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Antibacterial and Mosquito Repellent Potential of Eight Citrus Cultivars and Their Chemical Composition

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Abstract: Citrus fruit peels are a rich source of essential oils (EOs), which contain biologically active compounds; however, they are often discarded as waste, which causes pollution. The fresh peels of eight citrus cultivars growing in Pakistan were used to extract EOs through steam distillation. Gas chromatography-mass spectrometry (GC-MS) analysis of fresh peel EOs revealed that limonene was the most abundant compound, constituting 94.5%, 96.1%, 95.3%, 93.3%, 56.2%, 91.5%, 96.4%, and 96.7% of *Citrus jambhiri*, *C. aurantium*, *C. sinensis* var. Malta cv. Blood Malta, *C. sinensis* var. Malta cv. Shakri Malta, *C. limon*, *C. pseudolimon*, *C. reticulata* var. Mandarin cv. Feutrell's Early, and *C. reticulata* var. Mandarin cv. Kinnow, respectively. The dried peel EO of *C. reticulata* var. Mandarin cv. Kinnow contained 95.2% limonene. *C. limon* peel EO exhibited the highest antibacterial activity among all citrus peel EOs with the minimum inhibitory concentration of 312 µg/mL against *Staphylococcus aureus*. The *C. aurantium* and *C. sinensis* var. Malta cv. Shakri Malta peel EOs exhibited the highest mosquito repellent activity against *Ae. aegypti* females, providing protection for 45 min when tested at a concentration of 166 µg/cm^2. This study showed *C. aurantium* and Shaki Malta peel EOs could be used to formulate natural mosquito repellent.

Keywords: citrus; essential oil; repellent; *Aedes aegypti*; antibacterial; limonene

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1. Introduction

Most cultivated citrus are hybrids between two or more ancestral species of the genus *Citrus* (Sapindales: Rutaceae). They are distributed throughout the tropical, subtropical, and temperature zones with over 250 known commercial varieties [1], including orange, pomelo, grapefruit, kinnow, lemon, sweet orange, kumquat, lime, and others [2]. About 140 countries produce 70 million tons of citrus fruits annually. Pakistan is the 12th largest citrus producer, with an annual production of about 1,816,000 tons [3]. Swat, Mardan, Nowshera, Malakand, Lower Dir, Multan, Sahiwal, Sargodha, Bahawalpur, Toba Tek Singh, and Vehari are the citrus-producing districts in Pakistan [4].

Citrus peels comprise approximately 20–30% of the total weight of fruit [5]. Tons of solid citrus peel waste are produced during fruit processing, such as canning and juicing, and are often discarded as waste, contributing to significant environmental pollution with

a lack of practical reuse. Even though they are not edible, citrus peels could be used as fish feed, as a raw material for conventional paper, as an activated carbon adsorbent in cosmetics [6], and in bioethanol production [7]. Citrus peels are a good source of bioactive compounds such as ascorbic acid, carotenoids, and flavonoids [8]. Moreover, citrus peels are rich in essential oils (EOs). It is estimated that 0.5–3.0 g/kg of EO can be obtained from citrus fruit peels [9]. Citrus peel oil is produced by cold press, solvent extraction, or distillation. The cold press is the most commonly used industrial method for citrus EOs extraction, producing complex mixtures of about 400 compounds, 85–99% of which are volatile constituents, including several types of sesquiterpenes, hydrocarbons, and monoterpenes [10]. The composition of the mixture of terpenes varies from species to species and includes different compounds like limonene, α-pinene, β-pinene, β-myrcene, linalool, and terpinene [11]. Limonene is a major component of citrus peel EO, as it ranges between 32 and 98% [12].

Besides *Citrus* species, limonene is found in different proportions in diverse types of plant essential oils. Several previous studies demonstrated various biological activities of limonene. For example, a limonene racemic mixture and individual enantiomers exhibited antibacterial activity against different bacteria [13–16]. A study described the anti-fungal activity of limonene against food-spoiling yeast [17]. In another study, both enantiomers of limonene exhibited similar mosquito larvicidal activity against *Ae. albopictus*; however, in repellency bioassay, (-)-limonene showed higher activity, whereas (+)-limonene exhibited comparatively lower activity against female *Ae. albopictus* [18]. Miller et al. reported that limonene showed potential as an anti-cancer agent to treat breast cancer [19]. Vieira et al. (2018) reviewed several studies summarizing various health-beneficial effects of limonene, such as anti-inflammatory, anti-bacterial, anti-oxidant, and anti-cancer [20]. Both enantiomers of α-pinene and (-)-β-pinene exhibited moderate repellency, whereas (+)-β-pinene showed good repellency towards *Ae. albopictus* female mosquito [18]. A study showed the anti-bacterial activity of α-pinene and β-pinene against different bacteria [21]. A recent study reported the biological activity of α-pinene and β-pinene for controlling cattle tick *Rhipicephalus microplus* [22].

Infectious ailments are significant public health issues worldwide [23,24]. Though various antibiotic agents are available to treat microbial infection, microbes have acquired resistance against many antibiotics [24–26]. To overcome this problem, natural products from plants could be a better alternative to antibiotics since plants are the foremost source of bioactive compounds. Plant natural products are considered safe for personal use and are effective and readily available for treating various ailments [27,28]. Several studies reported that citrus peel EOs possessed a wide range of biological activities such as anti-viral [29,30], antibacterial [14,31–33], anti-inflammatory [34], antioxidant [35], anticancer [36], and anti-fungal activities [33,37,38].

Mosquitoes are significant carriers of several tropical diseases, including dengue, yellow fever, malaria, etc. An evident practical and most economical way of avoiding the spread of these diseases to people is the use of repellents [39]. Mosquito repellents are preferred to prevent insect-borne diseases and are a cost-effective healthcare practice. In the market, various synthetic and natural insect repellents are available, including the most famous formulation, *N*, *N*-diethyl-3-methylbenzamide (DEET), which has proven to show excellent repellency against mosquitoes and other blood-feeding insects [40]. However, several studies showed that prolonged use of DEET could pose some adverse effects. The research demonstrated that EOs-based mosquito repellents could be the best alternative to synthetic formulations as they are considered safe and show effective repellency against several mosquito species [41–43].

Numerous studies from various countries have reported the chemical composition of citrus peel EOs [12,44,45], as well as their antibacterial [32,33,44,46,47] and mosquito larvicidal [48,49] activities. However, a few studies in the literature describe the mosquito-repellent activities [50–53] of citrus peel EOs. To our knowledge, no previous study has reported the mosquito-repellent activity of *Citrus jambhiri*, *C. pseudolimon*, and *C. sinensis* var. Malta cv. Shakri Malta, *C. sinensis* var. Malta cv. Blood Malta, and *C. reticulata* (L.) var. Mandarin cv. Feutrell's early. Moreover, no detailed investigation has been carried out to compare the chemical composition and bioactivity of EOs extracted from the peels of various citrus cultivars, minimizing variations in sample preparation methods that affect the chemical composition and biological activities. To fill this knowledge gap, this study aimed to compare the chemical composition of EOs extracted from fruit peels of various citrus cultivars growing in Pakistan and to evaluate their mosquito-repellent activity against outdoor-biting *Aedes aegypti* as well as their antibacterial activity against pathogenic bacteria: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* PAO1. Moreover, the enantiomeric composition of limonene present in different citrus EOs was also investigated.

2. Materials and Methods

2.1. Collection and Maintenance of Citrus Peels

Different cultivars of citrus fruits were collected from their respective orchards located in various districts of Pakistan (Table 1). The plant specimens were identified by comparing the diagnostic morphological characters of the plant with those presented in the Flora of Pakistan and with those available from the literature sources [54–56]. In addition, the names of the plants were verified using World Flora Online [57] and the Flora of Pakistan [58]. Voucher specimens were submitted to the herbarium of the Department of Environmental Sciences, COMSATS University Islamabad, Abbottabad Campus, Abbottabad, Pakistan. The fruits were thoroughly washed with tap water and wiped with a cotton cloth. Afterward, the peels were carefully removed with a sharp knife, cut into pieces of 2–3 inches, and subjected to EO extraction on the same day or stored in the freezer at −30 °C until used for EO extraction within 24 h.

Table 1. Description and yield percentage of essential oils extracted from citrus cultivars.

EO Type	The Local Name of a Cultivar	Peels Condition	Voucher No	Latin Name	Location	% Yield
RL	Rough lemon	Fresh	CUHA-465	*Citrus jambhiri* Lush	Abbottabad	0.38 ± 0.04
SO	Sour orange	Fresh	CUHA-25	*Citrus aurantium* (L.)	Abbottabad	0.19 ± 0.02
BM	Blood malta	Fresh	CUHA-466-1	*Citrus sinensis* Osbeck var. Malta	Khanpur	0.12 ± 0.01
SM	Shakri malta	Fresh	CUHA-466-2		Khanpur	0.21 ± 0.01
DL	Desi lemon	Fresh	CUHA-467	*Citrus limon* (L) Osbeck	Multan	0.05 ± 0.00
GA	Galgal	Fresh	CUHA-468	*Citrus pseudolimon* Wester	Haripur	0.27 ± 0.02
FE	Feutrell's early	Fresh	CUHA-469-1	*Citrus reticulata* Blanco var. Mandarin	Sargodha	0.21 ± 0.01
KF	Kinnow	Fresh	CUHA-469-2		Sargodha	0.29 ± 0.02
KD	Kinnow	Dried	CUHA-469-2		Sargodha	0.35 ± 0.03

2.2. Extraction of EOs

Steam distillation was used to extract EOs from the fresh peels of citrus fruits using a previously reported method [42,59]. Weighed citrus fruit peels of 1500 g were subjected to

steam distillation in a stainless-steel distillation apparatus (Liaqat Engineering, Faisalabad, Pakistan). A 2 L of distilled water was added to the bottom of the stainless-steel vessel to avoid direct contact with the peels packed in a meshed container adjusted above water level. The vessel was then heated using an electric hotplate. The released steam passed through the packed peels, extracting the volatile compounds. The steam containing peel volatiles was cooled down using a water condenser connected externally to the top of the vessel. The distillate, consisting of water and peel volatiles, was collected in a 1 L glass separating funnel for 3 h. The lower water layer was disposed of, and the upper layer of EO was recovered through decantation and weighed using a digital analytical balance after removing traces of water over anhydrous $MgSO_4$ (Daejung Chemicals, Siheung-si, South Korea). The percentage yield of extracted EO was calculated by dividing the mass of EO by the mass of fresh peels and multiplying by a hundred. From each citrus peel sample, the EO was extracted in a triplicated manner. The extracted EOs were stored in glass vials at −20 °C until used for chemical analysis and bioassays.

2.3. Chemical Analysis of EOs by GC-MS

The chemical analysis of citrus peel EO was investigated using a Hewlett-Packard 6890 N gas chromatograph (GC) and an HP 5973 mass spectrometer (MS, Agilent Technologies Inc., Santa Clara, CA, USA). The GC was fitted with a DB-5 capillary column (Agilent Technologies Inc., Santa Clara, CA, USA) having 30 m length, 0.25 mm internal diameter, and 0.25 µm stationary phase film thicknesses. The parameters of GC and MS were set as previously reported by Azeem et al. [42]. In short, the GC injector was isothermally set at 225 °C. The initial temperature of the column oven was isothermally set at 40 °C for 2 min after that, increased at the rate of 4 °C/min to 230 °C, and finally isothermally set at 230 °C for 5 min. High-purity helium (99.99%) was used as the carrier gas that flowed at a steady rate of 1 mL/min through the column. Diluted solutions of EO samples were injected in a GC injector in the splitless mode set for 30 s. The parameters for the mass spectrometer were as follows: an electron ionization energy of 70 eV was used for ionization in positive mode. MS ion source temperature was isothermally set at 180 °C. Mass spectra of the separated compounds were acquired in the 30 to 400 m/z range. The GC peaks were used to calculate the percentage composition of every component of an EO. To identify the separated compounds, their mass spectra were first compared to those in the NIST-2008 (National Institute of Standard Technology) MS library, in the NIST webbook, and to published data [60]. The retention times of n-alkanes (C_9–C_{24}) were determined to calculate the retention indexes of the isolated compounds by applying the same GC-MS parameters used for the analyses of the EOs. The computed retention indices were compared to the published data to determine the elution sequence and identify the separated substances. Lastly, the identification of EO constituents was confirmed by injecting available pure reference compounds such as α-pinene, β-pinene, β-myrcene, limonene, and linalool, etc. (Sigma-Aldrich, St. Louis, MO, USA) using the identical GC-MS parameters applied for analyses of EOs.

The enantiomeric composition of limonene in EOs was determined by a Shimadzu GC-2010 Plus gas chromatograph equipped with an AOC-20i liquid autosampler, an FID detector (Shimadzu Corporation, Kyoto, Japan), and an Rt®-bDEXsm column (30 m × 0.25 mm × 0.25 µm) (Restek Corporation, Bellefonte, PA, USA). The stationary chiral phase of the column was composed of 2,3-di-O-methyl-6-O-tert-butyldimethylsilyl-β-cyclodextrin and cyanopropylphenyl/dimethylpolysiloxane. Cyclodextrin-based GC stationary phases provide excellent separation for a wide range of chiral compounds and are the most widely used [61]. The injector and the detector temperatures were set isothermal at 250 °C and 260 °C, respectively. The oven's initial temperature was 50 °C; afterward,

it ramped by 2 °C/min to 160 °C and then increased by 10 °C/min to 210 °C. Helium was used as the carrier gas at a 1.5 mL/min flow rate. Nitrogen was used as a make-up gas at a flow rate of 30 mL/min. Standards of (S)-(-)-limonene and (R)-(+)-limonene were obtained from Fluka Chemicals, Gillingham, UK.

2.4. Antibacterial Activity

To test the antibacterial potential of extracted EOs, four human pathogenic bacterial strains, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* (PAO1), *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 6538, were obtained from the National Institute of Health, Islamabad, Pakistan. The bacterial strains were streaked on nutrient agar (NA) Petri plates and grown overnight at 37 °C. The broth dilution method was used to find the minimum inhibitory concentration (MIC) against selected bacterial strains by adopting a reported method [62]. Briefly, freshly grown bacteria colonies were suspended in 4 mL of sterilized distilled water. The suspension's optical density was adjusted to the equivalent of 0.5 McFarland standard, which consisted of 10^8 colony-forming units per mL (CFU/mL). The bacterial suspension was further diluted serially in sterilized distilled water to obtain the required concentration of 10^4 CFU/mL. To determine the MIC of test substances, an aliquot of 10 µL of bacterial suspension was mixed in 990 µL of sterilized water to be used as a water reference to count the number of CFU originally added in any sample or control test tube. In another similar test tube, 980 µL of sterilized nutrient broth was taken, to which 10 µL of bacterial suspension and 10 µL of test substance solution were added. After overnight incubation at 37 °C, a 100 µL aliquot of the mixtures from the water reference and sample test tubes was spread evenly on separate NA Petri plates and incubated at 37 °C for 24 h, and viable CFUs were counted on each Petri plate. If the number of CFU in the test substance is less than or equal to the number of CFU in the water reference, then the concentration was considered MIC [62]. The concentrations ranging from 0.312 to 20 mg/mL were used in determining the MIC of test substances. In this experiment, ciprofloxacin was used as a positive control, whose two-fold dilutions of 2.5–40 µg/mL were employed. At least five replicates of each concentration of test or control samples were employed. The same bacterial strains were also used to find bacterial growth inhibition by adopting the reported method [62], with details presented in Supplementary Data.

2.5. Mosquito Rearing

Ae. aegypti colony was maintained under laboratory conditions as described earlier [42,63]. Briefly, *Ae. aegypti* eggs were added in distilled water maintained in a climate chamber set at 25 ± 2 °C and 80 ± 10% relative humidity at the photoperiod 12 h:12 h light: dark. The hatched larvae were fed with a fish diet (Osaka green fish food, Chennai, India). The larvae were observed daily, and the emerged pupae were transferred to a separate plastic container containing distilled water. The container was placed in the Plexiglas mosquito cages till the emergence of adults. Cotton soaked with 10% sucrose solution was placed in adult mosquito cages to provide food for adult mosquitoes. The mated female (4–5 days old) mosquitoes were fed with the blood of an immobilized pigeon. The polypropylene jars (200 mL) filled with distilled water and lined with wax paper were placed in each adult cage as egg-laying media. The eggs were shifted to fresh distilled water in a tray for hatching. The procedure was repeated until the number of adult mosquitoes was sufficient for mosquito repellency bioassays.

2.6. Mosquito Repellency Bioassay

The repellent activity of citrus peel EOs was investigated against adult female *Ae. aegypti* by using the human bait method previously described [42,50]. Briefly, 3–4 days old and blood-starved 20 female mosquitoes were released in a separate experimental

cage. Before the experiment, the volunteer hands were washed with fragrance-free soap and dried in the air. The volunteer wore gloves on both hands that covered the entire hand and arm except for a circular area of 30 cm^2 on the dorsal side of both hands. An aliquot of 100 µL solution of negative control (ethanol solvent) or test substance (1% or 5% *w*/*v*) solution was evenly applied to the exposed area of the hand. In this way, the concentration of pure EO on the test hand was 33.3 µg/cm^2 or 166 µg/cm^2. The solvent was evaporated for 3 min in the air before starting the repellency bioassay. The hand was exposed to female mosquitoes in an experimental cage for 5 min, and the number of mosquitoes' successful landings was counted on the negative control or sample-treated hand. To check the repellent persistence, the bioassay was carried out in the same way described above, except using the same treated hand after each 15 min period and counting females' landings for 5 min until the number of mosquito landings on control and treated hands became equal. The human subjects (3 volunteers) were informed about the test procedure, and informed consent was obtained before conducting repellency bioassays. Moreover, permission for human subjects use was obtained from the Ethical and Biosafety Committee of Bahauddin Zakariya University, Multan. The experiment was repeated five times for each test or control substance, and fresh mosquitoes were used in each replicate. The percent repellency was calculated using the formula: % Repellency = $[(M_c - M_t)/M_c] \times 100$, where M_c is the number of mosquito landings on the negative control and M_t is the number of mosquito landings on the test substance-treated hand.

2.7. Statistical Analysis

To determine the statistical difference between CFU percent inhibition (Supplementary Data) and the repellent effect of EO samples, the data were analyzed by one-way ANOVA with a post-hoc Bonferroni test. The statistical tests were performed using the computer software SPSS 20 (IBM, Armonk, NY, USA).

3. Results

3.1. Percentage Yield of EOs from Citrus Fruit Peels

Fresh fruit peels of different citrus cultivars produced 0.05–0.38% of EO. The fresh peels of *C. jambhiri* and *C. reticulata* yielded the highest quantity of EO, whereas *C. limon* peel yielded the least amount of EO compared to all other citrus cultivar samples (Table 1).

3.2. Chemical Composition of EOs

Limonene was the most abundant compound in the EOs of all citrus samples, composing over 90% of the oil content except *C. limon* EO, which comprised 56% of this monoterpene (Table 2, Figure S1). β-Myrcene was also found in the EOs of all citrus samples, and its proportion ranged from 0.8 to 2.7% (Table 2). β-Pinene composed 20.2% of *C. limon* EO, and its relative abundance was significantly higher than those determined in the EOs of all other samples (Table 2, Figure 1).

The chiral analysis of limonene present in different cultivar peel EOs showed that all citrus cultivars consisted of about 99% of (R)-(+)-limonene except *C. limon* EO, which contained 96.91% (R)-(+)-limonene and 3.09% (S)-(-)-limonene (Table 3).

Table 2. Chemical composition of citrus peel EOs.

Identified Compounds	RI	RL	SO	BM	SM	DL	GA	FE	KF	KD
α-Pinene	927	0.4 [1]	0.6	0.5	0.5	2.7	0.6	0.4	0.4	0.7
Camphene	942					0.3				
Sabinene	969	0.5	0.3	0.2	0.2	1.2	2.5	0.2	0.1	0.2
β-Pinene	972	0.2	0.1	tr	tr	20.2	0.4	0.1		tr
β-Myrcene	988	2.3	1.8	1.7	1.8	0.8	2.7	2.2	1.5	2.1
α-Phellandrene	1002	0.1	0.2	0.2	0.3	0.1	0.2	0.1	0.9	0.7
3-Carene	1008		tr	0.3	0.4	0.1				
α-Terpinene	1016		tr	0.1	0.1		0.1	tr		tr
p-Cymene	1021					8.3				
Limonene	1032	94.5	96.1	95.3	93.3	56.2	91.5	96.4	96.7	95.2
cis-β-Ocimene	1035					0.1				
trans-β-Ocimene	1047	0.1	0.1			0.2		0.1		tr
γ-Terpinene	1058	tr	tr	tr	0.1	1.6		tr		tr
Terpinolene	1088	0.1	tr	0.3	0.2	0.7	0.1	0.1	tr	tr
Linalool	1099	0.4	0.1	0.5	2.1	0.2	0.2	0.1		0.1
Nonanal	1104		0.1							0.1
Chrysanthenone	1106			0.2						
β-Citronellal	1153						0.1			tr
Terpinene-4-ol	1179	0.6	0.2			1.3	0.3	tr		tr
α-Terpineol	1192	0.3				1.6	0.1	tr		tr
Decanal	1201		tr							0.2
Carveol	1230					0.5				
α-Citral	1270					0.6				
δ-Elemene	1342					tr	0.1			
Copaene	1381		0.1							tr
trans-β-Caryophyllene	1426	0.1	tr	tr		0.4	0.1	tr		
trans-α-Bergamotene	1440					0.8	0.3			
Valencene	1500			0.6	0.4	tr		0.2		
β-Bisabolene	1514					1.3	0.4			
Spathulenol						0.2				

RI—retention index was determined using a DB-5 GC column; [1] value is percent; tr—traces; RL—*Citrus jambhiri*, SO—*Citrus aurantium*; BM—*Citrus sinensis* var. Malta, cv. Blood Malta; SM—*Citrus sinensis* var. Malta, cv. Shakri Malta; DL—*Citrus limon*; GA—*Citrus pseudolimon*; FE—*Citrus reticulata* var. Mandarin cv. Feutrell's early; KF—*Citrus reticulata* var. Mandarin cv. Kinnow (from fresh peel); KD—*Citrus reticulata* var. Mandarin cv. Kinnow (from dried peel).

Table 3. The relative abundance of limonene enantiomers in citrus peel essential oils.

EO Types	Enantiomeric Composition %	
	(R)-(+)-Limonene	(S)-(-)-Limonene
RL	99.51	0.49
SO	99.56	0.44
BM	99.51	0.49
SM	99.46	0.54
DL	96.91	3.09
GA	99.52	0.48
FE	99.36	0.64
KF	99.35	0.65
KD	99.33	0.67

RL—*Citrus jambhiri*, SO—*Citrus aurantium*; BM—*Citrus sinensis* var. Malta, cv. Blood Malta; SM—*Citrus sinensis* var. Malta, cv. Shakri Malta; DL—*Citrus limon*; GA—*Citrus pseudolimon*; FE—*Citrus reticulata* var. Mandarin cv. Feutrell's early; KF—*Citrus reticulata* var. Mandarin cv. Kinnow (from fresh peel); KD—*Citrus reticulata* var. Mandarin cv. Kinnow (from dried peel).

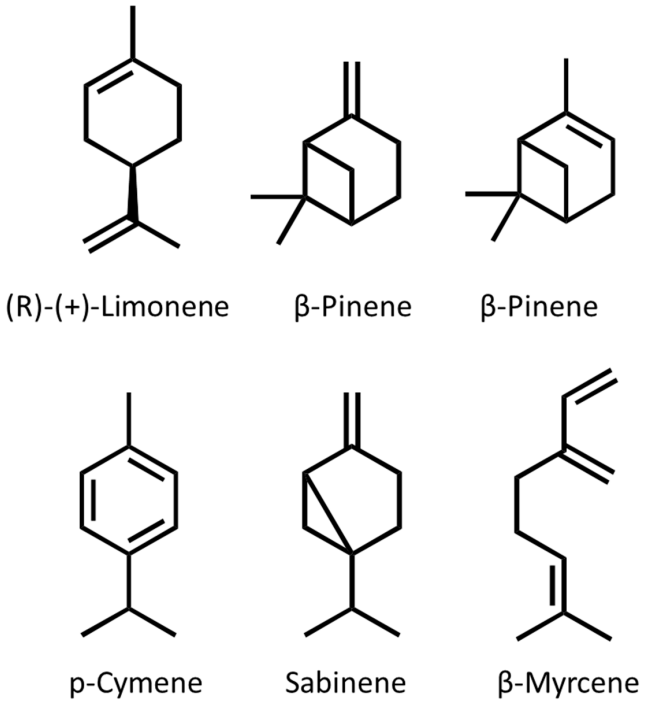

Figure 1. The chemical structures of components constitute over 2% of essential oils of citrus peels.

3.3. Antibacterial Activity of EOs

All citrus peel EOs showed antibacterial activity with varying degrees against tested bacterial strains. Among all citrus peel EOs, the *C. limon* EO was the most active, with MIC values ranging from 0.312 to 0.625 mg/mL against all bacteria except PAO1, against which this EO showed MIC 1.25 mg/mL. The EOs of *C. reticulata* KF and KD showed moderate activity with MIC values of 1.25 and 2.5 mg/mL against *E. coli*, whereas 2.5 and 1.25 mg/mL against *S. aureus*, respectively, both these EOs exhibited MIC of 2.5 mg/mL against the PAO1 (Table 4). EOs of *C. sinensis* BM and SM, *C. pseudolimon,* and *C. reticulata* FE showed the least activity against all tested pathogenic bacteria (Table 4). EO of *C. limon* showed the best inhibition of colony-forming units against all four bacteria species, i.e., *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (PAO1) compared to other EOs tested (Figure S2).

Table 4. The minimum inhibitory concentration (MIC) of different citrus peel EOs and Ciprofloxacin.

Sample	MIC (mg/mL)			
	Gram Positive		Gram Negative	
	B. subtilis	*S. aureus*	*E. coli*	*P. aeruginosa* (*PAO1*)
RL	10	20	5	10
SO	5	2.5	5	10
BM	20	20	5	20
SM	20	20	5	20
DL	0.625	0.312	0.625	1.25
GA	20	10	5	20
FE	20	10	5	20
KF	10	2.5	1.25	2.5
KD	5	1.25	2.5	2.5
Ciprofloxacin	0.01	0.01	0.005	0.02

RL—*Citrus jambhiri*, SO—*Citrus aurantium*; BM—*Citrus sinensis* var. Malta, cv. Blood Malta; SM—*Citrus sinensis* var. Malta, cv. Shakri Malta; DL—*Citrus limon*; GA—*Citrus pseudolimon*; FE—*Citrus reticulata* var. Mandarin cv. Feutrell's early; KF—*Citrus reticulata* var. Mandarin cv. Kinnow (from fresh peel); KD—*Citrus reticulata* var. Mandarin cv. Kinnow (from dried peel).

3.4. Repellent Activity of EOs Against Aedes aegypti Females

Mosquito repellent activity data showed that all citrus EOs showed activity against female *Ae. aegypti*. The statistical data analysis revealed that these EOs imparted significantly different bioactivity against female mosquitoes at 33.3 µg/cm^2 (df = 9, F = 171, $p < 0.0001$). Overall, *C. reticulata* KD exhibited the highest repellency, whereas *C. jambhiri* showed the least repellency among all tested EOs. After 5 min of sample application, EOs of *C. aurantium*, *C. reticulata* FE, and KD showed similar ($p > 0.05$) repellency. After the same time frame, the EO of *C. sinensis* SM peels showed 73% repellent activity that was similar ($p > 0.05$) to those of *C. aurantium* and *C. reticulata* FE but different ($p < 0.05$) from *C. reticulata* KD. The repellency of all EOs decreased over time, and after 30 min of exposure, only the EOs of *C. aurantium*, *C. sinensis* SM, and *C. reticulata* KD showed some repellency (Figure 2).

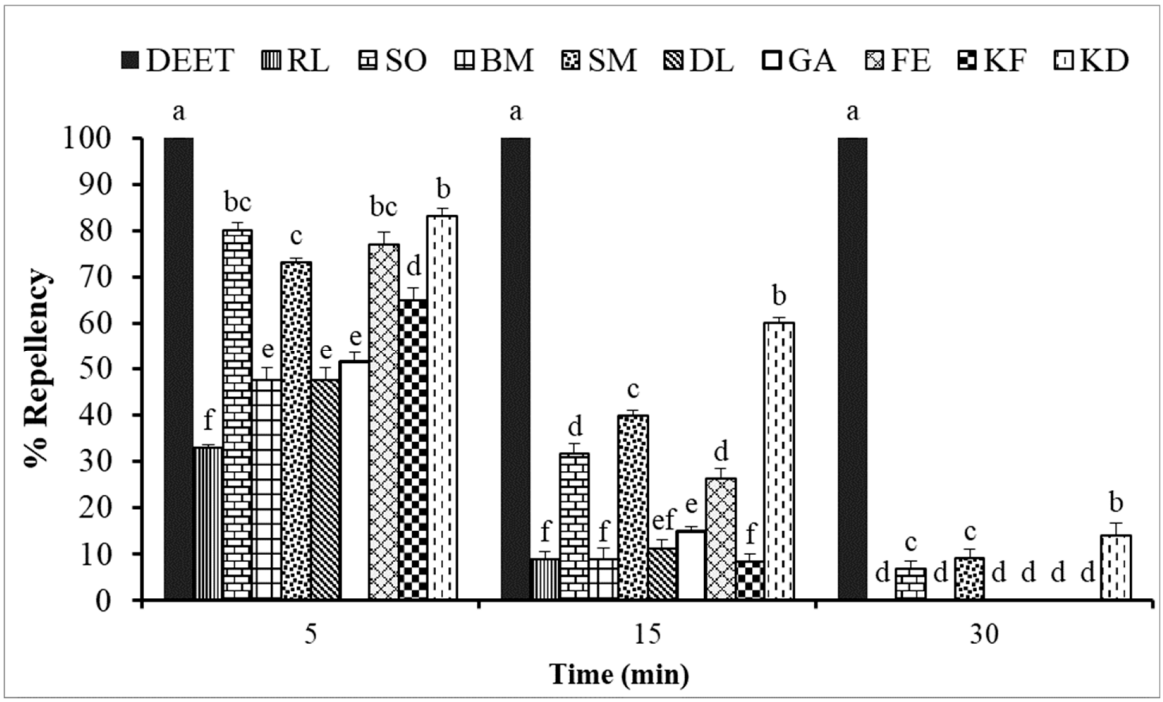

Figure 2. Repellent persistence of nine citrus EO samples and DEET tested at 33.3 µg/cm^2 against *Ae. aegypti* females. RL—*Citrus jambhiri*, SO—*Citrus aurantium*; BM—*Citrus sinensis* var. Malta, cv. Blood Malta; SM—*Citrus sinensis* var. Malta, cv. Shakri Malta; DL—*Citrus limon*; GA—*Citrus pseudolimon*; FE—*Citrus reticulata* var. Mandarin cv. Feutrell's early; KF—*Citrus reticulata* var. Mandarin cv. Kinnow (from fresh peel); KD—*Citrus reticulata* var. Mandarin cv. Kinnow (from dried peel). Bars having different letters depict significant differences ($p < 0.05$) among the repellency of test substances after different periods independently (ANOVA post-hoc Bonferroni test). Error bars denote the standard error (n = 5).

The ANOVA analysis revealed that at the higher concentration of 166 µg/cm^2, the tested EOs exhibited significantly different repellency against female mosquitoes when tested after 5 min (df = 9, F = 108, $p < 0.0001$) and 30 min (df = 9, F = 805, $p < 0.0001$). Among all tested citrus peels, EOs of *C. aurantium*, *C. sinensis* SM, *C. reticulata* FE, and *C. limon* showed 100% repellency, which was comparable ($p > 0.05$) to that of the positive control DEET (Figure 3). After 30 min of exposure, EOs of *C. aurantium*, *C. sinensis* SM, and *C. reticulata* KD showed 84%, 71%, and 56% repellency, respectively, whereas the repellency of all other samples decreased below 20%. After 75 min of exposure, only EOs of *C. aurantium* and *C. sinensis* SM displayed mosquito repellency of about 10% and 5%,

respectively, against *Ae. aegypti* females (Figure 3). The mosquito-repellent activity of *C. reticulata* KD was higher than that of *C. reticulata* KF throughout the testing period.

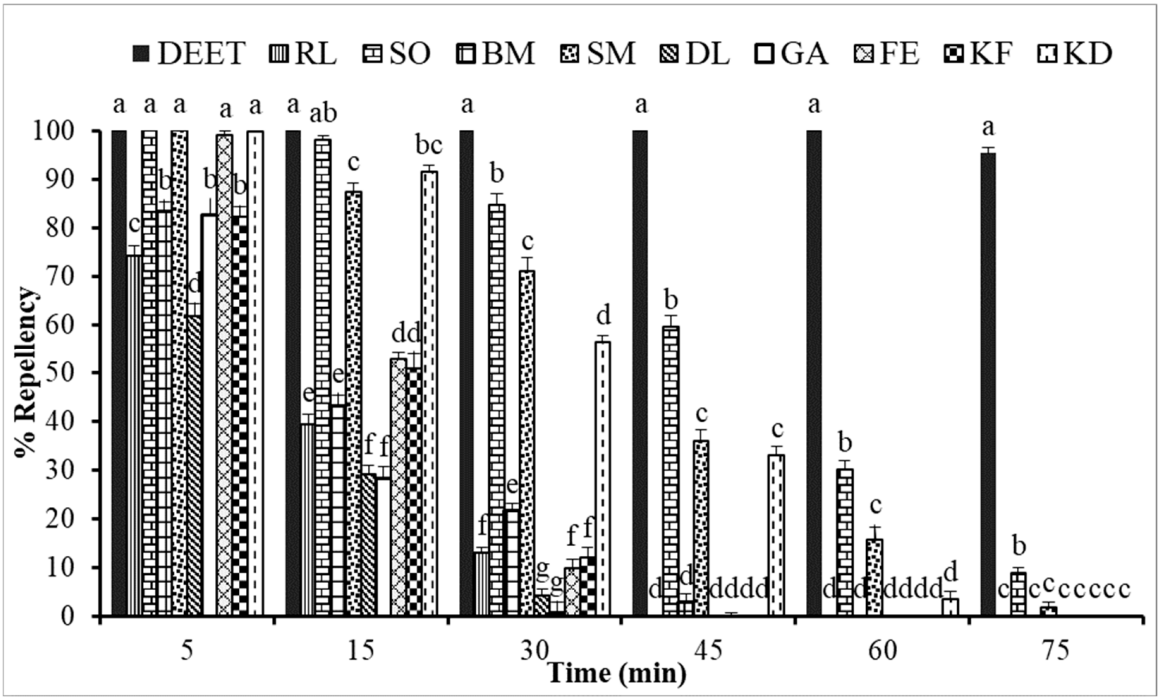

Figure 3. Repellent persistence of nine citrus EO samples and DEET tested at 166 μg/cm^2 against *Ae. aegypti* females. RL—*Citrus jambhiri*, SO—*Citrus aurantium*; BM—*Citrus sinensis* var. Malta, cv. Blood Malta; SM—*Citrus sinensis* var. Malta, cv. Shakri Malta; DL—*Citrus limon*; GA—*Citrus pseudolimon*; FE—*Citrus reticulata* var. Mandarin cv. Feutrell's early; KF—*Citrus reticulata* var. Mandarin cv. Kinnow (from fresh peel); KD—*Citrus reticulata* var. Mandarin cv. Kinnow (from dried peel). Bars having different letters depict significant differences ($p < 0.05$) among the repellency of test substances after different time periods independently (ANOVA post-hoc Bonferroni test). Error bars denote the standard error (n = 5).

4. Discussion

The yield of EOs distilled from peels of citrus cultivars was in the range of 0.05–0.38% of fresh peel mass. A previous study from Pakistan reported that *C. reticulata*, *C. paradisii*, and *C. sinensis* fresh peels produced 0.3%, 0.20%, and 0.24% of EOs [64]. Another study from Pakistan reported a 0.29% yield of *C. reticulata* peel EO [50]. An Iranian study revealed that *C. aurantium* produced 0.7% EO [65]. A recent study from Morocco reported that *C. limonum*, *C. reticulata*, and *C. paradisii* peels yielded 1.02%, 0.80%, and 0.90% of EOs, respectively [66]. In the current study and previous studies from Pakistan, large pieces of whole citrus peels were used for the extraction of EO, whereas in the study from Morocco, peel zest was utilized to extract EO, determining that the results of the current study are similar to previously reported studies from Pakistan and differ from those of Morocco. The variation in the percentage yield of EOs could be explained based on the differences in extraction method and the condition of the plant sample. Besides this, the growth conditions of plants, such as soil type, climate, and altitude, also affect the yield of an extracted EO [62,67,68].

In the current study, the major compound in the EO of *C. aurantium* peel was limonene, constituting more than 96%. A previous study from Greece reported that fresh peels of *C. aurantium* contained 94.7% limonene, 2.0% β-myrcene, and 0.7% linalool [69]. Another study from Iran described 81.6% limonene and 5.7% β-myrcene in EO of *C. aurantium* [44]. The chemical composition of *C. aurantium* determined in the current study is similar to that

described in Greece; however, it differs to some extent from that reported by the Iranian study. Our results showed that the EO of *C. jambhiri* peels comprised limonene, β-myrcene, and terpinene-4-ol. A study from Egypt reported 92.4% limonene, 1.5% β-myrcene, 0.6% sabinene, and 0.5% terpinene-4-ol in EO of *C. jambhiri* peel [45]. Another study from Sudan reported 84.5% limonene as well as sabinene, β-myrcene, and α-terpineol [70]. The chemical composition of *C. jambhiri* EO reported in the current study is similar to previously reported data. The EOs of *C. sinensis* BM and SM cultivars investigated in the current study contained limonene, β-myrcene, and linalool as main compounds. Previously, Tao et al. from China described that limonene (77.5%), β-myrcene (6.3%), α-farnesene (3.6%), and γ-terpinene (3.4%) were the major components in oven-dried sweet orange (*C. sinensis*) peels EO [71]. A recent study from Pakistan showed that limonene (95.8%), α-pinene (0.3%), and β-pinene (0.5%) were the major compounds in the EO of shade-dried C. sinensis peels [33]. Interestingly, the chemical composition of BM and SM peel EOs is very similar to each other except for the relative proportion of linalool and some other minor components. Moreover, the data of *C. sinensis* cultivars described in this study exhibited some similarities to a previous Pakistani study [33] while showing significant differences from that reported by Chinese [71] investigators.

The current investigation revealed that the EOs of *C. reticulata* cultivars were comprised of limonene, β-myrcene, and α-phellandrene. However, a study from Morocco showed that the main components of *C. reticulata* zest EO were 76.6% limonene, 2.3% β-myrcene, and 16.7% ρ-cymene [66]. A study from India reported that the EO of shade-dried C. reticulata peel contained 50.4% limonene, 3.0% β-myrcene, and 3.1% trans-carveol [72]. The data published from Bulgaria reported 85.2% limonene, 4.3% β-myrcene, and 1.3% α-pinene as the most abundant components of *C. reticulata* EO [73]. A recent study from Pakistan showed 92.7% limonene, 2.5% β-myrcene, and 1.6% sabinene, along with some minor compounds composed of *C. reticulata* peel EO [50]. The differences in the chemical composition of *C. reticulate* EOs reported in the current and previous studies carried out in Pakistan could be determined by different cultivars of this species used for the experiments as well as the growth conditions of the plants.

Besides other citrus cultivars studied, the chemical composition of *C. limon* EO was significantly different due to the presence of α-pinene, β-pinene, sabinene, limonene, γ-terpinene, terpinene-4-ol, α-terpineol, and citral. Several previous studies also reported similar chemical compositions. For example, a previous study from India identified limonene (29.0%), β-pinene (15.5%), γ-terpinene (8.6%), neral (4.2%), terpinen-4-ol (3.3%), and geranial (5.3%) as major constituents in EO of *C. limon* [74]. In 2014, Al-Jabri and Hossain [32] compared the chemical composition of Indian and Turkish lemon peel EOs. They found that the Indian lemon peel EO consisted of 53.6% limonene, 15.1% α-terpineol, 7.4% β-pinene, 4.3% α-terpinolene, and 3.6% citral, whereas in Turkish lemon peel EO there were 78.9% limonene, 5.1% β-pinene, 4.6% α-terpineol, and 0.9% citral [32]. A study from Greece reported the presence of 59.3% limonene, 13.4% β-pinene, 8.6% γ-terpinene, 3.5% β-myrcene, and 1.6% geranial in the EO of lemon [18]. The EO of *C. pseudolimon* is rarely studied for chemical composition and biological activities. The main compounds composing the EO of the cultivar Galgal were sabinene, β-myrcene, limonene, and β-bisabolene. The chemical composition of *C. pseudolimon* described in the current study is significantly different from a previous study conducted in Pakistan that reported the presence of 47.1% limonene, 10.2% eugenol, and 3.7% γ-terpinene in EO of *C. pseudolimon* extracted from peels collected from Sargodha district of Pakistan [75]. The difference in chemical composition could be due to the difference in cultivation area and the method of identification of compounds in both studies.

The enantiomeric composition analysis of limonene found in various citrus cultivars showed that (R)-(+)-limonene was the most abundant enantiomer in most of the citrus cultivars, constituting more than 99% except EO of *C. limon*, where the amount of (R)-(+)-limonene reached 97%. In literature, there are a few studies where the chemical composition of citrus EO and the enantiomeric composition of chiral compounds were described. For example, a study from Greece reported the presence of 99.2% (R)-(+)-limonene in *C. limon* EO [18], whereas a Norwegian study reported 99.9% (R)-(+)-limonene in commercial lemon oil and orange oil [76], which is significantly different from current reported data.

Overall, the tested EOs were more active against Gram-negative bacteria compared to Gram-positive bacteria. All citrus peel EOs showed antibacterial activity with varying degrees against different tested bacterial strains. Here, both cultivars of *C. sinensis* showed similar antibacterial activity against all tested bacteria. A previous study from China [71] showed that the MIC values of *C. sinensis* EO against *B. subtilis* were 9.33 µL/mL (~9.33 mg/mL), *S. aureus* 4.66 µL/mL, and *E. coli* 18.75 µL/mL. Another study from Pakistan showed that MIC values of *C. sinensis* EO against *E. coli*, *S. aureus*, and *S. agalactia* were 13.020, 10.410, and 6.510 mg/mL, respectively [33]. The MIC result of *C. senensis* cultivars investigated in the current study is similar to those previously reported in Pakistani and Chinese studies.

Our data demonstrated that among all tested citrus EOs, *C. limon* EO was the most active and showed low MIC values compared to other citrus samples. The possible reason for this difference might be the difference in the chemical composition of these EOs. In all other cultivars EOs, limonene is the most abundant compound, whereas, in the case of *C. limon*, there are several compounds, including limonene, β-pinene, *p*-cymene, and other minor compounds whose synergetic effect made *C. limon* EO comparably more active compared to all other EOs. A previous study from Morocco indicated that *C. limon* EO showed MIC values of 60 µg/mL against *S. aureus* and 750 µg/mL against *E. coli* [66]. A study from Egypt reported the MIC of *C. limon* EO against *B. cereus* (510 µg/mL), *E. coli* (260 µg/mL), *P. aeruginosa* (200 µg/mL), and *S. aureus* (430 µg/mL) using the microdilution method [77]. The antibacterial activity of *C. limon* EO, determined in the current study, differs from previously reported data. Moreover, the bioactivity of *C. limon* EOs presented in various studies also differs from that of other species. This might be due to the different chemical compositions of the EOs as well as the difference in susceptibility of bacterial strains tested in these studies.

In the current study, MIC values of *C. aurantium* peel EO were relatively lower compared to those reported in the previous study from Iran that showed 100 mg/mL MIC against *S. aureus* and 50 mg/mL against *E. coli, S. typhi*, and *B. cereus* [44]. Similarly, another study from Bulgaria described MIC in the range from 60 to >600 µg/mL for *C. aurantium* EO against *S. aureus*, *E. coli, P. aeruginosa*, and *B. subtilis*, which is lower than that reported in the present study against *S. aureus* and *B. subtilis*, whereas similar to that of *E. coli* and *P. aeruginosa* [73]. The difference in the bioactivity of different EO samples could be due to the difference in their chemistry as well as the varied susceptibility of microbial strains.

Among the EOs of three *C. reticulata* cultivars, the EO of the sample KD was the most active, whereas the EO of the FE cultivar possessed the least activity against all tested bacteria. A recent study from India reported 1250 µg/mL MIC values of *C. reticulata* EO against *E. coli* and *S. aureus* [78], which is similar to the bioactivity of EOs derived from the KF and KD cultivars determined in the current study. A study from Spain reported 1000 µg/mL MIC of *C. reticulata* EO against *S. aureus* and 5000 µg/mL against *E. coli and P. aeruginosa* [79]. The *P. aeruginosa (PAO1)* strain studied here is quite resistant under natural conditions due to its ability to biofilm formation [80]. Despite that, the EOs of *C. reticulata* inhibited the growth of the *P. aeruginosa* (PAO1) strain even at moderate

concentrations. Though the major compound in all *C. reticulata* cultivars was the same, however, the synergistic effect of minor compounds could be the reason for variations in their biological activity.

The effect of EOs on different pathogenic microbes includes degradation of a cytoplasmic membrane, destruction of the cell wall of pathogenic bacteria, destruction of membrane proteins, coagulation of the cytoplasm, and enhanced permeability of the cell membrane that causes the outflow of the essential cellular ingredients, decreasing the proton motive force or pressure and the cellular ATP by reducing the energy synthesis [81–83]. Though studies explained diverse types of mechanisms of action of essential oils on bacterial cells, most studies showed that essential oils are capable of degrading bacterial cell walls and causing damage to the bacterial cell structure [14,15,81,84] that leads to increased permeability due to the non-separable nature of EOs from the bacterial cell wall. In several studies, limonene was reported to destroy the cell morphology and structure by affecting the cell membrane [14–16], whereas a study reported a similar effect of *C. medica* EO on bacterial cells [85].

Interestingly, EO extracted from *C. aurantium* was among the least active EOs against bacteria; however, it showed the highest repellency against female *Ae. aegypti* that lasted for an extended period of time. The good mosquito repellent activity of *C. aurantium* EO could be due to the bouquet of monoterpenes, including limonene, occurring at a higher proportion compared to other EOs. To our knowledge, only a few publications reported the mosquito-repellent activity of *C. aurantium* EO. A study from India reported 50% bite protection activity of *C. aurantium* EO against *Ae. aegypti* females after four hours of application when a 1000 μg/cm^2 dose was tested [51]. A study from Thailand reported that *C. aurantium* EO exhibited 10 min protection time against *Ae. aegypti* mosquito bites when approximately 330 μg/cm^2 dose was applied [86]. The repellency results of the current study are different from the Thailand study, whereas they are similar to the Adhikari et al. [51] study from India. Here at a lowest tested dose of 33.3 μg/cm^2, *C. aurantium* EO exhibited moderate repellency after 15 min, whereas when the dose increased five times to 166 μg/cm^2, the repellent longevity increased four times, indicating the importance of a higher dose for a repellent activity for an extended period.

We have studied the repellent activity of EOs derived from two *C. sinensis* cultivars, i.e., SM and BM. The mosquito-repellent activity of the SM cultivar was significantly higher than that of BM. Though EOs of both cultivars contained almost similar proportions of limonene, however, the relative abundance of linalool was higher in the SM cultivar. Kline et al. [87] demonstrated that linalool showed similar spatial repellency against *Ae. aegypti* females compared to DEET; therefore, the repellency difference between these two cultivars of *C. sinensis* could be explained by a four times larger amount of linalool in the EO of a more repellent SM cultivar. A previous study from Thailand reported a 30 min protection time of 100 μL of pure *C. sinensis* EO against *Ae. aegypti*; however, this EO lost its activity when it was combined with ethanol [88]. Another study from Thailand reported the mosquito-repellent activity of *C. sinensis* EO against *Ae. aegypti* and *Culex quinquefasciatus* at 330 μg/cm^2 dose and reported mean protection times of 20.9 and 42.8 min, respectively [86]. The repellent results of the SM cultivar seem higher than the Phasomkusolsil et al. [88] report, whereas they are comparable to those of Soonwera [86]. The repellent activity of these studies points out that EO extracted from different cultivars of *C. sinensis* cultivated at different places showed diverse bioactivities against mosquitoes due to differences in their EO chemistry.

EO extracted from *C. limon* showed the least mosquito repellency among all tested EOs. The mosquito repellency of *C. limon* reported here is similar to a reported study from India where *C. limon* EO exhibited only 27% repellency when a 1000 μg/cm^2 dose was

tested after 4 h of application [51]. A study from Iran reported 71.1% mosquito repellency of *C. limon* while testing 1% EO solution against *Anopheles stephensi* [52]. A study from Greece showed moderate repellency of *C. limon* EO against *Ae. albopictus* when 0.2 µL/cm^2 (~200 µg/cm^2) dose was applied [18]. The repellent activity of *C. limon* studied here is in accordance with the reported data. Interestingly, the EO of *C. limon* was the most active against pathogenic bacteria but showed the least mosquito repellent activity compared to its counterpart EOs.

Among three samples of *C. recticulata* EOs, KD exhibited excellent mosquito repellency for about 60 min, whereas the other two EO samples, i.e., KF and FE, showed repellency for a shorter period of time against *Ae. aegypti* females. Interestingly, KF and KD were extracted from Kinnow peel collected from the same orchard, and the only difference between them was the condition of the peel, fresh or dried, which significantly changed the mosquito repellency of these two EOs. Effiom et al. [53] from Nigeria reported that *C. reticulta* peel extract did not show any repellency when 5–10% solution was applied, but the repellent longevity increased to 5 h when 25% solution was tested. A previous study from Pakistan reported mosquito repellent activity of fresh *C. reticulata* peel EO and demonstrated repellency for more than 90 min [50] at a similar dose used in the current study. The repellency of fresh Kinnow peel EO reported here is lower than previous Pakistani reported data, whereas it is higher than a Nigerian study. The difference in bioactivity could be explained based on the difference in the chemical composition of EOs reported in studies.

The mosquito-repellent activity of *C. pseudolimon* and *C. jambhiri* was similar, though the chemical composition of these two EOs was qualitatively the same, but the relative proportion of some minor components differed a little. There is no previous study reporting the mosquito repellency activity of *C. pseudolimon* and *C. jambhiri* EOs. Moreover, a previous study reported good insecticidal activity of *C. jambhiri* EO against stored grain beetle, *Tribolium castaneum*, when a 27 µL/L dose was tested in a fumigation bioassay [89].

5. Conclusions

Limonene was the most abundant compound, and (R)-(+)-limonene was the most abundant enantiomer in all EO samples. *C. limon* EO exhibited the highest antibacterial activity against tested bacterial strains, whereas 5% solutions of C. *aurantium* and *Citrus sinensis* var. Malta cv. Shakri Malta peel EOs exhibited 60% and 35% mosquito repellent activity against *Ae. aegypti* females for 45 min compared to DEET, which showed about 90% repellency for 75 min. The results showed that EOs extracted from *C. aurantium* and *Citrus sinensis* var. Malta cv. Shakri Malta peel has the potential to be used for formulating plant-based mosquito repellent.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11010009/s1, Figure S1: Citrus peel essential oils chromatograms; Figure S2: Percent bacterial growth inhibition with respect to negative control (DMSO) of different citrus peels essential oils against (a) *Bacillus subtilis*; (b) *Staphylococcus aureus*; (c) *Escherichia coli*; (d) *Pseudomonas aeruginosa* (PAO1). References [84,90] are cited in supplementary file.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical and Biosafety Committee of Bahauddin Zakariya University, Multan (protocol code No. 04 lURECl2022, approval date 21 September 2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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