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Antimicrobial Efficacy and Chemical Properties of *Caryophyllus aromaticus* and *Origanum majorana* Essential Oils Against Foodborne Bacteria Alone and in Combination

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Abstract

Background: Food products need to be protected against pathogenic and non-pathogenic microorganisms. One method is adding antimicrobial agents. Consumers' tendency to use synthetic additives is drastically decreasing due to their side effects and also the emergence of multidrug resistant microorganisms. Plant essential oils (EOs) are natural antimicrobial compounds which are widely used in food industry.

Objective: The objectives were to determine the chemical compositions of *Caryophyllus aromaticus* and *Origanum majorana* EOs and also to assess their antimicrobial activities against foodborne bacteria alone and in combination.

Materials and Methods: The EOs were analyzed by gas chromatography-mass spectrometry. Antibacterial activities of the EOs against foodborne bacteria were assessed using disc diffusion method. The minimum inhibitory concentration (MIC) values of the EOs were determined by microdilution broth method and then minimum bactericidal concentration (MBC) values were determined. Checkerboard synergy testing was performed to determine the fractional inhibitory concentration index. Then time-kill curves were drawn based on the bacterial population (CFU/mL) against time (h).

Results: The major constituents of *C. aromaticus* were eugenol and carvacrol, while *O. majorana* had carvacrol, thymol, trans-caryophyllene, and cymene as the main constituents. Zone of inhibition for *O. majorana* EO was greater than that for *C. aromaticus* EO. The inhibition zone of *O. majorana* EO against all the tested bacteria except for *Bacillus subtilis* was significantly greater than that of streptomycin ($P<0.05$). MIC value of the EOs against bacteria was 0.1% except for *O. majorana* EO against *B. subtilis* (0.3%). MBC values of *C. aromaticus* and *O. majorana EOs* ranged from 0.5% to 1.0% and 0.3% to 0.5% (v/v), respectively. The EOs were more effective on gram-positive bacteria than gram-negative ones. The combination of EOs revealed synergistic activity against *Listeria monocytogenes*, partial synergistic activity against *B. subtilis*, and additive effect against *Salmonella typhimurium*, *Escherichia coli* O157:H7 and *Staphylococcus aureus* were indifferent against the combination of EOs. Time–kill curves of the EOs demonstrated strong bactericidal effect against all foodborne bacteria at 6 and 24 hours either alone or in combination.

Conclusion: The synergistic, partial synergistic, and additive effects of the combination of *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

Waterborne and foodborne diseases are examples of the most important public health problems. It is estimated that over 200 microbial, chemical, and physical agents contribute to foodborne illnesses.[1] Approximately one in ten people experience food poisoning caused by bacteria each year in the United States.[2] Centers for Disease Control and Prevention (CDC) estimates that 76 million people get sick, more than 300 000 are hospitalized, and 5000 die from foodborne illnesses each year.[3] Furthermore, spoilage microorganisms can shorten the shelf-life of food, causing a remarkable concern.[4] Some of the most popular methods for food preservation are drying, freezing, irradiation, refrigeration, thermal

processing, adding antimicrobial agents, and modified atmosphere packaging.[5] There are many antimicrobial agents, however consumers' tendency to use synthetic additives is drastically decreasing due to their side effects and also the emergence of multidrug resistant microorganisms.[6] Therefore, food industry is seeking natural, safe, and effective alternatives.[7] Spices and herbs are generally recognized as safe (GRAS) and have been used as flavouring agents and food preservatives for thousands of years.[8] Plant essential oils (EOs) are widely used in food industry and their antimicrobial activities have been proved in numerous studies.[9-12] The addition of high concentrations of EOs to foods, however, in order to achieve antimicrobial activity has proven some adverse effects on food sensory acceptability including alteration in the taste, color, odor, and texture of food. One efficient procedure in order to reduce the required concentration of each EO is to benefit from combinational effects of EOs.[6,13] A number of studies have been conducted on the antimicrobial activity of EOs in combination.[14-16]

Caryophyllus aromaticus (clove) belongs to Myrtaceae family.[17] Clove EO contains antimicrobial constituents which have been used as traditional antimicrobial additives and flavouring agents in food and dental medicine.[18] Eugenol is the most important component of clove EO which exerts strong biological effects such as antimicrobial, insecticidal, and antioxidant activities. Clove EO has also remedial effects, including antiseptic, anti-vomiting, anti-carminative, analgesic, antispasmodic, and anti-phlogistic.[18,19] Previous studies have reported antimicrobial activity of clove EO against *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Escherichia coli* O157:H7, and *Pseudomonas aeruginosa*.[10] Some researchers have proved the synergistic effect of clove and cinnamon EOs against *L. monocytogenes*, *B. cereus* and *Y. enterocolitica*.[16,17]

Origanum majorana (marjoram) belongs to Lamiaceae family whose EO is used as a food flavour.[20] Marjoram has also been used in cosmetics and medications.[21] Major constituents of marjoram EO are terpinen-4-ol, (+)-cis-sabinene hydrate, γ-terpinene, and terpinolene.[22,23] Some studies have reported antimicrobial and antifungal activities of *O. majorana* EO.[21,23,24] Based on a report from Gutierrez et al,[15] the combination of thyme and marjoram shows additive effect against *E. cloacae*.

Objectives

The objectives of this study were to assess chemical compositions and to evaluate antimicrobial activities of *C. aromaticus* and *O. majorana* EOs against some foodborne gram-positive (*L. monocytogenes* ATCC 7644, *S. aureus* ATCC 65138, *B. subtilis* ATCC 11778) and gram-negative (*E. coli* O157:H7 ATCC 43895 and *Salmonella typhimurium* ATCC 14028) bacteria alone and in combination.

Materials and Methods

Plant Material and Extraction Procedure

Leaves of *C. aromaticus* and *O. majorana* were purchased from Pakan Bazr Company (Isfahan, Iran) and transferred to the Pharmacognosy Department, Faculty of Pharmacy, University of Tehran, Tehran, Iran for approval. The plants were placed in a Clevenger-type apparatus (Tajhizyar, Tehran, Iran) for hydrodistillation at 100°C for 5 hours. The EOs were isolated and dried over anhydrous sodium sulfate (Merck, Darmstadt, Germany) and then stored in dark glass bottles at 4°C until required.

Gas Chromatography-Mass Spectrometry Analysis

The EOs were 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, 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 EOs were identified tentatively by comparing their retention indices and mass spectra with those of Wiley 275 Registry of Mass Spectral Data and literature citations.[25,26]

Bacterial Strain and Inoculum Preparation

The frequently-reported foodborne 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) bacteria were supplied from Faculty of Veterinary Medicine, Amol University of Special Modern Technologies, Amol, Iran. Bacterial strains were cultured in sterile brain heart infusion (BHI) broth (Merck, Darmstadt, Germany) at 37°C for 18 hours. Then a loopful of the bacterial suspension was inoculated in sterile BHI broth and incubated at 37°C for 18 hours. The bacterial broth culture was placed in sterile cuvette and its optical density (OD) was adjusted to absorbance 0.1 at 600 nm, using T80+ UV/VIS Spectronic spectrophotometer (PG Instruments Ltd, Leicestershire, UK). The number of cells in the suspension was estimated by replica plating from 10-fold serial dilutions on BHI agar (Merck, Darmstadt, Germany) and counting the colonies after 18-hour incubation at 37°C.[27]

Disk Diffusion Assay

Antibacterial activities of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria were assessed using disc diffusion method. The OD of bacterial strains was adjusted to absorbance 0.1 at 600 nm, and according to the results of the previous section on colony counting, the bacterial suspensions were diluted to obtain10^5×1

colony forming units per mL (CFU/mL). One hundred microliters of bacterial suspension containing 1×10^5 CFU/mL were spread onto BHI agar containing 10% dimethyl sulfoxide (DMSO). The inoculated plates were left at room temperature for 5 minutes to dry. Then sterile paper disks inoculated with 10 µL of EO were placed on BHI plates containing chloramphenicol and streptomycin disks as positive controls and blank discs as negative controls. The plates were left at room temperature for 15 minutes to allow the diffusion of the EOs, and then were incubated at 37°C for 24 hours. In the end, the diameter of the clear zone around each disk was measured with a ruler and expressed in millimeters as its antimicrobial activity. EOs showed antimicrobial activities if inhibition zone was above 12 mm.[28,29]

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) values of the EOs were determined by microdilution broth method based on the document M7-A6 of CLSI[30] against foodborne bacteria. Sterile 96-well microplates were used for the assay. Stock solutions of the EOs were prepared in 10% (v/v) DMSO. Dilution series of EOs were prepared from 0.0031% to 1% (v/v) in BHI broth. Two hundred microliters of each dilution was transferred into 96-well microliter plate, 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) values of the EOs were determined according to the MIC values, based on Celiktas et al.[31] 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 EOs in which no viable bacteria were identified were 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 the EOs from 2 MIC to 1.64 MIC were prepared. One hundred microliter of *C. aromaticus* 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. 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, shaking at 125 rpm. The MIC of the EOs in combination was determined as described above. Each experiment was repeated 2 times. FICI was calculated as follows[32]:

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

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

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

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

EOs used in 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, shaking at 125 rpm. Each suspension was cultured on BHI agar after 0, 3, 6, and 24 hours of incubation and was then again incubated at 37°C for 24 hours. Time-kill curves were drawn based on the bacterial population (CFU/mL) against time (h).[13] Experiments were carried out in duplicate.

Statistical Analysis

Data from disk diffusion assay were subjected to Kruskal-Wallis test in SPSS software version 22.0. The Kruskal-Wallis test was also applied for the comparison of MIC and MBC of each EO on the evaluated bacteria. Furthermore, the Mann-Whitney U test was applied for the comparison of MIC and MBC of EOs on each evaluated bacterium. For all analyses, $P<0.05$ was considered statistically significant.

Results

Chemical Composition of the Essential Oils

The main constituents of *C. aromaticus* and *O. majorana* EOs are presented in Table 1. Eleven constituents were identified representing 99.71% of *C. aromaticus* EO. The major constituents of *C. aromaticus* EO were eugenol (96.81%) and carvacrol (1.74%). *O. majorana* EO consisted of 17 constituents representing 99.05% of the EO. The major constituents of *O. majorana* EO were carvacrol (57.86%), thymol (13.54%), trans-caryophyllene (11.52%), and cymene (6.78%).

Agar Disk Diffusion Assay

Antimicrobial activities of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria were evaluated by disk diffusion method (Table 2). The inhibition zones of the evaluated EOs against all the tested bacteria were

Table 1. Chemical Composition (%) of *Caryophyllus aromaticus* and *Origanum majorana* EOs Determined by GC-MS

No.	Constituent	Quantity (%)		Retention Time (min)
		Caryophyllus aromaticus	*Origanum majorana*	
1	Benzene, 1-methyl-4-(1-methylethyl)-	0.09		9.50
2	dl-Limonene	0.04	0.67	9.67
3	Camphor	0.03		14.64
4	Chavicol	0.18		20.87
5	Thymol	0.29	13.54	22.47
6	Carvacrol	1.74	57.86	22.80
7	Eugenol	96.81		25.97
8	trans-Caryophyllene	0.39	11.52	27.31
9	Alpha-humulene	0.07	1.57	28.51
10	cis-Jasmone	0.04		31.81
11	Globulol	0.03		35.35
12	Apha-thujene		0.08	5.94
13	Alpha-pinene		0.17	6.15
14	2-Beta-pinene		0.61	7.61
15	Beta-myrcene		0.10	8.19
16	Alpha-terpinene		0.09	9.17
17	Cymene		6.78	9.57
18	Gamma-terpinene		3.11	10.99
19	Alpha-cubebene		0.10	24.54
20	3-Allyl-6-methoxyphenol		1.98	25.09
21	Copaene		0.43	25.57
22	Delta-cadinene		0.17	30.90
23	Caryophyllene oxide		0.27	32.75
Total		99.71	99.05	

Table 2. Antimicrobial Activities (mm) of *Caryophyllus aromaticus* and *Origanum majorana* EOs Against Foodborne Bacteria Determined by Agar Disk Diffusion Assay

	Inhibition diameter (mm)					
Essential Oil	*E. coli* O157:H7	*S. typhimurium*	*S. aureus*	*B. subtilis*	*L. monocytogenes*	*P* Value
C. aromaticus	$14.16\pm0.14^{*aAC}$	15.66 ± 0.28^{aeAC}	15.58 ± 0.14^{adAC}	18.66 ± 0.57^{bcdeA}	15.56 ± 0.40^{acAC}	0.023
O. majorana	$23.58\pm0.38^{**abBCD}$	22.96 ± 0.25^{aBCD}	30.58 ± 0.72^{abBCD}	33.16 ± 0.76^{bBCD}	23.16 ± 0.38^{abBCD}	0.014
Streptomycin	12.33 ± 0.57^{aA}	13.66 ± 0.57^{adA}	12.00 ± 0.00^{aA}	23.00 ± 0.00^{bcdAC}	14.00 ± 0.00^{acA}	0.011
Chloramphenicol	17.66 ± 0.57^{aAD}	19.00 ± 0.00^{aeAD}	24.66 ± 0.57^{adAD}	29.00 ± 0.00^{bcdeAD}	22.66 ± 0.57^{acAD}	0.008
***P* value**	0.009	0.008	0.008	0.010	0.011	

*The inhibition zones of the EOs and antibiotics including 6 mm disc diameter are presented. Results are presented as mean ± SD of 2 replicates. Within the columns, significant differences are presented by different superscript capital letters ($P<0.05$). Within the rows, significant differences are represented by different superscript small letters ($P<0.05$).

greater than 12 mm, so the EOs showed remarkable antimicrobial effects. Range of inhibition zone for *O. majorana* EO (22.96-33.16 mm) was greater than that for *C. aromaticus* EO (14.16-18.66 mm). *C. aromaticus* EO was more effective than streptomycin against all the tested bacteria except for *B. subtilis*, but less effective than chloramphenicol against all the bacteria under study ($P>0.05$). *O. majorana* EO showed significantly greater antimicrobial effect against *B. subtilis* than *C. aromaticus* EO ($P<0.05$). The inhibition zone of *O. majorana* EO against all the tested bacteria except for *B. subtilis* was even significantly greater than that of streptomycin ($P<0.05$). Likewise, the inhibition zone of *O. majorana* EO against all the foodborne bacteria was greater than that of chloramphenicol ($P>0.05$). Furthermore, the most sensitive foodborne bacterium to both evaluated EOs was *B. subtilis*. On the contrary, the least sensitive foodborne bacteria to *C. aromaticus* and *O. majorana* EOs were *E. coli* O157:H7 and *S. typhimurium*, respectively ($P>0.05$).

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

MIC and MBC values of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria are shown in Table 3. MIC value of the EOs against foodborne bacteria was 0.1% except for that of *O. majorana* EO against *B. Subtilis*

Table 3. MIC and MBC Values (% v/v) of *Caryophyllus aromaticus* and *Origanum majorana* EOs Against Foodborne Bacteria Determined by Microdilution Broth Method

Essential Oil	*E. coli O157:H7*		*S. typhimurium*		*S. aureus*		*B. subtilis*		*L. monocytogenes*		*P* value	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
C. aromaticus	0.1^{*aA}	1.0^{*aA}	0.1^{aA}	0.8^{aA}	0.1^{aA}	0.8^{aA}	0.1^{aA}	0.5^{aA}	0.1^{aA}	0.5^{aA}	1.000	0.061
O. majorana	0.1^{aA}	0.5^{aA}	0.1^{aA}	0.3^{aA}	0.1^{aA}	0.3^{aA}	0.3^{aA}	0.3^{aA}	0.1^{aA}	0.3^{aA}	0.406	0.061
P value	1.000	0.333	1.000	0.333	1.000	0.333	1.000	0.333	1.000	0.333		

* Results are presented as mean of 2 replicates. Significant differences among MIC values in each row are presented by different superscript small letters ($P<0.05$). Significant differences among MBC values in each row are presented by different superscript small letters ($P<0.05$). Within the columns, significant differences are presented by different superscript capital letters ($P<0.05$).

(0.3%). MBC values of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria ranged from 0.5% to 1.0 % (v/v) and 0.3% to 0.5% (v/v), respectively. *O. majorana* EO had stronger bactericidal effect than *C. aromaticus* EO against foodborne bacteria ($P>0.05$). The highest MBC value was observed for both tested EOs against *E. coli* O157:H7.

Checkerboard Assay

The results of checkerboard synergy analysis of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria are shown in Table 4. FICI values for *C. aromaticus* EO plus *O. majorana* EO ranged from 0.500 to 2.000 against foodborne bacteria. The combination of *C. aromaticus* and *O. majorana* EOs showed synergistic interaction (FICI = 0.500) against *L. monocytogenes*. The MIC values of *C. aromaticus* and *O. majorana* EOs alone against *L. monocytogenes* were lowered from 0.100 to 0.025 in combination. The combination of *C. aromaticus* and *O. majorana* EOs showed partial synergistic activity (FICI = 0.583) against *B. subtilis* and additive effect (FICI = 1.000) against *S. typhimurium*. *E. coli* O157:H7 and *S. aureus* were indifferent against *C. aromaticus* plus *O. majorana* EOs. No antagonistic effect was observed for the EOs.

Time-Kill Assay

Time-kill assay was used to analyze the killing rate of *C. aromaticus* and *O. majorana* EOs alone and in combination against foodborne bacteria. The time-kill curves of *C. aromaticus* and *O. majorana* EOs (at MIC values) alone and in combination against foodborne bacteria are shown in Figure 1. Bactericidal effect of EOs is concluded when a reduction (equal to three or more) in bacterial counts is observed in time-kill curves and the bacteriostatic effect is concluded when EOs inhibit the bacterial growth. *C. aromaticus* showed bactericidal effect against *B. subtilis* and *S. aureus* at 6 hours. Moreover, the bactericidal effect of *C. aromaticus* EO against *S. typhimurium*, *L. monocytogenes*, and *E. coli* O157:H7 was observed at 24 hours. *O. majorana* EO showed bactericidal effect against *B. subtilis* at 3 hours, while the same effect was seen against *S. aureus* and *L. monocytogenes* at 6 hours. Accordingly, *O. majorana* EO showed bactericidal effect against *S. typhimurium* and *E. coli* O17:H7 at 24 hours. The combination of *C. aromaticus* and *O. majorana* EOs reduced the colony count of *B. subtilis* and *S. aureus* in comparison to *C. aromaticus* EO by 1 and 2 log, respectively at 3 hours. In the same way, the combination of *C. aromaticus* and *O. majorana* EOs reduced the colony count of *L. monocytogenes* and *E. coli* O157:H7 in comparison with *C. aromaticus* EO by 3 log at 6 hours. Furthermore, the combinational effect of *C. aromaticus* and *O. majorana* EOs caused a reduction in the colony count of *S. aureus* in comparison with *O. majorana* EO by 1 log at 3 hours. Additionally, the combination of both EOs reduced the colony count of *L. monocytogenes* and *E. coli* O157: H7 in comparison with *O. majorana* EO by 2 log at 6 hours. Similarly, the combination of *C. aromaticus* and *O. majorana* EOs diminished the colony count of *L. monocytogenes* in comparison with either of EOs by 4 and 3 log, respectively at 24 hours.

Discussion

The major constituents of *C. aromaticus* EO in the current study were eugenol and carvacrol, and those of *O.*

Table 4. Combinational Effects of *Caryophyllus aromaticus* and *Origanum majorana* EOs Against Foodborne Bacteria Using Checkerboard Assay

Microorganism	MIC (%v/v) of Each EO in Combination		FIC (%)		FICI	Outcome
	C. aromaticus	*O. majorana*	*C. aromaticus*	*O. majorana*		
E. coli O157:H7	0.100	0.100	1.000	1.000	2.000	Indifference
S. typhimurium	0.025	0.050	0.250	0.500	1.000	Additive
S. aureus	0.050	0.100	0.500	1.000	1.500	Indifference
B. subtilis	0.050	0.025	0.500	0.083	0.583	Partial synergism
L. monocytogenes	0.025	0.025	0.250	0.250	0.500	Synergism

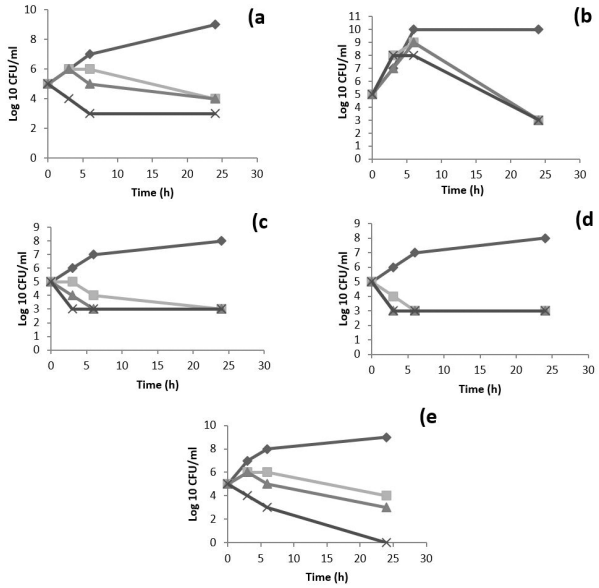

Figure 1. Time-Kill Curves of Control (♦), *C. aromatics* EO (■), *O. majorana* EO (▲) (at MIC value) and the Combination of *C. aromatics and O. majorana* EOs (×) Against Foodborne Bacteria (**a**=*E. coli* O157:H7, **b**= *S. typhimurium*, **c**=*S. aureus*, **d**= *B. subtilis* and **e**=*L. monocytogenes*).

majorana EO were carvacrol, thymol, trans-caryophyllene, and cymene. Similar findings have been reported by other investigators.[14] Clove EO had high eugenol (70%–90%) content.[9] Barbosa et al[33] found the major constituents of C. *aromaticus* EO to be eugenol (75.85%) and eugenyl acetate (16.38%). Daferera et al[34] reported the main constituents of *O. majorana* EO to be thymol, 3-carene, and terpinen-4-ol. Contrary to the results of the current study, the major constituents of *O. majorana* EO were linalool, terpinen-4-o1, and p-cymene in another study.[35] Differences in the chemical composition of EOs are the results of different factors including plant species, age of the plant, plant part used for extraction, geographic area, soil, weather conditions, harvest season, and extraction technique.[36,37] Culture conditions, crop processing, and post-harvest processing can be other explanations for variations in chemical composition of EOs.[38]

Caryophyllus aromaticus and *O. majorana* EOs showed antimicrobial effects against all foodborne bacteria as was evident from the large inhibition zones (inhibition zone >12 mm). *C. aromaticus* EO was more effective than streptomycin against all the tested bacteria except for *B. subtilis*, but less effective than chloramphenicol against all the bacteria under study ($P>0.05$). The inhibition zone of *O. majorana* EO against all the tested bacteria except for *B. subtilis* was significantly greater than that of streptomycin ($P<0.05$). According to the results of agar disc diffusion assay, *C. aromaticus* and *O. majorana* EOs were more effective against gram-positive bacteria (the most sensitive bacterium to both EOs was *B. subtilis*) than gram-negative ones (the least sensitive foodborne bacteria to both EOs were *E. coli* O157:H7 and *S. typhimurium*, respectively). In the study of Hoque et al,[9] clove EO showed antimicrobial effect against *L. monocytogenes*, *S. aureus*, *E. coli* O157:H7, *Salmonella enteritidis,* and *B. cereus*. Eugenol is the main constituent of *C. aromaticus* EO whose antimicrobial activity against foodborne pathogens and drug resistant microorganisms has been proved in other studies.[39] Mith et al[12] found that *O. majorana* EO had antimicrobial activity against *E. coli* O157:H7, *S. typhimurium*, *L. monocytogenes*, *Brochothrix thermosphacta*, and *Pseudomonas fluorescens*. Carvacrol as the main constituent of *O. majorana* EO, holding antimicrobial effects, has been recognized GRAS by the Food and Drug Administration (FDA).[40] EOs exert their antimicrobial effects through a number of mechanisms including inhibition of nucleic acid synthesis, disturbance of the cytoplasmic membrane, and energy metabolism.[33] In this study, it was found that the antimicrobial activity of *C. aromaticus* EO against all the tested bacteria was weaker than that of chloramphenicol. Similar result was found by Hoque et al[9] in that antimicrobial effect of clove EO against *L. monocytogenes*, *E. coli* O157:H7, *S. aureus*, *B. cereus*, and *S. enteritidis* was weaker than that of gentamicin. Mith et al[11] showed that the antimicrobial activity of thymol was greater than that of eugenol, thereby the antimicrobial activity of *O. majorana* being greater than that of *C. aromaticus*. Similarly, clove EO produced the largest inhibition zone against Gram positive bacteria especially *Bacillus* sp. (24 mm) and *B. subtilis* (19.5 mm)[10,14]; this can be attributed to the outer membrane structure having hydrophilic lipopolysaccharide and lipoproteins which form a barrier to restrict the entrance of hydrophobic compounds to gram-negative bacteria.[9,10,12,41]

In microdilution broth test, the MIC value of *C. aromaticus* and *O. majorana* EOs against all foodborne bacteria was 0.1% except for *O. majorana* EO against *B. Subtilis* (0.3%). MBC values of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria in the current study ranged from 0.5% to 1.0 % (v/v) and 0.3% to 0.5% (v/v), respectively. *E. coli* O157:H7 was the most resistant microorganism to bactericidal effects of both the EOs ($P>0.05$). Similar to the results of the previous section, *O. majorana* EO showed stronger bactericidal effect than *C. aromaticus* EO against foodborne bacteria ($P>0.05$). In two similar studies, the MIC value of clove EO against *S. aureus* was reported to be 0.09% (v/v).[42,43] Fu et al[14] found the MIC and MBC values of clove EO against foodborne microorganisms as 0.06%-0.50% (v/v) and 0.12%-0.50% (v/v), respectively. Contrary to the results of the present study, the lowest MIC and MBC values of clove EO against foodborne pathogens and spoilage bacteria were found to be1.25% and 2.5% (v/v) in the study of Hoque et al, respectively.[9] Gutierrez et al[13] showed that MIC value of marjoram against *Listeria innocua* was 0.5% (v/v). Similar to the results of the previous section, Gram

negative bacteria were less sensitive to bactericidal effect of *C. aromaticus* EO than Gram positives ones; this has been proved by other researchers.[24,41,44,45]

Antimicrobial combinational effect of *C. aromaticus* and *O. majorana* EOs against foodborne bacteria was studied for the first time. The present study revealed that the MIC value of each EO against *L. monocytogenes* was higher than the MIC value in combination. The combination of *C. aromaticus* and *O. majorana* EOs showed partial synergistic effect against *B. subtilis*, and additive effect against *S. typhimurium*. It was indifferent against *S. aureus* and *E. coli* $O_{157}H_7$. Goñi et al,[16] Probst et al,[17] and Nascimento et al[46] assessed the antimicrobial activity of *C. aromaticus* EO in combination with other antimicrobial agents. Clove plus rosemary EOs had additive antimicrobial effects against *S. epidermidis*, *S. aureus*, *B. subtilis*, *E. coli* $O_{157}H_7$, *Proteus vulgaris*, and *P. aeruginosa*.[14] Gutierrez et al[15] reported that combination of marjoram and oregano exerted additive effect against *B. cereus* and *E. coli* O157:H7, while it was indifferent against *L. monocytogenes* and *P. aeruginosa*. The combinations of marjoram and rosemary EOs, sage and basil showed additive effects against *L. monocytogenes*. Marjoram in combination with lemon balm and thyme was indifferent against *L. monocytogenes*.[15] Goñi et al[16] demonstrated that the combination of marjoram and oregano EOs can be used for controlling the growth of Gram positive and gram-negative bacteria. The synergistic or additive effects of EOs may decrease the need for usage of chemical additives, limit their adverse effects and antibiotic resistance, and also may reduce required doses and expand the spectrum of activity.[6,32] Furthermore, by the use of combinations of EOs, various chemical compounds of EOs could simultaneously inhibit microorganisms, and accordingly the combinational antimicrobial activity could be improved.[17]

Time–kill curves of *C. aromaticus* and *O. majorana* EOs showed strong bactericidal effects against all foodborne bacteria at 6 and 24 hours either alone or in combination. *O. majorana* EO was the only EO that showed bactericidal effect against *B. subtilis* at 3 hours. Contradictory results were reported by Fu et al[14] who studied the antimicrobial activity of clove and rosemary EOs against *S. epidermidis*, *E. coli* O157:H7, and *Candida albicans* at 1×MIC, and colony counts remained near the initial starting concentration after 30 hours. Barbosa et al[33] found that clove EO had bactericidal effect against *S. aureus* and bacteriostatic effect against *E. coli* O157:H7. Zu et al[47] studied the antimicrobial effect of 10 EOs (mint, ginger, rose, cinnamon, grapefruit, jasmine, lavender, chamomile, thyme, and lemon) at 0.25% v/v against *Propionibacterium acnes* and showed bactericidal effects at 30 minutes except for jasmine. Similar to the results of the current study, Probst et al[17] reported that the MIC value was bactericidal for clove EO against *S. aureus* and *E. coli* O157:H7 at 6 hours. The combination of *C. aromaticus* and *O. majorana* EOs in the current study reduced the colony count of *L. monocytogenes* at 24 hours in comparison with *C. aromaticus* and *O. majorana* EOs alone by 4 and 3 log, respectively. The results of time-kill assay proved the results of the previous section on the synergistic effect of *C. aromaticus* and *O. majorana* EOs against *L. monocytogenes*. Probst et al[17] showed that the combination of clove with cinnamon EOs reduced the colony count of *E. coli* O157:H7 by 1 log at 1.5 hours in comparison to clove EO alone.

According to the results of the current study, *C. aromaticus* and *O. majorana* EOs showed promising antimicrobial activities against the most important foodborne pathogens and spoilage bacteria. Gram positive microorganisms were more sensitive to *C. aromaticus* and *O. majorana* EOs than Gram negative ones. The combination of *C. aromaticus* and *O. majorana* EOs exhibited synergistic, partial synergistic and additive antibacterial activities depending on the corresponding microorganism. 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.

The combination of *C. aromaticus* and *O. majorana* EOs can be used as a potential alternative for synthetic additives, as food preservatives, and also in the production of herbal medicines. Further studies are required on the interaction of these EOs with food ingredients and their modes of action. To the best of our knowledge, this was the first study on the antimicrobial effects of *C. aromaticus* and *O. majorana* EOs in combination.

Authors' Contributions

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

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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