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FORMULATION AND CHARACTERIZATION OF OIL NANO-EMULSION BASED GEL FOR DERMATOLOGICAL APPLICATION

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ABSTRACT

Essential oil are one of the most notorious natural products used for medical purposes. Collective with their popular use in dermatology. The spontaneous emulsification, micro fluidization and ultra-sonication were compared at same Geranium oil (GO) %, at higher Geranium oil Concentrations (2, 4, 6, 8 and 10%), results were not significantly different for particle sizes. Moreover, for all GO concentrations, antimicrobial activity could not be enhanced by micro fluidization towards *S. aureus*. However, at higher GO concentration (8 and 10%), enhanced antimicrobial activity were obtained by spontaneous emulsification and ultra-sonication methods, and these results were not found to be significantly different ($p>0.05$). This study revealed that RO could successfully be utilized as carrier oil for preparing Nano-emulsions and stable Geranium oil Nano emulsions could be prepared at lower surfactant concentrations by the spontaneous emulsification method. It concludes the geranium oil was used as a potential antimicrobial agent for skin.

KEYWORDS: Geranium oil, Rosewood oil, Nano emulsion and Antimicrobial activity.

INTRODUCTION

Emulsions are heterogeneous systems composed by way of 2 immiscible liquids, one of which is equally discrete as excellent droplets during the other. These systems are thermodynamically unbalanced and their study and development is one of the most difficult and complex subjects in the pharmaceutical field.[1] Nevertheless, emulsions, in the form of creams, lotions or foams, are extensively used due to their therapeutic properties and as vehicles to deliver drugs and beauty agents through the skin Furthermore, these dosage forms facilitate drug permeation of and through the skin by their occlusive consequences and/or by using the combination of penetration-enhancing molecules.[2]

Nano emulsions may be categorised into three main types: water in oil (W/O), bi-continuous and O/W though a mixture of oil, water and surfactants will be able to generate a variety of structures and phases.[3,4] When similar amounts of oil and water are used, the structures formed are not well characterised and are assumed to be continuous.[5] While the formation of Nano emulsion depends on the capability of the surfactant system to reduce the surface tension, in practice almost all surfactants require the presence of additional co-surfactants. Excipients certain as like short-chain or long-chain alcohols or polyglycerol derivatives have been used to achieve low surface tension. Addition of a co-surfactant reduces the interfacial tension as well as the serious micelle concentration.[6]

Nano emulsions may also enhance topical and transdermal distribution mainly by increasing the solubilisation capacity for hydrophilic and lipophilic compounds, maintaining constant supply of the drug from the interior to the exterior phase and thus keeping the external phase saturated and promoting skin absorption. The method ingredients certain as the oil, surfactants, co-surfactants then entree enhancers can also increase drug diffusion by bettering partitioning through the skin. Also, the low interfacial tension required for Nano emulsion formations may be responsible for the superb wetting properties, which ensures surface contact between the membrane and the vehicle.[7] In addition to their positive diffusion development properties, Nano emulsions may also reduce skin irritancy of certain excipients.[8] Nano emulsion technology forms an encapsulating system for functional materials thus degradation can be prevented and bioavailability of compounds are increased.[9] Nano emulsions usually contains 2 immiscible liquids. One of these liquids is discrete as small globular drops and depending on the type of the continuous phase, Nano emulsions are classified as each oil-in water (O/W) or water-in-oil (W/O).[10]

Percutaneous absorption presents an eye-catching non-invasive way of direction for local topical or systemic effects of substances but is limited by the skin's intrinsic wall to penetration of some exogenous material. It is well-known so much the uppermost layer of the skin, the stratum corneum, is the main wall to such penetration but can be overcome to meet therapeutic and cosmetic aims by judicious considerations the active's potency, physicochemical properties, formulation and distribution systems.[11] Formulation methods consist of optimisation, usage regarding pro-drugs then incorporation of chemical or biological modifiers in conformity with transiently reduce stratum corneum barrier function. The range of transfer systems in recent use include: tansdermal patches, topical products, physical methods like micro needles and heat as well as other technologies, including iontophoresis, sonophoresis, and radiofrequency and laser ablation.[12]

Geranium oil (GO) or mauve is also recognised as a *pelargonium graveolens*, the origination of *pelargonium odorantissimum* tree from South Africa and the mauve is extracted from that tree. The pelargonium (Geraniaceae) genus is represented by many essential oil producing species inter alia: *P. odoranissimum*, *P. roseum*, *P. graveolens* and *P. zonale*. Mauve oil is obtained from flower, stalks and leaves by hydrodistillation or steam. The geranium oil is composed by various chemical constituents like citronellol, geraniol, linalool, and their esters.[13,14] The geranium oil is non-irritant, non-sensitizing, non-toxic and also it does not cause any other side effects, and the geranium oil have some therapeutic properties such as antiseptic, antidepressant and healing. It is used to diverse dermatological problems such as eczema, congested skin or oily and dermatitis.[15]

The rosewood oil extracted from the *Aniba rosaeodora* duke (Lauraceae family), it is a large tree and grown up to 30m height, occurring in the Amazon region. All part of the tree is fragrant although only the trunk wood is harvested and distilled. The oil is a colourless to pale yellow liquid with a woody-floral fragrance. The major constituent of rosewood oil is the linalool, monoterpene, which can be transformed into a number of derivatives of value to the flavour and fragrance industries.[16]

Nevertheless, the combination of GO and rosewood oil. The objective of the recent study was to investigate the Nano emulsions formation by low energy and high energy methods by using precise ideal system: non-ionic surfactant, oil, and water. In some additional information, there is no study in the literature about the optimizing of Nano emulsion preparation in terms of emulsion stability, emulsification efficiency (EE) and emulsion stability. The formation and characterization of Nano emulsions were analysed in term of physical properties like particle size and morphology.[17]

MATERIALS AND METHODS

Low energy method- Nano emulsion Preparation by Spontaneous Emulsification

Spontaneous emulsification was carried out by titration of organic phase containing Tween 80 and different amount of geranium and rosewood oil into aqueous phase while the system was continuously stirred at 750 rpm with magnetic stirrer at ambient temperature (25 °C). Experimentations were executed using standard method: 10wt % oil (geranium + rosewood), 10 wt % surfactant polyoxyethylene 20 (Tween 80) and 80wt% distillate water. To conduct particle size measurement, emulsions that include 2%, 4%, 6%, 8% and 10% geranium oil were prepared. In these samples, firstly oils were mixed together during 30 minutes and then surfactant was added and organic phase was mixed for another 30 minutes. The combination was titrated into aqueous phase at a speed of 1 ml /min.

High Energy Method- Pretreatment with Ultra-Turrax Homogenizer for Nano emulsion Formation

Nano emulsion was formed pre-homogenization of oil/water by mixing oil phase that include (geranium + rosewood oil + T-80) and aqueous phase (distillate water) at 10000rpm for 2 min.

Nano emulsion formation by Micro fluidization

To examine the consequence of micro fluidization on particle size, pre-homogenized Nano emulsions containing 10wt% oil and the ratio between (Geranium: rosewood oil- 2:8, 4:6, 5:5, 6:4, 8:2, 10:0) 10 wt % surfactant polyoxyethylene 20 (Tween 80) and 80wt% distillate water was subjected to high pressure micro fluidization. Then the sample was treated at 827 bar for 3 passes. The sample was passed through the micro fluidizer after sample passing if the particle measured.

Nano emulsion formation by Ultra sonication

To examine the effect of ultra-sonication on particle size of Nano emulsions, first, oil mixtures (2:8, 4:6, 5:5, 6:4, 8:2, 10:0, geranium oil: rosewood oil) were obtained following 30 minutes mixing with magnetic stirrer. After that, surfactant was added to the mixtures and obtained organic phase was mixed for another 30 minutes. Nano emulsion was formed when organic phase (10wt% (geranium oil + rosewood oil) + 10wt% Tween 80) and aqueous phase (distillate water) mixture was sonicated using an ultrasonicator for 10 minutes at 75 W.

Determination of the Critical Micelle Concentration (CMC) of Tween 80

To test if the surfactant used form micelles and inhibit Nano emulsion formation, critical micelle concentration of Tween 80 was determined. Tween 80 – water mixture containing 2%, 4%, 6%,8%, 10%,12%, 14%,16%,18% and 20% Tween 80 was titrated into oil phase with 6:4 CO to CNO ratio by using tensiometer to find surface tension between oil-water interface.

Characterization of Nano emulsions- Mean Particle Size Measurements

The distribution of particle size was determined by using dynamic light scattering and to measure the z-average (mean particle diameter). By using intensity time fluctuations of laser beam (633nm) that is scattered from sample with 173^0, the mean particle size of samples were determined. On an average, 15run was done for each individual measurement. Before the measurement, sample was diluted to avoid multiple scattering effects.

Antimicrobial Test- Agar Disc Diffusion Method

Before agar disc diffusion test, *E. coli* was inoculated on BHI agar with a loop from the stock culture. It was incubated at 37^0C for 20-24 hours. Following incubation, several colonies were selected and suspended in MHB. After incubating again in the incubator at 37^0C for 2 hours, turbidity of the suspensions was controlled with 0.5 McFarland standard by using white paper with black lines. If suspension showed same transparency with McFarland, it was ready for inoculation on MHA for agar disc diffusion test. Cotton swab is used for inoculation, and the whole plate was covered by streaking back and forth from edge to edge. Swabbing procedure was repeated three times while rotating plate 60^0.

Standard 6 mm paper discs that were obtained from filter paper were used for disc diffusion tests. 20µl of Nano emulsion having active compound was added on a disc. After 30 minutes the discs were placed on the inoculated plate while pressing each disc down family.

When the incubation was completed, zone diameters around the discs were measured. In order to control the accuracy of inhibition, antimicrobial disc such as Tetracycline (TE 30) were used as positive control and these zone diameters were compared with Clinical Laboratory Standards Institute (CLSI). Throughout this procedure, validation of the antimicrobial tests was conducted. Zone diameters were measured holding the plates on black nonreflecting surface. Measurements were done by using a rule.

RESULT AND DISCUSSION

Spontaneous Emulsification: Effect of Different Oil Combinations on the Mean Particle Size of Nano emulsion

The first factor studied on the Nano emulsions was the effect of oil phase composition on the characteristics of the emulsions. Oil phase included Geranium oil and Rosewood oil with different mass ratios (G: R = 2:8, 4:6, 6:4, 8:2, 10:0). For this analysis, total oil ratio of the emulsions was kept at 10% while surfactant (Tween 80) and aqueous phase were 10% and 80% respectively**.** Effect of oil composition on mean particle size of Nano emulsions are shown in Figure. 1.

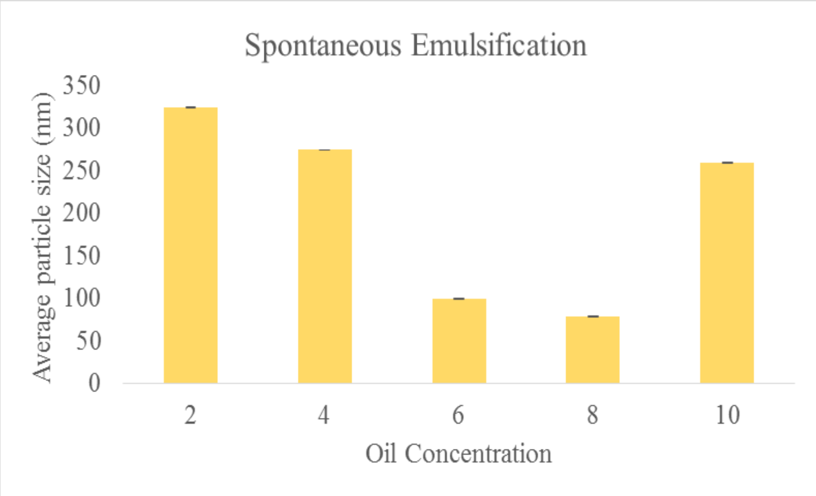

Figure 1: Effect of oil phase composition on particle size.

Our results indicated that by using spontaneous emulsification method, there was an optimal oil phase composition to obtain more stable, small droplets containing Nano emulsions. Although to form Nano emulsions with small droplets is possible by use of essential oil, Ostwald ripening (OR) is a problem due to water solubility properties.[18] OR can be inhibited by addition of the appropriate amount of highly hydrophobic materials into the oil phase.[19] As expected, addition of a second oil in the organic phase, decreased the droplet size to a certain extent.[20]

Effect of Surfactant to Oil Ratio (SOR) on the Mean Particle Size of Nano emulsions

Finding the minimum amount of surfactant that formed stable Nano emulsions which has small droplet size is very significant because of the safety, taste and economic reasons. In these experiments, effect of surfactant concentration on particle size of Nano emulsions was examined for three different concentrations (SOR: 0.5, SOR: 1 and SOR: 2) while the total amount of oil phase was held constant as 10 wt. % (Geranium oil: Rosewood oil 6:4 (w/w)).

Table 1 Showed that the mean particle diameter as measured by DLS decreased with increasing amount of surfactant.

Table 1: Effect of SOR (Surfactant to Oil Ratio) on Particle Size of Sample. Different letters represent significant difference ($p \leq 0.05$).

SOR	Average Particle Size (nm)
0.5	171±0.8
1	101±0.41
2	27±0.29

The surfactant amount to obtain smaller particle size can be associated with phase behavior of the surfactant-oil-water (SOW) system that was used to form Nano emulsions, since formation of ultrafine droplets by using spontaneous emulsification method occurs with only certain SOW compositions for each surfactant, oil and water combination.[21] Although, increasing surfactant amount reduces droplet size, this case is observed up to a point. When very high amount of surfactant is used, it can lead to droplet formation due to formation of micelles at Critical Micelle Concentration (CMC).

At the end of the study, usage of SOR: 1 was considered appropriate for other experiments since Nano emulsions with ≈100 nm mean particle size could be obtained with SOR: 1. Rather than obtaining smaller particle size with very high amount of surfactant, stable Nano emulsions were tried to be formulated at 10% Tween 80 concentrations by SE because more cost effective and safe.

Micro fluidization: Effect of Oil Phase Composition on the Mean Particle Size of the Nano emulsion

For this study; by using micro fluidization, effect of oil phase composition on the characteristics of emulsion were investigated. As in the spontaneous emulsification, oil phase composition included Geranium oil and Rosewood oil with different mass ratios (Geranium oil: Rosewood oil = 2:8, 4:6, 6:4, 8:2, 10:0). System components were kept constant as 10 % oil phase, 10 % surfactant (Tween 80) and 80 % aqueous phase.

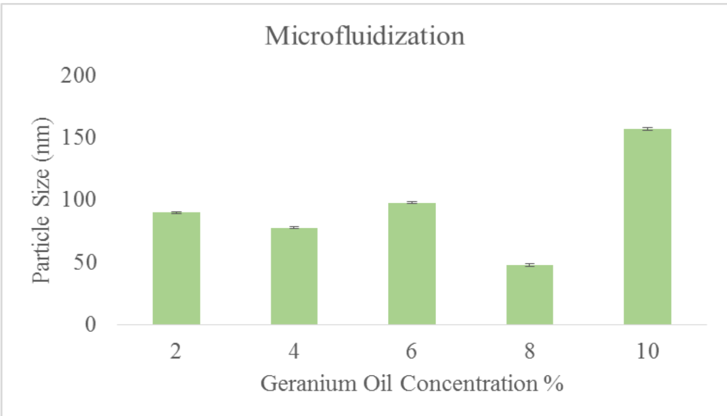

Figure 2: Effect of Oil Combination on Particle Size. Different letters represent significant difference ($p \leq 0.05$).

Similarly, for 8:2 G: R oil ratio, smaller mean particle size was obtained but this result was significantly different than others (Fig.2) ($p<0.05$). Generally, U shape curve.[22,23] is obtained for the graph of particle size vs. concentration, however there were no any regular trend for decreasing and increasing of particle size of droplets.

Ultra sonication: Effect of Oil Phase Composition on the Mean Particle Size of the Nano emulsion

For this study; by using an ultrasonic homogenizer, effect of oil phase composition on the characteristics of Nano emulsion were also investigated. Oil phase composition included Geranium oil and Rosewood oil with different mass ratios (Geranium oil: Rosewood oil = 2:8, 4:6, 6:4, 8:2, 10:0). System components were kept constant as 10 % oil phase, 10 % surfactant (Tween 80) and 80 % aqueous phase**.**

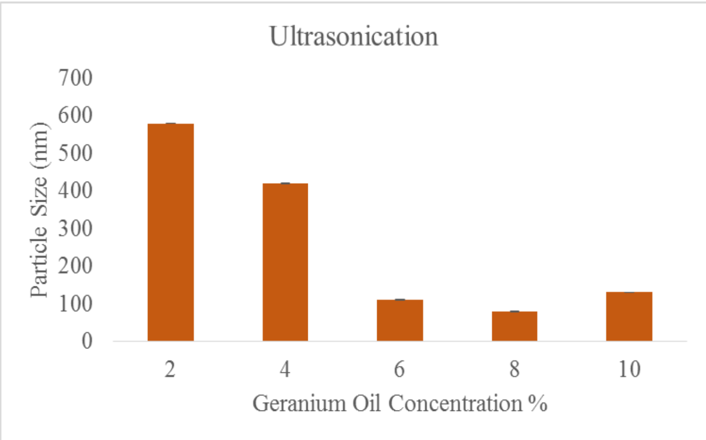

Figure 3: Effect of oil combination on particle size. Different letters represent significant difference ($p \leq 0.05$).

There were no significant difference between 6%, 8% and 10% CO concentrations in terms of mean particle size and smallest particle size (mean particle diameter) was obtained at 8% CO concentration as 75.4 nm (**Fig. 3**). However, formulation have a great effect on these results. In the study of Ghosh et al. (2013), almost same experiment procedure with this study was applied. Cinnamon oil Nano emulsions that included 6wt % CO, Tween 80 (1:1 CO: Surfactant) and distilled water, were obtained by using ultrasonicator at 750 W during 10 minute.[24] Higher energy was applied in this study however carrier oil was not used. This Nano emulsion had ~410nm mean droplet size, although 6wt % CO containing Nano emulsion in this thesis study has ~107nm. This big differences can be originated from effect of carrier oil in the system. Moreover, besides the concentrations of the individual components in Nano emulsion formulation, ultra sonication time and power could have effect on the particle size of final emulsion.[25] For this reason, if different process time and power was applied to system, different particle size results could be obtained with same formulations.

Comparison of Ultra sonication, Micro fluidization and Spontaneous Emulsification for the Mean Particle Size.

Table 2: Effect of Ultra sonication, Micro fluidization and SE on particle Size ($p \leq 0.05$).

Oil Concentration%	Particle Size (nm)		
	Ultra sonication	Micro fluidization	SE
2	580±0.9	90±0.16	325±0.1
4	420±0.86	78±0.6	275±0.25
6	110±0.1	98±0.21	100±0.51
8	80±0.16	48±0.32	79±0.32
10	130±0.28	157±0.16	260±0.28

To understand the change in particle size between Nano emulsions that prepared with spontaneous emulsification, micro fluidization and ultra-sonication processes, obtained results were analyzed. According to ANOVA results, at lower Geranium % (2% and 4 %) concentrations, significantly different results were obtained and minimum value was achieved by micro fluidization. However, at higher Geranium concentrations (6%, 8% and 10%), results were not significantly different, when three methods are compared at same Geranium % (Table.2).

Importance of Correct Dilution on Particle Size Detection of Nano emulsions by Using Dynamic Light Scattering

DLS working principle is based on the Brownian motion of the particles. Due to relatively small particle size, Brownian motion dominates the gravitational force, thus Nano emulsion has high stability.[26] If the emulsion shows instability, this means that inhomogeneity appears in the samples and this situation is detected with DLS through the particle random motion. This measurement depends on the detection of the translational diffusion coefficient (D) following the substitution on Stokes- Einstein equation.[27] However, for correct measurement, particle – particle interactions should be eliminated by dilution. Especially for opaque Nano emulsions and high concentrated samples, correct dilution factor should be found. To detect this factor, mean particle size of gradually diluted samples should be measured.

As a result, obtained mean particle size vs. concentration graphs should be plotted. With decreasing concentration, mean particle size increases to some extent and after that mean particle size value almost becomes fixed for Nano emulsions.

In this study, it was observed that from 1/5 dilution to 1/100 dilution, mean particle size increased and after 1/100 dilution mean particle size remained constant for 1/200, 1/500 and 1/1000 dilution. For this reason, in this study DLS measurements were conducted at 1/100 dilution. Effect of dilution on particle size difference can be seen in the **Figure 4**. With increasing dilution, mean particle size increased. The reason was that if adequate dilution was not done, particles would be in interaction with others. In other words, they would affect the random motion of each other's.[27]

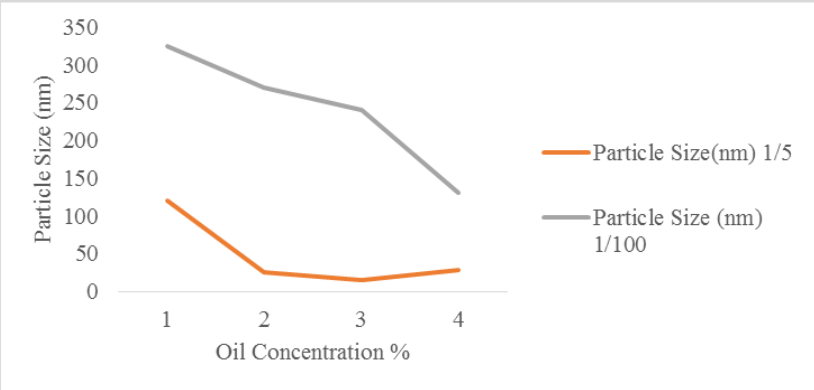

Figure 4: Effect of Dilution on Particle Size.

Effects of Essential Oils and Bioactive Compounds on Antimicrobial Activity towards Staphylococcus aureus

At the beginning of the study, effect of antimicrobial activity of some essential oils (100% concentrations) – Geranium oil, Rosewood oil, orange oil, peppermint oil, basil oil, lemon oil, camomile oil, thyme oil, fenugreek oil and some bioactive compounds (100% concentrations) – linalyl acetate and α – terpinene were investigated against *S. aureus* by agar disc diffusion method. Aim of these experiments was to screen the pure oil types or bioactive compounds for selecting the ones having higher antimicrobial effect against *S. aureus.* Results showed that, antimicrobial effect was only detected for Geranium oil (GO) and rosewood oil (RO). For other oils and active materials, no clear zone was observed on the plates.

Disc diffusion experiment results of pure Geranium oil and rosewood oil are given in Figure 5.

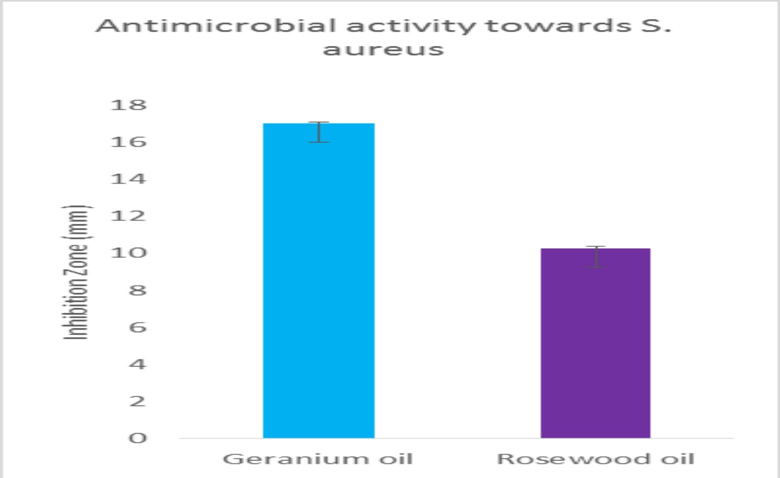

Figure 5: Antimicrobial Activity of Geranium Oil (GO) and Rosewood Oil (RO).

ANOVA results showed that there was significant difference between antimicrobial activities of cinnamon oil and rosemary oil towards *S. aureus* ($p \leq 0.05$).

According to this results, GO was found to have higher antimicrobial effect than RO. With pure GO, 17mm zone was obtained, whereas with pure RO, 10.25 mm zone was recorded. In this study, agar disc diffusion method was used for the antimicrobial activity tests. However different antimicrobial test procedures can lead to different results. For example, in the study of Bigos et al. (2012), direct addition of active materials on filter paper without any drying procedure was performed. Filter papers (6mm) were placed on the agar (sabouraud dextrose agar) and 10 ml of 100% essential oils were added to filter paper immediately. For this test, diameter of the inhibition zone obtained was 24.5 mm for Getranium oil against *S. aureus*.[28,29,30] If compared, the amount of Geranium oil was higher than the one used in this study. In addition to that, since Geranium oil was directly put on filter paper and not kept for drying, this procedure incapacitated the absorption of all essential oil by filter paper and allowed the direct diffusion of non-absorbed oil. That's why, high antimicrobial effect was observed.[29]

In addition to these studies, Afsharzadeh et al. (2013), investigated the microorganism sensitivity in diffusion methods (the disc diffusion, hole plate, and cylinder agar diffusion and agar dilution methods). Most antimicrobial resistant bacteria against extracts of conifer was found as *Staphylococcus aureus* among the other three: *Pseudomonas aeruginosa*, *E. coli* and *Candida albicans*. In accordance with this study, strong antimicrobial effect was found against *S.aureus* by hole plate agar diffusion method, although it demonstrated complete resistance in disc diffusion method. The reason of this was explained with low extract diffusion and inhibition of active material diffusion by precipitation of insoluble materials.[31]

CONCLUSION

Essential oils have strong antimicrobial properties. For this reason, in current years the usage of essential oils has been widespread instead of synthetic chemical compounds. Oil-in- water (O/W) Nano emulsions systems are most commonly used as colloidal delivery system for entrapping these functional components into the aqueous based food, beverage, cosmetic, pharmaceutical and chemical products. Geranium oil (GO) contains highly active compounds and to utilize these materials, Nano emulsions can be formulated. In this study, to understand the effect of Geranium oil in Nano emulsion systems; formulation, characterization and antimicrobial activity.

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