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DESIGN DEVELOP AND OPTIMIZE A NOVEL FUNCTIONAL EXCIPIENT FULVIC ACID APPENDED TOPICAL NANO EMULSION CONTAINING THYMOQUINONE FOR PROLONGED REMISSION OF PSORIASIS

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Abstract

The aim and objective of the proposed work, was to develop Fulvic acid and thymoquinone loaded nanoemulsion that could deliver the drug topically at the affected site of the diseased skin and to test the efficacy and safety of the formulated nanoemulsion through *in-vitro* and *in-vivo* study. Molecular docking studies showed the docking scores of thymoquinone and fulvic acid were -4.4 and -6.0 *kcal/mol* with interleukin-6 and the residues Leu11, Val118, Thr93, Ala175, Tyr183 form polar hydrogen bond with the ligands as well as hydrophobic bond interaction. 6.1, 6.2 and 6.3). After molecular docking study by Auto dock vina tools the results indicated that the fulvic acid have significant binding score (-6.0 and 6.4 *kcal/mol*) and high affinity with both receptors. Conclusively, the purported work explores a fulvic acid based thymoquinone nanoemulsion gel/ nanoemulgel using ultrasonic technique and "Quality by Design" (QbD) approaches. The concept of nanoemulgel was adopted to enhance solubility, absorption and bioavailability of a thymoquinone for topical as well as locoregional application. NE's size has resulted in combination therapy of TMQ and FA which utilised kalonji as the oil phase and Tween 80 as the surfactant, resulting as a perfect carrier for inclusion of poorly water-soluble medications (TMQ, FA), and enhanced penetration through the skin.

***Keywords*:** Quality by Design (QbD), Glycosaminoglycans (GAGS), Psoriasis, Fulvic Acid, Thymoquinone.

Introduction

The Skin: Structure and Function Skin

Skin is the body's exterior layer which acts as a protective shield for organs, and tissues from external environment. Skin's immune system gets altered when it is challenged by the bacteria, and fungus, etc., otherwise in undisturbed conditions it remains in an inactive state. At times, if there is a trigger in immune system, it leads to severe inflammation and hyper proliferation of skin (Aghmiuni and Khiavi 2017).

Biology of the skin

Skin is the largest and most important organ in the human body and comprises of 16% (between 2-6 kg) of body weight with surface area of approximately 1.8 m^2. It acts as a barrier for the deleterious external factors, provides shield against bacteria, other microorganisms, UV radiation, and mechanistic pressures to the tissues. However, skin is important because it is responsible for the various body functions which includes sense, touch, and body temperature regulation, provides vitamin D and waste product

elimination through sweating (Gola et al. 2015)(Lane et al. 2010).

Skin anatomy

Layers of Skin cells are continuously in a state of change and considered as a dynamic organ as it is shedding the outer most layers and replaced by the inner cells shifting to the upper surface. There are numerous appendages of skin which includes nails, hairs, sebaceous gland, apocrine glands etc (Lai-Cheong and McGrath, 2021; Monteiro-Riviere, 2010; Walters and Roberts, 2002). Skin thickness varies according to the location where it is situated such as sole of feet and palm of hands have comparatively thicker skin. Skin has three following essential layers as shown in Figure 1.1 which protect various organs of the body.

- •Epidermis
- •Dermis
- Subcutis (hypodermis)

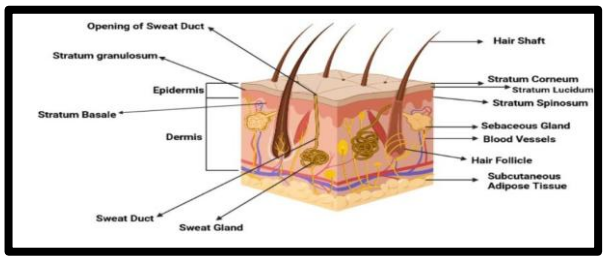
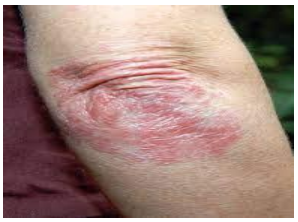
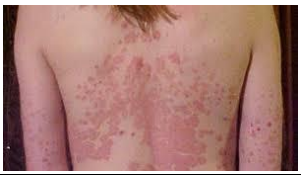
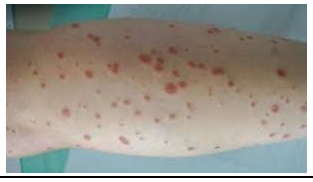


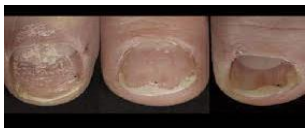
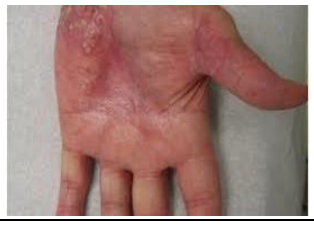
Figure 1: Diagrammatic presentation of skin layers

Epidermis and their layers

The outer most layer of skin is epidermis with approximately 0.1-1.4 mm thickness and its main cells which generate keratin proteins are keratinocytes. It also comprises other cells for instance Langerhans, Melanocyte and Merkel cells. There is a continuous process of proliferation and a lots of dead cells detaching from this layer. It is also anticipated that skin shred millions of cells at every 40 min which approximately around 18 kg of dead cell over a lifespan and this whole skin shedding process is termed as desquamation. This process takes 28-30 days (from stratum basale to stratum corneum) in the epidermis layer (Pak et al. 2003).

Classification of Psoriasis

Plaque psoriasis	Plaque psoriasis is a prevalent condition. It resembles regions of pink or red skin that are covered in silvery white scales (sometimes called plaques). The patches are slightly elevated above the skin's surface (Mason et al. 2013).	
Erythrodermic psoriasis	This is a severe and uncommon form of psoriasis. The majority or the entire surface of body's skin turns red and swollen. It requires immediate treatment (Rosenbach et al. 2010).	
Guttate psoriasis	This gives the appearance that the skin is covered with numerous tiny, scaly, red spots. These lesions may cover a significant portion of the skin (Telfer et al. 1992).	

Scalp, nail, facial and flexural psoriasis	Appears on particular body parts, and may be more difficult to treat. Flexural psoriasis affects the genitalia and manifests itself as skin folds, armpits, the area around the groin, beneath the breast, and between the buttocks (Merola et al. 2018).	 
Pustular psoriasis	This type of psoriasis can be extremely severe and causes the skin to develop numerous tiny blisters. It requires immediate medical care (Gooderham et al. 2019).	

Aim & Objectives

The aim and objective of the proposed work, was to develop Fulvic acid and thymoquinone loaded nanoemulsion that could deliver the drug topically at the affected site of the diseased skin and to test the efficacy and safety of the formulated nanoemulsion through in-vitro and in-vivo study.

The precise objective of the present research work was to design, develop and evaluate stable combined dual drug loaded nanoemulsion gel formulation for effective management of psoriasis. Further, specific objectives are as follows-

- To design, develop and optimize a novel functional excipient Fulvic acid, (FA) appended topical nanoformulation containing TMQ for prolonged remission of psoriasis.
- To assess the role of FA as a functional excipient in enhancing the pharmaceutical attributes of TMQ.
- To carry out the safety evaluation of the combination of FA and TMQ in a topical nanoformulation(s) using a suitable animal model.
- To investigate the efficacy of the prepared formulation and its comparison with a marketed product in a suitable animal model.
- To carry out the stability profiling of the optimized formulation as per the regulations/ICH guidelines.

Methodology

Physical characterization and identification of Thymoquinone (TMQ) and fulvic acid (FA)

The procured sample of Thymoquinone (TMQ) and Fulvic acid were characterized based on their physiochemical properties like odour, colour, solubility in aqueous and other solvents. DSC, FTIR, UV spectroscopy, and melting point determination were done and compared with the earlier reported literature.

Organoleptic Properties

TMQ and FA were characterized for its nature, colour and odour.

Melting point determination

Melting point apparatus was employed to determine melting point using the capillary rise technique. Sufficient drug was filled in the capillary at a height of 4-6 mm inside the column. Then capillary tube was placed along with the calibrated thermometer inside the melting point apparatus. The temperature was recorded at which drug melted.

Differential Scanning Calorimetry (DSC)

The DSC analysis of the samples pure drug (TMQ), FA, was performed using a Pyris 6 DSC, Perkin Elmer, (USA). 2 mg each sample was taken and separately subjected to a temperature range of 25–350 °C. The temperature was increased at a rate of 10 °C/min, and the nitrogen flow was maintained at 60 mL/min.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analysis of the pure drug (TMQ), FA, and lyophilized sample (using 5% w/v mannitol) of the optimized FTMQ-NE formulation was performed using FTIR spectroscopy.
First, a weighed quantity of the aforementioned samples (5 mg) was combined with potassium bromide (KBr) in a ratio of (1:1) (different pellets were used for each sample) and then compressed using a hydraulic press to form a flat pellet. The obtained pellets were then scanned between an infrared spectrum of light with a wavenumber of 4500–500 cm−1.

X-ray Diffraction (XRD)

The X-ray diffraction of the pure TMQ and FA was performed using an X-ray diffractometer (Philips X-ray generator Poznan, Holland) at a 35 kV electric potential, 30 mA voltage, and detector angle of 2θ (10–70 degree).
Analytical Methodology

Preparation of standard curve by Spectrophotometric method.

Calibration curve of TMQ in methanol.

10 mg of TMQ was dissolved in 10ml of methanol. 2ml was taken and the volume was made up to 100ml. From the stock of 20µg/ml different dilutions 1, 2, 3, 4, 6 to 8µg/ml were prepared with the same diluting medium and at 254 nm, the absorbance was measured. The plot was made between the mean absorbance of the samples verses concentration in µg/ml.

Calibration curve of TMQ in phosphate buffer pH 5.8

10 mg of TMQ and FA was dissolved in 10ml mixture of phosphate buffer 7.4. 1ml was taken and the volume was made up to 10ml. From the stock 100µg/ml different dilutions 1, 2, 3, 4, 6 to 18µg/ml were prepared with the same diluting medium and absorbance was recorded at 254 nm. The plot was made between the mean absorbance of these samples verses concentration in µg/ml.

RP-HPLC Analytical Method

For the analytical studies, a previously reported method was used. Different chromatographic conditions were followed for the TMQ as mentioned in the Table.5.4. The column was equilibrated with mobile phase for 30-40 minutes prior to analyte insertion. The injection volume was set at 20 L.

Results and Discussion

Preformulation study

In-silico Study

Molecular docking studies showed the docking scores of thymoquinone and fulvic acid were -4.4 and -6.0 *kcal/mol* with interleukin-6 and the residues Leu11, Val118, Thr93, Ala175, Tyr183 form polar hydrogen bond with the ligands as well as hydrophobic bond interaction (van der walls, alkyl, pi-alkyl, pi-pi T-shaped) with Pro54, Pro56, Pro209, Leu177 and Pro41 (Table 6.1) however, the docking score of both compounds with TNF were -4.8 and -6.4 *kcal/mol* and interacting residues Ala145, Phe144, Asp143, Phe144, Asp140 show the polar bonds and hydrophobic interactions with Phe144, Leu142 (interaction shown in Fig. 6.1,6.2 and 6.3). After molecular docking study by Auto dock vina tools the results indicated that the fulvic acid have significant binding score (-6.0 and 6.4 *kcal/mol*) and high affinity with both receptors.

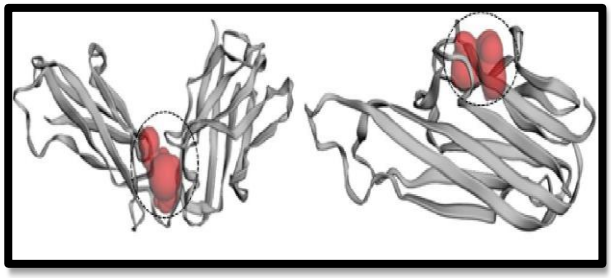


Figure 2: Pictorial presentation of binding pocket (Red circle with dotted line) of 4CNI and 6RMJ

Table: 1 Docking score, hydrogen and hydrophobic interaction of ligands with target receptors

Protein (PDB:ID)	Compound name (PubChem ID)	Docking score (*kcal/mol.*)	Hydrogen bonding interactions	Distance of hydrogen bond (Å)	Hydrophobic interactions
	Thymoquinone (10281)	-4.4	Leu11 N….O2	3.12	Pro154 (alkyl), Pro156 and Pro209 (pi-alkyl)
Interleukin- 6 (4CNI)	Fulvic acid (5359407)	-6.0	Val118 O…H24 Thr93 OG1…O23 Ala175 O…H26 Tyr183 OH….O2	2.93 2.91 2.12 3.08	Pro41and Leu177 (pi-alkyl)
Tumor necrosis factor (6RMJ)	Thymoquinone (10281)	-4.8	Ala145 NH…O2 Phe144 NH…O2	2.67 2.22	Phe144 (alkyl & pi-pi T-shaped), Leu142 (pi- alkyl)
	Fulvic acid (5359407)	-6.4	Asp143 OD1…H20 Asp143 HA…O19 Phe144 NH…O19 Asp140 HA….O25	2.41 2.84 2.14 3.05	Phe144 (pi-pi T-shaped)

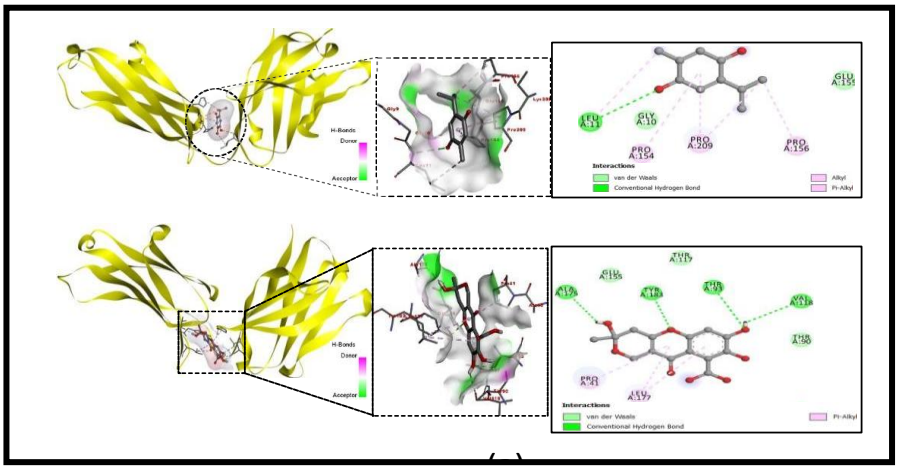


Figure 3: 3D interaction of Interleukin-6 (4CNI) with thymoquinone (a) and with fulvic acid (b). Physical characterization and identification of Thymoquinone (TMQ) and Fulvic acid (FA)

Organoleptic properties

TMQ and FA were assessed for organoleptic properties and the observations are shown in table.

Table 2: Organoleptic properties of TMQ and FA

S. No.	Parameters	TMQ	FA
1	Nature	Crystalline	Amorphous powder
2	Colour	Bright yellow	A white to off white
3	Odour	Odourless	odourless
4	Taste	Pungent	Tasteless

Melting point determination

By using capillary rise method, melting point was determined and it was found to be 48.281 °C and 223.417°C for TMQ and FA respectively.

Differential Scanning Calorimeter (DSC)

The DSC thermogram of TMQ and FA are shown in Figure. A sharp endothermic peak at 48.281 and 223.417°C was observed from DSC thermogram of TMQ and FA respectively. These were found to be in agreement with the value mentioned in the literature. Hence, on the basis of this, it can be said that the drug sample was authentic and pure.

Thermodynamic stability

Thermal stability studies, such as heating–cooling, centrifugation, and the freeze– thaw cycle, revealed that certain NE formulations were turbid, and some had shown phase separation. Such instability might occur due to Ostwald ripening, a free-surface energy process in which small droplets combine via diffusion to form aggregates or larger droplets (Elmowafy et al. 2016). The formulations which did not show any thermodynamic instability were subjected to further physical characterization, as highlighted in Table. The collated results of both the thermodynamic and physical characterizations revealed that formulation F-A2 was the most suitable and was considered to be optimized, FTQ-NE.

Table 3: Thermodynamic stability testing of selected nanoemulsion formulations using heating cooling (HC), freeze-thaw (FT), and Centrifugation (Cent).

Six ratios	Formulation coding	Percentage of component % (v/v)			Observation			Inference
		Oil	Smix	Water	Heating cooling	Freeze-thaw	Centrifu-gation	
Formulation P Smix = ratio 2:1	P-1	2.22	17.78	80	✓	✓	✓	Passed
	P-2	2	14	85	✓	✓	✓	Passed
	P-3	2.5	17.5	80	×	×	×	Failed
	P-4	7.84	47.06	45.1	✓	×	×	Failed
Formulation Q Smix = ratio 3:1	Q-1	1.67	13.33	85	×	✓	✓	Failed
	Q-2	1.87	13.12	85	×	×	×	Failed
	Q-3	2	14	85	✓	✓	✓	Passed
	Q-4	2.86	17.14	80	✓	×	×	Failed
Formulation R Smix = ratio 4:1	R-1	2.78	22.22	75	✓	✓	✓	Passed
	R-2	3.13	21.88	75	✓	✓	✓	Passed
	R-3	2.5	12.5	85	✓	×	×	Failed
	R-4	2	18	80	×	×	×	Failed

Table 4: Physical characterization studies of various nanoemulsion formulations.

Formulation coding	Particle size (nm) (Mean± SD) (N=3)		PDI (Mean± SD) (N=3)		pH (Mean± SD) (N=3)		RI (Mean± SD) (N=3		Viscosity (mp) (Mean± SD) (N=3)	
	Blank	TMQ-NE	Blank	TMQ-NE	Blank	TMQ-NE	Blank	TMQ-NE	Blank	TMQ-NE
P-1	150.6 ± 1.34	224.7 ± 1.30	0.24 ± 0.05	0.28 ± 0.03	5.9 ± 0.34	6.1 ± 0.44	1.401 ± 0.006	1.402 ± 0.008	43.31 ± 3.24	43.78 ± 2.42
P-2	41.13 ± 1.45	72.34 ± 1.43	0.16 ± 0.02	0.13 ± 0.03	5.8 ± 0.25	5.8 ± 0.32	1.405 ± 0.003	1.405 ± 0.007	134.0± 6.42	138.5 ± 3.08
Q-3	100.13 ± 1.74	187.6 ± 1.23	0.18 ± 0.04	0.23 ± 0.02	5.6 ± 0.13	5.7 ± 0.25	1.402 ± 0.002	1.368 ± 0.004	104.0 ± 3.40	106.0 ± 3.49

R-1	106.3 ± 2.33	159.3 ± 1.57	0.38 ± 0.03	0.33 ± 0.04	6.4 ± 0.65	6.6 ± 0.27	1.405 ± 0.005	1.402 ± 0.004	52.60 ± 2.70	53.51 ± 2.02
R-2	112.14 ± 2.74	171.2 ± 2.13	0.15 ± 0.06.	0.18 ± 0.02	6.3 ± 0.43	6.7 ± 0.14	1.403 ± 0.006	1.407 ± 0.004	157.2 ± 6.72	159.6 ± 5.52

Characterization of an optimized TMQ-NE

Dilution test

In the dilution test, the addition of oil leads to either cracking or phase separation. Thus, the optimized formulation, FTQ-NE (P-2), is of an o/w type (Figure). Following this, the filter paper and cobalt chloride test was performed, which also revealed a similar NE type (o/w), depicting rapid spreading (Figure) and change from blue to pink color on the filter paper (Figure), respectively. Furthermore, the %transmittance of the optimized FTQ-NE was found to be 98.99% ± 0.42, confirming its transparency as well as nano-globule size. Furthermore, the obtained refractive index (RI) of 1.415 ± 0.036 of the optimized FTQ-NE also highlighted a less dense or clear isotropic formulation (Hayat et al. 2011). Lastly, the viscosity and pH of the optimized FTQ-NE were 138.5 ± 3.08 mp and 5.8 ± 0.16 respectively.

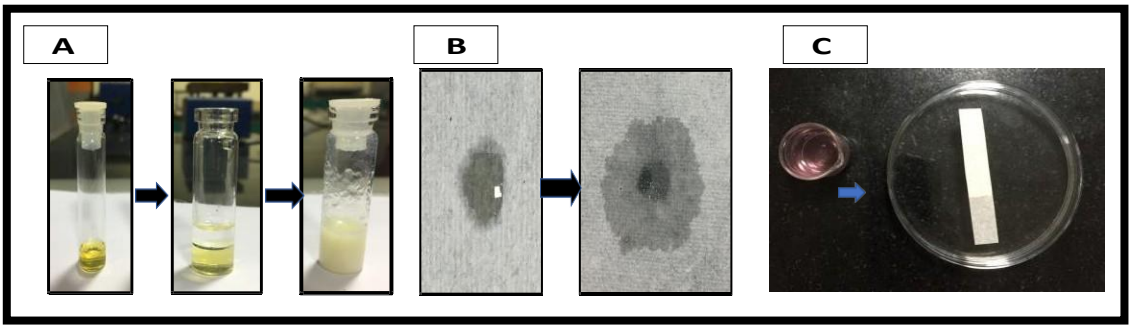


Figure 4: (A) Dilution test, (B) Filter paper test, and (C) Cobalt Chloride test.

SEM, TEM, & Fluorescent microscopy

The three different techniques were employed to analyze the structure and surface morphology of the final formulation. They collectively revealed a circular, globule- like structure with a size range below 100 nm (Figure). Furthermore, the obtained size range corroborated well with the observations of the zeta sizer. Thus, the optimized formulation is acceptable in terms of its shape and size, i.e., nanometric and globular size, respectively, for an efficient topical delivery permeation and retention.

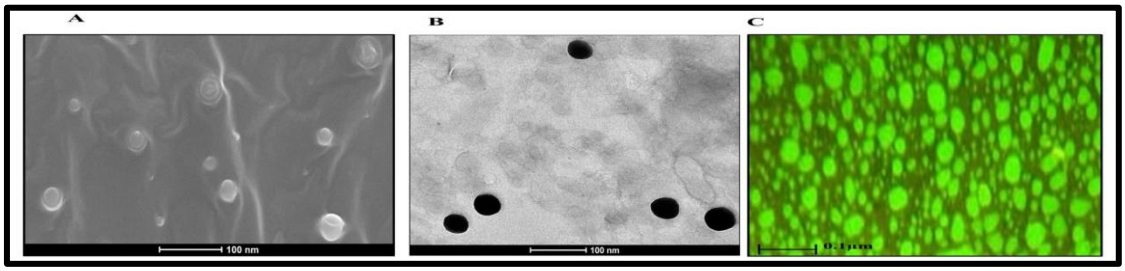


Figure: 5 (A) SEM, (B) TEM & (C) Fluorescent microscopy of optimized FTQ- NE.

Spreadability and Extrudability

Various placebo gel preparations with different concentrations of Carbopol 971® were evaluated, and the concentration of 1% showed acceptable results. The optimized FTQ-NEG did not have a coarse texture and was smooth and homogenous. The pH of the optimized gel was 5.8 ± 0.32, which could be inferred as safe and non-irritating to the skin. The spreadability and extrudability mainly contribute to an easy application at the desired site and expulsion from the packaging, respectively. Thus, they are critical for a high patient compliance. The optimized FTQ-NEG has a spreadability of 15.38 ± 1.65 g.cm/s and extrudability of 5.68 ± 1.03 g (Agarwal et al. 2008).

Conclusion

Conclusively, the purported work explores a fulvic acid (peat sourced) based thymoquinone nanoemulsion gel/ nanoemulgel using ultrasonic technique and "Quality by Design" (QbD) approaches. The concept of nanoemulgel was adopted to enhance solubility, absorption and bioavailability of a thymoquinone for topical as well as locoregional application. NE's size has resulted in

combination therapy of TMQ and FA which utilised kalonji as the oil phase and Tween 80 as the surfactant, resulting as a perfect carrier for inclusion of poorly water-soluble medications (TMQ, FA), and enhanced penetration through the skin. Our findings also showed that in the optimised NE formulation, No phase separation was found in the formulation after physical instability study. The result of TEM and, SEM suggested that the optimized formulation showed uniform shape and size of globules. The optimized formulation developed in to the transparent and uniform gel using Carbopol 971, for better holding on the skin surface. Particularly in the form of topical gel was tested for its texture and resulted in a uniform and transparent hydrogel, which is suitable for topical delivery. It showed better antipsoriatic effects than other groups due to the mucoadhesive nature, resulting in increased drug retention in the skin, which is required for psoriatic skin therapy. FTQNE gel was found to possess good texture profile and was homogenous in nature. Furthermore, skin irritation experiments have indicated that TQFNEG is safe for topical treatments with no potential for irritation, indicating the potential of long-term therapy. The findings are encouraging, proving that TQFNEG is safe and efficacious and demonstrating a viable method for developing topical psoriasis treatments. In an IMQ-induced psoriatic plaque model, topical administration of the TQFNEG decreased psoriatic symptoms. The findings of PASI scoring, histological investigation, and ELISA were compared to reveal that TQFNEG can cure psoriasis. To summarise, TQFNEG was shown to be more successful in treating psoriasis than commercially available treatments. The new formulation increased the local concentration of the two medications, lowering their systemic adverse effects and allowing for more penetration into the epidermal layers, which was previously impossible with standard formulations. In the current study, the combined treatment method promotes psoriasis recovery. When compared to the free drug in gel, the current formulation causes less skin irritation. In addition, as compared to the other trial groups, the local topical concentration improved. The developed system would provide a selective as well as acceptable safety profile. Long-term therapy is achievable with good patient compliance due to the formulation's lower irritation potential. This encourages the formulation's future applicability and commercialization. NE‘s nano size has resulted in combination therapy of TMQ and FA which utilised kalonji as the oil phase and Tween 80 as the surfactant, resulting as a perfect carrier for inclusion of poorly water-soluble medications (TMQ&FA), and enhanced penetration through the skin. Our findings also showed that the optimised NE formulation, particularly in the form of topical gel was tested for its texture and resulted in a uniform and transparent hydrogel, which is suitable in case of topical delivery. Because of its mucoadhesive nature, it had a better Antipsoriatic impact than other groups, resulting in increased drug retention in the skin that is predicted in the treatment of psoriatic skin. Furthermore, test conducted for skin irritation have directed towards the safety profile of FTQ-NEG via topical route and has no irritant potential, indicating the potential of long-term therapy. The findings are encouraging, proving that FTQ-NEG is safe and efficacious and demonstrating a viable method for developing topical psoriasis treatments. Topical application of the FTQ-NEG showed improvement in the symptoms of psoriasis in the IMQ-induced psoriatic plaque model. The findings of PASI scoring, histological investigation, and ELISA were compared to reveal that FTQ-NEG can cure psoriasis. To summarise, FTQ-NEG was shown to be more successful in treating psoriasis than commercially available treatments. The new formulation increased the site concentration of both the medicaments, lowering the systemic adverse effects and allowing for more penetration into the epidermal layers, which was previously impossible with standard formulations. In the current study, the combined treatment method promotes psoriasis recovery. When compared to the gel containing free drug, the current method causes less skin irritation. In addition, as compared to the other trial groups, the local topical concentration improved. This method would provide a customised as well as acceptable safety profile and the purported optimized formulation would be an acceptable drug delivery approach for a difficult to treat skin affliction like psoriasis.

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Conflict of Interest
No Conflict of interest

Informed Consent and Ethical Statement
Not Applicable.

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