Synergistic Effect of the Ethanol Extract of Alismatis rhizoma against Oral Pathogens

Keywords: Alismatis rhizoma; Antibacterial activity; Oral pathogen bacteria; Synergistic effect; Minimum inhibitory concentrations (MICs); Minimum bactericidal concentrations (MBCs)

Abstract

Background: Alismatis rhizoma or Alisma orientale (Zexie in Chinese), the dried rhizome of Alisma orientale Juzepzuk (Alismataceae), is known to have diuretic and damp-heat clearing actions and has been used for the treatment of dysuria, edema, urinary tract infections, the retention of fluid and phlegm, and vertigo in Asia and Europe.

Methods: In this study, the combination effect of the ethanol extract of Alismatis rhizoma (ARE) was evaluated against oral bacteria, either alone or with antibiotics, via broth dilution method and checkerboard and time kill assay.

Results: In these results, MIC/MBC values for ARE against all the tested bacteria ranged between 313-5000/625-5000 microg/mL for ampicillin 0.25-32/0.5-64 microg/mL and for gentamicin 4-32/8-64 microg/mL respectively. Furthermore, the MIC and MBC were reduced to one half-twelve as a result of the combination of ARE with antibiotics. 1-2 hours of treatment with 1/2 MIC of ARE 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.

Conclusions: These results suggest that the ARE is important in the antibacterial actions of oral pathogen agents.

Abbreviations

ARE: The Ethanol Extract of Alismatis rhizoma; MICs: Minimum Inhibitory Concentrations; MBCs: Minimum Bactericidal Concentrations; CFU: Colony Forming Unit; FIC index: Fractional Inhibitory Concentration index; FBC index: Fractional Bactericidal Concentration index

Introduction

Oral diseases are major health problems with dental caries and periodontal diseases among the most important preventable global infectious diseases [1,2]. Oral health influences the general quality of life and poor oral health is linked to chronic conditions and systemic diseases [3]. The association between oral diseases and the oral microbiota is well established [4]. The development of dental caries involves acidogenic and acid uric 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 [5,6]. In contrast, periodontal diseases are sub gingival conditions that have been linked to anaerobic gram-negative bacteria such as Porphyromonas gingivalis, Actinobacillus sp, Prevotella sp, and Fusobacterium sp. [7,8]. Antibiotics such as ampicillin, chlorhexidine, erythromycin, penicillin, tetracycline, and vanco- mycin have been very effective in preventing dental caries [9]. Of the selected putative periodontal species, strains of Prevotella intermedia, Fusobacterium nucleatum and to the first time, Tannerella forsythia, were β-lactamase positive, with P. intermedia being the most frequently detected enzyme positive species [10]. Sub gingival isolates of P. gingivalis, P. intermedia and F. nucleatum in a group of subjects increase in the MIC values of tetracycline [11]. This clinical observation led to studies that established metronidazole as an important antibiotic for anaerobic infection. Since then, this compound has also played an important role in treating anaerobe related infection in the oral cavity, abdomen, and female genital tract, among others [12]. Oral bacteria have been reported to show increased resistance towards common antibiotics such as penicillin, cephalosporin, erythromycin, tetracycline, and metronidazole which have been used therapeutically for the treatment of oral infection [13,14]. The increase in resistance and adverse effects has lead researchers to explore novel anti-infective herbal compounds which could be used for effective treatment of oral diseases [15,16].

Natural medicine is a precious resource of therapeutically active compounds and has increasingly attracted the attention of researchers [17]. Alismatis rhizoma or Alisma orientale (Zexie in Chinese), the dried rhizome of Alisma orientale Juzepzuk (Alismataceae), is known to have diuretic and damp-heat clearing actions and has been used for the treatment of dysuria, edema, urinary tract infections, the retention of fluid and phlegm, and vertigo in Asia and Europe [18]. Phytochemistry studies have shown that protostane triterpenes, guaiane sesquiterpenes, alisol B, polysaccharides, and kaurane diterpenes are the major chemical components [19,20], its components have been imported antbioactivities, including anti-hepatitis, anti-inflammatory, hypo-glycemic, and immunological effects [20-23]. A new triterpenoid from Alisma orientale exhibits their antibacterial effect against antibiotic resistant strains, Enterococcus faecium, Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa [23]. Many plant-derived medicines used in traditional medicinal
systems have been recorded in pharmacopeias as agents used to treat infections and a number of these have been recently investigated for their efficacy against oral microbial pathogens [24-26].

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

**Material and Methods**

**Plant material and preparation of crude plant extract**

*Alismatis rhizoma* was purchased from the herbal medicine cooperative association of Jeonbuk Province, Korea, in March 2005. The identity was confirmed by Dr. Bong-Seop Kil, College of Natural Science, and Wonkwang University. Voucher specimens (JD-JRG1) were deposited in the Herbarium of Institute of Jinan Red Ginseng. The dried and powered of *A. rhizoma* were extracted by refluxing the samples with ethanol (EtOH) for 4 h at 80 °C three times. The solvent extracts were removed under vacuum on a rotary evaporator at 40 °C. The extracts were then dissolved in 10% dimethyl sulfoxide (DMSO) for testing. All the extracts were kept at 4 °C in the dark until further use.

**Bacterial strains**

The oral bacterial strains used in this study were: cariogenic bacterial strains, *Streptococcus mutans* ATCC 25175 (American Type Culture Collection), *Streptococcus sanguinis* ATCC 10556, *Streptococcus sobrinus* ATCC 27607, *Streptococcus ratti* KCTC (Korean Collection for Type Cultures) 3294, *Streptococcus criceti* KCTC 3292, *Streptococcus parasanguinis* KCOM 1497 (Korean Collection for Oral Microbiology), *Streptococcus downei* KCOM 1165, *Streptococcus anginosus* ATCC 31412, and *Streptococcus gordonii* ATCC 10588 and periodontopathogenic bacterial strains, *Actinobacillus actinomycetemcomitans* ATCC 43717, *P梭杆菌 nucleatum* ATCC 10953, and *Porphyromonas gingivalis* ATCC 33277. Brain-Heart Infusion (BHI) broth supplemented with 1% yeast extract (Difco Laboratories, Detroit, MI) was used for cariogenic bacterial strains (facultative anaerobic bacteria). For periodontopathogenic bacterial strains (microaerophilic and obligate anaerobic bacteria), BHI broth containing hemin 1 μg/ml (Sigma, St. Louis, MO, USA) and menadione 1 μg/ml (Sigma) was used.

**Minimum inhibitory concentrations/minimum bactericidal concentrations assay**

The minimum inhibitory concentrations (MICs) were determined by broth dilution method [27], and were carried out in triplicate. The antibacterial activities were examined after incubation at 37 ℃ for 18 h (facultative anaerobic bacteria), for 24 h (microaerophilic bacteria), and for 1-2 days (obligate anaerobic bacteria) under anaerobic conditions. MICs were determined as the lowest concentration of test samples that resulted in a complete inhibition of visible growth in the broth. MICs and MBCs, defined as MICs at which, 50 and 90%, respectively of oral bacteria were inhibited, were determined. Following anaerobic incubation of MICs plates, the minimum bactericidal concentrations (MBCs) were determined on the basis of the lowest concentration of acacetin that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. Ampicillin (Sigma) and gentamicin (Sigma) were used as standard antibiotics in order to compare the sensitivity of ARE against oral bacteria.

**Checker-board dilution test**

The antibacterial effects of a combination of ARE, which exhibited the highest antimicrobial activity, and antibiotics were assessed by the checkerboard test as previously described [27,28]. The antimicrobial combinations assayed included ARE with ampicillin or gentamicin. Serial dilutions of two different antimicrobial agents were mixed in cation-supplemented Mueller-Hinton broth, which is the medium usually used for dilution antimicrobial susceptibility tests. This medium is supplemented with calcium and magnesium salts to produce correct MICs with aminoglycosides and *Pseudomonas aeruginosa* [29]. After 24-48 h of incubation at 37 ℃, 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 ARE that kills 99.9% of the test bacteria by plating out onto each appropriate agar plate. The fractional inhibitory concentration (FIC)/ fractional bactericidal concentration (FBC) index was calculated according to the equation: FIC/FBC index=FIC/FBC = (MIC/MBC of drug A in combination/MIC/MBC of drug A alone)+(MIC/MBC of drug B in combination/MIC/MBC of drug B alone). The FIC and FBC index are the sum of the FICs and FBCs of each of the drugs, which in turn is defined as the MIC and MBC of each drug when it is used in combination divided by the MIC and MBC of the drug when it is used alone. The interaction was defined as synergistic if the FIC and FBC index was less than or equal to 0.5, additive if the FIC and FBC index was greater than 0.5 and less than or equal 1.0, indifferent if the FIC and FBC index was greater than 1.0 and less than or equal to 2.0, and antagonistic if the FIC and FBC index was greater than 2.0.

**Time-kill curves**

Bactericidal activities of the drugs under study were also evaluated using time-kill curves on oral bacteria. Tubes containing Mueller-Hinton supplemented to which antibiotics had been added at concentrations of the 1/2 MIC were inoculated with a suspension of the test strain, giving a final bacterial count between 6~7×10^6 CFU/mL. The tubes were thereafter incubated at 37 ℃ in an anaerobic chamber and viable counts were performed at 0, 0.5, 1, 2, 3, 6, 9, 12, 18 and 24 h after addition of antimicrobial agents, on agar plates incubated for up to 48 h in anaerobic chamber at 37 ℃. 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 streptococc and BHI agar containing hemin and menadione for *P. gingivalis*.

**Results and Discussion**

The ARE was evaluated for their antimicrobial activities against twelve common bacterial species present in the oral cavity. The results of the antimicrobial activity showed that the ARE exhibited antimicrobial activities against cariogenic bacteria (MICs, 313 to 2500 μg/mL; MBCs, 625 to 5000 μg/mL), against periodonto pathogenic
bacteria (MICs, 2500 µg/mL; MBCs, 5000 µg/mL) and for ampicillin, either 0.25/0.5 or 32/64 µg/mL; for gentamicin, either 4/8 or 32/64 µg/mL on tested all bacteria (Table 1). The range of MIC_{50} and MIC_{90} of the ARE were from 313 to 2500 µg/mL and 313 to 2500 µg/mL, respectively. The ARE showed stronger antimicrobial activity against S. mutans, S. creceti, S. parasanguinis, and S. gordonii than another bacteria and the range of MIC_{50} and MIC_{90} were 313-625 µg/mL and 313-625 µg/mL.

Natural products are a major source of chemical diversity and have provided important therapeutic agents for many bacterial diseases [27,28,30,31]. Combinations of some herbal materials and different antibiotics might affect the inhibitory effect of these antibiotics [30,31]. The synergistic effects of the ARE alone or with antibiotics were evaluated in oral bacteria (Table 2 and Table 3). In combination with the ARE, the MIC for ampicillin was reduced ≥4-fold in cariogenic bacteria, S. mutans, S. sobrinus, S. criceti, S. parasanguinis, S. anginosus, and S. gordonii, producing a synergistic effect as defined by FICI ≤ 0.5. The MBC for ampicillin was shown synergistic effects in all cariogenic bacteria by FBCI ≤ 0.5, but not the periodontopathogenic bacteria by FBCI ≥ 0.75 (additive) (table 2). In combination with the ARE, the MIC for gentamicin was reduced

### Table 1: Antibacterial activity of the ethanol extract of Alismatis rhizoma (ARE) and antibiotics in oral bacteria.

| Samples          | ARE (µg/mL) | Antibiotic | Gentamicin (µg/mL) |
|------------------|-------------|------------|-------------------|
|                  | MIC_{50}    | MIC_{90}   | MIC/MBC (µg/mL)  |
| S. mutans ATCC25175 \(^1\) | 625/625    | 1250/2500  | 0.25/0.25         |
| S. sobrinus ATCC27607 | 625/1250   | 313/625    | 0.5/0.5           |
| S. sanguinis ATCC10556 | 1250/2500  | 313/625    | 0.5/0.25          |
| S. ratii KCTC3292 \(^4\) | 313/625    | 1250/1250  | 0.5/0.25          |
| S. parasanguinis KCOM1497 \(^3\) | 313/625    | 1250/1250  | 0.5/0.25          |
| S. downei KCOM1165 | 2500/5000  | 1250/2500  | 0.5/0.25          |
| S. anginosus ATCC31412 | 2500/5000  | 313/625    | 0.25/0.5          |
| S. gordonii ATCC10558 | 2500/5000  | 313/625    | 0.25/0.5          |
| A. actinomyceselegionitis ATCC43717 | 2500/5000  | 313/625    | 0.25/0.5          |
| F. nucleatum ATCC51190 | 2500/5000  | 313/625    | 0.25/0.5          |
| P. gingivalis ATCC33277 | 2500/5000  | 313/625    | 0.25/0.5          |

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

### Table 2: Synergistic effects of the ethanol extract of Alismatis rhizoma (ARE) with ampicillin against oral bacteria.

| Strains              | Agent   | MIC/MBC (µg/mL) | FIC/FBC | FICI/FBCI | Outcome     |
|----------------------|---------|-----------------|---------|-----------|-------------|
| S. mutans ATCC25175 \(^1\) | ARE 625/625 | 156/156        | 0.25/0.25 | 0.5/0.5 | Synergistic/Synergistic |
| S. sobrinus ATCC27607 | ARE 1250/2500 | 313/625        | 0.25/0.25 | 0.5/0.5 | Synergistic/Synergistic |
| S. sanguinis ATCC10556 | ARE 1250/2500 | 313/625        | 0.25/0.25 | 0.75/0.5 | Additive/Synergistic |
| S. ratii KCTC3292 \(^4\) | ARE 2500/5000 | 1250/1250      | 0.5/0.25  | 0.75/0.5 | Additive/Synergistic |
| S. parasanguinis KCOM1497 \(^3\) | ARE 625/1250 | 156/313        | 0.25/0.25 | 0.5/0.5 | Synergistic/Synergistic |
| S. downei KCOM1165 | ARE 1250/2500 | 313/625        | 0.25/0.25 | 0.75/0.5 | Synergistic/Additive |
| S. anginosus ATCC31412 | ARE 2500/5000 | 625/1250       | 0.25/0.25 | 0.5/0.5 | Synergistic/Synergistic |
Table 3: Synergistic effects of the ethanol extract of *Alismatis rhizoma* (ARE) with gentamicin against oral bacteria.

| Strains                  | Agent        | MIC/MBC (µg/mL) | FIC/FBC       | FICI/FBC<sup>5</sup> | Outcome               |
|--------------------------|--------------|----------------|---------------|-----------------------|-----------------------|
|                          |              | Alone          | combination<sup>a</sup> |                        |                       |
| *S. mutans* ATCC25175<sup>1</sup> | ARE          | 625/625        | 156/156       | 0.25/0.25             | 0.5/0.125             | Synergistic/ Synergistic |
|                          | Gentamicin   | 4/8            | 1/1           | 0.25/0.125            |                       |                       |
| *S. sobrinus* ATCC27607  | ARE          | 1250/2500      | 78/156        | 0.063/0.063           | 0.313/0.313           | Synergistic/ Synergistic |
|                          | Gentamicin   | 8/8            | 2/2           | 0.25/0.25             |                       |                       |
| *S. sanguinis* ATCC10556 | ARE          | 1250/2500      | 313/825       | 0.25/0.125            | 0.75/0.375            | Additive/ Synergistic  |
|                          | Gentamicin   | 32/64          | 16/16         | 0.5/0.25              |                       |                       |
| *S. rattii* KCTC3294<sup>4</sup> | ARE         | 2500/5000      | 1250/2500     | 0.5/0.5               | 1/1                   | Additive/ Additive    |
|                          | Gentamicin   | 32/32          | 16/16         | 0.5/0.5               |                       |                       |
| *S. criceti* KCTC3292    | ARE          | 313/1250       | 156/825       | 0.5/0.5               | 1/1                   | Additive/ Additive    |
|                          | Gentamicin   | 16/16          | 8/8           | 0.5/0.5               |                       |                       |
| *S. parasanguinis* KCOM1497<sup>3</sup> | ARE       | 625/1250      | 156/156       | 0.25/0.125           | 0.5/0.375             | Synergistic/ Synergistic |
|                          | Gentamicin   | 32/64          | 8/16          | 0.25/0.25             |                       |                       |
| *S. downei* KCOM1165     | ARE          | 1250/2500      | 313/825       | 0.25/0.25             | 0.5/0.5               | Synergistic/ Synergistic |
|                          | Gentamicin   | 32/64          | 8/16          | 0.25/0.25             |                       |                       |
| *S. anginosus* ATCC31412 | ARE          | 2500/5000      | 625/1250      | 0.25/0.25             | 0.5/0.5               | Synergistic/ Synergistic |
|                          | Gentamicin   | 32/64          | 4/16          | 0.25/0.25             |                       |                       |
| *S. gordonii* ATCC10558  | ARE          | 625/625        | 313/313       | 0.5/0.5               | 1/0.75                | Additive/ Additive    |
|                          | Gentamicin   | 16/32          | 8/8           | 0.5/0.25              |                       |                       |
| *A. actinomycetemcomitans* ATCC43717 | ARE  | 2500/5000    | 156/625       | 0.063/0.125           | 0.313/0.375           | Synergistic/ Synergistic |
|                          | Gentamicin   | 8/8            | 2/2           | 0.25/0.25             |                       |                       |
| *F. nucleatum* ATCC51190 | ARE          | 2500/5000      | 625/625       | 0.25/0.125            | 0.375/0.375           | Synergistic/ Synergistic |
|                          | Gentamicin   | 16/32          | 2/8           | 0.125/0.25            |                       |                       |
| *P. gingivalis* ATCC33277 | ARE         | 2500/5000      | 625/625       | 0.25/0.25             | 0.5/0.25              | Synergistic/ Synergistic |

<sup>1</sup>American Type Culture Collection (ATCC)
<sup>2</sup>Korean collection for Type Cultures (KCTC)
<sup>3</sup>Korean collection for Oral Microbiology (KCOM)
<sup>4</sup>The MIC and MBC of the ethanol extract of *Alismatis rhizoma* (ARE) with ampicillin
<sup>5</sup>The fractional inhibitory concentration (FIC) index/fractional bactericidal concentration (FBC) index

≥4-16-fold in all tested bacteria expect *S. sanguinis*, *S. rattii*, *S. cricetti*, and *S. gordonii* by FICI ≥ 0.75 and MBC in *S. rattii*, *S. cricetti*, and *S. gordonii* by FBCI ≥ 0.75 (Table 3).

Recently, interest in *A. rhizoma* is growing, because many of its components, such as triterpene, alisol B, and especially polysaccharides, have been reported to have important bioactivities, including anti-hepatitis, anti-inflammatory, hypoglycemic and immunological effects [20-22]. Four triterpenes (alisol A, alisol A monoacetate, alisol B, and alisol B monoacetate) isolated from the *A. rhizoma* have show anti-complementary and antibacterial activity against antibiotic resistant strains [20,32]. The polysaccharides of *A. rhizoma* have been documented for their effects of against lipid peroxidation, which are potentially important for human health [33]. Conventional methods, such as those by hot water, immersion...
The bacterial effect of the ARE with ampicillin or gentamicin against oral bacteria was confirmed by time-kill curve experiments. The ARE (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 ARE (1/2 MIC) with ampicillin (1/2 MIC) or gentamicin (1/2 MIC) (Figures 1 and 2). The ARE with ampicillin (1/2 MIC) combination was bactericidal effect up to and beyond 12 h exposure with all killing compared to ARE alone in S. sobrinus.

or Soxhlet, are often time-consuming and expensive, with low yield of the polysaccharides of A. rhizoma or even loss of some of their pharmacological activities [33,34].

Figure 1: Time-kill curves of MIC of ARE alone and its combination with 1/2 MIC of Amp or Gen against S. mutans, S. sanguinins, S. sobrinus, S. ratti, S. criceti, and S. parasanguninns. Bacteria were incubated with ARE (●), ARE + Amp (○), and ARE + Gen (▼) over time. Data points are the mean values±S.E.M. of six experiments. CFU, colony-forming units.
and *S. downei*. The ARE with gentamicin (1/2 MIC) combination was bactericidal effect in *S. sanguinis*, *S. downei*, *S. anginosus*, and *S. gordonii* with all killing up to 12 h exposure.

**Conclusion**

In conclusion, these findings suggest that a strong bactericidal effect of ARE was exerted in drug combinations and fulfills the
conditions required of a novel cariogenic bacteria and periodontal pathogens, particularly bacteroides species drug and may be useful in the future in the treatment of oral bacteria.

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