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Egyptian Citrus Essential Oils Recovered from Lemon, Orange, and Mandarin Peels: Phytochemical and Biological Value

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Abstract: Citrus peels are an important by-product of citrus processing industries, but a large part is considered waste. There has been increased attention in the last five years on these industrial by-products, especially those containing residual essential oils (EOs). Lemon, orange, and mandarin peels from Egypt were subjected to hydro-distillation to obtain EOs, which were analyzed via mass spectrometry (GC/MS) and by building Global Natural Products Social Molecular Networking (GNPS-MN) for the purpose of visually exploring the volatile components of citrus species. The constructed MN revealed that D-Limonene, α-pinene, and β-pinene are the dominant volatile constituents in the three Egyptian citrus species. The EOs from three citrus peels exhibited promising activities as antioxidants using two tested methods: 1,1-diphenyl-2-picryl-hydrazil (DPPH) and nitric oxide (NO) compared with vitamin C. Lemon EO proved excellent antimicrobial activity against Gram-positive and negative bacteria. Additionally, the three citrus EOs showed good activities against the yeast *Candida albicans*. Regarding the anti-inflammatory assay, the three citrus EOs showed promising activities as COX-1 and COX-2 inhibitors. This study concludes that EOs extracted from citrus peel waste can be valorized as an innovative strategy for food preservation or may be incorporated in cosmetics and pharmaceutical formulations in alignment with circular economy principles.

Keywords: citrus peels; essential oils; biological activities; antioxidant; antimicrobial; anti-inflammatory

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1. Introduction

The genus *Citrus* belongs to the family *Rutaceae* and is one of the most widely cultivated and consumed fruits worldwide. It represents an annual production of approximately 143 million tons, of which the most important are oranges, *Citrus sinensis* L. (76 million tons); mandarin, *Citrus reticulata* L. (37 million tons); and lemon (*Citrus limon*) and limes (*Citrus aurantifolia*) (20 million tons) [1,2]. Fresh production and processing generate a huge amount of waste. This waste includes all residues remaining after the juice extraction process, such as peels, seeds, and pulp [3], constituting between 50 and 65% of the total fruits' weight [4]. The management of citrus waste is the most critical concern in processing industries [5]. Many researchers have been trying to convert citrus waste into valuable products to avoid severe environmental pollution [6]. The food industry is trying to isolate the benefits of the bioactive compounds (BCs) obtained from waste to produce functional ingredients and

nutritional supplements with therapeutic potential for nutrition and health [7]. Citrus peels contain BCs, such as polyphenols, pigments (carotenoids), vitamins, sugars, dietary fiber (pectin, cellulose, hemicellulose, and lignin), and essential oils (EOs) [8].

Citrus EOs, mainly composed of D-Limonene, are complex mixtures of different compound classes that have shown an extensive range of biological activities, including antioxidant, anti-inflammatory, anxiolytic, antibacterial, and antifungal activities [9]. These biological activities are of great importance in many fields, from food chemistry to pharmaceutics and cosmetics. The EOs extracted from citrus peels are used as safe flavors and fragrances in cosmetic products [10]. These components have gained acceptance in the food industry, being generally recognized as safe (GRAS) by the US Food and Drug Administration [11,12]. Additionally, some studies have indicated that certain foods tolerate their presence [13,14].

The EOs from lemon peel (LP) are mostly composed of a higher percentage of monoterpenes (D-Limonene, citral, and carvone), sesquiterpenes (alcohols, aldehydes, ketones, and esters), and other compounds, which have been reported to have a wide spectrum of biological activities, such as antifungal, antibacterial, and anti-cancer properties [15]. Orange peel (OP) has been used in traditional drugs to treat many diseases, such as stomachaches, gastrointestinal digestive tract problems, cancer, diuretic issues, immune system diseases, viral and bacterial infections, and vitamin deficiencies [16]. Mandarin peel (MP) is known to be a valuable source of antioxidants, such as vitamin C, carotenoids, and phenolic compounds, as well as sugars, organic acids, amino acids, pectin, minerals, and volatile organic compounds. Essential oils are mostly found in the peel of mandarin fruits, with monoterpenes accounting for 86.62% (*w*/*w*) of the total oil. D-Limonene is the most common compound, followed by β-myrcene, 3-carene, α-pinene, and others [17].

According to the trends, consumer demand is changing, and interest in natural food products devoid of harmful additives is rising. In addition, consumers are looking for healthy, sustainable, and socially conscious food [18]. The Sustainable Development Goals (SDGs) of the United Nations are designed to ensure the global population's well-being and to preserve the environment in response to concerns about climate change and the scarcity of natural resources by 2030. SDG 12 focuses on promoting sustainable consumption, improving resource use efficiency, and reducing food loss through recycling and reusing [19].

Considering the efforts toward bio-circular green production, which are in line with the SDGs for sustainable development in the citrus industry, the current study aims to put into light the compositional analysis of three EOs recovered from citrus peels: lemon (*Citrus limon*), orange (*Citrus sinensis*), and mandarin (*Citrus reticulata*). The significance of the present study is highlighted by the extreme importance of comparative investigations into the biological properties of these by-products. Additionally, studies on citrus cultivars from Egypt are scarce.

In this context, it is of high relevance to investigate the differences between the aroma constituents among these three citrus species through the employment of modern and advanced molecular networking based on the GC/MS via the GNPS. The biological activities (antioxidant, antibacterial, antifungal, and anti-inflammatory) of these three Egyptian citrus species were also evaluated to establish a value-added upcycling of their by-products in a way that promotes the sustainability of the citrus value chain.

2. Materials and Methods

2.1. Plant Materials

Fresh fruits of lemon (*Citrus limon*), orange (*Citrus sinensis*), and mandarin (*Citrus reticulata*) were collected from a local Egyptian market from September to November 2021. The obtained samples (10 kg each) were homogeneous and harvested from the same farm.

2.2. Extraction of Essential Oils

The essential oils were extracted from the fresh peels of the three tested citrus. Two kg of fresh peels were subjected to hydro-distillation using a Clevenger-type apparatus for three hours, as mentioned in *Egyptian Pharmacopeia* 1984 [20]. The resulting essential oil was dehydrated with anhydrous sodium sulfate and kept in a deep freezer at −20 °C for GC/MS analysis.

2.3. Characterization of Essential Oils through GC/MS

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30 m, 0.251 mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with an ionization energy of 70 eV was used, and Helium gas was used as the carrier gas at a constant flow rate of 1 mL/min. The injector and MS transfer line temperature was set at 280 °C. The oven temperature was programmed at an initial temperature of 40 °C (hold 3 min) to 280 °C as a final temperature at an increasing rate of 5 °C/min (hold 5 min). The condition and method were performed as reported by Ibrahim et al. (2021) [21]. The identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the published data NIST, WILLY library data of the GC/MS system, and/or published data [22].

2.4. GC/MS Molecular Networking (GNPS-MN)

Using a documented workflow, a molecular network (MN) was constructed for the GC/MS data of the Eos obtained during hydro-distillation of the three citrus species under study [23]. Raw data files (raw format) were converted into the open format (.mzML) supported by the GNPS platform, using MS convert (http://proteowizard.sourceforge.io/download.html), accessed on 2 February 2022. The network spectra were then searched against GNPS-GC/MS spectral libraries. Cytoscape (version 3.8.2) was utilized to examine and display the created MN [24].

2.5. Total Phenolic Content (TPC) Determination

The total phenolic content (TPC) of citrus peel EOs was determined using a Folin–Ciocalteu reagent following the method of Farid et al. (2022) [25]. The absorbance was measured at 750 nm. Gallic acid was used as a standard for the calibration curve, and the results were calculated according to Equation (1) and expressed as milligram equivalent of gallic acid per gram of dry weight extract (mg GAE/g DW).

$$\text{TPC (mg GAE/g DE)} = \frac{C_{gallic\ acid} \times V \times m}{M} \quad (1)$$

$C_{gallic\ acid}$ is the standard (gallic acid) concentration established from the calibration curve; V is the dilution factor; m is the total extract weight (g); and M is the DW extract concentration.

2.6. Biological Activities

2.6.1. Antioxidant Activity

DPPH Radical Scavenging Assay

The citrus peel EOs were screened for free radical scavenging activity using a DPPH (1, 1-diphenyl-2-picryl-hydrazil) assay according to the reported method of Ibrahim et al. (2021) [21]. The absorbance was measured at 517 nm. DPPH radical scavenging activity was calculated following Equation (2).

$$\text{Inhibition}(\%) = \frac{Abs_{A0} - Abs_{EO}}{Abs_{A0}} \quad (2)$$

Abs_{A0} is the absorbance of the control, and Abs_{EO} is the absorbance of the treated sample with different concentrations of tested essential oils.

Nitric Oxide (NO) Radical Scavenging Assay

The principle of the assay is based on the generation of NO free radicals from sodium nitroprusside (SNP) in an aqueous solution, which changes at physiological pH to produce nitrite ions that can be measured by Greiss reagent (1% sulfanilamide in 5% ortho-H_3PO_4 and 0.1% naphthyl ethylene diamine dihydrochloride) [26]. The method was carried out as described by Ibrahim et al. (2021) [21]. The absorbance of these solutions was measured at 540 nm against the corresponding blank solution. NO radical scavenging activity was calculated following the Equation (2).

2.6.2. Antimicrobial Activity

Microbial Cultures

Qualitative evaluations were conducted in nutrient agar plates according to Mostafa et al. (2016) [27]. The inoculation of pathogenic and contaminant microorganisms used in this study were Gram-positive bacteria (Gm+; [*Bacillus cereus* (ATCC 6629), *Micrococcus luteus* (ATCC 10240), *Staphylococcus aureus* (ATCC 6538), and *Staphylococcus epidermidis* (ATCC 12228)]), Gram-negative bacteria (Gm−; [*Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 255566), and *Pseudomonas aeruginosa* (ATCC 27853)]), and yeast [*Candida albicans* (ATCC 10231)], which were prepared from fresh overnight broth cultures, using nutrient broth medium, that were incubated at 37 °C [28].

Disk Diffusion Assay

The inoculum of selected strains was prepared and adjusted to approximately 0.5 McFarland standard (1.5×10^8 CFU/mL) [29]. A 25.0 µL inoculum size of each microorganism strain was separately inoculated into each plate containing 20.0 mL of the sterile nutrient agar medium (NA). The EOs were applied on the 0.6 cm wells of the inoculated agar plates. These seeded plates were placed in a refrigerator for one hour for better diffusion of these samples, followed by incubation at 37 °C for 24 h, and zones of inhibition (ZI) were measured in mm [27].

2.6.3. Anti-Inflammatory Activity

COX-1 plays a role in the production of prostaglandins associated with normal physiologic function and is present in tissues such as the stomach, kidneys, and platelets. COX-2 was thought to be induced as the result of inflammation and responsible for generating prostaglandins such as prostaglandin E_2 [30]. Determination of the cyclooxygenase (COX-1 and COX-2) inhibition efficacy in the EOs of the three tested citrus species was performed as reported by Blobaum and Marnett (2007) [31], with procedures following the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, All rights reserved. Printed in USA). Indomethacin and celecoxib were used as standard anti-inflammatory compounds in examination against COX-1 and COX-2.

2.7. Statistical Analysis

To test the significance of variation in *Citrus* spp. peel on total phenolic compounds (TPC) and anti-inflammatory and antioxidant activities, one-way ANOVA and post hoc for multiple comparisons were used. The statistical analysis was carried out using the IBM-SPSS statistics program (version 25) at $p \leq 0.05$, a *t*-test (n = 3 replicates) was used for comparisons, and the significance of differences among means was determined at $p \leq 0.05$. The data were presented as mean ± standard deviation (SD).

3. Results and Discussion

3.1. Phytochemical Analysis

3.1.1. Chemical Composition of Essential Oils from Citrus Peels

A total of 74 volatile compounds were found among the three types of EOs, recovered from citrus peels (Table 1). The GC/MS analysis resulted in the identification of 44, 47, and 25 compounds constituting 97.55%, 94.22%, and 89.65% of the total peak area of identified compounds in lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*), respectively. The GC/MS chromatograms of these EOs are shown in Figure 1.

Table 1. GC/MS analysis of the EOs from lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*) peels.

No	Retention Time (R_t)	Base Peak (BP)	Molecular ion (M+)	RI *	RI **	Molecular Formula	Compounds	Area (%)		
								Lemon (*C. limon*)	Orange (*C. sinensis*)	Mandarin (*C. reticulata*)
1	7.73	85	172	830	827	$C_{10}H_{20}O_2$	Isovaleric acid	-	0.10	0.03
2	7.88	93	136	926	921	$C_{10}H_{16}$	Tricyclene	0.21	-	-
3	7.89	93	136	931	924	$C_{10}H_{16}$	α-Thujene	-	0.20	0.06
4	8.07	93	136	932	932	$C_{10}H_{16}$	α-Pinene	9.22	11.32	6.61
5	8.51	93	136	946	946	$C_{10}H_{16}$	Camphene	2.00	-	5.95
6	9.35	136	93	966	969	$C_{10}H_{16}$	Sabinene	-	1.89	2.55
7	9.41	93	136	980	974	$C_{10}H_{16}$	β-Pinene	31.38	4.48	8.97
8	9.99	69	136	987	988	$C_{10}H_{16}$	Myrcene	-	13.52	-
9	10.02	41	130	991	988	$C_8H_{18}O$	Octanol	-	0.25	-
10	10.50 7.72	128	41	998	998	$C_8H_{16}O$	Octanal	-	-	2.93
11	10.60	93	136	1000	1001	$C_{10}H_{16}$	Mentha-1(7),8-diene	1.51	-	-
12	10.62	93	136	1002	1002	$C_{10}H_{16}$	δ-carene 2	-	0.06	-
13	12.63	93	136	1003	1003	$C_{10}H_{16}$	β-Phellandrene	0.04	0.20	-
14	10.64	93	136	1007	1008	$C_{10}H_{16}$	δ-carene 3	-	-	0.05
15	10.69	93	136	1014	1014	$C_{10}H_{16}$	α-Terpinene	0.54	-	-
16	11.04	67/79	136	1025	1024	$C_{10}H_{16}$	D-Limonene	14.57	17.76	43.60
17	12.04	135	150	-	GNPS	$C_9H_{10}O_2$	2-Methoxy acetophenone	-	0.03	-
18	12.70	93	136	1054	1054	$C_{10}H_{16}$	γ-Terpinene	3.97	11.82	6.19
19	12.98	93	136	1086	1086	$C_{10}H_{16}$	Terpinolene	0.88	-	-
20	13.74	71	154	1090	1095	$C_{10}H_{18}O$	Linalool	1.57	2.39	0.98
21	13.75	81	154	1113	1114	$C_{10}H_{18}O$	α-Fenchol	0.13	-	-
22	15.69	71	156	1136	1134	$C_{10}H_{20}O$	Terpineol <trans-dihydro-β->	4.28	-	-
23	16.18	71	154	1140	1140	$C_{10}H_{18}O$	β-Terpineol	1.46	6.59	0.87
24	16.82	69	154	1152	1148	$C_{10}H_{18}O$	Citronellal	0.12	-	0.72
25	16.86	121	136	1155	1155	$C_8H_8O_2$	2′-Hydroxy acetophenone	-	0.03	0.01
26	16.88	154	71	1168	1174	$C_{10}H_{18}O$	Terpinen-4-ol	-	1.49	0.84
27	16.90	93	135	-	GNPS	C_8H_9NO	N-phenyl Acetamide	4.64	-	-
28	16.95	156	41	1201	1201	$C_{10}H_{20}O$	Decanal	-	1.38	5.47
29	18.22	109	152	1215	1215	$C_{10}H_{16}O$	*trans*-Carveol	0.22	-	-
30	18.25	69	156	1223	1223	$C_{10}H_{20}O$	β–Citronellol	0.21	2.09	1.01
31	18.27	69	152	1236	1235	$C_{10}H_{16}O$	Neral (z-citral)	1.63	0.19	-
32	18.49	69	150	1241	1239	$C_{10}H_{14}O$	Carvone	-	0.16	-
33	18.85	69	154	1250	1249	$C_{10}H_{18}O$	Geraniol	-	1.06	-
34	18.89 18.62	69	152	1266	1264	$C_{10}H_{16}O$	Geranial (E citral)	3.35	0.25	-
35	18.90	55	158	1267	1266	$C_{10}H_{22}O$	1-Decano	-	1.05	-
36	18.92	69	196	1288	1288	$C_{12}H_{20}O_2$	Lavandulyl acetate	0.04	-	-
37	20.28 22.41	81/95	198	1349	1348	$C_{12}H_{22}O_2$	Citronelly acetate	-	0.01	0.22
38	20.85	105/161	204	1350	1350	$C_{15}H_{24}$	α- Cubebene	-	0.24	-
39	20.59	196	41	1360	1359	$C_{12}H_{20}O_2$	Neryl acetate (Geranyl) acetate)	1.17	-	1.14

Table 1. *Cont.*

No	Retention Time (R_t)	Base Peak (BP)	Molecular ion (M+)	RI *	RI **	Molecular Formula	Compounds	Area (%)		
								Lemon (*C. limon*)	Orange (*C. sinensis*)	Mandarin (*C. reticulata*)
40	23.50 23.43	105/119	204	1373	1373	$C_{15}H_{24}$	α-Ylangene	0.10	-	-
41	23.57	93	204	1388	1389	$C_{15}H_{24}$	β-Elemenene	0.16	0.34	-
42	23.60	161	204	1406	1407	$C_{15}H_{24}$	Longifolene	-	0.03	-
43	23.64	41	184	1408	1408	$C_{12}H_{24}O$	Dodecanal	-	0.59	0.12
44	23.66 24.95	93	204	1410	1410	$C_{15}H_{24}$	Trans-Caryophyllene	0.27	0.29	-
46	23.70	161	204	1418	1419	$C_{15}H_{24}$	β-Cedrene	-	0.36	-
47	23.72	93	204	1432	1432	$C_{15}H_{24}$	α-Bergamotene	2.13	-	-
48	23.73	69	204	1440	1440	$C_{15}H_{24}$	β-Farnesene	-	0.45	-
49	23.75 26.16	93	204	1455	1452	$C_{15}H_{24}$	α–Humulene	0.14	-	-
50	23.76	161/105	204	1477	1478	$C_{15}H_{24}$	D-Germacrene	0.22	0.30	
51	23.77	161	204	1480	1480	$C_{15}H_{24}$	γ-muurolene	-	0.02	-
52	24.24	220	205	1485	1489	$C_{15}H_{24}O$	Butylated hydroxytoluene	-	0.32	0.25
53	24.76	204	204	1491	1495	$C_{15}H_{24}$	β-Selinene	0.34	-	-
54	24.80	105	204	1506	1505	$C_{15}H_{24}$	γ-Amorphene	-	7.11	-
55	24.90 29.03	69	204	1514	1514	$C_{15}H_{24}$	E-α-farnesene	4.48	0.26	-
56	24.94	161	204	1524	1522	$C_{15}H_{24}$	δ- cadinene	-	0.73	0.43
57	28.19	109/119	204	1531	1528	$C_{15}H_{24}$	Iso- γ-Bisabolene	0.06	-	-
58	28.20	222	59/93	1548	1548	$C_{15}H_{26}O$	Elemol	0.01	0.01	0.26
59	28.46 34.08	189	222	1630	1630	$C_{15}H_{26}O$	γ-selinenol	0.05	-	-
60	28.66	161	222	1643	1645	$C_{15}H_{26}O$	Cubenol	0.16	-	-
61	28.84	204/161	222	1653	1652	$C_{15}H_{26}O$	α-Cadinol	0.14	0.01	-
62	30.75	55	214	1672	1671	$C_{14}H_{30}O$	Tetradecanol	0.17	-	-
63	30.80 36.17	69	222	1681	1685	$C_{15}H_{26}O$	α-Bisabolol	0.41	-	-
64	30.81	93	218	1699	1699	$C_{15}H_{22}O$	β-Sinensal	-	2.70	-
65	30.84	95/204	222	-	GNPS	$C_{15}H_{26}O$	Selina-6-en-4-ol	0.45	-	-
66	30.86	93	218	1755	1755	$C_{15}H_{22}O$	α –Sinensal	-	0.66	-
67	30.92	41	218	1807	1806	$C_{15}H_{22}O$	Nootkatone	-	0.14	-
68	36.04	57	254	-	GNPS	$C_{18}H_{38}$	2,6,11-Trimethyl dodecane	0.19	-	-
69	38.83	57	282	2000	2000	$C_{20}H_{42}$	Eicosane	0.10	0.46	-
70	38.84	57	310	2200	2200	$C_{22}H_{46}$	Docosane	0.15	-	-
71	39.44	57	324	2300	2300	$C_{23}H_{48}$	Tricosane	2.75	0.88	0.09
72	40.60	57	366	-	GNPS	$C_{26}H_{54}$	5-Butyl docosane	-	0.19	-
73	42.00	57	338	2400	2400	$C_{24}H_{50}$	Tetracosane	1.18	0.18	-
74	42.58	57	352	2500	2500	$C_{25}H_{52}$	Pentacosane	0.78	0.29	0.07
Total identified (%)								97.55	94.22	89.65

RI *: Linear Retention Index; RI **: Linear Retention Index from the literature [22].

The percentage of different classes of terpenoid compounds varied in the EOs of the three species, as reported in Figure 2. The non-oxygenated monoterpenes represent (64.32%, 61.25%, and 73.98%), the oxygenated monoterpenes represent (12.94%, 14.26%, and 4.65%), the non-oxygenated sesquiterpenoids represent (7.90%, 9.89%, and 0.43%), the oxygenated sesquiterpenoids represent (1.22%, 3.52%, and 0.26%), and the monoterpene esters represent (1.21%, 0.01%, and 1.36%) in lemon, orange, and mandarin, respectively.

D-Limonene (14.57%, 17.76%, and 43.60%), α-pinene (9.22%, 11.32%, and 6.61%), β-pinene (31.38%, 4.48%, and 8.97%), and ɣ-Terpinene (3.97%, 11.82%, and 6.19%) represent the highest percentage of identified non-oxygenated monoterpenes in lemon, orange, and mandarin, respectively. Additionally, myrcene (13.52%) is exclusively present in orange, while it is absent in the other species. The major oxygenated monoterpenes identified are linalool (1.57%, 2.39%, and 0.98%), β-Terpineol (1.46%, 6.59%, and 0.87%), and β-Citronellol (0.21%, 2.09%, 1.01%) in the three citrus species.

The sesquiterpenes constituted a minor percentage of the identified compounds, as non-oxygenated sesquiterpenes constituted (7.90%, 9.89%, and 0.43%) and oxygenated sesquiterpenes constituted (1.22%, 3.52%, and 0.26%) in the three citrus species. E-α-farnesene (4.48%) in lemon EO and γ-amorphene (7.11%) in orange EO represent the highest percentage of non-oxygenated sesquiterpenes, while β-sinensal (2.70%) constituted a higher percentage of oxygenated sesquiterpenes in orange EO. The present study reveals that lemon (86.38%) and orange (88.92%) EOs boast the highest percentage of terpenoid compounds.

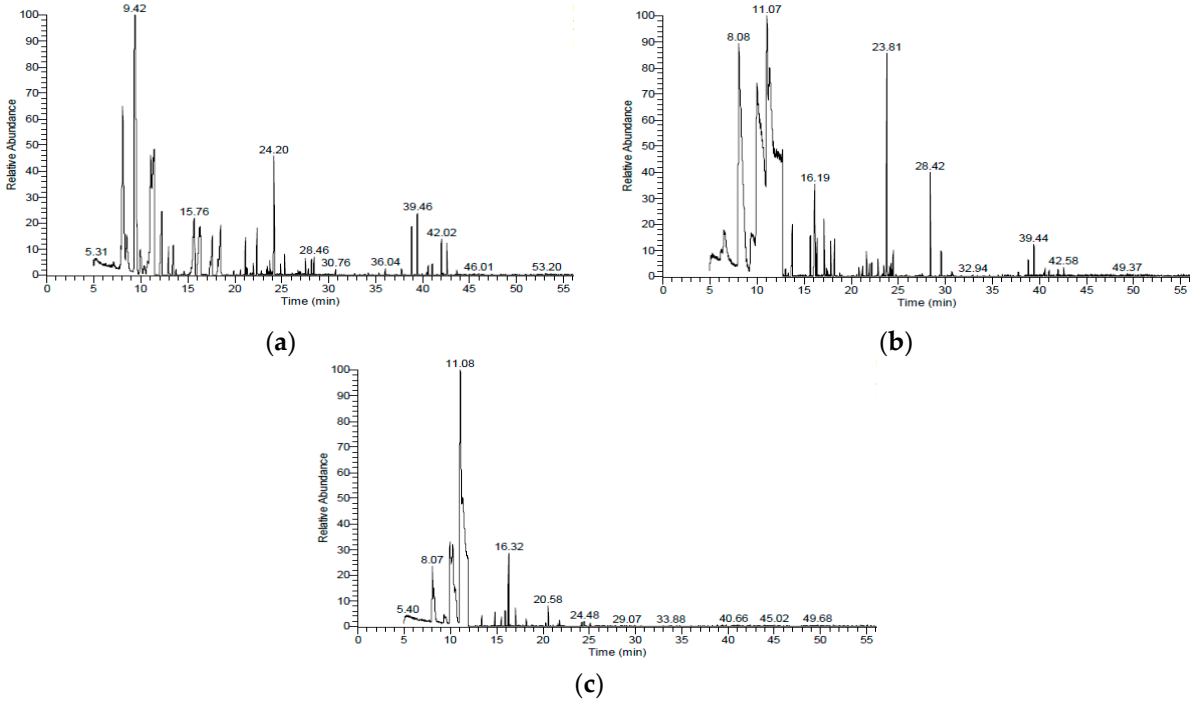

Figure 1. GC/MS chromatograms of EOs recovered from three *Citrus* spp. peels: (**a**) lemon (*C. limon*); (**b**) orange (*C. sinensis*); (**c**) mandarin (*C. reticulata*).

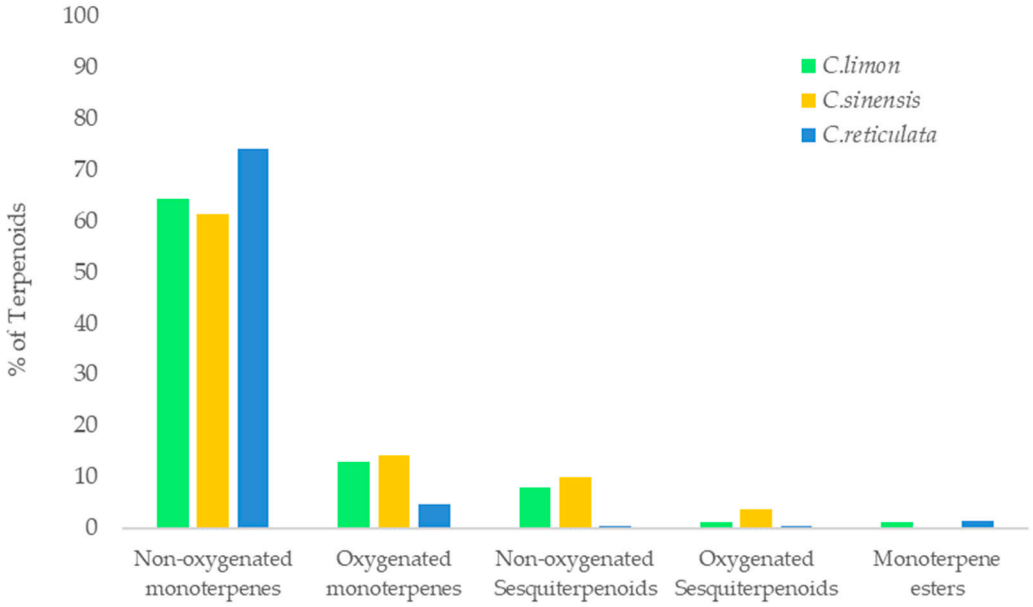

Figure 2. The percentage of terpenoid compounds from three *Citrus* spp. peels: lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*).

The identification of compounds was corroborated through mass spectral data (MS) and the relative retention time when compared to the existing literature [22], Wiley spectral library collection, and GNPS and NIST library databases. Significant variations between the three EOs of the citrus species were found by the GC-MS analysis and can be seen in the constructed molecular network (MN) (Figure 3).

Our findings align with observations made in citrus species from other countries. Additionally, other researchers have documented that lemon EO exhibited elevated levels of diverse types of monoterpenes, including alcohols, aldehydes, ester monoterpenes, and sesquiterpenes [32]. D-Limonene, classified as a monoterpene, stands out as a notable component of citrus plant oils. It is found in a range of citrus EOs, including orange, lemon, mandarin, lime, and grapefruit, making it one of the most prevalent inartificial monocyclic monoterpenes [33]. D-Limonene was the primary component in the studied EOs, with varying percentages. Moreover, according to the literature, D-Limonene has demonstrated efficacy against foodborne bacterial and fungal pathogens, such as *Aspergillus niger*, *Colletotrichum falcatum*, *Staphylococcus aureus*, and *Listeria monocytogenes* [34]. The terpenoid concentrations recovered from *Citrus aurantium* peels in Western Morocco were 90.9% [35]; in Iran, 94.81% [36]; and in Brazil, 98.66% [37]. Moosavy et al. (2017) mentioned that the main constituents of lemon peel were D-Limonene, γ-terpinene, and tricycline [38]. Similarly, D-Limonene and γ-terpinene were the major monoterpenes in mandarin and lemon EOs [39,40]. According to Benayad et al. (2021), the main constituents in the EOs of *C. aurantium* peel were D-Limonene (35.17%), β-myrcene (17.61%), and β-linalool (18.19%) [41]. The different concentrations of terpenoids in Egyptian citrus peel EOs can be influenced by the kind of soil, the location, and the climate in which the species are cultivated. These factors can contribute to the fluctuations in the percentage of EOs. Additionally, the terpenoid concentrations in citrus EOs may be influenced by factors such as harvest season, the maturity of the fruits, and the extraction methods [42].

3.1.2. Total Phenolic Content (TPC)

Phenolic compounds (PCs) are the most important group of bioactive compounds in both citrus fruit juices and by-products, determining their biological activity. The highest total phenolic content (TPC) was in lemon EO, followed by orange EO, and then mandarin EO, corresponding to 34, 24, and 16 mg GAE/g EO, respectively (Figure 4). Other researchers have reported the greatest phenolic contents in citrus EOs. Durmus M. et al. (2023) reported that the highest TPC was obtained from grapefruit EO (44.32 mg GAE/g), followed by lemon EO (35.52 mg GAE/g), mandarin EO (32.44 mg GAE/g), and then orange EO (31.99 mg GAE/g) [43]. Furthermore, numerous studies have examined the correlation between total phenol content and antioxidant activity in a variety of foods and vegetables, including citrus and its by-products [44,45]. The results show that a high concentration of total polyphenol content greatly boosts the ability to scavenge free radicals [46,47].

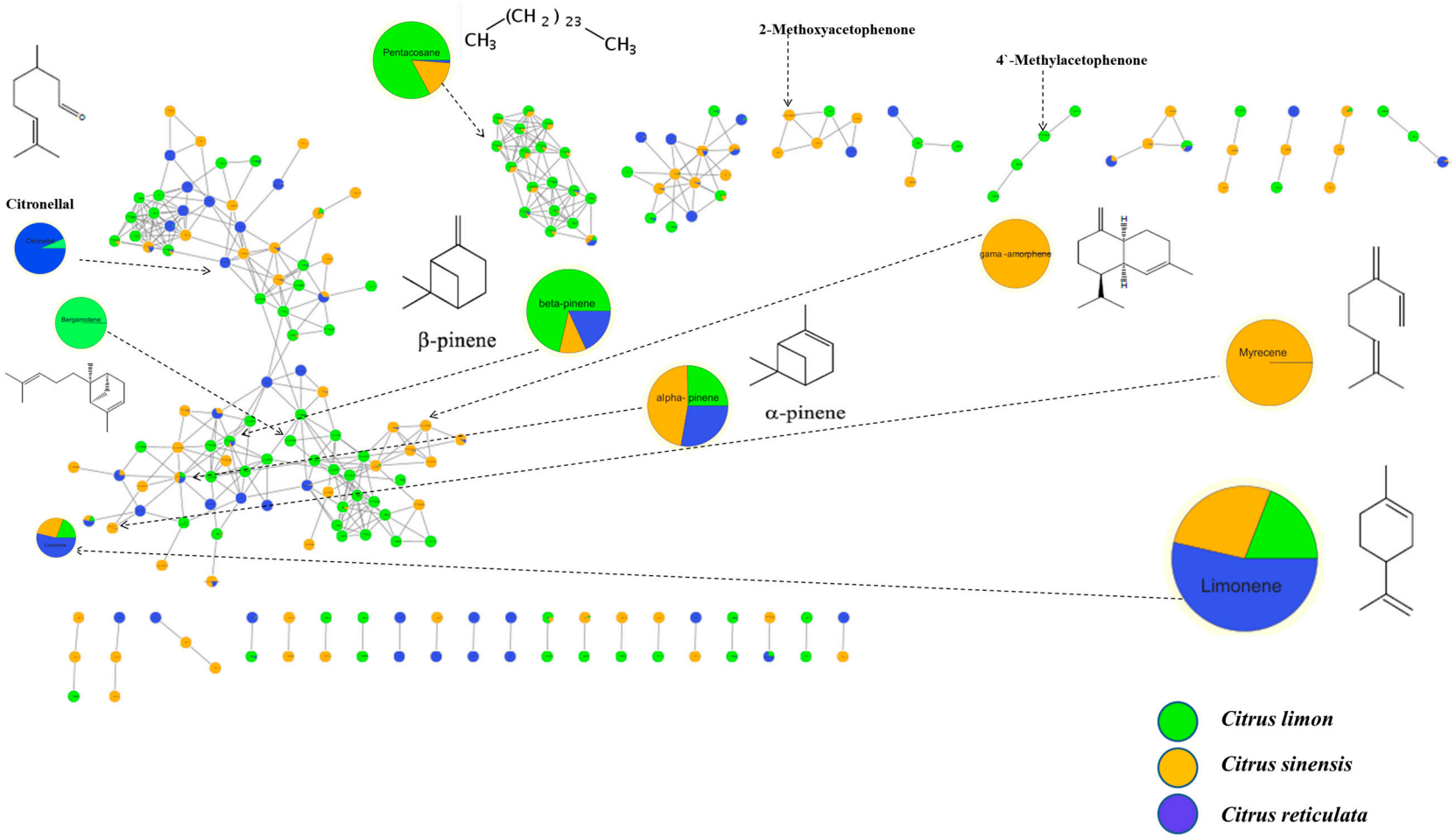

Figure 3. Molecular network of the GC/MS analysis of EOs of three *Citrus* spp. peels: lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*). Node size: the total sum of the intensity of the corresponding ion. Node color: distribution of ions among different citrus species. Nodes are labeled with spectral matches from the GNPS-GC/MS spectral libraries.

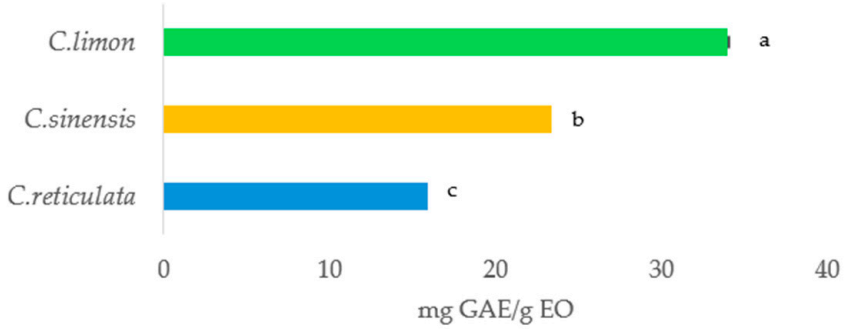

Figure 4. Total phenolic content (TPC) of EOs in lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*) peels. Values are represented by the average ± standard deviation. Different letters mean significant differences between extraction methods ($p \leq 0.05$).

3.2. Biological Studies

3.2.1. Antioxidant Activity

DPPH Assay

Antioxidant activity can be assessed using a DPPH assay, which measures the capacity of the essential oil to scavenge the stable free radical DPPH by the donation of a hydrogen atom or an electron [48]. Citrus EOs contain antioxidant properties that might delay or prevent cell damage induced by physiological oxidants [49,50]. Antioxidants react with DPPH, converting it to 1,1- diphenyl-2-picryl hydrazine, due to its rapid hydrogen accepting ability, which intercepts the spread of the free radical oxidation chain, forming stable end products that do not cause further lipid oxidation [51]. Free radicals are widely known for causing cell death and tissue damage, which leads to chronic illnesses [52]. Many studies have demonstrated the role of EOs regarding their free radical removal capacity [53], which is due to their beneficial antioxidant properties, allowing them to counteract cellular damage caused by physiological oxidants.

The effect of the different Egyptian citrus EOs on DPPH radical scavenging was compared to those of vitamin C, used as a positive control, and analyzed by the determination of the IC_{50} values (Table 2). The antioxidant activity of EOs from lemon, orange, and mandarin was concentration-dependent, which means that activity increased as the concentration of the EOs increased, from 250, 500, 1000, to 2000 µg/mL. EOs from lemon, orange, and mandarin showed moderate activity in the DPPH scavenging assay at 1000 µg/mL. The inhibition values of lemon, orange, and mandarin EOs at 1000 µg/mL were 51.3%, 45.45%, and 56.83%, respectively, compared with vitamin C (64.57%). The IC_{50} values were 947 µg/mL, 1073 µg/mL, and 878 µg/mL for lemon, orange, and mandarin peels, respectively, compared with vitamin C (734 µg/mL). Lemon EO had the highest antioxidant activity at 2000 µg/mL, with a percentage of 86.35%, compared to lower values in the other two tested citrus EOs.

Table 2. The percentage of inhibition of EOs extracted from lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*) in DPPH free radical at different concentrations (µg/mL) and their IC_{50}, compared with vitamin C (standard). Values are represented by the average ± standard deviation. Different letters mean significant differences between concentrations ($p \leq 0.05$).

Concentration (µg/mL)	Vitamin C	Lemon (*C. limon*)	Orange (*C. sinensis*)	Mandarin (*C. reticulata*)
250	$23.73^{e} \pm 0.53$	$26.90^{e} \pm 1.21$	$21.50^{f} \pm 1.27$	$27.30^{e} \pm 0.40$
500	$41.08^{d} \pm 0.52$	$38.20^{d} \pm 0.75$	$32.35^{d} \pm 0.38$	$39.86^{d} \pm 0.06$
1000	$64.57^{c} \pm 0.59$	$51.30^{c} \pm 0.75$	$45.45^{c} \pm 0.26$	$56.83^{c} \pm 0.28$
1500	$81.85^{b} \pm 0.46$	$61.20^{b} \pm 0.57$	$70.20^{b} \pm 0.46$	$68.67^{b} \pm 0.22$
2000	$96.09^{a} \pm 0.14$	$86.35^{a} \pm 0.25$	$76.15^{a} \pm 0.32$	$81.33^{a} \pm 0.32$
IC_{50}	735 ± 0.89	947 ± 1.78	1073 ± 2.45	878 ± 1.36

Other species within the *Citrus* genus have demonstrated antioxidant activity. Meryem S. et al. (2023) reported similar results for three tested Moroccan citrus peel EOs (*C. limonum*, *C. paradisi*, and *C. reticulata*). For the DPPH assay, the inhibition values were 65%, 72%, and 76%, respectively [54]. Kostova D. R. et al. (2021) documented that *C. paradisi* zest EO exhibited the greatest DPPH free radical inhibition, reaching 87.5% at a concentration of 1 mg/cm^3. *C. limon* zest oil also demonstrated notable DPPH radical capture at 86.1%. In contrast, *C. reticulata* zest EO displayed a slightly lower inhibition percentage of 78.0% [55]. Also, Sarrou et al. (2013) demonstrated a scavenging activity of 19.29% for *C. aurantifolia* peel EO [56].

Nitric Oxide (NO) Assay

A nitric oxide assay based on the scavenging of free radicals focused on nitrogen (•NO) was also used to evaluate antioxidant activity. EOs from lemon, orange, and mandarin also showed moderate activity in the NO scavenging assay at 1000 µg/mL. For the NO assay (Table 3), the inhibition values of EOs of lemon, orange, and mandarin peels at 1000 µg/mL were 63.81%, 50.27%, and 51.12%, respectively, compared with vitamin C (77.83%). The IC_{50} values for EOs of lemon, orange, and mandarin peels were 914 µg/mL, 1154 µg/mL, and 1066 µg/mL, respectively, compared with vitamin (263 µg/mL). To our knowledge, there are few studies on the purifying capacity of nitric oxide by citrus peel EOs. Recently, Manzur et al. (2023) investigated the scavenging activity of the NO assay. In this study, orange peel EOs were extracted with two different extraction technologies (the cold-press method and the cold-press method followed by steam distillation). The scavenging capacities of orange peel EOs were 0.35 mg/mL and 2.10 mg/mL, respectively [57].

Table 3. The percentage of inhibition of EOs extracted from lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*) against nitric oxide (NO) free radicals at different concentrations (µg/mL) and their IC_{50}, compared with vitamin C (standard). Values are represented by the average ± standard deviation. Different letters mean significant differences between concentrations ($p \leq 0.05$).

Concentration (µg/mL)	Vitamin C	Lemon (*C. limon*)	Orange (*C. sinensis*)	Mandarin (*C. reticulata*)
250	43.32 ^{e} ± 0.32	13.84 ^{e} ± 0.87	14.97 ^{e} ± 0.55	16.10 ^{e} ± 0.21
500	63.61 ^{d} ± 0.42	37.32 ^{d} ± 0.65	25.30 ^{d} ± 0.35	32.53 ^{d} ± 0.69
1000	77.83 ^{c} ± 0.51	63.81 ^{c} ± 0.25	50.27 ^{c} ± 0.20	51.12 ^{c} ± 0.26
1500	85.25 ^{b} ± 0.32	74.21 ^{b} ± 0.35	61.70 ^{b} ± 0.32	62.40 ^{b} ± 0.42
2000	92.94 ^{a} ± 0.28	83.98 ^{a} ± 0.41	79.43 ^{a} ± 0.75	83.91 ^{a} ± 0.35
IC_{50}	263 ± 1.85	914 ± 0.98	999 ± 2.05	995 ± 1.24

3.2.2. Antimicrobial Activity

Microbiological spoilage is a significant factor contributing to food waste, leading to illnesses and deaths caused by the consumption of contaminated food. It is imperative to explore new and effective methods to inhibit and eliminate contamination. Natural food preservatives with antimicrobial properties are being studied as safer alternatives to synthetic ones. Additionally, these natural food preservatives may prove effective against pathogens resistant to synthetic antibiotics [58]. The mode of action of antimicrobial agents toward the pathogens of human, animal, and plant origin depends on one of four main categories, based on their site of activity. This includes the inhibition of cell wall synthesis, protein synthesis, nucleic acid synthesis, and the disruption of cell membrane integrity [59].

The results of antimicrobial activity, measured by the disc diffusion method for the EOs from lemon, orange, and mandarin peels, as well as the bacteria standard (amoxycillin) and fungal standard (miconazole), are shown in Figure 5. The EO of LP showed good antimicrobial activity against Gm− bacteria, *Escherichia coli* (10 mm) and *Pseudomonas aeruginosa* (8 mm), and against Gm+ bacteria, *Bacillus cereus* (30 mm) and *Staphylococcus aureus* (21 mm). Lemon EO also had a specific spectrum toward the pathogenic yeast *Candida albicans* (40 mm) compared with the standard antifungal (10 mm). Lemon EO had

the best antimicrobial activity against the tested Gm+ and Gm− bacterial strains and the pathogenic yeast *C. albicans*, which may be an excellent, promising antimicrobial agent. The EO of MP had good antimicrobial activity toward Gm+ bacteria only, with an evident inhibition zone against *S. epidermidis*, *Micrococcus luteus*, *S. aureus*, and *B. cereus*, ranging from 9 to 14 mm. On the other hand, it exhibited no activity against Gm− bacteria (*E. coli* and *P. aeruginosa*) but demonstrated effectiveness against the yeast *Candida albicans* (15 mm). The EO recovered from OP displayed moderate activity against Gm+ bacteria, *B. cereus, M. luteus*, *and S. aureus* (16, 10, and 9 mm, respectively), and the yeast *C. albicans* (13 mm). Moreover, there was no activity observed against Gm− bacteria.

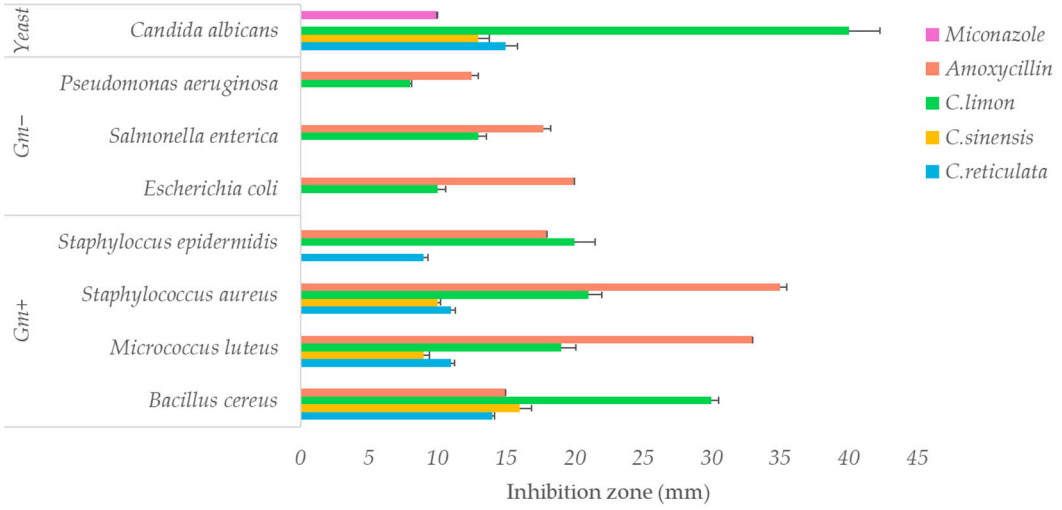

Figure 5. Antimicrobial (antibacterial and antifungal) activity of EOs from lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*) peels by disc diffusion assay.

The noted antimicrobial activity may probably be attributed to a combination of multiple constituents of EOs that exhibit synergistic effects [60]. Our results are in accordance with most studies investigating the action of EOs against food spoilage organisms and food-borne pathogens. These studies agree that EOs are generally slightly more active against Gm+ than Gm− bacteria [14]. It was observed that although LP EOs are effective against both groups of bacteria, their activity was higher in Gm+ compared to Gm− bacteria. These observations are in accordance with earlier studies that showed that Gm− organisms were less susceptible to herbal extracts than Gm+ isolates due to the presence of high lipid content in the outer membrane from the cell walls of Gm− bacteria [61]. Gm+ bacteria, such as *S. epidermidis* and *S. aureus*, contain teichoic acid in the peptidoglycan layer and are, therefore, inhibited by both citrus peel extracts and EO. Furthermore, the outer membrane of Gm− bacteria is recognized for forming a barrier that hinders the penetration of numerous antibiotic molecules. The periplasmic space within these bacteria contains enzymes capable of breaking down foreign molecules introduced from outside, thus enhancing greater resistance to them [61]. The antimicrobial activity could also be further attributed to the presence of a high percentage of oxygenated compounds and sesquiterpenes, including D-Limonene, α-pinene, β-myrcene, and caryophyllene. Furthermore, constituents like β-pinene, α-terpineol, γ-terpinene, and α-bergamotene may have also imparted synergistic effects along with limonene [62]. Although the mechanism of action of terpenes is not fully understood, it is thought to involve membrane disruption by the lipophilic compounds [14]. EOs containing terpenes, such as D-Limonene and carvone, have been reported to exhibit antimicrobial activity, aligning with the findings of our current studies. These compounds demonstrated efficacy against a broad spectrum of pathogenic fungi and bacteria [63].

3.2.3. Anti-Inflammatory Activity

Prostanoid substances (prostaglandins, prostacyclins, and thromboxanes) are created in the body as a result of the inflammatory response [30]. Cyclooxygenases (COX-1 and COX-2) are rate-limiting enzymes, as they serve as the major pathway or key for the formation of these prostanoids. COX-1, present in most body tissues, such as the gastrointestinal tract (GIT), has a useful role in maintaining the normal lining of the stomach, protecting the stomach from the digestive juices, and is also involved in kidney and platelet function [64]. COX-2 has been found at sites of inflammation common to nonsteroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen, aspirin, and indomethacin, which are prescribed to treat many types of arthritis [65]. The use of selective COX-2 inhibitors, such as celecoxib, targets pain and inflammation with fewer gastrointestinal side effects [30].

Table 4 shows the results of anti-inflammatory activity from citrus EOs. The EOs recovered from lemon, orange, and mandarin peels revealed activities as COX-1 and COX-2 inhibitors, with IC_{50} values of (12.5, 40.00, and 24.33) for COX-1 and (0.09, 0.63, and 0.31) µg/mL for COX-2, respectively. The EOs from citrus peels were compared to the reference standards celecoxib and indomethacin for COX-1 (97.5 and 6.25 µg/mL) and COX-2 (0.31 and 0.52 µg/mL), respectively. It was noticed from our results that the EOs of lemon, orange, and mandarin have strong activity as COX-2 inhibitors and are good inhibitors of COX-1, but while the EO of lemon showed selective activity as a COX-2 and COX-1 inhibitor, orange EO possessed weaker activity as a COX-2 inhibitor, compared with lemon, and good activity as a COX-1 inhibitor.

Table 4. In vitro anti-inflammatory activity against biomarkers COX-1 and COX-2 expressed in IC_{50} (ug/mL) for the essential oils obtained from lemon (*C. limon*), orange (*C. sinensis*), and mandarin (*C. reticulata*) peels. Celecoxib and indomethacin were used as standards. Data presented as mean ± SD. One-way ANOVA was used for data analysis (n = 3, $p \leq 0.05$). Different lower-case letters within the same column designate significant differences.

IC_{50} (µg/mL)	**COX-1**	**COX-2**
Celecoxib	97.50 ^{a} ± 0.10	0.31 ^{c} ± 0.01
Indomethacin	6.25 ^{e} ± 0.00	0.52 ^{b} ± 0.01
Lemon (*C. limon*)	12.50 ^{d} ± 0.20	0.09 ^{d} ± 0.00
Orange (*C. sinensis*)	40.00 ^{b} ± 1.00	0.63 ^{a} ± 0.00
Mandarin (*C. reticulata*)	24.33 ^{c} ± 0.85	0.31 ^{c} ± 0.01

Previous studies have shown that the lipophilicity of monoterpenes is promising for regulating inflammatory cytokines owing to their characteristic absorption and rapid response [66]. Monoterpenes decrease inflammatory responses and modulate the key chemical mediators of inflammation. Previous studies reported that monoterpenes, such as borneol, citral, and geraniol, exhibit anti-inflammatory activity by suppressing the LPS-induced production of proinflammatory cytokines and nitric oxide [67]. The anti-inflammatory effects of many natural compounds are due to a hydroxyl group in their structure. However, the exact mechanism of the effect of the hydroxyl group on the anti-inflammatory activity was not elucidated.

4. Conclusions

The current study investigated the efficiency of Egyptian citrus peel waste valorization through the production of EO with antioxidant, antibacterial, antifungal, and anti-inflammatory activities. A hydro-distillation technique for extracting the essential oil from the citrus peels was utilized. D-Limonene and β-pinene were the major compounds detected in the three EOs from lemon, orange, and mandarin peels. The antimicrobial activity of citrus essential oils was higher against Gm+ bacteria than the tested Gm− bacteria. The antioxidant activity of the citrus EOs was evidenced by two different antioxidant activity tests and showed moderate activity concerning the DPPH and NO scavenging

activities at 1000 µg/mL. Regarding the anti-inflammatory activity, it was noticed that citrus EOs have strong activity as COX-2 inhibitors. Therefore, citrus EOs represent natural and safe alternatives to extend the shelf life of food products by preventing oxidation and contamination by pathogens that spoil food, meaning that citrus EOs can be considered an innovative dual strategy for food preservation. Also, these EOs may be incorporated in cosmetics and pharmaceutical formulations due to highly valuable biological activities, such as antimicrobial, antioxidant, and anti-inflammatory ones, thus pivoting toward the application of circular economy principles.

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