Synergistic effect between cryptotanshinone and antibiotics in oral pathogenic bacteria

Jeong-Dan Cha[1], Mi-Ran Jeong[1], Kyung-Min Choi[1], Jeong-Hye Park[2], Su-Mi Cha[3], Kyung-Yeol Lee[3*]

[1]Department of Natural Products Research, Institute of Jinan Red Ginseng, Jinan, South Korea
[2]Department of Nursing, Dong-eui University, Busan, South Korea
[3]Department of Oral Microbiology and Institute of Oral Bioscience, Chonbuk National University, Jeonju, South Korea
Email: *kyleecnu@chonbuk.ac.kr

ABSTRACT

Cryptotanshinone (CT), a major tanshinone of medicinal plant *Salvia miltiorrhiza* Bunge, demonstrated effective in vitro antibacterial activity against all oral bacteria tested in this experiment. The antibacterial activities of CT against oral bacteria were assessed using the checkerboard and time-kill methods to evaluate the synergistic effects of treatment with ampicillin or gentamicin. The CT was determined against oral pathogenic bacteria with MIC and MBC values ranging from 0.5 to 16 and 1 to 64 μg/mL; for ampicillin from 0.0313 to 16 and 0.125 to 32 μg/mL; for gentamicin from 2 to 256 and 4 to 512 μg/mL respectively. The range of MIC_{50} and MIC_{90} were 0.0625 - 8 μg/mL and 1 - 64 μg/mL, respectively. The combination effects of CT with antibiotics were synergistic (FIC index < 0.5) against tested oral bacteria except additive, *Streptococcus sobrinus*, *S. criceti*, and *Actinobacillus actinomycetemcomitans* (FIC index < 0.75 - 1.0). The MBCs were shown reducing ≥4 - 8-fold, indicating a synergistic effect as defined by a FBCI of ≤0.5. Furthermore, a time-kill study showed that the growth of the tested bacteria was completely attenuated after 3 - 6 h of treatment with the 1/2 MIC of CT, regardless of whether it was administered alone or with ampicillin or gentamicin. The results suggest that CT could be employed as a natural antibacterial agent against cariogenic and periodontopathogenic bacteria.

1. INTRODUCTION

Dental caries and periodontal disease are prevalent worldwide. Bacteria existing in the dental plaque or biofilm play an important role in the development of both dental caries and periodontal disease [1,2]. Of the more than 750 species of bacteria that inhabit the oral cavity, a number are implicated in oral diseases [3]. The development of dental caries involves acidogenic and aciduric gram-positive bacteria (mutans streptococci, lactobacilli and actinomycetes) [4]. Periodontal diseases have been linked to anaerobic gram-negative bacteria (*Porphyromonas gingivalis*, *Actinobacillus*, *Prevotella*, and *Fusobacterium*) [5]. Antibiotics such as ampicillin, chlorhexidine, erythromycin, penicillin, tetracycline and vancomycin have been very effective in preventing dental caries [6]. Of the selected putative periodontal species, strains of *Prevotella intermedia*, *Fusobacterium nucleatum* and to the first time, *Tannerella forsythia*, were *β*-lactamase positive, with *P. intermedia* being the most frequently detected enzyme positive species [7]. Subgingival isolates of *P. gingivalis*, *P. intermedia and F. nucleatum* in a group of subjects increase in the MIC values of tetracycline [8]. This clinical observation led to studies that established metronidazole as an important antibiotic for anaerobic infection. Since then, this compound has also played an important role in treating anaerobe related infection in the oral cavity, abdomen, and female genital tract, among others [9]. Oral bacteria have been reported to show increased resistance towards common antibiotics such as penicillin, cephalosporin, erythromycin, tetracycline, and metronidazole which have been used therapeutically for the treatment of oral infection [10,11]. The increase in resistance and adverse effects has lead researchers to explore novel anti-infective herbal compounds which could be used for effective treatment of oral diseases [12,13].

Salvia miltiorrhiza Bunge (Danshen) is an herb commonly used in traditional oriental medicine the treatment of several pathologies including cardiovascular diseases, hepatitis, menstrual disorders, diabetes, and chronic renal failure [14-17]. Cryptotanshinone (CT) is one of the principal active constituents in Danshen extract and has several pharmacological effects, such as anti-inflamma-

tory, anti-oxidative, anti-bacterial, anti-angiogenic, anti-mutagenic, anti-platelet aggregation, anti-human hepato-cellular carcinoma effects, and anti-cyclooxygenase-2 (COX-2) functions [18-23]. CT exhibits antimicrobial activity against a broad range of Gram-positive, including *S. aureus*, and Gram-negative bacteria as well as other microorganisms [24]. Although CT exhibited fairly high levels of activity against *S. aureus*, there have been no reports related to the inhibitory mechanisms of CT against *S. aureus*. For over 100 years, chemical compounds isolated from medicinal plants have served as the models for many clinically proven drugs and are now being reassessed as antimicrobial agents [25]. Plant-derived antibacterials are always a source of novel therapeutics.

We have investigated the antibacterial activity of cryptotanshinone, a major bioactive constituent isolated from *Salvia miltiorrhiza* Bunge (Danshen) against oral pathogens when used alone and in combination with antibiotics.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

The cariogenic bacterial strains used in this study were: *Streptococcus mutans* ATCC 25,175, *Streptococcus sanguinis* ATCC 10,556, *Streptococcus sobrinus* ATCC 27,607, *Streptococcus ratti* KCTC (Korean collection for type cultures) 3294, *Streptococcus criceti* KCTC 3292,

Streptococcus anginosus ATCC 31,412, and *Streptococcus gordonii* ATCC 10,558 and the periodontopathogenic bacterial strains used: *Actinobacillus actinomycetemcomitans* ATCC 43,717, *Fusobacterium nucleatum* ATCC 10,953, *Prevotella intermedia* ATCC 25,611, and *Porphylomonas gingivalis* ATCC 33,277. Brain-Heart Infusion (BHI) broth supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI) was used for cariogenic bacterial strains. For periodontopathogenic bacterial strains, BHI broth containing hemin 1 μg/ml (Sigma, St. Louis, MO, USA) and menadione 1 μg/ml (Sigma) was used. (**Table 1**)

2.2. Minimum Inhibitory Concentrations/ Minimum Bactericidal Concentrations Assay

The minimum inhibitory concentrations (MICs) were determined for CT by the broth dilution method [26], 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_{50}s and MIC_{90}s, defined as MICs at which, 50 and 90%, respectively of oral bacteria were inhibited, were

Table 1. List of strains and culture media used for antimicrobial experiment.

Strains	Gram stain	Requires oxygen	Medium
Streptococcus mutans ATCC 25,175	+	facultative anaerobic bacteria	Brain-Heart Infusion (BHI) broth with 1% yeast extract
Streptococcus sanguinis ATCC 10,556	+	facultative anaerobic bacteria	BHI with 1% yeast extract
Streptococcus sobrinus ATCC 27607	+	facultative anaerobic bacteria	BHI with 1% yeast extract
Streptococcus ratti KCTC 3294	+	facultative anaerobic bacteria	BHI with 1% yeast extract
Streptococcus criceti KCTC 3292	+	facultative anaerobic bacteria	BHI with 1% yeast extract
Streptococcus anginosus ATCC 31,412	+	facultative anaerobic bacteria	BHI with 1% yeast extract
Streptococcus gordonii ATCC 10,558	+	facultative anaerobic bacteria	BHI with 1% yeast extract
Actinobacillus actinomycetemcomitans ATCC 43,717	−	microaerophilic bacteria	BHI with 1% yeast extract and 10% horse serum
Fusobacterium nucleatum ATCC 51,190	−	obligate anaerobic bacteria	BHI with hemin 1 μg/ml and menadione 1 μg/ml
Prevotella intermedia ATCC 25,611	−	obligate anaerobic bacteria	BHI with hemin 1 μg/ml and menadione 1 μg/ml
Porphyromonas gingivalis ATCC 33,277	−	obligate anaerobic bacteria	BHI with hemin 1 μg/ml and menadione 1 μg/ml

determined. Following anaerobic incubation of MICs plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of CT that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. Ampicillin (Sigma) and gentamicin (Sigma) were used as standard antibiotics in order to compare the sensitivity of CT against test bacteria.

2.3. Checker-Board Dilution Assay

The antibacterial effects of a combination of CT, which exhibited the highest antimicrobial activity, and antibiotics were assessed by the checkerboard test as previously described [26,27]. The antimicrobial combinations assayed included CT with ampicillin or gentamicin. Serial dilutions of two different antimicrobial agents were mixed in cation-supplemented Mueller-Hinton broth. After 24 h of incubation at 37°C, the MIC was determined to be the minimal concentration at which there was no visible growth. The fractional inhibitory concentration index (FICI) is the sum of the FICs of each of the drugs, which in turn is defined as the MIC of each drug when it is used in combination divided by the MIC of the drug when it is used alone. The interaction was defined as synergistic if the FIC index was less than or equal to 0.5, additive if the FIC index was greater than 0.5 and less than or equal 1.0, indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC index was greater than 2.0 [26,27].

2.4. Time-Kill Assay

A time-kill kinetic study against oral bacteria was performed using the broth macrodilution method [26]. The following samples were incubated in BHI medium at 37°C under anaerobic conditions: oral bacteria 5 - 7 × 10^6 CFU/mL + CT (MIC); oral bacteria 5 - 7 × 10^6 CFU/mL + CT (1/2 MIC) + Amp (1/2 MIC); and oral bacteria 5 - 7 × 10^6 CFU/mL + CT (1/2 MIC) + Gen (1/2 MIC). At 0, 30 min and 1, 2, 3, 4, 5, 6, 12, and 24 h, samples were taken and viable counts were determined as follows. Colony counts were performed in duplicate, and means were taken. The solid media used for colony counts were Brain-Heart Infusion (BHI) agar for streptococci and Brain-Heart Infusion agar containing hemin and menadione for *P. intermedia* and *P. gingivalis*.

2.5. Statistical Analysis

All the data are expressed as a mean ± standard error (SE) of triplicate experiments.

3. RESULTS

3.1. Antibacterial Activity of CT

In this study, CT was evaluated for their antimicrobial activities against eleven common bacterial species present in the oral cavity. The results of the antimicrobial activity showed that CT exhibited antimicrobial activities against cariogenic bacteria (MICs, 0.5 to 4 µg/mL; MBCs, 1 to 16 µg/mL), against periodontopathogenic bacteria (MICs, 8 to 32 µg/mL; MBCs, 16 to 64 µg/mL) and for ampicillin, either 0.125/0.5 or 64/64 µg/mL; for gentamicin, either 2/4 or 256/512 µg/mL on tested all bacteria (**Table 2** and **Figures 1-4**). The MIC_{50} and MIC_{90} determinations for cariogenic bacteria confirmed higher antibacterial activity of CT than periodontopathogenic

Table 2. Antibacterial activity of cryptotanshinone and antibiotics in oral bacteria.

Samples	Cryptotanshinone (µg/mL)			Ampicillin	Gentamicin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC	MIC/MBC (µg/mL)	
S. mutans ATCC 25,175[1]	0.25	1	1/2	0.0625/0.25	8/16
S. sanguinis ATCC 10,556	0.25	1	1/2	0.25/0.5	16/32
S. sobrinus ATCC 27,607	0.125	1	1/2	0.0313/0.125	16/32
S. ratti KCTC 3294[2]	2	16	4/16	0.125/0.5	8/16
S. criceti KCTC 3292	0.125	0.5	0.5/2	0.0313/0.125	8/16
S. anginosus ATCC 31,412	0.5	2	2/4	0.0625/0.25	8/16
S. gordonii ATCC 10,558	0.125	0.5	0.5/1	0.0625/0.25	16/32
A. actinomycetemcomitans ATCC 43,717	4	32	16/32	16/32	8/16
F. nucleatum ATCC 51,190	8	64	32/64	8/16	2/4
P. intermedia ATCC 49,049	2	16	8/16	1/2	32/32
P. gingivalis ATCC 33,277	4	16	8/16	0.5/0.5	256/512

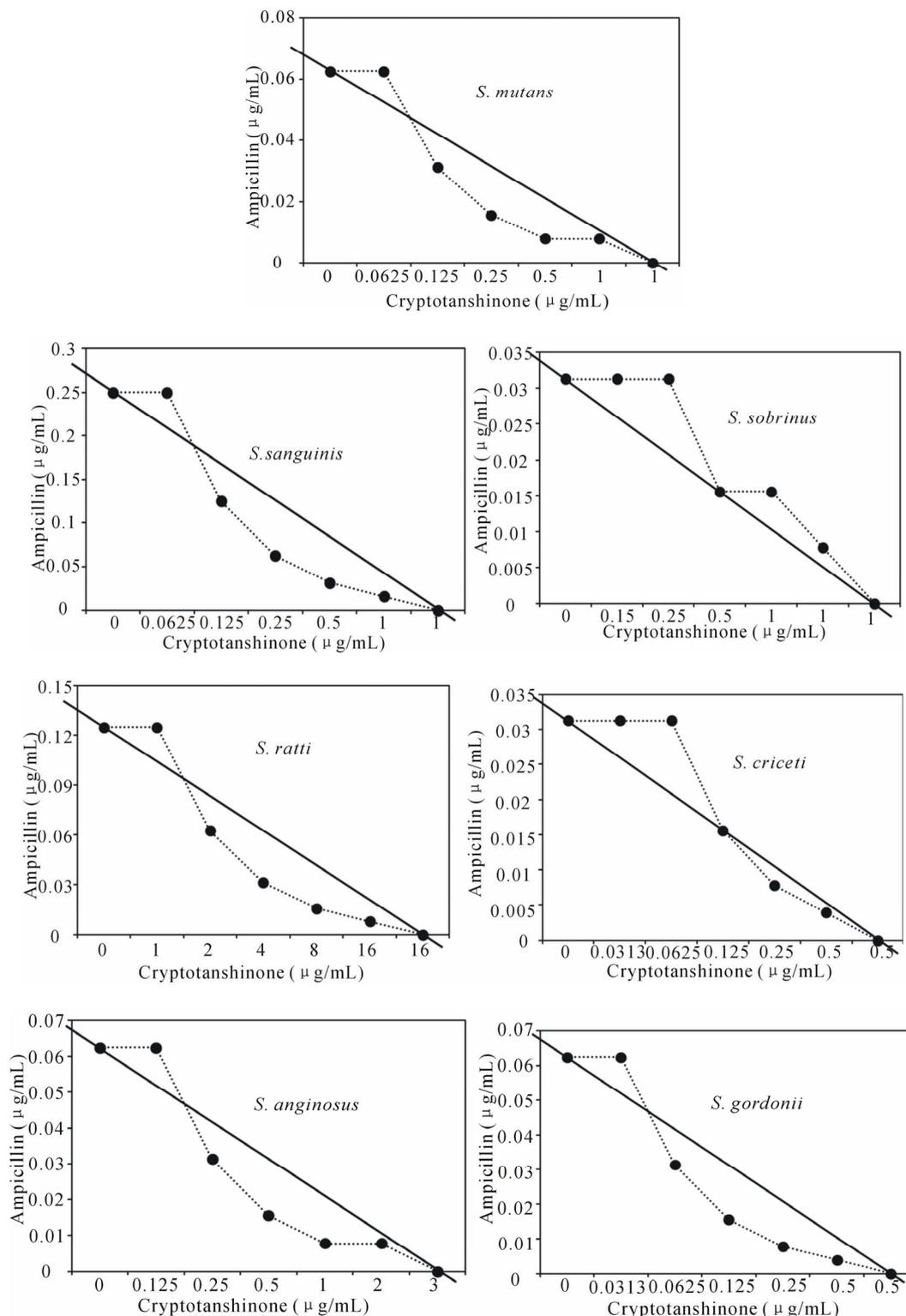[1]American Type Culture Collection (ATCC), [2]Korean collection for type cultures (KCTC).

Figure 1. Isobologram curve revealing the synergistic effect of cryptotanshinone (CT) with ampicillin against cariogenic bacteria, *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. anginosus*, *S. criceti*, and *S. ratti*.

bacteria. The range of MIC_{50} and MIC_{90} were from 0.125 to 8 μg/mL and 0.5 to 32 μg/mL, respectively. The CT showed the strongest antimicrobial activity against car- iogenic bacteria, *S. criceti* and *S. gordonii* (MIC/MBC, 0.5/1 - 2 μg/mL) and the range of MIC_{50} and MIC_{90} were 0.125 μg/mL and 0.5 μg/mL.
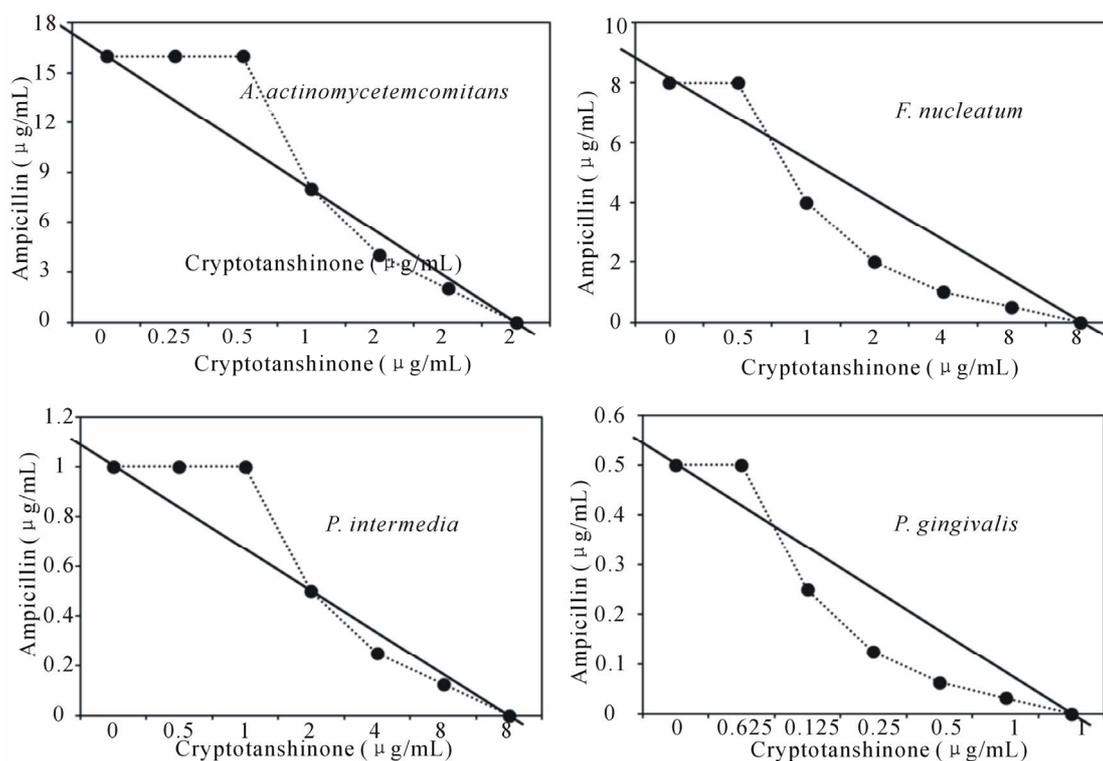
Figure 2. Isobologram curve revealing the synergistic effect of cryptotanshinone (CT) with ampicillin against periodontopathogenic bacteria, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, and *P. gingivalis*.

3.2. Synergistic Effect of CT with Antibiotics

Activity of antibiotics plus the plant extract was determined using the checkerboard technique [26,28]. The synergistic effect of CT with ampicillin or gentamicin in oral bacteria was presented in **Tables 3** and **4**, respectively. In combination of CT with ampicillin, the MIC ranges were observed in cariogenic bacteria at 0.125 μg/mL to 1 μg/mL and reduced ≥4-fold, producing a synergistic effect as defined by FICI ≤ 0.5, except additive effect in *S. sobrinus* and *S. criceti* by FICI ≤ 0.75 - 1.0. The MBC ranges (0.5 μg/mL to 4 μg/mL) of CT with ampicillin were reduced ≥4-fold in *S. ratti*, *S. anginosus*, and *S. gordonii* (**Table 3**). In periodontopathogenic bacteria, the MIC values of CT with ampicillin was also observed by ≥4-fold, producing a synergistic effect as defined by FICI ≤ 0.5, except additive effect in *A. actinomycetemcomitans* by FICI ≤ 0.75 and the MBC values (4 μg/mL to 16 μg/mL) of CT with ampicillin were reduced ≥4-fold in *F. nucleatum*. The combination of CT with gentamicin was observed resulted in the decrease ≥4-fold in MIC for most of tested bacteria, except *S. sanguinis*, *S. ratti*, *A. actinomycetemcomitans*, and *P. intermedia* by FICI ≤ 0.5 and in MBC for *S. mutans*, *S. criceti*, *S. anginosus*, and *P. gingivalis* by FBCI ≤ 0.5 but additive for *S. sanguinis*, *S. ratti*, *A. actinomycetemcomitans*, and *P. intermedia* by FIBI ≤ 0.75 (**Table 4**).

3.3. Time-Kill Curves

The synergistic effect of CT with ampicillin or gentamicin against oral bacteria was confirmed by time-kill curve experiments. The cultures of all bacteria, with a cell density of 10^5 CFU/ml, were exposed to MIC or 1/2 MIC of CT alone and with 1/2 MIC of ampicillin or gentamicin. We observed that CT with antibiotics resulted rate of killing increasing in CFU/ml at time-dependent manner (**Figures 1** and **2**). In order to assess the effects of combinations of CT and antibiotics, the MIC_{50} values of the antibiotics were determined as these provide the reference point for defining the interactions.

4. DISCUSSION

With the increase in the incidence of resistance to antibiotics, alternative natural products of plants could be of interest. Some plant extracts and phytochemicals are known to have antimicrobial properties, which could be of great importance in the therapeutic treatments [25-27, 29]. Many plants have been evaluated not only for direct antimicrobial activity but also as resistance-modifying agents [30,31]. In this study, the antibacterial activities and synergistic effects of CT or with antibiotics were exhibited in oral bacteria. The results of the antimicrobial activity showed that CT exhibited antimicrobial activities against cariogenic bacteria at 0.5 to 4 μg/mL of MICs
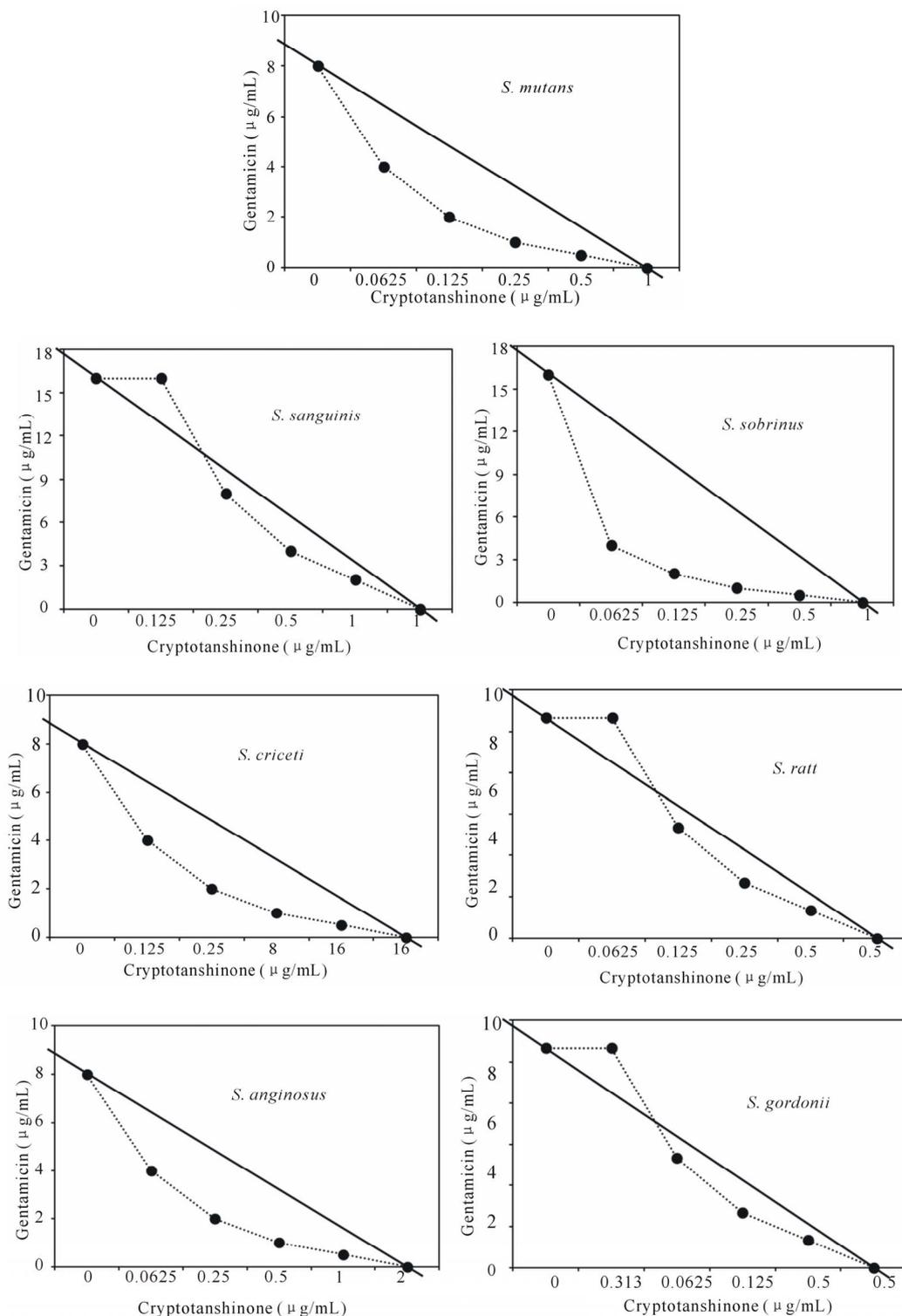
Figure 3. Isobologram curve revealing the synergistic effect of cryptotanshinone (CT) with gentamicin against cariogenic bacteria, *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. anginosus*, *S. criceti*, and *S. ratti*.

and 1 to 16 µg/mL of MBCs against periodontopathogenic bacteria at 8 to 32 µg/mL of MICs and 16 to 64 µg/mL of MBCs and the MIC_{50} and MIC_{90} determinations for cariogenic bacteria confirmed higher antibacte-

rial activity of CT than periodontopathogenic bacteria. The CT showed the strongest antimicrobial activity against cariogenic bacteria, *S. criceti* and *S. gordonii* (MIC/MBC, 0.5/1 - 2 µg/mL) and the range of MIC_{50}
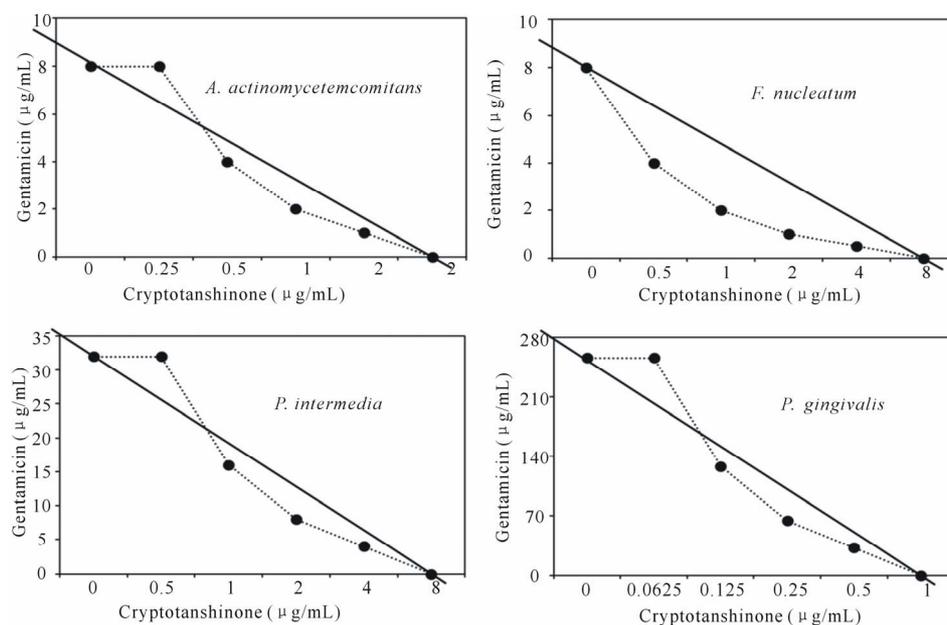
Figure 4. Isobologram curve revealing the synergistic effect of cryptotanshinone (CT) with gentamicin against periodontopathogenic bacteria, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, and *P. gingivalis*.

Table 3. Synergistic effects of cryptotanshinone with ampicillin against oral bacteria.

Strains	Agent	MIC/MBC (µg/ml)		FIC/FBC[2]	FICI/FBCI[3]	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25,175[4]	Cryptotanshinone	1/2	0.25/0.5	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.0625/0.25	0.0156/0.125	0.25/0.5		
S. sanguinis ATCC 10,556	Cryptotanshinone	1/2	0.25/0.5	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.25/0.5	0.0625/0.25	0.25/0.5		
S. sobrinus ATCC 27,607	Cryptotanshinone	1/2	0.5/1	0.5/0.5	1.0/0.75	Additive/Additive
	Ampicillin	0.0313/0.125	0.0156/0.0313	0.5/0.25		
S. ratti KCTC 3294[5]	Cryptotanshinone	4/16	1/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.125/0.5	0.0313/0.125	0.25/0.25		
S. criceti KCTC 3292	Cryptotanshinone	0.5/2	0.125/0.5	0.25/0.25	0.75/0.75	Additive/ Additive
	Ampicillin	0.0313/0.125	0.0156/0.0625	0.5/0.5		
S. anginosus ATCC 31,412	Cryptotanshinone	2/4	0.5/1	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.0625/0.25	0.0156/0.0625	0.25/0.25		
S. gordonii ATCC 10,558	Cryptotanshinone	0.5/1	0.125/0.25	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.0625/0.25	0.0156/0.0625	0.25/0.25		
A. actinomycetemcomitans ATCC 43,717	Cryptotanshinone	16/32	8/16	0.5/0.5	1.0/1.0	Additive/Additive
	Ampicillin	16/32	8/16	0.5/0.5		
F. nucleatum ATCC 51,190	Cryptotanshinone	32/64	8/16	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	8/16	2/4	0.25/0.25		
P. intermedia ATCC 49,049	Cryptotanshinone	8/16	2/8	0.25/0.5	0.5/1.0	Synergistic/ Additive
	Ampicillin	1/2	0.5/1	0.25/0.5		
P. gingivalis ATCC 33,277	Cryptotanshinone	8/16	2/4	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.5/0.5	0.125/0.25	0.25/0.5		

[1]The MIC and MBC of the cryptotanshinone with ampicillin. [2]The fractional inhibitory concentration (FIC)/The fractional bactericidal concentration (FBC). [3]The fractional inhibitory concentration index (FICI)/The fractional bactericidal concentration index (FBCI). [4]American Type Culture Collection (ATCC). [5]Korean collection for type cultures (KCTC).

Table 4. Synergistic effects of cryptotanshinone with gentamicin against oral bacteria.

Strains	Agent	MIC/MBC (μg/ml)		FIC/FBC[2]	FICI/FBCI[3]	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25,175[4]	Cryptotanshinone	1/2	0.125/0.5	0.125/0.25	0.375/0.5	Synergistic/ Synergistic
	Gentamicin	8/16	2/4	0.25/0.25		
S. sanguinis ATCC 10,556	Cryptotanshinone	1/2	0.5/0.5	0.5/0.25	0.75/0.75	Additive/ Additive
	Gentamicin	16/32	4/16	0.25/0.5		
S. sobrinus ATCC 27,607	Cryptotanshinone	1/2	0.125/0.5	0.125/0.25	0.25/0.5	Synergistic/ Synergistic
	Gentamicin	16/32	2/8	0.125/0.25		
S. ratti KCTC 3294[5]	Cryptotanshinone	4/16	2/4	0.5/0.25	0.75/0.75	Additive/ Additive
	Gentamicin	8/16	2/8	0.25/0.5		
S. criceti KCTC 3292	Cryptotanshinone	0.5/2	0.125/0.5	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	8/16	2/4	0.25/0.25		
S. anginosus ATCC 31,412	Cryptotanshinone	2/4	0.25/1	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	8/16	2/4	0.25/0.25		
S. gordonii ATCC 10,558	Cryptotanshinone	0.5/1	0.125/0.25	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	16/32	4/8	0.25/0.25		
A. actinomycetemcomitans ATCC 43,717	Cryptotanshinone	16/32	4/16	0.25/0.5	0.75/1.0	Additive/ Additive
	Gentamicin	8/16	4/8	0.5/0.5		
F. nucleatum ATCC 51,190	Cryptotanshinone	32/64	8/16	0.25/0.25	0.375/0.5	Synergistic/ Synergistic
	Gentamicin	2/4	0.25/1	0.125/0.25		
P. intermedia ATCC 25,611	Cryptotanshinone	8/16	4/8	0.5/0.5	0.75/1.0	Additive/ Additive
	Gentamicin	32/32	8/16	0.25/0.5		
P. gingivalis ATCC 33,277	Cryptotanshinone	8/16	2/4	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	256/512	68/128	0.25/0.25		

[1]The MIC and MBC of the cryptotanshinone with gentamicin. [2]The fractional inhibitory concentration (FIC)/The fractional bactericidal concentration (FBC). [3]The fractional inhibitory concentration index (FICI)/The fractional bactericidal concentration index (FBCI). [4]American Type Culture Collection (ATCC). [5]Korean collection for type cultures (KCTC).

and MIC_{90} were 0.125 μg/mL and 0.5 μg/mL.

These diterpene quinines are found exclusively in the genus *Salvia* and show antibacterial, antifungal, antioxidant, anti-inflammatory, and anti-platelet aggregation effects [32-34]. The cryptotanshinone and dihydrotanshinone I exhibit strong antimicrobial activity and MIC values of these on the spore germination of *M. oryzae* were 6.25 μg/mL and 3.13 μg/mL, respectively and on *A. tumefaciens*, *E. coli*, *P. lachrymans*, *R. solanacearum*, *X. vesicatoria*, *B. subtilis*, *S. aureus*, and *S. haemolyticus* ranged from 6.25 μg/mL to 100 μg/mL, and the median inhibitory concentration (IC50) values from 3.66 μg/mL to 57.38 μg/mL. Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics [26,27,29]. In combination of CT with ampicillin, the MIC ranges were observed in cariogenic bacteria at 0.125 μg/mL to 1 μg/mL and reduced ≥4-fold, producing a synergistic effect as defined by FICI ≤ 0.5 and in periodontopathogenic bacteria. The MIC values of CT with ampicillin was also observed by ≥ 4-fold, producing a synergistic effect as defined by FICI ≤ 0.5, except additive effect in *A. actinomycetemcomitans* by FICI ≤ 0.75 and the MBC values, 4 μg/mL to 16 μg/mL of CT with ampicillin were reduced ≥4-fold in *F. nucleatum*. The combination of CT with gentamicin was observed resulted in the decrease ≥4-fold in MIC and MBC for most of tested bacteria by FICI ≤ 0.5 but additive for *S. sanguinis*, *S. ratti*, *A. actinomycetemcomitans*, and *P. intermedia* by FIBI ≤ 0.75.

Such combinations would be synergistic if there is a decrease in the MIC of each agent of four-fold; partially synergistic if there is a MIC decrease for one drug of four-fold and a decrease of two-fold of the other agent; additive if there is a two-fold reduction in the MIC of both agents; indifference is all interactions not meeting the criteria listed above and not being antagonistic
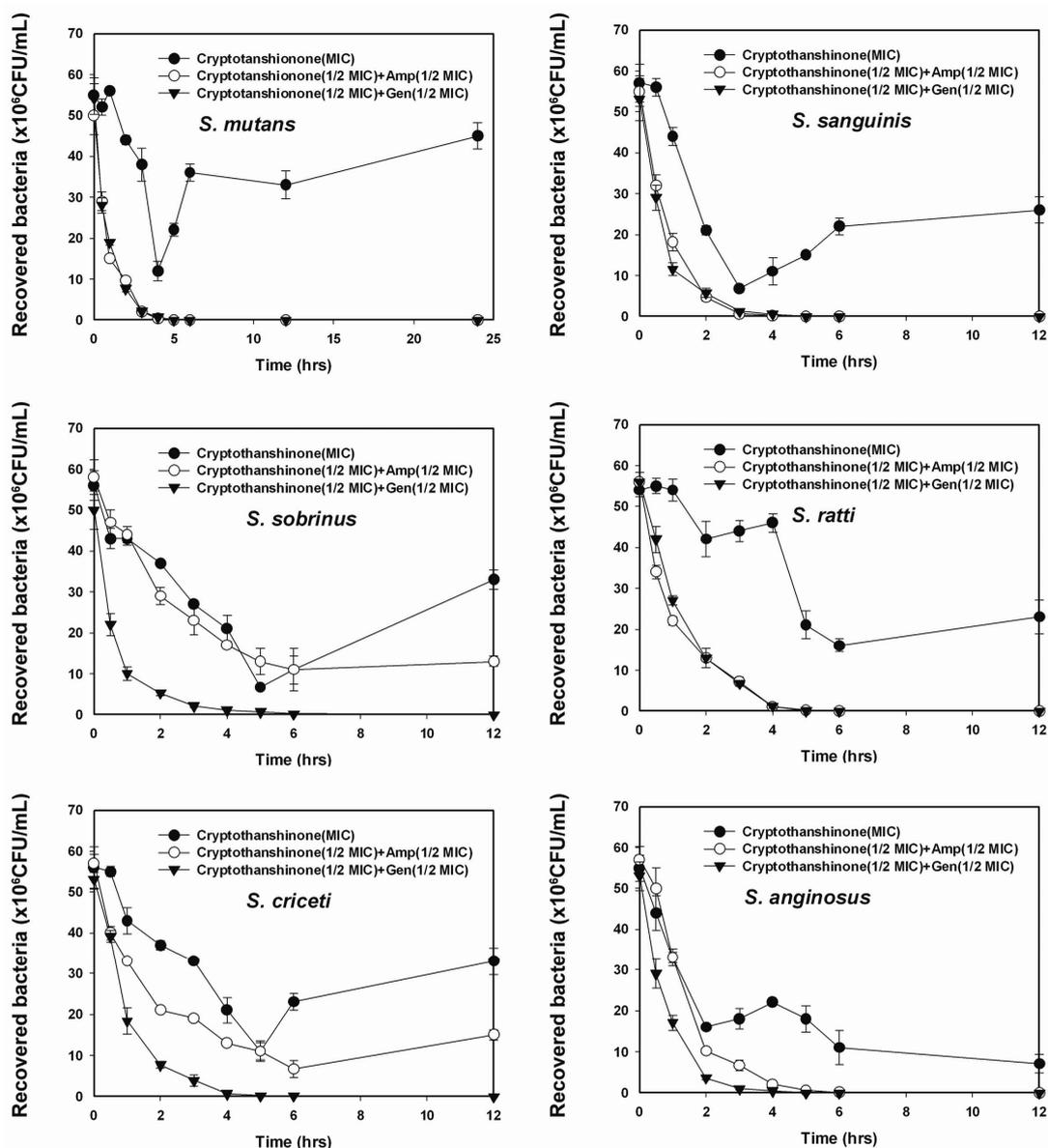
Figure 5. Time-kill curves of MIC or MIC_{50} of cryptotanshinon (CT) alone and its combination with MIC_{50} of Amp or Gen against *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. anginosus*, *S. criceti*, and *S. ratti*. Bacteria were incubated with MIC of cryptotanshinon (●), 1/2 MIC of cryptotanshinon + 1/2 MIC of Amp (○), and 1/2 MIC of cryptotanshinon + 1/2 MIC of Gen (▼) over time. CFU, colony-forming units.

[27,35]. Antagonistic response refers to where a MIC increase of four-fold for each drug would be observed in combination [36]. The synergistic effect of CT with ampicillin or gentamicin against oral bacteria was confirmed. 1 - 3 hours of treatment with 1/2 MIC of CT 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. Phenolic acids with a variety of bioactivities, including antimicrobial, antioxidant, anti-thrombosis, anti-hypertension, antivirus and antitumor properties, are widely distributed in the plant kingdom [37-39]. CT demonstrates effective *in vitro* antibacterial activity against all 21 *S. aureus* strains [24]. Affymetrix GeneChips are utilized to determine the global transcriptional response of *S. aureus* ATCC 25,923 to treatment with subinhibitory concentrations of CT [24]. Those results were found similar to our results which were evaluated as strong antibacterial activity against gram-positive bacteria, cariogenic bacteria.

In conclusion, the results suggest that combinations of CT with antibiotics should be investigated further for possible use in antibacterial products. Particularly, these may be useful in the future for the treatment of cariogenic bacteria.
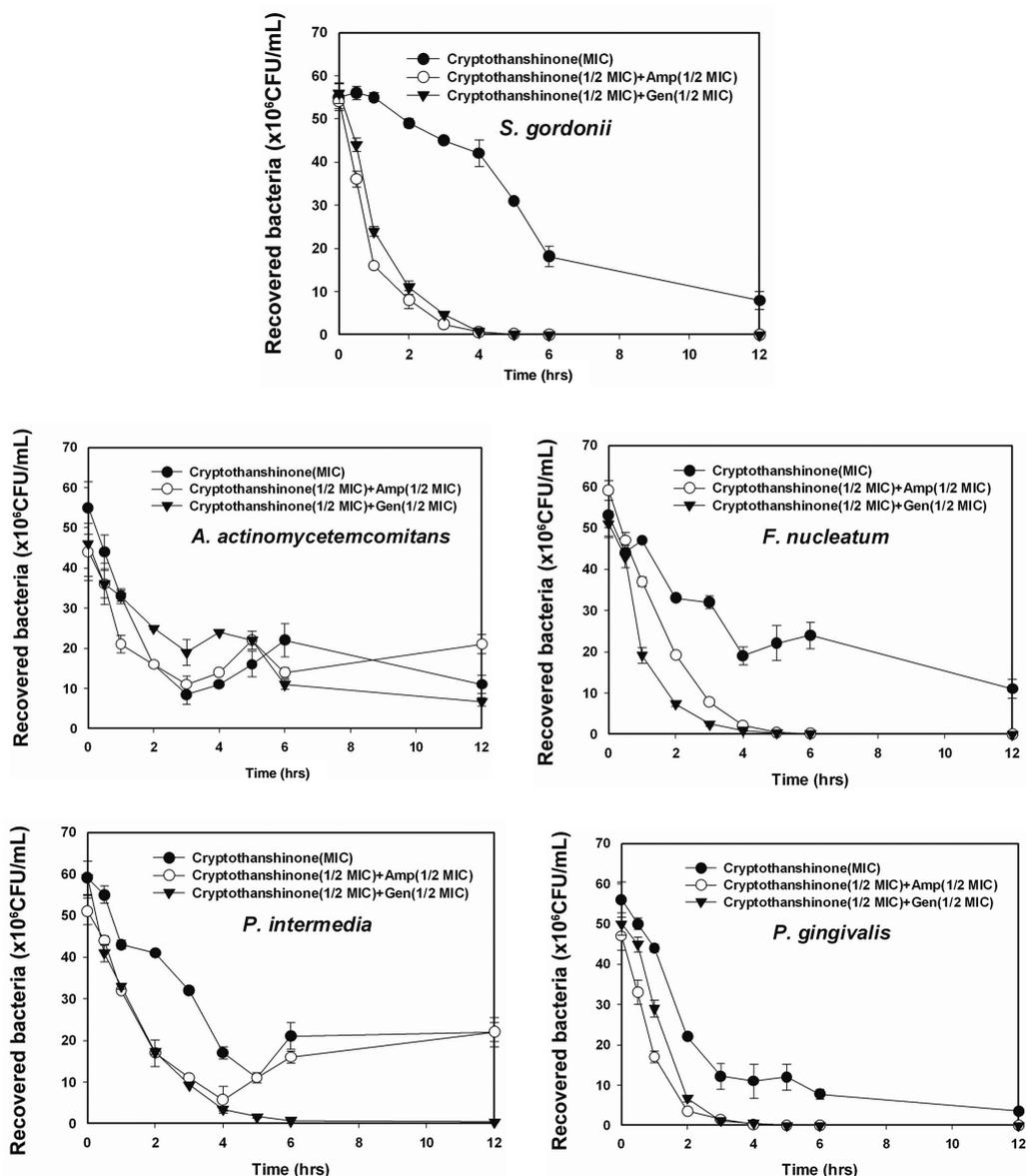
Figure 6. Time-kill curves of MIC of cryptotanshinon (CT) alone and its combination with MIC of Amp or Gen against *S. gordonii*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, and *P. gingivalis*. Bacteria were incubated with MIC of cryptotanshinon (●), 1/2 MIC of cryptotanshinon + 1/2 MIC of Amp (○), and 1/2 MIC of cryptotanshinon + 1/2 MIC of Gen (▼) over time. CFU, colony-forming units.

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