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Exploring the biological activities of the *Origanum majorana* essential oil nanoemulsion: antimicrobial, antibiofilm, antioxidant and anticancer activities

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The current study was aimed at preparing a nanoemulsion of the essential oil (EO) from *Origanum majorana* (*O. majorana*) and examining its different biological activities. Based on gas chromatography-mass spectrometry (GC-MS), the biologically effective ingredients in *Origanum majorana* essential oil (OEO) were identified. Additionally, transmission electron microscope (TEM) imaging was used to determine the shape and size of the prepared OEO nanoemulsion (ONE). The antimicrobial activities for OEO, ONE, and their combination were also assessed for numerous standard strains of pathogenic microbes. Furthermore, the capabilities of OEO, ONE, and their combination to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) were employed for a comparative evaluation of their antioxidant potential. Moreover, the potential cytotoxic effects of ONE on the Wi-38 normal line as well as its anticancer activity towards the Pc-3 and HepG-2 cancerous cell lines were investigated following the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Analysis of the TEM images also showed droplets with a spherical shape, a constant size of 49.2–60.6 nm, and a calculated polydispersity index (PDI) of 0.183. Moreover, our findings illustrated the inhibitory effect of OEO, ONE, and their combination against *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) biofilm formation. Upon completion of this research, we obtained biologically safe ONE with a characteristic tiny particle size and valuable antimicrobial, antibiofilm, as well as antioxidant activities.

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1. Introduction

Essential oil degradation is mostly caused by environmental variables, including their exposure to temperature, oxygen, and light. Based on nanotechnology approaches, nanoemulsification may retain the EOs' stability throughout their different applications.[1] The instability, limited water miscibility, and high volatility of EOs are associated with their difficult handling, subsequently requiring their direct integration within various commercial applications to retain their effectiveness.[2–6] As a result, the preparation of a nanoemulsion represents the most convenient way for the organization of an EO transport system for a variety of applications. These strategies also represent an appropriate tool that can boost the probability of EOs' biologically active constituents reaching the target areas.[7,8] Additionally, the successful preparation of the EO nanoemulsions essentially requires the presence of an emulsifying agent, energy as well as water and oils.[9] Emulsifiers play a significant role in the emulsification process by bringing the dissimilar aqueous and oil phases in direct contact *via* the hydrophilic and lipophilic localities, respectively.[10] Many emulsification methods can be used for the creation of nanoemulsions, including water-in-oil (W/O), oil-in-water (O/W), and multiple emulsions, such as oil-in-water-in-oil (O/W/O) and water-in oil-water (W/O/W).[11–13] Several techniques can be used for the creation of EO nanoemulsions including high-energy procedures such as high pressure, microfluidization, or sonication, as well as low-energy procedures such as the emulsion inversion point, phase inversion, and spontaneous emulsion.[14] Droplets of less than 100 nm size are described as nanoemulsion systems with thermodynamic non-equilibrium.[15]

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Although these systems are characterized by kinetic stability, there are diverse colloidal dispersions and one-phase droplets in an immiscible unceasing system enclosed with non-ionic surface active agents film of less than 100 nm particles.[16] Additionally, several lipophilic compounds, including antioxidant and antimicrobial agents, can be delivered using a nanoemulsion system in pharmaceutical, cosmetic, and food manufacturing.[17,18] The selection of a suitable nanoemulsion formulation is mainly correlated with their improvement of the solubility of oils and other water-immiscible compounds.[19] The utilization of non-ionic surface active agents for plant EO nanoemulsion formulation resulted in products with great stability and ecofriendly qualities.[20] The surface active agent stabilization of the nanoemulsion system was achieved by decreasing the tension that occurred at the water and oil droplet interface.[21]

The essential oil from *O. majorana* is classified as one the most globally popular EOs. These oils are obtained from *O. majorana* L. and are mostly composed of thymol and carvacrol.[22] Such structure gives OEOs the characteristic antioxidant, anticancer, as well as antibacterial activities.[23] Because of these bioactivities, the OEOs and its constituents have been extensively studied for its utilization in active packaging, food preservation, and the control of numerous illnesses, including infectious diseases.[24–28] The current study aims to prepare ONE and evaluate its biological activities, including antimicrobial effects against pathogenic strains, antioxidant potential *via* DPPH scavenging, and cytotoxicity on normal and cancerous cell lines by MTT assay. This study also aims to identify the active components present in the EO using GC-MS and TEM imaging to characterize the size and morphology of the nanoemulsion, ultimately demonstrating ONE's efficacy as a biologically safe agent with valuable therapeutic possessions.

2. Materials and methods

2.1. Essential oil analysis based on GC-MS

The active ingredients in OEO and their makeup were recognized by employing GC-MS analysis in accordance with previously published protocols.[29] The OEO sample was injected into a capillary column (30 m × 0.25 mm × 0.25 μm) using helium (He) as a carrier gas at 1 mL min^{-1} steady rate of flow. Following 1 min at 40 °C holding stage, the temperature was set to climb to 220 °C over the next 25 min at a rate of 3 °C min^{-1}. Finally, the temperature was retained at 250 °C for 10 min, the temperature of the ion source was kept at 230 °C, and the MS conditions were adjusted at 70 eV EI. Additionally, the atomic unit range of 35–350 was selected for the mass charge. Based on the retention durations as well as the eluted molecules mass spectra that were linked with a spectral library specific to known standard compounds, each target molecule was identified.

2.2. *Origanum majorana* oil-based nanoemulsion preparation

The OEO (10 mL) was mixed with distilled water (250 mL) and consequently centrifuged for 10 min at 5000 rpm at room temperature using a high-speed homogenizer (F6/10, Jingxin, Shanghai, China). At this time, the gentle addition of 20 mL Tween 80 was performed for the creation of a homogeneous emulsion.[30] The ultrasonicator (JY92-11D, Jingxin, Shanghai) was then used for 25 min to sonicate the obtained crude emulsion by providing strong disruptive forces, which reduced the size of the nanoemulsion droplets.[31]

2.3. Characterization of the prepared ONE

2.3.1 Dynamic light scattering (DLS). The ONE average particle size and the PDI of the nanodroplets were determined *via* DLS examination employing Zetasizer Ver. 7.12/MAL1029409 (Malvern Instruments Ltd). To reduce scattering effects, dilution of the tested ONE samples was conducted using distilled water and the experiment was performed in triplicate. Calculation of the *z*-average size was dependent on the distribution value intensity.[32]

2.3.2 Transmission electron microscope. The obtained ONE droplet size and morphology were determined using TEM. Distilled water was used for ONE dilution with subsequent filtration for exclusion of the inter-particle aggregation. The tested samples were then transferred onto copper grids, covered using carbon at room temperature for 1 min, and examined under TEM.[33]

2.4. Antimicrobial activity

The microbial growth inhibitory properties of OEO and ONE were evaluated based on agar diffusion.[34] The pathogenic bacterial strains were Gram-positive species [*S. aureus* ATCC 6538] and *Bacillus subtilis* (*B. subtilis* ATCC 6633) and Gram-negative species [*P. aeruginosa* ATCC 9027 and *Escherichia coli* (*E. coli*) ATCC 11229]. Eukaryotic unicellular fungi *Candida albicans* (*C. albicans* ATCC 10231) and multicellular fungi *Aspergillus brasiliensis* (*A. brasiliensis* ATCC 16404) were also examined. Initially, each tested bacterial, yeast, and *Aspergillus* strain growth was renewed *via* cultivation on nutritional agar and Capek's Dox agar plates, respectively, followed by incubation at 35 ± 2 °C for 24 h in the case of bacteria and yeast and at 28 °C for 72 h in the case of *A. brasiliensis*. Each freshly grown strain was then transferred to a Mueller-Hinton agar plate in the case of bacteria and yeast, or Capek's Dox agar for the tested *Aspergillus* strain. Three wells, each with 0.6 mm diameter, were then made on the agar surface using a sterile cork borer. Then, 100 μL from either OEOs, ONE, or its 1 : 1 mixture was added to the separate wells, followed by 1 h refrigeration. Consequently, the inoculated plates were incubated at 35 ± 2 °C for 24 h in the case of bacteria and yeast, and at 28 °C for 72 h in the case of *A. brasiliensis*. Each experiment was conducted in triplicate, and the clear zone formed around each well was considered as an initial inhibitory effect and its diameter was recorded.

Furthermore, microtiter 96-well polystyrene plates (SPL, Korea) containing tryptic soy broth in the case of fungi or Mueller–Hinton broth in the case of bacteria were utilized for evaluation of the minimum inhibitory concentration (MIC) for OEO, ONE, as well as their combination.[35] Various quantities of OEO, ONE, and their combination were added to each tested

well containing suitable broth and tested microbial suspensions. An overnight microbial culture dilution was then used to inoculate the plate, followed by incubation under the previously described conditions suitable for bacteria and yeast and *A. brasiliensis*. Lastly, the MIC of OEO, ONE, or their combination was recorded as its lowest concentration capable of preventing the growth of the tested bacterial or fungal strains. Duplicated experiments were carried out and a medium devoid of any tested emulsions was used as a control.[36] Data analysis was conducted following the established Clinical Laboratory Standard Institute (CLSI) procedures.[34]

2.5. Antioxidant activity

Comparative evaluation of the OEO and ONE capabilities for free-radical scavenging was conducted *via* employment of the DPPH assay. In this experiment, 1 mL of either methanolic DPPH solution, OEO, or ONE at various concentrations ranging from 0.78 to 200 μg mL^{-1} was prepared in test tubes. Once the mixture was thoroughly combined, incubation was conducted at 37 °C for 30 min in the dark with vigorous mixing at 100 rpm. Furthermore, an ascorbic acid (AA) antioxidant standard was included, with the same procedure and concentration gradients, as a positive control. An untreated negative control experiment was included. The absorbance of the produced color, at the end of incubation, was determined at 517 nm. Finally, the percentages of free radical scavenging were calculated using the following formula.[37]

$$\text{DPPH scavenging}\% = 1 - \frac{\text{absorbance of treatment}}{\text{absorbance of control}} \times 100$$

2.6. Antibiofilm

For evaluation of microbial biofilm inhibition by OEO and ONE, the tested bacterial strains were inoculated into tryptic soya broth containing glucose (1% w/v), followed by incubation for 24 h at 37 °C. The obtained microbial growth was then diluted and adjusted to a concentration equivalent to 0.5 McFarland standard turbidity. The wells of the flat-bottom microtiter plate (SPL, Korea) were filled using 200 μL of tryptic soya broth containing glucose (1% w/v), followed by the addition of the tested bacterial inoculum. The OEO/ONE at sub-inhibitory dosages were then applied to the plate with subsequent incubation for 24 h at 37 °C. After complete removal of the well contents, cleaning was conducted twice using phosphate buffered saline (PBS, pH 7.4). Subsequently, 200 μL of crystal violet (CV) solution (0.1% w/v) was applied at 37 °C for 20 min to stain the adhered biofilm. Each plate was rinsed twice using PBS after discarding the extra CV and allowed for drying at room temperature. The adhered dye was then solubilized using 30% acetic acid (200 μL). For quantification of the microbial biofilm suppression, a microplate reader (Statfax, USA) was used to measure the optical density (OD) at 492 nm.[35,38,39] Each experiment was conducted in triplicate, followed by calculating the percentage inhibition according to the following formula:

$$\%\ \text{inhibition} = 1 - \frac{\text{OD of treatment}}{\text{OD of control}} \times 100$$

2.7. Cytotoxicity

The potential cytotoxicity effect of ONE on the Wi-38 cell line, normal Lung fibroblast cells, as well as its anticancer activity

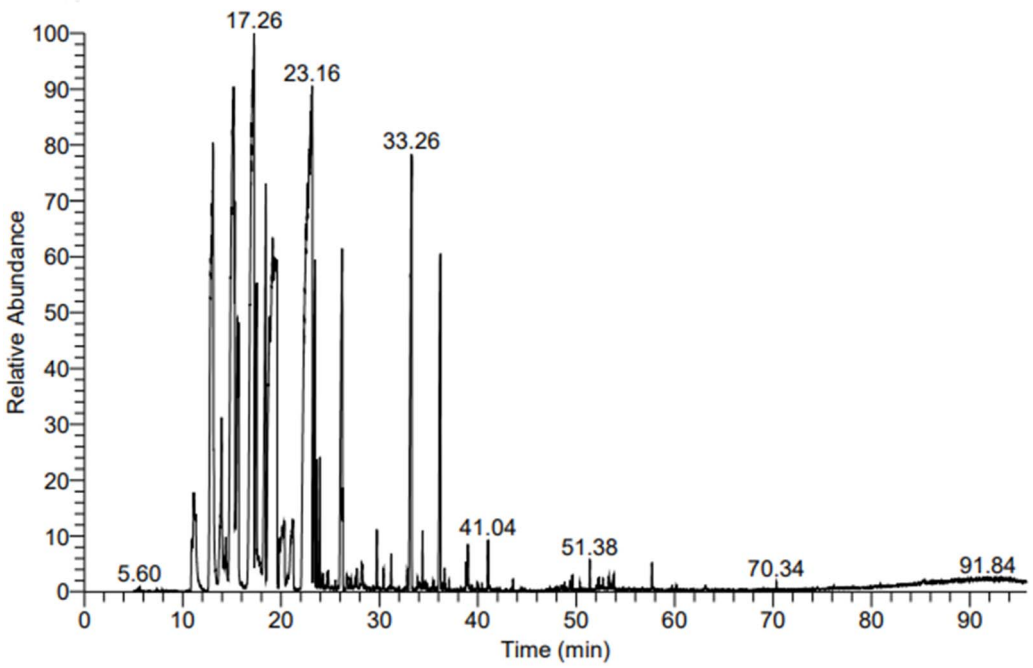

Fig. 1 The typical GC-MS chromatogram of the OEO.

towards Pc-3 (prostate cancer) and HepG-2 (liver cancer) cell lines, were investigated by MTT colorimetric assay. The cell lines were exposed to either 125, 62.5, or 31.25 μg mL^{-1} ONE in triplicate procedures. Briefly, the diluted samples were prepared using RPMI medium +2% serum and every dilution (0.1 mL) was added to different wells. Cell suspension wells containing no ONE were included as a negative control. The MTT solution (20 μL) was consequently added to each tested well, followed by plate incubation for 4 h at 37 °C in the presence of 5% CO_2. The OD was then determined at 560 nm to calculate the tested cells viability.[40]

3. Results and discussion

3.1. Chemical constituents of OEO

The indispensable analysis of EOs was conducted using GC-MS, as it precisely identifies their chemical constituents. Additionally, it verifies the authenticity, confirms compliance with safety standards, and supports regulatory requirements by providing a comprehensive chemical profile.[41] The GC-MS analysis results, represented graphically in Fig. 1, show the identified OEO volatile contents, which include more than 20 compounds. The findings revealed that the terpinene derivatives were the dominant component in the GC MS profile of OEO, where the

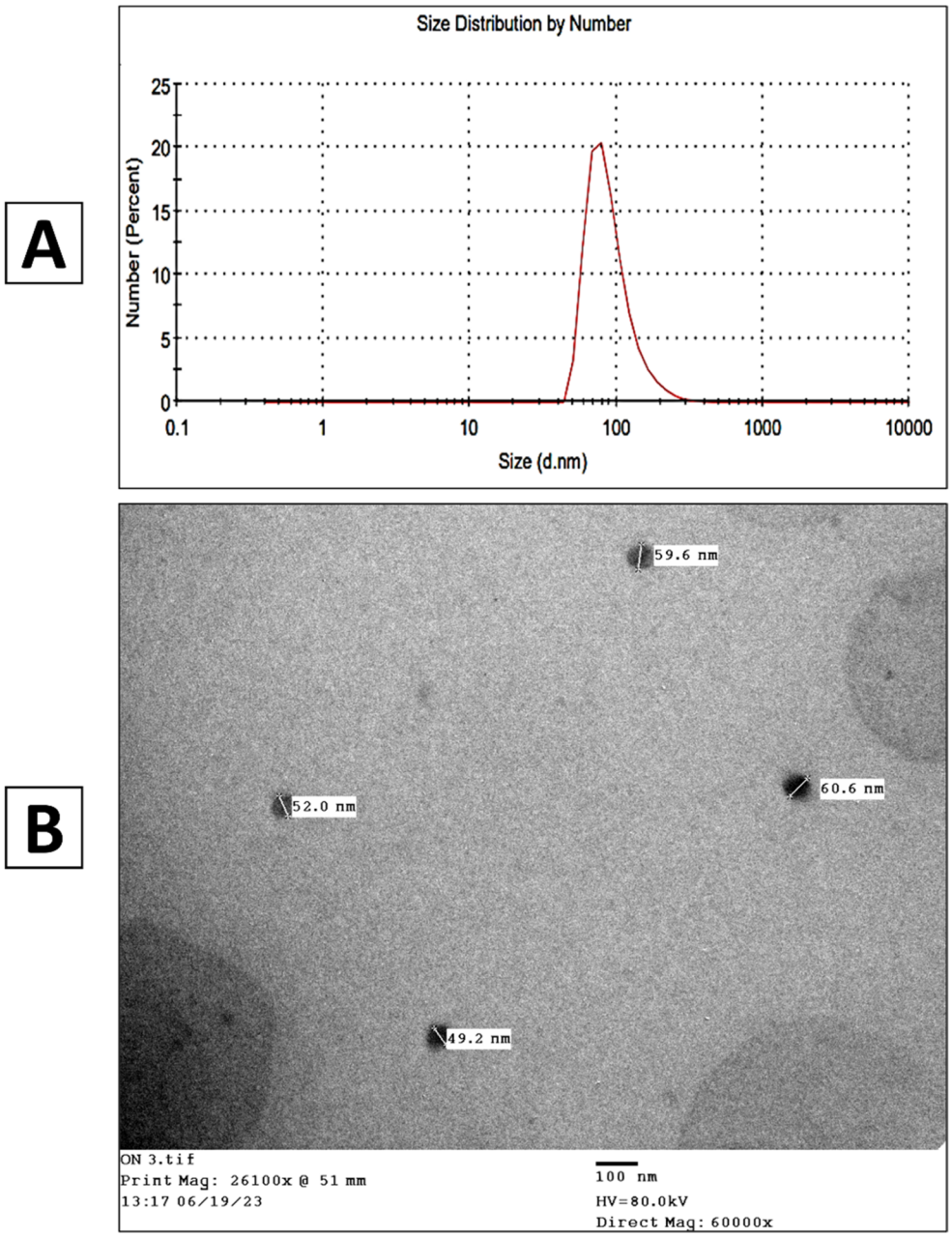

Fig. 2 The ultrasonication-prepared ONE showing the particle size distribution with the peak at 93 nm and PDI = 0.183 (A) and the TEM image of a 30 min sonication with the size range of 49.2–60.06 nm (B).

percentages of γ-terpinene, α-terpinene, terpinene 4-ol, terpinene 3-ol were 21.24%, 17.54%, 10.34% and 1.10%, respectively. Many other compounds also appeared, such as *p*-cymene, *o*-cymene, a-myrcene, pinene, elemene, humulene, caryophyllene, bicycloelemene, 1,6-octadien-3-ol, 3,7-dimethyl-, acetate, and D-limonene.

Monoterpenes, as well as sesquiterpenes, are the primary terpenes in OEOs.[42] The γ-terpinene, carvacrol, thymol, sabinene, sabinene hydrate, *p*-cymene, linalool, and β-ocimene, and 1,8-cineole represent the monoterpenes.[42,43] Sesquiterpenes comprise compounds like germacrene D, β-caryophyllene, caryophyllene oxide, and β-bisabolene.[44] Carvacrol and thymol, the main terpenes present in most European oregano varieties, are isomers known for their diverse biological activities. These compounds possess numerous useful effects, including antispasmodic, anticancer, antioxidant, insect-repellent, antiseptic, antibacterial, and antiviral activities.[45–48] β-Caryophyllene has an appreciated local anesthetic and is also valuable activity in various medical applications.[49] Germacrene D is also a significant flavor compound with applications as an insect attractant, insecticide, and antimicrobial agent.[50]

3.2. Droplet size and TEM imaging of ONE

The DLS findings (Fig. 2A) and low PDI value (0.183) of the ONE are confirmed by their TEM images that clarified the watery exterior phase with the internal uniformly dispersed W/O droplets. Additionally, the TEM images revealed the droplet size and the shape of the w/o/w nanoemulsion. The carefully investigated TEM picture (Fig. 2B) illustrates the constant size of the obtained droplet spanning from 49.2 to 60.06 nm with spherical shape. Significantly, the TEM observations, regarding the droplet particle sizes, were in close accordance with the DLS findings. These observations presented details about the nanoemulsion's droplet structural and stability characters, and emphasized the consequent w/o/w potential for a variety of nanotechnology applications.

3.3. Antimicrobial activity

The microbial inhibitory effects of OEO, ONE, and a mixture of them were analyzed *via* agar diffusion assay, illustrated their suppressing activity against the development of numerous pathogens. The findings verified an uppermost activity for ONE with inhibition zones of 27.17 ± 1.60, 27.0 ± 1.7, 23.0 ± 01.7, 21.67 ± 1.52, 29.67 ± 2.08, and 29.3 ± 1.53 nm for *S. aureus*, *B. subtilis*, *P. aeruginosa*, *E. coli*, *C. albicans*, and *A. brasiliensis*, respectively (Fig. 3). Regarding the OEO, a lower inhibitory effect was observed with decreased transparent zones diameter. The measured zones were 19.23 ± 1.62, 22.0 ± 2.64, 21.66 ± 0.5, 15.8 ± 3.3, 19.66 ± 2.08, and 16.13 ± 2.5 nm for *S. aureus*, *B. subtilis*, *P. aeruginosa*, *E. coli*, *C. albicans*, and *A. brasiliensis*, respectively.

Generally, EOS microbial inhibitory activity involves phenolic constituents, which interact with microbial cytoplasmic membrane porins. This interaction associated with ion and other intracellular constituents lead to leakage, eventually causing cell collapse.[51] The palmarosa, thyme, clove, or lemongrass EO-containing nanoemulsions showed elevated antimicrobial effects.[18] Thyme and other EOs containing thymol, as well as carvacrol terpenes, illustrated strong bacterial growth suppression activity.[52] Additionally, eugenol and citral which represent the main active constituents in clove, lemongrass, and rosewood EOs showed improved capability for wide range microbial inactivation.[53] Thus, the particular EOs

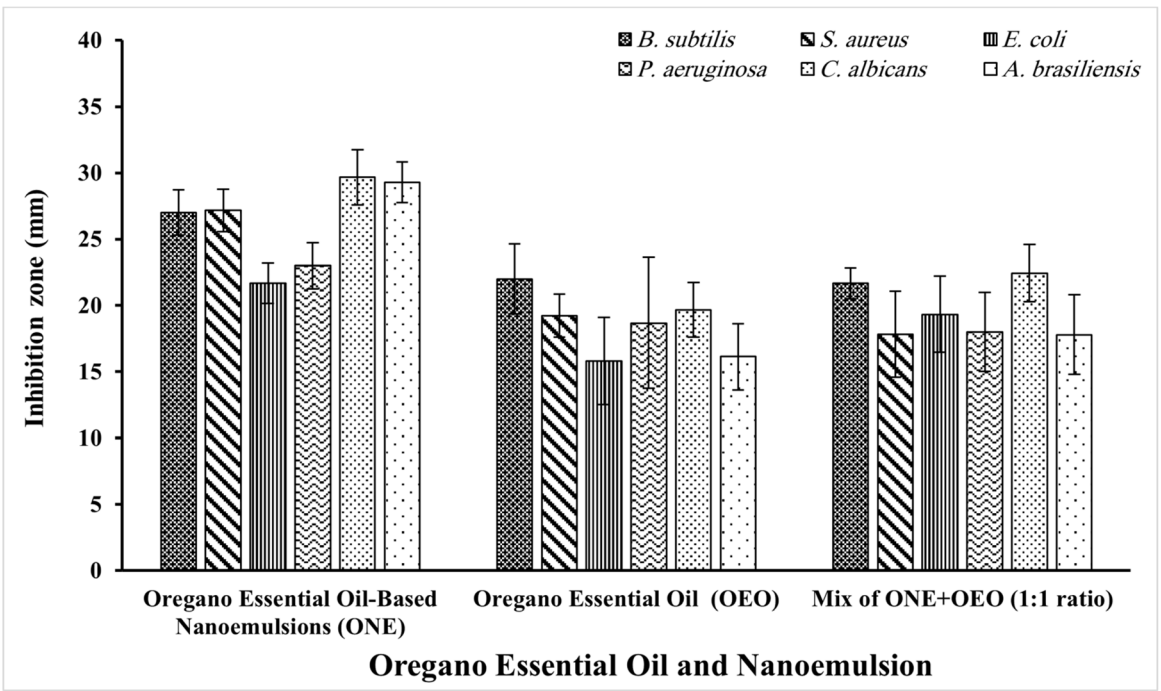

Fig. 3 Microbial inhibitory effect of the ONE, OEO, and its combination against several pathogens.

antibacterial effect controlled by their volatile molecule's composition and each molecule's concentration that controlled by the plant type, environmental growth condition, as well as the EO extraction procedures. In this survey, the elevated and robust nanoemulsions inactivation ability against all tested pathogenic strains demonstrated its considerable influence on EOs bacterial inhibitory effect.

The enhanced bacterial destructive activity of lipophilic antimicrobial agents in nanoemulsions is correlated to its prompt ability to cross the bacterial membranes. Porins, *P. aeruginosa* and *E. coli* and other Gram-negative bacterial outer membrane proteins form pores or channels which permit the entering of hydrophilic particles into the cells. Depending on the lipophilic molecular size, these porins act as an obstacle which limit their passage into microbial cells.[54] Consequently, it

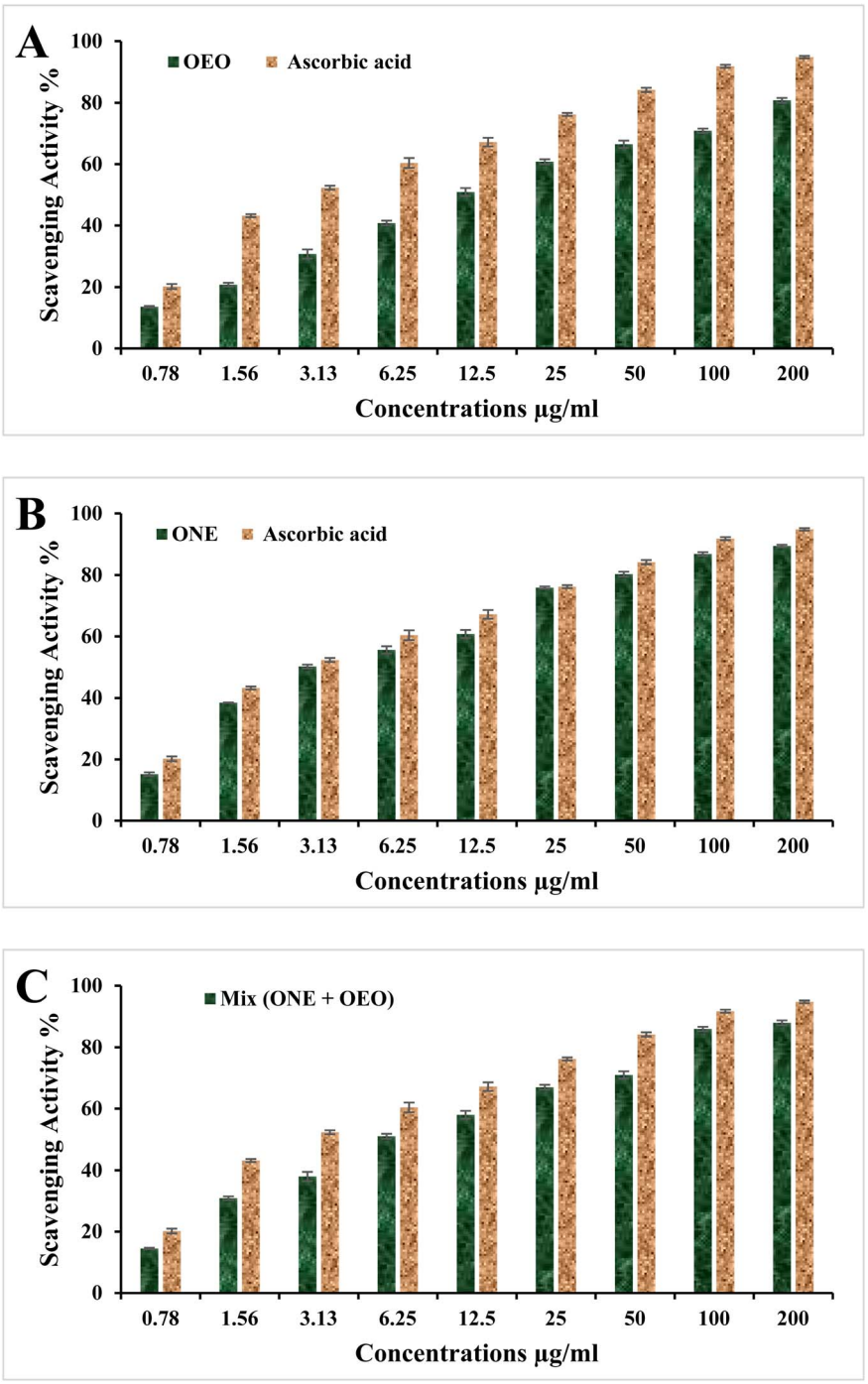

Fig. 4 Antioxidant behavior of the OEO (A), ONE (B), as well as OEO plus ONE OEO mixture (C) in comparison with the ascorbic acid positive control.

makes sense to believe that lipophilic antimicrobial agents solubilization in nanoemulsions will increase the binding sites number and enhance the interactions of their porins. Recently, limonene-containing nanoemulsions demonstrated an enhanced antibacterial activity, which is consistent with our findings.[55]

3.4. Antioxidant activity

The effectiveness of ONE, OEO, as well as their combination for free radicals scavenging was obvious after the assessment following DPPH method (Fig. 4). The free radical scavenging activity of ONE, OEO, and their combination was concentration-dependent with increased effect at the higher applied concentrations.[56] In this experiment, 200 μg mL^{-1} of OEO revealed the highest scavenging percentages (80.8% ± 0.8%). However, statistically significant difference ($P \leq 0.005$) was recorded in correlation with ascorbic acid that exerts 94.8% ± 0.4% scavenging action (Fig. 4A). At a concentration equal to 100 μg mL^{-1} OEO, a scavenging rate of 70.9% ± 0.6% was observed with a statistically significant difference ($P \leq 0.005$) between ascorbic acid which record rate of 91.8% ± 0.5%. Also, the other lower OEO concentrations, 0.78 μg mL^{-1} and 1.56 μg mL^{-1}, showed lower scavenging activities of 13.6% ± 0.23% and 20.8% ± 0.7% in comparing with the same ascorbic acid concentration scavenging percentages, 20.2% ± 0.8% and 43.2% ± 0.5%, respectively (Fig. 4A).

A relatively similar free radical scavenging pattern was observed for ONE. The ONE concentration of 200 μg mL^{-1} revealed the highest scavenging percentage (89.5% ± 0.5%). However, a statistically significant difference ($P \leq 0.001$) was recorded in comparison with ascorbic acid with a scavenging percentage of 94.8% ± 0.4% (Fig. 4B). Additionally, 100 μg mL^{-1} of ONE or ascorbic acid had a significantly different effect, where the reported scavenging rates were 86.9% ± 0.7% and 91.8% ± 0.5%, respectively. Furthermore, ONE at 0.78 μg mL^{-1} and 1.56 μg mL^{-1} displayed the lowest scavenging activity, 15.2% ± 0.7% and 38.5% ± 0.2%, in comparing with the effects of ascorbic acid, 20.2% ± 0.8% and 43.2% ± 0.5%, at equal concentrations, respectively (Fig. 4B).

However, 200 μg mL^{-1} of the mixture formed from combining OEO and ONE showed the maximum antioxidant effect, with scavenging rates of 88% ± 0.8%, according to the DPPH assay. Furthermore, 100 μg mL^{-1} of the mixture from OEO and ONE was compared with ascorbic acid, revealing a comparable free radical scavenging pattern, and the scavenging percentages were 86% ± 0.6% and 91.8% ± 0.5%, respectively. The lowest scavenging amounts, 14.5% ± 0.25% and 30.9% ± 0.8%, were seen with the tested mixture at 0.78 μg mL^{-1} and 1.56 μg mL^{-1}, respectively. Ascorbic acid at equal concentrations exhibited scavenging activities of 20.2% ± 0.8% and 43.2% ± 0.5%, respectively (Fig. 4C).

These novel compounds with free radical scavenging activity represent a great interest for health and therapeutic industries. Free radicals are associated with several illnesses including heart disease, neurological syndromes, and malignancy.[57] Subsequently, the introduction of new compounds with antioxidant characteristics creates novel approaches for the prevention and treatment of chronic disorders. Several NPs provide promising free radical scavenging activities due to their unique physical and chemical behaviors, such as the tiny size and elevated surface area-to-volume ratio. These characteristics are associated with enhanced activity, facilitating interactions

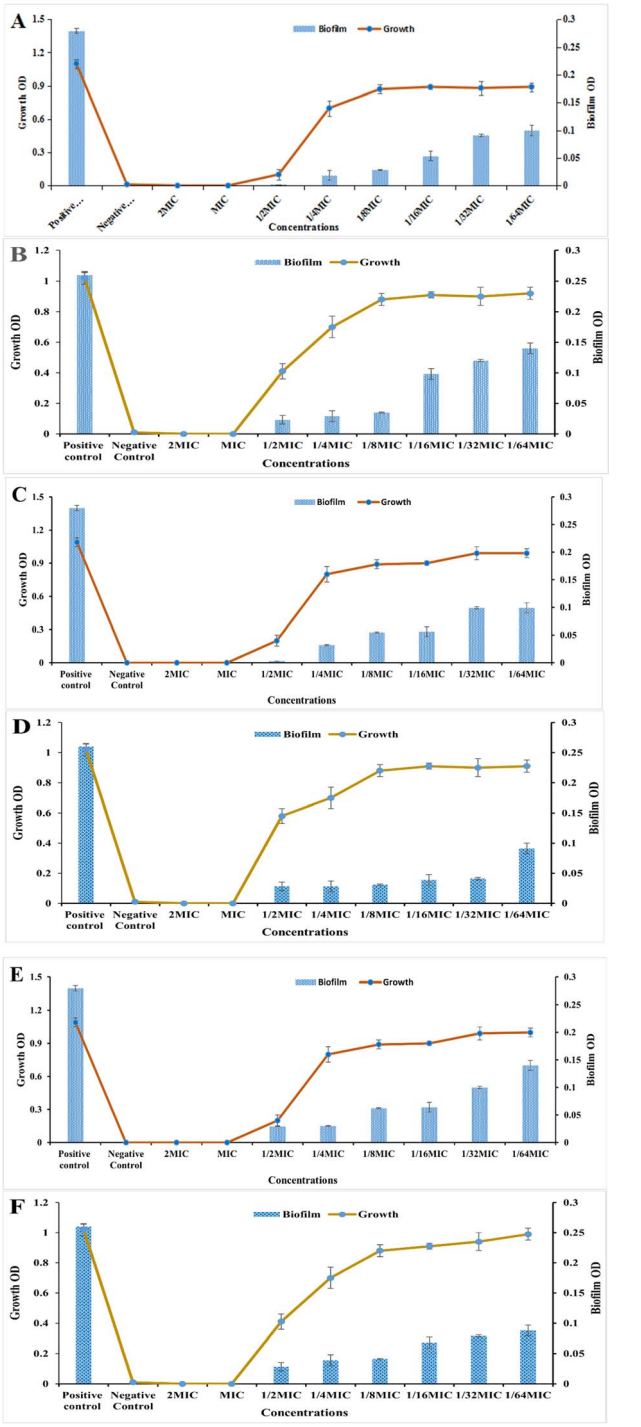

Fig. 5 Antibiofilm activity of the OEO against *S. aureus* (A) and *P. aeruginosa* (B). Antibiofilm activity of the ONE towards *S. aureus* (C) and *P. aeruginosa* (D). Antibiofilm activity of the ONE and OEO mixtures against *S. aureus* (E) and *P. aeruginosa* (F).

within the nanostructure biological systems and free radical neutralization.[58]

3.5. Antibiofilm activity

Upon testing the ONE, OEO, and their combination at sub-MIC, they demonstrated the concentration-dependent prevention of bacterial biofilm construction by strong biofilm-producing *S. aureus* and *P. aeruginosa*. The results shown in Fig. 5 demonstrate the OEO biofilm inhibitory effect on *S. aureus* and *P. aeruginosa* strains with no effect on the bacterial planktonic growth form. At 25, 12.5, 6.25, 3.125, and 1.56 μg mL^{-1}, which is equivalent to OEO 1/4, 1/8, 1/16, 1/32, and 1/64 MIC against the tested *S. aureus* strain, the biofilm development was diminished by 93.21%, 89.64%, 80.71%, 67.5%, and 64.28%, respectively (Fig. 5A). While OEO at 50, 25, 12.5, 6.25, 3.125, and 1.56 μg mL^{-1}, which is equivalent to its 1/2, 1/4, 1/8, 1/16, and 1/32 of MIC towards the *P. aeruginosa* strain, respectively. The bacterial biofilm construction was inhibited at a rate of 91.15%, 88.84%, 86.53%, 62.30%, 53.84%, and 46.15%, respectively (Fig. 5B).

Similarly, the results represented graphically in Fig. 5C and D prove the powerful biofilm inhibiting effect of ONE on *S. aureus* and *P. aeruginosa* tested pathogens. The ONE concentrations of 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 μg mL^{-1}, representing its 1/2,1/4, 1/8, 1/16, 1/32, and 1/64 MIC against *S. aureus*, inhibit its biofilm formation by 98.92%, 88.57%, 80.35%, 80.0%, 64.65%, and 64.65%, respectively (Fig. 5C). The concentrations of 6.25, 3.125, 1.56, 0.78, 0.39, and 0.195 μg mL^{-1}, equivalent to ONE 1/2, 1/4, 1/8, 1/16, and 1/32 of its MIC towards *P. aeruginosa*, respectively, inhibit bacterial biofilm development by 89.23%, 89.23%, 88.07%, 85%, 84.25%, and 65.80%, respectively (Fig. 5D).

The graphically presented results (Fig. 5E and F) also reveal the biofilm inhibitory activity of the ONE and OEO mixture towards the tested strains of *S. aureus* and *P. aeruginosa*. In the case of 25, 12.5, 6.25, 3.125, 1.56, and 0.78 μg mL^{-1} of the mixture (equivalent to 1/2,1/4, 1/8, 1/16, 1/32, and 1/64 MIC, respectively), the biofilm formation by the tested *S. aureus* strain was inhibited by 89.21%, 89.80%, 78.85%, 79.5%, 65.28%, and 52%, respectively (Fig. 5E). Furthermore, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 μg mL^{-1} (equal to the 1/2, 1/4, 1/8, 1/16, and 1/32, respectively) MIC of the mixture against the tested *P. aeruginosa* strain lowered the biofilm development by 90.23%, 86%, 84.23%, 73.84%, 69.61%, and 66.15%, respectively (Fig. 5F).

Anti-virulent agents are among the various effective approaches for combating antimicrobial resistance phenomenon. These agents act by modifying the focus from microbial survival (which is what drives the development of resistance genes in bacteria) to the microbial virulence factors, which are thought of as the agents' weapons that cause the pathogenicity to the host.[59] In general, these observations confirmed the inhibition of bacterial biofilm formation by ONE, OEO, as well as their mixture. However, the suppressive effect caused by OEO in a nanoemulsified form was notably greater than either the OEOs or ONE/OEO combination. Microbial biofilm development serves a key function in pathogen nullification of antibacterial agents' activity. The prevention of bacterial adhesion, the 1st first step in biofilm formation, represents the key for biofilm inhibition.[60] The EOs nanoemulsion's antibiofilm activities may be correlated to its capability to prevent bacterial attachment onto the surfaces. This inhibiting capacity represents the 1st stage towards the reduction of microbial pathogenesis.[33] The tested EOs, particularly its nanoemulsified form, exhibited substantial reduction in microbial biofilm development. Consequently, they could be vastly anticipated in food manufacturing.[61] As a result, EOs nanoemulsion forms used in food industries could represent an appropriate alternative that help in controlling infections caused by food-associated microorganisms.

3.6. Cytotoxicity

The MTT colorimetric assay was used for evaluation of the potential *in vitro* antiproliferative effect of nanoemulsions containing bioactive materials employing normal as well as cancer cell lines. The results showed similar viability between the nanoemulsions-treated cell lines and that in the untreated

Table 1 Inhibitory percentages of OEO and ONE concentrations for normal and tumor cell lines

		Inhibitory percentages		
Sample	Concentration (μg ml^{-1})	Wi-38	Pc-3	HepG-2
OEO	1000	97.81	97.02	97.24
	500	90.66	96.84	97.24
	250	78.56	93.00	96.94
	125	41.33	89.02	80.50
	62.5	2.56	54.70	48.47
	31.25	0.00	0.95	0.55
	IC50 ± SD	168.38 ± 1.2	74.74 ± 0.45	80.67 ± 1.75
ONE	1000	97.52	97.24	97.29
	500	89.89	96.75	97.24
	250	67.11	92.10	97.34
	125	35.77	87.44	67.72
	62.5	0.00	46.70	20.15
	31.25	0.08	1.67	1.70
	IC50 ± SD	189.38 ± 3.32	78.06 ± 0.42	101.08 ± 0.81

negative control experiment, indicating their non-toxic effect and consequent biosafety for various applications. The MTT technique is based on the enzyme efficiency of cellular oxidoreductase to convert the soluble purple colored MTT tetrazolium dye into formazan metabolite insoluble.[62] The viability of the tested cells increased with their growth, decreased with antiproliferative treatments, and remain unaffected upon cytostatic exposure. So, the percentage of cell viability is used for estimating based on the complete vitality of healthy cells in control sample.

The percentages of inhibition caused by OEO and ONE against all tumor and normal cells at six different treatments ranged from 1000 to 31.25 μg mL^{-1} showed a concentration dependent effect (Table 1). Using 125 μg mL^{-1} of OEO shown strong cytotoxic efficacy against the tested Wi-38, Pc-3, as well as HepG-2 cell lines, with inhibition percentage of 41.33%, 89.02%, and 80.50%, respectively. The cell growth percentages were charted against the ONE concentration to create the curves. Consequently, the findings illustrated that the decreased concentration and IC50 values were associated with increased cytotoxic activity. For the Wi-38, Pc-3, and HepG-2 cell lines, the ONE's IC50 values were 189.38 ± 3.32, 78.06 ± 0.42, and 101.08 ± 0.8 1 μg mL^{-1}, respectively. For a comparative study of the outcomes, the IC50 of OEO was 168.38 ± 1.2, 74.74 ± 0.45, and 80.67 ± 1.75 μg mL^{-1} for Wi-38, Pc-3, and HepG-2, respectively.

In a related study, the nanoemulsified clove oil cytotoxic effects against two cell lines, namely, BEAS-2B and L-02, were assessed. Following 24 h exposure to 0.025 mg L^{-1}, more than 90% survival rate was recorded by the exposed cell.[63] The emulsions observed by Kaur *et al.*[64] also recorded no cytotoxicity against the tested cell lines, with a reported viability of >85% at all tested dosages range of 3.125–50 μg mL^{-1}.

4. Conclusions

In our study, ONE was excellently designed, highlighting its significant biological activities and potential industrial applications. GC-MS testing recognized the fundamental terpinene derivatives as the primary bioactive components, confirming the oil's therapeutic potential. The TEM imaging demonstrated uniform spherical droplets with a size range of 49.2 to 60.6 nm, indicating effective nanoemulsion preparation. The antimicrobial assessments revealed that both OEO and ONE exhibit potent inhibitory effects towards various pathogenic microbes, including *S. aureus* and *P. aeruginosa*, with notable inhibition of biofilm formation. Furthermore, the antioxidant capabilities of the nanoemulsion were significantly enhanced, showcasing its potential in combating oxidative stress. Importantly, the cytotoxicity tests indicated that ONE is safe for normal cells, while also demonstrating anticancer effects against Pc-3 and HepG-2 tumor cell lines.

Author contributions

Amr H. Hashem and Salem S. Salem: conceptualization, validation, methodology, data curation, formal analysis, investigation, writing – original draft preparation, and writing – review and editing. Mohamed T. Selim, Nasser Ibrahim Issa, Karim M. Sobhy, Khaled M. Shaban, Ahmed A. Abdallah, and Ali M. Sabeq: methodology, data curation, formal analysis, investigation, writing – original draft preparation, and writing – review and editing. Bahaa M. Badr, Fathy M. Elkady, and Mahmoud M. H. Hassanin: investigation, writing – original draft preparation and funding acquisition. Nosiba S Basher, Sulaiman Abdullah Ali Alsalamah and Fahd A. Nasr: investigation, writing – review and editing and funding acquisition. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

Data availability

The data presented in this study are available on request from the corresponding author.

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