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Inhibitory effects of macrocarpals on the biological activity of *Porphyromonas gingivalis* and other periodontopathic bacteria

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Background/aims: Macrocarpals, which are phloroglucinol derivatives contained in eucalyptus leaves, exhibit antimicrobial activity against a variety of bacteria including oral bacteria. This study examined effects of macrocarpals A, B, and C on periodontopathic bacteria, especially *Porphyromonas gingivalis*.
Methods: Macrocarpals A, B, and C were purified from a 60% ethanol-extract of *Eucalyptus globules* leaves. To investigate antibacterial activity, representative periodontopathic bacteria were cultured in media with or without various amounts of macrocarpals; subsequently, the optical density at 660 nm was measured. Macrocarpal inhibition of *P. gingivalis* Arg- and Lys-specific proteinases was assessed by spectrofluorophotometric assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. The effect of macrocarpals on *P. gingivalis* binding to saliva-coated hydroxyapatite beads was examined with ^{3}H-labeled *P. gingivalis.*
Results: Growth of *P. gingivalis* was inhibited more strongly than growth of *Prevotella intermedia* or *Prevotella nigrescens* and *Treponema denticola* by macrocarpals, however, *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* were much more resistant. Macrocarpals inhibited *P. gingivalis* Arg- and Lys-specific proteinases in a dose-dependent manner. The enzyme-inhibitory effect of macrocarpals was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis in which hemoglobin degradation by *P. gingivalis* proteinase was inhibited by macrocarpals. *P. gingivalis* binding to saliva-coated hydroxyapatite beads was also strongly attenuated by macrocarpals.
Conclusions: Macrocarpals A, B and C demonstrated antibacterial activity against periodontopathic bacteria. Among tested bacteria, *P. gingivalis* displayed the greatest sensitivity to macrocarpals; additionally, its trypsin-like proteinase activity and binding to saliva-coated hydroxyapatite beads were inhibited by macrocarpals. These results indicate that eucalyptus leaf extracts may be useful as a potent preventative of periodontal disease.

Key words: macrocarpal; *Porphyromonas gingivalis*; eucalyptus leaf; periodontopathic bacteria; antibacterial activity

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Eucalyptus species have been utilized for medicinal purposes and as a food source. Extracts of *Eucalyptus globules* have been shown to have antibacterial effects against a variety of bacteria (3, 20, 28–30). Cineole, which is present in *Eucalyptus* essential oil, is a major component of eucalyptus extracts; however, our preliminary experiments revealed that the antibacterial activity of *Eucalyptus* oil components against cariogenic bacteria was less than that of extracts in which oil components were excluded. Previously, we reported that nonoil materials extracted from dried leaves of *E. globules* with 60% ethanol inhibited the growth of several cariogenic bacteria, including *Streptococcus mutans and Streptococcus sobrinus,* as

well as periodontopathic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Treponema denticola* (28, 30, 32). The growth of *P. gingivalis* was particularly inhibited even at low concentrations (10 μg/ml) of the crude eucalyptus extracts. Plants of the genus *Eucalyptus* contain many kinds of phloroglucinol, e.g. macrocarpals.

Macrocarpals, which are phloroglucinol-sesquiterpene-coupled compounds extracted from eucalyptus leaves, exhibit several interesting biological properties, including antibacterial and antiviral activities (39), antagonism to thromboxane A_2 and leukotriene D_4 (38), human immunodeficiency virus-reverse transcriptase inhibition (19, 24) and aldose reductase inhibition (17). Recently, Osawa et al. (29–31) isolated macrocarpals A, B, C, D, H, I, and J as well as eucalypton from ethanol extracts of *E. globules* leaves. Macrocarpals A, B, and C are major components of the extracts, which demonstrated relatively strong antimicrobial activity against a variety of bacteria, including cariogenic bacteria such as *S. mutans and S. sobrinus*; furthermore, they inhibit glucosyltransferase produced by *S. sobrinus* (30). Moreover, macrocarpals A, B, and C exert inhibitory effects on the growth of some gram-negative bacteria, including periodontopathic bacteria (30). However, little is known regarding the effects of macrocarpals on periodontopathic bacteria and their virulence factors.

P. gingivalis, which is a major etiologic agent of periodontal disease, possesses several potential virulence factors including fimbriae, proteinase, lipopolysaccharide and hemagglutinin (6, 14). Among these factors, arginine-specific cysteine proteinase (Arg-gingipain; RGP) and lysine-specific cysteine proteinase (Lys-gingipain; KGP) appear to be essential for growth and survival of *P. gingivalis* in periodontal pockets and to play critical roles in the virulence of this bacterium (7, 9, 21–23, 25). Fimbriae are thought to function in an important capacity in bacterial adherence and colonization (5).

The present study examined the inhibitory effect of macrocarpals A, B, and C on the growth of representative periodontopathic bacteria. Moreover, the effects of macrocarpals on *P. gingivalis* virulence factors were investigated.

Material and methods

Bacterial strains and culture conditions

P. gingivalis ATCC 33277 and W50, *P. intermedia* ATCC 49046, *Prevotella nigrescens* ATCC 25261, *T. denticola* ATCC 33520, *Actinobacillus actinomycetemcomitans* ATCC 29523, and *Fusobacterium nucleatum* ATCC 23726 were grown in prereduced trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD) containing 1 mg/ml yeast extract, 5 μg/ml hemin and 1 μg/ml menadione (TSB broth) in an anaerobic system 1024 (Forma, Marietta, OH) in an atmosphere of 80% N_2-10% CO_2-10% H_2 at 35°C.

Extraction and isolation of macrocarpals

Extraction and isolation of macrocarpals A, B, and C were performed according to the method reported previously (30). Briefly, oil components were removed from fine-cut *E. globules* leaves by hydrodistillation. Dried, ground leaves of *E. globules* were extracted with 60% ethanol at 80°C for 3 h. The extract was lyophilized and dissolved in ethyl acetate. The ethyl acetate-soluble fraction was subjected to silica gel column chromatography involving a mobile phase comprising a dichloromethane–methanol mixture (1 : 0 to 2 : 1 stepwise elution). A biologically active fraction was separated by silica gel HPLC (Senshupak silica-5251-S) employing the dichloromethane–methanol mixture (17 : 3 to 49 : 1). Each fraction was subjected to further purification by ODS HPLC (Senshupak ODS-5251-SS) utilizing methanol–water (9 : 1 to 1 : 0) and acetonitrile-water (9 : 1 to 1 : 0) to obtain macrocarpals A, B, and C.

Growth assay of periodontopathic bacteria

The aforementioned periodontopathic bacteria were grown in 10 ml of TSB broth anaerobically for 48 h. Bacterial culture (200 μl) was inoculated into 10 ml of TSB broth containing 0.1, 0.5, 1, 5, 10, 50 and 100 μg/ml of macrocarpals A, B, and C; subsequently, the mixture was cultured anaerobically at 35°C. After 48 h of incubation, growth was evaluated via measurement of the optical density at 660 nm with a UV-visible spectrophotometer UV-1600 (Shimadzu Co., Kyoto, Japan); the lowest concentration at which no growth ($OD_{600nm} \leqq 0.05$) was observed was defined as the minimum inhibitory concentration. Macrocarpals A, B, and C were dissolved in dimethyl sulfoxide (DMSO) and added to TSB broth at a final concentration of 2.5% DMSO. No growth inhibition by 2.5% DMSO was observed.

Measurement of enzymatic activity

Enzymatic activities were measured via the approach described previously with slight modifications (10, 11). *P. gingivalis* ATCC 33277 and W50 were cultured in TSB broth anaerobically for 48 h, after which culture supernatants were collected by centrifugation (High Speed Refrigerated Centrifuge SRX-201; Tomy Seiko Co., Ltd, Tokyo, Japan). Supernatant (100 μl) was mixed with synthetic substrate (100 μM) in 20 mM Tris-HCl buffer (pH 7.6) supplemented with 10 mM cysteine, 100 mM NaCl, and 5 mM $CaCl_2$ in a total volume of 1 ml. Benzoyl-L-arginine 4-methylcoumaryl-7-amide and *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methylcoumaryl-7-amide, which were obtained from Peptide Institute, Inc. (Osaka, Japan), served as synthetic substrates for RGP and KGP, respectively. Following incubation at 37°C for 15 min, the reaction was terminated by the introduction of 2 mM N^{α}-p-tosyl-L-lysine chloromethyl ketone hydrochloride. The released 7-amino-4-methylcoumarin was measured at 460 nm (excitation at 380 nm) with a spectrofluorophotometer RF-5300 PC (Shimadzu). To assess the inhibitory effects of macrocarpals A, B, and C, various concentrations of macrocarpals were added simultaneously to the mixture.

Degradation of hemoglobin by culture supernatant of *P. gingivalis*

To examine whether degradation of human hemoglobin by *P. gingivalis* could be inhibited by macrocarpals, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was used to confirm the inhibitory effect on *P. gingivalis* proteinase. Hemoglobin (5 μg) was incubated with 6 μl of 24-h culture supernatant of *P. gingivalis* W50 and macrocarpals A, B, and C (50 μg/ml) in 20 mM Tris-HCl buffer (pH 7.6) supplemented with 10 mM cysteine, 100 mM NaCl and 5 mM $CaCl_2$ at 37°C for 2 h in a total volume of 30 μl. Samples were boiled in reducing sample treatment buffer (0.125 M Tris-HCl, pH 6.8, containing 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue, and 5% β-mercaptoethanol) for 10 min and analyzed by SDS-PAGE (12.5% gel) in accordance with the method of Laemmli (12). Gel was stained with Coomassie Brilliant Blue. The 24-h culture supernatant of *P. gingivalis* W50 was boiled for 10 min and functioned as a negative control.

A low-molecular-mass calibration kit (Amersham Pharmacia Biotech, Little Chalfont, UK) was used to estimate molecular masses.

Binding assay of *P. gingivalis* to saliva-coated hydroxyapatite beads

Assay of *P. gingivalis* whole-cell binding to saliva-coated hydroxyapatite (HAP) beads was conducted according to the method described previously (18). Briefly, 2 mg of spherical HAP beads (BDH Chemicals, Poole, UK) were introduced to borosilicate culture tubes (12 × 75 mm; Asahi Techno Glass Co., Tokyo, Japan), washed once with distilled water and equilibrated in 1 ml of KCl buffer (50 mM KCl, pH 6.5, containing 1 mM KH_2PO_4, 1 mM $CaCl_2$, and 0.1 mM $MgCl_2$) under gentle oscillation in a rotator (RT-50; Taitek Co., Ltd, Koshigaya, Japan) at room temperature overnight. The HAP beads were washed twice with KCl buffer, treated with 150 µl of whole saliva (1.5 mg of protein/ml) and rotated at room temperature overnight. Unstimulated human whole saliva was collected in a chilled container via expectoration of a 29-year-old female donor. Enzyme inhibitor solution (10%, v/v; KCl buffer supplemented with 2% Na_2EDTA, 10% 2-propanol and 2 mM phenylmethylsulfonyl fluoride) was added to the saliva. Following centrifugation at 12,000 × *g* at 4°C for 30 min, the clarified saliva was dialyzed against KCl buffer. Saliva-coated HAP beads were washed twice with KCl buffer. For radiolabeling, *P. gingivalis* ATCC 33277 was incubated with 5 µCi/ml of [methyl-^{3}H] thymidine (Amersham Biosciences, Tokyo, Japan). After the culture was incubated at 35°C for 2 days anaerobically, the cells were harvested by centrifugation, washed three times with KCl buffer, and suspended in the same buffer. ^{3}H-labeled *P. gingivalis* (400 µl; 4 × 10^5 dpm/2 × 10^8 cells) in KCl buffer and 100 µl of macrocarpals at various concentrations were introduced to a series of tubes containing saliva-coated HAP beads simultaneously. The mixture was incubated in the rotator at room temperature for 1 h. After incubation, the reaction mixture was layered on 1 ml of Percoll (Sigma Chemical Co., St. Louis, MO). Unbound *P. gingivalis* cells floating on the Percoll layer were removed by aspiration. HAP beads with bound *P. gingivalis* whole cells were washed once with 0.5 ml of Percoll and twice with 1 ml of KCl buffer. KCl buffer (500 µl) was added to the tube; subsequently, the beads were transferred to a vial and radioactivity was determined with a Liquid Scintillation Counter LSC-5100 (Aloka Co., Ltd, Tokyo, Japan).

Results

Antibacterial activity of macrocarpals A, B, and C against periodontopathic bacteria

Macrocarpals A, B, and C inhibited growth of tested periodontopathic bacteria, with the exception of *F. nucleatum* ATCC 23726 (Table 1). Growth of *F. nucleatum* ATCC 23726 was detected upon introduction of 100 µg/ml of macrocarpal C; however, the growth was delayed (data not shown). *P. gingivalis* displayed the greatest sensitivity to macrocarpals among tested bacteria. Macrocarpals also demonstrated strong antibacterial activity against *P. intermedia* ATCC 49046, *P. nigrescens* ATCC 25261 and *T. denticola* ATCC 33520; in contrast, macrocarpal antibacterial activity against *A. actinomycetemcomitans* was weak. Growth of *P. gingivalis* ATCC 33277 was abolished by 1 µg/ml macrocarpals A and B, and by 0.5 µg/ml macrocarpal C. *P. gingivalis* W50 exhibited greater resistance to macrocarpals but its growth was abolished by 5 µg/ml macrocarpals A and B, and by 1 µg/ml macrocarpal C. Macrocarpal C exerted the strongest inhibitory effect on *P. gingivalis* growth among the macrocarpals tested.

Inhibitory effects of macrocarpals on *P. gingivalis* proteinase

Macrocarpals A, B, and C inhibited KGP and RGP activities associated with the culture supernatant of *P. gingivalis* ATCC 33277 and W50 in a dose-dependent manner (Fig. 1). Macrocarpals (50 µg/ml) inhibited the KGP and RGP activities of *P. gingivalis* ATCC 33277 by more than

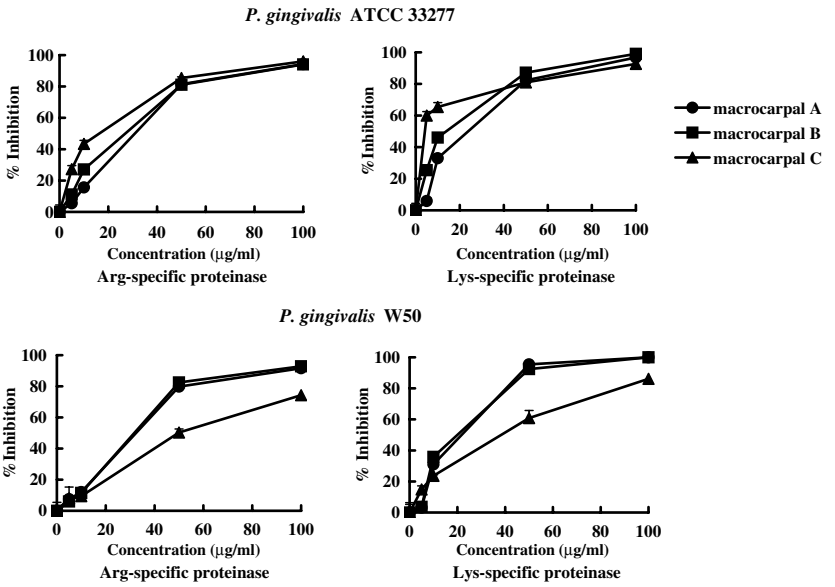

Fig. 1. Effects of macrocarpals A, B, and C on *P. gingivalis* Arg- and Lys-specific proteinases. Values are expressed as mean + SD derived from three experiments consisting of triplicate samples. Standard deviations for some mean values were small enough to be within the height of the symbols and are not evident in the figure.

Table 1. Antimicrobial activities of macrocarpals A, B, and C against periodontopathic bacteria

	MIC (µg/ml)		
Periodontopathic bacteria	macrocarpal A	macrocarpal B	macrocarpal C
Porphyromonas gingivalis ATCC 33277	1	1	0.5
Porphyromonas gingivalis W50	5	5	1
Prevotella intermedia ATCC 49046	10	10	1
Prevotella nigrescens ATCC 25261	10	10	10
Actinobacillus actinomycetemcomitans Y4	100	100	100
Fusobacterium nucleatum ATCC 23726	100	100	> 100
Treponema denticola ATCC 33520	10	10	1

80%. Macrocarpals A and B also attenuated the KGP and RGP activities of *P. gingivalis* W50 by more than 80% at 50 μg/ml, whereas macrocarpal C demonstrated approximately 50–60% inhibition at the same concentration.

Hemoglobin was degraded by *P. gingivalis* W50 proteinase excreted into the culture supernatant (Fig. 2, lane 4), and the degradation was attenuated by macrocarpals A, B, and C (lanes 5, 6, and 7, respectively). Heated *P. gingivalis* culture supernatant, which served as a negative control, demonstrated no hemoglobin degradation (lane 3).

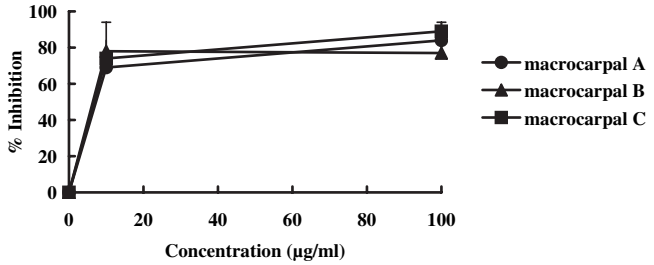

Fig. 3. Effects of macrocarpals A, B, and C on *P. gingivalis* binding to saliva-coated HAP beads. Values are expressed as mean + SD derived from three experiments consisting of triplicate samples. Standard deviations for some mean values were small enough to be within the height of the symbols and are not evident in the Figure.

Inhibitory effects of macrocarpals on *P. gingivalis* binding to saliva-coated HAP beads

Macrocarpals A, B, and C inhibited *P. gingivalis* ATCC 33277 binding to saliva-coated HAP beads to the same extent (Fig. 3). At 10 μg/ml they inhibited the binding 70–80%.

Discussion

In this investigation, macrocarpals A, B, and C abolished the growth of *P. gingivalis*, *P. intermedia*, *P. nigrescens*, and *T. denticola* at a concentration of less than 10 μg/ml. Sakanaka et al. (33) reported that green tea (*Camellia sinensis*) polyphenols, especially (–)-epigallocatechin gallate, inhibited the growth of *P. gingivalis* at concentrations of 250–500 μg/ml. Although simple comparison is difficult, *Eucalyptus* macrocarpals are more potent than green tea polyphenol in terms of antibacterial activity against *P. gingivalis.* Several studies pertaining to enzyme-inhibitory effects of polyphenols appear in the literature. Macrocarpals strongly inhibit the glucosyltransferase activity of *S. sobrinus* at a concentration of 100 μg/ml (30). Okamoto et al. (27) described inhibition of RGP and KGP activities of *P. gingivalis* by catechin derivatives. Attenuation of both eukaryotic and prokaryotic cell-derived collagenase activity (16) and protein tyrosine phosphatase in *P. intermedia* by catechin derivatives has been documented (26). The current investigation demonstrated macrocarpal inhibition of *P. gingivalis* KGP and RGP activities. RGP and KGP are considered essential for the growth and survival of *P. gingivalis* in periodontal pockets (7, 9, 21–23, 25). Thus, although the situation *in vivo* is different from that *in vitro*, inhibition of KGP and RGP activities by macrocarpals A, B, and C may be important mechanisms by which *Eucalyptus* extracts strongly attenuate growth of *P. gingivalis*.

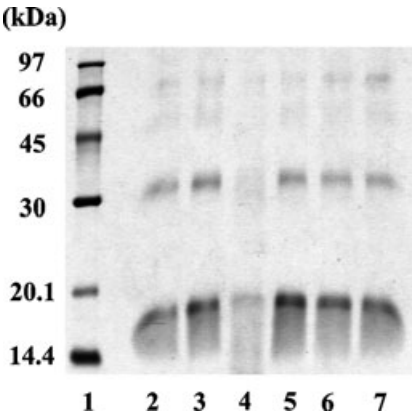

Fig. 2. Effects of macrocarpals A, B, and C on degradation of hemoglobin by *P. gingivalis.* Lane 1, molecular mass standards. Lane 2, hemoglobin control incubated in the absence of *P. gingivalis* culture supernatant. Lane 3, hemoglobin control incubated with heated *P. gingivalis* culture supernatant. Lane 4, hemoglobin incubated with *P. gingivalis* culture supernatant. Lane 5, hemoglobin incubated with *P. gingivalis* culture supernatant and macrocarpal A. Lane 6, hemoglobin incubated with *P. gingivalis* culture supernatant and macrocarpal B. Lane 7, hemoglobin incubated with *P. gingivalis* culture supernatant and macrocarpal C.

According to some reports, *P. gingivalis* binds and utilizes hemoglobin as an iron source (11, 35) and proteinase produced by the microorganism degrades hemoglobin (4, 36). These functions are considered essential for *P. gingivalis* with respect to acquisition of iron from hemoglobin. SDS-PAGE analysis revealed that macrocarpals A, B, and C inhibited degradation of hemoglobin by *P. gingivalis* proteinase, a finding that confirmed the enzyme-inhibitory effects of macrocarpals.

P. gingivalis fimbriae have been reported to play an important role in the adherence of this organism to salivary proteins (15), host epithelial cells (8) and other oral bacteria (1, 13). Among green tea polyphenols (–)-epigallocatechin gallate has been shown to inhibit adherence of *P. gingivalis* to buccal epithelial cells (33). In the present study, the inhibitory effects of macrocarpals on *P. gingivalis* binding to saliva-coated HAP beads were examined. Amano et al. (2) noted that fimbriae are responsible for *P. gingivalis* binding to saliva-coated HAP beads. *P. gingivalis* ATCC 33277 possess more fimbriae than W50 (37); as a result, *P. gingivalis* ATCC 33277 was selected for binding experiments. Macrocarpals A, B, and C inhibited *P. gingivalis* binding to saliva-coated HAP beads, which suggests that macrocarpals also attenuate *P. gingivalis* adherence. We attempted to clarify the effect of macrocarpals on *P. gingivalis* hemagglutination; however, this was not possible due to the hemagglutination activities of the macrocarpals themselves (data not shown).

Takahashi et al. (40) reported the anticaries activity in a 0.5% extract of *E. globules* in a gnotobiotic BALB/cA mouse model. Osawa et al. (31) reported that ethanol-extracts of *E. globulus* contained 2.22%, 2.29% and 3.96% macrocarpals A, B, and C, respectively. Although the concentration of the macrocarpals that might be delivered in an *in vivo* situation is unknown, Sato et al. (34) demonstrated that 0.3% eucalyptus-extract-containing chewing gum, which included macrocarpals A, B, and C, significantly inhibited plaque accumulation in comparison with control chewing gum without eucalyptus extract in human volunteers. Although the number of strains of periodontopathic bacteria tested in this investigation was limited, with respect to the following functions – antimicrobial activities against cariogenic and periodontopathic bacteria, glucosyltransferase-inhibitory and KGP- and RGP-inhibitory effects, and *P. gingivalis* binding-inhibitory activity – *E. globulus* extract containing macrocarpals has the potential to prevent both dental caries and

periodontitis. To assess the effect of *Eucalyptus* extract as a preventive substance against periodontitis, additional studies involving animal models and human volunteers are necessary.

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