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ORIGINAL ARTICLE

ANTIMICROBIAL AND ANTI-OXIDANT ACTIVITIES OF ESSENTIAL OILS DERIVED FROM SOME CITRUS PEEL

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Summary

Essential oils can be used in a variety of ways to treat microorganisms that have evolved antibiotic resistance. The research assessed the antimicrobial and antioxidant activities of essential oil obtained from *Citrus Limonum*, *Citrus reticulate*, and *Citrus sinensis* fresh peels using the hydro-distillation method. Their chemical compositions were analyzed by Gas Chromatography-Mass Spectrometer. Citrus oils had antimicrobial and antioxidant properties and their activity was increased with increasing concentrations. Oils had a significant antimicrobial effect on tested bacteria except on *P. aeruginosa* only *C. Limonum* had significant ($p \leq 0.05$) inhibitory effects at both 100 and 200 mg/ml. There was no significant ($p > 0.05$) difference in the inhibition zone of tested oils against *A. baumannii* and ciprofloxacin at 25 mg/ml, which was the same as against *E. coli* at 200 mg/ml. The oil inhibitory effect on *K. pneumoniae*, *P. mirabilis*, and *S. aureus* was less than that obtained from ciprofloxacin at concentrations used. At 100 mg/mL, *C. reticulate* oil had a 23 mm inhibitory zone, while *C. sinensis* oil had a 23 mm inhibitory zone at 200 mg/mL, which was the same as the inhibitory area of ciprofloxacin against *S. marcescens*. Oils had convergent antifungal activity against *Candida albicans* that increased with increasing concentrations. The extracts competed favorably with voriconazole being used as a positive control. Citrus oils had convergent scavenging activities at the concentrations used. The studies confirmed the medicinal and industrial use of citrus essential oils as a therapeutic and antioxidant agent.

Key words: citrus essential oils; antibacterial; antifungal; DPPH

Introduction

Plants create a diverse range of fragrances and odors that have a wide range of applications in daily life. According to several studies, essential oil (EO) may be found in about 3000 plant species (1). They are secondary metabolites produced by plants to protect them from pests and predators, attract pollinators, and distribute seeds (2). The volatile oil is another name for EO (3). Oils are distinguished by their high volatility and inability to saponify. EO is a concentrated hydrophobic liquid containing volatile fragrance molecules obtained from plants (3). They are soluble in organic dissolvent with lower intensity than water, such as benzene, acetone, toluene, methanol, and ethanol (4).

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Essential oils (EOs) can be found in many portions of the plant, including the roots, leaves, stems, flowers, seeds, and even fruits depending on the type (5). Especially for plant sections, the EO is retained in cells, glandular hairs, and secretory cavities (6). It can be extracted from practically any part of a plant using several commercial procedures. EOs are made up of a complex mixture of chemicals that give plants their distinct odor and flavor (1).

Although citrus peels are not edible, they offer a wide range of biological functions, including antibacterial, antioxidant, and anti-cancer effects (3). It was reported that the composition of EOs from similar plants varies depending on the geographical place in which they grow (3). Medical plants have long been utilized for curative purposes, and approximately 80 % of the world's residents consume herbal remedies to treat sickness, particularly infectious disorders (7). Due to microbial resistance to currently available antibiotics, conventional medicine can now be utilized as a medicinal normal agent (8). Higher plant natural products could provide a new exporter of antibacterial compounds with potentially different modes of activity (9). Citrus fruit peel contains a lot of flavonoids, glycerol, and varying amounts of volatile oils being extracted depending on the species. Many flavones have a variety of biological properties that are uncommon in those other (3). EOs were found to have antibacterial effects against a variety of microorganisms; including bacteria and fungus that are resistant to antibiotics. They may fight Gram-positive and Gram-negative bacteria, in the body, as well as, yeasts, and filamentous fungus. The reduction of microbial population is dependent on EO concentrations, increasing their concentrations resulting in high antibacterial activity (10). The research assessed the antimicrobial and antioxidant activities of essential oil obtained from *C. Limonum*, *C. reticulate*, and *C. sinensis* fresh peels using the Clevenger hydro-distillation method.

Material and methods

Collection of fruit peels: *Citrus Limonum*, *Citrus reticulate*, and *Citrus sinensis* were obtained from the local farmer and fruit sellers in Mosul, Iraq, and certified by plants scientist at the College of Agriculture and Forestry, Mosul University, Iraq. It was transferred to the laboratories in Pharmacy college, Mosul University, where it was cleaned with nonionized water, exfoliation its peels, and chopped into tiny parts before beginning the EO preparation.

Hydro-distillation for oil extraction: Hydro-distillation is a laboratory technique for extracting EOs from fresh peel samples. In hydro-distillation, the sample materials are immersed directly in distilled water; under atmospheric pressure in an alembic, the mixture is heated to boil which allows the odorous molecules in plant cells to release. Because of their density difference and immiscibility, these volatile fragrance chemicals and water form an azeotropic (azeotropes) mixture that could be vaporized at similar pressures, then concentrated in a Florentine flask. A cohobating system can also recycle distilled water. The Clevenger was used to extract EOs from fresh *C. sinensis*, *C. Limonum*, and *C. reticulate* peels according to the procedure of Harbone, J., 1998 with some modification (11). Using a blender, the shells of each species are ground and then 100 g of each is packed into the round bottom flask of the Clevenger device and 500 ml of distilled water is added to it separately. The Clevenger apparatus was installed on a thermostatic heating mantle and the temperature was set at 70-80 °C for 5 hours. The oil-water mixture was separated by water run-off and the oil reading is in the built-in calibration tube. The EO was separated from the oil and water mixture by adding magnesium sulfate ($MgSO_4$), which adsorbs water forming a precipitate so that the oil is above the precipitate which can be separated easily and collected in a glass container for further treatment. The percentage of oil extracts was calculated through this equation:

$$\text{Oil\% (\%v/w)} = \text{observed volume of oil (ml)/weight of sample (g)} \times 100.$$

The extraction process can be repeated several times, these oil peels extracts were employed to conduct additional biochemical and microbiological testing, as well as to calculate the oil density of each plant peel according to weight: volume ratio (12).

Gas Chromatography-Mass Spectrometry GC-MS: The EOs of three citrus fruits (*C. Limonum*, *C. reticulate*, and *C. sinensis*) were analyzed for chemical compositions using focus GC-MS (Gas Chromatography-Mass Spectrometer) (Agilent technologies GC- MS. CA, USA) according to Hazim I *et al.* 2020 and Behiry SI *et al.* 2020 with some modifications (13, 14). For gas chromatography and mass spectrometry examination, each EO was solubilized in n-Hexane (PubChem CID: 8058). Their compounds, formula, M. Wt. (Molecular Weight),

RT (Retention Time), SI (Standard Index), RSI (Reverse Standard Index), and most fragmentation were obtained with a quadruple detector and direct capillary column about 30 m × 0.25 mm inner diameter× 0.25 µm film thickness (TG–5MS) apparatus at Gazi Osman Pasha University, Tokat Province, Turkey. The oven temperature program's beginning at 40 °C, which was maintained for 4 minutes before escalating by 5 °C/min to 250 °C and remaining for 10 minutes. The temperature of the injector was 250 °C. Each oil samples volume was injected at about 1µl. In the MS, electron ionization (EI) was employed, and standard mass spectra with 70 EV ionization energy were taken from 0-500 m/z over one hour.

Antioxidant activity using DPPH radical scavenging assay

The radical scavenging activity of the various oil extracts was evaluated using the DPPH (1, 1-diphenil-2-picrylhydrazyl) radical as a reagent, as described by Kirby AJ, Schmidt RJ 1997 (15), with some modifications. In brief 5, 10, 25, 50, and 100 microliters of sample completely with distilled water to 1 ml then these (varying concentrations) were combined with 1 milliliter of a four percent (w/v) solution of 1, 1-diphenil-2-picrylhydrazyl radical in ethanol (PubChem CID: 702). The mixture was incubated in the dark for 20 minutes at room temperature. The absorbance at 517 nm was monitored spectrophotometrically to determine scavenging capacity. Higher free radical scavenging activity is indicated by the lower absorbance of the reaction mixture. Ascorbic acid was utilized as a control. The radical scavenging activity of DPPH (percent) was estimated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = [(\text{ODblank} - \text{ODsample}) / \text{ODblank}] \times 100.$$

The absorbance of the control reaction, which contains all reagents except the testing chemical, is denoted by ODblank. The absorbance of the test substance is denoted by ODsample. The tests were performed in duplicate.

Antimicrobial bioassay of essential oils

Sterilization of materials: all heat-sensitive materials were autoclaved for 15 minutes at 121°C.

Sterilization of culture media: Mueller Hinton's and Sabourod Dextrose Agar (Lab M Limited Topley House, UK) were autoclaved at 121°C and 15 psi for 15-30 minutes after being prepared according to the manufacturer's instructions. By swiping with Methylated Spirit, the workstation was kept sterile at all times.

Analyzed microorganisms: The bacterial strains *Acinetobacter baumannii* (*A. baumannii*), *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Proteus mirabilis* (*P. mirabilis*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Serratia marcescens* (*S. marcescens*), *Staphylococcus aureus* (*S. aureus*), and the fungal strain *Candida albicans* (*C. albicans*), were selected for their pharmacological and clinical significance. The pathogens were get from the Laboratory of Microbiology, Clinical Laboratory Sciences Department, Pharmacy College, Mosul University, Mosul, Iraq was used for estimating the antimicrobial efficacy of the tested EOs.

Pharmacological (antimicrobial) activity: *In vitro* antimicrobial assay of peels EO of three different citrus species against some human pathogenic bacteria and one fungi (yeast) was performed utilizing the well diffusion method. (16, 17). *S. aureus* as a model for Gram-positive bacteria, *E. coli*, *P. aeruginosa*, *S. marcescens*, *P. mirabilis*, *K. pneumoniae*, and *A. baumannii* as models for Gram-negative bacteria, and *C. albicans* as a yeast model. The study was conducted in the Microbiological Laboratory, Department of Clinical Laboratory Sciences, College of Pharmacy, Mosul University, Mosul, Iraq.

Citrus fruit oil extracts were diluted in a mixture of tween 20 (0.5 %) and dimethyl sulphoxide (DMSO) (0.5 %), then added distilled water up to 100 ml to make a series of dilutions (200, 100, 50, 25, 12.5 mg/ml). Sterile Petri-dish plates were flooded with 15-20 ml of sterile Mueller-Hinton agar or Sabourod Dextrose Agar and left to solidify. The test bacteria or yeast were prepared, this was accomplished by inserting one inoculation loop of the cultured pathogen (*S. aureus*, *E. coli*, *P. aeruginosa*, *S. marcescens*, *P. mirabilis*, *K. pneumoniae*, *A. baumannii*, and *C. albicans*) into 5 ml of Mueller Hinton broth for bacteria and Sabourod Dextrose broth solution for *C. albicans*, incubate at 37 °C for 24 hours in an incubator. It was also vortexed, and its turbidity was adjusted to 0.5 McFarland solution turbidity of 108CFU/mL. About 50 microliters of the bacterial or yeast solution were placed on the solidified culture

medium by using a sterile spreader or cotton swab. A cork borer was used to drill five holes measuring 6-8 mm in diameter, each containing a different concentration of citrus peel EOs. 0.5 % tween and 0.5 % DMSO were used as a negative control and 0.5 mg/ml of Ciprofloxacin and 0.1 mg/ml of voriconazole as a positive control. In general, an oil diffuses into the agar medium, inhibiting the growth of the test microorganism. The zone of inhibition (ZOI) is a circular region around the oil where microbial colonies do not grow. After 24 hours of incubation at 37 °C for bacteria and 24-48 hours for fungi, ZOI diameter was measured with a vernier caliper and a meter rule. The experiments were done in three replicates.

SPSS statistic software version 19 was used to analyze the data. One-way analysis of variance (ANOVA) was performed to differentiate the ZOIs means of the three oil tested, and Duncan Multiple Range Test (DMRT) was employed to differentiate means whether significant or not. P values of less than 0.05 were considered statistically significant.

Results

Hydro-distillation for oil extraction

Percentage of essential oil derived from citrus peels: Only 1 ml, 2.5 ml, and 1.5 ml of EO were recovered after Clevenger hydro-distillation extraction of EO from 100 g of *C. Limonum*, *C. reticulate*, and *C. sinensis* as shown in figure-1A. *C. reticulate* produced the highest yield of phytochemical oil extract (2.5 %), followed by *C. sinensis* (1.5 %) and *C. Limonum* (1 %) with a density of 0.71, 0.8, and 0.69 (wt. /vol.) respectively. The sensory evaluation found that they had a watery consistency, were colorless, and had a distinct smell which was used for further biochemical and antibacterial tests. The oil of *C. reticulate* and *C. sinensis* was transparent, in contrast to the *C. Limonum*, which appeared as opaque as shown graphically in figure-1B.

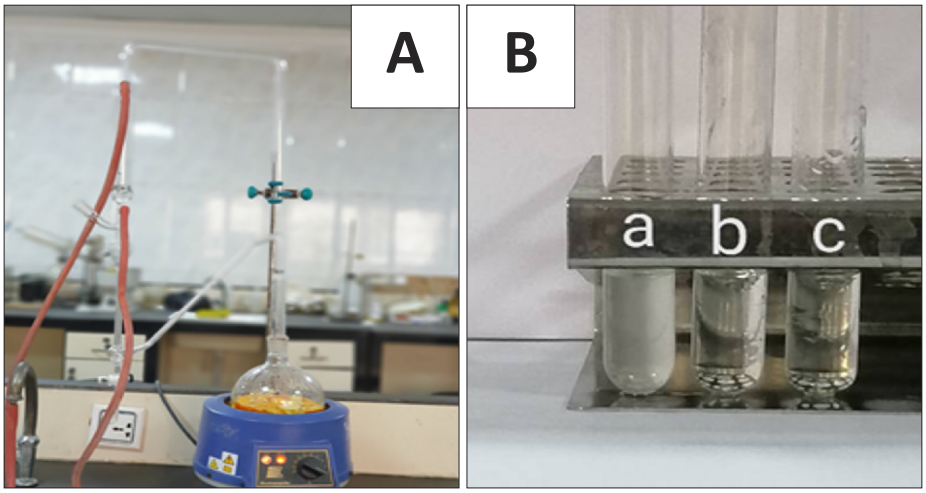

Figure 1. Hydro-distillation using Clevenger apparatus (A), the essential oil extracted from fresh peels (B) of three Citrus species (a) *C. Limonum*, (b) *C. reticulate*, and (c) *C. sinensis*.

Phytochemical analysis of citrus peel essential oils

Gas Chromatography-Mass Spectrometry (GC-MS): Some secondary metabolites were identified using GC-MS analysis of the three citrus peel EOs. Tables 1 demonstrate the different organic components found in EO extracts of *C. Limonum*, *C. reticulate*, and *C. sinensis* respectively. Nine chemical substances were identified through qualitative screening of phytochemical *C. Limonum* peel oil extracts. The peaks were recognized as Cyclohexene, 1-methyl-4-(1-methylethenyl), Decanal, Trans-anethole, e-Cadinene, Farnesol isomer B, Hexadecanoic acid, alpha-L-Rhamnofuranose, Tetra-o-acetyl, 5,8-Dimethoxy-1,4-anthraquinone, and Oleoamide. There were 8 primary

components found in the chemical composition of *C. reticulate* peels EO. The main chemical components were (+) – Lemonen, n-Decanal, Anethole, 1,1,4,7-Tetramethyldecahydro-1h-c yclopropa[e]azulen-4-ol, Carotol, Palmitic acid, Isopropyl 8-(3-octyl-2-oxiranyl)octanoate, and Oleoamide. For *C. sinensis* analysis by GC-MS, seven chemical components were identified. Those are endo-Borneol, alph-Fenchyl acetate, endo-1-bourbonanol, Palmitic acid, (E)-3,3'-Biindolyl-2-carbaldehyde oxime, 4,4a,5,9-Tetrahydro-4a,8-dimethyl-5,9-dioxoazu leno [6,5-b]furan-3-carboxylic Acid Methyl Ester, and n-Dotriacontane.

Table 1. Chemical components of *citrus* peel essential oil identified by GC-MS.

NO.	Compound	Formula	R.T.	M.Wt.	SI	RSI	%Hight	% Area
Citrus Limonum	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (R)	$C_{10}H_{16}$	6.8	136	939	940	22.36	30.48
	Decanal	$C_{10}H_{20}O$	9.39	156	870	948	1.31	1.09
	Trans-anethole	$C_{10}H_{12}O$	10.59	148	945	952	16.6	13.36
	ë-Cadinene	$C_{15}H_{24}$	13.69	204	883	922	2.46	2.72
	Farnesol isomer B	$C_{15}H_{26}O$	15.88	222	809	857	1.31	1.01
	Hexadecanoic acid	$C_{16}H_{32}O_2$	18.25	256	9.3	910	5.58	3.95
	à-L-Rhamnofuranose, tetra-o-acetyl	$C_{14}H_{20}O_9$	20.73	332	704	812	0.64	0.46
	5,8-Dimethoxy-1,4-anthraquinone	$C_{16}H_{12}O_4$	23.05	268	813	933	1.52	1.029
	Oleoamide	$C_{18}H_{35}NO$	25.64	281	761	829	2.44	3.4
Citrus reticulate	(+) - Lemonen	$C_{10}H_{16}$	6.79	136.2	933	934	12.67	20.33
	n-Decanal	$C_{10}H_{20}O$	9.4	156	788	899	0.33	0.33
	Anethole	$C_{10}H_{12}O$	10.6	148	818	832	0.95	1.05
	1,1,4,7-Tetramethyldecahydro-1h-c yclopropa[e]azulen-4-ol	$C_{15}H_{26}O$	14.38	222	800	801	7.72	5.2
	Carotol	$C_{15}H_{26}O$	15.74	222	822	843	4.93	3.36
	Palmitic acid	$C_{16}H_{32}O_2$	18.25	256	874	891	2.81	2.31
	Isopropyl 8-(3-octyl-2-oxiranyl)octanoate	$C_{21}H_{40}O_3$	20.15	340	664	702	1.35	1.42
	Oleoamide	$C_{18}H_{35}NO$	25.64	281	717	801	4.67	5.87
Citrus sinensis	endo-Borneol	$C_{10}H_{18}O$	8.98	154	928	929	3.61	5.32
	à-Fenchyl acetate	$C_{12}H_{20}O$	10.63	196	941	944	3.07	2.49
	endo-1-bourbonanol	$C_{15}H_{26}O$	14.47	222	821	894	2.95	9.67
	Palmitic acid	$C_{16}H_{32}O_2$	18.27	256	864	898	0.59	0.39
	(E)-3,3'-Biindolyl-2-carbaldehyde oxime	$C_{17}H_{13}N_3O$	20.17	275	848	981	1.69	1.07
	4,4a,5,9-Tetrahydro-4a,8-dimethyl-5,9-dioxoazu leno[6,5-b]furan-3-carboxylic Acid Methyl Ester	$C_{16}H_{14}O_5$	22.01	286	876	879	1.94	1.37
	n-Dotriacontane	$C_{32}H_{66}$	24.66	450	753	765	0.12	0.1

Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (R) was the most plentiful compound with 100% relative abundance in *C. Limonum* as shown in figure-2 A. On the other hand, (+) – Limonene and endo-1-bourbonanol were the most abundant compound (100%) in *C. reticulate*, and *C. sinensis* respectively as shown in figure-2 B and C.

DPPH radical scavenging assay: experimentally estimated the antioxidant activity of citrus extracts using DPPH (1, 1-diphenil-2-picrylhydrazyl) as a free radical scavenger. The absorption was measured spectrophotometrically at 517 nm, with ascorbic acid serving as a positive control. As demonstrated in Figure 3, At the concentrations tested, all oils demonstrated convergent scavenging activities and their activity was increased with increasing the concentration. At concentrations of 5, 10, 25, and 50 mg/ml, *Citrus sinensis* oil had the strongest scavenging activity (77-80.9 %) compared to the other oils, whereas *C. reticulate* oil had the maximum antioxidant activity (82.6 %) at a concentration of 100 mg/ml.

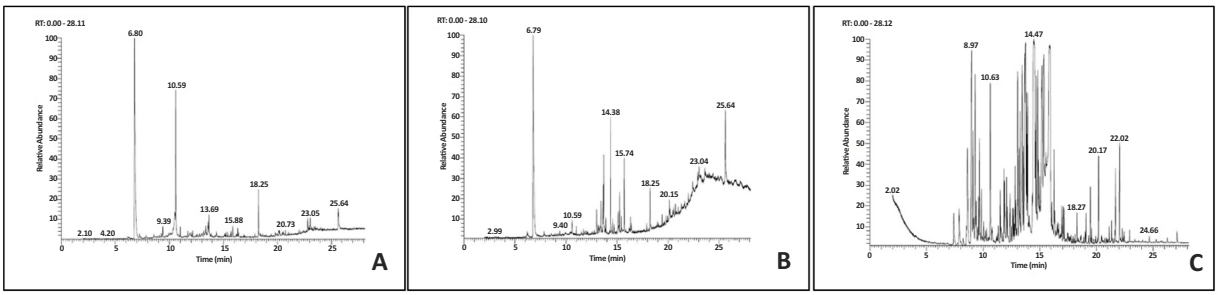

Figure 2. The gas chromatography mass spectrum of (A): *Citrus Limonum*, (B): *Citrus reticulate*, and (C): *Citrus sinensis* fresh peel essential oil.

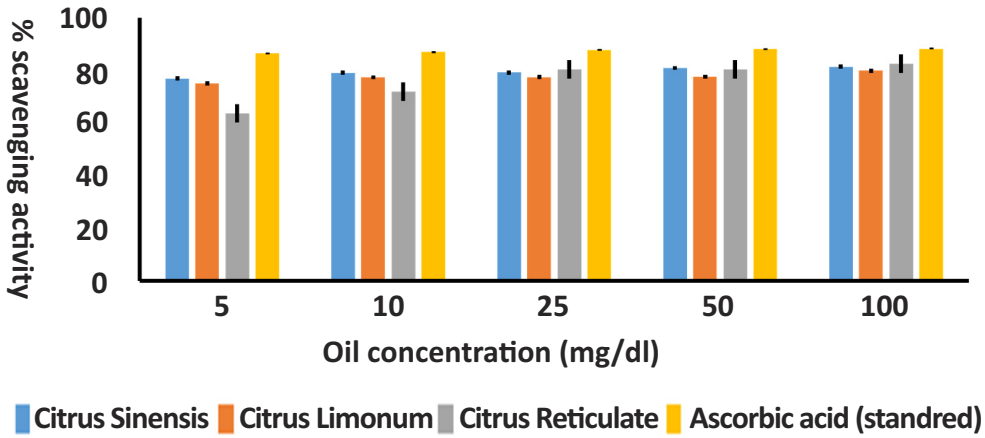

Figure 3. Antioxidant scavenging potential of three citrus fruits oils and ascorbic acid against DPPH radical.

Antimicrobial bioassay of essential oils: the effect of various EO extracts was investigated against different pathogens (seven bacteria and one yeast fungal) using an agar well diffusion assay. Means of ZOIs (mm) from the three replicate microbiological testing were recorded for each organism.

The result showed that Ciprofloxacin, a positive control, had antibacterial action against the tested reference microorganisms. The ZOI was 17.7, 25, 27, 25, 23, 23, and 40 mm on *A. baumannii*, *E. coli*, *K. pneumoniae*, *P. mirabilis*, *P. aeruginosa*, *S. marcescens*, and *S. aureus*. Where Voriconazole showed a ZOI of about 25 mm on *C. albicans*.

EO extracted from *C. Limonum* peels demonstrated a substantial significant effect on all bacterial isolates ($p \leq 0.05$) except on P. aeruginosa at concentrations 50, 25, 12.5 mg/ml that had no significant action ($p \geq 0.05$). As the concentration increased from 50-200 mg/ml, the ZOIs on *P. aeruginosa* raised significantly ($p \leq 0.05$) from 0.00 to 18 mm. In comparison to other bacteria examined, *C. Limonum* oil showed the strongest effect on *E. coli* (ZOIs of 24 mm) at 200 mg/ml. In addition, increasing oil concentrations on various pathogens raised the ZOI significantly ($p \leq 0.05$), as represented in table 2.

The finding revealed that *C. reticulate* oil had a significant ($p \leq 0.05$) action on all pathogens except on *P. aeruginosa* at all concentrations tested. There was a significant ($p \leq 0.05$) inhibitory effect on the remaining tested bacteria, which increased when the concentrations were raised. *C. reticulate* oil demonstrated both antifungal and antibacterial action. At 200 mg/ml, it had the strongest effect on *S. aureus* (ZOIs was 28 mm) in comparison to other bacteria tested as represented in table 2.

Table 2. Comparative effect of essential oil derived from various citrus peels on tested microorganisms at different concentrations.

Microorganism	Zone of inhibitions(mm)±SD					EO extraction	CPR	VCZ
	200	100	50	25	12,5			
A. baumannii	20±1.0^{a} C	19±0.00^{a} BC	18±0.76^{a} BC	18±0.00^{a}B	15±1.0^{a} A	CL	17.7	-----
	21±0.43^{ab} D	20±0.00^{b} C	19±0.00^{a} B	19±0.5^{b} B	18±0.00^{b} A	CR		
	22±0.00^{b} D	19±0.3^{a} C	19±0.00^{a} C	18±0.5^{a} B	14±0.1^{a} A	CS		
E. coli	24±1.0^{a} D	23±0.00^{a} C	23±0.00^{b} C	22±0.5^{c} B	18±0.00^{b} A	CL	25	-----
	24±0.56^{a} D	24±0.5^{b} D	22±0.26^{a} C	17±0.36^{a} B	16±0.34^{a} A	CR		
	24±0.44^{a} E	23±0.00^{a} D	22±0.00^{a} C	19±0.1^{b} B	18±0.00^{b} A	CS		
K. pneumoniae	21±0.00^{a} C	21±0.00^{a} C	21±0.86^{c} C	15±0.2^{a} B	14±0.2^{a} A	CL	27	-----
	24±0.5^{c} E	22±0.26^{b} D	19±0.00^{a} C	18±0.17^{c} B	16±0.00^{c} A	CR25		
	23±0.00^{b} E	22±0.4^{b} D	20±0.00^{b} C	17±0.26^{b} B	15±0.00^{b} A	CS		
P. mirabilis	22±0.00^{b} D	20±0.2^{b} C	18±0.00^{b} B	18±0.77^{b} B	15±1.0^{b} A	CL	25	-----
	23±0.5^{c} C	20±0.5^{b} B	20±0.00^{c} B	20±0.2^{c} B	17±0.3^{c} A	CR		
	17±0.36^{a} D	17±0.00^{a} D	16±0.17^{a} C	15±0.00^{a} B	13±0.00^{a} A	CS		
P. aeruginosa	18±0.5^{b} C	16±0.00^{a} B	0±0.00A	0±0.00A	0±0.00A	CL	23	-----
	0±0.00^{a}	0±0.00^{a}	0±0.00	0±0.00	0±0.00	CR		
	0±0.00^{a}	0±0.00^{a}	0±0.00	0±0.00	0±0.00	CS		
S. marcescens	22±0.00^{a} C	22±0.4^{a} C	21±0.00^{b} B	21±0.00^{b} B	20±0.45^{b} A	CL	23	-----
	25±0.45^{c} E	24±0.46^{b} D	23±0.17^{c} C	21±0.00^{b} B	15±0.00^{a} A	CR		
	23±0.45^{b} E	22±0.00^{a} D	20±0.00^{a} C	16±0.1^{a} B	15±0.00^{a} A	CS		
S. aureus	19±0.2^{a} C	17±0.00^{a} B	16±0.2^{a} A	16±0.00^{b} A	16±0.00^{b} A	CL	40	-----
	28±0.53^{c} E	18±0.53^{b} D	16±0.00^{a} C	15±0.5^{a} B	13±0.26^{a} A	CR		
	26±0.5^{b} D	23±0.00^{c} C	19±0.2^{b} B	18±0.00^{c} A	18±0.1^{c} A	CS		
C. albicans	28±0.5^{a} E	25±0.32^{c} D	24±0.34^{b} C	23±0.00^{b} B	20±0.00^{b} A	CL	-----	25
	31±0.91^{b} E	22±0.36^{b} D	20±0.00^{a} C	18±0.26^{a} B	17±0.00^{a} A	CR		
	29±0.2^{a} E	21±0.00^{a} D	20±0.00^{a} C	18±0.2^{a} B	17±0.00^{a} A	CS		

SD (standard deviation); Significant result at p ≤0.05; Vertically, different small letters reveal that the mean of the several groups differs significantly (p ≤0.05) at different concentrations. Values with the same superscript down the column are not significantly different (p>0.05); Horizontally, different capital letters reveal that the mean of the several groups differs significantly (p ≤0.05) at different concentrations. values with the same superscript in the same row are not significantly different (p>0.05). CPR=Ciprofloxacin, VCZ=Variconazole
CL=*Citrus Limonum*
CR= *Citrus reticulate*
CS= *Citrus sinensis*

All of the test isolates responded significantly ($p \leq 0.05$) to the *C. sinensis* peel oil extract, except for *P. aeruginosa*, which was resistant to *C. sinensis* EO at concentrations of 12.5, 25, 50, 100, and 200 mg/ml. The zones of the residual pathogens enhanced significantly ($p \leq 0.05$) as the *C. sinensis* oil concentration increased as represented in table 2. As with *C. reticulate* oil, *C. sinensis* oil had the strongest effect on *S. aureus* (ZOIs was 26 mm) in comparison to other bacteria tested at 200 mg/ml. At 200 mg/ml, the fungus strain *C. albicans* was strongly affected by the extract, with a zone of 29mm, which is larger than the positive control zone (Voriconazole). The extract competed successfully with the Voriconazole.

Comparative effect of various concentrations of *C. Limonum*, *C. reticulate*, and *C. sinensis* peel EO extracts on various isolates: On *A. baumannii*, The ZOI of the three citrus peel oil extracts tested at 25 mg/ml were larger

than ciprofloxacin (ZOI was 17.7 mm), i.e. the extract competed positively with the ciprofloxacin at 25 mg/ml and it's increased with increasing oils concentrations as represented in table 2.

The results revealed that the three citrus oil extracts tested had a significant inhibitory effect on *E. coli* at the concentrations used. Only at 200 mg/ml, there was no significant difference ($p>0.05$) of ZOIs between them approximately 24 mm as shown in table 2.

At 12.5, 25, and 200 mg/ml, *C. reticulate* EO had the highest significant inhibitory ($p\leq0.05$) effect on *K. pneumoniae* with a ZOIs of 16, 18, and 24 mm respectively, while *C. Limonum* oil had no significant differences ($p>0.05$) with ZOI of approximately 21 mm with increasing concentration from 50 to 200 mg/ml as represented in table 2. All oils had an effect on *K. pneumoniae* at the concentrations used but still less than that obtained by ciprofloxacin (ZOIs about 27 mm).

The result showed that the ZOI increased with increasing concentrations with all oils tested. Accordingly, the mean area of inhibition for *C. reticulate*, *C. Limonum*, and *C. sinensis* oil peel extract at 200 mg/ml concentration on *P. mirabilis* was 23, 22, and 17 mm respectively as represented in table 2. All of them are less than the effect of the Ciprofloxacin on it as ZOI reaches 25mm.

At 12.5, 25, and 50 mg/ml, there were no significant effects on *P. aeruginosa* growth of all three extracts. At 100 and 200 mg/ml, only *C. Limonum* oil extract had a significant difference ($p\leq0.05$) with an inhibition zone of 16 and 18 mm respectively. Although it was still less than that recorded with Ciprofloxacin with 23 mm. while there were no significant effects of *C. reticulate* and *C. sinensis* oil at all tested concentrations as represented in table 2.

There was a significant inhibitory ($p\leq0.05$) effect of the three tested citrus oil on *S. marcescens* in all concentrations being examined. At 50 mg/ml, *C. reticulate* oil had an anti-*Serratia* effect as ciprofloxacin that showed ZOIs of 23 mm. based on this, *C. reticulate* oil has a better effect on *S. marcescens* than Ciprofloxacin at both 100 and 200 mg/ml as represented in table 2. The result showed that *C. sinensis* oil extract had no significant difference as Ciprofloxacin effect at 200 mg/ml.

The EOs of various tested citrus peels had a significant ($p\leq0.05$) effect on *S. aureus*, but it was less than that obtained with Ciprofloxacin (ZOI 40 mm). *C. reticulate* oil had the highest significant ($p\leq0.05$) ZOI (28 mm) in comparison to *C. sinensis* and *C. Limonum* oil, 26 and 19 mm respectively at 200 mg/ml as shown in table 2.

The zones at 200 mg/ml of all three tested citrus peel oils were higher than that obtained from the positive control, voriconazole (ZOIs was 25 mm). The extracts competed favorably with the voriconazole as represented in table 2.

Discussion

According to the findings, citrus peels were shown to be a good source of oil and useful in folk medicine for treating a variety of ailments due to the presence of some active chemicals. The results of this study found that *C. reticulate* produced the highest yield of phytochemical oil extract (2.5%), followed by *C. sinensis* (1.5%) and *C. Limonum* (1%). It contrasted a similar study by Kamal S *et al*. 2011 (18), who found that *C. sinensis* produces the most oil of all the Citrus species investigated. followed by *C. reticulate* but still higher than that obtained from *C. Limonum*. This difference could be attributable to the method of extraction used, as Kamal S *et al*. used the Soxhlet extraction method and this study used the Clevenger hydro-distillation method. The findings correspond with those of Edogbanya P *et al*. 2019 (10), who found that *C. sinensis* had more oil than *C. Limonum*, noting that they used the cold maceration method. *C. sinensis* had the highest density (0.8 wt. /vol.) followed by *C. reticulate* and *C. Limonum* with 0.71 and 0.69 wt./vol respectively. These findings agreed with Edogbanya PRO *et al*. 2019 results (10).

According to the findings, ciprofloxacin and voriconazole both had inhibitory zones against the tested bacteria and *C. albicans*, which corresponds with Masadeh MM *et al*. 2016 results (19). When the antibacterial impact was compared, it was shown that all EOs tested exhibited consistent antimicrobial action, except for *C. Limonum*, which

could suppress P. aeruginosa growth at both 100 and 200 mg/ml. This result agrees with the findings of Okon OG et al. 2015 finding (20) but differs from Edogbanya P et al. 2019 (10) who found C. sinensis oil to be more effective against P. aeruginosa.

The ZOI on *A. baumannii* at 25mg/ml of the three oil extracts examined and ciprofloxacin showed no significant difference (P>0.05) (i.e. the extracts competed positively with the ciprofloxacin). The same results were shown with *E. coli*.

Citrus peels' chemical contents allowed them to be utilized in herbal medicine to treat a variety of illnesses, for example, D-Limonene is generated from the peels of citrus fruits that had antibacterial activity and some patients use this supplement to manage and control cancer (21). These findings corroborated previous research by Feger W, *et al*. 2003, who discovered that limonene is a significant component of tangerine that aids in its antibacterial action (22). Trans-anethole, endo-Borneol, and other flavonoids and phenolic constituents in *C. Limonum* and *C. sinensis* possess a powerful antimicrobial effect against bacteria, fungi, and yeasts (23, 24) The difference in sensitivity between Gram-positive bacteria and Gram-negative bacteria could be owing to variation in the cell wall structure, with the former having a peptidoglycan outer layer that acts as a protective barrier and the latter having a cell membrane only (10). Flavonoids, for example, are antibacterial agents, and several phenolic compounds have been proven to suppress bacterial development (25).

By comparing the antifungal properties of the tested citruses peel EO extracts and despite the convergence of ZOI diameter results, it was found that the *C. reticulate* oil extract was shown to be the most effective against *C. albicans* (31mm) followed by *C. sinensis* oil (29mm) while *C. Limonum* oil was found to be the least efficient (28mm). This work supports the work of Hasija S *et al*. 2015, who found that *C. Limonum* peel oil had a more inhibitory property than *C. sinensis* (26). This research's outcome is similar to the results of previous studies (27, 28, 29) in that the three EOs tested had a recorded inhibitory activity on *Candida alibican*.

Because the citrus samples used were obtained from the Mosul Market in Nineveh, Iraq, the discrepancy in findings could be due to varying environmental characteristics of different geographic sources, as Özcan M *et al*. 2005 have clearly stated that location produces variance in the chemical formulation of EOs (30). The type of extraction is also important to examine, as this study used the hydro distillation process. Although the area of inhibition for oils was less than that obtained from the drugs within the concentrations used in this research, increasing the concentrations could likely be a reason for citrus oils to be a good alternative to the drugs used in the treatment of various infections.

According to an antioxidant study, all citrus peel oils have a high capacity to convert DPPH radical to DPPH-H and showed convergent scavenging properties. For 100mg/ml, *C. reticulate* had the highest antioxidant activity, followed by *C. Limonum* and *C. sinensis*. These findings were consistent with the findings of Kamal *et al*. 2013 (31) and Javed S *et al*. (2014)(32), who found that *C. reticulate* had the most antioxidant potential while *C. sinensis* had the lowest. correspondingly, yang et al. (2010) found that limonen is a prominent ingredient of citrus peel oils with antioxidant activity comparable to a strong antioxidant (33). As noted by Jayaprakasha GK *et al*. 2005, these findings are most likely connected to phenolic content and flavonoids, which vary by plant species (34).

Conclusion

It can be concluded that citrus peels were shown to be a good source of oil. From this research, the results showed that *C. reticulate* yields a greater amount of oil followed by *C. sinensis* and *C. Limonum* respectively. They could be useful in folk medicine because they had antibacterial and antifungal properties. *C. Limonum*, *C. reticulate*, and *C. sinensis* peel EO demonstrated a significant ($p \leq 0.05$) effect on all bacteria tested except on *P. aeruginosa* only *C. Limonum* oil had inhibitory effects at both 100 and 200mg/ml. It can be concluded that EO concentrations influence the reduction of microbial population, increasing the concentrations lead to an increase in their antibacterial and antioxidant effect. At the concentrations tested, the citrus peel EOs possess a greater inhibitory effect on *C. albicans* than on bacterial strain. *C. reticulate* oil extract was found to be the most efficient against *C. albicans*, followed by *C. sinensis* oil and *C. Limonum* oil, with *C. Limonum* oil being the least effective. The extracts competed favorably with the voriconazole being used as a positive control for *C. albicans*.

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Conflict of Interest

There are no conflicts of interest declared by the authors for this study.

Adherence to Ethical Standards

Not applicable. This study is in vitro study involving no human or animal samples.

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