Inhibition of microbial adhesion to plastic surface and human buccal epithelial cells by Rhodomyrtus tomentosa leaf extract

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A B S T R A C T

Objective: The adherence of oral pathogenic microorganisms to host tissues is the initial step for successful process of oral diseases. This study aimed to determine the effect of the Rhodomyrtus tomentosa leaf extract and rhodomyrtone, an antibacterial compound from R. tomentosa leaf, on adhesion of some oral pathogens to polystyrene plastic surface and human buccal epithelial cells.

Methods: The minimum inhibitory concentration (MIC) was evaluated using broth micro-dilution method. The microbial adhesion to the plastic surface and buccal cells was determined using microtiter plate method and microscopy technique.

Results: The ethanol extract of leaf demonstrated antibacterial activity against oral microorganisms including Staphylococcus aureus ATCC 25923, Streptococcus mutans (clinical isolate), and Candida albicans ATCC 90028 with the MIC values of 31.25, 15.62, and 1000 μg/ml, respectively. Rhodomyrtone displayed activity with the MIC values of 0.78 and 0.39 μg/ml against S. aureus ATCC 25923 and S. mutans, respectively. The MIC value of the compound against C. albicans ATCC 90028 was more than 100 μg/ml which was the highest test concentration. All pathogenic microorganisms treated with the extract and rhodomyrtone at their subinhibitory concentrations resulted in a decrease in their adherence ability to both plastic surface and buccal cells.

Conclusion: It is suggested that R. tomentosa extract and rhodomyrtone may be useful in therapy or as prophylaxis in infections involving oral pathogens.

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1. Introduction

Infection is the process whereby the pathogen enters and detrimentally colonizes into its host. There are two essential steps in this process including: (1) entrance of the pathogen into the host, and (2) colonization of the pathogen within the host. The accomplishment of these two steps will lead to the result of progressive lesion. Adherence of pathogenic microorganisms to host tissues is believed to be the initial interaction between pathogens and hosts, providing the colonization and subsequent dissemination or cellular invasion. The adherence also is an important step in host cell killing and toxin delivery by the pathogens. Moreover, it is the first step for development of biofilm, a virulent structure which is found to be involved in wide variety infectious diseases and associated with antibiotic treatment failure or antibiotic resistance in many microbial infections. Therefore, the inhibition of microbial adherence to host tissues is the new ideal target that may lead to the development of new approaches for the therapy or prophylaxis in infectious diseases.

Interest in the study of inhibition and reduction of microbial adherence to various surfaces has increased tremendously in recent years. Medicinal plants have been studied by many workers as an alternative treatment for a number of infectious diseases, especially in oral diseases. A wide variety of plants and their active constituents have been reported for many biological activities which included anti-adherent properties, exclusively an inhibition on adherence ability and biofilm formation of oral pathogens. Downy rose myrtle, Rhodomyrtus tomentosa (Aiton) Hassk, is an evergreen shrub native to Southeast Asia and currently has no economic value. Our previous studies found that the leaf extract from this plant demonstrated good activity against many Gram-positive bacteria including a drugs resistance Rhodomyrtone which was isolated from the leaves of this plant displayed significant antibacterial activities against many bacterial pathogens. The recent studies from our group demonstrated anti-biofilm activity of this plant against some bacterial pathogens including, Streptococcus pyogenes, Staphylococcus spp. In order to provide more information about this potential plant, we investigated the inhibitory effect of this plant extract and its active compound on adherence ability of some oral pathogens to plastic surface and human buccal epithelial cells.

2. Materials and methods

2.1. Plant material

Classified reference voucher specimen of R. tomentosa was deposited at the Herbarium of Faculty of Traditional Thai Medicine, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The ethanol extract of leaves and rhodomyrtone were obtained from our previous studies. The extract and compound were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) before use.

2.2. Microbial strains and culture conditions

Staphylococcus aureus ATCC 25923 and Streptococcus mutans (clinical isolate) were grown onto brain heart infusion (BHI) agar (Difco, France) at 37 °C for 24 h (with 5% CO₂ for S. mutans). Candida albicans ATCC 90028 was cultured onto Sabouraud dextrose agar (SDA, Difco) at 37 °C overnight. All microorganisms were stored in BHI broth containing 20% glycerol at −80 °C until use.

2.3. Antimicrobial activities

A modified broth microdilution method according to Clinical and Laboratory Standards Institute Guidelines (CLSI, 2009) was used to determine the minimum inhibitory concentration (MIC) of the ethanol extract and rhodomyrtone. The extract and compound were dissolved in 10% DMSO and two-fold dilutions were made. Suspension of microorganisms in broth media was prepared from the overnight broth culture. The microbial suspension (180 μl) was mixed with the diluted test agents (20 μl) in 96 wells flat bottom microtiter plate (Corning Life Sciences, USA). The final microbial cell concentration was approximately 10⁵ cfu/ml. The final concentration of the extract and compound was ranging from 0.49 to 1000 and 0.049 to 100 μg/ml, respectively. 1% DMSO was used as a control. The microtiter plates were incubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration that produced a complete suppression of visible growth.

2.4. Adhesion to plastic surface

The effect of the R. tomentosa leaf extract and rhodomyrtone on microbial adhesion to plastic surface was investigated by a modified method from Rommohan et al. Briefly, the microbial suspensions (1.5 x 10⁵ cfu/ml) in their growth media were transferred to 96-well flat-bottom polystyrene microtiter plates (Costa, NY, USA) and the extract was added equal to their subinhibitory concentrations. The microtiter plate was incubated at 37 °C for 24 and 48 h. The planktonic microorganisms were removed by aspiration of the liquid media after desired incubation time points. The plates were washed twice with phosphate buffer saline (PBS) to remove non-adherent cells and then they were air-dried. The adherent cells were stained with 0.1% crystal violet (200 μl) for 30 min. The dye was removed and the plates were washed with water. After air-dried, the stained organisms were removed from the wells by adding DMSO (200 μl) and they were measured at OD 595 by microtiter plate reader. 1% DMSO was used instead of the extract as a control. In parallel experiments, unstained adherent cells and planktonic cells were mixed by vigorous vortexing, and microbial growth was quantified in a spectrophotometer at 660 nm.

2.5. Buccal cell collection and microbial suspension

Buccal epithelial cells were collected from a single healthy human volunteer subjects by gently rubbing the inside of the
cheeks with a sterile cotton swab. The cells were suspended in PBS and washed 6 times by washing-centrifugation procedure (2500 rpm for 3 min) to give buccal cell preparations virtually free of endogenous bacteria. Finally, the cells were adjusted to a density of approximately 10⁶ cell/ml by haemocytometer.

*S. aureus* ATCC 25923, and *S. mutans* were grown overnight in BHI broth at 37°C (with 5% CO₂ for *S. mutans*). *C. albicans* ATCC 90028 was cultured in Sabouraud dextrose broth (SDB) at 37°C for overnight. All microorganisms were harvested and washed three times with PBS by washing-centrifugation procedure (5000 rpm for 10 min). The bacteria were resuspended into PBS pH 7.2 and adjusted to the density of 0.5 McFarland standard (1.5 x 10⁶ cfu/ml). For *C. albicans*, the cells were adjusted to a density of 10⁶ cell/ml by haemocytometer.

2.6. Adhesion to buccal cells

The suspension of buccal cells (150 µl) was mixed with microbial suspension (300 µl) in plastic vials. The extract...
(50 μl) was added to give the final concentration at 1/2MIC, MIC, and 2MIC. 1% DMSO was used instead of the extract as a control. The vials were capped and gently rotated for 1.5 h at 37 °C. The suspensions were then centrifuged at low speed and suspended microorganisms were removed by pipetting. The washing-centrifugation procedure was repeated 6 times with PBS. Then, the suspensions were filtered through a filter paper (Whatman No. 1) and washed three times with PBS to remove loosely adherent organisms. Finally, the filter paper was placed in a upside down position on microscope slide and then the buccal cells were removed from the paper with a small drop of PBS. After heat fixing and staining with crystal violet, the adherent microorganisms were then counted by use of light microscopy. At least 100 buccal cells were evaluated for each assay. This assay was done in triplicate. Standard deviations were calculated and a Dunnett-ANOVA test was used to compare between the test and control.

2.7. Time-kill assay

Time-kill study was performed to determine the killing activity of the extract and compound on S. aureus ATCC 25923 and S. mutans at 0 and 2 h. An inoculum (10⁶ cfu/ml) of the growing cultures was added to broth media (1:10) supplemented with the extract and rhodomyrtone at concentrations of 1/2MIC, MIC, and 2MIC and incubated at 37 °C. Viable counts were determined by a drop plate method. Briefly, samples were collected at various time intervals and serially diluted tenfold. The experiment was carried out in duplicate and the results were presented as mean log numbers of organisms.

Fig. 2 – Sub-minimum inhibitory concentration (1/2- to 1/16MIC) effects of Rhodomyrtus tomentosa leaf extract (a, c and e) and rhodomyrtone (b, d and f) on microbial adherence to plastic surface, Staphylococcus aureus ATCC 25923 (a and b), Streptococcus mutans (c and d), and Candida albicans ATCC 90028 (e and f). Microbial adherence was quantified by measuring absorbance at 595 nm at 48 h. The MIC of rhodomyrtone on C. albicans ATCC 90028 was more than 100 μg/ml, the maximum tested concentration. The mean values of triplicate independent experiments and standard deviations are shown. *P < 0.05, Dunnett test shows the significant difference between the tests and the control (1%DMSO).
3. Results

3.1. Antimicrobial activities

The antimicrobial activity of *R. tomentosa* extract and rhodomyrtone on *S. aureus* ATCC 25923, *S. mutans*, and *C. albicans* ATCC 90028 are shown in Table 1. The extract demonstrated antimicrobial activity against *S. aureus* ATCC 25923, *S. mutans*, and *C. albicans* ATCC 90028 with the MIC values of 31.25, 15.62, and 1000 μg/ml, respectively. Rhodomyrtone displayed activity with the MIC values of 0.78 and 0.39 μg/ml against *S. aureus* ATCC 25923 and *S. mutans*, respectively. The MIC value of the compound against *C. albicans* ATCC 90028 was more than 100 μg/ml which was the highest test concentration.

3.2. Adhesion to plastic surface

The inhibitory effect of the *R. tomentosa* leaf extract and rhodomyrtone on microbial adhesion to plastic surface at 24 and 48 h is demonstrated in Figs. 1 and 2. All microorganisms treated with the plant extract and its active compound, rhodomyrtone, revealed a decrease in the adherence ability to plastic surface. Significant (P < 0.05) decreases in adherence of the microorganisms tested with the extract and rhodomyrtone were presented within 48 h more than 24 h. At 1/8MIC-1/2MIC of the extract after 48 h of incubation, all microorganisms revealed a significant decrease in their adherence ability. The ability of the extract to decrease the microbial adherence was higher than rhodomyrtone. At all concentration tests of both extract and compound, growth of all tested microorganisms was at the same level as that of the control (Figs. 3 and 4). This result suggested that inhibition of adherence ability to plastic surface did not result from inhibition of cell growth.

3.3. Adhesion to buccal cells

The inhibition of microbial adhesion to buccal epithelial cells by the plant extract and rhodomyrtone at 1/2MIC, MIC, and 2MIC is demonstrated in Fig. 5. Due to the MIC of rhodomyrtone on *C. albicans* ATCC 90028 was greater than 100 μg/ml (the highest tested concentration), the concentration of

![Fig. 3 – Sub-minimum inhibitory concentration (1/16- to 1/2MIC) effects of *Rhodomyrtus tomentosa* leaf extract (a, c and e) and rhodomyrtone (b, d and f) on microbial growth, *Staphylococcus aureus* ATCC 25923 (a and b), *Streptococcus mutans* (c and d), and *Candida albicans* ATCC 90028 (e and f). Microbial growth was quantified by measuring absorbance at 660 nm at 24 h. 1% DMSO was used as a control. The bacterial growth (%) mean values of triplicate independent experiments and SDs are shown. The MIC of rhodomyrtone on *C. albicans* ATCC 90028 was more than 100 μg/ml, the maximum tested concentration.](image-url)
In this study, we evaluated in vitro antimicrobial activity of the ethanolic extract of R. tomentosa leaf and its active component, rhodomyrtone, and they possessed antibacterial activity at a relatively low concentration. Especially noteworthy was the activity of rhodomyrtone against S. aureus and S. mutans with MIC values of 0.78 and 0.39 mg/ml, respectively. However, this compound did not exhibited activity against C. albicans. Differences between prokaryotic and eukaryotic cells may be responsible for this phenomenon. Previous studies found that Gram-negative bacteria are more susceptible to antimicrobial agents than Gram-positive bacteria. The differences in susceptibility might be due to differences in their cell wall composition.27,38

Adherence is required for the establishment of pathogenic microorganisms to human cells, an important first step in infection diseases. Human oral cavity consists of both hard (teeth) and soft (mucosa) tissues to which many oral pathogens may adhere. In the present study we found that the extract of R. tomentosa leaf and rhodomyrtone suppressed the adherence of oral microorganisms including S. aureus, S. mutans, and C. albicans to plastic surface (hard surface) and...
human buccal epithelial cells (soft surface). There are two possible mechanisms that may be involved: (1) inhibition of the cell growth and (2) interference with the microbial adherence process.39 Our results indicated that the extract and compound did not suppress the growth of all tested microorganisms and produce a significant killing activity, but obviously inhibit their adherence ability to both plastic surface and human buccal epithelial cells. Thus, the anti-adherence activity of the extract and compound did not result from growth inhibition or killing activity.

Our previous study found that rhodomyrtone caused changes in the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme of glycolysis pathway, in S. pyogenes.29 GAPDH is a major S. pyogenes surface protein with ability to bind to fibronectin, lysozyme, and cytoskeletal proteins.40 This fibronectin-binding protein has been involved in the adherence ability of S. aureus to fibronectin on mammalian cell surfaces.41 Therefore, GAPDH may play an important role in the initial steps of microbial mucosal colonization.29,42 The recent research proposed that the inhibitory effect of rhodomyrtone on the expression of fibronectin binding protein GAPDH may consequently affect the adherence ability of bacterial cell to mammalian cells and mucosal surfaces.29 This finding can at least partially explain the present observed anti-adherence activity of the R. tomentosa leaf extract and rhodomyrtone. However, the adherence process of microorganism to human tissues is very complicated. The process could be influenced by many factors, including microbial properties, material surface characteristics, and environmental factors.43 Therefore, further experiments are required to investigate the exact mechanism.

It is well recognized that adherence is the initial step for biofilm formation. Oral pathogenic microorganisms with an

**Fig. 5** - Inhibition of microbial adhesion to buccal epithelial cells by Rhodomyrtus tomentosa leaf extract (a) and rhodomyrtone (b). Buccal epithelial cells adherence expressed as number of microorganism/buccal epithelial cell. The data represent the mean from 100 buccal epithelial cells and standard deviations were calculated. *P < 0.05, Dunnett test shows the significant difference between the tests and the control (1% DMSO).

**Fig. 6** - Time-kill study of Staphylococcus aureus ATCC 25923 (a and b) and Streptococcus mutans (c and d) after treatment with Rhodomyrtus tomentosa leaf extract (a and c) and rhodomyrtone (b and d) at 0 and 2 h. The mean values of duplicate independent experiments and SDs are shown.
ability to form biofilms are responsible for acute and chronic infections. Examples of biofilm-associated diseases are dental caries, gingivitis, periodontitis, endocarditis, and prostatitis.

There is strong evidence indicating that the biofilm mode of life leads to increased tolerance or resistance to antimicrobial agents. Infections caused by microbial biofilms are persistent and extremely difficult to eradicate by conventional antimicrobial therapy. The higher resistance level of the cells in biofilm to antimicrobial agents is caused by a glue-like substance which acts as a diffusion barrier. It has been estimated that the embedded cells in biofilms are more than 1000-fold less susceptible to antimicrobial compounds than planktonic cells. The inhibitory effects of the ethanol extract and rhodomyrtone on staphylococcal biofilm formation and biofilm-grown cells have been reported. The ethanol extract was able to reduce biofilm formation to plastic surface more readily than rhodomyrtone. Similarly, we found that the anti-adherence activity of the extract was better than rhodomyrtone on S. mutans and C. albicans at 48 h of incubation. However, the ability of the extract and rhodomyrtone to decrease the microbial adherence to buccal epithelial cells was similar. Thus, the adherence inhibition of the extract and compound may inhibit the microbial biofilm formation and might be useful for preventing biofilm-associated diseases in oral cavity.

In conclusion, our results demonstrated that R. tomentosa leaf extract and rhodomyrtone displayed inhibitory effect on microbial adhesion against oral microorganisms including, S. aureus, S. mutans, and C. albicans. This finding suggests that the extract and compound might be a potential preventive and therapeutic agent in oral diseases.

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Conflict of interest

All listed authors have no conflict of interest to disclose.

Ethical approval

Not required.

Contributors

Authors SL, SH, SW, JS, and KM designed the study, wrote the protocol and performed the experiments. Authors SL, SC and SPV managed the literature searches and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.


