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RESEARCH ARTICLE

A Cosmetic Nanoemulsion Against Seborrheic Dermatitis: Development, Characterization and Effectiveness

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A Cosmetic Nanoemulsion Against Seborrheic Dermatitis: Development, Characterization and Effectiveness

SUMMARY

In this study, it was aimed to develop a topically applicable nanoemulsion (NE) that is expected to have an ameliorating effect in seborrheic dermatitis (SD). The main purpose of the formulation is to eliminate the disease factor, to repair the damage caused by the disease on the skin and to smooth the skin appearance by moisturization. For this reason, in vitro antimicrobial effect and in vivo effectiveness of the formulation were tested. For this aim; essential oils from tea tree, sage, cinnamon, oregano; extracts from Aloe vera, colloidal oatmeal, liquorice; vegetable oils from grape seed and sesame, and honey were used in a NE formulation. The NEs were prepared by ultrasonication method. Preliminary stability tests were applied to all formulations and then, pH, conductivity, viscosity, average droplet size, polydispersity index (PDI), and zeta potential measurements were taken on the selected NEs for 3 months. Finally, the antimicrobial effect and in vivo effectiveness of the optimum NE were tested. The average droplet size, PDI, and zeta potential value of the optimum formulation (F6P2) were 108.40 ± 0.90 nm, 0.195 ± 0.07, and -21.40 ± 1.45 mV, respectively. As a result, the moisture content of the skin increased significantly ($p < 0.001$), the sebum and redness values significantly decreased ($p = 0.008$ and 0.001, respectively) and there was no significant change in the pH of the volunteers' skin. Accordingly, it can be concluded that the optimum NE formulation developed in this study may be beneficial as a supplement for patients with SD.

Key Words: *Seborrheic dermatitis, nanoemulsion, herbal cosmetic, efficacy tests, essential oils, plant extracts, vegetable oils, honey*

Seboreik Dermatite Karşı Kozmetik Bir Nanoemülsiyon: Geliştirme, Karakterizasyon ve Etkinlik

ÖZ

Bu çalışmada seboreik dermatitte (SD) iyileştirici etkisi olması beklenen topikal olarak uygulanabilir bir nanoemülsiyon (NE) geliştirilmesi amaçlanmıştır. Formülasyonun temel amacı hastalık faktörünü ortadan kaldırmak, hastalığın ciltte verdiği hasarı onarmak ve nemlendirerek cilt görünümünü pürüzsüz hale getirmektir. Bu nedenle formülasyonun in vitro antimikrobiyal etkisi ve in vivo etkinliği test edilmiştir. Bu amaç için NE formülasyonunda; çay ağacı, adaçayı, tarçın, kekik esansiyel yağları; Aloe vera, kolloidal yulaf ezmesi, meyan kökü ekstreleri; üzüm çekirdeği ve susamdan elde edilen bitkisel yağlar ve bal kullanılmıştır. NE'ler ultrasonikasyon yöntemiyle hazırlanmıştır. Tüm formülasyonlara ön stabilite testleri uygulanmış ve ardından seçilen NE'ler üzerinde 3 ay boyunca pH, iletkenlik, viskozite, ortalama damlacık boyutu, polidispersite indeksi (PDI) ve zeta potansiyel ölçümleri alınmıştır. Son olarak, optimum NE'nin antimikrobiyal etkisi ve in vivo etkinliği test edilmiştir. Optimum formülasyonun (F6P2) ortalama damlacık boyutu, PDI ve zeta potansiyel değeri sırasıyla 108,40 ± 0,90 nm, 0,195 ± 0,07 ve -21,40 ± 1,45 mV olarak bulunmuştur. Sonuç olarak, cildin nem içeriği önemli ölçüde artmış ($p < 0,001$), sebum ve kızarıklık değerleri önemli ölçüde azalmış (sırasıyla $p = 0,008$ ve 0,001) ve gönüllülerin cildinin pH'ında önemli bir değişiklik meydana gelmemiştir. Buna göre, bu çalışmada geliştirilen optimum NE formülasyonunun SD'li hastalar için ek olarak faydalı olabileceği sonucuna varılabilir.

Anahtar Kelimeler: *Seboreik dermatit, nanoemülsiyon, bitkisel kozmetik, etkinlik testleri, uçucu yağlar, bitki ekstreleri, bitkisel yağlar, bal*

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INTRODUCTION

Seborrheic dermatitis (SD) is a common chronic recurring inflammatory disease that most commonly affects adults; however, a more transient infantile form also occurs (Del Rosso, 2011). SD is characterized by the presence of scratchy, erythematous areas with easily detachable, oily large scales. Although it can occur in a variety of anatomical locations, it tends to occur in areas containing multiple sebaceous glands, such as the scalp, face (nasolabial folds, ears, and eyebrows), upper chest, and back. Dandruff, a milder rash usually characterized by smaller dry, flaky scales, may occur on the scalp with SD (Berk & Scheinfeld, 2010). In some patients with SD, inflammatory erythematous folliculitis (possibly due to *Malassezia*) and blepharitis may develop. *Malassezia* species are thought to be the main factor in the development of SD. *Malassezia* species have been identified, seven of which have been associated with the human skin flora and SD. *Malassezia furfur, Malassezia pachydermatis, Malassezia restricta, Malassezia sympodialis, Malassezia globosa, Malassezia obobtusa* and *Malassezia slooffiae* have been detected in the affected skin (Vijaya Chandra, Srinivas, Dawson Jr, & Common, 2021).

Several treatment options can be effective in treating SD. The mechanism of action of the most common treatments includes inhibition of yeast colonization on the skin, reduction of itching and erythema, relaxation of crusts and scales, and reduction of inflammation (Berk & Scheinfeld, 2010). Mainstays of treatment for SD are antifungals, corticosteroids, calcineurin inhibitors, among other agents (Berk & Scheinfeld, 2010; Clark, Pope, & Jaboori, 2015; Gary, 2013). Current topical antifungals are largely safe and effective, but at times may be ineffective due to the increasing resistance of *Malassezia* to antifungal agents and some of these agents can cause irritant and allergic skin reactions. Moreover, even when their use is successful, there is still a high rate of recurrence in superficial skin diseases (Pintas & LIO, 2018). SD can also be treated with low or medium potency topical corticosteroids or calcineurin inhibitors (e.g., tacrolimus and pimecrolimus). These immunomodulatory agents are highly effective as anti-inflammatory agents, but long-term and prophylactic use seem to be inappropriate because of the chronic and recurrent nature of SD. Although steroids show efficacy and safety in short-term treatments, they could lead to frequent relapses shortly after the treatment discontinues. Also, they have the risk of causing addiction and steroid rosacea (du Vivier, 1976; Vardy, Cohen, Tchetov, Medvedovsky, & Biton, 1999; Rigopoulos, Ioannides, Kalogeromitros, Gregoriou, & Katsambas, 2004). Therefore, it may be important to identify novel treatments that may be more appropriate for long-term use without risk of these side effects (Pintas & Lio, 2018).

Natural ingredients with anti-*Malassezia* activity, have been anticipated to ensure patients with a wide range of treatments. Compliance is one of the main factors in the treatment of chronic conditions such as SD. Therefore, formulations containing natural ingredients with anti-*Malassezia* activity can be a prominent part of long-term treatment as an alternative and also for complementary management. In addition, it would be satisfactory to use them prophylactically in SD patients with mild symptoms (Han et al., 2017).

There are numerous advantages of using formulations containing natural ingredients for the treatment of SD, including patient compliance, fewer side effects, easy availability, low cost, and multiple modes of pharmacological action (Herman & Herman, 2016). Therefore, in this study, we developed a topical non-pharmacological formulation in NE form to be an alternative for synthetic drugs in SD therapy and to improve clinical outcomes of the disease. Essential oils from tea tree, sage, cinnamon, oregano; extracts from *Aloe vera*, colloidal oatmeal, liquorice; vegeta-

ble oils from grape seed and sesame, and honey were used in the NE formulation.

Tea tree oil is essential oil from the *Melaleuca alternifolia* tree, which is native to Australia (Beheshti Roy et al., 2014). This oil has been traditionally used for treatment of burns, infections and SD (Chandler & Osborne, 1998; Aburjai & Natsheh, 2003). Satchell et al. studied 5% tea tree (TT) oil to 63 patients with SD and observed a 41 percent improvement in symptom severity score compared to only 11 percent in the placebo group (Satchell, Saurajen, Bell, & Barnetson, 2002).

Essential oil of *Salvia officinalis L.* (sage) was shown to have antifungal activity against dermatophyte strains. Nitric oxide production stimulated by lipopolysaccharide in macrophages was inhibited by the essential oil without affecting cell viability, in concentrations up to 0.64 μL/mL. Consequently, it has been proposed as an appropriate bioactive agent in antifungal therapy (Abu-Darwish et al., 2013).

It has been confirmed that *Cinnamomum zeylanicum* (cinnamon) essential oil can be effectively down-regulated IL-1α , IL-6, IL-8 cytokines overproduced by the SD-inducing cocktail. Additionally, it also showed inhibitory effect on sebum lipid synthesis from primary sebocyte and growth inhibitory effect to Malassezia globosa yeast. As a result, *Cinnamomum zeylanicum* essential oil suggested as a natural herbal remedy to relieve or protect scalp SD (Kim, Kim, Lee, Jeon, & Park, 2012).

The essential oil from *Origanum vulgare L.* (oregano) possess strong inhibitory effects against a variety of phytopathogenic fungi and do not seems to cause the development of resistance in microorganisms (Vinciguerra, Rojas, Tedesco, Giusiano, & Angiolella, 2019). In a study conducted by Vinciguerra et. al., the antifungal activity of *Origanum vulgare* and *Thymus vulgaris* essential oils and carvacrol against 27 clinical isolates of *Malassezia furfur* were reported. Essential oils and carvacrol were found to be more actives against resistant or dose dependent to fluconazole *Malassezia furfur* isolates (Vinciguerra et al., 2019).

The compounds found in *Aloe vera* gel contain polysaccharides that are capable of reducing and restoring inflammation. It also has antimicrobial properties (Fozouni, Taghizadeh, & Kiaei, 2018). In a study with 44 patients with SD, 24 adults used an emulsion containing 30% *Aloe vera* extract, whereas 20 volunteers applied a placebo emulsion. After a four-week application period, there was a significant decrease in flaking (36.6% treatment vs. 17.6% placebo) and itching (21.5% vs. 5.3%) when compared with baseline. In addition, 58% of the patients had complete resolution or significant improvement in SD symptoms (Vardy et al., 1999).

Avena sativa (colloidal oatmeal) has been used for centuries to decrease itching in a variety of xerotic dermatoses (Sur, Nigam, Grote, Liebel, & Southall, 2008). Studies have revealed that the dermal formulations of natural colloidal oatmeal, especially avenanthramide, improves disease symptoms by restoring the cutaneous barrier. It may also play an important role in reducing the use of corticosteroids and calcineurin inhibitors in atopic dermatitis (Pigatto et al., 1997; Eichenfield, Fowler Jr, Rigel, & Taylor, 2007; Cerio et al., 2010).

Glycyrrhiza glabra (liquorice*)* has significant anti-inflammatory and anti-allergic activity. Glycyrrhizin, a kind of flavone found in licorice, reinforces cortisol's inhibition of antibody formation, stress reaction, and inflammation (Saeedi, Morteza-Semnani, & Ghoreishi, 2003).

Grape seed oil comes from the seeds of *Vitis vinifera.* It is rich in phenolic compounds, fatty acids, and vitamins. Grape seed oil has beneficial properties for health that are mainly detected by *in vitro* studies, such as anti-inflammatory, cardioprotective, antimicrobial, and anticancer properties. Phytosterols pres-

ent in grape seed oil which may prevent the release of proinflammatory mediators by oxidized low-density lipoprotein-stimulated macrophage during oxidative stress and eicosanoid synthesis (Garavaglia, Markoski, Oliveira, & Marcadenti, 2016; Lin, Zhong, & Santiago, 2018).

Sesame oil contains powerful, natural antioxidants (sesamin, sesamol, sesamolin, and phytosterol) which give the oil very good oxidative stability. It has pronounced regenerative action due to its high unsaponifiable content. Sesame oil has been reported to have healing effects on certain skin conditions such as seborrhea, eczema, psoriasis and sunburn (Rabasco Álvarez & González Rodríguez, 2000).

Honey has antibacterial, antifungal and antioxidants activities and has high nutrient value. In a clinical trial, crude honey has been demonstrated to improve SD and associated hair loss and prevent relapse when applied weekly (Al-Waili, 2001).

To develop innovative and superior dermatological and/or cosmetic skin care products containing bioactive compounds, it is necessary to depart from the basic technology and use key technologies. Recent advances in nanotechnology are promising for the potential use of poorly soluble, poorly absorbed and unstable plant extracts and phytochemicals in cosmetics. A modern perspective can improve both the aesthetics and effectiveness of a cosmetic product. The application of new techniques can also increase the effectiveness of herbs regarding their sustained effect on the human body (Altuntaş, Yener, & Özkan, 2019).

One of the key technologies that will provide innovative products is NE. Nanoemulsions (NEs) can also be termed as ultrafine emulsions, miniemulsions, and submicron emulsions due to the formation of droplets in the submicron range. The average droplet size of NEs can range from 20 to 500 nm (Guglielmini, 2008). The small size of the droplets gives them a natural stability against creaming, sedimentation, and aggregation, while ensuring effective delivery of active ingredients to the skin (De Vleeschauwer & Van der Meeren, 1999; Fang, Hong, Chiu, & Wang, 2001). NEs have attracted considerable attention in recent years for application in personal care products as potential vehicles for the controlled delivery of cosmetics and the optimized dispersion of active ingredients in particular skin layers (Guglielmini, 2008). The simplicity of NE fabrication also attracted interest towards increased loading, improved therapeutic efficacy and stability of herbal drugs as compared to conventional delivery systems (Harwansh, Deshmukh, & Rahman, 2019). Ultrasonic emulsification is a high energy method to develop NEs. It utilizes sound waves with frequency more than 20 kHz by using a sonotrode to cause mechanical vibrations followed by the formation of acoustic cavitation. Collapse of these cavities generates powerful shocks waves which breaks the coarse droplets (Ghosh, Mukherjee, & Chandrasekaran, 2013). Recent studies have shown that, in emulsification process, ultrasound had emerged as an excellent yet superior tool as compared to rotor stator in terms of obtaining a smaller droplet size and high energy efficiency (Abismaıl, Canselier, Wilhelm, Delmas, & Gourdon, 1999; Kentish et al., 2008). In addition, the results showed that the amount of surfactant required to produce an emulsion of the desired diameter was significantly reduced and the energy consumption was considerably lower than with other conventional mechanical devices (Tadros, Izquierdo, Esquena, & Solans, 2004). More interestingly, all of the acoustically formed emulsions are perfectly stable and more homogeneous compared to mechanical methods (Abismaıl et al., 1999; Tadros et al., 2004).

In light of the above, we aimed to develop a dermal NE formulation that contains the combination of oils and extracts of medicinal plants and honey for providing synergistic or potentiating effects in

the treatment of SD. For this purpose, dermatological NEs containing essential oils from tea tree, sage, cinnamon, oregano; extracts from *Aloe vera*, colloidal oatmeal, liquorice; vegetable oils from grape seed and sesame, and honey were prepared using ultrasonic emulsification method. The prepared NEs were characterised and physical stability of them was evaluated for 3 months. Its antimicrobial activity was tested on *Candida albicans* (*C.albicans*) strains. Finally, the optimized NE was tested clinically in terms of the efficacy and safety in the treatment of SD with non-invasive biophysical techniques and subjective assessment.

MATERIAL AND METHODS

Materials

Cinnamon and oregano essential oils were purchased from Arifoğlu Inc. (Turkey). Sage essential oil was provided from Vitarom Chemical Co. Ltd. (Turkey). Extracts of *Aloe vera*, colloidal oatmeal and liquorice and tea tree essential oil were supplied by Surya Chemical Co. Ltd. (Turkey). Flower honey were obtained from Balparmak (Turkey). Grape seed oil and sesame oil were kindly donated from Zade Naturel (Turkey). Poloxamer 188 and tocopherol acetate were obtained from Sigma (St. Louis, MO). Transcutol® HP, Labrafac™ CC were purchased from Gattefossé (France). Cremophor® A25 was provided from BASF (Germany). Neolone™ PE was acquired as a gift from Kale Kimya Inc. (Turkey). All other chemicals and solvents used were of pure analytical grade.

Preparation of Nanoemulsions

NEs containing oily phase with 3 different concentrations (%5, %6.68, %10 (w/w)) were prepared by ultrasonic emulsification method. Two different surfactant and cosurfactant mixtures (Poloxamer 188/ Transcutol® HP and Cremophor® A25/Transcutol® HP) with 1:1 (w/w) and 1:2 (w/w) surfactant and cosurfactant ratios were evaluated to find a stable NE formulation. Poloxamer 188 (5% w/w) and Transcutol® HP (10% w/w) was the only surfactant and cosurfactant combination that resulted in a stable formulation and therefore was chosen for further study. The formulations and manufacturing parameters of the selected NEs (1:2 (w/w) surfactant to cosurfactant ratio) are given in Table 1.

Initially, the ingredients of water phase and oily phase were weighed precisely in two separate beakers. Coarse emulsion was manufactured with/without pre-emulsification stage before ultrasonication process. During pre-emulsification, coarse emulsion was obtained using a rotor-stator type mixer (Witeg HG-15D, Germany) for 5 minutes at a mixing speed of 8100 rpm. The emulsion was then ultrasonically emulsified using a 20 kHz Sonicator (Ultrasonics, USA) (750W Ultrasonic power supply). The energy input was delivered via a sonotrode containing a piezoelectric crystal with a probe diameter of 13 mm. The ultrasonication process was started after the sonicator probe was placed vertically in the coarse emulsion. The process was applied with 50% amplitude, 30 s pulse on and 30 s pulse off, for two different sonication periods, 20 minutes or 30 minutes. To control the temperature of the emulsion during sonication, the beaker was placed in a larger container containing ice. Subsequently, characterization studies of formulated NEs were carried out and also the stability of selected NEs was investigated.

Table 1. The formulation compounds and the manufacturing parameters of the selected NEs.

	Ingredients	FORMULATION CODES		
	(% w/w)	F4 (P1-P4)	F5 (P1-P4)	F6 (P1-P4)
OIL PHASE	Tea tree essential oil	1	0.56	0.33
	Sage essential oil	1	0.56	0.33
	Oregano essential oil	1	0.56	0.33
	Cinnamon essential oil	0.50	0.28	0.17
	Grape seed oil	2	1.1	0.67
	Sesame seed oil	2	1.1	0.67
	Neolone™ PE	0.30	0.30	0.30
	DL-alpha tocopherol acetate	0.20	0.20	0.20
	Transcutol® HP	10	10	10
	Labrafac™ CC	2	2	2
AQUEOUS PHASE	Poloxamer 188	5	5	5
	Colloidal oatmeal extract	2	2	2
	Aloe vera extract	0.5	0.5	0.5
	Liquorice extract	2	2	2
	Honey	4	4	4
	Distilled Water (d.w.)	66.50	69.82	71.50
PROCESS	**Procedure 1 (P1):** 20 min sonication without pre-emulsification **Procedure 2 (P2):** 20 min sonication with pre-emulsification (5 min at 8100 rpm) **Procedure 3 (P3):** 30 min sonication without pre-emulsification **Procedure 4 (P4):** 30 min sonication with pre-emulsification (5 min at 8100 rpm)			

Characterization of Nanoemulsions

Particle Size Analysis

The mean droplet size and PDI of the produced NEs were measured by quasi-elastic light scattering technique based on the measurement of Brownian motion (movement in random direction) of sub-micron particles as a function of time. Light scattering was monitored at room temperature (25°C) at a scattering angle of 90° by Malvern Zetasizer Nano ZS (Malvern Instruments, UK). Samples of NEs were properly diluted with distilled water (1:100 v/v) prior to analysis to reduce the multiscattering effect (Rebolleda et al., 2015). Samples were considered polydisperse when the PDI was higher than 0.3 (He et al., 2011).

Zeta Potential Measurement

Zeta potential is measured using the aforementioned Malvern Zetasizer Nano ZS device. To measure zeta potential value of the NEs, samples were diluted with distilled water (1:100 v/v) and its value is estimated from the electrophoretic mobility of oil droplets (Rebolleda et al., 2015).

Rheological Characteristics

The viscosity of the NEs at different shear rates was studied using Brookfield cone and plate viscometer (Brookfield Engineering Laboratories, Inc., Mid-

dleboro, MA) at 25 ± 0.5 °C. The graphs were plotted between viscosity versus shear rate to evaluate rheological characteristics of the NEs (Shakeel, Ramadan, & Ahmed, 2009).

pH and Conductivity Measurement

The pH of the NEs was directly measured by a portable pH meter (Hanna Edge, USA) at 25 ± 2 °C. The electrical conductivity was determined at 25 ± 2 °C using the same device.

Preliminary Stability Tests

The preliminary stability of the NEs were evaluated by centrifugation, thermal stress and heating-cooling cycle tests. The NEs were analysed after centrifugation and thermal stress tests by visual observation regarding any physical instability issues. Afterwards, the stable NEs were taken for the heating-cooling cycle. These tests were carried out to predict the stable NEs in a short time before performing the long-term physical stability tests. Firstly, centrifugation was applied to 10 g of sample at 3500 rpm for 30 min with a laboratory centrifuge (ThermoScientific, USA) to accelerate possible instability issues (Altuntaş & Yener, 2015). Subsequently, thermal stress test was applied to the NEs that passed the centrifugation test successfully. Glass tubes containing 10 g of sample were submitted to a range of temperatures (from 40 up to 80°C, increasing by 5°C intervals) for 30 min at each temperature point using a thermostatic water-bath (Witeg WSB-30, Germany) (Altuntaş & Yener, 2015). A heating-cooling cycle test was performed to evaluate the NEs' stability at extreme temperature changes. For heating-cooling cycle test, the selected NEs were subjected to six cycles of heating and cooling. The samples were kept at 4°C for 48 hours and then at 40°C for 48 hours. The stability of the NEs as a result of the heating-cooling cycle was evaluated by visual observation, particle size analysis and zeta potential measurement (Shakeel et al., 2009). Only the NEs which represented no instability problems were selected for 3 months of physical stability tests.

Physical Stability Test

To investigate the physical stability of the selected NEs, the freshly prepared samples were stored at closed glass vials and placed at different temperatures for a period of 3 months: 25 ± 2 °C and 60% RH, 40 ± 2 °C and 75% RH, and 5 ± 3 °C. Samples were characterized with respect to their particle size, PDI, zeta potential, viscosity, pH and conductivity at predetermined time intervals. All experiments were performed in triplicate.

Microbiological Test

The antimicrobial effect of the selected NE formulation (F6P2) on yeasts was determined by the microdilution method reported by the Clinical Laboratory Standards Institute (CLSI) (Gong et al., 2019). As the yeast to be tested, *C. albicans* ATCC 10231 standard strain and four *C.albicans* strains from skin samples from Group Florence Nightingale Hospitals microbiology laboratories were used.

In this study, to determine the Minimum Inhibitor Concentration (MIC) value of test product against *C.albicans* strains, inoculums (0.5 - 2.5 x 10^3 cfu/ml) were prepared from fresh 24-48 hour cultures of standard and clinical *C.albicans* strains in MOPS buffered RPMI-1640 (Sigma) medium. After adding yeast suspensions to the test and market products, which were serially diluted in a 96-well U-bottom microplate, the microplates were incubated at 35°C for 48 hours. At the end of the incubation period, the lowest concentration without visible turbidity, which prevents the growth of *C.albicans* strains, was evaluated as MIC. In order to carry out a standardized study, the MIC values of fluconazole against the tested strains were also determined and these values were found to be within the quality control limits reported by CLSI.

In Vivo Studies

Study Design

In order to test the *in vivo* effectiveness of the selected NE formulation, the single-blind *in vivo* analyzes, compliance with the Helsinki Declaration, has been conducted after approval by the ethics committee of the Turkish Medicines and Medical Devices Agency affiliated to the Turkish Ministry of Health (Decision number: 58307721-512.99-44027). The study was initiated after 5 healthy volunteers aged 25-65 signed the written informed consent form. The selected volunteers applied the NE formulation as a thin layer to the SD area twice a day for 4 weeks. Inclusion and exclusion criteria for volunteers were determined as shown in the Table 2.

Table 2. The criteria for inclusion and exclusion of volunteers in the *in vivo* study.

The inclusion criteria	Persons must be male and female volunteers between the ages of 18 and 65 who have been diagnosed with SD.
	People should not have any other disease other than SD on their skin.
	Individuals must agree to sign a written informed consent form.
	No other product should be used at least seven days before the measurements.
	Volunteers must be available at the measurement times during the study.
	A different product should not be applied to the application area during the study period.
The exclusion criteria	Pregnant and lactating women.
	People using topical medication at the application site for any reason within the same period.
	Persons with known hypersensitivity to an ingredient in the formulation
	Persons with major hepatic and renal impairment.
	Persons involved in other clinical trials.
	Persons unable to adapt to work.
	Persons with hypersensitivity in the application area.

Dermatological Test (Skin Irritation Test)

In order to determine whether the formulation had any adverse effects on the skin of the volunteers, 0.2 grams of the test product was placed on the patch test material (IQ chamber) with an area of 1 cm^2, in contact with the forearm area, before the product was started to be applied. The volunteer was instructed to avoid contact with water and direct sunlight during the 48 hour observation period. At the end of the 48 hour period, the patch test material was removed and the volunteers' skin was checked for the presence of a skin reaction such as itching, redness, irritation, and edema (Altuntaş & Yener, 2015).

Preparation of The Measurement Environment

While taking measurements from the application areas of the volunteers based on the non-invasive biophysical methods, controlling the humidity and temperature of the measurement environment and preventing the skin of the volunteers from being exposed to direct sunlight are pivotal factors for the accuracy and reproducibility of the measurement results (Jansen van Rensburg, Franken, & Du Plessis, 2019). Therefore, the volunteers were tested after a 20-minute rest period in an air-conditioned room at 20 °C ± 2 temperature and 40 - 60% relative humidity in terms of dermal parameters such as skin pH, sebum, moisture and erythema.

Non-Invasive Biophysical Tests

Skin pH Measurement

To determine the effect of the test formulation on the skin pH of the volunteers, the pH of the skin was measured using the Skin-pH-Meter 900® (Courage & Khazaka, Electronic GmbH, Germany) (Altuntaş & Yener, 2015). At the beginning of the measurements,

the pH-meter was cleaned with distilled water. The probe tip was placed perpendicular to the skin and held on the skin for 3 seconds. After 3 seconds, the pH value was recorded. 5 different measurements were taken from the application area and the mean value were calculated.

Skin Sebum Measurement

The effect of the test formulation on the sebum secretion of the skin was determined by measuring with Sebumeter® SM 815 (Courage & Khazaka, Electronic GmbH, Germany) (Altuntaş & Yener, 2015). The cartridge was pressed lightly on the measuring device until the measuring time on the screen started counting down. When the countdown started, the cartridge was removed from the device and the measurement was started. During the 30 second time, the cartridge was held vertically in the measuring area on the skin. At the end of the period, the cartridge was re-inserted into the device and the measured value on the screen was recorded. 3 different measurements were taken from the application area and the mean value were calculated.

Skin Moisture Measurement

In order to determine the moisturizing effect of the test formulation on the skin, the water content of the skin was measured by Corneometer® CM 825 device (Courage & Khazaka, Electronic GmbH, Germany), which is based on the principle of electrical capacitance method. The water content value on the screen was recorded 1 second after the probe of the device came into contact with the skin. The results are given in arbitrary units (AU), where 1 unit of AU is estimated to correspond to 0.2 - 0.9 mg of water per gram of stratum corneum (De Melo & Maia Campos, 2018). 5 different measurements were taken from the application area and the mean value were calculated.

Skin Erythema Measurement

The pathological parameter, erythema (hemoglobin content), was evaluated photometrically with a Mexameter® 18 (Courage and Khazaka Electronic GmbH, Cologne, Germany) according to the principle of remission (Yilmaz & Borchert, 2006). The erythema value on the screen was recorded 1 second after the probe of the device came into contact with the skin. 3 different measurements were taken from the application area and the mean value were calculated.

Subjective Evaluation

After the end of the *in vivo* test period, the volunteers were also subjected to a subjective evaluation regarding the sensory and visual effects of the test formulation on skin. The volunteers were asked to score their preferences from 0 to 5 (dissatisfaction – most satisfaction) for the first 10 questions and for the last question, yes/no answers were received from the volunteers according to questionnaires specifically designed for the experiment.

Statistical Analysis

The *in vitro* data are expressed as means ± standard deviation (SD) (n = 3). The tests used for the statistical analysis of the results from the human study depended on the type of sampling distribution found, verified by tests of normality (Shapiro-Wilk test, Histogram, QQ-plot and box plot) of distribution and homogeneity of the variances involved in the experiment. Statistical differences were determined by the software package SPSS for Windows, Version 21 (SPSS Inc., Chicago, IL, USA)., using the Wilcoxon test to analyze the results from the human study. *P*-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Preparation and Characterization of Nanoemulsions

In our study, NEs containing oily phase with 3 different concentrations (%5, %6.68, %10 (w/w)) were successfully manufactured by ultrasonic emul-

sification method. Two different surfactant and cosurfactant mixtures (Poloxamer 188/Transcutol® HP and Cremophor® A25/Transcutol® HP) with 1:1 (w/w) and 1:2 (w/w) surfactant and cosurfactant ratios were tried. Poloxamer 188 (5% w/w) and Transcutol® HP (10% w/w) was the only surfactant and cosurfactant combination that resulted in a stable formulation and therefore was chosen for further study. The selected NEs were then characterized and the stability of them was studied. According to the results of the stability tests, among the tested NE formulations, the NE formulation with the least change in terms of particle size, PDI and, zeta potential values was selected as the optimum NE formulation.

There are various methods to making NEs, but these are usually inconvenient or costly and a phase diagram needs to be prepared to obtain a stable emulsion. However, NEs prepared with polymeric surfactants can be stably produced with small droplet sizes without a phase diagram (Wik, Bansal, Assmuth, Rosling, & Rosenholm, 2020). In this study, Poloxamer 188, a triblock copolymer, was chosen as surfactant to stabilize the NEs due to its well-known stabilization property and FDA approval status for human use. This polymeric surfactant has already been used in numerous studies to make a stable NE formulation (Wulff-Pérez, Gálvez-Ruíz, De Vicente, & Martín-Rodríguez, 2010; Wulff-Pérez, Torcello-Gómez, Gálvez-Ruíz, & Martín-Rodríguez, 2009). Transcutol®, a potent, non-toxic, biodegradable solvent, was also included as a co-surfactant to obtain a steady liquid interfacial film. Similar to our study, there are various researches in the literature using Transcutol® as a co-surfactant in their formulations (Goyal, Arora, & Aggarwal, 2012; Solanki, Sarkar, & Dhanwani, 2012).

As a result, all of the prepared formulations had low viscosity, and exhibited a milky and bluish appearance. Characterization study revealed that the average droplet diameter, PDI and zeta potential of all NEs were varied from 102.70 ± 3.10 nm to 126.30 ± 0.90 nm, 0.147 ± 0.01 to 0.253 ± 0.01 and - 21.40 ± 1.45 mV to - 34.10 ± 0.6 mV, respectively. In the prepared NEs, the droplet sizes of less than 200 nm of the dispersed oil phase, the PDI values of less than 0.3, and zeta potential values (ζ) of less than −20 mV, indicate their high kinetic stability and they are considered to be convenient for topical applications (Kumar, & Mandal, 2018; Pongsumpun, Iwamoto, & Siripatrawan, 2020).

Preliminary Stability Tests

NEs are thermodynamically unstable colloidal disperse systems, and thus stability issues such as creaming, sedimentation, coalescence or phase separation are likely to arise on long-term storage (Wik, Bansal, Assmuth, Rosling, & Rosenholm, 2020). Therefore, accelerated stability tests (centrifugation and thermal tests) were performed on NEs prepared with Poloxamer 188 and Transcutol® HP to accelerate emulsion degradation. The test result was recorded by observing any signs of physical stability issues. As a result of the centrifuge test, it was observed that all the tested formulations remained stable, indicating that the NEs were durable to centrifugal forces. However, thermal stress caused stability problems such as coalescence and creaming, which are frequently seen in colloidal systems in formulations prepared with 10% Poloxamer 188 and 10% Transcutol® HP in this study. On the other hand, it was determined that only the NEs containing 5% Poloxamer 188 and 10% Transcutol® HP remained stable after thermal stress test.

The heating/cooling cycle (H-C) test might be useful to evaluate the NEs stability at extreme temperature changes (Mota Ferreira et al., 2016). In our study, we observed that the NEs did not show any signs of instability issues after six H-C cycles. When the results were investigated, it was found that the droplet sizes of the NEs maintained at the desired nano-size range (min: 105.80 ± 0.95 nm and max: 140.70 ± 3.30)

after six H-C cycles. While the average droplet diameter of the selected NE formulation (F6P2) was 108.4 ± 0.90 nm before the test, it was found to be 128.00 ± 2.30 nm with a slight increase after the test (Figure 1). In addition, PDI values of the NEs remained under 0.3 after six H-C cycles. So, it can be concluded that all NEs tested had a similar droplet size distribution even under extreme conditions.

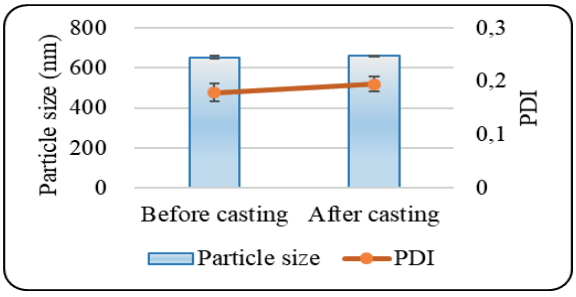


Figure 1. Comparison of average droplet size and PDI value of the selected NE formulation (F6P2) before and after the H–C test (means ± SD, n = 3).

Physical Stability Test

In order for the formulation to remain stable throughout its shelf life, an appropriate production process and formulation excipients should be selected, but the formulation should also be resistant to environmental conditions such as heat, humidity or light. To test the stability of the NEs, physical stability studies were conducted on selected NEs over a 3-month period in three different stability test environments. Organoleptic control, average droplet diameter, PDI, zeta potential, viscosity, pH and conductivity were monitored by taking measurements at pre-determined time points during the storage period.

All selected NE formulations in physical stability study remained in a homogenous state and showed no physical or phase changes after 3 months under all storage temperatures. According to Table 3, the droplet size, PDI, and zeta potential value of the selected NE did not change statistically over three months in three different storage environments. The droplet size of the selected NE was maintained between 120.90 and 129.80 nm for 3 months. All selected NE have low PDI values (below 0.2) during the 3-month test period, indicating a narrow droplet size distribution and superior stability of the system.The zeta potential of the selected NE scarcely changed in the range of -20.50 to -29.20 mV during the 3 months of the physical stability study. This indicated that the different storage temperatures did not have a significant effect on the repulsive forces between the droplets of the NEs.

If the pH values of the NEs measured at different time points are in the skin pH range (4 - 6), it can be stated that the formulations can be used safely on human skin (Muhammad, Akhtar, Haji, Mustafa, & Murtaza, 2014). In our study, it can be clearly seen that the change in pH of the selected NEs is negligible at different measurement points under different storage conditions and they are suitable for topical application. Conductivity measurement is a useful technique for determining emulsion type and observing changes during production or storage (Kildaci, Budama-Kilinc, Kecel-Gunduz, & Altuntas, 2021). Accordingly, the conductivity measurement results showed that there were no significant electrical conductivity changes in any of the NEs in three different stability environments during the 3 months. Korhonen et al. reported that oil-in-water emulsions have higher conductivity values compared to water-in-oil emulsions, since the outer phase is water (Korhonen, Niskanen, Kiesvaara, & Yliruusi, 2000). In line with this information, high conductivity values of the NEs produced in our study indicate the external phase of the NEs is aqueous.

Table 3. Physical stability test results of the selected NE formulation (means ± SD, n = 3).

Formulation	Temp.	Time	Average Droplet Size (nm)	Polydispersity Index (PDI)	Zeta potential (mV)	pH	Conductivity (µs/cm)
F6P2	5 °C	T30	128.90 ± 2.10	0.166 ± 0.01	-20.50 ± 0.70	4.57 ± 0.01	80.00 ± 1.24
		T60	127.40 ± 1.60	0.149 ± 0.01	-23.80 ± 1.00	4.52 ± 0.03	85.40 ± 2.57
		T90	123.10 ± 2.10	0.165 ± 0.04	-27.40 ± 0.90	4.51 ± 0.03	86.30 ± 3.44
	25 °C	T1	126.30 ± 0.90	0.147 ± 0.01	-21.40 ± 1.45	4.58 ± 0.02	74.20 ± 2.86
		T30	129.80 ± 1.00	0.159 ± 0.01	-23.30 ± 0.60	4.52 ± 0.01	93.80 ± 1.05
		T60	125.50 ± 2.30	0.160 ± 0.04	-26.40 ± 3.10	4.56 ± 0.01	114.60 ± 3.62
		T90	123.60 ± 0.50	0.133 ± 0.10	-27.20 ± 0.70	4.52 ± 0.02	129.50 ± 4.23
	40 °C	T30	125.90 ± 2.40	0.165 ± 0.017	-22.00 ± 0.90	4.52 ± 0.02	107.20 ± 2.21
		T60	120.90 ±0.70	0.165 ± 0.021	-29.20 ± 0.50	4.53 ± 0.03	152.30 ± 0.96
		T90	125.00 ± 2.80	0.159 ± 0.058	-25.80 ± 0.60	4.61 ± 0.01	157.30 ± 3.25

The results obtained from the rheological study for the selected NE formulation is represented in Table 4. It can be observed that the NE presented Newtonian behavior characterized by the independence of viscosity from the applied shear rate. Such rheological feature was observed in all NE formulations in this study. Our experiments are consistent with previous results (Shakeel, 2017; Erramreddy, Tu, & Ghosh, 2017).

Table 4. Viscosity measurements of the selected NE during the 3 months of the physical stability study at 5 ± 3 °C; 25 ± 2 °C and 60% RH; 40 ± 2 °C and 75% RH.

Speed (rpm)	Viscosity (cP)									
	T1	T30			T60			T90		
	25 °C	4 °C	25 °C	40 °C	4 °C	25 °C	40 °C	4 °C	25 °C	40 °C
70	6,63	7,01	6,45	6,76	6,46	6,64	6,74	6,45	6,68	6,98
75	6,62	6,71	6,38	6,35	6,46	6,79	6,02	8,11	6,54	7,15
80	6,45	6,54	6,25	6,37	6,36	6,76	5,97	7,85	6,21	7,03
85	6,39	6,46	6,26	6,29	6,32	6,8	5,92	7,54	6,08	6,85
90	6,39	6,25	6,18	6,31	6,21	6,77	5,89	7,34	6,10	6,69
95	6,47	6,26	6,18	6,25	6,20	6,74	5,92	7,30	5,92	6,68
100	6,42	6,29	6,10	6,26	6,12	6,71	5,89	7,06	5,89	6,67
105	6,42	6,18	6,18	6,28	6,10	5,69	5,85	7,10	5,85	6,66
110	6,43	6,18	6,06	6,23	6,00	6,67	5,89	7,08	5,83	6,60
115	6,43	6,14	6,06	6,26	5,96	6,71	5,86	6,99	5,52	6,65

Microbiological Test

The selected NE formulation was found to be effective on *C.albicans* in microbiological test. The MIC values of the tested formulation are as shown in Table 5. According to these results, the selected formulation F6P2 has showed good antifungal activity against *C. albicans*, especially clinical strains. *C. albicans* is an important pathogen that is representative for yeast fungi in many antifungal studies in the literature, and the antifungal activity obtained from that studies, indicates that the studied substance might be effective against many yeasts, including *M. furfur*. This efficacy was also similar to the antifungal activities of the active essential oils' in the NE formulation.

Table 5. *In vitro* susceptibility of *C.albicans* and its clinical isolates to the selected NE formulation by microdilution method.

Yeasts	MIC values (%)
	Test formula (F_6P_2)
***C.albicans* ATCC 10231**	6.75
Clinical isolate 1	1.6
Clinical isolate 2	0.4
Clinical isolate 3	0.8
Clinical isolate 4	3.1

***In Vivo* Studies**

The tested NE formulation did not cause any visual skin irritation after the skin irritation patch test at the end of the 48 hour period. Thus, it can be concluded that the tested formulation was well tolerated and compliance was achieved.

Non-invasive biophysical techniques are more preferred by consumers and cosmetic researchers, are more ethically acceptable and have great advantages such as reproducibility. They enable the detection of invisible changes in the skin or hair without causing pain and discomfort. They are also more suitable for statistical evaluation (Altuntaş & Yener, 2015). Therefore, we performed a single-blind *in vivo* assay to test the safety and efficacy of the selected NE formulation in SD patients using non-invasive biophysical techniques.

Some external factors such as soap, detergent and cosmetics can negatively change the normal pH of the skin. Changes in skin pH can cause irritation or inhibition of the keratinization process (Altuntaş & Yener, 2015). In order to investigate the effect of the formulation on skin pH, pH measurements were taken from the application sites of the volunteers before the test and after the 4-week product application period. Consequently, there was no significant difference between the skin pH index, before and 4 weeks after the application with the tested NE formulation ($p > 0.05$) (Table 6).

Suchonwanit et al. conducted a study to evaluate the biophysical and physiological profiles in scalp SD (Suchonwanit, Triyangkulsri, Ploydaeng, & Leerunyakul, 2019). As a result, they showed that the mean skin moisture content was significantly lower in SD patients compared to healthy volunteers ($p < 0.05$). Skin surface lipid was also measured significantly higher in SD group ($p < 0.05$). In line with this study, we detected low moisture and high sebum contents in the skin of patients with SD at the beginning of the study. While the mean skin hydration index significantly increased after the tested water based formula was applied (17.12 versus 27.04 AU; $p = 0.001$), the mean skin sebum content significantly decreased (25.80 versus 18.90 mg/cm^2; $p = 0.008$) (Table 6). The possible explanation for the increase in moisture content in the application area can be made as the changing epidermal barrier function of the skin resulting from the inflammatory process is restored to its healthy state with the emollients and humectants included in the formulation composition.

The increase in the skin moisture content was accompanied by a decrease in skin erythema. After 4 weeks of application of the test formulation, there was a statistically significant reduction of the mean skin erythema index compared to baseline (T0) (585.60 versus 567.30; $p = 0.001$) (Table 6).

Table 6. *In vivo* study variables results before and after treatment with the selected NE formulation.

Variable	Before administration (mean)	Four weeks after administration (mean)	*p* value
Skin pH	5.70 ± 0.2	5.60 ± 0.1	0.20
Skin sebum content (mg/cm^2)	25.80 ± 19.0	18.90 ± 12.2	0.008
Skin hydration index (AU)*	17.12 ± 17.1	27.04 ± 19.3	0.001
Skin erythema index (AU)*	585.60 ± 40.5	567.30 ± 34.0	0.001

*AU, arbitrary units

Also, when patients were asked to state their opinions about the test formulation, they agreed that the product was 80% effective in reducing skin redness, 84% in reducing dandruff, 72% in reducing skin itching, and 64% in reducing skin oiliness. 72% of the volunteers expressed their satisfaction with the test product they used.

Table 7: Questionnaire scores given by the volunteers (n = 5).

Evaluation criteria	The questions in the questionnaire	Score	percentage (%)
0 to 5 (dissatisfaction – most satisfaction)	Did the NE serum you used reduce the redness on your skin?	4.0	80
	Did the NE serum you used reduce the amount of dandruff on your skin?	4.2	84
	Did the NE serum you used reduce itching?	3.6	72
	Did the NE serum you used relief your skin?	3.4	68
	Did the NE serum you used moisturize your skin?	3.6	72
	Did the NE serum you used reduce the oiliness on your skin?	3.2	64
	Is the NE serum you used easily absorbed by your skin?	4.8	96
	Did the NE serum you used give your skin a soft feeling?	3.2	64
	Did the NE serum you used cause any allergic reaction such as redness, itching, burning on your skin?	0.2	4
	Are you satisfied with the NE serum you used?	3.8	76
yes/no	Would you consider using the NE serum again?	4 - yes 1- no	80

CONCLUSION

In this study, a herbal-based NE formulation was successfully developed against SD. For this purpose, a formulation in NE form with moisturizing, regenerating and healing effects was prepared in a stable form using constituents known to have both antifungal and antimicrobial activity (tea tree, sage, cinnamon and oregano essential oils, and honey) together with other antioxidant substances (extracts from *Aloe vera,* colloidal oatmeal, and liquorice; grape seed oil and sesame oil).

This formulation was subjected to an *in vitro* microbiological study and *in vivo* non-invasive instrumental analyzes and a subjective questionnaire test to demonstrate its therapeutic potential. When all findings were evaluated, the herbal based NE formulation was considered to be effective significantly against SD. Since SD is an incurable disease, this study will thought be a good reference for ongoing research on the subject.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

Developing hypothesis (F.G.Y., E.A.), experimenting (F.D., E.A., S.D.), preparing the study text (E.A.), reviewing the text (F.G.Y., E.A.), statistics, analysis and interpretation of the data (F.D., E.A.), literature research (F.D., E.A.).

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