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ORIGINAL ARTICLE

Anticancer, antioxidant, and antibacterial effects of nanoemulsion of *Origanum majorana* essential oil

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ABSTRACT

Background and Objectives: This study aimed to develop a natural nanoemulsion with antibacterial and anticancer properties.
Materials and Methods: The chemical composition of the *Origanum majorana* essential oil was investigated using GC–MS analysis. Besides, the successful loading of the essential oil in the nanoemulsion was confirmed using ATR-FTIR analysis. Moreover, nanoemulsion's anticancer, antioxidant, and antibacterial activities were investigated.
Results: Terpinen-4-o1 (46.90%) was identified as the major compound in the essential oil. The nanoemulsion with a 149 ± 5 nm droplet size and zeta potential of -11 ± 1 mV was prepared. The cytotoxic effect of the nanoemulsion against A-375 human melanoma cells (IC_{50} = 139 µg/mL) showed significantly more potency than A-549 human lung cancer cells (IC_{50} = 318 µg/mL). Interestingly, growth of *Staphylococcus aureus* (Gram-positive) and *E. coli* (Gram-negative) bacteria after treatment with 4800 µg/mL of nanoemulsion were obtained at 12 ± 2 and 6 ± 1%, respectively. However, the IC_{50} value of nanoemulsion against *E. coli* (580 µg/mL) was not significantly different ($P > 0.05$) from *S. aureus* (611 µg/mL).
Conclusion: A straightforward preparation method, high stability, and multi-biological effects are the main advantages of the prepared nanoemulsion. Therefore it could be considered for further investigation *in vivo* studies or complementary medicine.

Keywords: Nanotechnology; Skin neoplasms; Lung neoplasms; Anti-bacterial agents

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INTRODUCTION

Cancers are responsible for one in six deaths in 2020 (~ 10 million). With 2.21 and 1.5 million cases, lung and skin cancer were the second and fifth most common (1). Unfortunately, with increasing access to tobacco and industrialization in developing nations, lung cancer incidence is rising globally, with 1.8 m death in 2020 being the most dreadful cancer (1, 2). Furthermore, skin cancer is categorized into non-melanoma and melanoma (3). Melanoma is the most dreadful type of skin cancer that involves melanocytes and can be found throughout the skin or even in other organs such as the iris and rectum (4). Chemotherapy is the most common approach in cancer treatment; however, drug resistance and their side effects are serious challenges to the health system in the world (5). Moreover, drug resistance and side effects also exist in other health-threatening bioorganisms such as bacteria. *Staphylococcus aureus* and *Escherichia coli* are opportunistic bacteria threatening human health (6). *S. aureus* (gram-positive) is the cause of a wide variety of infections involving skin and soft tissues, endovascular sites, and internal organs (7). Moreover, in healthy and immunocompromised individuals, *E. coli* (gram-negative) causes diarrhea or extraintestinal diseases, stomach pain, nausea, and vomiting (8).

Essential oils (EOs) are natural oils that are secreted as secondary metabolites in many parts (especially bark, fruits, and flowers) of aromatic plants (9). They possess many biological properties, such as antibacterial and anticancer effects (10). Attempts to develop medicine using EOs have received much attention in recent years. For instance, *Origanum majorana L.* (*Lamiaceae* family) in folk medicine is used for cramps, depression, dizziness, gastrointestinal disorders, hay fever, toothache, migraine, nervous headaches, and paroxysmal coughs, and as a diuretic (11). Some important activities of *O. majorana* EO include antibacterial, antioxidant, and antifungal actions and increased liver and kidney function (12). However, despite the promising properties of EOs, their efficiency and stability need to be improved (13). Therefore, preparing nanostructures containing EOs as a promising stability and efficacy improvement approach has recently received much attention (14). Although advanced nanostructures such as liposome, noisome, autosome, and various polymer nanoparticles have been developed recently, nanoemulsions are still among the most suitable formulations (15). Nanoemulsions are a dispersion of oil in water (O/W) or water in oil (W/O) using amphiphilic material (surfactants) where the droplets are on the nanometer scale (16). High stability, high bioavailability, biocompatibility, and biodegradability are important advantages of nanoemulsions. Moreover, nanoemulsions are suitable for use in various ways, such as topical and spray (17).

Low-energy methods, such as spontaneous emulsification, and high-energy methods, such as ultrasonic, are two common nanoemulsions preparation approaches (18). In spontaneous emulsification by establishing a balance between the amount of oil, surfactant, and water, droplets with the desired size (< 200 nm) are formed, but in the next approach, by applying an external force such as ultrasound or a homogenizer, the droplets reach the desired size (18). Spontaneous emulsification is preferred in preparing nanoemulsions containing thermal-sensitive materials such as EO. In this study, the nanoemulsion containing *O. majorana* EO was thus prepared using this manner. Its antioxidant properties and anticancer effects were investigated against A375 (human melanoma cells) and A549 (human lung cancer cells) cell lines. Besides, its antibacterial activity standard strains of *S. aureus* and *E. coli* were investigated.

MATERIALS AND METHODS

A-375 Human melanoma and A-549 lung cancer cell lines (ATCC CRL-1619 and CCL-185) and *S. aureus* and *E. coli* (ATCC 25923 and 25922) supplied by Pasteur Institute of Iran. Bark-extracted *O. majorana* EO was bought from the Pharmaceutical Company Essential Oil Dr. Soleimani, Iran. Muller Hinton broth and tween 80 were purchased from Merck, Chemicals, Germany. Besides, MTT powder (Thiazolyl Blue Tetrazolium Bromide) and RPMI (Roswell Park Memorial Institute) cell culture medium were bought from Sigma-Aldrich (USA) and Shelmax (China), respectively.

Chemical composition of *O. majorana* EO. Gas chromatography/ quadrupole mass spectroscopy (GC-MS) system type Agilent 6890 with HP-1MS silica-fused columns (30 m × 0.25 mm; 0.25 µm film thickness) was used to characterize components of *O. majorana* EO as described in our previous study (19).

Briefly, n-Hexane and pure Helium (99.999%) were used as a diluent for the EO and gas carrier. Programming of the GC-MS column temperature was done from 40 to 250°C at 3ºC/min and remained isothermal for 60 min. The injector temperature was set at 250°C, and the detector was fixed at 230°C. Besides, system parameters, such as split flow (25 mL/min), septum purge (6 mL/min), and column flow rate (1 mL/min), were determined. Identification of the composition of *O. majorana* EO was performed based on comparing the retention indices (RIs) of sources to a homologous series of C6-C27 n-alkanes and mass spectra of standard components compared with available information in the computer library (Wiley7n.l MS). Besides, Peak normalization was used to quantify compounds.

Preparation of nanoemulsion containing *O. majorana* EO. Spontaneous emulsification was used to prepare nanoemulsions of *O. majorana* EO. A series of nanoemulsions (final volume 5000 µL) containing a fixed amount of *O. majorana* EO (50 µL) and different amounts of tween 80 (0-200 µL) were prepared as follows. The EO and tween 80 were mixed on a magnetic stirrer (500 rpm, 5 min). After that, the solution volume reached 5000 µL by adding distilled water dropwise. Then, the mixture was stirred at 2000 rpm for 30 min to form the nanoemulsion. DLS (K-One Nano Ltd. Korea) was recruited to measure the droplet size and droplet size distribution (SPAN) of nanoemulsions. SPAN was calculated by d90-d10/d50, d is diameter, and 10, 50, and 90 are the percentage of drops with sizes smaller than these numbers. Noted, droplet size and SPAN less than 200 nm and 1 were considered criteria for proper characteristics (20). Amongst the prepared samples, a nanoemulsion with lower droplet size and SPAN was selected for further characterization and bioassays. Moreover, a blank sample was prepared similarly to the selected nanoemulsion without *O. majorana* EO (100 µL tween 80 reached 5000 µL by distilled water).

Characterizations of the nanoemulsion containing *O. majorana* EO. The Zeta potential of the selected nanoemulsion was measured using a Zeta sizer. Moreover, Transmission electron microscopy (TEM) analysis was used to verify the size and shape of the nanoemulsion. A nanoemulsion droplet was poured on the carbon grid and subjected to the device (Philips EM 208S). Besides, the Attenuated Total Reflection-Fourier Transform InfraRed (ATR-FTIR, Bruker Company, Model Tensor II, USA) was employed to confirm the successful loading of *O. majorana* EO in the nanoemulsion.

Stability of the nanoemulsion containing *O. majorana* EO. The stability of the nanoemulsion was investigated in Short and Long terms analyses. Three different approaches were performed To screen the short-term stability of nanoemulsion. First, the nanoemulsion was centrifuged at 22,000 g for 30 min at three different temperatures (-4, +4, 25°C). After that, the nanoemulsion was subjected to a heating-cooling cycle and freeze-thaw cycle subsequently. In the heating-cooling cycle, six successive storage cycles for 48 hours, between 4°C (refrigerator) and 45°C (Bain-Marie), were done. In the freeze-thaw cycle, six cycles were accomplished to store the nanoemulsion between -25°C and room temperature (+25°C) for 48 hours. Finally, the nanoemulsion was visually checked for sedimentation, creaming, or biphasic, and droplet size was re-checked using DLS analysis. As the nanoemulsion showed stability in short-term screening, it was stored at 4°C and 25°C for six months. The samples were then surveyed for creaming, sedimentation, and bi-phasic changes.

Antioxidant assessment. For the investigation of the antioxidant effects of the nanoemulsion, the DPPH assay was used. DPPH solution (0.3 mM) was first prepared with ethanol as solvent. Then, 50 µL of DPPH solution and fifty microliters of nanoemulsion at a concentration of 37-4800 µg/mL were added to each well of 96-well plates. Three wells were treated with the blank as the negative control, and three were treated with nothing as a control group. Treated plates were incubated away from light for 30 min, and finally, the optical density of wells was read at 517 nm. The antioxidant effects at each concentration were calculated using the equation OD control – OD sample / OD control × 100.

Anticancer assessment. MTT test was used to investigate the cytotoxicity of samples as described in our previous study. Briefly, cell lines (A375 and A549) were cultured in RPMI, supplemented with 1% FBS and 1% antibiotics. After that, 50 µL of cell suspensions, containing 10000 cells/well, were added to the 96-well plate, and 24 h were incubated at 37°C in air containing 5% CO_2 for cell attachment. Next,

the liquid medium was discarded, and 50 µL of fresh medium and 50 µL of nanoemulsion with a concentration range of 37-1200 µg/mL were added to each well. Three wells were treated with the blank as the negative control, and three were treated with nothing as a control group. After 24 h incubation at the mentioned condition, the liquid medium of wells was discarded, and 50 µL MTT (0.5 mg/mL) that dissolved in RPMI medium was added to each well. After 5 h incubation, 100 µL/wells of DMSO was added, and the plate was shaken for 1 h. Finally, the optical density of wells at 570 nm was read using a plate reader (Synergy, HTX Multi-Mode Reader, USA). Cell viability at each concentration was calculated using the equation OD sample/OD control × 100.

Antibacterial assessment. The 96-well plate microdilution assay was used to investigate the antibacterial effects of the nanoemulsion, as described in our previous study. *S. aureus* and *E. coli* bacteria colonies suspensions with the standard density of 0.5 McFarland were first prepared. After that, 50 µL of the suspensions and 50 µL of nanoemulsion with a concentration range of 37-4800 µg/mL were added to each well and 24 h incubated. Three wells were treated with the blank as the negative control, and three were treated with nothing as a control group. Finally, the optical density of the wells at 630 nm was read using a plate reader. Using equation OD sample/OD control × 100, bacterial growth at each concentration was calculated.

Statistical analyses. All assays were repeated in triplicates, and the results are presented as mean and standard deviation. In addition, an Independent sample T-test with at least 0.05 significant levels was used to compare two samples (STATA, v11, StataCorp, USA).

RESULTS

Chemical composition of *O. majorana* EO. Compositions of *O. majorana* EO are listed in Table 1. Terpinen-4-o1, L-α-Terpineol, p-Cymene, Linalool, and sabinene with 46.90%, 6.72%, 6.35%, 4.00%, and 1.97% are five major compounds, respectively.

Size, zeta potential, and morphology of the nanoemulsion containing *O. majorana* EO. Ingredients and size characteristics of the prepared nanoemulsions are listed in Table 2. Sample 3 showed the best size characteristics and was selected for further investigation. Its nanoemulsion's droplet size and SPAN value are 149 ± 5 nm and 0.95 (Fig. 1A). Besides, its zeta potential was -11 ± 1 mV, as is depicted in Fig. 1B. Moreover, as the TEM image shows (Fig. 2), droplets are spherical.

Table 1. *O. majorana* EO identified composition via GC-MS analysis

NO.	RT	%	Components	KI	Type
1	11.03	0.26	α-Thujene	930	MH[1]
2	11.41	0.58	α-Pinene	939	MH
3	13.48	1.97	Sabinene	975	MH
4	13.75	0.32	ß-Pinene	979	MH
5	14.32	0.25	Myrcene	990	MH
6	15.80	0.43	α-Terpinene	1017	MH
7	16.31	6.35	Ƥ-Cymene	1024	MH
8	16.46	0.57	Limonene	1029	MH
9	16.59	0.78	ß-Phellandrene	1031	MH
10	18.00	1.61	ɣ-Terpinene	1059	MH
12	19.38	0.36	Terpinolene	1088	MH
13	20.22	4.00	Linalool	1096	MO[2]
14	20.35	1.18	Cis- ß-Terpineol	1132	MO
15	21.55	1.58	Cis-Para-Menth-2-en-1-o1	1139	MO
17	22.50	1.65	l-Terpineol	1140	MO
18	24.07	0.29	Borneol	1169	MO
19	24.47	46.90	Terpinen-4-o1	1177	MO
20	24.91	0.28	Ƥ-Cymen-8-o1	1182	MO
22	25.21	6.72	L- α-Terpineol	1192	MO
23	25.83	1.34	Cis-Para-Menth-1-en-3-o1	1211	MO
25	27.40	1.71	Linalool acetate	1257	MO
30	29.85	0.20	Thymol	1290	MO
41	35.13	1.46	E-Caryophyllene	1419	SH[3]
42	35.93	0.36	Aromadendrene	1441	SH
45	41.79	0.79	Espatulenol	1578	SO[4]
46	41.97	1.07	Caryophyllene oxide	1583	SO
47	42.12	0.33	Globulol	1590	SO
		83.33	Total Identification		

[1]Monoterpene Hydrocarbons, [2]Oxygenated Monoterpenes, [3]Sesquiterpene Hydrocarbons, and [4]Oxygenated Sesquiterpenes

ATR-FTIR. ATR-FTIR spectroscopy of the nanoemulsion was performed to identify the characteristic frequency of various functional groups and molecular interactions in the prepared nanoemulsion.

Table 2. Ingredients and size characteristics of the prepared nanoemulsions

No.	*O. majorana* EO	Tween 80 (µL)	Final volume (µL)	Droplet size (nm)	SPAN
1	50	0	5000	Not dispersed	
2	50	50	5000	220	0.94
3	50	100	5000	149	0.95
4	50	150	5000	185	1.5
5	50	200	5000	320	2.1

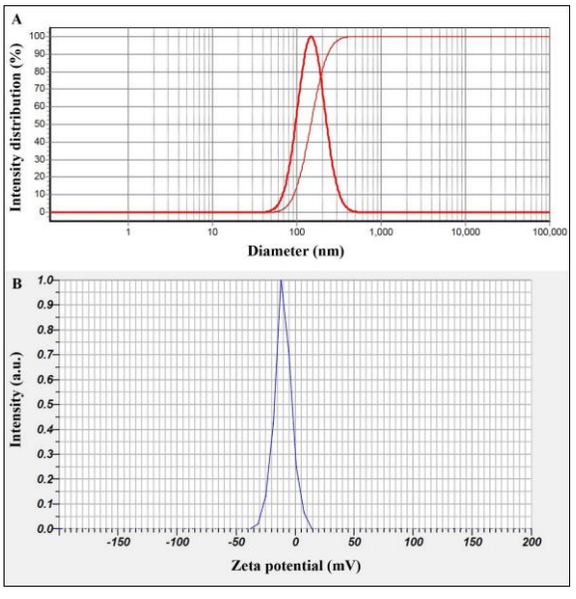

Fig. 1. A. DLS analysis of the nanoemulsion containing *O. majorana* EO and B. its zeta potential analysis

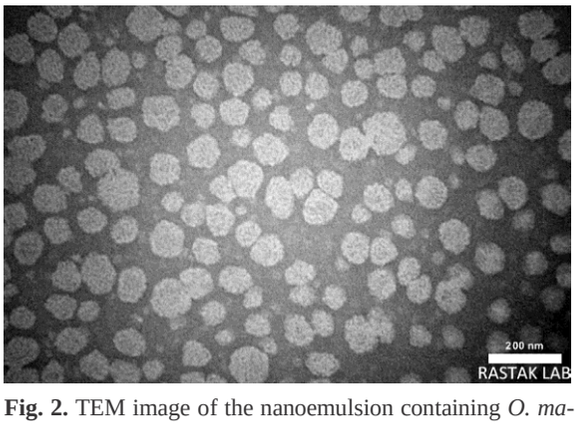

Fig. 2. TEM image of the nanoemulsion containing *O. majorana* EO

Fig. 3A shows the ATR-FTIR spectra of *O. majorana* EO. Concerning its chemical composition, the ATR-FTIR analysis proved the existence of alcohols, ethers, alkenes, aliphatic fluoro compounds, esters, carboxylic acids, and hydrogen-bonded alcohols. The broad characteristic band around 2960.11 cm^{-1} is attributed to the aromatic C–H stretching vibration, at 1514 cm^{-1} assigned to N–H bending, at 1463 cm^{-1}, where the CH_2 bending is detected. The absorption band at 1445 cm^{-1} is related to the C–C stretch of the aromatic ring. The band that appeared at 1249 cm^{-1} is assigned to the C–O–C stretching vibration, while the prominent peak at around 924 cm^{-1} indicates the absorption of the C–H ring (21). In the ATR-FTIR spectrum blank (Fig. 3B), peaks at 2924 and 2858 cm^{-1} are associated with asymmetric and symmetric stretching bands of ($-CH_2$) in tween 80. The characteristic band observed at 1732 cm^{-1} is related to the C=O ester group, and a major peak at 3497 cm^{-1} is assigned to the hydroxyl stretching vibration of tween 80 (22). In the ATR-FTIR spectrum of the prepared *O. majorana* nanoemulsion (Fig. 3C), characteristic bands of both *O. majorana* and tween 80 were observed, thus indicating the existence of the two components in the final nanoemulsion. However, several changes in the position and intensity of some peaks were detected, showing the molecular interactions between the components (21). These results agree with previous studies investigating essential oil-based nanoemulsions (23).

Stability analysis. Favorably, after short-term (centrifugation, heating-cooling, and freeze-thaw cycles) and long-term stability (storage at 4°C and 25°C for

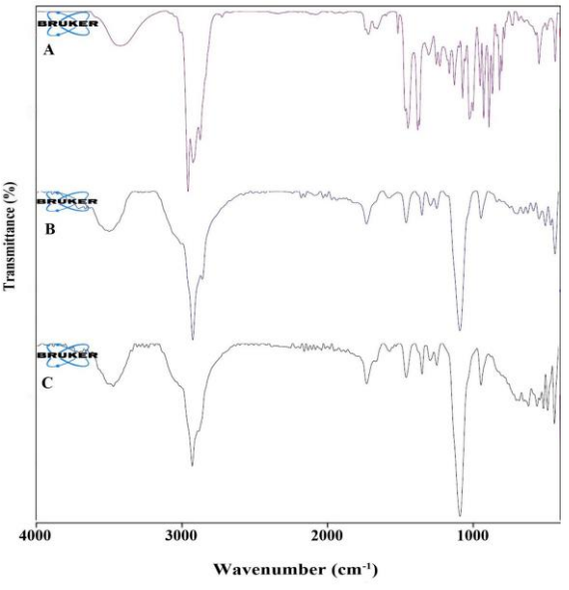

Fig. 3. ATR-FTIR spectra of A: *O. majorana* EO, B: blank, and C: nanoemulsion containing *O. majorana* EO

six months) analyses, the properties of the nanoemulsion was indicated without changes. No sedimentation, creaming, or bi-phasic was observed, so it confirmed its stability.

Antioxidant effect. The antioxidant effect of nanoemulsion containing *O. majorana* EO at a concentration range of 150-4800 is shown in Fig. 4. A dose-response effect was observed, and the best efficacy, i.e., 22.5%, was obtained at the highest concentration, 4800 µg/mL.

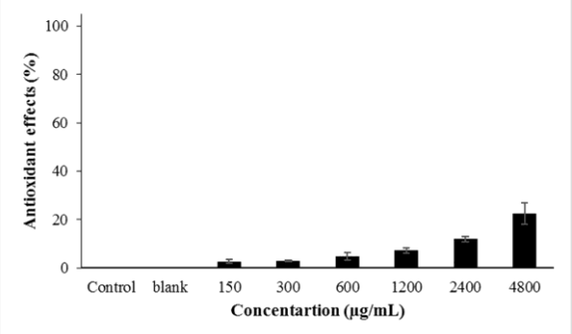

Fig. 4. Antioxidant effect of nanoemulsion containing *O. majorana EO*

Cytotoxicity effect. Fig. 5 shows the viability of cell lines A375 and A549 after being treated with the nanoemulsion. The decrease in cell viability after treatment with blank was negligible (<7%). Moreover, A375 cell viability was significantly decreased compared to A549 cells at 75 (P = 0.008), 150 (P < 0.001), 300 (P < 0.001), and 600 (P = 0.002) µg/mL. Besides, A-375 cells showed significantly (P < 0.05) more sensitivity to the nanoemulsion than A-549 cells; IC_{50} values were obtained as 139 (91-213) µg/mL and 318 (249-405) µg/mL (Table 3).

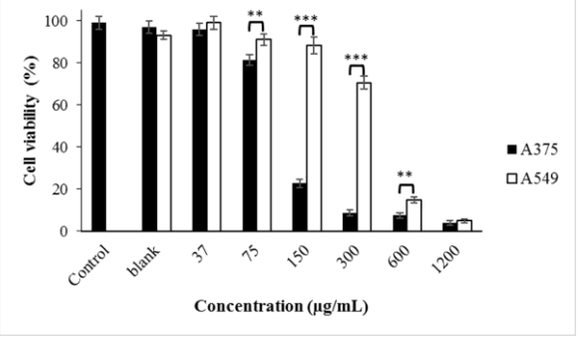

Fig. 5. Cytotoxicity activity of nanoemulsion containing *O. majorana* EO on A375 and A549 cells. **: P < 0.001 and ***: P < 0.0001

Table 3. Obtained IC_{50} values (µg/mL) of nanoemulsion containing *O. majorana* EO

Parameters	A375	A549	*S. aureus*	*E. coli*
IC_{50}	139	318	611	580
LCL[a]	91	249	349	407
UCL[b]	213	405	1070	827

A: Lower Confidence Limit, B: Upper Confidence Limit

Antibacterial effect. Bacterial growth after treatment with nanoemulsion containing *O. majorana* EO is presented in Fig. 6. Growth of *S. aureus* and *E. coli* after treatment with blank was observed at 75±4% and 82±5%. Interestingly, their growth after treatment with 4800 µg/mL of nanoemulsion was obtained at 12±2% and 6±1%. Besides, the growth of *E. coli* at 150 µg/mL was significantly more than *S. aureus* (P < 0.001). However, *S. aureus* growth at 300 (P = 0.009), 1200 (P < 0.004), 2400 (P < 0.006), and 4800 (P = 0.009) µg/mL was significantly more than *E. coli*. Moreover, as summarized in Table 3, no significant difference (P > 0.05) was observed between the efficacy of nanoemulsion on *E. coli* (IC_{50} = 580 µg/mL) and *S. aureus* (IC_{50} = 611 µg/mL).

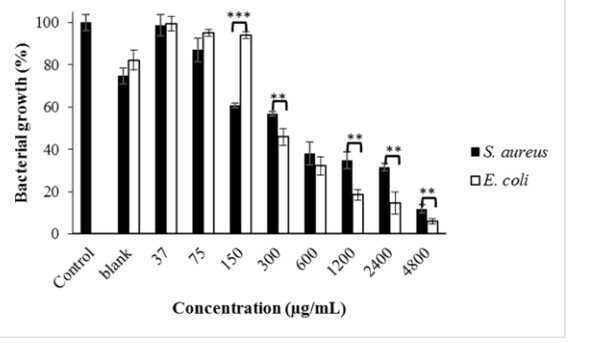

Fig. 6. Antibacterial activity of nanoemulsion containing *O. majorana* EO on *E. coli* and *S. aureus.* **: P < 0.001 and ***: P < 0.0001

DISCUSSION

This study used *O. majorana* EO as natural medicine. Terpinen-4-o1 (46.90%) was identified as the major compound of the EO in the current study; this finding is consistent with the literature (24, 25). Terpinen-4-ol is a monoterpene with anti-inflammatory, anticancer, and antitumor effects (25). Oxidants with two origins inside (ROS and NOX) and outside (Chemicals and Foods) the body have side effects on

human health. Some complications include premature aging, cardiovascular diseases, and cancers (26). Antioxidants help maintain health by neutralizing oxidants (27). This study first investigated the antioxidant effects of the prepared nanoemulsion. The nanoemulsion showed some antioxidant effects, which may be useful for preventing cancer, skin health, and inflammatory disease (28).

In this study, A-549 and A-375 cells were used. A-549 epithelial carcinoma cells account for 85-88% of all lung cancer; that has become a golden standard for lung cancer basic research and drug discovery (29). Furthermore, 42 melanoma cell lines are on the surface of transcriptomes. Studies indicate that the A-375 cell line is more aggressive with low sensitivity to chemotherapy treatment, so it is commonly used in melanoma research (30). Besides, some reports against A-549 cells have been found in the literature. For instance, C*innamomum cassia* nanoemulsion with an IC_{50} value of 18.5 µg/mL (31), *Zingiber ottensii* nanoemulsion with an IC_{50} value of 18.45 µg/mL (32), and *Citrus aurantium* with IC_{50} value 152 µg/mL (33). Moreover, results of the current study showed that A-375 cells (IC_{50} 139 µg/mL) were more sensitive to the nanoemulsion than A-549 cells (IC_{50} 318 µg/mL); nowadays, it is accepted that EOs have selectivity effects on cells (34). For instance, IC_{50} values of chitosan nanoparticles containing *Cinnamomum verum* were reported at 79 and 112 µg/mL against A-375 and MDA-MB-468 cells, respectively (35). Besides, 50% effects of *Zataria multiflora* EO against MDA-MB-468 and A375 were obtained at 600 and 75 µg/mL (36).

The results showed that the efficacy of the nanoemulsion against gram-negative bacteria (*E. coli*) was lower than gram-positive bacteria (*S. aureus*) in the lower concentrations. Gram-positive organisms are more sensitive to EOs than gram-negative bacteria (37). The cell wall of gram-negative bacteria is more complex and resistant than gram-positive bacteria (38). Gram-negative bacteria have an envelope consisting of three layers: the outer, middle, and inner membranes. Gram-positive bacteria do not have an outer membrane, distinguishing this type of bacteria from gram-negative bacteria (39). The thickness and lipid content of the cell walls of the gram-negative bacteria is higher than that of the gram-positive cell wall, such that the cell walls of gram-negative possess a much more complete range of amino acids, including aromatic, certain sulfur-containing amino acids, arginine, and proline (40). Therefore, the lower efficiency of nanoemulsion against gram-negative bacteria at low concentrations is due to the mentioned reasons. With the increase in nanoemulsion concentration, the gram-negative bacteria's cell wall was probably destroyed. However, more study is required.

CONCLUSION

This study proposed the nanoemulsion containing *O. majorana* EO with a wide range of biological properties, including anticancer effects (A-375 human melanoma cells and A-549 human lung cancer cells), antioxidant properties, and antibacterial activity (*E. coli* and *S. aureus*). Besides, stability analyses confirmed its proper stability. It could thus be used for further consideration against other cancer cell lines and pathogens.

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