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Antimicrobial activity of *Selaginella tamariscina* extract against oral bacteria

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

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

[2]Department of Oral Microbiology and Institute of Oral Bioscience, Chonbuk National University, Jeonju, 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

[#]Contributed equally to this work

Abstract

Selaginella tamariscina (*S. tamariscina*) (Beauv.) Spring (Selaginellaceae) has been used in oriental medicine for the treatment of dysmenorrhea, chronic hepatitis, hyperglycemia, amenorrhea, hematuria, prolapse of the anus and metrorrhagia. This study aimed to investigate the synergistic antibacterial activity with existing antimicrobial agents against oral pathogen. The synergistic effects of 50% ethanol extract of *S. tamariscina* (STE) were evaluated against oral bacteria, either alone or with antibiotics, via broth microdilution and time-kill method. The minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) values for STE, ampicillin and gentamicin against all the tested bacteria ranged between 6.25-50/50-200 μg/mL, 0.0625-16/0.25-32 μg/mL, and 4-128/16-256 μg/mL, respectively. STE in combination with ampicillin showed a strong synergistic effect against oral bacteria (fractional inhibitory concentration (FIC) index ≤0.5), whereas on combining with gentamicin, it reduced the on half-eighth times than used alone (FICI ≤ 0.5). Furthermore, a time-kill study showed that the growth of the tested bacteria was completely attenuated after 2-6 h of treatment with 1/2 MIC of STE 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. The results of this study demonstrate the antimicrobial and synergistic activity of STE and antibiotics against oral pathogens.

Abbreviations: STE: The ethanol extract of *Selaginella tamariscina*; MICs: Minimum inhibitory concentrations; MBCs: Minimum bactericidal concentrations; CFU: Colony forming unit; FIC index: Fractional inhibitory concentration; FBC index: Fractional bactericidal concentration index.

Introduction

Oral health problems, particularly periodontal diseases, dental caries and endodontic infections, are the most significant destructive processes in the oral cavity and are a costly burden to the public globally [1-3]. Dental caries (tooth decay or cavities) are the most common and widespread chronic oral diseases that affect children and adults [4]. They are irreversible infectious diseases of the teeth leading to cavities in the teeth structure, thus compromising the structure and function of the teeth [5]. Until a few decades ago, development of caries was ascribed to a few gram-positive bacterial species in the biofilm, i.e., the specific biofilm/plaque hypothesis, and *Streptococcus mutans*, *Streptococcus sobrinus* together with some Lactobacillus species were regarded as key pathogens [6,7]. Periodontal diseases are pathologic conditions of bacterial infection of the structures around the teeth (including the gums, the cementum that covers the root, the periodontal ligament and the alveolar bone) that can lead to tooth loss affecting more than half of all adults [8,9]. Generally, the etiological agents of periodontal diseases are Gram-negative rods including *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, Prevotella, Fusobacterium, and *Porphyromonas gingivalis* [9,10]. Recent reports have suggested a potential role for periodontal infections in more serious systemic diseases including cardiovascular disease, respiratory infections, and diabetes, which are pathologies that significantly affect the overall health of the infected individual [11].

Mechanical dental plaque removal is an efficient procedure to prevent periodontitis and caries [12]. 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 [13,14]. *Selaginella tamariscina* (*S. tamariscina*) (Beauv.) Spring (Selaginellaceae) has been used in oriental medicine for the treatment of dysmenorrhea, chronic hepatitis, hyperglycemia, cancer, amenorrhea, hematuria, prolapse of the anus, and metrorrhagia [15-17]. Pharmacological studies on *S. tamariscina* have reported its anti-inflammatory, antibacterial, anti-hypertensive and anti-hyperglycemic activities [17-20]. The investigation of the phytochemical constituents of *S. tamariscina* revealed it to be an abundant source of biflavonoids such as amentoflavone, hinokiflavone, isocryptomerine, sotetsuflavone,

Correspondence to: Jeong-Dan Cha, Ph.D., 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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and sumaflavone [18,21]. The biflavonoids isolated from *S. tamariscina* are known to display a variety of biological activities involving anti-inflammatory, anti-allergic, antitumor, antioxidant, antidiabetic, antiviral, and anticancer activities, and osteogenesis [15,18,20,21].

In this study, the antimicrobial activities of 50% ethanol extract of *Selaginella tamariscina* (STE) against oral bacteria were assessed using broth microdilution method and time-kill method for synergistic effect of the combination with antibiotics.

Materials and methods

Plant material and preparation of 50% ethanol extract of *Selaginella tamariscina* (STE)

Dried *Selaginella tamariscina* (P.Beauv.) Spring (*Selaginella Herba*, known in Korea as Kwon Baek or Boo Cheo Son) (100g) was macerated and were extracted in 8-fold volumes of 50% ethanol (800 mL) at 80°C for 4 h. The extract was then filtered, concentrated using a rotary vacuum evaporator (EYELA, Japan), lyophilized using a freeze dryer, and stored at 4°C. The yield of the lyophilized extract obtained was 18.5% (w/w) of dried *S. tamariscina*.

Bacterial strains

The oral bacterial strains used in this study were: *Streptococcus mutans* ATCC 25175 (American Type Culture Collection), *Streptococcus sanguinis* ATCC 10556, *Streptococcus parasanguinis* KCOM 1497 (Korean Collection for Oral Microbiology), *Streptococcus sobrinus* ATCC 27607, *Streptococcus ratti* KCTC (Korean Collection for type cultures) 3294, *Streptococcus criceti* KCTC 3292, *Streptococcus downei* KCOM 1165, *Streptococcus anginosus* ATCC 31412, *Streptococcus gordonii* ATCC 10558, *Aggregatibacter actinomycetemcomitans* ATCC 43717, *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) and menadione 1 μg/mL (Sigma) was used.

Minimum inhibitory concentrations/minimum bactericidal concentrations assay

The minimum inhibitory concentrations (MICs) were determined for 50% ethanol extract of *S. tamariscina* (STE) 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, used a mix of H2 and nitrogen (N2) (5/95%) or N2/carbon dioxide (CO2)/H2 (85/10/5 %) to remove oxygen. 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, defined as MICs at which, 50% of MIC of oral bacteria were inhibited, were determined. Following anaerobic incubation of **MICs** plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of STE 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 STE against oral bacteria.

Checkerboard dilution test

The antibacterial effects of a combination of STE and antibiotics were assessed by the checkerboard test as previously described [22,23]. The antimicrobial combinations assayed included STE with antibiotics, ampicillin, gentamicin, erythromycin, and vancomycin. Serial dilutions of two different antimicrobial agents were mixed in cation-supplemented Mueller-Hinton broth. After 24-48 h 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 STE 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:

$$\text{FIC index}=FIC_A+FIC_B=\frac{\text{Conc. Of A in MICs of A+B}}{\text{MIC of A alone}}+\frac{\text{Conc. Of B in MICs of A+B}}{\text{MIC of B alone}}$$

$$\text{FBC index}=FBC_A+FBC_B=\frac{\text{Conc. Of A in MBCs of A+B}}{\text{MBC of A alone}}+\frac{\text{Conc. Of B in MBCs of A+B}}{\text{MBC of B alone}}$$

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 [22].

Time-kill and growth inhibition curves assay

Bactericidal activities of STE and antibiotics 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 1/2 MIC 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*.

Results and discussion

Minimum inhibitory concentrations/minimum bactericidal concentrations of STE and antibiotics

The use of natural products and herbal medicines has been documented in the past. They have been reported to be effective in the management of many infections in general. Some of these have been assessed in the recent past for their antimicrobial potential against oral bacteria [24-26]. STE was evaluated for their antimicrobial activities against thirteen oral bacterial species present in the oral cavity. The results of the antimicrobial activity showed that STE exhibited antimicrobial activities against cariogenic bacteria at MICs, 6.25 to 50 μg/mL; MBCs, 25 to 100 μg/mL, against periodontopathogenic bacteria

at MICs, 12.5 to 50 μg/mL; MBCs, 50 to 200 μg/mL and for ampicillin, either MIC/MBCs 0.0625/0.25 or 16/32 μg/mL; for gentamicin, either MIC/MBCs 4/16 or 128/256 μg/mL on tested all bacteria (Table 1). The MIC_{50} and MIC_{90} ranges of STE were from 3.13 to 12.5 μg/mL and 12.5 to 50 μg/mL, respectively. The STE showed stronger antimicrobial activity against *S. mutans, S. criceti, S. gordonii*, and *P. gingivalis* at MIC/MBC, 6.25/25-12.5/50 μg/mL than another bacteria at MIC/MBC, 25-50/50-200 μg/mL. Isocryptomerin isolated from *Selaginella tamariscina* showed potent antibacterial activity against gram-positive and gram-negative bacterial strains including clinical isolates of multidrug-resistant bacteria [19,27].

Synergistic effect of STE with antibiotics

Many antimicrobial preparations, such as conventional antibiotics, chlorhexidine (CHX), phenolic compounds and triclosan, can inhibit bacteria biofilm effectively [28-30]. However, extensive use of these antimicrobial agents can lead to some side-effects, such as tooth staining, calculus formation, drug resistance and gastrointestinal reactions [30,31]. 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 [32]. The synergistic effects of STE with antibiotics or with antibiotics were evaluated in oral bacteria (Tables 2 and 3). In combination with ampicillin, STE was reduced ≥4-8 fold in all tested bacteria, except *S. sobrinus, S. criceti,* and *P. intermedia,* producing a synergistic effect as defined by FICI ≤ 0.5. The MBC for ampicillin was shown synergistic effects in *S. criceti*, *F. nucleatum, P. intermedia,* and *P. gingivalis* by FBCI ≤ 0.5 (Table 2). In combination with STE, the MIC for gentamicin was reduced ≥4-8-fold in all tested bacteria, except *S. sobrinus* by FICI ≤ 0.5 and MBC except *S. sobrinus*, *S. anginosus,* and *P. intermedia* by FBCI ≤ 0.75-1 (Table 3). Amentoflavone (AF) is a biflavonoid compound extracted from *S. tamariscina* Spring. as a representative biflavonoid with several pharmacological functions, the bioavailability of AF with intraperitoneal injection was 77.4%±28.0%. AF has pharmacological activities including anti-inflammatory, anti-microbial, and anti-oxidative [15,18,19].

Time kill of STE with antibiotics

Previous studies had reported the antimicrobial activities and mechanisms of STE against several kinds of bacteria [19,27]. However, the type of microorganisms and their cell membrane structure and composition could play an important role in the susceptibility to antimicrobials [33,34]. Overcome drug-resistance mechanisms are the use of combination of antibiotics, such as β-lactams together with β-lactamase inhibitors. β-lactam antibiotics are known to inhibit the synthesis of the bacterial cell wall by binding to the reactive Ser62 of the D-alanyl-D-alanine carboxypeptidase/transpeptidase, which catalyzes the final step in the cross-linking of the bacterial cell wall peptidoglycan [35,36]. The MIC value was similar to the results that MIC values of STE against *Staphylococcus aureus* and *Candida albicans* were 20 μg/mL and 50 μg/mL, respectively [27]. The bacterial effect of STE with antibiotics, ampicillin and gentamicin against oral bacteria was confirmed by time-kill curve experiments. The STE (MIC or 1/2 MIC) alone resulted rate of killing increasing or not changing in CFU/ml at time dependent manner, with a more rapid rate of killing by STE (1/2 MIC) with ampicillin or/and gentamicin (1/2 MIC) (Figures 1 and 2). A strong bactericidal effect was exerted in drug combinations.

Table 1. Antibacterial activity of *Selaginella tamariscina* ethanol extract (STE) and antibiotics in oral bacteria

Samples	STE (μg/mL)			Ampicillin	Gentamicin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC	MIC/MBC (μg/mL)	
S. mutans ATCC 25175[1]	3.13	12.5	12.5/50	0.25/0.5	4/16
S. sanguinis ATCC 10556	3.13	25	25/100	0.125/0.5	8/32
S. sobrinus ATCC 27607	12.5	25	25/50	0.125/0.5	16/32
S. ratti KCTC 3294[2]	12.5	50	50/100	0.25/1	16/32
S. criceti KCTC 3292	3.13	12.5	12.5/50	0.0625/0.25	8/32
S. anginosus ATCC 31412	12.5	50	50/100	0.25/1	8/16
S. gordonii ATCC 10558	1.56	6.25	6.25/25	0.125/0.5	16/64
A. actinomycetemcomitans ATCC 43717	12.5	50	50/100	16/32	4/16
F. nucleatum ATCC 51190	25	50	50/200	8/16	4/16
P. intermedia ATCC 49049	6.25	25	25/50	0.5/1	32/64
P. gingivalis ATCC 33277	3.13	12.5	12.5/50	0.5/2	128/256

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

Table 2. Synergistic effects of *Selaginella tamariscina* ethanol extract (STE) with ampicillin against oral bacteria

Strains	Agent	MIC/MBC (μg/mL)		FIC/FBC	FICI/FBCI[2]	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25175[3]	STE	12.5/50	3.13/12.5	0.25/0.25	0.5/0.75	Synergistic/Additive
	Ampicillin	0.25/0.5	0.0625/0.25	0.25/0.5		
S. sanguinis ATCC 10556	STE	25/100	6.25/25	0.25/0.25	0.5/0.75	Synergistic/Additive
	Ampicillin	0.125/0.5	0.0313/0.25	0.25/0.5		
S. sobrinus ATCC 27607	STE	25/50	12.5/25	0.5/0.5	0.75/0.75	Additive/Additive
	Ampicillin	0.125/0.5	0.0313/0.125	0.25/0.25		
S. ratti KCTC 3294[4]	STE	50/100	12.5/25	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Ampicillin	0.25/1	0.0313/0.25	0.25/0.25		
S. criceti KCTC 3292	STE	12.5/50	3.13/12.5	0.25/0.25	0.75/0.5	Additive/Synergistic
	Ampicillin	0.0625/0.25	0.0313/0.0625	0.5/0.25		
S. anginosus ATCC 31412	STE	50/100	12.5/50	0.25/0.5	0.5/0.75	Synergistic/Additive
	Ampicillin	0.25/1	0.0625/0.25	0.25/0.25		
S. gordonii ATCC 10558	STE	6.25/25	1.56/12.5	0.25/0.5	0.5/0.75	Synergistic/Additive
	Ampicillin	0.125/0.5	0.0313/0.125	0.25/0.25		
A. actinomycetemcomitans ATCC 43717	STE	50/100	12.5/25	0.25/0.25	0.5/0.75	Synergistic/Additive
	Ampicillin	16/32	4/16	0.25/0.5		
F. nucleatum ATCC 51190	STE	50/200	12.5/50	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Ampicillin	8/16	2/4	0.25/0.25		
P. intermedia ATCC 49049	STE	25/50	12.5/12.5	0.5/0.25	0.75/0.5	Additive/Synergistic
	Ampicillin	0.5/1	0.125/0.25	0.25/0.25		
P. gingivalis ATCC 33277	STE	12.5/50	3.13/12.5	0.25/0.25	0.375/0.5	Synergistic/Synergistic
	Ampicillin	0.5/2	0.0625/0.5	0.125/0.25		

[1]The MIC and MBC of the *Selaginella tamariscina* ethanol extract (STE) 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 *Selaginella tamariscina* ethanol extract (STE) with gentamicin against oral bacteria

Strains	**Agent**	**MIC/MBC (μg/mL)**		**FIC/FBC**	**FICI/FBCI[2]**	**Outcome**
		Alone	**Combination[1]**			
S. mutans ATCC 25175[3]	STE	12.5/50	3.13/12.5	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Gentamicin	4/16	1/4	0.25/0.25		
S. sanguinis ATCC 10556	STE	25/100	6.25/25	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Gentamicin	8/32	2/8	0.25/0.25		
S. sobrinus ATCC 27607	STE	25/50	6.25/25	0.25/0.5	0.75/1	Additive/Additive
	Gentamicin	16/32	8/16	0.5/0.5		
S. ratti KCTC 3294[4]	STE	50/100	12.5/25	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Gentamicin	16/32	4/8	0.25/0.25		
S. criceti KCTC 3292	STE	12.5/50	3.13/6.25	0.25/0.125	0.5/0.25	Synergistic/Synergistic
	Gentamicin	8/32	2/4	0.25/0.125		
S. anginosus ATCC 31412	STE	50/100	12.5/25	0.25/0.25	0.5/0.75	Synergistic/Additive
	Gentamicin	8/16	2/8	0.25/0.5		
S. gordonii ATCC 10558	STE	6.25/25	1.56/6.25	0.25/0.25	0.5/0.375	Synergistic/Synergistic
	Gentamicin	16/64	4/8	0.25/0.125		
A. actinomycetemcomitans ATCC 43717	STE	50/100	12.5/25	0.25/0.25	0.5/0.375	Synergistic/Synergistic
	Gentamicin	4/16	1/2	0.25/0.125		
F. nucleatum ATCC 51190	STE	50/200	12.5/50	0.25/0.25	0.5/0.375	Synergistic/Synergistic
	Gentamicin	4/16	1/2	0.25/0.125		
P. intermedia ATCC 25611	STE	25/50	6.25/25	0.25/0.5	0.5/0.625	Synergistic/Additive
	Gentamicin	32/64	8/8	0.25/0.125		
P. gingivalis ATCC 33277	STE	12.5/50	3.13/12.5	0.25/0.25	0.5/0.5	Synergistic/Synergistic
	Gentamicin	128/256	32/64	0.25/0.25		

[1]The MIC and MBC of the *Selaginella tamariscina* ethanol extract (STE) 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)

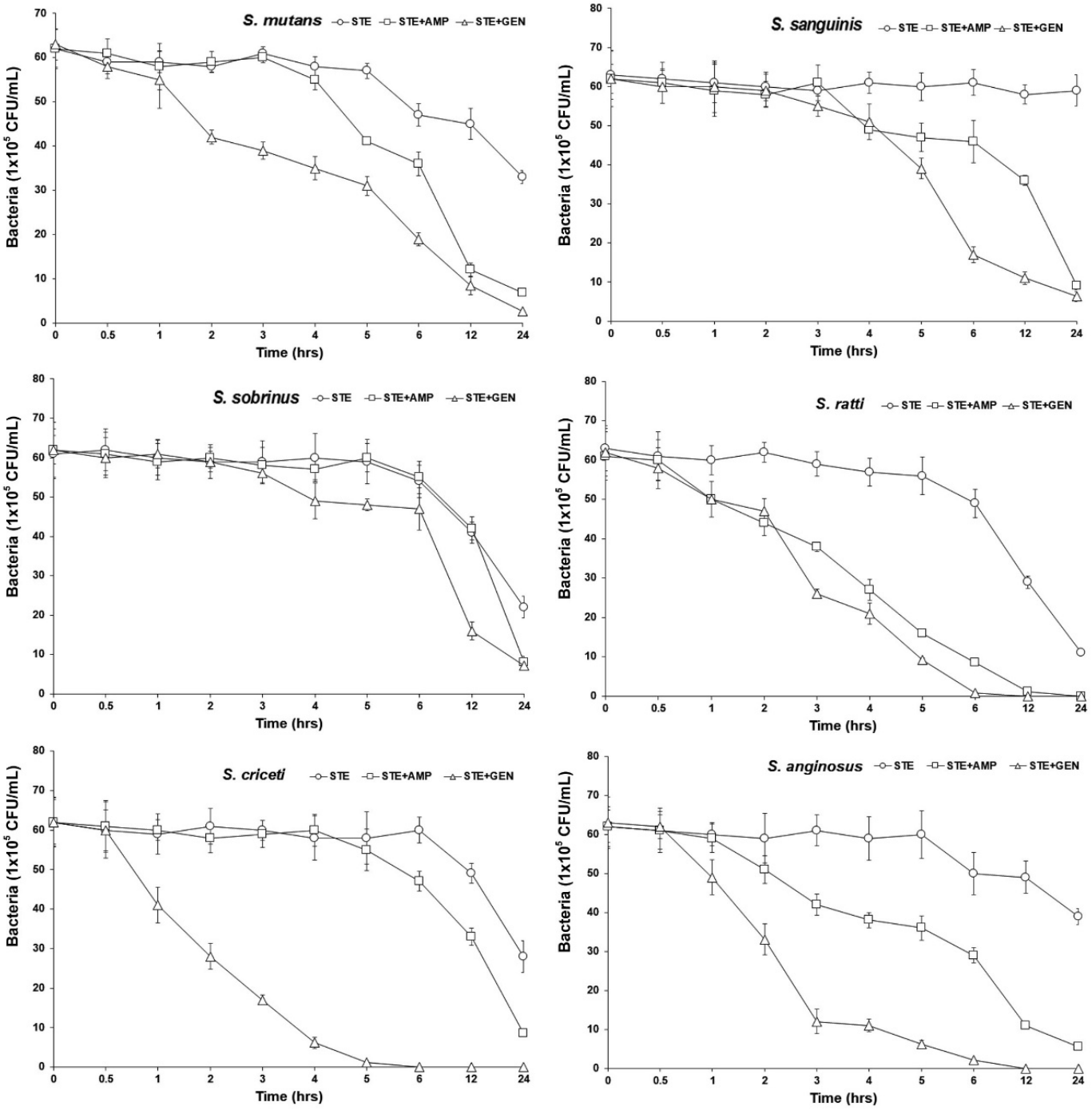

Figure 1. Time-kill curves of MIC of STE alone and its combination with 1/2 MIC of AMP and GEN against *S. mutans*, *S. sanguinis*, *S. sobrinus, S. ratti, S. criceti,* and *S. anginosus*. Bacteria were incubated with STE (○), STE + AMP (□), and STE + GEN (△) over time. CFU, colony-forming units

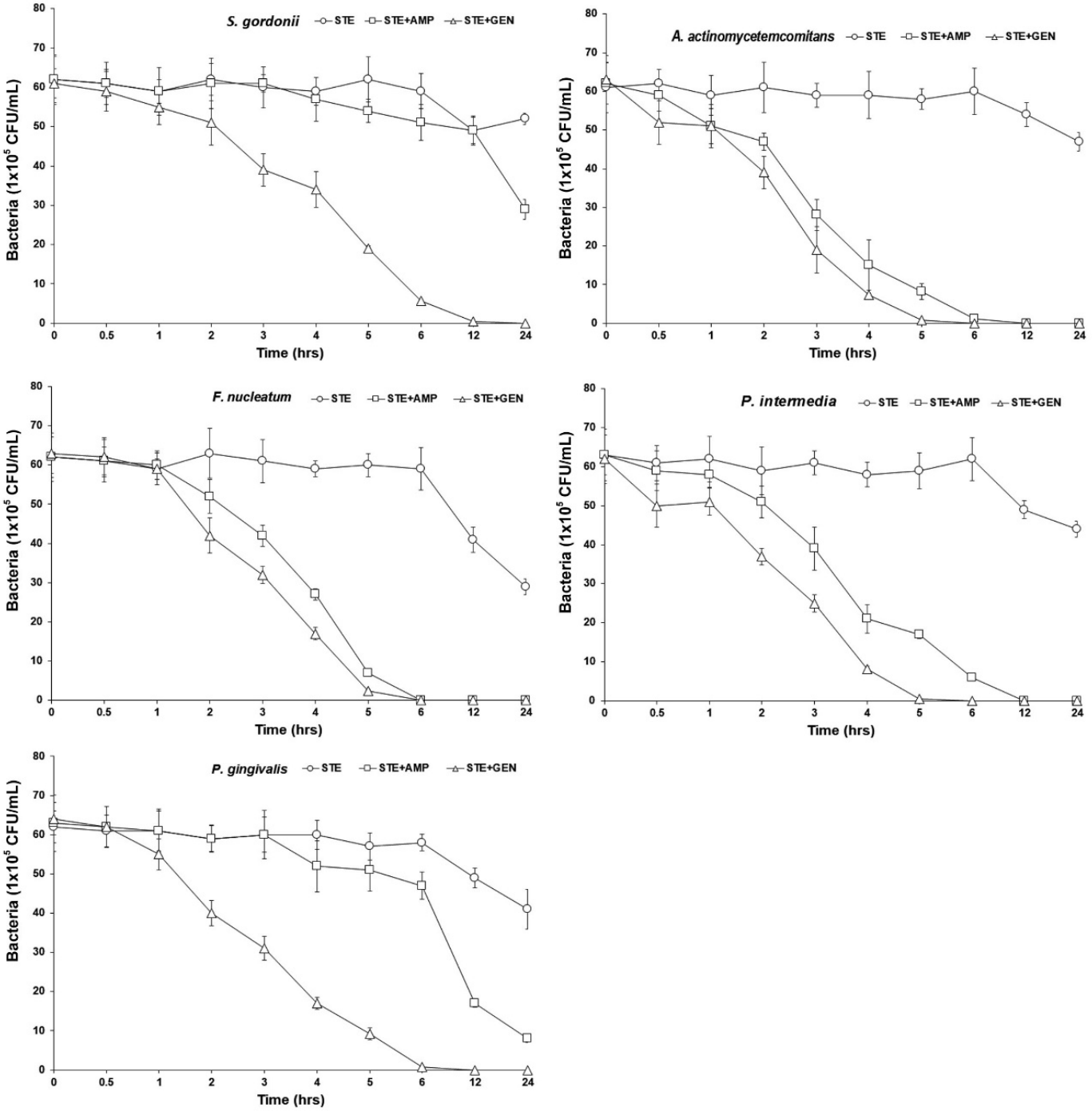

Figure 2. Time-kill curves of MIC of STE alone and its combination with 1/2 MIC of AMP and GEN against *S. gordonii*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia,* and *P. gingivalis.* Bacteria were incubated with STE (○), STE + AMP (□), and STE + GEN (△) over time. CFU, colony-forming units

In conclusion, these findings suggest that crude ethanol extract of *S. tamariscina* exhibited a wide range of pharmacological effects establish 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 biofilm.

Declaration of interest

The authors declare no conflict of interest.

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Ethical approval

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

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