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Research Article

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Antimicrobial Activity of *Angelica Dahurica* Ethanol Extract Against Oral Bacteria

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Abstract

Introduction: *Angelica dahurica (A. dahurica) has the protective activity against dexamehasone-induced disorders, liver protective activity, antimutagenic activity, anti-inflammatory, anti-microbial, anti-oxidative, anti-asthmatic, and anti-cancer effects.*

Aims of the Study: *This study aimed to investigate the synergistic antibacterial activity with existing antimicrobial agents against oral pathogen.*

Materials and Methods: *The synergistic effects of 50% ethanol extract of A. dahurica (ADEE) were evaluated against oral bacteria, either alone or with antibiotics, via broth microdilution and time-kill method.*

Results: *The minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) values for ADEE, ampicillin and gentamicin against all the tested bacteria ranged between 62.5-1000/250-2000 μg/mL, 0.0625-16/0.25-32 μg/mL, and 4-128/16-256 μg/mL, respectively. The ADEE displayed synergism with ampicillin and gentamicin, with 8-fold reductions in the MIC/MBC. Furthermore, a time-kill study showed that the growth of the tested bacteria was completely attenuated after treatment with 1/2 MIC of ADEE 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.*

Discussion and Conclusions: *The results of this study demonstrate the antimicrobial and synergistic activity of ADEE and antibiotics against oral pathogens.*

Keywords: Angelica dahurica, Antibacterial activity, Oral pathogen, Minimum inhibitory concentrations (MICs), Minimum bactericidal concentrations (MBCs), Synergistic effect

Background

Dental plaque is a complex biofilm formed on saliva coated tooth surfaces. Its development is dependent on adhesion of bacteria to salivary components adsorbed to the tooth surface [1,2]. Dental caries and periodontal diseases are among the most important global oral health problems, although conditions such as oral and pharyngeal cancers and oral tissue lesions are also significant health concerns [3]. The development of dental caries involves acidogenic and aciduric Gram-positive bacteria, primarily the mutans streptococci (*Streptococcus mutans* and *S. sobrinus*), lactobacilli and actinomycetes, which metabolize sucrose to organic acids (mainly lactic acid) that dissolve the calcium phosphate in teeth, causing decalcification and eventual decay [4,5]. 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 [6,7]. Generally, the etiological agents of periodontal diseases are Gram-negative rods including *Aggregatibacter actinomycetemcomitans, Tannerella forsythia, Prevotella, Fusobacterium,* and *Porphyromonas gingivalis* [8]. 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 [7,9].

Mechanical dental plaque removal is an efficient procedure to prevent periodontitis and caries. 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. Natural plant products are becoming increasingly popular treatments, even for oral health care [10-12]. The aromatic medicinal plant *Angelica dahurica* (Umbelliferae) grows wild in thickets in China, Japan, Russia, and Korea [13]. A number of studies have been reported that treatment of *A. dahurica* extract has the protective activity against dexamehasone-induced disorders, liver protective activity, antimutagenic activity, anti-inflammatory, anti-microbial, anti-oxidative, anti-asthmatic, and anti-cancer effects [14-18]. *A. dahurica* possesses various chemical composition including volatile oil, coumarins, glycosides, and steroids [14, 19-21]. Especially, coumarins are reported to have anti-inflammatory, anti-microbial anti-oxidative and anti-lipogenetic activities [22,23].

In this study, the antimicrobial activities of 50% ethanol extract of *A. dahurica* (ADEE) 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 A. dahurica (ADEE)

The dried radix of A. *dahurica* was purchased from Jinan Dang (Jinan, Korea). Dried *A. dahurica* roots (100g) were macerated and were extracted in 20-fold volumes of 50% ethanol (2000 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 28.3% (w/w) of dried A. *dahurica* roots.

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 and Minimum Bactericidal Concentrations Assay

The minimum inhibitory concentrations (MICs) were determined for 50% ethanol extract of *A. dahurica* (ADEE) 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 (N_2) (5/95%) or N_2/carbon dioxide (CO_2)/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. MIC50s, 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 ADEE 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 ADEE against oral bacteria.

Checkerboard Dilution Dest

The antibacterial effects of a combination of ADEE and antibiotics were assessed by the checkerboard test as previously described [24,25]. The antimicrobial combinations assayed included ADEE 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 ADEE 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:

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

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

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 [24,25].

Time-Kill and Growth Inhibition Curves Assay

Bactericidal activities of ADEE 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 5~7×10^6 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 and Minimum Bactericidal Concentrations of ADEE and Antibiotics

Plants have nowadays developed their own molecular antimicrobial strategies to survive, by producing secondary metabolites with synergistic action such as small antimicrobial peptides, alkaloids, coumarins, flavonoids, phenols, phenolic acids, quinones, saponins, tannins, and terpenoids [22, 26-29]. Active principles isolated from the genus Angelica (Family: Apiaceae) mainly include various types of coumarins, acetylenic compounds, chalcones, sesquiterpenes, and polysaccharides, have been used traditionally as anti-inflammatory, diuretic, expectorant and diaphoretic, and remedy for colds, flu, influenza, hepatitis, arthritis, indigestion, coughs, chronic bronchitis, pleurisy, typhoid, headaches, wind, fever, colic, travel sickness, rheumatism, bacterial and fungal infections and diseases of the urinary organs [13,16,17,19,20,22,30]. ADEE was evaluated for their antimicrobial activities against thirteen oral bacterial species present in the oral cavity. The results of the antimicrobial activity showed that ADEE exhibited antimicrobial activities against cariogenic bacteria at MICs, 62.5 to 500 µg/mL; MBCs, 250 to 1000 µg/mL, against periodontopathogenic bacteria at MICs, 125 to 1000 µg/mL; MBCs, 125 to 2000 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 (Figure 1, and 2 and Table 1). The MIC50 and MIC90 ranges of ADEE were from 15.6 to 125 µg/mL and 62.5 to 500 µg/mL, respectively. The ADEE showed stronger antimicrobial activity against S. gordonii at MIC/MBC, 6.25/125 µg/mL than another bacteria.

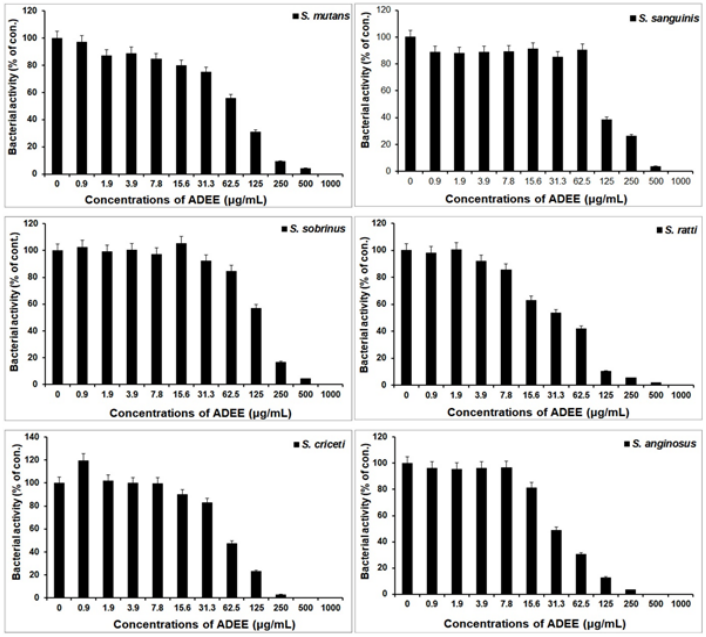

Figure 1: Antibacterial activity of different concentrations of ADEE against oral bacteria, *S. mutans, S. sanguinis, S. sobrinus, S. ratti and S. criceti*

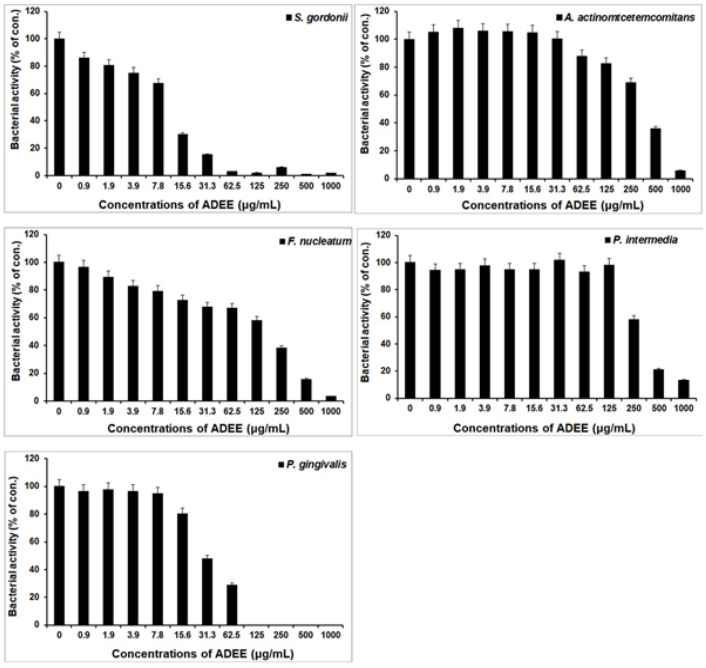

Figure 2: Antibacterial activity of different concentrations of ADEE against oral bacteria, *S. anginosus, S. gordonii, A. actinomycetemcomitans, F.nucleatum, P. intermedia, and P. gingivalis*

Table 1: Antibacterial activity of *Angelica dahurica* ethanol extract (ADEE) and antibiotics in oral bacteria

Samples	ADEE (μg/mL)			Ampicillin	Gentamicin
	$MIC_{50<}$	$MIC_{90<}$	MIC/MBC	MIC/MBC (μg/mL)	
S. mutans ATCC 25175[1]	62.5	250	250/500	0.25/0.5	4/16
S. sanguinis ATCC 10556	125	250	250/250	0.125/0.5	8/32
S. sobrinus ATCC 27607	125	500	500/1000	0.125/0.5	16/32
S. ratti KCTC 3294[2]	31.3	125	125/500	0.25/1	16/32
S. criceti KCTC 3292	62.5	250	250/500	0.0625/0.25	8/32
S. anginosus ATCC 31412	31.3	250	250/250	0.25/1	8/16
S. gordonii ATCC 10558	15.6	62.5	62.5/250	0.125/0.5	16/64
A. actinomycetem-comitans ATCC 43717	500	1000	1000/2000	16/32	4/16
F. nucleatum ATCC 51190	125	1000	1000/1000	8/16	4/16
P. intermedia ATCC 49049	250	1000	1000/2000	0.5/1	32/64
P. gingivalis ATCC 33277	31.3	125	125/125	0.5/2	128/256

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

Up to date, more than 100 coumarins have been obtained from A. dahurica, exhibiting notable and diverse pharmaceutical properties, such as anti-tumor, anti-inflammatory, anti-microbial, anti-oxidative, and acetylcholinesterase inhibitory activities [19,22]. These effects are believed to be attributed to the A. dahurica rich furanocoumarin compounds such as imperatorin and isoimperatorin [31]. Imperatorin has anti-inflammatory, anticonvulsant, hepatoprotective, myorelaxant, vasodilator, and anti-cancer effects [32,33]. Isoimperatorin has anti-inflammatory, antiallergic, and antimicrobial effects [34,35].

Synergistic Effect of ADEE with Antibiotics

Despite several agents being commercially available, these chemicals can alter oral microbiota and have undesirable side-effects such as vomiting, diarrhea and tooth staining. Hence, the search for alternative products continues and natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals. When ADEE was combined with ampicillin for the inhibition of tested bacteria, an important synergistic effect (FICI≤ 0.375-0.5) was observed when the ADEE and the antibiotics were combined at 8 and 16 times below their MIC/MBC values, respectively (Table 2 and 3). In combination with ampicillin, ADEE was reduced ≥4-8 fold in all tested bacteria, except *S. sanguinis* and *S. criceti*, producing a synergistic effect as defined by FICI ≤ 0.375-0.5. The MBC for ADEE with ampicillin was shown synergistic effects in *S. sobrinus, S. ratti, S. criceti, S. gordonii, F. nucleatum, and P. intermedia* by FBCI ≤ 0.375-0.5 (Table 2). In combination with gentamicin, the MIC for ADEE was reduced ≥4-8-fold in all tested bacteria, except *S. mutans, S. anginosus,* and *A. actinomycetamcomitans* by FICI ≤ 0.5 and MBC for ADEE was shown synergistic effects in *S. ratti, S. criceti, S. gordonii,* and *A. actinomycetemcomitans* by FBCI ≤ 0.375-0.5 (Table 3). Besides the pharmacological effects of furanocoumarins of *A. dahurica* such as imperatorin and isoimperatorin, root extracts contain a variety of phenolic compounds that are connected to its strong antioxidation, anti-inflammatory, antiproliferative, and antimicrobial effects [33,35,36].

Table 2: Synergistic effects of *Angelica dahurica* ethanol extract (ADEE) with ampicillin against oral bacteria

Strains	Agent	MIC/MBC (μg/ml)		FIC/FBC	FICI/FBCI2	Outcome
		Alone	Combination[1]			
S. mutans ATCC 25175[3]	ADEE	250/500	62.5/125	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	ADEE	250/250	62.5/125	0.25/0.5	0.75/0.75	Additive/ Additive
	Ampicillin	0.125/0.5	0.0625/0.125	0.5/0.25		
S. sobrinus ATCC 27607	ADEE	500/1000	125/250	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	0.125/0.5	0.0313/0.0625	0.25/0.125		
S. ratti KCTC 3294[4]	ADEE	125/500	31.3/125	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	0.25/1	0.0625/0.25	0.25/0.25		
S. criceti KCTC 3292	ADEE	250/500	62.5/125	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	ADEE	250/250	62.5/125	0.25/0.5	0.5/0.625	Synergistic/ Additive
	Ampicillin	0.25/1	0.0625/0.0625	0.25/0.125		
S. gordonii ATCC 10558	ADEE	62.5/250	15.6/31.3	0.25/0.125	0.5/0.375	Synergistic/ Synergistic
	Ampicillin	0.125/0.5	0.0313/0.125	0.25/0.25		
A. actinomyce-temcomitans ATCC 43717	ADEE	1000/2000	250/1000	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Ampicillin	16/32	4/8	0.25/0.25		
F. nucleatum ATCC 51190	ADEE	1000/1000	250/250	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Ampicillin	8/16	2/4	0.25/0.25		
P. intermedia ATCC 49049	ADEE	1000/2000	250/500	0.25/0.25	0.375/0.375	Synergistic/ Synergistic
	Ampicillin	0.5/1	0.0625/0.125	0.125/0.125		
P. gingivalis ATCC 33277	ADEE	125/125	31.3/62.5	0.25/0.5	0.5/0.625	Synergistic/ Additive
	Ampicillin	0.5/2	0.125/0.25	0.25/0.125		

1. The MIC and MBC of the *Angelica dahurica* ethanol extract (ADEE) 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 *Angelica dahurica* ethanol extract (ADEE) with ampicillin against oral bacteria

Strains	Agent	MIC/MBC (μg/ml)		FIC/FBC	FICI/FBCI2	Outcome
		Alone	Combination[1]			
S. mutans ATCC 251753	ADEE	250/500	125/250	0.5/0.5	1.0/0.75	Additive/ Additive
	Gentamicin	4/16	2/4	0.5/0.25		
S. sanguinis ATCC 10556	ADEE	250/250	62.5/125	0.25/0.5	0.5/0.625	Synergistic/ Additive
	Gentamicin	8/32	2/4	0.25/0.125		
S. sobrinus ATCC 27607	ADEE	500/1000	125/500	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Gentamicin	16/32	4/8	0.25/0.25		
S. ratti KCTC 32944	ADEE	125/500	31.3/125	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	16/32	4/8	0.25/0.25		
S. criceti KCTC 3292	ADEE	250/500	62.5/125	0.25/0.25	0.5/0.5	Synergistic/ Synergistic
	Gentamicin	8/32	2/8	0.25/0.25		
S. anginosus ATCC 31412	ADEE	250/250	125/125	0.5/0.5	0.75/0.75	Additive/ Additive
	Gentamicin	8/16	2/4	0.25/0.25		
S. gordonii ATCC 10558	ADEE	62.5/250	15.6/62.5	0.25/0.25	0.5/0.375	Synergistic/ Synergistic
	Gentamicin	16/64	4/8	0.25/0.125		
A. actinomyce-temcomitans ATCC 43717	ADEE	1000/2000	250/500	0.25/0.25	0.75/0.5	Additive/ Synergistic
	Gentamicin	4/16	2/4	0.5/0.25		
F. nucleatum ATCC 51190	ADEE	1000/1000	250/500	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Gentamicin	4/16	1/4	0.25/0.25		
P. intermedia ATCC 25611	ADEE	1000/2000	250/1000	0.25/0.5	0.5/0.625	Synergistic/ Additive
	Gentamicin	32/64	8/8	0.25/0.125		
P. gingivalis ATCC 33277	ADEE	125/125	31.3/62.5	0.25/0.5	0.5/0.75	Synergistic/ Additive
	Gentamicin	128/256	32/64	0.25/0.25		

1. The MIC and MBC of the *Angelica dahurica* ethanol extract (ADEE) 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)

Time Kill of ADEE with Antibiotics

Previous studies had reported the antimicrobial activities and mechanisms of ADEE against several kinds of bacteria [17]. However, the type of microorganisms and their cell membrane structure and composition could play an important role in the susceptibility to antimicrobials. Overcome drug-resistance mechanisms are the use of combination of antibiotics, such as β-lactam 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. The bacterial effect of ADEE with antibiotics, ampicillin and gentamicin against oral bacteria was confirmed by time-kill curve experiments. The ADEE (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 ADEE (1/2 MIC) with ampicillin or/and gentamicin (1/2 MIC) (Figure 3 and 4). ADEE containing ampicillin within 5 hours had the fastest killing rate and highest mortality rate in *P. gingivalis*. In ADEE containing gentamicin, *S. anginosus* and *P. gingivalis* and had the fastest and highest mortality rates. A strong bactericidal effect was exerted in drug combinations.

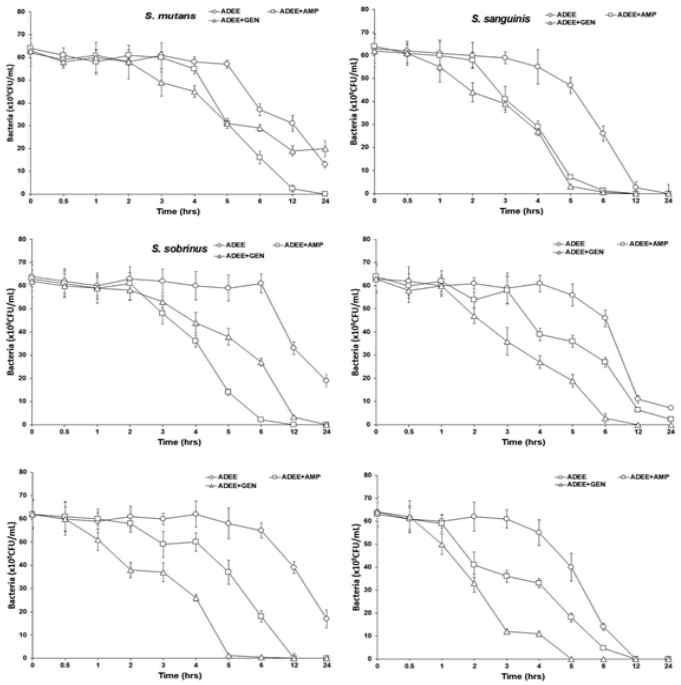

Figure 3: Time-kill curves of MIC of STE alone and its combination with 1/2 MIC of AMP and GEN against *S. mutans, S. sanguinis, S. sobrinus, S. ratti, S. criceti, and S. anginosus.* Bacteria were incubated with ADEE (○), ADEE + AMP (□), and ADEE + GEN (Δ) over time. CFU, colony-forming units

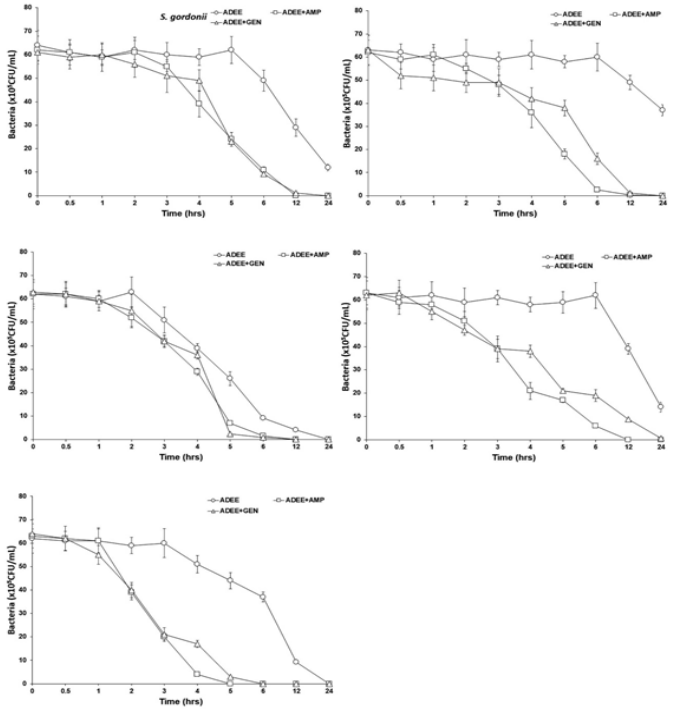

Figure 4: Time-kill curves of MIC of STE alone and its combination with 1/2 MIC of AMP and GEN against *S. gordonii, A. actinomycetemcomitans, F. nucleatum, P. intermedia, and P. gingivalis.* Bacteria were incubated with ADEE (○), ADEE + AMP (□), and ADEE + GEN (Δ) over time. CFU, colony-forming units

Conclusion

In conclusion, it is suggested that the crude ethanol extract of *A. dahurica* exerts a wide range of pharmacological effects that establish the necessary conditions for novel cariogenic and periodontal pathogens, especially bacteroid species drugs.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgement

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Su-Mi Cha carried out the experiments and analyzed the data. Jeong-Hun Seo and Sung-Mi Choi drafted the manuscript, read and proofread the manuscript. Kyung-Yeol Lee and Jeong-Dan Cha designed the study, performed statistical evaluation, contributed the discussion, wrote, read, and approved the final manuscript. All authors read and approved the final manuscript.

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