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RESEARCH ARTICLE

IN-VITRO ANTIBIOFILM ACTIVITY AND GROWTH INHIBITORY EFFECTS OF *Origanum onites* ESSENTIAL OIL AND CARVACROL AGAINST *Escherichia coli* ATCC 25922 AND METHICILLIN-RESISTANT *Staphylococcus aureus* (MRSA) ATCC 43300

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Abstract

The treatment of infections caused by *Escherichia coli* and Methicillin-resistant *Staphylococcus aureus* (MRSA) has become difficult due to the increasing development of resistance to the antibiotics used. Nowadays, in the fight against these infections, the importance of natural and inexpensive plant essential oils and their bioactive molecules with known antibacterial activity has been increasing. This study aimed to investigate the antibacterial and antibiofilm effects of *Origanum onites* essential oil (OEO) and carvacrol, to which bacteria cannot develop resistance, as an alternative to antibiotics. The chemical content of OEO was analyzed by GC-MS system. Antibacterial activity was analyzed by disk diffusion, macro broth dilution, and antibacterial curve assays, and also antibiofilm activity was analyzed by the quantitative crystal violet method. Carvacrol was defined as the major component in the OEO composition. The results showed that OEO and carvacrol exhibited antibacterial activity against *E. coli* and MRSA with the minimum inhibition concentration (MIC) of 100 µg/mL and 50 µg/mL, respectively. The antibacterial curve assay results showed that OEO and carvacrol exhibited bactericidal activity against *E. coli* and MRSA. OEO and carvacrol inhibited the biofilm formation of *E. coli* and MRSA in the range of 15.5%-80.7% at MIC, 1/2MIC, and 1/4MIC concentrations.

Keywords

Escherichia coli,
Origanum onites,
Antibiofilm activity,
Carvacrol,
Methicillin-resistant
Staphylococcus aureus

Time Scale of Article

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1. INTRODUCTION

Escherichia coli (*E. coli*) is largely found in the flora of the digestive tract and constitutes the largest proportion of Gram-negative facultative anaerobic bacteria in the intestines. As an opportunistic pathogen, it is the most frequent causative agent in urinary tract infections and community-acquired bacteremia [1]. It causes bloody diarrhea in the intestine. Apart from the intestine; it also causes urinary tract infections, neonatal meningitis, pneumonia, septic arthritis, skin and soft tissue infections [2]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a Gram-positive and facultative anaerobic bacterium that causes serious hospital acquired infections and threatens public health worldwide. MRSA infections are generally resistant to many antibiotics [3]. Diseases caused by pathogenic *E. coli* and MRSA strains in humans are generally treated with antibiotics, but the resistance developed by the strains against antibiotics increases the duration and cost of treatment, and sometimes the treatment fails. Unconscious approaches such as using the wrong dose of antibiotics, using antibiotics out of necessity, and not using the right antibiotics for treatment cause pathogenic bacteria to acquire multiple antimicrobial resistance instead of killing them. Although most of the resistance mechanisms developed by the *E. coli* and MRSA species against antimicrobials are known, an antimicrobial that can overcome

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all these mechanisms has not yet been developed [4]. Due to increasing resistance, it is now understood that the fight against pathogenic bacteria cannot continue with classical antibiotics. Therefore, it is necessary to develop alternatives to antimicrobials to which bacteria cannot rapidly develop resistance and to make them available for the treatment of bacterial infections as soon as possible. The fact that bioactive compounds in the composition of essential oils obtained from plants show antimicrobial and antibiofilm effects has increased the interest in essential oils in recent years [5, 6]. In addition, it is thought that they do not have toxic effects due to their natural structure and the development of bacterial resistance to chemicals in essential oils becomes difficult [7]. Essential oils can be an adjuvant and/or alternative to antibiotics because they do not harm human health and are both cheap and easily accessible.

There are 28 *Origanum* species in Turkey. *Origanum onites* are among the important export products of Turkey. *Origanum* species growing in Turkey are used as spices, condiments, and folk remedies. It has many uses due to its stomachic, sedative, antimicrobial, antitumor, antioxidant, antiseptic, antihelminthic, cardiovascular, and stimulant properties [8].

This study aimed to investigate the antibacterial and antibiofilm activity of the *O. onites* essential oil (OEO) and its bioactive compound (carvacrol) against *E. coli* and MRSA.

2. MATERIALS AND METHODS

2.1. Essential Oil

The OEO used in this study was obtained commercially which was obtained from *O. onites* leaves (country of origin; Turkey) by water vapor distillation. The OEO was dissolved in 10% dimethyl sulfoxide (DMSO) to prepare a 10 mg/mL stock solution. It was sterilized by filtration with a 0.22 µm Millipore filter.

2.2. Test Microorganism

Escherichia coli (ATCC 25922) reference bacterial strain and Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) reference strain were used in this research.

2.3. Antimicrobial Agent

Carvacrol (Sigma-Aldrich, W224511, ≥ 98.5% purity) was dissolved in 10% DMSO to prepare 10 mg/mL stock solution and then was sterilized by filtration by 0.22 µm Millipore filter.

2.4. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The chemical content of the OEO was analyzed by Shimadzu QP 5050 (Kyoto, Japan) GC-MS system. Varian CP WAX 52 CB capillary column (50 m x 0.32 mm ID, df: 1.2 µm) was used as the separation column. Helium (99.999%) was used as carrier gas with a constant column inlet pressure of 10 psi (1 psi = 6894.76 Pa). A volume of 0.01 mL was injected into the device. The GC oven was programmed as follows: The initial column temperature was set at 60^0C, maintained constant for 1 min, then increased to 220^0C with a temperature increase of 2^0C/min, and maintained constant at the final temperature of 220^0C for 20 min. Library searches were performed using Nist, Wiley, and Tutor libraries. The ion source temperature and injection block temperatures were set to 250 and 280^0C, respectively. The emission current of the ionization filament was set to an energy of 70 eV.

2.5. Disk Diffusion Method

The antibacterial activity of the OEO and carvacrol was determined *in vitro* using the disk diffusion method according to Clinical Laboratory Standards Institute Standards (CLSI) [9]. The OEO and carvacrol were prepared at 10, 2, 1, 0.5, and 0.25 mg/mL concentrations. *E. coli* and MRSA were incubated in Mueller Hinton Broth (MHB) medium at 37°C for 18-24 h and the turbidity of the prepared suspensions was adjusted to 0.5 McFarland ($1x10^8$ CFU/mL). *E.coli* and MRSA suspension (0.1 mL) were taken and sown on Mueller Hinton Agar (MHA) medium according to the spreading plate method and allowed to dry for 5-10 min. Sterile blank disks (6 mm diameter) were impregnated with 20 µL of the OEO and carvacrol prepared at five different concentrations. The 10% DMSO-impregnated disk was used to see the solvent effect (also as the negative control) and Gentamicin (10 µg, Oxoid) antibiotic disk was used as the positive control. The disks were placed on the surface of the agar medium using sterile forceps. The media were incubated in an incubator at 35°C for 24 h. After incubation, the zone diameter (mm), which indicates the zone of inhibition, was measured.

2.6. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The OEO and carvacrol showing an inhibition zone in the disk diffusion test were further tested to determine the MIC values by broth macro-dilution dilution method according to the recommendations of CLSI [10]. The turbidity of the bacterial suspensions, which were incubated in MHB medium at 37°C for 18-24 h, was adjusted to 0.5 McFarland ($1x10^8$ CFU/mL). 2 mL of stock OEO and carvacrol were added to the first test tubes containing 10% DMSO, and two-fold serial dilutions were prepared at a concentration range of 800-6.25 µg/mL. An equal volume of bacteria was added to the test mediums to make the final density of the bacteria per tube $\sim 5x10^5$ CFU/mL. Thus, the final concentrations of the OEO and carvacrol in the test tubes, diluted at a ratio of 1:2, were 400-3.125 µg/mL. Positive controls (bacteria + medium) and negative controls (medium alone and the essential oil alone) were also established. The lowest concentrations of the OEO and carvacrol that inhibited the visible growth of bacteria after overnight incubation at 37°C were recorded as the MIC values. MBC is the lowest concentration of antimicrobial required to kill a particular bacterium. To determine the MBC values, 0.1 mL of the dilutions without bacterial growth were spread on MHA media and incubated at 37°C for 24 h. At the end of the incubation, the OEO and carvacrol concentrations in the Petri dishes, where there was no bacterial growth, were recorded as the MBC values [11].

2.7. Antibacterial Curve Assay

The MIC and 1/2MIC values of the OEO and carvacrol were selected as the test concentrations. The OEO and carvacrol were added to the MHB mediums at the determined concentrations. Bacterial inoculums, adjusted to 0.5 McFarland, were added to the tubes to achieve a suspension containing $\sim 5x10^5$ CFU/mL. A tube containing medium and bacteria ($\sim 5x10^5$ CFU/mL) was also prepared as a growth control. The tubes were incubated at 35ºC at 120 rpm in a rotary incubator shaker. At certain interaction times (0, 4, 8, 12, 16, 20, and 24 h), 0.1 mL was taken from each tube and diluted in physiological saline at 1/10, 1/100, and 1/1000 rates. From each dilution, 0.1 mL was taken and spread on blood agar. The media was incubated at 35ºC for 18-24 h. Then, the colonies were counted and the number of viable bacteria (CFU/mL) was determined for each incubation period. The arithmetic means and the logarithms of the viable bacterial counts in the experimental and control series were calculated. The interaction times and the $\log_{10}$ values of the corresponding viable bacterial counts are shown in the graph.

2.8. Biofilm Assay

The effect of the OEO and carvacrol on biofilm formation was analyzed by using polypropylene plastic tubes and modifying the method reported by Gómez-Sequeda et al. [12]. *E. coli* and MRSA were grown

in Luria Bertani Broth medium at 37ºC for 16-18 h. The effect of the OEO and carvacrol on biofilm formation was tested at subMIC (1/2MIC, 1/4MIC, and 1/8MIC) concentrations. Positive control (bacteria + medium) and negative controls (medium alone and essential oil alone) were also established. Approximately 5×10^5 CFU/mL *E. coli* and MRSA suspensions interacted with the OEO and carvacrol (1/2MIC, 1/4MIC, and 1/8MIC final concentrations) and incubated at 37°C for 48 h. At the end of 24 h, the contents of the plastic tubes were poured out and washed 3 times with sterile saline (0.9%) and after the washing process was completed, the tubes were dried in an oven at 60°C for 45 min. Each tube was stained with 0.2 mL of 0.4% crystal violet and incubated for 15 min at room temperature. The tubes were then washed 3 times with 0.9% sterile saline solution to remove excess stain. After washing, 0.2 mL of 95% ethanol was added to the tubes. After 15 min, the absorbance values of the samples were read at 595 nm. Finally, the inhibition percentages of each of the OEO and carvacrol concentrations were calculated using the formula below.

Inhibition percentage= 100 x ($OD_{negative\ control} - OD_{experimental}$) / $OD_{negative\ control}$)

Statistics

All tests were performed in triplicate. All data obtained in the study were analyzed using the "SPSS (Statistical Package for the Social Sciences Inc., Chicago, IL, USA) 16.0" statistical program. The data were presented as arithmetic mean ± standard deviation. The conformity of the data to normal distribution was determined by the Shapiro-Wilk test. "One Way ANOVA" was applied to test the differences and $p<0.05$ was accepted as a statistical significance level.

3. RESULTS

3.1. GC-MS Analysis

The mass percentages and retention times of the components obtained as a result of GC-MS analysis of the OEO are given in Table 1. A total of 44 compounds were identified in different percentages. It was seen that the highest value belongs to carvacrol with 53%; followed by linalool with 13.05%, and p-cymene with 12.64% rate. The peak numbers in this table are also shown in the chromatogram in Figure 1.

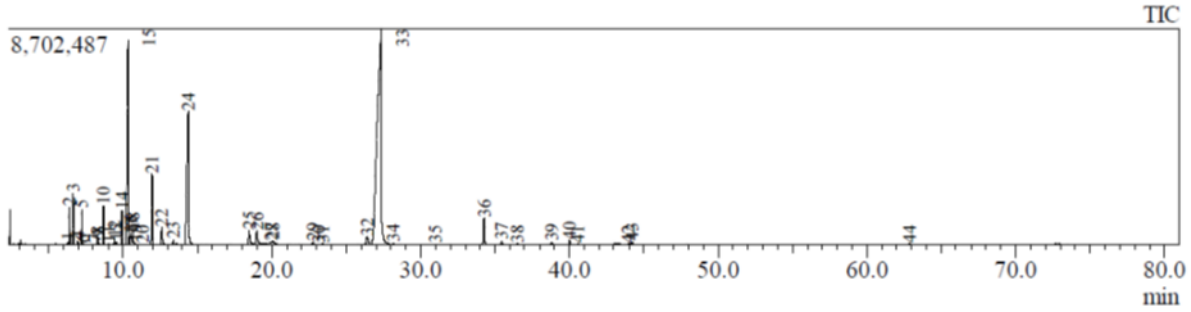


Figure 1. Chromatogram of the *O. onites* essential oil by GC-MS analysis. The x-axis represents the retention time and the y-axis represents the abundance.

Table 1. GC-MS analysis of *O. onites* essential oil

Peak	Retention time	Component	% Ratio
1	6.296	Tricyclene	0.07
2	6.381	α-Thujene	1.30
3	6.644	α-Pinene	1.82
4	6.968	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)-	0.12
5	7.212	Camphene	1.28
6	7.339	Verbenene	0.03
7	8.044	Sabinene	0.01
8	8.248	2-β-Pinene	0.34
9	8.309	1-Octen-3-ol (CAS) Oct-1-en-3-ol	0.25
10	8.693	β-Myrcene	1.52
11	9.046	3-Octanol (CAS) n-Octan-3-ol	0.03
12	9.430	l-Phellandrene	0.23
13	9.552	Delta-3-Carene	0.12
14	9.922	α-Terpinene	1.54
15	10.335	p-Cymene	12.64
16	10.508	Bornylene	0.49
17	10.569	β-Phellandrene	0.30
18	10.656	Eucalyptol (1,8-Cineole)	0.57
19	10.820	cis-Ocimene	0.06
20	11.338	B-Ocimene Y	0.02
21	11.957	γ-Terpinene	3.37
22	12.595	trans-Sabinene hydrate	1.10
23	13.381	α-Terpinolene	0.24
24	14.379	Linalool	13.05
25	18.464	Borneol L	0.97
26	19.002	4-Terpineol	0.92
27	19.945	B-Fenchyl Alcohol	0.17
28	20.085	B- Fenchyl Alcohol	0.33
29	22.755	Benzene, 1-methoxy-4-methyl-2-(1-methylethyl)-	0.16
30	23.146	Pulegone	0.02
31	23.434	2-Cyclohexen-1-one, 2-methyl-5-(1-methylethenyl)-, (R)-	0.05
32	26.409	Thymol	0.96
33	27.363	Carvacrol	53.00
34	28.179	2-Ethyl-5-N-propylphenol	0.05
35	30.994	Phenol, 5-methyl-2-(1-methylethyl)-, acetate	0.02
36	34.274	Caryophyllene	1.82
37	35.449	Aromadendrene	0.23
38	36.492	α-Humulene	0.07
39	38.808	Viridiflorene	0.14
40	40.014	Bisabolene <beta->	0.23
41	40.561	Cadinene <delta->	0.04
42	43.981	Spathulenol	0.05
43	44.205	(-)-Caryophyllene oxide	0.19
44	62.895	4a-methyl-1,2,3,4,4a,5,6,7-octahydronaphthalene	0.08
Total			100.00

The highest peak value seen in the chromatogram belongs to carvacrol and is peak number 33. The molecular structure of carvacrol is shown in Figure 2.

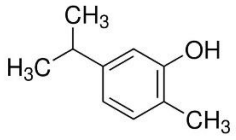


Figure 2. Molecular structure of carvacrol

3.2. Antibacterial Activity Test

In vitro antibacterial activities of the OEO and carvacrol were tested by the disk diffusion method. The zone diameter of the gentamicin antibiotic disk was measured as 23.00±1.00 mm and 24.00±1.00 against *E. coli* and MRSA, respectively. The 10% DMSO showed no inhibitory effect on *E. coli* and MRSA. For better determination of the antibacterial effect, stock OEO and carvacrol were diluted with 10% DMSO. The OEO and carvacrol were formed 48.23±0.50 mm, 45.80±1.77 mm, 38.73±1.38 mm, 30.06±0.40 mm, 22.06±1.44 mm and 49.96±2.45, 45.73±2.70, 39.26±2.80, 33.30±0.62, 24.00±1.58 inhibition zones against *E. coli* at 10, 2, 1, 0.5 and 0.25 mg/mL concentrations, respectively. The OEO and carvacrol were formed 52.70±0.26 mm, 47.03±1.68 mm, 41.00±1.22 mm, 34.23±0.66 mm, 22.00±1.60 mm and 54.36±0.77, 48.23±1.16, 42.33±0.97, 35.56±1.15, 23.33±1.13 inhibition zones against MRSA at 10, 2, 1, 0.5 and 0.25 mg/mL concentrations, respectively (Table 2).

3.3. Determination of MIC and MBC

The MIC values of the OEO and carvacrol, which were designated to show antibacterial activity against *E. coli* and MRSA. The lowest concentrations of the OEO that visually inhibited the growth of *E. coli* and MRSA were determined as 100 µg/mL and 50 µg/mL, respectively. The lowest concentrations of the carvacrol that visually inhibited the growth of *E. coli* and MRSA were determined as 100 µg/mL and 50 µg/mL, respectively (Table 3).

A 0.1 mL aliquot was taken from the tubes in which no visual growth was observed in the MIC test and spread inoculated on MHA medium. After incubation at 37°C for 24 h, OEO and carvacrol concentration in the Petri dishes without bacterial growth was determined as MBC. The MBC values of OEO against *E. coli* and MRSA were determined as 200 and 100 µg/mL, respectively. The MBC values of carvacrol against *E. coli* and MRSA were determined as 200 and 100 µg/mL, respectively (Table 4).

Table 2. Antimicrobial activity of *O. onites* essential oil and carvacrol.

	Dilution rates (w/v)	***E. coli***	**MRSA**
***O. onites* essential oil**	10 mg/mL	48.23±0.50**	52.70±0.26**
	2 mg/mL	45.80±1.77**	47.03±1.68**
	1 mg/mL	38.73±1.38**	41.00±1.22**
	0.5 mg/mL	30.06±0.40**	34.23±0.66**
	0.25 mg/mL	22.06±1.44ns	22.00±1.60ns
Carvacrol	10 mg/mL	49.96±2.45**	54.36±0.77**
	2 mg/mL	45.73±2.70**	48.23±1.16**
	1 mg/mL	39.26±2.80**	42.33±0.97**
	0.5 mg/mL	33.30±0.62*	35.56±1.15**
	0.25 mg/mL	24.00±1.58ns	23.33±1.13ns
Gentamicin		23.00±1.00	24.00±1.00
DMSO		-	-

The diameter of the zone of inhibition is expressed in millimeters. Values are presented as means±SD. Gentamicin (10 µg) antibiotic disk was used as the positive control. DMSO, Dimethyl sulfoxide was used as the negative control. Inhibition zones include the disk diameter (6 mm). *p≤0.01 compared with the positive control, **p≤0.0001 compared with the positive control, ns: non-significant (p≥0.05).

Table 3. MIC values of *O. onites* essential oil and carvacrol

	Dilution rates of *O. onites* essential oil (µg/mL)										
	400	200	100	50	25	12.5	6.25	3.125	PC	NC1	NC2
E. coli	-	-	-	+	+	+	+	+	+	-	-
MRSA	-	-	-	-	+	+	+	+	+	-	-
	Dilution rates of carvacrol (µg/mL)										
	400	200	100	50	25	12.5	6.25	3.125	PC	NC1	NC2
E. coli	-	-	-	+	+	+	+	+	+	-	-
MRSA	-	-	-	-	+	+	+	+	+	-	-

+, growth present; -, no growth (bactericidal); PC, positive control (bacteria + medium); NC1, negative control1 (medium alone); NC2, negative control2 (essential oil alone).

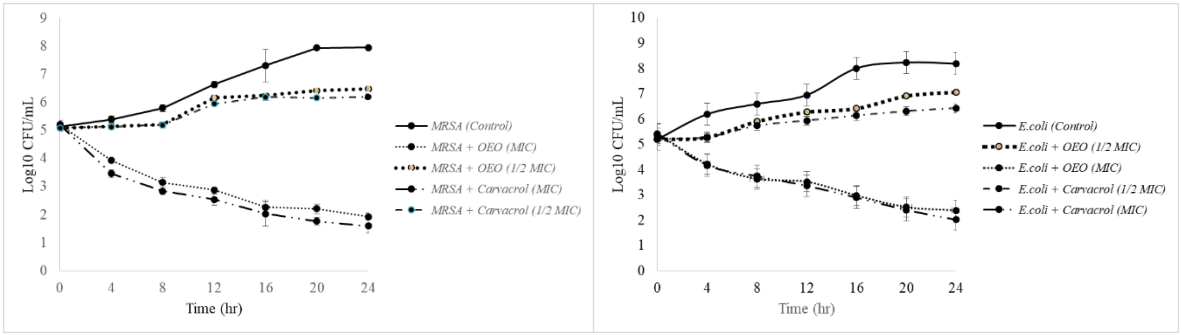
Table 4. MBC values of *O. onites* essential oil and carvacrol

	Dilution rates of *O. onites* essential oil (µg/mL)						
	50	100	200	400	PC	NC1	NC2
E. coli	nd	++	-	-	+++	-	-
MRSA	+	-	-	-	+++	-	-
	Dilution rates of carvacrol (µg/mL)						
	50	100	200	400	PC	NC1	NC2
E. coli	nd	+	-	-	+++	-	-
MRSA	+	-	-	-	+++	-	-

-, no growth (bactericidal); +, low growth; ++, moderate growth (bacteriostatic); +++, high growth (no antibacterial potential); PC, positive control (bacteria + medium); NC1, negative control1 (medium alone), NC2: negative control2 (essential oil alone); nd: not determined.

3.4. Antibacterial Curve Assay

The OEO and carvacrol prepared at MIC and 1/2MIC concentrations interacted with bacteria and their growth inhibitory effects were investigated. The arithmetic averages of the values of the number of viable bacteria at each interaction time were calculated. These values were accepted as the number of viable bacteria changing against time and $\log_{10}$ values were found. The same procedures were applied to the results in the control series (Figure 3A, B). The initial inoculums for *E. coli* and MRSA were determined as $1.6x10^5$ and $1.4x10^5$ CFU/ml. As a result of this assay, it was observed that the bacteria remained viable from 0 to 24 h with gradually decreasing amounts. When OEO interacted with *E. coli* at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24-hour was calculated as $2.9x10^2$ and $1.1x10^7$ CFU/mL, respectively. When carvacrol interacted with *E. coli* at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24 hours was calculated as $1.0x10^2$ and $2.7x10^6$ CFU/mL, respectively. When OEO interacted with MRSA at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24-hour was calculated as $8.6x10^1$ and $3.0x10^6$ CFU/mL, respectively. When carvacrol interacted with *E. coli* at MIC and 1/2MIC concentrations, the number of viable bacteria at the end of the 24 hours was calculated as $4.3x10^1$ and $1.5x10^6$ CFU/mL, respectively. In the control series, it was observed that the number of viable bacteria increased as the incubation time progressed.



(a) (b)

Figure 3. Plots of the mean values for the logarithm of colony forming units per milliliter versus time for *O. onites* essential oil and carvacrol tested on *E. coli* (a) and MRSA (b). The *O. onites* essential oil and carvacrol at MIC and 0.5xMIC concentrations were added at timepoint 0 and measurements were taken at 4-hour intervals for 24 hours. Each point represents the mean ± SD of three independent experiments.

3.5. Biofilm Assay

In this study, the effect of the OEO and carvacrol on biofilm formation was tested by the quantitative crystal violet method. The OEO and carvacrol were assessed for their ability to reduce the formation of bacteria biofilm using concentrations ranging from 1/2MIC, 1/4MIC, and 1/8MIC. The inhibitory effects of the OEO and carvacrol on biofilm formation in *E. coli* reached a statistically significant level at 25 µg/mL, and 12.5 µg/mL, respectively ($p \leq 0.0001$). The inhibitory effects of the OEO and carvacrol on biofilm formation in MRSA reached a statistically significant level at 6.25 µg/mL ($p \leq 0.001$ for OEO, $p \leq 0.0001$ for carvacrol). The formation of *E. coli* and MRSA biofilms was decreased with increasing the OEO and carvacrol concentrations (Figure 4 A, B).

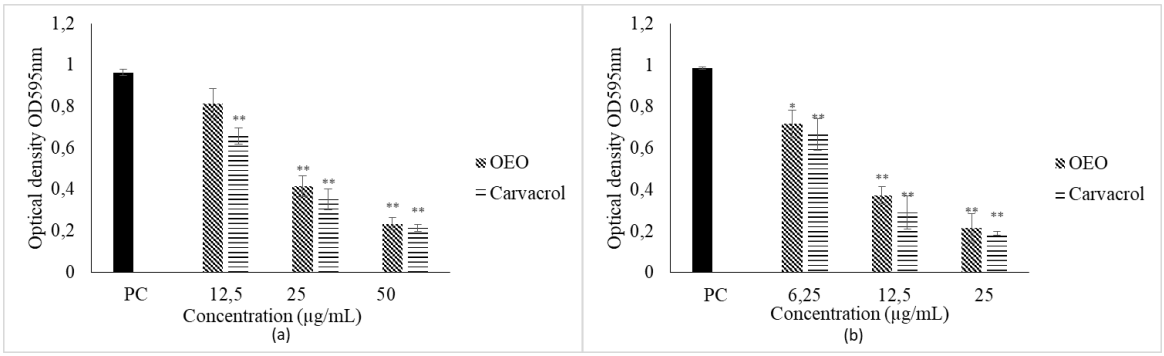


Figure 4. The effect of *O. onites* essential oil and carvacrol on biofilm formation in *E. coli* (a) and MRSA (b). PC, positive control (bacteria+medium). Each column represents the mean ± SD of three independent experiments. The asterisk indicates significant differences between the Optical Density (OD) values obtained against the different antibacterial agent concentrations evaluated and the control group (*$p \leq 0.001$, ** $p \leq 0.0001$).

The OEO inhibited *E. coli* biofilm formation at 50 µg/mL with 75.7% inhibition, followed by 25 and 12.5 µg/mL with 57% and 15.5% inhibition, respectively. Carvacrol inhibited *E. coli* biofilm formation at 50 µg/mL with 77.8% inhibition, followed by 25 and 12.5 µg/mL with 63.6% and 31.8% inhibition, respectively. The inhibition against MRSA biofilm formation was recorded for the OEO at 25 µg/mL with 78.3% inhibition, followed by 12.5 and 6.25 µg/mL with 62.1% and 27.3% inhibition, respectively. The carvacrol demonstrated inhibition of MRSA biofilm formation at 25 µg/mL with 80.7% inhibition, followed by 12.5 and 6.25 µg/mL with 70.6% and 32.4% inhibition, respectively (data not shown).

4. DISCUSSION

The chemical profile of the OEO was analyzed by GC-MS and a total of 44 components were characterized, representing 100% of the total chromatographic area. The most abundant phytochemical (53%) in the OEO was found to be carvacrol. The results of this study are compatible with the literature, as previous analyses have also concluded that carvacrol is typically the major constituent in the OEO. The carvacrol content in the oil ranges from approximately 50% to 85% [13, 14, 15]. The other main components identified in the OEO were linalool (13.05%), p-Cymene (12.64%), γ-Terpinene (3.37%), α-Pinene (1.82%), Caryophyllene (1.82%), α-Terpinene (1.54%), β-Myrcene (1.52%). These components have also been reported in other studies conducted with the OEO [13, 16, 17]. However, the levels of these components in essential oils have been found to vary. This variability is thought to be influenced by climatic and seasonal factors affecting the chemical composition and quantity of oils, as well as factors such as the type of plant, harvest period, geographical conditions, and distillation technique [18, 19].

In the disk diffusion test, the antibacterial activity of the OEO and carvacrol was investigated against *E. coli* and MRSA. The mean growth inhibition zones for the OEO against *E. coli* at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 22±1.4 mm, 30±0.4 mm, 38.7±1.4 mm, 45.8±1.8 mm, and 48.2±0.5 mm, respectively. The mean growth inhibition zones for the OEO against MRSA at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 22±1.6 mm, 34.2±0.6 mm, 41±1.2 mm, 47±1.6 mm, and 52.7±0.2 mm, respectively. The antibacterial activity of the OEO used in this study was higher than positive control at 0.5, 1, 2, and 10 mg/mL concentrations. It was observed that there was no statistically significant difference between the antibacterial activity of OEO against *E. coli* at a concentration of 0.25 mg/mL (22±1.4 mm) and the positive control ($p \geq 0.05$). In a previous study, ethanol extract obtained from *O. onites* was reported to show antibacterial activity with an inhibition zone diameter of 32.5 mm on *E. coli* at 1/50 concentration [20]. It was reported that the water extract obtained from *O. onites* formed an inhibition zone of 37 mm against *E. coli* ATCC25922 [21]. No study was found showing the antibacterial properties of OEO against MRSA used in this study.

The antibacterial activity of the carvacrol against *E. coli* at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 24±1.5 mm, 33.3±0.6 mm, 39.2±2.8 mm, 45.7±2.7 mm, and 49.9±2.4 mm, respectively. The antibacterial activity of the carvacrol against MRSA at concentrations of 0.25, 0.5, 1, 2, and 10 mg/mL were 23.3±1.1 mm, 35.5±1.1 mm, 42.3±0.9 mm, 48.2±1.1 mm, and 54.3±0.7 mm, respectively. The antimicrobial activity of the carvacrol used in this study was higher than positive control at 0.5, 1, 2, and 10 mg/mL concentrations. Similar to the results of this study, a previous study reported that 400 mg/mL carvacrol created a 35 mm inhibition zone against MRSA [22]. In a study, the mean growth of inhibition zones of carvacrol against *E. coli* at concentrations of 1000, 500, and 250 µg/mL were reported as 38 ± 5, 30 ± 4, and 18 ± 4 mm, respectively [23]. As a result of the disk diffusion test, it was determined that the inhibition zone diameters decreased in direct proportion to the decrease in the concentration of the OEO and carvacrol added to the test medium.

In the present study, the MIC value, which is the lowest OEO concentration that visually inhibits the growth of *E. coli*, was determined as 100 µg/mL. In addition, the MIC value for OEO was determined as 50 µg/mL against MRSA. The MBC value, which is the lowest essential oil concentration that kills 99.99% of bacteria, was determined as 200 µg/mL. In a study, the MIC value of *O. onites* methanol extract against *E. coli* ATCC11230 was reported as 1280 µg/mL [24]. In another study, the MIC value and the MBC value of oregano essential oil from Shanghai Sinopharm Chemical Reagent Co., Ltd (Shanghai, China) against MRSA were reported as 0.4 mg/mL [25]. In a study, it was found that the MIC and MBC of OEO against *S. aureus* were 0.125 and 0.25 mg/mL, respectively [26]. On the other hand, in the present study, MIC values of carvacrol against *E. coli* and MRSA were 100 and 50 µg/mL, respectively. This result was consistent with a previously reported study for the antibacterial properties

of carvacrol against *E. coli* (MIC of 100 µg/mL, and MBC of 100 µg/mL) [27]. In contrast, in another study, the MIC value of carvacrol against *E. coli* ATCC 25922 was reported as 256 µg/mL [28]. A previous study reported that carvacrol had a weak anti-staphylococcal activity against MRSA at a concentration of up to 1000 µg/disk [29]. However, another recent study reported that carvacrol had good anti-staphylococcal activity against MRSA with an MIC of 150 µg/mL [30]. This discrepancy may be due to the different techniques and concentrations used in these studies. The low MIC of carvacrol against Staphylococci was also supported by the present study. The results of these studies, together with the results in the current study, show that both carvacrol and OEO showed good antimicrobial activity against *E. coli* and MRSA. In addition, plant extracts are generally considered bactericidal if the MBC/MIC ratio is ≤4 and bacteriostatic if they are >4 [31]. In this study, the MBC/MIC values were determined as 2, so it can be said that OEO and carvacrol are bactericidal against *E. coli* and MRSA.

Antibacterial curve assay helps to understand the interactions that exist between antimicrobial agents and microbial strains. The assay indicates the time- or concentration-dependent effect of antimicrobial agents on microorganism species. It defines antimicrobial agents as bactericidal/fungicidal or bacteriostatic/fungistatic [32]. As a result of the antibacterial curve assay, it was observed that the bacteria remained viable from 0 to 24 h with gradually decreasing amounts. Bactericidal activity refers to a ≥3 $\log_{10}$ decrease in viability compared to the initial cultivation after 24 hours of exposure to the antimicrobial agent [33]. When OEO interacted with bacteria at MIC concentration, the number of viable *E. coli* and MRSA at the end of 24 hours was calculated as $2.9x10^2$ and $8.6x10^1$ CFU/mL, respectively. A decrease of 3 $\log_{10}$ CFU/mL were observed in *E. coli* and MRSA populations. Similarly, when carvacrol interacted with *E. coli* and MRSA at MIC concentration, the number of viable bacteria at the end of 24 hours was calculated as $1.0x10^2$ and $4.3x10^1$CFU/mL, respectively. A decrease of 3 $\log_{10}$ CFU/mL were observed in *E. coli* and MRSA populations. As a result, the bactericidal effects of OEO and carvacrol on *E. coli* and MRSA were confirmed using time-kill kinetics assay results. The present results were consistent with previous studies reporting that essential oils exhibited similar bactericidal activity with their phenolic compounds [34, 35].

Biofilm-associated infections are difficult to eradicate [36]. The use of antibiofilm agents that prevent bacteria from adhering to the surface may cause free-living cells to be easily attacked by the host immune system or antibiotic treatment [37]. Therefore, in recent years, the need for research focused on the discovery of new antibiofilm agents that prevent biofilm formation has increased. In the present study, the effects of OEO and carvacrol on biofilm formation of *E. coli* and MRSA were tested by the quantitative crystal violet method. The inhibitory effects of the OEO and carvacrol on the biofilm formation of *E. coli* reached a statistically significant level at 25 µg/mL, and 12.5 µg/mL, respectively ($p \leq 0.0001$). The inhibitory effects of the OEO and carvacrol on biofilm formation of MRSA reached a statistically significant level at 6.25 µg/mL ($p \leq 0.001$ for OEO, $p \leq 0.0001$ for carvacrol). The formation of *E. coli* and MRSA biofilms was decreased with increasing OEO and carvacrol concentrations.

OEO inhibited the biofilm formation in *E. coli* by 75.7%, 57%, and 15.5% at 1/2MIC, 1/4MIC, and 1/8MIC concentrations, respectively. In another study conducted with *E. coli* (Strain no: 97010), it was observed that commercially purchased *O. onites* essential oil reduced biofilm formation at subinhibitory concentrations. MIC and 1/2MIC doses have been reported to show a greater effect than 1/4MIC. It has been reported that a statistically significant decrease was observed even at the MIC level in biofilms treated with *O. onites* essential oil [38]. It has been reported oregano oil and carvacrol at sub-inhibitory concentrations (<0.01%, which is 0.2xMIC) showed antibiofilm activity against uropathogenic *E. coli* [39]. Carvacrol inhibited *E. coli* biofilm formation at 50 µg/mL with 77.8% inhibition, followed by 25 and 12.5 µg/mL with 63.6% and 31.8% inhibition, respectively. The inhibition against MRSA biofilm formation was recorded for the OEO at 25 µg/mL with 78.3% inhibition, followed by 12.5 and 6.25 µg/mL with 62.1% and 27.3% inhibition, respectively. The carvacrol demonstrated inhibition of MRSA biofilm formation at 25 µg/mL with 80.7% inhibition, followed by 12.5 and 6.25 µg/mL with 70.6% and 32.4% inhibition, respectively. In previous studies, it has been reported that the MIC value of

carvacrol against MRSA is 150 µg/mL and that 75 µg/mL of carvacrol inhibits biofilm formation by 93% [30]. In the presence of carvacrol (1/2MIC), the mean biofilm formation value for *S. aureus* were reported to be equal to 28.3% [40].

In this study, it was shown that OEO and carvacrol interfere with biofilm formation during planktonic growth. The reasons for this may be due to multiple factors acting synergistically or individually. The antimicrobial activity of oregano oil is mostly attributed to the action of its main phenolic component, carvacrol, which showed significant bactericidal activity when tested separately [41]. On the other hand, carvacrol interacts with the lipid bilayer of the cytoplasmic membrane due to its hydrophobic structure, causing loss of integrity and leakage of cellular materials such as nucleic acids, ATP, and ions [42].

Essential oils tend to be more potent compared to isolated compounds due to their ability to work synergistically, affecting multiple targets simultaneously, unlike pure compounds that typically have a singular mode of action. This multi-targeted approach can enhance the overall effectiveness of essential oils [43]. Other studies have also reported that essential oils rich in carvacrol, such as *O. onites*, have significant antimicrobial activity [44, 45]. Carvacrol, which has a monoterpene structure, is a phenolic compound found in essential oils. It can exhibit hydrophobic properties due to the aromatic chain in its structure and hydrophilic properties due to the phenolic -OH group. These properties in the chemical structure of carvacrol are considered to be the reason for its antimicrobial activity [46]. Carvacrol has also been shown to attack the external membrane of Gram-negative bacteria [47]. However, its main area of action is stated to be the cytoplasmic membrane, causing the transport of ions across the membrane without consuming energy. Cells exposed to carvacrol are thought to change the fatty acid composition of the cell membrane as an adaptation mechanism to maintain membrane structure and function [48, 49]. The use of phenolic compounds is suggested due to the high antimicrobial activity and the variance of phenolic composition in essential oils originating from different geographical locations. However, more researches are needed to determine the effective dosage and various mechanisms of action of OEO and carvacrol for clinical trials. In the light of these results, OEO and carvacrol can be new therapeutic agents for *E. coli* and MRSA.

5. CONCLUSION

One of the alternative strategies suggested to overcome the resistance problem is the use of plant essential oils with antimicrobial properties and the substances found in them. The OEO and carvacrol may be an effective source of natural supplements to treat infections caused by MRSA and *E. coli* bacteria. The findings also suggest that OEO and carvacrol can be used to treat diseases caused by *E. coli* and MRSA without allowing the development of resistance. However, *in vivo* antibacterial evaluation using experimental animals, toxicity tests, and pharmacokinetic properties of the active components (especially carvacrol) need to be investigated.

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CONFLICT OF INTEREST

The author stated that there are no conflicts of interest regarding the publication of this article.

CRediT AUTHOR STATEMENT

Demet Hançer Aydemir: Conceptualization, Investigation, Formal analysis, Writing – Original Draft, Visualization.

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