

Antibacterial Properties of Carvacrol against Antibiotic-Resistant Bacteria, Enteric Bacteria, and Oral Pathogens

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ABSTRACT: The present study investigated the antibacterial activity of carvacrol against Vancomycin-Resistant *Enterococci* (VRE), Carbapenem-Resistant *Enterobacteriaceae* (CRE), Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Streptococcus mitis*, *Streptococcus mutans* CNCTC 8/77 and *Streptococcus salivarius* CNCTC 64/59. According to the results of the disc diffusion method, carvacrol has extremely high antibacterial activity with zones of 28 mm, 38 mm, 39 mm, 36 mm, 50 mm, 41 mm, 47 mm, and 39 mm against *E. coli*, *E. faecalis*, VRE, CRE, *S. salivarius*, *S. mutans*, MRSA, *S. mitis*, respectively. In the present study, the Minimum Inhibition Concentration (MIC) was also investigated by using the Broth Microdilution method. Carvacrol has MIC of 7.62 mg/mL against *E. coli*, 15.25 mg/mL against VRE, 30.5 mg/mL against *E. faecalis*, CRE, and *S. salivarius*, 61 mg/mL against *S. mutans*, 1.90 mg/mL against MRSA and *S. mitis*. Carvacrol is a very strong antimicrobial agent and may be used in important applications in the food and pharmaceutical industry. It may also be a good candidate for developing alternative treatments. The results of the present study may be beneficial for further studies with this antibacterial agent for both academia and industry.

KEYWORDS: carvacrol; oral pathogens; antibacterial activity; antibiotic resistant bacteria; enteric bacteria

1. INTRODUCTION

The number of bacterial pathogens resistant to multiple antibacterial agents has dramatically increased in the last decade. Nowadays, multidrug-resistant bacteria are drawn attention to an emergent global disease and a major public health problem [1-2]. The antimicrobial resistance occurring in bacterial pathogens causes high morbidity and mortality [3]. The number of deaths would increase due to antimicrobial resistance globally to 10 million per year by the year 2050 [4].

Multiple varieties of antibiotics have been used for therapeutic purposes for decades. Besides, they have also been utilized prophylactically across other industries such as agriculture and animal husbandry. Antibiotics, which are used clinically, are often the same or similar to antibiotic compounds used in agriculture. The over-usage of them could also cause drug resistance [5] and environmental pollution [6].

The increase in the number of antibiotic-resistant bacteria and the serious problems encountered in the treatment of infectious diseases. Therefore, the research and development studies of new types of synthetic or natural antimicrobials become a requirement. World Health Organization (WHO) has triggered the increase in research with the theme "Combat drug resistance: no action today means no cure tomorrow" [3].

Essential oils that are isolated from plants are mainly utilized as flavours or fragrances. However, interest has been focused on plants rich in bioactive compounds and essential oils that both have antimicrobial properties. The antimicrobial activities of several essential oils result from the presence of phenolic compounds, like the efficiency of oregano was attributed to carvacrol [7].

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Carvacrol, called to have 5-isopropyl-2-methylphenol, is found in essential oils of oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), pepperwort (*Lepidium flavum*), wild bergamot (*Citrus aurantium bergamia*), and other plants [8]. Carvacrol is a phenolic monoterpene [8] with several properties, including antioxidative, anti-inflammatory, antibacterial, antifungal, antiprotozoal, anticarcinogenic, antidiabetic, antinociceptive, cardioprotective, and neuroprotective [9,10]. Carvacrol is insoluble in water due to its lipophilic property. However, carvacrol is highly soluble in acetone, diethyl ether, and ethanol [8]. The chemical structure of carvacrol was given in Figure 1.

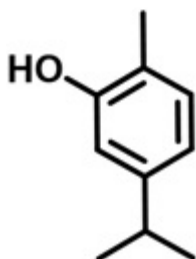


Figure 1. Chemical structure of carvacrol [10]

The ancient Egyptians used carvacrol as a protective agent for preserving the mummies. Carvacrol is also utilized as an active additive in food flavoring, perfumes, cosmetics, mouthwash, and topical ointments. Drugs formulated from carvacrol apply for caring for infections of the mouth and throat and preventing gingivitis. The antibacterial property of carvacrol depends on the capability of permeabilizing, depolarizing, and disrupting of the cytoplasmic membrane [9]. Carvacrol has been confirmed as GRAS (Generally Recognized As Safe) and approved for usage in food [11].

According to our literature research, no study has been conducted to investigate the antimicrobial properties of carvacrol against Vancomycin-Resistant *Enterococci* (VRE), Carbapenem-Resistant *Enterobacteriaceae* (CRE), Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis*. Thus, the present study is the first research study investigating the antibacterial activity of carvacrol against Vancomycin-Resistant *Enterococci* (VRE), Carbapenem-Resistant *Enterobacteriaceae* (CRE), Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis*.

2. RESULTS

This research study investigated the antibacterial properties of carvacrol against *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* CNCTC 8/77, *Streptococcus salivarius* CNCTC 64/59, *Streptococcus mitis* (clinic isolate), Vancomycin-Resistant *Enterococci* (VRE) (clinic isolate), Methicillin-Resistant *Staphylococcus aureus* (MRSA) (clinic isolate) and Carbapenem-Resistant *Enterobacteriaceae* (CRE) (clinic isolate) under aseptic conditions. The antibacterial activities of carvacrol against test microorganisms were shown in Table 1 and Figure 2. The results showed that carvacrol has an extremely high antibacterial activity against all the test microorganisms.

The inhibition zones of the carvacrol were determined to be 28 mm, 38 mm, 39 mm, 36 mm, 50 mm, 41 mm, 47 mm, and 39 mm against *Escherichia coli*, *Enterococcus faecalis*, VRE, CRE, *Streptococcus salivarius*, *Streptococcus mutans*, MRSA, *Streptococcus mitis*, respectively.

Clarithromycin (15 µg) and Amoxicillin (25 µg) were used as positive controls in this study. According to our findings, Clarithromycin and Amoxicillin have no antibacterial effects on Vancomycin-Resistant *Enterococci* and Carbapenem-Resistant *Enterobacteriaceae*. On the other hand, carvacrol has an extremely strong antibacterial activity against Vancomycin-Resistant *Enterococci* and Carbapenem-Resistant *Enterobacteriaceae* with 39 mm and 36 mm inhibition zone diameters, respectively. In addition to these, Clarithromycin and Amoxicillin have slightly antibacterial effects against MRSA with 23 mm and 11.5 mm inhibition zone diameters, respectively. However, carvacrol indicated an extremely strong antibacterial effect against MRSA with a 47 mm inhibition zone diameter. Hence, it can be concluded that carvacrol is strongly effective on Antibiotic Resistance Bacteria (Vancomycin-Resistant *Enterococci*, Carbapenem-Resistant *Enterobacteriaceae*, Methicillin-Resistant *Staphylococcus aureus*).

Table 1. Inhibition zone diameters of carvacrol against test microorganisms

Test Microorganisms	Inhibition Zone Diameter (mm)			
	Control	Carvacrol (10 µL/disc)	Clarithromycin (15 µg)	Amoxicillin (25 µg)
Vancomycin-Resistant <i>Enterococci</i>	0/0/0	36.2/41.7/40.1 39.3±2.8	0/0/0	0/0/0
Carbapenem-Resistant <i>Enterobacteriaceae</i>	0/0/0	37.2/38.5/32.4 36.0±3.2	0/0/0	0/0/0
Methicillin-Resistant <i>Staphylococcus aureus</i>	0/0/0	46.7/42.6/50.5 46.6±4.0*	23.2/23.7/23.2 23.4±0.3	11.4/11.3/11.9 11.5±0.3
<i>Streptococcus mitis</i>	0/0/0	41.1/38.8/38.3 39.4±1.5#	27.8/27.2/27.2 27.4±0.3	15.8/15.5/15.4 15.6±0.2
<i>Streptococcus salivarius</i> CNCTC 64/59	0/0/0	49.3/52.2/50.1 50.5±1.5*	34.6/34.6/34.4 34.5±0.1	39.9/40.1/40.3 40.1±0.2
<i>Streptococcus mutans</i> CNCTC 8/77	0/0/0	38.1/43.2/41.0 40.8±2.6	33.7/30.6/33.4 32.6±1.7	39.3/39.0/38.5 38.9±0.4
<i>Escherichia coli</i> ATCC 8739	0/0/0	28.5/27.6/27.9 28.0±0.5 [§]	11.7/11.9/12.0 11.9±0.2	20.9/21.0/20.6 20.8±0.2
<i>Enterococcus faecalis</i> ATCC 29212	0/0/0	36.4/40.1/38.3 38.3±1.9	0/0/0	17.5/16.8/16.6 17.0±0.5

±SD: Standard Deviation, 95 % confidence interval, critical ratio: $p < 0.05$.

*, #, &, §Different symbols in the same column correspond to significant differences by Tukey test

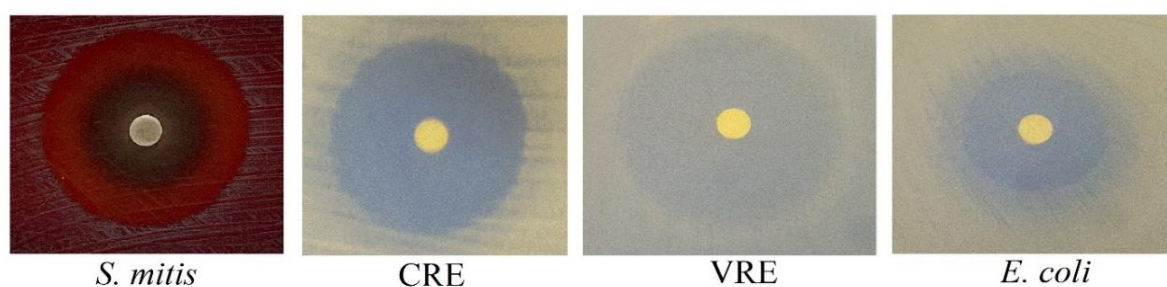


Figure 2. The inhibition zones of carvacrol against *S. mitis*, CRE, VRE, and *E. Coli*

The antibacterial effects of Clarithromycin and Amoxicillin were examined on Viridans Streptococci (*S. mitis*, *S. salivarius*, and *S. mutans*). It was determined that the inhibition zone diameters of Clarithromycin and Amoxicillin against *S. mitis* were 27 mm and 16 mm, orderly. When it was compared with carvacrol, the inhibition zone diameter of carvacrol was 39 mm. For *S. salivarius* the inhibition zone diameters of Clarithromycin and Amoxicillin were 34.5 mm and 40 mm, respectively. On the other hand, the inhibition zone diameter of carvacrol against *S. salivarius* was 50.5 mm. It was also found that the inhibition zone diameters of Clarithromycin and Amoxicillin against *S. mutans* were 33 mm and 39 mm, orderly. Whereas the

inhibition zone diameter of carvacrol for *S. mutans* was 41 mm. To sum up, carvacrol is much more effective than Clarithromycin and Amoxicillin on *S. mitis*, *S. salivarius*, and *S. mutans*.

The antibacterial effects of Clarithromycin and Amoxicillin were investigated on enteric bacteria (*E. coli* and *E. faecalis*). Although the inhibition zone diameters of Clarithromycin and Amoxicillin against *E. coli* were 12 mm and 21 mm, orderly, the inhibition zone diameter of carvacrol was 28 mm. For *E. faecalis*, Clarithromycin has no antibacterial activity. On the other hand, the inhibition zone diameter of Amoxicillin was 17 mm and the inhibition zone diameter of carvacrol was 38 mm. Therefore, the results showed that carvacrol is much more effective than Clarithromycin and Amoxicillin on *E. coli* and *E. faecalis*.

In the present study, the Minimum Inhibition Concentration (MIC) was also investigated by using the Broth Microdilution method. Carvacrol has MIC of 7.62 mg/mL against *Escherichia coli*, 15.25 mg/mL against Vancomycin-Resistant *Enterococci*, 30.5 mg/mL against *Enterococcus faecalis*, Carbapenem-Resistant *Enterobacteriaceae* and *Streptococcus salivarius*, 61 mg/mL against *Streptococcus mutans*, 1.90 mg/mL against Methicillin-Resistant *Staphylococcus aureus* and *Streptococcus mitis*. The MIC values were given in Table 2.

Table 2. Minimum Inhibition Concentration (MIC) (mg/mL)

Test Microorganisms	MIC (mg/mL)
<i>Enterococcus faecalis</i>	30.5
Vancomycin-Resistant <i>Enterococci</i> (VRE)	15.25
Methicillin-Resistant <i>Staphylococcus aureus</i> (MRSA)	1.90
<i>Escherichia coli</i>	7.62
Carbapenem Resistant <i>Enterobacteriaceae</i> (CRE)	30.5
<i>Streptococcus mutans</i>	61
<i>Streptococcus salivarius</i>	30.5
<i>Streptococcus mitis</i>	1.90

3. DISCUSSION

Bnyan *et al.* reported that carvacrol has great antibacterial activity against *Serratia* spp. (28 mm), *Enterobacter* spp. (25 mm), *Klebsiella pneumonia* (23 mm), *Proteus mirabilis* (22 mm), *S. aureus* (20 mm), *S. epidermidis* (22 mm) and *St. pneumonia* (16 mm). Also, Bnyan *et al.* investigated the antimicrobial activity of carvacrol against *E. coli* and determined the inhibition zone of carvacrol against *E. coli* as 26 mm [12]. In this study, we reported the inhibition zone of carvacrol against *E. coli* as 28 mm.

As well as, Wijesundara *et al.* determined that carvacrol showed growth inhibitory effects against strains of *S. pyogenes* with a MIC of 125 µg/mL [13]. In addition to this, Wong *et al.* demonstrated that carvacrol inhibited the growth of *M. avium* subsp. *paratuberculosis* with a MIC of 72.2 µg/mL [14].

Botelho *et al.* investigated the antimicrobial activity of carvacrol against oral pathogens (*S. mutans*, *S. sanguis*, *S. salivarius*, and *S. mitis*) by standard disc susceptibility tests. The inhibition zones of carvacrol against cariogenic bacteria ranged from 7.5 to 15 mm [15]. However, in this study, we determined that the inhibition zones of carvacrol to be 50.5 mm against *Streptococcus salivarius* and to be 41 mm against *Streptococcus mutans*.

Carvacrol caused a dose-related inhibition of the growth of *Bacillus cereus* [16]. Obaidat *et al.* determined that carvacrol, in the vapour phase was able to inactivate *Escherichia coli* O157:H7 [17]. Furthermore, carvacrol also possesses specific efficacy against biofilms of *Staphylococcus aureus* and *Staphylococcus epidermidis*. It makes an impact by targeting the viability of biofilm and the cell morphology in typical biofilm architecture [18].

Kunle reported that the isolated carvacrol from the hexane fraction has tremendous antimicrobial activity against *Staphylococcus aureus* ATCC 13709, *Escherichia coli* ATCC 9637, *Salmonella typhii*, clinical isolate, *Bacillus subtilis* isolated from canned food, and a fungus, *Candida albicans* ATCC 10231 [19].

Mathela *et al.* determined that carvacrol (100 µg/disk) has antibacterial activity against *Streptococcus mutans* MTCC 890 (30 ± 1.29 mm), *Staphylococcus aureus* MTCC 96 (25 ± 1.07 mm), *Bacillus subtilis* MTCC 121 (35 ± 1.41 mm), *Staphylococcus epidermidis* MTCC 435 (32 ± 1.39 mm), *Escherichia coli* MTCC 723 (35 ± 1.49 mm) [20].

Hasanvand *et al.* investigated the optimal time and concentration of carvacrol+ethanol against hospital environmental isolates of *P. aeruginosa* and *S. aureus*. It was determined that the concentration of 64 µl/mL for *P. aeruginosa* and 8 µl/mL for *S. aureus* after 1 h. Thus, they suggested that carvacrol may use as a natural disinfectant and may be an alternative to glutaraldehyde [21].

According to Yin *et al.*, carvacrol has an inhibitory effect on *Aspergillus flavus* and *Aspergillus parasiticus* growth and Aflatoxin production in potato dextrose broth and poultry feed. Yin *et al.* also put forward that carvacrol may use as a feed additive to control Aflatoxin contamination in poultry feed [22]. Also, Coelho *et al.* evaluated the antibacterial activity of carvacrol against shrimp pathogens and its use as a feed additive for the pacific white shrimp. They determined that the MIC of carvacrol was 0.078 mg mL⁻¹ for *Vibrio alginolyticus* and *Vibrio harveyi*. Also, the MIC of carvacrol was 0.156 mg mL⁻¹ for *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Escherichia coli*, and *Lactobacillus plantarum* [23].

Du *et al.* determined that carvacrol had strong antibacterial activity against pathogenic *Escherichia coli*, *C. perfringens*, and *Salmonella*. The MIC of carvacrol was 375 µg/mL for *Escherichia coli*, *Clostridium perfringens*, *Salmonella* Typhimurium, *Salmonella* Pullorum. Also, the MIC of carvacrol was 187.5 µg/mL for *Salmonella* Enteritidis [24].

Trevisan *et al.* investigated the antibacterial and antibiofilm activity of carvacrol against *Salmonella* Typhimurium. As a result of the research, it was found that carvacrol showed antibacterial and antibiofilm activity against *S. Typhimurium*. According to their results, carvacrol may be an alternative for the control of *S. Typhimurium* biofilms [25].

Souza *et al.* investigated the antimicrobial activity of carvacrol against multidrug-resistant *K. pneumoniae*. According to their results, it was found that the use of carvacrol as a therapeutic agent can carry out significant *in vitro* and *in vivo* antimicrobial activity against carbapenemase-producing *K. pneumoniae*, increasing animal survival and seriously decreasing bacterial loads [26].

4. CONCLUSION

In this study we reported that carvacrol has high antibacterial activity against *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* CNCTC 8/77, *Streptococcus salivarius* CNCTC 64/59, *Streptococcus mitis* (clinic isolate), Vancomycin-Resistant *Enterococci* (VRE) (clinic isolate), Carbapenem-Resistant *Enterobacteriaceae* (CRE) (clinic isolate) and Methicillin-Resistant *Staphylococcus aureus* (MRSA) (clinic isolate). The inhibition zone diameters of carvacrol for the test microorganisms were in the range of 28-50 mm.

Furthermore, the Minimum Inhibition Concentration of carvacrol for all the test microorganisms was found to be low and in the range of 1.9-61 mg/mL for *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* CNCTC 8/77, *Streptococcus salivarius* CNCTC 64/59, Vancomycin-Resistant *Enterococci* (VRE) (clinic isolate), Carbapenem-Resistant *Enterobacteriaceae* (CRE) (clinic isolate), Methicillin-Resistant *Staphylococcus aureus* (MRSA) (clinic isolate) and *Streptococcus mitis* (clinic isolate).

The present study exhibited that carvacrol is much more effective than Clarithromycin and Amoxicillin on Vancomycin-Resistant *Enterococci*, Carbapenem-Resistant *Enterobacteriaceae*, Methicillin-Resistant *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus mitis*, *Escherichia coli*, and *Enterococcus faecalis*.

In conclusion, carvacrol is an extremely strong antimicrobial agent and may be used in the food and pharmaceutical industry. It may also be a good candidate for developing alternative treatments. Carvacrol can be considered as an alternative antimicrobial agent against antibiotic-resistant pathogenic bacteria. The results of the present study may be beneficial for further studies about this antibacterial agent for the academia and industry.

5. MATERIALS AND METHODS

5.1. Material

Carvacrol (98%, 282197) was purchased from Sigma-Aldrich. *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Streptococcus mutans* CNCTC 8/77, and *Streptococcus salivarius* CNCTC 64/59 were supplied from İstanbul Medipol University, School of Pharmacy, Microbiology Research Laboratory. Vancomycin-Resistant *Enterococci* (VRE) (clinic isolate), Carbapenem-Resistant *Enterobacteriaceae* (CRE) (clinic isolate), Methicillin-Resistant *Staphylococcus aureus* (MRSA) (clinic isolate), and *Streptococcus mitis* (clinic isolate) were obtained from Medipol Mega Hospitals Complex Microbiology Laboratory.

5.2. Preparation of Overnight Bacterial Culture

Test microorganisms used in the present study were inoculated to Tryptic Soy Broth (Neogen, United Kingdom) and were incubated at 37°C for 24 hours. After the incubation period, microorganisms were inoculated to the 5% Sheep Blood Agar (Becton Dickinson GmbH, Germany). Then, for Viridans Streptococci, the incubation was performed at 37°C for 24 hours in 5-10% CO₂ conditions. Other test microorganisms were incubated at 37°C for 24 hours [27].

5.3. Determination of Antibacterial Activity

The overnight culture was used to obtain bacterial suspension and was adjusted to 0.5 McFarland by using a densitometer (Biosan). Disc diffusion method was applied to determine the antibacterial activity of carvacrol [27]. Sterile discs (6 mm in diameter) were impregnated with 10 µL of carvacrol. Next, bacterial suspension that was adjusted to 0.5 McFarland was inoculated to 5% Sheep Blood Mueller Hinton Agar (Becton Dickinson GmbH, Germany) for Viridans Streptococci and Mueller Hinton Agar (Neogen, United Kingdom) for another test microorganisms with sterile swabs. Finally, the impregnated discs were slightly pressed onto the inoculated Mueller Hinton Agars. The incubation was performed at 37°C for 24 hours. Also, the incubation step was performed under 5-10 % CO₂ condition at 37°C for 24 hours for Viridans Streptococci. After the incubation period, the diameters of the inhibition zone (IZs) were measured. Commercially available antibiotic discs (Clarithromycin 15 µg, Amoxicillin 25 µg) were used as positive controls and a non-impregnated disc was utilized as the negative control. Experimental studies were performed three times under aseptic conditions and the diameters of IZs were the average of the three replicates [27].

5.4. Determination of Minimum Inhibition Concentration (MIC)

The lowest concentration of an antimicrobial agent that is needed to inhibit the visible *in-vitro* growth of a microorganism was determined with the Minimum Inhibition Concentration (MIC) Test. Minimum Inhibition Concentrations of carvacrol for *Enterococcus faecalis*, *Escherichia coli*, CRE, VRE, MRSA, *Streptococcus salivarius*, *Streptococcus mutans*, and *Streptococcus mitis* were determined with Broth Microdilution Method by using 96 well-microplates. 100 µL of carvacrol was transferred to the wells. Two-fold dilution of carvacrol was carried out with physiological saline. The bacterial suspension was obtained from the overnight bacterial culture and was adjusted to 0.5 McFarland with a densitometer (Biosan) by using physiological saline. Then 5×10⁵ CFU/mL bacterial suspension was obtained by dilution. 50 µL of the prepared bacterial suspension (5×10⁵ CFU/mL) was transferred to the wells. Also, 50 µL of Mueller Hinton Broth was added to the wells. The incubation period was performed at 37±1°C for 24 hours. After the incubation period, the turbidity was

evaluated for determining the MIC of carvacrol for test microorganisms. The wells, which contain 100 µL of physiological saline, 50 µL of bacterial suspension, and 50 µL of Mueller Hinton Broth were used as controls [13].

5.5. Statistical analysis

In statistical analysis, one-way ANOVA variance analysis and post-hoc Tukey test were found as mean $\pm$ standard deviation for sample comparison using SPSS version 3.6. software ($p < 0.05$).

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Conflict of Interest

The authors declare no conflict of interest.

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