

Antimicrobial Activity of Ethanol Extracts of *Rosmarinus officinalis* against Oral Microorganisms

Yeon-Hee Kim¹, Jeong Hwan Kim², Hyung-Joo Jin² and Si Young Lee^{1*}

¹Department of Oral Microbiology, College of Dentistry, Research Institute of Oral Science and

²Department of Marine Molecular Biotechnology, College of Life Sciences, Gangneung-Wonju National University, Gangneung, 210-702, Korea.

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Rosmarinus officinalis is an edible evergreen shrub with fragrance native to the Mediterranean area. It is widely used for culinary and medicinal purpose around the world. In this study, the antimicrobial activity of ethanol extracts of *R. officinalis* on broad range of oral microbial species including streptococci, actinomyces, lactobacillus, candida and periodonto-pathogenic bacterial species was studied for possible usage of extracts of *R. officinalis* in dental care products for example mouth wash solutions and tooth pastes. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by a microdilution method in culture medium. The MICs of ethanol extracts of *R. officinalis* for oral streptococci were between 31.25 and 250 µg/ml and MBCs were between 31.25 and 500 µg/ml. *Actinomyces naeslundii* and *Actinomyces odontolyticus* showed MICs of 31.25 and 15.62 µg/ml, respectively, and MBC of 62.5 µg/ml. The MICs were 15.62 and 7.81 µg/ml for *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, respectively. The bactericidal activities of extracts of *R. officinalis* against susceptible bacterial species were dependent on concentration of extracts and incubation time. The treatment of bacteria with extract changed the cell surface texture of *Streptococcus mutans* and *P. gingivalis*. The data of our present study suggested that extracts of *R. officinalis* would be a useful compound for the development of antimicrobial agents against oral pathogens.

Key words: Antibacterial agents; Dental plaque; *Rosmarinus officinalis*.

The need for effective antimicrobial agents against pathogenic oral microorganisms necessitated a search for new antimicrobial agents. For this purpose, a lot of investigation on natural products as sources of new antimicrobial agents has been carried out¹. Several kinds of plant products were utilized for prevention and treatment of dental diseases^{2,3}. *Rosmarinus officinalis* an edible evergreen shrub with fragrance native to the Mediterranean area. It is widely used for culinary and medicinal purpose around the world. Previous study reported that extracts from *R.*

officinalis has antimicrobial properties against certain bacterial species such as *Escherichia coli*, *Salmonella typhi*, *Propionibacterium acnes* and several species of fungi including *Candida albicans*⁴⁻⁶.

Even though one study about antimicrobial activities of extracts of *R. officinalis* against *Streptococcus mutans* and *Streptococcus sobrinus* has been reported⁷, the study about the antimicrobial activity of extracts of *R. officinalis* against oral microorganisms is rare. In this study, the antimicrobial activity of ethanol extracts of *R. officinalis* on broad range of oral microbial species including actinomyces, lactobacillus, candida and periodontopathic bacterial species for possible usage of extracts of *R. officinalis* in dental care products for example mouth wash solutions

* To whom all correspondence should be addressed.
Tel.: +82-33-640-2455; Fax: +82-33-642-6410;
E-mail: siyoung@gwnu.ac.kr

and tooth pastes. We examined oral bacterial species which are related to oral diseases and frequently discovered from dental plaque and saliva. *S. mutans* and *S. sobrinus* are known as causative agents for dental caries and lactobacilli also play a role for progressing dental caries⁸⁻⁹. We examined some of mitis group streptococci such as *Streptococcus sanguinis* and *Streptococcus gordonii* as initial colonizers in dental plaque formation.¹⁰ Actinomyces are known to be significantly found at root caries lesion¹¹⁻¹². We also included bacterial species, such as aggregatibacter, fusobacterium and porphyromonas, which are suggested as causative agents for periodontal diseases^{13,14}. The antifungal activity of extracts of *R. officinalis* against *C. albicans* was also examined.

MATERIALS AND METHODS

Preparation of ethanol extract of *R. officinalis*

R. officinalis was purchased at a local market at Gangneung Korea in August, 2011. The powder of *R. officinalis* was made with an electric grinder and stored in sealed containers at -20°C. For each 20 mg sample, 1 ml of 100% ethanol was used to extract the ethanol-soluble fraction at room temperature for 1 day¹⁵. The ethanol extraction was repeated three times and combined. The ethanol fraction was transferred to a new tube and the extract weight was determined after completely evaporating ethanol under vacuum for 4 h at 30°C. For a stock solution of the ethanol-soluble fraction, 1 ml of ethanol was added for every 100 mg dried extract. After dissolving ethanol fraction, the solution was filtered through a 0.22 micron syringe filter before use.

Microbial Strains

S. sobrinus 6715, *S. gordonii* DL1, *S. sanguinis* ATCC 10556 and *Lactobacillus acidophilus* ATCC 4355 were laboratory strains stocked in Department of Microbiology and Immunology, College of Dentistry, Gangneung-Wonju National University. *S. mutans* KN405, *S. gordonii* KN180, *S. oralis* KN89 and *Streptococcus mitis* KN156 were isolated strains from Korean dental plaque and stocked in the same laboratory. *S. mutans* ATCC 25175, *Actinomyces naeslundii* CCUG 35333, *Actinomyces odontolyticus* ATCC 17929, *Candida albicans* KCTC 7270, *Aggregatibacter*

actinomycetemcomitans ATCC 33384, *Fusobacterium nucleatum* ATCC 23726 and *Porphyromonas gingivalis* ATCC 33277 were obtained from Korean Collection for Oral Microbiology (Chosun University, Gwangju, Korea).

Culture Conditions

Oral streptococci were grown in Brain Heart Infusion broth (BHI)(Becton, Dickinson and Company, Sparks, MD, USA) for 18 h at 37°C in aerobic condition supplemented with 5% CO₂ and actinomyces were cultured in BHI for 18 h at 37°C under an aerobic conditions (Bactron Anaerobic Chamber, Sheldon Manufacturing Inc. Cornelius, Oregon, USA) with an atmosphere of 90% N₂, 5% CO₂ and 5% H₂. Lactobacillus were grown in lactobacillus MRS medium (Becton, Dickinson and Company) for 18 h at 37°C in aerobic condition supplemented with 5% CO₂. *A. actinomycetemcomitans* was inoculated in BHI broth and *F. nucleatum* and *P. gingivalis* were cultured in prereduced trypticase soy broth (Becton, Dickinson and Company) containing 1mg/ml of yeast extract (Becton, Dickinson and Company), 5µg/ml of hemin (Sigma Chemical Co., St. Louis, MO, USA) and 1µg/ml of menadione (Sigma Chemicals Co.). These three species were incubated in an aerobic conditions (Bactron Anaerobic Chamber, Sheldon Manufacturing Inc.). Sabouraud Dextrose medium (Becton, Dickinson and Company) was used for culturing *C. albicans* and candida was incubated for 18 h at 25°C under aerobic condition. The culture media supplemented with bacteriological agar were used for solid agar plates. For plating *F. nucleatum* and *P. gingivalis*, sheep blood (5%) was added additionally to culture medium for these species.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) by Broth Dilution Method

The concentration of extracts of *R. officinalis* was 100 mg/ml. To determine the MIC of ethanol extracts of *R. officinalis*, a stock solution of extracts was prepared in microbial culture medium for each microbial species (final concentration 2 mg/ml). The MICs were determined by a microdilution method in culture medium which was modified from methods for antimicrobial susceptibility tests of Clinical and Laboratory Standards Institute (CLSI).¹⁶ Using a microbial culture in late log phase or stationary phase, a

suspension equivalent to that of the 0.5 McFarland standard (approximately 1×10^8 CFU/ml) in each microbial culture medium was prepared. The bacteria were inoculated into serially diluted extracts of *R. officinalis* solutions in 96 well round bottom microtitration plates for final concentrations of 5×10^5 CFU/ml. The final volume was 100 μ l in a microtitration plate well. The microdilution trays were incubated in the same conditions described in bacterial culture conditions. After incubation for 18 h for aerobic bacteria and 48 h for anaerobic bacteria, MIC was determined. The microtitration plates were read visually and the minimum concentration of the extracts showing no turbidity was recorded as the MIC. MBCs of extracts were determined by spreading a 100 μ l culture medium from the wells showing no visible growth on agar plates for each bacterial species. The agar plates were incubated for 3 days for aerobic bacteria and 7 days for anaerobic bacteria. The MBC was determined as the minimum concentration of extracts that showed $\geq 99.9\%$ reduction of the original inoculums. The experiments for determining MIC and MBC were repeated at least three times.

Effects of concentrations of extracts and incubation time on killing of bacteria

For examining the effects of concentrations of extracts of *R. officinalis*, serially diluted extracts were added in wells containing bacteria and wells were incubated for 1 h. In another set of experiment, the effect of incubation time on killing of bacteria by extracts of *R. officinalis* was investigated by increasing incubation time at *R. officinalis* extracts concentration of 125 μ g/ml. Each experimental group was consisted with duplicate wells. After incubation, each sample was serially diluted with PBS and 100 μ l of diluents was spread on duplicate agar plates for each species of microorganisms. The plates were incubated for 72 h at 37°C in culture conditions for each microorganisms and the number of CFU was determined by automatic colony counter (IUL, Barcelona, Spain).

Scanning electron microscopy (SEM)

Variable Pressure Field Emission Scanning electron microscopy (VP-FE-SEM) (SUPRA55VP, Zeiss, Germany) was used to examine the morphological changes in the *S. mutans* ATCC 25175 and *P. gingivalis* ATCC 33277.

The bacteria were treated with 250 μ g/ml of extracts of *R. officinalis* for 2 h at 37°C. The bacterial suspensions after treatment were centrifuged and bacterial pellets were fixed in 2.5% glutaraldehyde in PBS (pH 7.4) for 1 h at room temperature. The fixed samples were then washed three times with PBS for 10 min and dehydrated for 30 min in a graded series of ethanol. After critical point drying, the samples were mounted on stub, coated with gold and observed with SEM.

RESULTS

The MICs of ethanol extracts of *R. officinalis* for oral streptococci were between 31.25 and 250 μ g/ml and MBCs were between 31.25 and 500 μ g/ml (Table 1). *A. naeslundii* and *A. odontolyticus* showed MICs of 31.25 and 15.62 μ g/ml, respectively and MBC of 62.5 μ g/ml. The Gram-negative oral anaerobic bacteria which are related to periodontal diseases showed lower MICs and MBCs for extracts of *R. officinalis*. The MICs were 15.62 and 7.81 μ g/ml for *F. nucleatum* and *P. gingivalis*, respectively. The MIC for *C. albicans* was 500 μ g/ml and MBC was 2,000 μ g/ml. The MICs of ethanol were also determined for checking the suppressive effects of ethanol in

Table 1. MIC and MBC of ethanol extracts of *Rosmarinus officinalis* against oral microorganisms

Microorganism	<i>R. officinalis</i>	
	MIC	MBC
<i>S. gordonii</i> DL1	125 ^a	500
<i>S. gordonii</i> KN180	125	250
<i>S. mitis</i> KN156	31.25	31.25
<i>S. mutans</i> ATCC 25175	62.5	250
<i>S. mutans</i> KN405	62.5	250
<i>S. oralis</i> KN89	250	250
<i>S. sanguinis</i> ATCC 10556	31.25	62.5
<i>S. sobrinus</i> 6715	125	500
<i>A. naeslundii</i> CCUG 35333T	31.25	62.5
<i>A. odontolyticus</i> ATCC 17929	15.62	62.5
<i>L. acidophilus</i> ATCC 4355	62.5	125
<i>A. actinomycetemcomitans</i> ATCC 33384	250	500
<i>F. nucleatum</i> ATCC 23726	15.62	31.25
<i>P. gingivalis</i> ATCC 33277	7.81	7.81
<i>C. albicans</i> KCTC 7270	500	2000

^a μ g/ml.

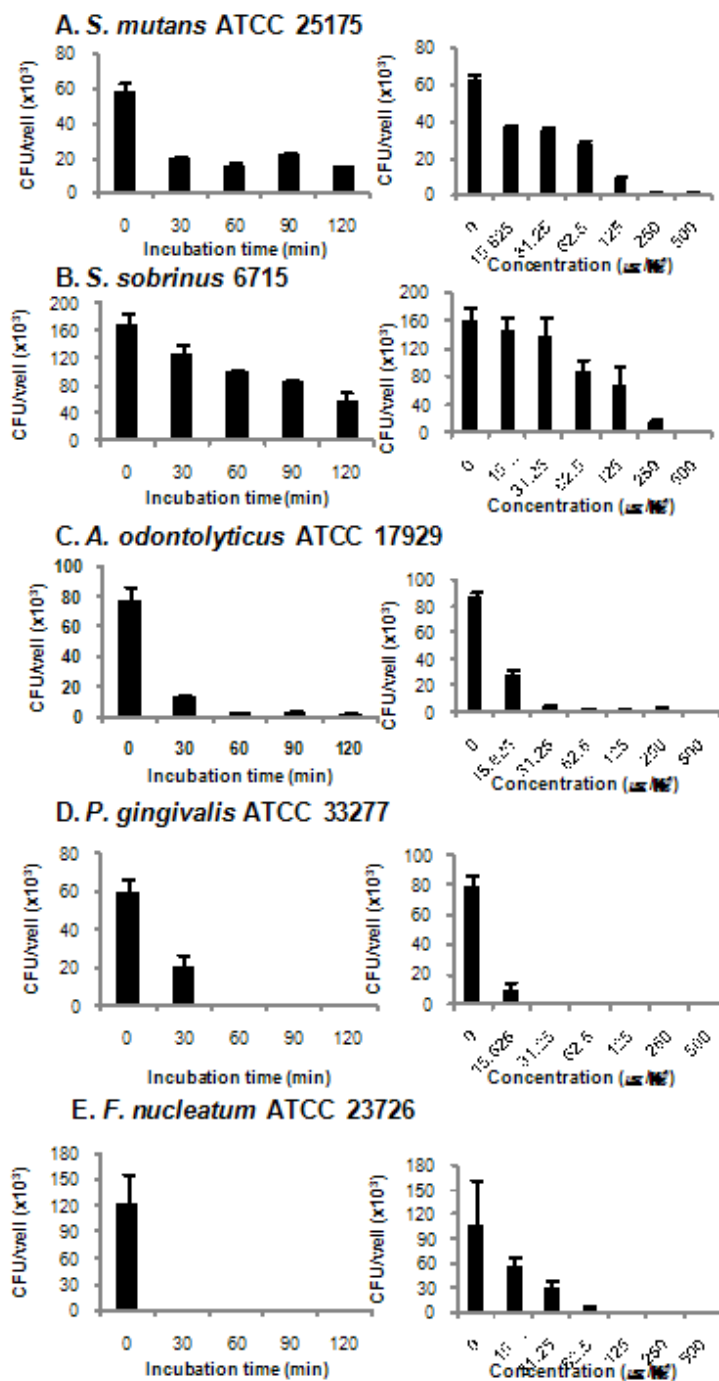


Fig. 1. Effects of concentrations of extracts and incubation time on killing of bacteria. Serially diluted extracts of *R. officinalis* were added in wells containing bacteria and wells were incubated for 1h at 37°C. The effect of incubation time on the killing of bacteria by extracts of *R. officinalis* was investigated by increasing incubation time at 125 µg/ml of final concentration of extracts. After incubation, each sample was serially diluted and diluents were spread on agar plates. The plates were incubated for 72 h and the number of CFU was determined. Values indicate means of duplicate determinations; standard deviations of the mean (error bars) are indicated by vertical lines.

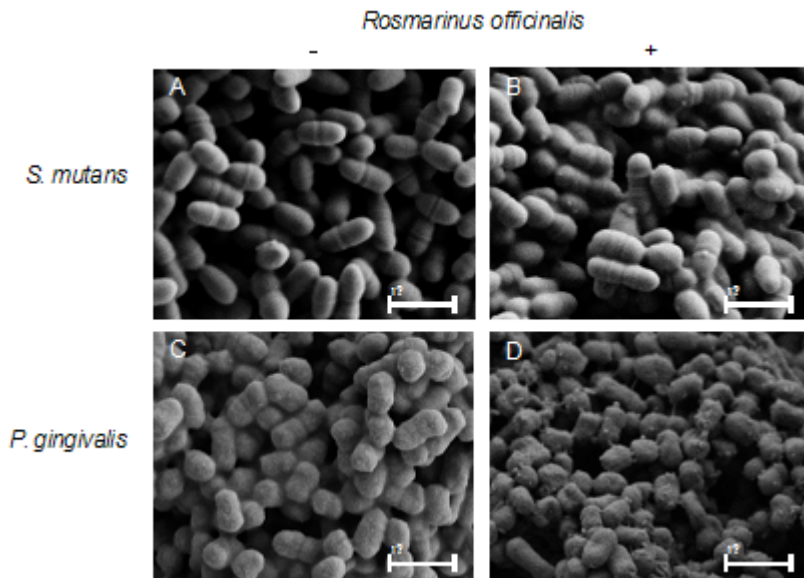


Fig. 2. Scanning electron microscopy (SEM). *S. mutans* ATCC 25175 and *P. gingivalis* ATCC 33277 were treated with 250 $\mu\text{g/ml}$ of extracts of *R. officinalis* for 2 h at 37°C. The bacterial suspensions after treatment were centrifuged and bacterial pellets were fixed in glutaraldehyde in PBS. The fixed samples were then washed and dehydrated in a graded series of ethanol. After critical point drying, the samples were mounted on stub, coated with gold and observed with SEM

extracts of *R. officinalis*. The MICs of ethanol for microbial strains used in this study were between 6.25 and 12.5% which were much higher than the concentration of ethanol (2%) in the highest concentration of extracts (2 mg/ml) used in this study.

S. mutans ATCC 25175 and *S. sobrinus* 6715 were killed by extracts of *R. officinalis* (Fig. 1A, B). At 250 and 500 $\mu\text{g/ml}$ of extract, the number of live *S. mutans* cell was reduced by 97% and 98%, respectively (Fig. 1A). Extract of 250 and 500 $\mu\text{g/ml}$ suppressed 89 and 98% of *S. sobrinus* 6715, respectively. In the experiment for examining the effect of incubation time on the killing of bacteria, the killing of *S. mutans* and *S. sobrinus* was dependent on incubation time (Fig. 1A, B). The incubation with extract for 1 h and 2 h at 125 $\mu\text{g/ml}$ of extract inhibited 74% of *S. mutans* ATCC 25175. The suppressive effects of extracts of *R. officinalis* against *A. odontolyticus*, *P. gingivalis* and *F. nucleatum* were higher than against streptococcal species (Fig. 1C,D,E). After 1 h incubation of bacteria with extracts of *R. officinalis* 125 $\mu\text{g/ml}$, almost 100% of bacteria were killed.

After *S. mutans* ATCC 25175 and *P. gingivalis* ATCC 33277 were treated with extracts of *R. officinalis* (250 $\mu\text{g/ml}$) for 2 h at 37°C, bacteria were observed with SEM (Fig. 2). The treatment of bacteria with extract changed the cell surface texture of both bacteria. Small bumps appeared on the surface of both bacteria after treatment with extract of *R. officinalis*. The surface of bacteria treated with extract was rougher than untreated bacteria and rugged in part of bacterial surface.

DISCUSSION

A number of previous studies have shown that plant extracts can inhibit formation of dental plaque.¹⁷⁻²¹ The extracts from *R. officinalis* were also utilized for preventing dental caries.^{7, 21, 22} In those studies, extracts from *R. officinalis* have antibacterial activities against oral streptococcal species including cariogenic *S. mutans* and *S. sobrinus*. It was reported that extracts from *R. officinalis* also inhibited the formation of dental plaque.²¹

In this study, it has been shown that extracts of *R. officinalis* possess antimicrobial activity against a wide variety of oral microbial species in addition to oral streptococci. Especially, the MICs and MBCs for oral anaerobic bacteria such as *F. nucleatum* and *P. gingivalis* which have been known as causative agents for inducing periodontal diseases were examined as lower than MICs for streptococci and actinomyces strains used in this study. These data might facilitate the possible usage of extracts of *R. officinalis* for suppressing the period onto pathogenic bacteria resulting in prevention of periodontal diseases. However, the further extensive study for the possible inhibitory roles of extracts of *R. officinalis* in pathogenesis of periodontal diseases should be conducted in future studies. The effects of extracts of *R. officinalis* on the virulence factors of oral pathogens, for example, bacterial adhesion on tooth surface, coaggregation with other bacteria, water-insoluble glucan formation and eventually formation of dental plaque, should be investigated in further studies. The investigation for searching effective components of extracts of *R. officinalis* for antimicrobial activity against oral pathogenic bacteria should be also conducted.

The antimicrobial activities of extracts of *R. officinalis* against a various species of bacteria have been well disclosed. The essential oil of *R. officinalis* exhibited strong antibacterial activity against *E. coli* and *P. aeruginosa*, and was also active against *Staphylococcus aureus*.²³ The antibacterial activity of rosemary essential oil against *Propionibacterium acnes* was observed with atomic force microscopy (AFM).⁵ The MIC of rosemary essential oil against *P. acnes* was 0.56 mg/ml. With increasing concentration of the essential oil, the bacterial bodies were severely damaged. With the increasing time at MIC, the bacteria length was reduced at 8 h, the width and height gradually became smaller, the shape of the cell became distorted, and finally led to cell wall damage and bacterial death at 8 h.⁵ In our examination of SEM in the present study, the reduction of bacterial width and height were not observed. However, the change of cell wall of bacteria was also observed as *P. acnes* treated with rosemary essential oil.

The oil- or water-soluble rosemary extracts have been shown to possess antibacterial activities

against gram-positive (bacillus and staphylococcus) and gram-negative (campylobacter and salmonella) bacteria.²⁴ Gram-positive bacteria were more sensitive than were gram-negative bacteria tested in their study. The other study also reported that the rosemary essential oil was found to be more active against the gram-positive pathogenic bacteria except *E. faecalis* and drug-resistant mutants of *E. coli*, compared to gram-negative bacteria.²⁵ In our study, Gram negative bacteria, *P. gingivalis* and *F. nucleatum*, showed lower MICs than those of Gram positive oral bacteria used in this study. The difference of this discrepancy might result from the difference of bacterial strains.

Antibacterial activity of the essential oil from *R. officinalis* and its major components against oral streptococci and *Enterococcus faecalis* was reported recently.⁷ It was shown that the pure major compounds were more active than the essential oil for these oral bacteria. The bioassay-guided fractionation of the leaf extract, which displayed the higher antibacterial activity than the stem extract, led to the identification of carnosic acid and carnosol as the major compounds in the fraction displaying the highest activity²². The antimicrobial and biofilm formation preventive properties of *R. officinalis* essential oils were assessed against *S. mutans* and *Streptococcus pyogenes*.²⁶ The other study also showed that the plant extracts, particularly those from *R. officinalis* L. and *S. officinalis* L., inhibited glucosyl transferase activity, glucan production and plaque formation in vitro²¹. Those data suggested that essential oils of *R. officinalis* may be considered as safe agents in the development of novel antibiofilm agents. It has been reported that extracts of *R. officinalis* also possess other biological activities in addition to antimicrobial property. *R. officinalis* essential oil exhibited the strongest cytotoxicity towards three human cancer cells²⁷. Antioxidant activity of extracts of *R. officinalis* was also shown as a free radical scavenging capacity⁴.

CONCLUSIONS

In this study, we showed that ethanol extracts of *R. officinalis* have antimicrobial activities against several oral pathogenic bacteria

such as actinomyces, porphyromonas and fusobacterium in addition to oral streptococcal species previously reported. The data of our present study suggested that extracts of *R. officinalis* would be a useful compound for the development of antimicrobial agents against oral pathogens and it has great potential for use in dental care products such as mouth wash and toothpaste for preventing and treating oral infections.

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