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Antimicrobial Activity of *Sophora Japonica* Extract Against Oral Bacteria

Keywords: *Sophora japonica*; Antibacterial activity; Oral pathogen; Biofilm formation; Synergistic effect; Minimum inhibitory concentrations (MICs); Minimum bactericidal concentrations (MBCs)

Abstract

Background: The flower and flower buds of *Sophora japonica L.* have the same medicinal uses, with significant biological activity, in the treatment of bleeding hemorrhoids, hematuria, hematemesis, hemorrhinia, uterine or intestinal hemorrhage, metrorrhagia, leukorrhea, conjunctivitis, pyoderma, arteriosclerosis, hypertension, and dizziness.

Methods: This study aimed to investigate the synergistic antibacterial activity with existing antimicrobial agents against oral pathogen. The synergistic effects and anti biofilm of 50% ethanol extract of *Sophora japonica* (SJEE) were evaluated against oral bacteria, either alone or with antibiotics, via broth microdilution method, time-kill method, and crystal violet assay.

Results: MIC/MBC values for SJEE, Ampicillin, Gentamicin, Erythromycin, and Vancomycin against all the tested bacteria ranged between 0.125-4/0.5-16 mg/mL, 0.0313-16/0.125-32 μg/mL, 2-256/4-512 μg/mL, 0.008-32/0.016-64 μg/mL, and 0.25-64/1-128 μg/mL, respectively. The synergistic effects were exhibited on SJEE with antibiotics against oral bacteria at Fractional Inhibitory Concentration Index (FICI)<0.5. Moreover, SJEE and antibiotics were found to synergistically reduce biofilm formation. 1-6 hours of treatment with 1/2 MIC of SJEE 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.

Conclusion: The SJEE was exerted a strong bactericidal effect in drug combinations against oral bacteria and biofilm formation.

Abbreviations: SJEE: *Sophora japonica* 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

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Introduction

More than 700 different bacterial species have been detected in the oral cavity of humans [1]. Saliva contains 10^8 to 10^9 bacteria per milliliter, and some of these adhere to the teeth and initiate formation of a dental biofilm, previously called dental plaque [2,3]. Dental caries are caused by demineralization of the enamel of the tooth by acid produced from dietary sugars by micro-organisms growing as a biofilm or plaque [4,5]. Until a few decades ago, development of caries was ascribed to a few gram-positive bacterial species in the biofilm, i.e. the specificbiofilm/plaque hypothesis, and *Streptococcus mutans*, *Streptococcus sobrinus* together with some *Lactobacillus* species were regarded as key pathogens [6]. While especially *S. mutans*, *Actinomyces*, and *Lactobacillus* species were previously regarded as responsible for caries, the list of caries-associated bacteria now includes species of the genera *Actinomyces*, *Lactobacillus*, *Dialister*, *Eubacterium*, Olsenella, *Bifidobacterium*, *Atopobium*, *Propionibacterium*, Scardovir, *Abiotrophia*, *Selenomonas*, and *Veillonella* in addition to carbohydrate fermenting oral streptococci [6,7].

Periodontitis is characterized by inflammation of the periodontal tissues. Generally, the etiological agents of periodontal diseases are Gram-negative rods including *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Prevotella*, *Fusobacterium*, and *Porphyromonas gingivalis* [8,9]. 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 [10-12].

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 products are a major source of new natural drugs and their use as an alternative medicine for treatment of various diseases has been increased in the last few decades [13]. *Sophora japonica L.* is a tree native to Korea, China, and Japan. Both the flower and flower buds have the same medicinal uses, with significant biological activity, in the treatment of bleeding hemorrhoids, hematuria, hematemesis, hemorrhinia, uterine or intestinal hemorrhage, metrorrhagia, leukorrhea, conjunctivitis, pyoderma, arteriosclerosis, hypertension, and dizziness [14-16]. Flavones from the buds and pericarp were discovered as haemostatic constituents [17,18]. Triterpenes, phospholipids, alkaloids, amino acids and fatty acids have been reported as the main chemical constituents of the seeds of this plant [19,20]. Among these compounds, kaempferol, quercetin, rutin, isorhamnetin, genistein, and sophoricoside are the major active constituents of *S. japonica* [15,17-20]. Rutin, in particular, is the most important and abundant constituent of *S. japonica* [15]. Crude extracts *in vitro*, the ethanol extract from flower buds of *S. japonica* exhibits a significant antibacterial activity against *Staphylococcus*

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aureus, *Propionibacterium avidum*, and *Propionibacterium* acnes under weak acidic conditions [21]. The Ethyl acetate (EtOAc)-soluble fraction is effective in inhibiting *Escherichia coli*, *Klebsiella pneumoniae*, and *S. aureus* [22].

In this study, the antimicrobial activities of 50% ethanol extract of *Sophora japonica* (SJEE) against oral bacteria were assessed using broth microdilution method, time kill method, and crystal violet assay for synergistic effect and biofilm formation of the combination with antibiotics.

Materials and Methods

Plant material and preparation of 50% ethanol extract of *Sophora japonica* (SJEE)

Dried flowers from *S. japonica* (2 kg) were macerated and extracted three times with 50% EtOH (10 L) for 4 h at 80 ˚C. The combined 50% EtOH extract (30 L) was clarified by filtration and evaporated to obtain a dark brown syrup (200 g). One hundred mg/mL of extract was dissolved in 10% dimethyl sulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) and then, diluted with bacteria culture medium for testing. All of the extract was kept at 4 ˚C in the dark until further use.

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 *Sophora japonica* (SJEE) 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 H_2 and nitrogen (N_2) (5/95%) or N_2/carbon dioxide (CO_2)/H_2 (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 SJEE that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. Ampicillin, gentamicin, erythromycin, and vancomycin (Sigma) were used as standard antibiotics in order to compare the sensitivity of SJEE against oral bacteria.

Checkerboard dilution test

The antibacterial effects of a combination of SJEE and antibiotics were assessed by the checkerboard test as previously described [23,24]. The antimicrobial combinations assayed included SJEE 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 SJEE 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:

$$\text{FIC} = \text{FIC of agent A} + \text{FIC of agent B}$$

$$\text{FIC of agent A} = \frac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}}$$

$$\text{FIC of agent B} = \frac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}}$$

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 [23].

Biofilm formation assay

Evaluation of the effect of SJEE on biofilm formation of oral bacteria by crystal violet biofilm formation assay was performed according to previous studies [25,26]. Briefly, 200 μL aliquots of treated oral bacteria (final concentration of 1.0×10^6 CFU/mL) at sub-lethal dose of SJEE (1/2 MIC) plus antibiotics (1/8 MIC) was transferred to a flat-bottomed sterile polystyrene micro plate, 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 micro plate reader (BMG LABTECH, USA).

Time-kill and growth inhibition curves assay

Bactericidal activities of SJEE 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,

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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 deviations (mean±SD) and were considered significant at the level of $p<0.05$ or/ and <0.01.

Results and Discussion

Minimum inhibitory concentrations/minimum bactericidal concentrations of SJEE 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 [27-29]. SJEE was evaluated for their antimicrobial activities against thirteen oral bacterial species present in the oral cavity. The results of the antimicrobial activity showed that SJEE exhibited antimicrobial activities against cariogenic bacteria at MICs, 0.125 to 4 mg/mL; MBCs, 0.5 to 16 mg/mL, against periodontopathogenic bacteria at MICs, 0.5 to 4 mg/mL; MBCs, 1 to 16 mg/mL and for ampicillin, either MIC/MBCs 0.0625/1 or 1/4 μg/mL; for gentamicin, either MIC/MBCs 2/8 or 256/512 μg/mL; for erythromycin, either 0.016/0.031 or 32/64 μg/mL; for vancomycin, either 0.25/1 or 64/128 μg/mL on tested all bacteria (Table 1). The MIC50 and MIC90 ranges of SJEE were from 0.313 to 1 mg/mL and 0.125 to 4 mg/mL, respectively. The SJFF showed stronger antimicrobial activity against S. sobrinus, S. gordonii, *A. actinomycetemcomitans* and *F. nucleatum* at MIC/MBC, 0.125/0.5-0.5/2 mg/mL than another bacteria at MIC/MBC, 1-4/1-16 mg/mL.

Synergistic effect of SJEE with antibiotics

Many antimicrobial preparations, such as conventional antibiotics, chlorhexidine (CHX), phenolic compounds and triclosan, can inhibit bacteria biofilm effectively [30,31]. However, extensive use of these antimicrobial agents can lead to some side-effects, such as tooth staining, calculus formation, drug resistance and gastrointestinal reactions [32,33]. 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 [34]. The synergistic effects of SJEE with antibiotics or with antibiotics were evaluated in oral bacteria (Tables 2-5). In combination with

Table 1: Antibacterial activity of 50% ethanol extract of *Sophora japonica* (SJEE) and antibiotics in oral bacteria.

Samples	SJEE		Ampicillin	Gentamicin	Erythromycin	Vancomycin
	$MIC_{50<}$	MIC/MBC (mg/mL)	MIC/MBC (μg/mL)			
S. mutans ATCC 25175[1]	0.5	2/2	0.125/0.25	8/16	0.063/0.125	1/2
S. sanguinis ATCC 10556	0.5	2/4	0.25/0.5	16/32	0.016/0.031	0.25/1
S. parasanguinis KCOM 1497[2]	0.125	1/2	0.5/1	16/32	0.125/0.5	2/4
S. sobrinus ATCC 27607	0.125	0.5/0.5	0.0313/0.125	16/32	0.031/0.063	1/2
S. ratti KCTC 3294[3]	1	4/16	0.125/0.5	8/16	0.008/0.016	0.5/1
S. criceti KCTC 3292	0.25	1/1	0.0313/0.125	8/16	0.125/0.25	1/4
S. downei KCOM 1165	0.5	2/4	1/4	16/64	0.25/0.5	4/16
S. anginosus ATCC 31412	0.125	1/4	0.0625/0.25	8/16	0.125/0.5	1/4
S. gordonii ATCC 10558	0.0313	0.125/0.5	0.0625/0.25	16/32	0.031/0.063	0.5/1
A. actinomyce temcomitans ATCC 43717	0.125	0.5/1	16/32	8/16	0.125/0.25	2/4
F. nucleatum ATCC 51190	0.125	0.5/2	8/16	2/4	32/64	64/128
P. intermedia ATCC 49049	1	4/16	1/2	32/32	16/32	16/36
P. gingivalis ATCC 33277	0.25	1/2	0.5/0.5	256/512	8-Feb	8/16

[1]American Type Culture Collection (ATCC)
[2]Korean collection for Oral Microbiology (KCOM)
[3]Korean collection for Type Cultures (KCTC)

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ampicillin, SJEE was reduced ≥4-fold in tested bacteria, except *S. ratti*, *S. criceti*, and *S. gordnii*, producing a synergistic effect as defined by FICI≤0.5. The MBC for ampicillin was shown synergistic effects in *S. parasanguinis*, *S. downei*, *S. anginosus*, *S. gordonii*, *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* by FBCI≤0.5 (Table 2). In combination with SJEE, the MIC for gentamicin was reduced ≥4-8-fold in all tested bacteria, expect *S. ratti* by FICI≤0.5 and MBC in *S. mutans*, *S. sobrinus*, *S. ratti*, *S. criceti*, *S. gordonii*, and *A. actinomycetemcomitans* by FBCI≤0.5 (Table 3). Moreover, the MIC for erythromycin with SJEE was reduced ≥4-fold in tested bacteria, except *S. sanguinis*, producing a synergistic effect as defined by FICI≤0.5 and the MBC for erythromycin was shown synergistic effects, except *S. mutans*, *S. sanguinis*, *S. sobrinus*, *S. rattii*, *S. criceti*, *S. gordonii*, and *A. actinomycetemcomitans* by FBCI≥0.75 (Table 4). In combination with SJEE, the MIC for vancomycin was reduced ≥4 fold in tested all bacteria, except *S. sanguinis*, *S. criceti*, *S. downei*, *P. intermedia*, and *P. gingivalis*, producing a synergistic effect as defined by FICI≤0.5. The MBC for vancomycin was shown synergistic effects in *S. sanguinis*, *S. sobrinus*, *S. criceti*, *A. actinomycetemcomitans*, and *P. gingivalis* by FBCI≤0.75 (Table 5).

Anti-biofilm formation of SJEE with antibiotics

Oral diseases, such as dental caries, periodontal disease are directly linked with the ability of bacteria to form biofilm [3,4,7,9]. Prenylated flavonoids are widely distributed in the plant world and prenylation elevates hydrophobicity on the basic structure of the molecule, enhancing flavonoid biological functions [35]. Prenylation facilitates flavonoid interaction with biofilm and uptake into bacteria through the membrane, enhancing inhibitory effects of the flavonoids on *P. gingivalis* growth and biofilm formation [36,37]. The isorhamnetin-3-O-β-D-ru-tinoside from the dried flowers of *S. japonica*, which significantly inhibited the action of sortase A (SrtA)

Table 2: Synergistic effects of 50% ethanol extract of *Sophora japonica* (SJEE) with ampicillin against oral bacteria.

Strains	Agent	MIC/MBC[1]		FIC/FBC	FICI/FBCI[3]	Outcome
		Alone	Combination[2]			
S. mutans ATCC 25175[4]	SJEE Ampicillin	2/2 0.125/0.25	0.25/1 0.0313/0.0625	0.125/0.5 0.25/0.25	0.375/0.75	Synergistic/ Additive
S. sanguinis ATCC 10556	SJEE Ampicillin	2/4 0.25/0.5	0.5/1 0.0625/0.25	0.25/0.25 0.25/0.5	0.5/0.75	Synergistic/ Additive
S. parasanguinis KCOM 1497[5]	SJEE Ampicillin	1/2 0.5/1	0.25/0.5 0.125/0.25	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic/ Synergistic
S. sobrinus ATCC 27607	SJEE Ampicillin	0.5/0.5 0.0625/0.125	0.125/0.25 0.0156/0.0313	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
S. ratti KCTC 3294[6]	SJEE Ampicillin	4/16 0.25/0.5	2/8 0.125/0.125	0.5/0.5 0.5/0.25	1/0.75	Additive/ Additive
S. criceti KCTC 3292	SJEE Ampicillin	1/1 0.0625/0.125	0.25/0.5 0.0313/0.0313	0.25/0.5 0.5/0.25	0.75/0.75	Additive/ Additive
S. downei KCOM 1165	SJEE Ampicillin	2/4 1/4	0.5/1 0.25/0.5	0.25/0.25 0.25/0.125	0.5/0.375	Synergistic/ Synergistic
S. anginosus ATCC 31412	SJEE Ampicillin	1/4 0.125/0.25	0.25/1 0.0313/0.0625	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic/ Synergistic
S. gordonii ATCC 10558	SJEE Ampicillin	0.125/0.5 0.0625/0.25	0.0313/0.0313 0.0313/0.0313	0.25/0.125 0.5/0.125	0.75/0.25	Additive/ Synergistic
A. actinomycetemcomitans ATCC 43717	SJEE Ampicillin	0.5/1 16/32	0.125/0.25 4/8	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic/ Synergistic
F. nucleatum ATCC 51190	SJEE Ampicillin	0.5/2 16/32	0.125/0.25 4/8	0.25/0.125 0.25/0.25	0.5/0.375	Synergistic/ Synergistic
P. intermedia ATCC 49049	SJEE Ampicillin	4/16 2/4	1/8 0.5/1	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
P. gingivalis ATCC 33277	SJEE Ampicillin	1/2 0.5/1	0.25/0.5 0.125/0.125	0.25/0.25 0.25/0.125	0.5/0.375	Synergistic/ Synergistic

[1]*Sophora japonica* (SJEE): mg/mL; Antibiotics: μg/mL
[2]The MIC and MBC of 50% ethanol extract of *Sophora japonica* (SJEE) with ampicillin 0.078-64 μg/mL
[3] The fractional inhibitory concentration index (FIC index)
[4]American Type Culture Collection (ATCC)
[5]Korean collection for Oral Microbiology (KCOM)
[6]Korean collection for type cultures (KCTC)

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Table 3: Synergistic effects of 50% ethanol extract of *Sophora japonica* (SJEE) with gentamicin against oral bacteria.

Strains	Agent	MIC/MBC[1]		FIC	FICI[3]	Outcome
		Alone	Combination[2]			
S. mutans ATCC 25175[4]	SJEE Gentamicin	2/2 8/16	0.5/1 2/4	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
S. sanguinis ATCC 10556	SJEE Gentamicin	2/4 16/32	0.5/0.5 4/8	0.25/0.125 0.25/0.25	0.5/0.375	Synergistic/ Synergistic
S. parasanguinis KCOM 14975	SJEE Gentamicin	1/2 16/32	0.25/0.5 4/8	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic/ Synergistic
S. sobrinus ATCC 27607	SJEE Gentamicin	0.5/0.5 16/32	0.125/0.25 4/8	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
S. ratti KCTC 3294[6]	SJEE Gentamicin	4/16 16/16	2/4 4/8	0.5/0.25 0.25/0.5	0.75/0.75	Additive/ Additive
S. criceti KCTC 3292	SJEE Gentamicin	1/1 8/16	0.25/0.5 2/4	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
S. downei KCOM 1165	SJEE Gentamicin	2/4 16/64	0.5/1 4/8	0.25/0.25 0.25/0.125	0.5/0.375	Synergistic/ Synergistic
S. anginosus ATCC 31412	SJEE Gentamicin	1/4 16/32	0.25/0.5 4/8	0.25/0.125 0.25/0.25	0.5/0.25	Synergistic/ Synergistic
S. gordonii ATCC 10558	SJEE Gentamicin	0.125/0.5 16/32	0.0313/0.0625 4/16	0.25/0.125 0.25/0.5	0.5/0.625	Synergistic/ Additive
A. actinomycetemcomitans ATCC 43717	SJEE Gentamicin	0.5/1 8/16	0.125/0.25 2/8	0.25/0.25 0.25/0.5	0.5/0.75	Synergistic/ Additive
F. nucleatum ATCC 51190	SJEE Gentamicin	0.5/2 4/8	0.125/0.25 1/2	0.25/0.125 0.25/0.25	0.5/0.375	Synergistic/ Synergistic
P. intermedia ATCC 25611	SJEE Gentamicin	4/16 32/32	1/4 4/8	0.25/0.25 0.125/0.25	0.375/0.5	Synergistic/ Synergistic
P. gingivalis ATCC 33277	SJEE Gentamicin	1/2 256/512	0.25/0.5 64/64	0.25/0.25 0.25/0.125	0.5/0.375	Synergistic/ Synergistic

Table 4: Synergistic effects of 50% ethanol extract of *Sophora japonica* (SJEE) with erythromycin against oral bacteria.

Strains	Agent	MIC/MBC[1]		FIC	FICI[3]	Outcome
		Alone	Combination[2]			
S. mutans ATCC 25175[4]	SJEE Erythromycin	2/2 0.0625/0.125	0.5/1 0.0156/0.0313	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
S. sanguinis ATCC 10556	SJEE Erythromycin	2/4 0.0156/0.0313	1/1 0.0078/0.0156	0.5/0.25 0.5/0.5	1/0.75	Additive/ Additive
S. parasanguinis KCOM 1497[5]	SJEE Erythromycin	1/2 0.125/0.5	0.25/0.5 0.0313/0.0625	0.25/0.25 0.25/0.125	0.5/0.375	Synergistic/ Synergistic
S. sobrinus ATCC 27607	SJEE Erythromycin	0.5/0.5 0.0313/0.0625	0.125/0.25 0.0078/0.0313	0.25/0.5 0.25/0.5	0.5/1	Synergistic/ Additive
S. ratti KCTC 3294[6]	SJEE Erythromycin	4/16 0.008/0.016	1/4 0.002/0.008	0.25/0.25 0.25/0.5	0.5/0.75	Synergistic/ Additive
S. criceti KCTC 3292	SJEE Erythromycin	1/1 0.125/0.25	0.25/0.5 0.0313/0.0625	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
S. downei KCOM 1165	SJEE Erythromycin	2/4 0.25/0.5	0.25/1 0.0625/0.125	0.125/0.25 0.25/0.25	0.375/0.5	Synergistic/ Synergistic
S. anginosus ATCC 31412	SJEE Erythromycin	1/4 0.125/0.5	0.25/1 0.0313/0.0625	0.25/0.25 0.25/0.125	0.5/0.375	Synergistic/ Synergistic
S. gordonii ATCC 10558	SJEE Erythromycin	0.125/0.5 0.0313/0.0625	0.0313/0.125 0.0078/0.0313	0.25/0.25 0.25/0.5	0.5/0.75	Synergistic/ Additive
A. actinomycetemcomitans ATCC 43717	SJEE Erythromycin	0.5/1 0.125/0.25	0.125/0.5 0.0313/0.0625	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
F. nucleatum ATCC 51190	SJEE Erythromycin	0.5/2 32/64	0.125/0.25 8/16	0.25/0.125 0.25/0.25	0.5/0.375	Synergistic/ Synergistic
P. intermedia ATCC 25611	SJEE Erythromycin	4/16 16/32	1/4 2/8	0.25/0.25 0.125/0.25	0.375/0.5	Synergistic/ Synergistic
P. gingivalis ATCC 33277	SJEE Erythromycin	1/2 2/8	0.25/0.5 0.5/2	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic/ Synergistic

[1]*Sophora japonica* (SJEE): mg/mL; antibiotics: μg/mL
[2]The MIC and MBC of 50% ethanol extract of *Sophora japonica* (SJEE) with erythromycin 0.002-512 μg/mL
[3]The fractional inhibitory concentration index (FIC index)
[4]American Type Culture Collection (ATCC)
[5]Korean collection for Oral Microbiology (KCOM)
[6]Korean collection for type cultures (KCTC)

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Table 5: Synergistic effects of 50% ethanol extract of *Sophora japonica* (SJEE) with vancomycin against oral bacteria.

Strains	Agent	MIC/MBC[1]		FIC	FICI[3]	Outcome
		Alone	Combination[2]			
S. mutans ATCC 25175[4]	SJEE Vancomycin	2/2 1/2	0.25/0.5 0.125/0.5	0.125/0.25 0.125/0.25	0.25/0.5	Synergistic / Synergistic
S. sanguinis ATCC 10556	SJEE Vancomycin	2/4 0.25/1	0.5/1 0.125/0.25	0.25/0.25 0.5/0.25	0.75/0.75	Additive/ Additive
S. parasanguinis KCOM 1497[5]	SJEE Vancomycin	1/2 2/4	0.25/0.5 0.5/1	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic/ Synergistic
S. sobrinus ATCC 27607	SJEE Vancomycin	0.5/0.5 1/2	0.125/0.25 0.125/0.5	0.25/0.5 0.125/0.25	0.375/0.75	Synergistic/ Additive
S. ratti KCTC 3294[6]	SJEE Vancomycin	4/16 0.5/1	1/2 0.125/0.25	0.25/0.125 0.25/0.25	0.5/0.375	Synergistic / Synergistic
S. criceti KCTC 3292	SJEE Vancomycin	1/1 1/4	0.5/0.5 0.25/1	0.5/0.5 0.25/0.25	0.75/0.75	Additive/ Additive
S. downei KCOM 1165	SJEE Vancomycin	2/4 4/16	0.5/1 2/4	0.25/0.25 0.5/0.25	0.75/0.5	Additive/ Synergistic
S. anginosus ATCC 31412	SJEE Vancomycin	1/4 1/4	0.25/0.5 0.25/1	0.25/0.125 0.25/0.25	0.5/0.375	Synergistic / Synergistic
S. gordonii ATCC 10558	SJEE Vancomycin	0.125/0.5 0.5/1	0.0313/0.125 0.125/0.25	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic / Synergistic
A.actinomycetemcomitans ATCC 43717	SJEE Vancomycin	0.5/1 2/4	0.125/0.5 0.5/1	0.25/0.5 0.25/0.25	0.5/0.75	Synergistic/ Additive
F. nucleatum ATCC 51190	SJEE Vancomycin	0.5/2 64/128	0.125/0.5 16/32	0.25/0.25 0.25/0.25	0.5/0.5	Synergistic / Synergistic
P. intermedia ATCC 25611	SJEE Vancomycin	4/16 16/36	2/4 4/8	0.5/0.25 0.25/0.25	0.75/0.5	Additive/ Synergistic
P. gingivalis ATCC 33277	SJEE Vancomycin	1/2 8/16	0.5/0.5 2/4	0.5/0.25 0.25/0.25	0.75/0.5	Additive/ Synergistic

Table 6: Biofilm quantity of SJEE and/or antibiotics preformed biofilms of oral bacteria

	Inhibition of biofilm (% of SJEE)												
	SM	***SS***	***SP***	***SS0***	***SR***	***SC***	***SD***	***SA***	***SG***	***AA***	***FN***	***PI***	***PG***
SJEE(1/8 MIC)	0	0	0	0	0	0	0	0	0	0	0	0	0
AMP (1/8 MIC)	0.84	1.31	0.44	-1.16	1.39	5.83	-0.44	-0.86	-1.83	-4.36	0.66	2.34	-0.65
GEN (1/8 MIC)	0.37	-2.47	2.08	-0.66	4.86	5.26	2.14	0.79	-1.05	-3.2	3.15	2.08	-5.65
ERY (1/8 MIC)	1.25	0.98	-0.04	-2.08	5.76	-1.14	2.73	1.59	2.06	-3.67	0.72	8.39	-0.1
VAN (1/8 MIC)	0.73	1.21	0.27	0	5.71	-0.28	3.31	0.46	0.74	-4.19	1.99	8.78	0.6
SJEE(1/8 MIC)+AMP (1/8 MIC)	57.39	22.75	59.76	88.55	36.92	47.11	34.99	59.56	48.93	66.34	52.29	38.93	72.04
SJEE(1/8 MIC)+GEN (1/8 MIC)	46.89	59.72	55.79	59.56	63.97	56.82	54.99	39.64	72.26	51.64	48.92	72.97	63.88
SJEE(1/8 MIC)+ERY (1/8 MIC)	39.22	38.88	54.37	52.78	66.85	37.06	48.56	32.43	66.34	77.92	82.83	55.67	87.74
SJEE(1/8 MIC)+VAN (1/8 MIC)	46.53	57.34	64.18	64.88	63.23	66.26	55.62	66.18	71.76	57.74	78.63	62.76	89.39

SM: *Streptococcus mutans*; *SS*: *Streptococcus sanguinis*; *SP*: *Streptococcus parasanguinis*; *SSO*: *Streptococcus sobrinus*; *SR*: *Streptococcus ratti*; *SC*: *Streptococcus criceti*; *SD*: *Sreptococcus downei*; *SA*: *Streptococcus anginosus*; *SG*: *Streptococcus gordonii*; *AA*: *Aggregatibacter actinomycetemcomitans*; *FN*: *Fusobacterium nucleatum*; *PI*: *Prevotella intermedia*; *PG*: *Porphylomonas gingivalis*

from *S. mutans* [15,40]. The maltol-3-O-(4'-O-cis-p-coumaroyl-6'-O-(3- hydroxy-3-methylglutaroyl))-β-glucopyranoside from the dried flowers of *S. japonica* showed the strongest inhibitory effect on saliva-induced aggregation in *S. mutans* with an IC50 value of 58.6 μM [38]. A gradual increase of biomass for oral bacteria was noted up to 24 h in both SJEE and antibiotics (Table 6 and Figure 1). *S. sobrinus* was the most susceptible to SJEE (1/8 MIC) plus AMP (1/8 MIC) with 88.1% inhibition as compared to SJEE (1/2 MIC) at the end of 24 h of incubation. *S. gordonii* indicated the most inhibition to SJEE (1/8 MIC) plus GEN (1/8 MIC) with 76.12%. SJEE (1/8 MIC) plus ERY (1/8 MIC) and SJEE (1/8 MIC) plus VAN (1/8 MIC) were shown with 89.42% and 94.03% inhibition for *P. gingivalis* biofilm, respectively.

Time kill of SJEE with antibiotics

Previous studies had reported the antimicrobial activities and mechanisms of SJEE against several kinds of bacteria [21,22].

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However, the type of microorganisms and their cell membrane structure and composition could play an important role in the susceptibility to antimicrobials [39,40]. The MIC value was similar to the results that MIC values of SJEE against *Staphylococcus aureus* and *Aeromonas hydrophila* were 20 μg/mL and 50 μg/mL, respectively [22,41,42]. The bacterial effect of SJEE with antibiotics, ampicillin, gentamicin, erythromycin, and vancomycin against oral bacteria was confirmed by time kill curve experiments. The SJEE (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 SJEE (1/2 MIC) with ampicillin, gentamicin, erythromycin, or/and vancomycin (1/2 MIC) (Figures 2-4). A strong bactericidal effect was exerted in drug combinations.

Conclusion

In conclusion, these findings suggest that crude extract of *S. japonica* 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.

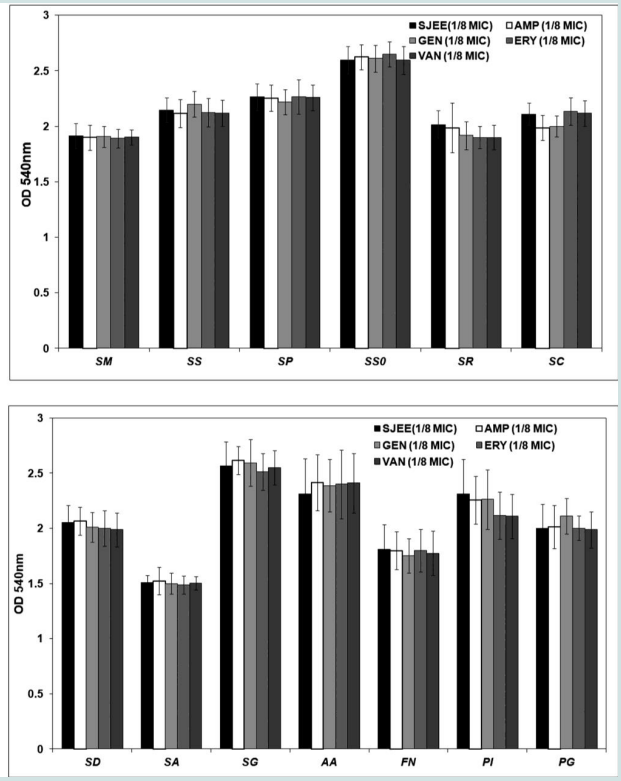

Figure 1: Anti-biofilm effect of different concentrations of SJEE alone or/and antibiotics on biofilm formation of oral bacteria, *SM*, *SS*, *SP*, *SSO*, *SR*, and *SC*. Cells stained with 0.1% (wt/vol) crystal violet solution for 15 min, washed with PBS, and de-stained with 96% ethanol 10 min in order 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 micro plate reader. Data points are the mean values±S.E.M. of six experiments.

SM: *Streptococcus mutans*; *SS*: *Streptococcus sanguinis*; *SP*: *Streptococcus parasanguinis*; *SSO*: *Streptococcus sobrinus*; *SR*: *Streptococcus ratti*; *SC*: *Streptococcus criceti*.

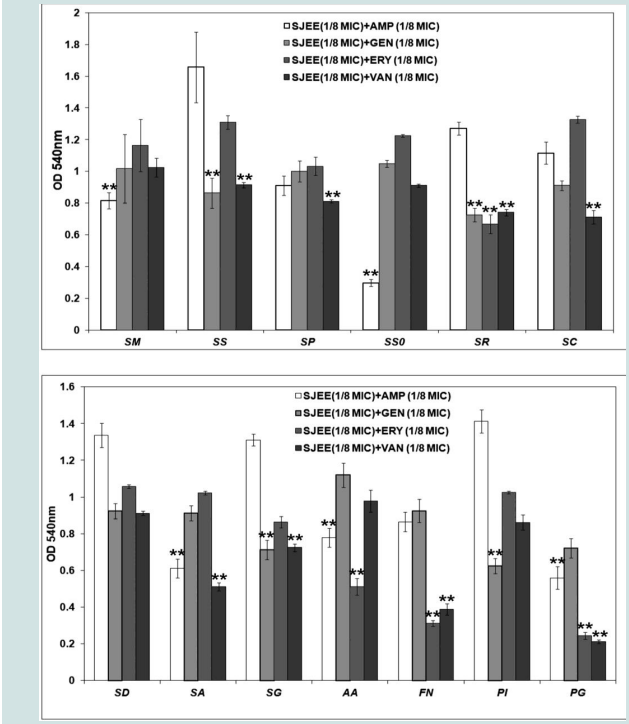

Figure 2: Anti-biofilm effect of different concentrations of SJEE alone or/and antibiotics on biofilm formation of oral bacteria, *SD, SA, SG, AA, FN, PI,* and *PG*. Cells stained with 0.1% (wt/vol) crystal violet solution for 15 min, washed with PBS, and de-stained with 96% ethanol 10 min in order to 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±S.E.M.

**Significantly different from the control group (1/8 MIC SJEE or antibiotics) (P<0.01) of six experiments.

SD*: Sreptococcus downei; SA: Streptococcus anginosus; SG: Streptococcus gordonii; AA: Aggregatibacter actinomycetemcomitans; FN: Fusobacterium nucleatum; PI: Prevotella intermedia; PG: Porphylomonas gingivalis*

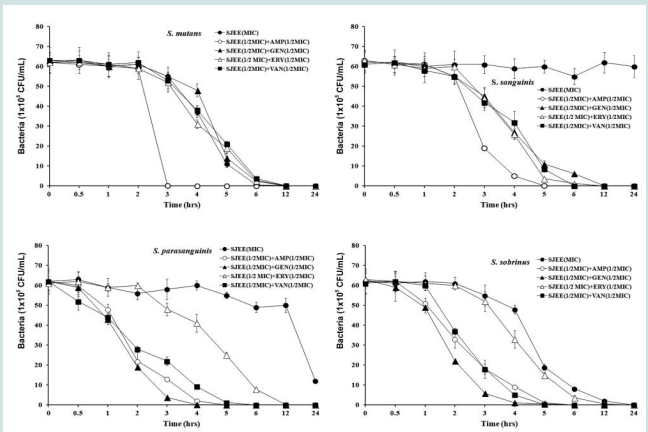

Figure 3: Time-kill curves of MIC of SJEE alone and its combination with 1/2 MIC of AMP, GEN, ERY, or/and VAN against *S. mutans*, *S. sanguinis*, *S. parasanguinis*, and *S. sobrinus*. Bacteria were incubated with SJEE (●), SJEE+AMP (○), SJEE+GEN (▲), SJEE+ERY (△), and SJEE+VAN (■) over time. Data points are the mean values±S.E.M. of six experiments. CFU, colony forming units.

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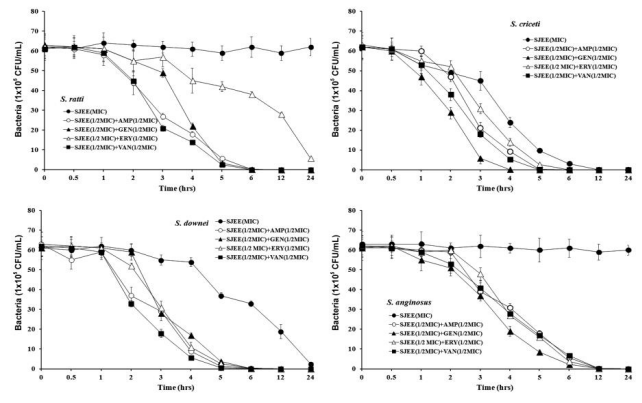

Figure 4: Time-kill curves of MIC of SJEE alone and its combination with 1/2 MIC of AMP, GEN, ERY, or/and VAN against *S. ratti*, *S. criceti*, *S. downei*, and *S. anginosus*. Bacteria were incubated with SJEE (●), SJEE+AMP (○), SJEE+GEN (▲), SJEE+ERY (△), and SJEE+VAN (■) over time. Data points are the mean values±S.E.M. of six experiments. CFU, colony forming units.

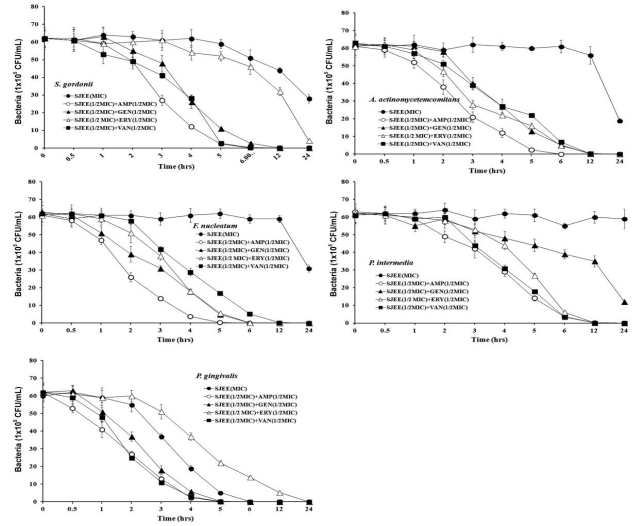

Figure 5: Time-kill curves of MIC of SJEE alone and its combination with 1/2 MIC of AMP, GEN, ERY, or/and VAN against *S. gordonii*, *A. actinomycetemcomitans*, *F. nucleatum*, *P. intermedia*, and *P. gingivalis*. Bacteria were incubated with SJEE (●), SJEE+AMP (○), SJEE+GEN (▲), SJEE+ERY (△), and SJEE+VAN (■) over time. Data points are the mean values±S.E.M. of six experiments. CFU, colony forming units.

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