their Levels in Box–Behnken Design for the Optimization of Metformin-Loaded Rosemary-Based Nanoemulsion

Independent Variables	**Levels (Actual Coded)**		
	Low	**Medium**	**High**
X1=Weight ratio of surfactant (%w/w)	30	40	50
X2=Weight ratio of co-surfactant (%w/w)	10	20	30
X3= Weight ratio of water (%w/w)	10	20	30
Transformed value	−1	0	+1
Studied Responses	**Goal**		
Y1= Particle size (nm)	Minimum		
Y2= Polydispersity index	Minimum		

Notes: X are the independent variables, and Y are the studied responses.
Abbreviations: % w/w, percentage weight per weight; nm, nanometer.

Entrapment Efficiency

For determining the entrapment efficiency (EE), the system was centrifuged for 60 min at 20,000 rpm using a high-speed centrifuge (Sigma- 4L42, Sigma Laboratory Centrifuges, Osterode am Harz, Germany). The supernatant was removed using a micropipette and filtrated before being diluted to determine the free metformin. Entrapment efficiency was calculated using the following equation.[16]

$$EE(\%) = \frac{(\text{Total amount of drug} - \text{Free drug in the supernatant})}{\text{Total amount of drug}} x100 \quad (1)$$

Fourier Transforms Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) was applied to assess the state of metformin and the possible interaction between metformin and the nanoemulsion components. FTIR spectrometer was used to record FTIR for rosemary oil, metformin, physical mixture, and the lyophilized metformin-loaded rosemary-based nanoemulsion (Bruker Vector 42, Billerica, MA, USA). Dry potassium bromide was mixed with the samples which were then compressed into a disc and scanned over a range of 200–4000 cm^{-1}.[8] It was performed under scan number 32 and resolution 4.

X-Ray Diffraction

X-ray diffraction (XRD) was carried out to analyze the solid states, amorphous or crystalline nature, of metformin-loaded rosemary-based nanoemulsion. XRD of the pure metformin drug and the lyophilized metformin-loaded rosemary-based nanoemulsion were performed using powder X-ray diffractometer (PANalytical 3 kW X'pert Powder, United Kingdom). Samples were placed in the sample stage and scanned from 2 to 60^{θ} with an operating voltage of 40 kV and a current of 40 mA.

Scanning Electron Microscope

The morphology of the particles was evaluated with a scanning electron microscope (SEM) using a MIRA 3 TESCAN. After lyophilization, samples were mounted on metallic stubs using conductive double-coated carbon tape. The particles were then sputter coated with a 12 nm thick layer of gold utilizing Quorum 150 V Plus, UK to be analyzed using SEM.[17]

Preparation of Metformin-Loaded Rosemary-Based Nanoemulgel

The prepared nanoemulsion was converted into a nanogel using Carbopol. Carbopol was swelled in distilled water overnight until a viscous gel was obtained. An equal amount of the prepared metformin-loaded rosemary-based nanoemulsion and Carbopol gel were mixed using a magnetic stirrer for 30 min. A few drops of triethanolamine (TEA) were added to adjust the pH value of the obtained nanoemulgel. The final concentration of Carbopol in the mixture was 1% w/v. The control gel was prepared simply by dissolving an equivalent amount of metformin in deionized water and then loading it into Carbopol matrix to form a gel.

Physicochemical Characterization of Metformin-Loaded Rosemary-Based Nanoemulgel

Rheological Properties

The rheological feature of the prepared gel was studied at room temperature using a Brookfield viscometer (DV-I + Pro, Brookfield AMETEK, Middleborough, MA, USA). The viscosity of the gel was measured at various shear rates (0.5 to 100 S^{-1}) and the construction of rheograms was carried out.

pH Measurement

The pH value of metformin-loaded rosemary-based nanoemulgel was determined by immersing the electrode on the gel surface using a pH digital meter (SED 12530 V Martini Instruments Co., Ltd., Beijing, China) at 25 ± 0.2°C.

Drug Content Uniformity

Drug content was analyzed by determining the amount of metformin in three different locations within the prepared gel

(top, bottom, and middle). Samples were first diluted in deionized water using water bath sonication (Sonica, Milano, Italy) for about 30 minutes at room temperature. The solutions were filtered and diluted. The concentration of metformin was calculated in the obtained solutions using a UV–visible spectrophotometer at λ_{max} 234 nm.[18]

In-Vitro Release

An In-vitro release study of free metformin, metformin-loaded rosemary-based nanoemulgel, and the control gel was performed using the dialysis bag technique over 24 hours. Samples of each formulation were placed into dialysis bags made up of semi-permeable membrane (14,000 cut-offs molecular weight, average diameter 27 mm, average flat width 43 mm, and 175 mL/ft, Sigma Aldrich D9527, Germany) which was pre-soaked overnight in ultra-pure water and immersed into vessels of the USP dissolution apparatus (Model DT 720, Erweka Ltd., Germany) containing phosphate buffer solution (pH 7.4). The bags were immersed under the following conditions: 34±0.5 °C temperature (simulating human skin temperature) and at 100 rpm paddle stirring. Samples were withdrawn at regular intervals (0.5 1, 2, 3, 4, 5, 6, and 24 hrs) and the sink condition was maintained by adding fresh phosphate buffer solution. The withdrawn samples were assayed for their metformin content spectrophotometrically at 230 nm at 25 °C.

The following equation determined the mean dissolution time (MDT) used to compare metformin release behavior from nanoemulgel and the control one.

$$MDT = \frac{\sum_{i}^{n} tmid\Delta c}{\sum_{i}^{n} \Delta c} \tag{2}$$

Mean dissolution time (MDT) is used in evaluating dissolution profiles, in which n is the number of release sample times, i is the sample number, t_{mid} is the mid-point time computed by $(t_i + t_{i-1})/2$, and ΔC is the additional concentration of the released drug between i and i-1.

To describe the kinetics of diclofenac release from the gels, mathematical models, namely, zero-order, first-order, Higuchi model, and Korsmeyer-Peppas were applied. The standard for selecting the most relevant model was based on a goodness-of-fit test.

In-vivo Assessment

Experimental Animals

A total of 50 Wistar albino rats of either sex weighing between 120 and 150 grams were obtained from the animal house of the Faculty of Pharmacy, Prince Sattam bin Abdulaziz University, Kingdom of Saudi Arabia. Animals were housed in clean cages with ventilation systems with enough space to move around comfortably (Rat IVC Blue Line, Techniplast, Buguggiate VA, Italy). Appropriate bedding materials (sawdust) that absorb moisture effectively were used. Rats were kept under controlled conditions of 12 h/12 h light/dark cycle, 25 ± 1 ◦C to ensure the comfort of the rats. All rats were fed a standard rat pellet. Water bottles equipped with stainless tubes were used to prevent contamination and ensure that the rats had access to clean, fresh water always. Cages were cleaned regularly, and bedding materials were changed to maintain cleanliness and prevent the buildup of ammonia and other contaminants. Animal handling during the work was carried out following the ARRIVE guidelines.

Animal Ethics Statement

All procedures described in the study were reviewed and approved by the Investigation Review Board (IRB) of Prince Sattam bin Abdulaziz University. The approval number is SCBR-183/2023.

The in-vivo experiment was carried out according to the Guidelines for the care and use of laboratory animals.[19]

Experimental Design

The experiment was conducted on 50 rats which were randomly divided into two equal main groups (A and B). Group A was used to study the effect of topical application of metformin-loaded rosemary-based nanoemulgel application on rats' hair growth. These rats were anesthetized at the beginning of the experiment before their hair was shaved by

injecting them with ketamine hydrochloride (5 mg/kg) and xylazine hydrochloride (2 mg/kg). Each animal's dorsal skin (9 cm^2 area) was shaved using an electric shaver 24 hours before starting the experiment. The skin was examined for any sign of injury or irritation before applying the tested gels.[20] Rats of the main group (A) were divided into five groups, each of 5 animals. The first group received no treatment representing the negative control, the second received metformin-loaded gel, the third was treated with unloaded rosemary-based nanoemulgel, the fourth group received metformin-loaded rosemary-based nanoemulgel and the last group received minoxidil serving as the positive control group. The sample size in each group at the beginning of the study (n=5) did not differ from the n numbers in the analysis. The tested formulations were applied topically once daily on the tested areas for 35 days in which animals were then sacrificed to detect hair follicle growth. The rats were closely monitored throughout the experiment for signs of health problems, distress, or behavioral changes. No animal was included or excluded during the experiment and no reuse of any of the animals occurred between experiments.

Animals of group B were divided and treated for 35 days with the same procedure as those in group A, but they were not sacrificed and left without any treatment for a further 35 days as a recovery period after which rats were sacrificed to assay the impact of recovery on hair follicles.

Histopathological Examination

At the end of the experiment, a skin sample was isolated from each group to detect hair follicle growth and skin tolerability using histopathological examination. First, skin samples underwent the fixation step using formaldehyde and were then embedded in paraffin, sectioned with microtome, and placed on microscopical slides. The slides were then de-waxed using xylene after drying and the tissues were hydrated with distilled water. After that, the tissues were stained with hematoxylin, and the excess background stain was removed using weak alcohol. Finally, the slides were stained with an alcoholic solution of eosin where a thin layer of polystyrene was applied.[16] The samples were then observed under a microscope to examine the hair follicles and skin integrity.

Stability Study

The stability of the prepared nanoemulgel was evaluated at two temperatures of 25 ± 2 ◦C (65 ± 5% RH) and 4 ± 2 °C (50 ± 5% RH) for 4 weeks, according to the ICH guidelines.[21] Samples were stored in glass vials and covered tightly with parafilm. Nanoemulgel was assessed for its appearance, viscosity, and drug content.

Statistical Analysis

The results of the various tests were expressed as means of standard deviations (SD) and analyzed using Student's *t*-test (for comparing two groups) and analysis of variance (ANOVA) test (for comparing three or more groups). When the *p-value* is less than 0.05, the data are considered statistically significant. For statistical analysis, Statistical Package for the Social Sciences (SPSS) version 22 was used.

Results and Discussion

Selection of Surfactant and Co-Surfactant

The properly selected surfactant and co-surfactant during nanoemulsion formulation is crucial since they are adsorbed at the interface, decrease the interfacial tension, emulsify the lipid system spontaneously, and provide steric hindrance-related stability to the system, and as a result, prevent droplets coalescence and fusion.[22] However, surfactants and co-surfactants should have safe profiles. A high concentration of surfactants may result in skin irritation when applied topically. Thus, cautious selection of surfactants, as well as co-surfactants, became mandatory. In this context, the selection of surfactant and co-surfactant in the current work was based on computing and comparing nanoemulsions percentage transmittance which reflects their ability to emulsify the lipid phase without any input energy. As displayed in Figure 1, Cremophor, has a primary ability to emulsify rosemary oil as it has the highest percentage transmittance (95.2±0.302%), followed by Labrasol and Tween with percentages transmittance of 88.5±0.32% and 87.9±0.203%, respectively. In coherent with this observation, Zeng et al stated that Cremophor EL was able, at low concentrations, to efficiently emulsify oil/water (O/W) nanoemulsion by spontaneous emulsification technique through contributing to the interfacial film formation and reduce the

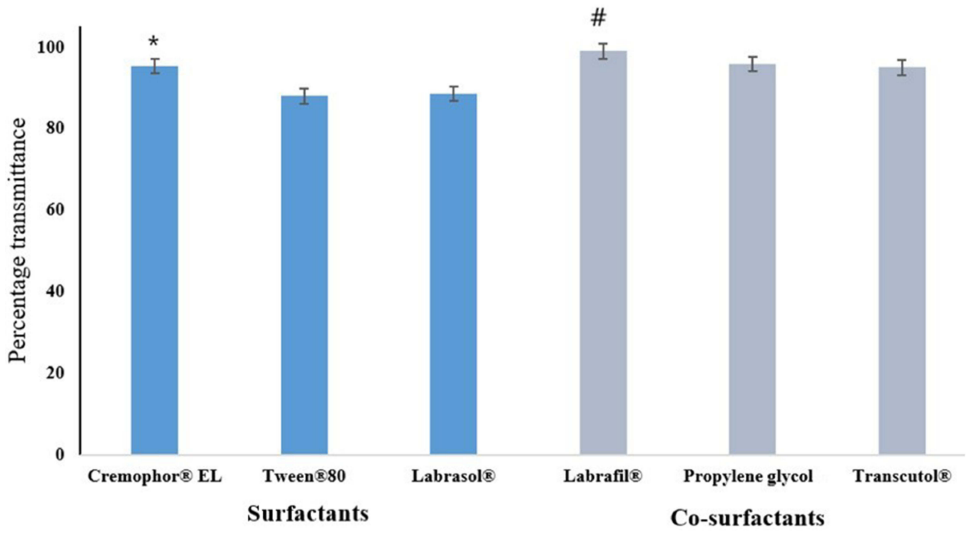


Figure 1 Emulsification efficiency of different surfactants (Cremophor, Tween, and Labrasol) and co-surfactants (Labrafil, Propylene glycol, and Transcutol) with rosemary oil as a step toward the formulation of rosemary-based nanoemulsion using spontaneous emulsification technique. *Significant difference with other surfactants. #Significant difference with other co-surfactants.

tension.[23] Cremophor is a non-ionic surfactant with hydrophilic-lipophilic balance between 12–14 and an approximate critical micelle concentration (CMC) at 0.02%w/w. Cremophor is characterized by its ability to solubilize, protect, and encapsulate a wide range of bioactive compounds.[24] Besides, many microemulsions, nanoemulsions, and self-nanoemulsifying drug delivery systems which are prepared based on Cremophor showed enhanced penetration, permeability, and bioavailability of camptothecin, triamcinolone acetonide, and silibinin have been reported.[25–27]

The selection of the co-surfactant is a critical step due to its ability to decrease the surfactant concentration, improve the polydispersibility of the system, provide a more stable nanoemulsion, and ensure the flexibility of the interfacial surfactant film.[28] To fabricate metformin-loaded rosemary-based nanoemulsion, three co-surfactants, namely, Labrafil, Transcutol, propylene glycol, were assessed for their emulsification potentials. As shown in Figure 1, the inclusion of Labrafil in the nanoemulsion, induced good emulsification with a high percentage transmittance (98.9±0.338%), followed by propylene glycol with a percentage transmittance of 95.870±0.209 to end with Transcutol (94.9±0.238%) ($P< 0.05$). From the previous results, Labrafil was selected for further study as a co-surfactant.

Fabrication and Optimization of Metformin-Loaded Rosemary-Based Nanoemulsion Using Box-Behnken Design

Nanocarriers can exhibit different characteristics in terms of their composition, physicochemical characteristics, and hair follicle penetration behavior. Among different types of nanovehicles, lipid-based nanocarriers, have gained attention as a trans-appendageal drug delivery system due to their ability to pass skin barriers and their safe profile.[29] Nanoemulsions have several advantages over other lipid-based nanocarriers. These advantages include kinetic stability, small droplet size with a large surface area, high loading capacity, the ability to solubilize both hydrophobic and hydrophilic drugs, and controlled drug release, in addition to a high permeation rate through the skin, and specifically, through hair follicles.[30]

The present investigation aimed to optimize the preparation of metformin-loaded rosemary-based nanoemulsion via spontaneous emulsification technique by applying Box-Behnken design. Each independent factor was examined at three levels, in addition to their binary interactions and their polynomial effects. The inspected features of the prepared systems were the droplet size and polydispersity index (PDI) which are depicted in Table 2. Quadratic mathematical models were applied to analyze the relationship between the independent factors and the studied responses as shown in the following equations:

Table 2 Observed Responses (Particle Size and PDI) from Seventeen Randomized Runs in the Box-Behnken Design*#

Run	Factor 1 Cremophor %w/w	Factor 2 Labrafil %w/w	Factor 3 Deionized Water %w/w	Response 1 Particle Size (nm)	Response 2 Polydispersity Index
1	40	10	30	232.59	0.325
2	**50**	**20**	**10**	**125.01**	**0.103**
3	40	20	20	138.25	0.212
4	40	20	20	139.25	0.210
5	40	30	30	237.11	0.310
6	30	10	20	252.11	0.24
7	40	20	20	135.25	0.211
8	40	20	20	134.11	0.215
9	30	20	10	245.23	0.128
10	30	30	20	249.35	0.230
11	40	30	10	238.9	0.120
12	40	10	10	230.88	0.115
13	50	30	20	145.28	0.216
14	30	20	30	246.11	0.320
15	50	20	30	130.25	0.321
16	50	10	20	140.85	0.211
17	40	20	20	136.58	0.212

Notes: *The results are expressed as mean ± standard deviation. #All the formulations included 20%w/w of metformin. The bolded values are the optimal concentration of Cremophor, Labrafil, and deionized water that resulted in the smallest size and PDI value. X are the independent variables, and Y are the studied responses.
Abbreviations: % w/w, percentage weight per weight; nm, nanometer.

Table 3 Regression Analysis for Particle Size (Y1) and Polydispersity Index (Y2) of Metformin Loaded Rosemary-Based Nanoemulsion

Response	Mathematical Model	Adequate Precision	R^2	R^2 Adjusted	R^2 Predicted	SD*	% CV*	Lack of Fit Value	*p-Value*
Y1	Quadratic	45.1595	0.93	0.9956	0.9749	3.61	1.94	5.52	<0.0001
Y2	Quadratic	89.2752	0.9991	0.9979	0.9881	0.00	1.50	5.79	<0.0001

Abbreviations: *SD, Standard deviation; CV, Coefficient of variation; P-value, probability value; R, resolution factor.

Particle size (response 1) = 136.688 −56.42625X1 +1.77625X2 +0.755X3+1.79749X1X2+1.09X1X3-0.875X2X3 +5.99475X1²+54.21475X2²+43.96725X3² (Equation 3)

PDI (response 2)= 0.212 −0.008375 X1 −0.001875 X2 +0.10125 X3 +0.00375 X1X2 +0.0065 X2X3 −0.005 X2X3 +0.006375 X1² +0.005875 X2² −0.00037 X3² (Equation 4)

As listed in Table 3, the obtained results proposed the quadratic model for the analysis of both the particle size and the PDI responses. *P-values* were less than 0.0001 revealing that the model terms are significant. Besides, Adequate precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. The ratios for responses Y1 and Y2 were 45.160 and 89.275, respectively, indicating adequate signal noise. Thus, the recommended model can be used to navigate the design space.

From the analysis of studied independent variables, it can be observed that Labrafil weight ratio (X3) showed minimal correlation and did not affect the investigated parameters, viz. particle size, and PDI, whether it was at its minimum or maximum level. The *p-values* computed in this study were less than 0.05, revealing the significant influence of the formulation variables, specifically the weight ratio of Cremophor and that of the deionized water on the studied responses. As shown in Figure 2A, upon increasing the weight ratio of Cremophor, both nanoemulsion particle size and polydispersity index decreased resulting in a more uniform smaller globule size distribution. This is coherent with what was stated by Goe et al where increasing Cremophor concentration reduced the particle size of cyclosporin-loaded

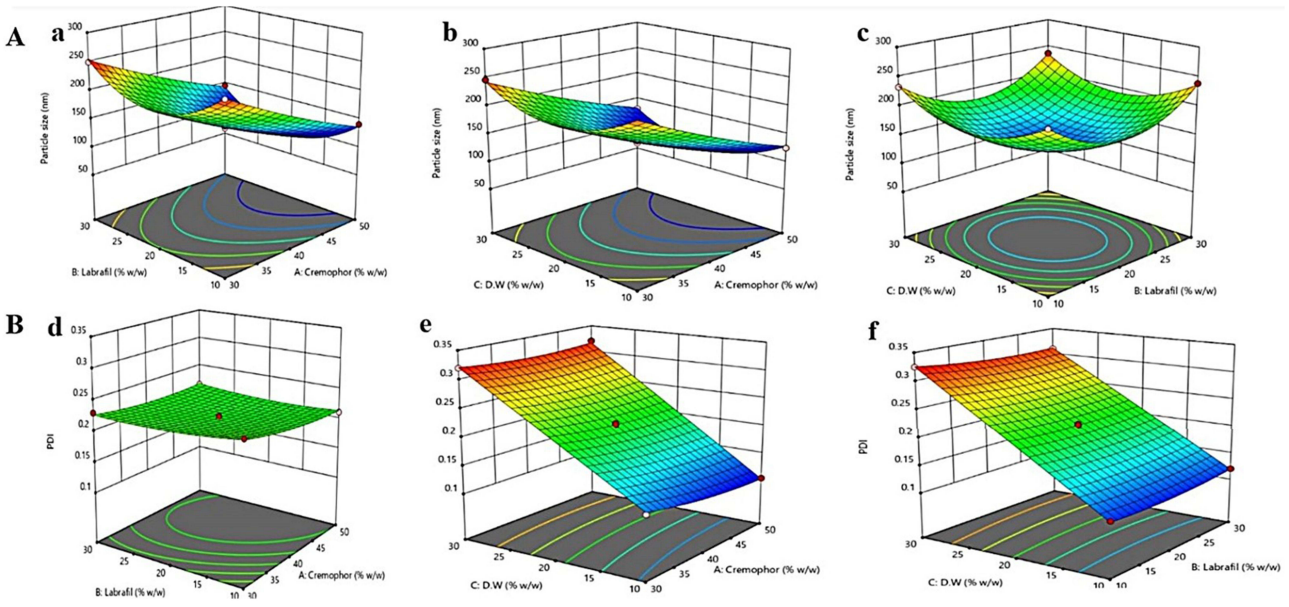


Figure 2 Three-dimensional figures depict the effect of independent variables as the percentage of surfactant (%w/w Cremophor), co-surfactant (%w/w Labrafil), and the deionized water (%w/w DW) on globule size (**A**) and polydispersity index (**B**) of the formed nanoemulsion. Individual responses of each ingredient on nanoemulsion size and PDI are graphed in a, b, c, d, e, and f.

microemulsion which was linked to the ability of Cremophor to cause the interfacial film to decrease and to be more stable, leading to a reduction in the droplet size.[31] Concerning deionized water, it was observed that as the water ratio increased in the formula composition, the formulation had a border size distribution as shown in F5, F14, and F15 (Table 2 and Figure 2B). This could be linked to the fact that increasing water content can reduce the rigidity of surfactants and co-surfactant film, leading to droplet coalescence, thus increasing the PDI of the system.[32] A similar result was reported by Su et al study in which increasing water content from 30% to 40% resulted in increasing the width of the particle distribution range of octyl dodecanol-based nanoemulsion.[33]

After experimentally executing the different 17 runs, the formula (F2) with the smallest particle size (125.01 nm) and lowest PDI was selected. This formula was obtained with 50%w/w Cremophor, 20%w/w Labrafil, and 10%w/w deionized water.

The data in Table 4 shows small residual values between the expected and the observed ones for particle size and polydispersity index. The measured values mirror close concession between the predicted values and the minimal standardized residuals to imply the validity of the developed mathematical model within the design space and its ability to interpret the effect of the surfactant, co-surfactant, and deionized water weight ratio of the formulated metformin-loaded rosemary-based nanoemulsion.

Table 4 Expected and Observed Values of the Optimized Metformin-Loaded Rosemary-Based Nanoemulsion

Factor	**Optimized Level**		
X1=Weight ratio of surfactant (%w/w) X2=Weight ratio of co-surfactant (%w/w) X3=Weight ratio of deionized water (%w/w)	50 19.20 10.40		-
Response	Expected	Observed	Residual
Y1= Size Y2= Polydispersity index	125.23 0.105	125.01 0.103	0.22 0.002

Notes: X are the independent variables, and Y are the studied responses.
Abbreviations: % w/w, percentage weight per weight; nm, nanometer.

Physicochemical Characterization of the Optimized Metformin-Loaded Rosemary-Based Nanoemulsion

As portrayed in Figure 3a., the optimal metformin-loaded rosemary-based NE displayed a nanometric size (125.01 ± 0.534 nm) with an unimodal particle size distribution value (0.103 ± 0.009) due to the inclusion of Cremophor and Labrafil which are acting as surfactant and co-surfactant, respectively. Their presence enhanced the entropy of the nanoemulsion system and reduced the oil/water interfacial tension, which in turn reduced the system's free energy. The droplet size and PDI are crucial factors for pharmaceutical nanocarriers to bypass skin barriers and diffuse through the hair follicles. Nanosized droplets exhibit, in general, a large interfacial surface area that boosts drug permeation through different layers of skin. Moreover, smaller particles less than 200 nm tend to enter the hair follicles through the trans-follicular route.[29] The homogeneity of the particle size distribution of nanoemulsion can be detected through careful examination of the polydispersity index (PDI). Unimodal particle size reflects the complete miscibility and the incorporation of the different nanoemulsions' components including the drug, and hence, minimizes agglomeration chance and maximizes drug loading. Relatedly, nanoemulsion was able to significantly improve the delivery of miconazole through the rats' skin compared to the marketed product, which was linked to the small droplet size of nanoemulsion that provided more efficient paracellular and transcellular transport across the skin layers as previously evidenced.[34]

Surface charge is another critical parameter owing to its ability to reflect the physical stability of a metformin-loaded rosemary-based NE system, where a zeta potential value around ± 30 mV can offer a high, packed energy barrier that limits droplet coalescence due to electrostatic repulsion. The prepared nanosystem showed a negative charge of value −19.9 ± 2.01 mV which is considered high enough to provide electrostatic stability for the prepared formulation (Figure 3b). The inclusion of surfactant and co-surfactant further improves the stability of the system by steric hindrance.[8] Even though metformin possesses a cationic charge, the observed negative charge of the system can be linked to the presence of the carboxylic acid group of rosemary oil which is the main component of the nanoemulsion oily phase.[35]

Entrapment efficiency (EE) of therapeutic cargoes is considered one of the crucial physicochemical characteristics assessed while developing a nanosystem. EE reflects the percentage of drugs that are successfully entrapped within the nanocarrier. High EE indicates high solubilization/encapsulation of the drug within the nanovehicle which in turn could guarantee a maximum efficacy of the loaded cargoes at lower doses compared to their free counterpart administration, limit the in-*vivo* adverse reactions associated with overuse of the drug and carrier building materials, and reduce the cost of scaling up and production.[36] The optimal formulation revealed a high entrapment efficiency of value 83.7 ± 3.77% despite the hydrophilicity of metformin. A similar result was reported where the nanoemulsion that was prepared to treat Type 2 diabetes displayed a high entrapment efficiency of metformin due to the ability of nanoemulsion to encapsulate both, hydrophilic and hydrophobic drugs.[37]

Fourier transform spectroscopy (FTIR) of rosemary oil, raw drug, metformin, and metformin-loaded rosemary-based nanoemulsion was performed to detect the chemical compatibility and any possible interaction between the drug and the

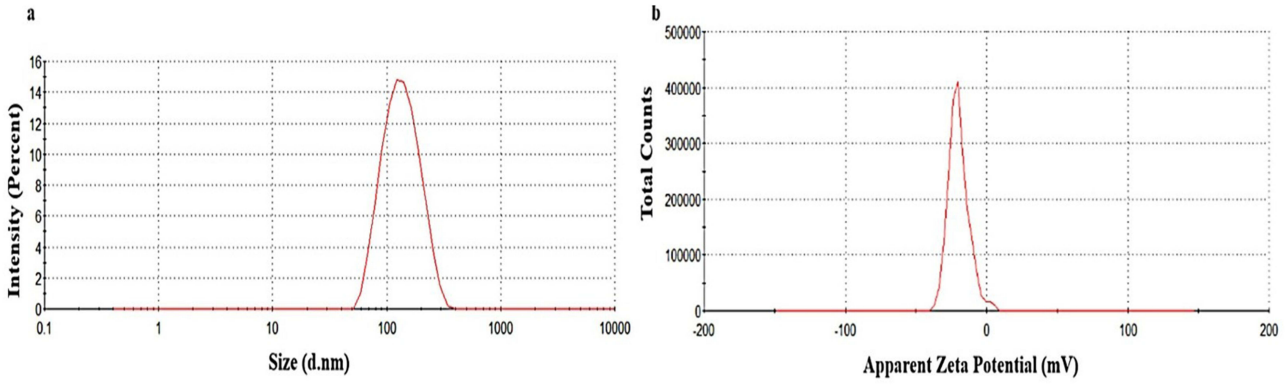


Figure 3 Particle droplet size (**a**) and zeta potential (**b**) of the optimized metformin-loaded rosemary-based nanoemulsion system. The measurements were carried out at ambient temperature and a scattering angle of 90 degrees.

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formulation components. As depicted in Figure 4, the metformin spectrum showed two peaks at 3292 cm^{-1} and 3173 cm^{-1} which are attributed to N-H symmetric stretching. Besides, the peaks at 1621 cm^{-1}, 1568 cm^{-1}, and 1417 cm^{-1} belonged to C=N stretching, N-H bending, and C-H asymmetric stretching respectively. Finally, the 1165 cm^{-1} peak reflects the C-N stretching metformin structure.[38,39] FTIR spectrum of the physical mixture and the prepared formulation revealed the retention of the characteristic functional groups of metformin indicating that the presence of other components particularly rosemary oil, Cremophor, and Labrafil, did not trigger any major shift in metformin principal peaks, revealing no chemical interaction between metformin and the used excipients in the fabricated nanoemulsion.

The molecular dispersion and solubilization of metformin in the lyophilized rosemary-based nanoemulsion was further verified using powder-XRD analysis. Typical diffraction patterns of metformin and lyophilized metformin-loaded rosemary-based nanoemulsion are depicted in Figure 5. The diffraction pattern of pure metformin revealed a crystalline structure that exhibited several diffraction reflections with four major sharp peaks at diffraction angles ($2\theta = 11°$, 25°, 27°, and 31°), indicating its crystalline state. These typical patterns were absent in the metformin-loaded rosemary-based NE indicating that the encapsulated drug is either molecularly dispersed or is in the amorphous state within the nanoemulsion which is characterized by its amorphous nature.[40] Similar results were observed in a previous study in which dispersing ketotifen fumarate in nanoemulsion decreased its crystallinity.[41]

Morphological and structural features of lyophilized metformin-loaded rosemary-based nanoemulsion were examined utilizing SEM, as shown in Figure 6. The micrograph displayed separate nano-droplets with smooth spherical outlines. Most nanoemulsion droplets had a diametric size in the range of 150 nm, verifying the data recorded by the Malvern Zetasizer. This could be linked to the adsorbed surfactant layer at the water/oil interface which provided the nanoemulsion stability.[42] Moreover, the addition of surfactants reduces interaction with the ice surface and provides steric stabilization during the freeze-drying process.[43] This result was coherent with Das et al study in which the scanning electron microscopy images of Nimodipine-loaded nanoemulsion displayed spherical droplets after lyophilization.[44]

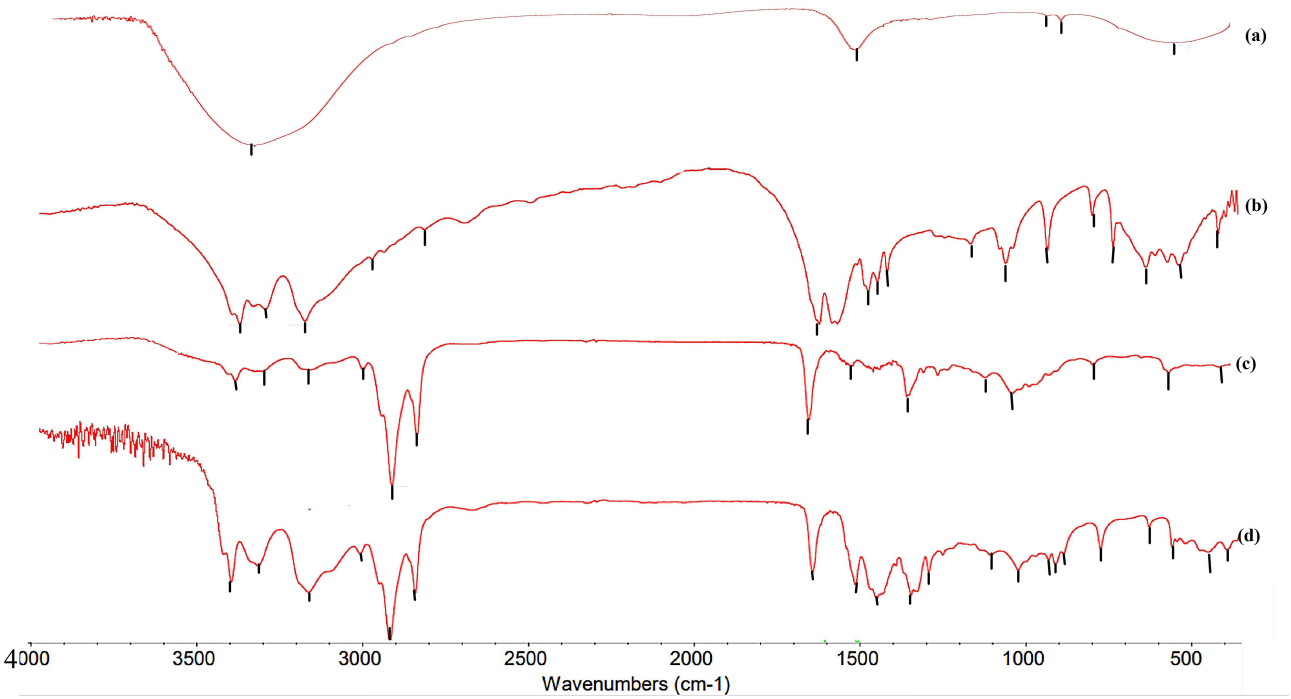

Figure 4 Fourier transforms spectroscopy of (**a**) rosemary oil, (**b**) raw metformin, (**c**) physical mixture, and (**d**) optimized metformin-loaded rosemary nanoemulsion.

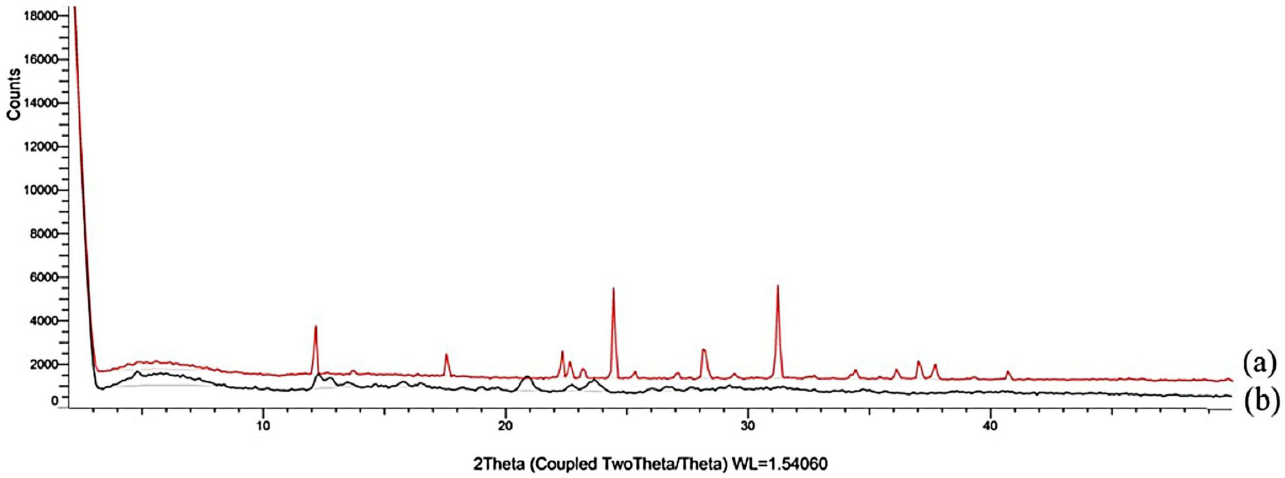

Figure 5 X-ray diffraction pattern of (**a**) raw metformin and (**b**) metformin-loaded rosemary-based nanoemulsion.

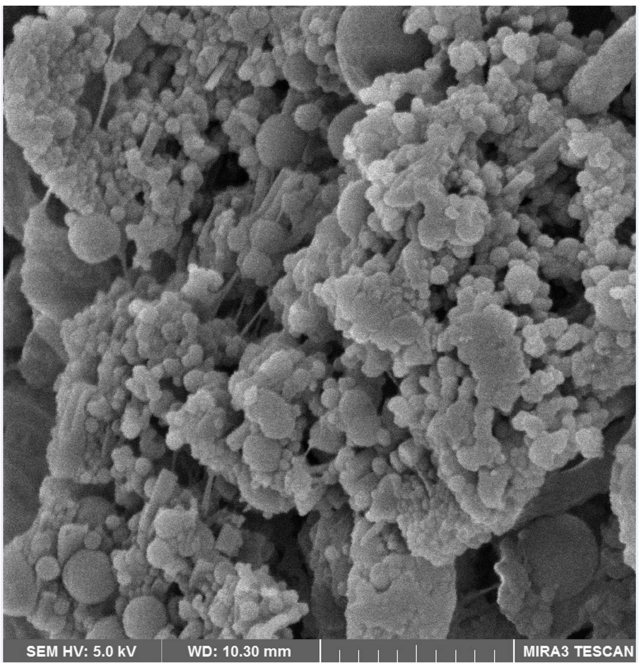

Figure 6 Scanning electron microscope image showing the morphological features of the lyophilized metformin-loaded rosemary-based nanoemulsion.

Preparation and Physicochemical Characterization of Metformin-Loaded Rosemary-Based Nanoemulgel

The application of nanoemulsions is inconvenient owing to their fluidity and low viscosity, thus, incorporation into hydrogel could overcome this problem and improve their permeation behavior. Hydrogels are three-dimensional polymeric networks that can absorb large amounts of biological fluids. Hydrogels are characterized by their biocompatibility, adhesiveness, consistency, and swelling behavior.[45] It has been reported that the viscosity of NE could be manipulated along with its skin permeation behavior by optimizing the thickening agent content in the gel matrix.[46] In the current study, Carbopol, an FDA-approved gelling agent, was selected owing to its safety profile, low cost, and ability to enhance NE permeation through the skin. It was stated that by increasing Carbopol concentration up to 3% w/v, the skin permeation rate of terbinafine decreased, the drug diffusion routes changed from skin appendages to mainly intercellular paths, and drug deposition in the epidermis/dermis increased.[47] Therefore, to ensure that the optimized formulation would be able to permeate into the

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underlying tissues without reaching systemic circulation, 1% w/v was selected as the proportion of Carbopol in the gel matrix.

The prepared metformin-loaded rosemary-based nanoemulgel displayed acceptable physicochemical characteristics. The efficacy of transdermal treatment relies greatly on the ability of the patient to spread the formulation as an even layer to distribute the required dose on a specific area. The optimal consistency of transdermal formulations ensures that the appropriate dose is applied and delivered. The prepared nanoemulgel followed pseudoplastic rheology with a shear-thinning attitude where viscosities decreased from 1980 ± 10.81 cP to 140 ± 12.83 cP with increasing the shear stress from 0.5 rpm to 100 rpm. Besides, the freshly prepared nanoemulgel displayed a pH value of 6.7±0.15. Despite the acidic nature of rosemary oil,[48] the inclusion of Cremophor EL and triethanolamine neutralized the acidity and increased its pH value, hence, it can be applied safely on the skin. Furthermore, the formulation exhibited a high drug content (92.9± 2.3%) with significant uniformity which lies within the accepted pharmacopeia range (90–110%) indicating that metformin is dispersed evenly within the semi-solid formulation.[8]

In-vitro Release Study

Product quality may be verified through in-vitro experimentation where an in-vitro release study is an accurate indicator. Dialysis technique is frequently used to determine the in-vitro drug release behavior of nanoparticulate drug delivery systems. The drug release profile generated from dialysis-based assays has been widely utilized to guide formulation development, predict the in-vivo performance of nanocarriers, and facilitate quality control and regulatory filing.[49] Despite that some reports have claimed that the dialysis technique lacks the reliability of the release technique since the drug is released first from the nanocarriers into the donor compartment solution and then permeates through the dialysis membrane to reach the dissolution medium in the receiver compartment, thus, the apparent drug release kinetics is computed by both the actual drug release kinetics and drug permeation kinetics.[50] Nevertheless, in practice, it is usually assumed that the membrane permeation process is rapid and is not rate-limiting so it can be neglected.[51] Hue et al, for example, revealed in their study that the dialysis bag technique was able to determine the real characteristics of a topical nano-formulation and could accurately indicate loperamide release from a topical formulation, particularly if it is conducted below the saturation point of the drug.[52]

In the current study, the in-vitro release behavior of metformin from rosemary-based nanoemulgel and the control gel was studied following the dialysis bag technique. As exhibited in Figure 7, the prepared nanoemulgel was able to control/ sustain the release of metformin over 24 hours whereas the drug-loaded gel released about 80% of the loaded metformin during the first four hours. Moreover, the mean dissolution time of metformin-loaded rosemary-based nanoemulgel was 3.45 ± 0.2h, which was significantly longer than that of the control gel (2.01 ± 0.08 h). The release efficiency at 6 hours

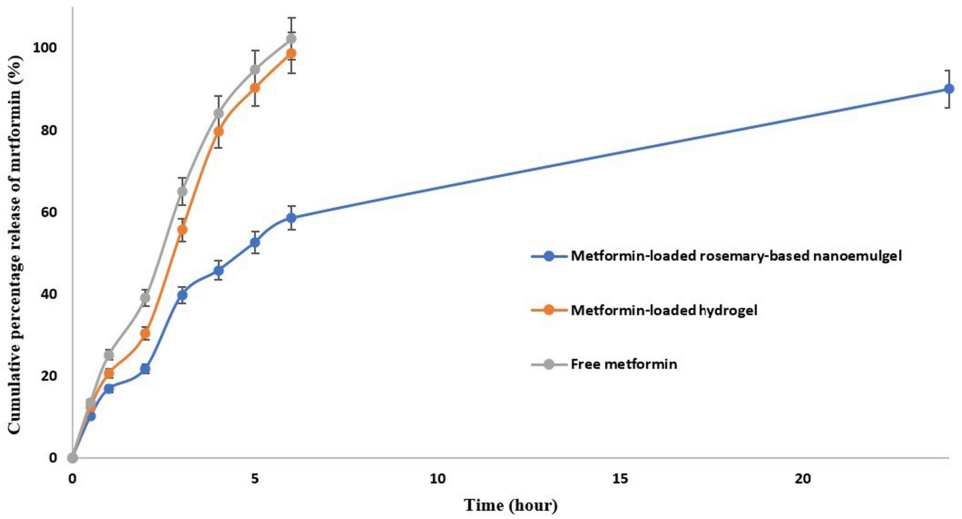

Figure 7 *The* in-vitro release pattern of free-metformin, metformin-loaded rosemary-based nanogel, and metformin-loaded Carbopol-based hydrogel over 24 hours into phosphate buffer solution (pH 7.4) at 34±0.5 °C using dialysis bag technique.

of metformin from the rosemary-loaded nanoemulgel was 60.3±2.8%, significantly lower than that of the control (96.6 ±4.3%). The extended-release of metformin from rosemary-based nanoemulgel may be attributed to the high solubilization and encapsulation efficiency of the drug within the nanoemulsion, whereby, to be diffused into the aqueous medium, metformin should diffuse through droplet–surfactant interfacial layer first, and then penetrate through the gel three-dimensional network structure, however, in the control gel, metformin should only diffuse through the gel matrix into the dissolution medium, hence its release was faster. A similar observation was recorded in Khurana et al study in which nanoemulsion-based gel displayed sustained release of meloxicam over 24 hours.[53]

Regarding the kinetics of the release process, the release of metformin from free solution andhydrogel followed zero-order revealing that the drug was released at a constant rate regardless of its initial concentration.[8] On the other hand, metformin release from rosemary-based nanoemulgel followed the first order with an R^2 value close to one, indicating that the metformin release mechanism is concentration-dependent. The amount of metformin release decreases with decreasing concentration gradient over time. This result was coherent with Yeo et al study in which the release of tocotrienols from naringenin-based nanoemulgel followed first order.[54] The "n" value determines the type of diffusion mechanism where n = 0.5 reveals the Fickian diffusion mechanism, whereas n > 0.5 means that the diffusion is anomalous or non-Fickian. The "n" value for the free solution and gels were in the range of 0.53–0.89, confirming non-Fickian or anomalous diffusion.[55]

In-vivo Histopathological Assessment and Dermatological Safety

To assess the effect of metformin-loaded rosemary-based nanoemulgel on skin and hair growth, a histopathological examination was performed at the end of the treatment (Figure 8) and 35 days after stopping the treatment (Figure 9). As depicted in Figure 8a, only a few hair follicles are sparsely distributed in the dermis and epidermis skin sections of the negative control group (rats that received no treatment) with a density of 3.12 new hair follicles/field. On the other hand, a higher number of hair follicles are observed in animals that were treated with metformin-loaded hydrogel (Figure 8b) and animals treated with rosemary-based nanoemulgel (Figure 8c) with hair densities of 2.87 and 3 new hair follicles/field, respectively. Animals that are treated topically with metformin-loaded rosemary-based nanoemulgel and minoxidil, respectively, displayed many hair follicles (> 3.5/field) that are distributed throughout the epidermis, dermis, and deeper layers, revealing the efficiency of the prepared formulation and minoxidil in improving hair growth compared to all other groups as illustrated in Figure 8d and e. In addition, the animals that were treated topically with the prepared metformin-loaded rosemary-based nanoemulsion showed preserved integral skin structure (stratum corneum, epidermis, and dermis) like the untreated animal group which reflects the safety profile of the developed nanoemulgel.

The two common transdermal pathways for drug skin penetration are the trans-epidermal penetration route via stratum corneum (SC) and through appendageal route in which molecules can penetrate the skin through hair follicles and glandular ducts (trans-appendageal). Through the trans-epidermal penetration route, cargo can pass directly around or across the corneocytes through the transcellular pathway. Due to SC nature, it provides a crucial barrier to large hydrophilic materials due to the corneocytes nature, which are anucleate cells filled with keratin and surrounded by a lipid envelope that hinders large molecules, with molecular weight higher than 500 Da, from passing. In the second pathway, molecules that are applied topically can penetrate via hair follicles to the underlying tissues beneath the epidermis.[29] The trans-appendageal route can provide a direct pathway to transport agents into the dermal microcirculation. Nevertheless, hair follicles and glandular ducts account for only approximately 0.05% of the total skin area which is filled with sebum. [56]

Metformin-loaded hydrogel was unable to penetrate through skin layer barriers due to its hydrophilic nature,[57] however, it is expected that a fraction of metformin was able to pass moderately through the appendageal route causing a moderate increase in hair follicles (Figure 8b) comparing the untreated group (Figure 8a). Metformin can augment the cellular proliferation of human dermal papilla and outer root sheath cells and increase AMP-activated protein kinase phosphorylation, which in turn suppresses β-catenin degradation and improves its nuclear accumulation, resulting in hair regeneration induction.[7] In the third group (Figure 8c) treated topically with rosemary-based nanoemulgel, there was also a moderate increase in the number of new hair follicles compared to the control group which is linked to the capability of nanoemulsion to penetrate effectively through skin layers due to numerous reasons. First, owing to the nanometric size and narrow size distribution of droplets, NE can diffuse efficiently through different pathways including the trans-appendageal and trans-epidermal routes in which nanoemulsion can be internalized by endocytosis via corneocytes[58]

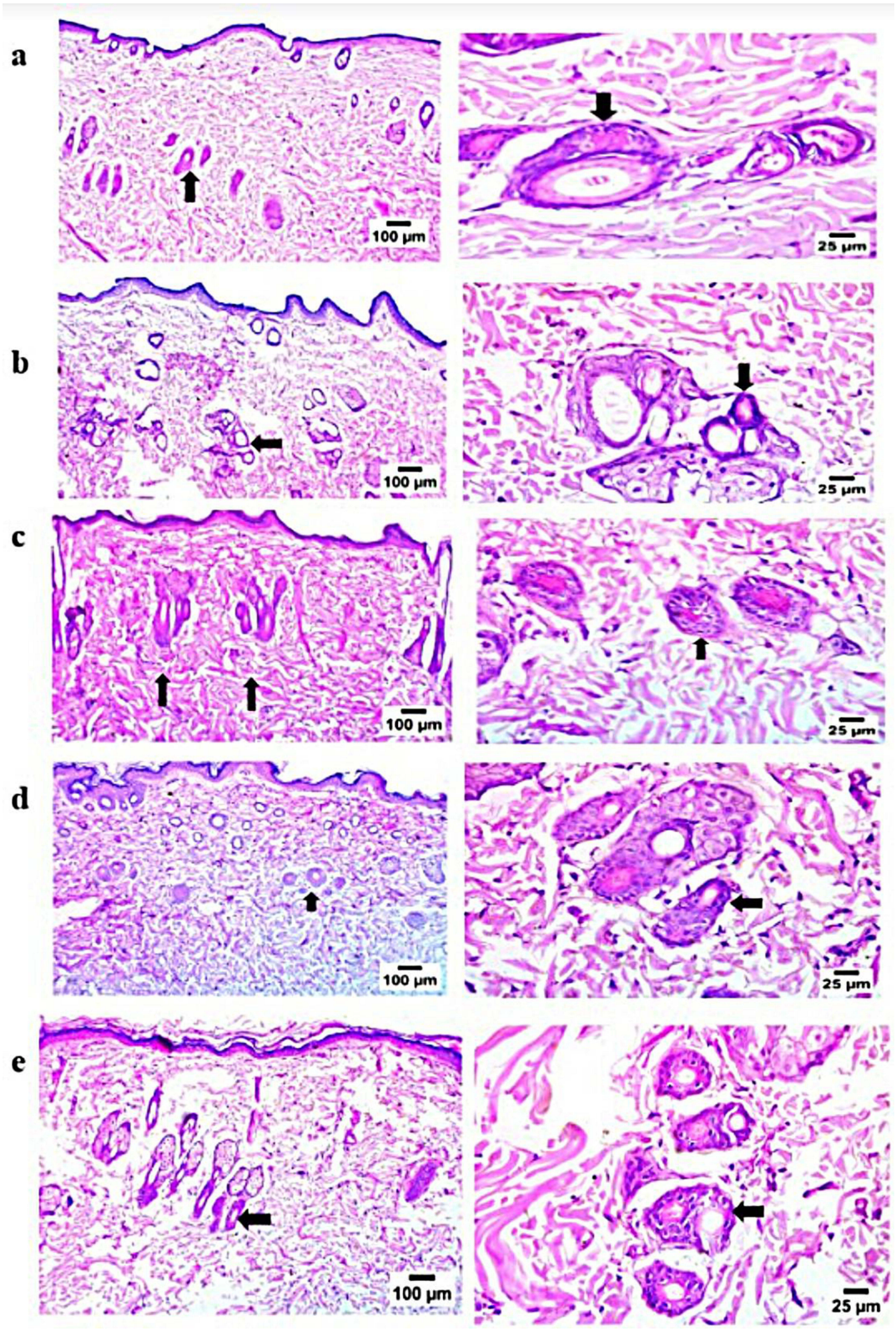

Figure 8 Photomicrographs of histopathological study of animals' skin that (**a**) received no topical treatment (negative control), (**b**) treated with topical metformin-loaded emulgel, (**c**) received unloaded-rosemary-based emulgel, (**d**) received metformin-loaded-rosemary-based nanoemulgel, and (**e**) and animals received minoxidil (positive control). Black arrows point out the new hair follicle.

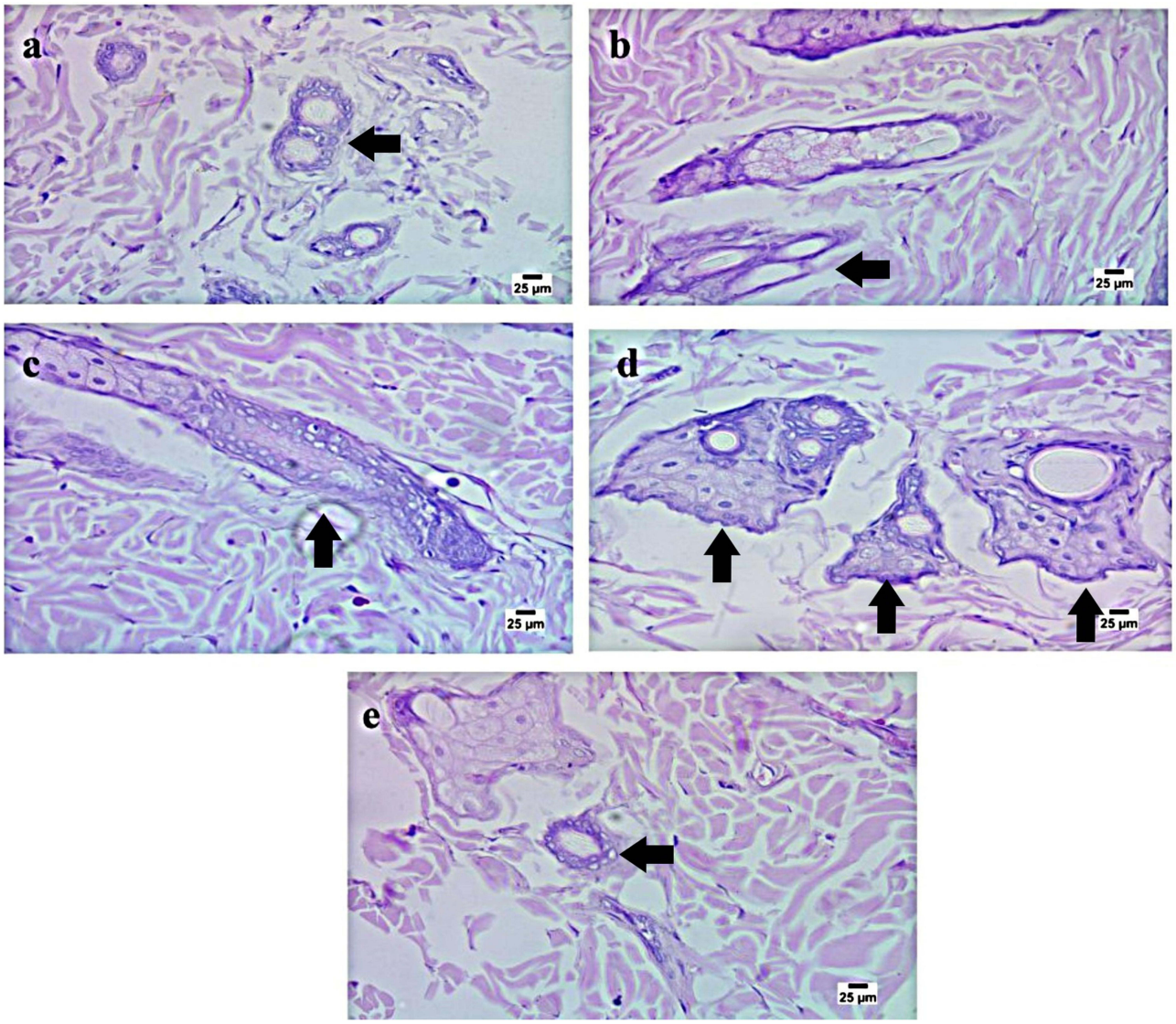

Figure 9 Photomicrographs of histopathological study of rat skin animals that (**a**) received no topical treatment (negative control), (**b**) treated with topical metformin-loaded emulgel, (**c**) received unloaded-rosemary-based emulgel, (**d**) received metformin-loaded-rosemary-based nanoemulgel, and (**e**) and animals received minoxidil (positive control). Black arrows display new hair follicle formation.

Besides, rosemary oil acts as a penetration enhancer. It was reported further that rosemary oil inhibits 5-alpha-reductase, improves vascularity, and increases blood flow, thus supplying hair follicles with the required nutrients needed for their proliferation.[59] Moreover, the non-ionic surfactant, Cremophor, is potentially able to partition into the intercellular domains of the SC lipidic matrix, hydrate SC, and increase its lipid fluidity, thus, diminishing its barrier function towards active compounds.[60] In the last group which was treated with metformin-loaded rosemary-based nanoemulgel (Figure 8d), there was an augmentation of hair growth which was evidenced by the number of new hair follicle formations compared with the other groups which could be explained, in addition to the previously favorable mentioned features of nanoemulsion, to the synergistic pharmacological effects on hair growth between rosemary oil and metformin. Despite that the prepared metformin-loaded rosemary-based nanoemulgel showed similar results to minoxidil (positive control) (Figure 8e), it cannot be excluded that the use of minoxidil has many disadvantages including change in hair texture, scalp irritation, temporary hair shedding, and facial hair growth in females.[61] Additionally, it is well known that after stopping minoxidil, there is a high risk of hair fall again because minoxidil only works while it is applied, and upon termination of use, the hair follicles will eventually return to their normal growth pattern, and this was depicted in

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Figure 9 in which there was a significant shrinkage in the hair follicle (Figure 9e) in contrast to other groups which maintain the size of hair follicles partially. Thus, it can be considered that the prepared formulation can be used as a safer and more effective alternative to the marketed minoxidil products.

Stability Study

The chemical and physical stability of the prepared metformin-loaded rosemary-based nanoemulgel were assessed at two different temperatures. The gels displayed insignificant differences in the evaluated parameters namely, appearance and viscosity compared with the fresh formulation. Additionally, there was an insignificant decrease in drug content from 92.9± 2.3% to 89 ± 1.6% after one month at both temperatures. Thus, it can be deduced that the prepared gel displays sufficient stability for four weeks.

Conclusion

A new generation of metformin-loaded rosemary-based nanoemulsion was prepared by spontaneous emulsification technique, optimized using the Box–Behnken Design, and displayed unique characteristics. The prepared nanoemulsion was successfully loaded into a polymeric gel which showed good physicochemical characteristics and was able to control the release of metformin over 24 hours. The histopathological examination of the fabricated metformin-loaded rosemary-based nanoemulgel evidenced its significant ability to accelerate hair follicle growth in rats compared with untreated animals which was attributed to the synergistic pharmacological effect of metformin and rosemary oil and the potential of nanoemulsion to penetrate deeper layers of skin. Therefore, metformin-loaded rosemary-based nanoemulgel can be a safe, convenient, and economical approach to boost hair follicle growth in patients suffering from androgenic alopecia.

Institutional Review Board Statement

All procedures described in the study were reviewed and approved by the Investigation Review Board (IRB) of Prince Sattam bin Abdulaziz University. The approval number is SCBR-183/2023.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest.

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