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Development of emulsified systems containing S-(-)-Limonene and evaluation of the antibacterial effect against Klebsiella pneumoniae strains

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Research Article

Keywords: Klebsiella pneumoniae, nanoemulsion, nanoemulgel, antibacterial effect, S-(-)-limonene, L-limonene

Posted Date: July 22nd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10348455/v1

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Additional Declarations: No competing interests reported.

Abstract

Klebsiella pneumoniae is associated, among other things, with skin wound infections. The objective was to develop and characterize emulsified systems and evaluate their antibacterial effect against Klebsiella pneumoniae strains. A nanoemulsion containing S-(-)-limonene was developed by ultrasonication, and a nanoemulgel by adding a gelling agent to the nanoemulsion. They were characterized in terms of droplet size and polydispersity index by dynamic light scattering, as well as their macroscopic characteristics and pH. Antibacterial activity was evaluated according to the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration. A stable nanoemulsion containing 10% S-(-)-limonene (droplet size of 47.47 nm and polydispersity index of 0.2561) was formulated up to day 90 without macroscopic changes. The nanoemulgel showed an increase in these parameters after the 30th day. When investigating the antibacterial activity of the nanoemulsion, a minimum inhibitory concentration of 1000 µg/mL was observed, as in the isolated product. The susceptibility test of the strains to the nanoemulgel revealed an antibacterial effect with the formation of a 20 mm inhibition halo. It is concluded that S-(-)-limonene is a candidate for the development of new pharmacological technologies for the treatment of skin lesions infected with Klebsiella pneumoniae, either alone or in formulations with emulsified systems.

1 Introduction

Bacteria belonging to the genus *Klebsiella spp*. are Gram-negative rod-shaped bacteria that can live in a wide variety of environments and a member of the Enterobacteriaceae Family (Santajit & Indrawattana 2016). The genus *Klebsiella* contains more than a dozen species, many of which cause opportunistic infections in humans. The species complex including *K. pneumoniae*, *K. quasipneumoniae*, *K. varicola* and *K. africanensis*, causes the greatest burden of disease in humans (Wyres & Holt 2018, Gonzalez-Ferrer et al. 2018).

Infections caused by *K. pneumoniae* (KP) include pneumonia, liver abscesses, bacteremia, soft tissue infections, urinary tract infections, endophthalmitis and meningitis (Paczosa & Mecsas 2016). Possibilities why *Klebsiella spp*. cause more frequent infections compared to other Gram-negative opportunistic pathogens include the ability to resist starvation, naturally resist antibacterials, outcompete other bacteria, readily exchange deoxyribonucleic acid with other members of the human microbiome and acquire mobile genetic elements encoding a wide range of antibacterial resistance and virulence-enhancing genes (Gonzalez-Ferrer et al. 2018).

Antimicrobial resistance is described as the ability of microorganisms to survive in the presence of antimicrobial agents in concentrations that are normally sufficient to inhibit or kill them. It can also be explained as a multifactorial process which, from the bacterial point of view, represents a combination of continuous exposure to antibacterials and the plasticity of the microbial genome (Watkins & Bonomo 2016, Gorrie et al. 2022).

The modes of antibacterial resistance in *K. pneumoniae* are either intrinsically encoded or acquired through mutation and acquisition of resistance genes. Several factors, including stress-induced acquisition of resistance determinants, altered membrane barrier functions and promotion of biofilm formation, can influence the adaptive response of *K. pneumoniae* (Ndlovu et al. 2023). The high rates of resistance to 3rd generation cephalosporins reported for *K. pneumoniae* mean that the treatment of infections likely to be caused by these bacteria in many settings must rely on carbapenems, the last resort for treating serious community-acquired and hospital-acquired infections. These antibacterials are more expensive, may not be available, and are also likely to further accelerate the development of resistance (World Health Organization 2022). This whole context points to the importance of developing new pharmaceutical products for the treatment of infections caused by *K. pneumoniae*.

Natural products have been shown to perform various biological functions in the human body, such as anticancer, anti-inflammatory, antioxidant and antimicrobial, among others. However, they have low stability, are highly sensitive to oxidation, have lower water solubility, low bioavailability and sustainability. To overcome these obstacles, which hinder their applications, various approaches have been introduced, among which, in recent years, the encapsulation strategy has received much attention as a prominent technique due to its wide range of health benefits and applications (Karim et al. 2022).

Nanoemulsions (NEs) of essential oils provided satisfactory results against a wide variety of microorganisms and their antimicrobial effect was even more acute when applied in a nanoemulsified form. This is because the smaller size of the nano-emulsified droplets increases the surface area that comes into contact with bacterial cells and promotes interaction between the antimicrobial agent and the microorganism's cell membrane (Garre et al. 2020). Encapsulation is considered a practical approach in which bioactives are coated with materials in the form of capsules or particles on a nanometric scale (Wang, Jung & Zhao 2017). Nanotechnology offers many advantages for drug release (Fernandes et al. 2021). Passive and active methods of increasing skin penetration have been successfully used to improve the efficiency of topical or transdermal administration. Nanometric drug carriers have attracted a lot of attention in the last decade as options in formulations for topical therapy. Nanoparticles (including nanospheres, solid lipid nanoparticles, micelles), liposomes and nanoemulsions are among the most studied systems (Zhang et al. 2013).

Nanoemulsions are heterogeneous isotropic mixtures of oil, water and surfactant in which the oil or water forms a dispersed phase and the other forms the continuous phase, they are stabilized by the interfacial film potential of the surfactant molecules (Ojha et al. 2022, Algahtani et al 2020). Also known as nanometric emulsions, they have droplets ≤ 200nm and are stabilized by the addition of emulsifiers (Preeti et al. 2023). Because they have a nanometric particle size, they are transparent or translucent. In addition, due to their smaller particle size, nanoemulsions are much more stable against flocculation, coalescence and crème formation (Moradi & Barati 2019). Although nanoemulsions have a high drug load and solubilization capacity, their transmembrane delivery is still slow due to their low viscosity and spreadability. To solve this problem, the researchers explored modifying the nanoemulsion into a nanoemulgel by adding a suitable gelling agent (Ojha et al. 2022).

Drugs can be incorporated into nanoemulsions that act as a targeted colloidal delivery system that can improve bioavailability and stability by reducing the overall size of the formulation. The nanoemulsion part of this delivery system protects the active portion from enzymatic degradation, hydrolysis etc., and the gel part provides thermodynamic stability to the system, reducing surface and interfacial tension and thus increasing overall viscosity and spreadability (Lal et al. 2023). The nanoemulgel has several advantages over lipid nanoparticles, microemulsions or liposomes in transdermal administration due to its high drug loading capacity, better diffusibility, permeability and low skin irritation (Kotta et al. 2014). In the process of developing various formulations of pharmaceutical importance, nanoemulgel has proved to be an excellent vehicle for administering drugs via transdermal, dental, ocular and vaginal application for the treatment of various types of disease (Setya et al. 2019, Srivastava et al. 2016).

When the nanoemulgel is compared to the pharmaceutical forms mentioned above, it has relatively improved stability, better penetration, less oiliness, ease of incorporation of hydrophobic drugs, high drug loading efficiency and better efficacy (Shankar et al. 2018, Sah et al. 2017). As with most new drug delivery systems, stability is a major issue, where the nanoemulgel can be considered an optimal system to overcome the instability problems associated with other drug delivery systems (Ojha et al. 2022).

Studies using nanoemulsions with limonene have also shown complete disruption of the cell structure leading to lysis and release of intracellular material in both Gram-positive and negative bacteria (Zhang et al. 2014, Bei et al. 2015, Zahi et al. 2015). However, the efficacy of limonene, as well as other antibacterial agents, may differ between Gram-positive and negative bacteria (Gupta et al. 2021).

2 Material and methods

2.1 Micro-organisms, test product, culture media and materials

S-(-)-Limonene was purchased from Sigma-Aldrich®, Methyl alcohol (CRC Produtos Químocos, São Paulo), Polysorbate 80 (Tween 80®), Sorbitan monooleate (Span® 80) and Carbopol were also purchased from Sigma-Aldrich (St. Louis, MO, USA). Propylene glycol (USP - Ely Martins, São Paulo). Ultrapure water was obtained using a reverse osmosis system (model OS50 LX, Gehaka Ins. Ltda., Brazil). The other reagents and products used were of analytical grade. The inocula were obtained from overnight cultures in Muller Hinton Agar at 37ºC and diluted in sterile saline solution to obtain a final concentration of approximately 1.5 x 108 colony-forming units per mL (CFU/mL), adjusted for turbidity when compared to the 0.5 tube of the McFarland scale (Bona et al. 2014). The culture media used in the tests to assess antimicrobial activity were Muller Hinton liquid medium and Muller Hinton Agar solid medium, purchased from Difco® and prepared according to the manufacturer's instructions.

2.2 Methodology for the development and characterization of nanoemulsion and nanoemulgel incorporated with S-(-)-Limonene

2.2.1 Identification of the hydrophilic-lipophilic balance (HLB) of S-(-)-Limonene

To determine the HLB of the oil, we used the method described by Griffin (1949), which consists of relating the masses of the surfactants Span® 80 and Tween® 80 to their respective HLB values (4.3 and 15) in different proportions, in order to obtain defined and scaled HLB values. The proportions of surfactants were calculated for HLB values between 09 and 15. The oil/water (O/W) nanoemulsions were formulated with 5% (w/w) oil, 5% (w/w) surfactants (Span® 80 and Tween® 80) and 90% (w/w) ultrapure water.

The formulations of each HLB were subjected to ultrasonication in an ice bath in an Eco-sonics Sonicator model QR550 (power 550W and frequency 20kHz) at 30% power for 2 minutes. After this procedure, the size of the droplet formed and the polydispersity index (PDI) were read by dynamic light scattering using a Zetasizer Ultra red device (Malvern Ins. Ltd, UK). Particle size ≤ 200nm and PDI ≤ 0.3 were considered. The lower the value obtained, preferably ≤ 0.3, the greater the degree of homogeneity of the sample (Preeti et al. 2023). The samples were diluted 1:10 (v/v) in ultrapure water.

2.2.2 Optimization of formulation parameters

In order to find the best conditions for obtaining stable S-(-)-Limonene nanoemulsions, 17 formulations of 1mL O/W nanoemulsions were produced, varying the concentration of S-(-)-Limonene (5 to 15%), surfactant (mixture of Twen 80 + Span 80 – 3% to 15%) and ultrapure water according to Table 2. The homogenization time was set for ultrasonication in an ice bath in an Eco-sonics QR550 Sonicator (550W power and 20kHz frequency) at 30% power for 2 minutes. The nanoemulsions obtained in this study were analyzed 24 hours after preparation for their average droplet size and PDI by dynamic light scattering using a Zetasizer Ultra Red device (Malvern Ins. Ltd, UK). The samples were diluted 1:10 (v/v) in ultrapure water and three readings were taken per sample. The formulation with the smallest droplet size and lowest PDI was chosen for further study.

2.2.3 Production of nanoemulgels incorporated with S-(-)-Limonene

The nanoemulgels (NGs) were prepared 24 hours after the formulation of the NEs with the best stability parameters presented in the study by adding 1% Carbopol to the NEs containing S-(-)-limonene and a gelling agent (AMP/TIPA 85%) under homogenization in a vortex for 3 minutes. The NGs were stored in hermetically sealed glass vials in a refrigerated environment (5 ± 2°C).

2.2.4 Characterization of nanoemulsions and nanoemulgels incorporated with S-(-)-Limonene

The formulations were evaluated in terms of average droplet size, PDI, hydrogen potential (pH) and macroscopic aspects. The average droplet size and PDI of the formulations (nanoemulsions and

nanoemulgels) were evaluated by dynamic light scattering. The nanoemulsion samples were diluted 1:10 (v/v) in ultrapure water and the nanoemulgel samples were diluted 1:20 (v/v) and analyzed at 25ºC with a detection angle of 173º and a wavelength of 633nm. The readings were taken on days 0, 1, 20, 30, 60 and 90, respectively, D0, D1, D20, D30, D60 and D90.

For macroscopic evaluation, the characteristics of appearance, color and homogeneity were analyzed in order to observe changes in appearance due to instability. The samples were analyzed to identify any instability, color change or phase separation (Montagner & Corrêa 2004). The criteria presented in Table 1 were used to analyze the results.

Table 1
Parameters for assessing macroscopic characteristics.

Parameters	Description
N	Normal
SCA	Slight Change in Appearance
SCC	Slight Change in Color
SOC	Slight Odor Change
MAC	Moderate Appearance Change
MCC	Moderate Color Change
MOC	Moderate Odor Change
IAC	Intense Appearance Change
ICC	Intense Color Change
IOM	Intense Odor Modification

Source: Adapted from Bühler and Ferreira (2008).

The pH measurements were carried out using a Nova Intruments® digital pH meter (model NI PHM, São Paulo, Brazil), previously calibrated with pH 4.0 and 7.0 buffer solutions at a temperature of 25ºC.

2.3 Antibacterial activity of nanoemulsion and nanoemulgel incorporated with S-(-)-Limonene

The microbiological test was carried out in order to determine whether the nanoemulsion and nanoemulgel containing the incorporated S-(-)-Limonene is capable of inhibiting the growth of the bacterium *Klebsiella pneumoniae*. In order to assess the antimicrobial activity of the nanoemulsion, the microdilution plate technique was used to determine the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC).

The MIC of the nanoemulsion was determined using the microdilution technique in a 96-well plate with a U-shaped bottom. 100µL of double-concentrated Mueller Hinton broth and 100µL of the nanoemulsion containing S-(-)-limonene were added to each well of the plate at concentrations of 1024 to 16µg/mL. The MIC was determined with 10µL of the inoculum previously prepared and added to each well. The growth control was also prepared in the penultimate well, containing 200µL of broth and the microorganism suspension, and the last well was the negative control with only 200µL of broth, and the entire test was carried out in duplicate. The plates were incubated at 35ºC for 24 hours, after which time the first reading of the results was taken.

Next, 20µL of sodium resazurin solution (Sigma Aldrich®) was added in sterile distilled water at a concentration of 0.01% (w/v), recognized as a colorimetric oxide-reduction indicator for bacteria, and incubated again at 37ºC. The reading was taken visually, by the absence or presence of growth of the microorganism through the formation of a cluster of cells (bud) and by observing the change in color of the solution from blue to pink, indicating growth of the microorganism. The MIC was determined as the lowest concentration of the product that inhibited the visible growth of the microorganism being tested, verified by a non-changing color of the indicator dye (Palomino et al. 2002, Ostrosky et al. 2008, Clinical and Laboratory Standards Institute 2012, Bona et al. 2014).

For determination of the Minimum Bactericidal Concentration (MBC) of the nanoemulsion, after reading the results of the MIC, inoculums (10µL) of three dilutions from the MIC were prepared for the Mueller-Hinton broth medium (100µL/cavity) in a sterile microdilution plate, then incubated at 37ºC for 24 hours, after which 20µL of resazurin was added. The tests were incubated at 37ºC for a further 24 hours to confirm the concentration capable of inhibiting the total growth of the bacterial species, which was verified by a non-change in the color of the indicator dye (Ncube et al. 2008, Guerra et al. 2012).

For the nanoemulgel, the antimicrobial activity was verified using the well diffusion method, i.e., 6 mm diameter holes were made in the Mueller-Hinton agar culture medium in Petri dishes, thus forming the wells. In this test, the Kp110 strain of Klebsiella pneumoniae was used. The plates were inoculated on the surface with microorganisms, and the wells were filled with 20 µL of the gel at the concentration to be tested. The plates were incubated at 35°C for 24 hours. The microbial growth inhibition halos were measured in millimeters using a millimeter ruler (Bona et al. 2014, Sumantri et al. 2021). For the disk diffusion test, halos with a diameter ≥ 6 mm were considered inhibitory activity (Bona et al. 2014).

3 Results

3.1 Development and characterization of emulsion systems incorporated with S-(-)-Limonene

Nanoemulsions were developed to aid in the application of S-(-)-Limonene in the treatment of infections in skin lesions caused by *K. pneumoniae*. The relationship between the droplet size of the emulsions and the HLB values is shown in Table 2.

Table 2
Average droplet size and PDI at different HLB values from 09 to 15 of nanoemulsions containing S-(-)-Limonene.

Component/ HLB	**09**	**10**	**11**	**12**	**13**	**14**	**15**
Tween80®	21,95µg	26,65µg	31,5µg	37µg	41,5µg	45µg	50µg
Span80®	28,95µg	23,55µg	18,5µg	13µg	8,5 µg	4,5 µg	-
S-(-)-Limonene	50µg	50µg	50µg	50µg	50µg	50µg	50µg
Ultrapure water	900µl	900µl	900µl	900µl	900µl	900µl	900µl
Average droplet size	470nm	212nm	130nm	189nm	307nm	140nm	264nm
PDI	0,4058	0,2700	0,2500	0,4667	0,6307	0,3801	0,6436
Appearance	LT[2]	LT	TL[3]	TL	TL	TL	TL

Legend: PI = polydispersity index. [2]LT = milky. [3]TL = translucent.

The results show that the average droplet size of the nanoemulsions can be affected by the HLB values. The formulation with HLB 11 had the smallest average droplet size (130nm) and the smallest PDI (0.25).

After identifying the ideal HLB, the formulation parameters of the 1ml O/W nanoemulsions containing S-(-)-Limonene were optimized. The results are shown in Table 3.

Table 3
Optimization of formulation parameters, droplet size and PDI.

Formulation	S-(-)-Limonene (%)	HLB - surfactants (%)	Ultrapure water (%)	Droplet size	PI
1	15%	5%	80%	349,9	0,8444
2	6%	6%	88%	402,6	0,8526
3	11%	10%	79%	174,4	0,3044
4	14%	14%	72%	190,4	0,3289
5	11%	10%	79%	136,4	0,2621
6	11%	15%	74%	112	0,2795
7	14%	14%	72%	194,4	0,3084
8	10%	7%	83%	274,4	0,3555
9	5%	13%	82%	58,72	0,2960
10	7%	15%	78%	55,72	0,2960
11	10%	10%	80%	156,5	0,2351
12	5%	3%	92%	135	0,4142
13	10%	3%	87%	190	031,23
14	10%	3%	87%	184,7	0,3309
15	5%	10%	85%	52,88	0,3524
16	15%	5%	80%	129,8	0,2853
17	15%	9%	76%	185,8	0,3983

Table 3 shows that Formulation 11 containing 10% S-(-)-Limonene (w/w), 10% surfactants (w/w) and 80% ultrapure water (w/w) had a droplet size of less than 200nm (156.5nm) and the lowest PDI (0.2351) of all the formulations. This last parameter assesses the degree of uniformity of the droplet size distribution within the system (Danaei et al. 2018). PDI values closer to zero indicate a monodisperse distribution (Mohd et al. 2020).

Formulation 11 (NEL) was monitored for 90 days in a refrigerated environment (5 ± 2°C). Over a 90-day period, dynamic light scattering analysis of the PDI and average droplet size, as well as macroscopic characteristics and pH were carried out, the results of which are shown in Table 4.

Table 4
Particle size, PDI and macroscopic characteristics of the nanoemulsion containing 10% S-(-)-Limonene (NEL).

Days	Droplet size	PI	pH	Macroscopic characteristics
D1	101,7	0,2728	5	Normal
D20	81,29	0,3631	4,5	Normal
D30	94,13	0,4439	4	Normal
D60	153,7	0,4319	3,5	Moderate Change in appearance
D90	807,7	0,864	3,5	Intense Change in Appearance
Mean	225,17	0,4655	--	--
Standard Deviation	± 286,44	± 0,2050	--	--

The average droplet size showed slight variations in the first 30 days, with a greater proportion from D60 onwards (droplet size = 153.7nm and PDI = 0.4319). Changes were also observed in the macroscopic characteristics from that day onwards with the formation of flocculation and in the pH, which tended to be more acidic, suggesting that the system was stable for 30 days. The nanoemulgel (NGL) was prepared 24 hours after the formulation of the NEL and the results of the average droplet size and PDI over 90 days are shown in Table 5.

Table 5
Droplet size, PDI, pH and macroscopic characteristics of the nanoemulgel containing 10% S-(-)-Limonene (NGL).

Days	Droplet size	PI	pH	Macroscopic characteristics
D0	125,7	0,4385	5	Normal
D20	239,9	0,3468	4,5	Normal
D30	434,8	0,5227	4	Normal Moderate
D60	548	0,7713	3,5	Moderate Appearance change
D90	465,9	0,6184	3	Intense Change in Appearance
Mean	323,36	0,5217	--	--
Standard Deviation	± 183,42	± 0,1529	--	--

It can be seen that the average size of the NGL droplets tended to increase throughout the stability study. Changes were also observed in the macroscopic characteristics from that day onwards, with a moderate change in appearance due to the formation of flocculation and the pH tending to be more acidic. To improve the long-term stability of the NEs and NGs, propylene glycol was added to the formulation at a rate of 5%, while maintaining the proportions of surfactants (HLB 11) and S-(-)-

Limonene, adjusting the proportion of ultrapure water. Thus, the new formulation (NEG) consisted of 10% surfactants, 10% S(-)-Limonene, 5% propylene glycol and 75% ultrapure water. Formulated following the same methodology, the results are shown in Table 6.

Table 6
Droplet size, PDI, pH and macroscopic characteristics of NEs with propylene glycol (NEG).

NEG	**D1**	**D20**	**D30**	**D60**	**D90**	**Mean ±Standard Deviation**
Droplet size	47,47	49,93	72,38	44,08	45,52	51,87 ± 11,67
PI	0,2561	0,3509	0,4192	0,3600	0,3816	0,3535 ± 0,0605
pH	5	4,5	4,5	4	4	-
Macroscopic characteristics	Normal	Normal	Normal	Normal	Normal	-

Table 6 shows that the nanoemulsion with 5% propylene glycol (NEG) had an average droplet size of 51.87nm (± 11.67) and remained more stable with a PDI of 0.3535 ± 0.0605. At the end of the 90-day follow-up period, NEG showed no changes in its macroscopic characteristics or pH. Experiments continued with NEG to formulate the nanoemulgel (NGG) and microbiological tests. Following these experiments and results, nanoemulgel (NGG) was prepared from NEG (10% surfactants, 10% S(-)-Limonene, 5% propylene glycol and 75% ultrapure water) 24 hours after its formulation, following the protocol described above (Table 7).

Table 7
Droplet size, PDI, pH and macroscopic characteristics of the nanoemulgel with propyleneglycol (NGG).

NGG	**D0**	**D20**	**D30**	**D60**	**D90**
Droplet size	176	90,09	92,39	201,1	547,9
PI	0,2946	0,3016	0,4247	0,3155	0,5172
pH	5	4,5	4,5	4	4
Macroscopic characteristics	Normal	Normal	Normal	Normal	Normal

The NGG showed variation in average droplet size and PDI during the stability study, as did the NG without propylene glycol (NGL - Table 5), however, it remained stable for 60 days (droplet size = 201.1nm). The behavior of the formulations during the stability study shows that the NGs tend to increase in the variables presented, but the NGG still has lower droplet size and PDI values. Therefore, the NEG and NGG formulations showed greater stability during follow-up when compared to the formulations without propylene glycol, including the pH which remained within the acceptable range for use on the skin, between 4 and 7 (Moradi & Barati 2019).

In many situations, one surfactant alone may not be able to reduce the oil/water interfacial tension sufficiently to prepare a nanoemulsion, so there is a need to use co-surfactants. These are also amphiphilic in nature, with a tendency to partition in large quantities into the interfacial monolayer of the surfactant, reducing the interfacial tension, increasing the fluidity of the interface and the entropy of the system (Setya et al. 2014)

In addition to co-surfactants reducing the interfacial tension necessary for the formation and thermodynamic stability of nanoemulsions, they also promote fluidization of the interfacial film formed by the surfactant, preventing a significant increase in the system's viscosity (Silva et al. 2015).

3.2 Antibacterial activity of nanoemulsion and nanoemulgel incorporated with S-(-)-Limonene

Following on from the tests, we investigated the MIC and MBC of the nanoemulsion incorporated with S-(-)-Limonene, the results of which are shown in Table 8. The MIC_{50} of 1000µg/mL was observed, as with the isolated product.

Table 8
MIC and MBC values obtained for the 10% S-(-)-Limonene nanoemulsion against *Klebsiela pneumoniae* strains.

Bacterial strains	MIC	MBC
Kp 101	1000µg/mL	> 1000µg/mL
Kp 103	1000µg/mL	> 1000µg/mL
Kp 104	1000µg/mL	> 1000µg/mL
Kp 105	1000µg/mL	> 1000µg/mL
Kp 110	128µg/mL	> 1000µg/mL

When testing the susceptibility of *K. pneumoniae* strains to nanoemulgel incorporated with S-(-)-Limonene, the Kp110 strain was used and an antibacterial effect was observed in view of the formation of a 20mm inhibition halo.

4 Discussion

Delivery systems based on nanoemulsions with incorporated constituents significantly increase antimicrobial activity compared to non-encapsulated systems and are promising because they are able to increase the concentration of bioactive compounds in areas where microorganisms are preferentially located (Lu et al. 2018). Surfactants are added to the system in order to produce stable emulsions, and their selection is fundamental to the adequate stability of the system. A significant consideration in selecting suitable surfactants is identifying the HLB value of the oil fraction. The authors go on to say that a simple methodology for determining the HLB of an essential oil is to analyze the short-term

stability of emulsions produced with the test product and surfactants of varying HLB values. The HLB value of the most stable emulsion is considered the required HLB of the product (Lopes et al. 2017).

Suitable HLB values of mixed surfactants are a key factor in the formation of emulsion droplets and during the formation of the O/W emulsion, lipophilic surfactants have more affinity with the dispersed emulsion droplets than the hydrophilic surfactant. They go on to state that an adequate HLB value is necessary to maintain the balance of the oil phase and the aqueous phase and, with ideal values, it is possible to stabilize and narrow the newly formed droplets during emulsification (Lu et al. 2014a).

The mixture of two surfactants has a synergistic effect on the formation of stable emulsions with identical head groups but different hydrocarbon tails. In addition, during emulsification by ultrasonication, cavitation helps to disperse the product into small droplets and then the mixed surfactant is rapidly adsorbed onto the surface of the newly formed droplets to accelerate the formation of nanoemulsions (Lu et al. 2014b).

The physical and chemical stability of nanoemulsions containing D-Limonene stored at different temperatures (ambient and refrigerated) was evaluated for a period of 30 days. The NEs showed an increase in droplet size from D1 to D30 with peaks ranging from 111nm to 433nm, corroborating the results obtained in this research. According to the authors, Ostwald ripening and coalescence are possible mechanisms that destabilize the growth in the size of oil droplets (Sohan et al 2023). Ostwald ripening is a common phenomenon observed in essential oils, short-chain triglycerides and flavoring oil-in-water emulsions. It occurs when oil molecules diffuse from small to large droplets through the aqueous phase, causing the larger droplets to consume the smaller ones (Piorkowski & MCclements 2014). The pH of emulsions prepared using different concentrations of oil showed stability throughout the pH range (3 to 9) without indicating droplet growth or noticeable instability. It had minimal influence on the stability of emulsions incorporating non-ionic emulsifiers (Sohan et al 2023, Cho et al 2009).

Nanoemulsions containing R-(+)-Limonene were formulated for microbiological testing with an average particle size of 116.60 ± 5.30nm and a polydispersity index of 0.205 ± 0.02. The pH of the optimized D-Limonene oil nanoemulsion was 6.50 ± 0.15. The relatively lower PDI value for nanoemulsions can be correlated with greater storage stability (Sonu et al 2018). Like the study mentioned above, other authors have had similar results when investigating the antioxidant, antimicrobial and antifungal capacities of the oily extracts of orange and mandarin peels (with 92% Limonene identified by Gas Chromatography coupled to Mass Spectrometer) encapsulated in NEs. The formulated NEs were stable with a low average droplet size (< 100nm) and PDI (< 0.2), however, the NE with the oil extracted from the orange peel (*Citrus sinensis L.*) increased the droplet size to accommodate the solubilized charge of the extracts. As previously discussed, the larger droplet size of NE and its lower stability is due to Ostwald ripening phenomena associated with the nature of the phenolic extract, which has hydrophilic and hydrophobic compounds (Sabry et al. 2024).

In many cases, surfactants alone can insufficiently reduce the interfacial tension between oil and water, forming small droplets. The addition of short- or medium-chain alcohols as co-tensides can further

reduce the oil/water interfacial tension. These are located between the hydrocarbon tails of the surfactant, which could stabilize the interfacial layer of the nanoemulsions. Thus, the surfactant and co-surfactant are considered important parts, which can influence the formation of the nanoemulsion in terms of particle size and stability (Panapisal 2016). Nanoemulsion instability is due to a few main factors, including cream formation, flocculation, coalescence and Ostwald ripening, the latter being the main factor (Sartoratto et al 2004).

When studying the antibacterial activity of the S-(-)-limonene nanoemulsion against *K. pneumoniae* strains, a moderate antibacterial effect was observed with an MIC50 of 1000µg/mL (Sartoratto et al. 2004). In a study evaluating the antimicrobial activity of a nanoemulsion containing D-Limonene against the Gram-positive strains *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633, the Gram-negative bacterium *Escherichia coli* ATCC 8739 and the yeast *S. cerevisiae* ATCC 9763 that the NE of D-Limonene exhibited high inhibitory effects with regard to growth in all four microorganisms, with an MIC of 7.5µg/mL against *S. aureus* and S. *cerevisiae*, while against *B. subtilis* and *E. coli* the MIC was 15µg/mL (Zhang et al 2014).

Previous results corroborate those presented in this research, where the authors found antimicrobial activity of NE containing 5% limonene against the microorganisms *B. cereus* (ATCC 14459), *E. faecalis* (NCDC 115), *E. coli* (ATCC 25922) and *Salmonella typhi* (NCDC 6017) and in all the strains tested bactericidal activity was observed with a MIC of 12.50µg/mL (Sonu et al. 2018).

This was followed by a study in which an edible coating incorporating different concentrations of R-(+)-Limonene emulsion was developed and the antimicrobial efficacy of edible coatings based on emulsions containing R-(+)-Limonene against different inoculated bacteria (*Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus)* on strawberries was evaluated. In this, the MIC of the R-(+)-limonene emulsions was 625µg/mL for *Bacillus subtilis* and *Escherichia coli* and 1250µg/mL for *Staphylococcus aureus*. Emulsions containing R-(+)-Limonene improved the microbial stability of strawberries and were effective in decontaminating the external pathogens tested (Shao et al. 2018).

With regard to the antibacterial effect of the nanoemulgel against *K. pneumoniae* strains, an antibacterial effect was observed through the formation of a 20mm inhibition halo (Bona et al. 2014). This result is in line with a previous study that developed an effective in-situ ocular nanoemulgel based on limonene to increase the efficacy of fluoroquinolones (levofloxacin) against ocular biofilm infection associated with methicillin-resistant Staphylococcus Aureus (MRSA). The particle size of the limonene-based nanoemulgel loaded with levofloxacin was 119.2 ± 0.321nm, with a unimodal size distribution characterized by a low polydispersity index (0.314 ± 0.05) reflecting the uniformity of the diameter and the homogeneity of the preparation (Mehanna et al. 2020).

The MIC of the limonene-based nanoemulgel alone was 25µg/mL, while that of the limonene-based nanoemulgel loaded with levofloxacin was 3.12 µg/mL, lower than that of the drug (6.2 µg/mL). In this case, limonene had a synergistic effect with the antibacterial tested in combating MRSA infections. The CBM values were 50µg/mL, 12.5µg/mL and 25µg/mL, respectively (Mehanna et al. 2020). The

antibacterial activity of the nanogel (droplet size 196 ± 8nm) incorporated with *Mentha spicata* essential oil, which has limonene as its second largest component, against Staphylococcus aureus and Escherichia coli was investigated. The authors observed the potency of the nanogel with a Mean Inhibitory Concentration (IC50) value of 55µg/mL, substantially lower than that of the isolated essential oil (997.4µg/mL). In addition, the growth of the bacterial strains tested after treatment with the 1000µg/mL nanogel decreased by around 50% compared to the control group (Rasti et al. 2022).

The antimicrobial activity of nanogels incorporated with *Citrus limon* essential oil against *Leishmania tropica* and *Leishmania major* was formulated and investigated. The main constituent of *C. limon* essential oil is limonene (61.83%) and the nanogel was formulated from NE with 4µg/mL of essential oil, which presented parameters acceptable to the authors (droplet size 146 ± 12nm and PDI of 0.96 ± 0.02). The leishmanicidal effect of the nanogel was observed in the viability of L. tropica after 24h of incubation, which fell from 80% to 40% when compared to the isolated essential oil. The viability of *L. major* was also significantly reduced when comparing the effect of essential oil alone and the nanogel incorporated with essential oil (from 82% to 43%). In addition, a perfect leishmanicidal effect (0% viability) was achieved against *L. major* and *L. tropica* using nanogels with 8 µg/mL of the oil (Zarenezhad et al. 2021).

Finally, chemical analysis of essential oil series has shown that the main antimicrobial active components are phenols, terpenes, aldehydes and ketones, and it is generally believed that essential oils act mainly against the cell cytoplasmic membrane. The main mechanisms of action of limonene are against the cytoplasmic membranes of microorganisms, causing loss of membrane integrity; inhibition of respiratory enzymes; and dissipation of the proton-motive force (Zhang et al. 2014). However, further research is needed to investigate the antibacterial effect of S-(-)-Limonene, either isolated or incorporated into emulsified systems, against other bacterial strains.

5 Conclusion

With regard to this research, it can be seen that emulsified systems incorporated with S-(-)-Limonene have been developed. The nanoemulsion with moderate antibacterial potential, as well as the nanoemulgel also with antibacterial potential, both formulations with stable particle size, PDI and macroscopic characteristics for 90 and 60 days, respectively. It is therefore concluded that S-(-)-Limonene is a candidate for the development of new pharmacological technologies for the treatment of skin lesions infected with *K. pneumoniae* alone or in formulations with emulsified systems (nanoemulsion and nanoemulgel).

Declarations

Author Contribution Statement

RL, EE and AA conceived and designed research. RL, AF and AA conducted experiments. RL, EE, HL and AA analyzed data. RL and MH wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors deny that there is any conflict of interest.

Funding

This study received financial support from the Coordination for the Improvement of Higher Education Personnel (CAPES).

Ethics Statement

Ethical approval was not required for this study, as it did not involve human or animal participants.

Data Availability

All data generated are included in the article.

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