



Evaluation of antimicrobial activity of natural antimicrobials against antibiotic-sensitive and resistant phytopathogens

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Abstract

Crop diseases caused by phytopathogens threaten a stable food supply. Although antibiotics are widely used to prevent these diseases, their use is becoming increasingly limited owing to their toxicity. In this study, we evaluated the efficacy of natural antimicrobials against both antibiotic-sensitive (S) and antibiotic-resistant (R) phytopathogens. The results showed that grapefruit seed extract (GSE) and several carboxylic acids exhibited broad-spectrum inhibitory effects against phytopathogens, irrespective of antibiotic resistance. Among these, GSE showed the highest antimicrobial activity against all strains with a minimum inhibitory concentration (MIC) value of $\leq 0.0781\%$. Our findings reveal that these natural antimicrobials could serve as substitutes for chemical pesticides; however, further studies are required to enhance their antimicrobial efficacy for practical applications in agriculture.

Keywords Phytopathogen · Antibiotic resistance · Natural antimicrobial · Grapefruit seed extract · Carboxylic acid

Introduction

Crop diseases caused by phytopathogens (plant pathogenic bacteria) reduce crop yield and quality, leading to significant economic losses worldwide. For example, *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) primarily causes soft rot in Chinese cabbage and potatoes, and *Pseudomonas syringae* pv. *actinidiae* (Psa) is the causal agent of bacterial canker in kiwifruit and is regarded as one of the most serious and economically significant diseases affecting this crop (Ares et al., 2021; Shen et al., 2025). *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) causes bacterial canker in tomatoes, and *Xanthomonas arboricola* pv. *pruni* (Xap) causes bacterial spot on various stone fruits such as peaches, plums, and almonds (Luo et al., 2022; Sedighian et al., 2025). It is estimated that 10–15% of major crops worldwide are lost annually because of plant diseases, resulting in economic losses amounting to hundreds of billions of

dollars annually (Khakimov et al., 2022). In recent years, the increasing frequency of extreme weather events owing to climate change has influenced the proliferation of phytopathogens and increased the risk of crop diseases (Juroszek et al., 2020). Furthermore, the spread of crop diseases is facilitated by various factors, including agricultural equipment, human activity, contaminated seeds, water, and international trade (Carezzano et al., 2017). Therefore, controlling phytopathogens is essential to minimize disease-related damage to crops.

Conventional chemical pesticides are widely used for crop protection owing to their low cost and convenience. However, the excessive use of chemical pesticides has led to the development of antibiotic-resistant (R) phytopathogens, which negatively affect human health and the environment (Valarmathi, 2020). In addition, pesticide residues in crops may pose health risks to humans because postharvest products containing toxic compounds can be consumed, thereby posing harmful effects on food safety (Srivastava et al., 2018). Consequently, global trends have shifted toward reducing the use of chemical pesticides, and a growing demand exists for environmentally friendly alternatives to synthetic chemical pesticides.

Natural antimicrobial compounds are well known as effective bacterial inhibitors against foodborne pathogens because of their strong growth-inhibitory properties, which

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contribute to the safety and nutritional quality of produce (Abdelhamid and El-DougDoug, 2020; Han et al., 2023). Therefore, safe, food-grade (edible) natural antimicrobials, such as plant extracts and essential oils (EOs), have emerged as safer alternatives to synthetic chemical pesticides in agricultural practice. However, studies investigating the efficacy of natural food-grade antimicrobials against phytopathogens are limited. Therefore, in the present study, natural antimicrobials were screened, followed by a comparison of their antimicrobial activities and an evaluation of their combined effects against both antibiotic-sensitive (S) and antibiotic-resistant (R) phytopathogens.

Materials and methods

Bacterial strains and antimicrobial agents

Table 1 summarizes the bacterial strains used in this study. Among them, *P. carotovorum* subsp. *carotovorum* (Pcc) KACC 10225 (S) and 10230 (R); *P. syringae* pv. *actinidiae* (Psa) KACC 10586 (S) and 10588 (R); *C. michiganensis* subsp. *capsici* (Cmc) KACC 18448 (S); *C. michiganensis* subsp. *michiganensis* (Cmm) KACC 17003 (R); and *X. arboricola* pv. *pruni* (Xap) KACC 19950 (S) and 19951 (R) obtained from Yeungnam University (Gyeongsan, Korea) were used in subsequent experiments. Bacterial strains were first grown on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, USA) at 28 °C for 24–48 h. A single colony was then inoculated into 5 mL of tryptic soy broth (TSB, Difco) and incubated at 28 °C for 24–48 h.

The various natural antimicrobials used in this study included EOs and their active compounds, plant extracts, and carboxylic acids. Detailed information is provided in Table 2.

Agar disc diffusion method

The antimicrobial activities of various natural antimicrobials against the eight phytopathogenic strains were determined using the agar disc diffusion method. A bacterial

suspension (100 µL) grown to 10^8 CFU/mL was uniformly swabbed onto the surface of TSA, and the inoculum was allowed to dry for 10 min. Sterilized filter paper discs (Whatman NO.2, England) containing 10 µL of each natural antimicrobial were then individually placed on the TSA surface. The inoculated plates were incubated at 28 °C for 24–48 h, and the diameters (mm) of the inhibition zones were measured.

Minimum inhibitory concentration (MIC)

The broth dilution method was used to determine the MIC. Serial two-fold dilutions of various natural antimicrobials, ranging from 1 to 0.0024%, were prepared in TSB-containing bacterial suspensions adjusted to 10^8 CFU/mL. After incubation at 28 °C for 24–48 h, the optical density at 600 nm (OD_{600}) was measured using a spectrophotometer (SpectraMax M2e, Molecular Devices, San Jose, CA, USA). MIC was defined as the lowest concentration that resulted in a 50% reduction in bacterial growth compared to the control, as determined by OD measurements.

Combined treatment with natural antimicrobials

The antimicrobial effects of the combined treatments were evaluated using grapefruit seed extract (GSE) in combination with acetic, malic, tartaric, propionic, phytic, lauric, and capric acids. Combinations of GSE and carboxylic acid were tested individually at their MIC and 1/2 MIC, as well as in combination (MIC + MIC, 1/2 MIC + 1/2 MIC, and 1/4 MIC + 1/4 MIC). Synergistic interactions were examined using the agar well diffusion method, in which the enlargement of the inhibition zone indicated a positive interaction. The interactions were further evaluated by determining the MIC values using the broth dilution method.

Table 1 Strains of phytopathogen used in this study

Scientific name	KACC No
Pcc	10225–10231, 10342–10347, 10401, 10402, 10416–10419, 10421, 10458, 10602, 11130, 11131, 13953, 13965, 13968, 14868–14870, 14873–14880, 14883–14885, 14887, 14888, 14890, 14891, 14893, 14894, 16999, 17004, 18385, 18387, 18645, 21701
Psa	10582–10584, 10586–10588, 10593–10595, 16841–16843, 16847–16849, 19139–19141, 19289, 19525, 19526
Cmc	18448
Cmm	16995, 16996, 17003, 18386
Xap	18153–18157, 19495, 19497, 19949–19953

Table 2 Natural antimicrobials used in this study

Compounds (n)	Antimicrobials	Conc (%)	Corporation
EOs and their active compounds (12)	Tea tree EO, thyme EO, lemongrass EO, peppermint EO, clove bud EO, oregano EO	1	Absolute aromas
	Onion EO, garlic EO	1	ES food
	Eugenol	1	Wako
	Thymol	1	Samchun
	Citral, carvacrol	1	Sigma
Phenolic compounds (6)	Gallic acid, salicylic acid, vanillin	1	Sigma
	Cinnamic acid	1	Junsei
	Caffeic acid, syringic acid	1	Tokyo chemical industry
Commercial plant extract (1)	GSE	1	ES food
Plant extracts (29)	<i>Mangifera indica</i>	2.24	Natural product central bank
	<i>Syzygium jambos</i>	1.76	
	<i>Phyllanthus urinaria</i>	2.44	
	<i>Lagerstroemia floribunda</i>	2.86	
	<i>Diospyros malabarica</i>	2.38	
	<i>Lagerstroemia cochinchinensis</i>	2.89	
	<i>Lagerstroemia floribunda J.</i>	2.58	
	<i>Lithocarpus truncates</i>	2.56	
	<i>Ilex umbellulata</i>	2.34	
	<i>Rheum franzenbachii</i>	2.12	
	<i>Maesa laxiflora</i>	2.28	
	<i>Alchornea trewioides</i>	2.44	
	Grape seed, <i>Aloe vera</i>	1	Chung-Ang university
	Clove, cinnamon, propolis, garlic, ginger, lemongrass, oregano, thyme, coriander, rosemary, turmeric, <i>Alchornea trewioides</i> , <i>Ilex umbellulata</i> , <i>Lagerstroemia floribunda</i> , <i>Erodium stephanianum</i>	20	Chung-Ang university
Carboxylic acids (10)	Citric acid, phytic acid, caprylic acid	1	Sigma
	Lactic acid, propionic acid, lauric acid	1	Daejung
	Acetic acid	1	Samchun
	Tartaric acid	1	Hayashi
	Malic acid	1	Junsei
	Capric acid	1	Yakuri
Others (4)	Chitosan	1, 10	Chung-Ang university
	Sodium hypochlorite	0.01	Daejung
	Calcium nitrate	1	Sigma

Results and discussion

Antimicrobial screening of natural antimicrobials

Based on the antibiotic resistance results described previously (Kim et al., 2023), four strains each of the antibiotic-S and antibiotic-R phytopathogens were selected for subsequent experiments (Table 3). Natural antimicrobials were screened using these phytopathogens. Overall, the antimicrobial effect was primarily observed in carboxylic acids compared to EOs, their active compounds, and plant extracts. Gram-positive bacteria were more sensitive to natural antimicrobials than Gram-negative bacteria. Among the

tested natural antimicrobials, 47% were effective against Cmm (R) and 26% were effective against Psa (R). This may be attributed to differences in cell membrane composition between Gram-positive and Gram-negative bacteria (Osonga et al., 2019). Gram-negative bacteria have an outer membrane that protects them against antimicrobial agents, thereby increasing their resistance to antibacterial activity (Radhi et al., 2021). Antibiotic resistance was associated with antimicrobial effectiveness, revealing that antibiotic-S strains, except for Pcc, tended to be more sensitive to natural antimicrobials than the antibiotic-R strains. This may be attributed to the fact that some antibiotic-R bacteria have acquired resistance to most available antimicrobial agents

Table 3 Resistance of selected phytopathogens to commercial antibiotics in agriculture for use in subsequent experiments

Strains	KACC No	Commercial antibiotics in agriculture								
		Oxytetracycline + streptomycin			Streptomycin			Validamycin A		
		1×	10×	100×	1×	10×	100×	1×	10×	100×
Pcc (S)	10225	—	—	—	—	—	—	+	+	±
Pcc (R)	10230	—	—	—	—	—	—	+	+	+
Psa (S)	10586	—	—	—	—	—	—	+	+	—
Psa (R)	10588	—	—	—	—	—	—	+	+	+
Cmc (S)	18448	—	—	—	—	—	—	—	—	—
Cmm (R)	17003	—	—	—	—	—	—	+	+	+
Xap (S)	19950	—	—	—	—	—	—	+	+	—
Xap (R)	19951	—	—	—	+	+	—	+	+	—

+ : distinct growth (clear morphology), ± : weak growth (tiny colonies), — : no visible growth

(Feßler et al., 2012; Kreausukon et al., 2012; Morar et al., 2015; Valenzuela et al., 2010).

Table 4 shows the diameters of the inhibition zones of natural antimicrobials, including EOs and their active compounds, plant extracts, and carboxylic acids. Among EOs and their active compounds, carvacrol and thymol exhibited broad antimicrobial spectra against phytopathogens. Carvacrol and thymol are generally safe and widely used as antimicrobial food flavorings and food additives because they have shown antimicrobial efficacy against a broad spectrum of foodborne and clinical pathogens (Hajibonabi et al., 2023), which is in agreement with our results.

Among the plant extracts, *Alchornea trewioides*, clove, grape seed, and GSE exhibited a wide range of inhibitory effects against phytopathogens. For example, clove extract showed a growth-inhibitory effect against all bacterial strains (≥ 7 mm). In addition, 1% GSE (≥ 10 mm) and 2.44% *A. trewioides* extract (≥ 7 mm) exhibited inhibitory effectiveness against all strains, except for Psa (R). Generally, the extent of antimicrobial activity is associated with the phenolic composition. GSE contains flavonoids with antimicrobial effects and has been shown to exhibit sufficient antibacterial activity to inhibit antibiotic-R bacteria (Han et al., 2021). Similarly, grape seed extract has inhibitory effects against both Gram-positive and Gram-negative bacteria, which can be attributed to the phenolic compounds present in the seed extract (Adámez et al., 2012). Among the strains tested, two strains of *C. michiganensis* were more susceptible than the other bacteria to various plant extracts. The susceptibility of Gram-positive bacteria to natural extracts may be linked to the absence of a lipopolysaccharide layer (Adámez et al., 2012).

The results for zones of inhibition for carboxylic acids revealed that 1% acetic acid (8–14 mm), 1% citric acid (9–12 mm), 1% lactic acid (8–10 mm), 1% tartaric acid (8–10 mm), 1% malic acid (8–10 mm), 1% propionic acid (7–23 mm), and 10% chitosan (8–10 mm) were effective

against most strains. Among these, six organic acids exhibited growth-inhibitory effects against all strains. Abdalla et al. (2019) reported that 5% acetic acid effectively inhibited the growth of phytopathogens, including Pcc. Although numerous reports have documented the antibacterial activity of organic acids against foodborne pathogens, few studies have focused on their effects against phytopathogens. Organic acids are well-known antimicrobial agents that decrease the intracellular pH or disrupt the electron transport system (In et al., 2013). It can be assumed that organic acids decrease the intracellular pH and inhibit the metabolic activity of phytopathogens. Meanwhile, for *C. michiganensis*, 1% lauric acid and 1% capric acid caused considerable inhibition zones of 20–25 and 14–17 mm, respectively. A similar tendency was reported in previous studies, which showed that these medium-chain fatty acids exhibited antibacterial activity, particularly against Gram-positive bacteria (Kabara, 1993).

Evaluation of MIC

The MIC values of the natural antimicrobials that showed inhibition zones for each strain in the agar disc diffusion method are presented in Fig. 1. For Pcc, GSE was the most effective at inhibiting microbial growth, as it exhibited the lowest MIC values. Similarly, GSE showed the lowest MIC values for Cmm and Xap, indicating a strong inhibitory effect against most phytopathogenic strains ($\text{MIC} \leq 0.0781\%$). Propionic and phytic acids also exhibited good antimicrobial activity against Psa. Natural antimicrobials that exhibited antimicrobial activity against each strain were grouped based on their MIC values, and those with $\text{MICs} < 0.02\%$ are summarized in Table 5. The antibiotic-R Psa strain tended to show higher MIC values than the other phytopathogens, indicating lower susceptibility to natural antimicrobials. Overall, strong antimicrobial activity was observed for GSE and several carboxylic acids, including

Table 4 Zones of inhibition (mm) around sterile discs loaded with natural antimicrobials against antibiotic-sensitive (S) and antibiotic-resistant (R) phytopathogens

Compounds	Antimicrobials	Pcc (S)	Pcc (R)	Psa (S)	Psa (R)	Cmc (S)	Cmm (R)	Xap (S)	Xap (R)
EOs and their active compounds	Carvacrol	7 (Δ)	9	–	7 (Δ)	–	8 (Δ)	7 (Δ)	8 (Δ)
	Clove bud EO	–*	7 (Δ)	–	–	–	–	–	–
	Eugenol	–	9 (Δ)	–	8 (Δ)	–	–	–	–
	Oregano EO	–	8	–	–	–	–	–	–
	Thyme EO	–	7 (Δ)	–	–	–	–	–	–
	Thymol	10	9	–	7 (Δ)	–	–	7 (Δ)	8 (Δ)
	Citral, garlic EO, lemongrass EO, onion EO, peppermint EO, tea tree EO	–	–	–	–	–	–	–	–
Plant extracts	<i>Alchornea trewioides</i>	7 (Δ)	7 (Δ)	24	–	7	13 (Δ)	27 (Δ)	31 (Δ)
	<i>Alchornea trewioides</i>	–	–	25	–	15	10	–	–
	Cinnamon	–	–	–	–	–	15	14	13
	Clove	11 (Δ)	10 (Δ)	28	14 (Δ)	8	8	15	14
	<i>Diospyros malabarica</i>	–	–	–	–	15	12	7	–
	<i>Erodium stephanianum</i>	–	–	15	–	–	–	–	–
	Garlic	–	–	–	–	10	11	–	–
	Ginger	–	–	–	–	–	18	–	–
	Grape seed	–	–	22	7	8	8	8	7
	GSE	10	10	10	–	12	14	11	10
	<i>Ilex umbellulata</i>	–	–	23	–	–	–	–	–
	<i>Lagerstroemia cochinchinensis</i>	–	–	22	14 (Δ)	14	18	14	14
	<i>Lagerstroemia floribunda</i>	–	–	16	–	9	9	10	9
	<i>Lagerstroemia floribunda J</i>	–	–	17	–	9	10	10	10
	Lemongrass	–	–	–	–	23	20	–	–
	<i>Lithocarpus truncates</i>	–	–	23	–	11	14	13	11
	<i>Maesa laxiflora</i>	–	–	11	–	15 (Δ)	14 (Δ)	–	–
	<i>Mangifera indica</i>	–	–	14 (Δ)	–	–	–	–	–
	Oregano	–	–	–	–	–	10	–	–
	<i>Phyllanthus urinaria</i>	9 (Δ)	7 (Δ)	24	–	8	8	–	–
	Propolis	–	–	–	–	25	25	–	–
	<i>Rheum franzenbachii</i>	–	–	14	–	13	12	11 (Δ)	11 (Δ)
	Rosemary	–	–	–	–	30	25	–	–
	<i>Syzygium jambos</i>	–	–	14	–	10	11	–	–
	Thyme	–	–	–	–	14	17	–	7
	Turmeric	–	–	–	–	14	13	–	–
	<i>Aloe vera</i> , Coriander, <i>Ilex umbellulata</i> –I, <i>Lagerstroemia floribunda</i>	–	–	–	–	–	–	–	–
Carboxylic acids and others	Acetic acid	8	8	14	9	12	8	10	9
	Caffeic acid	6	11 (Δ)	–	–	–	–	7	–
	Capric acid	–	–	–	–	17	14	–	9 (Δ)
	Caprylic acid	15 (Δ)	–	–	–	–	–	–	–
	Chitosan (10%)	10 (Δ)	8	10	–	10	9	9	8
	Cinnamic acid	14 (Δ)	11 (Δ)	–	6	–	–	7	–
	Citric acid	9	9	10	9	12	9	9	9
	Gallic acid	–	8 (Δ)	22	7 (Δ)	–	–	7	–
	Lactic acid	8	10	8	9	8 (Δ)	8	9	9
	Lauric acid	–	–	–	–	25	20	–	–

Table 4 (continued)

Compounds	Antimicrobials	Pcc (S)	Pcc (R)	Psa (S)	Psa (R)	Cmc (S)	Cmm (R)	Xap (S)	Xap (R)
	Malic acid	8	10	11	10	10	9	9	10
	Phytic acid	9	10	7	14 (Δ)	10	10	10	9
	Propionic acid	9	11	23	9	11	8 (Δ)	7 (Δ)	9
	Salicylic acid	12 (Δ)	10	–	9	–	–	8	8
	Sodium hypochlorite	9 (Δ)	8 (Δ)	–	–	–	–	–	–
	Syringic acid	–	–	–	–	–	–	8 (Δ)	7
	Tartaric acid	8	10	9	9	10	10	9	9
	Calcium nitrate, chitosan (1%), Vanillin	–	–	–	–	–	–	–	–

*, no inhibition zone

Δ, unclear inhibition zone

acetic, malic, tartaric, propionic, phytic, capric, and lauric acids. Juneja et al. (2006) reported that a GSE concentration of 200 ppm effectively inhibited the growth of *Clostridium perfringens*, which is consistent with the findings of this study. Similarly, spraying chicken skin inoculated with *Salmonella* Typhimurium and 0.5% GSE significantly reduced the bacterial population (Xiong et al., 1998).

Evaluation of the combined treatment of natural antimicrobials

It has been hypothesized that antimicrobial activity can be further enhanced by combining effective natural antimicrobials. Therefore, combined treatments with selected natural antimicrobials were evaluated using the agar disc diffusion test and broth dilution method. However, the combination of natural antimicrobials on the agar medium did not exhibit any synergistic or additive effects compared to a single treatment with GSE or carboxylic acids (data not shown). In addition, the antimicrobial activity did not increase when GSE was combined with carboxylic acids in the broth medium (data not shown). These results indicated that the diameters of the inhibition zone and MIC values

remained unchanged when GSE was used in combination with carboxylic acids. In contrast to our findings, Kim et al. (2009) reported that a mixture of 1% GSE and 10% lactic acid exhibited greater antimicrobial activity against food-borne pathogens than GSE alone. In addition, combined treatment with 0.5% GSE and 1% malic acid resulted in the greatest microbial reduction of *Escherichia coli* O157:H7 and *Listeria monocytogenes* compared to individual treatments (Kim et al., 2016). This discrepancy may be attributed to differences in the tested strains and the concentrations of the antimicrobial agents used.

In conclusion, the phytopathogens tended to exhibit high resistance to validamycin A, whereas no resistance was detected to oxytetracycline or streptomycin. Among the various natural antimicrobials, GSE and several carboxylic acids, such as phytic acid, show broad-spectrum antimicrobial activity, irrespective of antibiotic resistance. However, their individual effects were modest, and their combined effects were not synergistic. These findings indicate that the development of antibiotic resistance in phytopathogens is evident and may pose a significant threat to food security and plant disease management. Further investigations are

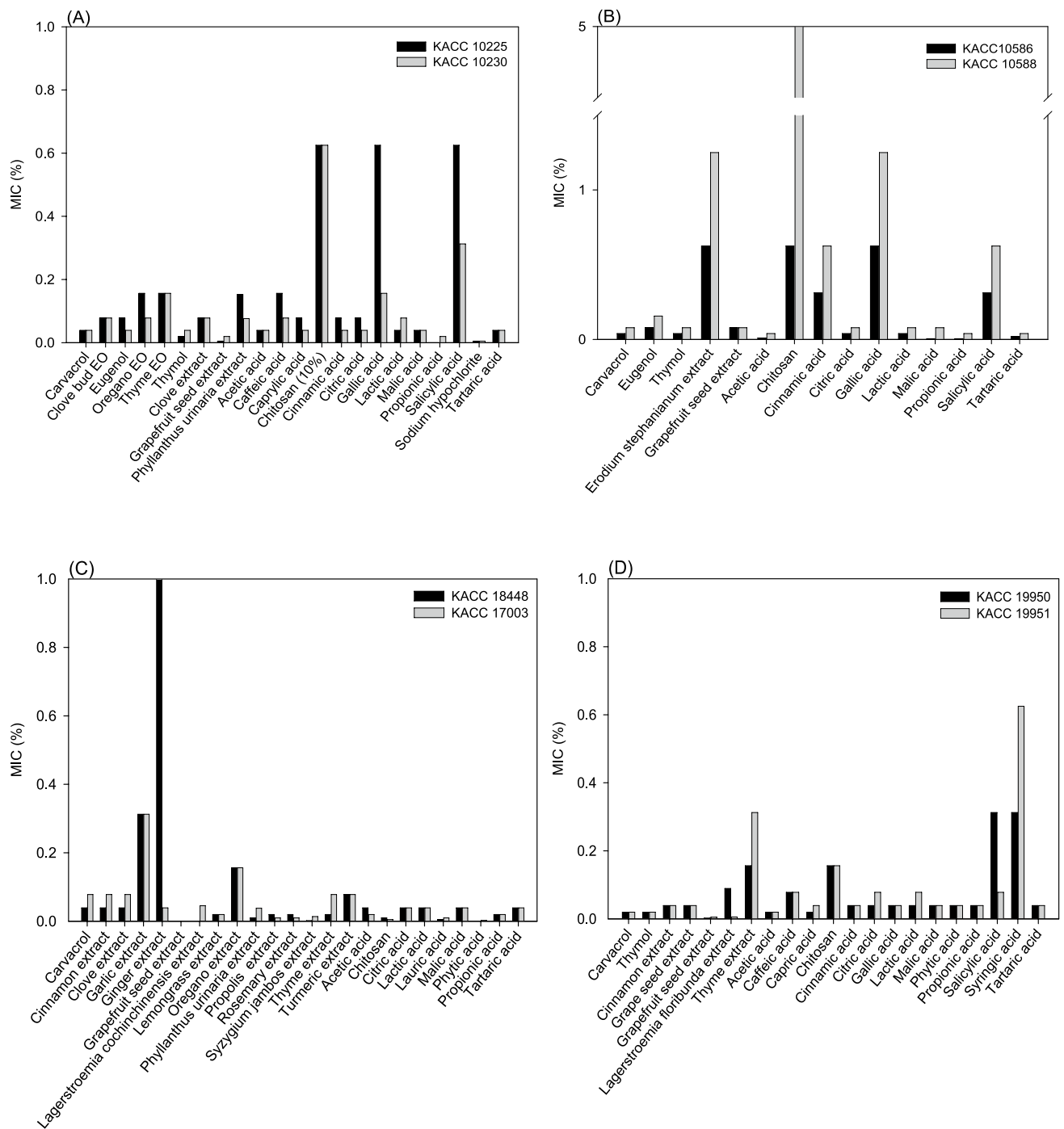


Fig. 1 Minimum inhibitory concentrations (MICs, %) of natural antimicrobials against antibiotic-sensitive (S; black bars) and antibiotic-resistant (R; gray bars) phytopathogens, including Pcc (A), Psa (B), Cmc and Cmm (C), and Xap (D)

Table 5 Natural antimicrobials with MIC values lower than 0.02% for each strain

Phytopathogens	Natural antimicrobials (MIC < 0.02%)	
Pcc (S)	Propionic acid	GSE
	Thymol	
Pcc (R)	GSE	Propionic acid
Psa (S)	Malic acid	Acetic acid
	Propionic acid	Tartaric acid
	Phytic acid	
	None	
Psa (R)	None	
Cmc (S)	GSE	Lauric acid
	Chitosan (10%)	<i>Syzygium jambos</i> extract
	Propolis extract	Lemongrass extract
	Rosemary extract	Acetic acid
	Propionic acid	Phytic acid
	GSE	Chitosan (10%)
	<i>Syzygium jambos</i> extract	Propolis extract
	Lauric acid	Lemongrass extract
	<i>Phyllanthus urinaria</i> extract	Thyme extract
	Propionic acid	Lagerstroemia cochinchinensis extract
Xap (S)	Rosemary extract	Phytic acid
	GSE	Acetic acid
	Thymol	Capric acid
	Carvacrol	
Xap (R)	GSE	Carvacrol
	Thymol	Acetic acid
	<i>Lagerstroemia floribunda</i> extract	

needed to enhance the efficacy of natural antimicrobials against phytopathogens for field applications.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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