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RESEARCH ARTICLE

Development of Innovative Cosmetic Formulations to Help Fungal Treatment and Testing the Efficiency of Formulations

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SUMMARY

In the fungus of hands and toenails, the thickening of the nail and its yellow color is the first signs of attention. The fungus of hands and toenails is mainly caused by Trichophyton rubrum dermatophyte. They have antifungal properties due to the components of lavender oil, geranium oil, and tea tree oil structures. Oral antifungal agents used the treatment of nail fungus can cause serious side effects, especially the liver. Therefore; topical applications have been given importance in recent years. However; in topical applications, antifungal agents have difficulties sending to the target area. Therefore; nanoemulsion technology was preferred in the study. Nanoemulsion formulations of essential oils were prepared using the ultrasonication method. Centrifugal and thermal tests were applied as preliminary stability to the formulations, the pH value, viscosity, droplet size, and polydispersity index of the formulations passing this step were measured, and organoleptic controls were performed. Antifungal efficacy and release studies were performed on the formulations F4P3-I (pelargonium), F4P3-L (lavender), F4P3-C (tea tree), and F4P3-K (mixture), which were successful as a result of all the tests. According to the study, it was concluded that F4P3-I, F4P3-L, F4P3-Ç, and F4P3-K formulations might help in the treatment of fungi.

Key Words: *Lavender oil, geranium oil, tea tree oil, nanoemulsion, ultrasonication*

Mantar Tedavisine Yardımcı Yenilikçi Kozmetik Formülasyonların Geliştirilmesi ve Formülasyonların Etkinliğinin Test Edilmesi

ÖZ

El ve ayak tırnaklarındaki mantarlarda genellikle tırnağın kalınlaşması ve sarı bir renk alması ilk dikkat çeken belirtilerdir. El ve ayak tırnaklarındaki mantara çoğunlukla Trichophyton rubrum dermatofiti neden olmaktadır. Lavanta yağı, ıtır yağı ve çay ağacı yağı yapılarında bulunan bileşenlerden dolayı antifungal özelliğe sahiptirler. Tırnak mantarı tedavisinde kullanılan oral antifungal ajanlar özellikle karaciğer üzerinde ciddi yan etkilere yol açabilmektedir. Bu yüzden son yıllarda topikal uygulamalara önem verilmiştir. Ancak topikal uygulamalarda da antifungal ajanların hedef bölgeye gönderilmesinde zorluklar yaşanmaktadır. Bu nedenle, çalışmada nanoemülsiyon teknolojisi tercih edilmiştir. Ultrasonikasyon yöntemi kullanılarak uçucu yağların nanoemülsiyon formülasyonları hazırlanmıştır. Formülasyonlara ilk önce santrifüj ve termal testler uygulanmıştır ve stabil kalan formülasyonların pH değeri, viskozite, damlacık boyutu ve polidispersite indeksi ölçülmüştür ve organoleptik kontrolleri yapılmıştır. Tüm testler sonucunda başarılı olan F4P3-I (ıtır), F4P3-L (lavanta), F4P3-Ç (çay ağacı) ve F4P3-K (karışım) formülasyonlarında antifungal etkinlik ve geçiş çalışmaları gerçekleştirilmiştir. Çalışmaya göre F4P3-I, F4P3-L, F4P3-Ç, F4P3-K formülasyonlarının mantar tedavisine yardımcı olabileceği sonucuna varılmıştır.

Anahtar Kelimeler: *Lavanta yağı, ıtır yağı, çay ağacı yağı, nanoemülsiyon, ultrasonikasyon*

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INTRODUCTION

Various methods are used in the treatment of hands and toenails fungus. These methods include chemical or mechanical removal of nails, treatment with systemic antifungal drugs, treatment with topical nail polishes, or a combination of these (Denning, 1995). In most cases, oral antifungal agents such as terbinafine, itraconazole, and fluconazole are used because they penetrate the nail bed and nail plate. However, the common side effects of these drugs include headaches, gastrointestinal symptoms, nausea, and rashes (Kreijkamp-Kaspers, 2017). As a topical treatment, amorolfin, ciclopirox, etc., products that are usually in solution form are used. Topical antifungal agents have the advantage of causing fewer side effects, while their effectiveness is limited, and treatment times are extended due to low nail plate penetrations (Elewski, 2013).

In recent years, nanoemulsion technology has been used in cosmetics, medicines, paints, etc. Nanoemulsions are oil-in-water (o/w) or water-in oil (w/o) emulsions with average droplet diameters ranging from 50-1000 nm (Shah, 2010). Nanoemulsions have several advantages, including high tolerability, rapid biodegradation, high bioavailability, good stability, and reasonable skin penetration rates of the active agents (Tadros, 2004; Aboofazeli, 2010). High energy or low energy methods are used to prepare of nanoemulsions (Anton, 2009). In high-energy methods, ultrasonics, microfluidizers, and high-pressure homogenizers are used (Graves, 2005; Mason, 2006; Jafari, 2007). In low-energy, phase inversion temperature, and phase inversion composition methods are used (Marszall, 1975; Shinoda, 1986).

This study aims develop formulations in the form of nanoemulsions, which can provide a more effective penetration of the active substances from the nail in the treatment of nail fungus and to examine their antifungal activity and release profiles with *in vitro* experiments.

MATERIAL AND METHODS

Materials

Lavandula angustifolia oil, *Melaleuca alternifolia* oil and *Pelargonium graveolens* oil were purchased Herbarom Laboratoire (Aouste-sur-Sye, France). Geogard Ultra is obtained from Lonza Group AG (Basel, Switzerland). Pluronic® F68 was obtained from BASF (Ludwigshafen, Germany). Transcutol® HP is obtained from Gattefosse (Lyon, France). Tween 20® (Polysorbate 20) and RPMI-1640 medium; were purchased from Merck KGaA (Darmstadt, Germany). *Trichophyton rubrum*, ATCC 28188, is purchased from ATCC (Virginia, USA). Ketoconazole is purchased from Liofilchem, Inc. (Italy). All organic solvents and other chemicals were analytical grade and obtained from Merck KGaA.

Preparation Method for Nanoemulsion Formulations

The essential oils are added to Transcutol® HP. Minimum inhibition concentrations (MIC) of essential oils against *Trichophyton rubrum* dermatophyte have been found in the literature and used in formulations at this rate (Shin, 2004). The oil phase is added into the water phase, which comprised a Pluronic® F68 and Geogard Ultra mixture at the same temperature (20 °C). To see whether the pre-mixing process affects on the characterization of nanoemulsion formulations, only half of the formulations had an ultrasonication method and the other half was pre-mixing and ultrasonication.

In the pre-mixing process, the formulations were mixed under constant stirring (8,100 rpm) and temperature (20°C) with an Ultra-Turrax (IKA T-25 Digital, Germany) for 5 m. In the ultrasonication process, the formulations were sonicated using a Hielscher UP200Ht probe-type sonicator (Hielscher®, Teltow Germany) at 50% amplitude level for 20 and 30 minutes to obtain a nanoemulsion.

Table 1. Preparation of Nanoemulsion Formulations of Essential Oils

Ingredients	F4P3-L (%)	F4P3-I (%)	F4P3-Ç (%)	F4P3-K (%)
Lavandula angustifolia oil	0.05	-	-	-
Melaleuca alternifolia oil	-	-	0.1	-
Pelargonium graveolens oil	-	0.05	-	-
Mixture (Lavender, Tea Tree Oil, Geranium)	-	-	-	0.2
Pluronic® F68	0.033	0.033	0.067	0.133
Transcutol® HP	0.067	0.067	0.133	0.267
Geogard Ultra	0.75	0.75	0.75	0.75
Water	99.1	99.1	98.95	98.65

Characterization of Nanoemulsion Formulations

Dynamic light scattering, called Photon Correlation Spectroscopy (PCS), is used to analyze fluctuations in the scattering intensity of droplets and particles due to Brownian motion (Ruth, 1995). Nanoemulsion droplet size, polydispersity, and zeta potential can be evaluated by PCS using a particle size analyzer. The polydispersity index indicates the quality or homogeneity of the dispersion (Li, 2011).

Particle Size and Zeta Potential Measurements

The mean diameter, polydispersity index (PI), and zeta potential of each sample were obtained using a Malvern Zetasizer Nano ZS (Malvern Instruments, U.K.) at 25°C. Before all measurements, the formulations were diluted with distilled water 1/100 in the flask.

***In Vitro* Release Study**

Active components (Linalyl acetate, Terpinene oil, Citronellol) of essential oils released from nanoemulsion formulations were performed using the dialysis bag technique. The dialysis bags (MWCO: 12-14 kDa, Spectrum Laboratories, Inc., CA) were soaked and preconditioned before the experiment. The required amount of formulation (10 ml) was placed into the preconditioned dialysis bag. Then, the dialysis bag is put in 150 ml phosphate-buffered saline (pH: 7.4) and incubated in a thermostatic reciprocating shaker maintained at 37 ± 0.5°C and continuously shaken at 300 rpm. An aliquot of 1 ml of release medium was withdrawn at predetermined time intervals (0.50, 1, 2, 3, 4, 5, 6, 7, and 8 h) and replaced immediately with the same volume of fresh medium to maintain the sink conditions. The concentration of active components in the aliquot was quantified using gas chromatography-mass spectrometry (GC/MS).

GC/MS Analysis

A pharmacopeia method is used for the GC/MS analysis of active components of essential oils during release studies. For this purpose, an Agilent Cary 60 system (Agilent Technologies, California, USA) consisting of an HP-5ms Ultra Inert, 30 m x 250 µm x 0,25 µm column compartment. The chromatograms were monitored and integrated using Agilent ChemStation software. While preparing the samples, 5 ml sample was diluted in 1.5 ml water: acetone mixture, and 2 ml sample were injected. Helium gas with a flow rate of 1.1 ml / min and a split ratio of 10:1 was used as the carrier gas. The injector temperature is 220°C. The initial and end temperatures of the analysis are 60°C and 260°C, respectively. The temperature increase rate was 3°C/min and the total analysis time took 66.6 min.

Disk Diffusion Method

The antifungal efficacy of the formulations against *Trichophyton rubrum* ATCC 28188 standard strain *in vitro* was evaluated using the disc diffusion method. For this purpose, a mixture of 200 ml of 2% glucose and 2% agar was sterilized in an autoclave, then added to RPMI 1640 medium (Applichem, Darmstadt, Germany) and distributed in Petri dishes. The inoculum of the microorganism used in the experiment was

prepared by CLSI (Clinical and Laboratory Standards Institute) M38-A criteria. The dermatophyte conidia suspension used in the study was prepared using 5 ml of sterile 0.9% saline.

The concentration of dermatophyte conidia suspension is $1.5x10^6$. The prepared conidia suspension was spread on the surface of Petri dishes containing sterile RPMI 1640 medium with 2% glucose. The Petri dishes were left to dry under aseptic conditions for 15 minutes. Then, 10 µl of formulation impregnated discs were added to the Petri plates. Discs containing 10 µg Ketoconazole (Liofilchem) are included in the study as a control. Petri dishes were incubated at 25°C for 4-7 days. The activity is evaluated by measuring the inhibition zones formed around the discs at the end of the incubation.

RESULTS AND DISCUSSION

In this study, nanoemulsion formulations of essential oils were successfully prepared using the ultrasonication method with the mixture of Pluronic® F68, Transcutol® HP, *Lavandula Angustifolia* oil, *Melaleuca alternifolia* oil, and *Pelargonium graveolens* oil. According to the literature research, studies have been found on pre-mixing while creating nanoemulsion formulations with this method (Hosseini, 2015; Carpenter, 2016). The formulations that were applied the pre-mixing process could not be stable. It is seen that when the ultrasonication process time increased, the particle size and polydispersity index (PDI) value of formulations decreased. When the droplet size and PDI values are examined, it was found that formulations with 30 minutes ultrasonication process, oil phase: surface- active substance ratio of 1:2 and Pluronic® F68 as surface-active substance and Transcutol® HP as co-surfactant were more suitable. The average particle size and polydispersity index of the formulations are shown in Table 2. The smallest average size (108.20 nm) was observed in the F4P3-K formulation. The PDI values of the formulations were lower than 0.3. PDI≤0.3 indicates excellent homogenous distribution (Salouti, 2014). The zeta potentials of the formulations are negative.

Table 2. Particle Size, PDI (polydispersity index), and Zeta Potential Values of the Formulations

Formulation code	Particle size (nm)	PDI	Zeta Potential
F4P3-L	182.4 ± 1.5	0.182 ± 0.05	-24,7 ± 0.79
F4P3-I	141.2 ± 0.9	0.220 ± 0.07	-29,8 ± 0.66
F4P3-Ç	188.1 ± 1.0	0.166 ± 0.04	-30,4 ± 1.22
F4P3-K	108.2 ± 0.3	0.111 ± 0.03	-37,2 ± 1.64

Antifungal activity test of nanoemulsion formulations that passed the long-term stability tests against *Trichophyton rubrum* was performed by disk diffusion method. The inhibition zone diameters of the formulations are given in Table 3.

Table 3. Inhibition Zone Diameters of the Formulations

Formulation code	Inhibiton Zone Diameter (mm)
F4P3-L	13,8
F4P3-I	12,9
F4P3-Ç	10,0
F4P3-K	11,0

Antifungal activity of nanoemulsion formulations of *Lavandula Angustifolia* oil, *Melaleuca alternifolia* oil, and *Pelargonium graveolens* oil has shown. However, the mixture of these oils did not show the expected antifungal activity.

CONCLUSION

Stability and characterization studies of nanoemulsion formulations have been successfully performed. The release of nanoemulsion and pure essential oil formulations was carried by the dialysis bag method in *in vitro* conditions. Figures 1-3 show the release profiles of nanoemulsion and pure formulations of essential oils. Active components of essential oils are examined in the release study. The release of the nanoemulsion formulation, which is a mixture of 3 essential oils from the membrane, is not examined due to having more than one essential oil. The antifungal efficacy test of nanoemulsion formulations against *Trichophyton rubrum* dermatophyte was performed by disc diffusion method under *in-vitro* conditions. The inhibition zone diameter of F4P3-L (lav-

ender nanoemulsion), F4P3-I (geranium nanoemulsion), F4P3-Ç (tea tree oil nanoemulsion), F4P3-K (lavender + geranium + tea tree oil nanoemulsion), and Ketoconazole (positive control) was 13.8, 12.9, 10.0, 11.0, 21,4 mm, respectively. While nanoemulsion formulations of lavender, geranium and tea tree oil showed antifungal activity, the nanoemulsion formulation in the form of the mixture (lavender + geranium + tea tree oil) did not show the expected antifungal activity.

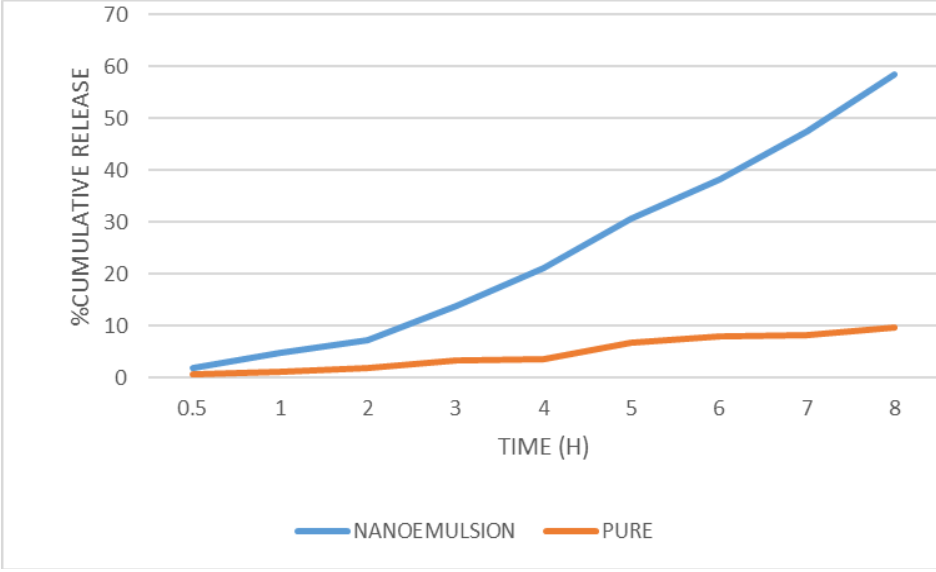


Figure 1. The cumulative release of nanoemulsion and pure lavender oil

Figure 1 shows the time-dependent release of nanoemulsion and pure lavender oil. The time-dependent release profile is determined according to the linalyl acetate substance, which is the significant component of lavender essential oil. After 8 hours, the nanoemulsion linalyl acetate component passed about 8.5 times more than its pure form.

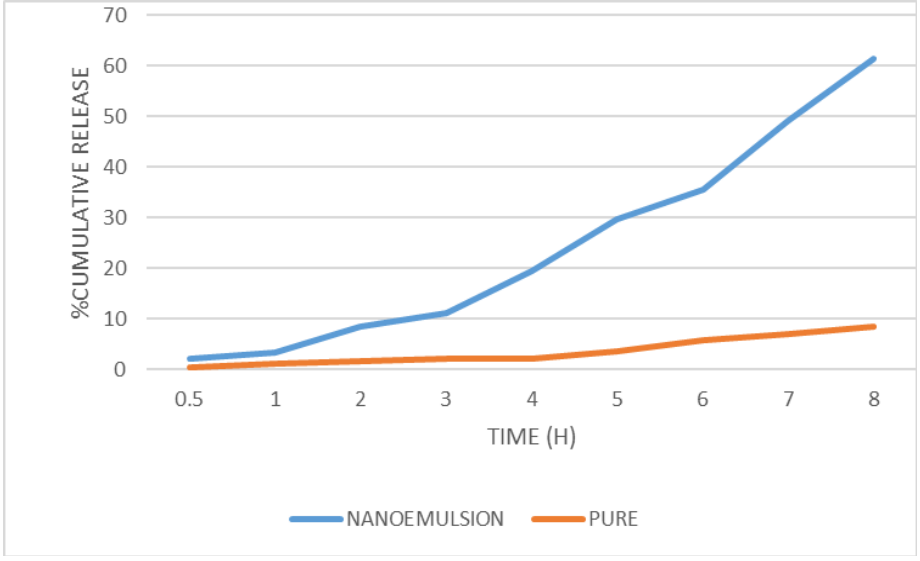


Figure 2. The cumulative release of nanoemulsion and pure tea tree oil

Figure 2 shows the time-dependent release of nanoemulsion and pure tea tree oil. The time-dependent release profile is determined according to the terpinenol substance, which is the significant component of tea tree essential oil. After 8 hours, the nanoemulsion terpinenol component passed about 7.5 times more than its pure form.

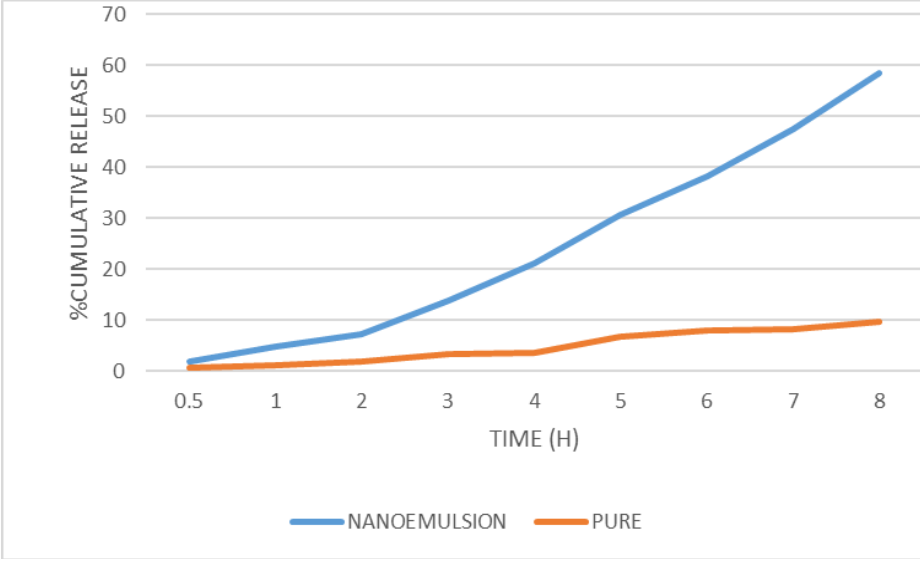


Figure 3. The cumulative release of nanoemulsion and pure geranium oil

Figure 3 shows the time-dependent release of nanoemulsion and pure geranium oil. The time-dependent release profile is determined according to the citronellol substance, which is the significant component of geranium essential oil. After 8 hours, the nanoemulsion citronellol component passed about six times more than its pure form.

As a result of all studies, it was concluded that F4P3-L, F4P3-I, F4P3-K, and F4P3-Ç formulations in nanoemulsion form could penetrate deeper and have better antifungal effects than their pure form.

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AUTHOR CONTRIBUTION STATEMENT

Initial literature survey, experimental design, data acquisition and analysis, interpretation of result, writing and revision of the manuscript (FGY). Initial literature survey, experimental design, sourcing for materials, laboratory work, data acquisition and analysis, interpretation of result (CA). Initial literature survey, experimental design, data acquisition and analysis, interpretation of result (BÖÇ). Initial literature survey, experimental design, sourcing for materials, laboratory work, data acquisition and analysis, interpretation of result (EMK).

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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