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Antibacterial activity and synergistic effects between *Machilus thunbergii* ethanol extract and antibiotics against oral pathogens

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Abstract

The cortex of *Machilus thunbergii*, which has been consumed as traditional herbal medicine for treatment of leg edema and abdominal distension and pain for a long period of time. In this study, the combination effect of *M. thunbergii* ethanol extract (MTEE) was evaluated against oral bacteria, either alone or with antibiotics, via broth dilution method and checkerboard and time kill assay. MIC/MBC values for MTEE against all the tested bacteria ranged between 12.5-50/50-200 microg/mL, for ampicillin 0.0625-8/0.125-32 microg/mL and for gentamicin 4-128/8-512 microg/mL respectively. Furthermore, the MIC and MBC were reduced to one half-eighth as a result of the combination of MTEE with antibiotics. 1-6 hours of treatment with 1/2 MIC of MTEE 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 MTEE is important in the antibacterial actions of oral pathogens agents.

Abbreviations: MTEE: *Machilus thunbergii* ethanol extract; 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 disease is one of the most important preventable infectious diseases, a major health problem in dental caries and periodontal disease [1,2]. Oral health affects the general quality of life and poor oral health is associated with chronic conditions and systemic diseases [3-5]. There are more than 750 bacteria in the oral cavity, many of which are related to oral diseases [6]. The development of dental caries includes acidogenic and aciduric gram-positive bacteria, mainly mutans streptococci (*Streptococcus mutans* and *S. sobrinus*), lactobacilli and actinomycetes, which metabolize sucrose into organic acids that dissolve the calcium phosphate in teeth, causing decalcification and eventual decay [6-8]. In contrast, periodontal disease is subgingival and gum diseases associated with anaerobic gram-negative bacteria such as *Porphyromonas gingivalis*, Actinobacillus sp., Prevotella sp., and Fusobacterium sp. [9-11]. In periodontal disease, gingival crevices or areas beneath the gingiva are infected, causing cellular inflammatory response of the gingiva and surrounding connective tissue [10,11]. These inflammatory reactions can be caused by gingivitis (extremely common and seen as bleeding of the gingival or gum tissues) or periodontitis (the inflammatory response results in loss of collagen attachment of the tooth to the bone and in loss of bone) [12-14].

Many plant-derived medicines used in traditional medicinal systems have been documented in pharmacopeias for the treatment of infections and a number of these have been recently proved effective against oral microbial pathogens [15-18]. *Machilus thunbergii* (Lauraceae) is widely distributed in Korea. The cortex of *M. thunbergii*, which has been consumed as traditional herbal medicine for treatment of leg edema and abdominal distension and pain for a long period of time [19-21]. Isoquinoline alkaloids have been obtained from the root , lignin, catechin and polysaccharides from the heartwood, polysaccharides, and essential oils from the leaves, volatile components from the fruits, and lignans and neolignans from the cortex [22-24]. Some of these compounds are antioxidants with hepatoprotective, and anti-bacterial activities, while a few other show inhibitory effects on nitric oxide synthesis inactivated macrophages and neuroprotective activity against glutamate-induced neurotoxicity [22,25, 26]. Machilin A (MA), one of the lignans shows biological activities, including stimulation of osteoblast differentiation *via* activation of p38 mitogen-activated protein(MAP) kinases in an *in vitro* osteoblasts [20].

In this study, we investigated the synergistic antibacterial activity of *M. thunbergii* ethanol extract (MTEE) in combination with existing antimicrobial agents against oral bacteria.

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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, *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 *M. thunbergiii* ethanol extract (MTEE) by the broth dilution method, and were carried out in triplicate (27). 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 determined. Following anaerobic incubation of **MICs** plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of MTEE 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 MTEE against oral bacteria.

Checker-board dilution test

The antibacterial effects of a combination of MTEE, which exhibited the highest antimicrobial activity, and antibiotics were assessed by the checkerboard test as previously described (27). The antimicrobial combinations assayed included MTEE with ampicillin or gentamicin. 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 MTEE 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 (27).

Time-kill curves

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 5~6.6×10^6 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 MTEE and antibiotics

MTEE evaluated the antimicrobial activities against eleven bacterial species present in the oral cavity.

The results of the antimicrobial activity showed that MTEE exhibited antimicrobial activities against cariogenic bacteria (MICs, 12.5 to 50 μg/mL; MBCs, 50 to 200 μg/mL), against periodontopathogenic bacteria (MICs, 25 to 50 μg/mL; MBCs, 50 to 200 μg/mL) and ampicillin showed a concentration of 0.0625/8 -0.125/32 μg/mL, while gentamicin showed a concentrations of 4/8-128/512 μg/mL on tested all bacteria (Table 1). The range of MIC_{50} and MIC_{90} were from 3.13 to 12.5 μg/mL and 12.5 to 50 μg/mL, respectively. The MTEE showed stronger antimicrobial activity against *S. gordonii* (MIC/MBC, 12.5/50 μg/mL) than another bacteria (MIC/MBC, 25/50-50/200 μg/mL) and the range of MIC_{50} and MIC_{90} were 3.13 μg/mL and 12.5 μg/mL.

Synergistic effect of MTEE with antibiotics

Natural products are a major source of chemical diversity and have provided important treatment agents for many bacterial diseases [16,27-29]. The combination of some natural products and antibiotics can increase the antimicrobial activity of antibiotics [30,31]. The synergistic effects of MTEE alone or with antibiotics were evaluated in oral bacteria (Tables 2 and 3). In combination with MTEE, the MIC for ampicillin was reduced ≥4-fold in all tested bacteria, producing a synergistic effect as defined by FICI ≤ 0.5, except *S. gordonii* by FICI≤0.75 and additive. The MBC for ampicillin was shown synergistic effects in all tested bacteria by FBCI ≤ 0.5, except *S. ratti*, *S. criceti*, and *P. gingivalis* by FICI≤0.75 and additive (Table 2). In combination with MTEE, the MIC for gentamicin was reduced ≥4-8-fold in all tested bacteria, except *S. criceti* and *P. gingivalis* by FICI ≥ 0.75 and MBC in all tested bacteria by FBCI ≤ 0.5, except *S. sanguinis, S. ratti*, *S. anginosus*, and *F. nucleatum* by FBCI ≤ 0.75 (Table 3).

Phytochemical constituents such as alkaloids, flavonoids, tannins, phenols, saponins, and several other aromatic compounds are secondary metabolites of plants that serve a defence mechanism against prediction by many microorganisms, insects and other herbivores [32-35]. Flavonoid complexes attach with extra cellular soluble protein and with bacterial cell wall [36,37]. Lignans, alkaloids, flavonoids, butanolides, and essential oils have been derived from *M. thunbergii*; some of these compounds are antioxidants with hepatoprotective and antibacterial activities [23,25,26]. Both the ethyl acetate fraction and water fraction

Table 1. Antibacterial activity of the *Machilus thunbergiii* ethanol extract (MTEE) and antibiotics in oral bacteria

Samples	MTEE (μg/mL)			Ampicillin	Gentamicin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC	MIC/MBC (μg/mL)	
S. mutans ATCC 25175[1]	6.25	25	25/100	0.125/0.25	8/16
S. sanguinis ATCC 10556	12.5	50	50/200	0.25/1	16/32
S. sobrinus ATCC 27607	6.25	25	25/50	0.0625/0.125	16/32
S. ratti KCTC 3294[2]	12.5	50	50/100	0.25/0.5	8/32
S. criceti KCTC 3292	6.25	25	25/100	0.0625/0.125	8/16
S. anginosus ATCC 31412	12.5	50	50/200	0.125/0.25	8/16
S. gordonii ATCC 10558	3.13	12.5	12.5/50	0.125/0.5	16/32
A. actinomycetemcomitans ATCC 43717	25	50	50/200	8/32	8/16
F. nucleatum ATCC 51190	6.25	25	25/100	8/16	4/8
P. intermedia ATCC 49049	12.5	50	50/100	1/2	32/64
P. gingivalis ATCC 33277	6.25	25	25/50	0.5/1	128/512

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

Table 2. Synergistic effects of *Machilus thunbergiii* ethanol extract (MTEE) 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]	MTEE	25/100	6.25/12.5	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	0.125/0.25	0.0313/0.0625	0.25/0.25		
S. sanguinis ATCC 10556	MTEE	50/200	12.5/50	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	0.25/1	0.0625/0.125	0.25/0.125		
S. sobrinus ATCC 27607	MTEE	25/50	6.25/12.5	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.0625/0.125	0.0156/0.0313	0.25/0.25		
S. ratti KCTC 3294[4]	MTEE	50/100	12.5/25	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.25/0.5	0.0625/0.25	0.25/0.5		
S. criceti KCTC 3292	MTEE	25/100	6.25/25	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.0625/0.125	0.0156/0.0625	0.25/0.5		
S. anginosus ATCC 31412	MTEE	50/200	12.5/50	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.125/0.25	0.0313/0.0625	0.25/0.25		
S. gordonii ATCC 10558	MTEE	12.5/50	3.13/12.5	0.25/0.25	0.75/0.5	Additive/ Synergistic
	Ampicillin	0.125/0.5	0.0625/0.125	0.5/0.25		
A. actinomycetemcomitans ATCC 43717	MTEE	50/200	12.5/50	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	8/32	2/8	0.25/0.25		
F. nucleatum ATCC 51190	MTEE	25/100	6.25/12.5	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	8/16	2/8	0.25/0.5		
P. intermedia ATCC 49049	MTEE	50/100	12.5/25	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	1/2	0.25/0.5	0.25/0.25		
P. gingivalis ATCC 33277	MTEE	25/50	6.25/12.5	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Ampicillin	0.5/1	0.125/0.5	0.25/0.5		

[1]The MIC and MBC of the *Machilus thunbergiii* ethanol extract (MTEE) 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)

of *M. thunbergii* bark and leaf show antimicrobial activity against all the tested Gram-positive bacteria, *Staphylococcus aureus* were 0.1 mg/mL and 0.5 mg/mL [38]. In this study, *M. thunbergii* ethanol extract shows susceptibility on gram-positive bacteria as well as gram-negative bacteria [38].

Time kill of MTEE with antibiotics

The bacterial effect of MTEE with ampicillin or gentamicin against oral bacteria was confirmed by time-kill curve experiments. The MTEE (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 MTEE (MIC_{50}) with ampicillin (MIC_{50}) or gentamicin (MIC_{50}) (Figures 1-3). A strong bactericidal effect was exerted in drug combinations.

In conclusion, these findings suggest that MTEE 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.

Table 3. Synergistic effects of *Machilus thunbergiii* ethanol extract (MTEE) 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]	MTEE	25/100	6.25/25	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	8/16	2/4	0.25/0.25		
S. sanguinis ATCC 10556	MTEE	50/200	12.5/50	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Gentamicin	16/32	4/16	0.25/0.5		
S. sobrinus ATCC 27607	MTEE	25/50	6.25/12.5	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	16/32	4/8	0.25/0.25		
S. ratti KCTC 3294[4]	MTEE	50/100	12.5/50	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Gentamicin	8/32	2/8	0.25/0.25		
S. criceti KCTC 3292	MTEE	25/100	12.5/25	0.5/0.25	0.75/0.5	Additive/ Synergistic
	Gentamicin	8/16	2/4	0.25/0.25		
S. anginosus ATCC 31412	MTEE	50/200	12.5/50	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Gentamicin	8/16	2/8	0.25/0.5		
S. gordonii ATCC 10558	MTEE	12.5/50	3.13/12.5	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	16/32	4/8	0.25/0.25		
A actinomycetemcomitans ATCC 43717	MTEE	50/200	12.5/25	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Gentamicin	8/16	2/8	0.25/0.5		
F. nucleatum ATCC 51190	MTEE	25/100	6.25/25	0.25/0.25	0.5/0.75	Synergistic/ Additive
	Gentamicin	4/8	1/4	0.25/0.5		
P. intermedia ATCC 25611	MTEE	50/100	12.5/25	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Gentamicin	32/64	8/8	0.25/0.125		
P. gingivalis ATCC 33277	MTEE	25/50	12.5/12.5	0.5/0.25	0.75/0.375	Additive/ Synergistic
	Gentamicin	128/512	32/64	0.25/0.125		

[1]The MIC and MBC of the *Machilus thunbergiii* ethanol extract (MTEE) 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)

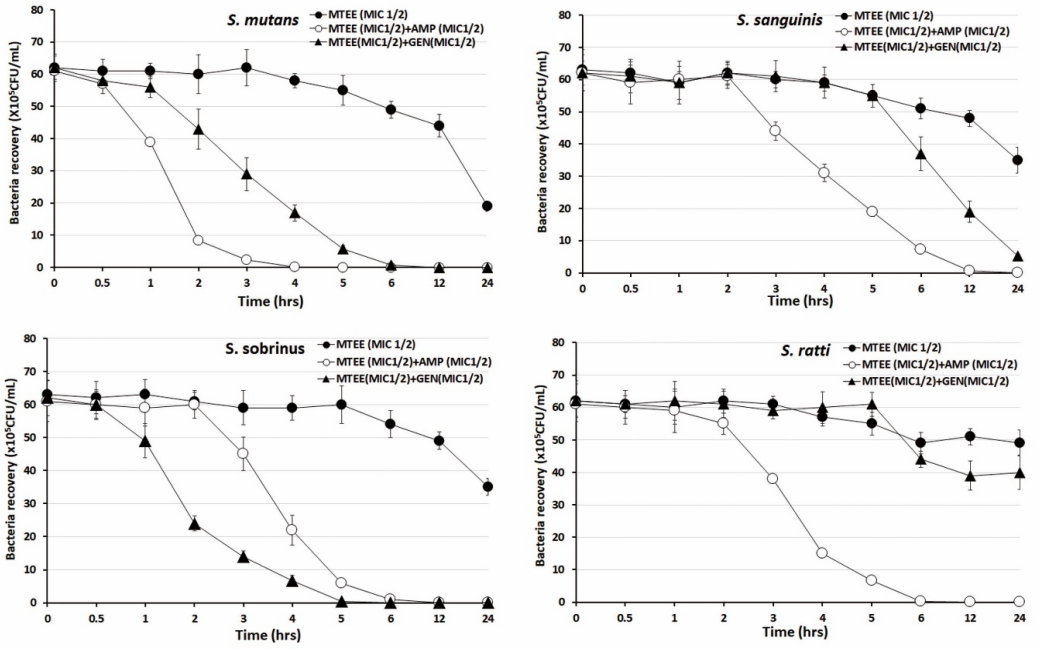

Figure 1. Time-kill curves of MICs of the *Machilus thunbergiii* ethanol extract (MTEE) alone and in combination with MICs of ampicillin or gentamicin against *S. mutans*, *S. sanguinis*, *S. sobrinus*, and *S. ratti*. Bacteria were incubated with METK along (●), METK with ampicillin (○), and METK with gentamicin (▲) over time. Data are presented as the mean ± SD of the four experiments. CFU, colony-forming units

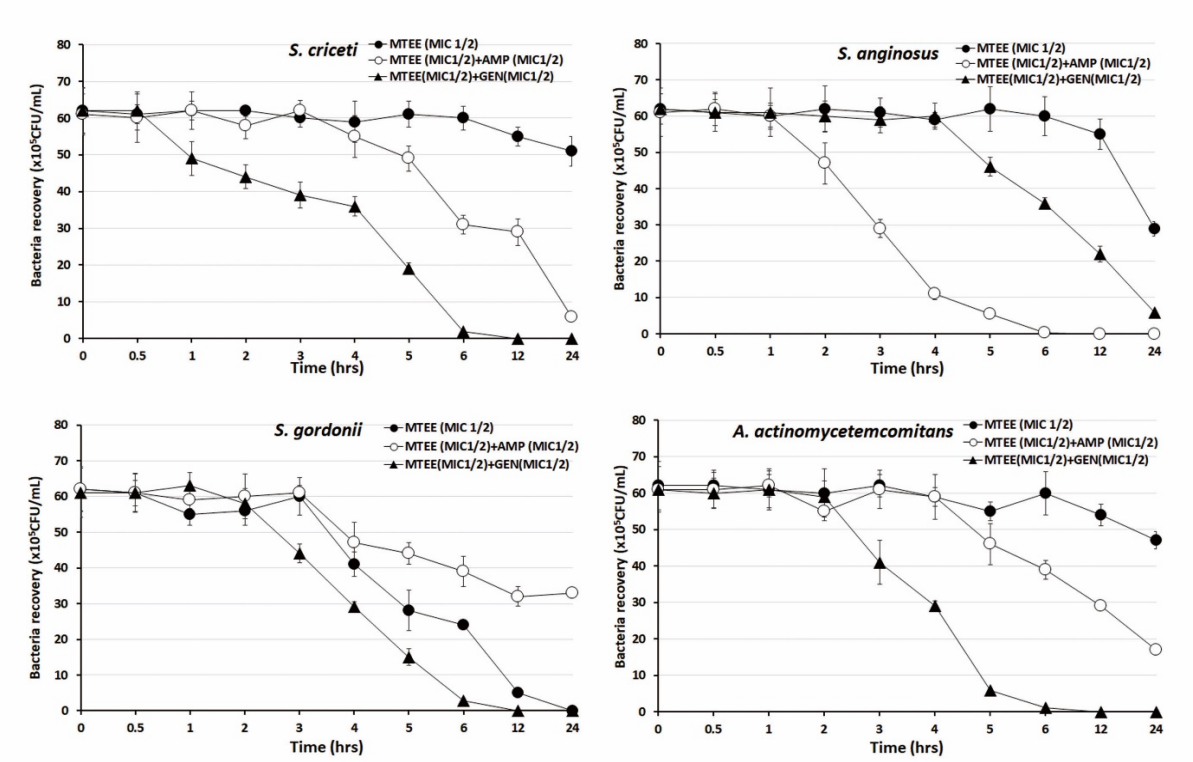

Figure 2. Time-kill curves of MICs of the *Machilus thunbergiii* ethanol extract (MTEE) alone and in combination with MICs of ampicillin or gentamicin against *S. criceti*, *S. anginosus*, *S. gordonii*, and *A. actinomycetemcomitans*. Bacteria were incubated with METK along (●), METK with ampicillin (○), and METK with gentamicin (▲) over time. Data are presented as the mean ± SD of the four experiments. CFU, colony-forming units

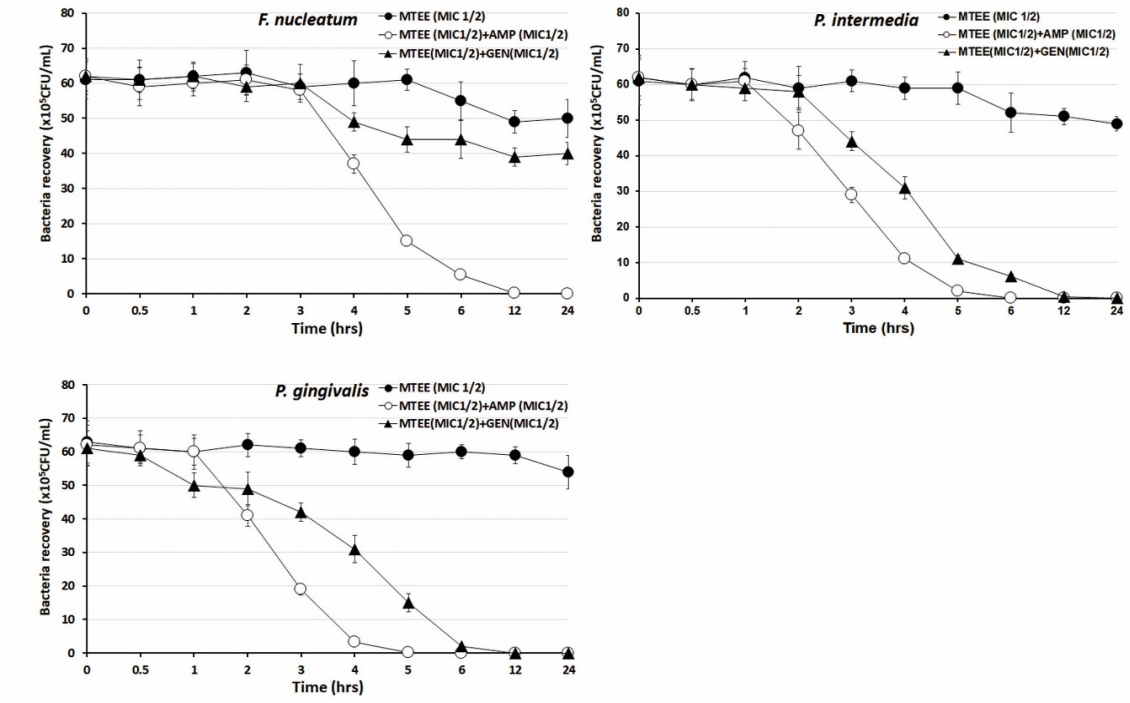

Figure 3. Time-kill curves of MICs of the *Machilus thunbergiii* ethanol extract (MTEE) alone and its combination with MICs of ampicillin or gentamicin against *F. nucleatum*, *P. intermedia*, and *P. gingivalis*. Bacteria were incubated with METK along (●), METK with ampicillin (○), and METK with gentamicin (▲) over time. Data are presented as the mean ± SD of the four experiments. CFU, colony-forming units

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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