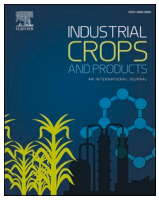
Industrial Crops & Products 210 (2024) 118096

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Chemical composition, cytotoxic effects and antimicrobial activity of combined essential oils from *Citrus meyeri, Citrus paradise,* and *Citrus sinensis* leaves

Mahmoud Hamdan [a], Nidal Jaradat [b], Nawaf Al-Maharik [c], Shurooq Ismail [d], Mohammad Qadi [d,*]

[a] PhD Program in Clinical Laboratory Science, Department of Medical and Health Sciences, Faculty of Graduate Studies, An-Najah National University, 7, Nablus, State of Palestine
[b] Department of Pharmacy, Faculty of Medicine and Health Sciences, An-Najah National University, 7, Nablus, State of Palestine
[c] Department of Chemistry, Faculty of Sciences, An-Najah National University, 7, Nablus, State of Palestine
[d] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, An-Najah National University, 7, Nablus, State of Palestine

ARTICLE INFO

Keywords:
Citrus meyeri
Citrus paradise
Citrus sinensis
Cytotoxic
Antimicrobial
Synergistic

ABSTRACT

Plants are the most abundant primary source of active phytochemicals, which are needed to treat a wide range of ailments. The current study aimed to investigate the chemical composition, cytotoxic, and antimicrobial activities of combined essential oils (EOs) from *Citrus meyeri, Citrus paradise,* and *Citrus sinensis* leaves. Gas chromatography-mass spectrometry identified the chemical composition, while the MTS technique was used to measure the EOs' cytotoxicity. The antimicrobial activities were assessed by broth microdilution assay against several fungal and bacterial strains. The EO content of *C. meyeri* revealed the presence of 27 compounds, totaling 97.5% of the EO. In comparison, *C. paradise* EO has 20 compounds, making up 99.8% of the composition, while *C. sinensis* EO contains 32 compounds, constituting 99.9% of its content. Limonene (43.2%), β-pinene (44.5%), and sabinene (55.9%) were the most abundant EOs identified in the three analyzed Citrus species namely *C. meyeri, C. paradise,* and *C. sinensis*, respectively. Moreover, MIC values of three EOs showed variable antimicrobial activity against the tested strains. On the contrary, the examination of the combined effects of the identified EOs showed synergistic results, as indicated by the Fractional Inhibitory Concentration (FIC) Index. Specifically, *C. meyeri* and *C. paradise* EOs exhibited synergistic effects (with FIC Index values of 0.73 and 0.08, respectively) against *Escherichia coli* and *Candida albicans*. Similarly, *C. meyeri* and *C. sinensis* EOs demonstrated synergy (with FIC Index values of 0.83 and 0.13, respectively) against *Proteus vulgaris* and *C. albicans*. Moreover, when *C. meyeri, C. paradise*, and *C. sinensis* EOs were combined, they synergistically affected *C. albicans*, with an FIC Index of 0.88. The cytotoxicity evaluation of the EO isolated from *C. meyeri* against HeLa, HepG2, and CaCo-2 cancer cell lines revealed its robust anticancer potentialities compared to the other studied Citrus species. Collectively, the achieved results support the possible therapeutic applications of the EOs from *C. meyeri, C. paradise,* and *C. sinensis,* and their combinations to be used as antimicrobial or anticancer agents.

1. Introduction

Herbal remedies refer to medications sourced from plants and are generally deemed safe when administered in appropriate dosages and align with cultural acceptance (Ugboko et al., 2020). Plants are nature's most abundant primary source of active drugs, which are essential for the medical treatment of diverse diseases (Jaradat, Abdallah et al., 2022). The biological activity of medicinal plants is usually attributed to the various phytochemicals contained in them (Jaradat, Qneibi et al., 2022). These phytochemicals have also been widely utilized in natural preservatives and cosmetics since ancient times (Mahmoud and Wafa, 2020). In fact, plants may contain potent bioactive molecules that have powerful antimicrobial, anticancer, wound-healing, analgesic, anti-inflammatory, and many other therapeutic properties

* Corresponding author.
E-mail addresses: mahmoud.hamdan@najah.edu (M. Hamdan), nidaljaradat@najah.edu (N. Jaradat), n.maharik@najah.edu (N. Al-Maharik), m.qadi@najah.edu (M. Qadi).

https://doi.org/10.1016/j.indcrop.2024.118096
Received 18 November 2023; Received in revised form 29 December 2023; Accepted 15 January 2024

(Elizondo-Luévano et al., 2022). The World Health Organization estimates that 80% of the world's population in developing countries uses folk medicine for their healthcare needs (Jubair et al., 2021). A plant's essential oil (EO) is a hydrocarbon compound and second metabolite containing multiple defensive and other properties (Jaradat et al., 2021). A plant's EOs can be found in leaves, fruits, seeds, flowers, rhizomes, and woods and are native to various types of plants (Altemimi et al., 2017). A growing body of research has demonstrated that EOs and their components have excellent antibacterial, antiparasitic, insecticidal, antiviral, antifungal, anticancer, and antioxidant properties (Ji et al., 2023).

Developed and developing countries consider bacterial infections one of the most significant causes of illness (Jubair et al., 2021). The treatment of various bacterial pathogens is traditionally accomplished with various conventional therapies, including antibiotics. However, the use of synthetic antimicrobials has been linked to hypersensitivity, allergies, asthma, gastrointestinal disorders, and cancer (Mahmoud and Wafa, 2020). Using synthetic antimicrobials can also lead to antimicrobial resistance and a reduction in harmless bacteria in the human intestines (Mahmoud and Wafa, 2020). Multidrug resistance has, however, exacerbated the severity of diseases caused by bacterial pathogens (Terreni et al., 2021). Even today, bacterial infections cause a significant proportion of human deaths. Multidrug-resistant bacteria kill 700,000 people each year worldwide, among the two million patients infected with various types of bacteria (Jubair et al., 2021).

In terms of mortality and morbidity, cancer is one of the leading causes worldwide (Wang et al., 2018). It is a chronic disease developed by the growth of abnormal cells in the body's tissues that destroy normal cells (Ulil Amna, Halimatussakdiah, Puji Wahyuningsih & Rosnani Nasution1, 2019). Among all the causes of death worldwide, cancer ranks second. Palestinians are also plagued by cancer, which is considered the second-leading cause of death. About 14% of deaths are caused by cancer after heart disease, which is about 30% of deaths in Palestine (Battat and Marie, 2022). In 2020, 19.3 million cancer cases and 10 million cancer deaths worldwide were reported, according to the World Health Organization. This is a significant increase in comparison to 2018 when 18.1 million cases and 9.6 million deaths were reported (Elizondo-Luévano et al., 2022). Given the inefficiency and toxicity of actual cancer treatments, there is a great need for safer and more effective anticancer drugs (Grewal et al., 2022).

Tropical and subtropical regions have given rise to many Citrus trees in the world (Lin et al., 2021). There are approximately 140 genera and 1300 species of Citrus trees in the Rutaceae family (Kamal et al., 2011). In the Rutaceae, *Citrus* genus belongs to the *Aurantioideae*, a monophyletic subfamily that includes important crops like oranges, lemons, pummelos, grapefruits, limes, etc. (Johnson et al., 2022). It is believed that the species of Citrus plants originated in the Himalayan foothills of Northern India, Northern Myanmar, Southeast Asia, and Southern China (Mahato et al., 2019). They were later introduced to other parts of the world and have become one of the most valuable fruits in the world. A fruit-bearing shrub with incredible nutritional attributes and economic importance, *Citrus sinensis* L. Osbeck (synonym *Citrus* ×*aurantium* L.) is widely cultivated with its fruit-bearing properties around the world (Gmitter et al., 2012; Kammoun et al., 2021). Several traditional dishes use *C. sinensis* leaves as an ingredient, especially as a tea flavoring agent (Hosni et al., 2013). *Citrus meyeri* N.L. Burman, (synonym *Citrus* ×*limon* (L.) Osbeck) or Meyer lemon, is one of several hybrid Citrus fruits originating in Beijing, China, which is a cross between *Citrus limon* (lemon) and *Citrus sinensis* (orange) (Miyake et al., 2012). Compared to other common lemon varieties, Meyer lemon has the highest juice yield per box, is thornless, and is well adapted to warm climates (Abbas et al., 2020). In 1821, Don Philippe introduced *Citrus paradise* (synonym *Citrus* × *aurantium* f. *aurantium*) to Texas after discovering it in Barbados in the Caribbean. Natural hybridization occurred between *Citrus maxima* (pummelo) and *Citrus sinensis* (orange) in this fruit (Paoli et al., 2016). The literature describes the chemical composition of *C. paradise* leaf EO as heterogeneous. Among the compounds extracted from grapefruit oils, sabinene is the most prevalent (Zanganeh et al., 2021). Different volatile compounds and biological activities are present in Citrus leaves, a plant biomass (Chi et al., 2020a). The Citrus's EO has a long history of human consumption, making it one of the major commercial products. In the pharmaceutical, sanitary, cosmetic, agricultural, and food industries, Citrus EOs exhibit antioxidant, antidiabetic, insecticidal, antifungal, and antibacterial properties (Fagodia et al., 2017). Due to their relatively safe nature and widespread consumer acceptance, Citrus EOs are one of the world's largest agro-industries (Lin et al., 2021).

Another strategy used to exploit the activity of EOs is the process in which chemistry and biology interact or combine in synergistic ways to produce effects that are greater than the effects of their components. This is known as synergy and it can be used to amplify the biological activities influence, attack multiple target sites, reduce the risk of side effects, and reduce the cost of using EOs (Gadisa and Usman, 2021). A recently proposed strategy to increase the antimicrobial spectrum and to overcome antimicrobial resistance is the combination of several antimicrobials and plant extracts (Aiyegoro et al., 2009). Because they target different pathways to produce multifaceted effects against powerful bacterial defenses, EOs, and antibiotics, combined, produce stronger bacterial inhibition than when they are administered individually (Chouhan et al., 2017). By combining these agents, synergism is achieved which will minimize the minimal inhibitory concentrations (MICs) of the agents, thus reducing both economic costs and sensory effects (Aleksic et al., 2014). Many combinations of antimicrobial agents have already been confirmed to be effective, including EOs/EOs, Extracts/Extracts, and Nanoparticles/Nanoparticles (Ncube et al., 2012).

Recently, several studies have focused on developing promising solutions to overcome antibiotic-resistant pathogens and cancer treatment problems. It is a promising alternative strategy to increase the antimicrobial and cytotoxic effects of two or more essential oil or their components by combining them or interacting their components with different drugs (Reda et al., 2017). It has been demonstrated that combinations of various antimicrobial agents enhanced their effectiveness, reduced the dosage used, produced fewer side effects, had better synergistic effects, attacked multiple target sites, and reduced risks over their use individually (Aleksic et al., 2014).

The current study aims to investigate the chemical composition, cytotoxic, and antimicrobial activities of combined EOs from *C. meyeri, C. paradise,* and *C. sinensis* leaves for possible applications in antimicrobial treatments or anticancer therapies.

2. Materials and methods

2.1. Plant Materials

The *C. meyeri, C. paradise,* and *C. sinensis* leaves were collected from Tulkarem city (Latitude: 31°56' 34'' N; Longitude: 35°15'25'' E) of Palestine in April 2023. Dr. Nidal Jaradat performed the characterization and deposition of the plants at An-Najah National University's Pharmacognosy Laboratory (voucher codes: Pharm-PCT-2803, Pharm-PCT-2804, and Pharm-PCT-2775, respectively). The gathered leaves were washed and dried at 25 °C and 55% relative humidity for approximately 25 days. After drying, the leaves were roughly crushed and preserved in amber glass jars to be extracted later.

2.2. Essential oils extraction

According to previous studies, the EOs were extracted by using hydrodistillation method with Clevenger apparatus running at atmospheric pressure by suspending One kilogram of the dried leaf powder in 5 L of distilled water with a 6 ml/min flow condensation rate for 3 h at 100 °C. The extracted EOs were subjected to chemical drying with calcium chloride, and the resulting percentage yield was assessed (Qadi

Table 1
Chemical composition of *C. meyeri*, *C. paradise*, and *C. sinensis* EOs from the Tulkarem region of Palestine.

Compound names	RT	RI	RI $_{lit}$	% of *C. meyeri* area	% of *C. paradise* area	% of *C. sinensis* area
α-Thujene	9.46	923	924	0.1	0.1	0.4
α − Pinene	9.78	931	932	0.5	5.3	2.3
Sabinene	11.55	970	971	0.4	4.1	55.9
β-Pinene	11.73	974	973	0.4	44.5	2.8
Myrcene	12.33	987	987	0.4	0.9	3.0
α-Phellandrene	13.01	1002	1003	ND	2.8	ND
δ-3-Carene	13.18	1006	1007	ND	ND	4.4
α-Terpinene	13.51	1014	1016	ND	ND	0.6
p-Cymene	13.83	1021	1023	0.5	ND	0.2
Limonene	14.05	1027	1029	43.2	ND	4.6
β- Phellandrene	14.08	1027	1030	ND	26.6	ND
1,8-Cineole	14.16	1029	1032	0.1	ND	ND
Cis-Ocimene	14.37	1034	1034	0.2	0.3	0.3
trans-Ocimene	14.81	1044	1044	5.2	10.0	8.4
γ -Terpinene	15.29	1056	1055	0.4	0.2	1.2
trans-4-Thujanol	15.82	1068	1070	ND	ND	0.6
p-Mentha-2,4(8)-diene	16.29	1079	1082	ND	ND	0.1
Terpinolene	16.45	1083	1083	ND	ND	1.1
Linalool	17.17	1100	1101	ND	ND	7.2
Citronellal	19.11	1149	1150	ND	ND	0.1
Terpinen-4-ol	20.24	1179	1179	ND	ND	2.2
α-Terpineol	20.78	1193	1193	ND	ND	0.4
Citronellol	21.97	1225	1225	ND	ND	0.1
β- Fenchyl acetate	22.35	1236	1226	ND	ND	0.3
Geranial	23.42	1265	1267	ND	ND	0.4
d-Elemene	25.75	1333	1337	0.8	ND	ND
Citronellyl acetate	26.19	1346	1347	ND	0.1	0.1
Neryl acetate	26.5	1355	1355	ND	0.2	0.1
Geranyl acetate	27.15	1375	1377	ND	0.3	ND
β-Elemene	27.57	1387	1393	0.4	ND	0.4
β-Caryophelene	28.55	1418	1418	2.9	2.1	0.4
γ-Elemene	28.96	1431	1432	0.1	ND	ND
α-Caryophellene	29.68	1454	1455	0.5	0.1	0.1
Germacrene D	30.48	1479	1478	0.8	ND	ND
Trans-β-ionone	30.49	1480	1484	ND	0.1	ND
Bicyclogermacrene	30.95	1495	1495	ND	1.5	0.2
Z-a-Bisabolene	31.28	1505	1505	1.2	ND	ND
Germacrene A	31.3	1506	1505	ND	ND	0.4
d-Cadinene	31.86	1525	1525	0.2	ND	ND
Trans-γ -bisabolene	32.06	1532	1530	0.1	ND	ND
Elemol	32.52	1547	1549	6.5	ND	ND
Germacrene B	32.85	1559	1558	6.6	0.2	ND
cis-3-Hexenyl benzoate	33.15	1569	1568	ND	ND	0.1
Spathulenol	33.4	1577	1577	ND	0.2	ND
Caryophellene oxide	33.54	1582	1582	0.1	ND	ND
Guaiol	33.95	1596	1596	2.2	ND	ND
Muurola-4,10(14)-diene-1-b-ol	34.71	1623	1625	0.9	ND	ND
γ-Eudesmol	34.87	1629	1632	5.2	ND	ND
α-Eudesmol	35.64	1656	1652	17.6	0.2	ND
Germacrone	36.65	1692	1693	ND	ND	1.2
α-Sinensal	38.14	1748	1748	ND	ND	0.4
Total				97.5	99.8	99.9
Phytochemical groups						
Hydrocarbon monoterpene				51.3	94.7	84.1
Oxygenated monoterpene				0.1	0.0	11.6
Hydrocarbon sesquiterpene				12.5	3.9	2.6
Oxygenated Sesquiterpene				32.4	0.4	0.4
Others				1.2	0.8	1.2
Total				97.5	99.8	99.9

*Note: Compounds are listed in order of their elution from a Perkin Elmer Elite-5-MS fused silica capillary column; RT: Retention Time; RI: Retention Index; RI $_{lit}$: retention index according to (NIST 08, 2008) and/or the (pherobase.com) library. ND: Not detected.

et al., 2023). The procedure was applied in the same way for all types of leaves. Finally, the extracted oils were stored in an amber-colored glass vial at 4 °C until further use (Jaradat, Abdallah et al., 2022).

2.3. Qualitative and quantitative analysis of the extracted oils

The chemical composition of the extracted oils was determined by GC–MS (Gas Chromatography-Mass Spectrometry) using the Perkin Elmer Clarus 500 Gas Chromatograph. A Perkin Elmer Clarus 560 mass spectrometer was connected to this apparatus. Separation of the samples was achieved by using Perkin Elmer Elite-5-MS fused silica capillary columns (film thickness 0.25 m, 30 m × 0.25 mm). As the column temperature rose from 50 °C to 280 °C, it was set to rise by 4 °C every minute. Helium flowed at a constant rate of 1 ml/min throughout the chromatographic run as the carrier gas. Using a split ratio of 1 to 50, one μL of the tested oils was dissolved in acetonitrile and injected in split mode at 250 °C. In addition, mass spectra of the chemical components were compared with reference spectra from NIST's MS Data Center, and their Kovats and retention indices were compared to literature values. Kovats Retention Index (KRI) for each compound was calculated by

Table 2
MIC values (mg/ml) of *C. meyeri, C. paradise, C. sinensis* EOs and the control antimicrobial agents (Fluconazole and Ciprofloxacin).

Isolates		MIC (mg/ml)				
		C. meyeri	*C. paradise*	*C. sinensis*	Ciprofloxacin	Fluconazole
S. aureus	ATCC 25923	3.1 ± 1.8	50 ± 21.7	25 ± 0	0.00078	-
MRSA	Clinical strain	25 ± 14	33.3 ± 14	41.7 ± 14	0.013	-
E. coli	ATCC 25922	50 ± 29	66.7 ± 29	66.7 ± 29	0.00156	-
K. pneumoniae	ATCC 13883	100 ± 0	50 ± 21.7	66.7 ± 29	0.000125	-
P. vulgaris	ATCC 8427	100 ± 0	66.7 ± 29	66.7 ± 29	0.015	-
P. aeruginosa	ATCC 9027	50 ± 29	83.3 ± 14	100 ± 0	0.0031	-
C. albicans	ATCC 90028	50 ± 29	83.3 ± 14	100 ± 0	-	0.00156

Note: Values represent mean ± standard deviation of experiments conducted in triplicate and the p values < 0.05.

using the retention time value from the Hydrocarbons Alkane standard (Jaradat, Abdallah et al., 2022).

2.4. Antimicrobial activity assays

2.4.1. Microbial strains and growth conditions

The microorganisms used in this research were obtained from the American Type Culture Collection (ATCC) in addition to one clinical isolate. The ATCC strains include Gram-positive, Gram-negative, and one-fungal strains including *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Proteus vulgaris* (ATCC 8427), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 90028). At the same time, the clinical isolate was Methicillin-resistant *Staphylococcus aureus* (MRSA) obtained from An-Najah National University Hospital. The mentioned bacterial strains, in all experiments, were inoculated and grown overnight on nutrient agar media (on sabouraud dextrose agar for *C. albicans*) at 37 °C. The next day, bacterial suspension was freshly prepared in Mueller Hinton Broth (MHB). Similar conditions were applied for *C. albicans*, but with Roswell Park Memorial Institute (RPMI) media instead of MHB.

2.4.2. Experimental framework for assessing the synergistic potential of EO combinations

Within the context of this study, the experimental design was structured to encompass a total of seven distinct trials, with the primary objective of evaluating the efficacy of EOs combinations. Each trial was explicitly dedicated to the application of either an individual EO or a carefully selected combination thereof. The trials were categorized as follows: (1) *C. meyeri*, (2) *C. paradise*, (3) *C. sinensis*, (4) *C. meyeri* + *C. paradise*, (5) *C. meyeri* + *C. sinensis*, (6) *C. paradise* + *C. sinensis,* and (7) *C. meyeri* + *C. paradise* + *C. sinensis.*

It is worth highlighting that trials 4, 5, 6, and 7 involved the meticulous blending of these EOs in equal proportions, thus forming a significant component of the experimentation (Rapper et al., 2021). This structured approach was employed to systematically investigate the potential synergistic effects arising from the combined application of these essential oils, offering a comprehensive evaluation of their antimicrobial properties.

2.4.3. Broth micro-dilution method

The evaluation of the antibacterial efficacy of the EOs was done as previously described and carried out employing the broth microdilution assay (Qadi et al., 2023; Jaradat, Abdallah et al., 2022). Each EO was dissolved in DMSO to an initial concentration of 200 mg/ml. Subsequently, the resulting solution underwent a serial micro-dilution process, wherein each was diluted by a factor of 2, repeated ten times, using sterile MHB. The dilution procedures were carried out in a sterile setting within the 96-well plate. The eleventh well consisted of MHB without EO, which served as a positive control to assess microbial growth. In contrast, well number twelve was filled with MHB that was devoid of EO and microbes. This particular well served as a negative control to assess the absence of microbial growth. The Wells numbers ranging from 1 to 11 were aseptically inoculated with the test microorganisms, which had been previously prepared in MHB, in order to achieve a standardized bacterial concentration. The experiment evaluating antimicrobial activity was conducted in triplicate. The plates that had been inoculated were placed in an incubator set at a temperature of 37 °C. In the case of C. *albicans*, a similar approach was employed, but with the substitution of RPMI medium in place of MHB. The duration of the incubation time was approximately 18–24 h for the plates that were inoculated with the test bacterial strains, whereas it extended to approximately 48 h for the plates that were inoculated with *C. albicans*. The least inhibitory concentration (MIC) of the studied extract was determined as the lowest concentration at which no apparent microbial growth was observed in the micro-well. The assessment of antimicrobial activity involved the utilization of established antimicrobial drugs, including Ciprofloxacin, which served as positive control for antibacterial activity. Additionally, Fluconazole was employed as a positive control to examine antifungal activity (Jaradat et al., 2020).

2.4.4. Determination of fractional inhibitory concentration (FIC) index of EOs combinations

The effect of EO binary combinations was also determined against the six bacterial strains and the *C. albicans*. Trials 4, 5, 6, and 7 represent the combination strategy using each of these trials as one EO. Fractional Inhibitory Concentration (FIC) indexes were calculated by using the conventional "checkboard" assay (Ayari et al., 2020). The FIC_{Index} was determined using the following formulas:

1. $FIC\ A = \frac{\text{MIC of A in the presence of B}}{\text{MIC of A individually}}$
2. $FIC\ B = \frac{\text{MIC of B in the presence of A}}{\text{MIC of B individually}}$
3. $FIC_{Index} = FIC\ A + FIC\ B.$

FIC Value of < 1.0, 1.0, or > 1.0 indicates that the tested EO combination is synergistic, additive, or antagonistic, respectively (Reyes--Jurado et al., 2016).

2.5. Cytotoxic effects

The seven trials of EOs were in vitro evaluated against four eukaryotic cell lines, including cervical cancer (HeLa), hepatocellular carcinoma (HepG2), hepatic stellate (LX-2), and colorectal adenocarcinoma (Caco-2) cells to determine the cytotoxicity of their IC_{50} (50% growth inhibition) values at various concentrations (62.5, 125, 250, 500, and 1000 μg/ml). Hence, Doxorubicin was used as a positive control and DMSO as a negative control. According to Jaradat et al. (2022), to cultivate cancer cells, RPMI-1640 media was supplemented with 1% L-glutamine, Penicillin/Streptomycin antibiotics, and 10% fetal bovine serum. At 37 °C, these cancer cells were cultured in a humidified environment with 5% CO_2 and seeded in a 96-well plate at 2.6×10^4 cells/well. After 24 h, cells were confluent, media was changed, and essential oils were incubated with cells for 24 h. According to the

Table 3
MIC values (mg/ml) of combined *C. meyeri, C. paradise,* and *C. sinensis* EOs and corresponding Fractional Inhibitory Concentration Index (FIC $_{Index}$).

Isolates	MIC (mg/ml)							
	C. meyeri + *C. paradise*	FIC Index	*C. meyeri* + *C. sinensis*	FIC Index	*C. paradise* +*C. sinensis*	FIC Index	*C. meyeri* + *C. paradise* +*C. sinensis*	FIC Index
S. aureus	10.4 ± 3.6		4.2 ± 1.8		20.8 ± 7.2		8.3 ± 3.6	
MRSA	20.8 ± 7.2		66.7 ± 29		25 ± 0.0		25 ± 0.00	
E. coli	20.8 ± 7.2	0.73	133 ± 58		100 ± 0.0		50 ± 0.00	
K. pneumoniae	41.7 ± 14		66.7 ± 29		100 ± 0.0		50 ± 0.00	
P. vulgaris	41.7 ± 14	1.0	33.3 ± 14	0.83	50 ± 0.0		41.7 ± 14	
P. aeruginosa	41.7 ± 14		NA		100 ± 0.0		133 ± 58	
C. albicans	2.6 ± 0.9	0.08	4.2 ± 1.8	0.13	NA		20.8 ± 7.2	0.88

Note: NA: No antimicrobial activity; values represent mean ± standard deviation of experiments conducted in triplicate

manufacturer's instructions, cell viability was determined using the Cell Tilter 96® Aqueous One Solution Cell Proliferation (MTS) Assay. The treatment was completed with the addition of 20 µL of MTS solution per 100 µL of media in each well, followed by 24-h incubation at 37 °C. At a wavelength of 490 nm, the absorbance was measured using a UV-Vis spectrophotometer (Jaradat, Abdallah et al., 2022). All experiments were carried out in three independent replicates.

2.6. Statistical analysis

A triplicate cytotoxicity and antimicrobial assays were performed on EOs, and the results were presented as means (±) standard deviation (SD). The Student t-test was used for statistical analysis. A p-value < 0.05 was considered statistically significant. For this, the SPSS21 (IBM, USA) computer program was used.

3. Results

3.1. Extracted EOs characteristics

The EOs extracted from *C. sinensis, C. paradise*, and *C. meyeri* leaves demonstrated average yields of 0.13%, 0.03%, and 0.4% (w/w), respectively. These EOs exhibited characteristic colors, with *the C. sinensis* oil displaying a bright yellow color, the *C. meyeri* oil presenting a greenish tint, and the *C. paradise* oil being colorless. Additionally, the *C. sinensis* EO emitted a robust aroma, while the *C. meyeri* and *C. paradise* EOs had more subtle fragrances.

3.1.1. Phytochemistry

The chemical compositions of three Citrus EOs were analyzed by GC-MS. A peak area normalization method was used to calculate the relative content of each component; a retention index, Mass Spectral Library data from NIST, and literature data were used to identify elements. On the Elite-5-MS fused silica capillary column, Table1 shows the elution sequence of the compounds that were discovered, as well as their percentages of area, retention times (RT), and Retention index (RIs) for the *C. meyeri*, *C. paradise*, and *C. sinensis* EO samples. GC/MS analysis identified 27, 20, and 32 compounds from *C. meyeri, C. paradise, and C. sinensis* EO samples, respectively. Based on the specified components of the tested EOs from the three areas, 97.5%, 99.8%, and 99.9% of the total EOs were identified, respectively. The primary constituents in the EO extracted from *C. meyeri* included limonene (43.2%), α- eudesmol (17.6%), germacrene B (6.6%), elemol (6.5%), and trans-ocimene (5.2%). On the other hand, the dominant components in the EO obtained from *C. paradise* consists of β-pinene (44.5%), β-phellandrene (26.5%), trans-ocimene (10.0%), α-pinene (5.2%), and sabinene (4.1%). Finally, the major constituents found in the EO of *C. sinensis* are sabinene (55.9%), trans-ocimene (8.4%), linalool (7.2%), limonene (4.6%), and δ-3-carene (4.4%).B.

As shown in Table 1, the components of the tested EOs were classified into five groups; hydrocarbon monoterpene, oxygenated monoterpene, hydrocarbon sesquiterpene, oxygenated sesquiterpene, and others. The hydrocarbon monoterpenes were the most abundant category among the examined EOs, where they accounted for 51.3%, 94.7%, and 84.1% of the total EOs composition of *C. meyeri, C. paradise, and C. sinensis*, respectively. The second abundant category was varied among the three analyzed citrus oils, with oxygenated sesquiterpenes comprising 32.4% of *C. meyeri* EO composition. While, hydrocarbon sesquiterpenes were almost 3.9% of *C. paradise* EO composition, and oxygenated monoterpenoids made up 11.6% of *C. sinensis* EO composition. On the other hand, the hydrocarbon sesquiterpene content of *C. meyeri* EO was more abundant (12.5%) than those in *C. paradise and C. sinensis* (3.9% and 2.6%, respectively). Moreover, oxygenated monoterpene (0 - 11.6%) and hydrocarbon sesquiterpene (2.6– 12.5%) comprise a minor proportion of the identified phytochemical groups. It is worth mentioning that only one oxygenated monoterpenoid, 1,8-cineole (0.1%), was characterized in the EO content of *C. meyeri* and there is no compound of this group in *C. paradise*.

3.2. Antimicrobial Activity of EOs tested individually

The antimicrobial activity was in vitro evaluated for EOs which were isolated and identified from Citrus plants collected from the Tulkarem region of Palestine. The antimicrobial activities of the tested EOs were assessed using a broth microdilution assay against one fungal strain and six bacterial strains. Table 2 shows that the three EOs had varying antimicrobial activity against all strains tested. A remarkable bacterial inhibitory effect of *C. meyeri* EO was observed against *S. aureus* ATCC 25923, with a MIC value of 3.1 ± 1.8 mg/ml. Moreover, *C. meyeri, C. paradise, and C. sinensis* EOs had potent antibacterial activity against the MRSA strain, with MIC values of 25 ± 14 mg/ml, 33.3 ± 14 mg/ml, and 41.7 ± 14 mg/ml, respectively. Furthermore, the three tested EOs of *C. meyeri, C. paradise,* and *C. sinensis* had conferred antimicrobial activity against *P. vulgaris* ATCC 8427 (MIC values of 100 ± 0, 66.7 ± 29, and 66.7 ± 29 mg/ml, respectively). It is worth noting that the antifungal activity of the tested citrus EOs against *C. albicans* fungal species is noted in our results.

3.3. Determination of Fractional Inhibitory Concentration (FIC) Index of EOs combinations

The antimicrobial activities of the EOs combinations were evaluated using the broth microdilution method. The MIC of the EOs combinations was determined against the same microbial strains in the previous section (Table 3). All EOs combinations displayed antibacterial activity against all tested microorganisms, except when *C. meyeri* and *C. sinensis* were combined, as well as, the *C. paradise* and *C. sinensis* EOs combination were tested against *P. aeruginosa* and *C. albicans*, respectively. The FIC $_{Index}$ results indicated synergistic effects against *E. coli* and *C. albicans* by combining equal volumes of *C. meyeri and C. paradise* EOs (FIC $_{Index}$ 0.73 and 0.083, respectively). Also, a synergistic effect was obtained by combining *C. meyeri* and *C. sinensis* EOs against *P. vulgaris* and *C. albicans* (FIC $_{Index}$ 0.83 and 0.13, respectively). Additionally, when *C. meyeri, C. paradise*, and *C. sinensis* EOs were combined, a

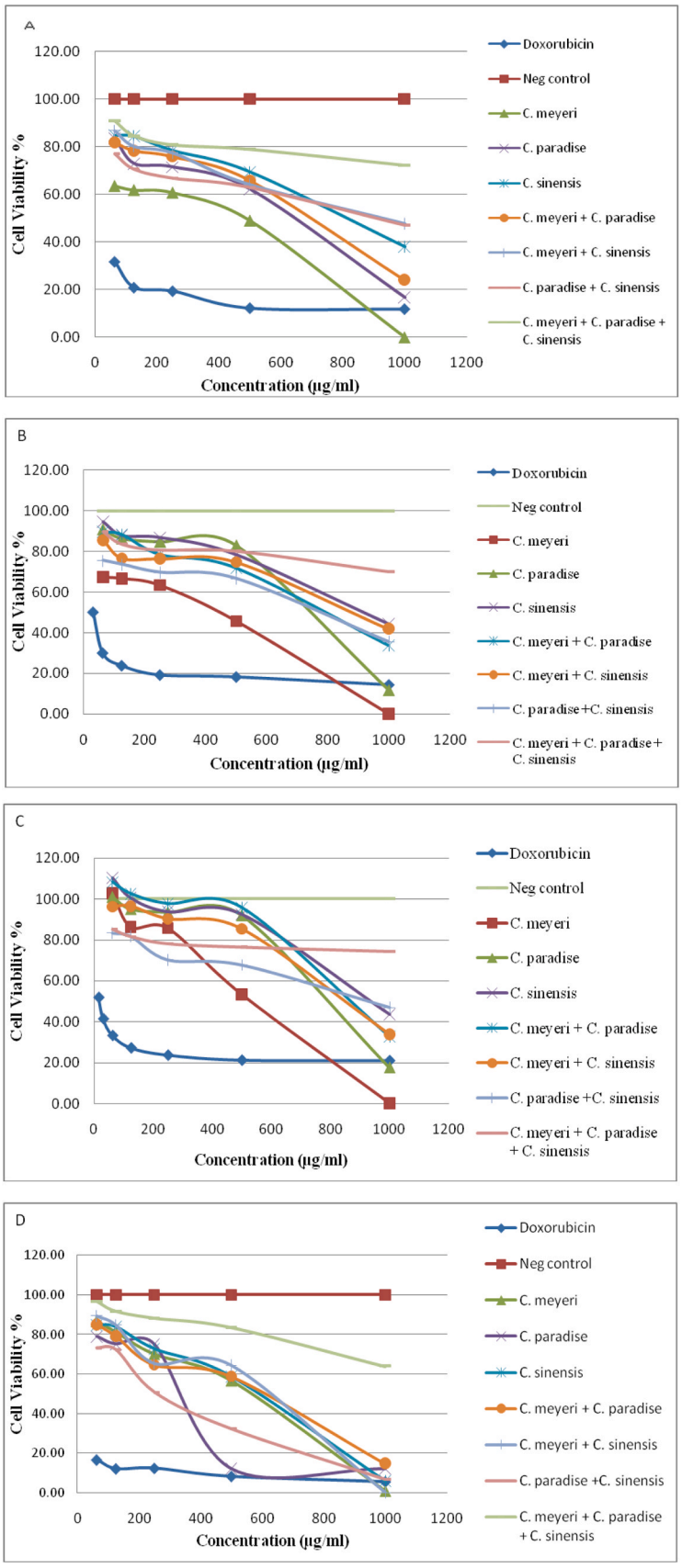


Fig. 1. Cytotoxic effect of seven citrus EOs combinations from *C. meyeri, C. paradise, and C. sinensis* against (A) HeLa, (B) HepG2, (C) CaCo-2, and (D) LX-2 cell lines (p values <0.05). Note: Cell viability (%) versus concentration doses (µg/ml) of EOs from citrus species and their combinations.

synergistic effect was observed against *C. albicans*, as indicated by an FIC $_{Index}$ of 0.88. An additive effect was observed against *P. vulgaris* using *C. meyeri* EO and *C. paradise* EO (FIC $_{Index}$ 1.0). The combination of *C. paradise* and *C. sinensis* EOs did not produce any noteworthy effects against the tested strains.

3.4. Cytotoxic Effects

The cytotoxic activities of the seven trials of EOs, Doxorubicin (positive control), and DMSO (negative control) were assessed on HeLa, HepG2, LX-2, and Caco-2 utilizing the MTS bioassay based on cell viability. Cancer cells were exposed to concentrations of EOs and Doxorubicin from 62.5 to 1000 μg/ml. The viability of cancer cell lines after treatment with different concentration doses of each citrus EO and their combinations is shown in Fig. 1. This study revealed a dose-dependent cytotoxic effect on all cancer cells for the seven Citrus EOs. In comparison to the positive control (Doxorubicin) and the negative control (DMSO), the EO from *C. meyeri* had better antiproliferative activity against HeLa, HepG2, CaCo-2 cancer cell lines, and LX-2 with a cell viability percentage of 0.04%, 0.05%, 0.24%, and 1.04% respectively, at a concentration of 1000 μg/ml in comparison with the positive control. A test of HeLa, HepG2, and CaCo-2 cancer cell lines showed that EO from *C. meyeri* had the highest cytotoxic activity than EO from other Citrus species with IC_{50} values of 338, 371, and 534 μg/ml, respectively. However, the eukaryotic cell line LX-2 was more sensitive to EO from the combination of *C. paradise* and *C. sinensis* with an IC_{50} of 347 μg/ml (Fig. 1D). Furthermore, the combination of *C. meyeri, C. paradise,* and *C. sinensis* EOs was shown to be less effective against cancer and normal tested cell lines.

4. Discussion

Herbal products serve as raw biomaterials in both modern and traditional medicine (Balekundri and Mannur, 2020). Infectious and non-infectious diseases have been treated and prevented with numerous plant species for centuries. Numerous pharmacologically active compounds are isolated in the pharmaceutical industry, either derived from crude plant extracts, essential oils (EOs), or pure isolates of active pharmacological substances (Najmi et al., 2022). Isoprene units are the main building blocks of EOs, which are hydrogenated, dehydrogenated, or oxygenated derivatives of hydrocarbons (Ninkuu et al., 2021). Due to their widespread commercialization, citrus EOs have been extensively studied for their chemical composition (Fagodia et al., 2017). Previous studies have also reported the chemical characterization of *C. meyeri, C. paradise,* and *C. sinensis* EOs. The results seem to agree with the findings of the present study, indicating that the major component of *C. meyeri* EO is limonene (Gulnaz et al., 2017). The study of Paoli *et al.* (Paoli et al., 2016) reported that the composition of twenty-four samples of EO isolated from leaves of *C. paradise* contained mainly monoterpene hydrocarbons and specially sabinene (up to 60.2%), (*E*)-β-ocimene (up to 15.0%) and punctually γ-terpinene (up to 56.1%), β-pinene (up to 30.9%) and p-cymene (up to 12.5%). The previous study is largely consistent with the results of the current study, which also showed the hydrocarbon monoterpenes were the most abundant (94.7%). The dominant components in the EO obtained from *C. paradise* consist of β-pinene (44.5%), β-phellandrene (26.5%), trans-ocimene (10.0%), α-pinene (5.2%), and Sabinene (4.1%). This is likely because *C. paradise* is known to produce large amounts of hydrocarbon monoterpenes. Monoterpenes are the building blocks for many EOs, and the abundance of these chemicals in *C. paradise's* EO suggests that it is a powerful source of these compounds (Noriega, 2021). The obtained results for *C. sinensis* are quite different from those found in Algeria by Youcef-Ettoumi et al. (2021) as they reported that limonene (94.4%) was the major component, followed by β- myrcene (2.2%) (Youcef-Ettoumi et al., 2021). It is significantly important to mention that there are several elements influencing the chemical composition of EOs, including plant components, genetic diversity, geographical location, soil characteristics, collecting time, and season (Li et al., 2022).

In response to the increasing microbial resistance to conventional synthetic antibiotics, phytogenic compounds are becoming more critical in terms of their antimicrobial properties. As a result, natural sources have been extensively screened for their powerful antibacterial, antifungal, and antiviral activities in the last three decades (Jaradat et al., 2020; Jaradat, Qneibi et al., 2022). Some EOs have demonstrated remarkable antimicrobial effects in this context, which is important in the cosmetic, agriculture, pharmaceutical, food supplement, and additive industries (Chouhan et al., 2017). It is clear from much of the documentation that EOs can be considered an effective alternative to synthetic drugs, particularly in cases where pathogenic microorganism strains have developed resistance to conventional antimicrobials (Wanda et al., 2019). In this research, the antimicrobial activities of the three citrus EOs and their combinations were assessed by broth microdilution assay against one fungal strain and six bacterial strains. The varying antibacterial activity in our results, as depicted by the percentages of phytochemical groups, could be attributed to differing quantities of various components. The percentage of phytochemicals varied depending on the type of plant used in the assay. This suggests that the chemical composition of plants plays an important role in their conferred antibacterial activity. The high hydrocarbon monoterpene content of *C. meyeri, C. paradise, and C. sinensis* EOs, which account for 51.3%, 94.7%, and 84.1%, respectively, may be responsible for their antibacterial activities. Hydrocarbon monoterpenes in EOs have been proven to alter cell permeability and inhibit respiration, resulting in cell integrity damage (Jaradat, Qneibi et al., 2022). The most notable antimicrobial efficacy was observed with the EO derived from *C. meyeri* against *S. aureus* ATCC 25923, yielding MIC of 3.1 ± 1.8 mg/ml. This outcome is consistent with the findings of Gulnaz *et al.*'s (2017) study. The results suggest that *C. meyeri* EO is a promising treatment for *S. aureus* infections. Further research is needed to determine the mechanism of action and to identify the optimal dosage and application method for *C. meyeri* EO. In this context, *C. meyeri, C. paradise,* and *C. sinensis* EOs furthermore observed good antibacterial activity against MRSA, with MICs of 25 ± 14, 33.3 ± 14, and 41.7 ± 14 mg/ml, respectively. MRSA isolates should be highlighted due to their renowned reputation as formidable pathogens in clinical settings (Turner et al., 2019). Previous reports suggest that gram-positive bacteria were generally more sensitive to the activity of the three citrus species (Saeb et al., 2016). On the flip side, all three EOs demonstrated antibacterial effectiveness against *E. coli*, *K. pneumoniae*, *P. vulgaris* and *P. aeruginosa*, with MIC values range from 50 to 100 mg/ml. This indicates that three EOs have the potential to be used as natural antimicrobial agents against gram-negative bacteria. Collectively, it can be concluded that the three EOs can relatively inhibit *S. aureus* and MRSA bacterial strains, in addition to the noticeable broad spectrum antimicrobial activity. Moreover, the antibacterial properties of EO from *C. meyeri* against *E. coli*, and *P. aeruginosa* were better than those from *C. paradise, and C. sinensis.* However, *K. pneumoniae* exhibited similar activity but demonstrated greater sensitivity to *C. paradise* compared to other species. The three EOs have significant antimicrobial properties against a wide range of microbial strains, as shown in Table 2. This suggests that EOs from the three citrus species could also be effective as alternative antimicrobial agents. Insufficient antimicrobial data currently exist for these citrus species in the extant literature. However, noteworthy antibacterial efficacy has been discerned in related studies involving *Citrus reticulata* and *Citrus japonica* against *Bacillus subtilis*, *E. coli*, and *Salmonella typhimurium* (Lin et al., 2021), as well as *Citrus aurantium* against *B. subtilis*, and *Stenotrophomonas maltophilia* (Kačániová et al., 2020). Interestingly, *Citrus grandis* exhibited heightened antimicrobial potency against *S. aureus, Bacillus cereus, Salmonella typhi*, and *P. aeruginosa* (Chi et al., 2020b). However, an EO's antimicrobial activity is dependent on its phytochemical content and its chemical characteristics, as well as, how each single compound of EO will interact with tested microbe (Jaafar et al., 2018).

In general, EOs' antimicrobial activity is influenced by their chemical composition, and they are primarily responsible for their activity if they contain more of their major components; so, if these components are more present, the antimicrobial activity will be enhanced (Reyes--Jurado et al., 2016). The antimicrobial combined effect of *C. meyeri, C. paradise,* and *C. sinensis* EOs has not been reported previously. In our study, the EOs combination of *C. meyeri, C. paradise, and C. sinensis* was found to have strong antifungal activity against *C. albicans* (MIC of 20.8 ± 7.2 mg/ml). The results suggested that the combination of *C. meyeri* with *C. paradise and C. sinensis* EOs is an effective antifungal treatment for *C. albicans*. Furthermore, the synergistic effect of the three EOs suggests that a combination of different EOs may have better antifungal activity than a single EO. Every combination of EOs demonstrated effectiveness in combating the tested microorganisms, except when *C. meyeri* and *C. sinensis* oils were mixed and when *C. paradise* and *C. sinensis* oils were blended against *P. aeruginosa* and *C. albicans*, respectively. This suggests that some EO combinations are more effective than others against certain microorganisms. Additionally, certain combinations may be more effective against certain microorganisms than others. Therefore, using the right combination of EOs when treating infections is important. This was evident in Rapper et al. (2021) study, which found that 57.1% of combinations had additive or synergistic effects, and only 5.4% showed antagonistic effects (Rapper et al., 2021). It can be attributed to the complex chemical compositions of EOs and their interaction, which causes antagonistic or synergistic effects to be observed in combinations of essential oils (Caesar and Cech, 2019). The FIC_{Index} findings revealed that when equal amounts of *C. meyeri* and *C. paradise* essential oils were combined, there synergistic effects were observed against *E. coli* and *C. albicans* (with FIC_{Index} values of 0.73 and 0.08, respectively). Additionally, when *C. meyeri* and *C. sinensis* EOs were mixed, a synergistic effect was noted against *P. vulgaris* and *C. albicans* (with FIC_{Index} values of 0.83 and 0.13, respectively). The FIC_{Index} results also suggest that the interaction between the two EOs was synergistic, with FIC_{Index} value of 0.73 for *E. coli* and 0.08 for *C. albicans*. This indicates that the synergistic effect of the two EOs was more potent than their individual effects. Minor components of EOs may play a more significant role in synergy than major compounds since major compounds do not only influence minor components. These minor components can also interact, creating more complex effects (De Azeredo et al., 2011).

Citrus EOs were found to exhibit dose-dependent cytotoxic effects on all tested cancer cell lines in this study (Fig. 1). According to the IC_{50} value of seven EOs, C. *meyeri* had better antiproliferative activities against HeLa, HepG2, and CaCo-2 cancer cell lines, with IC_{50} of 338 μg/ml, 371 μg/ml, and 534 μg/ml, respectively. However, compared to Doxorubicin (positive control), and DMSO (negative control), the LX-2 cells, which are considered normal, were less sensitive to C. *meyeri* EO with IC_{50} = 493 μg/ml, which indicates that *C. meyeri* EO is more selective against cancer cells than normal cells. To our knowledge, there are no studies about the cytotoxic effects of *C. meyeri* EO. In the current study, EOs of *C. meyeri* showed higher contents of oxygenated sesquiterpene such as elemol, muurola-4,10(14)-diene-1-b-ol, γ-eudesmol, and α-eudesmol, which are responsible for the cytotoxic activity of the EO, as reported in published literature (Jaafar et al., 2018; Saeb et al., 2016). These results suggest that the EO from *C. meyeri* has the potential to be used as a therapeutic agent in cancer treatment. Most citrus EOs - except *C. meyeri* - have a similar behavior of a cytotoxicity effect against cancer and normal cells. In this context, *C. sinensis* showed mild to moderate activity with IC_{50} range 810–975 μg/ml, and these results were similar to the results of the Kammoun et al. (2021) study that reported moderate cytotoxic activity of *C. sinensis* against MCF-7, HepG-2, and HeLa cells (Kammoun et al., 2021). In addition, Fig. 1 showed that the Caco-2 cell had the highest resistance to our seven trials according to IC_{50} value, lethal dose, and starting point of effectiveness.

5. Conclusion

The three citrus EOs examined in the current study had different chemical compositions. *C. meyeri* EO included the most Limonene, α-eudesmol, germacrene, and elemol, while *C. paradise* contained the most β-pinene, β-phellandrene, trans-Ocimene, and α-Pinene, but *C. sinensis* has sabinene, trans-ocimene, linalool, and limonene. The three EOs chave a potent antibacterial activity against gram-postive ATCC and clinical bacterial strains, in addition to noticeable general antimicrobial activity. A combination of *C. meyeri* and *C. paradise* EOs leads to synergistic effects against *E. coli* and *C. albicans*. Also, a synergistic effect was obtained by combining *C. meyeri and C. sinensis* EOs against *P. vulgaris* and *C. albicans.* Combining *C. meyeri* EOs with *C. paradise* and *C. sinensis* EOs could be considered an effective antifungal treatment for *C. albicans.* C. *meyeri* had better antiproliferative activities against HeLa, HepG2, and CaCo-2 cancer cell lines. *C. meyeri* EO is more selective against cancer cells than normal cells. Further research is needed to determine the mechanism of action and to identify the optimal dosage and application method for Citrus plant leaves EOs to be used as antimicrobial and anticancer therapeutic agents.

CRediT authorship contribution statement

Qadi Mohammad: Writing – review & editing, Supervision, Resources, Methodology, Investigation, Data curation, Conceptualization. **Ismail Shurooq:** Visualization, Methodology, Conceptualization. **Al-Maharik Nawaf:** Methodology, Formal analysis. **Jaradat Nidal:** Resources, Methodology, Conceptualization. **Hamdan Mahmoud:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

References

Abbas, M.W., Raza, A.B.M., Dageri, A., Arshad, M., Khan, M.A., Ullah, M.I., Afzal, M., 2020. Consequences of leaf biochemical characters for citrus leafminer, phyllocnistis citrella stainton (Lepidoptera: Gracillariidae) along the microclimatic gradient of citrus plants. Kuwait J. Sci. 47, 4.

Aiyegoro, O.A., Afolayan, A.J., Okoh, A.I., 2009. Synergistic interaction of Helichrysum pedunculatum leaf extracts with antibiotics against wound infection associated bacteria. Biol. Res. 42 (3) https://doi.org/10.4067/S0716-97602009000300007.

Aleksic, V., Mimica-dukic, N., Simin, N., Stankovic, N., Knezevic, P., 2014. Phytomedicine Synergistic effect of Myrtus communis L. essential oils and conventional antibiotics against multi-drug resistant Acinetobacter baumannii wound isolates. Eur. J. Integr. Med. 21 (12), 1666–1674. https://doi.org/10.1016/j.phymed.2014.08.013.

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D.G., Lightfoot, D.A., 2017. Phytochemicals: extraction, isolation, and identification of bioactive compounds from plant extracts. Plants 6 (4). https://doi.org/10.3390/plants6040042.

Amna, Ulil, Halimatussakdiah, Puji, Wahyuningsih, N.S., Nasution1, Rosnani, 2019. Evaluation of cytotoxic activity from Temurui (Murraya koenigii [Linn.] Spreng) leaf extracts against HeLa cell line using MTT assay. J. Adv. PharmTechnol 10 (*51-5.*), 10.

Ayari, S., Shankar, S., Follett, P., Hossain, F., Lacroix, M., 2020. Potential synergistic antimicrobial efficiency of binary combinations of essential oils against Bacillus cereus and Paenibacillus amylolyticus-Part A. Microb. Pathog. 141 https://doi.org/10.1016/j.micpath.2020.104008.

Balekundri, A., Mannur, V., 2020. Quality control of the traditional herbs and herbal products: a review. Future J. Pharm. Sci. 6 (1) https://doi.org/10.1186/s43094-020-00091-5.

Battat, M.M.K., Marie, M., 2022. Rehabilitation interventions for depression symptoms among cancer patients in Palestine: a systematic review. Front. Rehabil. Sci. 3 https://doi.org/10.3389/fresc.2022.978844.

Caesar, L.K., Cech, N.B., 2019. Synergy and antagonism in natural product extracts: when 1 + 1 does not equal 2. *Nat. Prod. Rep.* Vol. 36 (Issue 6) https://doi.org/10.1039/c9np00011a.

Chi, P.T.L., Van Hung, P., Le Thanh, H., Phi, N.T.L., 2020a. Valorization of citrus leaves: chemical composition, antioxidant and antibacterial activities of essential oils. Waste Biomass-.-. Valoriz. 11 (9), 4849–4857. https://doi.org/10.1007/s12649-019-00815-6.

Chi, P.T.L., Van Hung, P., Le Thanh, H., Phi, N.T.L., 2020b. Valorization of citrus leaves: chemical composition, antioxidant and antibacterial activities of essential oils. Waste Biomass-.-. Valoriz. 11 (9), 4849–4857. https://doi.org/10.1007/s12649-019-00815-6.

Chouhan, S., Sharma, K., Guleria, S., 2017. Antimicrobial activity of some essential oils—present status and future perspectives. Medicines 4 (3). https://doi.org/10.3390/medicines4030058.

De Azeredo, G.A., Stamford, T.L.M., Nunes, P.C., Gomes Neto, N.J., De Oliveira, M.E.G., De Souza, E.L., 2011. Combined application of essential oils from Origanum vulgare L. and Rosmarinus officinalis L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. Food Res. Int. 44 (5), 1541–1548. https://doi.org/10.1016/j.foodres.2011.04.012.

Elizondo-Luévano, J.H., Gomez-Flores, R., Verde-Star, M.J., Tamez-Guerra, P., Romo-Sáenz, C.I., Chávez-Montes, A., Rodríguez-Garza, N.E., Quintanilla-Licea, R., 2022. In Vitro cytotoxic activity of methanol extracts of selected medicinal plants traditionally used in mexico against human hepatocellular carcinoma. Plants 11 (21). https://doi.org/10.3390/plants11212862.

Fagodia, S.K., Singh, H.P., Batish, D.R., Kohli, R.K., 2017. Phytotoxicity and cytotoxicity of Citrus aurantiifolia essential oil and its major constituents: Limonene and citral. Ind. Crops Prod. 108 (July), 708–715. https://doi.org/10.1016/j.indcrop.2017.07.005.

Gadisa, E., Usman, H., 2021. Evaluation of antibacterial activity of essential oils and their combination against multidrug-resistant bacteria isolated from skin ulcer. Int. J. Microbiol. 2021 https://doi.org/10.1155/2021/6680668.

Gmitter, F.G., Chen, C., Machado, M.A., de Souza, A.A., Ollitrault, P., Froehlicher, Y., Shimizu, T., 2012. Citrus genomics. Tree Genet. Genomes Vol. 8 (Issue 3). https://doi.org/10.1007/s11295-012-0499-2.

Grewal, J., Kumar, V., Rawat, H., Gandhi, Y., Singh, R., Singh, A., Babu, G., Srikanth, N., Mishra, S.K., 2022. Cytotoxic effect of plant extract-based nanoparticles on cancerous cells: a review. Environ. Chem. Lett. 20 (4), 2487–2507. https://doi.org/10.1007/s10311-022-01422-z.

Gulnaz, O., Kacar, Y.A., Bozkurt, T., Gülnaz, O., Kaçar, Y.A., 2017. Chemical composition of the essential oils from some citrus species and evaluation of the antimicrobial activity. IOSR J. Environ. Sci. 11 (10), 29–33. https://doi.org/10.9790/2402-1110032933.

Hosni, K., Hassen, I., M'Rabet, Y., Sebei, H., Casabianca, H., 2013. Genetic relationships between some Tunisian Citrus species based on their leaf volatile oil constituents. Biochem. Syst. Ecol. 50 (2013), 65–71. https://doi.org/10.1016/j.bse.2013.03.035.

Jaafar, M., Mitri, S., Nawas, T., 2018. Inhibition of gram negative bacterial growth and biofilm formation by alpha thujone. IOSR J. Pharm. Biol. Sci. (IOSR-JPBS 13 (2), 40–47. https://doi.org/10.9790/3008-1302024047.

Jaradat, N., Qadi, M., Abualhasan, M.N., Al-lahham, S., Al-Rimawi, F., Hattab, S., Hussein, F., Zakarneh, D., Hamad, I., Sulayman, I., Issa, L., Mousa, A., 2020. Carbohydrates and lipids metabolic enzymes inhibitory, antioxidant, antimicrobial and cytotoxic potentials of Anchusa ovata Lehm. from Palestine. Eur. J. Integr. Med. 34 https://doi.org/10.1016/j.eujim.2020.101066.

Jaradat, N., Hawash, M., Abualhasan, M.N., Qadi, M., Ghanim, M., Massarwy, E., Ammar, S.A., Zmero, N., Arar, M., Hussein, F., Issa, L., Mousa, A., Zarour, A., 2021. Spectral characterization, antioxidant, antimicrobial, cytotoxic, and cyclooxygenase inhibitory activities of Aloysia citriodora essential oils collected from two Palestinian regions. BMC Complement. Med. Ther. 21 (1), 1–11. https://doi.org/10.1186/s12906-021-03314-1.

Jaradat, N., Abdallah, S., Al-Maharik, N., Altamimi, M., Hawash, M., Qneibi, M., Abu Khair, A., Zetawi, A., Jabarin, L., 2022. Constituents, antibacterial adhesion, cytotoxic and in vitro metastasis blocking properties of salvia fruticosa essential oils from three palestinian localities. Chem. Biodivers. 19 (4) https://doi.org/10.1002/cbdv.202100872.

Jaradat, N., Qneibi, M., Hawash, M., Al-Maharik, N., Qadi, M., Abualhasan, M.N., Ayesh, O., Bsharat, J., Khadir, M., Morshed, R., Yaaqbeh, S., Marei, S., Hamayel, S., Mousa, A., Daqqa, M., Bdir, S., 2022. Assessing Artemisia arborescens essential oil compositions, antimicrobial, cytotoxic, anti-inflammatory, and neuroprotective effects gathered from two geographic locations in Palestine. Ind. Crops Prod. 176 (November 2021), 114360 https://doi.org/10.1016/j.indcrop.2021.114360.

Ji, J., Shankar, S., Royon, F., Salmieri, S., Lacroix, M., 2023. Essential oils as natural antimicrobials applied in meat and meat products—a review. Crit. Rev. Food Sci. Nutr. 63 (8), 993–1009. https://doi.org/10.1080/10408398.2021.1957766.

Johnson, J.B., Batley, R., Manson, D., White, S., Naiker, M., 2022. Volatile compounds, phenolic acid profiles and phytochemical content of five Australian finger lime (Citrus australasica) cultivars. Lwt 154, 112640. https://doi.org/10.1016/j.lwt.2021.112640.

Jubair, N., Rajagopal, M., Chinnappan, S., Abdullah, N.B., Fatima, A., 2021. Review on the antibacterial mechanism of plant-derived compounds against multidrug-resistant bacteria (MDR). Evid. -Based Complement. Altern. Med. 2021 https://doi.org/10.1155/2021/3663315.

Kačániová, M., Terentjeva, M., Galovičová, L., Ivanišová, E., Štefániková, J., Valková, V., Borotová, P., Łukasz Kowalczewski, P., Kunová, S., Felšöciová, S., Tvrdá, E., Žiarovská, J., Benda Prokeinová, R., Vukovic, N., 2020. Biological activity and antibiofilm molecular profile of citrus aurantium essential oil and its application in a food model. Molecules 25 (17). https://doi.org/10.3390/molecules25173956.

Kamal, G.M., Anwar, F., Hussain, A.I., Sarri, N., Ashraf, M.Y., 2011. Yield and chemical composition of Citrus essential oils as affected by drying pretreatment of peels. Int. Food Res. J. 18 (4), 1275–1282.

Kammoun, A.K., Altyar, A.E., Gad, H.A., 2021. Comparative metabolic study of Citrus sinensis leaves cultivars based on GC–MS and their cytotoxic activity. J. Pharm. Biomed. Anal. 198, 113991 https://doi.org/10.1016/j.jpba.2021.113991.

Li, C., Cai, Q., Wu, X., Tan, Z., Huang, S., Wei, C., Zhang, W., Chen, Z., Zhang, L., Xiang, H., 2022. Variation in compositions and biological activities of essential oils from four citrus species: citrus limon, citrus sinensis, citrus paradisi, and citrus reticulata. Chem. Biodivers. 19 (4) https://doi.org/10.1002/cbdv.202100910.

Lin, X., Cao, S., Sun, J., Lu, D., Zhong, B., Chun, J., 2021. The chemical compositions, and antibacterial and antioxidant activities of four types of citrus essential oils. Molecules 26 (11), 1–12. https://doi.org/10.3390/molecules26113412.

Mahato, N., Sinha, M., Sharma, K., Koteswararao, R., Cho, M.H., 2019. Modern extraction and purification techniques for obtaining high purity food-grade bioactive compounds and value-added co-products from citrus wastes. *Foods* Vol. 8 (Issue 11). https://doi.org/10.3390/foods8110523.

Mahmoud, S.H., Wafa, M.M., 2020. The antibacterial activity of Laurus nobilis leaf extract and its potential use as a preservative for fresh lamb meat. Afr. J. Microbiol. Res. https://doi.org/10.5897/ajmr2020.9405.

Miyake, Y., Ito, C., Itoigawa, M., 2012. A novel trans-4-hydroxycinnamic acid derivative from Meyer lemon (Citrus meyeri). Food Chem. 135 (4), 2235–2237. https://doi.org/10.1016/j.foodchem.2012.07.020.

Najmi, A., Javed, S.A., Al Bratty, M., Alhazmi, H.A., 2022. Modern approaches in the discovery and development of plant-based natural products and their analogues as potential therapeutic agents. Molecules Vol. 27 (Issue 2). https://doi.org/10.3390/molecules27020349.

Ncube, B., Finnie, J.F., Van Staden, J., 2012. In vitro antimicrobial synergism within plant extract combinations from three South African medicinal bulbs. J. Ethnopharmacol. 139 (1), 81–89. https://doi.org/10.1016/j.jep.2011.10.025.

Ninkuu, V., Zhang, L., Yan, J., Fu, Z., Yang, T., Zeng, H., 2021. Biochemistry of terpenes and recent advances in plant protection. *Int. J. Mol. Sci.* Vol. 22 (Issue 11) https://doi.org/10.3390/ijms22115710.

Noriega, P. (2021). Terpenes in Essential Oils: Bioactivity and Applications. https://doi.org/10.5772/intechopen.93792.

Paoli, M., de Rocca Serra, D., Tomi, F., Luro, F., Bighelli, A., 2016. Chemical composition of the leaf essential oil of grapefruits (Citrus paradisi Macf.) in relation with the genetic origin. J. Essent. Oil Res. 28 (4), 265–271. https://doi.org/10.1080/10412905.2016.1140090.

Qadi, M., Jaradat, N., Al-Maharik, N., Hawash, M., Abdalrazeq, M., Fuqha, A., Jabareen, D., Atamni, N., Zarour, A., 2023. Anticandidal effects and chemical compositions of volatile oils extracted from Origanum syriacum, Clinopodium serpyllifolium subsp. fruticosum and Thymbra capitata from Palestine. Chem. Biol. Technol. Agric. 10 (1), 1–10. https://doi.org/10.1186/s40538-023-00459-8.

Rapper, S.L., De, Viljoen, A., van Vuuren, S., 2021. Essential oil blends: The potential of combined use for respiratory tract infections. Antibiotics 10 (12). https://doi.org/10.3390/antibiotics10121517.

Reda, F.M., El-zawahry, Y.A., Omar, A.R., 2017. Synergistic effect of combined antibiotic and methanol extract of eucalyptus camaldulensis leaf against staphylococcus aureus and pseudomonas aeruginosa, 5, 486–497. https://doi.org/10.3126/ijasbt.v5i4.18620.

Reyes-Jurado, F., López-Malo, A., Palou, E., 2016. Antimicrobial activity of individual and combined essential oils against foodborne pathogenic bacteria. J. Food Prot. 79 (2), 309–315. https://doi.org/10.4315/0362-028X.JFP-15-392.

Saeb, S., Amin, M., Seyfi Gooybari, R., Aghel, N., 2016. Evaluation of antibacterial activities of citrus limon, citrus reticulata, and citrus grandis against pathogenic bacteria. Int. J. Enteric Pathog. 4 (4) https://doi.org/10.15171/ijep.2016.13.

Terreni, M., Taccani, M., Pregnolato, M., 2021. New antibiotics for multidrug-resistant bacterial strains: Latest research developments and future perspectives. *Molecules* Vol. 26 (Issue 9). https://doi.org/10.3390/molecules26092671.

Turner, N.A., Sharma-Kuinkel, B.K., Maskarinec, S.A., Eichenberger, E.M., Shah, P.P., Carugati, M., Holland, T.L., Fowler, V.G., 2019. Methicillin-resistant Staphylococcus aureus: an overview of basic and clinical research. Nat. Rev. Microbiol. Vol. 17 (Issue 4) https://doi.org/10.1038/s41579-018-0147-4.

Ugboko, H.U., Nwinyi, O.C., Oranusi, S.U., Fatoki, T.H., Omonhinmin, C.A., 2020. Antimicrobial importance of medicinal plants in Nigeria. Sci. World J. 2020 https://doi.org/10.1155/2020/7059323.

Wanda, M., Czubaszek, A., Szumny, A., 2019. Essential oils as antimicrobial agents-myth or real alternative? Molecules 1–21.

Wang, Z., Qi, F., Cui, Y., Zhao, L., Sun, X., Tang, W., Cai, P., 2018. An update on Chinese herbal medicines as adjuvant treatment of anticancer therapeutics. *Biosci. Trends* Vol. 12 (Issue 3). https://doi.org/10.5582/bst.2018.01144.

Youcef-Ettoumi, K., Zouambia, Y., Moulai-Mostefa, N., 2021. Chemical composition, antimicrobial and antioxidant activities of Algerian Citrus sinensis essential oil extracted by hydrodistillation assisted by electromagnetic induction heating. J. Food Sci. Technol. 58 (8) https://doi.org/10.1007/s13197-020-04808-5.

Zanganeh, H., Mortazavi, S.A., Shahidi, F., Alizadeh Behbahani, B., 2021. Evaluation of the chemical and antibacterial properties of Citrus paradise essential oil and its application in Lallemantia iberica seed mucilage edible coating to improve the physicochemical, microbiological and sensory properties of lamb during refrigerated storage. J. Food Meas. Charact. 15 (6) https://doi.org/10.1007/s11694-021-01129-9.