The antimicrobial and antibiofilm potential of *Citrus aurantium* and *Artemisia annua* essential oils nanoemulsions

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ABSTRACT

Antimicrobial resistance has posed considerable health and economic burdens globally (approximately five million deaths annually), particularly among developing countries. The estimated annual treatment costs in the United States include US$4.6 billion. Vast antibiotic resistance among Gram-negative and Gram-positive bacterial species has spread from healthcare to the environment, community, and animals. These conditions have limited and sometimes failed the infection eradication choices and facilitated the distribution of drug-resistant organisms. The spread of drug-resistant bacterial infections is a huge human health concern, hence seeking novel antibacterial agents is crucial. This study used the nanoemulsions of *Citrus aurantium* and *Artemisia annua* essential oils (EOs) as natural antibacterial agents. Gas chromatography mass spectrometry (GC–MS) analysis showed that limonene (31.4%) and artemisia ketone (26.2%) were major components, respectively. After that, their nanoemulsion dosage forms with a mean droplet size of 181 ± 7 and 160 ± 5 and with zeta potential values 3.1 ± 0.8 and -4.9 ± 0.5 mV were prepared. Meanwhile, successful loading of the EOs in nanoemulsion was confirmed by Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) analysis. *A. annua* nanoemulsion with 40% antioxidant effect was significantly more potent than *C. aurantium* nanoemulsion. Meanwhile, nanoemulsions' antibacterial and antibiofilm activity against clinical and standard strains, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia*, were investigated. The best efficiency was related to the effect of *C. aurantium* nanoemulsion against *S. aureus*; minimum inhibitory and bactericidal concentrations (respectively MIC and MBC) were 500 and > 2000 µg/mL. Besides, no biofilm was formed after treatment by both nanoemulsions. Therefore, *C.*

٤٧ *aurantium* and *A. annua* EO nanoemulsions may act as natural antioxidant and antibacterial
٤٨ agents in complementary medicine.

٤٩ **Keywords:** Antibacterial Agents, Essential Oil, Reactive Oxygen Species, Phytochemicals

1. Introduction

Vast antibiotic resistance among Gram-negative and Gram-positive bacterial species has spread from healthcare to the environment, community, and animals. These conditions have limited and sometimes failed the infection eradication choices, resulting in the distribution of drug-resistant organisms (1, 2) such as those non-susceptible to last-resort antibiotics of carbapenems and glycopeptides. As alternatives to chemotherapies, natural resources such as essential oils (EOs) have been suitable alternatives (3-5). In addition, nanoformulation of EOs by optimization in particle size, increasing solubility, and improving bioavailability and stability causes medicines to be more permeable into cells, such as bacteria or cancer cells (6-10). The *citrus* genus includes several species of citrons within the family *Rutaceae*, utilized as herbal medicines, fruits, juice, and additives. *Citrus* fruits contain vitamins C and B, minerals, nutrients, and bioactive compounds such as phenolic compounds, volatile oils, and terpenoids. *Citrus aurantium* (*C. aurantium*) L. cultivar has exhibited anti-inflammatory, hypoglycemic, antimicrobial, anticancer, pain-relief, and organ protective effects (11). Although the species has bioactive compounds and biological activities, its pharmacological effects, traditional usage, and exact bioactive compounds have not been uncovered (12). On the other, *C. aurantium* L. dietary supplementation has not exerted side effects (13). Various extracts of *C. aurantium* L. leaves, including aqueous, alcoholic, or chloroform portions, have demonstrated antibacterial effects against various agents (11, 13). In addition, the antimicrobial effects of its EOs have unraveled potential activities against *Agrobacterium tumefaciens*, *Dickeya solani*, and *Erwinia amylovora* (14, 15). *Artemisia annua* grows globally in Europe, Asia and North America with preferred arid and semi-arid climates and provides considerable health benefits such as anticancer and antimicrobial traits. Bioactive compounds of the herb mainly include artemisia ketone, 1,8-

cineole, germacrene D, and camphor (16). This study aimed to investigate the antibacterial and antibiofilm effects of nanoemulsions of *A. annua* L. and *C. aurantium*.

2. Materials and Methods

2.1. Materials

C. aurantium and *A. annua* EOs were bought from Iranian companies, Tabib Daru Company and Pharmaceutical Company Essential Oil Dr. Soleimani. Bacterial standard strains included *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumonia* ATCC13883 were cultured from bacterial stock preserved at -20 °C in the microbiology laboratory of Fasa University of Medical Sciences, Iran. Moreover, clinical isolates were collected from patients. Mueller Hinton broth, trypticase soy broth, crystal violet, tween 20, and tween 80 were purchased from Merck Chemicals, Germany. The standard analytical solution of Alkanes (C9-C24) was bought from Sigma-Aldrich (USA).

2.2. Chemical composition of EOs

Gas chromatography mass spectrometry (GC–MS) analysis was used for the chemical compositions of the EOs. For this purpose, the gas chromatographic device (Agilent 6890, USA) with HP-5MS silica fused columns coupled to a network mass selective detector (Agilent 5973, USA) was used. The major constituents of EOs were identified by comparing their retention indices to homologous C9-C24 n-alkanes, as described in our previous reports (17, 18).

2.3. Preparation and characterizations of nanoemulsions

A. annua EO (0.4% v/v) was mixed with tween 80 (0.5% v/v), and *C. aurantium* (0.4% v/v) was mixed tween 20 (0.5% v/v), separately, for 3 minutes at room temperature at 2000 rpm. Afterward, distilled water was added drop by drop to reach the final volume of 5 mL, and the

mixture was stirred for 40 minutes at room temperature at 2000 rpm. The prepared nanoemulsions' droplet size and droplet size distribution (SPAN) were measured using a DLS (Dynamic Light Scattering, DLS9900, K-ONE, Korea) device. SPAN was calculated using the relationship d90-d10/d50; in this equation, d is the diameter, 90, 10, and 50 percent of particles with a size smaller than the values mentioned. The droplet size below 200 nm and SPAN below one were necessary conditions to confirm the appropriate size characteristics. TEM (Transitional Electron Microscopy, Philips, TEM, EM 208s, Netherland) was used to confirm the droplet size and determine their morphology. ATR-FTIR (Attenuated Total Reflection-Fourier Transform Infrared) analysis is used to evaluate the successful loading of the EOs in the nanoemulsion. Spectra of the EOs, nanoemulsion (-oil), and nanoemulsion were recorded in 400 - 4000 cm^{-1} using a spectrometer (Tensor II model, Bruker Co, Germany). Furthermore, the stability of nanoemulsions was investigated. Nanoemulsions were centrifuged at −4, +4, and +25°C (14,000 g, 30 min) to investigate stability against precipitation. Besides, nanoemulsions were stored at +45°C and room temperature for six consecutive intervals of 48 hours for thermal stability analysis. Moreover, nanoemulsions were placed at −20°C and room temperature for six consecutive 48-hour intervals for cryogenic stability. In addition, nanoemulsions were placed at 4^0 C and room temperature for six months for long-term stability analysis. After each test, the nanoemulsion was visually checked for sedimentation, creaming, or biphasic.

2.4. Investigation of antioxidant properties of EOs and nanoemulsions

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to measure antioxidant properties. First, serial dilutions of the nanoemulsions (62.5-2000 µg/mL) were prepared in ethanol. Next, 50 µL/well of each prepared dilution and 0.2 mM DPPH solution was added to a 96-well plate, and it was incubated for 30 minutes away from light at room temperature. Finally, the wells' OD

(optical density) was read at 517 nm using a plate reader (Synergy HTX Multi-Model Reader, USA).

The antioxidant activity was calculated using OD test/OD control×100.

2.5. Minimum inhibitory concentration

Micro-dilution test was implemented. The range of concentrations of nanoemulsions (250, 500, 1000, 1500 and 2000 µg/mL) was prepared by PBS containing 0.5% DMSO as solvent. Antibacterial effects of EOs and nanoemulsion were investigated using 96-well broth micro-dilution, as described in our previous study (19). Briefly, 40 µL of each was inoculated into wells of 96 well plates containing 50 µL of Mueller Hinton broth. Afterward, 10 µL/well of each bacterial suspension (0.5 McFarland standard turbidity, 1.5×10^8 CFU/mL) was added to each well. The plates were incubated for 24 hours at 37 ºC, and then the OD of the wells was read at 630 nm. Bacterial growth was obtained using OD sample/OD control×100. The minimum inhibitory concentration (MIC) of *A. annua* and *C. aurantium* single and nanoemulsion forms against bacterial strains were determined using concentrations ranging from 250-2000 µg/mL. Moreover, the bacterial suspension equal to 0.5 McFarland standard was prepared. The test was performed the same as that of the broth micro-dilution method.

2.6.Biofilm formation

Anti-biofilm effects of EOs and nanoemulsions were assessed against clinical isolates. biofilmThe biofilm formation with and without exposure to the nanoemulsions was performed into 96-well plates using a microtitre tissue plate assay. Briefly, an overnight culture of bacterial strains was obtained into the trypticase soy broth (TSB) medium containing 1% glucose and diluted 1:100. For each bacterial suspension, 20 µL was taken and inoculated into wells containing 180 µL of the TSB medium in triplicate and incubated for 5 hours for exposed (2000

µg/mL of each nanoemulsion) group and 24h for the un-exposed group. The un-exposed group medium was exchanged with each nanoemulsion and incubated for 24 hours. Next, the wells were washed using double distilled water and fixed using methanol. Then, 0.1% crystal violet or safranin was added for 15 min. After repeated washing, the absolute ethanol (200 µL) was added to solubilize bacterial contents and read using the ELISA reader at 490 nm. The biofilm formation levels (strong, moderate, weak, or non-adherence) were calculated using Table 1. This study compared biofilm formation levels among groups, including the control (unexposed) and *A. annua* and *C. aurantium* EOs nanoemulsion-treated groups.

Table1. The calculation of biofilm formation levels

Biofilm formation ability	Calculation of cut-off level	OD calculated results	Reference
Strong	OD>ODc×4	0.33296>OD	(20, 21)
Moderate	ODc×2≤OD<ODc×4	0.16648≤OD<0.33296	
Weak	ODc≤OD<2×ODc	0.083324≤OD<0.16648	
No binding	OD≤ODc	0.08324≤OD	

OD: optical density, ODc: mean OD of control wells

2.7.Data analysis

All experiments were done in triplicates. The data was analyzed using the SPSS version, from which Chi-Square and analysis of variance (ANOVA) tests were applied to determine differences at a *p*-value cut-off of 0.05.

3. Results

The main components of the *C. aurantium* were limonene (31.4%), sabinene (15.6%), ɣ-terpinene (6.0%), linalool (5.6%) and cis-nerolidol (5.1%) (Table 2). As well as the main components of the *A. annua* were artemisia ketone (26.2%), camphor (19.2%), 1,8-cineole (12.3%), *trans*-caryophyllene (4.5%), and camphene (4.4%) (Table 2).

Table 2. Identified compounds in the EOs using GC-MS analysis

Retention Time (min)	Compound	*C. aurantium*		*A. annua*		Retention Index
		Area	%	Area	%	
9.46	α-pinene	58484827	1.7	122296938	4.1	932
10.06	camphene	--	--	132788539	4.4	954
11.14	sabinene	542668432	15.6	44039171	1.5	975
11.24	β-pinene	43301938	1.2	29556997	1.0	979
11.96	β-myrcene	108784770	3.1	29379228	1.0	988
12.50	Yomogi alcohol	--	--	41103264	1.4	999
13.08	α-terpinene	59177141	1.7	--	--	1014
13.81	1,8-cineole	--	--	368453758	12.3	1026
13.89	limonene	1088445097	31.4	--	--	1029
14.67	*cis*-ocimene	162728160	4.7	--	--	1037
15.13	ɣ-terpinene	207125216	6.0	--	--	1054
15.48	artemisia ketone	--	--	784989266	26.2	1062
16.31	artemisia alcohol	--	--	33487881	1.1	1083
17.16	linalool	192637034	5.6	--	--	1095
19.22	camphor	--	--	576552736	19.2	1146
20.12	borneol	--	--	28483988	1.0	1169
20.63	4-terpineol	66692764	1.9	33586786	1.1	1177
23.55	cuminic aldehyde	128003231	3.7	--	--	1239
29.38	α-copaene	--	--	47639328	1.6	1376
31.26	*trans*-caryophyllene	--	--	136219018	4.5	1419
33.78	germacrene D	--	--	84811438	2.8	1481
33.99	β-selinene	--	--	90133377	3.0	1490
37.11	*cis*-nerolidol	178234586	5.1	--	--	1532
37.76	caryophyllene oxide	--	--	35663606	1.2	1583
42.89	*cis*-farnesol	34776356	1.0	--	--	1698

3.1. TEM analysis of *C. aurantium* and *A. annua* EO nanoemulsions

The mean droplet diameter and zeta potential of *C. aurantium* nanoemulsion were 181 ± 7 nm and 3.1 ± 0.8 mV, respectively (**Figure 1 A & B**). The *A. annua* nanoemulsions showed droplet diameter at 160 ± 5 nm and zeta potential values -4.9 ± 0.5 mV (**Figure 2 A & B**). The TEM analysis (**Figures 1C and 2C**) revealed that both nanoemulsions were spherical, size < 100 nm.

Furthermore, the stability of nanoemulsions was investigated. Nanoemulsions were centrifuged at −4, +4, and +25°C (14,000 g, 30 min); no sedimentation or bi-phasic was observed. Besides, nanoemulsions were stored at +45°C and room temperature for six consecutive intervals of 48 hours for thermal stability analysis; no sedimentation or bi-phasic was observed. Moreover, nanoemulsions were placed at −20°C and room temperature for six consecutive 48-hour intervals for cryogenic stability; no sedimentation or bi-phasic was observed. In addition, nanoemulsions were placed at 4 and room temperature for six months for long-term stability analysis; no sedimentation or bi-phasic was observed.

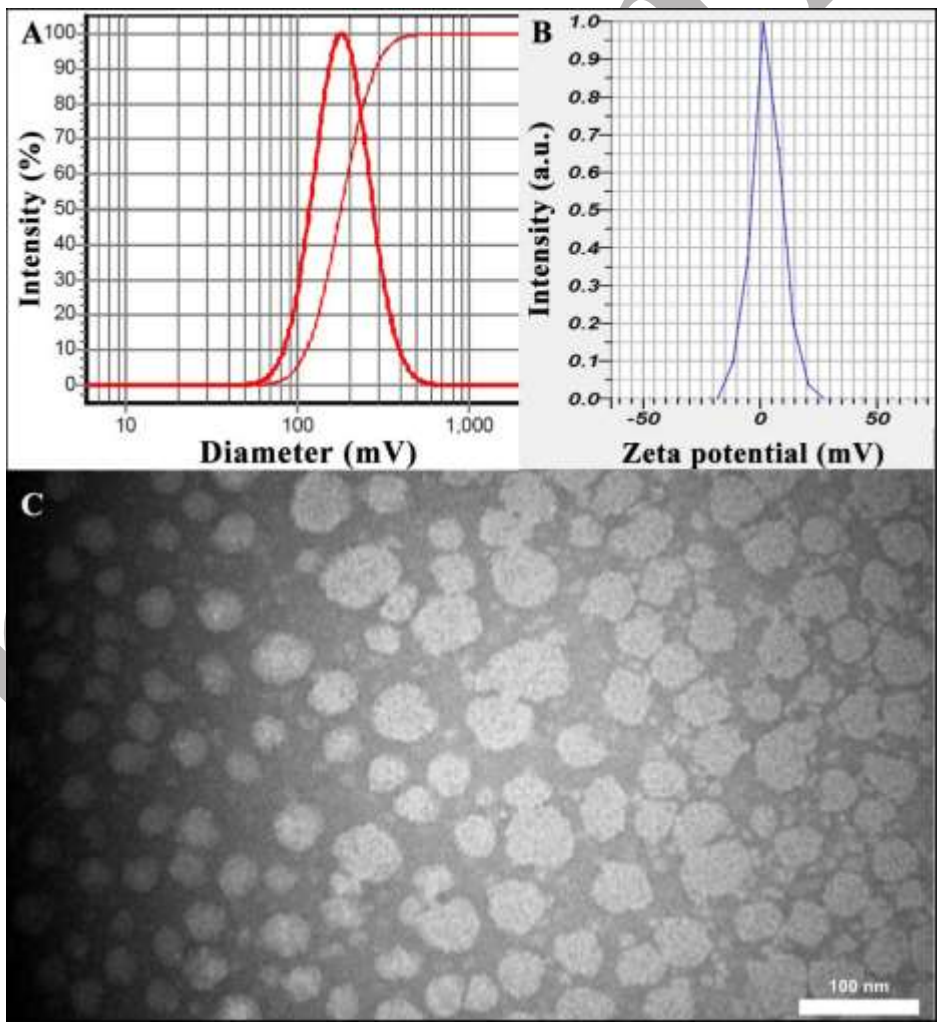

Figure 1. Characterization of *C. aurantium* EO nanoemulsion, A: DLS profile, B: zeta potential profile, and C: TEM image

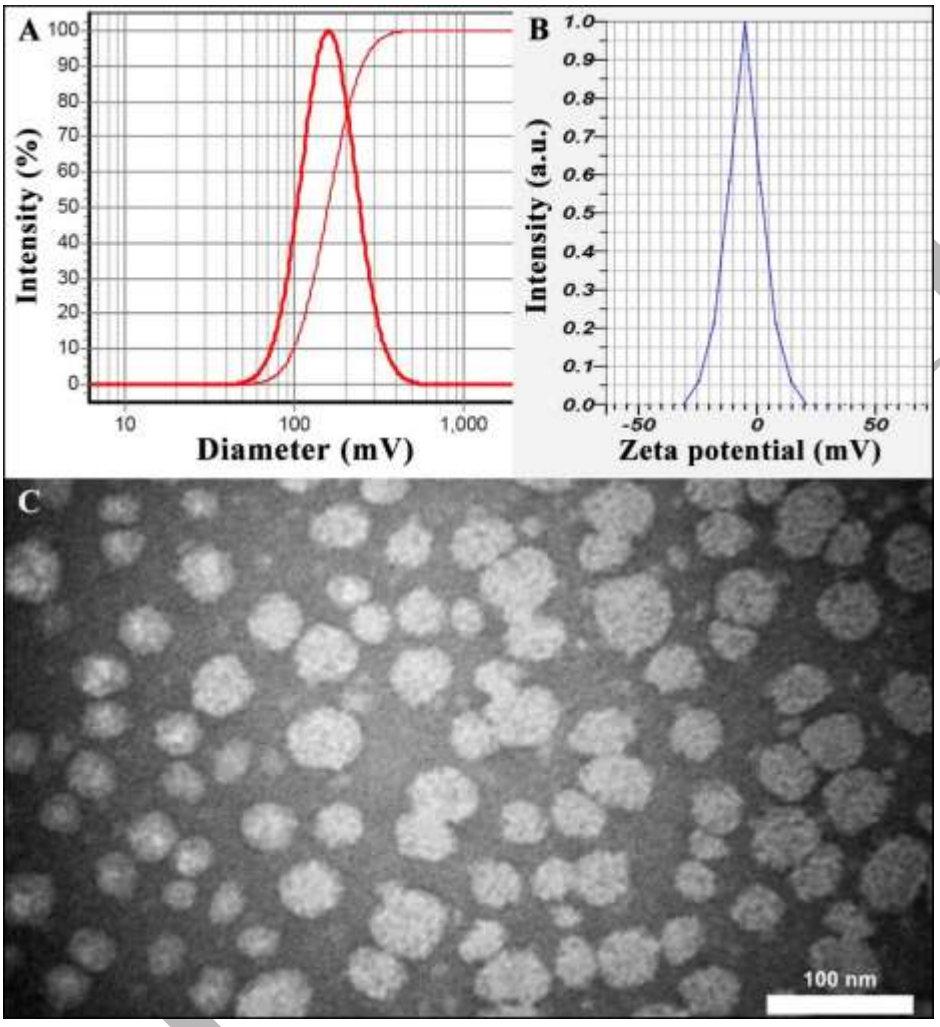

Figure 2. Characterization of *A. annua* EO nanoemulsion, A: DLS profile, B: zeta potential profile, and C: TEM image

3.2. ATR-FTIR analysis of *C. aurantium* and *A. annua* nanoemulsions

ATR-FTIR analysis confirmed EO loading in nanoemulsion (**Figure 3**). The spectra of *C. aurantium* EO displayed in Figure 3A, broadband at 3469 cm^{-1} can be related to stretching vibration of the hydroxyl group due to hydrogen bonding in alcoholic and phenolic bioactive compounds in EO, spectra at 3076 cm^{-1} can be corresponded to stretching vibration of CH in sp^2 groups and the bands at 2961, 2923 and, 2872 cm^{-1} can be attributed to stretching vibration of

CH in sp^3 groups, the band at 1706 and 1676 cm^{-1} can be related to stretching vibration of carbonyl groups. Bands at 1108 and 1052 cm^{-1} showed stretching vibration of C-O groups. The peak at 989 cm^{-1} is related to C-H bending absorption, and the strong peak at 758 cm^{-1} is allocated to benzene rings C-H vibration absorption. A peak at 689 cm^{-1} is attributed to the vibration absorption of alkenes. The spectrum of nanoemulsion without *C. aurantium* EO displayed in Figure 3B, the broad peak at about 3493 cm^{-1} can be attributed to OH stretching vibration due to hydrogen bonding between water and tween 20. Spectra at 2924 cm^{-1} corresponded to C-H stretching in tween 20. A strong band at 1733 cm^{-1} attributed to C=O stretching representing the carbonyl group in tween 20. Characteristic band at around 1462 cm^{-1} can be related to CH_2 bending tween 20. Characteristic and sharp peak at 1091 cm^{-1} is assigned to C-O stretching. FTIR of *C. aurantium* EO nanoemulsion spectrum (**Figure 3C**) showed the broadband at 3518 cm^{-1} attributed to OH stretching vibration due to the strong hydrogen bonding between water, tween 20, and phenolic and alcoholic compounds in EO. Any band at 2969, 2924, and 2856 cm^{-1} is related to C-H stretching due to sp^3 hybrid in tween 20 and EO. Strong band at 1728 cm^{-1} showed carbonyl stretching (C=O) tween 20 and EO. The absorption at around 1456 cm^{-1} corresponded to CH_2 bending tween and EO. A sharp and strong peak at about 1093 cm^{-1} can be attributed to C-O stretching.

Spectrum of the *A. annua* EO has been demonstrated in **Figure 3D**, a broad and characteristic band at about 3520 cm^{-1}, can be attributed to the hydroxyl functional groups in EO, and a band at 3084 cm^{-1}, allocated to the stretching vibration of =C-H groups from olefins in sp^2 hybrid. Peaks at 2963, 2928, and 2872 cm^{-1}, related to stretching vibrations of –CH in sp^3 hybrid, the spectra at around 1743 cm^{-1} related to C=O, the absorption around 1620 and 1415 cm^{-1} assigned to C = C, the bands at 1215 and 1167 cm^{-1} are related to (C-O-C) bonds and the band at 876 cm^{-1} can be

allocated to angular deformations of CH_2 groups. Spectra of nanoemulsion without *A. annua* EO has been shown in **Figure 3E**. A characteristic and broad peak between 3200 to 3600 cm^{-1} corresponds to OH stretching vibration due to the hydrogen bonding between water and tween 80. Spectra at 2964 and 2925 cm^{-1} are related to C-H stretching. Absorption at 1740 cm^{-1} was attributed to carbonyl stretching (C=O) in tween 80 and at about 1456 cm^{-1} can be allocated to CH_2 bending. A strong and characteristic band at 1080 cm^{-1} corresponded to C-O stretching. ATR-FTIR of nanoemulsion containing *A. annua* EO has been displayed in **Figure 3F**. A peak at about 3422 cm^{-1} is attributed to OH stretching vibration due to hydrogen bonding between EO, tween 80, and water and at 2924 cm^{-1} corresponds to C-H stretching of EO and tween 80. Absorption at 1733 cm^{-1} attributed to C=O stretching representing the carbonyl group in EO and tween. A characteristic peak at about 1462 cm^{-1} outlined CH_2 bending in EO and tween 80. A strong and sharp peak at 1091 cm^{-1} was assigned to C-O stretching. The presence of other bands in EOs and blanks confirmed the successful loading of EOs in the prepared nanoemulsion.

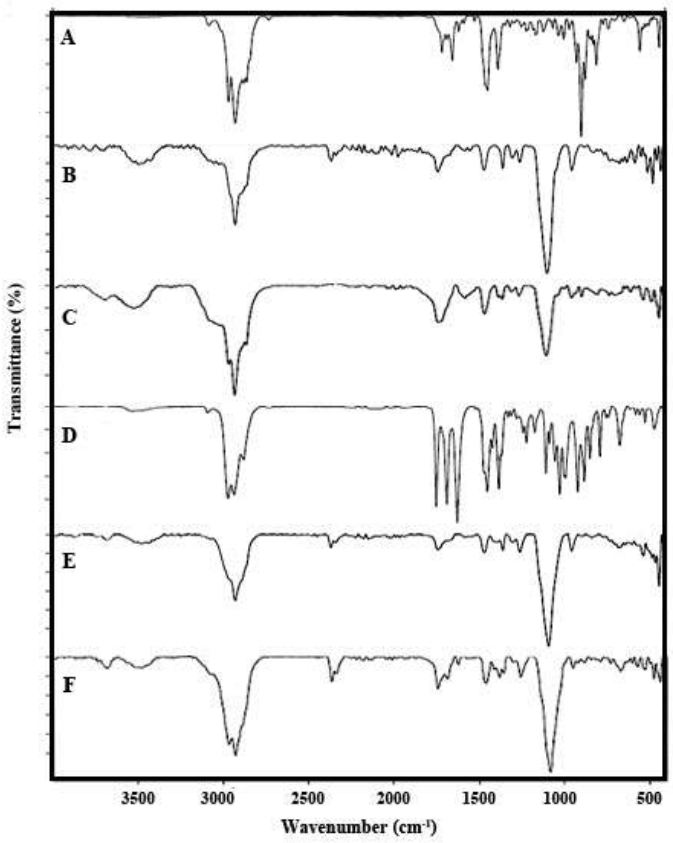

Figure 3. ATR-FTIR spectra of A: *C. aurantium* EO, B: nanoemulsion without *C. aurantium* EO, C: nanoemulsion containing *C. aurantium* EO, D: *A. annua* EO, E: nanoemulsion without *A. annua* EO, F: nanoemulsion containing *A. annua* EO

The *C. aurantium* and *A. annua* nanoemulsions were evaluated for their antioxidant effect by DPPH assay. As shown in **Figure 4**, the most potent free radical scavenging activity was obtained from *A. annua* EO nanoemulsion, 40 % at 2000 µg/mL.

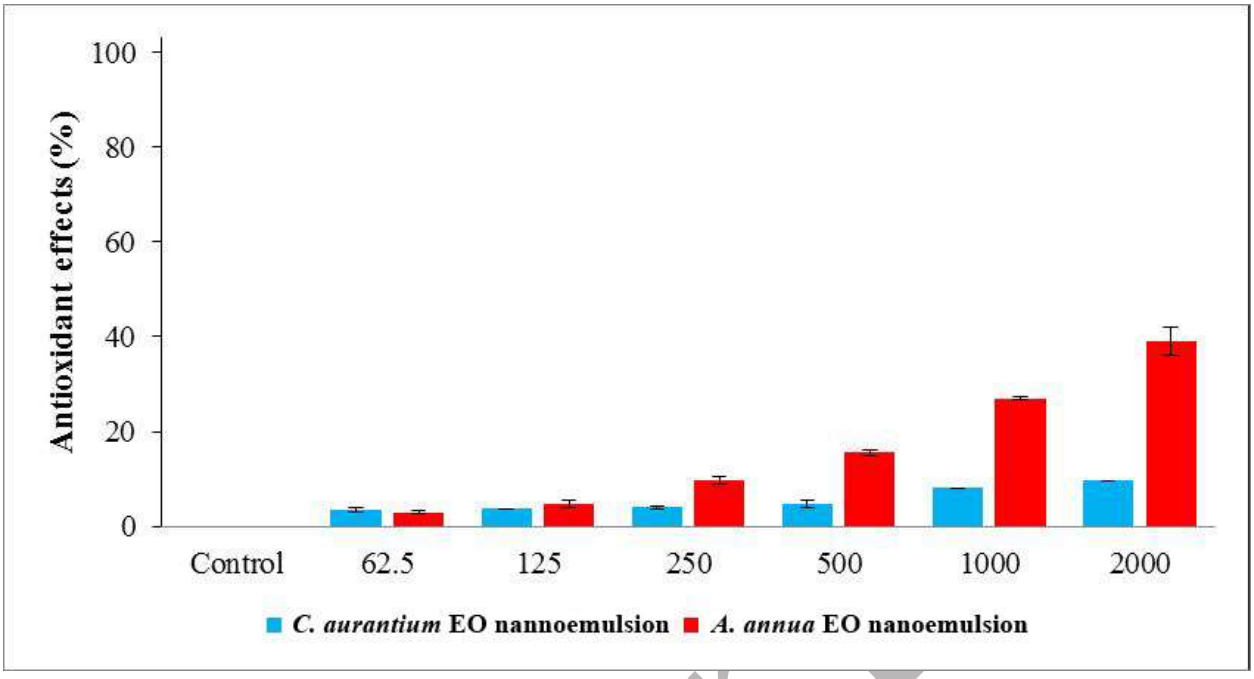

Figure 4. The antioxidant effects of samples

3.3. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

From Table 3, the *C. aurantium* EO nanoemulsion MIC against *S. aureus*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* included 500 µg/mL, 1000 µg/mL, 1000 µg/mL, and 1000 µg/mL, respectively. The MBC values against bacterial strains also included >2000µg/mL. Moreover, The MIC values of *A. annua* nanoemulsion EO against *S. aureus*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* included 1000 µg/mL, 2000 µg/mL, 2000 µg/mL, and 2000 µg/mL, respectively. The MBC values included >2000 µg/mL for all the tested bacterial strains.

Table 3. The MIC and MBC levels (µg/mL) samples

Samples	*S. aureus* (MIC, MBC)	*E. coli* (MIC, MBC)	*P. aeruginosa* (MIC, MBC)	*K. pneumonia* (MIC, MBC)
C. aurantium EO nanoemulsion	500, >2000	1000, >2000	1000, >2000	1000, >2000

A. annua EO nanoemulsion	1000, >2000	2000, >2000	2000, >2000	2000, >2000

3.4.Antibacterial effects

The bacterial growth inhibitory effect of *C. aurantium* nanoemulsion EO was concentration-dependent. The highest bactericidal effect was observed against *S. aureus* at 2000 µg/mL, in which 56% of growth was inhibited (**Figure 5**).

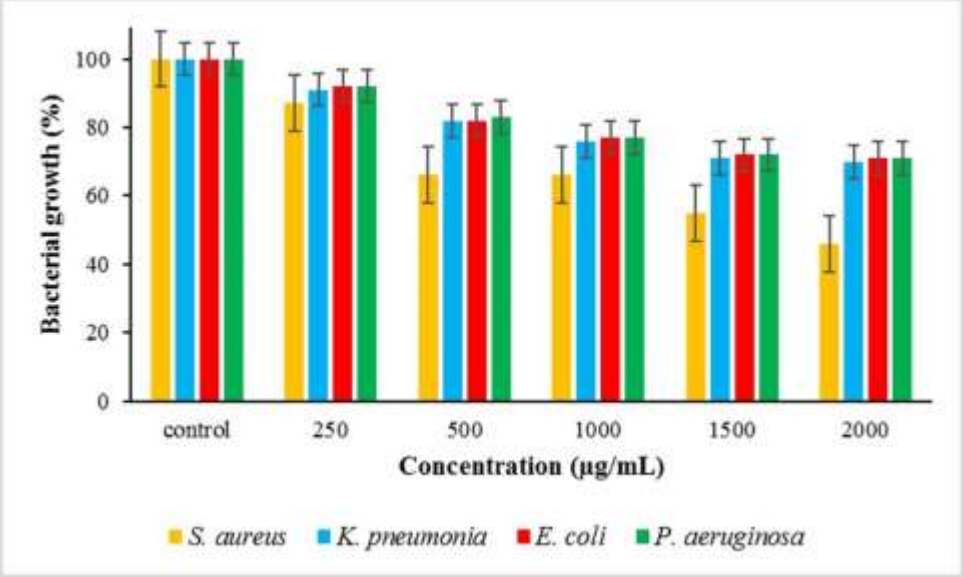

Figure 5. The bacterial growth in exposure to *C. aurantium* nanoemulsion EO

The growth inhibitory effect of *A. annua* nanoemulsion EO was mostly against *S. aureus* at 2000 µg/mL, at which ~20 % of bacterial growth was inhibited (**Figure 6**).

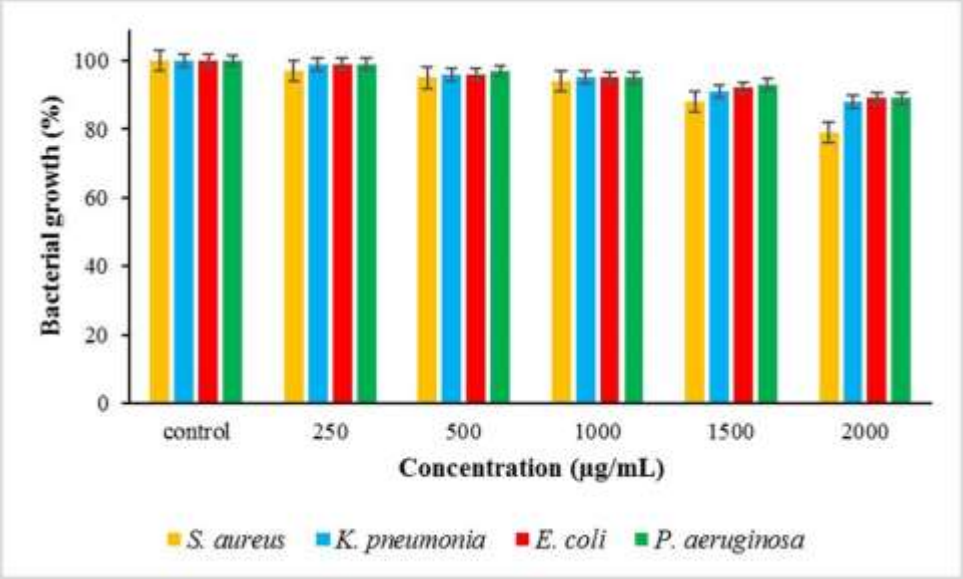

Figure 6. The bacterial growth in exposure to *A. annua* nanoemulsion EO

3.5.Anti-biofilm effects

As shown in **Figure 7**, biofilms in the control group all bacteria were formed (OD > 0.083). However, after treatment with both nanoemulsions, no biofilm (OD < 0.083) was formed by all examined bacteria, i.e., *S. aureus*, *K. pneumonia*, *E. coli*, and *P. aeruginosa*.

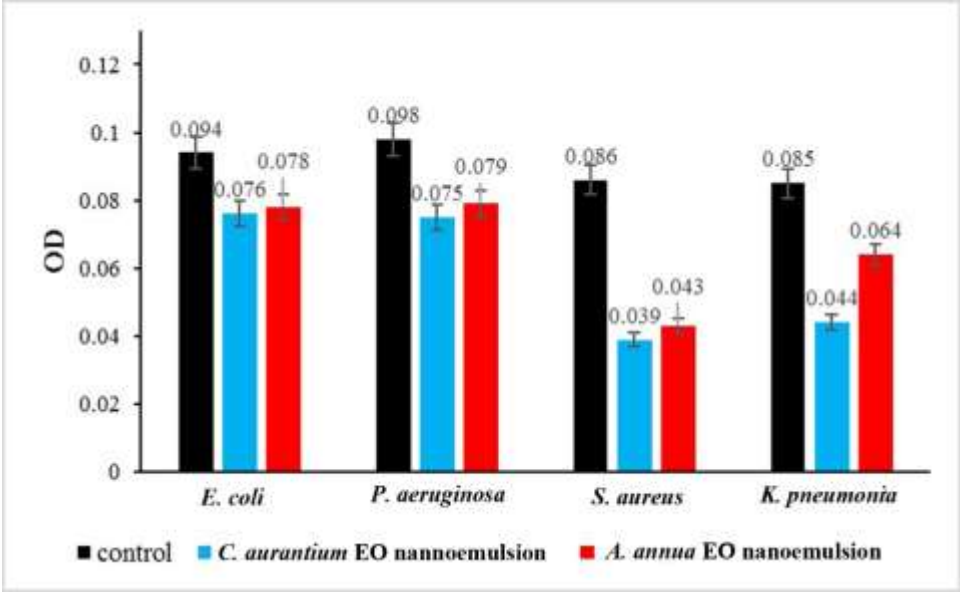

Figure 7. The anti-biofilm effects of (mean OD value) samples

4. Discussion

In our study, nanoemulsions of *C. aurantium* and *A. annua* EOs were prepared using spontaneous emulsification. Meanwhile, their antimicrobial and antibiofilm against selected bacterial pathogens were investigated. Some previous studies have investigated antimicrobial and insecticide properties of *C. aurantium* and *A. annua* extracts and EOs against different microorganisms, but our study has been carried out mostly on their nanoemulsion forms (22). For example, the biological activity and antibiofilm molecular profile of *C. aurantium* EO were investigated by Kačániová et al., that concluded that *C. aurantium* EO had potent antibacterial activity against *Stenotrophomonas maltophilia* and *Bacillus subtilis* followed by *Penicillium crustosum* (15). In addition, EOs extracted from the peel of *C. aurantium*, as noted by Madhuri et al., can be used against infectious agents such as *K. pneumoniae* and *Bacillus cereus* (23). *In vitro* studies by Marinas et al. also showed that *A. annua* EO has selective antipathogenic activity on Gram-positive and Gram-negative bacterial strains (24). Its antibacterial and anti-biofilm activities have been also unveiled (25, 26). In addition, Mariadosset et al. fabricated the selenium

nanoparticles using *A. annua* (AaSeNPs) of 109.2 nm in size and the characterized AaSeNPs indicated an antibacterial activity against multidrug-resistant pathogens such as *S. aureus*, *B. subtilis*, *Proteus mirabilis*, and *E. coli* (27). As well as the results obtained by Das et al. showed stronger antimicrobial activity of Pickering nanoemulsion of the *Artemisia* essential oil (26). In our study, antimicrobial and antibiofilm effects of nanoemulsions of *C. aurantium* and *A. annua* EOs against selected bacterial pathogens were confirmed by MIC and MBC tests with the highest activity against *S. aureus*. Exposure of pathogens with nanoemulsions resulted in a significant reduction in the biofilm formation by all examined bacteria i.e., *S. aureus*, *K. pneumonia*, *E. coli*, and *P. aeruginosa*. This study firstly investigated the chemical composition of *C. aurantium* and *A. annua* EO. Their nanoemulsion dosage forms were then prepared. The antimicrobial and antibiofilm activities of nanomulsions were unraveled. Both nanoemulsions inhibited the growth of *S. aureus*. Interestingly, no biofilms of *S. aureus*, *K. pneumonia*, *E. coli*, and *P. aeruginosa* were observed after treatment with these nanoemulsions. Moreover, *A. annua* nanoemulsion inferred potent antioxidant properties. Limitations of this study mostly included low number of bacterial pathogens, lack of *in silico* assessment of binding of bioactive compounds to bacterial targets and lack of *in vivo* study.

Acknowledgements

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Authors' contributions

MO and AG conceptualized and designed the study. AG performed antibacterial tests and revised the MS. HA drafted MS in cooperation with MO. MAH and BM prepared nanoemulsions. EZ interpreted ATR-FTIR. MZ performed the DPPH assay. MO designed the

study, supervised the project, and drafted the MS. All authors contributed to drafting MS and approved the final version.

Conflict of interest

None to declare.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Ethics

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Reference

1. Mestrovic T, Aguilar GR, Swetschinski LR, Ikuta KS, Gray AP, Weaver ND, et al. The burden of bacterial antimicrobial resistance in the WHO European region in 2019: A cross-country systematic analysis. The Lancet Public Health. 2022;7(11):e897-e913.
2. Laxminarayan R. The overlooked pandemic of antimicrobial resistance. The Lancet. 2022;399(10325):606-7.
3. Noorpisheh Ghadimi S, Sharifi N, Osanloo M. The leishmanicidal activity of essential oils: A systematic review. Journal of Herbmed Pharmacology. 2020;9(4):300-8.
4. Qasemi H, Fereidouni Z, Karimi J, Abdollahi A, Zarenezhad E, Rasti F, et al. Promising antibacterial effect of impregnated nanofiber mats with a green nanogel against clinical and standard strains of Pseudomonas aeruginosa and Staphylococcus aureus. Journal of Drug Delivery Science and Technology. 2021:102844.
5. Kanwar R, Rathee J, Salunke DB, Mehta SK. Green nanotechnology-driven drug delivery assemblies. ACS omega. 2019;4(5):8804-15.
6. Swain SS, Paidesetty SK, Padhy RN, Hussain T. Nano-technology platforms to increase the antibacterial drug suitability of essential oils: A drug prospective assessment. OpenNano. 2022:100115.
7. Rahchamani R, Zarooni S, Borhani MS. The Chemical Composition and Antibacterial Effect of Essential Oils of Rosemary and Basil in Milk. Iranian Journal of Veterinary Medicine. 2024. DOI:10.22059/IJVM.2024.3 2443 7 .100 7 5 51.
8. Farzaneh M, Fadaei V, Gandomi H. Antioxidant, Syneresis, and Sensory Characteristics of Probiotic Yogurt Incorporated With Agave tequilana Aqueous Extract. Iranian Journal of Veterinary Medicine. 2023;17(3):243-52.

9. Ali Anvar SA, Nowruzi B, Afshari G. A Review of the Application of Nanoparticles Biosynthesized by Microalgae and Cyanobacteria in Medical and Veterinary Sciences. Iranian Journal of Veterinary Medicine. 2023;17(1).

10. Khaji L, Noori N, Jebelli Javan A, Khanjari A, Ghandomi Nasrabadi H. Antimicrobial effect of Cuminum Cyminum essential oil on Iranian white soft cheese in air and modified atmosphere packaging during refrigerated storage. Iranian Journal of Veterinary Medicine. 2023.

11. Maksoud S, Abdel-Massih RM, Rajha HN, Louka N, Chemat F, Barba FJ, et al. Citrus aurantium L. Active Constituents, Biological Effects and Extraction Methods. An Updated Review. Molecules. 2021;26(19).

12. Gao L, Zhang H, Yuan CH, Zeng LH, Xiang Z, Song JF, et al. Citrus aurantium 'Changshan-huyou'-An ethnopharmacological and phytochemical review. Frontiers in Pharmacology. 2022;13:983470.

13. Liu S, Lou Y, Li Y, Zhang J, Li P, Yang B, et al. Review of phytochemical and nutritional characteristics and food applications of Citrus L. fruits. Frontiers in Nutrition. 2022;9:968604.

14. Okla MK, Alamri SA, Salem MZ, Ali HM, Behiry SI, Nasser RA, et al. Yield, phytochemical constituents, and antibacterial activity of essential oils from the leaves/twigs, branches, branch wood, and branch bark of Sour Orange (Citrus aurantium L.). Processes. 2019;7(6):363.

15. Kačániová M, Terentjeva M, Galovičová L, Ivanišová E, Štefániková J, Valková V, et al. Biological activity and antibiofilm molecular profile of Citrus aurantium essential oil and its application in a food model. Molecules. 2020;25(17):3956.

16. Anibogwu R, Jesus K, Pradhan S, Pashikanti S, Mateen S, Sharma K. Extraction, Isolation and Characterization of Bioactive Compounds from Artemisia and Their Biological Significance: A Review. Molecules. 2021;26(22).

17. Bilia AR, Santomauro F, Sacco C, Bergonzi MC, Donato R. Essential Oil of Artemisia annua L.: An Extraordinary Component with Numerous Antimicrobial Properties. Evidence Based Complementary and Alternative Medicine. 2014;2014:159819.

18. Mirzaei-Najafgholi H, Tarighi S, Golmohammadi M, Taheri P. The Effect of Citrus Essential Oils and Their Constituents on Growth of Xanthomonas citri subsp. citri. Molecules. 2017;22(4):591.

19. Osanloo M, Ghaznavi G, Abdollahi A. Sureveying the chemical composition and antibacterial activity of essential oils from selected medicinal plants against human pathogens. Iranian Journal of Microbiology. 2020;12(6):505-12.

20. Catania AM, Di Ciccio P, Ferrocino I, Civera T, Cannizzo FT, Dalmasso A. Evaluation of the biofilm-forming ability and molecular characterization of dairy Bacillus spp. isolates. Frontiers in Cellular and Infection Microbiology. 2023;13.

21. Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. Journal of Microbiological Methods. 2000;40(2):175-9.

22. Durán Aguirre CE, Pratissoli D, Damascena AP, Romário de Carvalho J, de Araujo Junior LM. Lethal and sublethal effects of Citrus aurantium and Citrus sinensis essential oils and their major component limonene on Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae). Journal of Essential Oil Bearing Plants. 2024;27(3):838-48.

23. Madhuri S, Hegde AU, Srilakshmi N, Prashith Kekuda T. Antimicrobial activity of Citrus sinensis and Citrus aurantium peel extracts. Journal of Pharmaceutical and Scientific Innovation (JPSI). 2014;3(4):366-8.

24. Marinas IC, Oprea E, Chifiriuc MC, Badea IA, Buleandra M, Lazar V. Chemical composition and antipathogenic activity of Artemisia annua essential oil from Romania. Chemistry & Biodiversity. 2015;12(10):1554-64.

25. Al-Mothafar NA, Al-Shahwany AW. Phenolic compounds from Thymus vulgaris, Artemisia annua extracts and pure Thymol were tested against twenty Pseudomonas spp. strains for antibacterial and anti-biofilm activities. IOP Journal of Physics Under review. 2022
26. Das S, Vörös-Horváth B, Bencsik T, Micalizzi G, Mondello L, Horváth G, et al. Antimicrobial activity of different Artemisia essential oil formulations. Molecules. 2020;25(10):2390.
27. Mariadoss AVA, Saravanakumar K, Sathiyaseelan A, Naveen KV, Wang M-H. Enhancement of anti-bacterial potential of green synthesized selenium nanoparticles by starch encapsulation. Microbial Pathogenesis. 2022;167:105544.