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RESEARCH

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Phytochemical profiling and bioactivity analysis of *Citrus japonica* leaves volatile oil from Palestine

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Abstract

Background Exploring the therapeutic potential of unutilized plant parts from agricultural crops represents a promising strategy for discovering novel medications with high positive economic value. This study aimed to characterize the composition, antidiabetic, anti-obesity, antioxidant, and cytotoxic effects of volatile oil (VO) extracted from the leaves of *Citrus japonica* trees. This is the first research to assess the *C. japonica* VO's anti-obesity, anti-lipase, and cytotoxic properties. Using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays and gas chromatography–mass spectrometry (GC–MS) analysis, the components of VO and its capacity to suppress the growth of cancer and other abnormal cells were ascertained, respectively. Stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals, *p*-nitrophenyl butyrate (PNPB), and dinitrosalicylic acid (DNSA) assays were employed to determine antioxidant, anti-obesity, and antidiabetic activities, respectively.

Results The *C. japonica* leaf showed significant diversity in type and percentage of VO molecules. Overall, 45 compounds were identified in the VO, constituting 99.69% of the total oil composition. γ-Muurolene (28.12%), β-eudesmol (10.93%), γ-eudesmol (8.44%), germacrene B (7.39%), and elemol (7.27%) are the major characterized molecules. According to the inhibition percentage results of DPPH free radicals, porcine pancreatic lipase, and α-amylase, the VO exhibits strong antioxidant properties and weak inhibitory effects on lipase and α-amylase enzymes. The *C. japonica* VO showed a moderate cytotoxic effect against Hep3B and considerable activity on B16F1, CaCo-2, HeLa, MCF-7, and HepG2, with IC_{50} doses in the range of 69.7–171.96 µg/mL. The VO cytotoxic effect IC_{50} against the normal cell line LX-2 was 224.95 µg/mL.

Conclusion The current study collectively presented the chemical constituents of *C. japonica* leaf VO from Palestine for the first time and demonstrated its inhibitory effects against DPPH free radicals, porcine pancreatic lipase, and α-amylase. The results suggest that *C. japonica* leaf VO has the potential to be used as a natural supplement to prevent or treat cancer, as well as in the food industry as a natural antioxidant.

Keywords *Citrus japonica*, Volatile oil, Phytoconstituents, Antioxidant, Anti-obesity, Antidiabetic, Cytotoxicity

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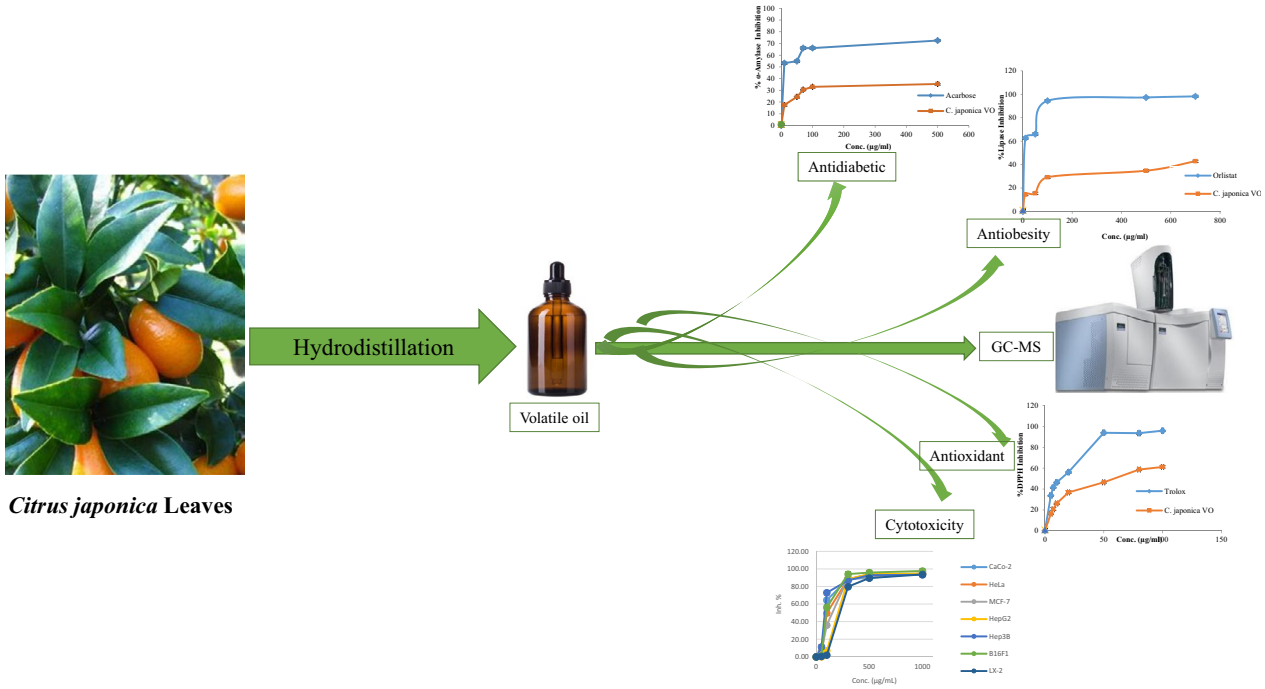
Graphical Abstract



***Citrus japonica* Leaves**

Introduction

Volatile oils (VOs) have been an important part of medicine for generations, offering a natural and integrative approach to wellness and healing. The medicinal qualities of these purified plant oils range from stress reduction and pain treatment to anti-inflammatory and antimicrobial actions. The versatility of VOs in supplementing traditional treatment and their ability to improve persons' general health and vigor make them a valuable tool in modern healthcare [1–3]. VOs have made their way into mainstream medicine in addition to their usage in aromatherapy. They are becoming more well-known for their antibacterial, antifungal, and antiviral characteristics, which have led to their inclusion in therapies for various ailments. Furthermore, studies have revealed that several VOs have analgesic effects, offering them a natural alternative for pain relief. The use of VOs in modern medical practices indicates rising respect for the potential advantages of natural remedies, providing patients with various options that correspond with an integrated healthcare strategy while also helping develop novel medicines [4–6].

Citrus japonica Thunb. is also referred to synonymously as *Fortunella japonica* (Thunb.) Swingle belongs to the Rutaceae family and is native to the Asia–Pacific and South Asia. It has many traditional therapeutic benefits: expectorant, carminative, antivirus, and inflammatory [7].

Cancer is a serious global medical issue that impacts individuals and communities on a personal and emotional level, as well as affecting societal, economic, and psychological aspects of life. It transcends borders, cultures, and demographics, highlighting the need for collaborative efforts in research, prevention, and access to quality healthcare. Addressing both the quantitative aspects of cancer, such as incidence and mortality rates, and the qualitative aspects is essential, as the effects of cancer extend far beyond a medical diagnosis [8, 9]. Diabetes mellitus, a chronic condition characterized by high blood glucose levels, is a serious global health issue [10]. It poses significant challenges for healthcare systems, requiring extensive research and effective mitigation strategies [11]. The number of people with diabetes has surged from 108 million in 1980 to 422 million in 2014, driven by sedentary lifestyles, poor nutrition, and an aging population [12]. This condition imposes a substantial financial burden on healthcare systems and individuals due to the costs of prescriptions, hospitalizations, and treatments for complications [13].

Obesity has emerged as a crucial global healthcare problem, posing significant health hazards and putting pressure on healthcare systems across the world

[14, 15]. According to the World Health Organization (WHO), approximately 650 million adults and 340 million children were obese in 2016 [16]. Oxidative stress, caused by an imbalance in the body's free radicals and antioxidants, has emerged as a widespread element in the development of numerous illnesses, posing a severe worldwide healthcare problem [17]. Cardiovascular disorders, neurological ailments such as Alzheimer's and Parkinson's diseases, cancer, and inflammatory disorders are all associated with oxidative stress [18]. The effect of oxidative stress on cellular damage and dysfunction emphasizes its importance in the etiology of various diseases.

C. japonica a kumquat species cultivated in Egypt, is known for its sweet and fragrant rind, making the whole fruit enjoyable to eat [19]. It can be made into marmalade, added to fruit salads, or preserved in sugar syrup for the food industry [20]. Traditionally, it has been used in folk medicine to treat colds, coughs, and respiratory tract inflammations. Despite its traditional uses, its phytoconstituents and biological activities are poorly understood [21]. Previous studies identified C-glycosyl flavonoids, flavanones, and methylated aglycones as the primary phenolic compounds in *C. japonica*. These compounds have shown various biological effects, including antioxidant, anti-inflammatory, and anticancer properties [22].

Investigating the therapeutic potential of underutilized plant parts derived from crops is a viable approach for identifying innovative drugs with significant economic benefits [23]. Besides, Palestine is among the world's largest exporters of Citrus fruits [24]. Given the significant global healthcare challenges posed by diabetes, cancer, obesity, and diseases associated with oxidative stress, this study aims to comprehensively profile the phytochemical constituents of *C. japonica* leaf volatile oil (VO) from Palestine and assess its cytotoxic, antidiabetic, anti-obesity, and antioxidant activities.

Materials and methods

Plant material

C. japonica plant was recognized in An-Najah National University's Herbal Product Laboratory by a pharmacognosist, Professor Nidal Jaradat, which was then placed within a voucher specimen code (Pharm-PCT-2803). Next, fresh *C. japonica* leaves were gathered in the Palestinian governorate of Jenin in July 2023. After being thoroughly cleaned with distilled water to avoid contamination, the gathered leaves were dried in the absence of sunshine at a regulated temperature of no more than 25 °C and a normal humidity level. Following drying, the plant dry material was roughly ground into powder and kept in bags for later use.

Chemicals and instruments

Frutarom (Clacton-on-Sea, UK) provided the following chemical and biological materials: methanol, trolox, NaCl, Na_2HPO_4/NaH_2PO_4, DPPH, 3,5-dinitro salicylic acid (DNSA), porcine pancreatic lipase, α-amyalse, starch, and Acarbose. Besides, dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich in Germany. In addition, the instruments, namely a shaker apparatus (Memmert, Buchenbach, Germany), a UV–visible spectrophotometer (Jenway 7135, Staffordshire, UK), a grinder (Moulinex, model LM2211, UNO, Shanghai, China), a balance (Rad wag, AS 220/c/2, Radom, Poland), gas chromatography (PerkinElmer, Clarus 500, Waltham, Massachusetts, USA), and mass spectrometer (Shelton, CT105484, Massachusetts, USA) were used in this investigation.

Volatile oil extraction

The *C. japonica* VO was extracted by hydrodistillation using a Clevenger device. One liter of distilled water was added to a round-bottom flask holding six hundred grams of plant leaves. This mixture was then hydrodistilled for about 4 h. The extracted oil was dried on anhydrous magnesium sulfate [25]. The extracted VO was sealed in opaque glass vials and stored at 4 °C until being utilized. On a weight-per-weight basis (w/w), the VO output was 2.22%.

Gas chromatography–mass spectrometry

Using gas chromatography connected to a Perkin Elmer Clarus 560 mass spectrometer, the phytochemical components of *C. japonica* VO were ascertained both qualitatively and quantitatively. A Perkin Elmer Elite-5 fused-silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm) was used to separate. The column's starting temperature was set at 50 °C and maintained there for 5 min. After that, it was programmed to increase by 4 °C per minute until it reached its ultimate temperature of 280 °C. Helium was used as a carrier gas, and its flow rate was maintained at 1 mL/min for each chromatographic run. 0.2 µL of the obtained *C. japonica* VO was added to a split chamber at 250 °C with a splitter rate of 1:50. By comparing the mass spectra of the phytochemical components of *C. japonica* VO with reference samples that were already in the analyzer library (National Institute of Standards and Technology; NIST), its elements were determined. Using the homologous series of n-alkanes C_6–C_{28} as a standard, each compound's retention index (RI) was calculated using the Kovats technique and compared to standards found in the literature [26]. The retention index was calculated from Eq. (1):

$$\mathrm{RI} = 100 * \left[n + \left((\mathrm{RT}(\mathrm{Samble}) - \mathrm{RT}(n))/(\mathrm{RT}(N) - \mathrm{RT}(n))\right)\right]. \quad (1)$$

n: the number of the preceding carbon atom of the sample in a mixture of *n*-alkanes. *N*: the number of the following carbon atoms of the sample in a mixture of *n*-alkanes. RT: the individual retention time.

Free radical scavenging activity

We assessed the VO's capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals using a method previously reported [27]. A stock solution was serially diluted in this experiment to provide different concentrations (5, 7, 10, 20, 50, 80, and 100 µg/mL). Next, each sample's absorbance was evaluated at 517 nm in wavelength. A substance-free solution was combined with equal quantities of DPPH solution and methanol. Trolox was employed as the reference standard at the mentioned VO concentration. The absorbance of this solution was measured to facilitate the computation of the inhibition percentage.

$$\text{DPPH inhibition \%} = (\mathrm{B} - \mathrm{Z})/\mathrm{B} \times 100\%. \quad (2)$$

B = absorbance of blank, Z = absorbance of the tested samples.

Porcine pancreatic lipase suppressant method

This work used the porcine pancreatic lipase inhibition test developed based on a prior investigation [28]. Five different solutions with 10, 50, 100, 500, and 700 µg/mL concentrations were diluted with methanol from a stock solution of plant VO at 1 mg/mL in 10% DMSO. The pancreatic lipase enzyme was prepared as a 1 mg/mL stock solution just prior to use. Furthermore, 20.9 mg of p-nitrophenyl butyrate (PNPB) was dissolved in 2 mL of acetonitrile to generate a stock solution. Porcine pancreatic lipase (1 mg/mL) and 0.2 mL of various VO concentrations (10, 50, 100, 300, and 400 µg/mL) were combined in separate test tubes.

The Tris–HCl solution (pH 7.4) was added, and the mixtures were incubated at 25 °C for 15 min to reach a final volume of 1 mL. Each test tube was filled with 0.1 mL of PNPB solution after incubation. The mixes were incubated at 37 °C for 30 min once more. Using a UV–visible spectrophotometer, the hydrolysis of p-nitrophenyl butyrate to p-nitrophenol at 405 nm was measured to evaluate the inhibitory activity of pancreatic lipase. Orlistat was used as a positive control, and the same protocol was followed with the previously specified concentrations. Every test was run in triplicate.

$$\text{Porcine pancreatic lipase inhibition \%} = (\mathrm{AB} - \mathrm{AE})/\mathrm{AB} \times 100\%. \quad (3)$$

AB is the blank solution's recorded absorbance, and AE is the recorded absorbance of the oil sample solution.

α-Amylase inhibitory method

The plant-derived VO was first dissolved in a small amount of dimethyl sulfoxide (DMSO) and then diluted in a buffer solution (NaCl at 0.006 M, NaH_2PO_4/NaH_2HPO_4 at 0.02 M, and pH adjusted to 6.9) to achieve a concentration of 1 mg/mL. Subsequently, 10, 50, 70, 100, and 500 µg/mL dilutions were produced, and the reference standard Acarbose was used in the same mentioned concentrations. The following formula was used to calculate the percentage of inhibition for the α-amylase inhibitory activity:

$$\%\ \text{of}\ \alpha\text{-amylase inhibition} = (B - S)/B \times 100\%, \quad (4)$$

where B is the absorbance of a blank; S is the absorbance of a tested sample [29].

Cytotoxicity screening

The research sought to assess the anticancer potential of *C. japonica* VO against different cell lines. The cell lines used in this experiment included breast cancer (MCF-7), hepatocellular carcinoma (Hep3B, HepG2), cervical cancer specimen (HeLa), and colon carcinoma (Caco-2) cells were cultured in RPMI 1640 media, while human hepatic stellate (LX-2) and skin cancer (B16F1) cells were cultured in Dulbecco's modified Eagle's medium (DMEM). After incubating all cells at 37 °C in a humid condition with 5% CO_2, 2.6×10^4 cells/mL were seeded into a 96-well plate. Cells were treated for 48 h to various concentrations of the investigated VO (1000, 500, 300, 100, and 50 µg/mL) and 5-flurouracil medication (300, 100, 50, 10, and 1 µM). The Cell-Tilter 96® Aqueous One Solution Cell Proliferation (MTS) bioassay was used to assess cell viability after culture. The MTS assay was based on adding 90 µl of medium and 10 µL of MTS solution to each well, and the plates were incubated for 24 h at 37 °C before the absorbance was determined using a UV–Vis spectrophotometer at 490 nm. Equation (5) was used to calculate the cell viability percentage of VO:

$$\text{Cell viability}(\%) = \frac{\mathrm{ODsample}}{\mathrm{ODcontrol}} \times 100\%, \quad (5)$$

where OD: optical density.

The selectivity index (SI) calculations were established by dividing the IC_{50} value into normal cells by the IC_{50} value on cancer cells:

$$\text{Selectivity index (SI)} = \frac{\text{IC50 of } VO \text{ on normal cells}}{\text{IC50 of } VO \text{ on cancer cells}}. \quad (6)$$

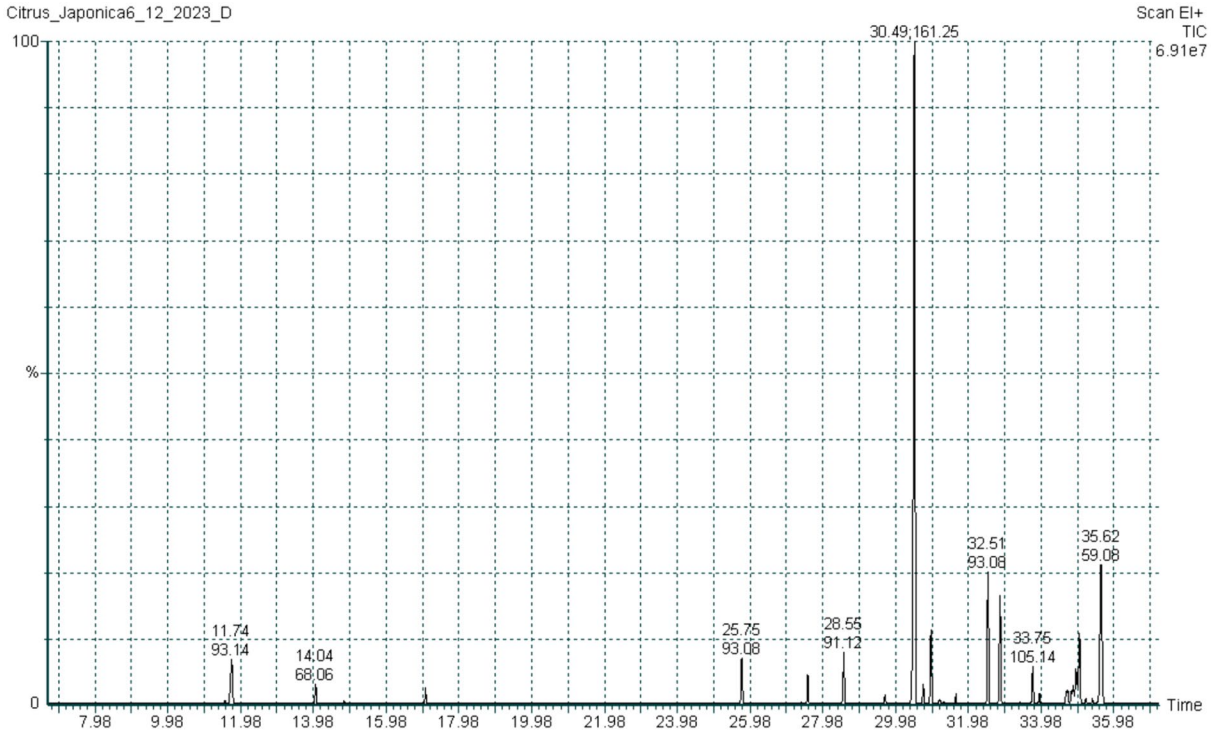


Fig. 1 Gas chromatography–mass spectrometry chromatogram of *C. japonica* leaf volatile oil

The SI value indicates the sample's selectivity to the cell lines tested. Samples with a SI > 3 was considered to have a high selectivity for tumorous cells [30].

Statistical analysis

All the experiments were carried out in triplicates. The results are presented as means ± standard deviation (SD). The statistical significance of the difference was evaluated by variance analysis (ANOVA) using an Excel program. Only *p*-values less than 0.005 were deemed significant.

Results and discussion

Phytochemical analysis

VOs consist of a complex combination of natural molecules that vary in concentration, ranging from 20 to 60 components, less or more. The primary components of VOs are primarily responsible for their biological and chemical characteristics. These components belong to two categories of biosynthetic origins, classified mainly from terpenoids and terpenes, which are low molecular weight aliphatic or aromatic components [31].

The yield of VO obtained for *C. japonica* was 2.22%. Instrumental GC–MS technology branded 45 elements, accounting for 99.69% of the total VO. The predominant molecules are *γ*-muurolene (28.12%), *β*-eudesmol (10.93%), γ-eudesmol (8.44%), germacrene B (7.39%), and elemol (7.27%) (Fig. 1, Table 1). Regarding the phytochemical classification of *C. japonica* VO, hydrocarbon sesquiterpene is the major class, representing 58.66%, and its major components were *γ*-muurolene (28.12%), germacrene B (7.39%), and elixene (5.40%). The second class of VO is oxygenated sesquiterpenoid (33.18%), and its trend molecules are *β*-eudesmol (10.93%), γ-eudesmol (8.44%), and elemol (7.27%). The next class is hydrocarbon monoterpene (6.38%), and the least phytochemical class is oxygenated monoterpenoid (1.46%), whose main constituent is linalool (1.30%). The hydrocarbon sesquiterpene class represented 21 compounds, followed by hydrocarbon monoterpene, oxygenated sesquiterpenoid, and oxygenated monoterpenoid, which have 10, 9, and 5 molecules, respectively.

Satyal et al. reported that the VO of *C. japonica* from Nepal was obtained in 0.075% yield, and 42 elements accounted for 99.6% of the oil. The major compounds in the VO were linalool (35.1%), geraniol (12.7%), and *trans*-isomer nerol (5.3%) [32].

A study conducted by Sutour et al. found that *C. japonica* VO from France was dominated by germacrene D (14.9%), *β*-elemol (9.1%), *cis*-guai-6-en-10p-ol (6.3%), *β*-eudesmol (5.5%), and *δ*-elemene (5.2%) [33].

C. japonica leaves VO from Thailand; the major components were *β*-pinene (47.4%), limonene (10.2%), and linalool (9.8%. [34]. While the major leaf components

Table 1 Chemical constituents identified in the volatile oil of *C. japonica* leaf from Palestine

#	Name	RT	Area	KRI	L KRI	VO, (%)	Classifications
1	*α*-Pinene	9.76	398,828	930	930	0.17	Hydrocarbon monoterpene
2	Sabinene	11.55	881,472	970	970	0.37	Hydrocarbon monoterpene
3	*β*-Pinene	11.73	7,655,249	974	974	3.20	Hydrocarbon monoterpene
4	*β*-Myrcene	12.33	169,527	987	987	0.07	Hydrocarbon monoterpene
5	3-*β*-Carene	13.13	87,446	1005	1006	0.04	Hydrocarbon monoterpene
6	Limonene	14.03	4,571,687	1026	1026	1.91	Hydrocarbon monoterpene
7	1,8-Cineole	14.15	84,454	1029	1030	0.04	Oxygenated monoterpenoid
8	Z-*β*-Ocimene	14.38	24,033	1034	1035	0.01	Hydrocarbon monoterpene
9	E-*β*-Ocimene	14.82	1,368,325	1045	1044	0.57	Hydrocarbon monoterpene
10	*γ*-Terpinene	15.29	72,071	1056	1056	0.03	Hydrocarbon monoterpene
11	Terpinolene	16.44	31,721	1083	1083	0.01	Hydrocarbon monoterpene
12	Thujone, *cis*-	16.75	119,332	1090	1090	0.05	Oxygenated monoterpenoid
13	Linalool	17.05	3,111,230	1097	1097	1.30	Oxygenated monoterpenoid
14	Terpinen-4-ol	20.2	89,191	1171	1172	0.04	Oxygenated monoterpenoid
15	*α*-Terpinol	20.76	86,989	1184	1185	0.04	Oxygenated monoterpenoid
16	*δ*-Elemene	25.75	7,779,617	1333	1333	3.25	Hydrocarbon sesquiterpene
17	*α*-Cubebene	26.15	104,603	1345	1345	0.04	Hydrocarbon sesquiterpene
18	*α*-Ylangene	26.91	173,996	1367	1367	0.07	Hydrocarbon sesquiterpene
19	Isoledene	27.12	378,275	1374	1374	0.16	Hydrocarbon sesquiterpene
20	β-Bourbonene	27.39	730,680	1382	1382	0.31	Hydrocarbon sesquiterpene
21	β-Elemene	27.57	5,560,726	1387	1387	2.33	Hydrocarbon sesquiterpene
22	Caryophyllene	28.56	9,600,674	1418	1418	4.02	Hydrocarbon sesquiterpene
23	*γ*-Elemene	28.86	1,083,900	1428	1428	0.45	Hydrocarbon sesquiterpene
24	*α*-Guaiene	29.24	913,038	1440	1439	0.38	Hydrocarbon sesquiterpene
25	Spirolepechinene	29.48	580,434	1447	1446	0.24	Hydrocarbon sesquiterpene
26	*α*-Humulene	29.69	2,158,624	1454	1454	0.90	Hydrocarbon sesquiterpene
27	*γ*-Muurolene	30.53	67,231,776	1481	1481	28.12	Hydrocarbon sesquiterpene
28	*β*-Selinene	30.75	4,426,777	1488	1488	1.85	Hydrocarbon sesquiterpene
29	Elixene	30.96	12,912,011	1495	1492	5.40	Hydrocarbon sesquiterpene
30	*trans*-*β*-Guaiene	31.19	2,357,774	1502	1502	0.99	Hydrocarbon sesquiterpene
31	Germacrene A	31.31	1,003,050	1506	1506	0.42	Hydrocarbon sesquiterpene
32	*γ*-Cadinene	31.48	388,522	1512	1513	0.16	Hydrocarbon sesquiterpene
33	*β*-Cadinene	31.64	3,014,578	1517	1517	1.26	Hydrocarbon sesquiterpene
34	*γ*-Selinene	32.07	1,191,625	1530	1532	0.50	Hydrocarbon sesquiterpene
35	Elemol	32.54	17,382,082	1546	1545.7	7.27	Oxygenated sesquiterpenoid
36	Germacrene B	32.85	17,670,646	1555	1555	7.39	Hydrocarbon sesquiterpene
37	Longipinanol	33.29	295,875	1570	1571	0.12	Oxygenated sesquiterpenoid
38	Spathulenol	33.4	1,260,471	1573	1573	0.53	Oxygenated sesquiterpenoid
39	Viridiflorol	33.76	6,099,252	1585	1585	2.55	Oxygenated sesquiterpenoid
40	Guaiol	33.95	2,404,007	1591	1590	1.01	Oxygenated sesquiterpenoid
41	10-epi-*γ*-Eudesmol	34.71	4,464,588	1622.9	1621	1.87	Oxygenated sesquiterpenoid
42	γ-Eudesmol	35.05	20,177,178	1635.1	1635	8.44	Oxygenated sesquiterpenoid
43	Hinesol	35.22	1,112,465	1641.2	1640	0.47	Oxygenated sesquiterpenoid
44	*β*-Santalene	35.41	978,227	1645.3	1644	0.41	Hydrocarbon sesquiterpene
45	*β*-Eudesmol	35.65	26,122,264	1654.3	1654	10.93	Oxygenated sesquiterpenoid
	SUM		239,054,452			99.69	
	Yield						

Table 1 (continued)

	Phytochemical classifications	%	Compounds account
	Hydrocarbon monoterpene	6.38	10
	Oxygenated monoterpenoid	1.46	5
	Hydrocarbon sesquiterpene	58.66	21
	Oxygenated sesquiterpenoid	33.18	9
	Total	99.69	45

RT retention time, *KRI* Kovats retention index, *L KRI* literature Kovats retention index

from Vietnam were elemol (17.7%), *β*-eudesmol (16.6%), and *epi*-bicyclosesquiphellandrene (16.6%) [35]. In contrast, the *C. japonica* leaf VO from Egypt, the major constituents were elemol (19.4%), *δ*-cadinene (16.0%), *γ*-eudesmol (11.9%), guaiol (11.7%), and *τ*-cadinol (11.1%) [36].

Obviously, our studied leaf VO primary ingredients are *γ*-muurolene (28.12%), *β*-eudesmol (10.93%), *γ*-eudesmol (8.44%), germacrene B (7.39%), and elemol (7.27%), which are drastically different from Egyptian, France, Vietnam, and Nepalese leaves VOs.

The chemical compositions of VOs are a complex and dynamic interplay of various factors, and previous studies reported that VOs chemical ingredients can vary considerably based on the plants' location, soil composition, rainfall level, height, temperature, humidity, and sunshine exposure [37].

Porcine pancreatic α-amylase, lipase, and DPPH free radicals' inhibitory effects

Hyperlipidemia/hypercholesterolemia and obesity are two of the main risk factors for developing signs of diabetes mellitus in people. In numerous developing regions, herbal medicine is still a popular alternative treatment. Various elements of herbal plants, including VOs, are utilized to treat diabetes and obesity [38]. Moreover, for those who have diabetes and obesity, oxidative stress is a serious risk that exacerbates their health issues. Under these circumstances, there is an increase in the risk of inflammation, insulin resistance, neurological damage, and cardiovascular diseases due to elevated levels of reactive oxygen species. Controlling oxidative stress becomes essential to halting further problems and enhancing the general health of persons with diabetes and obesity [39]. Previous investigations demonstrated that VO had antioxidant, α-amylase, and lipase inhibitory effects to help treat oxidative stress, diabetes, and obesity [40, 41].

α-Amylase inhibitory activity

Figure 2 displays the percentage of α-amylase inhibition at different concentrations and showed that both samples exhibited a rise in α-amylase inhibition with an increased concentration. At a concentration of 500 µg/mL, Acarbose inhibited the action of α-amylase by 72.54 ± 2.1%, while *C. japonica* VO inhibited the effect of α-amylase by 35.45 ± 0.11% (p-value < 0.005).

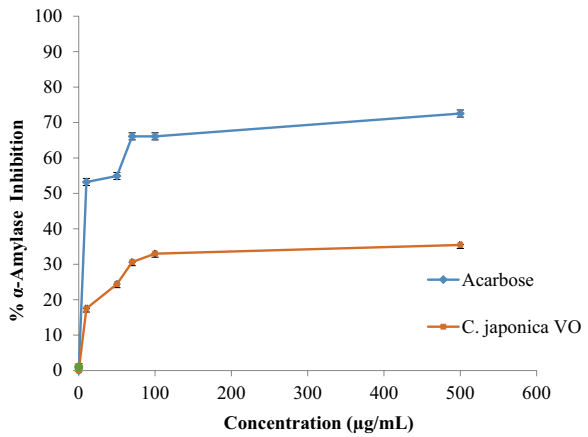


Fig. 2 α-Amylase inhibitory activity by the volatile oil of *C. japonica* leaf and Acarbose drug

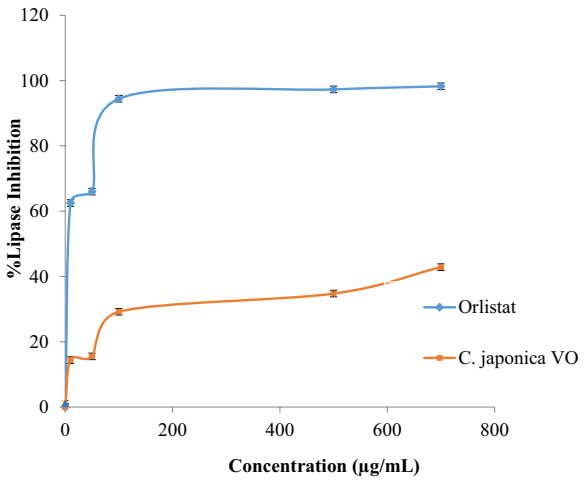


Fig. 3 Porcine pancreatic lipase suppressant action by *C. japonica* VO and Orlistat

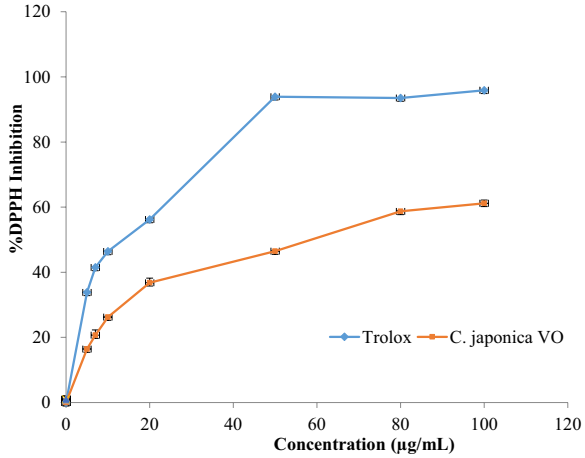


Fig. 4 DPPH free radical scavenging activity by *C. japonica* leaf volatile oil and Trolox

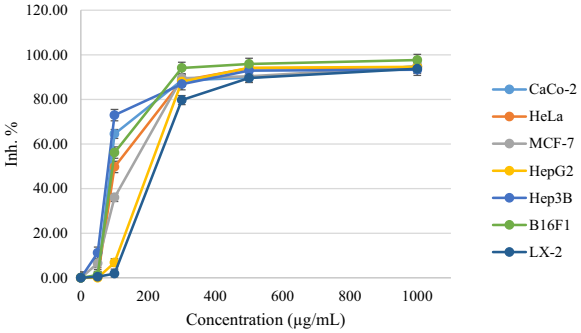


Fig. 5 Cytotoxic effects of *C. japonica* leaf volatile oil against a series of cells

Table 2 Cytotoxicity test IC_{50} (µg/mL) values and selectivity index (SI) of *C. japonica* volatile oil and 5-flurouracil

Cell lines	IC_{50} (µg/mL)	
	C. japonica VO	5-Flurouracil
CaCo-2	94.15 ± 1.99	3.72 ± 0.84
HeLa	98.29 ± 2.54	2.16 ± 1.01
MCF-7	116.29 ± 1.79	1.29 ± 0.45
HepG2	171.96 ± 1.71	4.23 ± 1.78
Hep3B	69.7 ± 2.55	13.28 ± 2.21
B16F1	73.42 ± 1.51	12.91 ± 1.36
LX-2	224.95 ± 2.03	0.74 ± 0.33

All tests were repeated three times (p value < 0.05)

Pancreatic lipase inhibitory results

Regarding porcine pancreatic lipase, Fig. 3 reflects the *C. japonica* VO and Orlistat anti-obesity medication. The results showed that *C. japonica* VO has lipase inhibition action in a dose-dependent manner. However, at a concentration of 700 µg/mL, *C. japonica* VO inhibited the effect of porcine pancreatic lipase by 42.86 ± 0.16. At the same concentration, Orlistat suppressed the action of lipase enzyme by 98.25 ± 0.58% ($p < 0.005$). The concentration used from *C. japonica* VO in this study is almost similar to the one used earlier by Liu et al. [42] from protopanaxadiol type of ginsenosides that is isolated from the leaves of the American ginseng to inhibit the action of lipase. A concentration ranging from 250–1000 µg/mL was used to inhibit the activity of pancreatic lipase in the assay system.

The use of anti-obesogenic drugs is increasing, with reported side effects from dry mouth, blood pressure induction, headache, constipation, and insomnia [43]. In addition, these drugs are not affordable for everyone in the developing countries. Therefore, developing new treatments using natural medicines derived from unused parts of plants is highly advantageous. These drugs will have reduced costs and minimal or without adverse side effects [44].

Antioxidant results

Figure 4 depicts the free DPPH inhibitory actions of *C. japonica* leaf VO and Trolox. Trolox, a vitamin E analog, has a potent antioxidant effect and is utilized therapeutically as an official oxidative stress-reducing medication [45]. Figure 4 indicates that the *C. japonica* leaf VO in a depending manner and at a concentration of 100 µg/mL, the tested VO exhibited 61.17 ± 1.01% of DPPH free radical scavenging property compared with Trolox which has 95.87 ± 0.01% of DPPH free radical suppressant effect with IC_{50} value of 39.8 ± 1.01 and 10.23 ± 0.12 µg/mL, respectively ($p < 0.005$).

Bunrathep et al. [34] examined the antioxidant properties of *C. japonica* leaves and peels by measuring the percentage of DPPH radical inhibition and IC_{50} values (µg/mL). These results were then compared to synthetic vitamin E and butylated hydroxytoluene. The researchers discovered that the leaf and peel VOs exhibited antioxidant activity, with IC_{50} values of 53.11 and 102.11 µg/mL, respectively. These results agreed with the Bunrathep et al. study, as our study's stable DPPH assay results had strong activity compared with Trolox with 39.8 ± 1.01 and 10.23 ± 0.12 µg/mL, respectively.

Investigators led by Dawidowicz [46] found that the antioxidant properties of VO are not always linked to the antioxidant activity of its main ingredient. It is important to understand synergistic, antagonistic, and additive effects.

The IC_{50} calculation revealed that the VO extracted from *C. japonica* leaves exhibits potent antioxidant properties and moderate inhibitory effects on lipase and

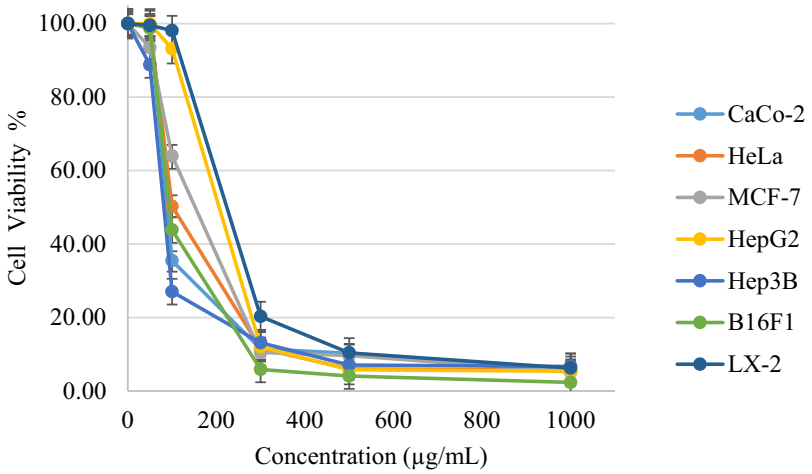


Fig. 6 Cell viability percentages of *C. japonica* volatile oil against CaCo-2, HeLa, MCF-7, HepG2, Hep3B, B16F1 cancer cells, and LX-2 normal cells at various concentrations

α-amylase enzymes, as demonstrated by the DPPH, porcine pancreatic lipase, and α-amylase inhibitory assays.

Cytotoxic effects

Citrus fruits were explicitly examined for their potential in inhibiting mammalian DNA polymerase (pol), impeding the growth of cancer cells (particularly in human colon carcinoma, HCT116), showcasing antiallergic properties, and acting as inhibitors of anti-β-hexosaminidase release in rat basophilic leukemia RBL-2H3 cells subjected to calcium ionophore A23187 treatment. The primary focus of the investigation centered on elucidating the anticancer activity exhibited by citrus fruits [47]. Figure 5 shows that *C. japonica* leaf VO at a concentration of 1000 µg/mL inhibited the growth of all used cancer cell lines by a percentage higher than 90%.

The cytotoxic IC_{50} results (Table 2) highlight the notable cytotoxic effects of *C. japonica* VO across the various cell lines compared with the 5-flurouracil chemotherapeutic agent. In the IC_{50} calculations, the *C. japonica* VO showed the highest cytotoxic effect against Hep3B, followed by B16F1, CaCo-2, HeLa, and MCF-7, and the lowest was HepG2, with IC_{50} doses of 69.7 ± 2.55, 73.42 ± 1.51, 94.15 ± 1.99, 98.29 ± 2.54, 116.29 ± 1.79, and 171.96 ± 1.71 µg/mL, respectively. At the same reading, 5-Fu has IC_{50} doses of 13.28 ± 2.21, 12.91 ± 1.36, 3.72 ± 0.84, 2.16 ± 1.01, 1.29 ± 0.45, and 4.23 ± 1.78 µg/mL, respectively. Regarding the IC_{50} calculations for *C. japonica* VO, the results showed that the tested VO is not as toxic as at a normal cell line (LX-2) compared with the 5-Fu drug, which has IC_{50} values of 224.95 ± 2.03 and 0.74 ± 0.33 µg/mL, respectively. Thus, the *C. japonica* VO is not toxic to normal cells but has a potential cytotoxic effect against all screened cancer cells.

Cell viability percentage was calculated for CaCo-2, HeLa, MCF-7, HepG2, Hep3B, B16F1, and LX-2 cells at various concentrations, as shown in Fig. 6. Cancer cells with the lowest cell viability at 100 µg/mL concentration were Hep3B (27.04%), CaCo-2 (35.48%), B16F1 (43.84%), and HeLa (50.27%). At the same time, they showed cell viability for MCF-7 and HepG2 cancer cells at 63.98 and 93.16%, respectively. These results indicate that the VO has significant anticancer activity, especially against liver, colon, and skin cancer cells, as this oil kills these cells by 72.96, 64.52, 56.16, and 49.73%, respectively. At the same concentration, the VO was almost not toxic to the LX-2 normal cells, as the cell viability percent was 99.99, which means that the VO inhibited the growth of the normal cells by 0.01%.

The comparative analysis reveals the noticeable cytotoxic effect of *C. japonica* VO compared with 5-flurouracil drugs across diverse cell lines. These findings contribute to our understanding of this natural substance's relative efficacy and potential therapeutic applications.

All these potential bioactivities of investigated *C. japonica* VO may be due to the presence of *γ*-muurolene VO, the major component of our study, which has been demonstrated in previous research to have potential antioxidant and anti-inflammatory activities. It has also been shown to inhibit protein targets involved in cancer cell proliferation [48, 49]. Moreover, this may be due to the presence of hydrocarbon sesquiterpene, the most abundant phytochemical class in *C. japonica* VO (58.66%), which is shown in numerous studies to have strong antioxidant properties [50]. In addition, recent efforts in the research and development of new drugs derived from natural products have identified various sesquiterpenes

that possess promising anti-carcinogenic activities [51]. Sun et al. reported that sesquiterpenes had potential cytotoxic activity against adenocarcinoma (A549), stomach cancer (BGC-823), hepatoma (Bel 7402), colon cancer (HCT-8), and HELA cell lines, with IC_{50} values of 1.68, 1.22, 1.91, 1.77 and 1.61 µg/mL, respectively [52]. Moreover, previous investigations have shown that sesquiterpenes have a variety of biological activities, including anticancer, antidiabetic, antimicrobial, anti-inflammatory, immune system stimulant, and antioxidant properties, making them potential targets for developing new therapeutics and medical applications [53].

It is worth mentioning that VO's bioactivities are generally not always linked to its main ingredient, and it is important to understand the synergistic, antagonistic, and additive effects of the VO mixtures in plants [49].

To the best of our knowledge, no previous studies have reported the cytotoxic, anti-obesity, and anti-lipase effects of VO extracted from the leaves of *C. japonica,* and the current study is the first one. Further advanced phytochemical procedures to separate the active constituents of *C. japonica* leaf VO, as well as in vivo and preclinical research, are required to validate the findings of our study and develop appropriate pharmaceutical dosage forms. In addition, pharmacodynamic investigations are needed to delineate the underlying mechanisms governing the observed biological effects of *C. japonica* VO.

Conclusion

Palestine is one of the world's largest producers of various kinds of citrus fruits, and the beneficial roles of different citrus VOs have been widely reported. However, the chemical constituents and bioactivities of some of the unutilized parts of citrus plant have not been investigated. In this study, *C. japonica* VO was extracted via the hydrodistillation technique, and its chemical constituents, antioxidant, cytotoxic, anti-obesity, and antidiabetic activities were investigated. The results revealed strong cytotoxicity against cancer cells and safety against normal cells. It also showed that the VO has strong antioxidant activity and activity against porcine pancreatic lipase and α-amylase enzymes in a dose-dependent manner. The results showed that *C. japonica* VO has potential for future pharmaceutical and food use. Further in vivo and preclinical investigations on *C. japonica* VO bioactivities are needed to validate these promising findings of our study.

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Author contributions
Nidal Jaradat conceived and designed the present study. Nidal Jaradat, Ruba Atiani, Ghufran Omariyah, Lama Hamodi, Heba Mosa, Fatima Hussein, Linda Issa and Shurooq Sobuh experimented. Nidal Jaradat, Nawaf Al-Maharik, Mohammed Hawash, Trobjon Makhkamov, Komolitdin Sultonov, Marah S. Shakhshir, and Nilufar Abdullayeva analyzed the data. Nidal Jaradat wrote and revised the manuscript. All authors reviewed the paper and read and agreed to the published version of the manuscript..

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request. The datasets supporting the conclusions of this article are included in the manuscript. The raw data and materials of the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The collection of the plant material was complying with the Guidelines for the Assessment of Herbal Medicines and Legislation.

Consent for publication
The authors of the current work gave consent for publication to Professor Nidal Jaradat.

Competing interests
The authors declare no competing interests.

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