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The anthraquinone rhein exhibits synergistic antibacterial activity in association with metronidazole or natural compounds and attenuates virulence gene expression in *Porphyromonas gingivalis*

Jabrane Azelmat, Jade Fournier Larente, Daniel Grenier *

Groupe de Recherche en Écologie Buccale (GREB), Faculté de Médecine Dentaire, Université Laval, Quebec City, Quebec, Canada

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ABSTRACT

Objective: Rhein is a major anthraquinone found in rhubarb root. As a continuation of our ongoing studies aimed to identify beneficial properties of this anthraquinone for periodontal disease, in this study, we investigated the ability of rhein to (i) exhibit antibacterial synergy towards the periodontopathogen *Porphyromonas gingivalis* when used in combination with metronidazole or polyphenols belonging to different families, and (ii) attenuate virulence factor gene expression in *P. gingivalis*.

Methods: The minimal inhibitory concentrations (MIC) of compounds under investigation were determined by a broth microdilution assay. The synergistic effects of rhein in association with either metronidazole or polyphenols of various families were evaluated using the chequerboard technique to determine the fractional inhibitory concentration index (FICI). The effect of rhein on virulence factor gene expression in *P. gingivalis* was determined by quantitative RT-PCR.

Results: Rhein showed a MIC of 2.5 μg/mL, which was similar to that of metronidazole. Except for the association with epigallocatechin-3-gallate that gave an additive effect, all the other combinations (licochalcone A, glabridin, myricetin, and metronidazole) resulted in synergistic effects. The strongest synergy was observed when rhein was used in association with myricetin (FICI = 0.12) and licochalcone A (FICI = 0.19). At a sub-MIC of rhein (0.5 μg/mL), a significant decrease in the expression of *fimA*, *hagA*, and *hagB* genes, which are involved in host colonization, was observed. Moreover, the expression of *rgpA* and *kgp*, two protease genes related to inactivation of host defense mechanisms, tissue destruction, and nutrient acquisition, was also down-regulated.

Conclusion: The data presented in our study indicate that rhein possessed antibacterial activity, which can be potentiated in combination with metronidazole or other polyphenols. In addition, rhein can impair the pathogenicity of *P. gingivalis* by reducing transcription of genes coding for important virulence factors.

* *Corresponding author at*: Groupe de Recherche en Écologie Buccale (GREB), Faculté de Médecine Dentaire, Université Laval, 2420 Rue de la Terrasse, Quebec City, Quebec, Canada G1V 0A6. Tel.: +1 418 656 7341; fax: +1 418 656 2861.
E-mail address: Daniel.Grenier@greb.ulaval.ca (D. Grenier).

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1. Introduction

Periodontal diseases are common chronic inflammatory disorders and are separated into infections affecting only the gingivae (gingivitis) and those affecting the underlying structures of the periodontium, including the alveolar bone (periodontitis).[1] Periodontal diseases are complex multifactorial disorders with two distinct but interconnected etiologic components: a limited number of Gram-negative anaerobic bacteria colonizing subgingival sites, and an uncontrolled host inflammatory response to these periodontopathogens.[1] Depending of the age groups, up to 15% of the population is affected by severe forms of the disease[2] which, if left untreated, may result in tooth loss and systemic complication such as cardiovascular diseases and preterm low birth weight babies.[3] While as many as 700 different bacterial species may be present in subgingival plaque samples, strong evidence has accumulated to implicate *Porphyromonas gingivalis*, a Gram-negative anaerobic bacterium often found in association with *Tannerella forsythia* and *Treponema denticola*, as one of the key pathogens in the chronic form of periodontitis.[4,5] *P. gingivalis* produces a broad array of virulence factors (adhesins, proteases, haemolysin, etc.) involved in tissue colonization and destruction, as well as in host defense perturbation.[6–8] The lipopolysaccharide of *P. gingivalis* is also known to induce the secretion of pro-inflammatory mediators and matrix metalloproteinases (MMPs) by mucosal and immune cells.[8] Given that *P. gingivalis* plays a central role in the pathogenesis of chronic periodontitis, it is considered as a key target for preventive and therapeutic strategies directed against this disease.

In the last decade, many plant-derived molecules have been investigated with regard to their potential for preventing/controlling periodontal disease.[9] In a recent study,[10] we reported on the ability of a rhubarb root extract and its anthraquinone constituents to inhibit the growth of *P. gingivalis*. Among the anthraquinones tested, rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid) showed the highest antibacterial activity towards *P. gingivalis*. Moreover, rhein was found to reduce the proteolytic activity of the bacterium.[10] Additional properties of rhein that further support the therapeutic interest of this natural compound for periodontal disease have been reported by other groups.[11–14] More specifically, studies have shown that rhein can (i) exert anti-inflammatory activities,[11,12] (ii) enhance the synthesis of matrix components such as type II collagen and aggrecan,[13] and (iii) inhibit the activity of MMPs.[14]

As a continuation of our ongoing studies aimed to identify beneficial properties of rhein for periodontal disease, in this study, we investigated its capacity to (i) exhibit antibacterial synergy towards *P. gingivalis* when used in combination with metronidazole or polyphenols belonging to different families, and (ii) attenuate virulence factor gene expression in *P. gingivalis*.

2. Materials and methods

2.1. Bacterial strain and growth conditions

The reference strain *P. gingivalis* ATCC 33277 was used in this study. Bacteria were routinely grown in Todd Hewitt broth (BBL Microbiology Systems, Cockeysville, MA, USA) supplemented with 0.001% hemin and 0.0001% vitamin K (THB-HK) and incubated at 37 °C in an anaerobic chamber (N_2:H_2:CO_2 75:10:15).

2.2. Compounds

Rhein was purchased from Chromadex Inc. (Irvine, CA, USA) while the other compounds (licochalcone A, glabridin, myricetin, epigallocatechin-3-gallate [EGCG], and metronidazole) were obtained from Sigma–Aldrich Canada Co. (Oakville, ON, Canada). Stock solutions were prepared by dissolving 10 mg of each compound in 1 mL of ethanol.

2.3. Determination of minimal inhibitory concentrations

A 24-h culture of *P. gingivalis* was diluted in fresh THB-HK to obtain an optical density at 660 nm (OD_{660}) of 0.2. Equal volumes (100 μL) of bacteria and serial dilutions of the above compounds in culture medium were mixed into the wells of 96-well microplates. Control wells with no bacteria or no compounds were also prepared. After an incubation of 24 h at 37 °C under anaerobic conditions, bacterial growth was recorded visually. Minimal inhibitory concentration (MIC) values (μg/mL) were determined as the lowest concentration at which no bacterial growth occurred. All assays were performed in triplicate in two independent experiments to ensure reproducibility. Preliminary experiments showed that at the dilutions used, no inhibitory effects were associated with the presence of ethanol.

2.4. Synergistic interactions of rhein with metronidazole or polyphenols

The potential synergistic antibacterial effects of rhein in combination with metronidazole or various polyphenols were evaluated using the chequerboard technique.[15] Rhein was serially diluted in THB-HK (100 μL) along the ordinate of a 96-well microplate, while metronidazole or polyphenols were serially diluted in THB-HK (100 μL) along the abscissa. Cell suspensions of *P. gingivalis*, prepared in THB-HK and adjusted to an OD_{660} of 0.2, were used as inoculum (100 μL). The microplates were incubated at 37 °C for 24 h under anaerobiosis. Wells with no bacteria or compounds were included in the assay. After the incubation period, bacterial growth was assessed visually. The lowest concentration at which no growth occurred was considered the MIC. The fractional inhibitory concentration index (FICI) was calculated as follows: $FICI = FIC_A + FIC_B = (MIC_{rhein}$ in combination/MIC_{rhein} alone) + ($MIC_{compound}$ in combination/$MIC_{compound}$ alone). An FICI $\leq$0.5 was considered as indicating a synergistic effect, an FICI $>$0.5 and $\leq$1.0 as an additive effect, an FICI $>$1.0 and $\leq$4.0 as no effect, and an FICI $>$4.0 as an antagonistic effect. All assays were performed in triplicate in two independent experiments to ensure reproducibility.

2.5. Effect of rhein on virulence factor gene expression in *P. gingivalis*

The effect of rhein on the expression of several *P. gingivalis* virulence factor genes involved in host colonization (*fimA*,