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Synergistic Effect of Methanol Extract of Salvia Miltiorrhiza and Antibiotics against Dental Caries Pathogens

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Salvia miltiorrhiza Bunge (*S. miltiorrhiza*) is a traditional Korean medicine that is commonly used for the treatment of inflammatory diseases such as edema, arthritis, and hepatitis. The present study investigated the antimicrobial activity of methanol (MeOH) extract of *S. miltiorrhiza* roots against oral bacteria using broth the microdilution method and the checkerboard and time-kill methods evaluated the synergistic effects of treatment with antibiotics. The MeOH extract was demonstrated as a higher antibacterial activity (MICs, 8 to 64 μg/mL; MBCs, 16 to 64 μg/mL) against all tested oral bacteria. Additionally, the extract was observed to have a synergistic effect with ampicillin or gentamicin. A time-kill study evaluating the effects of the extract indicated that the extract treatment in combination with ampicillin or gentamicin showed rapid bactericidal activity. The results suggest that MeOH extract of *S. miltiorrhiza* could be employed as a natural antibacterial agent against dental caries.

Key words: *Salvia miltiorrhiza*, Antibacterial activity, Dental caries, Oral bacteria, Minimum inhibitory concentrations/minimum bactericidal concentrations, Time-kill curves, Fractional inhibitory concentrations

Introduction

Salvia miltiorrhiza Bunge (*S. miltiorrhiza*, Labiatae, Danshen) has long been used in traditional oriental herbal medicine for the treatment of a variety of diseases, including arthritis, hepatitis, hypertension, chronic renal failure, dysmenorrheal, and antibacterial activity [5, 11, 13, 16, 21, 24]. The aqueous extracts of the fresh leaves, dried leaves, and bark have been administered as a counteracting agent for the treatment of insecticide and ethyl alcohol poisoning, and the dried roots have been utilized as an anti-inflammatory agent and antipyretic [9, 11, 23, 26]. It has recently been reported that the extracts of *S. miltiorrhiza* leaves and roots exert a protective effect against ethanol-induced based on hepatic lipid peroxidation, blood ethanol concentration, and alcohol dehydrogenase and aldehyde dehydrogenase activity [21, 23, 24]. Over 50 chemical constituents isolated and identified from *S. miltiorrhiza* include two constituent groups of hydrophilic phenolic acids, salvianolic acid B and lithospermic acid, as well as lipophilic tanshinones such as tanshinone I, tanshinone IIA, tanshinone IIB, and cryptotanshinone [2, 7, 14, 22, 27]. These lipophilic tanshinones have been shown to inhibit platelet-aggregation and protect the myocardium against ischemia-induced derangement, as well as to protect liver microsomes, hepatocytes, and erythrocytes against oxidative damage [1, 4, 15, 18, 24]. Specifically, the phenolic acids have been found to have significant bioactivities including antioxidant, antiblood coagulation, and cell protection, as well as a wide variety of other activities including anti-ischemia-reperfusion, antihypertension, antivirus, antibacterial, and anti-tumor effects [2, 5, 14, 17, 25, 27, 28].

In the present study, the antimicrobial activities of MeOH extract of *S. miltiorrhiza* roots against dental caries pathogens were assessed using the checkerboard and time-kill methods to evaluate the synergistic effects of treatment with ampicillin or gentamicin.

Materials and Methods

Plant material and preparation of MeOH crude plant extract

S. miltiorrhiza was purchased from the herbal medicine

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cooperative association of Jeonbuk Province, Korea, in March 2005. The identity was confirmed by Dr. Bong-Seop Kil, College of Natural Science, Wonkwang Unibersity. Voucher specimens (DJ-JC05A) were deposited in the Herbarium of the College of Natural Science, Wonkwang University. The dried and powered roots of *S. miltiorrhiza* were extracted by refluxing the samples with MeOH for 4 h at 80°C three times. The solvent of the extract was removed under vacuum on a rotary evaporator at 40°C. The extract was then dissolved in 10% dimethyl sulfoxide (DMSO) for testing. The extract was kept at 4°C in the dark until further use.

Bacterial strains

Antimicrobial activites of the extract of *S. miltiorrhiza* roots against some oral bacteria were determined by the broth dilution method. 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, and *Streptococcus gordonii* ATCC 10558. Brain-Heart Infusion broth supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI) was used for all bacterial strains.

Minimum inhibitory concentrations/minimum bactericidal concentrations assay

The antimicrobial activity of the MeOH extract of *S. miltiorrhiza* roots against oral bacteria was determined through the broth dilution method carried out in triplicate. The minimum inhibitory concentrations (MICs) were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. Following anaerobic incubation of MICs plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of the MeOH extract of *S. miltiorrhiza* roots that kill 99.9% of the test bacteria by plating out onto each appropriate agar plate.

Checker board dilution test

The antibacterial effects of a combination of the MeOH extract, which exhibited the highest antimicrobial activity, and antibiotics were assessed by the checkerboard test as previously described [3]. The antimicrobial combinations assayed included the MeOH extract plus ampicillin or gentamicin. The interaction was defined as synergistic if the FIC (fractional inhibitory concentrations) index was less than or equal to 0.5, additive if the FIC index was greater than 0.5 and less than or equal 1.0, indifferent if the FIC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC index was greater than 2.0 [3].

Time-kill curves

Bactericidal activities of the drugs under study were also evaluated using time-kill curves on oral bactreria. Tubes containing Mueller-Hinton supplemented to which antibiotics had been added at concentrations of the MBCs were inoculated with a suspension of the test strain, giving a final bacterial count between $0.5\sim1\times10^6$ CFU/mL. The tubes were thereafter incubated at 37°C in 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 Brain-Heart Infusion (BHI) agar for streptococci.

Results and Discussion

The results of the antibacterial activity showed that the MeOH extract of exhibited antimicrobial activities against all oral bacteria tested (MICs, 8 to 64 μg/mL; MBCs, 16 to 64 μg/mL) in Table 1. Especially, *S. gordonii* (MIC/MBC, 8/16 μg/mL) were showed as the strong antimicrobial activity than *S. ratti* and *S. criceti* (MIC/MBC, 64/64 μg/mL). The MICs/MBCs for ampicillin were found to be either 1/2 or 32/32 μg/mL; for gentamicin, either 2/4 or 16/32 μg/mL.

The synergic effects of the MeOH extract administered in conjunction with ampicillin or gentamicin are shown in Tables 1, 2. When administered in combination with the extract, the MICs for ampicillin were reduced ≥4-8-fold when tested against all of the bacteria evaluated in this study, with the exception of *S. ratti* and *S. criceti* (≥2-fold), producing a synergistic effect as defined by FICI ≤0.375-0.5. The administration of gentamicin with the extract induced a ≥4-fold reduction against *S. sanguinis*, *S. ratti*, *S.*

Table 1. Antibacterial acitvity of the MeOH extract of *S. miltiorrhiza* with ampicillin in oral bacteria.

Strains	Agent	MIC /MBC (μg/mL)		FIC (mg/mL)	FICI[3]	Outcome
		Alone	Combination[2]			
S. mutans ATCC 25175	MeOH[1]	32/32	8/8	0.25	0.5	Synergistic
	Ampicillin	4/4	1/2	0.25		
S. sanguinis ATCC 10556	MeOH	16/32	4/4	0.25	0.375	Synergistic
	Ampicillin	32/32	4/4	0.125		
S. sobrinus ATCC 27607	MeOH	16/16	4/4	0.25	0.5	Synergistic
	Ampicillin	2/4	0.5/1	0.25		
S. ratti KCTC 3294	MeOH	64/64	32/32	0.5	1.0	Additive
	Ampicillin	4/4	2/2	0.5		
S. criceti KCTC 3292	MeOH	64/64	32/32	0.5	1.0	Additive
	Ampicillin	4/4	2/2	0.5		
S. anginosus ATCC 31412	MeOH	16/32	4/4	0.25	0.5	Synergistic
	Ampicillin	4/4	1/2	0.25		
S. gordonii ATCC 10558	MeOH	8/16	1/2	0.125	0.375	Synergistic
	Ampicillin	1/2	0.25/0.5	0.25		

[1)] the methanol extract
[2)] the methanol extract with ampicillin
[3)] the FIC index

Table 2. Antibacterial acitvity of the MeOH extract of *S. miltiorrhiza* with gentamicin in oral bacteria.

Strains	Agent	MIC /MBC (μg/mL)		FIC (mg/mL)	FICI[3]	Outcome
		Alone	Combination[2]			
S. mutans ATCC 25175	MeOH[1]	32/32	8/8	0.25	0.75	Additive
	Gentamicin	8/8	4/4	0.5		
S. sanguinis ATCC 10556	MeOH	16/32	4/4	0.25	0.5	Synergistic
	Gentamicin	8/16	2/4	0.25		
S. sobrinus ATCC 27607	MeOH	16/32	8/8	0.5	0.75	Additive
	Gentamicin	4/4	1/1	0.25		
S. ratti KCTC 3294	MeOH	64/64	16/16	0.25	0.5	Synergistic
	Gentamicin	4/4	1/2	0.25		
S. criceti KCTC 3292	MeOH	64/64	32/32	0.5	1.0	Additive
	Gentamicin	8/16	4/4	0.5		
S. anginosus ATCC 31412	MeOH	16/32	4/8	0.25	0.5	Synergistic
	Gentamicin	16/32	4/4	0.25		
S. gordonii ATCC 10558	MeOH	8/16	2/4	0.25	0.5	Synergistic
	Gentamicin	2/4	0.5/1	0.25		

[1)] the methanol extract
[2)] the methanol extract with gentamicin
[3)] the FIC index

anginosus, and *S. gordonii*, producing a synergistic effect as defined by FICI ≤0.5. The bacterial effects of the MeOH extract with ampicillin or gentamicin against oral bacteria were confirmed by time-kill curve experiments (Fig. 1). The cultures of all bacteria, with a cell density of 10^6 CFU/mL, were exposed to MBCs of the MeOH extract alone and with ampicillin or gentamicin. The result was found that treatment in a combination of the extract with ampicillin or gentamicin produced a more rapid decrease in the concentration of bacteria CFU/mL than treatment with the extract alone, and that these effects occurred in a time dependant manner. The growth of the tested bacteria was

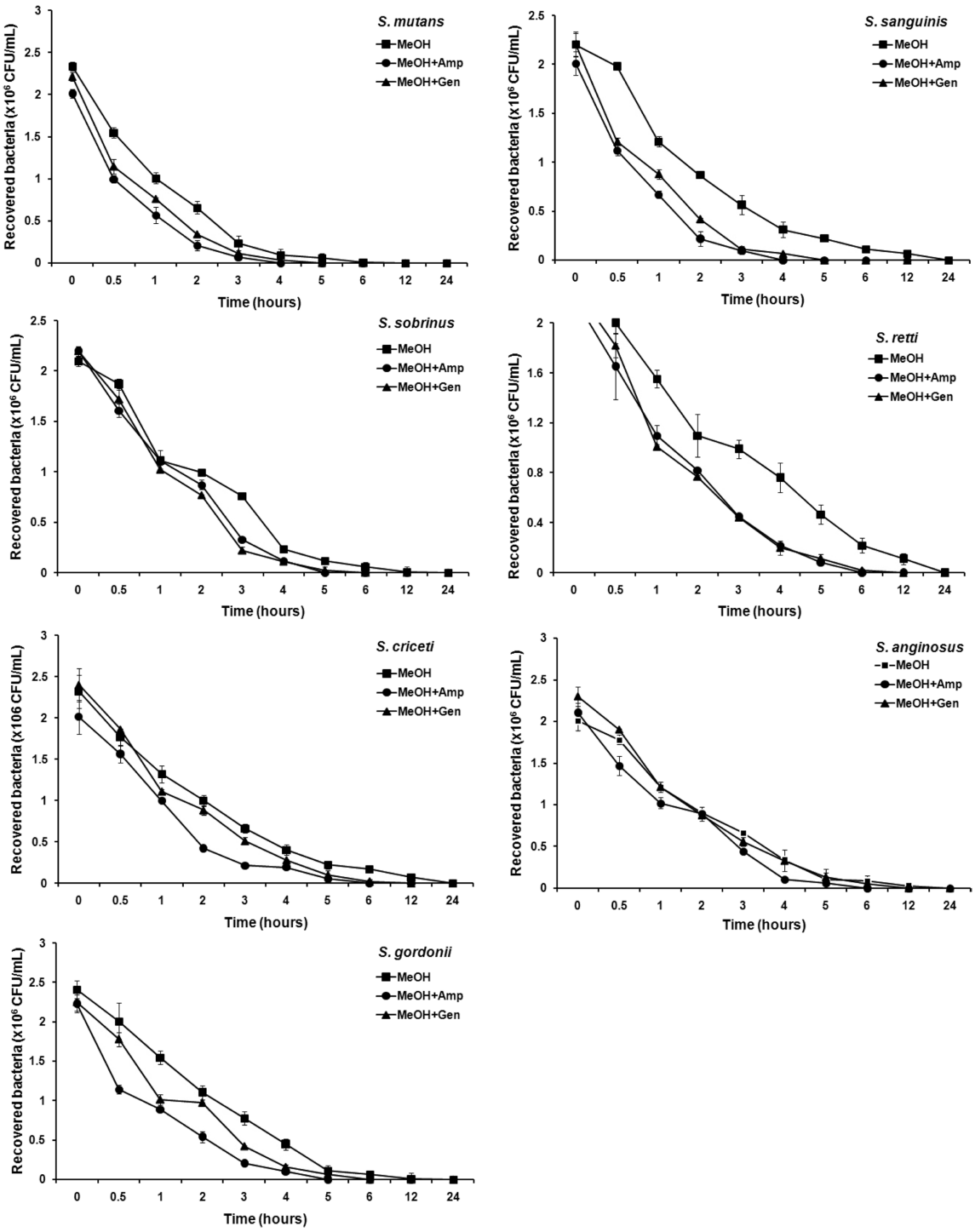

Fig. 1. Time-kill curves of MBC of the MeOH extract alone and its combination with MBC of ampicillin or gentamicin against *S. mutans, S. sanguinis, S. sobrinus, S. ratti, S. criceti, S. anginosus*, and *S. gordonii*. Bacteria were incubated with the MeOH extract alone (■) and with ampicillin (●) or with gentamicin (▲) over time. Data points are the mean values±S.E.M. of six experiments. CFU, colony-forming units.

completely attenuated after 5-12 h of treatment with the MBC of the extract, regardless of whether it was administered alone or with ampicillin or gentamicin. A strong bactericidal effect is exerted in drug combinations.

Various pharmacological studies conducted *in vitro* and *in vivo* have concentrated on *S. miltiorrhiza* components

such as hydrophilic phenolic compounds, danshensu, salvianolic acid B, and the lipophilic diterpene compounds known as tanshinones [2, 7, 13, 14, 22]. These compounds are known to have bioactivities that make them useful for the treatment of coronary heart disease, myocardial infarction, hypertension, chronic hepatitis, liver fibrosis, and bone loss, as well as to possess antioxidant, antiinflammation, antitumor, antivirus, and antibacterial activity [1, 5, 10, 15, 16, 20, 21, 24]. Some tanshinones in *S. miltiorrhiza* exert strong antimicrobial activity against a broad range of Gram-positive, including *S. aureus*, and Gram-negative bacteria as well as other microorganisms [6, 20]. Several studies have demonstrated that phenolic compounds produced by plants exert antibacterial activity and interfere with the inflammatory process [8, 17, 19]. Furthermore phenolic compounds inhibit plaque formation and developing gingivitis in oral cavity [17]; therefore, it is likely that the phenolic compounds in *S. miltiorrhiza* may be related, in part, to the antibacterial effects observed in the present study.

In the present study, the antibacterial activity of *S. miltiorrhiza* extract based on their inhibition of the growth of oral bacteria. Furthermore, the results demonstrated that these compounds exerted synergistic effects when administered with ampicillin or gentamicin. These findings suggest that *S. miltiorrhiza* is effective against dental caries bacteria, and that it may prove useful in the treatment of dental caries.

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국문초록

치아우식증유발세균에 대한 단삼 메탄올추출물과 항생제와의 병용효과

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단삼(*Salvia miltiorrhiza* Bunge)은 우리나라에서 전통적으로 부종, 관절염, 간염 등과 같은 염증질환의 치료에 사용해왔다. 본 연구에서는 단삼 메탄올 추출물을 이용하여 구강미생물에 대한 항균활성을 확인하였다. 그 결과 메탄올추출물은 실험 되어진 모든 구강미생물에서 강한 항균활성을 나타내었다(MICs, 8 to 64 μg/mL; MBCs, 16 to 64 μg/mL). 추가적으로 ampicillin이나 gentamicin과의 병용투여서 최소억제농도(MIC)와 최소살균농도(MBC)가 감소하는 병용효과를 나타내었다. 더불어 메탄올추출물 단독 사용시 보다 항생제와 병용투여시 빠른 사멸효과를 보였다. 결론적으로 단삼 메탄올추출물이 치아우식을 유발하는 많은 세균들에 대한 항균효과가 뛰어남을 확인 할 수 있었으며, 안정성이 높은 천연 치아우식예방제로서의 개발가능성을 확인하였다.