https://doi.org/10.31925/farmacia.2024.5.9 **ORIGINAL ARTICLE**

LEMON OIL NANO EMULSION NEW FORMULATION FOR TOPICAL APPLICATION AND *IN VITRO* EVALUATION FOR BIOLOGICAL ACTIVITY

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Abstract

This study investigates the formulation, physical properties and antibacterial efficacy of a water-in-oil (W/O) nanoemulsion using lemon oil. The nanoemulsion was prepared using Tween-80 as a surfactant, polyethylene glycol as a co-surfactant and water as an aqueous phase. The detailed physical characterization of the nanoemulsion included measurements of droplet size, dispersion properties, viscosity, refractive index, acid concentration and electrical conductivity, which provide a basis for future research improvements. The results showed that the average droplet size of the A2 formulation ranged from 2.992 to 155.6 nm, with a polydispersity index of 0.280 and a zeta potential of -14.1 ± 5.52 mV. Other properties measured were refractive index (1.330 ± 0.014), viscosity (4.487 ± 0.131 cP), pH (5.62 ± 0.0143) and conductivity (364.34 ± 6.23 s/cm). The antibacterial activity of the nanoemulsion was tested against various bacterial strains, with the following order of effectiveness: *Staphylococcus aureus* ATCC 512477 > *Salmonella choleraesuis* ATCC 10708 > *Escherichia coli* ATCC 25922 > *Klebsiella pneumoniae* ATCC 700603 > *Enterococcus faecalis* ATCC 29212 > *Proteus mirabilis* ATCC 299. These results underline the potential of the nanoemulsion as an antibacterial agent.

Rezumat

Acest studiu se concentrează pe formularea și determinarea proprietăților fizice și a eficacității antibacteriane a unei nanoemulsii apă în ulei (W/O) folosind ulei de lămâie. Nanoemulsia a fost preparată folosind Tween-80 ca surfactant, polietilen glicol ca și co-surfactant și apă. Caracterizarea fizică detaliată a nanoemulsiei a inclus măsurători ale dimensiunii picăturilor, proprietăților de dispersie, vâscozitate, indice de refracție, concentrație de acid și conductivitate electrică, care oferă o bază pentru îmbunătățirile viitoare ale cercetării. Rezultatele au arătat că dimensiunea medie a picăturilor a formulării A2 a variat de la 2,992 la 155,6 nm, cu un indice de polidispersitate de 0,280 și un potențial zeta de -14,1 ± 5,52 mV. Alte proprietăți măsurate au fost indicele de refracție (1,330 ± 0,014), vâscozitatea (4,487 ± 0,131 cP), pH-ul (5,62 ± 0,0143) și conductivitatea (364,34 ± 6,23 s/cm). Activitatea antibacteriană a nanoemulsiei a fost testată împotriva diferitelor tulpini bacteriene, cu următoarea ordine de eficacitate: *Staphylococcus aureus* ATCC 512477 > *Salmonella choleraesuis* ATCC 10708 > *Escherichia coli* ATCC 25922 > *Klebsiella pneumoniae* ATCC 700603 > *Enterococcus faecalis* ATCC 29212 > *Proteus mirabilis* ATCC 299. Aceste rezultate subliniază potențialul nanoemulsiei ca agent antibacterian.

Keywords: nano emulsion, lemon oil, Droplet size distribution, viscosity, conductivity, anti-bacterial activity

Introduction

Acne is a dermatosis that affects the hair follicles and sebaceous glands on the face, chest and back. Acne affects up to 85% of people [1]. Although there is no immediate danger, patients with psychological well-being can suffer from acne. *Staphylococcus, Propionibacterium, Streptococcus, Corynebacterium* and *Malassezia* are some of the most important microbial genera associated with skin health. Human skin, which is the largest organ in the body, also consists of a variety of other microorganisms. A common bacterial cause of acne is *Cutibacterium acnes* (*C. acnes*) [2]. Therefore, elimination of the acne-causing bacterium *C. acnes*, has already been identified as an important treatment target in acne. The skin microbiota species *C. acnes* is found in areas with a high concentration of sebaceous glands, such as the face and trunk. It is known to be a commensal bacterium and contributes to maintaining healthy skin. However, because it is involved in numerous infections, it has become an opportunistic pathogen. *C. acnes* are the main factors implicated in the pathophysiology of acne. Antibiotics

are usually used to treat moderate to severe acne that does not respond to other treatments. Erythromycin and clindamycin are the most prescribed antibiotics for the treatment of *C. acne* [3].

The use of antibiotics to treat acne can have several negative consequences. Among others, it can lead to dry scalp, rashes, irritation, skin discoloration and an increase in bacterial resistance [4]. Studies on bacteriostasis have found that traditional medicinal plant preparations and extracts have antifungal properties. Acne can be treated with antibacterial essential oils (EO) obtained from plant extracts. Citrus essential oils contain a variety of bioactive compounds [5].

Lemon essential oil (LEO) is used for flavouring and in aromatherapy. LEO has FDA approval for both safety and flavouring [6]. According to previous studies, citrus essential oils have antimicrobial properties against *Escherichia coli*, *Salmonella enteritidis*, *Staphylococcus epidermidis*, *Listeria monocytogens*, *Acinebacter baumannii* and *Mycobacterium smegmatis*. The antimicrobial activity of citrus against *Candida albicans* suggests that it could be used as a natural antimicrobial substitute, and its product properties make it attractive to those concerned about skin health [7]. The ability of the skin to absorb an effective amount of EO is the main obstacle to effective treatment [8]. The nanotechnology-based drug carrier has unique properties that can significantly improve the administration of topical drugs [9]. Hydrogels are adaptable biomedical materials with modifiable physicochemical and biological properties. Although hydrogels have potential benefits as topical applications, their properties are limited, such as uniform drug dispersion, swelling ability and formulation stability [10]. An emerging drug delivery method known as nano emulgel is being developed with the goal of im-proving the therapeutic profile of lipophilic drugs.

Nanoemulsions with a size between 50 and 500 nm are tiny emulsions that improve the delivery of active pharmaceutical ingredients. They are formed by mixing two incompatible liquids into a stable phase with the help of surfactants and co-surfactants. Because they can absorb large amounts of insoluble drugs, plant-based nano emulsions offer prolonged drug release and versatile routes of administration, including oral and transdermal applications. Due to their small particle size, nanoemulsions penetrate deep into the skin and improve the absorption and efficacy of oily drugs while minimizing irritation. They are ideal for solubilizing hydrophobic drugs and ensure a controlled, gradual release of the active ingredient, thereby improving treatment compliance and reducing side effects. This study focuses on the development of a lemon oil nano-emulsion known for its therapeutic potential, especially for the topical treatment of skin problems. The aim of the research was to develop and evaluate a method to produce this emulsion to formulate a non-toxic, safe product for the treatment of acne and to investigate its efficacy in *in vitro* and *in vivo* studies.

Materials and Methods

Lemon oil (Safa Almurooj Est. of KSA) was purchased from the local market in Jazan, Saudi Arabia. PEG Tween-80, Tween-20 and ethanol (Merck, USA). The remaining chemicals were of analytical grade.

Excipient Screening

Solubility/miscibility of lemon oil in co-surfactants and surfactants is an important criterion for screening components. Tween-20, Tween-60, Tween-80 and Labrasol, as well as the co-surfactants Transcutol-P, ethanol, Plurol oleique, PEG-200 and PEG-400 were used in a 1:1 ratio (oil:surfactant/co-surfactant) to test the incompatibility of lemon oil. The miscibility tests were performed visually. The clear mixtures prepared in the ratio 1:1 (v/v) was selected for further studies.

Phase studies

The surfactant Tween-80 and the co-surfactant PEG were selected for the preparation of the lemon oil nano emulsion based on the solubility/miscibility of the experiments. Milli-Q water was used as aqueous medium to avoid surface active impurities. Surfactant and co-surfactant were mixed in a ratio of 1:1 (Smix). To cover the maximum ratio for the study, sixteen different oil and Smix combinations were used (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 3:7, 1:2, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1). Using the titration method in aqueous phase, pseudo ternary phase diagrams were obtained to determine the presence of a nano emulsion zone. For each ratio of oil and Smix, a slow titration process was performed with the aqueous phase, and visual observations of a transparent and easily flowable oil-in-water (O/W) nano emulsion were made. To represent the physical state of the nano emulsion, a pseudo ternary phase diagram was used in which the aqueous phase, the oil phase and a mixture of surfactant and co-surfactant with fixed weight ratio were plotted on a line (Smix ratio).

Formulation selection for physical stability testing

Pseudo ternary phase diagrams were used to create many different formulations for different types of nano emulsions. Formulations with oil content ranging from 5% to 25% were selected. Physical stability tests were performed with the selected formulations.

Physical stability evaluation and optimum nano emulsion selection

To address the issues with metastable nano emulsions, a physical stability evaluation was performed. The selected nano emulsion preparation was centrifuged at 5000 rpm for 30 minutes. No phase separation was observed during the heating and cooling cycles. At each temperature, samples were stored for at least 48 hours over the course of six cycles, beginning with storage at 4°C and ending at 45°C. Stability was tested by placing the formulations in a cooler at -20°C for

24 hours and then removing them and returning them to room temperature. The experiments were repeated three times, each time taking 2 - 3 minutes for the physically stable formulations to return to their original state. In this study, nano emulsions with high oil content and low surfactant content were selected because they are easier to process.

Dynamic light scattering (DLS) analysis

Surface charge, particle size and poly-dispersity index (PDI) are required for physical characterization of injectable nanoparticles. Zeta potential (ZP) measurement determines the surface charge of nanoparticles in the nano emulsion. DLS was used to determine the nanoscale particle size (NS) and polydispersity index (PDI) of the nano emulsion. In this study, a nano- ZS Zetasizer (Malvern Instruments, UK) was used to measure ZP, NS and PDI. The emulsion was placed in a folded capillary cell without air bubbles. The emulsion formulation was tested according to the recommendations in the Malvern Instruments manual.

Determination of viscosity and pH

The rheological properties of the emulsion were determined using a Brookfield digital viscometer (model LVDV-E, USA). Spindle S63 was used to determine the viscosity of the samples. A clean, maintained sample holder was used to hold 25 mL of the emulsion, which was then allowed to settle for five minutes. The viscosity was then determined at room temperature and a rotational speed of 30 rpm. The pH of a sample indicates its acid or alkali content. The pH was determined using an Oakton pH 700 Benchtop Meter (United States).

Bacterial strains used and standardization of bacterial cultures

The bacterial strains used in the study were *Staphylococcus aureus* ATCC 512477, *Escherichia coli* ATCC 25922, *Salmonella choleraesuis* ATCC 10708, *Proteus mirabilis* ATCC 299, *Klebsiella pneumoniae* ATCC 700603 and *Enterococcus faecalis* ATCC 29212. 24-hour cultures were prepared and standardised using nutrient broth gradient dilutions ranging from 10^{-1} to 10^{-7}. The viability of bacterial cultures was determined by counting colony forming units (CFU) *per* millilitre (mL). Antimicrobial susceptibility testing was performed as previously described. Muller-Hinton agar plates were prepared for antibacterial testing. Bacterial subcultures were prepared from the stock culture, and after 24 hours of incubation, the culture was tested for the antibacterial properties of the nano emulsion. Agar well diffusion method was used for sample analysis and disk diffusion was used for standard ciprofloxacin disk (5 µg/disk). Inoculation was performed by inserting a clean cotton swab into a standardised culture containing specific organisms and then spreading the culture evenly and individually on the agar plate MH, while rotating the Petri dish. After the plates had dried for approximately 10 min, the agar well diffusion procedure was performed by punching holes in the inoculated MH agar plates with a conventional sterile stainless-steel drill. Sample analytes were individually added to the corresponding wells of the plates. After incubation for 24 hours, the plates were examined to determine the antibacterial spectrum. This was done by at 37°C observing the growth of inhibition zones on the plates.

Statistical analysis

Each experiment was performed three times (n = 3), and data were subjected to one-way analysis of variance (ANOVA). The statistical significance level was $p < 0.05$. Statistical analyses were performed using the Prism 9 Graph Pad Instat software system, Boston, MA, USA. Values for the test samples were compared with those for the standard preparation using the Tukey-Kramer test (post-hoc analysis).

Results and Discussion

Excipient Selection Criteria

Pharmaceutically acceptable excipients that are safe, non-irritating and non-sensitizing were selected for their skin compatibility. A key criterion was their ability to dissolve in oil to ensure the stability of the nano-emulsion. When selecting surfactants, their potential skin irritation and safety perception are decisive; non-ionic surfactants are preferred due to their lower toxicity. The hydrophilic-lipophilic balance (HLB) is crucial in the selection of surfactants as it balances the water-attracting and oil-attracting parts. Surfactants with HLB values of 8 to 18 are commonly used in oil-in-water (O/W) emulsions [11]. A pseudo ternary phase diagram can be used to show the connection between the phase behaviour of a mixture and its composition. The lemon oil nano emulsion in this research was created using the Smix ratio. (1:1). An earlier study showed that increasing the surfactant concentration in the Smix ratio 1:1 was effective in developing o/w nano emulsion, which could be due to increased interfacial tension and fluidity of the interface [12]. Stable nano-emulsions are formed when the oil phase, aqueous phase, HLB and surfactant concentration are sequentially matched [13]. Combining the surfactant with appropriate proportions with low and high HLB values results in a stable nano emulsion formulation. Co-surfactants are excipients that are added to emulsions to further stabilize the interfacial film and avoid droplet coalescence (Table I). Greater penetration of the oil phase was observed in the hydrophobic region of the surfactant monomers, as was reported earlier [14].

Phase studies and Formulation selection

Pseudo ternary phase diagrams of oil, Smix and aqueous phase were developed using the aqueous titration method. It can be used to show the relationship between the phase behaviour of a mixture and its composition. It was prepared separately for each case to obtain the Smix ratio of the O/W nano emulsion

(Figure 1). The epidermis of the skin serves as an effective barrier that protects the body from the penetration of molecules and microorganisms from the environment, as well as from excessive water loss, which the body needs to maintain its homeostatic state [15].

Due to the relationship between high surfactant concentration and skin toxicity and irritation, it is crucial to use a low concentration of surfactants and co-surfactants. The aim of the present study was to develop topical delivery systems that improve skin permeation. The role of surfactants and co-surfactants in the formulation of nano emulsions is self-explanatory in Table I.

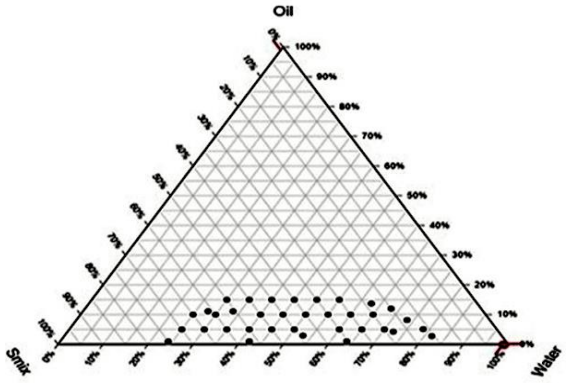

Figure 1.
Diagram of Pseudo-ternary phase for Smix (1:1)

Table I
Miscibility of lemon oil with surfactants

Miscibility of Lemon Oil				
Sr. No.	**With surfactant (1:1)**	**Observation with surfactant**	**With co-surfactant (1:1)**	**Observation with co-surfactant**
1	Tween-20	Turbid	Ethanol	Turbid
2	Tween-80	Clear	Transcutol P	Turbid
3	Lecithin	Turbid	PEG	Clear
4	Unitop 100	Turbid	Pleurol oleique	Turbid

Physical stability studies

Nano emulsion preparations are kinetically stable systems in which no phase separation, creaming or cracking is expected at a given oil, surfactant and water concentration. The phase diagram of selected nano emulsion preparations and various evaluations of stability under stress were performed, such as the heating and cooling cycle, the centrifuge and the freeze-thaw cycle. Some nano emulsion preparations became turbid during the physical stability test, while others showed phase separation. In addition, when the temperature is lowered during the loading stability evaluation, the nano emulsion becomes unstable because the oil phases separate, and the change in free energy curvature favours droplet distributions with smaller sizes. Nano emulsions with minimal phase separation, creaming, cracking, coalescence and phase inversion were selected for further investigation during the load stability test (Table II).

Physical characterization of nano emulsion

Physical stability was established for three formulations (A2, A4 and A5). A2 was selected as the best candidate for further investigation due to its high oil content and low Smix ratio (5% oil: 45% Smix: 55% total water). The physical characterization of the nano emulsion is shown in Table III.

Table II
Physical stability studies of drug-loaded nano emulsion formulations

Formulation Code	Heating and Cooling Cycles	Freeze and Thaw cycles	Centrifugation Studies
A1	√	x	x
A2	√	√	√
A3	x	x	√
A4	√	√	√
A5	√	√	√
A6	x	√	x

√: Passing the test; x: Failing the test

Table III
Physical characterization

Characteristics	Parameters								
	pH	**Viscosity grade (cP)**	**Zeta (mV)**	**Particle size (z.d.n*)**	**Particle size (d.nm**)**	**PDI**	**% Poly dispersity**	**Conductivity mS/cm**	**Refractive index**
Formulation A2	5.62 ± 0.0143	4.487 ± 0.131	-14.1 + 5.52	4.784 ± 2.530	2.992 to 155.6	0.280	52.9	364.34 ± 06.23	1.330 ± 0.014

*z d.nm, zeta average size, diameter in nanometer; **d.nm, diameter in nanometer

The results demonstrate the successful formulation of an O/W nano emulsion. The viscosity of an emulsion is an important parameter for determining stability. In a previous study, it was found that viscosity grades of 225 ± 25 cP were constant throughout the experiment and did not separate or become creamy during centrifugation [16]. In contrast, the viscosity of the developed nano emulsion in this study was 4.487 ± 0.131 cP and proved to be a stable nano emulsion. The zeta potential of the nano emulsion was determined to be -14.1 mV with a single peak (Figure 2).

According to the zeta potential study, the nano emulsion was a stable preparation. In the present study, the distribution of droplet size was not homogeneous. The average droplet size was 2.992 d.nm with 74.4% of the distribution and 25.6% of the average particle size was 155.6 (Figure 3A). The z d.nm of the nano emulsion was found to be 4.789 z d.nm (Figure 3B). Figure 3C shows the intensity peaks of the percent size distribution, which are self-explanatory. From the analysis, most droplet sizes were inhomogeneous, with the maximum peak ranging from 2 to 8 d.nm and the average size being 2.992 ± 0.914. Of the droplets, 50% had an average size of 3.33 d.nm. The short peak represents the size of the droplet distribution, and the average droplet size was determined to be 155.6 ± 87.28 d.nm was determined and 90% of the droplets had a size of 156 d.nm. Interestingly, the PDI of the A2 formulation was 0.28, with a percent PDI of 52.9 showing good consistency. The size distribution of nanodroplets based on the mass fraction (in d.nm) of a Nano emulsion system is shown in Figure 3D. The study showed 100% uniform size distribution in the emulsion system.

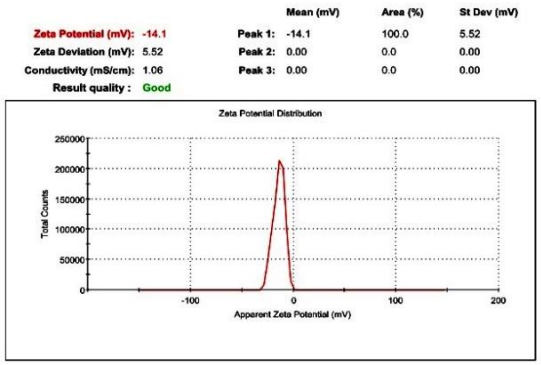

Figure 2.
Physical characterization: zeta potential analysis of nano emulsion

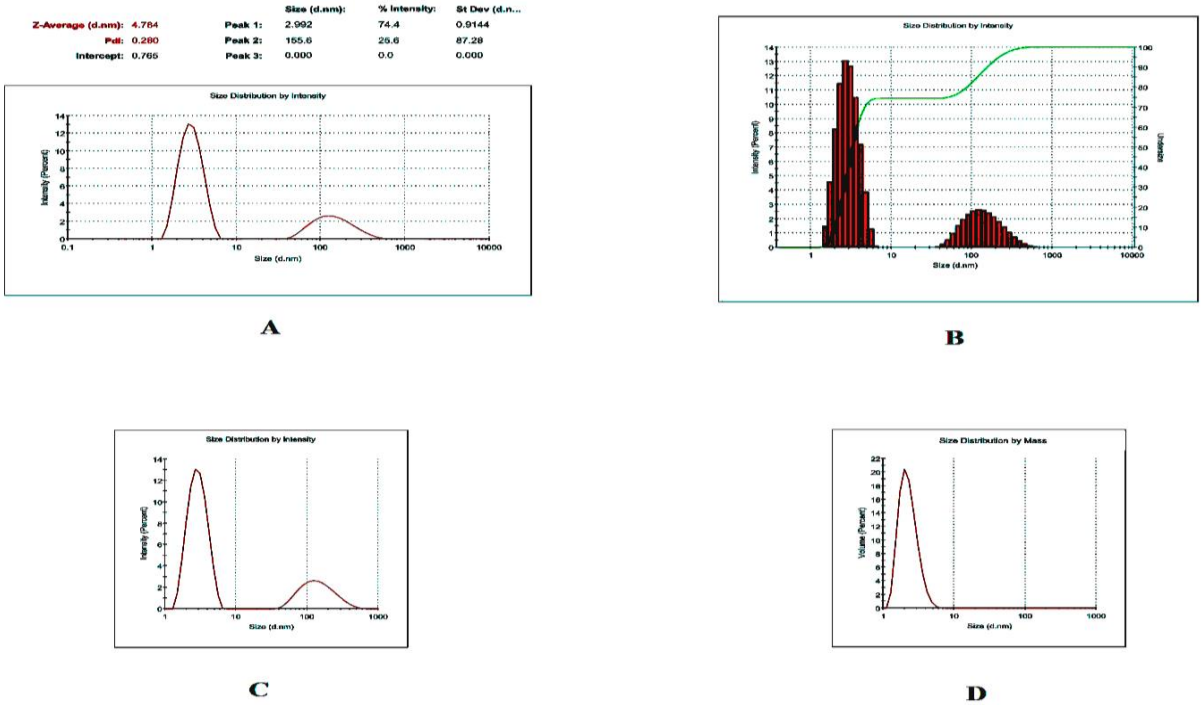

Figure 3.
Physical characterization of nano emulsion: (A) Various size distribution analysis through the percent intensity (B) Percent intensity of size (d.nm, diameter in nanometer) distribution of nano droplets (C) Size distribution analysis by intensity (D) Size distribution analysis by mass

In addition, Figure 4A presents the cumulative fit analysis of the droplet distribution in the nano emulsion system. The nano-droplets showed good quality in dispersive system with 90% linearity. The results show that the A2 formulation is of good quality. On the other hand, the droplet distribution analysis showed 100% agreement, indicating a successful formulation of the Nano emulsion (Figure 4B). The surface

charge and the size of the nano formulations are two parameters that are essential for targeting bacterial cells and determine the degree to which the nano formulations can penetrate the bacterial cell. In addition, anionic droplet sizes have been shown to cause less damage to bacterial cell walls and membranes than their cationic counterparts, which have been the subject of numerous studies [17]. The surface charge and particle size of nanodroplets of a nano emulsion affect their pharmacokinetic properties for biological distribution. The small particle size and a ZP of -14 mV in the current study suggest passive targeting of bacterial cells [18]. Similarly, a previous study found that the zeta potential of nano emulsions with lemon oil was -14.9 mV, suggesting that the electrokinetic effect and surface charge of the particles have a higher degree of stability. Laxmi *et al*. (2015) reported the development of a nano emulsion and the determination of a zeta potential of -15 mV and found that the nano emulsion exhibited excellent electrokinetic stability [19].

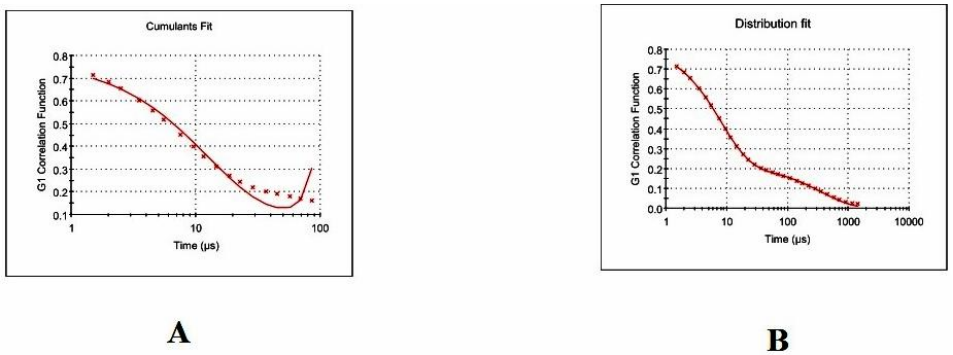

Figure 4.

The analysis of nano emulsions: (A) Cumulative fit analysis (B) Size distribution fit analysis

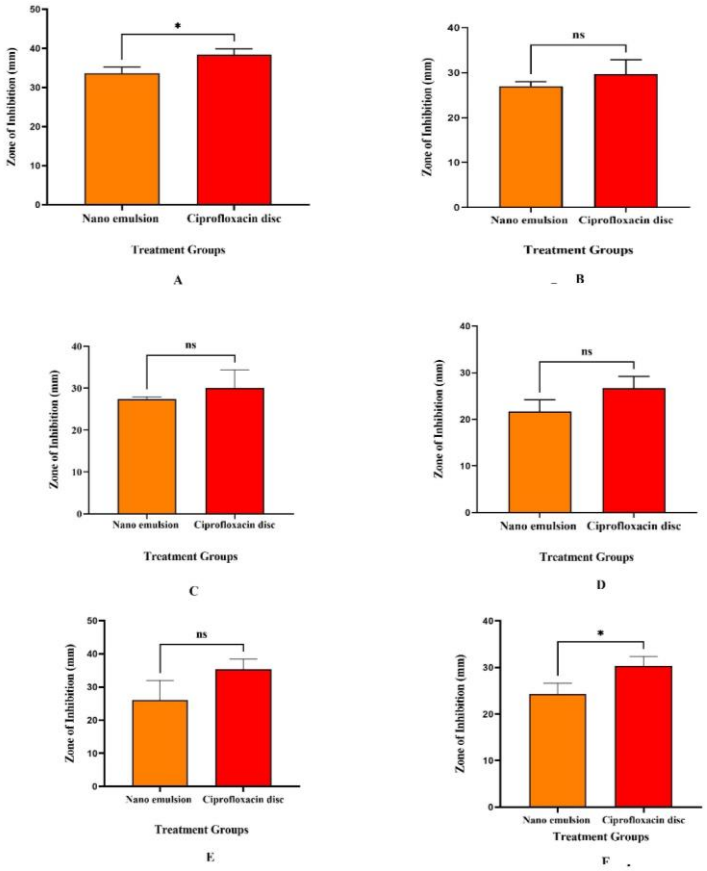

Figure 5.

The antibacterial activity of nano emulsion on comparing with standard ciprofloxacin disc against test organisms

* Significant at $p < 0.05$ (A) *Staphylococcus aureus* ATCC 512477 at 2×10^4 CFU / mL concentration (B) *Escherichia coli* ATCC 25922 at 4×10^5 CFU / mL concentration (C) *Salmonella choleraesuis* ATCC 10708 at 2×10^4 CFU / mL concentration (D) *Proteus mirabilis* ATCC 299, at 3×10^6 CFU / mL concentration (E) *Klebsiella pneumoniae* ATCC 700603, at 2×10^3 CFU / mL concentration (F) *Enterococcus faecalis* ATCC 29212, at 3×10^3 CFU / mL concentration

Recently, geraniol nano emulsion was developed. The zeta potential was measured to be -17.95 ± 5.85 mV, indicating an ideal zeta potential for a stable nano emulsion, and implying that the greater the zeta potential leads to mutual repulsion between particles and the greater the stability of the dispersion system [20]. An earlier study reported that one of the most important factors that determines the conductivity of particles is their mobility inside a colloidal injectable system. Particles with sizes fewer than 20 nm have significant mobilities in the colloidal system and can affect the surface charge of particles [21]. In the present study showed that the conductivity of droplet was 364.34 ± 06.23 mS/cm indicated high mobility of droplets of the nano emulsion which was reflected in anti-bacterial study (Figure 5).

The mobility influences the conductivity of the droplets of nano emulsion. The pH of a formulation was determined to be 5.62, making it suitable for topical application (skin pH is 5.5 to 6.5). The refractive index is very close to that of water (1.333), indicating that water constitutes the majority of the A2 formulation.

Anti-bacterial studies

The results are shown in Figure 5, which is self-explanatory that the nanoemulsion showed good activity and sequenced as *Staphylococcus aureus* > *Klebsiella pneumoniae* > *Salmonella choleraesuis* > *Escherichia coli* > *Enterococcus faecalis* > *Proteus mirabilis*. The activity of the samples showed a good spectrum of activity against the organisms studied, but their efficacy was significantly lower than that of the standard ciprofloxacin disk (Table IV).

Table IV

Anti-bacterial study

Organisms	Concentration of 24 h culture, CFU/mL		Zone of Inhibition (mm)		
		Nano emulsion	Tween 80	PEG	Ciprofloxacin 5 µg/disc
Staphylococcus aureus ATCC 512477	2×10^{-4}	33.7 ± 1.53	-	-	38.33 ± 1.5
Escherichia coli ATCC 25922	4×10^{-5}	27 ± 1	-	-	29.6 ± 3.2
Salmonella choleraesuis ATCC 10708	2×10^{-4}	27.3 ± 0.57	-	-	30 ± 4.36
Proteus mirabilis ATCC 299	3×10^{-6}	21.7 ± 2.52	-	-	26.6 ± 2.5
Klebsiella pneumoniae ATCC 700603	2×10^{-3}	26 ± 6	-	-	35.3 ± 3
Enterococcus faecalis ATCC 29212	3×10^{-3}	24.3 ± 2.31	-	-	30.3 ± 2

[#] Each value is the mean of 3 batches with standard deviation. All the values are compared to standard ciprofloxacin disc

Staphylococcus aureus is a bacterium that can cause contagious infections of the skin and soft tissues, as Gram-negative folliculitis is caused by *Proteus mirabilis, Klebsiella aerogenes, Pseudomonas aeruginosa*, *Serratia marcescens* and *Escherichia coli* in acne patients [22]. In another study, the antimicrobial activity of lemon oil against human pathogens *Staphylococcus aureus, Enterococcus faecalis* and *Salmonella paratyphi A* was found and suggested that lemon oil nano emulsion is a natural antimicrobial agent. This study also suggests that lemon oil nano emulsion could be used as a food additive. The antibacterial study showed that the nano emulsion had excellent activity against *Staphylococcus aureus*. A previous study showed that the pores of *S. aureus* ranged from 50 to 500 nm in size and their diameter ranged from 5 to 50 nm [23]. In the present study, the droplet sizes varied from 2 to 156 nm and successfully penetrated the cell membrane and triggered the effect. Therefore, the formulation of nano emulsion with the optimal size of nanodroplets enables passive targeting of nano emulsion to bacterial cells. The therapeutic significance of nano emulsion can be realised through a process of cellular uptake determined by the size of the particles, which ultimately leads to therapeutic efficacy. Figure 6 shows the comparative antibacterial efficacy of the nano emulsion against the tested bacterial organisms.

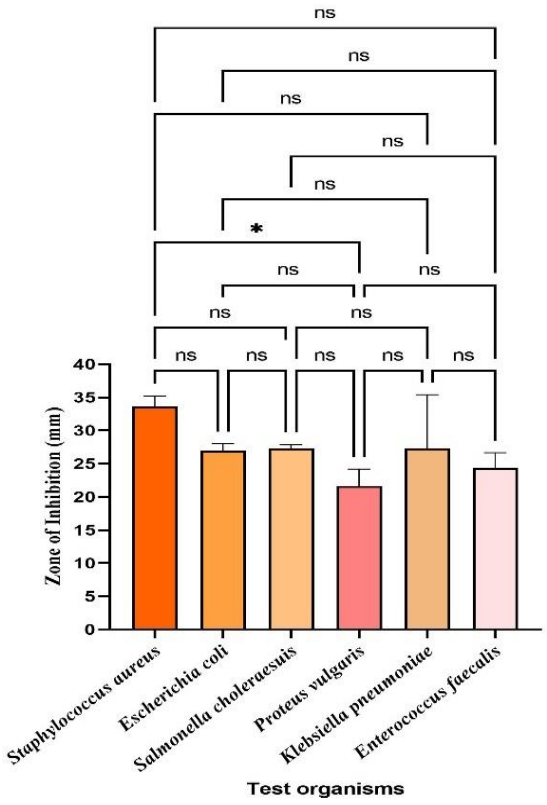

Figure 6.

A comparative anti-bacterial study of nano emulsion against the tested organisms

* Significant at $p < 0.05$

The study proves that nano emulsion has a good spectrum of antibacterial activity against the tested bacteria. Hamouda *et al*. demonstrated that nano emulsions with a size ranging from 100 to 800 nm showed a good spectrum of activity against the studied bacteria (24). The present study shows that the size of the nano emulsion was about 200 nm and penetrated well into the bacterial cell and exhibited a good spectrum of activity.

Conclusions

The present study demonstrated the successful formulation of a lemon oil nano emulsion (O/W) that was stable and suitable for topical application. Moreover, the lemon oil nano emulsion formulated in this study had excellent inhibitory activity against the bacteria studied. Therefore, the lemon oil nano emulsion could be beneficial as a suitable antibacterial formulation against human pathogenic bacteria. Although we studied some human pathogenic bacteria associated with the development of acne, we still need to study very specific bacteria such as *Cutibactrium acnes* and *Klebsiella aerogenes*, which is in progress for future studies. Although the O/W nano emulsion system for lemon oil is successful, it should also fulfil very challenging tasks, such as formulation and processing parameters to obtain a stable emulsion, diffusion properties, biodistribution through tissue barriers at the site of application and pharmacokinetic profile. In addition, the toxicity of nano emulsions should be determined to ensure safe use of the product and avoid local allergic reactions. The balance between proinflammatory and anti-inflammatory cytokine mediators should also be thoroughly investigated. Therefore, further work on standardization, optimization and *in vivo* animal study is underway to better understand the efficacy of lemon oil nano emulsion as a topical formulation.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Bienenfeld A, Nagler AR, Orlow SJ, Oral antibacterial therapy for Acne vulgaris: An evidence-based review. *Am J Clin Dermatol.*, 2017; 18(4): 469-490.
2. Chien AL, Qi J, Rainer B, Sachs D, Helfrich YR, Treatment of acne in pregnancy. *J Am Board Fam Med.*, 2016; 29(2): 254-262.
3. Karadag AS, Aslan Kayran M, Wu CY, Chen W, Parish LC, Antibiotic resistance in acne: changes, consequences and concerns. *J Eur Acad Dermatol Venereol.*, 2021; 35(1): 73-78.
4. Winkelman WJ, Aromatherapy, botanicals and essential oils in acne. *Clin Dermatol.*, 2018; 36(3): 299-305.
5. Liu T, Gao Z, Zhong W, Fu F, Li G, Guo J, Shan Y, Preparation, characterization and antioxidant activity of nano emulsions incorporating lemon essential oil. *Antioxidants*, 2022; 11: 650.
6. Man A, Santacroce L, Jacob R, Mare A, Man L, Antimicrobial activity of six essential oils against a group of human pathogens: A Comparative Study. *Pathogens*, 2019; 8(1): 15.
7. Pinna R, Filigheddu E, Juliano C, Palmieri A, Manconi M, D'hallewin G, Petretto G, Maioli M, Caddeo C, Manca ML, Solinas G, Bortone A, Campanella V, Milia E, Antimicrobial Effect of *Thymus capitatus* and *Citrus limon* var. *Pompia* as Raw Extracts and Nanovesicles. *Pharmaceutics*, 2019; 11(5): 234.
8. Abdo L, Esentürk-Güzel İ, Topuzoğlu S, Gürbüz Yurtsever A, Erdal MS, Algın Yapar E, Development of herbal bioactive loaded nanoparticles for topical application in vitiligo. *Farmacia*, 2023; 71(6): 1305-1315.
9. Miastkowska M, Kulawik-Pióro A, Szczurek M, Nano emulsion Gel Formulation Optimization for Burn Wounds: Analysis of Rheological and Sensory Properties. *Processes*, 2020; 8(11): 416.
10. Mikulcová V, Kašpárková V, Humpolíček P, Buňková L, Formulation, Characterization and Properties of Hemp Seed Oil and Its Emulsions. *Molecules*, 2017; 22(5): 700.
11. Mukesh G, Tejal, S, Lal H, Asha P, Nayan P, Development and optimization of plant extract loaded nano emulsion mixtures for the treatment of inflammatory disorder. *Curr Res Drug Dis.*, 2014; 1(2): 29-38.
12. Llinares R, Santos J, Trujillo-Cayado LA, Ramírez P, Muñoz J, Enhancing rosemary oil-in-water micro fluidised nano emulsion properties through formulation optimization by response surface methodology. *Food Sci Technol.*, 2018; 97: 370-375.
13. Kohsaku K, Takayoshi Y, Yasushi M, Eri K, Koji T, Yoshitaka N, Kazuyoshi M, Microemulsion formulation for enhanced absorption of poorly soluble drugs: I. Prescription design. *J Control Release.*, 2022; 81(1-2): 65-74.
14. Effendy I, Maibach HI, Surfactants and experimental contact dermatitis with irritants. *Contact Dermatitis*, 1995; 33(4): 217-225.
15. Arianto A, Cindy C, Preparation and evaluation of sunflower oil nano emulsion as a sunscreen. *Open Access Maced J Med Sci.*, 2019; 7(22): 3757-3761.
16. Abouelhag HA, Sivakumar SM, Bagul US, Mohamed Eltyep E, Safhi MM, Preparation and physical characterization of cisplatin chitosan nanoparticles by zeta nano sizer "prime step for formulation and development". *IJPSR*, 2017; 8(10): 4245-4249.
17. Sultan MH, Moni SS, Alqahtani SS, Makeen HA, Madkhali OA, Bakkari MA, Menachery SJ, Almoshari Y, Salawi A, Alshamrani M, Safhi AY, Mohan S,

Elmobark ME, Formulation, characterization and biological evaluation of injectable nanocrystals from stem exudate gel of *Caralluma retrospiciens* (Ehrenb) - Part C. *Arabian J Chem.*, 2022; 15(2): 103579.
18. Harleen K, Pranav P, Ramneek K, Shriya A, Manisha S, Synthesis and characterization of *Citrus limonum* essential oil based nanoemulsion and its enhanced antioxidant activity with stability for transdermal application. *J Biomater Nanobiotechnol.*, 2020; 11(4): 215-236.
19. Laxmi M, Bhardwaj AS, Mehta A, Development and characterization of nano emulsion as carrier for the enhancement of bioavailability of artemether. *Artif Cells Nanomed Biotechnol.*, 2015; 43(5): 334-344.
20. Feng X, Feng K, Zheng Q, Tan W, Zhong W, Liao C, Liu Y, Li S, Hu W, Preparation and characterization of geraniol nano emulsion and its antibacterial activity. *Front Microbiol.*, 2022; 13: 1080300.
21. Razie R, Elham Z, Abbas A, Marjan N, Samira F, Najmeh N, Mahmoud O, Nano emulsion and Nanogel Containing *Cuminum cyminum* L. Essential Oil: Antioxidant, Anticancer, Antibacterial and Antilarval Properties. *J Trop Med.*, 2023; 2023: 5075581.
22. Ito T, Sun L, Bevan MA, Crooks RM, Comparison of nanoparticle size and electro-phoretic mobility measurements using a carbon-nanotube-based coulter counter, dynamic light scattering, transmission electron microscopy and phase analysis light scattering. *Langmuir*, 2004; 20(16): 6940-6945.
23. Böni R, Nehrhoff B, Treatment of Gram-Negative Folliculitis in Patients with Acne. *Am J Clin Dermatol.*, 2003; 4: 273-276.
24. Hamouda T, Myc A, Donovan B, Shih AY, Reuter JD, Jr Baker JD, A novel surfactant nano emulsion with a unique non-irritant topical antimicrobial activity against bacteria, enveloped viruses and fungi. *Microbiol Res.*, 2001; 156: 1-7.