

Effects of Carvacrol on *Staphylococcus aureus* isolated from bulk tank milk

ERHAN KEYVAN, HIDAYET TUTUN*

Department of Food Hygiene and Technology, *Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Burdur Mehmet Akif Ersoy University, Burdur, 15030, Turkey

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Keyvan E., Tutun H.

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Summary

The occurrence of multidrug-resistant *Staphylococcus aureus* is an important causative agent of mastitis in cattle and of foodborne diseases. It is a worldwide concern, making it essential to develop alternative treatments to fight against the bacteria. Thus, the aim of this study is to determine the ability of carvacrol to inhibit the growth of *S. aureus* isolated from bulk tank milk in Turkey's Burdur Province. All strains (n = 31) were used to investigate the antimicrobial activity of carvacrol, including the methicillin-resistant *S. aureus* and strains from the American Type Culture Collection and England's National Collection of Type Cultures. The minimum inhibitory concentration (MIC) values were determined via a microdilution method, and the antimicrobial susceptibility profiles via a disk diffusion method. Antibiotic resistance was detected in 20 strains (64.5%). Multidrug resistance was observed in 8 strains (25.8%). Carvacrol exhibited strong antimicrobial activity, with MIC value at 0.058-0.234 mg/ml, in the microdilution method. Inhibition zones of carvacrol were in the range of 19 to 45 mm. The results of this study emphasize the promising role of carvacrol among new antibacterial agents that can combat *S. aureus* strains.

Keywords: Carvacrol, drug-resistant bacteria, MIC, *Staphylococcus aureus*

Milk is an essential protein source for humans, and it is also a suitable medium for bacterial growth (31, 33). Milk microbiota affect the quality of dairy products (22). *Staphylococcus aureus* is a human and animal pathogen that causes osteoarticular, endocardial, soft tissue and pulmonary infections (44). *S. aureus* can produce a variety of toxins called staphylococcal enterotoxins, which cause symptoms of food poisoning such as abdominal cramps, diarrhea and vomiting in humans (3, 19, 20). In the dairy industry, bovine mastitis, an inflammation of the udder, is still a global concern because of the costs of antibiotics and other treatments (45). *S. aureus* is a significant cause of mastitis in dairy cows (42, 50). The impact of the bacteria is amplified by increasing drug resistance to beta-lactam antibiotics (β -lactams), especially in the strain called methicillin-resistant *S. aureus* (MRSA) (13). The occurrence of drug-resistant *S. aureus* in raw milk itself and dairy products is a common and worldwide problem (25). Extensive usage of antibiotics, especially in dairy cows, for the treatment or prevention of mastitis or other infections has led to antibiotic-resistant bacteria (37). Due to the adverse effects of antibiotics, natural antimicrobial compounds have been used more and more in recent years (16).

Because resistance to existing antibiotics is increasing, it is necessary to develop alternative strategies or more effective treatment agents. Researchers have proposed using plant extracts containing antimicrobial compounds to prevent the occurrence of multidrug-resistant bacteria and to treat diseases caused by antibiotic-resistant bacteria (4, 48). Carvacrol [2-methyl5-(1-methylethyl)phenol] is the major constituent of essential oils which are found in many plant species such as thyme and oregano. It is known for its wide spectrum of antimicrobial activities (24, 27, 28, 48). It acts on bacteria by increasing the permeability of the cytoplasmic membrane and inhibiting ATPase enzymes that catalyze energy production in living cells (5). In addition, carvacrol exhibits multiple properties, such as antioxidant (32), anti-inflammatory (11), analgesic (49), antifungal (7), antiparasitic (43), insecticidal (8, 18), anticarcinogenic (51), antidiabetic (5), cell-protective (39) and antiplatelet (41) characteristics.

A study (27) has reported a susceptibility to carvacrol among several drug-resistant *S. aureus* strains isolated from pasteurized milk. Nevertheless, very little is known about carvacrol's effect on drug-resistant isolates of *S. aureus*. The objective of this study was to investigate the effectiveness of carvacrol in fighting various strains of *S. aureus*.

Material and methods

Strains origin. In the current study, American Type Culture Collection (ATCC) standard strain *Staphylococcus aureus* ATCC 25923, strains of MRSA ATCC 43300 and National Collection of Type Cultures (NCTC) 13552 (newly *mecA* homologue soon to be assigned *mecC*) were used. Moreover, 31 isolates of *S. aureus* were isolated from different bulk tank milk samples in Burdur province of Turkey.

Identification of *S. aureus* strains. Bulk tank milk samples (0.1 mL) were plated on Baird Parker Rabbit Plasma Fibrinogen agar medium (BP-RPF, Oxoid, Italy) and incubated at 37°C for 24-48 h. Colonies developing a typical coagulase halo on BP-RPF agar were considered suspected of *S. aureus*. Some biochemical tests (Gram staining, catalase reaction, β hemolysis, DNase and ability to coagulate rabbit plasma) were used for the characterization of the isolates (17).

Overnight cultures in Brain Heart Infusion broth (BHI, Oxoid, Italy) were used for the DNA isolation. For this purpose, 2 ml of the broth cultures were centrifuged at 5,000 g. 10 min and the supernatant was discarded. Bacterial pellet was washed twice with 1 mL of the saline solution and centrifuged again. Bacterial pellets were resuspended in 180 μ L Tris EDTA buffer (Sigma-Aldrich, 93283) containing 18 μ L of lysostaphin (0.5 U/ μ L, Sigma, L7386) and incubated at 37°C for 1 h (1). Genomic DNA was extracted according to GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA) manufacturer's protocol.

Gradient polymerase chain reaction (PCR) for *nuc* gene primers (sense, 5'-ATAGGGATGGCTATCAGTAATGT-3', antisense, 5'-GACCTGAATCAGCGTTGTCTT C-3') designed by Lem et al. (21) with temperatures ranging from 54.5 to 57°C was performed. The optimal annealing temperature was detected as 54.5°C. Extracted DNA was confirmed by PCR using the primers. All samples were run in duplicate. The reaction was carried out with the following steps: initial denaturation step for 4 min at 94°C, 30 s at 94°C, 30 s at 54.5°C and 40 s at 72°C for 35 cycles and a final elongation at 72°C for 10 min. Amplified PCR products of each samples were visualized on 1.5% agarose gel electrophoresis.

Antimicrobial susceptibility testing. Minimum antimicrobial susceptibility profile of all milk isolates (n = 31) were determined by using disk diffusion method on Mueller Hinton agar (Oxoid, Italy). The following antibiotics discs were used: chloramphenicol (30 μ g), gentamicin (10 μ g), clindamycin (2 μ g), erythromycin (15 μ g), trimethoprim-

sulfamethoxazole (25 μ g). Antibiotic resistance was detected by analyzing the inhibition zones, measured and compared according the standards set by the Clinical and Laboratory Standards Institute (CLSI) (9) which classified the strains as sensitive, intermediate or resistant to the antibiotics.

Antibiogram to carvacrol. The antimicrobial activity of carvacrol (98%, Cat no.: 923459, J&K) was tested by using the disc diffusion test on Mueller Hinton agar. Sterile paper discs (6 mm in diameter) were soaked with 98% carvacrol (10 μ L). The antimicrobial effect was detected by the appearance of the inhibition zones surrounding the disks. The inhibition zone diameters were measured in millimeters. A disk soaked with phosphate buffer solution (10 μ L) was used as a negative control and tetracycline (30 μ g) was used as positive control (48).

Determination of MIC value. The MIC of carvacrol on standard and 31 isolated strains of *S. aureus* was determined by using the microdilution method according to the CLSI guidelines (10). Stock solution of 50% (v/v) carvacrol were prepared in ethanol (Absolute, Merck). The carvacrol was prepared in concentration of 0.058 mg/ml to 30 mg/ml by a serial 2-fold dilution in the BHI broth media. The turbidity of the bacterial suspension used adjusted to 0.5 McFarland standard for *S. aureus*. Triplicate wells were used for each concentration. Three control tubes were maintained for each test batch (media control, organism control and extract control) After incubation at 37°C for 24 h, microbial growth (turbidity) was determined at 600 nm using a microplate reader (Epoch, BioTek, USA). MIC was the lowest concentration at an OD600 value of 0 in which there was no measureable growth.

Results and discussion

Mastitis is one of the most economically devastating problems in cattle and a difficult disease to control because a wide variety of pathogens can infect the udder. These infections alter milk composition and reduce milk yield (30). Antibiotics are used extensively in the treatment or prevention of bovine mastitis. As mastitis-causing organisms develop resistance to antibiotics, the treatment of this disease becomes more difficult (34, 35). Milk and other dairy products can harbor many organisms that are resistant to conventional antibiotics and can be significant sources of foodborne pathogens (29). Therefore, alternative methods are needed for the treatment and control of infectious diseases associated with drug-resistant pathogens.

Some essential oils of plants, the extracts of these oils (when added to various solvents) and their active substances have antimicrobial effects and are not harmful to human or animal health. They can be used to fight infectious diseases (23, 26). Plants and their essential oils are sources of compounds showing antimicrobial activities against a wide variety of bacteria (23, 46). In particular, oxygenated monoterpenes, which are widespread components of essential oils such as thymol and carvacrol, exhibit strong antimicrobial properties (40).

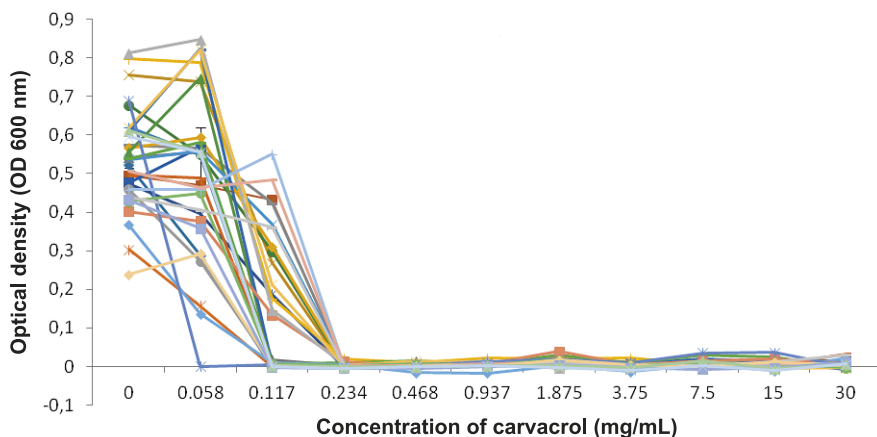


Fig. 1. MICs of carvacrol for *S. aureus* isolates

Carvacrol is recommended as Generally Recognized as Safe (GRAS) by the US Food and Drug Administration (FDA) (12), used as a natural food preservative for foods (e.g. baked goods, sweets, beverages) and as a component of feed supplement to improve health and performance of animals (38).

S. aureus has been known as one of the most common bacteria capable of causing mastitis in dairy cows (15, 47). In addition, it was reported that *S. aureus* is the one of the main causes of antimicrobial treatment applications (14). Thirty one of the isolates were confirmed as *S. aureus* by PCR method with the species-specific *nuc* gene in the current study.

Antibiotic resistance was detected in 20 strains (64.5%). Although a multidrug-resistant profile was observed in 8 strains (25.8%), 11 strains (35.4%) did not develop any resistance against the tested antibiotics in the current study. The most common resistance to the isolates with clindamycin (54.8%), 6.4%, 9.6% and 9.6% of the isolates showed resistance rates to gentamicin, erythromycin and trimethoprim-sulfamethoxazole, respectively. Several studies have shown that multidrug-resistant strains of *S. aureus* are found in foods (6, 44, 48). Occurrence of the multidrug-resistant strains in milks are related to non-discriminatory and excessive use of antimicrobials in the treatment of mastitis in cattle (36).

Inhibition zones of carvacrol (in the range of 19 to 45 mm) showed that all strains of *S. aureus* were susceptible to carvacrol. Inhibition zones of tetracycline were in the range of 8 to 35 mm (Tab. 2). In this study, sensitivity to tetracycline with a zone of 26-30 mm was observed in 14 (45.1%) of the strains obtained from the milk samples. Twenty of the isolates (64.5%) subjected to the carvacrol showed a zone size of larger than those of tetracycline. According to antibiogram, the results of this study are similar to those of Vasconcelos et al. (48).

It was found that all *S. aureus* strains were susceptible to carvacrol in this study. Carvacrol inhibited the bacteria in very small concentrations. The results of carvacrol's antibacterial activity are shown in Tab. 1. It exhibited strong antimicrobial activity with the MIC value at 0.058-0.234 mg/mL in the microdilution method (shown in Fig. 1). MICs of carvacrol for *S. aureus* strains were determined as 0.058 mg/mL in a strain, 0.117 mg/mL in 18 strains and 0.234 mg/mL in 15 strains in the current study. The MIC values obtained are in the range of those performed by Aligiannis et al. (2), Nostro et al. (27) and Nostro et al. (28).

The present study verifies the information on the antimicrobial activity of carvacrol and provides MIC values of carvacrol and multi-drug resistant profile for *S. aureus* isolated from bulk tank milk samples in Burdur province of Turkey. The results may indicate the possibility of adding carvacrol to antimicrobial drug formulations or using it alone as pharmaceutical active ingredient for drugs to treat udder infections caused by *S. aureus* in dairy cows.

Tab. 1. Resistance of *S. aureus* strains to various antimicrobial agents and MICs of carvacrol for *S. aureus* strains

Isolate number <i>S. aureus</i>	Antibiogram	Carvacrol MIC (mg/mL)
1 (ATCC 25923)		0.234
2 (MRSA ATCC 43300)		0.117
3 (NCTC 13552)		0.117
4	Cli	0.117
5	Gen, Cli, Cp	0.117
6	Cp	0.234
7	Cli	0.234
8	-	0.234
9	-	0.234
10	Ery, Cli, Tmp-smx	0.117
11	-	0.117
12	Gen, Cli	0.234
13	-	0.234
14	-	0.117
15	Cp	0.117
16	Cli	0.234
17	-	0.117
18	Cli	0.117
19	Cli, Tmp-smx	0.234
20	Cli	0.117
21	Tmp-smx	0.117
22	-	0.117
23	Cli	0.234
24	Cli, Ery	0.234
25	-	0.234
26	Cli	0.058
27	Cli, Cp	0.117
28	Cli	0.234
29	Cli	0.234
30	Cli, Ery	0.234
31	-	0.117
32	-	0.117
33	Cli, Cp	0.117
34	-	0.117

Explanations: Cli – clindamycin; Cp – chloramphenicol; Ery – erythromycin; Tmp-smx – trimethoprim-sulfamethoxazole; Gen – gentamicin

Tab. 2. Distribution of the *S. aureus* strains according to inhibition zone size against carvacrol and tetracycline

Inhibition halos interval (mm)	<i>S. aureus</i> strains (n = 31)	
	Carvacrol (%)	Tetracycline (%)
8-20	4 (12.9%)	6 (19.3%)
21-25	9 (29%)	7 (22.5%)
26-30	3 (9.6%)	14 (45.1%)
31-35	9 (29%)	4 (12.9%)
36-40	3 (9.6%)	0
41-45	3 (9.6%)	0

Explanations: tetracycline 30 µg, carvacrol 10 µl

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Corresponding author: Asst. Prof. Dr. Erhan Keyvan, Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Burdur, 15030, Turkey; e-mail: erhankeyvan@mehmetakif.edu.tr