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The Impact of Nanoencapsulation on Volatile Constituents of *Citrus sinesis* L. Essential Oil and their Antifungal Activity

Amr Farouk[1], Amal S. Hathout[2], May M. Amer[2], Omaima A. Hussain[2], Ahmed S.M. Fouzy[2*]

[1]*Flavor and Aromatic Chemistry Department, National Research Centre, Cairo, Egypt*

[2] *Food Toxicology and Contaminants Department, National Research Centre, Cairo, Egypt*

Abstract

The agricultural waste peels have been considered as an ecological burden on society. Therefore, this study focuses on the use of *Citrus sinensis* L. peel oil and nanoemulsion as an antifungal agent, along with the effect of high-intensity ultrasound on the chemical constituents and the activity of the oil nanocapsules. The chemical composition was determined using gas chromatography/mass spectrometry. A total number of 20 components were identified, representing 99.65%, of the total essential oils, while 14 and 6 compounds were identified in nanoemulsions and dried peels accounting for 90.61 and 74.21%, respectively. Limonene was predominant in all investigated samples but with a quantitative difference, while the encapsulation leads to identify other predominates like linalool, carveols, mentha-2,8-dienol, carvone, and limonene aldehyde. Meanwhile, the sun-dried technique is negatively affected the active constituents of the oil. The mean particle size of the *Citrus sinensis* L. nanoemulsion was 97.22 nm, with a poly dispersibility index (PDI, 0.016), while the zeta potential value was -16.31 ± 2.54 mV, which is consistent with the pH 5.18. The viscosity of the prepared nanoemulsion was 1.37 mPa/sec. showing non-significant differences, and higher stability formula. The transmission electron microscope showed that the nanoparticles were spherical, uniformly distributed, discrete, and non-aggregated. A varying degree of antifungal activity of both *Citrus sinensis* L. peel essential oil and nanoemulsion was observed. *Citrus sinensis* L. peel essential oil exhibited antifungal activity against *A. niger*, *A. ochraceus*, *Fusarium* spp., and *Penicillium* spp. Meanwhile, nanoemulsion displayed lower antifungal activity, against *A. flavus*, *A. niger*, and *A. ochraceus*. The incorporation of *Citrus sinensis* L. peel essential oil into nanoemulsions provided an improved method for delivering this oil while retaining its bioactivity.

Keywords: *Citrus sinensis* L. peels; essential oil; nanoemulsion; antifungal activity.

1. Introduction

The fruit and vegetable industry and home cooking produced a large amount of peel waste, causing huge nutritional and economic losses, as well as environmental problems [1]. Each fruit contains 15 to 50% of the peel, which is discarded as waste after using its fleshy part (that is, the mesocarp). In some cases, the amount of waste obtained is greater than the product itself [2-4]. Nowadays, the valorization of agro-industrial waste, especially the lignocellulosic ones, has become an economic and environmental necessity, whereas many economic materials such as essential oils, pectin, animal feed, activated carbons, pollutants adsorbents, fuels, and energy have been extracted and reported in the literature [5, 6]. The valorization of fruit peel waste has a great potential for the transition toward a bio-economy. Additionally, the negative impact of the fruit peel processing industry on the environment makes valorization even more important [7].

Orange (*Citrus sinesis* L.) is a rich source of phenolic compounds, flavonoids, soluble sugars, dietary fibers (cellulose, hemicelluloses, pectin), vitamins, and essential oils [8, 9]. Orange juice is an important agro-industrial economic sector, which is why a large amount of *Citrus sinensis* L. peel waste is processed [10]. Juice constitutes about half of the fresh weight of orange fruit while the remains are pulp, peel, and seeds [11]. Approximately 95% of these wastes are made of peels, which is a big problem for this industry because their management requires economic and energy resources, and there is a risk of air, water, and soil pollution. The valorization of *Citrus sinensis* L. peel waste is a

*Corresponding author e-mail: amoursy@hotmail.com.; (Prof. Ahmed S.M. Fouzy).

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potential target due to its composition of essential oil which is widely used in chemicals, food, cosmetics, and perfumery industries, biofuels, biorefinery, pectin extraction, and animal feed [12].

Orange constitutes the main citrus crop in Egypt with about 80% of the total citrus area, whereas the total planted orange area is 168,000 ha [13]. Therefore, regarding the total amount of waste that could generate due to the processing of orange crops in Egypt, thus the treatment and exploitation of *Citrus sinensis* L. peel waste have become a potential issue to avoid environmental pollution.

The orange essential oil has been reported to have antioxidant, anti-cancer, anti-inflammatory, cardio-protective, neuroprotective, anti-bacterial, and anti-mycotic activities [14]. Many attempts have been undertaken to retain essential oils' bioactive compounds, one of which is the production of an essential oil nanoemulsion. Nanoemulsions are the stabilized biphasic dispersion of two immiscible liquids that are either oil-in-water (o/w) or water-in-oil (w/o) by a surfactant that is amphiphilic [15].

The above information is the main motivation behind this study that aimed to use dried *Citrus sinensis* L. peel waste, its essential oil, and nanoemulsion as antifungal activity. Formulation of nanoemulsion can be limited by instability due to the poor dispersibility of the essential oils in hydrophilic media, and sensitivity towards oxidation, heat, and light. Therefore, this study opens perspectives for safer solutions employing agricultural byproducts of the food industry associated with nanotechnology for application in environmental purposes.

2. Materials and methods

2.1. Plants and chemicals

The ripened fresh fruits of sweet orange of Washington Navel orange (*Citrus sinensis* L.) were obtained from the farms of the Egyptian Ministry of Agriculture on December, 15th 2020. Diethyl ether was purchased from Fisher Chemicals (Pittsburgh, USA). The mixture of n-alkanes C6–C26, authentic compounds, polysorbate 20, and sodium sulfate anhydrous was obtained from Sigma Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of *Citrus sinensis* L. peel

Sweet orange fruits were checked for defects, insect damage, disease, surface color change, and other defects to ensure the final product's quality. Orange was washed thoroughly with distilled water to get rid of any dust or dirt that adheres to the *Citrus sinensis* L. peel, and wiped dry. *Citrus sinensis* L. peels were separated manually and cut into small parts for about 2 × 2 cm, then dried using solar energy at 50°C for 96 hours. The fruit peels were then ground thoroughly and passed through a 0.25 mm mesh.

2.3. Extraction of the volatile components

Extraction of the volatile compounds from dried *Citrus sinensis* L. peel was carried out by hydrodistillation for 3 h, using a Clevenger type apparatus. The volatile-compounds extract was dried using anhydrous sodium sulfate, stored in airtight glass vials covered with aluminum foil at -20°C until analysis [16].

2.4. Preparation of nanoemulsion from *Citrus sinensis* L. peel essential oil by high-intensity ultrasound

Nanoemulsion (*Citrus sinensis* L. peel essential oil in water) (5%) was prepared using Polysorbate 20. The organic phase was added to the aqueous phase and subjected to sonication using an ultrasonicator. A probe diameter of 13 mm at a high frequency of 20 kHz and a power output of 750 W was used. The energy was given through a sonicator probe and ice was used to reduce energy [17].

2.5. Physiochemical characterization of nanoemulsion

2.5.1. Dynamic light scattering (DLS)

The particle size distribution of nanoemulsions was measured by a dynamic light scattering (DLS) with a Zetasizer NanoZS laser diffractometer. This instrument determines the particle size from intensity-time fluctuations of one laser beam (633nm, 2 mV power respectively) scattered from a sample at angles of 173°. Before measurements, all samples were diluted 10 times (1:10) using citrate buffer solution (5 mM, pH 3.5) to avoid multiple scattering effects and stirred continuously during the tests to ensure that homogeneous emulsions are obtained [18].

2.5.2. Zeta potential measurements

The zeta potential was measured at 25°C by phase-analysis light scattering (PALS) with a Zetasizer NanoZS laser diffractometer. Samples were diluted 1:200 (v/v) with 0.22 μm filtered sample buffer. Before zeta potential measurements all samples were sonicated for 5 minutes [19].

2.5.3. Viscosity measurement

Viscosity measurements were performed using Kinexus Rotational Rheometer. The measurements

were carried out at the temperature of 25.0 °C ± 0.5 °C at a 5 s^{-1} shear rate [20]. The values of viscosity were recorded after 1, 7, 14, and 21 days.

2.5.4. Titrable Acidity and pH

Titrable acidity was calculated as the percentage of citric acid by titrating 10 mL of the nanoemulsion with a solution of NaOH (0.1 N) till pH 8.1. The pH was measured by a pH meter.

2.6. Transmission electron microscope (TEM)

The morphology of nanoemulsion was studied by transmission electron microscope. Before the analysis, the nanoemulsion sample was diluted in distilled deionized water (1:1000). The sample was adsorbed onto carbon-coated copper/palladium grids for 1 min., then the phosphotungstic acid solution (2%, pH = 6.7) was used for staining. The grids were washed three times by floating it face-down on drops of sterilized, deionized water for 1 min. The replica was lifted to dry at room temperature (27°C) and then the image was visualized at 80 KV accelerating voltages [21].

2.7. Gas chromatography-mass spectrometry (GC/MS)

The components of the dried *Citrus sinensis* L. peel powder, essential oils extracted from *Citrus sinensis* L. peels, and nanoemulsion, were analyzed by GC/MS apparatus. The separation was performed on a Trace GC Ultra Chromatography system (Thermo Scientific, USA) equipped with an ISQ-mass spectrometer (Thermo Scientific, USA) with a 60 m × 0.25 mm × 0.25 μm-thick TG-5MS capillary column (Thermo Scientific, USA). The column separation was programmed from 50°C with a holding time of 3 min, and then the temperature was increased at a rate of 4°C per min to 140°C with a holding time of 5 min. After that, the temperature was increased at 6°C per minute to 260°C for a 5-min isothermal holding time. The injector temperature was 180°C, the ion source temperature was 200°C, and the transition line temperature was 250°C. The carrier gas was helium with a constant flow rate of 1.0 ml/min. The mass spectrometer had a scan range from m/z 40–450, and the ionization energy was set at 70 eV. The identification of compounds was based on matching with the MS computer library (NIST library, 2005 version) and comparing with those of authentic compounds and published data [22]. The relative percentage of the oil constituents was calculated from the GC peak areas. Kovat's index was calculated for each compound, using the retention times of a homologous series of C6–C26 n-alkanes and matching with the literature [23-26].

2.8. Antifungal activity

The antifungal activity was evaluated against five fungal species *Aspergillus flavus*, *A. niger*, *A. ochraceus*, *Penicillium* spp., and *Fusarium* spp. These fungal strains were obtained from Food Toxicology and Contaminants Department, National Research Centre. Antifungal activity of essential oil and nanoemulsion was performed using a well diffusion agar assay. Sterilized Potato Dextrose Agar (PDA, NEOGEN, Lansing, MI 48912 USA) was poured into Petri plates and allowed to dry. Fungal cultures spore suspensions (104/mL) were inoculated over the dried surface of the PDA plate. The wells were cultured with a cork borer of 8 mm diameter, and 100 μl of the essential oil and nanoemulsion were poured into each well and incubated at 25 °C for 72 h [27]. The size of the inhibition zone formed in millimeters was reported.

2.9. Statistical analysis

Results are expressed as mean ± SD. Statistical analysis of the data was carried out using Microsoft Excel 2010 statistical program. A one-way analysis of variance (ANOVA) was performed, in which $P<0.05$ was considered statistically significant.

3. Results and Discussion

3.1. The effect of high-intensity ultrasound encapsulation on the volatile constituents of *Citrus sinensis* L.

The chemical composition of the volatile oils from peels of *Citrus sinensis* L. was characterized by GC-MS (Table 1, Figure 1A). A total number of 20 components were identified, representing 99.65%, of the total oils. Limonene (92.36%), γ-terpinene (1.47%), octanal (1.12%), α-myrcene (1.08%), and α-pinene (1.05%) were the dominant compounds in the *Citrus sinensis* L. peel essential oil. Similar results were reported by Xu et al. [23]; Ayala et al. [24]; Golmohammadi et al. [25]; and Ibrahim and El-Sawi [26], who studied the same species in Egypt, Iran, Spain, Mexico, and the USA. The differences in the major components of the essential oil could be attributed to different growing conditions, geographic origins, seasonal variation, and extraction processes [28].

The findings of the chemical analysis of the nanoemulsions by GC/MS showed a great difference from that of the essential oil. Only 14 constituents were identified in nanoemulsions, representing 90.61% of the total nanoemulsion oil. Like essential oil, limonene was the predominant but with a quantitative difference (71.40%) (Table 1, Figure 1B). Some of the oxygenated terpenes,

e.g., linalool and trans- carveol, were increased in the nanoemulsion, whereas others, such as mentha-2,8-dienol, limonene oxide, cis- carveol, nerol, carvone, and limonene aldehyde, were not detected in the essential oil but found as predominates in the nanoemulsion (Table 1). Most of the studies dealt with the encapsulation of oils or flavors and focused on the physical stability and biological activity of the microparticles or nanoparticles but not on the changes in the volatile constituents of the encapsulated oils. Few studies have reported that the formulation based on energy-intensive techniques may lead to Ostwald ripening, flocculation, or coalescence of the emulsion with changes in its physical stability and biological activity [29]. High-pressure homogenization, high-intensity ultrasound, and high shear homogenization represent examples of energy-intensive techniques that result in the decomposition of the active constituents of essential oils and the accumulations of others [30]. Interestingly, an increase of oxygenated terpenes in the oil nanoemulsion was at the expense of non-oxygenated concentrations detected in the essential oil (Table 1). Therefore, further studies can be conducted to examine the stability of different aroma and volatile compounds during microencapsulation techniques, especially under severe conditions, and discover the mechanisms of the transfer of such volatile compounds to others.

Only 6 volatile compounds were identified in the sun-dried *Citrus sinensis* L. peels accounted for 74.21% (Table 1, Figure 1C). Limonene was predominant with 69.59%, while most of the monoterpenes detected in peels essential oil were absent in the dried one. Sun or solar drying is one of the oldest drying methods applied for many agricultural products, such as medicinal plants and aromatic herbs in most tropical or sub-tropical countries. Sun-drying may not be a suitable method for some types of herbs due to substantial color and aroma degradation in dried herbs. For example, the amount of major volatile components in roman chamomiles such as isobutyl isobutyrate, 3-methyl butyl isobutyrate, and propyl tiglate was lower in the sun-dried herb in comparison to air-dried one. The same scenario was observed in lemongrass and basil [31].

3.2. Physiochemical characterization of nanoemulsion

Microemulsion characterization must also involve particle size analysis to confirm the ultra-fine size (< 100 nm). Data in Table (2) indicated that the mean particle size of the *Citrus sinensis* L. nanoemulsion was 97.22±0.13 nm, with a poly dispersibility index (PDI, 0.016). Results in Figure (2) indicated that the particle size of the formulated orange peel nanoemulsion was a monomodal size distribution pattern. The increase of particle size is evidence of micelles swelling to accommodate the solubilized load of extracts. The degree of swelling reached depends on the type of surfactant or surfactant mixture applied. One should consider that the nature of the oil, which is another factor that can contribute to the difference in particle size of their emulsions. Terpenes in *Citrus sinensis* L. oil emulsion interacts with the surfactant micelles in a defined mode in comparison to single components mentioned in literature like citral which showed a particle size of 20±0.4 nm for single surfactant and 5.8±0.01 nm for mixed surfactants, respectively. Meanwhile, in another study lemongrass oil recorded a higher particle size in the same study due to its nature which is agreed with our results [32].

Table 1: Identification of the volatile constituents of dried *Citrus sinensis* L. peel using gas chromatography/mass spectrometry

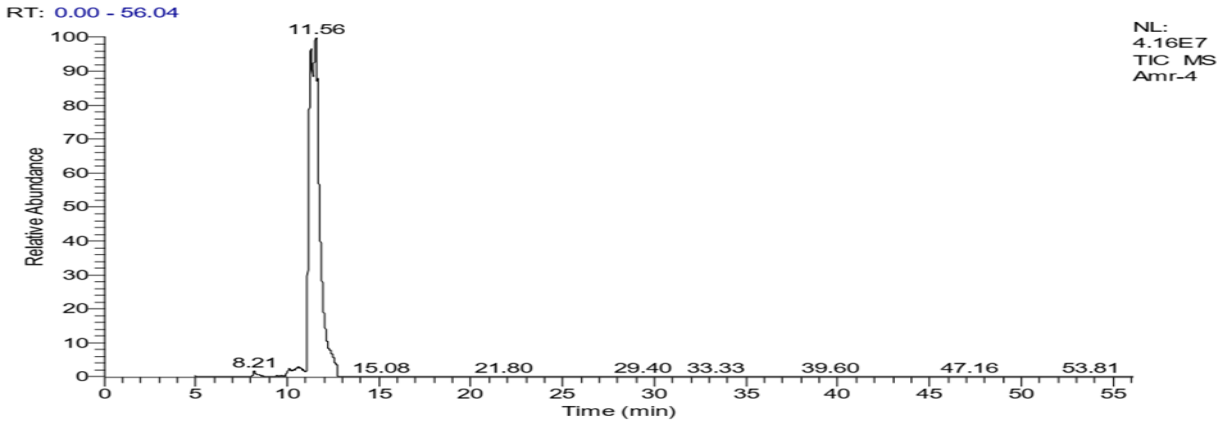
S/N	Compound	RI [a]	LRI [b]	Area%			Identification method [c]
				Essential oil	Nanoemulsion	Dried peel	
1	α- Thujene	932	931	0.08	0.21	-	RI, MS
2	α- Pinene	937	939	**1.05**	0.56	-	RI, MS, STD
3	Sabinene	978	976	0.36	0.23	-	RI, MS, STD
4	α- Myrcene	986	991	**1.08**	0.93	-	RI, MS, STD
5	Octanal	1008	1006	**1.12**	-	-	RI, MS
6	δ- Carene	1015	1011	0.97	0.30	-	RI, MS
7	Limonene	1034	1031	**92.36**	**71.40**	**69.59**	RI, MS, STD
8	γ- Terpinene	1070	1062	**1.47**	-	-	RI, MS, STD
9	Terpinolene	1091	1088	0.1	-	-	RI, MS
10	Linalool	1100	1098	0.14	**1.94**	-	RI, MS
11	*trans-p*-Mentha-2,8-dienol	1113	1110	-	**2.70**	-	RI, MS
12	Limonene oxide	1139	1130	-	0.51	-	RI, MS
13	Terpin-4-ol	1180	1177	0.12	-	-	RI, MS
14	α- Terpineol	1191	1189	0.08	-	-	RI, MS
15	Decanal	1209	1204	0.07	-	-	RI, MS
16	*trans* - Carveol	1220	1217	0.11	**3.23**	-	RI, MS
17	*cis*- Carveol	1225	1226	-	**1.22**	-	RI, MS, STD
18	Nerol	1228	1229	-	0.57	-	RI, MS
19	Carvone	1239	1243	-	**5.54**	0.60	RI, MS, STD
20	Limonene aldehyde	1325	1328	-	**1.27**		RI, MS
21	*Z*- Citral	1381	1383	0.07	-		RI, MS
22	*E*- Citral	1392	1391	0.09	-		RI, MS
23	Dodecanal	1412	1407	0.1	-		RI, MS
24	β- Caryophyellene	1428	1418	0.05	-	0.55	RI, MS
25	α- Patchoulene	1444	1454	-	-	0.70	
26	Valencene	1505	1491	0.12	-	-	RI, MS
27	β- Sinensal	1707	1695	0.11	-	-	RI, MS
28	Hexadecanoic acid, butyl ester	2180	2188	-	-	0.91	RI, MS
29	Docosane	2200	2200	-	-	**1.86**	RI, MS, STD
-	**Total**	-	-	**99.65**	**90.61**	**74.21**	-

[a] RI: retention indices calculated on DB-5 column using alkanes standards.

[b] LRI: retention indices according to literature.

[c] Confirmed by comparison with the retention indices, the mass spectrum of the authentic compounds, and the NIST mass spectra library data.

A.



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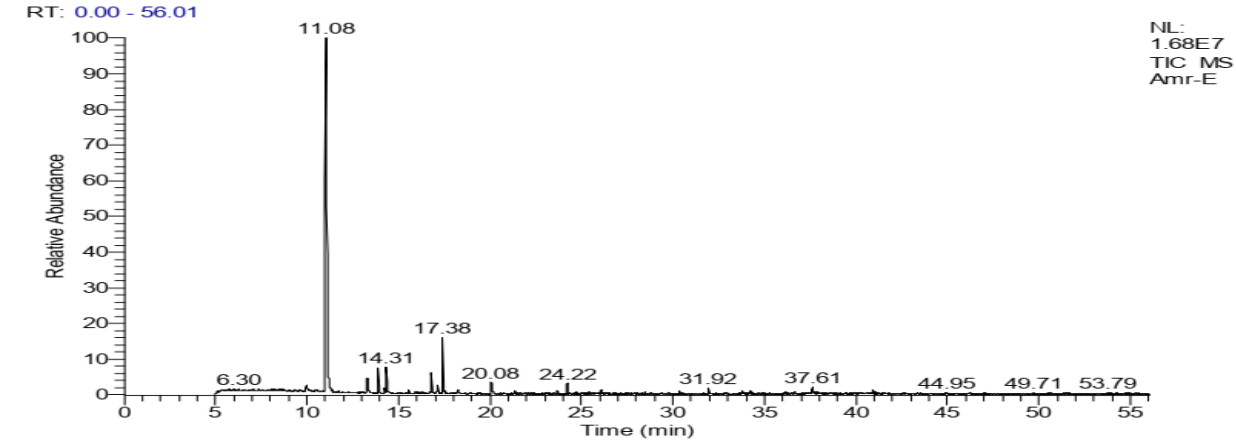

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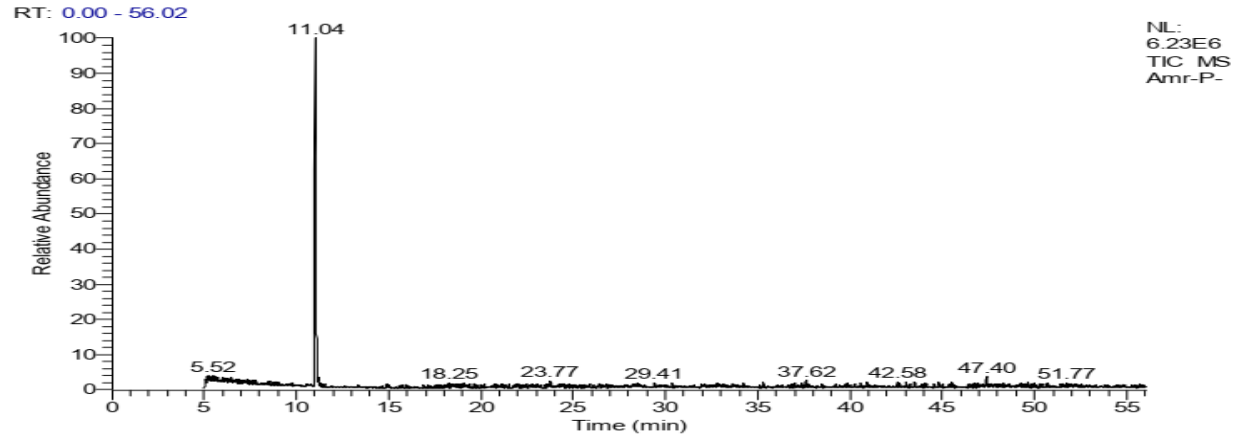

Figure 1: Volatile chromatogram for A) *Citrus sinensis* L. essential oil, B) nanoemulsion and C) dry peel

Table 2: Physiochemical characterization of *Citrus sinensis* L. nanoemulsion

Characterization	*Citrus sinensis* L. nanoemulsion
Particle size (nm)	97.22±0.13
Zeta potential	- 16.31±2.54
PDI	0.016±0.006
Viscosity Day 1 (mPa/sec) Day 7 (mPa/sec) Day 14 (mPa/sec) Day 21 (mPa/sec)	 1.37±0.026 1.31±0.027 1.49±0.033 1.51±0.041
pH	5.18±0.04
Acidity as (g citric / L)	1.17±0.08

Results are mean ± SD

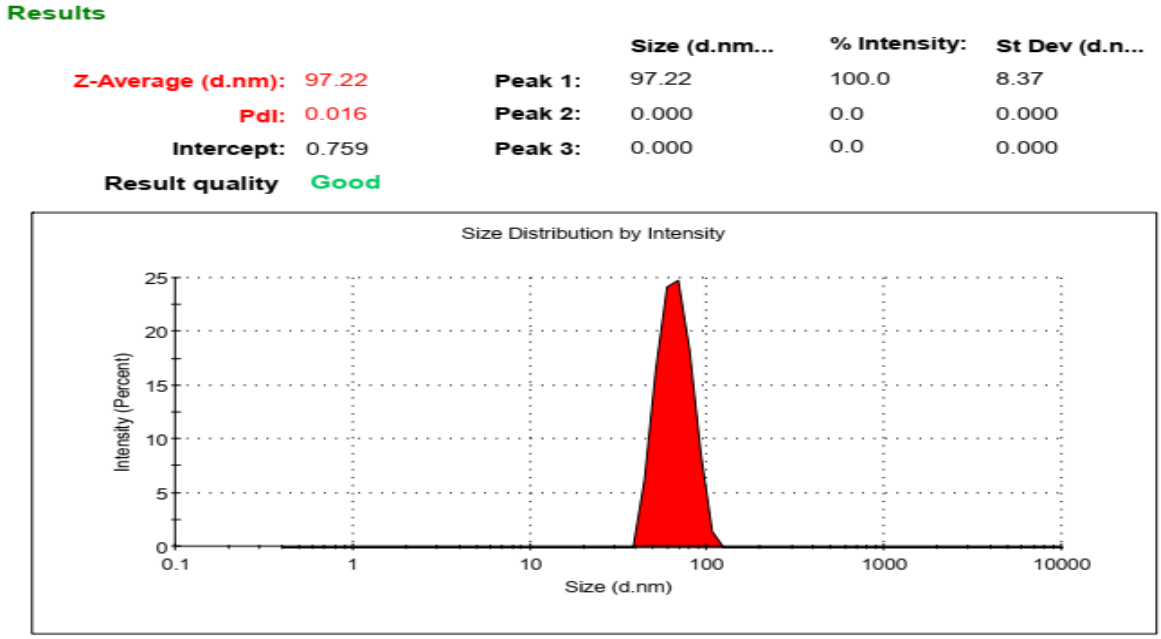

Figure 2: Particle size distribution of *Citrus sinensis* L. nanoemulsion

If all the particles in suspension have a large negative or positive zeta potential, they will tend to repel each other, and there will be no tendency for the particles to come together. However, if the particles have low zeta potential values, there will be no force to prevent them from coming together and flocculating. To obtain electrostatically stabilized nanoemulsions, zeta potential values should be in the range of ± 30 mV [33]. In the current study zeta potential value of *Citrus sinensis* L. nanoemulsion was −16.31 ± 2.54 mV, which is consistent with the pH value listed as 5.18 in Table (2). The negative charge of the nanoemulsion may be due to the anionic groups of the fatty acids and glycols present in the surfactant applied [34]. Therefore, such stability means simply a suitable shelf-life for the nanoemulsion which opens prospects to be used in many fields with an efficient and extend action.

Results in Table (2) showed the viscosity over 21 days for homogenized emulsions. Viscosity is an important criterion as it measures the physical stability of nanoemulsion and it affects the size of capsules and the thickness of their walls [35]. The viscosity of the prepared nanoemulsion was low compared to other formulas reported in the literature [34, 36] with 1.37 mPa/sec. Differences in viscosity over 21 days showed non-significant differences which revealed higher stability in the formulated nanoemulsion.

The transmission electron microscope (TEM) characterization of the nanoemulsions gave the

actual size and shape, whereas the droplets in the nanoemulsions appeared dark (Figure 3). The TEM micrographs showed that the nanoparticles were spherical, uniformly distributed, discrete, non-aggregated, and had a different average diameter in nm based on Image J software that automatically adjusted the contrast between the background and the particles and calculated the diameter of the rounded nanoparticles. Differences in diameter could be related to the Ostwald ripening phenomenon where the oil or extract phase exhibited a mild solubility in the surrounding aqueous phase of the emulsion system which transfers from small to large droplets [29].

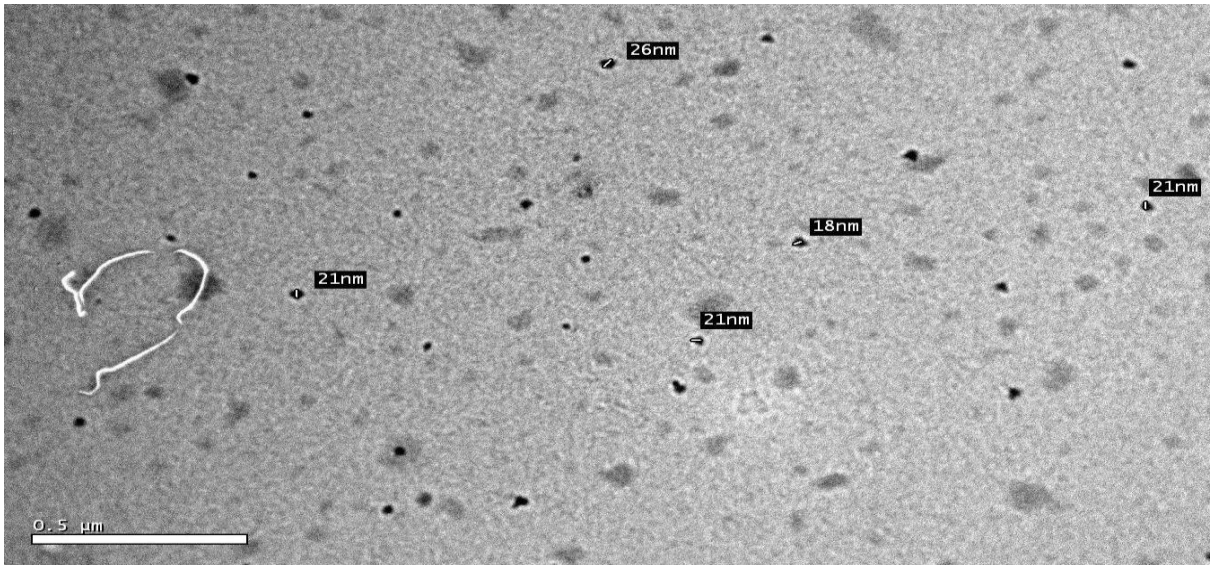

Figure 3: Transmission electron microscope of *Citrus sinensis* L. nanoemulsion

3.3. Antifungal activity

Data in Table (3) and Figures (4 and 5) showed a varying degree of antifungal activity of both *Citrus sinensis* L. peel essential oil and nanoemulsion. *Citrus sinensis* L. peel essential oil exhibited antifungal activity against *A. niger*, *A. ochraceus*, *Fusarium* spp., and *Penicillium* spp. Meanwhile, nanoemulsion displayed lower antifungal activity, against *A. flavus*, *A. niger*, and *A. ochraceus*. Similar observations stated that *Citrus sinensis* L. essential oil was effective against a broad spectrum of organisms, such as *A. niger* [37], *A. flavus*, *A. fumigate* [38], and *P. chrysogenum* [39]. The antifungal activity of *Citrus sinensis* L. peel essential oil could be due to the presence of the following components limonene, γ-terpinene, linalool, and terpinen-4-ol [40]. Jing et al. [41] reported that there is a relationship between antimicrobial activity and the chemical structures of the most abundant compounds in the essential oils. In agreement, Viriato [42] added that essential oil is rich in terpenes, such as limonene, which are considered to be a natural antifungal due to their apolar chemical structures, with hydrophobic and lipophilic characteristics, which facilitate the interaction with the elements of the fungal cell membrane. This was explained by Kedia et al. [43] who described that the essential oils cross the cell wall and the cytoplasmic membrane, interfere with the structure of the different layers of polysaccharides, fatty acids, and phospholipids, and interact with the enzymes and membrane proteins responsible for the biosynthesis of ergosterol to produce a flow of protons to the exterior of the cell. Concurrently, the rupture of the membrane occurs and causes an imbalance in permeability that reflects a leakage of the cellular content and results in the death of the fungus. Recently, Magalhães et al. [14] stated that membrane permeability and fungal mycelial integrity were altered by the essential oil and thus caused the leakage of cellular components.

Results also revealed that nanoemulsion showed lower antifungal activity compared to the essential oil. Similar results were reported by Mossa et al. [44] who indicated that *Citrus sinensis* L. essential oils and their nanoemulsions ranged from no effect to encourage mycelial linear growth of *Alternaria*

tenuissima. In agreement, Ribes et al. [45] described that encapsulation of citrus essential oils in nanoemulsions generally reduces antifungal activity, which may be due to the hydrophobic effect of nanoemulsions on the main components of citrus essential oils. They added that in the case of citrus oil nanoemulsions, the limited driving force to release the oil into the aqueous phase reduces the resulting antibacterial activity. However, although the initial inactivation rate of encapsulated essential oil is lower than that of free essential oil, Majeed et al. [46] reported that emulsions and nanoemulsions can significantly extend the antibacterial activity by ensuring sustained release for prolonged periods.

Table 3: Antifungal activity *of Citrus sinensis* L. peel essential oil and nanoemulsion

Fungi	Essential oil	Nanoemulsion
	Zone inhibition (mm)	
Aspergillus flavus	ND	17.5±0.71
Aspergillus niger	11.5±0.71	10.5±2.12
Aspergillus ochraceus	23.0±0.00	11.0±0.00
Fusarium spp.	22.0±0.00	ND
Penicillium spp.	18.0±2.83	ND

Results are mean ± SD
Within each column, means showed no significant difference $P > 0.05$.
Within each raw; means showed no significant differences $P>0.05$.

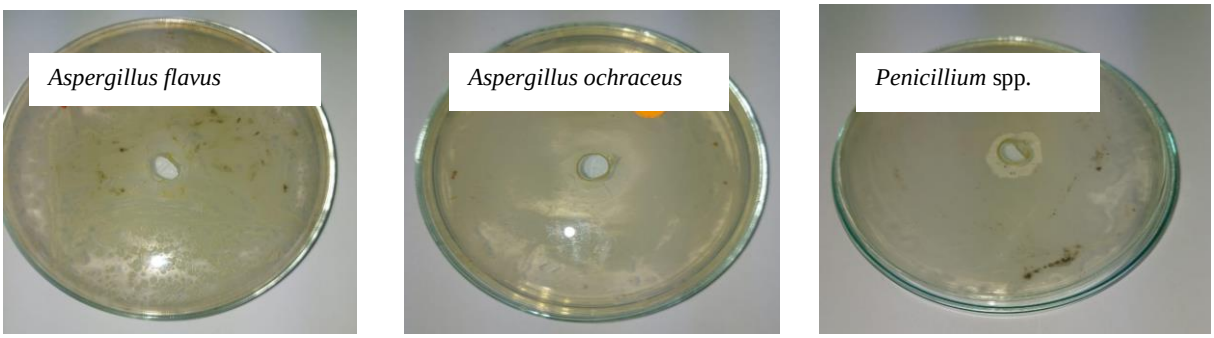

Figure 4: Zone Inhibition of the *Citrus sinensis* L. essential oil against fungi

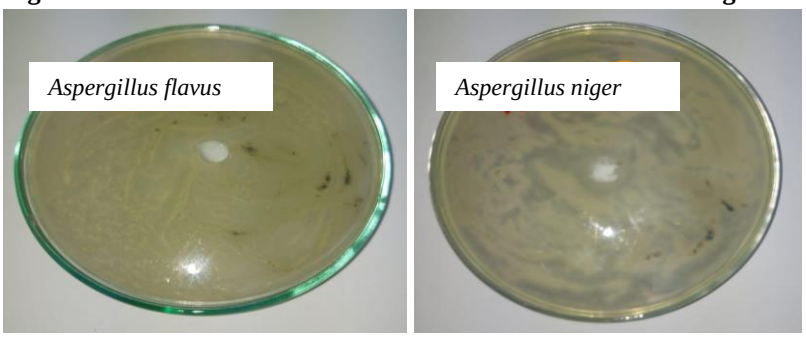

Figure 5: Zone Inhibition of the nanoemulsion against fungi

4. Conclusion

The use of high-intensity ultrasound homogenization to load the *Citrus sinensis* L. oil in nanosystems has affected the volatile constituents of the oil and its nanoemulsions and led to a difference in tested antifungal activity. Non-oxygenated terpenes were affected dramatically by such homogenization technique, while the sun-dried method is negatively affected the potential volatile constituents of the *Citrus sinensis* L. Further studies are necessary to explain the behavior of chemical constituents during different encapsulation processes despite the physical stability proved in the nanoemulsion of the current study.

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