Essential Oils from Citrus Fruit Peels to Control Foodborne Bacteria in Fresh-cut Guava Fruits

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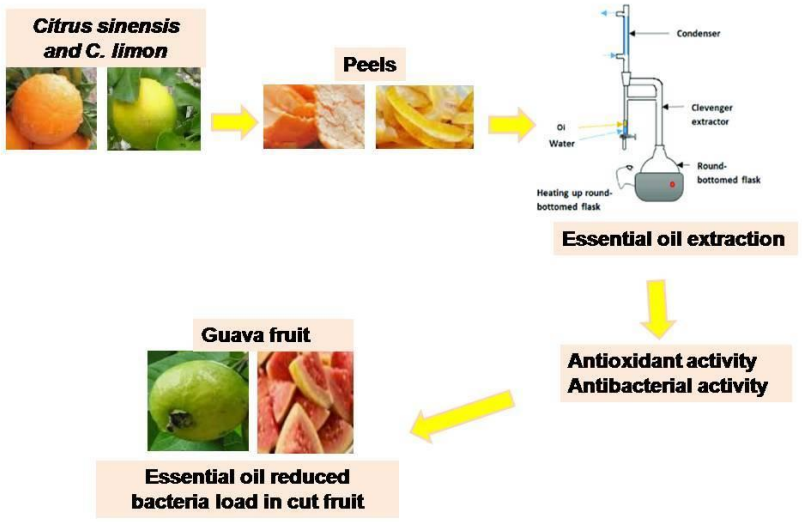
GRAPHICAL ABSTRACT



Essential Oils from Citrus Fruit Peels to Control Foodborne Bacteria in Fresh-cut Guava Fruits

Asathurai Marithangam,[a,b] Velayuthan Pillai Sarojini,[c,*] Dunia A. Al Farraj,[d] Mohamed S. Elshikh,[d,*] and Selvaraj Arokiyaraj [e]

Peels from *Citrus sinensis* and *C. limon* were used for the preparation of essential oils. The hydrodistilled citrus peels presented various compounds, including cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)- (91.8%) and 7-methyl-3-methylene-1,6-octadiene(3.40%). Compared with the essential oils isolated from *C. limon*, the *C. sinensis* essential oil showed maximum radical scavenging activity, with an IC_{50} value of 4.31 µg/mL. *Bacillus subtilis* growth was generally inhibited by essential oils, and the zone of inhibition was 21 ± 1 mm, while the zone of inhibition was 20 ± 2 mm against *Escherichia coli.* The minimum inhibitory concentration ranged from 12 ± 1 to 128 ± 2.6 µg/mL. Similarly, essential oils presented lower minimum bactericidal concentrations against *Bacillus subtilis*, followed by *Escherichia coli*. The antimicrobial activity was tested using packed samples of fresh-cut guava fruit stored under refrigeration. The essential oil-treated guava fruit presented a decreased viable cell count. After 2 days of *C. sinensis* and *C. limon* essential oil treatment, the reduction in *B. subtilis* was approximately 1.7 log CFU/g compared with that of the control. In cut fruits treated with *L. monocytogenes*, the essential oils significantly reduced the bacterial population, and a 7 log CFU/g reduction was achieved after 8 days of treatment ($p<0.05$).

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Keywords: Citrus peels; Essential oil; Antioxidant; Antibacterial; Food storage

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INTRODUCTION

Approximately 100 million tons of several types of citrus fruits are used in orange juice production, and >50 million tons of citrus peel waste are generated annually (Anwar *et al*. 2008). Orange peels are rich in various bioactive compounds, including phenolic compounds, pectin, and antioxidants (Ghasemi *et al*. 2009). They are also rich sources of flavonoids that have several biological properties, including anti-inflammatory, antitumour, and antibacterial properties, with protection against various diseases, including heart diseases (García-Lafuente *et al*. 2009; Boukroufa*et al*. 2015; Chagas *et al*. 2022). The major flavonoids in citrus are hesperidin, narirutin, eriocitrin, and naringin (Zhang *et al*. 2015). The bioactive compounds in citrus fruits have potential antioxidant properties. Citrus fruit peel has been used for the production of animal feed, fuel production, and

bioactive compounds (Li *et al*. 2006).Generally, certain byproducts are prepared from mixtures of orange varieties such as grapefruit, lime, and orange. The annual demand for orange fruit is approximately30,000 tons, which accounts for 4% to5% of the total citrus fruit (Yeoh *et al*. 2008). Orange is one of the major citrus fruits and contributes to>60% of citrus production worldwide. In fruit processing industries, more byproducts are generated, especially peels, accounting for approximately 45% of the total mass (Farhat *et al*. 2011). The waste generated from the food industry cause several environmental problems, especially water pollution resulting from biomaterials from peels, mainly sugars, essential oils, and pectins (Bousbia *et al*. 2009; Taneja *et al*. 2023; Yadav *et al*. 2022). Valorizing these byproducts increases income and decreases environmental pollution. Citrus essential oil is an important byproduct of citrus peel and is used for the preparation of various products for several purposes, such as cosmetics, the production of perfumes, and pharmaceutical products. Moreover, the biological effects, aroma quality, and yield of citrus essential oils vary, depending on the extraction method used (Bouabdallah *et al*. 2022).

Citrus essential oils are prepared *via* distillation methods (steam distillation, hydrodistillation, and dry distillation) and nonthermal extraction methods (supercritical CO_2 fluid extraction, cold pressing, and solvent extraction) (Filly *et al*. 2016). Moreover, combined extraction methods, such as ultrasound-assisted extraction and solvent-free microwave extraction, have also been used to improve essential oil yields and flavors in citrus (Chemat *et al*. 2020). The amount of volatile compounds in essential oils is mainly based on the type of extraction method. However, hydrodistillation and cold pressing methods are considered conventional methods. During the hydrodistillation method, citrus peels are boiled with water, and the released essential oil is harvested by water evaporation (Bayramoglu *et al*. 2008).

Bacterial infections cause severe mortalities in humans and were one of the leading causes of death worldwide in 2019 (Murray *et al*. 2022). Recent analysis revealed a total of 5 bacterial pathogens that cause serious infections in humans. They include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*. These five bacterial pathogens constitute approximately 50% of the total bacterial disease caused among individuals. Compared with that in developed nations, the mortality rate has been high in poor countries due to infectious diseases (Murray *et al*. 2022). The essential oils extracted from citrus fruit peels contain several antimicrobial and volatile monoterpenes and other nonvolatile compounds that are rich in antimicrobial, anticancer, and antioxidant activities (Bora *et al*. 2020). Essential oils are rich in terpenes, flavonoids, coumarins, and carotenes, which have various bioactive properties and are responsible for antimicrobial effects, showing their potential against drug-resistant organisms (Lawani *et al*. 2023). The emergence of new bacterial variants that are multiresistant to available antibacterial agents is associated with increased mortality and morbidity due to the high cost of new generation antibiotics and their nonavailability to poor people (Murray *et al*. 2022). Therefore, there is an urgent need to explore effective, inexpensive, and plant-based antibacterial agents to combat drug-resistant bacterial diseases in developing countries.

In addition, food spoilage is a serious issue worldwide. Food spoilage bacteria are involved in disease outbreaks, resulting in death and illness. Fresh-cut fruits can function as vehicles for transmitting various pathogenic bacteria, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp., and *Escherichia coli*. Listeriosis outbreaks have been reported in several countries, and most of the outbreaks were associated with fruits

such as caramel apples, melons, plums, and nectarines (Akinsemolu and Onyeaka 2024). Because of their high water content, fresh-cut fruits favor the growth of several spoilage bacteria. The natural products used to control decay and improve the shelf-life of foods, including essential oils, have received much more attention in recent years. Limonene is widely used in the cosmetic industry as well as for the preparation of pharmaceutical agents. Limonene has low toxicity, but it has been identified as a serious skin contact allergen. The widespread application of D-limonene in detergents, foods, and cosmetics effectively increases the risk of skin allergies in various countries (Matura *et al*. 2002). Exposure of D-limonene to dermal tissues resulted in a purpuric rash or irritation on the skin, and the level of irritation depends on the degree of oxidation of D-limonene (Api *et al*. 2022). In experimental rats, D-limonene showed respiratory sensitization, carcinogenicity, and nephrotoxicity in rats (Ravichandran *et al.* 2018). Humans lack α_{2u}-globulin proteins; hence, the toxic effect of D-limonene to rats are not applicable to humans (Kim *et al*. 2013).Essential oils are broadly recognized as safe for human health and the environment. The antimicrobial properties of essential oils make them suitable alternatives to replace synthetic preservatives. Essential oils (EO) extracted from plants have been applied as food additives to extend the shelf life of foods and improve taste by preventing rancidity and affecting microbial growth (Atif *et al*. 2020; Al-Ansari *et al*. 2021). Indeed, these EOs can affect the development of pathogenic bacteria because of their primary phenolic compounds and secondary materials, terpenes, aliphatic alcohols, iso-flavonoids, ketones, acids, and aldehydes (Singh 2018). These EOs are generally considered safe and can be considered effective food-preserving agents. EOs consist of various bioactive compounds, including oxygenated terpenoids, and hydrocarbons, such as monoterpene, aliphatic, and sesquiterpene hydrocarbons and these compounds have shown antimicrobial activities (Bassolé and Juliani 2012). Pinto *et al*. (2011) reported that supplemented EOs affected the permeability of the cell membrane of bacteria, causing cell wall destruction, cell content leakage and cell structure disruption. In *Citrus* fruits, d-limonene is a major compound, and geraniol, linalool, and nerolare compounds have been found to exhibit antimicrobial activity against food-borne pathogens (Geraci *et al*. 2017).The bioactive properties of EOs varied based on orange varieties and chemical compositions of EOs.The application of essential oils in food preservation is a suitable approach; however, the presence of aroma compounds is a limitation. Hence, the use of combinations of essential oils from different sources, because of their synergistic activity, could significantly reduce the required concentration and, subsequently, the negative impact without affecting the antibacterial activity. The objective of this work was to analyze the chemical composition of essential oils from two citrus fruits (*Citrus sinensis* and *C. limon*) and their ability to improve the shelf-life of perishable fruits.

EXPERIMENTAL

Citrus Fruits

The citrus fruits used in this study (*C. sinensis* and *C. limon*) were collected from the market. Approximately 5 kg of fresh fruits were collected, washedwith tap water, and peeled. Then orange peels were separated and further washed with double distilled water, and adhered materials were removed. Fresh peels were used for the extraction of essential oils.

Extraction of Essential Oils

Essential oils (EO) were extracted from *C. sinensis* and *C. limon via* the hydrodistillation method. One hundred grams of orange peels were mixed with 500 mL of double distilled water. These mixtures were hydrodistilled using a Clevenger apparatus for 4 h. The extraction process was monitored, and this process was extended until no supplemental EO was extracted. The collected EO was dried over anhydrous sodium sulfate and stored at 4 °C until use (Atif *et al*. 2020).

Gas Chromatography and Mass Spectrophotometry Analysis

The chemical compounds present in the hydrodistilled EO were analyzed *via* gas chromatography coupled with mass spectrometry(Thermo Scientific, Waltham, MA, USA). Helium was used as the carrier gas, and the flow rate was 1.0 mL/min. The temperature of the column was set at 40 °C for 8 min, the temperature was increased to 180 °C at a rate of 3 °C/min, and the temperature was increased to 230 °C at 20 °C/min. The mass spectra of the separated compounds were detected, and the results were compared with the National Institute of Standards and Technology-Mass Spectra libraries (Al-Ansari *et al*. 2021).

Physical Properties of Essential Oils

The extracted EO was subjected to physical and chemical property analysis. The specific gravity, refractive index, solubility in 95% ethanol, and optical rotation were analyzed at 28 ± 1 °C.

Antioxidant Activity

The antioxidant activities of *C. sinensis* and *C. limon* were determined through free radical assay using 1-diphenyl-2-picrylhydrazyl (DPPH) reagent. The EO was diluted in ethanol (2 mg/mL) and used for the antioxidant assay. It was mixed with 1.0 mL of DPPH solution (0.004%) prepared in ethanol. The mixture was incubated for 30 min at room temperature in darkness, and the absorbance was read at 517 nm (Zhang *et al*. 2020)using a UV-visible spectrophotometer (Thermo Fisher Scientific, New York, USA). The percentage antioxidant activity was calculated *via* Eq. 1,

$$\mathrm{AA}(\%) = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \quad (1)$$

where A_0 denotes the absorbance of the blank and $A_1$is the absorbance of the sample. Ascorbic acid was used as a positive control.

Determination of Antioxidant Compounds from the Fruit Peels

Total phenolic compounds

The total phenolic content of the EO was determined as described previously (Ahmed *et al*. 2019). Briefly, 100 µL of EO was mixed with 250 µL of Folin-Ciocalteu reagent (1 N). The mixture was incubated for 5 min, and 1.25 mL of aqueous sodium carbonate solution (20%) was added. The color development was observed for 30 min, and the absorbance was read at 725 nm against a reagent blank. The content of phenolic compounds in the essential oil was analyzed based on a gallic acid standard curve, and the result was expressed as mg of gallic acid equivalent (GAE)/g of essential oil.

Total flavonoid contents

The total flavonoid content of the EO was tested *via* the spectrophotometric method using aluminum chloride. Briefly, 100 µL of EO was mixed with 600 µL of 5% sodium nitrite solution and incubated for 5 min. Then, aluminum chloride solution (600 µL, 10%) was added. The volume of the solution was adjusted to 2.5 mL using double distilled water and incubated for 6 min. Then, 3 mL of NaOH (1 M) solution was added, and the mixture was centrifuged at 4000 ×*g* for 10 min. The absorbance of the clear solution was read at 510 nm, and the result was compared with that of the blank solution. The flavonoid content in the EO was analyzed with catechin as a standard. The results are expressed as mg of catechin equivalent/g of sample (mg CE/g).

Antibacterial Activity of Essential Oils

The antibacterial activity of the EO was analyzed *via* the disc diffusion method. Briefly, 0.1 mL of bacterial suspension (6×10^5 colony-forming units/mL) was spread on Mueller–Hinton agar medium after solidification on a Petri dish. The selected pathogenic strains were *Staphylococcus aureus, Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*. Then, 50 µL of EO was loaded on a sterile disc (6-mm diameter), placed on the solid medium surface, and incubated for 24 h at 32 ± 1 °C. To determine the synergistic activity of the EO, 25 µL of essential oils from both sources (*C. sinensis* and *C. limon*) were mixed and assayed. The zone of inhibition was expressed in millimeters (mm) (Baazeem *et al*. 2021).

Minimum Inhibitory Concentration (MIC)

A microdilution method was used to determine the MIC values of the essential oils. Briefly, 200 µL of Mueller–Hinton broth medium was loaded in a 96-well plate, 10 µL of bacterial inoculum was added, and the final concentration of the culture was 10^6 CFU/mL. Individual and combined EO were subsequently diluted from 512 to 0.250 µg/mL. To the negative control, essential oils were not added. The microtiter plates were incubated for 24 h at 37 °C. The MIC value was defined as the minimum amount of EO that inhibited the visible growth of bacteria, and the optical density was measured at 600 nm. The results are expressed as the means ± standard deviations.

Effect of Aflatoxin Reduction Potential of Essential Oils in Liquid Culture

A fungal strain, *Aspergillus flavus* (MTCC 2798), was inoculated in an Erlenmeyer flask containing 50 mL of liquid medium. The culture media contained yeast extract sucrose. To the positive control, a mixture of EO prepared from *C. sinensis* and *C. limon*, about 1% solution, was introduced. The Erlenmeyer flasks were incubated for 8 days at 30 ± 1 °C. After 8 days, the fungal mycelia were filtered through Whatman no. 1 filter paper and dried at 80 °C until a constant weight was achieved. The cell-free extract was subjected to determination of mycotoxins. The cell-free extract (20 mL) was mixed with 20 mL of chloroform. The mixture was shaken vigorously and then left undisturbed for 10 min. The lower phase of the mixture was dried and evaporated under nitrogen. It was dissolved in acetonitrile, and 0.1 mL of solution was mixed with 10 mL of double distilled water. The aflatoxin in the extract was purified using an Afla-test immune affinity column. Approximately 2.0 mL of sample was loaded and washed with double distilled water (two volumes). Then, methanol was added, and aflatoxin was eluted at 0.5 mL/min. The contents of aflatoxins (aflatoxin B and G) in the medium were analyzed *via* high-performance liquid

chromatography (Agilent 1100; Agilent Technologies, Santa Clara, CA, USA) (Rammanee and Hongpattarakere 2011).

Analysis of the Antibacterial Activity of Essential Oils on Cut Fruits

The antimicrobial activity upon using a combination of EO was tested in packed samples of fresh-cut guava fruit. A total of 10 µL (4×10^6 CFU/mL) of bacterial suspension (*B. subtilis*, *E. coli,* and *L. monocytogenes*) was individually mixed with 25 mL of sterile double distilled water, and it was used as inoculum. The surface of the fresh guava fruit was washed with sterile double distilled water, and it was cut into several pieces (40±2 g). The cut fruit was dipped in inoculums and incubated for 10 min (immersion treatment). It was kept in laminar air chamber and air dried. It was subsequently submerged in 1% essential oil prepared in 1% sucrose containing double distilled water. For the control, EO treatment was not treated but inoculated with bacterial suspension (*B. subtilis*, *E. coli,* and *L. monocytogenes*). The fruits were aseptically packed in plastic bags and stored at 4 °C, and the microbial load was determined frequently (2 to 10 days). The stored bags were cut open, and a 10 g sample was ground with 99 mL of peptone water. It was homogenized and used as the sample. To determine the bacterial load, the sample was poured into nutrient broth medium and counted directly. The mixture was incubated for 48 h at 37 °C, and the viable cells were expressed as CFU/g (Alfarhan *et al*. 2024; Gao *et al*. 2024).

Statistical Analysis

The analysis of data was performed using one-way analysis of variance (ANOVA), and a p-value ≤0.05 was considered statistically significant. A statistical Package for Social Sciences (SPSS, IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA) was used for data processing.

RESULTS AND DISCUSSION

Essential oil Yield

The yield of *C. sinensis* and *C. limon* EO was 4.02% and 3.29%, respectively. The yield obtained in this study was higher than in a few previous reports. A cold press extraction method was used to extract citrus EO, and 0.5% yield was obtained (Pultrini *et al*. 2006). A hydrodistillation method showed a 0.6% EO yield from the citrus peel (Ezejiofor *et al*. 2011). Sharma and Tripathi (2008) extracted EO from Indian sweet orange Osbeck epicarp using the hydro-distillation method and the yield was about 1.8%. Bustamante *et al*. (2016) extracted EO from Valencia late orange peels using the hydro-distillation method and the yield was a little higher (2.3%) than these earlier reports. The EO yield obtained in the present study was little lower than the report of Palazzolo *et al*. (2013) and achieved 4.40%, and 3.47% yield after steam distillation, and water distillation, respectively.

Chemical Composition of *C. sinensis and C. limon* Essential Oil

A total of 21 volatile compounds were determined from the EO of *C. sinensis* (Table 1). The major constituents were found to becyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)- (91.83%), 7-methyl-3-methylene-1,6-octadiene(3.402%), 1-caprylaldehyde(0.521%), (1S,5S)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene(0.429%), and 3,7-dimethyl-1,6-octadien-3-ol (0.392).

Table 1. Chemical Composition of Essential Oils from *C. sinensis* and *C. limon*

***C. sinensis* EO**				***C. limon* EO**			
No.	**Compound**	**Retention Time (min)**	**Composition (%)**	**No.**	**Compound**	**Retention Time (min)**	**Composition (%)**
1	(1S,5S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	11.29	0.43	1	α-Phellandrene	11	0.182
2	3-Isopropyl-6-methylene-1-cyclohexene	14.02	0.08	2	(1S,5S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene	12	4.8
3	7-Methyl-3-methylene-1,6-octadiene	15.43	3.4	3	7-Methyl-3-methylene-1,6-octadiene	14	2.94
4	Ocimene	16.06	0.04	4	n-Tetradecane	15	0.035
5	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-	18.48	91.8	5	β-Pinene	15	4.7
6	α-Terpinene	19.41	0.05	6	n-Nonyl acetate	15	*0.076*
7	Citronellal	23.05	0.08	7	Sabinene	15	1.42
8	3,7-Dimethyl-1,6-octadien-3-ol	25.02	0.39	8	p-Cymene	16	0.86
9	γ-Terpineol	27.94	0.07	9	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-	18	69.09
10	β-Citral	29.05	0.13	10	α-Terpinene	20	1.62
11	Germacrene-D	29.85	0.03	11	3,7-Dimethyl-1,6-octadien-3-ol	25	0.28
12	β-Cubebene	30.25	0.02	12	1-Methyl-4-(propan-2-yl)cyclohexa-1,4-diene	25	9.29

13	δ-Cadinene	31.49	0.03	13	Terpinen-4-ol	26	0.49
14	Valencene	32.98	0.21	14	α-Terpineol	26	0.39
15	Nonanal	33.92	0.12	15	Geraniol	27	0.11
16	*1-Caprylaldehyde*	34.59	0.52	16	Camphor	28	0.15
17	n-Dodecanal	35.04	0.03	17	Neryl acetate	29	0.11
18	β-Pinene	35.13	0	18	(E)-Caryophyllene	30	0.25
19	Camphor	36.05	0.05	19	β-Bisabolene	30	0.05
20	n-Nonanol	36.17	0.01	20	α-Trans-bergamotene	31	0.001
21	Geraniol	36.23	0.01	21	n-Nonanol	33	0.011
				22	n-Decanal	34	0.192

The other volatile compounds in the EO were present in lower amounts (<0.2%). In this study, a total of 22 chemical compounds were detected in *C. limon*, which was similar to the results of Djenane (2015) in Algerian *C. sinensis* EO. However, the reported major monoterpene hydrocarbon, cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-, was less common than in the present study (7.37%). The other detected components were 3-isopropyl-6-methylene-1-cyclohexene(1.9%), β-pinene (3.45%), 1-caprylaldehyde (1.24%), naphtalene (1.41%), linalol (1.21%), (1S,5S)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (0.76%), and decanal (0.82%). The EO extracted from *C. sinensis* presented variations in the percentages of volatile compounds. The percentage compositions of the compounds were isopiperitenone (3.58%), γ-muurolene (4.44%), citronellol (4.88%), 3,7-dimethyl-1,6-octadien-3-ol (9.73%), cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-(17.7%), and α-terpineol (35.4%) (Egharevba *et al*. 2016). The chemical profile of citrus EO has been reported, and the major contributor was monoterpenes (>90%), followed by other compounds such alcohols, aldehydes, and esters.

The chemical composition of the EO from *C. limon* was determined, and oxygenated monoterpenes contributed high amounts (>75%). Moreover, a reduced level of oxygenated compounds was detected in this study due to variations in the limonene oxidation products, such as terpinene, cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-, and phellandrene (Table 1). The results obtained in this study revealed that cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-was the major compound in the hydrodistilled essential oil from *C. limon*. Limonene (cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S), a monoterpene hydrocarbon, is a major compound (91.8%). This result agreed with previous findings on *C. limon* EO, and monoterpene hydrocarbons were reported as the major compounds. Like in the present study, cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)-was recognized as the major chemical compound (Bourgou *et al*. 2012; Ben Hsouna *et al*. 2017). Moreover, the amount of chemical compounds present varies depending on the variety of fruit, method of extraction, and maturity stage of the fruits used (Golmakani and Moayyedi 2015; Himed *et al*. 2019; Yao *et al*. 2023).

Physical Parameters of the Essential Oils

The physical parameters of the EO are shown in Table 2. The EO extracted by the hydrodistillation method had a tangy smell and was brownish-yellow in color.

Table 2. Physical Properties of *Citrus* Peel Oils

Parameters	Results	
	Citrus sinensis	*Citrus limon*
Color	Brownish yellow	Brownish yellow
Odor	Tangy smell	Tangy smell
Water solubility	Insoluble	Insoluble
Density (g/cm^3)	0.733	0.692
Specific gravity (g/cm^3)	0.857	0.816

The EO was water insoluble, the specific gravity of *C. sinensis* was 0.857 g/cm^{3,} and the oil density was 0.733 g/cm^3. In *C. limon*, the specific gravity was 0.816 g/cm^3, and the density was 0.692 g/cm^3. Similarly, the EO extracted from grapefruit is brownish yellow in color (Katsuda *et al*. 2008). The specific gravity EO extracted from *C. sinensis*

and *C. limon* was <1 and was less dense than distilled water, and these results are in agreement with previous findings (Bhuyan *et al*. 2015; Samavat *et al*. 2019). However, the physical properties of essential oils are influenced by various factors, including monoterpenes, sesquiterpenes, and their oxygenated derivatives (Singh *et al*. 2021).

Antioxidant Activity

The antioxidant activities of *C. sinensis* and *C. limon* EO were analyzed *via* the DPPH method. The DPPH scavenging potentials of the EO extracted by the hydrodistillation method revealed that the essential oils have the potential to reduce DPPH to DPPH-H based on the concentration of the EO in the reaction mixture. In the present study, the potential of essential oils to reduce DPPH free radicals was analyzed by their concentration, which provided 50% inhibition (IC_{50}). Compared with the EO isolated from *C. limon* (4.54 µg/mL), the EO isolated from *C. sinensis* showed higher radical scavenging activity, with an IC_{50} value of 4.31 µg/mL (Fig. 1).

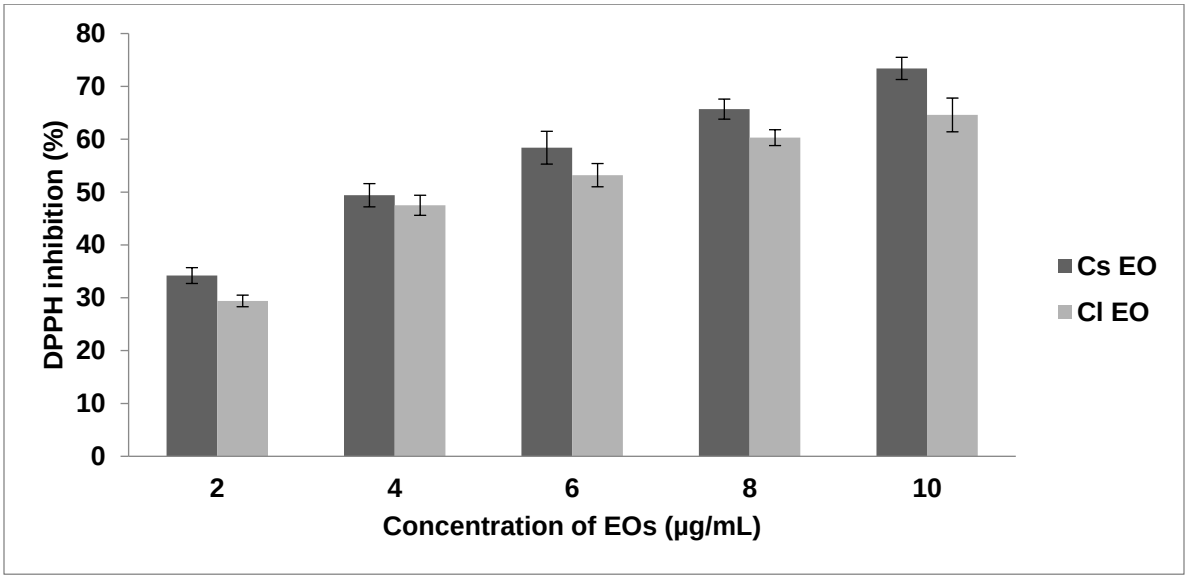


Fig. 1. Antioxidant activity of essential oils extracted from *C. sinensis* and *C. limon* fruit peels

The increased antioxidant activity of *C. sinensis* essential oil might be because this EO contains increased amounts of monoterpenes, especially limonene and 7-methyl-3-methylene-1,6-octadiene. Wei and Shibamoto (2007) previously reported the antioxidant properties of monoterpenes in essential oils. Previous studies reported the presence of flavonoid compounds, such as γ-terpinene, terpinolene, 7-methyl-3-methylene-1,6-octadiene, and β-pinene in the essential oils involved in the antioxidant activity of citrus fruits. Frassinetti *et al*. (2011) reported the essential oils of sweet orange, orange bitter, mandar, and lemon. The reported antioxidant activity of EOranges from 20% to 80%, and the concentration of EOranges from 50 to 1000 µg/mL. This variation in the previous findings can be attributed mainly to the methods of oil extraction, geographical location of the selected plant variety, and method of the antioxidant assay. In agreement with the present findings, Raspo *et al*. (2020) reported the antioxidant activity of EO extracted from Argentinian orange *via* the hydrodistillation method. Similarly, Toscano-Garibay *et al*. (2017) reported the antioxidant activity of EO from *Citrus latifolia* and *Citrus sinensis*. The extracted EO exhibited antimutagenic and antioxidant activities. Guo *et al*. (2018)

reported the antioxidant activity of EO from citrus fruits from varieties of citrus fruits, and the antioxidant activity varied widely. The antioxidant level of essential oils varies based on the source of the EO. The antioxidant EO was recently extracted from *C. sinensis* and reported to preserve food against spoilage bacteria (Al-Dhabi *et al*. 2020; Manzur *et al*. 2023).

Total Phenolic and Flavonoid Contents

The total phenolic content of *C. sinensis* EO was 40.21 ± 1.9 mg GAE/g DW, which was lower than that of *C. limon* EO (32.4 ± 2.2 mg GAE/g DW). The flavonoid content of *C. sinensis* EO was 83.2 ± 2.2 mg CE/g, which was higher than that of *C. limon* (76.5 ± 2.2 mg CE/g). Zhang *et al*. (2014) studied approximately 14 wild mandarin citrus genotypes, and the total phenolic content ranged from 29.4 to 51.1 mg GAE/g DW; the reported phenolic content was close to that in the current study. The maximum flavonoid content of the orange peel extract was similar to that reported by Shehata *et al*. (2021), and the ethanolic orange peel extract increased the flavonoid content. Different drying methods significantly affect flavonoid yield, and oven drying affects the stability of flavonoid compounds, such as diosmin, narirutin/isonaringin, and didymin/neoponcirin, in fallen citrus fruits (Kumar *et al*. 2021). The total flavonoid content of certain Chinese wild mandarins ranges between 7.95 and 20.66 mg RE/g DW in fruit peels (Zhang *et al*. 2014), which revealed that the amount of flavonoid compounds detected in this study was greater than that reported in previous studies. Previous results revealed that the variation in total flavonoid content in peels can be explained by various factors, including climate, genetic background, tree age, and cultivation system (Gil-Izquierdo *et al*. 2004; Cheng *et al*. 2009; Schmidt *et al*. 2010). The increased amounts of phenolic and flavonoid compounds detected in the citrus peel EO in this study were similar to those reported in the studies of Guimarães *et al*. (2010). The phenolic and flavonoid contents of the citrus EO analyzed in this study may be correlated with their antioxidant properties. The increased phenolic and flavonoid contents of *C. sinensis* fruit peels were correlated with increased antioxidant activity compared with those of *C. limon* fruit peel essential oils. A correlation between phenolic content and antioxidant properties has been reported in various sources, including fruits and vegetables, revealing that the free radical scavenging power varies with the total polyphenol content (Atif *et al*. 2020).

In vitro Antibacterial Activity

The antimicrobial properties of essential oils from *C. sinensis* and *C. limon* and the combination of these two essential oils were analyzed. *C. sinensis* EO affected the proliferation of both Gram-positive bacteria and Gram-negative bacteria. The results revealed that *B. subtilis*, *E. coli*, and *Salmonella typhi* were the bacterial strains most affected by orange peel essential oils, followed by *S. aureus* and *L. monocytogenes*. EO exhibited a very strong inhibition against *B. subtilis* (21 ± 1 mm) and strong inhibition (20 ± 2 mm) against *E. coli.* Similarly, *C. limon* EO affected *L. monocytogenes* growth, and the zone of inhibition was 17 ± 1 mm (Table 3). The present findings revealed that, compared with those of *C. limon*, the EO of *C. sinensis* presented significant antibacterial activity. The synergistic effects of the essential oils of *C. sinensis* and *C. limon* were tested, and synergistic effects were observed against *B. subtilis*, *L. monocytogenes*, *E. coli*, and *S. typhi*. The GC–MS analysis revealed that the essential oils derived from *C. sinensis* and *C. limon* contained several antimicrobial chemical compounds. These secondary metabolites exhibit antimicrobial properties against various food-borne pathogens. Based on the

current findings, *C. sinensis* and *C. limon* may be utilized for their antimicrobial effects (Park *et al*. 2012; Pulaj *et al*. 2016; Guo *et al*. 2018). The EOs function as plant defense mechanisms and protect against pathogenic microorganisms. The efficacy of the antimicrobial properties of the EO varies based on the type of extraction process applied, the source of the fruit peels, the type of orange peel, and the type of bacteria being tested. In addition, essential oils could be evaluated as possible bioactive compounds for treating microbial infections when combined with other preservation technologies.

Table 3. Antibacterial Activity of Essential Oils from *Citrus sinensis*, *Citrus limon*, and the Combination of *C. sinensis*, and *C. limon*

Bacteria	Antibacterial Activity (mm zone of inhibition)		
	Citrus sinensis	***Citrus limon***	**Cs+ClEssential Oil**
Staphylococcus aureus	16 ± 2[a]	14 ± 1[b]	18 ± 2[c]
Bacillus subtilis	21 ± 1[a]	15 ± 2[b]	23 ± 1[c]
Listeria monocytogenes	14 ± 1[a]	17 ± 1[b]	18 ± 1[b]
Escherichia coli	20 ± 2[a]	14 ± 1[b]	21 ± 2[a]
Pseudomonas aeruginosa	17 ± 0[a]	10 ± 0[b]	16 ± 1[a]
Salmonella typhi	19 ± 2[a]	17 ± 0[b]	21 ± 0c

Cs+Cl Essential Oil: Mixture of *Citrus sinensis* and *Citrus limon*essential oils; mm zone of inhibition: Millimeter zone of inhibition

Determination of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the essential oils (*C. sinensis* and *C. limon*) were tested, and the EO showed synergistic activity. These findings confirmed the potential antimicrobial activity against both Gram-negative and Gram-positive bacteria, as revealed by the broth dilution test (Table 4).

Table 4. MIC and MBC of Essential Oils from *C. sinensis* and *C. limon*

Bacteria	MIC (µg/mL)	MBC (µg/mL)
Staphylococcus aureus	64 ± 2.5	128 ± 2
Bacillus subtilis	12 ± 1	24 ± 2
Listeria monocytogenes	32 ± 1	64 ± 2
Escherichia coli	12 ± 1	32 ± 1
Pseudomonas aeruginosa	64 ± 1.5	128 ± 1.5
Salmonella typhi	128 ± 2.6	240 ± 1.5

The MIC values ranged from 12 ± 1 µg/mL to 128 ± 2.6. Similarly, EO presented lower MBC values against *Bacillus subtilis* (24 ± 2 µg/mL), followed by *Escherichia coli* (32 ± 1 µg/mL). The antimicrobial activity of essential oils from *Citrus* fruit has been reported previously (Bora *et al*. 2020). Yi *et al*. (2018) reported the bactericidal activity of citrus EO on *Escherichia coli*, which is moderately sensitive to citrus essential oils. In the present study, the combination of *C. sinensis* and *C. limon* EO resulted in higher activity than previously reported. In addition, the current study revealed the potential activity of

citrus EO against *B. subtilis*. Deng *et al*. (2020) reported the sensitivity of bacterial strains, such as *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, and *S. typhimurium*, to essential oils. These previous findings are in good agreement with the results of the present study, which revealed the bactericidal activity of EO against *B. subtilis* and *E. coli*. *L. monocytogenes* is a food-borne pathogen, and the essential oil extracted in the present study showed antibacterial activity, similar to previous findings. Ambrosio *et al*. (2017) reported that EO from citrus have significant bactericidal activity against bacteria such as *S. aureus*, *E. coli*, *L. monocytogenes*, and *E. faecalis*. The sensitivity of bacteria to EO varies among Gram-positive and Gram-negative bacteria.

Effect of Peel Essential Oils on Aflatoxin Produced by *A. flavus*

In this study, the effect of EOon aflatoxin biosynthesis was detected, and the results are depicted in Table 5. According to the table, the mixture of EO at 4% concentration significantly affected aflatoxin production compared to the control ($p<0.05$). The culture with 1% EOinhibited 7.24% mycelial growth, 5.4 ± 0.15% aflatoxin B, and 24.7 ± 0.15% aflatoxin G, respectively. The amount of EO improved inhibition of aflatoxin production. At 4% EO concentration, 74.8 ± 1.1% mycelial growth inhibition was achieved and 71 ± 1.18% aflatoxin G inhibition was achieved at 4% EOin the mixture. The findings show that the selected fungi were highly sensitive to the EO and this result is in agreement with earlier findings (Atanda *et al*. 2007). The mode of action of EOs on fungi was established. Essential oils affect microbial structure and denatured enzymes, which affects fungal growth and morphogenesis (Ghfir *et al*. 1997). In this study, the EO exhibited fungicidal and antiaflatoxigenic activity that was similar with recent research (Gwad *et al*. 2024).

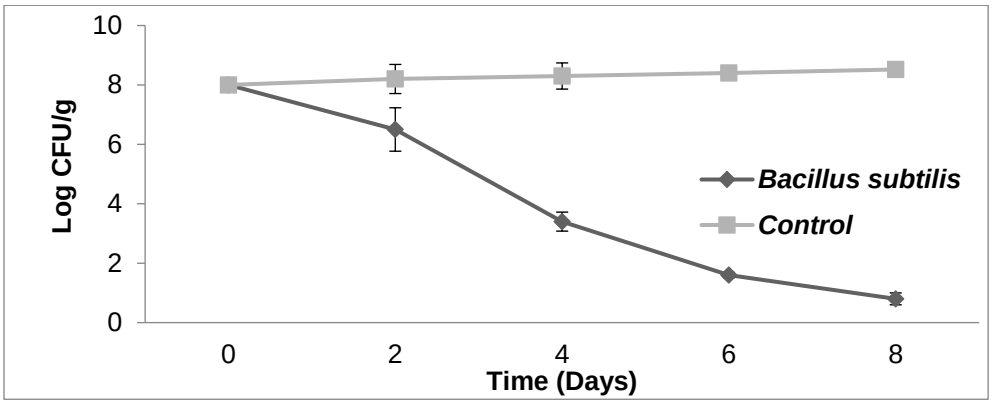
Table 5. Effect of Essential Oil on Fungal Growth Inhibition and Aflatoxin Inhibition at Various Concentrations

Essential Oil Conc. (%)	Aflatoxin B (µg/mL)	Inhibition (%)	Aflatoxin G (µg/mL)	Inhibition (%)	Fungal Hyphae	Inhibition (%)
1	18.2 ± 0.21	5.4 ± 0.15	20.5 ± 0.08	24.7 ± 0.15	3.9 ± 0.12	7.24 ± 0.17
2	13.9 ± 0.32	27.78 ± 0.11	18.7 ± 0.05	31.25 ± 0.16	2.8 ± 0.08	33.33 ± 0.83
3	10.1 ± 0.18	47.5 ± 0.13	12.5 ± 0.11	54.1 ± 0.19	1.5 ± 0.47	64.3 ± 1.9
4	6.1 ± 0.11	68.3 ± 0.68	7.9 ± 0.02	71 ± 1.18	1.08 ± 0.28	74.8 ± 1.1
5	18.2 ± 0.22	5.4 ± 0.16	20.5 ± 0.09	24.7 ± 0.16	3.9 ± 0.13	7.24 ± 0.18
Control	19.22 ± 0.21	100 ± 0	27.2 ± 0.12	100 ± 0	4.2 ± 0.59	100 ± 0

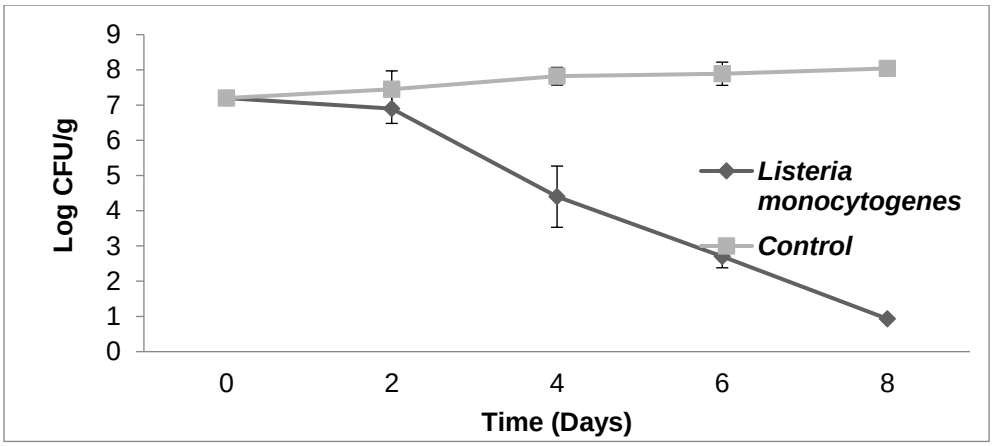
Analysis of the Antibacterial Activity of Essential Oils on Cut Fruits

The antimicrobial activity of the combination of EO was tested in packed samples of fresh guava fruit stored under refrigeration. The EO-treated guava fruit presented a decreased viable cell count after 48 h. After 2 days, for *C. sinensis* and *C. limon* EOs, the reduction in *B. subtilis* was approximately 1.7 log CFU/g, compared with that of the control (8.2 ± 0.2 log CFU/g) (Fig. 2a).

a



b



c

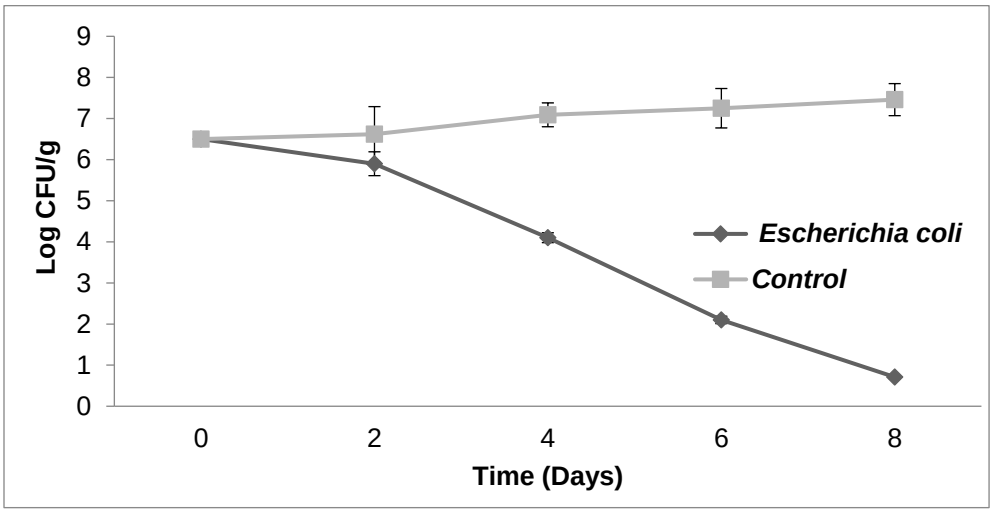


Fig. 2. Microbial viable counts (log CFU/g) of cut guava fruit treated with an essential oil mixture of *C. sinensis* and *C. limon*: (a) *B. subtilis* viable counts, (b) *L. monocytogenes* viable counts, (c) *E. coli* viable counts; 2 X MIC concentrations were selected in this study, and the results are presented as the means ± standard deviations.

In cut fruits treated with *L. monocytogenes*, the EO significantly reduced the bacterial population (0.55 log CFU/g) after 4 days, and a 7 log CFU/g reduction was achieved after 8 days of treatment (Fig. 2b). To the control fruits, substantial level of CFU/g

due to the presence of *B. subtilis*, *E. coli*, and *L. monocytogenes*. Similarly, treatment of the cut fruits with *E. coli* and the combination of essential oils resulted in a reduction of approximately 0.72 CFU/g after 2 days, and the log 6.5 CFU/g bacterial count decreased after 8 days (Fig. 2c). Fruit skin is a natural barrier used to control fruit spoilage caused by bacteria. The neutral pH value of cut fruits serves as a suitable environmental condition, thus favoring the growth of pathogenic bacteria.

In the present study, synergistic antimicrobial activity was detected among the major food-borne pathogens *L. monocytogenes* and *E. coli*. Essential oils have shown potential antibacterial activity against food-borne pathogens (Xu *et al*. 2016). The major compounds of citrus EO are limonene, 7-methyl-3-methylene-1,6-octadiene, 1-caprylaldehyde, (1S,5S)-2,6,6-trimethylbicyclo[3.1.1]hept-2-ene and 3,7-dimethyl-1,6-octadien-3-ol.These compounds have bactericidal activity, as they affect membrane function and inhibit cell wall biosynthesis and certain enzymatic activities. The EOexhibited activity against Gram-positive and Gram-negative bacteria, including *B. cereus*, *E. coli*, and *S. typhimurium*. Other studies have demonstrated the antimicrobial activity of cinnamon EOagainst spoilage bacteria such as *E. coli*, *Y. enterocolitica*, *S. aureus*, *B. cereus*, *Salmonella enterica*, *L. monocytogenes*, and *P. aeruginosa* (Trajano *et al*. 2009; Nanasombat and Wimuttigosol 2011).

The increased antibacterial activity and reduction in the bacterial load on cut fruits caused by *C. sinensis* and *C. limon* EO were due mainly to synergistic activity, which was significantly reduced at the concentrations used. Purkait *et al*. (2020) reported synergy between cinnamon EO and clove EO against spoilage bacteria. The combination of more than one EOin food applications could minimize the negative olfactory effect on customers. The emergence of multidrug-resistant food-borne bacterial pathogens in the food chain is a serious problem, and fresh-cut fruits could represent a suitable environment for rapid virulence gene transfer *via*a horizontal gene transfer mechanism, which is responsible for a significant level of antimicrobial resistance. As described earlier, essential oils are natural compounds capable of modulating the sensitivity of drug-resistant bacterial pathogens, including biofilm-forming bacteria (Iseppi *et al*. 2021, 2023).

CONCLUSIONS

1. Citrus peel is a low-cost biomass for the production of essential oils (EO). The hydrodistillation method is simple method, and it is suitable for the preparation of citrus peel EOs.
2. The extracted essential oils (*C. sinensis* and *C. limon*) exhibited synergistic interactions against the tested bacterial and fungal isolates. The citrus peel EOs inhibited fungal growth and aflatoxin production.
3. This high antimicrobial activity may be related to their bioactive components. Essential oils are considered natural preservative agents. EOs, which are currently used as flavoring agents, could also serve as food preservatives.

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