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Synergistic bactericidal effects of carvone and β-lactams against *Xanthomonas campestris* pv. *vesicatoria*

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Abstract

Xanthomonas campestris pv. *vesicatoria* (Xcv) causes brown spots on the leaves, stems, and fruits of plants, called bacterial leaf scorch (BLS). For the control of pathogens, antibiotics have been used frequently, and they can develop the resistance. In this study, the bactericidal and synergistic effects of caraway oil and its main components against the pathogen (Xcv) were investigated. The tested caraway oil consisted of 58.4% of carvone and 31.1% of limonene. The minimum inhibitory concentration (MIC) of caraway oil and carvone was the same as 125 μg mL^{-1}, and the minimum bactericidal concentration (MBC) was 1000 μg mL^{-1} for caraway oil and 500 μg mL^{-1} for carvone, while limonene showed no inhibition below 1000 μg ml^{-1}. In the growth of Xcv, carvone treatment over 31.3 μg mL^{-1} inhibited dose-dependently, and the bactericidal effect showed after 18 h more than 250 μg mL^{-1}; It was agreed with the release of intracellular components over 250 μg mL^{-1}, especially. Furthermore, carvone damaged the plasmid DNA of Xcv, and it would be the reason for the bactericidal activity. The synergistic effect of carvone was found with β-lactams selectively; the fractional inhibitory concentration (FIC) indexes of carvone with ampicillin or amoxicillin were below 0.5, and the mixture of carvone (125 μg mL^{-1}) and ampicillin (500 μg mL^{-1}) showed the bactericidal activity as well.

Keywords *Xanthomonas campestris*, Caraway oil, Carvone, Synergistic effect, DNA damage, Bactericide

Introduction

The development of most plant diseases occurs as a result of various bacterial infections [1]. Bacterial leaf scorch (BLS) is caused by bacterial pathogens such as *Xylella fastidiosa*, *Xanthomonas campestris pv. vesicatoria* and *Xanthomonas arboricola pv. pruni* [2–4]. BLS primarily affects the leaves of plants, causing them to turn brown or develop brown spots. The development of spots is also known to occur on branches, fruits, and entire aerial parts, depending on the infecting pathogen, and results in reduced productivity [5, 6]. *Xanthomonas campestris* pv. *vesicatoria* (Xcv), a gram-negative bacterium, is mainly responsible for BLS affecting pepper and tomato, and antibiotics such as streptomycin and oxytetracycline were applied to control Xcv [7–11]. However, the eventual emergence of antibiotic-resistant Xcv has been reported [8–11], and to suppress the resistance development, research on the discovery of alternative antibacterial substances from natural sources is currently underway [12–15]. These studies have evaluated the

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natural substances from *Lippia gracilis* [12], *Lentinula edodes* [13], and *Metasequoia glyptostoboides* [14] which exhibit antibacterial activity against Xcv, while the studies of the antibacterial substances from caraway and its major metabolites against Xcv have not been reported.

Caraway (*Carum carvi*), an herbaceous plant, is a member of the *Apiaceae* family and is widely native to Asia, Europe, and Africa [16]. It is used as an ingredient in food, and its leaves and roots are mainly consumed as vegetables worldwide [17]. Although the seeds are also used in brewing and spices, they are best known for their use in essential oils known as caraway oil [18]. According to a previous study, the main components of caraway oil are carvone and limonene, which account for more than 60% and 30% of its total content, respectively [19]. Carvone and limonene, which are terpenoids, are listed as food flavoring by the United States Food and Drug Administration (FDA) [20, 21]. Their use as antibacterial activity against *Bacillus*, *Staphylococcus*, *Pseudomonas*, and *Salmonella* has also been reported [22, 23]. They can donate and accept electrons because of their structural characteristics along with their potential to function as inducers of reactive oxygen species (ROS) [24], and can destroy the cell membrane and penetrate the cytoplasm of bacteria, resulting in ROS [25], because intracellular ROS exerts a bactericidal effect through its involvement in DNA damage, lipid peroxidation, protein denaturation, and enzyme inactivation [26–28].

Accordingly, this study aimed to evaluate the antibacterial potential of caraway oil and its main components, carvone and limonene, against Xcv, and the bacterial sensitivity to Xcv was investigated with the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). In addition, the bactericidal activity of carvone was observed with a time-killing assay in growth, the release of the intracellular components, and the plasmid DNA damage assay. In addition, the synergistic antibacterial effect of carvone with several conventional antibiotics, including β-lactams, was investigated with the fractional inhibitory concentration (FIC) index.

Materials and methods

Chemicals and instruments

Measurement of bacterial turbidity in treated or untreated reagents was performed using an iD3 multimode microplate reader (Molecular Devices, CA, USA). Caraway oil, which originated in Hungary, was purchased from a local market. Standard materials (R)-(-)-carvone and (±)-limonene were purchased from Tokyo Chemical Industry Co., LTD (Tokyo, Japan). Ampicillin, tetracycline, validamycin, oxolinic acid, amoxicillin, penicillin G, and streptomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bacterial culture conditions

Xanthomonas campestris pv. *vesicatoria* were donated by the Jeonnam Bioindustry Foundation (Gokseong, Korea). Xcv in Luria–Bertani (LB) broth was cultured at 37 °C for 48 h in a shaking incubator. The initial concentration of Xcv was adjusted to approximately 1×10^8 cfu mL^{-1}, and measurement of bacterial turbidity at 600 nm was performed to confirm the concentration.

Composition analysis by GC–MS

The oil sample (0.10 g) was diluted with acetone, and the diluted solution was filtered using a syringe filter (0.23 μm). Quantitative and qualitative analyses of the oil were performed using gas chromatography-mass spectrometry (GC–MS; Shimadzu Co., Ltd. Kyoto, Japan). Qualitative analysis was initially performed in the total ion chromatogram (TIC) mode, and the candidates were compared with the NIST library and identified using standard materials. Quantitative analyses of carvone and limonene were performed in the selected ion mode (SIM). Detailed analytical conditions are listed in Table 1.

Determination of MIC and MBC

Caraway oil, carvone, and limonene were progressively diluted with DMSO, and 10 μL of the diluted samples and 15 μL of 1×10^8 cfu mL^{-1} Xcv were added to 975 μL of LB broth. The treated broth was incubated at 35 °C for 48 h in a shaker incubator. Following incubation, the lowest concentration of antibacterial samples on the transparent medium where Xcv growth was not observed was recorded as the MIC. The culture medium (300 μL) was

Table 1 Instrumental condition for the analysis of components in caraway oil

Instrument	**GCMS-2010 (Shimadzu Co. Ltd., Kyoto, Japan)**		
Injection	**1.0 uL (splitless)**		
Column	**DB-WAX (30 m × 0.25 μm × 0.25 mm)**		
Flow	**1.0 mL/min**		
Injector temperature	**200 ℃**		
Oven condition	Rate(℃/min)	Temp(℃)	Hold time(min)
	Initial	80	2
	10	170	2
	2	190	0
	10	240	5
Mass spectrometer condition			
Ion source	EI		
Selected ion (m/z)	Compound	Quantitative	Qualitative
	Carvone	93	108, 106
	Limonene	93	107, 121

then spread on LB agar plates and incubated for 48 h at 35 °C. Monitoring for colony formation on the plate was performed to determine the MBC.

Measurement of the bacterial growth curve

Xcv usually reaches the stationary phase at least 40 h after incubation. Therefore, turbidity measurements were performed at 3 h intervals during culturing for 48 h after treatment with carvone at different concentrations (0, 31.25, 62.5, 125, 250, and 500 μg mL^{-1}) in Xcv broth. Briefly, 25 μL of Xcv (1×10^8 cfu mL^{-1}) was added, and the initial optical density (OD) value was 0.09. The broth was incubated in a shaking incubator at 35 °C, and turbidity was measured at 600 nm.

Determination of FIC by micro checkerboard method

The synergistic effects of the combination of carvone and representative antibiotics (ampicillin, tetracycline, validamycin, oxolinic acid, and streptomycin) were evaluated using the fractional inhibitory concentration (FIC) index [29]. These combinations were prepared by serial twofold dilutions using DMSO according to the treatment concentration. The combination (10 μL) was added to 990 μL of Xcv broth (approximately 1×10^8 cfu mL^{-1}) and incubated for 48 h to determine whether the concentration affected the extent of Xcv growth. The FIC index is the sum of the individual FICs included in the combination. The FIC index indicated a synergistic effect (values ≤ 0.5), an additional effect (0.5 < values < 1), an indifferent effect (1 < values < 4), and antagonistic effect (values ≥ 4) [30]. The FIC index values were derived as follows: FIC index = $FIC_{carvone} + FIC_{antibiotics.}$ Where $FIC_{carvone}$ or $FIC_{antibiotics}$ are the values obtained by dividing the MIC of carvone or antibiotics in the combination by the MIC in treated alone, respectively.

Time-killing kinetics

Time-killing kinetic curves were obtained by counting colony-forming units per 1 mL every 6 h during the growth of Xcv. Similar to the bacterial growth curve test, 990 μL of diluted Xcv broth (approximately 1×10^3 cfu mL^{-1}) was incubated with 10 μL of carvone at 0, 31.25, 62.5, 125, 250, and 500 μg mL^{-1} at 35 °C. During the incubation period, the mixture was collected and the number of colonies at each concentration of carvone was counted every 6 h.

Intracellular components release experiments

The experiments were conducted based on the principle that nucleic acids, such as DNA and RNA, are detected at a maximum absorption wavelength of 260 nm. Treatment of 990 μL LB broth containing 1×10^3 cfu mL^{-1} Xcv and 10 μL of different concentrations of carvone (0, 31.25, 62.5, 125, 250, and 500 μg mL^{-1}). The mixture was incubated at 35 °C and harvested after 0, 6, 12, 36, and 48 h. The collected mixtures were centrifuged at 11,000 rpm for 10 min. Measurement of the supernatant, except for the cell pellet, was performed at 260 nm to monitor the intracellular components.

Effects of carvone on damage to plasmid DNA extracted from Xcv

For plasmid DNA damage measurement, 48 h incubated Xcv in LB broth was treated carvone as a final concentration of 125 μg mL^{-1} and additionally incubated for 4, 8, and 12 h. Extraction and purification of DNA from Xcv were performed using alkaline lysis methods [31]. Briefly, harvested pellet was resuspended by 250 μL of S1 buffer containing RNase A, 250 μL of S2 buffer, and 350 μL of G3 buffer sequentially for the completely lysis. The mixture lysate was injected into a filter column to wash and remove the other cellular components using 700 μL of PW buffer. Afterwards, the DNA samples were secured by elution with 50 μL of EB buffer. 5 μL of DNA sample and 1 μL of 6 × DNA loading buffer (Enzynomics, Daejeon, Korea) were mixed and loaded into a 0.8% agarose gel containing a DNA dye solution (1 × RedSafe, Intron Biotechnology, Gyeonggi, Korea) in Tris–acetate EDTA (TAE) buffer. Agarose gel electrophoresis was performed for 30 min at 100 V, followed by visualization using a gel imaging system (Azure 200, Azure Biosystems, Dublin, CA, USA).

Statistical analysis

All experiments were performed in triplicates. Data were acquired using the Sigma Plot (version 14.0). Statistical significance was set at a 5% confidence interval to identify the differences among the treatments.

Results and discussion

Composition of caraway oil

The components of the tested caraway oil were identified and quantified with GC–MS. Carvone (64.2%), limonene (33.5%), dihydrocarvone (< 1%), *cis/trans*-carveols (< 1%), and *cis/trans*-limonene-1,2-epoxides (< 1%) were detected in the caraway oil (Table 2). It was similar to the previously reported results of the essential oil [19].

Antibacterial activity of caraway oil and its metabolites

The antibacterial activity of caraway oil and its main components, carvone and limonene, against Xcv was evaluated using MIC and MBC. As shown in Table 3, almost no difference in MIC values was observed between caraway oil (MIC = 125 μg mL^{-1}) and carvone (MIC = 125 μg mL^{-1}), whereas limonene exhibited no inhibitory activity with a MIC of more than 1000 μg mL^{-1}. Caraway oil and

Table 2 Carvone and limonene contents in the tested caraway oil

	Composition (%)
R-(-)-Carvone	58.4
Limonene	31.1
Dihydrocarvone	< 1
cis-carveol	< 1
trans-carveol	< 1
cis-limonene-1,2-epoxide	< 1
cis-limonene-1,2-epoxide	< 1

Table 3 MIC and MBC of caraway oil, carvone, limonene, and antibiotics against Xcv

Samples	MIC[a] (μg mL^{-1})	MBC[b] (μg mL^{-1})
Essential oil		
Caraway oil	125	1000
Main component		
Carvone	125	500
Limonene	> 1000	NT[c]
Antibiotics		
Ampicillin	500	> 1000
Amoxicillin	500	NT
Penicillin G	500	NT
Tetracycline	0.24	NT
Validamycin	NI[d]	NT
Oxolinic acid	0.98	NT
Streptomycin	31.3	NT

[a] MIC indicates the minimal inhibitory concentrations against Xcv

[b] MBC indicates the minimal bactericidal concentrations against Xcv

[c] NT means not tested

[d] NI means no inhibitory effect against Xcv

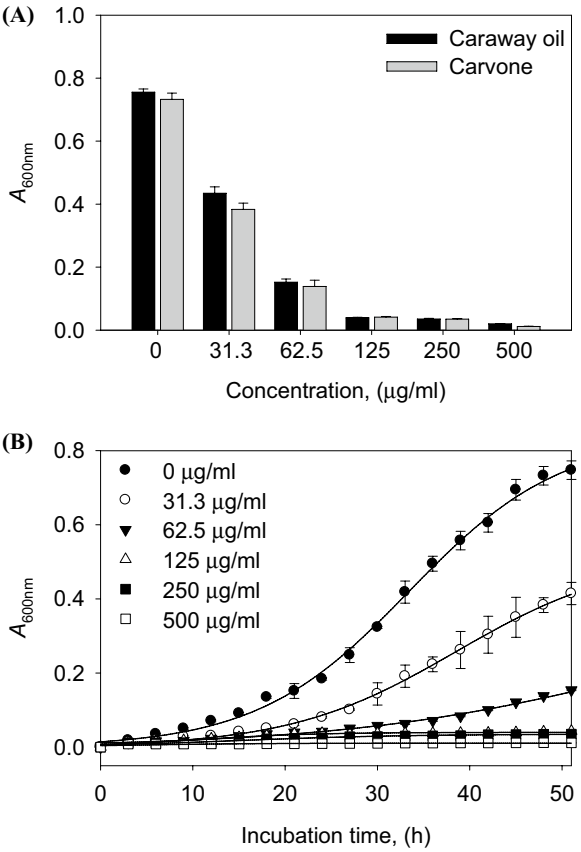

Fig. 1 (**A**) Antibacterial effects of treatment with caraway oil and carvone at different concentrations (0, 31.3, 62.5, 125, 250, and 500 μg mL^{-1}) at 48 hr incubation against *X. campestris*, (**B**) Growth curve for Xcv with influenced carvone at 0, 31.3, 62.5, 125, 250, and 500 μg mL^{-1}.

carvone inhibited Xcv growth in a dose-dependent manner (Fig. 1A). Specifically, the results confirmed that the growth of Xcv decreased with increasing carvone concentration (Fig. 1B).

The MBC values for caraway oil and carvone were 1000 μg mL^{-1} and 500 μg mL^{-1}, respectively. Thus, carvone was expected to be the bactericidal compound in caraway oil. In the comparison of antibacterial activity between carvone and various antibiotics including ampicillin (Amp), tetracycline (Tcc), validamycin (Val), oxolinic acid (Oxo), and streptomycin (Stp), carvone was more effective than Amp (MIC = 500 μg mL^{-1}) and Val (MIC = 1000 μg mL^{-1}). However, Tcc (MIC = 0.24 μg mL^{-1}), Oxo (MIC = 0.98 μg mL^{-1}), and Stp (MIC = 31.3 μg mL^{-1}) showed greater sensitivity than carvone.

Synergistic effect of carvone and antibiotics

To reduce the development of antibiotic resistance, the developments of alternatives and synergists are necessary. In here, the synergistic effects of carvone combined with representative antibiotics, including Amp of β-lactams, Tcc of tetracyclines, Val and Stp of aminoglycosides, and Oxo of quinolones were evaluated using the FIC index.

As shown in Table 4, Amp/Car combination indicated a strong synergistic effect (FIC index ≤ 0.5) with 0.41 at Amp (15.6 μg mL^{-1})/carvone (46.9 μg mL^{-1}). Carvone (46.9 μg mL^{-1}) was 2.5-fold more effective than carvone alone (MIC = 125 μg mL^{-1}), and Amp usage (15.6 μg mL^{-1}) was decreased 30-fold compared with

Table 4 FIC index of the combination between antibiotics and carvone against Xcv

Group	Antibiotics (μg mL^{-1})	FIC index against Xcv						
		Carvone (μg mL^{-1})						
		0	**15.6**	**23.4**	**31.3**	**46.9**	**62.5**	**125**
β-lactams	Ampicillin							
	0							1.00
	15.6					0.41	0.53	1.03
	31.3					0.44	0.56	1.06
	50					0.48	0.60	1.10
	100				0.45	0.58	0.70	1.20
	250		0.63	0.69	0.75	0.88	1.00	1.50
	500	1.00	1.13	1.19	1.23	1.38	1.50	2.00
	Amoxicillin							
	0							1.00
	31.3							1.06
	50					0.48	0.60	1.10
	100					0.58	0.70	1.20
	250		0.63	0.69	0.75	0.88	1.00	1.50
	500	1.00	1.13	1.19	1.25	1.38	1.50	2.00
	Penicillin G							
	0							1.00
	100							1.20
	250					0.88	1.00	1.50
	500	1.00	1.13	1.19	1.25	1.38	1.50	2.00
Aminoglycosides	Validamycin							
	1000	NI[a]	NI	NI	NI	NI	NI	NI
	Streptomycin							
	0							1.00
	15.7							1.25
	23.4		0.88	0.94	1.00	1.12	1.25	1.75
	31.3	1.00	1.13	1.19	1.25	1.38	1.50	2.00
Quinolones	Oxolinic acid							
	0							1.00
	0.73		0.87	0.93	0.99	1.12	1.25	1.75
	0.98	1.00	1.13	1.19	1.25	1.38	1.50	2.00
Tetracyclines	Tetracycline							
	0							1.00
	0.12							1.50
	0.24	1.00	1.13	1.19	1.25	1.38	1.50	2.00

[a] NI means no inhibitory effect against Xcv

Amp alone (MIC = 500 μg mL^{-1}). The lowest FIC_{index} for Oxo (0.73 μg mL^{-1})/Car (15.6 μg mL^{-1}) combination and Stp (23.4 μg mL^{-1})/Car (15.6 μg mL^{-1}) were 0.87 and 0.88 against Xcv, respectively. Otherwise, Tcc showed unremarkable effects (FIC index > 1.0) in all combinations with carvone, and Val was inactive against Xcv both alone and in combination. Thus, we extensively investigated the synergistic effect of carvone with other β-lactams such as amoxicillin (Amo) and penicillin G (PenG). Amp and Amo belong to broad-spectrum antibiotics used for gram-negative bacteria having relatively thick cell membranes and gram-positive bacteria, while PenG is natural penicillin to have efficacy for gram-positive bacteria [32, 33]. All three β-lactams (Amp, Amo, and PenG) had the same

MIC value of 500 μg mL^{-1} (Table 3). Whereas, as shown in Table 4, carvone combinations were displayed in two types as follows; synergistic effect with Amp (FIC index = 0.41) and Amo (FIC index = 0.48), and an additional effect with PenG (FIC index = 0.88) were presented on Xcv. For the best FIC index on the carvone (46.9 μg mL^{-1}) combination, the MICs of Amp, Amo, and PenG exhibited 15.6, 50.0, and 250 μg mL^{-1}, respectively; these MICs were shown as reduced 32-fold for Amp, tenfold for Amo, and twofold for PenG than their usage alone. As the results, carvone only exhibited the synergistic effect with β-lactam antibiotics specifically. These effects was presumed that the β-lactam can be interrupt bacterial cell wall production to increase the uptake of the carvone into the membrane of Xcv. Therefore, carvone could be used as a synergist for β-lactams, and it effectively reduce the antibiotics use for controlling Xcv.

Time-kill kinetics assay

The time-dependent bactericidal activity of carvone was investigated with the time-killing kinetic curve in culture media (Fig. 2A). The curve exhibited a significant dose-dependent bactericidal activity against Xcv treated with carvone ranging from 31.3 to 500 μg mL^{-1}. At the MIC, the number of bacterial colonies counted (around 1.5×10^3 cfu mL^{-1}) was equal to the incubation time from 0 to 54 h. Concentrations below the MIC showed a pattern similar to that of the negative control (1×10^8 cfu mL^{-1}), however, the counted colonies were 1.2×10^7 cfu mL^{-1} for 31.25 μg mL^{-1} and 3.16×10^6 cfu mL^{-1} for 62.5 μg mL^{-1} after 48 h incubation; these were slightly lower than the control. Concentrations above the MIC (125 μg mL^{-1}) showed an outstanding bactericidal effect, as a reduction in the initial number of bacteria was observed. Specifically, the number of bacteria after incubation was close to zero at 500 μg mL^{-1} carvone after 42 h. The colonies number was maintained until 18 h incubation, and a difference was observed according to the concentration after 18 h.

To confirm the bactericidal effect, the release of intracellular component was investigated time-dependently. As shown in Fig. 2, carvone showed no inhibition effect on bacterial counts until 18 h after incubation in a time-killing curve, related to no intracellular nucleic acid release was observed at this time as well. However, carvone led to an increase in absorbance (Abs_{260}) and a decrease in the number of bacteria after 18 h. The absorbance was not significantly changed ($Abs_{260} < 0.1$) below the MIC (125 μg mL^{-1}), whereas it drastically increased

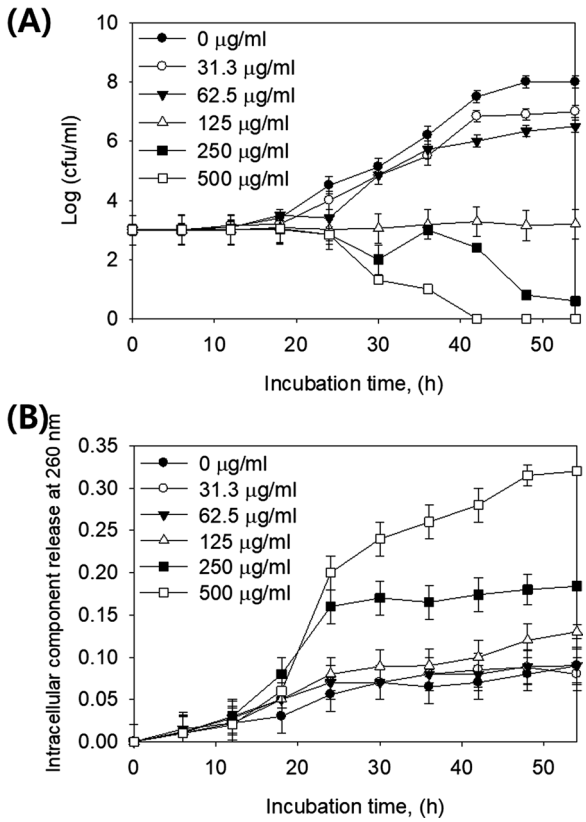

Fig. 2 **(A)** Time killing kinetic curves and (**B**) intracellular components release of Xcv treated with carvone at 0, 31.3, 62.5, 125, 250, and 500 μg mL^{-1} during an incubation period of 54 h. All the data are presented as the mean ± SD of independent experiments

up to Abs_{260} 0.32 above the MIC in a dose-dependent manner. Cell membrane disruptors such as β-lactames had been reported that the bactericidal effect and the release of intracellular components showed within several hours [34, 35]. While, the component release and the bactericidal activity after carvone treatment was shown on Xcv after 18 h, thus cell membrane disruption was not expected to be the reason but the result of cell death.

In addition, the bactericidal potential of the synergistic combinations of carvone with Amp was confirmed as well. The time-killing curve for Amp/carvone combination were presented at diversiform concentrations with 500/0 μg mL^{-1}, 500/125 μg mL^{-1}, 250/62.5 μg mL^{-1}, 125/62.5 μg mL^{-1}, 25/62.5 μg mL^{-1}, and 15.6/46.9 μg mL^{-1} of Amp/carvone (Fig. 3). Colony number of all the combination of carvone with Amp except the combination of Amp (500 μg mL^{-1})/carvone (125 μg mL^{-1}) were observed almost the same before and after incubation. The significant bactericidal effects

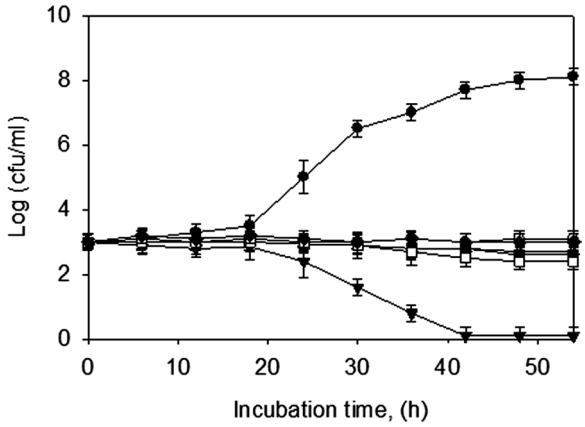

Fig. 3 Time killing kinetic curves of the mixture of ampicillin and carvone. ● control; ○ Amp 500 μg mL^{-1}; ▼ Amp 500 and Car 125 μg mL^{-1}; △ Amp 250 and Car 62.5 μg mL^{-1}; ■ Amp 125 and Car 62.5 μg mL^{-1}; □ Amp 25 and Car 62.5 μg mL^{-1}; ◆ Amp 15.6 and Car 46.9 μg mL^{-1}. The data are presented as the mean ± SD of independent experiments

were shown at the concentration of Amp (500 μg mL^{-1})/carvone (125 μg mL^{-1}). The colonies showed a noticeable decrease compared to other treatments from 24 h after Xcv culture.

Plasmid DNA damage effect of carvone

To find the mode-of-action of bactericidal activity of carvone, the plasmid DNA damage effect was investigated on Xcv in 4, 8, and 12 h of the exposure time at 125 μg mL^{-1} of carvone. As shown in Fig. 4, the extracted plasmid DNA showed the lowest position and a clear band in lane 2 for non-carvone treatment, whereas the DNA band appeared from a high position in lanes 3–5 for carvone treated for 4, 8, and 12 h time respectively. Based on the results, carvone exhibited DNA damage reaction even before reaching the killing time of Xcv (18 h). Previous reports have demonstrated that the damaged plasmid DNA by compounds converts supercoiled (scDNA) to open circular (ocDNA), linear (lnDNA), and further nicked forms, and it induced the cell death by the loss of metabolic control [36, 37]. Thus, the plasmid DNA damage of Xcv by carvone would be the reason for the bactericidal activity. The bactericidal effect of carvone (500 μg mL-1) and its combination with Amp (Amp 500/Car 125 μg mL-1) dramatically decreased the bacterial count after 24 hrs of exposure. In addition, the release of intracellular components by carvone treatment was confirmed by measuring nucleic acids in the supernatants at 260 nm. Moreover, the damage of isolated plasmid DNA from Xcv in the presence of carvone was also revealed by visualizing the electrophoresis. As a result, carvone exhibited antibacterial and bactericidal effects against Xcv to relate

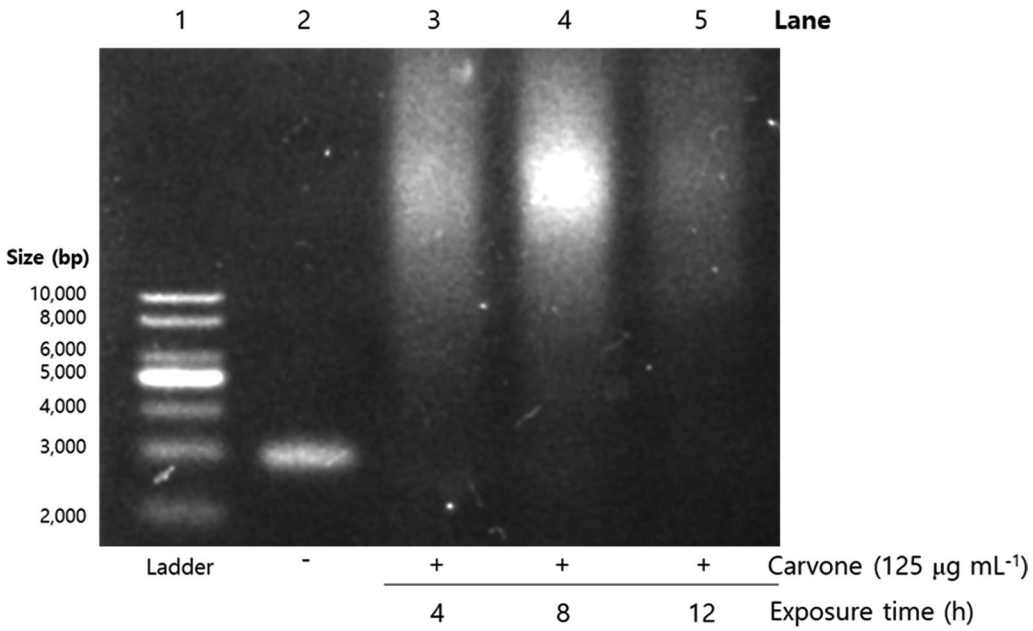

Fig. 4 DNA damage effect of carvone (125 μg ml^{-1}) on Xcv. Lane 1 for ladder (1 kb), Lane 2 for DNA without carvone, Lane 3–5 for DNA exposed to carvone after incubation with 4, 8, and 12 h, respectively

to DNA damage. It also possessed the selective synergistic potential with β-lactam antibiotics to expect the reducing antibiotic use.

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Author contributions
Ju-Yeon Kim, Park JY, and Seo MK performed the antibacterial activity test and the data gathering; Kim JS and Shin MK performed the composition analysis and synergistic combinations; J-YK performed the bactericidal activity and discussed the result; Kim JH designed this study and supervised all the results. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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