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Citrus essential oils' nano-emulsions: formulation and characterization

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RESEARCH ARTICLE

Abstract

The use of citrus essential oils nano-emulsions (CEO-NE) plays a crucial role in enhancing food safety. This is achieved through various mechanisms such as the enhancement of organoleptic properties, increased bioavailability of bioactive components from essential oils, controlled release of key compounds for long-term efficacy, and extension of the shelf life of food and beverages (antioxidant and antimicrobial activity). In this work, CEO-NEs, formulated with different types of citrus essential oils, Tween 80, and alcohol, were obtained by magnetic stirring combined with ultrasound method. The main component of CEO-NEs was D-limonene (31.37 % bergamot-loaded nano-emulsions, 48.81 % tangerine-loaded nano-emulsions, 87.82 % orange-loaded nano-emulsions, 87.09 % grapefruit-loaded nano-emulsions, 48.53 % lemon-loaded nano-emulsions). The selected formulations demonstrated a mean droplet diameter of 47.285 nm. The nano-emulsions were stable even after 30 days of storage (except tangerine nano-emulsions). The bergamot-loaded nano-emulsion had the highest antibacterial activity against *Escherichia coli*, while *Staphylococcus aureus* and *Listeria monocytogenes* seemed to be more resistant to citrus nano-emulsions.

Keywords: citrus essential oils, antioxidant activity, antimicrobial activity, cytotoxicity

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INTRODUCTION

In recent years, essential oils (EOs) have captured the focus of world research because of their bioactive properties that make them suitable for a variety of potential applications, in different industries (agricultural, cosmetic, pharmaceutical, and food). Furthermore, due to antibacterial, antioxidant, cardioprotective, and anticancer properties associated with essential oils (as supported by many literature research), there has been an increasing interest in their application within a variety of products (H. Ahari, Naeimabadi, M., 2021; H. Ahari & Nasiri, 2021; Asadinezhad, 2019; Ashaolu, 2021; Azmi, Elgharbawy, Motlagh, Samsudin, & Salleh, 2019; N. Dasgupta, Ranjan, S., 2018; Franklyne, Iyer, Ebenazer, Mukherjee, & Chandrasekaran, 2019; Giunti, 2019; Harwansh, Deshmukh, & Rahman, 2019; Islam et al.; Jesser et al., 2020; Jianguo Fenga & Seid Mahdi Jafarib, 2020; Juliana Junqueira Pinelli, 2021; N. Kumar, Verma, A., Mandal, A., 2021; Maninder Meenu a, 2023; Mohammed, Muhialdin, & Hussin, 2020; Monica Yumnam a, 2023; Musazzi, Franze, Minghetti, & Casiraghi, 2018; Nishad,

2021; Nishala Erandi Wedamulla a, 2022; Pereira, Barroso, Mourao, & Fernandes, 2021; Rai, Mishra, Yadav, & Yadav, 2018; Salam, 2021; Tarhan & Spotti, 2021; S. Yalcinoz & Ercelebi, 2018; Yesim ¨Ozogul 2021; Zhong et al., 2021)

However, citrus essential oils themselves have no constant physicochemical stability over time because of environmental factors. pH, humidity, oxygen exposure, temperature, or light could damage the stability and properties of pure citrus essential oils; therefore, to increase their stability over time and to enhance the antioxidant, antimicrobial, and antimutagenic properties, essential oils can be introduced into nanosized colloidal systems called nano-emulsions (NEs). Nano-emulsions are biphasic systems that contain two immiscible liquids, usually oil and water. Oil in water NE is formed through the dispersibility of oil droplets into the water phase. A simple emulsion becomes a nano-emulsion when it reaches dimensions of less than 200 nm (Choi, 2020; N. Kumar, Mandal, A., 2018; Naseema, Kovooru, Behera, Kumar, & Srivastava, 2021; Wen-Chien Lu & Jen-Chieh Tsai, 2018; S. Yalcinoz, Ercelebi, E., 2020). Encapsulation into nano-emulsions has the potential to enhance the handling, water-dispersibility, chemical stability, and effectiveness of the functional compounds (Islam et al.; Tarhan & Spotti, 2021; Wani, Masoodi, Jafari, & McClements, 2018; S. Yalcinoz & Ercelebi, 2018).

In recent times, there has been a notable rise in consumer inclination towards natural ingredients within the realms of both the food and pharmaceutical sectors. The aforementioned tendency has led to a noteworthy surge in the desire to replace synthetic chemicals with natural ones. Citrus essential oils represent natural materials that have demonstrated useful properties (Elsherif & Al Shrief, 2021; Jianguo Fenga & Seid Mahdi Jafarib, 2020; Roberta Bento, 2020; Sabeena Manzoor c, 2021; Yesim ¨Ozogul 2021).

Another aspect that needs to be noted is the uncontrolled public health concern, caused by the contamination of food with microorganisms. This issue has prompted the food sector to implement novel methodologies for the regulation of these microorganisms. One notable aspect to consider is the use of antibacterial compounds. There has been a notable rise in the number of studies examining the antimicrobial properties of naturally derived compounds in recent years. This trend may be attributed to the growing aversion among contemporary consumers towards synthetic chemicals, as well as the greater consumption of minimally processed or natural food products. These chemicals are utilized both singly and in combination, with the aim of assessing potential synergistic effects. Within the realm of natural chemicals, several substances have demonstrated noteworthy antibacterial properties. Bacteriocins, exemplified by constituents found in essential oils, are particularly notable in this regard (Asadinezhad, 2019).

In this context, there is a constant need for functionalized formulations ready to be used in food as green additives or in medicine for non-invasive treatments. The use of nano-emulsions is an important step for food safety: the controlled release of key compounds helps long-term efficiency against microorganisms, increasing the shelf life of food and beverages. Furthermore, the organoleptic properties are improved and bioavailability during intake is increased due to the protected bioactive components from the essential oils, (Guliani, 2018; Shatabdi Das & Mondal, 2020).

Thus, the aim of the present study was the formulation and characterization of 5 types of NEs containing bergamot (Be), tangerine (Ta), orange (O), grapefruit (G), and lemon (Le) essential oils. The obtained citrus essential oils nano-emulsions (CEO-NEs) were intended to be used as a functional ingredient for food applications and biomedical uses (e.g. gastric dressings). The ultrasonication method was used to form the nano-emulsions, and several techniques such as laser diffraction, scanning electron microscopy, transmission electron microscopy, spectrophotometry, minimum inhibitory concentration have been used for their characterization, to evaluate their stability, antioxidant and antimicrobial activities.

The major objective of this study was to emphasize the significance of a suitable composition of nano-emulsions to ensure optimal stability and enhance the understanding of essential oil utilization. To achieve this objective, we develop our method for the formulation of nano-emulsions, using both a magnetic stirrer and an ultrasonication bath. Combining these two methods and adapting the amount of each mixture component we obtained a novel formulation for the NEs.

MATERIALS AND METHODS

Citrus essential oils, microbial strains and reagents

Bergamot (*Citrus bergamia*) - Be, lemon (*Citrus limon*) - Le, tangerine (*Citrus reticulata*)- Ta, sweet orange (*Citrus sinensis*)- O and grapefruit (*Citrus paradisi*) - G essential oils were purchased from Herbes del Molí, S.L. (Alicante, Spain). These oils are organic, 100% pure, and certified. Tween 80 and ethanol were used as surfactant and co-surfactant respectively and were purchased from Nordic Chemicals. Also, for the NEs preparation distilled water was used.

Chemical analysis of citrus essential oils

The volatile composition of the EOs and CEO-NEs was determined using GC-MS analysis coupled with headspace solid phase micro-extraction (HS-SPME) as described in Pérez-Marin paper (Pérez-Marín et al., 2021). Briefly, 1 g

of sample was weighed and added into a 20 mL vial with polypropylene caps and PTFE/silicone septa. The vial was placed in an AOC-6000 Plus autosampler (Shimadzu Corporation, Kyoto, Japan) and, after 5 min of equilibration time, a 50/30 μm DVB/CAR/PDMS fiber (1 cm) was exposed to the sample headspace for 50 min at 40°C (with agitation, 250 rpm). The separation and identification of compounds were carried out by GC2030 (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), in a Sapiens X5MS column (Teknokroma, Barcelona, Spain), 30 m × 0.25 mm i.d., 0.25 μ m film thickness, and coupled with a mass spectrometer detector (TQ8040 NX triple quadrupole mass spectrometer; Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). Identification and quantification of CEO-NEs constituents were done based on their retention times, the peak area of chromatograms, and their mass spectra, which were confirmed by comparison with data from Wiley mass spectral library.

Nano-emulsion preparation, droplet characterization and stability

The process of nano-emulsion formation combined spontaneous emulsification with the ultrasound method to obtain particles with better properties. Firstly, spontaneous emulsification was performed by titration of the aqueous phase (distilled water) into an organic phase (containing 8% essential oil, ethanol, and Tween 80) drop by drop, under continuous stirring at room temperature. After this stage, the coarse emulsion was obtained. The prepared coarse emulsion was subjected to sonication for 15 minutes at 72 amplitudes to obtain the functionalized nano-emulsion (Gharibzahedi, 2018; Shatabdi Das & Mondal, 2020). All formulations resulted are oil-in-water nano-emulsions, those systems being frequently used in the food industry (Choi, 2020). All of the nano-emulsion components (essential oil, alcohol, water, and Tween 80) are GRAS substances that are permitted as food additives in the European Union. Tween 80 (T80), alternatively known as Polysorbate 80 is widely utilized due to its safety profile and has received approval from the European Union for its application in food products (Sanchez, 2022). The hydrophobic tail of Tween 80 has a curled conformation, which leads to optimal curvature and packing parameters. This characteristic aids in the efficient formulation of nano-emulsions (N. Dasgupta & Ranjan, 2018; S. Yalcinoz & Ercelebi, 2022).

The droplets present in oil-in-water (O/W) nano-emulsions consist of a hydrophobic core composed of oil molecules, surrounded by a hydrophilic shell composed of emulsifier molecules. The molecules of the emulsifier align in a manner that their polar regions extend into the aqueous phase, while their non-polar regions extend into the center of the oil droplets. The oil droplets in nano-emulsions have an electrical charge when they are coated by ionized surface-active substances, such as ionic surfactants, phospholipids, proteins, or polysaccharides. The physical stability of nano-emulsions is influenced by the electrical properties of the oil droplets, which in turn affect the colloidal contacts between the droplets and their propensity to assemble. Furthermore, these compounds have the ability to influence the chemical stability of nano-emulsions through their capacity to modify the interaction between the oil droplets and other constituents present in the food matrix (Choi, 2020). Choosing the right emulsifier is an important step to obtain stable and useful nano-emulsions. Hydrophilic-lipophilic balance (HLB) and surfactant concentration must be taken into consideration when a surfactant is chosen (Donsi, 2018). The turbidity, morphology, and bioactive properties of the NEs were investigated and their stability was monitored under different environmental conditions (storage at room temperature, at 37oC, refrigeration, freezing). The physical stability of CEO-NEs was assessed after 1 month of storage under low light conditions by visually inspecting the samples. Each emulsion exhibited different degrees of gravitational separation, depending on the chemical composition and storage conditions. The turbidity experiments were performed using the method from Liew et. al. with some modifications. This parameter was determined by measuring the absorbance of 30 times diluted nano-emulsions at a wavelength of 600 nm using a UV-VIS Spectrophotometer (Shimadzu UV-1700). Distilled water served as the standard solution for this essay. Each sample was measured in triplicate for 12 days. The stability of nano-emulsions was assessed by analyzing the changes in structural uniformity and the emergence of destabilizing phenomena, in four different environmental conditions (room temperature, fridge, freezer, and 37 degrees) during 30 days of storage. The average droplet size and zeta potential were determined by a dynamic light scattering instrument (DLS) (Zetasizer Nano, Malvern). These qualitative analyses evaluated the droplet surface charge, as indicated by the zeta potential (z) values (electrophoretic mobility of emulsions), and the droplet dimension, expressed average size of the tested formulations at 25°C. These physical characteristics are useful for determining the quality of the nano-emulsions developed and predicting their stability over time. Low Z-average sizes (<100 nm) guarantee the formation of nano-metric droplets; it is generally accepted that smaller droplet sizes correspond to superior nano-emulsions (Campolo, 2020). Droplet size was determined at the beginning of the experiment.

DPPH and ABTS radicals scavenging activity

For the antioxidant assays the procedures followed the method of Jia-jing Guo (2018) with some modifications. DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) are free radicals that have hydrogen acceptor capability to antioxidants. For the first method, 100 μL essential oil nano-emulsion was mixed with 3.9 ml DPPH working solution (0.0025% in methanol), the mixture was incubated in the

dark, at room temperature for 30 minutes and after this, the absorbance was read at 515 nm. For the second method, 150 µL essential oil nano-emulsion was mixed with 2.850 mL ABTS working solution (mixing ABTS-7.4 mM and potassium persulfate-2.6 mM stock solutions with methanol), the mixture was kept in the dark for 2 hours, and after the absorbance was read at 734 nm (Thaipong K., 2006). The absorbances were recorded at room temperature (≃20°C) at different time intervals (30 minutes for DPPH and 2 hours for ABTS).

Transmission and Scanning Electron Microscopy

The morphology of the nano-emulsions was evaluated by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). TEM provides a two-dimensional representation of the sample by producing a cross-sectional view, while SEM generates a three-dimensional picture (Choi, 2020). The TEM experiments were carried out at an operating voltage of 80 kV, employing equipment manufactured by ZEISS in Germany. The emulsion specimens were applied onto a carbon-coated copper grid. The samples were subsequently subjected to air-drying prior to the ultimate analysis (Jyoti Nishada, 2021).

Antimicrobial activity of citrus essential oils nano-emulsions

The antimicrobial activity was performed using minimum inhibitory concentration (MIC)(Semeniuc, Pop, & Rotar, 2017) and nano-emulsions antimicrobial activity was tested against some standard strains: Escherichia coli ATCC 25922, Salmonella enteritidis ATCC 13076, Listeria monocytogenes ATCC 19114 and Staphylococcus aureus ATCC 6538P. Gentamicin (0.04 mg/mL in saline solution) was used as a positive control, while the nano-emulsion without essential oil was the negative control. The wells of a 96-well microplate were filled with 100 µL of sterile nutrient broth. Then, 100 µL of samples were added to the first well, and 11-fold serial dilutions were made by transferring 100 µL from well to well along each row. The 100 µL surplus in the final well in the series was discarded. Then, 10 µL of an inoculum containing 1.5×CEO-NEs104 CFU/mL was added to each well.

After incubating the microplates for 20-22 hours at 37°C, 20 µL of 0.2 mg/mL resazurin aqueous solution was added to each well. The microplates were then incubated for 2 hours at 37°C. After this period, resazurin was oxidized to resorufin (the color turns from blue to pink) wherever viable bacterial cells were present. Thus, the concentration in the final well of each raw that remained blue was deemed to completely inhibit bacterial growth; this concentration was determined the MIC.

Cytotoxic activity

Cell Culture

The metastatic B16-F10 murine melanoma cell line was purchased from ATCC (Rockville, MD, USA) and grown under standard conditions. More specifically the cells were cultivated in DMEM (Dulbecco's Modified Eagle Medium) medium containing 4.5 g/L glucose, 10% fetal bovine serum (FBS) supplemented with 2 mM glutamine, 1% penicillin, and streptomycin. The non-tumor model (Hs27 human fibroblast cells, ATCC Rockville, MD, USA) was also cultivated in DMEM (Dulbecco's Modified Eagle Medium) medium containing 4.5 g/L glucose, 10% FBS supplemented with 2 mM glutamine, 1% penicillin, and streptomycin. Both cell lines were maintained under standard conditions at 37 ^{0}C, 5% CO2 and 95% relative humidity.

At approx. 80% confluence, cell lines were detached using 0.25% (w/v) trypsin–0.53 mM EDTA solution and were seeded in 96-well microplates at a concentration of 5×104 cells per well in 150 µL culture medium. After 24 h, 0, 5, 10, 15, 20, 25 µg/mL for each essential oil nano-emulsion were added to the culture medium and cells were incubated for the next 24 h under the same conditions. Tween 80 was used as a solvent for EONEs (10%) and the cytotoxic activity of Tween 80 was also tested at the maximum concentration. Working dilutions were freshly prepared on the day of testing. At the end of incubation time, cells of each well were examined in contrast phase microscopy (Zeiss LSM 710).

Analysis of Cell Proliferation

The cytotoxicity assay was assessed by using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide reagent (MTT). After a PBS washing step, cells were incubated with 150 µL/well MTT solution (5 mg/mL) for 1 h at 37^{0}C. The resulting formazan crystals were dissolved in 150 µL/well dimethyl sulfoxide (DMSO). The absorbance values were measured using wavelengths of 550 nm and 630 nm with an HT BioTek Synergy microplate reader (HT BioTek Synergy, BioTek Instruments, Winooski, VT, USA). Cell viability was expressed as a percentage of control (cells incubated in normal medium only).

Statistical analysis

All experiments were performed in triplicates and data were reported as mean ± standard deviation (SD), resulting from the assays. The statistical significance of the variations was investigated by Minitab Software using

one-way variance analysis (ANOVA) and Tukey`s honestly significant difference (HSD) test with a confidence interval of 95% or 99%. Statistical significance was accepted at a level of $p<0.05$.

RESULTS AND DISCUSSIONS

Chemical compositions of CEOs and CEO-NEs

As we mentioned above, we explored the chemical compositions of the pure essential oils (Table 1) and oily systems, respectively (Table 2). The pure oils and citrus oils nano-emulsions were analyzed by GC-MS. Depending on the sample, 28 to 39 compounds were identified in pure EOs and 21 to 36 compounds were identified in CEO-NEs. These compositions represent 99.94-99.99% of the essential oils, 99.48-99.91% of the CEOs-NEs, respectively. D-limonene was the predominant constituent, with a concentration varying between 46.18-87.94% for EOs and 31.37-87.82 % in citrus essential oil nano-emulsions. According to the results, the use of nanotechnology to formulate stable nano-emulsion equilibrates the chemical composition and enhances the compound's stability over time, without affecting the overall phytochemical profile of essential oils. The main compounds identified in our CEO-NEs are listed in Table 2. Bergamot (BeNEs), tangerine (TaNEs), orange (ONEs), grapefruit (GNEs) and lemon (LeNEs) essential oils nano-emulsions were characterized by a high percentage (up to 50%) of monoterpenes hydrocarbons. BeEO nano-emulsions also contained a significant ammount of oxygenated monoterpenes (12.94%) and esters (27.2%). The most abundant compounds were linalool (11.41 ± 0.16 %) and linalyl acetate (27.02 ± 1.02 %), along with γ-terpinene (10.85 ± 0.26%) in BeNEs, 23.59±0.09% in TaNEs and 13.25±0.08% in LeNEs. The chemicals detected in CEO-NEs distinguish them in terms of physical, chemical and sensory properties. Aldehydes have a significant role in the characteristics of citrus flavors. Saturated aliphatic aldehydes (octanal and decanal) contribute to the bright green color and have citrus- and peel-like scents, making them likely candidates for citrus flavor. Linalool gives the fragrant scent of citrus. The obtained data are consistent with the previously published studies that characterized the composition of nano-emulsions based on different type of citrus essential oils (Abdelsamed I. Elshamya, 2020; Balwinder Singh, 2021; Do, Nguyen, Pham, Trieu, & Luu, 2020; Giunti, 2019; Monica Yumnam a, 2023; Ortiz-Zamora et al., 2020; Zhiwei Kang a, 2022).

Table 1: Essential oils composition; Be-bergamot, Ta-tangerine, O-orange, G-grapefruit, Le-lemon

No.	Identified compound	Retention time (min)	Flavoring compound, % from total peaks area				
			Be	Ta	O	G	Le
1	alpha-Thujene	3.948	0.25	2.05	0.01	0.03	0.93
2	alpha-Pinene	4.108	1.14	5.06	2.09	1.82	3.66
3	Camphene	4.41	0.03	0.04	0.01	0.01	0.13
4	Sabinene	4.762	0.89	0.67	0.78	1.13	3.83
5	5-Hepten-2-one, 6-methyl-	4.838	0.01	-	-	-	-
6	β-Pinene	4.915	9.49	3.72	0.06	0.40	13.83
7	beta-Myrcene	4.976	1.28	3.21	5.51	4.91	2.95
8	Octanal	5.237	0.02	0.08	0.74	0.95	0.11
9	alpha-Phellandrene	5.434	0.02	0.13	0.11	0.08	0.08
10	Terpilene	5.509	0.02	-	0.36	-	-
11	3-Carene	5.668	0.11	0.66	0.01	-	0.45
12	o-Cymene	5.834	1.65	5.06	0.03	-	1.52
13	D-Limonene	5.996	**29.73**	**48.80**	**87.07**	**87.94**	**46.18**
14	β-cis-Ocimene	6.273	0.30	0.12	0.15	-	0.22
15	gamma-Terpinene	6.667	10.09	23.15	0.03	0.31	12.54
16	Sabinene hydrate	6.982	0.07	0.17	0.03	0.02	0.04
17	Terpinolene	7.423	0.35	1.75	0.08	0.03	0.88
18	Linalool	7.741	12.40	0.37	1.35	0.20	0.26
19	trans-Sabinene hydrate	7.845	-	0.36	0.11	0.12	0.25
20	cis-p-Mentha-2,8-dien-1-ol	8.824	0.08	0.13	0.04	0.10	0.09
21	Limonene oxide, trans-	8.977	0.08	0.15	0.07	0.12	0.07
22	p-Mentha-trans-2,8-dien-1-ol	9.139	-	0.24	-	-	0.05
23	Camphor	9.354	-	0.04	0.12	0.08	0.16
24	L-trans-Pinocarveol	9.796	-	0.09	-	-	0.02
25	cis-Pinocarveol	9.952	-	0.07	-	-	0.02

26	trans-Carveol	10.355	-	0.04	-	-	-
27	Terpinen-4-ol	10.464	-	0.13	-	-	0.15
28	alpha-Terpineol	10.939	0.11	0.75	0.19	0.11	0.51
29	Decanal	11.261	0.02	0.07	0.50		0.07
30	Acetic acid, octyl ester	11.377	0.07	-	-	0.58	-
31	cis-Carveol	11.777	-	-	-	0.04	-
32	Linalool, formate	11.956	0.03	0.06	-	0.03	0.13
33	Carveol	12.266	-	-	-	0.02	-
34	β-Citral	12.459	0.31	-	0.11	0.09	2.00
35	D-Carvone	12.7	0.13	0.16	0.03	0.05	
36	Linalyl acetate	12.953	29.25				0.11
37	3,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-	13.345	0.15	-	-	0.19	3.88
38	α-Citral	13.569	0.61	-	0.27	0.04	0.07
39	Perillal	13.927	-	0.08	0.07	-	-
40	Thymol	14.402	-	0.21	-	-	-
41	α-Terpineol acetate	16.718	0.15	1.78	-	-	-
42	Methyl m-methylanthranilate	19.066	-	0.19	-	-	-
43	Isocaryophyllene	19.733	0.28	-	0.06	0.47	0.7
44	α-Bergamotene	20.246	0.25	-	-	-	1.62
45	α-Himachalene	20.403	-	-	-	-	0.06
46	(E)-.beta.-Famesene	21.034	-	-	-	-	0.09
47	Humulene	21.173	-	-	-	0.06	0.05
48	β-Santalene	21.315	-	-	-	-	0.04
49	gamma-Muurolene	22.947	-	-	-	0.01	0.15
50	alpha-Farnesene	23.06	-	0.39	-	-	-
51	beta-Bisabolene	23.201	0.62	-	-	-	2.06
	TOTAL:		**99.99**	**99.98**	**99.99**	**99.94**	**99.96**

Table 2: Citrus essential oil nano-emulsions composition; Be-bergamot NEs, Ta-tangerine NEs, O-orange NEs, G- grapefruit NEs, Le-lemon Nes NEs type

No.		**Be**	**Ta**	**O**	**G**	**Le**
Compound name		**I. Monoterpenes hydrocarbons**				
1	α-Thujene	Retention time (min)	3.948			
		Concentration (%)	0.27 ± 0.01	1.76 ± 0.02	0	0	1.06
		Physical properties	Boiling point: 150-152 °C Solubility: alcohol/water 2.91 mg/L 25°C				
		Fragrance	wood, green, herb				
2	α-Pinene	Retention time (min)	4.108			
		Concentration (%)	1.23 ± 0.03	4.35 ± 004	1.82 ± 0.05	1.89 ± 0.03	4.03 ± 0.06
		Physical properties	Melting point: -64 - -62 °C Boiling point: 155-156°C Solubility: alcohol/water 2.49 mg/L 25 °C				
		Fragrance	pine, turpentine				
3	Camphene	Retention time (min)	4.41			
		Concentration (%)	0.03	0.03 ± 0.01	0	0	0.15 ± 0.01
		Physical properties	Melting point: 51 °C Boiling point: 78-79°C Solubility: alcohol/water 4.6 mg/L 25 °C				
		Fragrance	camphor				
4	Sabinene	Retention time (min)	4.762			
		Concentration (%)	0.99 ± 0.03	0.66 ± 0.01	0.72 ± 0.03	1.20 ± 0.03	4.29 ± 0.07
		Physical properties	Boiling point: 163-165°C Solubility: alcohol/water 2.494 mg/L 25 °C				
		Fragrance	pepper,turpentine,wood				
5	β -pinene	Retention time (min)	4.915			
		Concentration (%)	9.76 ± 0.13	3.56 ± 0.03	5.48 ± 0.20	5.39 ± 0.06	13.98 ± 0.19
		Physical properties	Melting point: - 61.5 °C Boiling point: 165-166°C				

			Solubility: alcohol/water 7.06 mg/L 25 °C				
		Fragrance	pine,resin, turpentine				
6	β -myrcene	Retention time (min)	4.976				
		Concentration (%)	1.40 ± 0.06	3.37 ± 0.09	0	0	3.37 ± 0.05
		Physical properties	Melting point: -10 °C Boiling point: 65-66°C Solubility: alcohol/water 6.923 mg/L 25 °C				
		Fragrance	balsamic, must, spice				
7	α -Phellandrene	Retention time (min)	5.434				
		Concentration (%)	0.02	0.13 ± 0.01	0.12 ± 0.03	0.08	0.10
		Physical properties	Boiling point: 93-94°C Solubility: alcohol/water 2.862 mg/L 25 °C				
		Fragrance	dill				
8	α -terpinene	Retention time (min)	5.509				
		Concentration (%)	0.03	0	0.36 ± 0.03	0	0
		Physical properties	Melting point: <25 °C Boiling point: 173-175°C Solubility: alcohol/water 5.915 mg/L 25 °C				
		Fragrance	refreshing, lemony-citrus				
9	3-carene	Retention time (min)	5.668				
		Concentration (%)	0.13	0.57 ± 0.02	0	0	0.54 ± 0.02
		Physical properties	Melting point: <25 °C Boiling point: 169-174°C Solubility: alcohol/water 4.581 mg/L 25 °C				
		Fragrance	lemon,resin				
10	o-cymene	Retention time (min)	5.834				
		Concentration (%)	1.89 ± 0.07	5.6 ± 0.09	0	0.03 ± 0.03	1.81 ± 0.04
		Physical properties	Boiling point: 177-179°C Solubility: alcohol/water 23.3 mg/L 25 °C				
		Fragrance	-				
11	**D-Limonene**	**Retention time (min)**	**5.996**				
		Concentration (%)	**31.37 ± 0.68**	**48.81 ± 0.31**	**87.82 ± 0.41**	**87.09 ± 0.38**	**48.53 ± 0.81**
		Physical properties	**Melting point: -96 °C Boiling point: 175-177°C Solubility: alcohol/water 13.8 mg/L 25 °C**				
		Fragrance	**citrus,mint**				
12	β -cis-ocimene	Retention time (min)	6.273				
		Concentration (%)	0.4 ± 0.06	0.07	0.18 ± 0.05	0.45 ± 0.09	0.25 ± 0.01
		Physical properties	Boiling point: 177 °C Solubility: alcohol/water 2.012 mg/L 25 °C				
		Fragrance	sweet, herb				
13	γ-terpinene	Retention time (min)	6.667				
		Concentration (%)	10.85 ± 0.26	23.59 ± 0.09	0.04 ± 0.01	0.03 ± 0.01	13.25 ± 0.08
		Physical properties	Melting point: - 10 °C Boiling point: 181-183°C Solubility: alcohol/water 8.68 mg/L 25 °C				
		Fragrance	gasoline,turpentine				
14	Terpinolene	Retention time (min)	7.423				
		Concentration (%)	0.40 ± 0.02	2 ± 0.02	0.09 ± 0.01	0.02	1.04 ± 0.02
		Physical properties	Melting point: <25 °C Boiling point: 186-187°C Solubility: alcohol/water 9.5 mg/L 25 °C				
		Fragrance	sweet,pine				
II. Oxygenated monoterpenes							
15	Octanal	Retention time (min)	5.237				
		Concentration (%)	0.03	0.09	0.73±0.02	1.04	0.12 ± 0.01
		Physical properties	Melting point: 12-15 °C Boiling point:171-173 °C Solubility: alcohol/water 570 mg/L 25 °C				
		Fragrance	fat, soap, lemon, green				
16	Sabinene hydrate	Retention time (min)	6.982				
		Concentration (%)	0.05	0.11 ± 0.01	0.02	0.01 ± 0.01	0.01 ± 0.01

		Physical properties	Melting point: 58-62 °C Boiling point: 200-201 °C Solubility: alcohol/water 440.5 mg/L 25 °C				
		Fragrance	wood, balsamic				
17	Linalool	Retention time (min)	7.741				
		Concentration (%)	11.41 ± 0.16	0.39 ± 0.02	1.18 ± 0.01	0.21	0.15 ± 0.14
		Physical properties	Boiling point: 117-118°C Solubility: alcohol/water 1590 mg/L 25 °C				
		Fragrance	flower, lavender				
18	trans-Sabinene hydrate	Retention time (min)	7.845				
		Concentration (%)	0	0.23 ± 0.01	0.12 ± 0.02	0.15 ± 0.01	0.25
		Physical properties	Boiling point: 201-202°C Solubility: alcohol/water 440.5 mg/L 25 °C				
		Fragrance	wood balsamic				
19	cis-p-Mentha-2,8-dien-1-ol	Retention time (min)	8.824				
		Concentration (%)	0	0.02	0	0.03	0
		Physical properties	-				
		Fragrance	-				
20	Limonene oxide, trans-	Retention time (min)	8.977				
		Concentration (%)	0.06 ± 0.02	0.19	0.06± 0.01	0.11	0.05
		Physical properties	Boiling point: 198-199 °C Solubility: alcohol/water 137.2 mg/L 25 °C				
		Fragrance	fruit				
21	p-Mentha-trans-2,8-dien-1-ol	Retention time (min)	9.139				
		Concentration (%)	0	0.15 ± 0.01	0	0	0
		Physical properties	-				
		Fragrance	-				
22	Camphor	Retention time (min)	9.354				
		Concentration (%)	0	0.05	0.11 ± 0.01	0.09	0.16
		Physical properties	Boiling point:207 °C Solubility: alcohol/water 100 mg/L 25 °C				
		Fragrance	camphor				
23	L-trans-Pinocarveol	Retention time (min)	9.796				
		Concentration (%)	0	0.07	0	0	0
		Physical properties	Boiling point:215-217 °C Solubility: alcohol/water 958.1 mg/L 25 °C				
		Fragrance	woody				
24	cis-Pinocarveol	Retention time (min)	9.952				
		Concentration (%)	0	0.08	0	0	0
		Physical properties	Boiling point:217-218 °C Solubility: alcohol/water 958.1 mg/L 25 °C				
		Fragrance	flower				
25	Trans-carveol	Retention time (min)	10.355				
		Concentration (%)	0	0.05	0	0	0
		Physical properties	Boiling point:231-232 °C Solubility: alcohol/water 519.7 mg/L 25 °C				
		Fragrance	spicy				
26	Terpinen-4-ol	Retention time (min)	10.464				
		Concentration (%)	0.03	0.22 ± 0.01	0	0	0.16 ± 0.01
		Physical properties	Melting point: 88-90 °C Boiling point: 209 °C Solubility: alcohol/water 386.6 mg/L 25 °C				
		Fragrance	turpentine, nutmeg, must				
27	a-Terpineol	Retention time (min)	10.939				
		Concentration (%)	0.09 ± 0.01	0.72 ± 0.01	0.13 ± 0.01	0.07 ± 0.01	0.35 ± 0.03
		Physical properties	Melting point: 40-41 °C Boiling point: 214-218°C Solubility: alcohol/water 710 mg/L 25 °C				
		Fragrance	must				
28	Decanal	Retention time (min)	11.261				
		Concentration (%)	0.02	0.10	0.51 ± 0.05	0.76 ± 0.07	0.06 ± 0.01
		Physical properties	Melting point: 17-18 °C Boiling point: 207 - 209°C Solubility: alcohol/water 43.52 mg/L 25 °C				

		Fragrance	soap, orange peel, tallow				
29	Cis-carveol	Retention time (min)	11.777				
		Concentration (%)	0.04 ± 0.01	0.02	0	0	0
		Physical properties	Boiling point: 231.46°C Solubility: alcohol/water 519.7 mg/L 25 °C				
		Fragrance	spicy				
30	Linalool formate	Retention time (min)	11.956				
		Concentration (%)	0.03	0.06	0	0	0
		Physical properties	Boiling point: 101 - 103°C Solubility: alcohol/water 28.25 mg/L 25 °C				
		Fragrance	fresh, citrus, green, herbaceous, bergamot-like				
31	Carveol	Retention time (min)	12.266				
		Concentration (%)	0.02	0	0	0	0
		Physical properties	Boiling point: 226-227°C Solubility: alcohol/water 519.7 mg/L 25 °C				
		Fragrance	fresh, spearmint, caraway				
32	β-Citral	Retention time (min)	12.459				
		Concentration (%)	0.32 ± 0.01	0.01	0.09 ± 0.01	0.10 ± 0.01	1.80 ± 0.05
		Physical properties	Boiling point: 103-104°C Solubility: alcohol/water 1340 mg/L 25 °C				
		Fragrance	lemon				
33	D-carvone	Retention time (min)	12.7				
		Concentration (%)	0.13 ± 0.01	0.02	0	0	0
		Physical properties	Boiling point: 98°C Solubility: alcohol/water 1300 mg/L 25 °C				
		Fragrance	mint				
34	3,6-Octadien-1-ol, 3,7-dimethyl-, (Z)- (isogeraniol)	Retention time (min)	13.345				
		Concentration (%)	0.13 ± 0.01	0	0	0	0
		Physical properties	Melting point: -15°C Boiling point: 103-105°C Solubility: alcohol/water 255.8 mg/L 25 °C				
		Fragrance	rose				
35	α -citral	Retention time (min)	13.569				
		Concentration (%)	0.58 ± 0.05	0	0.23 ± 0.01	0.19 ± 0.03	3.02 ± 0.12
		Physical properties	Boiling point: 103-104°C Solubility: alcohol/water 1340 mg/L 25 °C				
		Fragrance	lemon				
36	Thymol	Retention time (min)	14.402				
		Concentration (%)	0	0.05 ± 0.03	0	0	0
		Physical properties	Melting point: 49-51 °C Boiling point: 231-232°C Solubility: alcohol/water 900 mg/L 25 °C				
		Fragrance	herbal				
		III. Sesquiterpenes hydrocarbons					
37	(+)-trans-Chrysanthenyl acetate	Retention time (min)	12.584				
		Concentration (%)	0	0.27 ± 0.02	0	0	0
		Physical properties	Boiling point: 232-233°C Solubility: alcohol/water 30.19 mg/L 25 °C				
		Fragrance	-				
38	Isocaryophyllene	Retention time (min)	19.733				
		Concentration (%)	0.23 ± 0.04	0.23 ± 0.03	0	0.72 ± 0.21	0.26 ± 0.07
		Physical properties	Boiling point: 266-268°C Solubility: alcohol/water 0.05011 mg/L 25 °C				
		Fragrance	wood				
39	α-Bergamotene	Retention time (min)	20.246				
		Concentration (%)	0.18 ± 0.05	0	0	0	0.48 ± 0.15
		Physical properties	Boiling point: 259-260°C Solubility: alcohol/water 0.02985 mg/L 25 °C				
		Fragrance	wood, warm, tea				
40	Humulene	Retention time (min)	21.173				
		Concentration (%)	0	0	0	0.08 ± 0.02	0
		Physical properties	Melting point: <25 °C Boiling point: 166-168°C Solubility: alcohol/water 0.01396 mg/L 25 °C				

		Fragrance	woody				
41	a-Farnesene	Retention time (min)	23.06				
		Concentration (%)	0	0.25 ± 0.06	0	0	0
		Physical properties	Boiling point: 260-262°C Solubility: alcohol/water 0.01053 mg/L 25 °C				
		Fragrance	wood,sweet				
42	b- Bisabolene	Retention time (min)	23.201				
		Concentration (%)	0.47 ± 0.22	0	0	0	0.60 ± 0.18
		Physical properties	-				
		Fragrance	balsamic				
IV. Ketones							
43	5-Hepten-2-one, 6-methyl-	Retention time (min)	4.838				
		Concentration (%)	0.01	0	0.05	0.11	0
		Physical properties	Melting point: - 67 °C Boiling point: 72-73°C Solubility: alcohol/water 3351 mg/L 25 °C				
		Fragrance	fatty, green citrus-like				
44	Perillal	Retention time (min)	13.927				
		Concentration (%)	0	0.13	0.07	0.06 ± 0.01	0.05 ± 0.01
		Physical properties	Boiling point: 238-240°C Solubility: alcohol/water 160.7 mg/L 25 °C				
		Fragrance	fat				
V. Esthers							
45	Octyl ester	Retention time (min)	11.377				
		Concentration (%)	0.07	0	0	0	0
		Physical properties	Melting point: -38.5 °C Boiling point: 210°C Solubility: alcohol/water 33.9 mg/L 25 °C				
		Fragrance	fruity, orange-like, jasmine-like				
46	Linalyl acetate	Retention time (min)	12.953				
		Concentration (%)	27.02 ± 1.02	0	0	0	0
		Physical properties	Boiling point: 115-116°C Solubility: alcohol/water 20.12 mg/L 25 °C				
		Fragrance	sweet, fruit				
47	α-Terpineol acetate	Retention time (min)	16.718				
		Concentration (%)	0.11 ± 0.02	0	0	0	0
		Physical properties	Boiling point: 220°C Solubility: alcohol/water 18.97 mg/L 25 °C				
		Fragrance	sweet, refreshing, herbaceous				
48	Methyl m-methylanthranilate	Retention time (min)	19.066				
		Concentration (%)	0	1.88 ± 0.19	0	0	0
		Physical properties	Melting point: 18.5-19.5 °C Boiling point: 255°C				
		Fragrance	grapes,orange peels				
Monoterpenes hydrocarbons			58.77	94.5	96.18	96.18	92.4
Oxygenated monoterpenes			12.94	2.63	3.18	2.76	6.12
Sesquiterpenes hydrocarbons			0.88	0.75	0	0.8	1.34
Ketones			0.01	0.13	0.12	0.17	0.05
Esthers			27.2	1.88	0	0	0
Total			**99.8**	**99.89**	**99.48**	**99.91**	**99.91**

Characterization and stability of citrus essential oils nano-emulsion

Based on the data from the literature, we assumed that Tween 80 is the most suitable surfactant, to obtain stable and significantly reduced size for NEs, so we used it for CEOs-NEs preparations. (Enayatifard, 2021). Tween 80 is often regarded as the most advantageous surfactant for the formulation of nano-emulsion owing to its superior HLB in comparison to other polysorbates. The absorption of surfactant results in a reduction in interfacial tension at the oil-water interface, hence facilitating the generation of ultrafine particles (Shatabdi Das & Mondal, 2020). Surfactants with a low HLB value have the ability to create emulsions where water is dispersed as droplets within an oil phase. Conversely, surfactants with a high HLB value are capable of forming emulsions where oil is dispersed as droplets within a water phase (Chong et al., 2018).

TEM and SEM analysis of CEO-NEs revealed the spherical and uniform particle shape and also, confirmed the results of the particle size analysis using dynamic light scattering with a diameter between 43 and 50 nm (Figure 1). The value for zeta potential was -10.4 mV which indicates moderate stability. The size of droplets in a nano-emulsion plays a crucial role in determining their physicochemical and functional characteristics, including its visual attributes, consistency, storage stability, and behavior inside the gastrointestinal system (Choi, 2020).

The morphology of the CEO-NEs was revealed by employing TEM analysis. As seen in Figure 1 (b), the NE displayed a clear and noticeable characteristic. The most central layer was determined to be a droplet of water, surrounded by an emulsifier that served as an interface. The contact was encompassed by an external layer of oil, exhibiting a shell-like configuration. It is widely assumed that this shell consists of a two-layer covering. The confirmation of the effective encapsulation of polyphenolic chemicals within oil was achieved by the observation of the existence of these bioactive particles located at the core of the nano-emulsion.

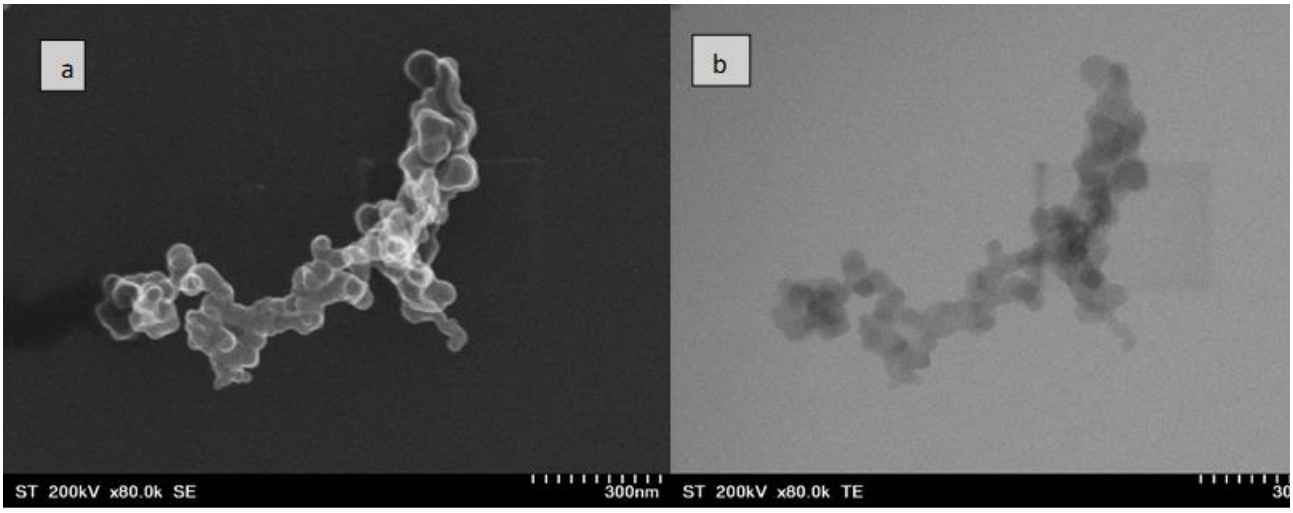

Figure 1. Morphological aspect of citrus essential oils nano-emulsions through
(a) SEM and (b) TEM.

Changes in turbidity are shown in Figure 2 as a function of the storage time of CEO-NEs stored in different environmental conditions (storage at room temperature (R.T.), at 37oC, refrigeration (4°C), freezer (-20°C)) (Guliani, 2018). Each emulsion exhibited different degrees of gravitational separation, the ones stored at 37°C being the most unstable, showing phase separation (all the nano-emulsions stored at 37°C exhibited the same decreasing turbidity trend).

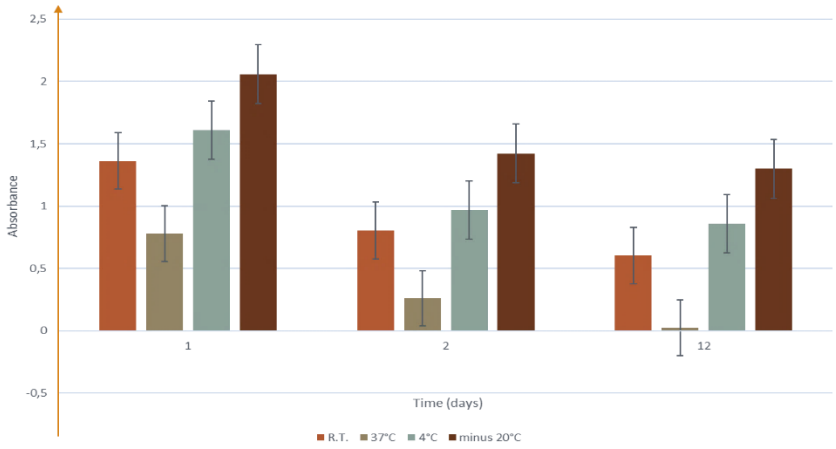

Figure 2: Stability depending on turbidity

Oil in water nano-emulsions are obtained by dropwise addition of the aqueous phase into the organic phase. The lamellar liquid crystal phase is successively transformed into the gel phase during inversed phase emulsification, and the gel phase's low interfacial tension and high viscosity are conducive to inhibiting the coalescence of ultrafine oil droplets (Jianguo Fenga & Seid Mahdi Jafarib, 2020).

On the other hand, for the TaNEs was observed the occurrence of an oily ring at the upper phase of NE after 30 days of storage (Figure 3). The observed phenomenon can be attributed to the ineffective action of the surfactant in safeguarding the interface against various destabilization mechanisms, including coalescence, Ostwald ripening, and creaming. Also, an increase in the droplet size leads to gravitational separations. Therefore, in this study we have two classifications regarding the stability of NEs: one is based on the turbidity results, and the other one on the visual perception.

Figure 3: Stability depending on appearance: TaNEs after 30 days of storage at R.T., 4°C, at 37°C, -20°C (from left to right)

Antioxidant testing assays of citrus essential oils nano-emulsions

Monoterpene-rich essential oils nano-emulsions are recognized as natural antioxidants. Antioxidant activities for CEO-NEs obtained using DPPH and ABTS assays from a single sample were measured three times to test the reproducibility. The activities were measured on days 2, 7, and 30 upon synthesis, in different temperature conditions (-20°C, 4°C, RT, and 37°C). The physicochemical properties and functional aspects of nano-emulsions are significantly influenced by the interfacial characteristics of the droplets.

According to the results **(Tables 3, 4)**, we conclude that CEO-NEs have a better response for the DPPH assay, in terms of reproducibility and uniformity of the results. The results obtained through ABTS assay presented a non-uniform evolution over time. DPPH and ABTS radicals react differently with the compounds from NEs, for this reason, CEO-NEs seem to be more susceptible to the DPPH test than to ABTS. Anyway, there is a need for several optimization steps for these methods.

The antioxidant activity of the five varieties of CEO-NEs by the DPPH method ranged from 0.169 to 0.415 μM TE/mL, depending on sample, temperature, and analysis day. On day 2, the antioxidant activity range was 0.169-0.233 μM TE/mL, while on day 30 the DPPH antioxidant activity values ranged between 0.291 to 0.393 μM TE/mL. In all cases, the storage temperature had a minimal impact on the antioxidant properties of various nano-emulsions. This was valid both for DPPH and ABTS assays.

The TaNEs exhibited superior antioxidant properties among all tested samples, as evidenced by both DPPH and ABTS assays, making it the most promising candidate in this regard.

The different behavior of CEOs loaded nano-emulsions was due to their different chemical compositions. Other studies also reported the relation between chemical composition and the antioxidative properties of CEOs loaded nano-emulsions (Balwinder Singh, 2021; Jia-jing Guo, 2018; Jyoti Nishada, 2021; Monica Yumnam a, 2023; Ros-Chumillas, Garre, Mate, Palop, & Periago, 2017; Sabeena Manzoor c, 2021)

The high amounts of naturally occurring antioxidants in the essential oil in the present study in terms of free radical scavenging may be attributed to D-limonene, one of the primary components in the oil, which has antioxidant properties. Unfortunately, D-limonene presents susceptibility to external factors such as heat, light, and humidity. The results obtained using the ABTS method show that sudden and uneven variations in antioxidant activity may be due to the instability of the main compound in CEO-NEs. D-limonene instability leads to instability mechanisms in nano-emulsions, over time and in different storage conditions (Ros-Chumillas et al., 2017). D-limonene undergoes initial oxidation to provide D-limonene hydroperoxide, which subsequently undergoes additional reactions leading to scission reactions resulting in the formation of epoxide, ketone, and alcohol. Surprisingly, this procedure led to the depletion of its lemon-like taste and the generation of distinctive odors, hence diminishing antibacterial effectiveness and altering the flavor of food in varying ways (Zhiwei Kang a, 2022) These sensorial changes were observed using human perception.

Table 3: Antioxidant activity through DPPH assay

DPPH µM Trolox equivalents /mL sample																			
NEs sample	Le -20°C	Le 4°C	Le R.T.	Le 37°C	Ta -20°C	Ta 4°C	Ta R.T.	Ta 37°C	G -20°C	G 4°C	G R.T.	G 37°C	O -20°C	O 4°C	O R.T.	O 37°C	Be -20°C	Be 4°C	Be R.T.	Be 37°C
Day 2	0.171 ± 0.002	0.169 ± 0.005	0.176 ± 0.001	0.153 ± 0.002	0.219 ± 0.013	0.228 ± 0.028	0.224 ± 0.052	0.233 ± 0.001	0.166 ± 0.034	0.169 ± 0.005	0.169 ± 0.000	0.170 ± 0.01	0.171 ± 0.0056	0.169 ± 0.007	0.176 ± 0.003	0.153 ± 0.026	0.182 ± 0.005	0.190 ± 0.0045	0.190 ± 0.000	0.192 ± 0.0297
Day 7	0.291 ± 0.006	0.292 ± 0.016	0.292 ± 0.031	0.304 ± 0.05	0.343 ± 0.104	0.335 ± 0.153	0.332 ± 0.097	0.328 ± 0.125	0.281 ± 0.142	0.277 ± 0.037	0.284 ± 0.023	0.288 ± 0.056	0.296 ± 0.016	0.284 ± 0.001	0.275 ± 0.003	0.278 ± 0.019	0.300 ± 0.000	0.311 ± 0.0242	0.308 ± 0.009	0.316 ± 0.0052
Day 30	0.359 ± 0.005	0.360 ± 0.006	0.367 ± 0.004	0.358 ± 0.009	0.366 ± 0.079	0.375 ± 0.066	0.415 ± 0.05	0.399 ± 0.132	0.357 ± 0.046	0.350 ± 0.005	0.370 ± 0.005	0.393 ± 0.033	0.317 ± 0.025	0.296 ± 0.024	0.291 ± 0.01	0.323 ± 0.000	0.373 ± 0.0887	0.376 ± 0.000	0.378 ± 0.000	0.393 ± 0.000

Table 4: Antioxidant activity through ABTS assay

ABTS µM Trolox equivalents /mL sample																				
NEs sample	Le -20°C	Le 4°C	Le R.T.	Le 37°C	Ta -20°C	Ta 4°C	Ta R.T.	Ta 37°C	G -20°C	G 4°C	G R.T.	G 37°C	O -20°C	O 4°C	O R.T.	O 37°C	Be -20°C	Be 4°C	Be R.T.	Be 37°C
Day 2	820 ± 2.45	784 ± 1.00	807 ± 1.16	865 ± 2.79	1971 ± 140.525	2030 ± 280.425	1985 ± 8.673	2016 ± 0.001	822 ± 11.72	836 ± 2.886	652 ± 2.556	720 ± 4.612	173 ± 12.74	360 ± 7.259	449 ± 6.421	586 ± 7.601	707 ± 4.03	948 ± 2.68	1235 ± 0.06	854 ± 20.6
Day 7	1176 ± 4.95	1203 ± 0.022	1161 ± 0.078	1194 ± 2.082	1705 ± 52.013	1643 ± 112.86	1663 ± 120.8	1643 ± 150.4	437 ± 10.59	345 ± 22.45	323 ± 38.90	450 ± 30.11	326 ± 0.004	208 ± 0.243	203 ± 0.271	722 ± 2.518	694 ± 9.29	836 ± 4.08	1055 ± 33.3	1170 ± 40.7
Day 30	837 ± 0.05	822 ± 5,900	695 ± 25.121	739 ± 0.029	1882 ± 121.24	2020 ± 298.94	2063 ± 203.25	1912 ± 315.12	730 ± 5.022	735 ± 4.894	887 ± 4.337	836 ± 4.564	826 ± 4.910	430 ± 2.894	415 ± 19.07	445 ± 2.93	699 ± 12.73	1587 ± 7.33	730 ± 0	791 ± 0

-20°C – freezer ; 4°C - fridge : R.T.-room temperature

The intensity of relationships between the DPPH and ABTS was determined with Pearson's correlation with a 95% confidence interval. Statistically significant correlations were established ($p \leq 0.05$) between the DPPH and ABTS free radical scavenging activity in the samples subjected to analysis) using Pearson's correlation test (Table 5).

Table 5: Pearson correlation coefficients for each pair of 2, 7 and 30 days stored sample through DPPH and ABTS

Correlation between		R	p	N
$DPPH^{Le, freezer, 7th day}$	$DPPH^{Le, freezer, 2nd day}$	0.494	0.506^{ns}	12
$DPPH^{Le, freezer, 30th day}$	$DPPH^{Le, freezer, 2nd day}$	0.840	0.160^{ns}	12
$DPPH^{Le, freezer, 30th day}$	$DPPH^{Le, freezer, 7th day}$	0.855	0.145^{ns}	12
$ABTS^{Le, freezer, 7th day}$	$ABTS^{Le, freezer, 2nd day}$	-0.032	0.968^{ns}	12
$ABTS^{Le, freezer, 30th day}$	$ABTS^{Le, freezer, 2nd day}$	-0.215	0.785^{ns}	**12**
$ABTS^{Le, freezer, 30th day}$	$ABTS^{Le, freezer, 7th day}$	0.484	0.516^{ns}	12
$DPPH^{Ta, freezer, 7th day}$	$DPPH^{Ta, freezer, 2nd day}$	0.723	0.277^{ns}	12
$DPPH^{Ta,freezer, 30th day}$	$DPPH^{Ta, freezer, 2nd day}$	-0.331	0.669^{ns}	12
$DPPH^{Ta, freezer,30th day}$	$DPPH^{Ta, freezer, 7th day}$	-0.804	0.196^{ns}	12
$ABTS^{Ta, freezer, 7th day}$	$ABTS^{Ta, freezer, 2nd day}$	-0.408	0.592^{ns}	12
$ABTS^{Ta, freezer,30th day}$	$ABTS^{Ta,freezer, 2nd day}$	0.087	0.913^{ns}	12
$ABTS^{Ta, freezer,30th day}$	$ABTS^{Ta, freezer, 7th day}$	0.492	0.508^{ns}	12
$DPPH^{G, freezer, 7th day}$	$DPPH^{G, freezer, 2nd day}$	-0.884	0.116^{ns}	12
$DPPH^{G,freezer, 30th day}$	$DPPH^{G, freezer, 2nd day}$	-0.205	0.795^{ns}	12
$DPPH^{G, freezer,30th day}$	$DPPH^{G, freezer, 7th day}$	0.624	0.376^{ns}	12
$ABTS^{G, freezer, 7th day}$	$ABTS^{G, freezer, 2nd day}$	-0.097	0.903^{ns}	12
$ABTS^{G, freezer,30th day}$	$ABTS^{G, freezer, 2nd day}$	-0.997	0.003^{**}	12
$ABTS^{G, freezer,30th day}$	$ABTS^{G, freezer, 7th day}$	0.074	0.926^{ns}	12
$DPPH^{O, freezer, 7th day}$	$DPPH^{O, freezer, 2nd day}$	-0.359	0.641^{ns}	12
$DPPH^{O,freezer, 30th day}$	$DPPH^{O, freezer, 2nd day}$	-0.485	0.515^{ns}	12
$DPPH^{O, freezer,30th day}$	$DPPH^{O, freezer, 7th day}$	0.843	0.157^{ns}	12
$ABTS^{O, freezer, 7th day}$	$ABTS^{O, freezer, 2nd day}$	0.584	0.416^{ns}	12
$ABTS^{O, freezer,30th day}$	$ABTS^{O, freezer, 2nd day}$	-0.815	0.185^{ns}	12
$ABTS^{O, freezer,30th day}$	$ABTS^{O, freezer, 7th day}$	-0.067	0.933^{ns}	12
$DPPH^{Be, freezer, 7th day}$	$DPPH^{Be,freezer,2nd day}$	0.523	0.477^{ns}	12
$DPPH^{Be,freezer,30th day}$	$DPPH^{Be, freezer, 2nd day}$	-0.984	0.016^{ns}	12
$DPPH^{Be,freezer,30th day}$	$DPPH^{Be, freezer, 7th day}$	-0.612	0.388^{ns}	12
$ABTS^{Be, freezer, 7th day}$	$ABTS^{Be, freezer, 2nd day}$	0.694	0.306^{ns}	12
$ABTS^{Be,freezer,30th day}$	$ABTS^{Be, freezer, 2nd day}$	0.002	0.998^{ns}	12
$ABTS^{Be,freezer,30th g}$	$ABTS^{Be, freezer, 7th day}$	0.152	0.848^{ns}	12

R—Pearson correlation coefficient; p—is the probability of obtaining an F-ratio as large or larger than the one observed, assuming that the null hypothesis of no difference amongst group means is true; N—number of samples. Significance of effect: ns—not significant, $p > 0.05$; *** extremely significant $p \leq 0.001$, ** extremely unsignificant $p \geq 0.001$.

Antimicrobial activity of CEO-NEs

In the present investigation, the MIC of the five CEO-NEs was evaluated against several microorganisms known as foodborne pathogens, namely the Gram-positive (Staphylococcus aureus and Listeria monocytogenes) and Gram-negative (Escherichia coli and Salmonella enteritidis). The bacterial strains used in this investigation are susceptible to each CEO-NE to varying degrees. The results indicated that the antimicrobial activity was dependent on the essential oil contained in the nano-emulsion. The antimicrobial activity of citrus essential oils loaded nano-emulsions against the four microorganisms using gentamicin as positive control is shown in Table 6. Unfortunately, tangerine and lemon-loaded nano-emulsions presented a negative response for all the strains used. Gram-positive bacteria are more resistant to grapefruit and orange essential oil nano-emulsions than to bergamot nano-emulsions. GNEs and ONEs manifest similar behaviors against Escherichia coli ATCC 25922.

BeNEs prevented the growth of all tested organisms. BeNEs had maximum antimicrobial activity against Escherichia coli with a minimum inhibition concentration of 4.109 µL/mL, followed by Salmonella enteritidis (MIC 8.63 µL/mL). The lowest antimicrobial activities of BeNEs were on Staphylococcus aureus and Listeria monocytogenes with a MIC concentration of 18.13 µL/mL. On the other hand, GNEs were most effective against Salmonella enteritidis strain, with a MIC of 8.63 µL/mL, followed by Staphylococcus aureus (MIC 38.09 µL/mL for both GNEs and ONEs) and Escherichia coli (MIC 18.03 for ONEs and 38.09 µL/mL for GNEs).

The antimicrobial activity varies in a dose dependent manner on the NEs concentration. Bioactive compounds act against the cytoplasmic membrane of the cell, altering its structure by influencing the unsaturated fatty acid on the bacterial membrane. Terpene compounds such as limonene cause membrane permeability and proton-motive force dissipation. Orange and grapefruit NEs contain considerable concentrations of D-limonene 87.82%, and 87.09 % respectively, having a good minimum inhibitory activity against Salmonella enteritidis (Ros-Chumillas et al., 2017). Bergamot and orange contain an important amount of linalool, which possesses good antibacterial activity against different kinds of microorganisms (Zhong et al., 2021).

Terpene alcohol exhibits significant antibacterial efficacy against various bacterial strains, particularly S. aureus and E. coli. Furthermore, α-pinene, β-pinene, sabinene, and limonene possess significant antibacterial properties through their ability to disrupt the structural integrity of bacterial or fungal membranes, as well as hinder ion transport processes and respiration (Monica Yumnam a, 2023). The key response obtained from performing this analysis is that the antimicrobial activity is not the same for all types of essential oils nano-emulsions, it is related to their chemical composition and the individual properties of each compound that the chemical composition has. Generally, Gram positive-bacteria are more susceptible to essential oils compared to Gram Negative bacteria. Gram positive bacteria do not have an outside membrane like Gram-negative bacteria. This outer membrane of Gram-negative bacteria comprises polar termini and lipopolysaccharides. As a result, it is difficult for EONEs to enter the cells of Gram-positive bacteria. The increased inhibitory impact of CEO on *E. coli* observed in this experiment may be attributed to the distinct functional groups of the compounds in CEO that target one or more sites, as well as the ability of certain compounds to broke and cross the outer membrane of *E. coli* (Ruimin Ran a, 2023).

Table 6: Antimicrobial activity of CEO-NEs against Gram-positive and Gram-negative bacteria

Sample	***Salmonella enteritidis* ATCC 13076**	***Staphylococcus aureus* ATCC 25923**	***Escherichia coli* ATCC 25922**	**Listeria monocytogenes *ATCC 19114***
	µL/mL			
BeNEs	8.63 ± 0.01^{A}	18.13± 0.01^{B}	**4.109 ± 0.004^{C}**	18.13 ± 0.00^{B}
GNes	**8.63 ± 0.01^{A}**	38.09 ± 0.00^{A}	38.09 ± 0.00^{A}	-
ONEs	**8.63 ± 0.01^{A}**	38.09 ± 0.01^{A}	18.13 ± 0.01^{B}	38.09 ± 0.00^{A}
Gentamicin	0.00024	0.0005	0.00024	0.00152
Sig	p=0.579	p=0	p=0	-

Values are expressed as the mean of three replicates. Values that do not share a letter in the same column are significantly different (Sig.) Bolted results are the representative ones.

Cytotoxic activity of CEO-NEs

The tested nano-emulsion exhibited a cytotoxic effect on melanoma cells. Approximately 40-45% inhibition of melanoma cell growth was observed across most concentrations tested. This suggests a potential anti-cancer activity of the essential oil blend against melanoma cells. Importantly, the nano-emulsion was not cytotoxic to normal fibroblast cells. The inhibition rate for these cells was very low, ranging from 10-12%. Studies often highlight the need for treatments that can selectively target cancer cells while sparing normal cells, which is aligned with your findings.

This differential effect, where cancer cells are inhibited significantly more than normal cells, is a desirable trait in cancer treatment as it implies lower toxicity to healthy cells. The control test with the emulsion base (without essential oils) showed no cytotoxicity. This indicates that the observed cytotoxic effect on melanoma cells is likely due to the essential oils and not the emulsion base.

Bergamot oil nano-emulsion showed that at applied concentrations (0-25 µg/mL), it had minimal cytotoxic effects on normal fibroblast cells (Hs-27), maintaining high cell viability. However, the effect on melanoma cells

(B16-F10) was variable across these concentrations. Notably, at a high concentration of 50 µg/mL, the nano-emulsion significantly reduced the viability of melanoma cells while still having minimal impact on normal fibroblast cells. This suggests that the bergamot oil nano-emulsion, at higher concentrations, is potentially effective in selectively targeting melanoma cells with limited toxicity to normal cells (Figure 4). The same results were also observed for OEO-NEs, who displayed low cytotoxicity towards normal fibroblast cells (Hs-27) across all concentrations, maintaining high viability. In contrast, melanoma cells (B16-F10) showed significant cytotoxicity at the highest concentration (50 µg/mL), with varied responses at lower concentrations (Harleen Kaur1, 2020; Marchese, 2020).

Studies often report that certain essential oils and their components exhibit cytotoxic effects on various cancer cell lines. For instance, compounds like limonene and citral, found in citrus oils, have been reported to induce apoptosis and inhibit cell proliferation in cancer cells (Fatouma Mohamed Abdoul-Latif 1; Yousefian Rad, 2020).

Our results suggest that the essential oils nano-emulsion has a selective cytotoxic effect, primarily affecting melanoma cells while sparing normal fibroblast cells. This selective toxicity is crucial in cancer therapy to minimize damage to healthy tissues. The significant inhibition of melanoma cell growth by the essential oil's nano-emulsion indicates its potential as a therapeutic agent. However, further studies are needed to understand the mechanism of action and to evaluate its efficacy and safety in vivo (Mol1, 2023).

The use of nano-emulsion as a delivery system for essential oils might have contributed to the effectiveness in targeting cancer cells. Nano-emulsions are known for improving the solubility, stability, and bioavailability of therapeutic compounds (Josef Jampilek 1, 2022). Research in this area often shows enhanced efficacy of essential oils against cancer cells when used in nano-emulsion form.

Additional research, including molecular studies, would be beneficial to understand how the essential oils induce cytotoxicity in melanoma cells. This could involve investigating pathways related to inflammation, apoptosis, cell cycle arrest, or other mechanisms of cell death.

In summary, the obtained results are promising and suggest a potential anti-cancer property of the tested essential oils nano-emulsions against melanoma, with minimal effects on normal cells. Further detailed studies could pave the way for developing a novel therapeutic approach for melanoma treatment.

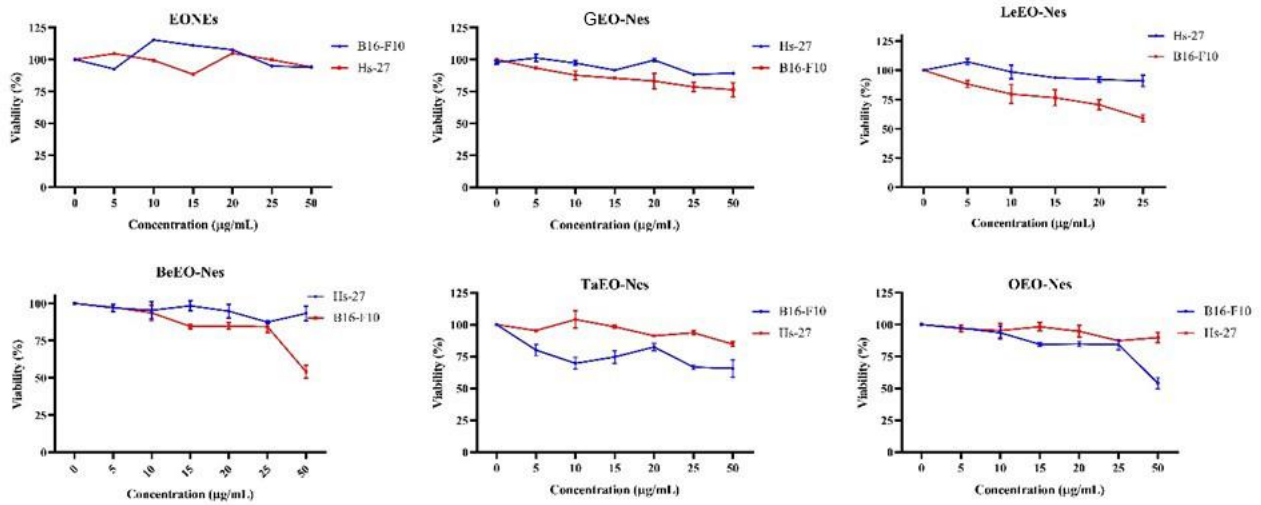

Figure 4: Effect of CEO-NEs on Hs-27 and B16-F10 cell proliferation

CONCLUSIONS

The five citrus essential oils nano-emulsions obtained, containing bergamot, tangerine, orange, grapefruit and lemon essential oils had remarkable antioxidant and antimicrobial activities. The TaNEs were most efficient as antioxidants (0.228 µM TE/mL by DPPH and 2030 µM TE/mL by ABTs at 4°C), while the BeNEs, GNEs, and ONEs had good antimicrobial properties. BeNE was most effective against Gram Negative strains (MIC of 4.109 µL/mL and 8.63 µL/mL against E. coli and S. enteridis respectively), and GNEs ad ONEs were most effective against S. enteridis (MIC 8.63 µL/mL). Another notable finding with potential for future applications is the selective cytotoxic effect, the CEO-NEs primarily affecting the cellular viability of B16-F10 melanoma cells, while showing little effect on normal Hs-27 fibroblast cells. The inhibition of melanoma cell growth indicates its potential as a therapeutic agent. However, further studies are needed to understand the mechanism of action and to evaluate its efficacy and safety in vivo.

This study revealed that the formed citrus essential oils nano-emulsions provide many benefits in specific applications due to the distinctive physicochemical and functional attributes associated with their reduced droplet size. This research demonstrates that NEs may effectively encapsulate citrus essential oils, resulting in enhanced water dispersibility, stability, and bioavailability of their bioactive compounds. To achieve desirable functional features, it is imperative to exercise control over the lipid phase composition, interfacial properties, and particle size. The achievement of this objective can be facilitated by the manipulation of nano-emulsion composition and optimization of homogenization conditions. However, the area of nano-emulsions research remains largely unexplored due to the inherent difficulties associated with modifying their surface properties. For this reason, the potential of nano-emulsions as customizable therapeutic carriers for targeted drug delivery represents a challenge.

Furthermore, it is plausible that the antimicrobial properties of CEONEs are associated with their chemical composition, with limonene being identified as the primary constituent of the oil.

The current experimental findings are helpful in fabricating stable nanometric lipidic systems with potential applicability in food as green additives or in medicine as non-invasive treatments.

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Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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