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Inhibition of gingipains and *Porphyromonas gingivalis* growth and biofilm formation by prenyl flavonoids

T. Kariu[1], R. Nakao[2], T. Ikeda[3], K. Nakashima[3], J. Potempa[4,5], T. Imamura[6]

[1]Department of Life Science, Shokei University, Kumamoto, Japan, [2]Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan, [3]Division of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan, [4]Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland, [5]Department of Oral Immunology and Infectious Disease, University of Louisville, School of Dentistry, Louisville, KY, USA and [6]Department of Molecular Pathology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

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Background and Objective: Porphyromonas gingivalis is considered a major pathogen of chronic periodontitis, which also may be implicated with systemic diseases such as atherosclerosis. Secreted cysteine proteases, gingipains Rgp and Kgp, are essential for *P. gingivalis* virulence. Some polyphenols and flavonoids are known to inhibit gingipain activity and interfere with biofilm formation by *P. gingivalis*. Many bioactive compounds have been isolated from *Epimedium* species, but availability of these compounds on gingipains and *P. gingivalis* is still unclear. Therefore, the aim of this study was to evaluate natural products from medical plants to develop a new therapeutic agent against periodontal disease.

Material and Methods: Prenylated flavonoids were isolated from *Epimedium* species plant using column chromatographies. The inhibitory effect of the prenylated flavonoids against protease activity of gingipains were examined using purified gingipains and fluorogenic substrates. Anti-*P. gingivalis* activity was evaluated to analyze planktonic growth and biofilm formation in brain heart infusion medium in the presence of the prenylated flavonoids.

Results: We isolated 17 prenylated flavonoids (Limonianin, Epimedokoreanin B, etc.) from *Epimedium* species. We found that some prenylated flavonoids inhibited gingipain activity in a non-competitive manner with K_i values at μM order. The prenylated flavonoids also hindered growth and biofilm formation of *P. gingivalis*, in a manner independent of gingipain inhibition by the compounds.

Conclusion: The results indicated an inhibitory effect of the prenylated flavonoids against *P. gingivalis* and would provide useful information for future development of periodontitis treatment that suppresses gingipains, *P. gingivalis* growth and biofilm formation.

Toru Kariu, PhD, Department of Life Science, Shokei University, 2-6-78 Kuhonji, Chuo-ku, Kumamoto 862-8678, Japan
Tel: +81 96 362 2011
Fax: +81 96 363 2975
e-mail: tkariu@shokei-gakuen.ac.jp
and
Takahisa Imamura, MD, PhD, Department of Molecular Pathology, Faculty of Life Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan
Tel: +81 96 373 5306
Fax: +81 96 373 5308
e-mail: taka@kumamoto-u.ac.jp

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Porphyromonas gingivalis, a gram-negative oral anaerobe, is a major etiologic factor in the onset of adult periodontitis, a disease characterized by breakdown of the tooth supporting tissues and resultant tooth loss (1,2). Gingipains, cysteine proteases secreted by *P. gingivalis* occur in cell membrane-bound and soluble forms and are the main virulence factors of this bacterium (3,4). Based on substrate specificity, gingipains are classified into arginine-specific (Rgp) and lysine-specific (Kgp) proteases (5). Gingipains contribute to acquisi-

tion of nutrients, processing of outer membrane proteins, biofilm formation and evasion of the host defense system through proteolytic inactivation of key components of this system (3,4). Moreover, gingipains are implicated to play a role in development of systemic diseases, such as atherosclerosis (6,7). Therefore, inhibition of gingipains may impede progression of periodontitis and related systemic diseases. *P. gingivalis* forms biofilm, matrix-enclosed structure in subgingival plaque. Bacteria enclosed in the biofilm structure are highly resistant to attack by the host immune system and antibacterial agents (8,9), which enables persistent infection. Thus, inhibition of biofilm formation facilitates *P. gingivalis* elimination.

Flavonoids are natural, low molecular weight polyphenolic compounds composed of two aromatic rings (A and B) connected with three carbons that form an oxygenated heterocircle (C-ring). Some polyphenols and flavonoids are present in plant extracts and are able to inhibit gingipain activity and interfere with biofilm formation by *P. gingivalis*. For examples, a high-molecular weight fraction from the cranberry extract that contains polyphenols inhibited biofilm formation (10–12) by *P. gingivalis,* and a flavonoid catechin and its derivatives from green tea suppressed the inflammatory reaction (13,14). Both efficiently inhibited activity of gingipains. Prenylated flavonoids are widely distributed in the plant world and prenylation elevates hydrophobicity on the basic structure of the molecule, enhancing flavonoid biological functions (15,16). Prenylation may facilitate flavonoid interaction with biofilm and uptake into bacteria through the membrane, enhancing inhibitory effects of the flavonoids on *P. gingivalis* growth and biofilm formation.

Many bioactive compounds have been isolated from the *Epimedium* species, a genus of the family *Berberidaceae* (17). From a flavonoid fraction of the extract of *Epimedium* species, we purified 17 prenylated flavonoids and the derivatives. To explore an inhibitory effect of prenylated flavonoids on periodontitis, we examined these compounds for inhibition of gingipains, *P. gingivalis* growth and biofilm formation.

Material and methods

Reagents and materials

Fluorogenic substrates, *t*-butyloxycarbonyl-L-valyl-L-prolyl-L-arginine-4-methylcoumaryl-7-amide (Boc-Val-Pro-Arg-MCA) for 50 kDa Rgp (RgpB) and *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine (Boc-Val-Leu-Lys-MCA) for Kgp were purchased from the Peptide Institute (Minoh, Japan). *Epimedium* species was purchased from Uchida Wakan-yaku Co. Ltd. (lot number: C1S1504; Tokyo, Japan). Quercetin was purchased from Wako Pure Chemical Industries (Osaka, Japan). Luteolin was obtained from Cayman Chemical (Ann Arbor, MI, USA). Epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC) and epigallocatechin gallate (EGCg) were purchased from Nagara Science Corp. (Gifu, Japan). The flavonoids were defined as ≧ 98% purity by the supplier (luteorin, EC, ECg, EGC and EGCg) or high-performance liquid chromatography using Triart C18 (YMC Co. Ltd., Kyoto, Japan) (quercetin).

Extraction and isolation of flavonoids

The aerial parts of *Epimedium* species (3.0 kg) were extracted with methanol by sonication for 6 h (30 min × 12) at room temperature. The extract was concentrated with an evaporator to obtain a residue (485.0 g). The residue was partitioned between *n*-hexane and 80% methanol, and the 80% methanol layer was concentrated with an evaporator to give a residue (408.1 g), which was loaded on to a MCI gel CHP20P column (ϕ50 × 300 mm; Mitsubishi Chemical Co., Tokyo, Japan) and eluted stepwise with H_2O-methanol (1.5 L of 0%, 50% and 100% methanol solutions) to give three fractions. The second fraction (46.5 g, eluted by 50% methanol) was further applied to a MCI gel CHP20P column (ϕ50 × 300 mm) and eluted stepwise with H_2O-methanol (1.5 L of 50%, 60%, 70%, 80% and 100% methanol solutions) to give five fractions (Fr. 2-1 to 2-5). Fr. 2-3 (5.3 g, eluted with 70% methanol) and Fr. 2-4 (1.1 g, eluted with 80% methanol) were applied to a Sephadex LH-20 column (ϕ20 × 1000 mm; GE Healthcare Bioscience Co., Uppsala, Sweden), then separated with a μ-Bonda Pak C_{18} (ϕ25 × 200 mm; Waters Co., Milford, MA, USA), eluted stepwise with H_2O-methanol (135 mL of 60%, 70%, 80% methanol solutions). In these procedures, eluted solutions were divided by silica gel thin layer chromatography using pre-coated silica gel 60 F_{254} (Merck Ltd., Frankfurter, Germany) and compounds were detected by spraying with 10% H_2SO_4 followed by heating. The solutions containing prenyl flavonoids from Fr. 2-3 and Fr. 2-4 were applied to a COSMOSIL AR-II column (5 μm, ϕ10.0 × 250 mm; Nacalai Tesque Inc., Kyoto, Japan) at a flow rate 2.0 mL/min at 40°C and eluted with 70% methanol to give compounds **5** (4.5 mg) and **4** (1.5 mg), respectively. The third fraction (65.0 g, eluted by 100% methanol) was further applied to a MCI gel CHP20P column [ϕ50 × 300 mm, eluted with H_2O-methanol (1.5 L of 0%, 50%, 60%, 70%, 80%, 90%, 100% methanol solutions)] to give seven fractions (Fr. 3-1 to 3-7). Fr. 3-4 (9.0 g, eluted by 70% methanol from MCI gel) was loaded on to a Sephadex LH-20 column (ϕ20 × 1000 mm, eluted with methanol), then separated with a μ-Bonda Pak C_{18} [ϕ25 × 200 mm, eluted stepwise with H_2O-methanol (135 mL of 60%, 70%, 80% each methanol solutions)]. Eluted solutions were loaded on to a silica gel column [(ϕ10 × 100 mm, 230–400 mesh; Merck Ltd.), eluted with $CHCl_3$/methanol/H_2O = 9 : 1 : 0.1 (v/v)], and then separated with a Sunfire Prep C_{18} column (5 μm, ϕ10.0 × 250 mm; Waters Co.) by elution with 70% methanol for compounds **1** (28.9 mg) and **14** (47.3 mg), with an X-Bridge Prep C_{18} column (5 μm, ϕ10.0 × 250 mm; Waters Co.) by elution with 65% methanol for compound **17** (6.3 mg). Fr. 3-5

(10.4 g, eluted by 80% methanol from MCI gel) was subjected to a Sephadex LH-20 column (ϕ20 × 1000 mm, eluted with methanol), and then separated with a COSMOSIL $5C_{18}$ AR-II column by elution with 70% methanol for compounds **2** (3.3 mg) and **3** (28.1 mg), with an X-Bridge Prep C_{18} column by elution with 70% methanol for compound **10** (4.0 mg), with a COSMOSIL π-Nap column (5 μm, ϕ10.0 × 250 mm; Nacalai Tesque Inc.) by elution with 80% methanol for compounds **9** (1.2 mg) and **15** (17.1 mg). Fr. 3-6 (12.2 g, eluted by 90% methanol from MCI gel) was subjected to a Sephadex LH-20 column (ϕ20 × 1000 mm, eluted with methanol) and then separated with a μ-Bonda Pak C_{18} column [ϕ25 × 200 mm, eluted stepwise with H_2O-methanol (135 mL of 70%, 80%, 90% methanol solutions)]. Eluted solutions were further separated with a COSMOSIL $5C_{18}$ AR-II column by elution with 85% methanol for compounds **6** (7.7 mg), **7** (4.6 mg), **8** (33.7 mg) and **11** (4.0 mg), with a Triart PFP column (5 μm, ϕ4.6 × 150 mm; YMC Co. Ltd., Kyoto, Japan) by elution with 75% methanol for compound **13** (2.5 mg). Compounds **12** and **16** were obtained by an enzymatic hydrolysis of glucosides **2** and **5**, respectively. Compound **2** (2.0 mg) in acetate buffer (1.0 mL, pH 5.0, 100 mM) was incubated for 12 h at 37°C in the presence of β-glucosidase from Almond (5.0 mg, EC 3.2.1.21; Merck KGaK, Darmstadt, Germany). The reaction was quenched by adding methanol and the solvent was evaporated *in vacuo* to obtain a residue. The residue was separated with a silica gel column [ϕ8 × 40 mm, eluted with $CHCl_3$/methanol = 20 : 1 (v/v)] to afford compound **12** (1.0 mg). In the same manner described above, compound **16** (1.0 mg) was prepared from compound **5** (2.0 mg).

^{1}H- and ^{13}C-NMR spectra of compounds were measured with a ECA 500 NMR spectrometer (JOEL Ltd., Akishima, Tokyo, Japan). HR-ESI-MS was recorded with a JEOL JMS-T100LP spectrometer. According to the spectrum data, the chemical structure of compounds **1**–**17** were identified to Icariin, Ikariside I, Ikariside II, Ikariside A, Epimedoside C, Limonianin, 8,5′-diprenyl apigenin, Epimedokoreanin B, Neophellamuretin, 8-prenyl luteolin, Broussonol D, Anhydroicaritin, Euchrestaflavanone A, Sagittatoside A, Korepimedoside A, Desmethylicaritin, Epimedokoreanin C, respectively, using the authentic data of respective compounds. These compounds were dissolved in dimethyl sulfoxide to make a 1 mM stock solution.

Purification and activation of gingipains

RgpB and Kgp were purified from culture media of *P. gingivalis* (HG66 strain) and activated with cysteine according to the method described previously (18). The activated proteases were diluted with 0.1 M Tris-HCl (pH 7.6) buffer containing 50 mM NaCl and 5 mM $CaCl_2$ directly before assays.

Protease inhibition assay

Fifty μL of flavonoid sample in 0.1 M Tris-HCl (pH 7.6) buffer containing 50 mM NaCl and 5 mM $CaCl_2$ and 50 μL of 5 nM RgpB or Kgp were mixed in a 96-well plate. After preincubation at 37°C for 5 min, 50 μL of substrate solution (500 μM) was added to the mixture. Release of aminomethyl-coumarin was measured using a fluorescence spectrophotometer (Wallac 1420 ARVO Multilabel Counter; Perkin Elmer Inc., Waltham, MA, USA) with an excitation at 380 nm and emission at 440 nm. The linear increase of aminomethyl-coumarin release was recorded for 10 min. Residual activity in the presence of a sample was expressed as relative protease activity for the activity in the absence of the sample.

The inhibition pattern of compound **8** against gingipains was determined by the Dixon plot ($[S]/v$ vs. $[I]$), where $[I]$ is a compound **8** concentration, v is the substrate cleaving velocity and $[S]$ is the substrate concentration. K_i values were calculated by non-linear analysis using PRISM 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

P. gingivalis growth assay

P. gingivalis ATCC 33277 was grown in brain heart infusion (BHI) broth supplemented with hemin and menadione (HM) or on BHI blood agar plate with HM in an anaerobic chamber (miniMACS anaerobic workstation; Don Whitley Scientific Ltd., Shipley, UK) in 80% N_2, 10% H_2 and 10% CO_2. Influence of prenylated flavonoids on *P. gingivalis* growth was investigated by measuring the turbidity of bacterial suspension in a 96-well microplate format (3595; Corning, New York, NY, USA). Two μL of a prenylated flavonoid at various concentrations was added to *P. gingivalis* suspension standardized at 2×10^7 CFU in 200 μL of BHI-HM broth (1×10^8 CFU/mL) in the wells. Absorbance at 620 nm was measured at different times using a microplate reader (Multiskan Ascent; Thermo Electron Oy, Vantaa, Finland).

Biofilm formation assay

To examine the effect of prenylated flavonoids on biofilm formation, *P. gingivalis* was assayed using a method described previously (19) with modifications. Briefly, to produce biofilms, 2×10^7 CFU of *P. gingivalis* in 200 μL of BHI-HM broth (1×10^8 CFU/mL) was added to microtiter plate wells (3595; Corning). After the plates were anaerobically incubated at 37°C for 48 h, planktonic cells in liquid medium were discarded and the plates were washed twice with distilled water. The plates were then air-dried and attached biofilms were stained with 200 μL of 0.25% safranin for 30 min. Then, the plates were rinsed twice with distilled water to remove excess dye and air-dried. All dye associated with the attached biofilms was dissolved with 200 μL of 100% ethanol, and then absorbance at 492 nm was measured with a microplate reader (Multiskan Ascent; Thermo Electron Oy) to determine the amount of biofilm formation.

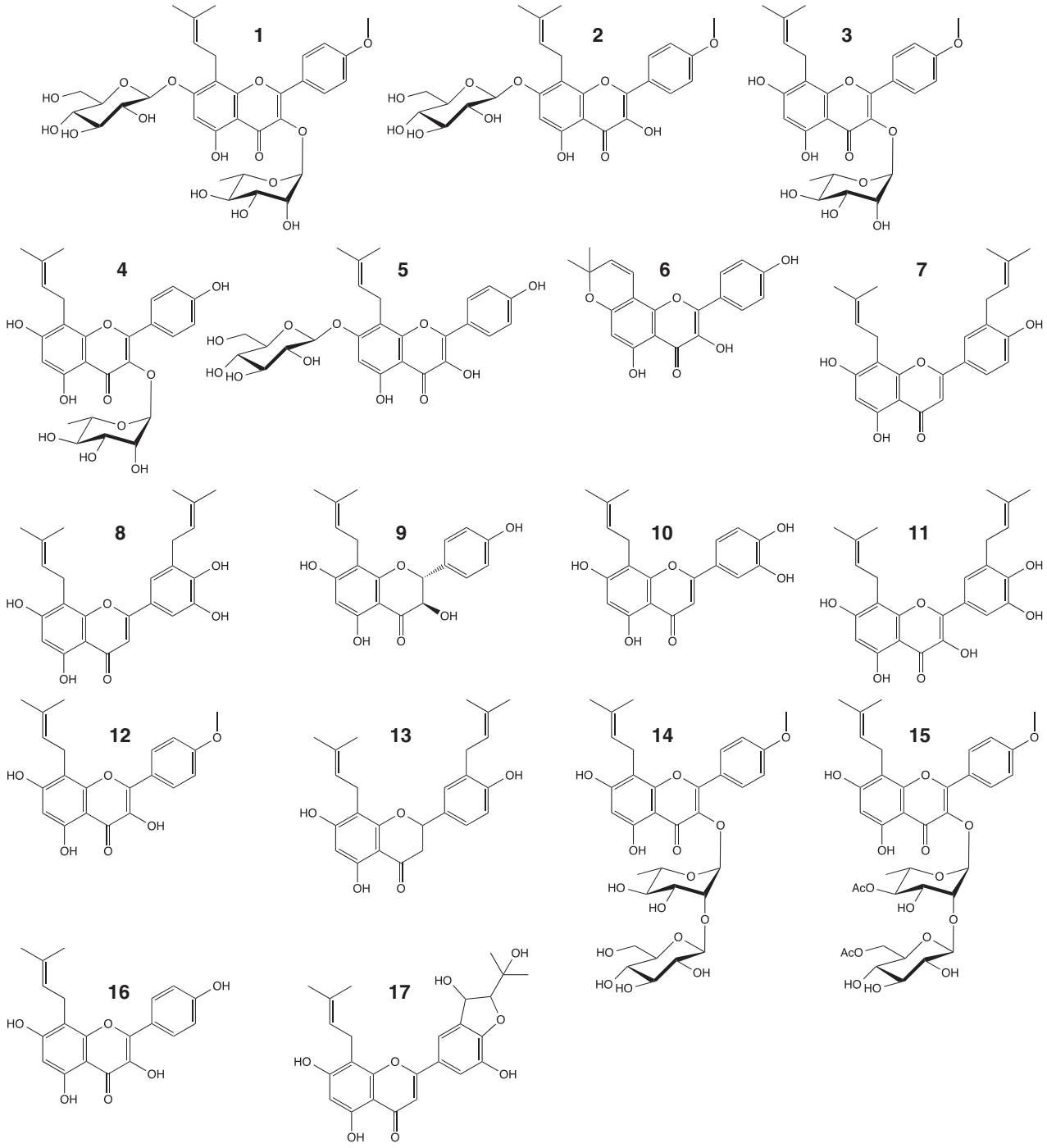

Fig. 1. Structure of flavonoids from *Epimedium* species. Compound **1**, Icariin; **2**, Icariside I; **3**, Icariside II; **4**, Icarisoside A; **5**, Epimedoside C; **6**, Limonianin; **7**, 8-5′ diprenyl apigenin; **8**, Epimedokoreanin B; **9**, Neophellamuretin; **10**, 8-prenyl luteolin; **11**, Broussonol D; **12**, Anhydroicaritin; **13**, Euchresta flavanone A; **14**, Sagittatoside A; **15**, Korepimeside A; **16**, Desmethylicaritin; **17**, Epimedokoreanin C.

Results

Inhibition of gingipains by prenylated flavonoids

From the extract of *Epimedium* species, we isolated more than 30 flavonoids, including glycosylated flavonoids (Icariin, compound **1**; Icariside I, compound **2**; etc.), non-glycosylated flavonoids (Limonianin, compound **6**; Epimedokoreanin B, compound **8**; etc.) (Fig. 1) and novel flavonoids (data not shown). All of the isolated flavonoids were prenylated. In our preliminary experiments, some of the prenylated flavonoids showed an inhibitory effect against gingipains at few to 30 μM order in a dose-dependent manner (data not shown). Screening of the known flavonoids for gingipain inhibition activity revealed that at 10 μM concentration compounds

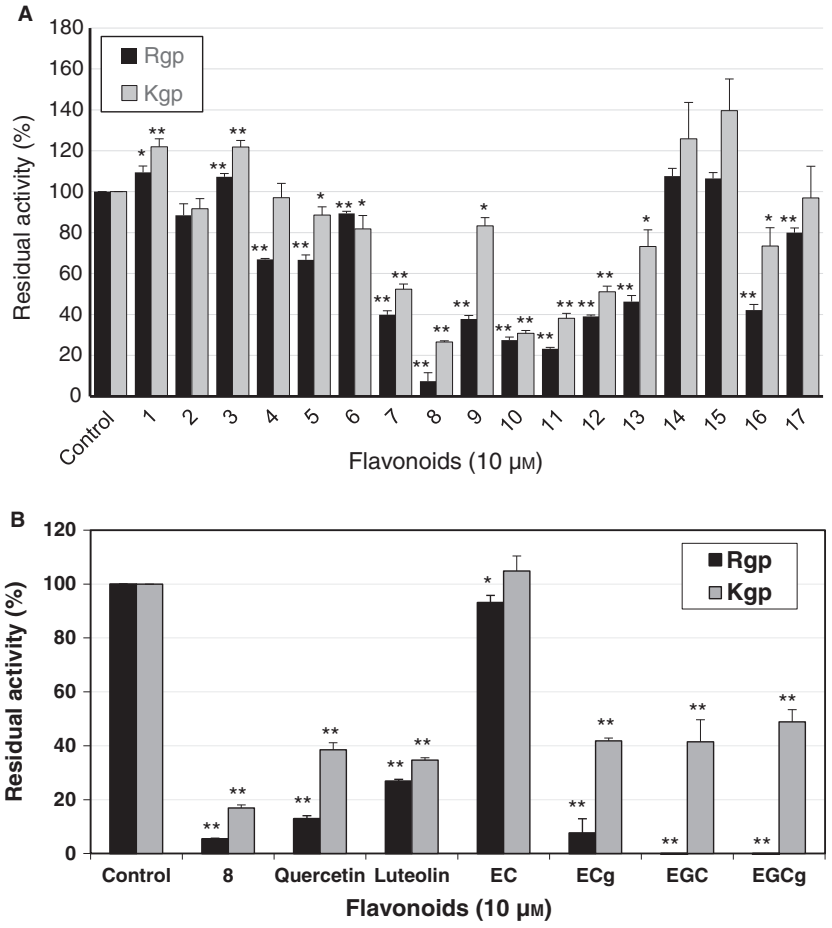

Fig. 2. Inhibition of Rgp and Kgp by prenylated flavonoids isolated from *Epimedium* species (A) and selected flavonoids (B). Rgp and Kgp (each at 1.67 nM final concentration) were preincubated with 10 μM of prenylated flavonoid and residual enzyme activity was measured. The enzyme activity in the absence of flavonoid was taken as 100%. All values are expressed as average ± standard error in triplicate assay. *$p < 0.05$ and **$p < 0.01$ significant difference of the values vs. control values. EC, epicatechin; ECg, epicatechin gallate; EGC, epigallocatechin; EGCg, epigallocatechin gallate.

8, **10** and **11** inhibited gingipains in this order of efficiency (Fig. 2A). The inhibitory activity of compound **8** against Rgp was more potent than that of quercetin, luteolin and EC ($p < 0.01$) and compound **8** was a more potent Kgp inhibitor ($p < 0.05$) than any of the unprenylated flavonoids (Fig. 2B). Compared with these unprenylated flavonoids, compound **8** was a potent inhibitor against both Rgp and Kgp.

Gingipain inhibition pattern of compound 8

To determine the mode of the gingipain inhibition by compound **8**, the kinetic of inhibitory reaction was analyzed. The plot of $[S]/v$ vs. $[I]$ intersected the x-axis, which indicates that compound **8** is a non-competitive inhibitor of the gingipains (Fig. 3A and 3B). From the x-axis intersection points the K_i values were determined as 1.67 ± 0.07 μM for Rgp and 2.71 ± 0.22 μM for Kgp (Fig. 3).

Inhibition of *P. gingivalis* growth and biofilm formation by prenylated flavonoids

Gingipains are essential for acquisition of nutritious peptides by *P. gingivalis*, which are used as a source of carbon and energy in the asaccharolytic fermentative metabolism. Thus, we have next investigated the effect of 17 prenylated flavonoids on the growth of the bacterium in BHI-HM broth medium. Compounds **6**, **7**, **8** and **13** inhibited completely *P. gingivalis* growth at 12.5 μM while compound **3** exerted a partial effect (Fig. 4A–C). Other compounds showed a negligible inhibitory effect on the *P. gingivalis* growth.

P. gingivalis exists *in vivo* in biofilms called subgingival plaques that accrete to the surface of the tooth root (20,21). Host defense systems are not effective in the elimination of *P. gingivalis* in biofilms, thus inflammatory responses to the pathogen continue to result in tissue destruction and ultimately in tooth loss (22). Because gingipains directly and indirectly (prefimbrillin processing and fimbriae assembly) participate in colonization of a subgingival plaque (23), we investigated the effect of prenylated flavonoids on biofilm formation by *P. gingivalis*. The biofilm formation was assessed in 96-well microplates in the presence or absence of compound **1**, **6** or **8** at 1.25 μM. These compounds are a representative of glycosylated prenylated flavonoid, aglycone with moderate inhibitory activity and aglycone with strong inhibitory activity against gingipain, respectively. The concentration at which these compounds do not affect the *P. gingivalis* growth was tested whether these flavonoids would inhibit biofilm formation (Fig. S1 A–C). This assay revealed that compounds **6** and **8**, but not compound **1**, significantly inhibited biofilm formation by *P. gingivalis* (Fig. 4D).

Discussion

Gingipains play critical roles in growth and biofilm formation of *P. gingivalis* thus are essential virulence factors in the periodontal disease pathology, such as tissue destruction, host-defense system dysregulation, vascular permeability induction, etc. (3,4). Therefore, inhibitors targeting these cysteine proteinases can be promising therapeutic agents for periodontitis. Prenyl flavonoids are widely distributed in the plant kingdom and some of them are well-known bioactive components of

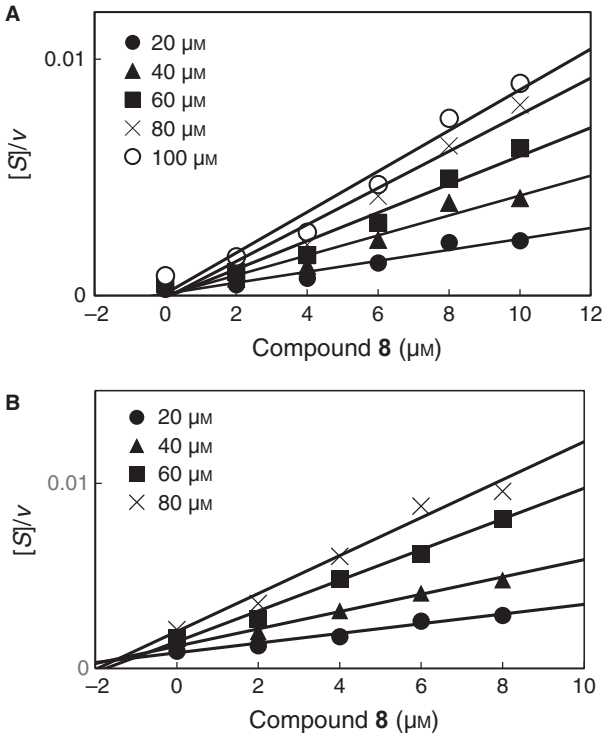

Fig. 3. Determination of the mode of inhibition of RgpB (A) and Kgp (B) by Epimedokoreanin B (compound **8**). The velocity (v) of the reaction was determined at the substrate concentration [S] in the range from 20 to 100 μM in the presence of various concentrations (0–10 μM) of Epimedokoreanin B and the ratio of [S]/v for each inhibitor concentration [I] was plotted (the Dixon plot).

medicinal plants (24,25). Prenylation enhances hydrophobicity of flavonoids and augments flavonoid bioactivity and tissue accumulation (15,26). In contrast, glycosylation increases the molecular size and polarity of flavonoids upsetting their planar structure thus negatively affecting their inhibition activity against some enzymes (27). Here, we analyzed gingipain inhibition by prenylated flavonoids (Fig. 2). The gingipain inhibition profile revealed the following structures as favorable for inhibition: (i) catechol group in the benzenoide (B-ring of flavonoid) as shown from comparison between compounds **7** and **8**; (ii) flavone (compounds **7** and **8**) rather than flavonol (compound **11**) and flavanone (compound **13**); (iii) prenylation (addition of isoprenyl group) at the C5′ carbon of the B-ring, as shown from comparison of luteolin with compounds **8** and **10**; and (iv) non-glycosylated flavonoids rather than *O*-glycosylated flavonoids as shown from comparison between compounds **4** and **16**, and of compound **12** with compounds **3**, **14** and **15**. These findings can be used to design synthesis of modified prenyl flavonoids to increase their inhibitory potency against gingipains.

Gingipain-deficient *P. gingivalis* that lacks the protease genes *rgpA*, *rgpB* and *kgp* is unable to grow in a defined medium containing bovine serum albumin as a sole carbon/energy source (28). In addition, small peptide analogs (KYT inhibitors) and compounds in a rice protein extract, which are known to inhibit gingipains, suppress growth of wild-type *P. gingivalis* in the defined medium (29,30). These reports indicate that proteolysis of extracellular protein by gingipains is essential for bacterial growth. Compounds **6**, **7**, **8** and **13** inhibited the planktonic growth of *P. gingivalis* in this order, followed by compound **3** (Fig. 4A–C). Thus, these prenyl flavonoids and related compounds are potent suppressors of *P. gingivalis* growth. However, the BHI-HM broth used in this study is a nutrition-rich media in which gingipain null-*P. gingivalis* mutants grow as well as the wild-type strain. This result strongly suggest that prenylated flavonoids may target to *P. gingivalis* other than nutrition acquisition with gingipain proteolytic activity or, more likely they also hinder vital metabolic pathways of *P. gingivalis*. In addition, it needs to be kept in mind that besides gingipains *P. gingivalis* produces a number of extracellular and cell-associated proteinases (31). This corroborates with the finding by Blankenvoorde *et al*. (32) that inhibition of cysteine proteases did not cause any suppression of *P. gingivalis* growth, suggesting an involvement of proteases other than cysteine proteases in the growth of this bacterium. It is likely that prenylated flavonoids also inhibit these proteases. The structure–activity relationship of prenyl flavonoid in the inhibition of *P. gingivalis* planktonic growth is unclear. The exception is the strongly reduced growth-inhibitory activity of compound **4** in comparison to **16**, which is caused by *O*-glycosylation. Of note, some prenylated flavonoids (compounds **6** and **13**) with weak inhibitory activity of gingipains (Figs 2 and 4A–C) strongly suppressing planktonic growth. This supports a contention that the prenylated flavonoids inhibit *P. gingivalis* growth by undefined mechanism(s) independent of gingipain inhibition. Taken together, prenylated flavonoids can be considered as potential novel therapeutic agents capable of inhibiting both gingipain-dependent virulence and bacterial growth in periodontitis sites where proteins are the sole source of nutrition.

The location of *P. gingivalis* in subgingival biofilm in a periodontal pocket facilitates evasion of host immune responses and resistance to antibacterial agents. The finding that biofilm formation by *P. gingivalis* was significantly reduced in the presence of compound **6** or **8** (Fig. 4D) also indicates therapeutic applicability of these compounds in the treatment of periodontitis. Interestingly, compound **6** suppressed *P. gingivalis* biofilm formation at 1.25 μM (Fig. 4D) but did not affect the bacterium growth even at

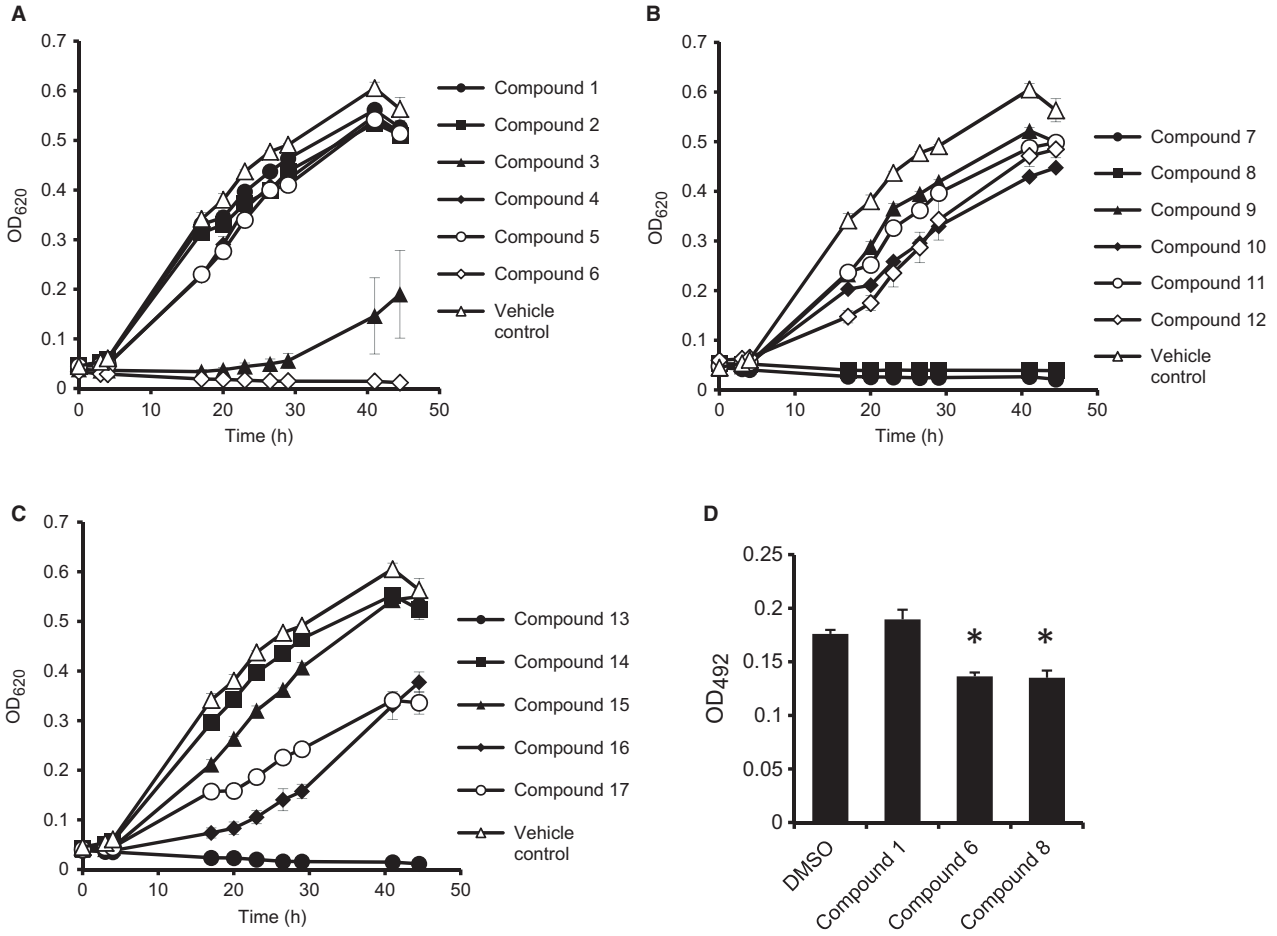

Fig. 4. Inhibitory effect of prenylated flavonoids on growth and biofilm formation of *P. gingivalis*. (A–C) *P. gingivalis* growth in the presence of prenylated flavonoids at 12.5 μM is shown by measuring absorbance at 620 nm different time points. Results were similar in each of three independent experiments and representative data are shown as means ± SD in a triplicate assay. (D) Biofilm formation (absorbance at 492 nm after staining with safranin) was assessed after a 48 h culture in the presence of a compound at 1.25 μM. All the compounds at 1.25 M had no effect on growth of *P. gingivalis*. Results were similar in three independent experiments and representative data are shown. Results are expressed as means ± SD in a triplicate assay. Data were statistically analyzed by one-way analysis of variance followed by the Dunnett's multiple comparison test. *Significant difference ($p < 0.05$) from values obtained in the absence of flavonoids. DMSO, dimethyl sulfoxide.

2.5 μM concentration (Fig. S1 B). This result indicates that some flavonoids exert efficient suppression of the biofilm formation via mechanisms other than gingipain inhibition and growth inhibition. The similar finding was described for tea catechin, lactoferrin and cranberry proanthocyanidin, all of which hampered the *P. gingivalis* biofilm formation independent of gingipain inhibition (11,13,33). Using gingipain-null mutants, Kuboniwa *et al*. (34) showed that gingipains affect the biofilm structure and maturation, although they are dispensable for biofilm formation *per se*. Cumulatively, apart from inhibition of gingipain and *P. gingivalis* growth by flavonoids and prenylated flavonoids they may act through an unknown mechanism, which impedes biofilm formation. Further studies are necessary to elucidate the mechanism. The present study provides useful information for future development of periodontitis treatment using prenylated flavonoids that suppress gingipains, *P. gingivalis* growth and biofilm formation.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Inhibition of *P. gingivalis* growth one in the presence of various concentrations of compounds **1**, **6** and **8**.

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