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RESEARCH PAPER

Lavender essential oil nanoemulsion gel as skin lightener: green formulation, full characterization, anti-melanogenesis effect, and in-vitro/in-vivo safety profile assessment

Mahmoud Omidi[1,2], Katayoun Morteza-Semnani[3], Majid Saeedi[4], Mina Frahmand[5], Marzie Kamali[6], Esmaeil Mohammadian [1,7], Seyyed Mohammad Hassan Hashemi[1,8]**

[1]Food Health Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[2] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[3]Department of Medicinal Chemistry, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
[4]Department of Pharmaceutics, Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran
[5]Student Research Committee, Faculty of Pharmacy, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[6]Molecular Medicine Research Center, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[7]Department of Medicinal Chemistry, School of Pharmacy and Pharmaceutical Sciences, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[8]Department of Pharmaceutics, Faculty of Pharmacy, Hormozgan University of Medical Sciences, Bandar Abbas, Iran

ABSTRACT

Objective(s): In this study, to improve anti-melanogenesis properties, the green method based on an ultrasonic approach was utilized to prepare lavender essential oil (LEO) nanoemulgel.

Material and Methods: Nano-emulsion green preparation, physico-chemical characterization, inspection morphology, animal safety study, cellar toxicity and anti-pigmentation test were performed.

Results: The obtained results showed that with a decline in HLB value (Hydrophilic–lipophilic balance), the mean particle size declined from 157.600±3.798 to 85.566± 2.227 nm (P< 0.05). The evaluations performed with the accelerated stability test (freeze-thaw cycle) showed stability for nanoemulgel. Other factors, such as pH, spreadability, and, viscosity were also measured. In vitro cytotoxicity studies for the revealed LEO-nanoemulsion, on the HFF normal cell line showed less toxicity (73%) than pure LEO. Moreover, LEO-nanoemulsion had a higher cytotoxic impact on B16F10 melanoma cancer cells than pure LEO. The inhibitory activity of this LEO-nanoemulsion was evaluated in melanin content, which indicated that melanin synthesis is more inhibited than LEO. Furthermore, the evaluation of the inhibitory activity presented that the inhibition of L-dopa auto oxidation (87.33± 2.45%) is better than that of LEO (74.22± 3.26%) at a concentration of 5000 µg/ml. It should be noted that no skin irritation was observed with the histo-pathological examination on Wistar rat and the dermal irritation study for the LEO-nanoemulgel.

Conclusion: The results of the present study can be very useful for the introduction and local administration of LEO nanoemulgel for the management of hyperpigmentation conditions.

Keywords: *Lavender, Essential oil, Nanotechnology, Green method, Anti-melanogenesis*

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INTRODUCTION

Melanin as a type of biological pigment is found throughout the body's skin (1). Melanin production in the body is carried out using several enzymes from the melanocytes in a complex process. The tyrosinase enzyme, which contains copper, is one of the important enzymes in the process of melanin synthesis. Tyrosinases are known by synonyms such as polyphenol oxidase, catecholase, cresolase, and phenolase (2, 3).

* *Corresponding authors: Email: e.mohamadyan1390@gmail.com, smhhashemipharma@gmail.com.* Note. This manuscript was submitted on July 04, 2024; approved on August 26, 2024

Pigmentation disorders, as a prevalent dermatological issue, are created due to the improper distribution, function, and structure of melanocytes in the skin. One of the most important challenges for people, especially women, is the treatment of these problems. middle-aged women can develop abnormal skin pigmentation due to a variety of exogenous and endogenous factors (4). Among pigmentation disorders, hyperpigmentation involves excessive pigmentation, while hypopigmentation occurs when pigmentation is reduced. There can be psychological, cognitive, cosmetic, and psychosocial consequences for patients who suffer from skin pigmentation problems. Furthermore, cutaneous pigmentation abnormalities can also lead to freckles (ephelides) and chloasma (melasma) (5) (6, 7) Inhibiting the biosynthesis of melanin by skin lightening agents is useful for treating hyperpigmentation. Melanogenesis inhibitors have recently become increasingly popular for both food quality and human health reasons, particularly for skin lightening in Eastern cultures. Plant extracts are promising sources of bioactive compounds for industries seeking natural alternatives. A variety of applications, including medicinal and cosmetic, have been provided by herbal preparations throughout history (8).

The Lavender plant as known as the perennial evergreen shrub (Latin name: Lavandulais), is native to the Mediterranean region. Today, this species exists naturally in the following areas: Europe, North Africa, the United States, and Australia. Traditionally, for the treatment of pain, parasitic infections, burns, hyperpigmentation, insect bites, cramps and muscle spasms, *L.angustifolia* (Lavander), as the most valuable medicinal, is used (9). LEO has been found to have depigmenting properties on human skin due to its tyrosinase-blocking effect. A study showed that the oil reduced skin pigmentation by more than a third from the initial value, indicating its potential as an effective depigmenting agent (10). One of the disadvantages of essential oils (volatile) is that their stability is affected by light, heat, moisture, and oxygen, which reduces their effectiveness (11). On the other hand, the low solubility of essential oils in water has necessitated the need for new formulations in the industry.

Nowadays, nanolipid systems are of great interest as one of the new options for pharmaceutical targeting. Strategies based on nanolipids include the following: liposomes, solid lipid nanoparticles, nanostructured lipid carriers, microemulsions, and nanoemulsions. Among the mentioned cases, nanoemulsions are more effective strategies for drug delivery of lipophilic active compounds (12). Research has shown that nanoemulsions have high performance for skin applications. Nanoemulsions can carry many lipophilic ingredients and effectively deliver these ingredients to the skin (13). Also, Nanoemulsions are highly effective in improving the delivery and stability of LEO. They protect LEO from oxidation, temperature, and light, thereby extending shelf life and maintaining quality. The small droplet size in nanoemulsions enhances absorption, bioavailability, and targeted delivery, leading to improved therapeutic effects of LEO(14).

Existing investigation aimed to generate stable green nanoemulsions from LEO via an ultrasonic manner the employed of organic solvents is excluded. Also, a binary surfactant with diverse HLB values has not been investigated in any study for its effect on the development of nanoemulsions containing LEO. In the next step, their anti-melanogenesis properties were investigated. Also, LDOPA auto-oxidation and melanin count have not been studied using nanoemulsions containing LEO in previous publications. Nanoemulsions containing LEO have also not been tested for the possibility of causing dermal sensitivity in preceding publications. The development of LEO nanoemulsions in gel form using ultrasonic technology was investigated for their *in vitro* cellular viability on the normal fibroblast and B16F10 cell line. Consequently, nanoemulsions containing LEO gel were developed as cosmeceutical products as alternatives to traditional carriers applied locally to the skin.

MATERIALS AND METHODS

Materials

Carbopol 940, Triethanolamine, formalin, Tween 20, Tween 80, Span 80, and Lavender essential oil (LEO) were purchased from BF Goodrich Co (UK), Sigma- Aldrich (St Louis, MO), Merck (Darmstadt, Germany), and Barij Essence Pharmaceutical Company (Kerman Province in Iran), respectively.

Essential oil analysis

The chemical components of LEO were analyzed using a fused-silica (30 m 0.25 mm, sheet

Table 1. Physicochemical properties of formulation. Each data is reported as average± SD after three repetitions (n = 3).

Formulation	LEO (mg)	Tween 80 (mg)	Span 80 (mg)	Tween 20 (mg)	HLB	Particle size (nm)	PDI	Zeta potential (mv)
F1	200	0	0	600	16.7	157.60± 3.79	0.386± 0.005	-4.31± 0.56
F2	200	600	0	0	15	138.50± 3.17	0.334± 0.009	-5.55± 0.25
F3	200	530	70	0	12.85	120.36± 2.28	0.298± 0.005	-7.36± 0.82
F4	200	400	200	0	11.43	103.10± 4.63	0.284± 0.007	-9.37± 0.39
F5	200	300	300	0	9.65	89.03± 2.14	0.256± 0.006	-11.94± 0.62
F6	200	200	400	0	7.78	85.56± 2.22	0.248± 0.003	-3.66± 0.60

width 0.25 m) capillary DB-5 gas chromatography (GC) interfaced with a 5975°C mass spectrometer (Agilent Technologies, Palo Alto, CA). Helium at a discharge rate of 1 mL/min was used as the transport gas. In split mode (split percentage of 1:100), 1 µL of essential oil was introduced into the GC. The temperature related to the injector and detector both remained at 250 °C. The intended procedure called for preheating the oven to 50 °C for 5 min, then, gradually increasing the temperature to 200 °C at a rate of 5 °C per min. The temperature was continued for 5 min before being steadily increased to 280 °C (rate of 10 °C per min). Mass spectrum matching to several datasets (including NIST08 and Wiley7n.l) and an evaluation of the retention index for n-alkanes allowed for the identification of the components. The percentage of the substances was derived from the region of the GC peak without the application of a correction factor.

Preparation of nanoemulsions

By dispersing LEO into the deionized water and Tween 80, Tween 20, or a mixture of Tween 80/Span 80 as emulsifiers, a coarse emulsion was obtained. During the experiment, the surfactant concentration remained constant at 3 wt%. CE nanoemulsion was prepared in the range of HLB values from 9.65 to 16.7, and then the effect of the HLB value was examined (Table 1). The high-speed homogenizer (Silent Crusher M, Heidolph, Germany) at 8000 rpm for 20 min was used to homogenize the mixture. Then, we sonicated an emulsion for 10 minutes using an ultrasonic sonicator (HD 3200, Bandelin, Germany) equipped with an amplitude (AM) of 100%, a high-grade titanium tip, and a constant frequency of 20 kHz. An outer ice-water bath was used throughout the processing to keep the nanoemulsions below 15°C.

Characterization of nanoemulsions

In the dynamic light scattering (DLS) assay, the size, zeta potential, and polydispersity index (PDI) of the obtained nanoemulsion were calculated with Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK equipped with a disposable capillary cuvette (DTS 1060). PDI and an intensity- weighted mean diameter (Z-average) were provided with DLS technology. Fixed scattering angle of 90° at 25 °C were the conditions for experimenting. All tests were repeated three times, and all values were reported as mean ± standard deviation (15). Also, the zeta potential (through electrophoretic mobility) measurements were studied with the Malvern Zetasizer. It should be noted that the same cuvette was used for the zeta potential measurement.

Transmission electron microscopy (TEM)

The TEM assay was used to assess the surface morphology of obtained nanoemulsions. Briefly, after placing a drop of the nanoemulsion formulation on the copper surface, 2 percent phosphotungstic acid was used for negative staining. The samples were dried using air, and filter paper was applied to remove any remaining traces of solution. A 100 kV tungsten source with Philips EM 208S (Netherlands) was used for testing.

Preparation of nanoemulgel

200 mg essential oil as a nanoemulsion dispersion, including carbopol 941 (1% w/v) was diluted with preserved water including 0.1% sodium benzoate, and stored at room temperature for 24 hours before use. As a net result of neutralization, 100 mg of triethanolamine was added to the carbopol combination in the nanoemulsion formulation by a propeller homogenizer (500 rpm).

Release test

The experiments were performed using Cellulose membranes (12 kDa) in Franz cells, which

featured a 33 mL receptor segment and a diffusion zone of 3.8 $cm^{2.}$ The experiments were carried out at a temperature of 32 °C ± 1 °C. The receptive liquid consisted of a phosphate buffer (pH 5.5) mixed with ethanol in an 80:20 ratio. Specimens were discarded at consistent periods (2, 4, 6, 8, and 24 hours) and assayed using HPLC at 207 nm for linalool detection. The receptive segment was replenished with fresh phosphate buffer (pH 5.5) mixed with ethanol in an 80:20 after each sampling. Linalool was quantified using a Kneuer HPLC system (Germany) equipped with an Agilent RP C18 column (4.6×150 mm, 5µm). The mobile phase was an acetonitrile: water mixture (55:45 v/v) at a flow rate of 1 ml/min.

Freeze–Thaw cycle

Six freezings and six thawings were performed over 12 days with the optimum formulation gel. In each period, the temperature related to the samples was specifically controlled for 24 h. The temperature of the refrigerator and stability chamber were kept 4 ±2 °C, and 40 °C, respectively.

Centrifugation test

A tapered test tube was used to centrifuge 10 g of the formulation. The 3–30 K devise (Sigma, Germany) was used for centrifuging (at 3000 rpm) the samples at room temperature.

Spreadability

The spreadability of the optimum formulation gel was determined on a glass plate based on the following steps: i) spreading 0.5 g of the gel on a pre-marked circle of 2 cm diameter and ii) 0.5 kg weight was put on the upper second glass plate for 5 min. Then, the radius of the circle was measured after spreading the gel.

Spreadability%=(A2/A1)×100

A1 = 2 cm and A2 = after spreading.

pH calculation

The pH of the optimum formulation was determined using a digital pH meter. Calibration was done for the glass electrode, and the solutions with pH equal to 4 and 7 were used in this process. The experiment to determine the pH of the formulation was repeated three times, and the mean output was computed.

Evaluation of rheological manners and viscosity

The Brookfield viscometer (Brookfield, DV-II +, USA) equipped with spindle S5 was used to measure the viscosity of the prepared nano-emulsion gel. At various speeds, including 5, 10, 20, 50 and 100 rpm, the gel viscosity was determined.

Accelerated stability and physicochemical investigation of optimum formulation gel

The optimum formulation gel was subjected to accelerated stability tests. During the experiments to measure the color, odor, pH, and viscosity of the gel, the temperature of the gel was kept in the range of 20 to 25°C. In order to evaluate the resistance to the freeze–thaw cycle related to the formulation gel, stored at temperatures of 4, 25, and 40 °C for 12 days was performed. 6 months after preparing the formulations, relevant tests were performed. The weight of each sample in the tests was 30 grams, and all the tests were repeated three times.

Stability studies

During three months and according to ICH guidelines, the stability of the best nano-emulsion preparation was assessed at 4, 25 and 40 °C. In addition to testing the physical stability of the formulation, the impacts of temperature and time were monitored for changes in color, precipitate aggregation, and lipid ingredients.

Non-specific cytotoxicity assessment

Using HFF cells (Human Foreskin Fibroblast and B16F10 melanoma cells obtained from the National Cell Bank in the Pasteur Institute of Iran; Tehran, Iran) the in vitro cell toxicity of the obtained nanoemulsion was determined. The cells in microplates (Nunclon) with a density of 5000 cells/well were incubated for 24 h with different concentrations of nanomicelles included without LEO, LEO in the concentrations of 5000, 1000, 500, 400, 200, 100 and 50 µg/ml and a control sample. As soon as the components were removed, the cells were rinsed with PBS, and the colorimetric MTT assay was used to assess cell viability. In the next step, MTT was added to the samples at a concentration of 0.5 mg/mL, and incubation was done at 37 °C for 4 h. Next, after removing the supernatant, 100 µL of DMSO was used to dissolve the remaining formazan crystals. A multi-well spectrophotometer was employed to determine the optical density (560 nm) after shaking the plates for 20 minutes. In the presence

of six additional controls (cells in media) and different concentrations of nanomicelles, including 5000, 1000, 500, 400, 200, 100 and 50 µg/ml, cell viability was measured.

Inhibitory L-DOPA auto oxidation assay

Based on the earlier identified approach, the inhibitory effect of the pure drug and the obtained formulations on the auto-oxidation of the L-dopa compound was measured. Briefly, the various concentrations of i) LEO nanoemulsion, including 5000, 1000, 500, 400, 200, 100, and 50 µg/mL, plain nanoemulsion without ii) LEO, and iii) LEO in the 0.1 M sodium phosphate buffer at pH=6.5, were prepared individually in the 96-well plate. Then, sodium phosphate buffer (in the concentration of 0.1 M) at pH= 6.5, including 10 mM L-DOPA, was added to the plate with a total volume of 300 µL, and it was incubated in the next step at 37 °C for 15 min. The activity was evaluated by measuring the absorbance at 475 nm.

Melanin content assay

From the National Cell Bank Pasteur Institute of Iran (Tehran, Iran), murine B16F10 melanoma cells were purchased. For 24 hours in RPMI medium including 10% fetal bovine serum (Gibco, USA), penicillin (100 units/mL), and streptomycin (0.1 mg/mL), cells were grown in a 12-well plate. After 24 h, different concentrations of LEO nanoemulsion, including i) free nanoemulsion of LEO, ii) a plain nanoemulsion without LEO, and iii) LEO at concentrations of 5000, 1000, 500, 400, 200, 100 and 50 µg/ml were used for the treatment of cells. Then, a solution of 100 µL of NaOH (2 M) was used to dissolve the cells for 30 min at 100 °C. Measure the absorbance at 405 nm using a Microplate Reader (BioTek, Winooski, USA) to evaluate the melanin content. In this experiment, a comparison with the control group was made for all samples.

In-vivo assessments of nanoemulsions containing LEO Animals

This experiment was performed on male Wistar rats weighing between 120 and 150 g. Rats were placed in a light-dark cycle so that the temperature was 21 ± 2 °C and the time was 12 h. It should be noted that the lights were turned on from 07:00 to 19:00 h in plastic cages at the animal facility. On the other side, except for the duration of the experiment, water and food were always available to the animals and each animal was tested only once.

Dermal sensitivity assessments

In the animal test, we followed an ethically approved protocol (Guidelines of the Center Animals Ethical Panel). One day before performing all the experiments, the hair of the Wistar rats was trimmed. The test was performed on 5 groups (n = 5) of rats: i) control group; ii) treated with the nanoemulsions containing LEO gel; iii) treated with LEO; iv) given the plain nanoemulsions gel; v) reacted with an aqueous solution of formalin (0.8% (v/v)) as a typical irritant. Fresh preparations were used for three consecutive days. A visual grading system was used to evaluate the application sites by the same viewer.

Histopathology

After three days of surgery, all the tested animals were euthanized for histological studies. Using surgery, dermal tissue samples were removed, and in the next step, they were immediately fixed in a 10% formalin solution. From the obtained samples, 4-7 µm parts were prepared and placed in paraffin blocks. All samples were cut and stained with hematoxylin and eosin (H&E). Pathological changes in stained sections were examined using an optical microscope,.

Ex-vivo cutaneous permeation

Male rats with a mass variety of 130-160 g were sedated by ketamine/ xylazine. The skin was discarded and the fat attached to the epidermis region was eliminated and flooded in normal saline overnight. The cutaneous was sited in the Franz cells, which had a diffusional region of 3.8 cm2, for penetration assay. The Franz cells were maintained at a temperature of 32±0.5 °C, and the temperature of the modified water was controlled by a jacket around the cell bodies during the studies. On the hairless side of the donation segment, LEO (1%) nanoemulsion gel (1g) and plain LEO (1%) gel (1g) were massaged regularly. At fixed intervals (2, 4, 6, 8 & 24 h), 1 mL of the solution was reserved from the receptor segment, and an identical extent of new buffer phosphate pH 5.5 (normal skin pH): ethanol 80:20 was inserted to the receptor segment. The linalool extent (one of the main components in LEO has an anti-melanogenesis effect) in the samples was evaluated at 207 nm with HPLC instrument

Table 2. Composition percentage of LEO

No.	Compounds	RI[a]	GC area (%)	No.	Compounds	RI[a]	GC area (%)
1	1,8-Cineole	1026	32.7	11	(E)-β-Farnesene	1456	1.32
2	Linalool	1095	23.57	12	(Z)-β-Ocimene	1032	1.22
3	Borneol	1165	9.58	13	Myrcene	988	1.09
4	Camphor	1141	5.24	14	Lavandulyl acetate	1288	1.07
5	β-Pinene	974	3.01	15	Cryptone	1183	0.9
6	α-Terpineol	1186	2.9	16	δ-3-Carene	1008	0.74
7	α-Pinene	932	2.72	17	Camphene	946	0.72
8	Terpinen-4-ol	1174	2.63	18	Caryophyllene oxide	1582	0.64
9	Linalool acetate	1254	1.93	19	Lavandulyl isovalerate	1509	0.49
10	α-Bisabolol	1685	1.63	20	(E)-Caryophyllene	1417	0.46

(section 2.7).

Statistic evaluation

All outputs obtained were described as the average ± SD (with at least three repetitions). SPSS 22.0 (IBM Co., USA) was used to assess the statistics. The ANOVA, followed by the Tukey test and LSD's post-hoc test, was evaluated for specified parameters. $P< 0.05$ was stated as statistically significant.

RESULTS AND DISCUSSIONS

LEO assessment

Table 2 displays the 34 compounds found in *Lavandula angustifolia*, which account for 98.74 % of the essential oil content. The GC-MS study showed that there was a significant number of oxygenated monoterpenes and monoterpene hydrocarbons in the essential oil. 1,8-Cineole (32.70%), Linalool (23.57%), Borneol (9.58%), and Camphor (5.24%) were the major compounds of the *Lavandula angustifolia*.

The findings of this investigation were consistent with those of other research. 1,8-Cineole, Linalool, Borneol, and Camphor were found to be one of the most common compounds in *Lavandula* species (16).

1,8-Cineole, Linalool, and α-Pinene are major secondary metabolites in the LEO that help to anti-pigmentation impact according to previous studies(17, 18). When these components are encapsulated in a nanoemulsion, the therapeutic efficiency is expected to increase.

Characterization of nanoemulsion

The size distribution for LEO nanoemulsion droplet was illustrated in Table 1. Having a suitable HLB value as a major driving forces, is effective on the stability of nanoemulsions (19). Table 1 demonstrates that a reduction in the HLB value from 16.7 (F1) to 8 (F6) related to binary mixture of surfactants, leding to a decrease in the particle size of the nanoemulsion from 157.600±3.798 to 85.566± 2.227 nm ($P< 0.05$). In the present study, due to the use of ultrasonics, the range of particle distribution is narrow, so that the range of droplet size was obtained from 85.566± 2.227 to 157.600±3.798 nm. The biggest droplet nanoemulsion size was 157.600±3.798 nm for a blend of surfactants with the required HLB equal to 16.7. When the amount of HLB related to the emulsifier is nearer to HLB of oil, the smallest droplets can be obtained with the narrow distribution of size (20). It is necessary to mention that the final HLB of 7.78 for a mixture of surfactants generates the minimal nanoemulsion droplet diameter. On the other side, the outcomes displayed that the minimum droplet diameter with narrow distribution can be produced with a combination of non-ionic surfactants with a maximum HLB alterations. This phenomenon is justified as follows in the existence of a surfactant with a minimum HLB that dissolves in the oil phase and one with a maximum HLB that dissolves in the aqua phase. When two surfactants work well together, the result is that HLB values are closer to each other (19, 21). Furthermore, greater surface area was related to small-sized nanoemulsions, so that, higher concentrations of Span 80 (lipophilic) were required in order to stabilize nanoemulsions by using the surface coverage of nanoemulsion droplets, (20).

Nanoemulsion stability is generally determined by the PDI, which is a principal indicator of

uniformity (22, 23). One of the main instability pathways in nano-emulsion is Ostwald ripening, since the small droplets provide a greater driving force (24). Ostwald ripening is heavily influenced by the PDI (25). It is proven that PDI equal to 0.7 represents the extended distribution of droplets, while PDI score among 0.08 and 0.70 provides an adequate particle diameter distribution (26, 27). The PDI values in Table 1 indicate that all the formulations had PDI values in the range of 0.248±0.003 (F6) and 0.386±0.005 (F1). Comparing PDI to the various HLB values showed similar trends with particle size (Table 1). In the studies conducted, the smallest PDI was related to the state where HLB was equal to 7.78. In their extensive analysis of nanoemulsions based on anise cumin essential oil, Morteza Semnani et al. determined the appropriate HLB for these nanoemulsions. Nanoemulsions with the different amounts of HLB were obtained based on binary mixture different from emulsifers. Their research on the nanoemulsions based on anise myrtle and lemon myrtle essential oils revealed that the minimum droplet diameter and PDI were found for HLB value equal to 9.65 (11). On the other hand, in the study by Enayatifard et al., various ratios of Tween 60, Span 80, and Tween 20 were used to create a nanoemulsion of oregano essential oil. The results showed that in the presence of 80 as a surfactant, the lowest particle size and PDI were found. Also, two important factors, including the type of emulsifier and the HLB value of the nanoemulsion affect the formulation, morphology, and interfacial layer charge covering oil particles (28).

As shown in Table 1, Zeta potential related to the various formulations has been obtained in the range of -3.66 mV to -11.94 mV. The obtained results show that by decreasing the HLB value of nanoemulsion from 16.7 to 7.8, the entire zeta potential was amplified from -4.31± 0.56 mV (F1) to -11.94 ± 0.62 mV (F5) with P value < 0.05 (more negative). Evidence shows that compared to the single surfactant, the tendency of binary mixtures of Tween and Span increased so that more condensed barrier formed around colloidal particles. Also, more surfactants around nanoparticles led to more negatively charged. It was already mentioned that the presence of non-ionic surfactants created a negative charge around the colloidal particles. This phenomenon did not apply to the F6 formulation because the amount of Tween in the formulation was lower than the Span. The results show that the important factor on zeta potential was the ratio of Span to Tween. In the presence of an equal ratio of these two surfactants, the zeta potential reached its maximum value. Furthermore, these two surfactants also affected the size of nanoparticles as well as their impact zeta potential (Table 1). In other words, the presence of more surfactant around the nanoparticles led to smaller sizes and more negative charge. According to Table 1, F5 and F6 had the different zeta potentials, and there was a reduction in zeta potential may be due to the lack of surfactants around nanoparticles by a film layer of Tween formed by Span at high concentrations, . The surface coverage of nanoemulsions led to a reduction of the electrophoretic mobility of the particles and decreased the zeta potential (29, 30). According to the critical micelle concentration of Tween 80 about 0.015 mM, probably low concentration of Tween 80 monomers is in the dispersion medium (31). This fact indicates that rather than forming micelles, surfactant monomers are adsorbed on the hydrophobic surfaces of lipids.

When surfactants come into contact with each other in immiscible liquids, such as water and oil, the hydrophobic parts of the surfactant are oriented towards the oil side, and the hydrophile parts are oriented towards the water side (30). Diminution in the zeta potential of nanoemulsion from -11.94 ± 0.62 mV to -3.66 ± 0.60 mV was observed after reduction in the HLB of Tween: Span mixture to 9.65 and 7.87 related to F5 and F6, respectively. Observations show that nonionic surfactants covered the entire surface while in the presence of great amount of a second surfactant (in this case, Span), the ability to completely cover of the first surfactant was lost (30). Based on the results obtained from the ratio of binary surfactant, the great amount of the second surfactant disturbed the surfactant film around the particles, as a result, the zeta potential decreased. It should be noted that in this research work, the optimal formulation was selected based on zeta potential as the main parameter. The tendency to stability in non-ionic surfactants like Tween and Span 80 was caused by superior steric stabilization (despite minimum zeta potential) and less electrostatic stabilization (30). Also, it has been reported that the dipole nature of the ethoxy groups of nonionic surfactants leads to an increased negative zeta potential nearby in

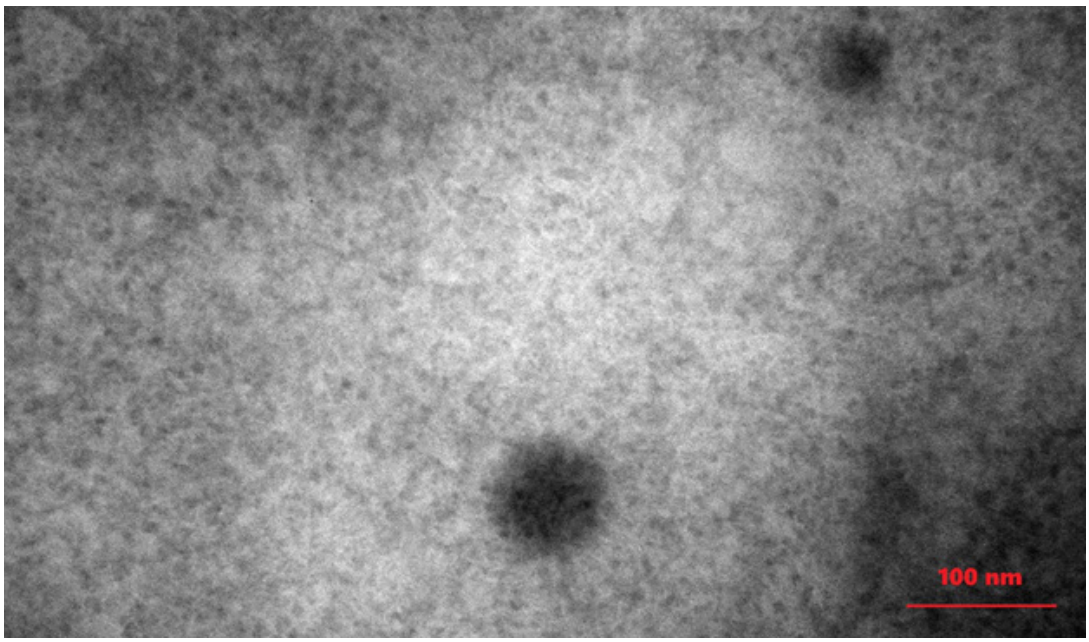

Fig. 1. TEM image of F6.

particles (32).

It should be noted that it is difficult to create nanoparticles with a suitable particle size and narrow particle size distribution range (considered as optimal condition). According to the investigations, F6 with reasonably acceptable particle dimension of 85.56± 2.22nm, zeta potential of -3.66± 0.60 mV and PDI of 0.248± 0.003 were selected for further investigation.

TEM assessment

One of the most important characteristics of the nano-emulsion platform is the structure of its droplets. Morphology of LEO nanoemulsion (has mixed surfactant with HLB equal to 7.78) was compiled. As shown in figure 1, TEM images with negatively stained samples were studied. Spherical nanoparticles with a particle size of about 100 nm were obtained. The results obtained with the TEM technique confirmed the previous droplet size analysis obtained with the DLS assay.

Physicochemical analysis of nanoemulgel

Color and other characteristics as qualities and macroscopic properties of the nanoemulgel formulation were evaluated. The findings showed that smooth, white color, homogeneous texture and typical LEO odor were the characteristics of the nanoemulgel formulation. These properties of the nanoemulgel remained constant for 6 months after production. On the other side, before and after the freeze–thaw cycle, no notable alteration was detected in the properties of the nanoemulgel. Between 5.98 ± 0.05 to 5.99 ± 0.01, different values for pH of the nanoemulgel were obtained. These results show that a nanoemulgel with this pH value does not irritate the cutaneous. The pH number of the nanoemulgel was constant during the experiment, so there was no significant difference in freezing–thawing period. With the mentioned features, these nanoemulgels can be employed locally. In table 3 different physicochemical properties of nanoemulgel before and after the freeze-thawing period (12 days) were shown.

Because of its low time spread, the

Table 3. Outcomes of the assessment of the initial stability of F6 gel (nanoemulgel) before and after the freeze-thaw cycle

Sample	Appearance	pH	Centrifugation
F6 gel(nanoemulgel)-before	Homogeneous, white, with typical LEO odor	5.98 ± 0.05	No noticeable instability in the formulation
F6 gel(nanoemulgel)-- after	Homogeneous, white, with typical LEO odor	5.99 ± 0.01	No noticeable instability in the formulation

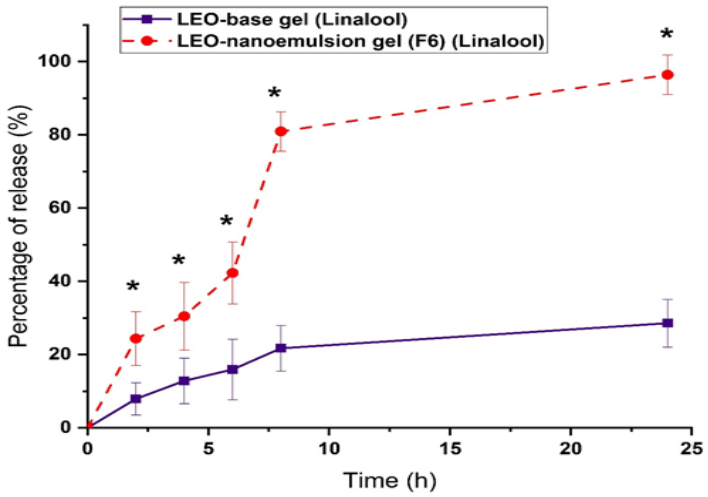

Fig. 2. Profile of linalool release from LEO-nanoemulsion gel and LEO-base gel. (n=3)* (P<0.05)

spreadability of nanoemulgel was great. The therapeutic efficacy of a gel is determined by gel spread. According to this argument, the spreading of the gel on the skin influences its spread evenly. For prepared gels in topical application, having spreadable and encountering the optimal standard are critical. This feature is an important factor in drawing the patient's attention to the treatment process. In the present study, the spreadability of the formulation was found to be 401.6%. It is well known that topical formulations leave a thin layer on the skin, so the consistency of the material and the viscosity of the desired gel have a great effect on controlling the diffusion of the pharmaceutical substance into the dermis. Figure 2 shows the results of checking the viscosity of the carbopol 941 (1% w/v) and nanoemulgel at 5, 10, 20, 50, and 100 rpm (with three repetitions).

In general, consistency is shown by the viscosity of the gel formulations (33). Based on non-Newtonian flow or shear thinning, with enhancing shear, the viscosity of these semi-solid diminishes. The obtained manner is adequate because minimum stream resistance exists in high shear conditions (34). As shown in figure 3, the possible pseudoplastic manner noticed in the nano-emulsion gel leads to a decrease in viscosity, so that when employing some force, it

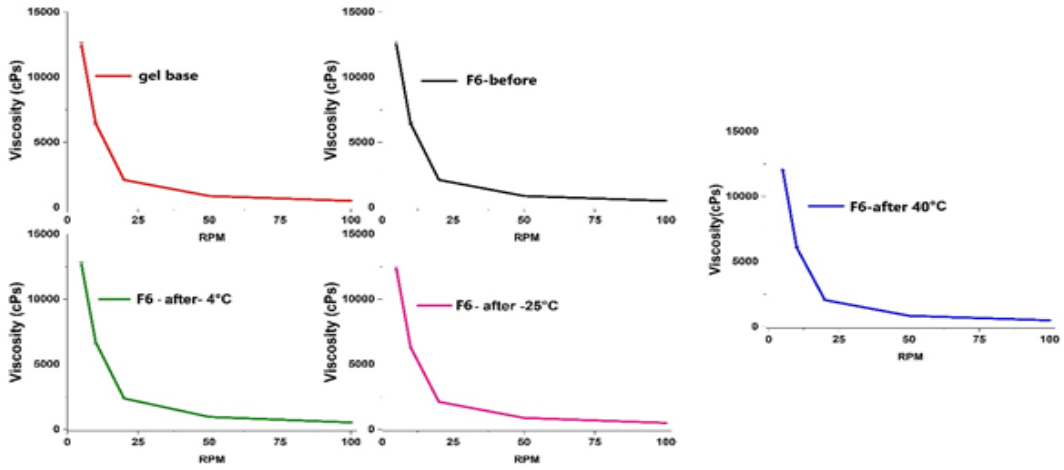

Fig. 3. Shear rate effect on F6 gel (nanoemulgel) viscosity.

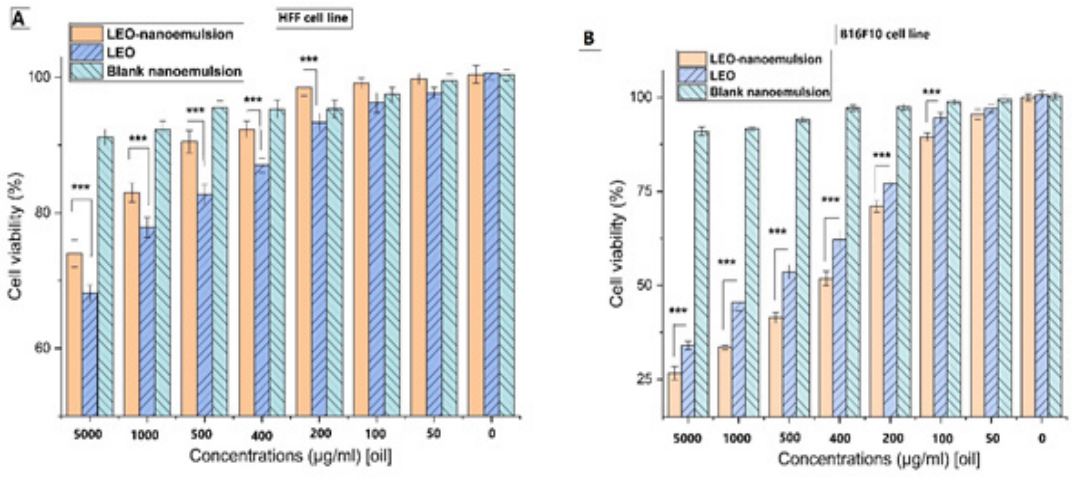

Fig 4. A) Viability of cells at the varied concentrations of LEO, blank nanoemulsion, and F6 (LEO-nanoemulsion) ***(p<0.001) (data are mean ± SD, n =6).Fig 4a. After 24 h exposure, LEO-nano-emulsion (F6) had fewer cytotoxicity effect on HFF cells than pure LEO meaningfully ***(p<0.001). B) After 24 h exposure, LEO-nano-emulsion (F6) had higher cytotoxicity effect on B16F10 melanoma cell than pure LEO significantly ***(p<0.001)

demonstrates high spreadability. Therefore, it can be seen that properties are preserved at the region of touch without leaking (35).

Release trial

An in vitro analysis was conducted to examine the release characteristics of LEO-nanoemulsion gel (containing linalool) and LEO-simple gel (also containing linalool). Given the limited solubility of linalool in aqueous environments, a release medium consisting of phosphate buffer at pH 5.5 supplemented with 20% ethanol was chosen for the study. The results showed that the nanoemulsion gel formulation released around 30% of the LEO content within the initial 4 hours, with a subsequent escalation in the drug's dissolution rate, reaching approximately 96% by 24h (Fig. 2). Notably, the LEO-nanoemulsion gel exhibited a significantly higher percentage of drug release compared to the LEO-base gel over the 24 hours that was a statistically significant difference ($p<0.05$).

According to preceding experimental research, Nano emulsions enhance drug release by increasing surface area for faster release, improving drug solubility and bioavailability, enabling controlled and enhanced drug permeation through biological membranes, and protecting sensitive drugs from degradation. This leads to improved therapeutic outcomes and efficacy(36).

Cytotoxicity test

The HFF normal fibroblast cell line and B16F10 cell line were tested for the cytotoxicity effect of the LEO, F6, and blank nanoemulsions at a range of concentrations from 50 to 5000 µg/mL (Fig. 4). As shown in Fig. 4A, the cell survival of HFF normal fibroblast significantly decreased over a period of 24 h after testing by F6 and LEO ($p < 0.001$). After 24 h exposure to the formulations of F6 and LEO in the HFF cell line, cell viability was obtained 73% and 68%, respectively. This result illustrated that LEO-nano-emulsion (F6) had fewer cytotoxicity effects than pure LEO meaningfully. Additionally, using F6 and LEO on B16F10 melanoma cells resulted in significant reductions in cell survival over 24 h compared to negative control ($p < 0.001$) (Fig.4b). The cell survival percentage for 24 h exposure to the formulations in the B16F10 cell line related to F6 and LEO was found to be 26% and 33%, respectively. This outcome illustrated that LEO-nano-emulsion (F6) had higher cytotoxicity effect on B16F10 melanoma cell than pure LEO significantly ($p<0.001$). Furthermore, the IC50 of F6 and LEO on HFF normal fibroblast cells and the B16F10 cell line was 783.100±27.900 µg/mL and 487.300±23.400 µg/mL and 247.700±17.800 µg/mL and 317.900±20.500 µg/mL, respectively.

Small-scale particles can easily be transfected into cells and cause cell death. Drug nanoparticles are commonly recognized as hazardous to tissue. It is also possible that nanovesicles might cause

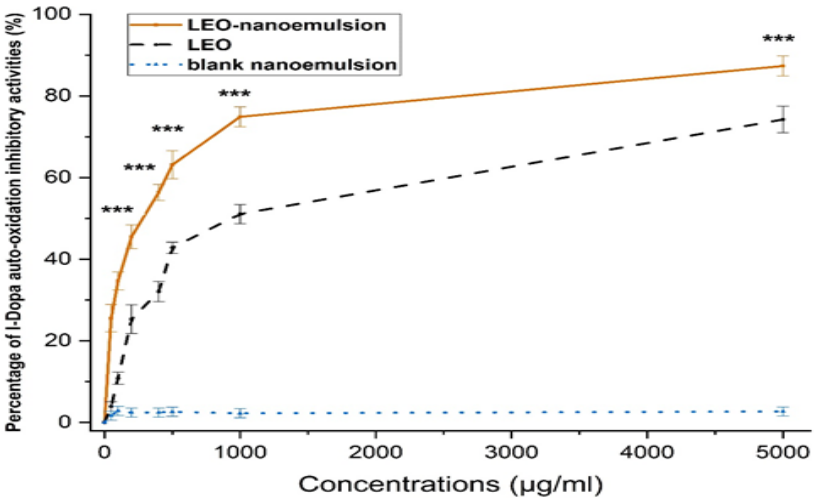

Fig. 5. L-Dopa auto-oxidation inhibitory activities of LEO, LEO-nanoemulsion (F6), and blank nanoemulsion. ***(p<0.001)

oxidative stress and enhance their interaction with cells and proteins due to the surface electrical charge on the surface, which can lead to reduce viability of cells (37, 38). Due to the enzymatic effect on the ester bonds, non-ionic ester surfactants, such as Span and Tween, are biocompatible in formulations (blank nanoemulsion and F6) (39). As a result, nanoemulsion formulations reduced cell death (40). In another theory, the reduced toxicities can be attributed to the appropriate size of the alkyl chain of the surfactant.

Inhibitory L-DOPA auto oxidation analysis

Patients' cosmetic quality of life is significantly affected by skin hyperpigmentation, which requires effective anti-melanogenic products (41). In this study, concentration-dependent inhibition of oxidation of L-DOPA were studied for both LEO-nanoemulsion (F6) and LEO. The data in Fig. 5 shows that the maximum inhibitory concentration of LEO (5000 µg/mL) was found to be 74.22± 3.26%. Additionally, higher F6 concentrations resulted in greater inhibition (87.33± 2.45%) (p<0.001).

The antioxidant properties of LEO can indirectly affect L-DOPA auto-oxidation, despite not being a direct inhibitor. As part of LEO's mechanism, free radicals are scavenged, chain reactions are terminated, metal ions are chelated, L-DOPA is stabilized, and oxidative stress is reduced (42). Based on these findings, LEO might be useful as an agent for skin lightening when incorporated into non-ionic surfactant nanoemulsions (F6) to enhance the process of inhibitory auto-oxidation.

Melanin content analysis

A murine melanoma cell line B16F10 was measured for intracellular melanin release in a melanin content assay. The results clarified that in a concentration-dependent manner, both LEO and F6 inhibited melanin synthesis without cytotoxicity. Blank nanoemulsion, however, did not have any significant impact on melanin content, regardless of its concentration. After treatment with the various concentrations of LEO and F6 (5000, 1000, 500, 400, 200, 100, and 50 µg/mL) for 24 h, a decrease in melanin content was demonstrated with a spontaneous melanogenesis assay (Fig. 6) so that, the maximal inhibition of 38.89± 1.54% and 21.95± 1.08% (at 5000 µg/mL) were found respectively (p<0.001).

The F6 nanoemulsion possessed greater suppression of melanin content than either LEO or blank nanoemulsion during normal melanin production. LEO is not directly involved in the regulation of melanin, but it had the potential to exert indirect effects on melanin production. Constituents of LEO, including linalool and linalyl acetate, exhibit anti-inflammatory and antioxidant properties, which may have the capacity to mitigate hyperpigmentation resulting from inflammatory responses and oxidative stress. Furthermore, the anxiolytic effects of LEO could indirectly contribute to melanin balance by averting hormonal disruptions associated with stress. Additionally, LEO

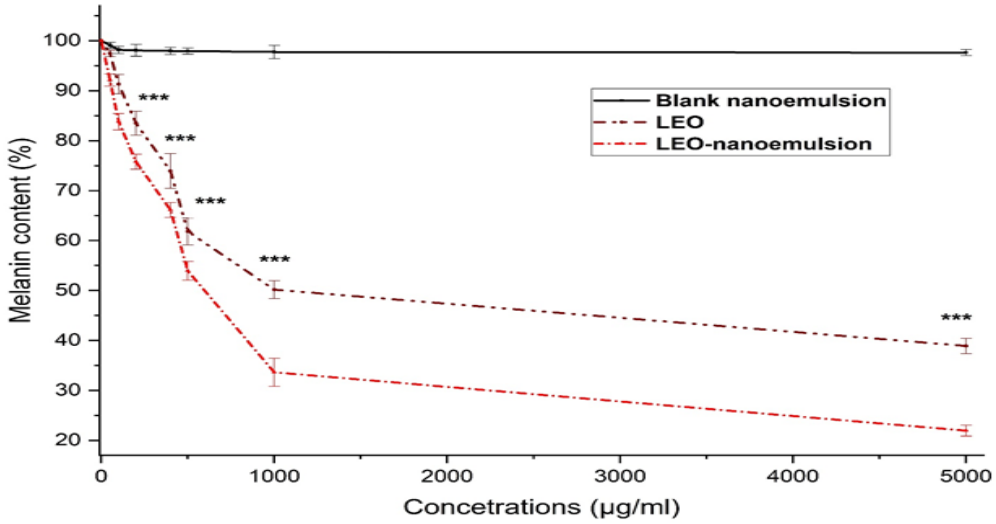

Fig. 6. Percentage of melanin content of LEO, LEO-nanoemulsion, and blank nanoemulsion. *(p<0.001)

may protect against ultraviolet (UV) radiation and is occasionally incorporated into skin-lightening products designed to address hyperpigmentation, although the precise mechanisms underpinning these effects require further elucidation (43). These findings demonstrate that integrating LEO into a non-ionic surfactant nanoemulsion (F6) can potentially inhibit melanin synthesis, making it a beneficial choice for cosmetic applications as a highly effective skin lightening agent.

Dermal absorption test

In pharmacological skin absorption research, murine dermis is frequently employed due to its analogous structural and permeability characteristics with human skin. Murine dermis is more readily obtainable for investigative purposes, and its diminutive size facilitates handling in laboratory environments. Moreover, the utilization of murine models aids in comprehending potential interactions between a drug and human skin before proceeding to clinical trials (44).

Fig. 7 exhibits the cumulative quantities of LEO (specific linalool) penetrated through the rat skin (transdermal targeting) for LEO-nanoemulsion gel and LEO-base-gel. The LEO-nanoemulsion gel demonstrated a higher penetration into and across the cutaneous layers revealing that it

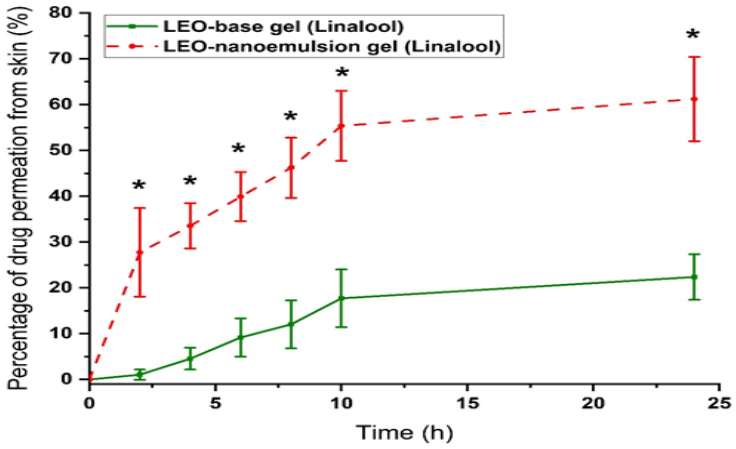

Fig 7. Total percentage of linalool across from skin. The total percent of linalool (LEO-nanoemulsion gel) in receptive segment was higher than LEO-base-gel *(p<0.05).

Table 4. Skin irritation scores following topically administration

	control		F6gel (nanoemulgel)		LEO		blank nanoemulgel		Formalin	
Rat num	[a]Erythema	[b]Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema
1	0	0	0	0	0	0	1	1	4	3
2	0	0	0	0	0	0	0	0	3	4
3	0	0	0	0	0	0	0	1	3	3
4	0	0	0	0	1	1	0	0	4	3
5	0	0	0	0	1	0	0	0	3	3
6	0	0	1	1	0	1	1	0	3	4

was highly appropriate for transdermal delivery compared to LEO-base-gel. The greatest level of linalool detected in the receptor compartment for LEO-base-gel (22.378±4.972% or 6.773± 1.505µg/cm2) was significantly lower than that of the LEO-nanoemulsion gel (61.206± 9.183% or 18.525± 2.779µg/cm2) ($p < 0.05$).

Nanoemulsions are increasingly popular in cosmetics due to their ability to enhance the delivery and effectiveness of active ingredients in skincare products. Nanoemulsions in cosmetics work by enhancing the delivery of active ingredients through the skin's outer layer due to their small droplet size, which allows deeper penetration and improves effectiveness of ingredients like vitamins and peptides. They also stabilize and protect sensitive ingredients, increasing their solubility and shelf life. Nanoemulsions provide a non-greasy lightweight texture, enhance skin hydration by reducing water loss, and improve the overall appearance and feel of products, leading to better consumer satisfaction and product performance (45). Also, Essential oils (in this case LEO) enhance medicine absorption in formulations by disrupting the lipid matrix of the stratum corneum, the outermost skin layer, making it more permeable. This increased permeability allows drug molecules to penetrate the skin more effectively, improving the delivery of active ingredients. Essential oils, rich in compounds like terpenes and phenols, further support this process by altering skin permeability and enhancing overall drug absorption (46).

These findings suggested that a nanoemulsion gel formulation would be the best alternative for topical administration than a conventional gel preparation.

Irritation assessment on cutaneous

In addition to releasing medication, medication delivery platforms must also ensure that they are irritant-free, toxicity-free, and immunogenic-free. For LEO-nanoemulgel to be considered safe for cosmetic use, they must prevent allergic reactions on the skin (47). Cutaneous sensitization as one of the most typical side effects of local remedy formulation has a direct relationship with the concentration of the active substance. The use of a controlled drug release formulation may ultimately allow reducing cutaneous diffusion and enhance intra-follicular absorption, thus reducing the adverse effects of a formulation (48).

The impact of various preparations on the cutaneous sensitization score, such as edema and erythema, are shown in Table 4. Based on the findings per Woodward, Draize, and Calvery, materials with a value of two or less can be classified as non-sensitive. Consequently, the F6 gel, with features that included no discomfort, increased patient adherence, and epidermis compatibility, was obtained. It is a true assumption that F6 gel, as the optimum nanoemulgel, is safe for two cases, including dermatological use on human skin and treatment of hyperpigmentation symptoms. Hussein O. Ammar et al.'s findings showed that nanoemulsions based on dorzolamide were nonirritant (49).

Histological evaluation

The investigation of the therapeutic effect of F6 gel was carried out in the first stage hematoxylin-eosin for staine of rat dermis and then, dermal sensitivity or harmful effects were studied using a microscope the histopathological alterations. Also, histopathological images versus negative control was compared (Fig. 8). Based on the factors included i) severity of epidermal and sub-epidermal cellular disruption, ii) the presence of prominent blood vessels, iii) granulation tissue, iv) inflammatory cell infiltration, and v) collagen deposition, the following ranking was conceivable for preparations: control < F6 gel (nanoemulgel) < Lavender essential oil (LEO) < blank nanoemulgel < formalin solution. Consequently, the nanoemulsions can be introduced as a reliable and practical tool for skin drug delivery without skin irritation. The observed results for histopathological examinations are similar to those previously reported for dorzolamide, so

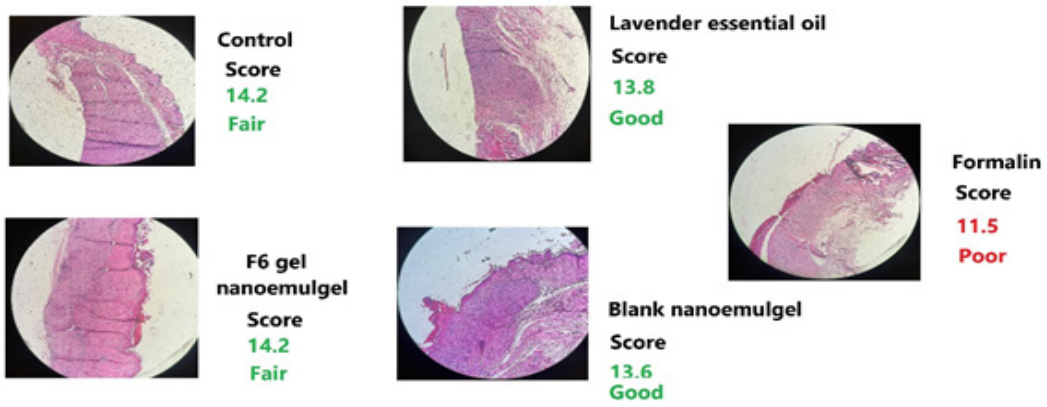

Fig 8. Histological analysis of hematoxylin and eosin (H&E) stained cutaneous sample from diverse administration groups on day 3 post-treatment.

the nanoemulsions presented in this study are promising in terms of safety (50).

Stability studies

As shown in Table 5, evaluation of stability with factors such as zeta potential, particle size, and PDI were performed for three months at 4, 25, and 40 °C. The results showed that for nanoemulsions kept at these three temperatures, the same colloidal nanometer range was found. Investigations carried out for nanoemulsions stored at 4, 25, and 40°C clrarify that no significant increments were found for PDI as wider distribution due to particle growth. No significant change in size, PDI, and zeta potential for nanoemulsion kept at 4, 25, and 40 °C proved they are that suitable storage temperature for nanoemulsion

Nanoemulsions have a pronounced positive impact on the stability of essential oils. By reducing essential oil droplets to nanoscale particles and encapsulating them with surfactants, nanoemulsions enhance the solubility and dispersion of essential oils in aqueous systems, thereby preventing phase separation and ensuring even distribution. Furthermore, they protect essential oil constituents from oxidation, light exposure, and heat, extending the shelf life of the oils (51, 52).

CONCLUSION

In this study, using the ultrasonication method as a green technology for the incorporation of LEO into nanoemulsions (a blend of nonionic surfactants) was positively developed. The obtained nanoemulsion had the following the characteristic including small droplet size (85.566± 2.227 nm), PDI (0.248±0.003), zeta potential (-3.66±0.60) and spherical morphology. LEO-nanoemulsion illustrated fewer cytotoxicity impacts on HFF normal cells than pure LEO. Also, LEO-nanoemulsion displayed a higher toxic effect than pure LEO on B16F10 melanoma cancer cell. After treatment on both cell lines with F6, about 73% and 23% of the HFF cells and

Table 5. F6 nano-emulsion stability results after 3 months

Storage condition	Time (month)	Particle size (nm)	PDI	Zeta potential (mV)	Organoleptic
Initial	-	89.03± 2.14	0.256± 0.006	-11.94± 0.62	Stable-milky
4°C	1	89.96± 1.15	0.25± 0.004	-11.36± 0.40	Stable-milky
	2	89.83± 1.25	0.258± 0.002	-11.53± 0.25	Stable-milky
	3	90.03± 0.89	0.261± 0.002	-11.46± 0.58	Stable-milky
25°C	1	89.76± 1.85	0.255± 0.004	-11.66± 0.59	Stable-milky
	2	89.76± 1.96	0.258± 0.004	-11.93± 0.59	Stable-milky
	3	90.06± 1.87	0.259± 0.003	-11.93± 0.59	Stable-milky
40°C	1	90.90± 1.27	0.261± 0.004	-12.56± 0.50	Stable-milky
	2	91.70± 1.05	0.262± 0.004	-12.66± 0.58	Stable-milky
	3	91.93± 0.95	0.263± 0.005	-12.73± 0.64	Stable-milky

B16F10 survived, respectively. Also, F6 gel was found to be safe in a cutaneous sensitivity assay. The usefulness of nanoemulgel for topical drug targeting by histological evaluation was confirmed. Nanoemulgel loaded with LEO showed some anti-hyperpigmentation activities with suppression of melanin formation and L-Dopa auto-oxidation inhibitory and also showed maximum impact in matched to LEO and blank nanoemulgel. This study suggests that non-ionic surfactants nanoemulgel loaded with LEO can have potential anti-hyperpigmentation activity in pharmaceutical and cosmetic products.

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ETHICAL APPROVAL

The Ethics approval board for Animal Investigation at HUMS provided their approval to all animal experiments which were confirmed a registration number =IR.HUMS.REC.1402.206.

DISCLOSURE STATEMENT

No potential conflict of interest was reported by the authors.

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