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Synergistic effects of *Chrysanthemum indicum* ethanol extract with antibiotics against oral pathogens

Kyung-Yeol Lee[1#], Su-Mi Cha[1], Sung-Mi Choi[2], Eun-Jin Jang[3#], Jeong-Hun Seo[4] and Jeong-Dan Cha[4*]

[1]Department of Oral Microbiology and Institute of Oral Bioscience, Chonbuk National University, Jeonju, Republic of Korea

[2]Department of Dental Hygiene, Daegu Health College, Daegu, Republic of Korea

[3]Department of Dental Technology, Daegu Health College, Daegu, Republic of Korea

[4]Research Manager, Material Development Team, R&D Center, General Bio Co., Ltd., Namwon, Republic of Korea

[#]Kyung-Yeol Lee and Sung-Mi Choi contributed equally to this work

Abstract

Wild chrysanthemum (*Chrysanthemum indicum*) is one of well-known medicinal plants traditionally used in Korea. This study aimed to investigate the synergistic antibacterial activity of *C. indicum* ethanol extract (CIE) with existing antimicrobial agents against oral pathogen. The synergistic effects and anti-biofilm of CIE were evaluated against oral bacteria, either alone or with antibiotics, via broth dilution method and crystal violet assays. MIC/MBC values for CIE, ampicillin, and gentamicin all the tested bacteria ranged between 2-32/4-64 microg/mL, 0.0625-16/0.125-32 microg/mL, and 2-256/8-512 microg/mL, respectively. The synergistic effects were exhibited on CIE with antibiotics against oral bacteria at fractional inhibitory concentration index (FICI) <0.5. Moreover, CIE and antibiotics were found to synergistically reduce biofilm formation. 1-6 hours of treatment with 1/2 MIC of CIE with 1/2 MIC of antibiotics resulted from an increase of the rate of killing in units of CFU/mL to a greater degree than was observed with alone. These results suggest that the CIE is important in the antibacterial actions of oral pathogen agents.

Introduction

Many oral diseases have been known as chronic bacterial infectious diseases, such as caries, periodontitis, and periimplantitis [1,2]. Some microorganisms, such as *Streptococcus, Lactobacillus,* and *Actinomyces* species, can colonize and form pathogenic plaque biofilms on the tooth surface, and have proven to be the main cause of oral infectious diseases [3,4]. Effectively inhibiting the growth of planktonic pathogenic bacteria and the formation of biofilms on the tooth surface is the basis for the prevention and treatment of oral diseases [5,6]. Virulent biofilms firmly attached to the oral surface are the main cause of infection diseases in the oral cavity, including dental caries and periodontal disease [7,8]. Recent reports have suggested a potential role of periodontal infections in more serious systemic diseases, including cardiovascular disease, respiratory infections, and diabetes, which have a significant impact on the overall health of infected individuals [9-11]. Mechanical dental plaque removal is an efficient procedure to prevent periodontitis and caries [12,13]. However, the use of chemical compounds as a complementary method is also necessary and has proven to be a valuable tool to decrease tooth biofilm formation [14].

Natural products are a major source of new natural drugs and can help increase the use of alternative medicines for health care over the past decades [15,16]. *Chrysanthemum indicum* (L.) des monl. (compositae), spreading widely in Korea, is a well-known herb with small yellow flowers [17]. Oriental traditional medicine has used the aerial parts (stems, leaves and flowers) of *C. indicum* to treat vertigo, several infectious diseases such as pneumonia, colitis, stomatitis, carbuncle, and fever and human diseases, including inflammatory diseases, oxidant stress, respiratory diseases, cancer, and hypertension [17-22]. Its flowers are also commonly used as tea to treat some eye diseases in Asia traditional medicine [23]. Several chemical compounds isolated from *C. indicum* flowers were also found to exhibit inhibitory activity against nitric oxide (NO) in lipopolysaccharide-activated macrophages and rat lens aldose reductase [24-26]. In recent years, the chemical composition of essential oil of air-dried *C. indicum* flowers has been studied, antibacterial activity of its oil has also been confirmed against *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus pneumoniae* [27,28].

In this study, the antimicrobial activities of aerial parts (stems, leaves and flowers) of *C. indicum* ethanol extract (CIE) against oral bacteria were assessed using broth microdilution method and time-kill methods and crystal violet assay for synergistic effect and biofilm formation of the combination with antibiotics.

Materials and methods

Bacterial strains

The oral bacterial strains used in this study were: *Streptococcus mutans* ATCC 25175, *Streptococcus sanguinis* ATCC 10556, *Streptococcus sobrinus* ATCC 27607, *Streptococcus ratti* KCTC (Korean collection for type cultures) 3294, *Streptococcus criceti* KCTC 3292, *Streptococcus anginosus* ATCC 31412, *Streptococcus gordonii* ATCC 10558, *Aggregatibacter actinomycetemcomitans* ATCC 43717,

Correspondence to: Jeong-Dan Cha, Research Manager, Material Development Team, R&D Center, General Bio Co., Ltd., 254 Yongtusna-ro, Songdong-myeon, Namwon-si, Jeollabuk-do, 55793 Republic of Korea, Tel. +82-63-263-0001; Fax: +82-70-5101-1563; E-mail: joungdan@jbnu.ac.kr

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Fusobacterium nucleatum ATCC 10953, *Prevotella intermedia* ATCC 25611, and *Porphylomonas gingivalis* ATCC 33277. Brain-Heart Infusion (Difco Laboratories, Detroit, MI) broth supplemented with 1% yeast extract (Difco) was used for all bacterial strains except *P. intermedia* and *P. gingivalis*. For *P. intermedia* and *P. gingivalis*, BHI broth containing hemin 1 μg/mL (Sigma, St. Louis, MO, USA) and menadione 1 μg/mL (Sigma) was used.

Minimum inhibitory concentrations/minimum bactericidal concentrations assay

The minimum inhibitory concentrations (MICs) were determined for CIE by the broth dilution method and were carried out in triplicate. The antibacterial activities were examined after incubation at 37°C for 18 h (facultative anaerobic bacteria), for 24 h (microaerophilic bacteria), and for 1-2 days (obligate anaerobic bacteria) under anaerobic conditions. MICs were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. MIC_{50S} and MIC_{90}s were defined as MICs in which 50 and 90% of oral bacteria were inhibited, respectively. Following anaerobic incubation of **MICs** plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of CIE that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. Ampicillin and gentamicin (Sigma) were used as standard antibiotics in order to compare the sensitivity of CIE against oral bacteria.

Checker-board dilution test

The antibacterial effects of a combination of CIE, which exhibited the highest antimicrobial activity, and antibiotics were assessed by the checkerboard test as previously described [29,30]. The antimicrobial combinations assayed included CIE with antibiotics, ampicillin and gentamicin. Serial dilutions of two different antimicrobial agents were mixed in cation-supplemented Mueller-Hinton broth. After 24-48 hr of incubation at 37°C, the MICs were determined to be the minimal concentration at which there was no visible growth and MBCs were determined on the basis of the lowest concentration of CIE that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. The fractional inhibitory concentration (FIC)/ fractional bactericidal concentration (FBC) index was calculated according to the equation: $FIC/FBC\ index=FIC/FBC_A+FIC/FBC_B$=(MIC/MBC of drug A in combination/MIC/MBC of drug A alone)+(MIC/MBC of drug B in combination/MIC/MBC of drug B alone). The FIC and FBC index are the sum of the FICs and FBCs of each of the drugs, which in turn is defined as the MIC and MBC of each drug when it is used in combination divided by the MIC and MBC of the drug when it is used alone. The interaction was defined as synergistic if the FIC and FBC index was less than or equal to 0.5, additive if the FIC and FBC index was greater than 0.5 and less than or equal 1.0, indifferent if the FIC and FBC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC and FBC index was greater than 2.0.

Biofilm formation assay

Evaluation of the effect of CIE on biofilm formation of oral bacteria by crystal violet biofilm formation assay was performed according to previous studies [14, 31]. Briefly, 200 μL aliquots of treated oral bacteria (final concentration of 1.0×10^6CFU/mL) at sub-lethal dose of CIE (1/4 MIC) plus antibiotics (1/8 MIC) was transferred to a flat-bottomed sterile polystyrene microplate, and incubated for 24-48 h at 37°C under anaerobic conditions to form biofilm. Then, cells were washed with phosphate-buffered saline (PBS), stained with 0.1% (wt/vol) crystal violet solution for 15 min, washed with PBS, and de-stained with 96% ethanol 10 min in order to fix the cells. Thereafter, the wells were rinsed and air-dried. 33% (vol/vol) acetic acid was then added to each well and biofilm formation was quantified by measuring the absorbance of the solution at 540 nm using a microplate reader (BMG LABTECH, USA).

Time-kill and growth inhibition curves assay

Bactericidal activities of the drugs under study were also evaluated using time-kill curves on oral bacteria. Tubes containing Mueller-Hinton supplemented to which antibiotics had been added at concentrations of the MIC_{50} were inoculated with a suspension of the test strain, giving a final bacterial count between $5\sim7\times10^6$ CFU/mL. The tubes were thereafter incubated at 37°C in an anaerobic chamber and viable counts were performed at 0, 0.5, 1, 2, 3, 4, 5, 6, 12 and 24 h after addition of antimicrobial agents, on agar plates incubated for up to 48 h in anaerobic chamber at 37°C. Antibiotic carryover was minimized by washings by centrifugation and serial 10-fold dilution in sterile phosphate-buffered saline, pH 7.3. Colony counts were performed in duplicate, and means were taken. The solid media used for colony counts were BHI agar for streptococci and BHI agar containing hemin and menadione for *P. intermedia* and *P. gingivalis*.

Statistical analysis

Experiments were performed three times and statistical analyses were performed with parametric tests (two-way analysis of variance [ANOVA] and Tukey's test) using commercial software (SPSS 22.0). The results were expressed as mean values ± standard errors (mean ± SE) and were considered significant at the level of $p < 0.05$.

Results and discussion

Minimum inhibitory concentrations/minimum bactericidal concentrations of CIE and antibiotics

The main etiological factor of dental caries and periodontal disease is dental plaque [3,7,32,33]. Therefore, it is reasonable to search for natural products that have antiplaque properties and antimicrobial activity against oral pathogens [15,31,34,35]. The active molecules in *C. indicum* are glycosides and flavonoids; the plant has the ability to act as antibiotic against many species of bacterial pathogens [18,27,36,37]. CIE was evaluated for their antimicrobial activities against eleven common bacterial species present in the oral cavity. The results of the antimicrobial activity showed that CIE exhibited antimicrobial activities against cariogenic bacteria at MICs, 4 to 32 μg/mL; MBCs, 4 to 64 μg/mL, against periodontopathogenic bacteria at MICs, 2 to 32 μg/mL; MBCs, 8 to 64 μg/mL and for ampicillin, either 0.0625/0.125 or 16/32 μg/mL; for gentamicin, either 2/8 or 256/512 μg/mL on tested all bacteria (Table 1). The MIC_{50} and MIC_{90} ranges of CIE were from 0.5 to 16 μg/mL and 2 to 32 μg/mL, respectively. The CIE showed the strongest antimicrobial activity against *S. gordonii* and *P. gingivalis*, at MIC/MBC, 2/4-8 μg/mL than other bacteria at MIC/MBC, 8-32/32-64 μg/mL.

Synergistic effect of CIE with antibiotics

Many antimicrobial preparations, such as conventional antibiotics, chlorhexidine (CHX), phenolic compounds and triclosan, can inhibit bacteria and biofilm effectively [38-42]. However, extensive use of these antimicrobial agents can lead to some side-effects, such as tooth staining, calculus formation, drug resistance and gastrointestinal reactions [42, 43]. Therefore, searching for new antimicrobial molecules, which exhibit few or no side-effects and long-term retention in oral cavity, has been intensified in recent years [44]. Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics [34,45]. The synergistic effects of

CIE alone or with antibiotics were evaluated in oral bacteria (Tables 2 and 3). In combination with CIE, the MIC for ampicillin was reduced ≥4-fold in tested bacteria, except *S. ratti* and *S. criceti*, producing a synergistic effect as defined by FICI ≤ 0.5. The MBC for ampicillin was shown synergistic effects in *S. sanguinis*, *A. actinomycetemcomitans*, and *P. gingivalis* by FBCI ≤ 0.5 (Table 2). In combination with CIE, the MIC for gentamicin was reduced ≥4 fold in all tested bacteria expect *A. actinomycetemcomitans* and *F. nucleatum* by FICI ≤ 0.5 and MBC in *S. anginosus*, *P. intermedia,* and *P. gingivalis* by FBCI ≤ 0.5 (Table 3).

Anti-biofilm formation of CIE with antibiotics

Oral diseases, such as dental caries, periodontal disease are directly linked with the ability of bacteria to form biofilm [1,13,46]. Biofilm in the form of supragingival and subgingival plaque is the etiologic agent in dental caries and periodontal diseases [4,32]. Flavonoids and proanthocyanidins isolated from fresh cranberry fruit were found to inhibit growth and biofilm formation of same periodontal pathogens *P. gingivalis*, *S. mutans* and other oral streptococci [35,47,48]. *C. indicum* extracts have also been shown to exhibit antibacterial, antiviral, immunomodulatory, hepatoprotective, and bone remodeling activities, which may be due to the presence of flavonoids, terpenoids, and phenolic compounds in the extracts [17, 19, 22, 23, 26]. In this study, CIE 1/4 MIC with ampicillin 1/8 MIC effectively inhibited the biofilm formation of *S. sanguinis, A. actinomycetemcomitans,* and *P. gingivalis*, with ≤25% inhibition, for gentamicin 1/8 MIC at the inhibited the biofilm formation of *S. anginosus*, *P. intermedia*, and *P. gingivalis* with ≤25% inhibition (Figure 1). The CIE showed strong antibiofilm formation against cariogenic and periodontic bacteria.

Table 1. Antibacterial activity of CIE and antibiotics in oral bacteria

Samples	CIE (μg/mL)			Ampicillin	Gentamicin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC[3]	MIC/MBC (μg/mL)	
***S. mutans* ATCC 25175**[1]	4	16	16/32	0.125/0.25	16/16
***S. sanguinis* ATCC 10556**	2	8	8/32	0.125/0.5	16/32
***S. sobrinus* ATCC 27607**	16	32	32/32	0.0625/0.25	16/32
***S. ratti* KCTC 3294**[2]	4	16	16/32	0.25/0.5	16/32
***S. criceti* KCTC 3292**	8	32	32/64	0.0625/0.125	8/16
***S. anginosus* ATCC 31412**	4	8	8/32	0.125/0.25	8/32
***S. gordonii* ATCC 10558**	1	4	4/4	0.125/0.5	16/32
***A. actinomycetemcomitans* ATCC 43717**	8	32	32/64	8/32	16/32
***F. nucleatum* ATCC 51190**	2	16	16/32	16/32	2/8
***P. intermedia* ATCC 49049**	2	8	8/32	2/4	32/64
***P. gingivalis* ATCC 33277**	0.5	2	2/8	0.25/0.5	256/512

[1]American Type Culture Collection (ATCC)
[2]Korean collection for type cultures (KCTC)
[3]Minimum inhibitory concentration/minimum bactericidal concentration (MIC/MBC)

Table 2. Synergistic effects of CIE with ampicillin against oral bacteria

Strains	Agent	MIC/MBC (μg/mL)		FIC	FICI[2]	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25175[3]	CIE	16/32	4/16	0.25/0.5	0.5/1.0	Synergistic/ Additive
	Ampicillin	0.125/0.25	0.0313/0.125	0.25/0.5		
S. sanguinis ATCC 10556	CIE	8/32	2/8	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.125/0.5	0.0313/0.125	0.25/0.25		
S. sobrinus ATCC 27607	CIE	32/32	8/16	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.0625/0.25	0.0156/0.0625	0.25/0.25		
S. ratti KCTC 3294[4]	CIE	16/32	4/8	0.25/0.25	0.75/0.75	Additive/ Additive
	Ampicillin	0.25/0.5	0.125/0.25	0.5/0.5		
S. criceti KCTC 3292	CIE	32/64	8/16	0.25/0.25	0.75/0.75	Additive/ Additive
	Ampicillin	0.0625/0.125	0.0313/0.0625	0.5/0.5		
S. anginosus ATCC 31412	CIE	8/32	2/8	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.125/0.25	0.0313/0.125	0.25/0.5		
S. gordonii ATCC 10558	CIE	4/4	1/2	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.125/0.5	0.0313/0.0625	0.25/0.25		
A. actinomycetemcomitans ATCC 43717	CIE	32/64	8/16	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	8/32	2/4	0.25/0.125		
F. nucleatum ATCC 51190	CIE	16/32	4/8	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	16/32	4/16	0.25/0.5		
P. intermedia ATCC 49049	CIE	8/32	2/8	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	2/4	0.5/2	0.25/0.5		
P. gingivalis ATCC 33277	CIE	2/8	0.5/2	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.25/0.5	0.0625/0.125	0.25/0.25		

[1]The MIC and MBC of the CIE with ampicillin
[2] The fractional inhibitory concentration (FIC) index/fractional bactericical concentration (FBC) index
[3]American Type Culture Collection (ATCC)
[4]Korean collection for type cultures (KCTC)

Table 3. Synergistic effects of CIE with gentamicin against oral bacteria

Strains	Agent	MIC/MBC (μg/mL)		FIC	FICI[2]	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25175[3]	CIE	16/32	4/8	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Gentamicin	16/16	4/8	0.25/0.5		
S. sanguinis ATCC 10556	CIE	8/32	2/8	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Gentamicin	16/32	4/16	0.25/0.5		
S. sobrinus ATCC 27607	CIE	32/32	8/16	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Gentamicin	16/32	4/8	0.25/0.25		
S. ratti KCTC 3294[4]	CIE	16/32	4/8	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Gentamicin	16/32	4/16	0.25/0.5		
S. criceti KCTC 3292	CIE	32/64	8/16	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Gentamicin	8/16	2/8	0.25/0.5		
S. anginosus ATCC 31412	CIE	8/32	2/4	0.25/0.125	0.5/0.25	Synergistic/ Synergistic
	Gentamicin	8/32	2/4	0.25/0.125		
S. gordonii ATCC 10558	CIE	4/4	1/2	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Gentamicin	16/32	4/8	0.25/0.25		
A. actinomycetemcomitans ATCC 43717	CIE	32/64	16/16	0.5/0.25	1.0/0.75	Additive/ Additive
	Gentamicin	16/32	8/16	0.5/0.5		
F. nucleatum ATCC 51190	CIE	16/32	4/8	0.25/0.25	0.75/0.5	Additive/ Synergistic
	Gentamicin	2/8	1/2	0.5/0.25		
P. intermedia ATCC 25611	CIE	8/32	2/4	0.25/0.125	0.5/0.25	Synergistic/ Synergistic
	Gentamicin	32/64	8/8	0.25/0.125		
P. gingivalis ATCC 33277	CIE	2/8	0.5/2	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Gentamicin	256/512	64/64	0.25/0.125		

[1]The MIC and MBC of the CIE with gentamicin
[2] The fractional inhibitory concentration (FIC) index/fractional bactericical concentration (FBC) index
[3]American Type Culture Collection (ATCC)
[4]Korean collection for type cultures (KCTC)

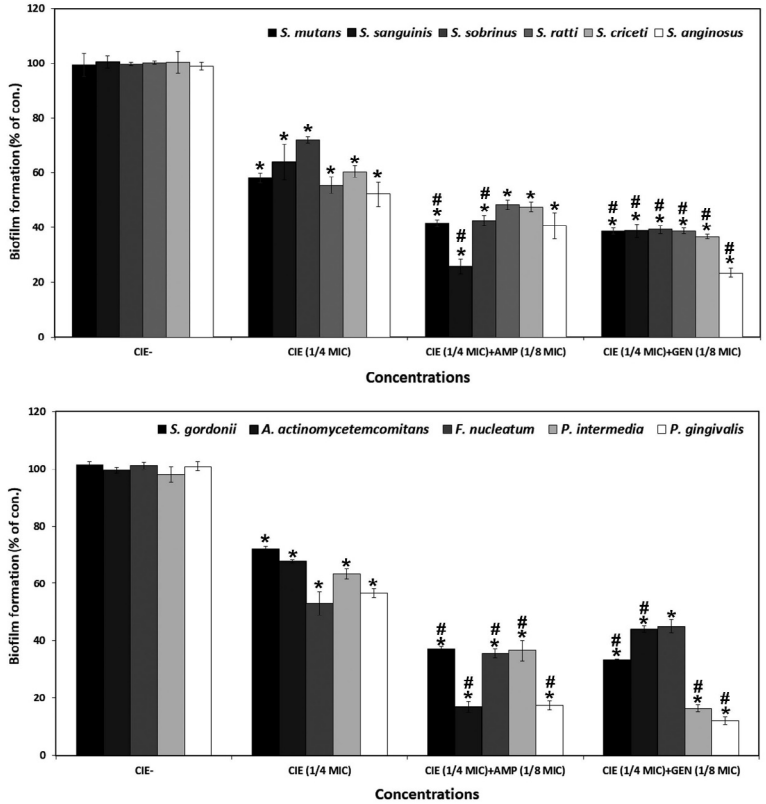

Figure 1. Anti-biofilm effect of different concentrations of CIE alone or/and antibiotics on biofilm formation of oral bacteria. Cells stained with 0.1% (wt/vol) crystal violet solution for 15 min, washed with PBS, and de-stained with 96% ethanol 10 min in order to fix the cells. Thereafter, acetic acid was then added to each well and biofilm formation was quantified by measuring the absorbance of the solution at 540 nm using a microplate reader. Data points are the mean values±SE of six experiments. *p<0.05: significantly different from CIE- and *#p<0.05*: significantly different from CIE (1/4 MIC)

Time kill of CIE with antibiotics

Among several bioactive compounds isolated from *C. indicum*, acetylenic compounds and flavonoids potently inhibited NO production in macrophages [26]. Due to the potent antibacterial and anti-inflammatory activities, *C. indicum* extracts have been shown to effectively attenuate the development of skin diseases, such as atopic dermatitis and ear edema, in animal models [23]. The bacterial effect of CIE with antibiotics, ampicillin and gentamicin against oral bacteria was confirmed by time-kill curve experiments. The CIE (MIC or MIC_{50}) alone resulted rate of killing increasing or not changing in CFU/ml at time dependent manner, with a more rapid rate of killing by CIE (MIC_{50}) with ampicillin and gentamicin (MIC_{50}) (Figures 2 and 3). A strong bactericidal effect was exerted in drug combinations.

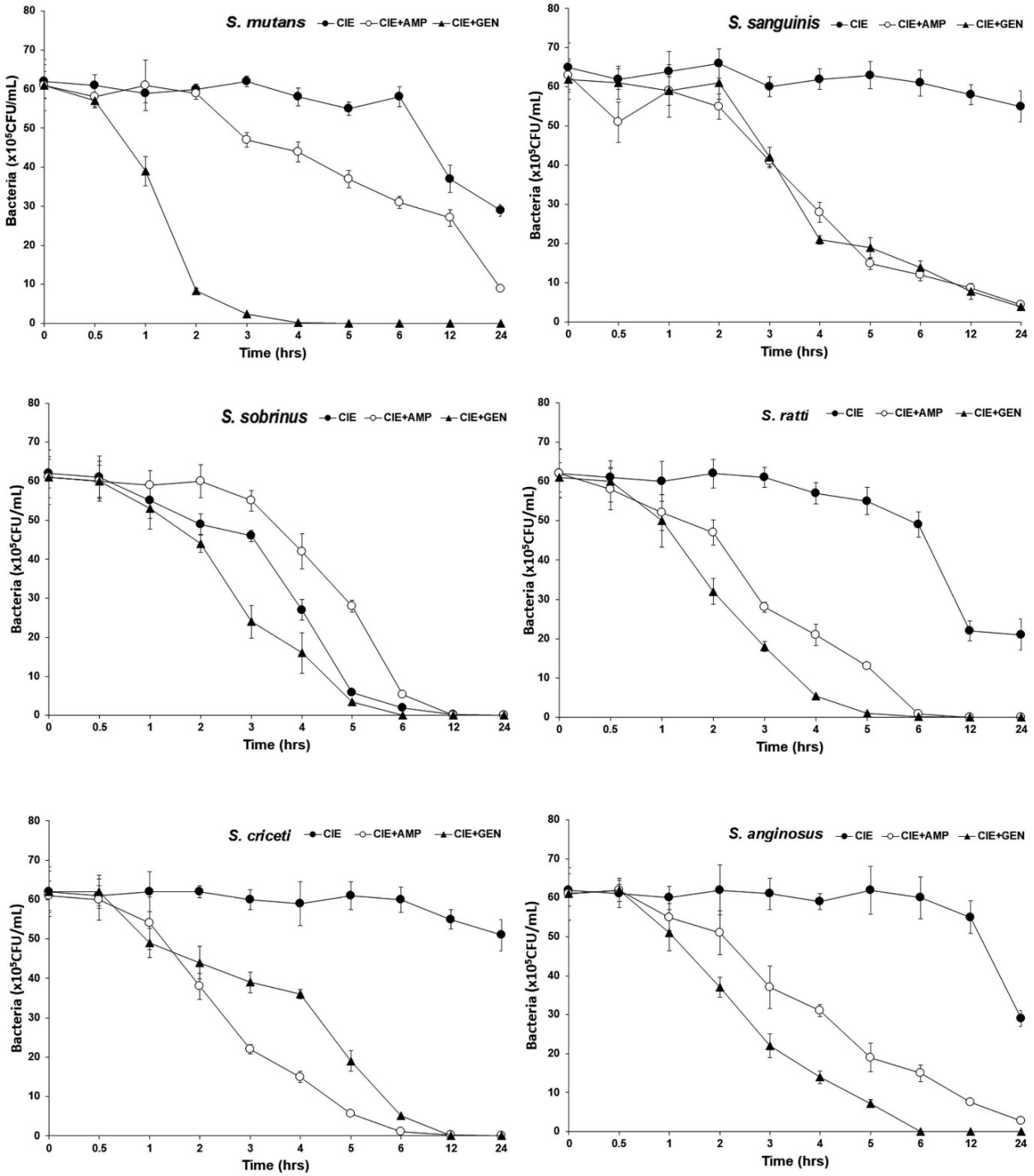

Figure 2. Time-kill curves of MIC of CIE alone and its combination with MIC_{50} of AMP or/and GEN against *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. anginosus*, *S. criceti*, and *S. ratti*. Bacteria were incubated with CIE (●), CIE + AMP (○), and CIE + GEN (▲) over time. Data points are the mean values±SE of six experiments. CFU, colony-forming units

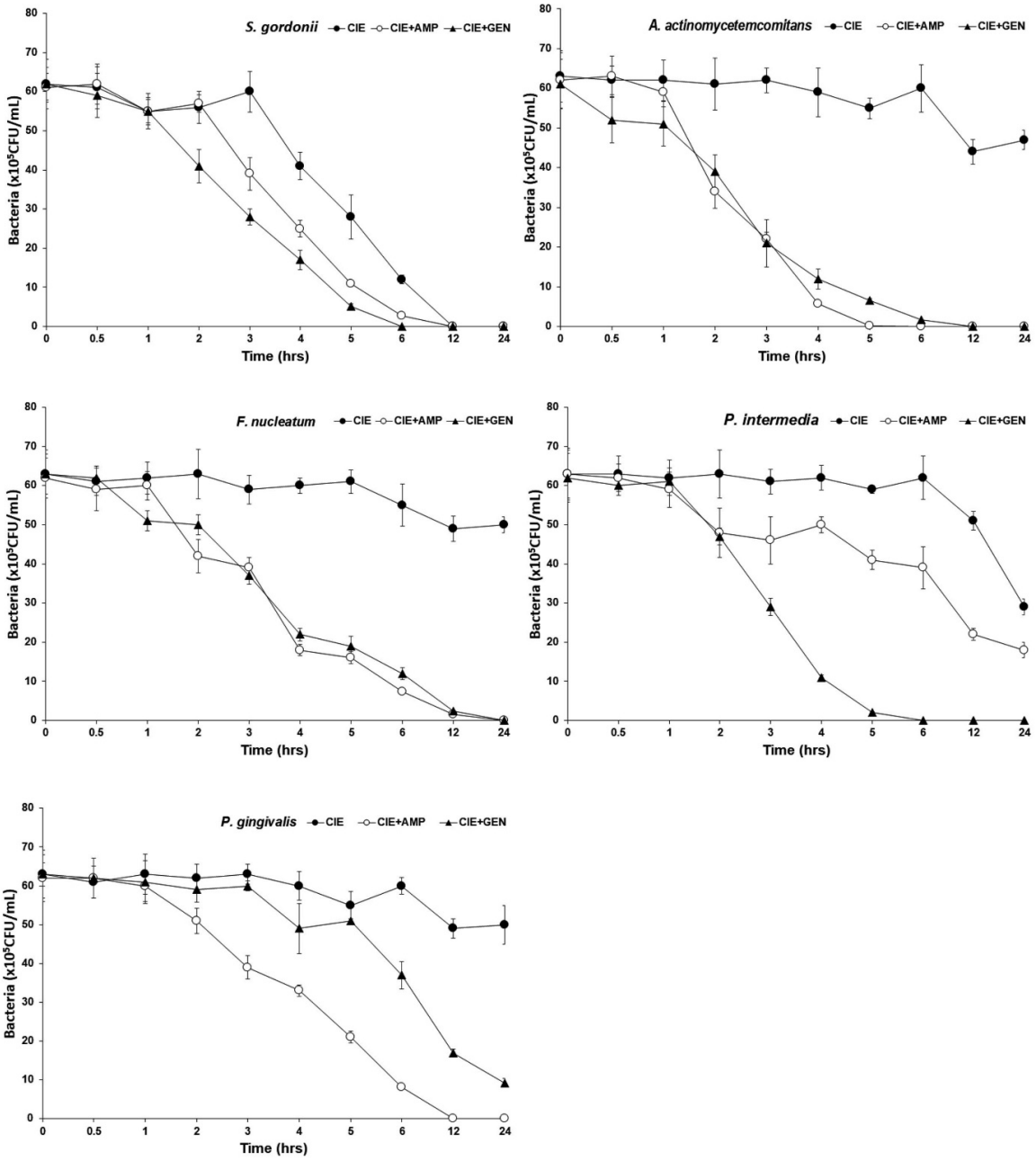

Figure 3. Time-kill curves of MIC of CIE alone and its combination with MIC_{50} of AMP or/and GEN against *S. gordonii*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia,* and *P. gingivalis*. Bacteria were incubated with CIE (●), CIE + AMP (○), and CIE + GEN (▲) over time. Data points are the mean values±SE of six experiments. CFU, colony-forming units

In conclusion, these findings suggest that CIE fulfills the conditions required of a novel cariogenic bacteria and periodontal pathogens, particularly bacteroides species drug and may be useful in the future in the treatment of oral bacteria.

Declaration of interest

The authors declare no conflicts of interest.

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This study was not funded by any organization.

Ethical approval

This study has no need for prior approval by an ethics committee.

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