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Study of the antimicrobial activity of carvacrol and its mechanism of action against drug-resistant bacteria

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Abstract

Drug-resistant bacterial infections have been one of the critical health issues encountered worldwide currently because most conventional antibiotics are losing their effectiveness in clinical treatments. It is thus urgently to identify new antibiotics or alternatives against drug-resistant bacteria. For this purpose, we attempted to seek active compounds from commercially available natural products, which may be one of the fast-tracks to address the drug-resistant bacterial infections. In the present study, we investigated the antibacterial activity, antibacterial mechanism and synergistic effects of carvacrol against a panel of drug-resistant bacteria, including some clinical isolates. The results show that carvacrol (*cymophenol*), a monoterpenoid phenol, has excellent antibacterial activity. The MIC values against the bacteria examined are found to be 4–16 μg/mL. Our results also suggested that carvacrol might not likely to induce drug-resistance. More importantly, when carvacrol combined with first-line antibiotics, it exhibited good synergistic effects against drug-resistant bacteria. Moreover, in morphological studies, carvacrol could cause *B. subtilis* 168 elongation and *S. aureus* BAA-41 enlargement, which may suggest an antibacterial mechanism possibly correlated with the inhibition of bacterial cell division. We further demonstrated that carvacrol facilitated the polymerization of FtsZ that is a critically important protein for regulating bacterial cell division. Furthermore, molecular modeling predicted that carvacrol could interact with T7-loop of FtsZ. The findings of this study suggest that carvacrol may be a potential inhibitor of FtsZ and it could be utilized to combat drug-resistant bacteria in combination with existing antibiotics.

Keywords

Drug-resistant bacteria; Carvacrol; Antibacterial activity; FtsZ inhibitor

1. Introduction

Antibiotics are crucial medicines for treating various bacterial infections in clinical practice. Nonetheless, bacteria have evolved resistance against most clinically used antibiotics, leading to the emergence of antibiotic crisis. The drug-resistant bacteria are well-known for causing high morbidity and mortality rates and they have been a serious public health threat worldwide [1,2]. Therefore, there is an urgent need to search for novel antibacterial agents and/or effective treatment methods to combat drug-resistant bacteria.

Bacterial proliferation requires a critical process of binary fission, in which the cell replicates its genetic material, then forms a transverse septum in the center of the cell, and finally divides into two daughter cells [3]. Bacterial division is a highly precise regulatory process, coordinated by a variety of division proteins [4]. Filamenting temperature-sensitive mutant Z (FtsZ) is one of the essential division proteins in bacteria, serving as a signal processing center that coordinates the synthesis of cell walls at the site of division. FtsZ is a structural homolog of the eukaryotic cytoskeleton protein tubulin and is highly conserved [5,6]. In the presence of GTP and Mg^{2+}, FtsZ subunits will be aligned head-to-tail to form the protofilaments [6,7]. Treadmilling by the dynamic polymerization of FtsZ, peptidoglycan will be recruited to the septum [7,8]. Eventually, a new cell wall will be formed, completing cell division process [7]. When FtsZ activity is inhibited, bacteria

form elongated narrow filaments or enlarged spherical structures. Bacterial proliferation is thus inhibited. For this reason, FtsZ is considered a potential drug target to inhibit bacterial cell division. In recent years, some FtsZ inhibitors have been reported, including cinnamaldehyde [9] and berberine [10] identified from natural products and some synthetic small-molecule compounds such as PC190723 [11]. These compounds have been demonstrated to show strong antibacterial activity.

Many natural products possess unique pharmacological and may show biological activities and they could provide valuable resources for drug discovery [12,13]. Carvacrol (Fig. 1A) is a natural phenolic monoterpenoid and is readily available from certain essential oils (EOs) of the *Lamiaceae* family, including oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), and other species [14]. Carvacrol exhibits diverse biological activities including anticancer, antioxidant, and anti-inflammatory properties [[14], [15], [16], [17]]. Notably, carvacrol demonstrates significant antimicrobial activity against clinically relevant pathogenic microorganisms [18]. This antimicrobial potential has prompted researchers to investigate its mode of action [[18], [19], [20]]. Furthermore, carvacrol is recognized as safe by regulatory authorities including the U.S. Food and Drug Administration (FDA) and the European Commission, being approved for use as both a flavoring agent and food additive [[21], [22], [23]]. Because carvacrol shows potential to be drug candidate, in the present study, we thus evaluated its *in vitro* antimicrobial activity against a panel of drug-resistant bacteria. To understand the biofunction and activity of carvacrol, we conducted a number of cell-based assays including the study of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), time-killing curves, synergistic effects with conventional antibiotics, and the evaluation of drug resistance. In addition, we studied the antibacterial mechanism of carvacrol and evaluated the effect of carvacrol in inhibiting *S. aureus* FtsZ protein activity.

2. Materials and methods

2.1. Bacteria and reagents

The information on the strains used in this article is listed in Table S1. The following experiments used bacterial culture conditions of inoculating bacteria in TSB at 37 °C shaking at 200 rpm. Carvacrol (50 mg/mL, 98 %) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted to a stock solution (25.6 mg/mL) in DMSO.

2.2. Antimicrobial susceptibility test

According to the Clinical and Laboratory Standards Institute (CLSI) [24], the MIC was determined by the broth microdilution method. Appropriate concentrations of carvacrol and bacterial suspension were added to 96-well plates. The lowest concentration of carvacrol that resulted in no visible bacterial growth was defined as the MIC. Then, 10 µL of the solution was evenly spread onto TSA, and the plates were incubated at 37 °C for 24 h to observe bacterial growth. The lowest concentration of bacterial growth on the plate with <5 colonies was defined as the MBC.

2.3. Time-killing curve assay

Different concentrations of carvacrol were added to make the final concentration 0.5 × , 1 × , 2 × , and 4 × MIC, with 1 % (v/v) DMSO as the control. At different time points, 200 µL of the corresponding bacterial suspension was taken out and the growth curve was determined by measuring the OD_{600}. Additionally, the bacterial suspension was diluted with sterile PBS to an

appropriate concentration, 100 µL was then added to a TSA and spread evenly. Finally, colonies were counted and the CFU/mL was calculated [25].

2.4. Drug resistance study

The initial carvacrol MIC of *S. aureus* ATCC 29213 was determined. Vancomycin and norfloxacin were used as controls. The bacterial solution at the concentration of 0.5 × MIC was passaged to new Mueller-Hinton broth (MHB) and cultured to the logarithmic growth phase to determine MIC of the next passage. The process was repeated for 20 passages consecutively and each MIC result was recorded [26].

2.5. Checkerboard assay

The combined susceptibility of carvacrol and conventional antibiotics against drug-resistant bacteria were determined by checkerboard assay [27]. With a 96-well plate, each antibacterial agent was serially diluted from the concentration of 4 × MIC to 1/16 × MIC. The lowest concentration of the two drug combinations in the wells with no bacterial growth was defined as the MIC of the combined susceptibility. Finally, the Fractional Inhibitory Concentration Index (FICI) was calculated [28]. FICI = CombinedMICAIndividualMICA + CombinedMICBIndividualMICB. “synergistic” if the FICI is ≤ 0.5, “additive” if FICI is > 0.5 and ≤1, “indifferent” if FICI is > 1 and ≤4, “antagonistic” if FICI is > 4.

2.6. Visualizations of bacterial morphology

B. subtilis 168 was diluted by TSB, 0.5 × MIC of carvacrol was added and incubated for 4–5 h. The supernatant was removed by centrifugation, and the precipitate was resuspended in PBS. 10 µL was aspirated onto a glass slide, and the bacterial morphology was observed by a microscope (Zeiss, Germany) at a 40 × objective lens.

0.5 × MIC of carvacrol was added to *S. aureus* BAA-41 and incubated for 12 h. The supernatant was removed by centrifugation, and the precipitate was resuspended in PBS. 2.5 % (v/v) glutaraldehyde fixative was added and fixed at 4 °C. The precipitate was subjected to a series of ethanol gradients and finally resuspended in anhydrous ethanol. The samples were dried and sputter-coated with gold. Images were taken with a scanning electron microscope (JEOL, Japan).

2.7. Polymerization assay

According to the previous protocol [26], purified *S. aureus* FtsZ protein (12 µM) (Cytoskeleton, USA) was prepared in PBS containing 50 mM KCl and 5 mM $MgCl_2$, and carvacrol with different concentrations (final concentrations: 8, 16, 32 µg/mL, respectively) were added, with 1 % (v/v) DMSO as the control. Then, 1 mM GTP was added to initiate the reaction. Reaction mixtures were incubated at 37 °C for 1 h under static conditions, followed by centrifugation at 14,000×*g* for 60 min. Supernatants were collected and subjected to analysis through 12 % SDS-PAGE with Coomassie Brilliant Blue staining.

2.8. GTPase activity assay

The effect of carvacrol on the GTPase activity of FtsZ was measured in 96-well plates using the ATPase/GTPase Activity Assay Kit (Sigma-Aldrich, USA), following the manufacturer's protocol with modifications as previously described [29,30]. Purified *S. aureus* FtsZ protein (12 µM) was incubated with 1 % DMSO or carvacrol (0.125–32 µg/mL) at 25 °C for 30 min. Subsequently, 10 µL of 400 µM GTP solution was added to each reaction mixture, followed by 30 min incubation. Reactions were

terminated by adding 200 µL of reagent buffer, and OD_{620} was measured after 30 min. GTPase activity levels were calculated using a standard curve, and the activity was compared with that of the control group.

2.9. Molecular docking

The molecular modeling was performed using MolSoft ICM 3.8.4 software. The X-ray crystal structure of *S. aureus* FtsZ was downloaded from the PDB database (PDB entry: 4DX) [31]. The protein and carvacrol were prepared for docking using a preparation protocol of DS. The automated docking study was carried out using DSCDocker protocol in the Discovery Studio. The highest-scoring poses were visually inspected.

3. Results

3.1. Antibacterial activities of carvacrol

In this study, our primary aim is to evaluate the potential of carvacrol as a natural antibacterial agent against drug-resistant bacteria. The antibacterial activity of carvacrol was determined via MIC and MBC against a panel of selected bacteria. Since the antibiotic of the first choice for clinical treatment will depend on the species of bacteria, we used different conventional antibiotics as a control for gram-positive and gram-negative bacteria, respectively. Moreover, the MIC values obtained suggest that carvacrol generally shows reasonable antibacterial activity (Table 1).

In a comparison study, the selected gram-positive bacteria were treated with carvacrol, β-lactam antibiotics methicillin and ampicillin, and the glycopeptide antibiotic vancomycin, respectively. Compared methicillin, ampicillin and vancomycin against different bacteria, for methicillin-resistant *Staphylococcus aureus* (MRSA), MICs of methicillin were >128 µg/mL; for vancomycin-intermediate *Staphylococcus aureus* (VISA), MICs of vancomycin were 4–8 µg/mL; for vancomycin-resistant *Enterococcus* (VRE), MICs of vancomycin were >128 µg/mL. Compared to these clinical drugs, carvacrol generally shows better potency against the drug-resistant bacteria tested as indicated by the MICs that were found in the range of 4–16 µg/mL.

Carbapenem antibiotics are commonly used to treat *Enterobacteriaceae*, we thus used meropenem for testing the MIC of gram-negative bacteria while using ampicillin as a control. We find that carvacrol is also effective against carbapenem-resistant *Enterobacteriaceae* (CRE) which are resistant to meropenem (with MIC >32 µg/mL). The MIC experiments may reveal that carvacrol could have a broad-spectrum antibacterial activity and it also suggests both compounds could have the same or similar antibacterial action mechanism.

The definition of MBC is the lowest drug concentration that kills 99.9 % of the test microorganism. We determined the MBC of carvacrol against the test bacteria by plate counting and calculated the ratio of MBC to MIC (Table 2). The MBC and MIC values of carvacrol for all the tested bacteria are extremely comparable, being only 1–2 folds higher. According to the CLSI, when MBC/MIC ≤2, the tested compound is regarded as a bactericide [24]. The experiment results show that carvacrol has excellent bactericidal effects and is regarded as a bactericide.

3.2. Time-killing curve

We further determined the time-killing curves of four different drug-resistant bacteria with carvacrol and explored the dynamic antibacterial regulations at different concentrations of

carvacrol against these selected bacteria. From Fig. 1B, MRSA BAA-41 was treated with 0.5 × MIC of carvacrol, it showed no antibacterial effect. However, when using carvacrol at 1 × MIC, the curve shows a slow decline within 8 h, indicating that the bacterial proliferation may be inhibited by carvacrol. Then, after 8 h, the inhibitory effect was found disappeared. The bacteria enter the recovery and regeneration period. Under the condition with 2 × MIC carvacrol applied, there is a significant bactericidal effect and a reduction in colony number of 2-3log CFU/mL is observed. At 4 × MIC, carvacrol shows a more rapid bactericidal effect. In addition, we observe that there is a slight increase in bacterial count after 4 h, and only 1–2 colonies grow after 24 h. The growth curve (Fig. S1C) also reflects the same trend, where a 0.5 × MIC logarithmic growth phase is delayed by only 2 h, after which bacterial numbers increase rapidly. While a 1 × MIC concentration, it causes the culture to become turbid at 16 h after inoculation. Both 2 × MIC and 4 × MIC concentrations remain clear at 24 h.

Furthermore, we find that carvacrol exhibits a similar TKC and growth curve against CRE strain *K. pneumoniae* 18104 (Fig. 1C and S1D) and BAA-41. After 1 h, at 2 × MIC and 4 × MIC, no detectable growth of live bacteria was observed, indicating a good bactericidal efficacy of carvacrol. In contrast, the bacterial count in *E. coli* 52769 showed a rebound at 2 × MIC after 24 h (Fig. S1A). From the growth curve (Fig. S1F), we find that *E. faecium* DYVRE002 at 1 × MIC only delays the entry into the logarithmic phase by 4 h. Although the bacterial suspension is still clear under 2 × MIC after 24 h, the TKC exhibits a weak antibacterial effect (Fig. S1B). At 4 × MIC, no live bacteria are detected in 1–2 h, but the bacteria enter the recovery and regeneration phase after 4 h. Therefore, a higher carvacrol concentration may be needed to achieve better antibacterial and bactericidal effects against VRE. Taken together, these results may indicate that carvacrol could have good antibacterial and bactericidal effects against most drug-resistant bacteria tested.

3.3. Drug resistance study

Bacterial resistance to conventional antibiotics is a major challenge in the clinical treatment of infectious diseases [32]. To assess the induction of drug resistance by carvacrol, we exposed *S. aureus* ATCC 29213 for 20 passages in the presence of 0.5 × MIC of carvacrol, with norfloxacin and vancomycin as the controls (Fig. 1D). We observe that the bacteria become resistant to norfloxacin soon after exposure, with the MIC increasing by 128-fold at the sixth passage. In contrast, the resistance to carvacrol and vancomycin is not as serious as norfloxacin. Both compounds show a 2-fold increased MIC value over 20 passages. The results suggest that the development of resistance to carvacrol is low. Carvacrol could be considered an active antibacterial agent.

3.4. Synergistic effects of carvacrol with conventional antibiotics

Combination therapies can achieve the treatment goal while reducing the concentration of antibiotics used, thereby achieving multitarget engagement and diminishing the emergence of spontaneous resistance [33]. To investigate whether the combination of carvacrol and conventional antibiotics could exhibit synergistic antibacterial activity against resistant bacteria, we conducted checkerboard assay and calculated the FICI. We tested the combined antibiotic susceptibility of carvacrol with β-lactam antibiotics (methicillin/ampicillin) against 3 MRSA, meropenem against 3 CRE, and vancomycin against 3 VRE.

We find that carvacrol generally has synergistic effects by restoring the antibacterial activity of antibiotics against the drug-resistant bacteria tested. Carvacrol showed synergistic effects with

methicillin (Fig. 2A and S2A) or ampicillin (Fig. 2B and S2B) against the 3 selected MRSA, with FICI ranging from 0.5 to 0.75 (Table 3). It also has additive effects with meropenem against CRE (Fig. 2C and S2C) and vancomycin against VRE (Fig. 2D and S2D), with the FICI found in the range of 0.5–1.

3.5. Effects of carvacrol on bacterial morphology

One of the features of FtsZ inhibitors is that they could interfere with the polymerization of FtsZ and prevent normal cell division [25]. To investigate whether carvacrol affects the normal division of bacteria, we used *B. subtilis* 168 as the study object and the bacteria were treated with carvacrol (0.5 × MIC). We find that the control group of *B. subtilis* 168 (treated only with DMSO) exhibits short cylindrical morphology (Fig. 3A). In contrast, bacteria treated with carvacrol show a trend toward elongation (Fig. 3B). The lengths of the bacteria under the microscope were analyzed statistically. The average length of the control group bacteria is about 7.2 μm (n = 301), while the length of the bacteria treated with carvacrol is 66 μm (n = 134) that is almost 10 times longer compared to the control (Fig. 3C). The length difference is statistically significant ($P < 0.0001$).

In addition, we used *S. aureus* BAA-41 as the study object and the bacteria were treated with carvacrol (0.5 × MIC). The cell morphology was observed under SEM. By statistically analyzing the average diameters of bacteria, we find that the average bacterial diameter of control group treated only with DMSO is 0.71 μm (n = 194). While the average bacterial diameter treated with carvacrol is 0.8 μm (n = 262). The diameter difference is statistically significant ($P < 0.0001$). The SEM analysis shows that carvacrol may significantly enlarge the *S. aureus*. Concerning literature, similar results are also found with reported FtsZ inhibitors, such as quindoline derivatives B10 and celastrol [25,34]. Morphological studies suggest that carvacrol may cause bacteria elongation or enlargement. These results may suggest carvacrol could interrupt bacterial cell division.

3.6. Effects of carvacrol on FtsZ protein polymerization and GTPase activity

To investigate further whether carvacrol affects bacterial division by interrupting the function of FtsZ protein, we treated *S. aureus* FtsZ with different concentrations of carvacrol. The supernatants were subjected to SDS-PAGE followed by grayscale analysis (Fig. 3G). The result suggested that the relative FtsZ protein content remaining in the supernatant treatment with carvacrol at the MIC (8 μg/mL) is approximately 68.84 % (Fig. 3H). This value dramatically decreased to 2.69 % when exposed to 16 μg/mL carvacrol, while negligible residual FtsZ was detected in the supernatant after treatment with 32 μg/mL. These findings demonstrate a dose-dependent enhancement of FtsZ polymerization, as evidenced by the progressive reduction of FtsZ in supernatants with increasing carvacrol concentrations.

Apart from FtsZ polymerization assay, we also investigated the effect of carvacrol on the GTPase activity of FtsZ. However, the result shows that carvacrol exerts little or no impact on the GTPase activity of *S. aureus* FtsZ.

3.7. Binding mode of carvacrol and FtsZ protein

To predict the binding mode of carvacrol and FtsZ protein, molecular modeling was performed to identify the potential binding site. An *X*-ray crystal structure of *S. aureus* FtsZ was utilized as a model to dock with carvacrol. The highest docking score suggests that carvacrol may bind near the T7-loop of FtsZ (Fig. 4A). The molecular docking result also predicts that the hydroxy group of carvacrol could form a hydrogen bond with residue VAL203 of FtsZ. Additionally, carvacrol forms multiple non-polar

interactions with the surrounding hydrophobic residues LEU209, LEU200, and VAL297. These interactions may further enhance the ligand-FtsZs interaction (Fig. 4B).

4. Discussions and conclusions

In the current situation of bacterial drug resistance, there is an urgent need to explore novel antibacterial agents and effective treatment methods to combat drug-resistant bacteria. Carvacrol is considered to be one of the main bioactive constituents in essential oils from oregano and thyme [35,36]. Carvacrol (5-isopropyl-2-methylphenol) is a monoterpenoid compound and it was found that it has antibacterial effects due to its unique chemical structure [37,38]. In this study, we demonstrated that carvacrol exhibits potent antibacterial effects against drug-resistant bacteria and shows low levels of drug-resistance induction through a series of *in vitro* experiments. The checkerboard assay also shows that carvacrol may have synergistic or additive effects in combination with different antibiotics such as methicillin, ampicillin vancomycin and meropenem against drug-resistant bacteria. In a previously described study, carvacrol nanoparticles also have revealed the synergistic effects with quinolone antibiotics ciprofloxacin and doxorubicin [39]. In general, carvacrol could be a potent antibacterial agent for combined treatment with existing antibiotics against infectious diseases.

Similar to other natural products, carvacrol exerts its antimicrobial effects through multi-target mechanisms currently proposed to include: cell membrane damage, proton motive force collapse, inhibition of efflux pumps, and reduction of biofilm formation [38,[40], [41], [42], [43], [44], [45]]. Wijesundara et al. demonstrated that exposure of *Streptococcus pyogenes* to carvacrol at 1/4 × MIC (31.25 μg/mL) induced abnormally elongated shapes with ruptured or broken cell walls, indicating that that membrane damage that carvacrol induces cytoplasmic content leakage represents a key antibacterial mechanism [42]. In our investigation, using *B. subtilis* 168 and *S. aureus* BAA-41 as model organisms, exposure to carvacrol at a relatively lower concentration of 1/2 MIC (4 μg/mL) compared to the literature resulted in an interesting observation: while no bacterial membrane rupture was detected under this concentration, significant morphological alterations including cellular elongation and enlargement were evident. These morphologies may reveal a new antimicrobial mechanism by carvacrol not previously reported, potentially involving interference with bacterial cell division, which prompted our further investigation into the effects of carvacrol on essential cell division protein FtsZ. An *in vitro* FtsZ polymerization assay revealed that carvacrol enhances the polymerization of the cell division protein FtsZ in a dose-dependent manner. However, no significant effect of carvacrol on GTPase activities of FtsZ was found. Molecular docking predicted a potential binding site for carvacrol near the T7-loop region of FtsZ. This observation aligns with reported FtsZ inhibitors such as TXY436 [46], 3-aminobenzamide derivatives [47], and thiazole-quinoline derivatives [48], which similarly exhibit no effect on GTPase activity while binding to the interdomain cleft of FtsZ. Collectively, our findings propose a novel antibacterial mechanism wherein carvacrol promotes FtsZ polymerization by binding to the T7-loop without interfering with its GTPase activity, which lead to abnormal cell division.

In conclusion, we considered that carvacrol exhibits potent antibacterial effects against drug-resistant bacteria, and that carvacrol has the potential to delay the development of bacterial resistance because of its low levels of drug-resistance induction and synergistic or additive effects in combination with different antibiotics. In addition, the antibacterial mechanism of carvacrol may

be related to the inhibition of bacterial cell division protein FtsZ, which could be further studied as a potential inhibitor of FtsZ.

Declaration of competing interest

All authors have read and approved to submit it to your journal. There is no conflict of interest of any authors in relation to the submission.

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Table 1. Minimum inhibitory concentration (MIC, μg/mL) of carvacrol against bacterial strains.

Bacterial strains	Carvacrol	Methicillin	Ampicillin	Vancomycin	Meropenem
(+) *S. aureus* ATCC 29213[a]	8	1	2	1	
(+) *S. aureus* ATCC 43300[b]	8	<32	32	0.5	
(+) *S. aureus* ATCC 33591[b]	8	256	256	0.5	
(+) *S. aureus* BAA 41[b]	8	128	128	2	
(+) *S. aureus* BAA 1720[b]	8	1024	512	0.5	
(+) *S. aureus* BAA 1747[b]	4	256	<0.25	0.5	
(+) *S. aureus* XN108[c]	16	512	>1024	8	
(+) *S. aureus* Mu50[c]	8	>1024	256	4	
(+) *E. faecium* DYVRE002[d]	8	>1024	>1024	>128	
(+) *E. faecium* DYVRE003[d]	8	>1024	>1024	>128	
(+) *E. faecium* DYVRE004[d]	8	>1024	>1024	>128	
(+) *E. faecium* DYVRE005[d]	8	>1024	>1024	>128	
(+) *E. faecium* DYVRE006[d]	8	>1024	>1024	>128	
(+) *E. faecium* DYVRE007[d]	16	>1024	>1024	>128	
(+) *B. subtilis* 168	8	<8	<2	<0.25	
(-) *E. coli* ATCC 25922	16		>1024		<4
(-) *E. coli* ATCC 35218	16		>1024		<4
(-) *E. coli* 58030[e]	8		>1024		32
(-) *E. coli* 52769[e]	8		>1024		128
(-) *K. pneumoniae* ATCC 700603	16		>1024		<4
(-) *K. pneumoniae* 18104[e]	8		>1024		512
(-) *K. pneumoniae* 55581[e]	8		>1024		64

(+) Stand for Gram-positive bacteria; (-) Stand for Gram-positive bacteria;

[a] Methicillin-Sensitive *Staphylococcus aureus* (MSSA)

[b] Methicillin-Resistant *Staphylococcus aureus* (MRSA)

[c] Vancomycin-Intermediate *Staphylococcus aureus* (VISA)

[d] Vancomycin-Resistant *Enterococcus* (VRE)

[e] Carbapenem-Resistant *Enterobacteriaceae* (CRE)

Table 2. MBC/MIC ratios of carvacrol against testing bacterial strains

Bacterial strains	MIC (μg/mL)	MBC (μg/mL)	MBC/MIC
S. aureus ATCC 29213	8	8	1
S. aureus ATCC 43300	8	8	1
S. aureus ATCC 33591	8	8	1
S. aureus BAA 41	8	8	1
S. aureus BAA 1720	8	8	1
S. aureus BAA 1747	4	4	1
S. aureus XN108	16	16	1
S. aureus Mu50	8	16	2
E. faecium DYVRE002	8	16	2
E. faecium DYVRE003	8	16	2
E. faecium DYVRE004	8	16	1
E. faecium DYVRE005	8	16	2
E. faecium DYVRE006	8	16	2
E. faecium DYVRE007	16	32	2
B. subtilis 168	8	8	1
E. coli ATCC 25922	16	16	1
E. coli ATCC 35218	16	16	1
E. coli 58030	8	8	1
E. coli 52769	8	16	2
K. pneumoniae ATCC 700603	16	16	1
K. pneumoniae 18104	8	8	1
K. pneumoniae 55581	8	8	1

Table 3. The synergistic effect of carvacrol in combination with conventional antibiotics against the drug-resistant bacteria selected.

	Alone (μg/mL)		Combination (μg/mL)		FIC index
	methicillin	Carvacrol	methicillin	Carvacrol	
S. aureus BAA-1720	1024	8	256	4	0.75
S. aureus BAA-41	128	8	32	2	0.5
S. aureus ATCC 33591	256	8	64	2	0.5
	Ampicillin	Carvacrol	Ampicillin	Carvacrol	
S. aureus BAA-1720	512	8	128	4	0.75
S. aureus BAA-41	128	8	32	2	0.5
S. aureus ATCC 33591	256	8	64	4	0.75
	Meropenem	Carvacrol	Meropenem	Carvacrol	
K. pneumoniae 18104	512	8	256	4	1
K. pneumoniae 55581	64	8	16	4	0.75
E. coli 52769	128	8	64	4	1
	Vancomycin	Carvacrol	Vancomycin	Carvacrol	
E. faecium DYVRE002	2048	8	1024	1	0.625
E. faecium DYVRE005	1024	8	512	4	1
E. faecium DYVRE007	2048	16	512	4	0.5

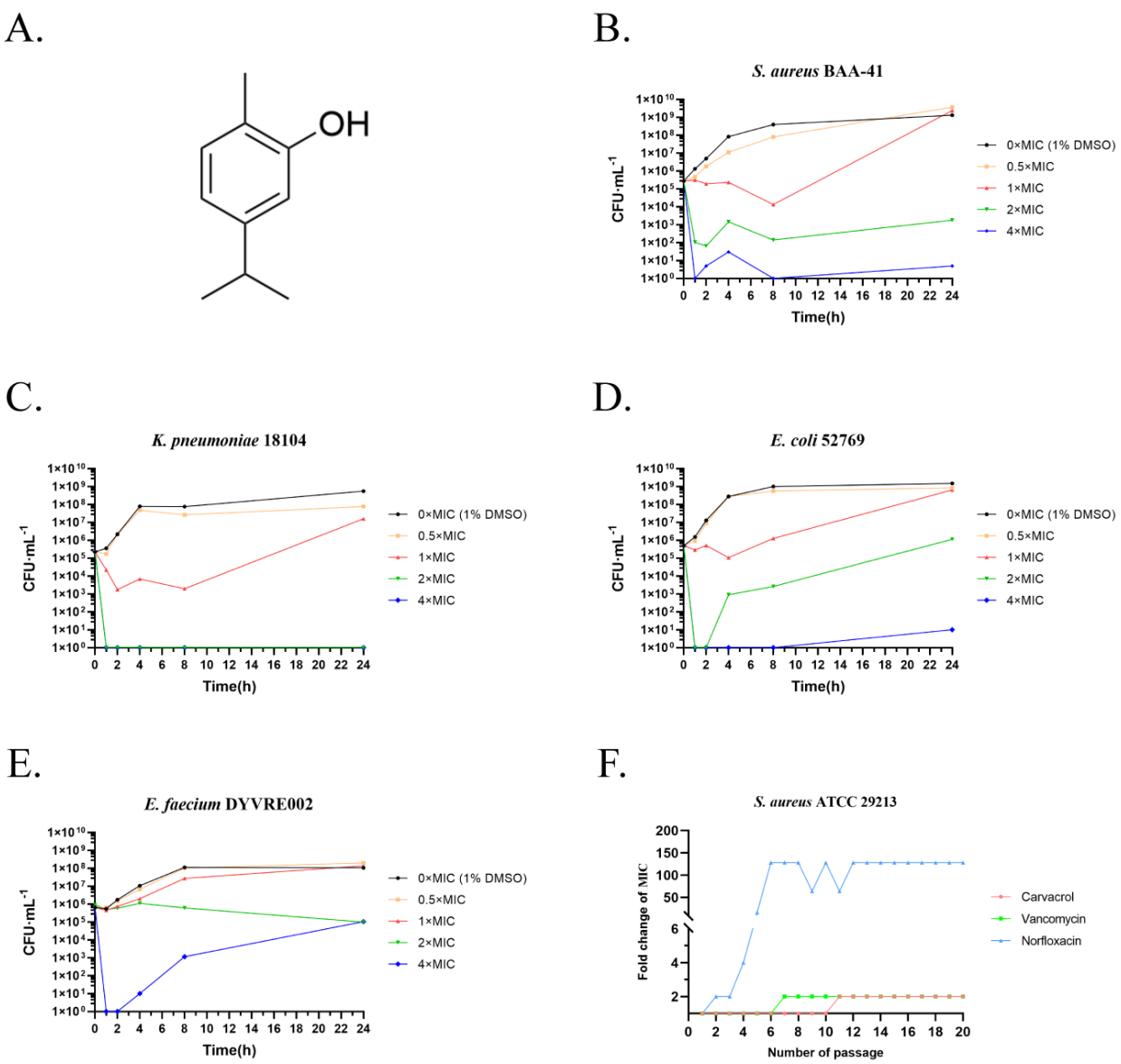

Fig. 1. (A) Chemical structure of carvacrol; Time-killing curves of carvacrol at concentrations of 0.5 × MIC (4 μg/mL), 1 × MIC (8 μg/mL), 2 × MIC (16 μg/mL), and 4 × MIC (32 μg/mL) against MRSA S. aureus BAA-41 (B), CRE K. pneumoniae 18104 (C), 1 % (v/v) DMSO as the control; (D) Drug resistance of carvacrol.

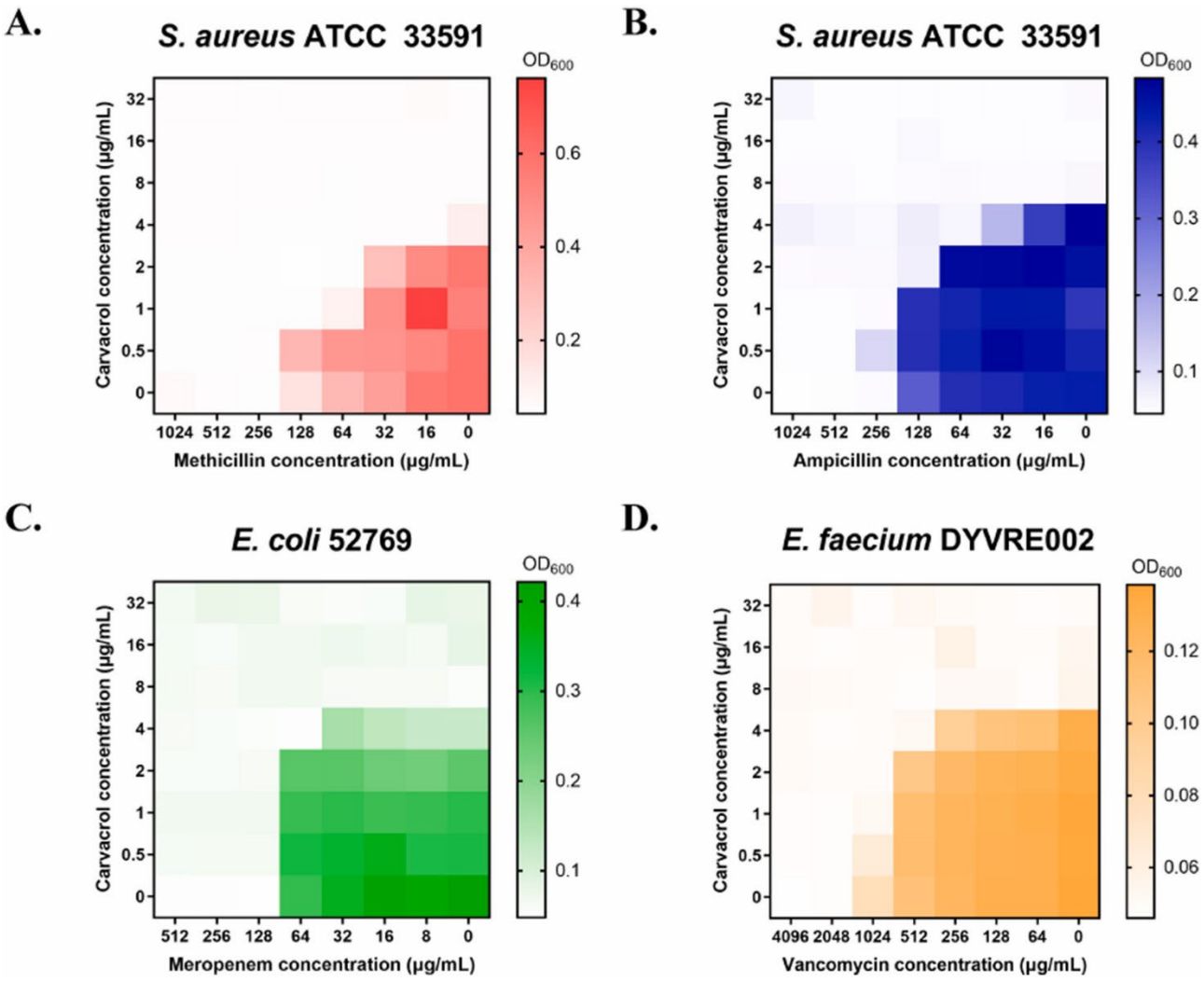

Fig. 2. The thermogram results of carvacrol combined with conventional antibiotics using a checkerboard assay. The synergistic effects of carvacrol with methicillin (A) and ampicillin (B) against MRSA, with meropenem against CRE (C) and with vancomycin against VRE (D).

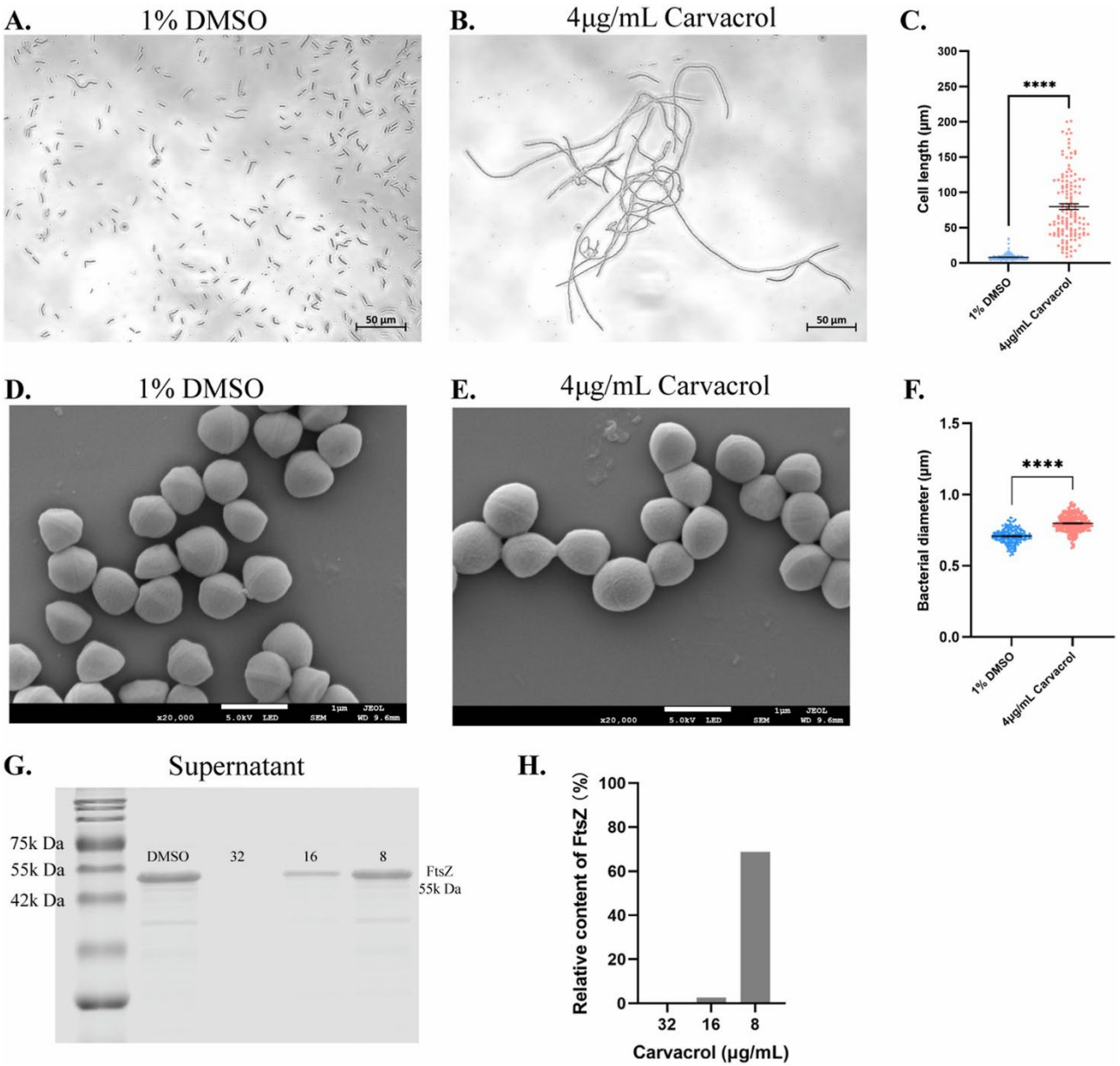

Fig. 3. The morphology of *B. subtilis 168* under the microscope (40 ×) after treatment with 1 % (v/v) DMSO (A) and 0.5 × MIC of carvacrol (4 μg/mL) (B); (C) Statistical graph of the lengths of *B. subtilis* 168 in DMSO and carvacrol; SEM observation of *S. aureus* BAA-41 treated with 1 % (v/v) DMSO (D) and 0.5 × MIC of carvacrol (4 μg/mL) (E); (F) Statistical graph of the diameters of *S. aureus* BAA-41 in DMSO and carvacrol; (G) SDS-PAGE of protein *S. aureus* FtsZ in supernatant treated with 1 % (v/v) DMSO or 8, 4, 2 μg/mL of carvacrol; (H) Graph of relative content of FtsZ in supernatant (%).

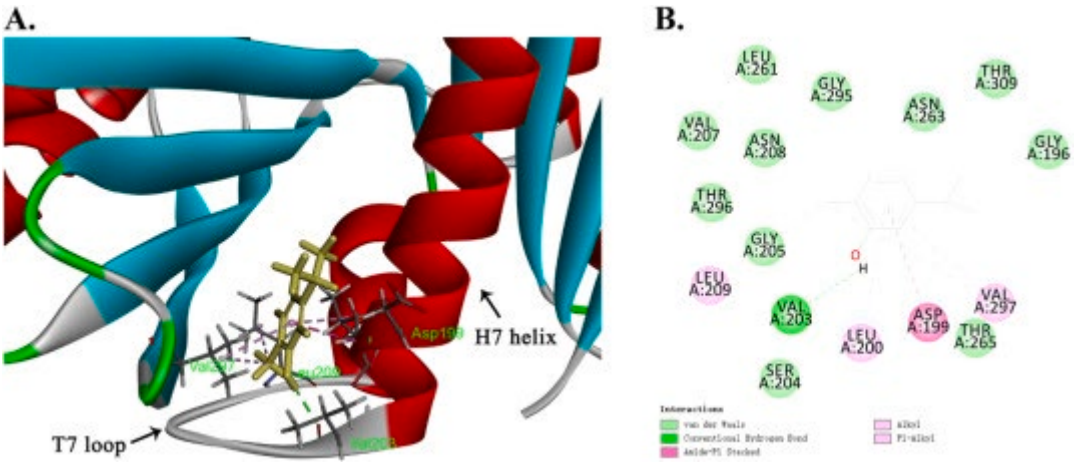

Fig. 4. The predicted binding mode of carvacrol in *S. aureus* FtsZ (PDB ID: 4DXD). (A) carvacrol in the interdomain cleft of FtsZ. (B) Predicted interactions between carvacrol and amino acids of FtsZ.