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RESEARCH

Preparation and Fabrication of Nanoemulsions of *Spent Coffee Oil* and *Ganoderma lucidum Spore Oil* for Skin Whitening and Anti-Wrinkle Applications: In Vitro and In Vivo Evaluations

Maryam Rahimi[1] · Majid Azizi[2] · Hamid Soorgi[3] · Fatemeh Gheybi[4,5] · Ali Nokhodchi[6] · Amir Amani[1,7] · Fatemeh Oroojalian[1,7]

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Abstract
Spent coffee ground is an inexpensive and readily available by-product of the food industry which contains a high concentration of antioxidants. The purpose of this study was to use spent coffee oil (an abundant natural product) and *Ganoderma lucidum* spore oil (a natural skin care substance) to make nanoemulsions for use in cosmetic products for whitening skin and also as anti-aging. To this end, nanoemulsions of *Ganoderma* spore oil and spent coffee oil and nanoemulsions containing both oils (mix nanoemulsions) were made by spontaneous emulsification method. To investigate the effect of the manufactured nanoemulsions, the shaved back skin of mice was exposed to UVA radiation to create pigment followed by topical application of the formulations. The analysis of skin factors was checked by the skin analyzer device. From the results, the nanoemulsion (NE) of the oils as well as the NE containing both oils (Mix NE) showed improved performance compared to their bulk forms. The cell viability tests did not show cytotoxicity in the examined concentrations. The penetration studies on the skin using the Franz diffusion cell method indicated enhanced penetration of NE into the skin. The melanocyte cell culture demonstrated a reduction in melanin production, following treatment with the NEs. The animal studies exhibited a significant reduction in acne score of spent coffee oil NE and Mix NE when compared with the control group. Furthermore, the spots detected on the skin reduced significantly using Mix NE compared with hydroquinone cream and the control group. In conclusion, the developed nanoemulsion containing spent coffee oil and *Ganoderma lucidum* spore oil showed great potential as a skin whitening and anti-aging product in vivo.

Keywords Nanoemulsions · Spent coffee oil · *Ganoderma lucidum* spore oil · Skin whitening · Anti-wrinkle

1 Introduction

In the personal care and beauty market, the simultaneous use of cosmetics and food products has gained attraction in recent years, impacting different aspects of lifestyle (e.g., eating habits, use of nutritional supplements, and use of food-based cosmetics). Ingredients originating from natural food sources are touted for their numerous skin-benefitting properties [1–3]. Today, consumers are driving innovation in cosmetics by using safe components. They are more likely to spend more for a cosmetic that offers greater skin benefits and are more likely to select cosmetics comprised of natural ingredients [4]. To find new sources of active ingredients for beauty products, several research groups have recently begun processing food or

✉ Fatemeh Oroojalian
f.oroojalian@ut.ac.ir; f.oroojalian@gmail.com

[1] Department of Medical Nanotechnology, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran

[2] Department of Horticultural Science and Landscape, Faculty of Agriculture, Ferdowsi University of Mashhad (FUM), Mashhad, Iran

[3] Department of Internal Medicine, School of Medicine, North Khorasan University of Medical Sciences, Bojnurd, Iran

[4] Department of Medical Biotechnology and Nanotechnology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

[5] Nanotechnology Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran

[6] School of Life Sciences, University of Sussex, Brighton, UK

[7] Natural Products and Medicinal Plants Research Center, North Khorasan University of Medical Sciences, Bojnurd, Iran

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plant wastes to produce antioxidants, antimicrobials, and anti-aging agents [5]. Cosmetics and cosmeceuticals with herbal active ingredients are commonly recognized as having minimal adverse effects, which has made them very popular and desirable in the market, both in personal care (e.g., hair treatment, wrinkles, and hyperpigmentation) and medical disorders (e.g., psoriasis, bloating, and hair loss) [6]. Recently, there has been an increase in interest regarding the application of nanoparticles across various biomedical fields, including targeted drug and gene delivery [7, 8], bioimaging [9, 10], and the development of biosensors [11]. Nanoemulsions (NEs) are dispersions of oil, surfactant, co-surfactant, and aqueous phase which are optically isotropic and have promising features for topical or transdermal administration [12] with transparent or semi-transparent appearance and sizes below 100 nm [13]. Compared to traditional formulations, NEs offer several advantages, such as improved drug solubility, stability, and penetration through different biological barriers [14]. A large number of medication categories, including proteins [15], antibiotics, and nonsteroidal anti-inflammatory drugs, have been successfully evaluated to be loaded in NEs [6]. Due to their simplicity in preparation, ability to control particle size, and appropriate stability, NEs are suitable delivery systems for use in cosmetics [16]. Because NEs readily create a thin lipid film, which promotes improved performance and greater bioavailability, formulations specifically intended for topical use can be administered with ease [17]. A popular beverage worldwide is coffee [18]. Spent coffee ground is an inexpensive and readily available by-product of the food industry which contains a high concentration of antioxidants. The primary components of coffee that can display antioxidant activity are melanoidins, caffeine, and chlorogenic acid [19]. It is hugely left over from retail establishments, private coffee cafes, and instant coffee factories [20]. Spent coffee grounds could be used to produce new products for other sectors such as cosmetics [21]. Coffee oil has the potential to serve as a raw material for cosmetics manufacturing due to its ability to relieve skin irritation and aging which could be due to the actions of both linoleic acid and palmitic acid [22]. An emulsion formulation of coffee oil showed that coffee oil is effective in mitigating skin damage from UVB (280–320 nm) radiation as indicated by sun protection factor (SPF) [23]. Since ancient times, mushrooms have been prized as traditional sources of organic bioactive substances. More recently, they have been investigated as possible ingredients in cosmetics. It has long been recognized that a variety of mushrooms and their components may be beneficial for keeping skin and hair healthy. Compounds such as phenolics and polyphenolics, vitamins, polysaccharides, volatile organic compounds, terpenoids, and selenium serve as ingredients in personal care products. Such products are alternatives to chemical cosmetics because of their anti-aging, anti-wrinkle, skin-whitening, and hydrating properties [24]. Among the mushrooms, *Ganoderma lucidum* has attracted global interest due to its diverse range of biological activities, particularly those intended for skin whitening [25, 26]. Given the fact that *G. lucidum* extract has a strong whitening effect and low cytotoxicity, it is not surprising that several whitening commercially available face mask products contain its extract. The term *Ganoderma* originates from the Greek words *ganos*, which conveys the concepts of brightness and sheen, and derma, which signifies skin [27]. Natural products are recognized as safe ingredients, beneficial for the skin, and popular with consumers [28, 29]. Spent coffee oil is rich in antioxidant activity (due to melanoidin, caffeine, and chlorogenic acid) [19] and can help improve inflammation and reduce skin aging (due to linoleic acid and palmitic acid) [22]. *G. lucidum* extract has a strong whitening effect and low cytotoxicity [27]. Also, *Ganoderma* protein has many biological functions, including antioxidant, antibacterial, antiviral, and anti-inflammatory properties, and also inhibits melanin formation [30]. Due to these properties, these two natural substances have the potential to be used in cosmetic products. The purpose of this study was to use spent coffee oil as an abundant natural product with economic value, as well as *Ganoderma lucidum* spore oil as a natural skin care substance to make nanoemulsion for use in cosmetic products as skin whitening and anti-aging.

2 Materials and Methods

2.1 Materials

Spent coffee ground oil (SCO) and *Ganoderma lucidum* spore oil (GSO) were obtained from Ferdowsi University of Mashhad, Faculty of Agriculture. Surfactants (Tween 80, Span 80, Tween 20) and co-surfactant (ethanol) were from Merck (Germany). Melanoma cell lines (B16-F10) and human fibroblast cell lines (L929) were procured from Ferdowsi University of Mashhad.

2.2 Extraction of Oil from Spent Coffee Ground and *Ganoderma lucidum* Spore

Oil extraction from spent coffee ground [31] and *Ganoderma lucidum* spore [32] was performed according to previously described methods.

2.3 Preparation of Nanoemulsion (NE)

All formulations were prepared using the spontaneous emulsification method. By testing different amounts of oil, surfactants (Tween 80, Tween 20, Span 80), co-surfactant (ethanol), and distilled water, the optimal formulation was recognized to be (3% (w/w) oil, 22% (w/w) Tween 80, 6% (w/w) Span 80, 14% (w/w) Tween 20, 5% (w/w) ethanol, and 50% (w/w) distilled water). To prepare the NE of SCO and GSO, initially, the oils (SCO and GSO) were mixed with the surfactant system and

co-surfactant. Then, distilled water was added dropwise and stirred at 30 °C for 60 min to obtain a clear dispersion, representing the NE. To obtain the Mix NE, 1.5% (w/w) of SCO and 1.5% (w/w) of GSO were mixed thoroughly followed by the same procedure mentioned above for the preparation of NE. The final composition of each formulation is shown in Table 1.

2.4 Droplet Size and PDI Analysis

Non-diluted samples were analyzed for droplet size and PDI via dynamic light scattering (DLS) at a scattering angle of 90° using a DLS-9900 system (Scatteroscope I, K-One, South Korea). PDI and droplet size of each formulation are shown in Table 1.

2.5 Nanoemulsion Stability Test

To evaluate the stability of the obtained NEs, the formulations were stored at various temperatures (4, 25, and 40 °C). At different time intervals (1, 7, 21, 30, and 60 days), the properties of the NE were investigated in terms of clarity, phase separation, and droplet size distribution.

2.6 MTT Assay

The cell suspension of L929 cells was seeded in a 96-well plate and cultured for cell adhesion (24 h). Subsequently, different concentrations of NE (100, 50, 25, 12.5, 6.25 µg/mL = µg of oil/mL of medium) were used for the treatment of NE for 72 h. The cells incubated in a culture medium without NE were used as a negative control. Then, the MTT solution (0.5 mg/mL) was incubated for 4 h in 5% CO_2 at 37 °C. After the incubation, the culture medium was removed, followed by dissolving formazan crystals by adding 100 µL of DMSO. Absorbance in a 96-well plate was recorded at 570 nm by an ELISA plate reader, and cell viability was reported [7, 33–35].

2.7 Skin Penetration Test

Penetration performance was assayed by Franz diffusion cell system in vitro (Fig. 1). Ethics approval was granted by the Research Ethics Committee of Laboratory Animals at the North Khorasan University of Medical Science (Approval ID: IR.NKUMS.AEC.1401.005, dated: 2022–08–17). After anesthetizing the mice (5 weeks old) and shaving the back hair, the skin of the shaved area was separated and washed with normal saline. The separated skins were mounted on diffusion cells in such a way that the visceral part was facing the recipient compartment. The selected formulation was placed in the donor phase compartment, and the receiver phase contained phosphate buffer saline solution (pH 7.4). The contents of the receiver phase were stirred using a magnet at a speed of 250 rpm, and the temperature was maintained at 37 °C. To prevent the evaporation of the donor phase, the top of the cells was closed by parafilm. At different time intervals

Table 1 The composition, droplet size, and polydispersity index (PDI) of the three selected nanoemulsions prepared for studying droplet size

Sample	Ethanol % (w/w)	Tween 20 % (w/w)	Span 80 % (w/w)	Tween 80 % (w/w)	Oil % (w/w)	Distilled water % (w/w)	Droplet size (nm)	PDI
SCO NE	5	14	6	22	3	50	102 ± 3.2	0.32 ± 0.02
GSO NE	5	14	6	22	3	50	105 ± 0.81	0.32 ± 0.01
Mix NE	5	14	6	22	3	50	110 ± 0.4	0.32 ± 0.02

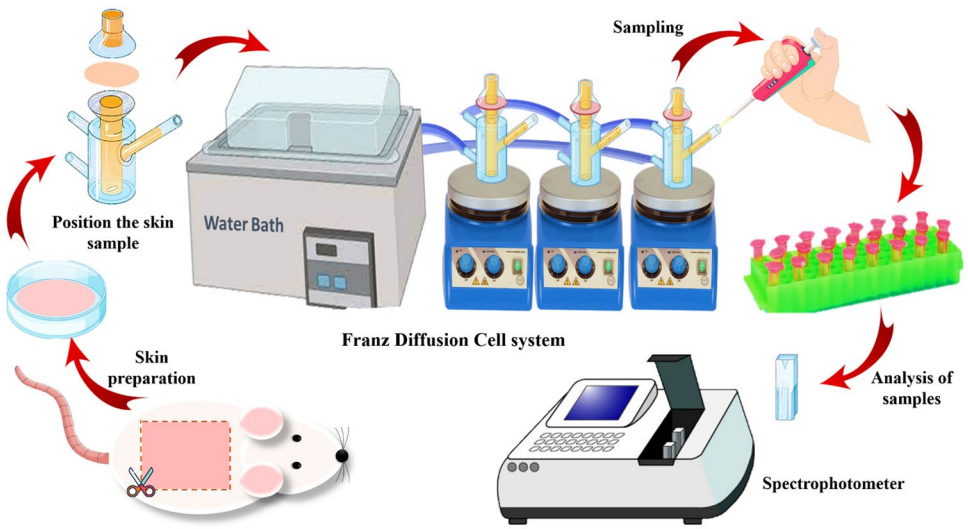

Fig. 1 Schematic representation of the skin penetration test

(0, 1, 3, 6, and 12 h), 2 mL of the samples were withdrawn. The withdrawn samples were replaced with fresh PBS (equal volume) to maintain the volume in the receiver phase constant. The absorbance of the samples was determined at a wavelength of 231 nm for spent coffee ground oil (SCO) and 214.5 nm for *Ganoderma lucidum* spore oil (GSO) by UV–Vis spectrophotometer (CECIL, England), and the skin penetration percentage was calculated [36].

2.8 Measurement of Intracellular and Extracellular Production of Melanin by B16-F10 Melanoma Cells

Melanoma cells (B16-F10, as the cells producing melanin) were seeded in a 24-well plate in DMEM without phenol red to culture for 24 h for cell adhesion. Then, NEs (50 and 100 µg/mL) were exposed to melanocyte cell aggregates for 48 and 72 h. To measure intracellular melanin, each well received 100 µL of NaOH (1 N, containing 10% DMSO), which was then heated for 90 min at 80 °C. Then, the absorbance was recorded using an ELISA reader at 490 nm. A 96-well plate was filled with 100 µL of cell culture media, and the absorbance was measured to determine the amount of extracellular melanin present. The experiments were repeated three times, and the average absorbance was used. To determine the depigmenting index (DI) in the treated samples relative to the untreated control, the following equation was used [37].

$$\text{Depigmenting index (DI)} = \frac{\text{melanin}_{\text{untreated}} - \text{melanin}_{\text{treated}}}{\text{melanin}_{\text{treated}}} \times 100$$

2.9 Animal Experiments

A total of 35 mice (5 weeks old) (Ethical approval code: IR.NKUMS.REC.1397.107) were divided into seven groups, based on the treatment received. These include control (aloe vera gel, which was used as a preservative and thickener of NEs), control + (hydroquinone cream, 2%), SCO NE (gelified nanoemulsion of spent coffee ground oil), SCO (gelified spent coffee ground oil), GSO NE (gelified nanoemulsion of *Ganoderma lucidum* spore oil), GSO (gelified *Ganoderma lucidum* spore oil), and Mix NE (gelified mixture of SCO NE and GSO NE, having 1.5% of each oil).

Twenty-four hours before the studies, the dorsal area of the trunk of the mice was shaved. Then, UVA was radiated on the skin of the mice on days 2, 4, and 6 with a dose of 6 J/cm^2 for 3 min. After that, the treatments were applied daily for 2 weeks. Using a skin analyzer (VanClear, China), the following skin parameters were determined on days 0, 7, and 21.

UV acne: acne severity score, determined using UV
UV spot: skin pigmentation score, determined using UV
PL spot: skin pigmentation score, determined using polarized light
Wrinkle: size and density of wrinkles, determined using visible light

2.10 Skin Irritation Test

Four New Zealand white rabbits were used to assess acute cutaneous irritation using the Draize model. The rabbits' dorsal trunk was shaved about 24 h before the experiment. Next, 2 × 2 cm gauze patches having 0.5 g of each formulation were applied, and the rabbit trunk was wrapped. Following the removal of the patch, skin irritation was noted and categorized as erythema and edema reactions. A Draize scoring system was employed to score the reactions. The Primary Dermal Irritation Index (PDII) was calculated based on the following equation [38]:

$$PDII = \frac{[(\Sigma\ erythema\ grade\ at\ 1/24/48/72h + \Sigma\ edema\ grade\ at\ 1/24/48/72h)]}{4 \times \text{ number of rabbits}}$$

The degree of irritation was categorized as non-irritation, slight irritation, moderate irritation, or severe irritation according to the PDII values (i.e., 0–0.5, 0.5–2.0, 2.1–5.0, and 5.0–8.0, respectively).

2.11 Statistical Analysis

At least three repetitions were considered for each experiment. GraphPad Prism 10 was used to analyze the data, which were described by mean and standard deviation. All the experimental data were analyzed by one-way analysis of variance, followed by a post-hoc Tukey test.

3 Results and Discussion

3.1 Preparation and Characterization of Nanoemulsion Formulations

In this study, nanoemulsions (NEs) were prepared by spontaneous emulsification. Upon the addition of water,

translucent liquid dispersions from SCO NE, GSO NE, and Mix NE emerged. Table 1 shows the composition of each formulation plus DLS results for SCO NE, GSO NE, and Mix NE. The DLS results showed that there is no big difference in the droplet size of the NE prepared and their droplet size is in the range of 102–110 nm. The PDI of the NE formulations varied from 0.32 ± 0.01 to 0.32 ± 0.02, indicating a homogeneous droplet size distribution.

The stability test (formulations were stored at 4, 25, and 40 °C up to 60 days) was also performed on these three NE formulations, and the effects of storage conditions on droplet size, clarity, and PDI were investigated. Droplet size and PDI were set up in triplicate, and average values were calculated. The effect of time and temperature on the droplet size and PDI of the obtained nanoemulsions is listed in Table 2. The NE formulations remained completely clear and stable throughout the test, and no phase separation or color change was observed. Images of the NE formulations after 60 days of storage at 4, 25, and 40 °C are shown in Fig. 2.

The results shown in Table 2 and Fig. 3 indicate that the obtained NE formulations are quite stable in terms of droplet size as no significant changes were obtained in the droplet size at time zero with other storage times of the stored NE formulations under different conditions.NE formulations had monodispersed size distribution (PDI < 0.33). The PDI value represents the size distribution of nanoparticles. Samples with homogenous and equally sized particles would have a lower PDI, indicating uniform particle

Table 2 Effects of storage conditions on the droplet size and PDI of the NE formulations and stability after 60 days at 4°C, 25 °C, and 40 °C (mean ± SD, $n = 3$)

PDI	Droplet size (nm)							Storage condition	Sample
	Day 60	Day 30	Day 21	Day14	Day 7	Day 1	Day 0		
0.32 ± 0.03	100 ± 1.2	103.0 ± 1.6	103.2 ± 0.89	103.5 ± 1.02	103.6 ± 1.07	102.4 ± 0.54	102 ± 0.81	4 °C	SCO NE
0.32 ± 0.02	101.0 ± 3.09	104.2 ± 2.6	104.0 ± 2.8	103.6 ± 3.02	103.0 ± 3.02	103.0 ± 2.9	102 ± 3.2	25 °C	
0.32 ± 0.02	102.0 ± 0.4	107.0 ± 0.81	105.5 ± 0.4	103.0 ± 1.2	102.5 ± 0.81	102.2 ± 0.16	102 ± 1.2	40 °C	
0.32 ± 0.04	103.5 ± 0.4	105.0 ± 0.24	105.8 ± 1.1	105.4 ± 0.98	105.7 ± 1.1	105.5 ± 1.02	105 ± 0.9	4 °C	GSO NE
0.32 ± 0.01	102.0 ± 0.81	106.3 ± 1.7	106.0 ± 1.4	105.3 ± 1.6	105.1 ± 0.32	105.0 ± 1.06	105 ± 0.81	25 °C	
0.32 ± 0.03	104.0 ± 0.4	107.0 ± 1.06	106.9 ± 0.7	106.0 ± 1.6	105.5 ± 1.2	105.3 ± 1.06	105 ± 0.7	40 °C	
0.32 ± 0.03	108.2 ± 1.4	110.2 ± 0.97	111.1 ± 0.81	111.2 ± 0.65	111.5 ± 1.02	110.8 ± 0.43	110 ± 0.81	4 °C	Mix NE
0.32 ± 0.02	108.0 ± 0.73	110.5 ± 1.06	110.9 ± 0.81	110.7 ± 1.06	110.2 ± 0.89	110.1 ± 0.4	110 ± 0.4	25 °C	
0.32 ± 0.04	110.2 ± 0.81	112.4 ± 0.32	111.5 ± 1.2	110.9 ± 1.1	110.0 ± 1.63	109.5 ± 0.62	110 ± 0.73	40 °C	

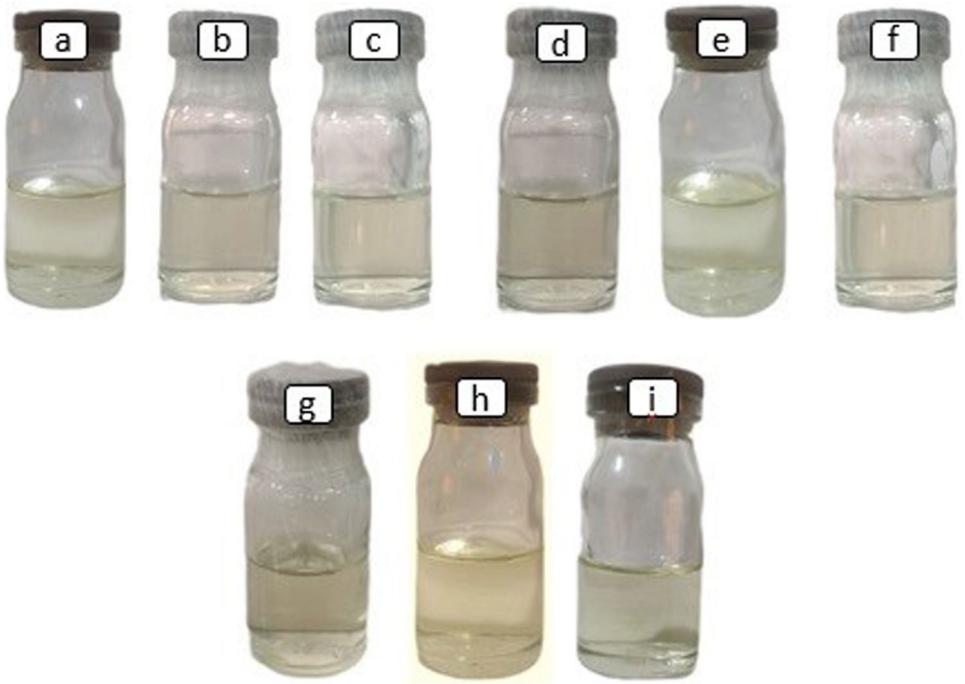

Fig. 2 NE formulations after 60 days of storage at 4, 25, and 40 °C. **a–c** SCO NE, spent coffee oil nanoemulsion; GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; and Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil, respectively, of storage conditions 4 °C. **d–f** SCO NE, GSO NE, and Mix NE, respectively, of storage conditions 25 °C. **g–i** SCO NE, GSO NE, and Mix NE, respectively, of storage conditions 40 °C

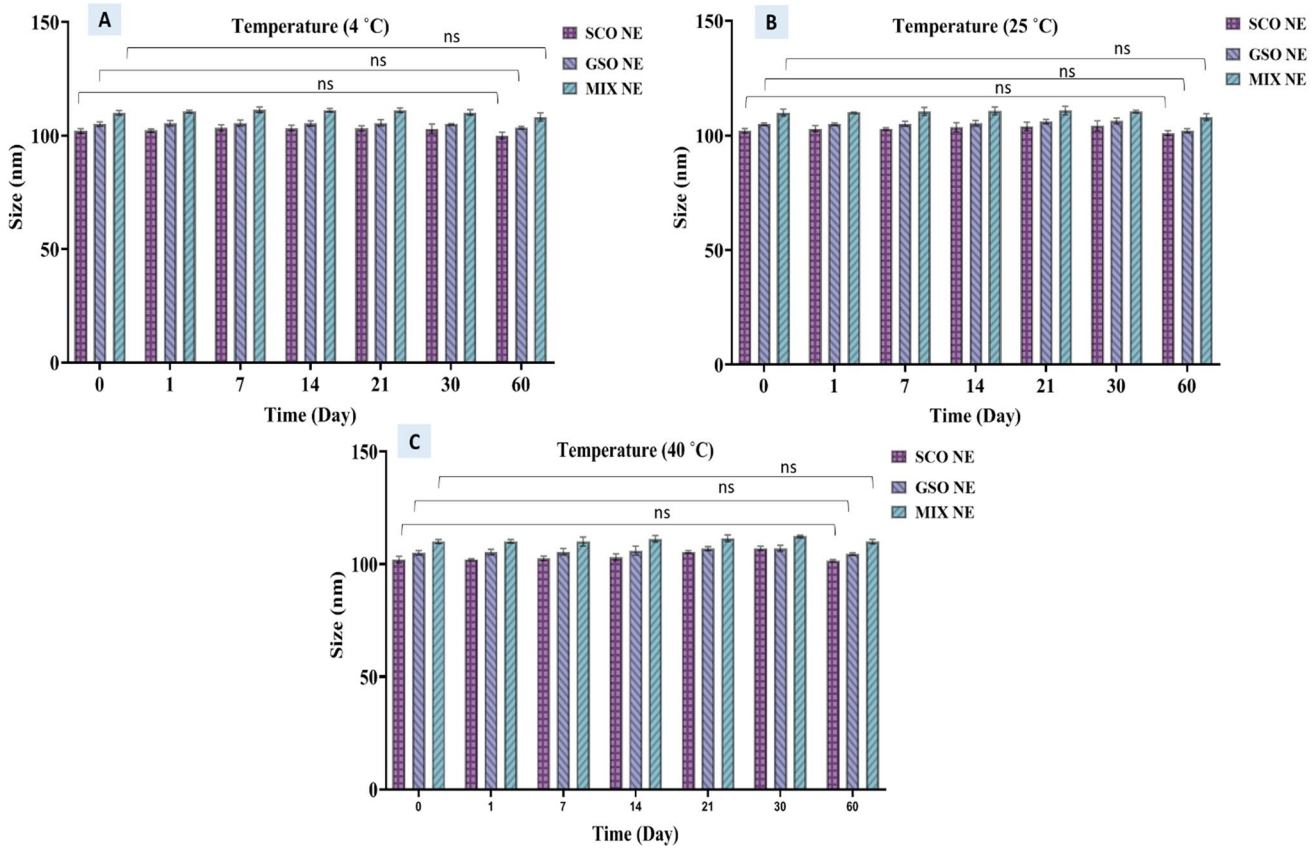

Fig. 3 Results of comparing droplet size at time zero with other storage times under different storage conditions. **A** NE formulations under storage conditions of 4 °C. **B** NE formulations under storage conditions of 25 °C. **C** NE formulations under storage conditions of 40 °C. SCO NE, spent coffee oil nanoemulsion; GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil

distribution, whereas samples with a larger range of particle sizes would have a higher PDI [39]. As demonstrated in Table 2, all nanoemulsions had a PDI of 0.32, indicating a homogeneous particle distribution. Overall, the results showed that the manufactured NE formulations should be stable during the shelf-life which is important for nanoparticles in cosmeceutical preparations. Nanoemulsions present several advantages over conventional emulsions due to their small droplet size, high optical clarity, good physical stability against gravitational separation and aggregation, and enhanced bioavailability of encapsulated substances. Depending on the desired formulation, the preparation method should be tailored to optimize droplet size distribution given the profound impact on stability. Systems with droplet diameters less than 200 nm with a monomodal distribution often have a homogeneous structure (i.e., evenly dispersed and not forming flocs) that can remain stable for up to 6 months, explaining why nanoemulsions are more stable than traditional emulsions carrying the same cargo [40]. Furthermore, nanoemulsions have outstanding biological and physical properties, such as skin dispersion and hydration, making them widely popular as cosmetic and beauty medications [6]. Increased stability for unsaturated fatty acids, vitamins, and antioxidants which are loaded in nanoparticles has been reported previously [41]. By manipulating the concentration of ingredients and particle size of nanoemulsion formulations, the stability can be changed, especially when environmental variables such as pH and temperature may vary from batch to batch [42]. Our findings showed acceptable stability (minimum 2 months) for the manufactured NEs.

3.2 Cytotoxicity

The viability results of the L929 cell line which were exposed to the NEs for 72 h are shown in Fig. 4. IC_{50} obtained for GSO, GSO NE, SCO, SCO NE, and Mix NE were 846.2, 1250, 564.5, 864.6, and 624.1 µg/mL, respectively. From the MTT assay data, it is evident that the prepared NEs are not cytotoxic. The concentrations used for the MTT assay were below the IC_{50} values obtained in the current study. It is important to note that due to the break-up

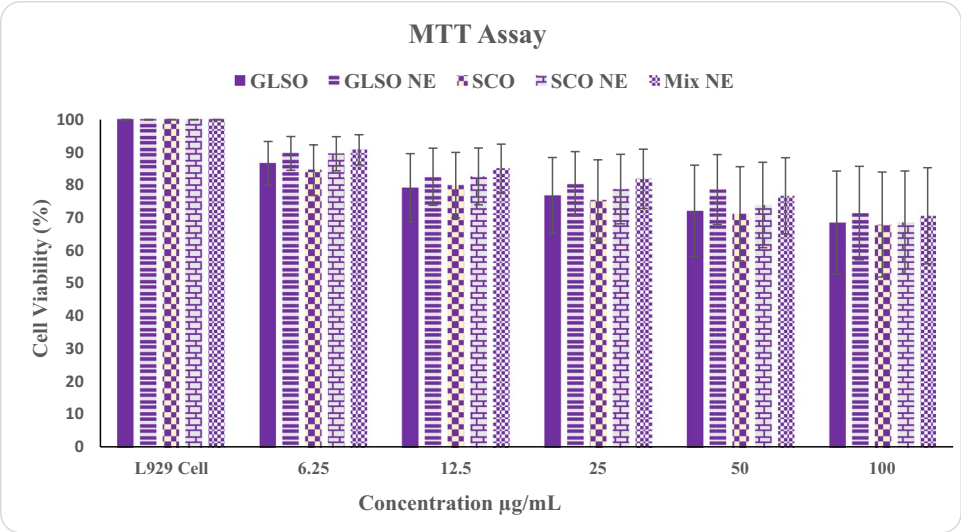

Fig. 4 Results of cell viability studies in different concentrations of the preparations. GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil

of the particles upon dilution, the MTT results should be considered with caution in NEs [7, 14].

3.3 Skin Penetration

Figure 5 displays the outcomes of the in vitro permeation studies of the NEs, employing Franz diffusion cell. Penetration studies showed that the percentages of oil penetration for GSO at different time intervals of 1, 3, 6, and 12 h were 17.74%, 38.22%, 74.36%, and 83.1%, respectively. In the case of GSO NE, these values were 6.07%, 36.61%, 43.18%, and 94.09%, respectively, for the same time intervals. The amount of oil passed through the membrane in the case of SCO was 6.40%, 8.40%, 17.40%, and 59.28% for the time interval of 1, 3, 6, and 12 h, whereas these values in the case of SCO NE were 23.49%, 44.05%, 58.74%, and 79.41%, respectively. Penetration of oil through the membrane for Mix NE was 44.97%, 58.4%, 72.16%, and 97.78%, at the time intervals of 1, 3, 6, and 12 h, respectively.

When the penetration data after 12 h were compared (Fig. 5), the results showed that, overall, NE preparations enhanced penetration compared with that of bulk oils (GSO 83.1%, GSO NE 94.09%, SCO 59.28%, and SCO NE 79.41%). It is apparent from the figure that the mix formulation is more effective than other NE formulations. Nanoemulsions have an oil (lipid) phase that makes the two lipid layers of the stratum corneum of the skin fluid and thus increases the penetration of the drug into the skin [43]. Here, the mix formulation can increase the penetration of the drug into the skin due to the synergistic effect of the combination of two oils.

Compared to common formulations, nanoemulsions have various advantages over conventional emulsions such as increased drug solubility, high thermodynamic stability, and improved drug transdermal penetration. The inherent properties

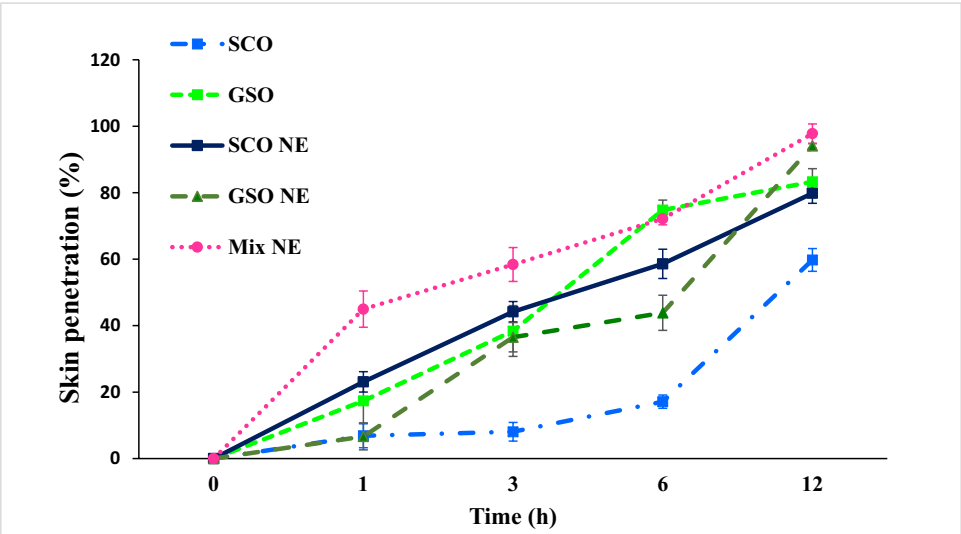

Fig. 5 Penetration percentage of the oil in Franz diffusion cell. GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil

of oil-in-water (O/W) emulsions can increase the absorption of lipophilic drugs when applied topically on the skin. Encapsulation of lipophilic drugs in the oily core of nanoemulsions can improve their bioavailability [42]. Also, the presence of surfactants in the formulations can increase drug loading, increase skin hydration, and hence drug permeability [6].

In another study, nanoemulsion gel containing meloxicam demonstrated an enhanced permeation of the drug in percutaneous absorption tests. Deep permeation of the drug (up to 130 µm) was reported, indicating the effectiveness of the nanoemulsion for transdermal delivery [44]. Using mouse skin, different nanoemulsions have shown the potential to enhance the permeation rate of ketoprofen [45].

3.4 Melanin Decrease in Melanoma Cells

The lightening effect of Mix NE (nanoemulsion containing spent coffee oil and *Ganoderma* spore oil) on B16F10 melanocyte cells after 48 and 72 h for intracellular melanin and extracellular is shown in Figs. 6 and 7. The decrease in melanin, 48 and 72 h after contact with Mix NE (50 and 100 µg/mL) was read by an ELISA reader at a wavelength of 490 nm. The details show that extracellular melanin and intracellular melanin decreased with time, depending on the concentration of Mix NE. Furthermore, the amount of melanin absorption in the untreated cells was higher than in the treated cells, as represented in the depigmenting index.

The skin-lightening effect of the nanoemulsion was also determined in vitro. The depigmenting index increased in Mix NE after 48 and 72 h. A nanoemulsion of coffee oil-algae oil was shown to be effective against the growth of melanoma cell B16-F10, in which the cell cycle was arrested at the G2/M phase [13]. Ganodermanondiol, a bioactive compound isolated from *Ganoderma lucidum*, can inhibit the expression of cellular tyrosinase, tyrosinase-related protein-1 (TRP-1), TRP-2, and microphthalmia-associated transcription factor (MITF), thus, a reduction in the production of melanin [25]. In

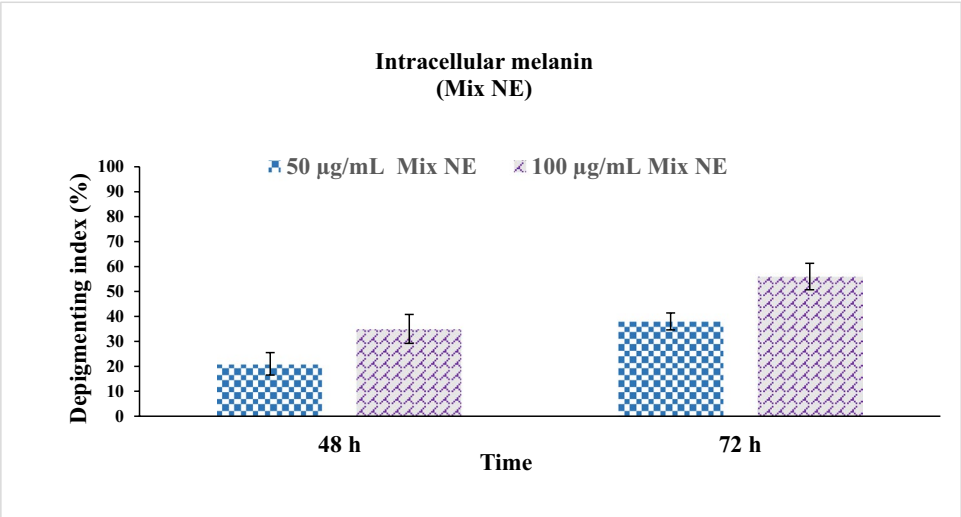

Fig. 6 Lightening effect of Mix NE (nanoemulsion containing spent coffee oil and *Ganoderma* spore oil) on B16F10 melanocyte cells after 48 and 72 h (intracellular melanin)

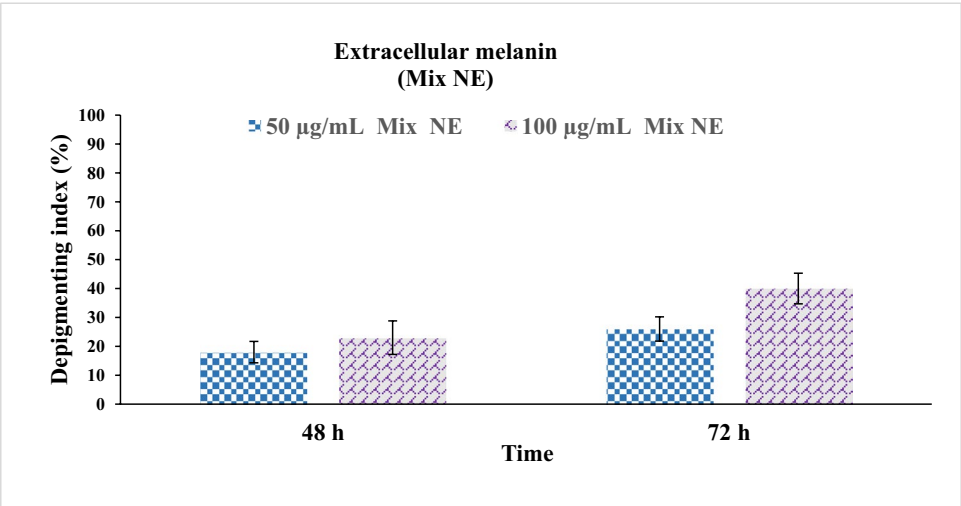

Fig. 7 Lightening effect of Mix NE nanoemulsion containing spent coffee oil and *Ganoderma* spore oil) on B16F10 melanocyte cells after 48 and 72 h (extracellular melanin)

another study, spent coffee oils showed a promising increase in skin elasticity, reducing wrinkle appearance, and boosting skin brightness. Tyrosinase and TRP-2 were significantly inhibited by the oil, which also showed a remarkable ability to reduce cellular melanogenesis, outperforming the inhibitory effects of kojic and linoleic acids [20].

3.5 Animal Studies

Figure 8 shows an example of mice skin before and after UV radiation and after treatment with the groups understudy, and the quantitative results are illustrated in Figs. 9, 10, 11, and 12.

As you can see in Fig. 8, and also Figs. 9, 10, 11, and 12, skin damage caused by UV radiation improved after the treatment with NEs. From the results, NE formulations (GSO NE and SCO NE) as well as the NE containing both oils (Mix NE) showed an improvement in performance compared to their bulk forms. This showed the feasibility of using coffee oil in the manufacture of cosmetics products. The increase in the performance could be attributed to the presence of both linoleic acid and palmitic acid in the formulation as both of these chemicals can reduce skin irritation and the aging of the skin [46]. A different study that concentrated on the emulsion formulation with coffee oil as the main component showed that coffee oil is effective in mitigating skin damage from UV radiation as indicated by the sun protection factor (SPF) [23]. Kanlayavattanakul et al. [20] showed that spent coffee oils had unique qualities that make them ideal for increasing skin elasticity, reducing wrinkle appearance, and boosting skin brightness. Several studies showed that the coffee oil has antioxidant properties, and our current results are in agreement with these studies [30].

3.5.1 UV Acne Analysis Results

Figure 9 shows the results of UV acne before and after UV radiation, as well as after treatment with the different groups understudy. From the results, in the control + and SCO NE groups, the UV acne decreased significantly after the treatment compared with UV radiation. Additionally, in GSO NE and Mix NE groups, the UV acne decreased significantly compared to treatment after UV radiation.

3.5.2 UV Spot Analysis Results

The results of the UV spot assessment are briefed in Fig. 10. The findings show that a significant decrease in UV spots is detected in GSO NE and Mix NE compared to subjects treated with UV radiation. GSO NE also showed a significant reduction in UV spots after treatment compared with before UV radiation.

3.5.3 PL Spot and Wrinkle Analyses

PL spot variation and wrinkle analysis in the groups under study are shown in Figs. 11 and 12, respectively. From the details, a significant reduction in PL spot is evident in Mix NE and GSO NE after treatment compared with before or after treatment with UV radiation (Fig. 11).

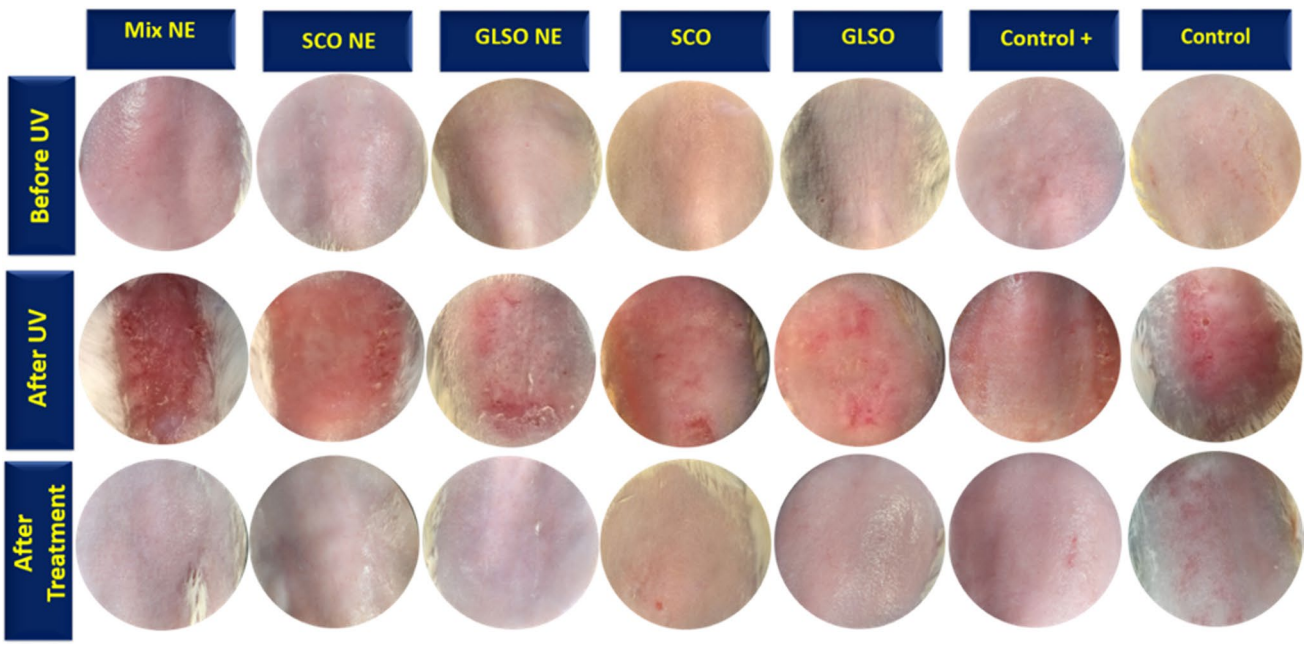

Fig. 8 The skin of the studied mice before and after UV radiation and after treatment

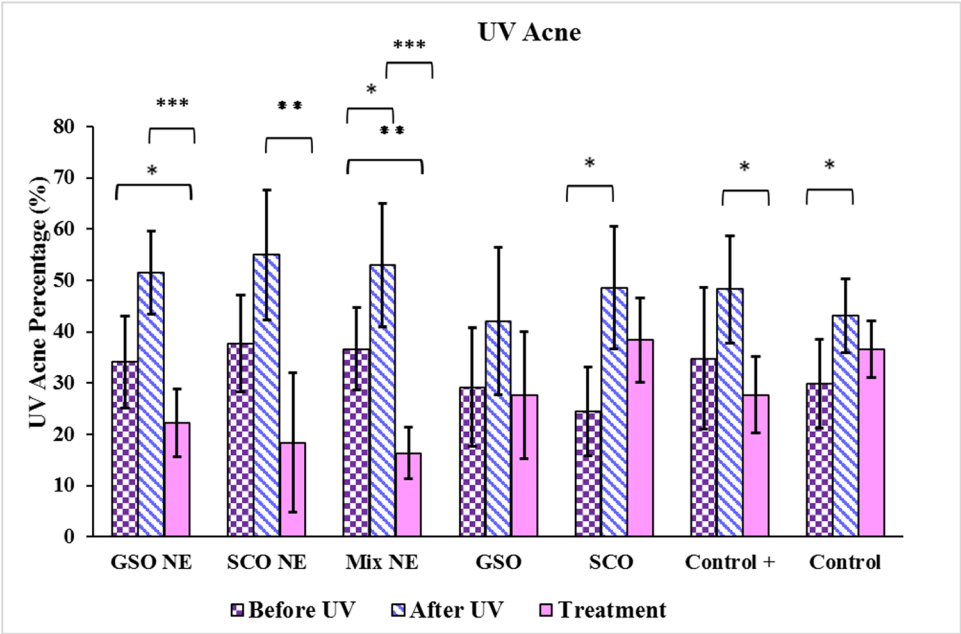

Fig. 9 The effect of the formulations used on the UV acne. GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

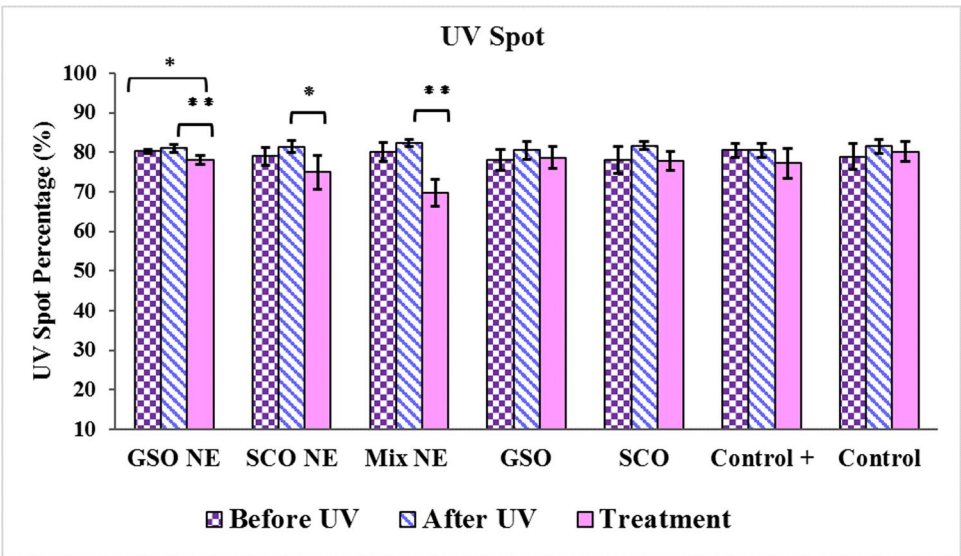

Fig. 10 The effect of the formulations used on the UV spot factor. GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively

Figure 12 shows a significant decrease in wrinkles after and before treatment in groups treated with GSO NE, SCO, and control +. From the results of our animal studies, the nanoemulsions contribute to soothing the UV effects. The positive effects of coffee oil and *Ganoderma lucidum* spore oil on the skin have already been documented elsewhere [20, 23, 30, 46, 47]. Although the cited references have not formulated spent coffee oil *and Ganoderma lucidum* spore oil as nanoemulsion formulations, they showed that the presence of fatty acid compounds and antioxidant properties in spent coffee oil can have a hydrating effect, reduce aging of the skin, increased skin elasticity, reduced wrinkle appearance, and boosted skin brightness. They also showed a whitening effect and antioxidant due to the presence of protein in *Ganoderma lucidum*. It seems that formulating spent coffee oil and *Ganoderma lucidum* spore oil as nanoemulsion formulation improves their performance even further which is discussed below. In another study, by analyzing the spent coffee oil based on physicochemical properties, it was stated that due to the beneficial fatty acid compounds, this oil has a hydrating and nourishing effect on the skin [48]. The biological effects of GLSO were investigated using the *Drosophila melanogaster* model. The results showed that GLSO may effectively scavenge free radicals and increase lifespan in *Drosophila* [49].

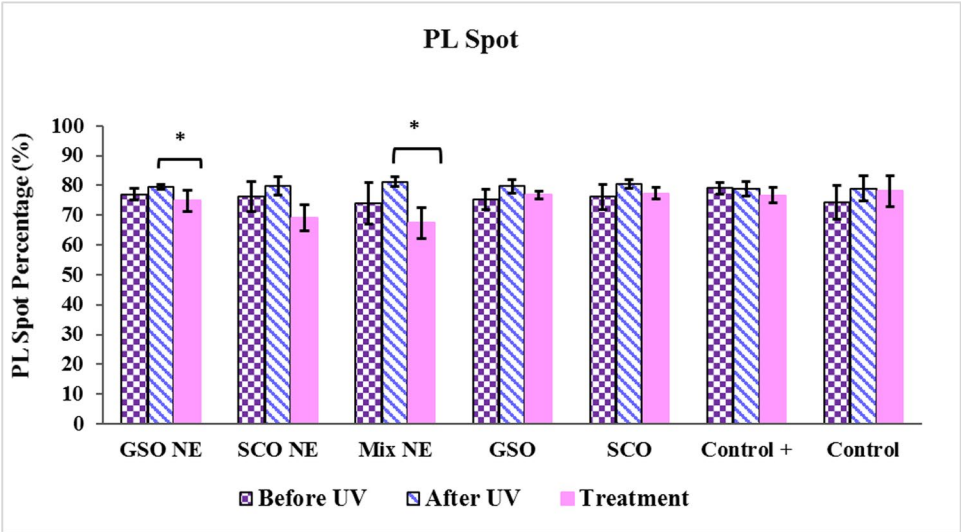

Fig. 11 The effect of the formulations used on the PL spot factor. GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil. * indicates $p < 0.05$, respectively

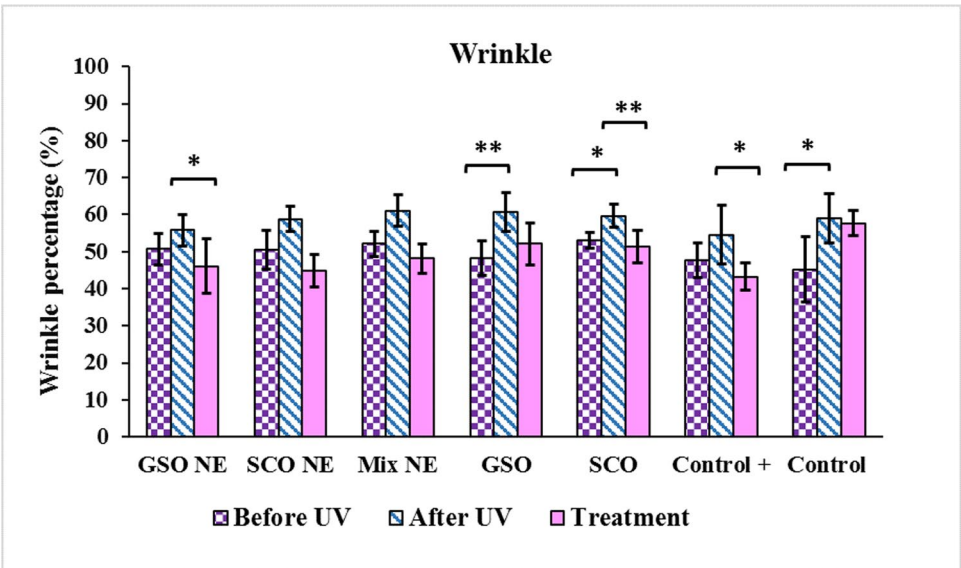

Fig. 12 The effect of the formulations used on the wrinkle factor. GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively

In a different investigation, spontaneous emulsification was used to successfully create a new tea tree oil nanoemulsion with 0.1% adapalene. The dermal transport of the drug through the skin and its antibacterial activity were both significantly improved by a combined therapy involving tea tree oil and adapalene. Various reasons have been proposed in the literature regarding the effect of nanoemulsions on improving the topical application of the active ingredients, namely, (a) by enhancing the solubility of poorly water-soluble agents [50], (b) by reducing particle size [51], (c) the presence of water to hydrate the skin (also mentioned as a penetration enhancer) [52], (d) by facilitating interaction with skin lipids due to the presence of surfactants [53], and (e) by disrupting the structure of stratum corneum (SC) [43].

To summarize the findings of animal experiments and simplify the comparison between the groups understudy, the change in UV acne, UV spot, PL spot, and wrinkle has been compared in the different treatments after UV radiation and after treatment (Fig. 13). The drop in UV acne of SCO NE and Mix NE is significantly higher, compared with SCO and control. Additionally, the reduction of PL spot in Mix NE is significantly more than that of GSO, control +, and control.

3.6 Skin Irritation Test

A skin irritation test was employed to ascertain the safety of the formulations for topical use. The scores for erythema and edema were documented with respect to PDII. The associated data on skin irritation are given in Table 3 and Fig. 14. From the details, the formulations exhibited no signs of cutaneous irritation. In another study, a cosmetic formulation incorporating spent coffee ground oil

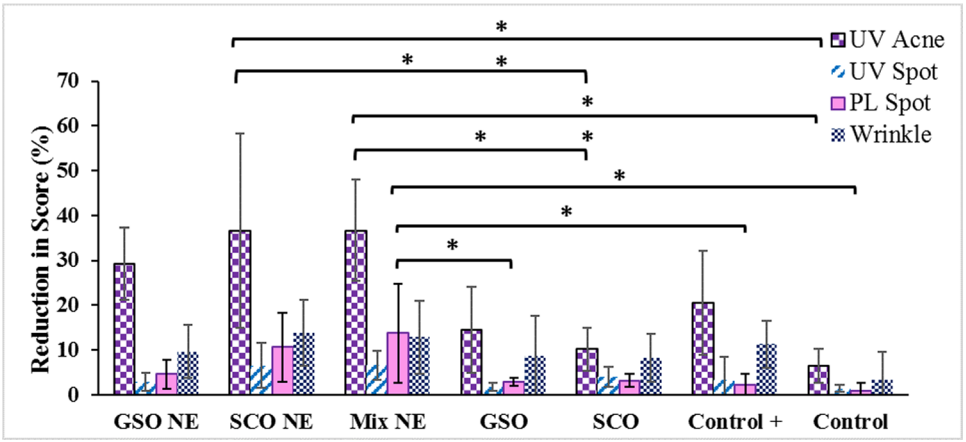

Fig. 13 The difference between studied skin parameters after treatment and before treatment (after UV radiation). GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively

Table 3 Primary Dermal Irritation Index (PDII) and observed irritation response in rabbit skin

Sample	PDII	Observed skin reaction
SCO NE	0	Not irritated
GSO NE	0	Not irritated
Mix NE	0	Not irritated
Control	0	Not irritated

GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; *SCO NE*, spent coffee oil nanoemulsion; *Mix NE*, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil

did not exhibit any signs of skin irritation throughout the experimental assessments. Based on their findings, creams that contained spent coffee grounds were categorized as nonirritant, indicating their safety for topical application, including use on facial regions [54].

4 Conclusion

In the present study, the effects of the nanoemulsions of *Ganoderma* spore oil and spent coffee oil on skin whitening and reduction of skin wrinkles were shown. The nanoemulsion containing both oils showed a greater effect on the skin. Also, the lack of cytotoxicity, and suitable skin penetration of the formulation, without any skin

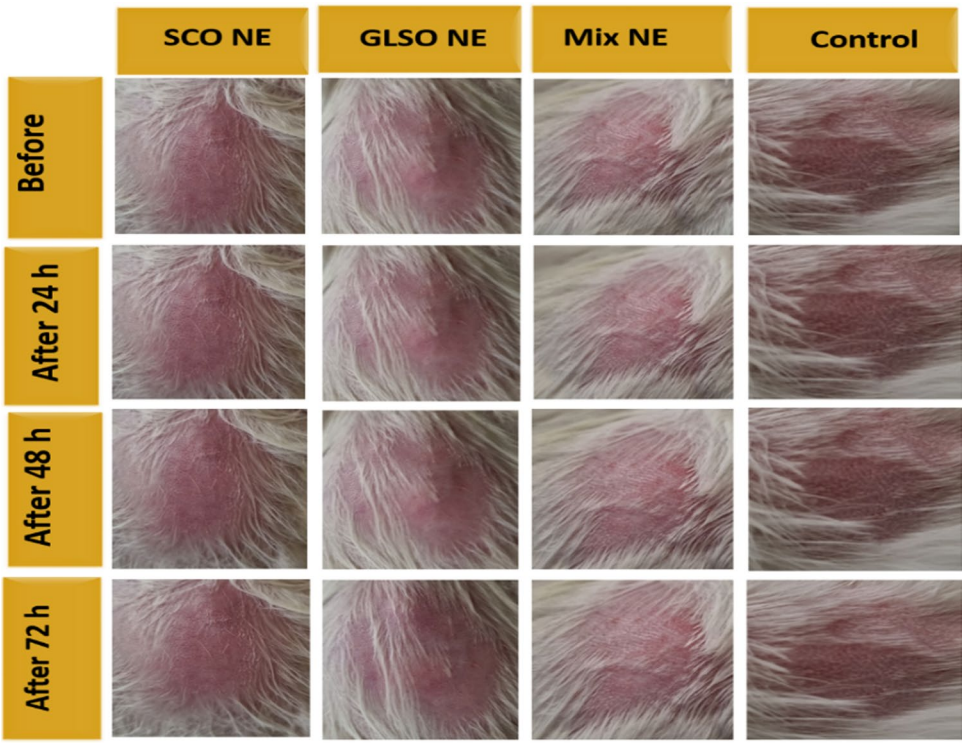

Fig. 14 Skin irritation reaction of rabbits before and after the test. GSO, *Ganoderma lucidum* spore oil (bulk); GSO NE, *Ganoderma lucidum* spore oil nanoemulsion; SCO, spent coffee oil (bulk); SCO NE, spent coffee oil nanoemulsion; Mix NE, nanoemulsion containing *Ganoderma lucidum* spore oil and spent coffee oil

irritation, indicated that the nanoemulsions from *Ganoderma* spore oil and coffee waste oil are valuable products with promising results for skin whitening and reduction of skin wrinkles. Overall, the combination of spore oil of *Ganoderma* with spent coffee oil in nanoemulsion form acts as an excellent potentiator for the development of high-performance cosmetic products related to skin whitening and wrinkle reduction. Thus, it can create immediate and long-term skincare benefits.

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Author Contribution MR, Writing the original draft, Methodology, Formal analysis; MA, Methodology, Formal analysis; HS, Methodology, Formal analysis; FGh, Formal analysis; AN, Writing- Reviewing and editing, AA, Formal analysis; FO, Supervision, Funding, Writing-Reviewing and editing. All authors reviewed the manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate The present research was approved by the Ethics Committee of the North Khorasan University of Medical Science, Bojnurd, Iran (Ethical approval code: IR.NKUMS.REC.1397.107).

Consent for Publication Not applicable.

Research Involving Humans and Animals Statement This study did not involve human participants. All animal experiments were conducted in accordance with institutional and national guidelines for the care and use of laboratory animals. Ethical approval was obtained from the Ethics Committee of the North Khorasan University of Medical Science, Bojnurd, Iran (Ethical approval code: IR.NKUMS.REC.1397.107).

Informed Consent None.

Competing Interests The authors declare no competing interests.

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