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Evaluation of Antimicrobial Activity of *Cymbopogon citratus* Essential Oil Alone and in Combination with *Origanum majorana* and *Caryophyllus aromaticus* Essential Oils against Some Foodborne Bacteria

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Abstract

Background: Food spoilage and foodborne diseases are two important problems in the food industry. On the other hand, consumers' tendency to use natural additives is increasing. Hence, plant essential oils (EOs) can be safe alternatives in this regard.

Objective: The objectives were to determine the chemical composition and to evaluate the antimicrobial activity of *Cymbopogon citratus* EO against some foodborne bacteria alone and in combination with *Origanum majorana* and *Caryophyllus aromaticus* EOs.

Materials and Methods: Chemical composition of *C. citratus* EO was analyzed by gas chromatography-mass spectrometry. Further, antibacterial activity of the EO against foodborne bacteria was assessed using disk diffusion method. In addition, the minimum inhibitory concentration of the EO was determined by microdilution broth method and then the minimum bactericidal concentration value was determined. Checkerboard synergy testing was also performed to determine the fractional inhibitory concentration index. Finally, time-kill curves were drawn based on the bacterial population (CFU/mL) against time (h).

Results: The major compounds of *C. citratus* EO were isothymol, thymol, trans-caryophyllene, and cymene. The most and the least sensitive foodborne bacteria to *C. citratus* EO were *Staphylococcus aureus* and *Bacillus subtilis*, respectively. The minimum inhibitory concentration (MIC) values of *C. citratus* EO against all the evaluated bacteria were 0.1% and The minimum bactericidal concentration (MBC) values ranged between 0.1 and >2% (v/v). The combination of *C. citratus* and *O. majorana* EOs showed a synergistic activity against *Salmonella typhimurium* and partial synergism against *B. subtilis*, *Escherichia coli* O157:H7, *S. aureus*, and *Listeria monocytogenes*. Moreover, the combination of *C. citratus* and *C. aromaticus* EOs demonstrated partial synergism against *S. aureus* and *L. monocytogenes*, and additive interaction against *S. typhimurium*; however, the combination was indifferent against *E. coli* O157:H7 and *B. subtilis*. Furthermore, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs showed a bactericidal effect against *S. typhimurium* after 24 hours in the time-kill assay.

Conclusion: In general, the synergism, partial synergism, and additive effects of *C. citratus* in combination with *C. aromaticus* and *O. majorana* EOs strengthen the antimicrobial activity, expand the spectrum of activity, reduce the concentrations required, decrease the side effects, and prevent the alteration of organoleptic properties of food.

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Background

Foodborne diseases are among the causes of hospitalization and/or deaths all over the world every year.[1,2] In the meanwhile, foodborne bacteria can shorten the shelf life of food.[3] Food industry is seeking for natural, safe, and effective alternatives to synthetic food additives because of their side effects.[4,5] Essential oils (EOs) are the secondary plant metabolites, which are liquid, volatile, and soluble in fat,[6] and are reported to do an antimicrobial activity, as well.[7-11] Further, they are hydrophobic compounds, which

accumulate in the lipid layer of bacterial cell membrane and cause membrane damage.[12-15] Thymol, carvacrol, and eugenol as the major compounds of some plant EOs affect the function and permeability of cell membranes.[14-16] A number of studies have assessed the antimicrobial activities of EOs in combination.[12,17] Using EOs alone in food industry necessitates high concentrations of EOs and have some adverse effects on food sensory acceptability such as alteration in the taste, color, odor, and texture of food. However, using EOs in combination reduces the required concentration of each EO.[18,19]

Cymbopogon citratus (lemon grass) belongs to Poaceae family. *C. citratus* is native to tropical and subtropical areas of the world especially India and Sri Lanka.[20,21] Lemon grass is also cultivated in some other regions like Jiruft, Dezfool, Sari, and Masjed Soleiman in Iran.[22,23] It is used as diuretic, sedative, antispasmodic, and antibacterial, as well as being used in the treatment of neurological and gastrointestinal disorders, acne, and pimples.[20,21] Furthermore, lemon grass has antiamebic, antidiarrheal, antifilarial, antifungal, and anti-inflammatory effects.[11,24-26] The major compounds of *C. citratus* EO are geranial, neral, myrecene, and β-pinene.[27] The antimicrobial and antifungal activities of *C. citratus* EO alone and in combination with other EOs have been proved in some studies.[11,25,26,28,29] *Origanum majorana* originates from eastern Mediterranean region and grows in the north and northwest of Iran.[30] This plant has been used as tonic, diuretic, sedative, and antiseptic, as well as being used in wound healing.[31] *Caryophyllus aromaticus* is native to tropical areas especially Indonesia and India.[32,33] The most important usage of *C. aromaticus* in traditional medicine is the treatment of toothache and gingivitis with its antibacterial effect against oral bacteria.[34,35]

The objectives of this study were to assess chemical compositions and to evaluate antimicrobial activity of *C. citratus* EO against some gram positive (*Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 65138, *Bacillus subtilis* ATCC 11778) and gram negative (*Escherichia coli* O157:H7 ATCC 43895 and *Salmonella typhimurium ATCC* 14028) foodborne bacteria alone and in combination with *O. majorana* and *C. aromaticus* EOs.

Materials and Methods

Plant Material and Extraction Procedure

Leaves of *C. citratus* were purchased from Pakan Bazr Company (Isfahan, Iran). The plant was taxonomically identified at the Pharmacognosy Department, Faculty of Pharmacy, University of Tehran, Tehran, Iran. The plant was submitted to hydrodistillation in a Clevenger-type apparatus at 100°C for 5 hours. The EO was isolated and dried over anhydrous sodium sulfate and then stored in dark glass bottles at 4°C until required. *O. majorana* and *C. aromaticus* EOs were provided from the previous study.[36]

Gas Chromatography-Mass Spectrometry Analysis

C. citratus EO was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) (Thermoquest 2000, Manchester, UK). The chromatograph was equipped with DB5 capillary column (30 m × 0.25 mm ID× 0.25 µm film thickness) and the data were acquired under the following conditions: initial temperature 50°C, program rate 2.5°C per minute, final temperature 265°C, and injector temperature 250°C. The carrier gas was helium and the split ratio was 1:120. The mass spectrum (MS) was run in the electron ionization mode, using an ionization energy of 70 eV. The components of *C. citratus* EO were identified tentatively by comparing their retention indices and mass spectra with those of Wiley 275 Registry of Mass Spectral Data and literature citations.[37,38] The chemical composition of *O. majorana* and *C. aromaticus* EOs have been determined in the previous study.[36]

Bacterial Strain and Inoculum Preparation

Standard strains of Gram positive (*L. monocytogenes* ATCC 7644, *S. aureus* ATCC 65138, *B. subtilis* ATCC 11778) and Gram negative (*E. coli* O157:H7 ATCC 43895 and *S. typhimurium* ATCC 14028) foodborne bacteria were supplied from Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran. Bacterial strains were refreshed twice in sterile brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) at 37°C for 18 hours. The bacterial broth culture was placed in sterile cuvette and its optical density (OD) was adjusted to the absorbance at 600 nm of 0.1, using T80+ UV/VIS Spectronic spectrophotometer (PG Instruments Ltd, Leicestershire, UK). The number of cells in the suspension was estimated by duplicate plating from 10-fold serial dilutions on BHI agar (Merck, Darmstadt, Germany) and counting the colonies after incubation at 37°C for 18 hours.[39]

Disk Diffusion Assay

Antibacterial activity of *C. citratus* EO against foodborne bacteria was assessed using disk diffusion method. One hundred µL of bacterial suspension containing 1×10^5 colony forming units per mL (CFU/mL) was spread onto BHI agar containing 10% dimethyl sulfoxide (DMSO). The inoculated plates were put at room temperature for 5 minutes to dry. Then sterile paper disks inoculated with 10 µL of the EO were placed on BHI plates with chloramphenicol and streptomycin disks as positive controls and blank disks as negative controls. The plates were left for 15 minutes at room temperature to allow the diffusion of the EO, and were incubated at 37°C for 24 hours. At the end of the period, the diameter of the clear zone around each disk was measured with a ruler and expressed in millimeters as its antimicrobial activity. The EO would have antimicrobial activity if inhibition zone was more than 12 mm in diameter.[40,41]

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) of *C. citratus* EO was determined by microdilution broth method based on the document M7-A6 of CLSI (CLAI, 2015)[42] against foodborne bacteria. Sterile 96-well microplates were used for the assay. Dilution series of the EOs were prepared from 0.0031% to 1% (v/v) in BHI broth. The stock solutions of the EOs contained 10% (v/v) DMSO. Two hundred microliters of each dilution was transferred into 96-well microtiter plates, followed by the addition of 20 µL of respected standardized microorganism suspension containing 1×10^5 CFU/mL. Growth control consisted of BHI broth, 10% (v/v) DMSO, and bacterial suspension. After incubation at 37°C for 24 hours, the lowest concentrations without visible growth were defined as the concentrations that completely inhibited bacterial growth (MICs). The minimum bactericidal concentration (MBC) of the EO was determined according to the MIC values, based on Celiktas et al.[43] Ten microliters of each well that showed complete absence of growth was transferred to BHI agar plates and incubated at 37°C for 24 hours. The lowest concentrations of the EO where no viable bacteria were identified were recognized as the MBCs.

Checkerboard Assay

Checkerboard synergy testing was performed to determine the fractional inhibitory concentration index (FICI). Checkerboard assay was done by the microdilution broth method. In brief, serial double dilutions of *C. citratus*, *O. majorana*, and *C. aromaticus* EOs from 2 MIC to 1/64 MIC were prepared. The MIC of *O. majorana* EO was 0.1% against all tested bacteria except for *B. subtilis* (0.3%) and the MIC of *C. aromaticus* EO was 0.1% against all tested bacteria.[36] One hundred microliters of *C. citratus* dilutions were added to the rows of a 96-well microtiter plate in diminishing concentrations and 100 µL of *O. majorana* dilutions were also added to the columns in diminishing concentrations. Moreover, 100 µL of *C. citratus* dilutions were added to the rows of another 96-well microtiter plate in diminishing concentrations and 100 µL of *C. aromaticus* dilutions were also added to the columns in diminishing concentrations. A 20-µL suspension of the bacterial strains adjusted to 1×10^5 CFU/mL was added to each well and incubated at 37°C for 24 hours, with shaking at 125 rpm. The MIC of *C. citratus* EO in combination was determined as described above. Each experiment was repeated two times. FICI was calculated as follows[44]:

$$\text{FIC of } C.\ citratus = \frac{\text{MIC of } C.citratus \text{ in combination}}{\text{MIC of } C.citratus \text{ alone}}$$

$$\text{FIC of } O.\ majorana = \frac{\text{MIC of } O.\ majorana \text{ in combination}}{\text{MIC of } O.\ majorana \text{ alone}}$$

FICI= FIC of *C. citratus* + FIC of *O. majorana*

$$\text{FIC of } C.\ citratus = \frac{\text{MIC of } C.citratus \text{ in combination}}{\text{MIC of } C.citratus \text{ alone}}$$

$$\text{FIC of } C.\ aromaticus = \frac{\text{MIC of } C.\ aromaticus \text{ in combination}}{\text{MIC of } C.\ aromaticus \text{ alone}}$$

FICI= FIC of *C. citratus* + FIC of *C. aromaticus*

FICI was interpreted as follows: synergism, FICI ≤0.5; partial synergism, 1.0 >FICI >0.5; additive effect, FICI = 1.0; indifference, 1.0<FICI ≤ 4.0; and antagonism, FICI >4.0.

Time-Kill Assay

The EOs used in the time-kill assay had concentrations equivalent to 1×MIC. The final concentration of the bacterial suspension in BHI tubes was adjusted to 1×10^5 CFU/mL. A growth control without EO was included. The suspensions were incubated at 37°C for 24 hours, with shaking at 125 rpm. Each suspension was cultured on BHI agar after incubation for 0, 3, 6, and 24 hours and was then incubated at 37°C for 24 hours. Time-kill curves were drawn based on the bacterial population (CFU/mL) against time (h).[17] Experiments were carried out in duplicate.

Statistical Analysis

Data from disk diffusion assay were subjected to Kruskal-Wallis test using SPSS statistical package, version 22.0. For comparison of MIC and MBC of *C. citratus* EO on the evaluated bacteria, the Kruskal-Wallis test was used. For all analyses, $P<0.05$ was considered statistically significant.

Results

Chemical Composition of the EO

The main constituents of *C. citratus* EO are presented in Table 1. *C. citratus* EO consisted of 15 compounds representing 99.03% of the EO. The major compounds of *C. citratus* EO were isothymol (59.42%), thymol (15.23%), trans-caryophyllene (10.18%), and cymene (5.82%).

Agar Disk Diffusion Assay

Antimicrobial activity of *C. citratus* EO was evaluated by disk diffusion method (Table 2). The evaluated EO had remarkable antimicrobial effect (inhibition zone >12 mm). Based on this evaluation, the most and the least sensitive foodborne bacteria to *C. citratus* EO were *S. aureus* and *B. subtilis*, respectively. Moreover, the inhibition zone of *C. citratus* EO against all the tested bacteria except for *B. subtilis* were even greater than that of streptomycin ($P>0.05$). Furthermore, the inhibition zone of *C. citratus* EO against *E. coli* O157:H7 and *S. typhimurium* was greater than that of chloramphenicol ($P>0.05$).

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The MIC and MBC values of *C. citratus* EO against foodborne bacteria are shown in Table 3. The MIC values

Table 1. Chemical Composition (%) of *Cymbopogon citratus* EO Determined by GC-MS

No.	Component	Quantity (%)	Retention Time (min)
1	2-beta-Pinene	0.53	7.61
2	Cymene	5.82	9.57
3	gamma-Terpinene	2.70	10.99
4	Thymol	15.23	22.56
5	alpha-Cubebene	0.09	24.54
6	Copaene	0.38	25.57
7	trans-Caryophyllene	10.18	27.36
8	alpha-Humulene	1.41	28.48
9	delta-Cadinene	0.16	30.90
10	1S alpha-Pinene	0.13	6.15
11	beta-Phellandrene	0.22	9.69
12	trans-Anethole	0.72	21.66
13	Isothymol	59.42	23.58
14	Eugenol	1.79	25.12
15	(-)-Caryophylleneoxide	0.25	32.76
Total		99.03	

of *C. citratus* EO against all the evaluated bacteria were 0.1% and the MBC values ranged between 0.1 and >2% (v/v).

Checkerboard Assay

The results of checkerboard analyses for *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs against foodborne bacteria are shown in Tables 4 and 5, respectively. FICI values of *C. citratus* plus *O. majorana* EOs against foodborne bacteria ranged from 0.500 to 0.750. The combination of *C. citratus* and *O. majorana* EOs showed a synergistic interaction (FICI≤0.5) against *S. typhimurium*. According to the analysis, the MIC of *C. citratus* and *O. majorana* EOs alone against *S. typhimurium* was lowered from 0.100 to 0.025 (% v/v) in combination. Moreover, the combination of *C. citratus* and *O. majorana* EOs showed partial synergism (1.0>FICI>0.5) against *B. subtilis, E. coli* O157:H7, *S. aureus*, and *L. monocytogenes*. Likewise, FICI values for *C. citratus* plus *C. aromatics* EOs ranged from 0.75 to 1.25 against foodborne bacteria. The combination of *C. citratus* and *C. aromaticus* EOs showed partial synergism (1.0 >FICI >0.5) against *S. aureus* and *L. monocytogenes* and an additive interaction (FICI = 1.0) against *S. typhimurium*. While, the combination of *C. aromaticus* and *C. citratus* EOs was indifferent against *E. coli* O157:H7 and *B. subtilis*. Finally, no antagonistic effect was observed for *C. citratus*.

Time-Kill Assay

Time-kill assay was used to analyze the killing rate of *C. citratus* EO alone and in combination with *O. majorana* and *C. aromaticus* EOs against foodborne bacteria. The time-kill curves of *C. citratus* EO alone and in combination with *O. majorana* and *C. aromaticus* EOs (at MIC values) against foodborne bacteria are shown in Figure 1. Bactericidal effects of EOs are concluded when a three or more reduction in bacterial counts is observed in time-kill curves and the bacteriostatic effect when EO inhibits the bacterial growth.[45,46] *C. citratus* EO showed a bacteriostatic effect against foodborne bacteria. The combination of *C. citratus* and *C. aromaticus* EOs reduced the bacterial colony count of *E. coli* O157:H7 in comparison to *C. citratus* EO by 2 log after 6 hours. *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs showed bactericidal effects against *S. typhimurium* after 24 hours. Furthermore, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs reduced the bacterial colony count of *S. typhimurium* in comparison to *C. citratus* EO by 3 log after 24 hours. In

Table 2. Antimicrobial Activity (mm) of *Cymbopogon citratus* EO Against Foodborne Bacteria as Detected by Agar Disk Diffusion Assay

EO	Inhibition Diameter (mm)					*P* value
	E. coli O157:H7	*S. typhimurium*	*S. aureus*	*B. subtilis*	*L. monocytogenes*	
C. citratus	$20.16\pm0.28^{bcD*}$	20.16 ± 0.57^{bcD}	20.66 ± 0.28^{bC}	18.66 ± 0.57^{cD}	19.33 ± 0.76^{bcCAB}	0.033
Streptomycin	12.33 ± 0.57^{bcC}	13.66 ± 0.57^{bcdeC}	12.00 ± 0.00^{bdC}	23.00 ± 0.00^{eCD}	14.00 ± 0.00^{bcdeCA}	0.011
Chloramphenicol	17.66 ± 0.57^{cCD}	19.00 ± 0.00^{cbCD}	24.66 ± 0.57^{cbC}	29.00 ± 0.00^{bC}	22.66 ± 0.57^{cbB}	0.008
P value	0.025	0.023	0.023	0.020	0.023	

*Inhibition area including 6 mm disk diameter.
Results are mean ± SD of 3 replicates.
Within the columns, significant differences are represented by different superscript capital letters ($P<0.05$). Within the rows, significant differences are represented by different superscript small letters ($P<0.05$).

Table 3. MIC and MBC values (% v/v) of *Cymbopogon citratus* EO Against Foodborne Bacteria Determined by Microdilution Broth Method

	Foodborne Bacteria					*P* Value
	E. coli O157:H7	*S. typhimurium*	*S. aureus*	*B. subtilis*	*L. monocytogenes*	
MIC	0.1^{a}	0.1^{a}	0.1^{a}	0.1^{a}	0.1^{a}	1.000
MBC	0.1^{a}	0.1^{a}	0.1^{a}	$>2^{b}$	$>2^{b}$	0.061

Note: Within the rows, significant differences are represented by different superscript small letters ($P<0.05$).

Table 4. Effects of 2 EO Combinations (*C. citratus* and *O. majorana*) Against Foodborne Bacteria Using Checkerboard Assay

Microorganism	MIC (%v/v) of each EO in combination		FIC (%)		FICI	Outcome
	C. citratus	*O. majorana*	*C. citratus*	*O. majorana*		
E. coli O157:H7	0.025	0.050	0.250	0.500	0.750	Partial synergism
S. typhimurium	0.025	0.025	0.250	0.250	0.500	Synergism
S. aureus	0.025	0.050	0.250	0.500	0.750	Partial synergism
B. subtilis	0.050	0.012	0.500	0.041	0.541	Partial synergism
L. monocytogenes	0.050	0.025	0.500	0.250	0.750	Partial synergism

Table 5. Effects of 2 EO Combinations (*C. citratus and C. aromaticus*) Against Foodborne Bacteria Using Checkerboard Assay

Microorganism	MIC (%v/v) of Each EO in Combination		FIC (%)		FICI	Outcome
	C. citratus	*O. majorana*	*C. citratus*	*O. majorana*		
E. coli O157:H7	0.025	0.1	0.25	1	1.25	Indifference
S. typhimurium	0.05	0.05	0.5	0.5	1	Additive
S. aureus	0.025	0.05	0.25	0.5	0.75	Partial synergism
B. subtilis	0.0124	0.1	0.041	1	1.041	Indifference
L. monocytogenes	0.025	0.05	0.25	0.5	0.75	Partial synergism

addition, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs reduced the bacterial colony count of *S. aureus* in comparison to *C. Citratus* EO by 1 log after 6 hours. It was also found that the combination of *C. citratus* and *C. aromaticus* EOs reduced the bacterial colony count of *L. monocytogenes* in comparison to *C. Citratus* EO by 1 log after 6 hours.

Discussion

In the present study, the main components of the EO of *C. citratus* leaves were found to be isothymol, thymol, trans-caryophyllene, and cymene. The major components of whole plant of *C. citratus* collected from Kenya were geranial, neral, myrecene, and geraniol.[47] Oliveira et al reported that the main components of EO of *C. citratus* fresh leaves collected from Brazil were geranial, neral, and myrcene.[48] The factors such as characteristics of plant species, plant part used for extraction, and extraction technique, as well as environmental, seasonal, and geographical conditions are the reasons for the differences in the chemical composition of plant EOs.[49,50] Thymol causes a distortion of the membrane physical structure and increases the microbial cytoplasmic membrane permeability.[51]

It was found that *C. citratus* EO had a remarkable antimicrobial effect (inhibition zone > 12 mm). *S. aureus* and *B. subtilis* were the most and the least sensitive bacteria to *C. citratus* EO, respectively. The inhibition

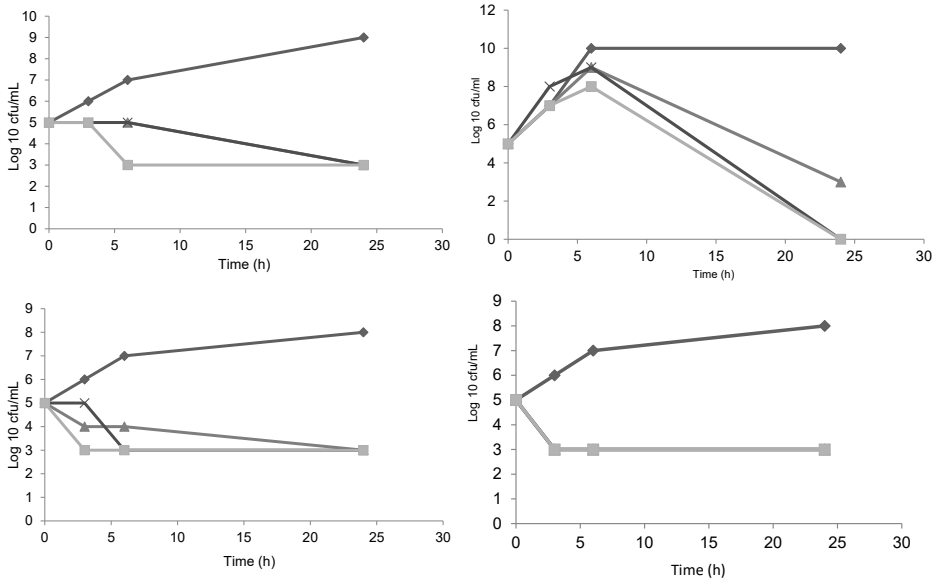

Figure 1. Time-kill Curves of Control (♦), *C. citratus* EO (▲), *C. citratus Plus O. majorana* EOs (×) and *C. citratus Plus C. aromaticus* EOs (■) (at MIC Value) Against Foodborne Bacteria (**a**=*E. coli* O157:H7, **b**= *S. typhimurium*, **c**=*S. aureus*, **d**= *B. subtilis* and **e**=*L. monocytogenes*).

zone of *C. citratus* EO against all the tested bacteria except for *B. subtilis* were even greater than that of streptomycin ($P > 0.05$). And the inhibition zone of *C. citratus* EO against *E. coli* O157:H7 and *S. typhimurium* was greater than that of chloramphenicol ($P > 0.05$). Bassol et al showed the antimicrobial effect of *C. citratus* EO against *E. faecalis, S. aureus, L. monocytogenes, E. aerogenes, E. coli, S. typhimurium, S. dysenteriae*, and *P. aeruginosa*, and reported larger inhibition zone for *C. citratus* EO against other microorganisms.[28] This difference can be attributed to the main components of the EO which were geranial and neral. Naik et al studied the antimicrobial effect of *C. citratus* EO against *S. aureus, Bacillus cereus, B. subtilis, E. coli, Klebsiella pneumonia*, and *P. aeruginosa* by disk diffusion method. *S. aureus* and *P. aeruginosa* were the most and the least sensitive bacteria, respectively. Akin to the results of the present study, zone of inhibition (mm) of *C. citratus* EO (30% (v/v)) against *S. aureus* was 29.66 mm.[52] EOs exert their antimicrobial effects through a number of mechanisms including inhibition of nucleic acid synthesis, disturbance in the cytoplasmic membrane properties and energy metabolism.[45] EOs attack cell membrane phospholipids and increase the permeability of the cell wall and cause cytoplasmic leakage.[12-15]

The MIC values of *C. citratus* EO against all foodborne bacteria were 0.1% (v/v). Likewise, the MBC values of *C. citratus* EO against foodborne bacteria were 0.1% except for *B. subtilis* and *L. monocytogenes* (MBC >2%). This result was proved by disk diffusion assay which showed the least inhibition zones for *B. subtilis* and *L. monocytogenes*. Bassolé et al found the MIC values of *C. citratus* EO, ranged from 0.1% for *Enterococcus faecalis* to 8% for *P. aeruginosa*.[28] Chaftar et al also found the MIC value of *C. citratus* EO against *E. coli* O157:H7 and *S. typhimurium* (>0.4%).[53]

In this study, the antimicrobial effect of *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs against foodborne bacteria were studied for the first time. The combination of *C. citratus* and *O. majorana* EOs showed a synergistic effect against *S. typhimurium*. Furthermore, the combination of two EOs (*C. citratus* and *O. majorana*) showed partial synergism against *B. subtilis, E. coli* O157:H7, *S. aureus*, and *L. monocytogenes*. The combination of *C. citratus* and *C. aromaticus* EOs also showed partial synergism against *S. aureus* and *L. monocytogenes* and an additive interaction against *S. typhimurium*. In addition, the combination of two EOs (*C. citratus* and *C. aromaticus*) was indifferent against *E. coli* O157:H7 and *B. subtilis*. Yet no antagonistic effect was observed for *C. citratus*. Bassolé et al showed that the combination of *C. citratus* and *Cymbopogon giganteus* EOs had a synergistic effect against *S. aureus, L. monocytogenes, S. typhimurium*, and *E. aerogenes*.[28] Gutierrez et al reported that the combination of *O. majorana* and *Origanum vulgare* EOs were indifferent against *L. monocytogenes* and *P. aeruginosa*; while an additive effect was seen against *B. cereus* and *E. coli* O157:H7.[19] Tserennadmid et al reported that the combination of *O. majorana* and *Juniperus communis* EOs had a synergistic effect against *E. coli*.[54] *C. aromaticus* plus *Rosmarinus officinalis* EOs had additive antimicrobial effects against *S. epidermidis*, *S. aureus*, *B. subtilis*, *E. coli* $O_{157}H_7$, *P. vulgaris*, and *P. aeruginosa*.[17] Using the combinations of EOs with synergistic or additive effects may decrease the need for chemical additives, limit their adverse effects and antibiotic resistance, may reduce required doses, and expand the spectrum of activity.[18,44] Furthermore, by using EOs in combination, the microorganisms were inhibited through the simultaneous effects of various chemical compounds, thereby improving antimicrobial properties.[46]

In the current study, *C. citratus* EO alone did not show bactericidal effect against foodborne bacteria in the time-kill assay. While, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs showed a bactericidal effect against *S. typhimurium* after 24 hours. Furthermore, *C. citratus* plus *O. majorana* EOs and *C. citratus* plus *C. aromaticus* EOs reduced the bacterial colony count of *S. typhimurium* in comparison to *C. citratus* EO by 3 log after 24 hours. Khan and Ahmad studied the antimicrobial effect of *C. citratus* and *Syzygium aromaticum* EOs against *Candida albicans* by time-kill assay and reported that more than 60% reduction in viable count of *C. albicans* was exhibited by *C. citratus* EO after 10–12 hours which was more effective than amphotericin B and fluconazole.[29] Oliveira et al studied the antimicrobial effects of *Origanum vulgare* and *O. majorana* EOs at the MIC values against *S. aureus, Proteus* spp., and *Klebsiella* spp. by the time-kill assay. The most potent inhibitory effect was shown by *O. majorana* EO against *Proteus* spp. which killed the initial inoculum after 4 hours.[51] The results of time-kill assay of the current study verified the abovementioned results on the synergistic effect of *C. citratus* and *O. majorana* EOs and additive effect of *C. citratus* and *C. aromaticus* EOs against *S. typhimurium*.

Conclusion

In general, the current study showed that *C. citratus* EO had an antimicrobial activity against the most important foodborne bacteria. Therefore, the combination of *C. citratus* EO with *O. majorana* and *C. aromaticus* EOs can be used as an alternative for synthetic additives to reduce their side effects and also to decrease antibiotic resistance. The combination of these EOs, depending on the corresponding microorganism, exhibited additive, synergistic, and partial synergistic, as well as indifferent interaction. These interactions strengthen the antimicrobial activity, expand the spectrum of activity, reduce the concentrations required, decrease the side effects, and prevent the alteration of organoleptic

properties of food. To the best of our knowledge, this is the first study on the antimicrobial effects of *C. citratus* in combination with *O. majorana* and *C. aromaticus* EOs. Further studies on the interaction of these EOs with food ingredients, their modes of action, and their components' mechanisms of action are required.

Authors' Contributions

RP: Designing the study, writing the manuscript; FT: Drafting the manuscript; ZB: Conducting the statistical analyses; AS: Obtaining the samples.

Ethical Approval

All procedures performed in this study were in accordance with the ethical standards of the National Research Committee.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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