# Evaluation of a Mouthwash Containing *Sambucus Williamsii var. Coreana* Extract for Prevention of Periodontal Disease - a Randomized Placebo-controlled Clinical Trial

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# Abstract

***Background:*** The purpose of this study is to verify the clinical applicability by applying a mouthwash containing *Sambucus williamsii var. coreana* extract for preventing periodontal disease.

***Methods:*** A randomized, double-blind, placebo-controlled study was conducted on 64 patients, excluding those with insufficient data, who visited M dental clinic located in Busan, Korea. Thirty-two people were assigned respectively to the saline solution gargle group and the *Sambucus williamsii var. coreana* extract gargle group to conduct O'Leary index, plaque index (PI), gingival index (GI), and subgingival plaques. For the homogeneity of the two groups, scaling was carried out one week before the experiment, and the participants were taught for oral care to conduct during the study period. SPSS 24.0 for Windows (IBM Corp., Armonk, NY, USA) was used to compare the saline solution gargle group and the *Sambucus williamsii var. coreana* extract gargle group as well as to analyze Baseline (before gargle application), Treatment (immediately after gargle application), and After 5 Days (5 days after gargle application).

***Results:*** When Baseline was measured, there was no difference between the two groups in terms of O'Leary index, PI, GI (indicators related to periodontal disease), and subgingival plaques. However, there was a significant difference in Treatment and After 5 days($p<0.05$). Also, the periodontal-related indexes improved as the application time increased in the *Sambucus williamsii var. coreana* extract gargle group. The antibacterial effect was also shown for gram-positive bacteria and gram-negative bacteria in subgingival plaques as the application time increased.

***Conclusion:*** The use of the mouthwash containing *Sambucus williamsii var. coreana* extract was found to be effective for oral periodontal-related indicators and bacteria causing periodontal disease. Therefore, using a mouthwash containing *Sambucus williamsii var. coreana* extract, a natural drug, will possibly maintain healthy periodontal health by inhibiting and preventing the progression of periodontal disease.


# Background

Periodontal disease is a non-communicable chronic disease in which soft and hard tissues are destroyed due to local bacterial infection and an imbalance between the host immune responses [1]. As periodontal disease progresses, the collagen fibers of the periodontal ligament are destroyed to form a periodontal pocket, which creates a favorable ecological environment for anaerobic bacteria to thrive [2]. As a characteristic of the bacterial habitat, most periodontal disease bacteria are anaerobic bacteria sensitive to oxygen, so they are distributed in the subgingival gingival biofilm, where oxygen supply is poor compared to the supragingival gingival biofilm [3]. The anaerobic bacteria associated with such periodontal disease include *Parvimonas micra (P. micra)*, Porphyromonas gingivalis (P. gingivalis), Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans), Treponema denticola (T. denticola), Prevotella nigrescens (P. nigrescens), Fusobacterium nucleatum (F. nucleatum) and others [4].

The lipopolysaccharides (LPS) endotoxins or metabolites formed by these anaerobic bacteria increase the secretion of proinflammatory cytokines from tissues and immune cells [5]. In particular, Interleukin-1

(IL-1β) and Tumor necrosis factor-α (TNF-α) are representative cytokines involved in tissue destruction. IL-1β induces the following: recruitment of inflammatory cells, priming and degranulation of polymorphonuclear leukocyte, production of inflammatory mediators such as prostaglandin, production of matrix metalloproteinases (MMP), inhibiting collagen synthesis, activating T and B lymphocytes [6]. TNF-α increases cellular apoptosis, bone resorption, MMP secretion, intercellular adhesion molecule (ICAM) expression, and IL-6 production [7]. In addition, IL-6 is involved in the tissue destruction process by promoting osteoclast formation, bone resorption, and T lymphocyte differentiation [8]. Therefore, it is crucial to suppress the early colonization of anaerobic bacteria to prevent periodontal disease.

As a method of inhibiting bacteria, the use of simple and portable mouthwash is increasing because mechanical plaque control methods such as toothbrushing are more cumbersome than chemical plaque control methods such as mouthwash [9]. Above all, the SARS-CoV-2 virus of COVID-19, which is causing the pandemic, was reported in 91.7% of patients' saliva samples [10]. Accordingly, interest in mouthwash increased as it was recommended as an effective measure to reduce the risk of viral infection [11]. Generally used mouthwash has intensive chemical agents, so there is a high possibility of removing pathogens and resident bacteria in the mouth. Therefore, natural extracts are recommended rather than synthetic chemical agents to suppress pathogens only while maintaining healthy oral bacteria [12].

Recently, as research on the utility of natural extracts has been active, natural antibacterial action is being used in various ways [13]. *Glycyrrhiza uralensis* extract [14] and bamboo extract [15] have been reported as effective natural agents showing antibacterial effects related to periodontal disease. It was reported that onion extract inhibited strains of periodontal pathogens *P. gingivalis* and *Prevotella intermedia (P. intermedia)* at a concentration of 40 μg/ml [16]. In addition, pulsatilla extract has an antibacterial effect in the layer separated by butyl alcohol [17]. Although research on the antibacterial effect of various natural medicine continues, studies on the suppression and prevention of periodontal disease in the oral cavity by clinical studies are insufficient. Therefore, it is necessary to prove its clinical effect to develop a natural medicine as a mouthwash.

Williams Elder (*Sambucus williamsii var. coreana*) is a deciduous broad-leaved tree distributed in Korea, China, and Japan. It is used as a medicine good for bones and musculoskeletal injuries in traditional oriental medicine [18]. There were studies of triterpenes, phenols, and lignans isolated from *Sambucus williamsii var. coreana* used in proliferation experiments on mouse osteocytes (UMR106) and alkaline phosphatase (ALP) activity experiments on UMR106 cells [19]. Based on these research results related to osteogenesis, there found the potential for the regenerative ability for the destroyed alveolar bone and periodontal tissue caused by periodontal disease. In terms of a study checking the relationship between *Sambucus williamsii var. coreana* and oral disease, there was a study showing the antibacterial effect of *Sambucus williamsii var. coreana* extract against oral pathogens [20]. However, studies have not yet been conducted to verify the clinical usefulness based on clinical effectiveness in the dental field.

Therefore, this study evaluated the clinical applicability of mouthwash using phytotherapeutic medication, *Sambucus williamsii var. coreana* extract, to develop an effective medicine against

periodontal-related clinical indicators and pathogens that cause periodontal disease in the oral cavity.

## Materials And Methods

# 1. Ethical consideration

This study followed the International Council for Harmonization of Technical Requirements for Pharmaceuticals for human use (ICH) guideline. The Silla University Institutional Review Board (1041449-202008-HR-001, Busan, Korea) had approved the human study and WHO International Clinical Trial Registry Platform was registered by clinical trial registration (10/03/2022, registration number: KCT0007064; https://cris.nih.go.kr/cris/search/detailSearch.do/21436). All participants had noticed relevant information (purpose, procedures, and risks) of this study. Participants were free to withdraw from the study at any time. An informed consent form was provided to all participants prior to enrollment in the clinical trial and written informed consent was obtained.

# 2. Study participants

The G* Power 3.1 program calculated sample sizes. 68 participants were required for an independent t-test with significance level a = 0.05 two-tailed, power = 0.8, and effect size = 0.7. An initially planned sample size was 96 to consider a dropout rate of 40%, and actual participants were 98 in this study. The dropout rate was set high as the subjects were college students or working adults. A total of 98 subjects were screened, but 74 participants were randomly assigned to the saline solution gargle group or *Sambucus williamsii var. coreana* extract gargle group after excluding 24 subjects who did not meet inclusion criteria or refused to participate during 5 days. 64 subjects were selected as final subjects, excluding 6 subjects who did not follow the guidelines for 5 days and 4 subjects who did not complete the final analysis (Fig. 1).

# 3. Study design and protocol

An experiment was randomized, double-blind, and placebo-controlled. In this study, a dental hygienist with more than ten years of experience directly stated the aim of the study to the patients who visited M dental clinic in Busan during October 2020 ~ June 2021. These patients agreed to participate in the study on December 15, 2020. Among the subjects, the following subjects who met the exclusion criteria were excluded from this study: a person with severe dental disease (e.g., periodontitis, dry mouth, dental caries), a person treated for a general disease that may cause bad breath (e.g., Sjogren's syndrome, rheumatism, renal disease, and hepatic disease), a smoking person, a person diagnosed with sinusitis and/or rhinitis, a person taking antibiotics, a person with tongue problems (e.g., tongue cancer, glossitis), a person received scaling within two months. Among dental caries, patients with enamel caries were eligible to participate in the study, but patients with more than one dentin caries were excluded. Accordingly, the subjects of this study were those who agreed to complete the questionnaire, were not included in the exclusion criteria, and had 16 or more remaining teeth. 74 patients who satisfied the

inclusion criteria were selected as the study subjects. Finally, the participants in this study were analyzed with a result of 64 subjects.

# 4. Clinical examination

Participants who agreed to this study visited the M Dental Clinic in Busan one week before the start and received an oral examination by a dentist. Two dental hygienists trained under the guidance of a dentist performed light scaling to the participants for the same oral environment and provided the same toothbrush and toothpaste to them. After a recovery period of 1 week after scaling, the study started. As representative teeth for an oral examination, maxillary right first molar (#16), maxillary left central incisor (#21), maxillary left first premolar (#24), mandibular left first molar (# 36), mandibular right central incisor (#41), and mandibular right first premolar (#44) were selected and measured.

After scaling, two dental hygienists trained under the guidance of a dentist obtained the following data: before the application of mouthwash as Baseline, immediately after the application of mouthwash as Treatment, and five days after the application of mouthwash as After 5 Days. Participants were provided with labeled items so that they could not tell whether they were in the experimental group or the control group. They were also educated on how to use mouthwash and how often and how to brush teeth using the provided toothpaste and toothbrush. For five days, the experimental group held 15 ml of mouthwash containing *Sambucus williamsii var. coreana* extract for 30 seconds and then spitting it out before going to bed after brushing, while the control group did the same with 15ml of saline solution. After gargling, intake of food including water was prohibited. O'Leary index, plaque index (PI), and gingival index (GI), and microbiological analysis were evaluated as indicators related to periodontal disease.

## 4.1. O’Leary index

We conducted O'Leary, Drake, and Naylor’s dental plaque test (O'Leary index) [21], where all teeth in the oral cavity were discolored with a dental surface discoloration agent and estimated the status of adherence (%) using the plaque control score (O'Leary index). 1 point scored if the dental plaque adheres to the dental surfaces of 4 teeth (mesial, efferent, facies, and lingual), and score 0 if not.

## 4.2. PI

Using the PI technique of Loe and Silness [22], dividing the tooth surface into two parts; the gingival margin and the tooth surface. Then, measuring plaque accumulation and its thickness with a red colorant. The evaluation criteria are as follows; 0 = no plaque; 1 = thin plaque attached to the gingival margin and visible when rubbing gently with a probe or applying a tooth colorant; 2 = moderate plaque visible along the gingival margin; 3 = thick plaque collection in gingival margin and tooth surface as well as in the gingival pockets. PI is the average quantity of plaque per measured tooth surface and its score for each tooth was calculated using the average value.

## 4.3. GI

Gingival health status at the proximal, distal, buccal, and lingual regions was evaluated using the GI technique [23]. Each section was assigned from score 0 to 3: 0 = normal gingiva; 1 = a slight color change and swelling of gingivitis showing no bleeding with a mild stimulus; 2 = red and swollen gingivitis showing bleeding with a mild stimulus; 3 = remarkably red and swollen due to advanced inflammation showing the possibility of ulceration and natural bleeding. The values for each tooth were added up to calculate the individual's total mean GI.

# 4.4. Microbiological analysis

To obtain subgingival microbiome samples from periodontal pockets, sterile #15 paper points were placed in the gingival sulcus at 4 sites: 2 maxillary teeth (#16 and #21) and 2 mandibular teeth (#36 and #41) with pocket depth (PD) less than 4 mm for 10s. They were then placed in sterile 1.5 mL tubes and frozen at -20°C until right before an analysis. DNA was dragged from collected #15 paper points using the AccuPrep Universal RNA Extraction Kit (Bioneer, Daejeon, Korea) following the manufacturer's instructions. As shown in Table 1, we used three oligonucleotides (forward primer, reverse primer, probe) and OligoMix (YD Global Life Science Co., Ltd., Seongnam, Korea) that react particularly to each bacterium [24]. Also, to prepare reaction samples of polymerase chain reaction (PCR), we combined the following: 9 μL of OligoMix, 10 μL of 2x probe qPCR mix (Takara Bio Inc., Shiga, Japan), 1 μL of template DNA. A 96-well plate with PCR reaction samples was placed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, USA) for DNA amplification. The cycle requirements of PCR were as follows: PCR initial activation step at 95°C for 30 s, denaturation at 95°C for 10 s, annealing at 62°C for 30 s with 40 times of repeated cycles. The Bio-Rad CFX Manager Software program was used to calculate the cycle threshold (Ct) parameter, and Ct values were plotted on a standard curve for each bacterium to figure out the number of copies.

Table 1
Primers and probes used in the real-time PCR assays

| Bacteria | Target genes | Primers/Probe sets | Amplicon size (bp) |
|---|---|---|---|
| *Parvimonas micra* | 16S ribosomal RNA gene | 5'-GAGGAATACCGGTGGCGAAG-3'<br>5'-GGCACCGAGATTTGACTCCC-3'<br>5'-FAM-GGTACGAAAGCGTGGGGAGCA-BHQ1–3' | 148 |
| *Staphylococcus aureus* | clumping factor A (clfA) gene | 5'-GCGCAAGTAACGAAAGCAAAA-3'<br>5'-GATTTTGCGCCACACTCGTT-3'<br>5'-FAM-TGCTGCACCTAAAACAGACGACACA-BHQ1–3' | 132 |
| *Eubacterium nodatum* | hypothetical protein | 5'-TGCTTGCCGGTGACTTAGGA-3'<br>5'-AAACCGGGCTCAACAACCAT-3'<br>5'-Texas Red-TTGAGGAGCCGGTGACTTTGG-BHQ2–3' | 130 |
| *Porphyromonas gingivalis* | hemagglutinin (phg) gene | 5'-ACACGGTGTATCGTGACGGC-3'<br>5'-GCCGGCTGCGTACTTAACCT-3'<br>5'-HEX-CGACCTACCGCGATGCAGGA-BHQ1–3' | 119 |
| *Treponema denticola* | oligopeptidase B (opdB) gene | 5'-AGAAAGGCTTTGGGCGACAG-3'<br>5'-GCTGGAGCCGTAGCTTCCAT-3'<br>5'-Cy5-CGGGTCCTCACCCGCTCTTC-BHQ2–3' | 127 |
| *Fusobacterium nucleatum* | 16S ribosomal RNA gene | 5'-GGCTGTCGTCAGCTCGTGTC-3'<br>5'-CTCATCGCAGGCAGTATCGC-3'<br>5'-FAM-AACGAGCGCAACCCCTTTCG-BHQ1–3' | 114 |
| *Prevotella intermedia* | hemagglutinin (phg) gene | 5'-CACACGCTGGCGAAACCTAC-3'<br>5'-CACGTGGCGTTGCTTCTTTC-3'<br>5'-HEX-CCGAAGATGCGCCGTTGAAC-BHQ1–3' | 143 |

| Bacteria | Target genes | Primers/Probe sets | Amplicon size (bp) |
|---|---|---|---|
| *Prevotella nigrescens* | gyrase subunit B (gyrB) gene | 5'-AGCAAGCTGTAGGCGAGGCT-3’<br>5'-GCTGAACACTTTCGCGTGCT-3’<br>5'-Texas Red-GCTCGTATTGCAGCCCGCAA-BHQ2–3’ | 132 |
| *Eikenella corrodens* | proline iminopeptidase (pip) gene | 5'-GCCAACTGCTGCTGGAAGTG-3’<br>5'-GCCGCTGATTTCGGAGAGTT-3’<br>5'-HEX- ACAGCCATCGGCACAGGCAT-BHQ1–3’ | 110 |
| *Campylobacter rectus* | groEL gene | 5'-AAATTTAAGCGGCGACGAGG-3’<br>5'-TCCTTGCTCACGCTTACGGA-3’<br>5'-HEX-GGCTTTGACGCGGGCGTAGT-BHQ1–3’ | 132 |

# 5. Statistical analysis

An analysis of frequency was conducted on the demographic characteristics of the participants in this study. SPSS 24.0 for Windows (IBM Corp., Armonk, NY, USA) was used to verify the significance of the measured O'Leary index, PI, GI, and subgingival plaques between the saline solution gargle group and the *Sambucus williamsii var. coreana* extract gargle group performing independent t-test at level 5%. One-way ANOVA was conducted on Baseline, Treatment, and After 5 Days, which are changes according to the application time of gargling. Tukey's test was analyzed as a posteriori test.

## Results

# Population characteristics

Table 2 shows the general features of the study subjects. The control group consisted of 19 females and 13 males, and the experimental group consisted of 20 females and 12 males, so there was no significant difference in gender distribution ($p > 0.05$). The control group was 36.13 ± 16.48 years old while the study group was 38.06 ± 17.68 years old, so there was no significant difference between the two groups concerning the average age of the subjects ($p > 0.05$). Also, there was no significant difference between the two groups in systemic disease and marriage ($p > 0.05$).

Table 2
Characteristics of the subjects in the saline solution group and *Sambucus williamsii var. coreana* group

| Characteristics | | N (%) | | |
|---|---|---|---|---|
| | | Saline solution group | *Sambucus williamsii var. coreana group* | *p*-value |
| *Gender | Male | 13 (40.6) | 12 (37.5) | 0.424 |
| | Female | 19 (59.4) | 20 (62.5) | |
| Age (mean ± SD) | | 36.13 ± 16.48 | 38.06 ± 17.68 | 0.647 |
| *Systemic disease | No disease | 29 (90.6) | 29 (90.6) | 0.634 |
| | Have a disease | 3 (9.4) | 3 (9.4) | |
| *Marriage | Single | 17 (53.1) | 17 (53.1) | 0.497 |
| | Married | 15 (46.9) | 15 (46.9) | |

*p*-values are determined by independent t- test, **p*-values are determined by chi-square test, ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

# Clinical indicators related to periodontal disease

Table 3 shows the measured results for O'Leary index, PI, and GI of the saline solution gargle group and the *Sambucus williamsii var. coreana* extract gargle group. There was no difference concerning the Baseline ($p > 0.05$), but there was a significant difference between the two groups concerning Treatment and After 5 Days ($p < 0.05$). When comparing the two groups concerning periodontal disease-related indexes, such as O'Leary index, PI, and GI, the application of mouthwash containing *Sambucus williamsii var. coreana* extract resulted in a lower value, indicating that the clinical improvement of the oral environment. In the case of the saline solution gargle group, all indicators in Baseline, Treatment, and After 5 days did not show a significant difference ($p > 0.05$). On the other hand, in the *Sambucus williamsii var. coreana* extract gargle group, all clinical indicators showed significantly lower values as the application time increased ($p < 0.05$).

Table 3
Clinical outcomes observed between two groups

| Variables | Group | Mean ± SD | | | |
|---|---|---|---|---|---|
| | | Baseline | Treatment | After 5days | *p-value |
| O'Leary index | Saline solution | 50.82 ± 8.85[a] | 48.50 ± 9.62[a] | 40.29 ± 8.23[a] | 0.099 |
| | *Sambucus williamsii var. coreana* | 50.50 ± 9.50[a] | 23.00 ± 7.86[b] | 17.10 ± 5.22[b] | **0.000** |
| | †p-value | 0.947 | **0.000** | **0.000** | |
| Plaque index (PI) | Saline solution | 1.72 ± 0.41[a] | 1.61 ± 0.37[a] | 1.46 ± 0.34[a] | 0.120 |
| | *Sambucus williamsii var. coreana* | 1.93 ± 0.33[a] | 0.67 ± 0.21[b] | 0.44 ± 0.13[b] | **0.000** |
| | †p-value | 0.586 | **0.000** | **0.000** | |
| Gingival index (GI) | Saline solution | 0.01 ± 0.21[a] | 1.01 ± 0.16[a] | 0.96 ± 0.10[a] | 0.083 |
| | *Sambucus williamsii var. coreana* | 1.01 ± 0.22[a] | 0.61 ± 0.15[b] | 0.32 ± 0.15[c] | **0.000** |
| | †p-value | 0.163 | **0.000** | **0.000** | |

†*p*-values are determined by independent t- test, * *p*-values are determined by one-way ANOVA and Duncan tests ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

# Gram-positive bacteria in subgingival plaques

Gram-positive bacterial data in subgingival plaques was detected as three types of bacteria (Table 4): *P. micra, Staphylococcus aureus (S. aureus), and Eubacterium nodatum (E. nodatum)*. When comparing the saline solution gargle group and the *Sambucus williamsii var. coreana* extract gargle group against *P. micra*, there was a significant difference only in the mandible at the time of Treatment and After 5days ($p < 0.05$). There was a significant difference in After 5days in the maxilla ($p < 0.05$) regarding *S. aureus*, and it was not detected in both groups in the mandible. There was no significant difference between the two groups for *E. nodatum* in the oral cavity ($p > 0.05$). Also, based on the Baseline, there was a significant difference from Treatment to After 5days in the mandible of the *Sambucus williamsii coreana var.* extract

gargle group in the case of *P. micra* ($p < 0.05$). As for *E. nodatum*, there was a significant difference in both groups in the maxilla ($p < 0.05$).

Table 4
Gram-positive bacterial measurements in subgingival plaque (3 types)

| Variables | | Group | Mean ± SD | | | |
|---|---|---|---|---|---|---|
| | | | Baseline | Treatment | After 5days | *p-value |
| *Parvimonas micra* | Maxilla | Saline solution | 921.43 ± 654.03[a] | 879.34 ± 1752.96[a] | 112.88 ± 104.13[a] | 0.331 |
| | | *Sambucus williamsii* *var. coreana* | 957.03 ± 1666.78[a] | 88.30 ± 116.49[a] | 93.12 ± 56.66[a] | 0.159 |
| | | †p-value | 0.957 | 0.222 | 0.674 | |
| | Mandible | Saline solution | 1708.80 ± 2011.47[a] | 1646.82 ± 1007.24[a] | 1291.93 ± 371.47[a] | 0.824 |
| | | *Sambucus williamsii* *var. coreana* | 1832.78 ± 1689.04[a] | 29.38 ± 26.88[b] | 357.00 ± 336.00[b] | **0.007** |
| | | †p-value | 0.901 | **0.000** | **0.000** | |
| *Staphylococcus aureus* | Maxilla | Saline solution | 18.63 ± 21.43[a] | 18.13 ± 20.99[a] | 11.13 ± 13.91[a] | 0.697 |
| | | *Sambucus williamsii* *var. coreana* | 22.50 ± 24.29[a] | 16.30 ± 19.58[a] | 0.00 ± 0.00[a] | 0.065 |
| | | †p-value | 0.745 | 0.862 | **0.037** | |
| | Mandible | Saline solution | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | - |
| | | *Sambucus williamsii* *var. coreana* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | **-** |
| | | †p-value | - | - | - | |
| *Eubacterium nodatum* | Maxilla | Saline solution | 50.27 ± 50.97[a] | 13.70 ± 19.27[a,b] | 9.37 ± 14.24[b] | **0.032** |

* *p*-values are determined by one-way ANOVA and Duncan tests ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

| | | | | | |
|---|---|---|---|---|---|
| | *Sambucus williamsii var. coreana* | 25.60 ± 30.15[a] | 0.67 ± 0.80[a,b] | 5.80 ± 7.87[b] | **0.022** |
| | *p*-value | 0.260 | 0.074 | 0.551 | |
| Mandible | Saline Solution | 9.21 ± 18.87[a] | 68.20 ± 104.05[a] | 4.72 ± 4.20[a] | 0.770 |
| | *Sambucus williamsii var. coreana* | 9.50 ± 8.64[a] | 3.30 ± 2.22[a] | 3.12 ± 3.81[a] | 0.055 |
| | *p*-value | 0.970 | 0.178 | 0.478 | |

* *p*-values are determined by one-way ANOVA and Duncan tests ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

# Gram-negative bacteria in subgingival plaques

The following seven types of bacteria were detected as gram-negative bacteria in the oral cavity (Table 5): *P. gingivalis*, *T. denticola*, *F. nocleatum*, *P. intermedia*, *P. nigrescens*, *Eikenella corrodens (E. corrodens)*, and *Campylobacter rectus (C. rectus).* When comparing the saline solution gargle group and the *Sambucus williamsii var. coreana* extract gargle group, *P. gingivalis* showed a significant difference in Treatment and After 5days in maxilla and mandible ($p < 0.05$). *T. denticola* was detected only in the mandible, and there was a significant difference between the two groups at the time of Treatment and After 5days ($p < 0.05$). *F. nocleatum* differed between groups only in the maxilla in After 5days ($p < 0.05$). As for *P. intermedia*, there was a significant difference between the groups in Treatment and After 5days only in the mandible ($p < 0.05$). In the case of *P. nigrescens*, there was a significant difference between the two groups in After 5days in both the maxilla and the mandible ($p < 0.05$). *E. corrodens* was not significantly different between the two groups in both the maxilla and the mandible. *C. rectus* showed a difference between the two groups in Treatment and After 5days in the case of the maxilla, but showed a significant difference only in Treatment in the mandible ($p < 0.05$). The change according to the application time was not significantly different in the saline solution gargle group from Baseline to After 5 days except for *F. nocleatum* in the maxilla and *E. corrodens* in the mandible, among the 7 types of gram-negative bacteria ($p > 0.05$). On the other hand, in the *Sambucus williamsii var. coreana* extract gargle group, all bacteria in the maxilla and mandible were significantly reduced according to the application time, except for *F. nocleatum* in the mandible, *P. intermedia*, and *P. nigrescens* of the maxilla. In particular, based on Baseline, *P. gingivalis*, and *C. rectus* showed a marked difference from Treatment to After 5 days in both the maxilla and mandible, verifying that gram-negative bacteria were definitely reduced. From Treatment to After 5 days, *T. denticola, P. intermedia*, and *E. corrodens* were clearly reduced in the mandible only, and *T. denticola* was not even detected in Treatment and After 5 days.

Table 5
Gram-negative bacterial measurements in subgingival plaque (7 types)

| Variables | | Group | Mean ± SD | | | |
|---|---|---|---|---|---|---|
| | | | Baseline | Treatment | After 5days | *p-value |
| *Porphyromonas gingivalis* | Maxilla | Saline Solution | 109.30 ± 133.68[a] | 47.67 ± 24.08[a] | 50.93 ± 38.28[a] | 0.262 |
| | | *Sambucus williamsii var. coreana* | 57.67 ± 53.35[a] | 7.93 ± 10.45[b] | 6.15 ± 6.89[b] | **0.005** |
| | | †p-value | 0.352 | **0.000** | **0.004** | |
| | Mandible | Saline Solution | 171.10 ± 140.72[a] | 206.47 ± 146.23[a] | 238.55 ± 151.38[a] | 0.681 |
| | | *Sambucus williamsii var. coreana* | 168.53 ± 122.87[a] | 10.33 ± 6.26[b] | 10.60 ± 7.12[b] | **0.000** |
| | | †p-value | 0.970 | **0.001** | **0.000** | |
| *Treponema denticola* | Maxilla | Saline Solution | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | - |
| | | *Sambucus williamsii var. coreana* | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | - |
| | | †p-value | - | - | - | |
| | Mandible | Saline Solution | 10.50 ± 12.29[a] | 6.40 ± 7.22[a] | 8.07 ± 9.04[a] | 0.716 |
| | | *Sambucus williamsii var. coreana* | 11.43 ± 10.62[a] | 0.00 ± 0.00[b] | 0.00 ± 0.00[b] | **0.003** |
| | | †p-value | 0.876 | **0.022** | **0.021** | |

* *p*-values are determined by one-way ANOVA and Duncan tests ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

| | | | | | | |
|---|---|---|---|---|---|---|
| *Fusobacterium nocleatum* | Maxilla | Saline Solution | 412231.93 ± 237792.45[a] | 316335.28 ± 249760.27[a] | 33533.67 ± 24271.80[b] | **0.004** |
| | | *Sambucus williamsii var. coreana* | 333965.80 ± 322845.65[a] | 104002.86 ± 144013.44[a,b] | 11462.04 ± 4562.88[b] | **0.016** |
| | | *p*-value | 0.608 | 0.069 | **0.038** | |
| | Mandible | Saline Solution | 279433.90 ± 210103.94[a] | 273512.70 ± 212041.39[a] | 158732.78 ± 126279.75[a] | 0.409 |
| | | *Sambucus williamsii var. coreana* | 265363.90 ± 202138.62[a] | 129837.11 ± 145893.87[a] | 95250.00 ± 92088.18[a] | 0.110 |
| | | *p*-value | 0.896 | 0.139 | 0.315 | |
| *Prevotella intermedia* | Maxilla | Saline Solution | 390.59 ± 4783.59[a] | 3941.66 ± 6515.62[a] | 471.63 ± 625.49[a] | 0.300 |
| | | *Sambucus williamsii var. coreana* | 40268.10 ± 61402.20[a] | 1720.47 ± 3149.98[a] | 173.54 ± 208.53[a] | 0.057 |
| | | *p*-value | 0.117 | 0.406 | 0.248 | |
| | Mandible | Saline Solution | 774.43 ± 617.20[a] | 413.86 ± 362.89[a] | 275.15 ± 269.36[a] | 0.108 |
| | | *Sambucus williamsii var. coreana* | 818.38 ± 528.18[a] | 81.40 ± 114.22[b] | 54.37 ± 50.66[b] | **0.000** |
| | | *p*-value | 0.886 | **0.027** | **0.049** | |
| *Prevotella nigrescens* | Maxilla | Saline Solution | 1021.38 ± 909.20[a] | 754.78 ± 342.72[a] | 801.04 ± 279.63[a] | 0.679 |

* *p*-values are determined by one-way ANOVA and Duncan tests ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

| | | | | | | |
|---|---|---|---|---|---|---|
| | | *Sambucus williamsii var. coreana* | 1093.63 ± 1158.02[a] | 1064.70 ± 1542.44[a] | 293.89 ± 275.80[a] | 0.338 |
| | | *p*-value | 0.901 | 0.596 | **0.002** | |
| | Mandible | Saline Solution | 971.58 ± 788.81[a] | 851.69 ± 924.14[a] | 500.36 ± 232.09[a] | 0.482 |
| | | *Sambucus williamsii var. coreana* | 959.30 ± 969.86[a] | 219.78 ± 187.43[a,b] | 97.29 ± 98.77[b] | **0.025** |
| | | *p*-value | 0.980 | 0.088 | **0.000** | |
| *Eikenella corrodens* | Maxilla | Saline Solution | 24.73 ± 19.52[a] | 21.40 ± 16.45[a] | 17.96 ± 15.61[a] | 0.759 |
| | | *Sambucus williamsii var. coreana* | 53.80 ± 52.30[a] | 20.40 ± 21.15[a,b] | 8.30 ± 10.74[b] | **0.033** |
| | | *p*-value | 0.162 | 0.919 | 0.186 | |
| | Mandible | Saline Solution | 184.89 ± 179.89[a] | 68.20 ± 104.05[a,b] | 7.73 ± 10.71[b] | **0.019** |
| | | *Sambucus williamsii var. coreana* | 197.11 ± 255.86[a] | 23.40 ± 35.70[b] | 15.56 ± 15.59[b] | **0.040** |
| | | *p*-value | 0.919 | 0.272 | 0.280 | |
| *Campylobacter rectus* | Maxilla | Saline Solution | 71.23 ± 74.59[a] | 57.40 ± 56.93[a] | 29.40 ± 31.02[a] | 0.356 |
| | | *Sambucus williamsii* | 64.97 ± 60.09[a] | 4.00 ± 4.55[b] | 2.78 ± 2.67[b] | **0.001** |
| | | *p*-value | 0.860 | **0.016** | **0.026** | |

* *p*-values are determined by one-way ANOVA and Duncan tests ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

| | | | | | |
|---|---|---|---|---|---|
| Mandible | Saline Solution | 167.83 ± 57.83[a] | 115.70 ± 140.49[a] | 111.97 ± 137.50[a] | 0.693 |
| | *Sambucus williamsii* | 189.97 ± 191.03[a] | 6.22 ± 8.97[b] | 38.40 ± 73.79[b] | **0.010** |
| | *p*-value | 0.826 | **0.042** | 0.210 | |

* *p*-values are determined by one-way ANOVA and Duncan tests ($p < 0.05$).; Values are means ± standard deviations.; significant (bold).

# Discussion

About 700 types of microorganisms live in the oral cavity. Because the supply of nutrients necessary for the growth of microorganisms is continuous, the proliferated bacteria form a dental plaque, which is the mechanism that causes bacterial oral diseases [25]. Therefore, the best way to prevent this is to remove the dental plaque, representatively using Tetracyclines, Metrodinidazole, Penicillin, Clindamycin, and Ciprofloxacin out of the various types of antibacterial agents [26]. Chemical antibacterial agents inhibit bone loss and are effective for periodontitis [27]. But their use is limited due to side effects such as the generation of resistant bacteria and mycosis [28].

Therefore, this study verified O'Leary index, PI, and GI as periodontal-related indicators and antibacterial effects on related pathogens that cause periodontal diseases to develop a natural mouthwash using *Sambucus williamsii var. coreana* extract, which has a natural antibiotic effect preventing periodontal disease. As a result of this study, O'Leary index, PI, and GI all showed a marked decrease from Treatment to After 5 days compared to the saline solution gargle group when applying mouthwash containing *Sambucus williamsii var. coreana* extract. Also, as the application time of the mouthwash containing *Sambucus williamsii var. coreana* extract increased, the values of periodontal-related indicators were effectively lowered, verifying that there was an effect of improving the oral environment. According to a previous study, *Sambucus williamsii var. coreana* extract had antioxidant and anti-inflammatory effects and had no cytotoxicity [29]. Therefore, it is considered appropriate to apply it in the oral cavity as a mouthwash to suppress and prevent occurring periodontal disease.

*P. micra*, which is known as a bacterium associated with periodontal disease, is a gram-positive, non-motile, anaerobic coccus [30] that is more common in patients with severe or moderate periodontitis than those with gingivitis and mild periodontitis [31]. In this study, a mouthwash containing *Sambucus williamsii var. coreana* extract was effective in the mandible. The effect appeared immediately after application, and the antibacterial effect showed up over time until After 5 days. *P. micra* is known to act as a core pathogen in developing the pathogenesis of the periodontal disease [32], and signaling molecules derived from *P. micra* stimulate the expression of the *P. gingivalis* proteolytic gingipain enzyme thirteen times more to enhance the growth and toxicity of Porphyromonas gingivalis, a major periodontal pathogen [33]. *P. gingivalis*, a gram-negative anaerobic bacterium, is associated with periodontal

destruction and increased risk of disease recurrence [34] and is the foremost periopathogen for periodontal disease progression [35]. After applying the mouthwash containing *Sambucus williamsii var. coreana* extract to *P. gingivalis*, it decreased sharply in the maxilla and the mandible compared to the saline solution gargle group. The mouthwash was verified to be very effective against *P. gingivalis* as it decreased continuously from the immediate application. Therefore, a mouthwash using *Sambucus williamsii var. coreana* extract can inhibit and block the growth of core pathogens of periodontal disease. *P. gingivalis* is also considered the major pathogen because of its ability to change the normal microflora into a dysbiotic community [36]. Among dysbiotic species, the increase in *P. gingivalis*, a major periodontal disease-related pathogen, is accompanied by various bacterial species including *P. intermedia* and *F. nucleatum* [37]. Since the antibacterial effect was shown by marked reduction of *P. gingivalis* in this study, the use of mouthwash containing *Sambucus williamsii var. coreana* extract will play a huge role in reducing various anaerobic bacteria. *P. intermedia*, one of the anaerobic black bacteria, is known to be one of the powerful causative agents of adult-type periodontitis and various types of periodontal disease [38]. In addition, since the expression frequency of *P. nigrenscens* was reported to be more than double that of *P. intermedia* in the inflammatory site of patients with periodontal disease [39], dealing with *P. nigrenscens* is also necessary. Comparing with the saline solution gargle group, *P. intermedia* was effectively reduced in the mandible immediately after applying a mouthwash containing *Sambucus williamsii var. coreana* extract. There was no effect on *P. nigrenscens* immediately after using a mouthwash containing *Sambucus williamsii var. coreana* extract, but it was found to be effective in both maxilla and mandible after using the mouthwash for 5 days. Regarding the change according to the application time, both *P. intermedia* and *P. nigrenscens* in the mandible were more effectively reduced over time by a mouthwash containing *Sambucus williamsii var. coreana* extract. As a result, a mouthwash containing *Sambucus williamsii var. coreana* extract reduced *P. gingivalis* immediately after application. *P. nigrenscens* and *P. intermedia* also decreased as the application time increased. *F. nocleatum* is one of the oral microflora and also a causative agent of periodontitis. It is also related to preterm birth, colorectal cancer, inflammatory bowel disease, respiratory tract infections, cardiovascular disease, rheumatoid arthritis, and Alzheimer's disease [40]. *T. denticola*, a bacterium that causes progressive periodontitis, was reported that be associated with loss of attached gingiva and bleeding when the depth of the periodontal pocket and the periodontal pocket were measured [3]. In the results of this study, *F. nocleatum* was reduced compared to the saline solution gargle group in After 5 days in the maxilla upon using a mouthwash containing *Sambucus williamsii var. coreana* extract. On the other hand, in the case of *T. denticola*, the mouthwash containing *Sambucus williamsii var. coreana* extract was found to be effective until After 5 days as the bacteria completely disappeared from Treatment in the mandible. In addition, *E. corrodens* is a bacterium that causes acute periodontitis and promotes complex infection. *C. rectus* is known as a bacterium involved in root canal infection and periodontal disease [41]. Both bacteria appear in patients with severe periodontal disease. When comparing the saline solution gargle group and the *Sambucus williamsii coreana var.* extract gargle group, there was no significant difference between the two groups regarding *E. corrodens*. In the case of the saline solution gargle group, there was a significant difference in the mandible in After 5 days when the application time increased. It means that if the application time increases, the reduction of acute periodontitis-causing bacteria can

occur even with a saline solution. The number of *C. rectus* was reduced in *Sambucus williamsii var. coreana* extract gargle group showing the antibacterial effect. In both the maxilla and the mandible, there were continuous differences immediately after applying a mouthwash containing *Sambucus williamsii var. coreana* extract. Based on these oral clinical results, *Sambucus williamsii var. coreana* extract was found to be an excellent natural substance with antibacterial effects for improving periodontal disease.

As periodontal disease progresses, bacterial species diversify [42], so continuous management of anaerobic bacteria related to periodontal disease is required. Based on the results of the study, a mouthwash containing *Sambucus williamsii var. coreana* extract reduced bacteria in the oral cavity such as *P. gingivalis, C. rectus, T. denticola, P. intermedia, and E. corrodens* immediately after application. As the application time of a mouthwash containing *Sambucus williamsii var. coreana* extract extended, the more marked antibacterial effect appeared, which means it is excellent in improving periodontal disease. According to the results of this study, mouthwash was more effective in the mandible. It seems that the teeth and periodontal tissues of the mandible were in contact with and stored more in the mouthwash when keeping the mouthwash in the mouth. Therefore, to maximize the effect of mouthwash, it will be more effective to gargle mouthwash enough to cover the entire oral cavity rather than simply holding it.

Based on this study verifying the practicality and development of oral health care products using *Sambucus williamsii var. coreana* extract, a mouthwash containing *Sambucus williamsii var. coreana* extract can inhibit and prevent periodontal disease. As a natural ingredient with sufficiently excellent effects, *Sambucus williamsii var. coreana* extract can be used for oral health by improving periodontal disease.

# Conclusion

A mouthwash containing *Sambucus williamsii var. coreana* extract affects oral hygiene by reducing the O'Leary index, PI, and GI. It also has a potential advantage in inflammation control by reducing periodontal disease-causing bacteria, so alternative oral care products as the adjuvant treatment of periodontal disease can be possible. In addition, medicine using *Sambucus williamsii var. coreana* extract rather than chemicals can be a promising field for treating gingivitis.

# List Of Abbreviations

Plaque index, PI; gingival index, GI; *Parvimonas micra, P. micra; Staphylococcus aureus, S. aureus; Eubacterium nodatum, E. nodatum; Parvimonas micra, P. gingivalis; Treponema denticola, T. denticola; Fusobacterium nucleatum, F. nucleatum; Prevotella intermedia, P. intermedia; Prevotella nigrescens, P. nigrescens; Eikenella corrodens, E. corrodens; Campylobacter rectus, C. rectus.*

# Declarations

i. Ethics approval and consent to participate

The study was approved by Institutional Review Board of Silla University (1041449-202008-HR-001, Busan, Korea). Informed consent was obtained from all individual participants included in the study. All methods have been carried out in accordance with the relevant guidelines and regulations.

ii. Consent for publication

Not applicable.

iii. Availability of data and material

The data sets generated and/or analyzed during the current study are not publicly available for reasons of personal and organizational integrity but are available from the corresponding author on reasonable request.

iv. Competing interests

The authors declare that they have no competing interests.

v. Funding

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vi. Authors' contributions

Y.R Kim and S.H Nam participated in experiments, data analysis, and interpretation of the results. All authors read and approved the final manuscript.

vii. Acknowledgements

Not applicable.

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# Figures

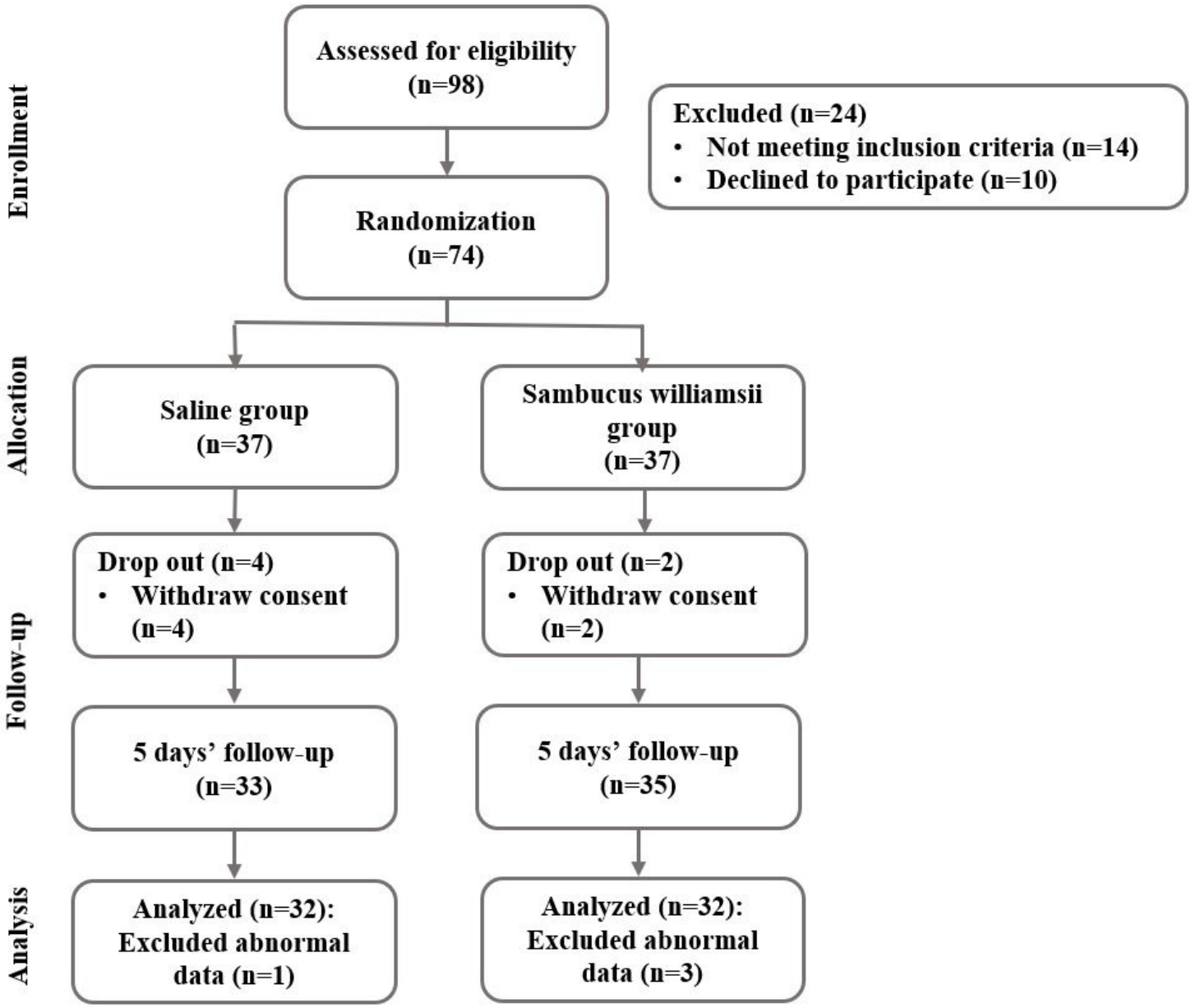


**Figure 1**

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