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Research paper

Chemical analysis and bioactivity evaluation of *Citrus limon* leaves volatile oil from Palestine: investigating phytochemical, anti-inflammatory, antimicrobial, and cytotoxic properties

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ABSTRACT

Introduction: Scientists consider repurposing discarded waste into valuable resources for the pharmaceutical, cosmetic, and food flavoring manufacturing industries a challenge to generate new revenue streams, lower production costs, and reduce waste. Therefore, the current study aimed to identify *Citrus limon* leaf volatile oil (VO) constituents from Palestine and assess its in vitro antimicrobial, anti-inflammatory, and cytotoxic properties.

Methods: The components of the VO were analyzed using Gas Chromatography/Mass Spectrometry (GC/MS). The antimicrobial activity of VO was assessed using a microdilution technique. The cyclooxygenases (COX-1 and COX-2) assay was utilized to assess the anti-inflammatory effect. An aqueous one solution cell proliferation (MTS) assay was employed to determine the cytotoxic effect of the VO.

Results: Thirty-six molecules were identified in the oil, and geranial, neral, and limonene are the most abundant molecules, comprising 31.06%, 23.98%, and 14.32%, respectively. The antimicrobial results showed that the VO has notable inhibition against *Proteus vulgaris,* Methicillin-resistant *Staphylococcus aureus*, and *Candida albicans*. The COX IC_{50} calculations revealed that it has high potency against COX-2 IC_{50} = 9.32 ± 0.88 μg/ml. The most potent cytotoxic effect of *C. limon* VO was noticed against MCF-7, with an IC_{50} dose of 162.90 ± 1.85 mg/ml.

Conclusion: The GC-MS analyses revealed that geranial, neral, and limonene are the predominant compounds in the VO of *C. limon* leaves from Palestine. The biological test results demonstrated that the VO has potential inhibitory actions against some cancer cells, bacterial and fungal species, and COX-2 enzyme, suggesting it may be a viable choice for treating or preventing microbial infections, cancer, and inflammatory illnesses.

Introduction

Volatile oils (VOs) are natural compounds extracted mainly through distillation, characterized by distinctive fragrances originating from plants or other sources. They occur in the chloroplasts of leaves, the vesicular layer of cell walls, or through the hydrolysis of certain glycosides (Sharifi-Rad et al., 2017). These naturally occurring chemicals significantly contribute to health and possess bioactive capabilities, including antibacterial, antiviral, antioxidant, and antidiabetic. Furthermore, it is essential in inhibiting cancer and facilitating

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chemoprevention. Furthermore, VOs play a crucial role in inhibiting cancer and facilitating chemoprevention (Tanu and Harpreet, 2016).

The genus *Citrus* (Rutaceae) is believed to have originated on the Asian continent. It comprises 140 genera and about 1 300 flowering species commonly found in the warm temperate regions, including the Mediterranean Sea basin and semi-tropical and tropical countries. Citrus plant species are known as one of the world's most famous fruit crops (Mabberley, 2008). *Citrus limon* (L.) Osbeck, also known as lemon, is a flowering evergreen tree or fragrant shrub that can grow up to 6 m tall and has thick spines. The leaves are oval, dark, leathery, and green (Nguyen et al., 2009).

C. limon fruits have demonstrated anticancer, anti-inflammatory, sedative, antioxidant, and antiseptic properties (Klimek-Szczykutowicz et al., 2020). Numerous global studies have looked at the analgesic, insecticidal, anti-leishmanial, antibacterial, and antioxidant properties of various *C. limon* leaf VOs (Petretto et al., 2023).

Cancer ranks among the foremost causes of mortality globally; however, its effects differ significantly. Worldwide, lung, breast, liver, stomach, breast, and colon cancers are the top five leading causes of cancer-related death in 2020 (Cao et al., 2021). Over the year 2020, an estimated 19.3 million new cases of cancer were reported, leading to over 10.0 million fatalities (Hawash et al., 2024). Several variables, including genetic, cultural, demographic, environmental, and ecological variables, contribute to the heterogeneity of cancer incidence and mortality (Qiu et al., 2021).

Antimicrobial-resistant (AMR) illnesses represent a worldwide emergency. Globally, an estimated 1.3 million fatalities were directly caused by AMR infections in 2019 (Tamma et al., 2024). Sometimes, microorganisms that ordinarily respond well to an antibiotic's action can become resistant or less sensitive. Antibiotic-resistant bacteria are commonplace and are a severe clinical risk (Munita and Arias, 2016). Microorganisms can become resistant to antibiotics through any one of the following processes: transference, phage transduction, mutation, and selection. Microbial resistance can be inherent in the organism or acquired through environmental exposure (Al-Masri et al., 2021). Bacterial resistance to antimicrobial drugs is still a problem because of factors like overuse, underdosing, illogical attitudes on the part of prescribers, patient demands, and antimicrobial agents in agriculture and horticulture (Verma et al., 2022).

All nonsteroidal anti-inflammatory drugs (NSAIDs) raise the risk of gastrointestinal bleeding, stroke, and myocardial infarction. NSAIDs decrease prostaglandin synthesis, with COX-1 and COX-2 inhibition levels varying depending on the drug (Jahnavi et al., 2019). All NSAIDs raise the risk of cardiovascular disease and bleeding (Waleed M et al., 2004). However, selective COX-2 inhibitors are more likely to result in cardiovascular conditions, whereas less selective NSAIDs are more likely to result in bleeding in the gastrointestinal tract. Common adverse responses to NSAIDs include ecchymosis, tinnitus, fluid retention, increased Alanine transaminase and Aspartate aminotransferase liver enzymes, rash, drowsiness, dizziness, headache, constipation, abdominal pain, nausea, and dyspepsia (Yousefifard et al., 2020). An individual using an NSAID is likely to experience one or more of these undesirable effects regularly. None of these conventional responses are immediately lethal. Nevertheless, they can be problematic as they may compel the client to consult a physician for further prescriptions to mitigate the harmful effects (Davis and Robson, 2016).

In fact, *C. limon* leaves, despite their traditional medicinal uses, are frequently discarded as waste, and unused parts of plants can be repurposed as valuable resources, rendering them ideal for use in the pharmaceutical, cosmetic, and food flavoring manufacturing industries. Utilizing these leaves can reduce waste, lower production costs, and generate new revenue streams (Petretto et al., 2023; Russo et al., 2021). Furthermore, the circular economy, characterized by the efficient reuse of resources, is facilitated by the sustainable use of plant by-products, which contributes to economic resilience and innovation. This approach also supports environmental conservation efforts (Arruda et al., 2021; Galvão et al., 2018).

No prior studies have examined the phytochemical, anti-inflammatory, antimicrobial, and cytotoxic properties of *C. limon* leaf VO from Palestine. The present study seeks to investigate *C. limon* leaf VO's chemical composition in Palestine and assess its antimicrobial, anticancer, and anti-inflammatory properties for potential applications in the pharmaceutical, cosmetic, and food flavoring industries.

Material and methods

Plant materials and volatile oil extraction

In July 2022, the leaves of *C. limon* were collected from the Jenin governorate of Palestine. Professor Nidal Jaradat, a pharmacognosist at An-Najah National University, established the plant deposition and identification in the Herbal Products Laboratory, which is associated with the voucher specimen code (Pharm-PCT-2741). The leaves were washed multiple times with tap water and subsequently dried in the shade at 25 ± 6 °C and 55 ± 2% humidity for approximately 11 days. Subsequently, the plant material was coarsely crushed and stored in tightly sealed bags for later use. The VO from the *C. limon* plant was extracted using the Clevenger apparatus's hydrodistillation method. In a round-bottom flask, 0.1 kg of fresh leaves was combined with 1 l of distilled water and boiled at 100 °C for 240 minutes. The organic layer (VO) was dried out with magnesium sulfate ($MgSO_4$), the liquid was poured off, and the *C. limon* VO was kept at 2–8 °C for later use. It made up 1.24% ± 0.11 of the total weight.

Gas chromatography/mass spectrometry (GC-MS)

A Perkin Elmer Clarus 500 gas chromatograph was used to analyze the VO contents of the fresh leaves of the *C. limon* plant. This device was equipped with a Perkin Elmer Clarus 560 mass spectrometer. A Perkin Elmer Elite-5 fused silica capillary column (film thickness 0.25 μm, 30 m x 0.25 mm) was employed to separate the samples. At a rate of 4 °C/Min, the column temperature increased from 50 °C to 280 °C for 5 minutes. Helium was pumped through the chromatographic apparatus at a 1 ml/Min rate throughout each run. Purified *C. limon* VO was added in split mode at 250 °C with a splitting ratio 1:50. Mass spectra were employed to compare the sample components to those in the library or with standards. The matches were supported by GC retention times and indices (Zhao et al., 2005). The following equation was used to calculate the RI for the phyto-components of *C. limon* VO.

$$RI = 100 \times (((tR(i) - tR(z))/(tR(z + 1) - tR(z))) + z)$$

Where the carbon atom numbers are in the smaller *n*-alkane, and tR (i), tR(z), and tR(z + 1) are the retention times of the desired molecule, the smaller *n*-alkane, and the larger *n*-alkane, respectively. The VO composition was identified by comparison of their retention indices (RI) to the RT of a series of (C_6–C_{27}) *n*-alkanes (Sigma, Milan, Italy) with those reported in the literature (Adams, 2007)

Antimicrobial activity

The antimicrobial efficacy of *C. limon* VO was assessed via the broth-based microdilution procedure as instructed previously (Qadi et al., 2020). Fluconazole and ciprofloxacin drugs were employed as antifungal and antibacterial agents to validate this method. From the American Type Culture Collection, the antibacterial effect of the VO was established using five virulent bacterial species, including *Escherichia coli* (25922-ATCC), *Klebsiella pneumonia* (13883-ATCC), *Proteus vulgaris* (8427-ATCC), *Pseudomonas aeruginosa* (9027-ATCC), and *Staphylococcus aureus* (25923-ATCC). From An-Najah National University Hospital, Methicillin-resistant Staphylococcus aureus (MRSA) was utilized as a clinical isolate. Besides, the antifungal characteristics of *C. limon* VO were also assessed against the growth of *Candida albicans*

(90028-ATCC). Around 200 µl/ml of Dimethylsulfoxide was dissolved into a solution for the tested VO. Two-fold serial micro-dilutions were done ten times in sterile water from each of the resultant solutions in the Mueller-Hinton broth. The dilutions were carried out in 96-well plates in aseptic conditions, and these mentioned 10 wells would contain a gradient of concentrations of each VO mixed with an average microbial amount (Balouiri et al., 2016).

Cytotoxicity assay

Cervical cancer (HeLa), hepatocellular carcinoma (Hep3B), human hepatic stellate (LX-2), and breast cancer (MCF-7) cells (ATCC, Rockville, MD, USA) were grown in RPMI-1640 media and supplemented with 1% streptomycin/penicillin, 1% l-glutamine, and 10% fetal bovine serum. Before seeding 2.5 x 10^4 cells per well in 96-well plates, the cells were cultured at 37 ˚C in a 5% CO_2 environment. The measured VO concentrations (500, 250, 125, 62.5, and 31.25 µg/ml) were examined for 24 hours after 48 hours of culture. The CellTilter 96®Aqueous One Solution Cell Proliferation (MTS) Assay was used to determine the viability of the screened cells evaluated following the manufacturer's instructions (Promega Corporation, Madison, USA). To finish the technique, 100 microliters of media were mixed with 20 µl of MTS solution, and the medium was then incubated for two hours at 37 ˚C. The absorbance was measured at 490 nm (Jaradat et al., 2024).

Cyclooxygenase inhibitory effect

The anti-inflammatory effect of *C. limon* VO was evaluated using bovine COX-1 and human recombinant COX-2. Arachidonic acid was converted to prostaglandin H_2 employing a COX inhibitor screening test kit in accordance with the manufacturer's guidelines (Cayman Chemical, Michigan, USA). *C. limon* VO was evaluated in triplicate for its 50% inhibitory concentration (IC_{50}) on COX-1/COX-2 activity. The produced multiple regression best-fit line from the kit instructions was utilized to compare the inhibition of the tested plant VO sample against a standard curve comprising eight distinct doses of prostaglandin, along with a non-specific binding sample and a maximum binding sample. The IC_{50} concentration was determined based on the inhibition percentage at the specified concentration (Jaradat et al., 2021).

Statistical analysis

There were three replicates of each established experiment. The findings are shown as means with standard deviation (SD). Only when the *P*-values were less than 0.005 were the findings deemed significant.

Results and discussion

GC-MS characterization of C. limon VO

The chemical composition of *C. limon* VO from Palestine was qualitatively and quantitatively recognized using the GC-MS apparatus. Thirty-six molecules were identified, representing 100% of the total oil. The most abundant compounds in the investigated *C. limon* VO are geranial, neral, and limonene, representing 31.06, 23.98, and 14.32%, respectively. The GC-MS chromatogram (Supplementary Fig. 1) and Table 1 show the chemical components, retention index (RI), and retention time (RT), along with their concentrations (%). In addition, oxygenated monoterpenoids and hydrocarbon monoterpene phytochemical classes were identified as the main phytochemical groups of *C. limon* VO, accounting for 80.14 and 15.75%, respectively.

The major compounds in our study are geranial and neral, which represent a mixture of *cis*- and *trans*-isomers of citral (3,7-dimethyl-2,6-octadienal). Phytochemically, geranial, and neral are monoterpene aldehydes are classified as oxygenated monoterpenoids (Molińska et al., 2015). Table 2 summarizes the major components of *C. limon* leaves from different countries.

Table 2 indicates that the primary chemical components of VOs differ based on geographical locations, harvesting time, extracted plant parts, developmental stages, agricultural methods and practices, soil types, climates, and growth conditions (Moghaddam and Mehdizadeh, 2017; Şanli and Karadoğan, 2017).

Antimicrobial activity

The exponential increase in the prevalence of multidrug-resistant microbes has contributed to the global spread of antibiotic resistance. Misuse of antibiotics is a major factor in this problem (Hattab et al., 2021). Antiseptic, antibacterial, antiviral, and antifungal properties in VOs have been widely observed. Therefore, VOs may be an effective tool for combating the spread of antibiotic-resistant microbes (Chouhan et al., 2017).

The analysis of VOs from various plants has shown that oxygenated monoterpenes, particularly geranial and neral, are more effective as antibacterial agents than standard monoterpene hydrocarbons. Citral "neral and geranial isomers" have received approval from the US Food and Drug Administration for their safety as natural preservatives and flavoring agents. This is because they exhibit antibacterial activity against gram-negative bacteria such as *Escherichia coli* and positive bacteria such as *Staphylococcus aureus* (El-Kased and El-Kersh, 2022). Table 3 showed that *C. limon* VO showed an outstanding inhibition against *P. vulgaris,* MRSA, and *C. albicans*. Furthermore, it affects mildly *S. aureus* and *E. coli*, as well as weakly *P. aeruginosa* and *K. pneumoniae*. The minimum inhibitory concentrations (MICs) by optical density assay for *P. vulgaris,* MRSA, and *C. albicans* were 1.56 ± 0.09, 6.25 ± 0.12, and 0.39 ± 0.08 µl/ml, respectively. A significant number of studies have concentrated on investigating plants that contain geranial (trans-citral, citral A) and neral (cis-citral, citral B) compounds. These compounds are present in the leaves and fruits of *citrus* plants. Citral has been shown to impact both the cytoplasmic/outer membrane and the stress response regulated by the sigma factor RpoSin *E. coli* (Nuță et al., 2021).

Asker et al. investigated *C. limon* VO from Egypt and found a significant inhibitory effect against *P. aeruginosa* and *S. aureus*, with inhibition zones of 49 ± 0.01 mm and 32 ± 0.01 mm, respectively. Simultaneously, it did not impact *Bacillus cereus* or *E. coli*. The MIC was determined by optical density assay for *P. aeruginosa* and *S. aureus* (Asker et al., 2020).

Citral occurs in various plant sources and is classified as Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA). Recent investigations have shown that this compound displays various biological activities, including antibacterial, antifungal, antibiofilm, antiparasitic, antiproliferative, anti-inflammatory, and antioxidant properties, as evidenced by *in vitro* assays. The proposed mechanisms of citral's antimicrobial action are inhibiting respiratory enzymes, dissipation of the proton-motive force, and interaction with the cytoplasmic membrane, resulting in membrane integrity loss (Mokarizadeh et al., 2017).

Furthermore, citral incorporation into various food matrices has been shown to decrease the microbial load of pathogenic microorganisms and prolong shelf life. It has suitable drug-like properties and adheres to Lipinski's rules, making it a viable candidate for drug development. The evidence indicates that citral may serve as a promising compound for the formulation of food additives aimed at prolonging the shelf life of both animal and vegetable-based foods, as well as for the development of pharmaceutical products (Gutiérrez-Pacheco et al., 2023).

Cyclooxygenase inhibitory effects

Since NSAIDs have undesirable effects on the gastrointestinal and urinary tracts, these drugs are often replaced with medicinal herbs.

Table 1
The chemical constituents of *C. limon* volatile oil from Palestine.

#	Name of the compounds	RT	RI	Area	Components (%)
1	Sabinene	11.561	970	37 585	0.14
2	*β*-Pinene	11.742	974	224 037	0.82
3	Myrcene	12.34	987	25 358	0.09
4	*o*-Cymene	13.84	1 022	38 643	0.14
5	Limonene	14.04	1 026	3 910 742	14.32
6	1,8-Cineole	14.17	1 029	187 746	0.69
7	trans-ocimene	14.82	1 045	65 267	0.24
8	Linalool	17.05	1 097	233 632	0.86
9	Nonanal	17.25	1 102	21 580	0.08
10	*Cis*-Limonene oxide	18.35	1 130	21 963	0.08
11	Exoisocitral	18.75	1 141	13 319	0.05
12	Citronellal	19.11	1 150	60 157	0.22
13	Terpinen − 4-ol	20.19	1 177	166 352	0.61
14	*α*-Terpineol	20.76	1 193	129 929	0.48
15	Methyl chavicol	20.85	1 194	45 823	0.17
16	*n*-Dodecane	20.97	1 197	57 784	0.21
17	trans-Carveol	21.69	1 217	13 239	0.05
18	Nerol	21.93	1 224	106 249	0.39
19	Neral	22.34	1 236	6 548 353	23.98
20	4-Methoxybenzaldehyde	22.92	1 252	276 452	1.01
21	Geranial	23.42	1 265	8 481 555	31.06
22	*E*-Anethole	24.05	1 283	2 696 175	9.87
23	Tridecane	24.55	1 297	107 584	0.39
24	Citronellyl acetate	26.18	1 346	173 902	0.64
25	*neo*-Dihydro carveol acetate	26.26	1 348	24 087	0.09
26	Neryl acetate	26.49	1 355	2 144 142	7.85
27	Geranyl acetate	27.14	1 374	839 204	3.07
28	Anisyl methyl ketone	27.26	1 378	52 844	0.19
29	*n*-Tetradecane	27.89	1 397	91 180	0.33
30	*β*-Caryophyllene	28.55	1 417	255 053	0.93
31	*α*-trans-Bergamotene	28.96	1 431	30 410	0.11
32	*n*-Pentadecane	31.03	1 497	67 341	0.25
33	*(Z)*-α-Bisabolene	31.27	1 505	44 592	0.16
34	Caryophyllene oxide	33.52	1 581	61 887	0.23
35	*n*-Hexadecane	33.99	1 597	40 334	0.15
36	Heptadecan	36.79	1 697	15 324	0.06
	Total			27 309 824	100.00
	Yield				1.24% ± 0.11
	Phytochemical groups				
Hydrocarbon monoterpenes					15.75
Oxygenated monoterpenoids					80.14
Hydrocarbons sesquiterpenes					1.21
Oxygenated sesquiterpenoid					0.23
Others					2.67
Total					100.00

RI, retention index; RT, retention time.

Toxicity and recurrence of symptoms after discontinuing the use of potent synthetic pharmaceuticals are the most significant drawbacks of these medications (Ukrainets et al., 2006). Numerous investigations into medicinal plants in search of anti-inflammatory medications highlight the need for screening and developing pharmaceuticals with anti-inflammatory action (Nunes et al., 2020; Shawarb et al., 2021). The cyclooxygenase family's iso-enzymes COX-1 and COX-2 catalyze the synthesis of autacoid mediators known as prostaglandins. These prostaglandins impact almost all known physiological and pathological processes (Fitzpatrick, 2004). Since aspirin and many other NSAIDs inhibit COX enzymes, these enzymes are crucial in clinical settings. Inflammatory, pyretic, thrombotic, neurodegenerative, and oncological conditions are alleviated by COX inhibitors (Sweileh et al., 2004). Furthermore, it was shown that the alcoholic extract of lemongrass, which contains citral as its primary component, including geranial and neral, decreased the production of Tumor necrosis factor-α in bronchoalveolar macrophages that were stimulated with Lipopolysaccharides. This increased the anti-inflammatory effect of citral, suggesting that regulating the COX-2 and Tumor necrosis factor-α genes may be one of the mechanisms underlying this activity (Idrees et al., 2019) (Fig. 1).

In the current investigation, COX suppressant testing of the *C. limon* VO from Palestine showed that the percentage inhibition at 300 μg/ml is more than that at 50 μg/ml against both COX enzymes. However, the VO showed more inhibition potency against COX-2 than COX-1. Moreover, the

Table 2
C. limon leaves volatile oil chemical components from various regions.

Region	Major chemical constituents	References
Iran	Linalool (30.62%), geraniol (15.91%), *α*-terpineol (14.52%), and linalyl acetate (13.76%).	(Hojjati and Barzegar, 2017)
Egypt	Sabinene (29.5%), *β*-ocimene (8.27%), limonene (7.86%), and 3-carene (7.18%).	(Asker et al., 2020)
Tunisia	Limonene and β-pinene, represented 39.74 and 25.44%, respectively.	(Ben Hsouna et al., 2017)
India	Limonene (13.5%), geranial (6.4%), citronellic acid (7.0%), neryl acetate (5.4%) and geranyl acetate (6.2%)%	(Tomer et al., 2010)
Nigeria	Limonene (31.5%), sabinene (15.9%), citronellal (11.6%), linalool (4.6%), neral (4.5%), and geranial (4.5%)	(Owolabi et al., 2018)

Table 3
Antimicrobial effects MIC values of *C. limon* volatile oil.

Examined samples	Bacterial strains						Fungal strain
	S. aureus	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*P. vulgaris*	MRSA	*C. albicans*
C. limon VO (µl/ml)	6.25 ± 0.1	6.25 ± 0.01	25 ± 0.05	12.5 ± 0.11	1.56 ± 0.09	6.25 ± 0.12	0.39 ± 0.08
Fluconazole (µg/ml)	NA	NA	NA	NA	NA	NA	1.56 ± 0.02
Ciprofloxacin (µg/ml)	0.78 ± 0.001	1.56 ± 0.005	3.12 ± 0.01	0.125 ± 0.001	15 ± 0.16	12.5 ± 0.93	NA

MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus; VO, volatile oil.*

Fig. 1. The leaves of *C. limon* plant.

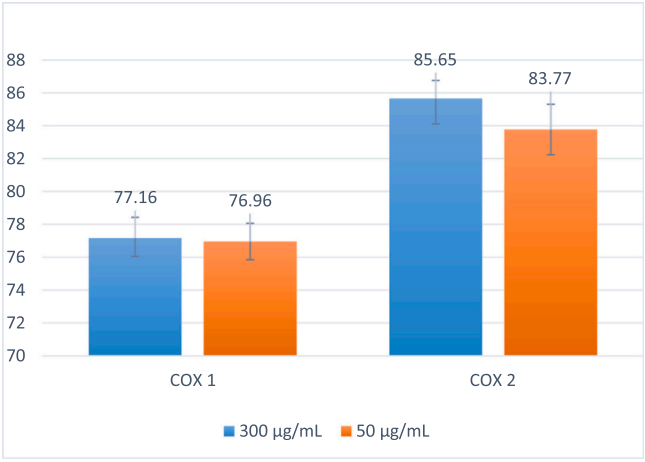

Fig. 2. Inhibition percentages of COX-1 and COX-2 by the *C. limon* VO at 50 and 300 µg/ml (*P* values < 0.005). COX, cyclooxygenases; VO, volatile oil.

data showed that at a dose of 300 µg/ml, the tested VO inhibited COX-1 and COX-2 with 77.16 ± 1.27% and 85.65 ± 1.1%, respectively, as illustrated in Figure 2. The IC_{50} calculations showed that *C. limon* VO has high potency against COX-2 (IC_{50} = 9.32 ± 0.88 µg/ml) while having a mild cyclooxygenase suppressant effect against COX-1 (IC_{50} = 19.1 ± 0.96 µg/ml). To our knowledge, no previous studies investigated the COX inhibitory effect by *Citrus* species.

Cytotoxic activity

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay is a prevalent colorimetric method for assessing cellular metabolic activity. This assay is frequently employed to assess the viability and cytotoxicity of diverse agents, including pharmaceuticals, chemicals, and natural compounds. The MTS assay offers several advantages for assessing cytotoxicity: it is susceptible, easily adaptable for high-throughput screening, enabling rapid analysis of numerous samples; it is non-radioactive, ensuring safety for researchers and the environment; it accommodates various cell types and can be modified to evaluate different parameters of cell viability and cytotoxicity, such as proliferation, apoptosis, and necrosis. It is relatively economical compared to other cytotoxicity assays, rendering it a cost-effective choice for many research applications, and it necessitates minimal sample preparation, thereby conserving time and minimizing potential experimental variability. Overall, the MTS assay is a reliable and versatile method for evaluating cytotoxicity and is widely used in academic and industrial research settings. The anticancer potential of *C. limon* VO and its major components (limonene, linalyl acetate, geranial, and neral) has attracted researchers' interest. Additionally, a prior investigation showed the capacity of citral (geranial, neral) to inhibit cell growth by raising intracellular reactive oxygen species (ROS) and reducing mitochondrial membrane potential in HeLa cells (Othman et al., 2022). Furthermore, citral demonstrated cytotoxic properties and the ability to induce apoptosis in many cancer cell lines (Petretto et al., 2023), as well as LS174T human colon cancer cells, were driven to undergo apoptosis by D-limonene through the mitochondrial death pathway, and the inhibition of the PI3K/Akt pathway (Klimek-Szczykutowicz et al., 2020).

The cytotoxicity MTS assay outcomes in the current study revealed that *C. limon* VO at a concentration of 500 µg/ml inhibited the growth of MCF-7, Hep3B, HeLa, and LX-2 at 96.88, 95.61, 93.96, and 85.79%, respectively, as illustrated in Figure 3.

From the following equations: MCF-7: y = 37.231 ln(x) – 139.49; HeLa: y = 98.111 ln(x) – 500.76; Hep3B: y = 38.643 ln(x) – 150.44; and LX-2: y = 35.096 ln(x) – 118.41, the cytotoxicity IC_{50} values were calculated, and the results showed that *C. limon* VO has varied cytotoxic effects against all cancer cell lines. The *C. limon* VO was the most active product against MCF-7 with an IC_{50} of 162.90 ± 1.85 mg/ml, but compared with the potent anticancer drug Doxorubicin (DOX), it seemed relatively weak, as demonstrated in Table 4.

Regarding the cell viability after the treatment with *C. limon* leaf VO, the results showed that the cell viability was inhibited strongly with 250 µg/ml rather than 62.5 µg/ml.

As presented in Figure 4, at a concentration of 62.5 µg/ml, the MCF-7, Hep3B, HeLa, and LX-2 cells' viability were inhibited by 98.56, 96.74, 99.55, and 90.42%, respectively.

A study conducted by Al Othman et al. investigated the cytotoxic effect effects of *C. limon* leaves VO from Negeri Sembilan and found that it has potent antiproliferative activity on the HeLa cell line, with an IC_{50} value of 11.66 µg/ml (Othman et al., 2023).

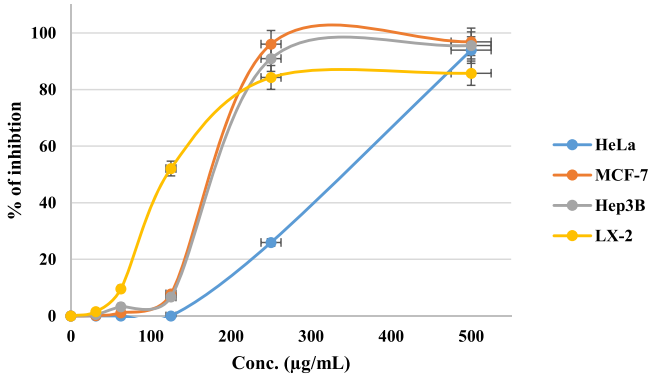

Fig. 3. MCF-7, Hep3B, HeLa, and LX-2 cells lines inhibition percentages by *C. limon* VO. VO, volatile oil.

Table 4
The cytotoxicity test IC_{50} values (mg/ml) of *C. limon* VO and Doxorubicin (DOX).

Tests	IC_{50} (mg/ml)			
	MCF-7	HeLa	Hep3B	LX-2
C. limon VO	162.90 ± 1.85	275.174 ± 3.21	179.631 ± 2.01	121.126 ± 2.42
DOX	0.314 ± 1.08	0.84 ± 1.1	1.21 ± 1.0	> 0.05

VO, volatile oil.

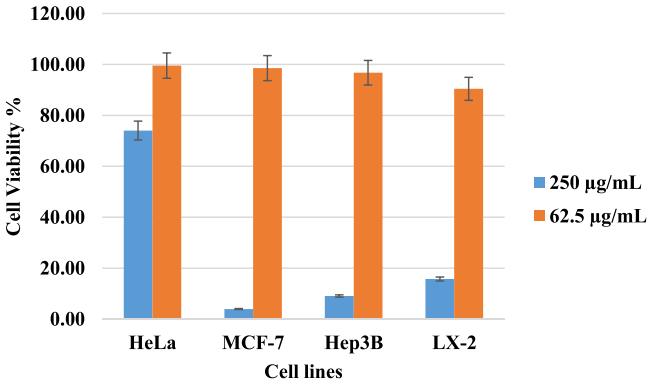

Fig. 4. MCF-7, Hep3B, HeLa, and LX-2 cells' viability percentage after the treatment with 62.5 µg/ml and 250 µg/ml *C. limon* leaf VO. VO, volatile oil.

Geranial is the principal compound of the investigated volatile oil, recognized as a lipophilic substance that is deemed safe and used across various sectors of the pharmaceutical and food industries (Jaradat, 2015). Although geranial offers various health benefits, such as antibacterial, antifungal, immunosuppressive, and anti-inflammatory effects, its mechanism of action remains inadequately understood (Gómez-Sequeda et al., 2020; Kwiatkowski et al., 2022; Lira et al., 2020). Moreover, Chueca et al. (2014) elucidated the mechanism by which limonene induces mortality of *E. coli*, a prevalent component of *C. limon* VO. The inactivation of *E. coli* by this phytochemical was attributed to Fenton-mediated hydroxyl radical generation, which resulted in oxidative DNA damage (Chueca et al., 2014).

The present study has several limitations that warrant attention in future research. Notably, this investigation concentrated solely on the volatile organic compounds of *C. limon* leaves, neglecting other plant parts, such as the bark or fruit peels, which may harbor additional bioactive molecules. Furthermore, although the cytotoxic, anti-inflammatory, and antimicrobial properties were evaluated, the precise mechanism of action of the examined VO on these bioactivities was not thoroughly investigated. Furthermore, the antimicrobial and cytotoxicity assessments were restricted to particular microbial species and cancer cell lines. Future research is necessary to evaluate the wider range of antimicrobial and cytotoxic effects against various microbial strains and cancer cells.

Conclusion

The GC-MS method found 36 molecules in Palestine's *C. limon* leaf VO. These molecules comprised 100% of the total oil, with geranial, neral, and limonene leading oil components. The investigated oil showed remarkable antimicrobial activity, especially against *P. aeruginosa*, MRSA, and *C. albicans*. In addition, the *C. limon* leaf VO showed high potency against COX-2 and a mild suppressant effect against COX-1. Moreover, it demonstrated a potent cytotoxic effect against the MCF-7 cell line. This study can serve as an a priori reference for developing bioactive food supplements from *C. limon* VO that can provide health-promoting properties after pharmacodynamics and pharmacokinetics studies, in addition to possible applications in cosmetics and food flavoring agents' industries.

Ethics approval

Hereby, I am Nidal Jaradat/consciously assured that for the manuscript "Chemical analysis and bioactivity evaluation of *C. limon* leaves volatile oil from Palestine: investigating phytochemical, anti-inflammatory, antimicrobial, and cytotoxic properties, the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' research and analysis in a truthful and complete manner.
4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
5) The results are appropriately placed in prior and existing research context.
6) All sources used are properly disclosed (correct citation).
7) All authors have been personally and actively involved in substantial work leading up to the paper and will take public responsibility for its content.

I agree with the above statements and declare that this submission follows the policies outlined in the Guide for Authors and the Ethical Statement.

Author contributions

Aya Qabaha: Visualization, Methodology, Investigation. **Khariya Amarneh:** Visualization, Methodology, Investigation. **Raneen Qabaha:** Visualization, Methodology, Investigation. **Mohammad Qadi:** Validation, Formal analysis, Data curation. **Trobjon Makhkamov:** Writing – review & editing, Data curation. **Linda Issa:** Visualization, Methodology, Investigation. **Sama' Qassarwi:** Visualization, Methodology, Investigation. **Nawaf Al-Maharik:** Validation, Formal analysis, Data curation. **Murad Abualhasan:** Validation, Formal analysis, Data curation. **Abdumurod Sattarov:** Writing – review & editing, Data curation. **Mohammed Hawash:** Validation, Formal analysis, Data curation. **Nilufar Ergasheva:** Writing – review & editing, Data curation. **Nidal Amin Jaradat:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Data curation, Conceptualization.

Availability of data

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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.

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Appendix A. Supporting material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.hermed.2024.100954.

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