https://doi.org/10.31925/farmacia.2023.6.22 **ORIGINAL ARTICLE**

DEVELOPMENT OF HERBAL BIOACTIVE LOADED NANOPARTICLES FOR TOPICAL APPLICATION IN VITILIGO

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Abstract

Vitiligo is characterised by the appearance of white lesions on the skin, which are mainly caused by depigmentation of the skin due to the destruction of selective melanocytes. *Ammi visnaga* L. is a plant that grows widely in Mediterranean climate regions and khellin is one of its major components. Khellin stimulates the migration and proliferation of melanocytes in hair follicles. Both oral and dermal use of khellin is available, but some systemic side effects such as nausea and hepatotoxicity can be accompanied by oral administration. In the case of dermal application, poor skin penetration of khellin related to its lipophilicity needs to be overcome. In this study, khellin loaded cellulose acetate phthalate nanoparticles were developed to be used in the topical treatment of vitiligo by using Taguchi Orthogonal Array Design in the preliminary formulation studies. The characterization studies were carried out by determination of the size, zeta potential and encapsulation efficiency of nanoparticles and *in vitro* drug release and *ex vivo* penetration studies were carried out to evaluate their performance. The optimum nanoparticle formulation was found as an average size of 217.8 nm, PDI value of 0.11, zeta potential of -18.4 mV and encapsulation efficiency of 34.2%. Khellin loaded nanoparticles released 75% of khellin during 24 hours in a controlled manner. In addition, incorporation of khellin nanoparticles into HPMC gel increased the amount of khellin accumulated in the deep layers of the skin.

Rezumat

Vitiligo este o afecțiune comună a pielii care provoacă depigmentare din cauza distrugerii melanocitelor. *Ammi visnaga L.* este o plantă care crește pe scară largă în regiunile cu climă mediteraneană, iar khellinul este una dintre componentele sale majore. Khellin stimulează migrarea și proliferarea melanocitelor în foliculii piloși. Este disponibilă atât utilizarea orală, cât și cutanată a khellinei, dar unele efecte secundare sistemice, cum ar fi greața și hepatotoxicitatea, pot apărea după administrare orală. În cazul aplicării cutanate, trebuie îmbunătățită penetrarea khellinei prin piele în corelație cu lipofilia sa. În acest studiu, au fost dezvoltate nanoparticule cu khellin pentru utilizare topică în vitiligo. Studiile de caracterizare au fost efectuate prin determinarea mărimii, potențialului zeta și eficienței de încapsulare a nanoparticulelor. Au fost efectuate studii *in vitro* de cedare a khellinei și de penetrare *ex vivo*. Formularea optimă a nanoparticulelor a generat o dimensiune medie de 217,8 nm, o valoare de PDI de 0,11, potențial zeta de -18,4 mV și eficiență de încapsulare de 34,2%. Nanoparticulele încărcate cu khellin au eliberat 75% din substanță timp de 24 de ore. În plus, încorporarea nanoparticulelor de khellin în gelul HPMC a crescut cantitatea de substanță acumulată în straturile profunde ale pielii.

Keywords: *Ammi visnaga L.*, khellin, nanoparticle, topical, vitiligo

Introduction

Ammi visnaga L. is a plant that grows widely in Mediterranean climate regions such as North Africa, East Asia and Europe [18]. The plant is used in the treatment of kidney diseases, cardiovascular diseases and asthma, and also has antidiabetic, anti-inflammatory, antispasmodic and antimicrobial pharmacological effects. *Ammi visnaga* L. contains γ-pyrones, coumarins, flavonoids as well as essential oils. Khellin, which has a γ-pyron structure, is one of the major components of the plant. It has been proven by studies that khellin, the major substance of the plant, is very effective in the treatment of vitiligo [15, 18].

Vitiligo is a common skin disorder that causes loss of pigment in the skin and is classified as an autoimmune disease, which can also lead to serious psychological problems [13]. The estimated prevalence of vitiligo in the worldwide population is 0.5 - 2% [4]. It is characterized by the appearance of white lesions on the skin and is sometimes accompanied by a whitening of the hair [4, 11]. Although the lesions can be seen anywhere on the body, they are usually seen on the hands, face and genitals. Furthermore, friction may trigger vitiligo in areas such as the neck, elbows and ankles [11]. Current vitiligo treatment options include topical and oral corticosteroids, calcineurin inhibitors, topical vitamin D3 analogue, psoralens, statins, anti-oxidants, intravenous immunoglobulin, methotrexate,

immunosuppressive agents, biological drugs such as rituximab, physical therapies such as phototherapy and laser therapy and surgical treatments. However, the treatment of vitiligo is challenging due to the safety profiles and contraindications of some treatment strategies, for example, acute side effects of psoralens are mostly erythema, pruritus, nausea and headache, and in chronic administration phototoxicity, xerosis, hypertrichosis, hyperpigmentation, cutaneous malignancies may occur. Additionally, psoralens are contraindicated in children with xeroderma pigmentosum, lupus erythematosus, bullous pemphigoid diseases, family history of melanoma, and children under 12 years of age [8, 24, 30]. Khellin is considered to be a useful alternative in the treatment of vitiligo, since it is similar in structure to psoralen, but has less phototoxicity and less effect on DNA mutation [10]. In the 1980s, effect of khellin in the oral treatment of vitiligo was demonstrated and successful results were obtained [1, 34]. However, due to the side effects of oral khellin application such as nausea and hepatotoxicity, classical topical formulations were studied in the 1990s, but successful results were not obtained [33, 40]. For this reason, various studies have been conducted to develop topical applications of khellin using different drug carrier systems [12, 22, 23, 38, 42, 46].

The main source of melanocytes in the epidermis is formed by the differentiation of melanocytes in the bulge region of hair follicles and their migration to the epidermis [31]. Khellin not only stimulates melanogenesis, but also ensures the migration and proliferation of melanocytes in hair follicles. Therefore, studies have shown that khellin is very effective in the treatment of vitiligo by repairing the loss of melanocytes and melanin [5, 45]. Accordingly, to achieve the desired effect, khellin should be targeted to the bulge area of the hair follicle [31, 38]. Nanoparticles are stable, targetable and modifiable nanocarrier systems. Due to their ability to improve solubility, nanoparticles are ideal carrier systems for both hydrophobic and hydrophilic drugs [35]. Nanoparticles can reach deep areas of the hair follicle and accumulate there [17, 41]. According to a study, the residence time of nanoparticles in the hair follicle is 10 times longer than the residence time in the *stratum corneum* [21]. Nanoparticles with a size of 200 - 600 nm are more likely to reach the bulge region than particles with smaller or bigger sizes [37, 47]. In addition, it is known that pH levels increase from the surface to deeper layers of the hair follicles [17]. This suggests that suitable-sized nanoparticles composed of cellulose acetate phthalate (CAP), a polymer that releases substances in high pH environments, could be a proper drug delivery system for the treatment of vitiligo with khellin.

In the preliminary studies, an experiment design called Taguchi Orthogonal Array Design can be used. Experiment designs are structured and organized approaches used to figure out the relationship between parameters of a process and responses [39]. Using Taguchi Orthogonal Array Design, parameters with complex interrelationship could be analysed at the same time and each parameter could be assessed independently of all the other parameters with only few experimental runs [28]. This reduces the number of trials and significantly affects the total cost of the study. Many previous studies have used this experimental design in the optimization studies of various pharmaceutical formulations including micro and nanoparticles [26, 28, 44].

In this study, khellin loaded nanoparticles were developed to be used in the topical treatment of vitiligo. Taguchi Orthogonal Array Design was used in the preliminary studies. The characterization studies were carried out by determination of the size, zeta potential and encapsulation efficiency of the nanoparticles and *in vitro* drug release and *ex vivo* skin penetration studies were performed to evaluate their properties.

Materials and Methods

Materials

Khellin, cellulose acetate phthalate, Tween 80 and hydroxypropyl methylcellulose were purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany). Acetone, ethanol and methanol were supplied from ISOLAB (Istanbul, Türkiye). All other reagents used were analytical grade.

Methods

Nanoparticle preparation

Nanoparticles were prepared by using nanoprecipitation method. The preformulation studies parameters are given in Table I.

Optimization of the formulation and experimental design

The basic nanoprecipitation parameters such as the type of organic solvent, the type of surfactant and the concentration of the polymer were optimized in the preliminary studies. Thereafter the remaining parameters were adjusted by using Taguchi Array L16 (4^4) Experimental Design Method. The experiment design was constructed with 16 experiments using four factors: concentration of excipient (0.5%, 1%, 2% or 3% Tween 80), the ratio of organic phase to the aqueous phase (organic:aqueous - 3:10, 5:10, 8:10, or 10:10), stirring speed (500, 700, or 1000 rpm) and pumping rate of the organic phase (3, 5, 7, or 9 mL/h). With the advantage of this design, the optimum parameters were selected according to the particle size, PDI values and zeta potential results.

Preparation of optimum formulation

3 mL of acetone containing 0.26 mg of khellin and 1.25% (w/v) CAP polymer was used as the organic phase. The aqueous phase was prepared with 10 mL of distilled water adjusted to pH 4 and containing 3% (w/v) Tween 80. The organic phase was added drop-

wise to the aqueous phase at a rate of 9 mL/h while the aqueous phase was stirred at room temperature at 250 rpm using a magnetic stirrer. Then, acetone was removed using the fume hood (Figure 1).

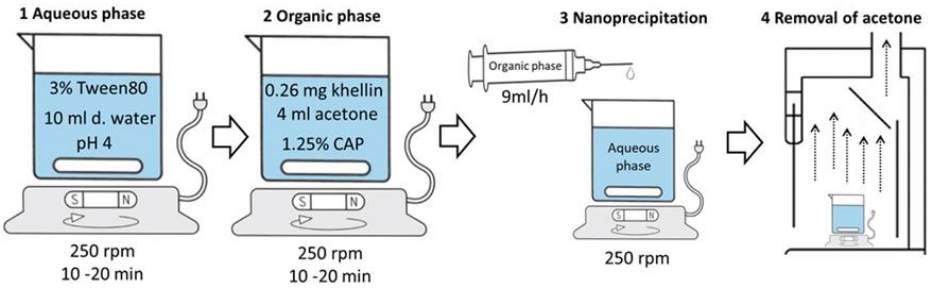

Figure 1.
Preparation steps of nanoparticles

Table I
Parameter changes in the preformulation studies of nanoparticles

Preformulation parameters	Changes applied to parameters
Polymer (CAP) concentration (w/v)	0.25% - 0.5% - 0.75% - 1% - 1.5% - 2%
Organic solvents	50% acetone:50% ethanol - 100% acetone
Surfactants	PVA - Tween 80 - Kolliphor EL
Concentration of excipient (w/v)	0.5% - 1% - 2% - 3% Tween 80
The ratio of organic phase to aqueous phase (organic phase:aqueous phase)	3:10 - 5:10 - 8:10 - 10:10
Stirring speed (rpm)	250 - 500 - 750 - 1000
The pumping rate of the organic phase (mL/h)	3 - 5 - 7 - 9

Nanoparticle Characterization

Size, polydispersity index and Zeta-potential determination

The size of the nanoparticles was analysed using the dynamic light scattering method (Malvern Zetasizer, Malvern, UK). The polydispersity index (PDI) was recorded to determine the homogeneity of the nanoparticle size distribution. Electrophoretic light scattering method (Malvern Zetasizer, Malvern, UK) was used to determine the zeta potential values of the nanoparticles.

Determination of the encapsulation efficiency

To determine the amount of active substance loaded in the nanoparticles, the formulations were centrifuged at 15000 rpm for 6 minutes at 10°C. Then, the supernatant was collected to determine the amount of the active substance that is not loaded into nanoparticles. The total amount of khellin in the nanoparticles was determined by dissolving nanoparticle colloidal dispersion before centrifugation with methanol. After the addition of methanol, the resulting mixture was kept in an ultrasonic water bath for 1 minute and vortexed. All collected samples were then diluted with HPLC mobile phase and filtered using a 0.45 µm polytetrafluoroethylene polymer (PTFE) membrane filter to be analysed by HPLC. The following formula was used to calculate the encapsulation efficiency [43]:

$$Encapsulation\ efficiency = \frac{Total\ amount\ of\ active\ substance - Amount\ of\ unloaded\ active\ substance}{Total\ amount\ of\ active\ substance}\ x\ 100\%.$$

Preparation of control gel and gel-dispersed nanoparticle formulation

In the preparation of the control gel formulation, 1.5% (w/v) hydroxypropyl methylcellulose (HPMC) was chosen as the polymer. The polymer material was mixed with distilled water with a magnetic stirrer until a homogeneous gel base was obtained. The same amount of khellin found in the final formulation was dissolved in propylene glycol and mixed with the pre-prepared gel. The gel-dispersed nanoparticle formulation was prepared by dispersing the nanoparticles in a 1.5% (w/v) HPMC gel.

In vitro drug release

Franz diffusion cells (Permegear V6A Stirrer, Hellertown, USA) were used to evaluate *in vitro* khellin release from the formulations. Nitrocellulose membranes were used in the study. The prepared formulations were placed in the donor chamber of the Franz diffusion cells with a receptor volume of 12 mL and a diffusion area of 1.77 cm^2. pH 7.4 PBS solution was used as the receptor phase to simulate the physiological conditions of the hair follicle [9]. One g of each nanoparticle formulation, gel-dispersed nanoparticle formulation and control gel were loaded in the donor chambers of the Franz diffusion cells. The receptor phase was stirred continuously at 250 rpm with a magnetic stirrer at 32°C. 1 mL samples were withdrawn at predefined time intervals (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} and 24^{th} hours) and replaced by fresh medium. All collected samples were filtered using a 0.45 µm PTFE membrane filter to be analysed by HPLC.

Ex vivo penetration

Franz diffusion cells (Permegear V6A Stirrer, Hellertown, USA) were used to evaluate the *ex vivo* skin penetration

property of nanoparticles. The diffusion cells were assembled with dorsal pig skin separating the donor compartment from the receptor compartment. The receptor compartment was filled with 12 mL of phosphate buffer, pH 7.4. 1 mL of nanoparticle formulation or 1 g of gel-dispersed nanoparticle formulation was added to the donor compartment, which was in direct contact with the receptor solution. The experiments were conducted for 24 h. At the end of the experiment, the skin was removed from the diffusion cell and placed onto a flat surface with the *stratum corneum* (SC) facing up. Some of the skin samples were reserved to be analysed by FTIR. The remaining skin samples were cleaned with a water-soaked gauze pad and cut finely in a tube with a lid used for extraction. Hereafter, 3 mL methanol was added and vortexed before being exhaustively extracted on a shaker over a 24 h period. Finally, khellin content in the skin was determined using HPLC.

Fourier transform infrared spectroscopy (FTIR)

ATR-FTIR spectroscopy was used to determine the interaction between the formulation components and skin samples used in the previously described *ex vivo* penetration study. The formulation and the receptor phase residues on the skin surface were cleaned gently with a paper. Then, skin samples were placed on an ATR-crystal-bearing Fourier Transform Infrared Spectroscopy (FTIR, Nicolet™ 6700 Continuum™ Infrared Microscope, Thermo Fisher Scientific, Switzerland) tray with the *stratum corneum* surface in contact with the crystal. A constant force (60 N) was always applied to the skin to ensure adequate contact between the crystal and the skin sample. Analyses were performed in the frequency range of 4000 - 550 cm^{-1}, with a spectral resolution of 4 cm^{-1}.

HPLC analysis

HPLC analysis method of khellin was modified from the study of Badr *et al.* [3]. A Shimadzu LC 10 A system including an LC-20AT pump, CTO-10AS VP column oven and a DAD detector were used. The sample injection volume was 100 µL. The analysis was performed using an isocratic method with a flow rate of 1 mL/min, a mobile phase consisting of 3% THF, 47% MeOH and 50% ultra-pure water using a C18 column (250 mm × 4.6 mm, 5 µm particle size) at 35°C. Khellin was detected at 13 minutes retention time and 247 nm wavelength. The method was linear over a range of 0.0625 - 6.25 µg/mL (r^2 = 0.9998). Calculations and analysis were done using the Microsoft Excel program.

Stability

Nanoparticle formulation samples were stored at 2 - 8°C and 25 ± 2°C/60 ± 5% conditions, being evaluated periodically during 20 weeks in terms of size, PDI and zeta potential values using Zetasizer (Malvern Zetasizer, Malvern, UK).

Results and Discussion

Preparation and characterization of nanoparticles

effect of polymer concentration on formulation

Formulations containing the polymer concentrations of 0.25%, 0.5%, 0.75%, 1%, 1.5% or 2% (w/v) were evaluated. As a result, it was found that the polymer with 1% concentration had the most suitable size, PDI and zeta potential (Table II). After optimization of the other parameters, the polymer concentration parameter was re-evaluated with 1%, 1.25% and 1.5% CAP ratios (Table III). As a result, while precipitates were observed in the formulation containing 1.5% polymer, it was determined that no precipitation was formed in the 1% and 1.25% formulations. While similar results were obtained in the zeta and PDI values of these two formulations, the size of 1.25% was larger than 1%. Since nanoparticles of larger size would penetrate deep into the hair follicle [35, 47], which is the target region, the amount of polymer for the final formulation was determined as 1.25%.

Effect of organic solvents used as organic phase

Mixtures of ethanol: acetone (1:1) and 100% acetone were evaluated as organic solvents. Although both solvents gave good results, acetone was preferred in subsequent studies due to the lower PDI value of the formulation prepared with 100% acetone (Table IV).

Table II

Average sizes, zeta potentials and PDI values of nanoparticles containing 0.25 - 2% CAP

CAP% / Parameter	0.25%	0.50%	0.75%	1%	1.50%	2%
PDI	0.445 ± 0.02	0.341 ± 0.02	0.430 ± 0.02	0.201 ± 0.02	0.282 ± 0.02	0.234 ± 0.02
Z-average (nm)	124.9 ± 2.0	106.1 ± 2.00	160.9 ± 2.0	117.5 ± 2.0	327.8 ± 2.0	249.5 ± 2.0
Zeta potential (mV)	-2.758 ± 0.5	-4.046 ± 0.5	-0.007 ± 0.5	-13.59 ± 0.5	-1.145 ± 0.5	-3.213 ± 0.5

Table III

Average sizes, zeta potentials and PDI values of nanoparticles containing 1%, 1.25% and 1.5% CAP

CAP% / Parameter	1%	1.25%	1.5%
PDI	0.142 ± 0.02	0.155 ± 0.02	0.218 ± 0.02
Z-average (nm)	179 ± 2.0	194.5 ± 2.0	193 ± 2.0
Zeta potential (mV)	-13.35 ± 0.5	-11.19 ± 0.5	-10.19 ± 0.5

Table IV

Average sizes, zeta potentials and PDI values of nanoparticles containing 100% acetone or mixtures of ethanol:acetone (1:1)

CAP% / Parameter	100% acetone	50% ethanol:50% acetone
PDI	0.032 ± 0.02	0.145 ± 0.02
Z-average (nm)	141.5 ± 2.0	113 ± 2.0
Zeta potential (mV)	-11.49 ± 0.5	-9.787 ± 0.5

Types of excipients

PVA, Tween 80 and Kolliphor EL were evaluated at 0.5% (w/v) concentrations as excipients. In the size distribution graph, it was observed that the surfactant that provided the most appropriate peak was Tween 80 (Table V).

Table V

Average sizes, zeta potentials and PDI values of nanoparticles containing 0.5% PVA, 0.5% Tween 80 and 0.5% Kolliphor EL as surfactants

CAP% / Parameter	0.5% PVA	0.5% Tween 80	0.5% Kolliphor EL
PDI	0.399 ± 0.02	0.472 ± 0.02	0.576 ± 0.02
Z-average (nm)	329.6 ± 2	352.9 ± 2	361.6 ± 2
Zeta potential (mV)	-19.69 ± 0.5	-9.172 ± 0.5	-0.268 ± 0.5

Effect of the ratio of organic phase to aqueous phase, mixing speed, pumping speed of the organic phase and surfactant concentration

The ratio of the organic phase to the aqueous phase, mixing speed, pumping speed of the organic phase and surfactant concentration were evaluated using Taguchi Experimental Design Method. This method was used to evaluate these parameters more effectively, faster and with a minimum number of experiments.

It has been observed that the size increases as the mixing speed decreases. The mixing speed was minimized in the final formulation.

While a positive correlation was observed between Tween 80 concentration and particle size, the zeta potential approaches zero with the increase of concentration. Although it is undesirable for the zeta potential to approach zero, since large sizes are more suitable for the target area, Tween 80 concentration was increased to 3% in the final formulation. To get the desired zeta potential, the range and other parameters were adjusted. Even though the size was not affected much by changing the addition speed, positive results were obtained in PDI and zeta potential values. The highest addition speed rate (9 mL/h) was chosen.

While the organic phase: aqueous phase ratio gave insignificant results in terms of size, positive results were observed in zeta potential values. The organic phase: aqueous phase ratio was determined as 3:10 (Figure 2).

Determination of the final formulation

The final formulation was determined based on the results obtained from the preformulation studies. The parameters were settled as follows: polymer concentration as 1.25% (w/v), organic phase solvent as 100% acetone, organic phase to liquid phase ratio as 3:10, Tween 80 concentration as 3% (w/v), addition speed as 9 mL/h and stirring rate as 250 rpm. The final formulation was prepared by nanoprecipitation method. Using these parameters, a whitish nanoparticle dispersion was obtained (Figure 3). The final formulation had a PDI of 0.11, an average size of 217.8 nm (Figure 4). The obtained PDI values show that the formulation is perfectly homogeneous and has excellent size distribution. Narrow size distribution is very important to achieve optimal clinical results [7]. Achieved average particle size is also ideal for the vitiligo treatment objectives. It was previously proven in the literature that nanoparticles with a size of 200 - 600 nm are more likely to penetrate through the hair follicle than particles with smaller or larger sizes [37, 47]. Moreover, according to the results obtained from the previous study, particles with a size near 230 nm are expected to target the sebaceous gland right above the bulge region of the follicles [37]. Khellin release in this area will ensure that the active substance reaches the area where the main source of melanocytes in the epidermis is located [31]. Therefore, khellin could stimulate the migration and proliferation of melanocytes, thus repairing the loss of melanocytes and melanin and providing an effective treatment of vitiligo [5, 45].

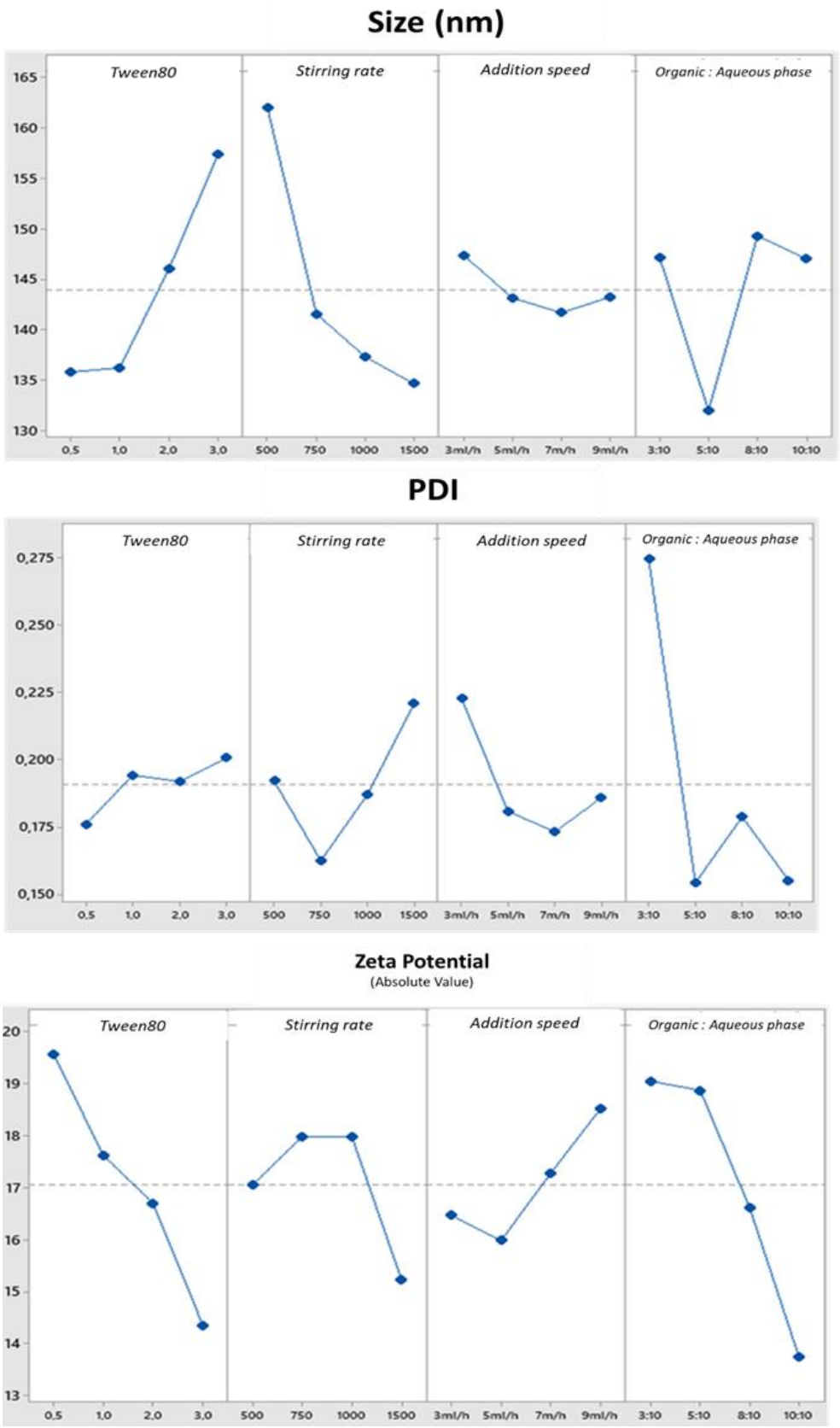

Figure 2.

Size, PDI and zeta potential values of Tween 80, mixing speed, addition rate and organic phase: aqueous phase ratios

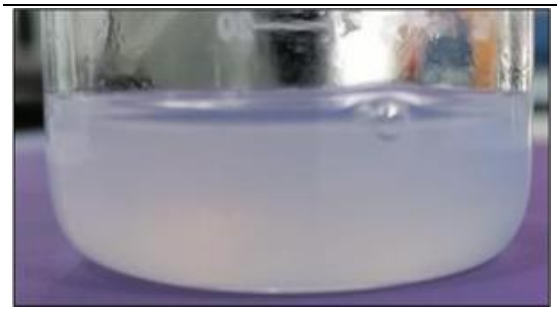

Figure 3.
Image of the final formulation

The Zeta potential of the formulation was recorded as -18.4 mV. Cellulose acetate phthalate is a polymer with a pKa value of 5.5 [27]. In an acidic environment, this polymer will stay uncharged, hence less soluble. This will lead to more uniformly shaped nanoparticles. However, a very acidic formulation is not suitable for topical applications because of the possibility of irritation. In the final formulation of this study, a medium with a pH value of 4.5 was used. Using this pH, a less irritating formulation and fairly negatively charged particles were obtained which will grant a more stable formulation.

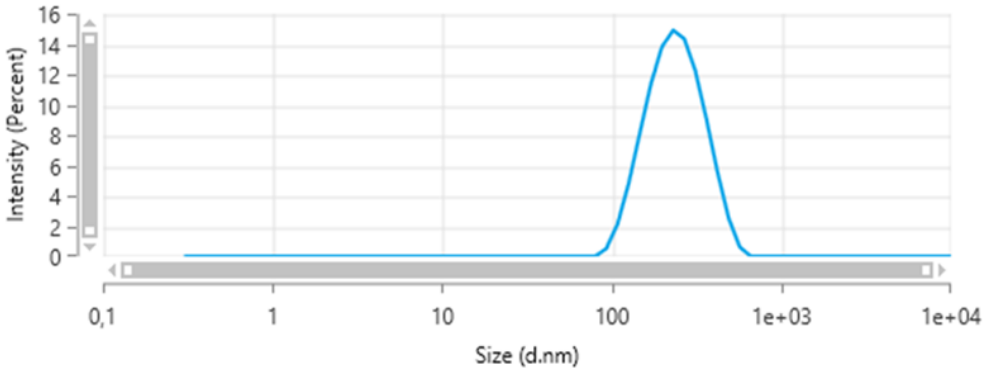

Figure 4.
Size distribution graph of the final formulation

Encapsulation efficiency of the final formulation

The encapsulation efficiency of the final formulation was calculated as 34.2%. In the literature, the encapsulation efficiency was found to be in the range of 10 - 20% for the nanoparticles, and this range was interpreted as good encapsulation efficiency [6, 25]. Accordingly, it can be said that khellin is effectively loaded into the nanoparticles.

In vitro drug release study and release kinetics

Khellin-containing control gel formulation released only 33% of total khellin during 24 hours, while 75% of khellin was released from the nanoparticles in a controlled manner during the same period. When nanoparticles were dispersed in the gel, khellin release was reduced to 62% due to the barrier formed by the gel. After the first hour, the nanoparticle formulation showed a release of 27% of the drug while the gel-dispersed nanoparticle formulation and the control gel released 16% and 9% of the drug, respectively. This shows that the nanoparticle formulation has a straighten release character compared to the other formulations. These results showed that the nanoparticle formulation had superior release properties (Figure 5).

Regression coefficients of the release kinetics matched the Korsmeyer-Peppas model and the Higuchi model (Table VI). The Higuchi release kinetic model is a model that describes the release rate of drugs through diffusion from porous matrix systems [20].

The Korsmeyer-Peppas model is a simple model describing drug release from a polymeric system. It defines release mechanisms simultaneously, such as diffusion of water into the matrix, swelling of the matrix and dissolution of the matrix [19]. The calculated 'n' number is less than 0.4 indicating that the release model is in a diffusion-controlled model [29].

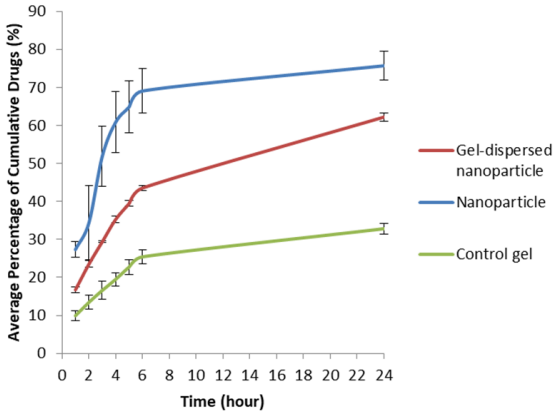

Figure 5.
Graph showing the mean cumulative drug percentage *versus* time of nanoparticle, nanoparticle dispersed in gel and control gel formulations

Table VI

Kinetic evaluation of nanoparticles, nanoparticle dispersed gel and control gel formulations

Model Parameter	Zero Order r^2	First Order r^2	Hixon-Crowell r^2	Higuchi r^2	Korsmeyer-Peppas r^2/n
Nanoparticle	0.427	0.357	0.38	0.627	0.794 0.353
Gel-dispersed nanoparticle	0.758	0.585	0.646	0.904	0.942 0.413
Control gel	0.738	0.585	0.638	0.876	0.932 0.392

Kinetic evaluation of investigated nanoparticles, nanoparticle dispersed gel and control gel formulations, which were presented in Table VI, showed that the highest determination coefficient (r^2) values were obtained for Korsmeyer-Peppas kinetic model for all formulations. According to diffusion exponent values, all of the formulations were fitted to Fickian diffusion due to $n \leq 0.45$ values [2]. Therefore, the mechanism involved in the release of khellin from the nanoparticle system was diffusion controlled. This suggests that nanoparticles will function as drug reservoirs for controlled drug diffusion after penetration which could help in reducing dose and frequency during clinical applications.

Ex-vivo skin penetration study

While khellin loaded to nanoparticles in suspension form accumulated in the skin at an amount of 0.521 ± 0.045 μg/cm^2, the gel-dispersed nanoparticle form achieved a 1.001 ± 0.146 μg/cm^2 amount of accumulation. This means that khellin in the gel-dispersed nanoparticle formulation had 2 times better penetration than khellin in the nanoparticle dispersion formulation. Similar results were obtained in the study of Patzelt *et al*., which compared the penetration of nanoparticles into hair follicles in gel and suspension forms. The study revealed that gel form of the nanoparticles significantly increased permeation depths and enhanced penetration when compared to the suspension form [36]. While increasing the penetration of particles, gel formulations will also provide a more comfortable use for patients when practically applied compared to suspensions.

Fourier transform infrared spectroscopy (FTIR)

ATR-FTIR spectroscopy is a method used to determine the molecular sequence of the lipid structure in the *stratum corneum* [14]. The interaction of the formulation components with the alkyl chains of the intercellular lipids in the *stratum corneum* cause the wave numbers of the carbon-hydrogen (C-H) symmetric (2850 cm^{-1}) and asymmetric (2920 cm^{-1}) stretching bands to shift to higher values [16]. As shown in Table VII, carbon-hydrogen (C-H) symmetric (2850 cm^{-1}) and asymmetric (2920 cm^{-1}) stretch bands increased in all samples. This increase was higher in the gel-dispersed nanoparticle formulation compared to the nanoparticle aqueous dispersion. Higher interaction between formulation and the lipid structure in the *stratum corneum* grants a higher skin penetration [32]. This result is consistent with the findings of the *ex vivo* skin penetration study; both exhibit a consistent result that nanoparticles dispersed in HPMC gel are expected to have better skin penetration results when applied to the patients' skin. This could be related to the high viscosity and low fluidity of the gel formulation which improved the interaction of the drug with the skin.

Table VII

Results of FTIR spectrum carbon-hydrogen symmetric and asymmetric stretch bands

Sample	Carbon-hydrogen (C-H) symmetric stretch band (cm^{-1})	Carbon-hydrogen (C-H) asymmetric stretch band (cm^{-1})
Control skin	2850.40	2918.40
Nanoparticle aqueous dispersion	2852.34	2921.74
Gel-dispersed nanoparticle formulation	2852.46	2922.42

Stability of nanoparticles

The stability results of nanoparticle formulations at 2 - 8°C and 25 ± 2°C/60 ± 5% storage conditions for 20 weeks are given in Table VIII. At 25 ± 2°C/60 ± 5% storage conditions it was observed that the formulation remained stable during the first 8 weeks. During this time period, no significant change was observed in size, PDI or zeta potential. However, at the 12th week a rise in the zeta potential occurred followed by an increase in the size and PDI values in the following weeks. This indicates that, when used practically, the formulation would be expected to begin to lose its stability after 2 months of storage at room conditions which could be considered as a limitation of this formulation. On the other hand, no significant change in size, PDI or zeta potential occurred when the formulation was stored at 2 - 8°C throughout the test period. This shows that the nanoparticle formulation must be stored and transferred at cold conditions when used as a pharmaceutical product. Long term stability tests are still required to confirm these results.

Table VIII

Stability results evaluated by light scattering method

	25°C			2 - 8°C		
Time (weeks)	**Size (nm)**	**PDI**	**Zeta potential (mV)**	**Size (nm)**	**PDI**	**Zeta potential (mV)**
0	206.9 ± 2.1	0.14 ± 0.02	-14.4 ± 0.6	206.9 ± 2.05	0.14 ± 0.02	-14.4 ± 0.6
1	205 ± 4.5	0.15 ± 0.03	-13.8 ± 0.4	204.2 ± 1.4	0.11 ± 0.02	-15.5 ± 0.2
2	202.4 ± 1.6	0.11 ± 0.03	-14.5 ± 0.6	205.4 ± 0.3	0.13 ± 0.01	-14.2 ± 0.4
4	199.6 ± 2.6	0.12 ± 0.02	-13.6 ± 0.4	201.3 ± 3.3	0.14 ± 0.03	-15 ± 0.4
8	204 ± 1.7	0.11 ± 0.01	-12.5 ± 0.3	207.2 ± 6.2	0.14 ± 0.02	-15.2 ± 1.2
12	203.5 ± 1.8	0.13 ± 0.04	-9.6 ± 0.5	202.2 ± 2.7	0.12 ± 0.04	-16.1 ± 1.1
16	218.767 ± 2.811	0.179 ± 0.006	-7.917 ± 0.111	196.467 ± 2.845	0.159 ± 0.029	-16.467 ± 0.306
20	229.067 ± 3.337	0.233 ± 0.007	-6.307 ± 0.082	196.533 ± 1.58	0.128 ± 0.016	-15.167 ± 0.368

Conclusions

Khellin, the major substance of the *Ammi visnaga* L. plant, is a very effective herbal bioactive in the treatment of loss of skin pigment or vitiligo. Khellin cures vitiligo by stimulating melanogenesis and enhancing the migration and proliferation of melanocytes which normally forms in the hair follicles. Accordingly, to achieve the desired effect, khellin should be targeted to the hair follicles. Hair follicle targeting is best achieved using particles that are 200 - 600 nm in size and capable of triggering drug release at basic pH. Cellulose acetate phthalate nanoparticles with suitable properties were developed in the scope of this study. *In vitro* release studies showed that khellin-containing control gel released only 33% of total khellin during 24 hours, while 75% of khellin was released from the nanoparticles in a controlled manner during the same period, which shows that the nanoparticle formulation has superior release properties. Release kinetics presented a Fickian diffusion, which is in accordance with the gel dosage forms. The skin penetration and FTIR skin interaction studies showed that gel-dispersed nano-particle formulation had the best skin penetration and skin interaction results. In the optimum formulation, the high viscosity of the gel led to better interaction between the formulation and the skin, whereas the dispersed nanoparticle improved drug release. In conclusion, gel dispersed khellin-loaded CAP nanoparticles are promising nanocarrier systems for vitiligo treatment in terms of *in vitro* drug release and *ex vivo* skin penetration studies.

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Conflict of interest

The authors declare no conflict of interest.

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